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Evolution, Biodiversity and a Reassessment of the Hygiene Hypothesis

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Evolution, Biodiversity and a Reassessment of the Hygiene Hypothesis

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Preface

The development and physiological functions of most, probably all, multicellular organisms require interactions with microorganisms. These interactions include signals that modulate organ development and function, data for the immune system, and colonisation; DNA of the microbiota encodes a huge, flexible repertoire of metabolic functions. It is broadly accepted that modern lifestyles have diminished or distorted some of these essential microbial interactions, and that this has contributed to simultaneous increases in a range of localised chronic inflammatory disorders (allergies, autoimmunity, and inflammatory bowel diseases) and also to systemic chronic inflammatory states with persistently raised biomarkers of inflammation, predisposing to metabolic, cardiovascular, and psychiatric problems. This volume explores all of these issues.

The hygiene hypothesis was born in the field of allergic disorders. Awareness of an increase in allergies associated with wealth and urbanisation can be traced back to the nineteenth century when Blackley noted that hay fever was less prevalent amongst the common people than amongst city dwellers [1]. Then in 1989 David Strachan observed that hay fever was less common in children with multiple older siblings, and suggested this could be explained if allergic diseases were prevented by infections in early childhood [2].

This suggestion became known as the hygiene hypothesis, initially focussed on allergic disorders and on the possible role of reduced exposure to the common infections of childhood. But those infections of early childhood (measles, etc.) are mostly *Crowd Infections* that did not enter human populations until recently; measles, for example, probably transferred to humans during the Roman Empire. Moreover, epidemiological studies have shown that the common infections of childhood make allergies worse, not better.

We cannot be in a state of evolved dependence on the crowd infections of childhood. Nevertheless, those infections, or the vaccines that now prevent them, might play roles in priming immunoregulation. Moreover, some authors believe that we are suffering from the absence of infections such as *Helicobacter pylori* and helminths that, unlike the crowd infections, were often present in our hunter-gatherer

ancestors. These controversial issues are discussed in a broad outline chapter in this volume [3].

So which microorganisms do we need to encounter? We do not have a detailed answer, but we do have two broad principles. First, we know that exposure to the microbiota of mother (and other family members) is crucially important. This probably explains the observations of Strachan. . .the older siblings enhanced exposure to the family microbiota. Development of the child's microbiota is discussed in detail [4].

Secondly, major advances in our understanding have come from comparing disease incidences in rural versus urban populations, or farming versus non-farming, or animal-exposed versus not animal exposed [5]. These studies have revealed that exposure to the microbiota of the natural environment is crucial, particularly for activation of immunoregulatory mechanisms [5], and that such exposures are being reduced, while the environmental microbiota itself is being modified by human activity [6, 7].

What about the modern home? Unfortunately, the media seized upon the word "hygiene", and encouraged the view that our homes have become "too clean for our own good". This is misleading and dangerous. Until recently humans built homes with materials taken from the environment (natural timber, thatch, rendered with mud, straw and dung). The microbiota of such homes was similar to that of the natural environment in which humans evolved. But modern homes are built with biocide-treated timber, plasterboard, plastic, and concrete so they have a bizarre microbiota, and if damp they harbour organisms that make secondary metabolites that are toxic to humans because we didn't evolve with them, and these organisms can lead to "sick building syndrome". We have not evolved to require contact with the microbiota of the modern home, so exposure to it may not be beneficial unless it resembles that of the natural environment, which is more likely if the home is rural, on a traditional farm, has a garden, or contains pets. Clearly socio-economic status (SES) has a large influence on exposure of urban populations to the natural environment and this issue, and other SES-related effects on the microbiota (such as diet and stress) are discussed [3].

So the evidence points to the importance of organisms from mother and from the natural environment and provides little support for a damaging effect of domestic hygiene. Clearly this knowledge does not lead to a precise definition of the relevant organisms. This book therefore attempts to broaden our thinking, and to put the inappropriately named hygiene hypothesis into an evolutionary, biological, and mechanistic context. We include a chapter on the regulation of organ development and function by microorganisms in non-human species in order to alert the medical profession to the possibility of additional microbe-driven effects in humans [8]. Similarly we provide a chapter on the nature and functions of skin microbiota in non-human vertebrates [9]. But what about mechanisms? We have detailed chapters on the progress that has been made towards unravelling how microorganisms modulate the immune [10] and metabolic systems [11].

Finally, one of the most dramatic recent developments is the realisation that in the absence of a microbiota the gut and the central nervous systems fail to develop or

function normally. Awareness of this gut-brain axis is likely to revolutionise our understanding of psychiatric conditions. Thus this volume includes chapters on the effects of the microbiota on brain structure and function [12] and the links between microbiota and neurodegeneration [13].

The bottom line is that we should be guided by our evolutionary history, which points to the need for contact with the microbiota of mother and family, and with the microbiota of the natural environment. Understanding the latter has led to novel strategies for reducing the prevalence of allergic disorders, and our final chapter provides a hopeful vision of the exploitation of this understanding in the field of allergy [14]. The term "hygiene hypothesis" is extremely misleading. It might be wise to replace it with the Old Friends and/or Biodiversity hypotheses discussed in this volume.

We express our profound gratitude to all the authors who have contributed chapters.

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Contents

From Observing Children in Traditional Upbringing to Concepts of Health

Erika von Mutius

Abstract Studies in rural areas of developing countries and the farm studies have generated a rich texture of investigations on how traditional lifestyles in natural environments can protect children from developing asthma and allergic diseases. Many environmental exposures, be it from plants, animals, parasites, or microbial sources, are likely to contribute to protection, but the underlying pathways have not been fully understood. While involvement of the first line of defense, i.e., the multiple components of innate immunity, has been described to some extent, the interaction with and the consequences for adaptive immunity eventually deviating from asthma- and allergy-related Th2 immunity are not understood. The human skin, airway, and gut microbiome, as the interface between the external environment and its microbiome, and the host's immune responses are likely to play an essential mediating role in this process. The findings call for novel avenues to prevention since both epidemiology and the associated experimental studies have reproducibly shown that mice and children can be almost fully protected from these conditions. The best approaches to prevention are however still debated, and this chapter will address a number of potential options.

Keywords Children · Farm · Asthma · Allergies · Microbiome

1 Rural–Urban Differences

Childhood asthma and allergic diseases have been called the epidemic of the twenty-first century. Since the middle of the last century, a sharp rise in the prevalence of these conditions has been documented in many countries of the western world [1] and is still being observed in middle-low income countries [2]. Many environmental exposures have been scrutinized as potential causative factors, but the culprit for

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these rising trends has not been found. It seems likely that not one, but many exposures in varying combinations and contexts will have contributed to the observed temporal changes.

In turn, it seems remarkable that in many rural areas rates of childhood asthma and allergies are very low to exceedingly low. Wong and colleagues documented that in rural China the prevalence rates of wheeze and physicians' diagnosed asthma amounted to 1.0% and 1.1%, respectively, which were significantly lower than in urban children in Beijing (7.2% and 6.3%, respectively, $p < 0.0001$). A positive allergy test was 3.2 times more frequent in urban as compared to rural children ($p < 0.0001$) [3]. In Mongolia, the prevalence of allergic rhinoconjunctivitis and allergic sensitization was assessed in villages, rural towns, and the capital city of Ulaanbaatar and was shown to significantly rise with increasing urbanization ($p = 0.02$ and $p = 0.003$, respectively) [4]. In South Africa, the prevalence of food allergy was assessed by skin prick tests confirmed by open oral food challenges in toddlers aged 12–36 months in urban Cape Town and in the rural Eastern Cape. The prevalence of food allergy was 5 times higher in Cape Town as compared to the rural areas after taking ethnicity into account (2.9% vs. 0.5%, $p = 0.007$) [5]. These findings point toward early age when allergy trajectories fall apart in urban and rural environments.

Recently differences in the prevalence of childhood asthma, allergic sensitization, and particularly, aeroallergen sensitization were given for the province of Zealand, a westernized, densely populated area of Denmark [6]. Asthma at age 6 years was seen in 26.6% of included children who had spent their first year of life in urban environments as compared to 16.2% of children who had grown up in rural environments in the first year of life ($p = 0.0015$). The difference for aeroallergen sensitization amounted to 29.5% vs. 16.3%, respectively ($p = 0.0008$). While these differences are highly significant, the overall prevalence in Danish rural areas exceeds by far prevalences reported from less westernized areas.

In Poland, two subsequent cross-sectional surveys including all inhabitants older than 5 years in the small town of Sobotka with 4000 inhabitants and the surrounding small villages were conducted before and after the accession to the European Union. In the first survey, allergic sensitization was very uncommon (7%) among villagers at all ages but not among the urban population (20%, $p < 0.001$). Interestingly, the lower rates were mostly seen in subjects below the age of 40 years, i.e., those born after 1960, [7] which coincides with the general increase of asthma and allergies in the westernized world in these decades [1, 8]. After accession to the European Union, prevalence rates of allergic sensitization assessed in exactly the same manner as in the first survey remained unchanged in the small town of Sobotka, but more than doubled in the small villages (7.9% vs. 17.8%, $p < 0.0001$) within a 9-year period. This temporal change was observed for all age groups suggesting a maintained responsiveness to environmental exposures also in adulthood.

Another very remarkable example describes differences in two geographically adjacent rural areas, i.e., Finnish and Russian Karelia. After the Second World War, the Karelian population was divided by the "iron curtain" with Russian Karelia belonging to the Soviet Union until 1991 resulting in tremendous differences in

standards of living and environmental exposures on both sides. The gross domestic product (GDP) per capita differed substantially between both regions whereby the Russian GDP amounted to 24% of the Finnish GDP in 1996 and this gap may have widened since [8]. In children and their mothers, the rate of any positive reaction to an allergen skin prick test differed considerably: 42.5% vs. 15.7%, $p < 0.0001$, and 32.2% vs. 17.4%, $p < 0.0001$, respectively, between Finnish and Russian Karelia. As in the Polish studies, a second survey 10 years later was performed among adults 25–54 years. Rates of any allergic sensitization increased from 21.5% to 27.1% ($p = 0.01$), with a particular increase in polysensitization to more than one allergen (7.8% to 15.0%, $p < 0.001$) and sensitization to birch and grass pollen and cat dander [9].

Likewise, in Moscow (Russia) and Tallinn (Estonia) a lower prevalence of wheeze and asthma in school age children had been seen in the early ISAAC surveys when compared to Helsinki in Finland [10]. In the subsequent DIABIMMUNE birth and young children cohort in Russia, Estonia and Finland specific IgE levels were measured at age 18 and 24 months, and 3, 4, and 5 years, respectively [11]. Already at the age of 24 months a pronounced gradient for hen's egg and cow's milk sensitization from Espoo in Finland (27.8%; 25.0%) over Tartu in Estonia (15.7%; 19.6%) to Petrozavodsk in Russia (8.3%; 8.3%) was seen [12]. This allergy gap increased at older ages and was accompanied by significantly lower rates of wheeze, allergic rhinitis, and atopic eczema in Estonia as compared to Finland (no data were published from Russia) suggesting that divergent environmental exposures already impact on the allergy trajectories very early in life.

2 Determinants of Urban–Rural Disparities

Some authors have investigated potential environmental determinants for the discrepant rates between urban and rural areas. Exposure to animals and farming practices has been identified in Mongolia [4] (herd animals plus dung heating), China (crop farming [13]), South Africa [5] (farm animals), Karelia [14] (cats, parental farming), and Poland [7] (farm animal contact). Furthermore, differences in environmental microbial exposures were seen in Karelia with respect to gram-positive bacteria and animal-related species being more abundant in Russia and bacterial contamination of drinking water being documented on the Russian side [15]. In China almost fivefold higher levels of endotoxin, a component of the cell wall of gram-negative bacteria, were found in beds of children living in the rural area of Conghua as compared to urban Guangzhou [13]. The endotoxin levels were furthermore inversely related to asthma rates in these children. Additionally, regional differences in human microbiota composition were reported from several of these investigators. In the DIABIMMUNE cohort in Russia, Estonia, and Finland, the compositional structure of the gut microbiome differed already significantly in the first year of life [12]. Furthermore, differences in skin and nasal microbiota composition were found very early in life between Estonian and Finnish children of this

cohort [11] which will be described in more detail in a later section of this chapter (page xxx). Differences in airway microbiota composition were also detected in Danish urban and rural infants in their first year of life [6].

These observations overall hint at the importance of human exposures to animals, farming practices, and environmental microbial exposures which may shape an infant's microbiome. In this context, biodiversity of plant exposures may also come into play as demonstrated in rural Finnish Karelia [16, 17]. Environmental biodiversity was characterized by the vegetation cover of the yards and the major land use types (forest, agriculture, water bodies, built areas) within 3 km of the homes of adolescent study subjects inhabiting a small town, villages of different sizes, and isolated houses. Biodiversity (forest and agriculture land use; flowering plants in the backyard) was inversely related to allergic sensitization and positively with skin microbiota, in particular gammaproteobacteria and more precisely with *Acinetobacter*. In turn, *Acinetobacter* in skin swabs was inversely related to allergic sensitization suggesting that the protective effect of biodiversity might be mediated by alterations of the skin microbiome. Additional chapters in this book will discuss the biodiversity hypothesis in more detail.

3 Farm Studies

Additional insight has been gained in studies investigating the prevalence of asthma and allergic conditions of children who have grown up on family run farms in rural areas of westernized countries. Farms may be seen as residual pockets of former ubiquitous traditional lifestyles and environments with very close ties to domesticated animals such as cows, pigs, horses, sheep, goats, poultry, cats, and dogs as well as plant material for feeding and bedding the animals. In large parts of the world, such upbringing was standard living for self-supply before industrialization took over. Meanwhile farmers have become minorities in rural villages in many westernized countries. There is a large body of evidence repeatedly documented in numerous countries worldwide and nicely summarized and reviewed elsewhere [17] showing that children growing up on farms have significant protection from asthma, hay fever, allergic sensitization, and atopic dermatitis when compared to other village children without farm-related exposures. Traditional full-time run farms were related to stronger protective effects than farms where parents were only involved half-time [18]. The farm effect on hay fever and atopy is very robust as documented in meta-analysis, [17] whereas the effect on asthma is less pronounced and mostly seen for farm children whose parents cultivate fields in addition to keeping cattle [19, 20]. Intriguingly, the farm effect on asthma is not confined to allergic asthma, but is also associated with wheeze among non-allergic subjects and viral-associated wheeze [21] (Fig. 1). The anti-infectious component of the "farm effect" has furthermore been found in the European multicenter PASTURE birth cohort enrolling pregnant women half of them actively involved in farm activities. A detailed diary over the first year of life weekly documenting nutrition, environmental

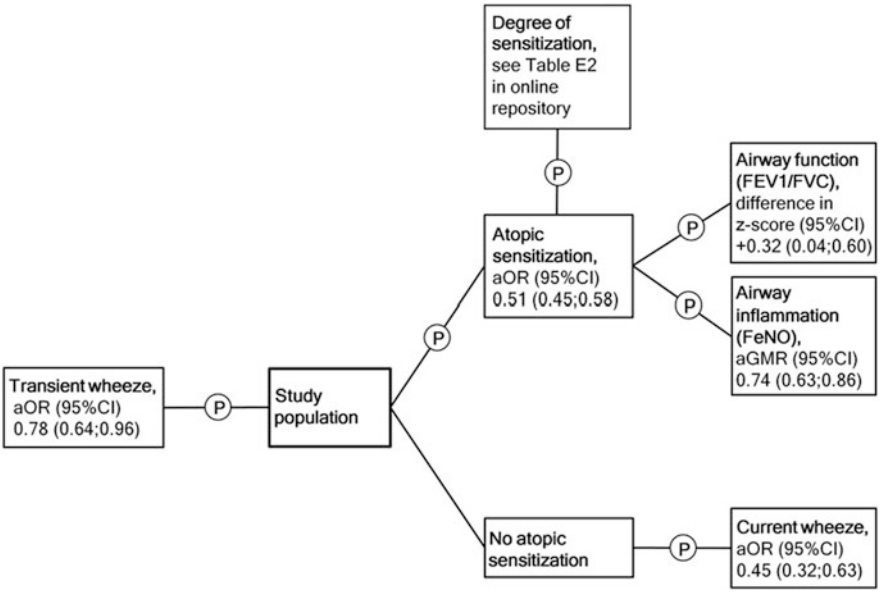

Fig. 1 Multifaceted protection of a farm environment on allergic sensitization, airway function, airway inflammation, transient wheeze, and current wheeze according to the findings from the GABRIEL Study (Fuchs et al. JACI 2012). *P* protection

exposures, symptoms, and treatments of enrolled infants showed reduced risk of otitis, rhinitis, and fever with farm-related exposures [22, 23]. Moreover, in the Marshfield Epidemiologic Study Area or "MESA" study in rural Wisconsin, the prevalence of severe respiratory illnesses in the first 2 years of life such as pneumonia, respiratory syncytial virus (RSV) infection, and bronchiolitis was assessed in children aged 5 to 17 years born onto dairy farms and compared with children who grew up in similar rural areas but lacked farm exposure. A significant reduction in severe respiratory illnesses was seen among the farm children [24]. Thus, upbringing on a traditional farm affects multiple endpoints and at least three main avenues to disease: allergic sensitization, viral infections, and (upper and lower) airway inflammation.

Farm lifestyles differ considerably from lifestyles of surrounding families. In a number of cross-sectional studies and in the PASTURE birth cohort, the main pillars of the farm effect have repeatedly been identified as the consumption of unprocessed cow's milk [25] and the children's exposure to animal sheds, in particular cow sheds, which explain the farm effect on asthma and to a large but not full extent on allergic sensitization [20]. A large array of other potential lifestyle factors such as delivery mode, family size, pet keeping, parental education, maternal smoking and passive smoke exposure, exercise, infant nutrition, health-related quality of life, levels of house dust mite and cat allergen exposure, and markers of infectious exposures such as serology to *Toxoplasma gondii* and *Helicobacter pylori* [26, 27] have been

scrutinized as potential confounders in the European farm studies. While some of these exposures have additive effects, they do not substantially confound the main exposures, unprocessed cow's milk and exposure to cow sheds, and do not explain the farm effect on asthma, hay fever, and allergic sensitization. This notion is further supported by the finding that both main exposures are also inversely related to asthma, hay fever, and allergic sensitization in neighboring children not living on a farm, but exposed either through their peers or their parents buying milk directly on the farm.

The importance of traditional as compared to industrialized farming practices has been highlighted in a study comparing children from Amish and Hutterite families. The Amish and Hutterite populations arose during the Anabaptist movement in sixteenth-century Switzerland (Amish) and South Tyrol (Hutterites). Both groups were persecuted because of religious beliefs and eventually emigrated to the United States. The Amish settled on single family farms, whereas the Hutterites built up communal farms. Both populations have comparable lifestyles such as large sibship sizes, similar diets rich in fat, salt, and raw milk, long durations of breast-feeding, minimal exposure to tobacco smoke and air pollution, and taboos against indoor pets, TV, and, the Internet. However, they completely differ with respect to farming practices. A journey to the Amish is like a journey back in time. They farm like European farmers before World War One without any modern technology, using horses for fieldwork and transportation, and keeping a large variety of a few other farm animals such as poultry, rabbits, and pigeons. Importantly, animal sheds are in immediate vicinity of the family's home, and all children are constantly exposed very early in life, often barefoot bringing these exposures into the home. In contrast, the Hutterites live on large communal farms and embrace modern technology, and their industrial-sized animal sheds which are outside of the families' living area can house up to 100,000 turkeys, 20,000 hogs, or 600 cows. Young Hutterite children are not allowed into these barns until boys only learn the farming business from puberty on. The prevalence of asthma, hay fever, and allergic sensitization is strikingly different. Hay fever is almost non-existent among Amish school age children [28]. A positive allergy test was found in 7.2% of the Amish children as compared to 33.3% of the Hutterite children; asthma was reported for 5.2% of Amish and 21.3% of Hutterite children [29]. Importantly, Amish and Hutterite children have similar genetic ancestries pointing to the fact that these large discrepancies in disease rates are attributable to environmental exposures. Likewise, no major genetic differences exist between Finnish and Russian Karelian subjects [30] nor between farm and nonfarm European populations (unpublished data).

Overall, the farm studies recapitulate and corroborate the observations made in traditional rural upbringings. Both main exposures are composite exposures containing a large number of ingredients be it in the milk whey, fat and carbohydrate compartment, or the multiple contributors such as cows, cats—which like to raise their kittens in warm animal sheds—plant material such as hay and silage as fodder and straw as bedding material, and bacteria, fungi, viruses, and phages to animal shed exposures. It seems therefore unlikely that "one needle in the haystack" will

explain these already diverse protective effects on allergy, risk of respiratory infections, and airway inflammation in asthma.

Before further attempting to better understand the environmental impact on these diseases, we should focus our attention on the nature of these interrelated conditions. Asthma is not one disease but rather a complex illness made up of a combination of multiple traits such as allergy, impaired lung function, eosinophilia, susceptibility to rhinovirus and respiratory syncytial virus infections, airway inflammation, Th2 skewed immunity, and weakened antiviral defenses to name a few [31]. Comorbidity of asthma with hay fever and atopic eczema occurs in some but not all children. All conditions occur in certain windows of pediatric development. For example, the rate of viral infections is greatest in infant–toddler years and decreases by school age. Allergic sensitization often has a temporal, age-dependent sequence of events where sensitization to foods appears in the first 1–2 years followed by mite and cat sensitization and then pollen sensitization at school age and beyond. Naturally not all children show such a course, but many do. Atopic dermatitis often occurs in infants shortly after weaning or even while being breastfed. The majority of children diagnosed with asthma at school age will have developed symptoms in the first 3–4 years, many already in the first year of life. Hay fever typically occurs in teenager years, but the onset of disease seems to move to younger age in recent decades. Moreover, immune responses and microbiomes in the respiratory tract and the gut mature over childhood years. Therefore, the environmental impact of traditional upbringing will dynamically influence and interfere with developmental processes. Unfortunately, we still know very little about the driving forces of pediatric development.

However, an environment protecting from the development of illness will have to occur before the onset of disease or the onset of clinically unapparent disease-associated traits. Given that for the conditions under consideration here, the onset is early in life, exposures early in life or during pregnancy will have to be scrutinized. There is in fact evidence that the maternal farming environment impacts on disease development in the offspring. Both in Wisconsin farmers in the USA—a group of emigrated German farmers—and in the European farm studies, maternal exposure to an increasing number of animals (cows, pigs, cats, poultry, horses) was incrementally related to decreased risk of atopic dermatitis [32, 33]. Furthermore, maternal farm activities translate into alterations of immune responses in cord blood of newborn children be it cytokine profiles, IgE antibodies, the number and function of regulatory T cells, and epigenetic changes in cord blood (reviewed in [34]). Maternal farming was furthermore related to gene expression of microbial recognition receptors TLR2, TLR4, and CD14 at school age [35]. These findings suggest that a mother's farm exposures affect her child's early setup of immunity which either translates into decreased risk of disease, i.e., atopic dermatitis, or sets the stage for balanced immune trajectories which may then contribute to tolerance of environmental allergens (plants, animals, mites, food) and avert excessive airway inflammation. In the following sections I will discuss in more detail the effects of the main pillars of exposure: unprocessed cow's milk and animal sheds.

4 Unprocessed Cow's Milk

Milk that we buy in supermarkets has undergone numerous processing steps. The strongest concern about raw cow's milk is the infectious risk from, for example, *Mycobacterium bovis*, *Brucella abortus*, enterohemorrhagic *Escherichia coli* (EHEC), *Salmonella*, and *Listeria monocytogenes*. Pasteurization, heating to 72 °C–75 °C for 15–30 s, kills these pathogens and is in most countries the minimal requirement for microbial safety with some regulatory differences between areas. Pasteurization does however not kill all bacteria and will not destroy spores. Therefore pasteurized milk has to be kept cool to slow microbial growth, but will spoil after about 6–10 days. For extended shelf life, sterilization, i.e., heat treatment at high or ultra-high temperature, is performed in various specifications by different dairies in different areas. Heat treatment will however not only affect microbial viability and growth but will also denature milk proteins that are contained in the whey fraction of the milk depending on the level and duration of heat treatment. Milk proteins are made up of a number of immunologically active substances such as lactoferrin and immunoglobulins.

Besides heat treatment, cow's milk usually passes several additional processing steps including centrifugation, filtering, and homogenization. In whole milk, the fat separates from the water and is found at the top of a milk bottle. To prevent creaming up, the milk is forced at high pressure through small holes thereby breaking up and reducing the milk fat globules in size to achieve uniform dispersion in the milk. These milk droplets (0.1–10 mm) are coated with a trilayer of phospholipids and proteins. The milk goblets contain around 400 different fatty acids and mono-, di-, and triglycerides, phospholipids, cholesterol, fat-soluble vitamins, and hundreds of different proteins. During homogenization, the globule structure is destroyed, and the trilayer of phospholipids and proteins is mostly replaced by milk protein. Eventually homogenization allows the sale of non-separating milk at any fat content.

In a comprehensive meta-analysis Brick and colleagues [25] have summarized the findings from eight studies on potential allergy- and asthma-protective effects of the consumption of unprocessed cow's milk. This meta-analysis corroborated the findings from single studies and has shown a 25%–42% reduction in risk of asthma and current wheeze, hay fever or allergic rhinitis, and allergic sensitization in farm but also neighboring non-farm children. An important observation in the farm studies has been the abolishment of protection when boiled cow's milk is consumed by the child hinting at the importance of heat-sensitive ingredients. However, cow's milk is a complex liquid with more than 2000 constituents with lipids, proteins, carbohydrates, and many other low-abundant components, such as vitamins, minerals, and miRNAs. Therefore, in population-based studies the potential to identify important single constituents is limited. Nevertheless a number of candidates have been suggested.

Obviously both microbial content and milk proteins have been discussed because of the heat-sensitive nature of the protective effect. In the farm studies no clear effect of either total bacterial count nor of the selected microbiological groups

pseudomonades, Enterobacteriaceae, micrococci plus staphylococci, lactobacilli, yeast plus mold, bacilli plus endospores, psychrotrophic bacteria, and human pathogens has been seen [22]. Broader approaches such as 16S rRNA gene sequencing have, however, not been applied. The prebiotic potential of unprocessed cow's milk consumption has only recently been addressed in the PASTURE birth cohort. At the age of 12 months, the consumption of unprocessed cow's milk was associated with higher richness and a higher Shannon index of the gut microbiome as compared to children drinking processed "shop" milk (Pechlivanis et al., submitted).

Proteins are another group of major components accounting for 3% to 4% of the milk. The bioactive whey proteins (20%) are generally present as single globular proteins dissolved in the water phase. They undergo profound changes upon heat exposure. The best known proteins are the milk allergens α-lactalbumin, ß-lactoglobulin, and bovine serum albumin which have been inversely associated with asthma in the cross-sectional GABRIEL survey, in which milk components had been measured in milk samples collected from study participants. Other less abundant whey proteins such as lactoferrin, lactoperoxidase, different enzymes (e.g., alkaline phosphatase and lipase) which lose their bioactivity after heating, and cytokines such as transforming growth factor beta (TGF-ß) may also play a role.

The fat content may also matter. Unprocessed cow's milk contains 3% to 6% fat, whereas commercially available milk is generally standardized to a fat content of, for example, 3.5% or 1.5%. In the cross-sectional PARSIFAL study, a reduced asthma risk was found for children consuming full-cream milk or farm-produced butter [25]. In the PASTURE birth cohort, higher n-3 polyunsaturated fatty acid levels and a lower n-6/n-3 polyunsaturated fatty acid ratio in raw cow's milk as compared with industrially processed milk were also inversely associated with asthma. Finally, carbohydrates are the most abundant constituents in milk, and among those lactose may act as a prebiotic constituent. Carbohydrates are however not affected by heat treatment and do not differ substantially between raw and processed milk samples.

More insight into single milk components may in the future arise from murine experimental studies. In a house-dust mite model of allergic asthma, mice fed 0.5 mL of raw cow's milk three times a week were protected from house dust mite-induced airway hyperresponsiveness and eosinophilic inflammation [36]. In turn, the feeding of heated raw milk did not, thus corroborating the epidemiological observations.

Intriguingly, a very small pilot study was furthermore reported by these investigators [37]. Nine children with parent-reported and physician-confirmed cow's milk allergy had been recruited from the Reha Klinik, Interdisciplinary Centre for Dermatology, Pneumology and Allergology in Neuharlingersiel, Germany. Each child underwent a double-blind placebo-controlled oral provocation test with raw and processed milk (a conventional pasteurized and homogenized milk standardized at 3.8%) according to standard procedures. While all children tolerated the raw milk up to a maximum of 50 mL, 8/9 of these children developed symptoms after challenge with the processed milk resulting in the premature stop of the provocation. While these data are very, very preliminary and must be confirmed before any firm conclusions can be drawn, they may suggest that heat treatment may increase

allergenicity of milk proteins such as α-lactalbumin, ß-lactoglobulin, and bovine serum albumin.

5 Exposure to Animal Sheds

Since the early ALEX farm study, we have repeatedly found that exposure to animal sheds is protective against the development of asthma and hay fever, but not atopic dermatitis and allergic sensitization [19, 20]. The type of animals kept seems to play an important role. There was no effect or even a slightly increased risk associated with exposure to sheep, goats, hares, and rabbits. In contrast, pigs and poultry were reported to be inversely related to atopic sensitization, [19] and exposure to cow sheds was reported to protect from asthma and hay fever [20]. As discussed above the timing of the exposure may matter. In fact, in the PASTURE birth cohort, the stay of infants in animal sheds as assessed by weekly diaries throughout month 2–12 of the first year of life was significantly inversely associated with wheeze [38] (Fig. 2). Wheeze in the first year of life is relatively common, and not all children with wheezes develop asthma. There is, however, a genetic locus on chromosome 17q21 that confers significant risk of subsequent asthma in early life wheezers as demonstrated in three birth cohorts, the COAST, COPSAC studies, and also the PASTURE cohort [38]. This genetic make-up allows defining young children with wheeze as being at risk of subsequent asthma, and it is in these children that the

Fig. 2 Exposure to a traditional cow shed in a European farm

exposure to animal sheds was particularly protective. Moreover, a clear dose response was seen as children staying on average more than 20 min showed an 80% risk reduction for asthma symptoms as compared to children staying for shorter time periods (40% risk reduction).

These observations have initiated experimental studies in murine models of allergic asthma [29, 39, 40]. To mimic the infants' exposure, dust was collected from cow sheds and extracted in an aqueous solution thereby washing out substances that may confer protection. For both OVA- or house dust mite–induced asthma models, the nasal instillation of cow shed dust extracts resulted in the almost complete prevention of eosinophilia in the bronchoalveolar lavage and of airway hyperresponsiveness, both hallmarks of allergic asthma in mice and men. These experimental studies thus corroborate the epidemiological observations and allow further understanding of underlying mechanisms.

6 The Environmental Microbiome

A cow shed is not a clean place but rather an area of intense and diverse microbial exposure to bacteria, fungi, phages, viruses, protozoa, parasites, animal dander, and plant debris. In an elegant work by the French microbiologists of the GABRIEL team, the entry of bacterial and fungal exposure from cow sheds and barns into the children's homes was thoroughly assessed. This work clearly showed that microorganisms are transported from animal sheds and barns into farm homes [41]. Farm children are thus exposed to more and a more diverse mixture of microorganisms both in their home and when present in animal sheds and barns. For most of the fungal groups, the ratio between arithmetic mean exposures indoors compared to the animal shed was between 1:10 and 1:40. When extrapolating these data, a child that has spent 10–40 h in its bedroom will have had an equivalent exposure than if it had stayed 1 h in an animal shed. Findings for bacterial exposures were similar.

This notion is further supported by the finding of high levels of endotoxin, a cell-wall component of gram-negative bacteria which is often used as a general marker of environmental bacterial exposure, found in homes of Amish but not Hutterite children [29]. Likewise, levels of endotoxin, and of two markers of fungal exposures—extracellular polysaccharides (EPS) and ß (1->3)-glucans—were significantly higher in farm homes as compared to non-farm homes [42, 43]. While endotoxin levels were inversely associated with allergic sensitization but not asthma, the opposite was seen for EPS: protective association with asthma but no relation with allergic sensitization [19, 43]. These data suggest that not all exposures have the same effect on asthma as they have on allergy outcomes.

Endotoxin, extracellular polysaccharides, and glucans are merely markers of bacterial and fungal exposure, but the findings support the notion that the environmental microbiome may play an important role in asthma and allergy protection as found in farm environments. They do, however, not allow understanding which bacteria or fungi may underlie these protective effects. With the advent of

sequencing methods targeting the bacterial 16S rRNA gene and the fungal nuclear ribosomal internal transcribed spacer (ITS) region, such differentiation has become possible, though the depth of analysis is somewhat limited—rarely down to the species level—depending on the methods used.

7 Environmental Microbiome and Asthma

The studies investigating the environmental microbiome in the PARSIFAL and the GABRIEL study have shown that the diversity of the bacterial and fungal exposure is protective for asthma development [44] (Fig. 3). While in farm homes many low abundance cattle-associated bacterial taxa were found, overall less taxa but originating from human rather than animal sources were detected in non-farm homes [45]. The diversity scores explained the effect of the farming environment on asthma partially in the PARSIFAL study and almost completely in the GABRIEL survey. In turn, the diversity scores explained only 1% and 19% of the strong farm effect on allergic sensitization in the PARSIFAL and GABRIEL study, respectively. These data clearly suggest that other environmental exposures matter for allergic sensitization.

Diversity of exposure may be interpreted as a quantitative association, i.e., "the more the better," and will certainly exclude the possibility of the "one magic bullet" explaining these associations. Alternatively, a certain mix of ingredients within the "exposure soup" may be relevant. The data from the GABRIEL and PASTURE studies hint toward the second possibility. Sequencing methods were rather

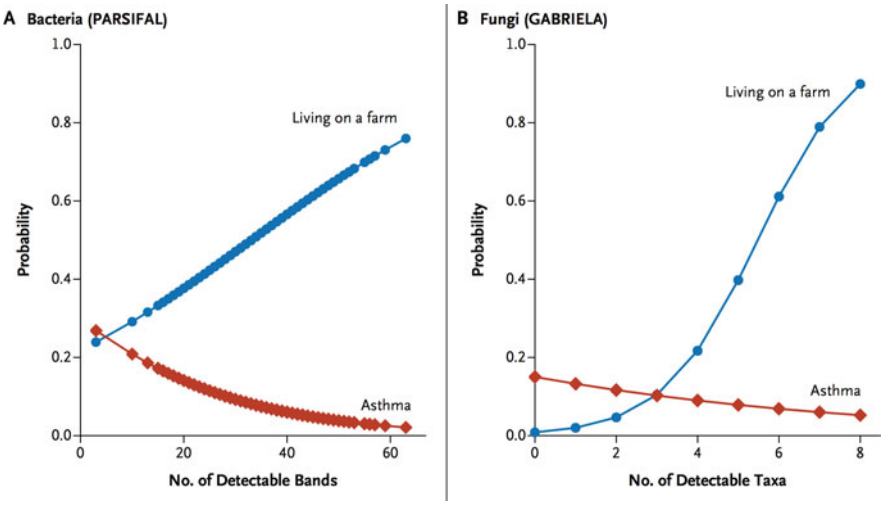

Fig. 3 The diversity of microbial (bacterial and fungal exposure) protects from the development of asthma in the PARSIFAL and the GABRIEL Study, respectively (Ege et al., NEJM 2011)

expensive at the beginning of the microbiome era. Therefore, a screening method (PCR-SSCP) was employed in the GABRIEL cross-sectional survey in which fragments of the bacteria-specific 16S rRNA gene isolated from environmental samples were amplified by PCR, digested to single-stranded DNA, and subjected to electrophoresis [44, 46]. The resulting bands were associated to health outcomes adjusting for potential confounders; the bands were then isolated and sequenced. Thereby, single bands can contain a number of 16S rRNA gene fragments which were then compared to a database to identify distinct bacteria. Bacteria which were identified as potential asthma-protective microbes were numerous: *Corynebacterium mycetoides*; *Zoogloea* sp.; *Duganella* sp.; *Aurantimonas ureolytica* sp.; *Gardnerella vaginalis*; *Lactobacillus curvatus*, *Lactobacillus sakei*; *Streptococcus* sp.; *Moraxella* sp.; *Staphylococcus sciuri* sp.; *Jeotgalicoccus* sp.; *Salinicoccus* sp.; *Macrococcus brunensis*; *Corynebacterium mucifaciens*, *C. freiburgense*, *C. variabile*, C. sp. *Triatoma infestans*; *Neisseria meningitidis*, *N. mucosa*, and *N. subflava.* Such a list is at most suggestive and should be replicated in other farm studies before firm conclusions can be drawn.

However, one of these candidates, *Staphylococcus sciuri*, was detected in significantly higher amounts in mattress dust of the GABRIEL farm children as compared to the GABRIEL nonfarm children [47]. Furthermore, *Staphylococcus sciuri* W620 was tested in two independent murine models of experimental asthma (OVA and house dust mite). In both models intranasal application abrogated airway eosinophilia, a hallmark of allergic airway inflammation. These experiments validate the PCR-SSCP method to some extent as a valid screening system to elucidate potential asthma-protective candidates.

The diversity of fungal indoor exposures was also inversely related to asthma in the GABRIEL study [48]. Much less is however known about the fungal kingdom, and culture and sequencing methods only cover a small proportion of all existing environmental fungi. In the GABRIEL study culture-based methods were used. Of the diversity score built on 15 fungal taxa, only *Eurotium* sp. and *Penicillium* sp. were inversely associated with asthma. In the GABRIEL study the fungal exposure was furthermore assessed by the PCR-SSCP sequencing method. The inverse association between *Penicillium* and asthma was confirmed and additional asthma-protective candidates were detected: *Metschnikowia*, *Aureobasidium*, *Epicoccum*, and *Galactomyces*. Molds produce a variety of bioactive compounds with detrimental but also beneficial immune-regulatory capacities, which may underlie these associations.

Besides the environmental microbial diversity and certain "exposure cocktails" within them which reflect the presence (or absence) of certain taxa, the relative abundance, i.e., the quantity of exposure to certain taxa may matter. In the PASTURE birth cohort and the associated LUKAS cohorts, the relative abundance of the indoor microbiome was characterized by 16S rRNA gene sequencing in farm and in non-farm homes [45]. The investigators modelled differences in house dust microbiota composition between farm and non-farm homes and then observed that in children growing up in non-farm homes asthma risk decreased as the similarity of their home bacterial microbiome composition to that of farm homes increases. In

other words, indoor exposures can protect from childhood asthma if children do not live on a farm, but live in homes where the relative abundance of the indoor microbiome resembles that of farm homes. The protective microbiota had a low abundance of *Streptococcaceae* relative to outdoor-associated bacterial taxa. However, most of the taxa could not be traced back to any distinct source, and more work will have to elucidate where protective environmental microbiomes stem from in non-farm environments. Intriguingly, the observation in PASTURE and LUKAS was replicated in the GABRIEL study with some taxa being protective in both settings and others only in PASTURE/LUKAS or GABRIEL.

8 Environmental Microbiome and Allergic Sensitization

In contrast to asthma, the diversity of the bacterial and fungal exposure did not matter much for allergic sensitization. In the GABRIEL study only the detection of gram-negative rods and two potential candidates were identified by the PCR-SSCP sequencing approach for protection from allergic sensitization, i.e., *Lactobacillus iners* and *Acinetobacter lwoffii* [44]. The latter gram-negative bacterium has also been identified in other studies as protecting from allergic sensitization. Among high-risk, inner-city children of the URECA birth cohort in the USA indoor dust specimens were collected at 3 months of age to undergo 16S rRNA gene sequencing, and children were followed up to age 7 years to assess a diagnosis of asthma [49]. Dust from the homes of children that did not develop asthma was among others enriched in *Acinetobacter*, but this taxon was not further characterized as potentially being *Acinetobacter lwoffii*. As described in a previous section (page xxxx), young individuals with allergic sensitization in rural Finnish Karelia had significantly lower generic diversity of gammaproteobacteria, in particular *Acinetobacter* on their skin as compared to healthy individuals [16]. In the healthy Karelian subjects, the abundance of the gammaproteobacterial genus *Acinetobacter* on the skin was positively correlated with gene expression of the anti-inflammatory cytokine interleukin (IL)-10. Again *Acinetobacter* was not further characterized down to the strain level. A third comparison of geographic disparity in rates of allergic sensitization was seen between Finland and Estonia as discussed in this previous section (page xxx). In the DIABIMMUNE birth cohort, infants with HLA susceptibility to type 1 diabetes were followed up from birth to the age of 3 years in Finland, Estonia, and Russia [30]. In the Finnish and Estonian population skin, nasal and fecal samples at the age of 6 months underwent 16S rRNA gene sequencing. In skin and nasal samples, *Acinetobacter lwoffii* and the genus in general was substantially more abundant and genetically more diverse in Estonian than Finnish infants. These findings thus suggest that *Acinetobacter*, in particular *Acinetobacter lwoffii*, a gram-negative bacterium found in farm yards and soil, may play an important role in allergy protection.

A number of experimental studies support this notion. The Finnish group of investigators used *Acinetobacter lwoffii* strains from blood culture isolates for

intradermal injection in the sensitization phase of a murine OVA model of experimental allergic asthma [50]. This application resulted in protection from allergic sensitization and lung inflammation. Others have used intranasal application of *Acinetobacter lwoffii* F78, an isolate from a farm environment, which prevented experimental allergic asthma in adult and neonatal mice using an OVA and house dust mite protocol, respectively [51, 52]. Moreover, maternal prenatal administration of *Acinetobacter lwoffii* F78 prevented the development of an asthmatic phenotype in the progeny, and this protection was dependent on epigenetic mechanisms [53]. Further experimental studies in vitro demonstrated that the allergy-protecting effects of *Acinetobacter* lwoffii F78 were attributable to the activation of a TH1-polarizing program in human dendritic cells which in turn was mediated by the lipopolysaccharide of *Acinetobacter* lwoffii F78 [54]. Overall, the data suggest that the development of allergic sensitization may be more influenced by environmental endotoxin, i.e., LPS exposure, e.g., through environmental exposure to *Acinetobacter lwoffii* potentially through the skin and/or nasal route. In contrast asthma may be determined by a certain "cocktail" of bacterial and environmental microbial exposures which awaits further detailed characterization.

9 Plant-, Animal-, and Parasite-Derived Substances

However not only microbial exposures may matter given the strong protective signals found for silage, hay, and straw in the farm studies [19, 20, 55] . In fact pollen exposure occurs in animal sheds throughout the year without clear seasonal patterns as it is related to feeding and bedding the animals [55]. Farmers and their children who attend cowsheds during the feeding sessions are thus perennially exposed to high pollen concentrations together with high microbial exposures. Whether this context of pollen exposure matters for the protective effect on hay fever must be further investigated. Plants many also be the source of immunomodulatory substances such as arabinogalactan which stems from the grass *Alopecurus pratensis* used as fodder for animals. Treatment of murine dendritic cells with grass arabinogalactan resulted in autocrine IL-10 production and intranasal application either with arabinogalactan or with whole grass extract protected mice from allergic sensitization, allergic airway inflammation, and airway hyper-responsiveness in an OVA model [56]. Animals may furthermore not just be sources of microbial exposures but give rise to immunomodulatory substances such as N-glycolylneuraminic acid (Neu5Gc), a sialic acid found in non-human mammalian glycoproteins, but not bacteria [57]. Such mammalian substances may thus stimulate the immune responses as "non-self antigens." Antibody levels against Neu5Gc were higher in farm children and inversely associated with wheeze and asthma in non-allergic subjects. Exposure to Neu5Gc in mice also resulted in reduced airway hyperresponsiveness and inflammatory cell recruitment to the lung.

Another approach to identify further allergy- and asthma-protective substances from farming environments is to collect dust from the main pillar of farm exposure,

i.e., the cow shed. As described above (page xxx) dust was collected from cow sheds and extracted in an aqueous solution thereby mimicking a child's exposure and washing out substances that may confer protection. A serine protease from the midgut of *Tenebrio molitor* larvae, which is known as a stored product pest living on traditional farms, was detected [58]. This protease was isolated from the midgut of *Tenebrio molitor* larvae, and it was shown to induce the release of biologically active complement factor C5a in murine bronchoalveolar lavage fluid. C5a was in turn applied in different doses to mice during the sensitization phase and first OVA challenge. C5a dampened important parameters of allergic airway inflammation, such as infiltration of eosinophils, lymphocytes, and neutrophils in a dose-dependent way into lung tissue, and likewise Th2 cytokine secretion by lung cells.

Another substance of animal origin is ß-lactoglobulin found in the whey fraction of cow's milk and via cow's urine also in cow sheds. Beta-lactoglobulin belongs to the lipocalin protein family to which many major allergens from mammalian sources belong. They all show a highly conserved fold despite having very low amino acid sequence homology among one another. The ligand or the cargo a lipocalin carries may be decisive for either an allergenic or tolerogenic activity. Recent work showed that binding of ß-lactoglobulin to iron-quercetin 2 (FeQ2) complexes resulted in prevention of experimental allergic sensitization and anaphylaxis in mice, whereas free ß-lactoglobulin without bound FeQ2 did not [59]. Furthermore, ß-lactoglobulin associated with zinc was detected in cow shed and bed dust of children from dairy farms which had been eluted in PBST buffer (Pali–Schöll et al. in revision). These findings overall suggest that lipocalins may be Janus-faced carrier molecules abundant in the natural environment where the environmental cargo(s) and presumably the receptor(s) to which these complexes bind determine an allergy-inducing or tolerogenic activity. This theme should become an area of increased scientific scrutiny in the future.

10 Impact on the Human Microbiome

A child growing up in traditional rural and farm environments will be exposed to the environmental microbiome through the skin, the upper and lower airways, and the oro-digestive tract. As described above (page xxx) compositional differences in the skin, nasal, and gut microbiome of children growing up in Russia versus Estonia versus Finland, in Russian versus Finnish Karelia, and in rural versus urban Denmark have been documented. This difference in compositional structure of Russian infants' fecal samples as compared to Finnish infants' fecal samples was related to a higher relative abundance of *Escherichia coli* LPS versus *Bacteroides* LPS in Russia [60]. *Escherichia coli* LPS is structurally distinct from *Bacteroides* LPS and induces endotoxin tolerance thereby potentially protecting from allergy and asthma. Thus, environmentally induced changes in the gut microbiome composition will not only affect its metabolic but also its immune-stimulatory function.

In the farm context, the increased diversity of the bacterial environmental exposure is mirrored by an increased diversity of the respiratory tract microbiome, particularly in those with a high exposure to farming characteristics such as exposure to cows and straw [61, 62]. Alterations in nasal but not throat microbiota were associated with asthma. Higher α- and ß-diversity of the nasal microbiota was related to decreased asthma risk, whereas asthma risk was increased in non-farm children harboring *Moraxella* OTU 1462 in their nose. Interestingly, *Moraxella* was not associated with asthma risk in farm children potentially due to competing richness of the nasal microbiota in farm children.

Farm and other environmental exposures also impact the gut microbiome as documented in the Karelian and DIABIMMUNE studies. In the PASTURE cohort fecal samples were obtained at the age of 2 and 12 months, and the compositional structure of the microbiome in this first year of life was related to school age asthma [63]. Axis 3 of the Principal Component Analysis (PCA) at the age of 2 months was associated with reduced asthma risk whereby breastfeeding at month 2 contributed to the beneficial effect. In turn Caesarian section and maternal smoking in pregnancy were both negatively associated with PCA axis 3 suggesting an adverse effect on the asthma-related gut microbiome composition at age 2 months. The gut microbiome undergoes profound changes in the first year of life and up to age 3 years when it starts resembling adult structures. Therefore, the dynamic process of gut microbiome maturation between 2 and 12 months of age was scrutinized. A delayed maturational process was associated with asthma risk, whereas beneficial accelerated maturation was determined by a number of environmental exposures such as presence of at least 2 siblings, keeping cats, growing up on a farm, exposure to animal sheds, and a number of nutritional factors such as consumption of cow's milk and eggs. Intriguingly, the farm effect on asthma was explained by 19% by the accelerated maturational process of the gut microbiome in the first year of life. The resulting compositional structure at age 12 months was suggestive of a role of bacterial metabolites in asthma risk reduction. In particular, the short chain fatty acids butyrate and propionate may contribute additionally to the maturational process and the compositional structure at age 2 months and determine asthma risk. Thus a number of different facets of the gut microbiome are likely to independently and additively impact on the development of childhood asthma. These facets were unrelated to the development of allergic sensitization whereas bacterial richness at age 12 months was inversely related to hay fever at age 10 years (Pechlivanis et al., submitted) again suggesting divergent pathways to asthma and allergy.

11 Involved Immune Mechanisms

A multitude of alterations in immune responses among farm children have been reported which have been beautifully summarized in [64, 65]. As described above (page yyy) the maternal exposure to a farm environment already prenatally impacts diverse immune responses at birth, i.e., in cord blood of the offspring, and the

development of atopic dermatitis in the first year of life. These prenatal influences in pregnancy do, however, not suffice to fully protect the child from developing upper and lower airway disease. Rather postnatal, inhaled and ingested continued exposure to cow sheds and unprocessed farm milk is necessary for the maturation of immune responses and protection from disease.

Innate stimulation from environments rich in microbial exposures has been shown in a number of farm studies. For example, increased gene expression of TLR downstream signaling molecules such as IRAK-1, IRAK-2, and RIPK1 as well as HLA-DRA, and SOCS-4 was found among farm children, whereby the expression of IRAK-1, IRAK-2, and RIPK1 partially mediated the protective farm effect on asthma [66]. These data suggest that activation of innate immunity is associated with both farm exposure and reduced asthma risk. In primary bronchial epithelial cells, another element of innate immunity, i.e., epithelial barrier function, was enhanced after exposure to cow shed dust extracts which in turn was associated with reduction of rhinovirus infection in primary epithelial cells [67]. These findings suggest that tightening of epithelial barrier function might be an essential element of asthma and also antiviral effect of farm exposures.

In Amish children an enrichment in innate immunity genes involved in the response to microbes, both bacteria and viruses, was found [68]. Ingenuity Pathway Analysis (IPA) was used to construct unsupervised protein-protein interaction networks. Major hubs in this network were tumor necrosis factor (TNF) and IRF7, key proteins in the innate immune response to microbial stimuli. Among the genes in these networks was TNFAIP3, which encodes A20, an ubiquitin-editing enzyme critical to limit the activity of multiple NF-κB-dependent inflammatory pathways. In turn, expression of A20 in the airway epithelium had previously been shown to be essential for the asthma-protective effect of dust extracts from cowsheds [40]. The protective effect of Amish environmental exposure on experimental allergic airway disease also disappeared in MYD88/TRIF knockout mice [29]. These findings collectively suggest that protection from asthma requires appropriate stimulation of innate immunity. However, the precise nature of such stimulation—which innate immune elements by which stimuli at which age—needs further elucidation. The concept of trained immunity [69] may pave the way to improved understanding.

As reverse conclusion one might argue that childhood asthma is a disease of aberrant or inadequate innate immune responses. Recently in nested case-control studies decreased TNFAIP3 gene and protein expression was demonstrated in urban asthmatic patients which was restored to healthy levels ex vivo by farm dust or LPS stimulation and which reversed NF-kB signaling–associated gene expression to an anti-inflammatory state. Interestingly, newborns having developed asthma at school age showed already reduced TNFAIP3 expression at birth [70, 71].

A number of downstream adaptive immune mechanisms have also been reported [71]. Amish children had increased activated regulatory CD41 T-cell phenotypes, which were associated with an increase in inhibitory receptors on monocytes. Intriguingly, the Amish children had a high proportion of CD28null CD8 T cells, and the proportion of these cells correlated with high T-cell interferon gamma (IFN-γ) production and low serum IgE levels. Moreover, the number of CD28null

CD8 T cells was increased in children with high expression of the innate genes TNF and TNFAIP3 in peripheral blood leukocytes. The unique feature to the farm studies is, however, a downregulation of all cytokines in peripheral blood regardless of Th1, Th2, or Th17 origin [29, 43, 45].

As discussed in previous sections, a number of murine models of allergic asthma have been performed by various labs and investigators. While a number of different microbes and substances eventually all resulted in prevention of airway eosinophilia and airway hyperresponsiveness, the involved receptors differed: TLR2, TLR4, NOD1, and NOD2 for *Acinetobacter lwoffii*; TLR2 and NOD2 for *Lactococcus lactis* and *Staphylococcus sciuri*; DC-SIGN and MMR-1 for arabinogalactan. Thus multiple, redundant pathways result in prevention of experimental allergic asthma and presumably also of childhood asthma, yet the common final paths to which the diverse receptor-ligand interactions converge to are still poorly understood.

12 Interpretation of Findings and Concepts of Health and Prevention

The studies described in this chapter very clearly show that virus-associated wheeze, asthma, hay fever, and allergic sensitization are strongly determined by environmental exposures up to the point of being almost non-existent in populations with intense traditional lifestyles such as among Amish or rural China and rural South African populations. These consistent findings around the globe may allure one to speculate about a common cause. When studying these populations in more detail on a coarse lifestyle, meta-level similarities such as contact to animals and their products (dung, milk), parasites, plants and their products (hay, silage, straw), and soil as in outdoor activities and crop farming arise which may be best summarized under the broad umbrella term biodiversity. However, biodiverse environments such as sheep or goat farming have not been associated with protection from these conditions. Atopic dermatitis, allergic sensitization, hay fever, asthma, and severe and mild viral respiratory diseases can manifest in various combinations of comorbid trajectories, but also as single, distinct illnesses. Yet, a biodiverse environment affects any individual or combination of these outcomes. This notion may already challenge the "one size fits all" perspective. Rather the characteristics pertaining to a protective biodiversity must first be identified.

When leaving the lifestyle meta-level and describing the environmental exposures on a molecular level such as bacteria, fungi, and parasite-, plant-, and animal-derived substances, an amazing diversity and complexity of exposures is revealed with divergent associations with asthma and allergic sensitization. Yet, most of this environmental complex molecular world is unknown to us today. We have just started to interrogate it, which feels comparable to the first observations made with the newly developed microscope in former times.

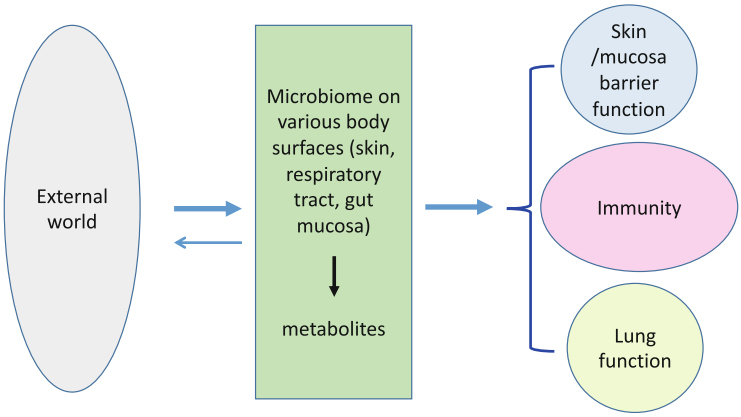

Fig. 4 The human microbiome as interface between the external world and the host's response systems

Our bodies' surfaces such as skin and mucosal surfaces of the airways and gut are in constant exchange with the outside environment. The interface between us and the outside world is the complex system of the human microbiome deeply interconnected with the host's immune responses. This complex system "digests" signals from environmental exposures such as family size, pet keeping, delivery mode, maternal smoking, farm environment, antibiotics, and nutrition into structural changes and maturational processes of the skin, airway, and gut microbiome which in turn induces structural and maturational processes in the interconnected innate and adaptive immune system (Fig. 4).

What can we learn from the observations we made so far? How do they inform us about the nature of the diseases under scrutiny? There is agreement that these conditions are complex illnesses. Such nomenclature might be interpreted as complicated illnesses which will be understood once we have identified all molecular pathway of all subtypes of disease. An analogy would be the molecular dissection of anemia, a clinically rather uniform condition with pallor and fatigue, where the differential diagnosis will reveal the one underlying cause, e.g., vitamin B12 deficiency, iron deficiency, sickle cell disease, etc., which determines therapy. I have proposed that complex diseases can in most cases not be reduced to unicausal entities, but manifest in highly complex combinations of underlying traits and pathways in individual patients in a multitude of ways [31].

A crude visualization of this concept may be an onion with layers of complex microbial wraps, additional layers of redundant, overlapping, and interconnected innate and adaptive immune responses, and several convergent pathways to allergic, inflammatory skin and airway disease in the inner core. Endocrine, nervous system, and other functions are likely to add to such complex texture. From such a perspective, risk can be conceptualized as holes in different layers that may sum up and depending on their location, quality and quantity result in measurable pathologic traits and clinically recognizable disease. Protection may in turn shield the "onion"

and its various layers from damage. A wide range of regulatory and antiregulatory circuits are likely to be involved.

While such considerations reflect a bird's eye view and are likely to still lack essential elements, they may matter when thinking about translational approaches to maintain health, i.e., absence of the complex diseases discussed herein. One possibility is to restore the environmental habitat to former shape by, e.g., restoring natural biodiversity in all aspects of our daily lives. Given the alarming loss of biodiversity in general on our planet, such an approach is certainly warranted and should be pursued wherever possible. A number of recent approaches to improve environments, for example, in day care settings by covering part of the gravel with forest floor and sod which diversified both the environmental and skin gammaproteobacterial communities and resulted in changes of plasma immune markers, are interesting approaches [72]. A recent intervention trial with only pasteurized, but not otherwise processed full cow's milk as compared to an extended shelf life low fat cow's milk as primary prevention for asthma and allergy in young children, the MARTHA trial, is another example [25].

However, in many highly populated areas this strategy is limited by numerous aspects of urbanization. In such settings we must translate the above-described observations into applicable, cost-conscious prevention strategies. From all findings reported above, it seems unlikely that there will be the one "magic bullet," i.e., the one substance that will protect all individuals in genetically admixed populations in mega-cities around the globe. Rather mixtures of exposures, substances, microbes, or microbial metabolites protecting the numerous and individually diverse vulnerable sites of the "onion layers" will be needed if protection for populations is sought after. We will in the future have to learn how such non-redundant mixtures can be identified, manufactured, and how the target population can be identified with appropriate biomarkers.

While primary prevention targets whole populations, such approach may be prohibitive and unacceptable to all members of a community. Thus, targeting individuals at risk, i.e., secondary prevention of individuals with first symptoms of illness, a family history of asthma, hay fever and/or atopic dermatitis, or a genetic background such as the chromosome 17q12–21 locus in young children, may seem more realistic. The epidemiological data at hand can, however, only guide such an attempt under the assumption that the same mechanisms will operate before onset and after the first clinical appearance of the illness.

A potential scientific and translational approach is to learn how to reduce redundancy in environmental exposures, in the interface of the human microbiomes and in the interconnected networks of innate and adaptive immunity. Such a reductionist approach decreases the incredible richness and lavish diversity nature has created over millennia embedding humans in both hazardous and protective environments. When developing novel preventive approaches, we must maintain diversity in protective exposures and develop mixtures rather than a single substance to target all relevant functions of complex diseases in genetically diverse populations. Recent microbiome research identifying a limited number of core

members and core functions of taxonomically diverse microbiota may guide the way [73, 74].

13 Conclusions

The farm studies have generated a rich texture of still limited insight into how traditional lifestyles in natural environments can protect children from developing asthma and allergic diseases. It seems likely that many components of the environment be it from plant, animal, parasite, or microbial sources can contribute to such protection, but the underlying pathways have not been fully described. While the first line of defense, i.e., the multiple components of innate immunity, are likely to be involved in various combinations, the interaction with and the consequences for adaptive immunity eventually deviating from asthma- and allergy-related Th2 immunity are not understood. The human skin, airway, and gut microbiome as the interface between the external environment and the host's immune responses is likely to play an essential mediating role in this process. The strong protection seen in the many population-based studies reported herein calls for novel avenues to prevention since both epidemiology and derived experimental studies have reproducibly shown that mice and children can be almost fully protected from these conditions. The best approaches to prevention are however still debated. Interventions with unprocessed cow's milk as in the MARTHA study or environmental interventions in day care settings attempt to restore components which have been lost in modern food processing or buildings. Alternatively, or in addition non-redundant mixtures of protective metabolic or immune response functions must be identified and translated into science-based, safe, and efficient novel avenues into prevention.

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Human Evolution, Microorganisms, Socioeconomic Status and Reconciling Necessary Microbial Exposures with Essential Hygiene

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Abstract Exposure to microorganisms and colonisation by them influence the development and function of essentially all organs, notably the immune, metabolic and central nervous systems. We therefore need to maintain essential microbial exposures, but this is often thought to conflict with the need to maintain hygiene to avoid disease-causing pathogens. This chapter suggests a framework for solving this conundrum. First, an evolutionary approach illuminates the two-way dialogue between host and microbiota and helps us to determine which exposures really are essential, and to question the role of the unnatural microbiota of the modern home. The evolutionary approach also helps us to understand the mechanisms of the health benefits derived from microbial inputs and the lifestyle changes that are distorting them. Importantly, distorted microbial exposures may explain much of the health deficit associated with low socioeconomic status (SES). By combining these insights with new understanding of the inherent Th2-adjuvanticity of some cleaning agents, and the non-specific immune system-modifying role of pathogens and of their replacement by the "trained immunity" effects of vaccines, it is possible to construct a framework for targeting hygiene and domestic cleaning in such a way that they protect us from pathogens while maintaining the essential microbial exposures.

Keywords Immunoregulation · Microbiota · Infection · Vaccines · Socioeconomic status · Biodiversity · Allergy · Autoimmunity

1 Introduction

The awareness of the fact that modern lifestyles are associated with altered patterns of human disease can be traced back at least as far as the nineteenth century, when Blackley noted that hay fever was most prevalent among wealthy urban citizens

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[1]. Another major landmark was the observation, published in 1989, that the incidence of hay fever was reduced in children who had older siblings [2]. Strachan suggested that exposure to the common infections of childhood provided protection against hay fever and that modern hygiene was reducing such exposures. So the "Hygiene Hypothesis" was born. The media rapidly picked up this concept, and journalists began exposing us to a flow of publications telling us that we are "too clean for our own good". Meanwhile the field became increasingly confused as some authors confined their arguments to allergic diseases where the immune system is targeting inherently harmless molecules using the Th2 response pattern, while others sought to explain the simultaneous increases in autoimmunity or inflammatory bowel diseases mediated by different components of the immune system that attack other "forbidden targets" such as self-components (e.g. multiple sclerosis (MS)) or gut contents (e.g. inflammatory bowel diseases). Finally, others have been more concerned with the simultaneously increasing prevalence of disorders accompanied by chronically raised systemic background inflammation, often manifested as raised C-reactive protein, that predispose to metabolic, cardiovascular and psychiatric problems. The result has been a proliferation of hypotheses, each one concentrating on one particular aspect of the problem. For example, many authors confine their hypotheses to the effects of the gut microbiota, and so neglect the effects of molecular signals and DNA provided by organisms that do not necessarily colonise the host, or that act via molecular sensing systems in the skin, gut or airways. Many of these points are summarised in Fig. 1.

This chapter attempts to draw all these strands together, starting with an evolutionary approach to the relationship between vertebrates and microorganisms, and to the identification of the microbial communities on which we may be in a state of evolved dependence. This includes a brief discussion of some of the mechanisms of the health benefits that microorganisms provide, and the sources from which we obtain them.

I then turn to the fact that infections whether transient (and survived by the host) or persistent and intermittently active also have lasting effects on the immune system, some of which can be mimicked by vaccines. Thus the changing patterns of exposure to pathogens and to the vaccines that block infectious disease are becoming major themes, particularly in this era of COVID-19.

It then emerges that socioeconomic status (SES) has major effects on microbial exposures, and indeed an inappropriate pattern of microbial exposures might explain much of the health deficit seen in individuals of low SES.

Finally, this chapter suggests that by drawing these strands of thought together, it becomes possible to provide a framework that might enable us to solve the "too clean for our own good" conundrum. We suggest that maintaining essential microbial exposures is not inevitably in conflict with domestic cleaning and personal hygiene.

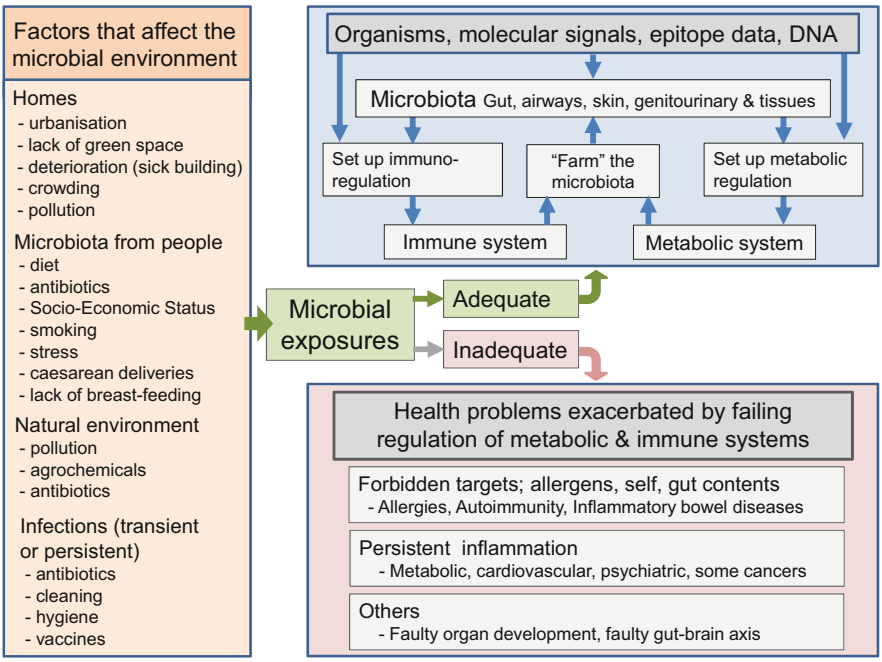

Fig. 1 Some of the factors that modulate our microbial exposures, and the health consequences of adequate or inadequate microbial inputs

2 Evolution of Our Relationship to Microorganisms

2.1 Our Microbial Origins: Genes

The first cellular life forms evolved on earth approximately 3.8 billion years ago. Eukaryotic life followed about 1.5 billion years ago when an endosymbiotic event led to an organism resembling an alpha-proteobacterium starting to live inside another organism. This event gave rise to the mitochondrion [3]. This seems to have occurred only once, so ultimately humans, like all eukaryotic life forms, evolved from a blend of 2 or more microbes. We now know that about 65% of human genes originated in Bacteria, Archaea and eukaryotic microbes [4]. This is strikingly true of the genes enabling synthesis of the neurotransmitters that are crucial to the brains of which we are so proud [5]. So we evolved from Bacteria, Archaea and eukaryotic microbes, and we took most of our genes from them.

2.2 Gut and the Gut Microbiota

Not only did we evolve from microorganisms, but we live in a microbial world. A recent study, which expressed the biomass of each major kingdom of life in terms of the carbon they contain, estimated that bacteria (~7 Gigatons of carbon; Gt C) are second only to plants in terms of total biomass, whereas humans constitute only about 0.06 Gt C. Moreover there are about 10^{30} bacteria, archaea and fungi on our planet, compared to only 7×10^9 humans, implying that there are about 10^{20} bacteria for each human.

Our guts contain symbiotic organisms (the microbiota) that are at least as numerous as the human cells in our bodies, and 30% or more of the small molecules in our peripheral blood, many of which have profound effects on our physiology, are products of the metabolism of these microbes [6]. How did this situation evolve? Early in evolution the organisms that inevitably found their way into the gut were separated from the host by a chitin barrier [7], a structure that persists in arthropods and annelids. In chordate invertebrates, such as tunicates, the chitin mesh is embedded in a mucin gel, and the gut bacteria are still rigorously separated from the gut epithelium. In the most primitive vertebrates (the ray-finned fish), a more substantial mucus layer is secreted by intestinal goblet cells, and this mucus covers the epithelium. However the mucus layer is still separated from the lumen by a chitin membrane. Finally, in mammals the chitin layer is lost entirely, and complex mucus layers interact with and nourish organisms, many of which adhere to the mucus and modulate the function of the underlying cells [7]. It is interesting that this parallels the situation in plants where organisms are attracted and nourished by molecules secreted from the roots, and then take part in symbiotic two-way signalling and exchange of nutrients [8].

2.3 Evolution Turns the Inevitable into a Necessity

Does this evolutionary history enable us to deduce which microbial exposures and inputs are essential because we are in a state of evolved dependence on them? Crucial functions can be outsourced to "inevitably present" microorganisms. The classic example is a laboratory experiment where a culture of amoebae became infected with a bacterium. At first both species in this mixed culture were handicapped, but after 5 years the two organisms became mutually dependent and could no longer survive alone [9]. Each of them had lost some crucial genes because the encoded functions could be outsourced to the other organism, which was *inevitably* present. However, evolved dependence can clearly arise in two different ways. First, a species might evolve in the presence of something (e.g. our need for oxygen), and so incorporate that something into its physiology from the start. Alternatively, as in the experiment with the amoeba described above, something might appear later and cause the pre-existing germline-encoded function to become

redundant. For example, most mammals can synthesise vitamin C, but in humans and some other species the gene encoding an essential enzyme is corrupted [10]. We lost an enzyme required for making vitamin C because the diet of evolving humans "inevitably" contained adequate supplies of it. Unfortunately for sailors on long sea voyages before vitamin C was recognised, the presence of vitamin C in the diet turned out not to be inevitable after all, and scurvy was common.

Both mechanisms apply to the human need for collaboration with, and exposure to, microorganisms, and this concept helps us to identify the essential organisms and their physiological roles, as will be outlined later.

2.4 Evolution Avoids Turning the Non-inevitable into a Necessity

So evolution turns the inevitable into a necessity, but it is equally important to take note of the reverse concept: evolution tends not to *turn the non-inevitable* into a necessity because this can lead to gene-environment mismatch. Sometimes human development results in lifestyle changes that evolution could not "predict" (such as the long sea voyages without sources of vitamin C), but sometimes we scientists assume inevitability that was not there. For example, the belief that we are in a state of evolved dependence on infection with helminths seems to be an error of this type. Helminths need to keep the host alive, so they downregulate inflammation in order to avoid fatal immunopathology. So it was argued that humans accumulated mutations to partly offset the immunoregulatory strategies of helminths, with the consequence that without helminths our immune systems are too pro-inflammatory [11]. But different helminth species live in blood, tissues, bladder or gut, and each species downregulates inflammatory responses via a different mechanism. Moreover loads of helminths differ wildly between individuals, even when they live in similar geographical locations. So there is no constant "inevitable" factor that could drive germ-line encoded dependence on helminths [discussed in [12]]. Rather than becoming written into germline mutations, intermittent or temporary environmental or infectious stresses are coped with via epigenetic adaptations that can fade over several generations, or be renewed if required. Therefore it is not a surprise that allowing MS patients to become infected with helminths they would have encountered in childhood while their immune systems were developing can stop progression of the disease [13], whereas trials of helminth therapy for MS in locations where helminths have not been endemic for several generations are failing [14, 15]. Evolution turns the occasional into an option (via epigenetics), not into a germline-encoded necessity.

2.5 The Two-Way Dialogue Between Host and Microbiota

A logical extension of the realisation that vertebrates co-evolved with their microbiota is the suggestion that the host and the microbiota might be exchanging signals that modulate gene expression in both, and that enhance the symbiotic relationship. For example, the host generates neurotransmitters, hormones and cytokines that can affect microbial metabolism, while the microbiota modulates these signals and also produces a repertoire of microbe-derived signals that can modulate the gut and brain [16, 17].

Recent attention has been focussed on small non-coding RNAs present in exosomes (membrane-bound extracellular vesicles; EV) derived from the host gut epithelium and similar small non-coding RNAs present in membrane vesicles (MVs) from Gram-positive bacteria or outer-membrane vesicles (OMVs) from Gram-negative bacteria. Host-derived microRNAs (miRNA) in EV can mediate post-transcriptional modification of microbial gene expression, and the crucial role of this function was demonstrated by creating mice in which the miRNA-processing enzyme, Dicer, was not expressed in gut epithelial cells so that no miRNAs were formed. These animals developed uncontrolled gut microbiota and exacerbated colitis, which could be corrected by administrating faecal miRNA [18]. So the host regulates gene expression in the gut microbiota.

Does the reverse pathway exist? Does the microbiota use a similar strategy to modulate gene expression in the host? There is suggestive evidence. Small non-coding RNAs in bacterial OMVs align with some human genes, and such signals might epigenetically modulate host gene expression, perhaps particularly in relation to development of the gut to ensure a local host environment favourable to host-microbe symbiosis and to the maintenance and integrity of the holobiont [19]. Signals to the brain also seem likely. It is suggested that the need for horizontal transmission of microbiota and of small non-coding RNA might have played a role in driving the evolution of sociality among vertebrates, and in generating a kin-specific odour to enhance group recognition and cohesion [Reviewed in [20]]. Finally, when diet or environment changes, it is obvious that the rate at which the genome of the microbiota can evolve and adapt is an order of magnitude faster than the rate at which the host genome can do so. Perhaps host evolution is initially driven by rapid change in microbial RNA signals to the host, followed by epigenetic adaption in the host, leading eventually to germline incorporation of the new strategy in the host genome.

3 Evolution of the Immune System

We can consider the evolution of the immune system in the light of this theoretical evolutionary background. The innate immune system relies on inherited germline encoded pattern recognition receptors (PRR), so rapid bacterial evolution can give

rise to pathogens with structures not recognised by existing PRR. The innate immune system can try to catch up by duplicating the gene for a PRR and selecting a modification of its structure that is able to recognise the new pathogen, but clearly this process is slow. Moreover it results in the genome becoming cluttered with large numbers of duplicated PRR genes. The development of the adaptive immune system in vertebrates provided a way to create a very large repertoire of different receptors with a minimal increase in genetic complexity. This is achieved by somatic hypermutation involving the genes encoding the receptors of B lymphocytes and T lymphocytes. These random mutations create large numbers of distinct T and B lymphocyte clones bearing a huge diversity of receptors. This keeps the genetic load low, but it creates several other problems. For example, random mutation could result in vast numbers of useless lymphocytes that recognise nothing and so waste metabolic resources and space. Worse still, there might be lymphocytes that recognise the host's tissues and so mediate autoimmunity. However the diversified receptors generated by mutation are expressed clonally. Each lymphocyte clone expresses only one receptor, so that if that receptor turns out to be useless or autoreactive, the relevant cell line can be eliminated. The autoreactive cells are mostly eliminated in the thymus, where self-antigens are expressed. However, in order to decide which other lymphocyte clones to keep for managing and tolerating the microbiota, while eliminating pathogens, the adaptive immune system requires data from the microbiota that is picked up from mother and family, and data from the environment [21]. The subtlety of this arrangement is that each new individual develops an immune repertoire that is matched to the microbial world into which he or she is born.

3.1 Types of Information Acquired from Microbial Inputs

Therefore, like the brain, the immune system is a learning system, and like the brain, it must receive appropriate inputs, and these must be received early in life, and then maintained and updated throughout life. A simple classification of the types of information that the immune system receives from microorganisms is suggested in Table 1, and several of the items listed are discussed briefly below. The emphasis in this chapter is on the mechanisms that lead to immunoregulation rather than to enhanced protection from infection because the disorders that are increasing in rich urban societies are associated with failing immunoregulation.

3.1.1 Organisms and a Biodiverse Microbiota

Contact with mother, other people and the natural environment helps to populate the microbiotas (see also the discussion of spores in Sect. 4.4.1) and increases their biodiversity. There is a strong correlation between this biodiversity and health [48]. Most illnesses are accompanied by reduced biodiversity of the gut microbiota,

Table 1 Types of microbial input and their functions

Input	Mechanism	Immune system	Refs.
Organisms			
Diverse organisms	Populate microbiota	Organ development and digestive function, short-chain fatty acids. Set up regulation of metabolic and immune systems	[22–24]
Spores, gut-adapted strains	Trapped by mucus and cilia, swallowed	Expand or restore the gut microbiota	[25]
Low-dose pathogens	Disarm in airways, swallow	Immunity to common pathogens	[26, 27]
Signals			
Microbial components (e.g. some LPS and muramic acid derivatives)	Signals via pattern recognition receptors (PRR)	Tolerance, immunoregulation, innate immune system regulation. Drive release of *TNFAIP3* (A20)	[28–31]
Data			
Diverse microbial epitopes	Select and expand diverse repertoire of useful lymphocyte clones	Expanded lymphocyte repertoire to recognise novel pathogens	[21, 32, 33]
Microbial epitopes from microbiota transported to thymus	Select lymphocyte clones that recognise gut microbiota	Recognise and manage symbiotic partner organisms	[34]
Pathogens and vaccines			
Infection with pathogens	Death or immunity to the pathogen	Non-specific post-survival benefit after some infections or during persistent infection	[35–38]
Vaccines	Immunity to the pathogen	Non-specific survival benefit Epigenetic adjustments to adaptive immune system	[39–41]
Other inputs			
Phages	Phages	Phage-driven regulation of gut microbiota	[42, 43]
	Antibody to bacteriophage?	Modify phage-driven regulation of strain abundance in gut microbiota	[42]
DNA	Horizontal gene transfer	Adapt gut ecosystem and metabolic repertoire to diet	[44–47]

Note: The table provides examples only, and omits the numerous microbial metabolites, some derived from tryptophan and tyrosine, that also modulate host immune, metabolic and central nervous systems

and a progressive reduction in biodiversity heralds decline in the elderly [22, 49]. Why is this so? It is possible that it is not the diversity per se, which is important, but rather the increased likelihood of the presence of essential species driving, for example, regulatory T cells (Treg) and other types of immunoregulation

[23, 25] or production of short-chain fatty acids (SCFA) [24], or other necessary functions not yet discovered.

Another possibility is the greater stability of complex ecosystems. Ecologists have observed that species diversity buffers against excessive change-induced damage to environmental ecosystems, because the presence of many species increases the probability that these include some that can adapt quickly to the new conditions [50].

A third possibility is that biodiversity inhibits potentially dangerous biofilm formation. The physiology of an organism changes when it switches to biofilm mode, and some organisms become pathogenic as well as resistant to antimicrobials and resistant to the immune system. For example, most pathology caused by *Candida albicans* follows the switch from yeast to hyphal forms that occurs when it makes biofilm [51]. In patients with IBD, gut microbiota can bypass the mucus barrier, and abnormal biofilm is found adherent to the epithelial surface. Organisms from such biofilm can translocate across human intestinal epithelial cell monolayers in vitro, whereas bacteria from the microbiota of healthy donors do not [52]. It is possible that high gut microbiota biodiversity affects the quorum sensing signals, and stops potential pathogens from switching to biofilm.

3.1.2 Signals

At least some of the establishment of immunoregulatory mechanisms is driven by exposure to microbial components that trigger PRR such as TLR4, TLR2, TLR9, or the aryl hydrocarbon receptor (AhR) or PI3K/Akt/mTORC1 signalling systems. More details on this topic will be found in chapter "Regulation of Host Immunity by the Gut Microbiota" of this volume. Briefly, most of the microbial components that drive these immunoregulatory circuits are well-known to trigger inflammation. However this inflammation is often an acute phenomenon, and the eventual result of these exposures, or of repeated low-dose exposures, may be the priming of anti-inflammatory mechanisms. This is at least partly due to the fact that most triggers of inflammation cause release of IL-1β, and this cytokine is able to induce tolerance both to itself and to endotoxin [53]. Thus exposure to these microbial signals is important for informing the immune system about the microbial environment and triggering epigenetic adjustments of the innate immune system (discussed in greater detail in Sect. 5) that include immunoregulatory pathways. Endotoxin (LPS) is a classic example. For example, in an animal model LPS was shown to induce Treg via tolerogenic dendritic cells and TGF-β [54]. In human farmers endotoxin in dust protects against developing allergic responses by inducing A20 in lung epithelial cells [55]. A20, the product of the *TNFAIP3* gene, is a potent inhibitor of the NF-κB signalling pathway, and this and other biomarkers of immunoregulation are increased in Amish farmers using traditional farming methods [28, 29].

Similarly, in mice a TLR2 agonist led to reduced Th17 cells, an increase in splenic type 1 regulatory T cells and attenuation of Experimental Autoimmune Encephalomyelitis (EAE) [56]. The same authors found that patients suffering

from MS had abnormally low circulating levels of a bacterium-derived TLR2 agonist, when compared to healthy donors [56].

TLR9 is an intracellular PRR that detects unmethylated CpG motifs. These are relatively common in microbial genomes, and they usually drive an inflammatory response. However, there may be variants of the CpG motif and of other microbial DNA sequences that have lost their pro-inflammatory effects, or become anti-inflammatory [57, 58], and this seems to be true of many *Lactobacillus* species [59]. This might explain why the probiotic effects of lactobacilli require the presence of TLR9, which is well expressed in the gut and airways [60].

In fact the airways contain a number of cellular sensor systems that can monitor the content of biogenic aerosols in inhaled air, whether derived from plants or from microorganisms. Thus plant polyphenols such as quercetin, resveratrol and curcumin can also exert anti-inflammatory effects via the aryl hydrocarbon receptor (AhR) [61], as can microbial pigments such as phenazines and naphthoquinones, and these have been shown to regulate inflammation and anti-bacterial responses [30]. Similarly, in addition to products of bacteria and fungi, molecules from algae and higher plants can inhibit the activities of protein kinases of the PI3K/Akt/mTORC1 signalling system, and the overall effect is thought to be anti-inflammatory [31].

3.1.3 Data

As explained above in Sect. 3, the immune system needs data in the form of antigenic epitopes in order to enable the process that selects and retains potentially useful lymphocytes from the randomly generated clones. Thus each individual develops a custom-made repertoire based on the antigens present in his or her environment. The diversity of this repertoire needs to be large. All biological entities are built from variants of the same building blocks, often reflecting their microbial origins, so the more diverse the lymphocyte repertoire, the more likely it is to include memory cells that by chance recognise a novel virus, such as HIV [32]. Such T cells are not found in the blood of newborns, and seem to be induced by cross-reactivity of the T cell receptor with environmental antigens.

This extended repertoire of lymphocytes is also needed for controlling and tolerating the gut microbiota. The innate immune system is clearly involved in the "farming" of the microbiota [62]. But as outlined above, evolutionary biologists suggest that the adaptive immune system evolved precisely in order to assist the innate immune system with this task [discussed in [12]]. In mice at least there is evidence that in very early life gut organisms are transported by intestinal dendritic cells to the thymus where they enable selection of T cells that recognise these partner organisms [34]. Thus if dendritic cells (DC) lack MHC Class II so that they cannot activate the T cells of the adaptive immune system, there is rapid and severe gut inflammation (unless the animals are germ-free) that can be mitigated by antibiotic treatment [33]. Clearly the precise farming of the microbiota requires the specificity of the adaptive immune system.

3.1.4 Pathogens and Vaccines

Pathogens and vaccines exert immunomodulatory effects. It is now clear that the common infections of childhood are mostly "Crowd Infections" to which humans were not exposed until relatively recently, when large human populations appeared [63]. Thus we may not be in a state of evolved dependence on these organisms, and it is not surprising that epidemiological studies have shown that these infections do not protect from allergic disorders [64–66]. Nevertheless recent work suggests that some infections, if you survive them, do provide a lasting health benefit [35]. Other infections may persist in a subclinical state and continuously activate or modulate the immune system [36–38]. Finally, it has emerged during the last 20 years that the vaccines that replace these infections also exert significant immunomodulatory effects [39–41]. These important topics are explored in greater detail in Sect. 5.

3.1.5 Phages

Bacteriophages are the most numerous biological entities in the gut, and approximately 90% of the human gut virome consists of virulent bacteriophages predicted to target major taxonomic groups of gut bacteria [67]. Some phages (lytic phages) lyse the bacteria or archaea that they infect, while others (temperate phages) can either trigger lysis, or alternatively, integrate into the host DNA or persist within the host as a plasmid. Either way the phage exerts profound effects on the function and survival of the host organisms. Integrated prophages can express genes that increase the fitness of the bacteria and protect them from infection by lytic phages. They may also supply bacteria with genes that are involved in the metabolism of toxins and polysaccharides, or in antibiotic resistance. Some phages cause changes in the O-antigen component of the LPS of Gram-negative bacteria [42].

So does intake of phages influence our gut microbiota? There are about 10^9 phages/gm of soil [68], and they are also found in drinking water where their presence is used as a test for contamination with human-derived waste. It is suggested that every day ~30 billion bacteriophage particles cross the gut epithelium and enter human tissues [42], and many phages can be identified in human blood. Therefore phage intake from the environment must be massive, and likely to include phages of human gut-adapted bacteria and archaea. These phages could directly modify the composition of the gut microbiota and the effects of the microbiota on the host. For example, the rate of replication of different organisms in the gut is enormously varied [69]. An organism that is present in low numbers because rapid proliferation is counteracted by rapid phage-mediated lysis might be providing more molecular signals to the physiology of the human host than an organism present in greater numbers, with a low replication rate and low turnover.

More evidence of the crucial role of phages may be emerging from the observation that *Clostridioides difficile* infection can be treated using bacteriologically sterile (0.2-μm filter) faecal filtrates [43]. This study did not identify the components

of the sterile filtrate responsible for the cure, but phages must be strong candidates. However small non-encoding RNAs of either host or microbial origin described in Sect. 2.5 are also clear candidates.

Phages also induce an immune response, and phage-neutralising antibodies are commonly found in the blood of humans and other animals. Specific IgA in the gut, but also IgG and IgM can all inactivate phages and decrease the titre of active phages in the faeces [42]. It is therefore possible that not only the phages themselves, but also antibody-mediated disturbances of gut phage populations might be factors involved in some instances of dysbiosis.

3.1.6 DNA

In addition to microbial molecular signals, and colonising organisms, the natural environment can provide microbial genes [44, 45]. For example, enzymes acquired by horizontal gene transfer (HGT) from marine seaweed-associated bacteria enable the gut microbiota of Japanese individuals to metabolise seaweed carbohydrates [44]. The frequency of genes in the human microbiome that appear to have been acquired by HGT is remarkably high [46, 47], and transfer can occur between species that diverged in evolution millions of years ago. The natural environment thus constitutes a resource of genetic diversity for the microbiota and facilitates adaptation to a changing diet [45, 70]. HGT must also help organisms from the natural environment to adapt quickly to the gut, so such environment-derived gut strains might appear to diverge from the environmental precursor [71].

4 Sources of Microbial Inputs

So what are the sources of the microbial inputs that are essential for health and that provide the signals, data and DNA discussed above? These topics, and the changes in our environment and lifestyle that lead to deficient inputs, are dealt with in detail in chapters “Biodiversity, Microbiomes, and Human Health”, “The Development of the Gut Microbiota in Childhood and Its Distortion by Lifestyle Changes”, and “Distortion of the Microbiota of the Natural Environment by Human Activities” of this volume, but certain aspects that are particularly relevant to the development of a framework for reconciling hygiene and essential microbial exposures are outlined below. Notably, the role of the microbiota of the modern home is a complex issue clearly relevant to the “hygiene conundrum”, as is the evidence that exposures to certain infections exert profound effects on our health and immune systems. Finally it seems that much of the health benefit derived from infections, if you survive them, can be replaced more safely by appropriate vaccine schedules.

4.1 Mother and Family

Mother-to-infant (and sibling-to-infant) transfer of microbiota is crucial for the development of the infant's microbiota, as well as for development of the immune and metabolic systems [72]. The major lifestyle factors that reduce this transfer and correlate with increased immunoregulatory disorders are caesarean deliveries, lack of breast feeding and lack of mother-baby intimacy [72–74], together with antibiotic use and poor diet (see chapter "The Development of the Gut Microbiota in Childhood and Its Distortion by Lifestyle Changes" for further detail). Some components of the child's microbiota appear later in infancy and are still accumulating at 5 years of age [75]. These organisms must be picked up from the father and other family members, and from children and personnel at day-care centres as well as from the natural environment. Studies of social networks have demonstrated person-to-person transmission of microbial strains both within and outside the home [76, 77]. These findings suggest that the transfer occurs mostly via normal social and mother-infant interactions, and is reduced by modern lifestyles.

4.2 Microbiota of the Home

Does the microbiota of the modern home provide a necessary microbial input? We can approach this question by considering human evolution. Early humans lived in caves or shelters built with natural products such as stones, mud, branches and leaves. These shelters later evolved into houses constructed with the same natural products reorganised for human convenience. Walls were built with straw, timber, mud or stone and rendered with mixtures of straw, soil, clay and animal dung, while roofs were covered with thatch or turf. The microbiota of such a home would not differ greatly from that of the natural environment, and even when damp and deteriorating, the organisms present would be those with which humans co-evolved. In contrast, modern homes, built with synthetic products including biocide-treated timber, plywood and synthetic gypsum board, develop an unusual microbiota that bears little resemblance to that of the natural environment [78, 79]. This difference is exacerbated if the home is urban and remote from nature [80]. Moreover when a modern home is damp and deteriorating, as homes of low socioeconomic status frequently are, its bacterial and fungal microbiota can produce secondary metabolites that are toxic to humans, resulting in various degrees of "Sick Building Syndrome" [81–83], and a greater risk that children will be hospitalised for respiratory infections [84]. It is therefore unlikely that this unnatural microbiota of the modern home is a necessary, or even a desirable microbial exposure for infants. On the other hand, the microbiota of the home does become beneficial when it resembles that of farms and the natural environment, at least where asthma and other disorders associated with faulty immunoregulation are concerned [85–87]. For example, the peripheral blood cells of children from homes with farm-like

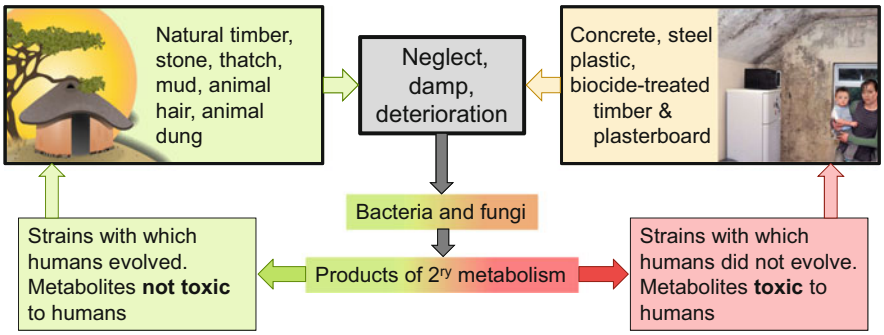

Fig. 2 Microbial hazards of modern compared to traditional homes. A traditional home consists of natural products reorganised for human convenience. When a traditional home deteriorates the microbiota is that of the natural environment in which humans evolved. In contrast, the biocide-treated components of the modern home can develop a microbiota that is toxic to humans

microbiomes released lower levels of inflammatory cytokines in response to bacterial cell wall components in vitro [87] (Fig. 2).

4.3 Natural Environment

The importance to health of exposure to the natural environment has become clear. (See chapters "Biodiversity, Microbiomes, and Human Health", "Distortion of the Microbiota of the Natural Environment by Human Activities", and "Clinical Application of the Biodiversity Hypothesis in the Management of Allergic Disorders" of this volume for further detail.) At least some of the data reveal mechanisms and strongly suggest a role for the microbiota. The three disorder types discussed below show particularly convincing negative associations with exposure to green space.

4.3.1 Immunoregulatory Disorders

In the late nineteenth century, it was noted that farmers were less likely to develop hay fever than were city dwellers [1]. Since then hundreds of epidemiological studies have confirmed that exposure to the farming environment in early life diminishes the risk of allergic disorders [28, 85], while other studies have shown that merely living in proximity to green spaces lowers the risk of allergic sensitisation [88]. Importantly, some of these papers include immunological findings that strongly indicate cause and effect, rather than merely chance association [28, 29, 88]. Biomarkers of immunoregulation were increased in Amish farmers who use traditional methods, and have very low prevalence of allergic disorders, when compared to industrialised Hutterite farmers [28, 29]. Similarly, deliberately exposing children to biodiversity

from the natural environment in their school playgrounds resulted in increases in peripheral blood biomarkers of immunoregulation [89].

While the direct evidence for the protective effect of exposure to the natural environment is weaker for the other chronic inflammatory disorders, there is suggestive evidence for inflammatory bowel diseases [90], and for autoimmune diseases [25].

4.3.2 Metabolic Syndrome, Obesity and Cardiovascular Disease

Metabolic syndrome, obesity and cardiovascular disease constitute another major group of health problems that plague modern humans. A longitudinal study based on four clinical examinations over a 15-year period of 6076 individuals, aged 45–69 years at baseline, who participated in the Whitehall II study revealed a 13% lower risk of metabolic syndrome in people living within 500 m of green space [91]. Such longitudinal data reinforce the classical study of approximately 40×10^6 UK citizens which suggested that living in proximity to green space reduced the risk of cardiovascular disease and prolonged overall survival [92].

4.3.3 Psychiatric Disorders

Psychiatric disorders have also increased in rich urban societies. A study of approximately one million Danish citizens found that living close to high levels of green space during childhood reduced the risk of most mental illnesses later in life. In contrast, for those most deprived of green space during childhood the risk of mental illness was up to 55% higher [93], and similar results for depression and anxiety were reported in a large study in the Netherlands [94]. Clearly there are many possible explanations for this relationship, but evidence suggesting roles for the microbiota in the development, function and pathology of the brain is presented in chapters "The Influence of the Microbiota on Brain Structure and Function: Implications for Stress-Related Neuropsychiatric Disorders" and "Neurodegenerative Diseases and the Gut Microbiota" of this volume.

4.4 Soil as a Source of Microbiota from the Natural Environment

While we are not aware of any data that directly link consumption of soil with health, it is obvious that soil is the source of much of the microbial intake during exposure to the natural environment (see chapters ""Biodiversity, Microbiomes, and Human Health" and "Distortion of the Microbiota of the Natural Environment by Human Activities" of this volume). Soil organisms can enter the air via dust in dry

conditions, but raindrops impacting soil cause tiny explosions of soil organisms to enter the air, so organisms are always present in the air we breathe [95]. They will also settle on the food that we eat, especially in farmers' markets where food has been subjected to little, if any, washing or packaging. But it turns out that soil consumption (geophagy) is an evolved behaviour, and extremely ancient in an evolutionary sense. It is likely that all vertebrates do it, especially in early life, and the green iguana has been much studied in this context [96]. More relevant to humans is the fact that many primate species eat soil, including the closest relatives of humans, gorillas, orangutans and chimpanzees [97, 98]. In the 1990s it was noted that in western Kenya ~70% of 207 schoolchildren aged 5–18 years consumed soil on a daily basis (median ingestion of 28 g/day, range 8–108 g) [99]. This behaviour was more prevalent in girls and continued into adolescence. It has been noted in numerous other cultures, on all continents [98, 99]. Geophagy is also common in pregnancy, not only in undeveloped rural cultures, but also as a manifestation of "pica" in Westernised ones, where it is usually regarded as pathological.

4.4.1 Spores

Soil is an important source of spores, which had been neglected until recently. Spores are remarkably resistant, and can remain viable in the environment for hundreds, perhaps thousands of years [reviewed in 100]. It now seems that about 60% of the bacterial genera in the gut make spores, including some genera not previously thought to do so [101, 102]. They play an essential role by enabling strictly anaerobic organisms essential to human health to be transmitted from one individual to another via the environment [101, 102]. Direct transmission of these organisms via the oxygen-rich air would be inefficient, and spore-forming organisms are likely to be among the components of the child's microbiota that appear later in infancy and are still accumulating at 5 years of age [75]. Many spore-forming members of the microbiota are derived from the environment, presumably as spores, rather than via direct contact with mother [102]. They are crucial because many of them drive formation of SCFA which have numerous essential physiological roles [103], and expansion of Treg populations [25, 104].

Human faeces contain up to 10^4 spores/g while soil contains approximately 10^6 spores/g [105, 106], so wherever humans have lived the environment is inevitably seeded with human gut-adapted bacterial strains. Therefore, it is possible that when a spore-forming gut organism becomes extinct as a result of dietary inadequacy or antibiotic misuse [107], it can be "reinstalled" via spores from the environment. This raises an interesting issue. We humans have distorted the spore content of the environment in which we live. At least 90% of mammal biomass is now human or domesticated livestock, while at least 70% of all bird biomass is now domesticated poultry [108]. Livestock and pets distribute about 14×10^{12} kg/year of faeces into our environment, while humans contribute only about 1×10^{12} kg/year [109]. The human output is relatively small, and in the modern world it is no longer deposited in the spaces in which we live, but rather is flushed away to treatment

plants. We need to consider the possibility that the human environment in rich developed countries now contains few human-gut-adapted strains, so that we might be tending to be colonised with strains better adapted to other species.

We also need to be aware that many other soil-derived spore-forming organisms such as *Bacillus* spp. can germinate and replicate in the intestinal tracts of insects and other animals [100]. There is therefore a growing view that *B. subtilis* and other environmental spore-forming species should be regarded as gut commensals rather than soil microorganisms [106]. For example, *B. subtilis* is an important stimulus for development of the gut-associated lymphoid tissue (GALT) in rabbits and sporulation of live bacilli within the GALT was considered critical to this process [110].

4.5 Low-Dose Infection Via the Airways

The microbial diversity of air is comparable to that of seawater, soil and the human gut, but in a recent study only 9% to 17% of the sequences were identifiable [111]. At least some of the organisms in respired air will be potential pathogens. However the dose will usually be very low, and will drive an immune response rather than disease. This is likely to be one of the ways in which microbial exposures are beneficial to health. The nasal mucosa responds to LPS, acting via TLR4, by releasing exosomes containing inducible nitric oxide synthase. These exosomes transfer the enzyme to neighbouring epithelial cells which increase release of nitric oxide [112]. Bacterial attachment also leads to release of cathelicidin (also known as LL-37) which is taken up by infected cells. Cathelicidin is one of well over 100 human antimicrobial peptides (AMP) that also include defensins, β-defensins, lysozyme, lactoferrin, secretory leukocyte proteinase inhibitor, elafin and RNase 7 [26]. Entry of bacteria and cathelicidin into the host cell triggers activation of the NLRP3 inflammasome and a cascade of events including the activation of caspase 1, death of some infected cells and release of the pro-inflammatory cytokines IL-1β and IL-18. These events enhance inflammation and recruit neutrophils. Under the influence of cathelicidin and other AMP, the neutrophils form networks of extracellular fibres consisting mainly of DNA to which some AMPs such as neutrophil elastase and cathepsin G adhere. These AMP-armed Neutrophil Extracellular Traps (NET) contribute to inactivation of microorganisms [26]. Thus these mechanisms in airways kill or disarm the respired organisms which are then taken in by the lymphoid tissue of Waldeyer's ring, or exposed to acid in the stomach before being sampled by the dendritic cells in the small bowel [27]. Thus inspired low doses of pathogens may provide useful data to the immune system, including the priming of immunity to potential pathogens encountered at low sub-infectious doses in respired air.

5 "Beneficial" Infections and Vaccines

It has been known for decades that infection with one pathogen can protect from another unrelated infection. This can be due to persistence of the infection, leading to a continuous or intermittent stimulus to the immune system, or to epigenetic changes discussed later, or both. For example, by studying herds of cattle, and experiments with guinea pigs, Pullinger showed in 1936 that *M. tuberculosis* conferred resistance to *Brucella abortus* and suggested that the phenomenon was attributable to activated monocytes [113]. It was then confirmed that immunising rabbits with either BCG or a mutant *Brucella* strain caused the monocytes from the immunised animals to become resistant to both *M. tuberculosis* and virulent *Brucella melitensis* [114]. Subsequent workers amplified this concept by showing cross-protection between unrelated parasite species, between bacteria and parasites or between *Listeria monocytogenes* and influenza virus [115].

5.1 Non-Specific Benefits of Persistent Infections

Some infections confer non-specific health benefits by persisting and continuously or intermittently activating the immune system. Three examples are given below.

5.1.1 Herpes Viruses

Several types of herpes virus are extremely widespread among humans. More than 90% of adults have been infected with at least one of the five commonest species (HSV-1, HSV-2, varicella zoster, Epstein–Barr virus, cytomegalovirus). Herpesviruses tend to remain latent, and periodic reactivation can influence the state of the immune system. Mice latently infected with either murine gammaherpesvirus 68 or murine cytomegalovirus were found to be resistant to infection with the bacterial pathogens, *Listeria monocytogenes* and *Yersinia pestis* [36]. This is likely to be due to intermittent reactivation and to consequent cytokine-mediated activation of macrophages. It seems probable that a similar phenomenon happens in humans, but this does not seem to have been investigated.

5.1.2 *Mycobacterium tuberculosis*

M. tuberculosis is another organism that might exert such effects. Latent tuberculosis infection (LTBI) is extremely common in developing countries, and the persistent presence of the organisms in multiple tissues has been demonstrated by in situ PCR [116]. There is a small risk of developing clinical tuberculosis. Interestingly, treating latent tuberculosis in non-HIV-infected individuals reduces the incidence of

tuberculosis, but fails to provide an overall survival benefit because of increased mortality from other causes [38]. Similarly the Bacillus of Calmette and Guérin (BCG), an attenuated live vaccine derived from a mycobacterium, also has non-specific immunomodulatory effects, discussed below in Sect. 5.2.

5.1.3 *Helicobacter pylori*

Helicobacter pylori is another persistent infection that some consider beneficial because it might suppress allergies. Epidemiological surveys revealed an inverse relationship between *H. pylori* seropositivity, and childhood asthma [37]. The authors pointed out that *H. pylori* has been carried by humans throughout much of our evolutionary past but that antibiotic use has caused *H. pylori* seroprevalence to fall below 10% in native-born citizens of Western urbanised countries. Experiments in mice confirm this allergy-blocking effect and suggest that it is mediated via the expansion of Treg subsets expressing CXCR3 or RORγt, and demethylation of the FOXP3 locus [117].

As will be discussed below, it is now clear that vaccines can also exert non-specific protective effects that may be able to replace many of the immune system modulatory effects of infections, so this topic is now very relevant to a discussion of which microorganisms we need to be exposed to in the modern world.

5.2 *Non-Specific Effects of Vaccines*

So infections, if you survive them, may provide non-specific health benefits. However vaccines may be able to replace some of the beneficial non-specific effects of infections without subjecting the individual to the risk of disease. In the 1980s it began to be reported that vaccination with a live measles vaccine in Africa reduced overall childhood mortality to a degree that could not be explained by the incidence of measles itself. By the early 2000s the same claim was being made for BCG vaccination, and multiple repeat studies have led to the conclusion that several live vaccines (measles, polio, smallpox, BCG) enhance resistance to unrelated infections, particularly when given early in the presence of maternal antibody [39, 40]. A number of epidemiological studies had added weight to these observations, culminating in a recent clinical trial in which it was shown that BCG administered to individuals with a mean age of ~80 years was able to protect them from respiratory infections [118]. (This observation appears compatible with the suggestion made above that latent tuberculosis provides a non-specific survival benefit [38].)

5.2.1 Trained Immunity

Recent work has confirmed that non-specific and cross-protective effects such as those outlined above can be mediated by several components of the innate immune system including natural killer (NK) cells and above all, by monocytes [41], as suggested in the 1930s [113]. This phenomenon is now often called "Trained Immunity" [41, 119]. But monocytes are relatively short-lived cells, so why do these effects last for months or years, as clearly shown by the epidemiology? Moreover what can explain the importance of the sequence of exposures that is discussed below in Sect. 5.2.2? The answers to these questions lie in the epigenetic mechanisms underlying Trained Immunity that operate not only at the level of monocytes, but also at the level of the haemopoietic stem cells that generate the monocytes [120]. Epigenetic mechanisms will not be reviewed in full here, but briefly, DNA is coiled round histone octamers to form nucleosomes. This complex of DNA and the associated histones is known as chromatin. The accessibility of different sections of the DNA in the chromatin can be modified by methylations and demethylations of the DNA itself, and by methylation or acetylation of the histones. These changes in the accessibility of the DNA modify both the possibility that the genes in any given stretch of DNA will be transcribed, and also the likelihood that any part of DNA/histone complex can undergo further modifications of their methylation or acetylation state. These mechanisms occurring at the level of haemopoietic stem cells can explain the long duration of trained immunity, and also the relevance of the sequence of exposures. For example, if the first exposure causes modifications of the chromatin that "hide" the chromatin that would otherwise have been modified by exposure to a second stimulus, then the effect of exposure to the second stimulus will not be the same as it would have been if it had been the first stimulus. A remarkably dramatic example of this is the experimental observation that a first exposure to LPS + IFNγ greatly enhances the cytokine response to a second stimulus of LPS alone. But if the sequence is reversed so that the first stimulus is LPS alone, a subsequent exposure to LPS + IFNγ yields a greatly reduced response [120]. This may be relevant to the issue of the timing of infections in childhood, and also to the order in which vaccinations are given.

5.2.2 Sequence of Exposures to Infections and Vaccines Can be Important

As outlined in the previous section, work on non-specific effects of vaccines has revealed that the sequence of exposure to different vaccines can be crucial. The non-specific effects of non-live vaccines are variable, and can oppose the non-specific survival benefit of live ones, particularly in females, and if given after the last live vaccine [40], suggesting that the sequence determines that nature of the epigenetic modifications. This phenomenon, probably attributable to the way in

which epigenetic modifications are applied, as explained above in Sect. 5.2.1, may be equally relevant to infections [120], though other explanations are possible.

It is suggested that delayed infection with an agent, perhaps a virus, that would in our evolutionary past have been encountered very early in childhood, plays a role in the aetiology of acute lymphatic leukaemia (ALL). A recent "two hit" hypothesis suggests that a pre-leukaemic B lymphocyte clone develops in utero by fusion gene formation or hyperdiploidy, and that a subsequent dysregulated immune response to an infection drives further mutations leading to leukaemia [121]. It may be relevant that ALL is more common in situations where exposure to microorganisms in early life is reduced (caesarean delivery, lack of breast feeding and lack of older siblings). This is reminiscent of the early life factors that are associated with an increased risk of immunoregulatory disorders such as allergies [72, 74]. Interestingly, immunisation against *Haemophilus influenzae* type B in infancy appears to protect against ALL. Thus it is suggested that ALL is an example of the consequences of delayed infection with something that would, during our evolutionary past, have occurred very soon after birth.

A similar idea involving delayed exposure to rotavirus has been proposed to explain the increase in type 1 diabetes (T1D) that accompanied the development of the modern Western lifestyle, and the subsequent fall in prevalence after the introduction of vaccines of which the first dose was given at 2 months [122]. Examination of the sequence of the virus suggests the possibility of molecular mimicry with islet autoantigens. It had been observed that in mice rotavirus infection in the neonatal period prevents diabetes whereas infection at weaning accelerates it [Reviewed in [122]].

6 Link to Low SES

It is well-known that health and life expectancy are closely linked to socioeconomic factors [123]. Interestingly many aspects of life at the lower end of the socioeconomic spectrum are known to lead to distorted or deficient microbiota, and some of these are listed in Table 2. This raises the possibility that at least some of the health deficit in low SES populations is mediated via changes in the microbiome. Some of the factors that contribute to this SES-linked defective microbiota were described in Sect. 4.2 in relation to the abnormal microbiota of damp crowded, poorly constructed modern homes, and the increased risks of immunoregulatory, metabolic, cardiovascular and psychiatric disorders that appear in populations deprived of exposure to green space (Sect. 4.3). However Table 2 lists several other microbiome-changing SES-associated factors.

Table 2 Low socioeconomic status (SES) and distortion of microbial exposures

SES-associated problem		Effects	References
Pollution	Traffic, air pollution	Direct effects on microbiota, and indirect effects via host immune system and damaged epithelia	[8, 124, 125]
	Agrochemicals		
	Damp, sick-building	Toxic microbial secondary metabolites	[81, 82, 84, 126]
	Exposure to cleaning and hygiene products	Th2 adjuvant effects	[127–129]
Lack of green space	Little exposure to strains and spores from nature	Low biodiversity of microbiota, and reduced immunoregulation-driving strains Increased psychiatric disorders	[89, 93, 130]
	Less sunlight, vitamin D	Defective immunoregulation, altered microbiota	[131, 132]
Stressors	Drug abuse, violence, heat, noise, sleep disorders	Changes to microbiota and reduced biodiversity via signals within the gut-brain axis	[133–136]
Poor diet	Processed, unvaried	Low biodiversity of microbiota	[137]
	Low fibre	Low SCFA including butyrate and others	[103]
	Low vitamins	Potential deficiencies	[138]
	Obesity	Metabolic problems	[139]
	Sugars, artificial sweeteners	Distorted microbiota. Raised glycaemic response	[140]
Education	Smoking	Switch from aerobes to anaerobes, more biofilm and *Clostridioides difficile*	[141]
	Antibiotic misuse	Exposure in utero or in early life correlates with metabolic and immunoregulatory problems	[142]
	Vaccine hesitancy and refusal	Infection risk and lack of beneficial non-specific vaccine effects	[143]
	Cleaning products	Probable Th2 adjuvant effect	[144]

6.1 Pollution: Traffic and Agrochemicals

Metabolic dysfunction and type 2 diabetes are increased in populations exposed to air pollution from traffic [124]. However these metabolic disturbances are also associated with abnormal gut microbiota [145], suggesting that pollution might damage health at least partly via the microbiota. Many pollutant chemicals with anti-bacterial properties and the ability to alter microbiota can be detected in the blood or urine of most people [146, 147]. For example, glyphosate, which was initially patented as an anti-microbial [148], was detected in 93% of a cohort of pregnant women in the USA [149]. More importantly, polycyclic aromatic hydrocarbons (PAH derived from coal, crude oil, vehicle exhaust, cigarette and wood smoke and fumes from asphalt roads) accumulate in urban soils where concentrations can be 10–100 times higher than in unpolluted rural soils [150]. The microbiota of such soils is markedly altered [151]. Some reports have concluded that exposure

to air pollution can lead to changes in the gut microbiota potentially relevant to metabolic dysfunction and type 2 diabetes [124, 125]. Another example is the use of un-purified reclaimed waste water to irrigate parks in China. The levels of antibiotics in this water were sufficient to modify the soil microbiota [152].

6.2 Diet

6.2.1 Artificial Sweeteners

Non-caloric artificial sweeteners have direct effects on murine gut microbiota in vitro, and the modified microbial community causes glucose intolerance when transferred into germ-free mice [140]. Similarly, saccharin consumption induced changes in the microbiome of a subset of human volunteers who also developed an elevated glycaemic response. This glycaemic response was replicated in germ-free mice that received transplants of microbiota from these individuals [140].

6.2.2 Fructose

There has been a 100-fold increase in the consumption of fructose during the last century, particularly in fruit juices. Excessive consumption is linked to non-alcoholic fatty liver disease, obesity and diabetes [153]. The microbiota of the small intestine metabolises fructose, and so blocks uptake [153], but if the small gut is overloaded, fructose enters the colon and distorts the microbiota [154]. The accompanying metabolic disturbances can be corrected by antibiotics or faecal transplantation in rat models [155].

6.2.3 Vitamin D

Many authors consider that low vitamin D levels may predispose to systemic autoimmune conditions. We know that vitamin D promotes Tregs, inhibits differentiation of Th1 and Th17 cells and reduces activation of monocytes [132]. Vitamin D3 signalling also modulates epithelial tight junctions. Effects on the immune system and on epithelial permeability would be expected to impact the microbiota. Small human studies in patients with Crohn's disease, ulcerative colitis or MS have noted that vitamin D3 supplementation induces significant but inconsistent changes in the gut microbiome [132]. However a suggestive pilot study performed in Vancouver, Canada, during the season when Ultraviolet B (UVB) radiation is essentially absent, found that whole body skin exposure to Narrow Band UVB caused enrichment of several microbial families in the microbiota in women who had not been taking vitamin D supplements [131]. Notably there was enrichment of Clostridia known to promote induction of Treg. Other authors noted that the changes seen resulted in an

increased similarity to the microbiomes of the Yanomami, a people living in the Amazon forests with little or no clothing [156].

6.3 *Stress*

Stress alters the microbiota of experimental animals [157], and the same is true of the microbiota of severely stressed critically ill humans, where the changes are rapid and prolonged [158]. Stress also affects the development of the microbiota in early life. In a rat model the stress of maternal separation in the neonatal period had long-term effects on the diversity of the microbiota that were still apparent when the pups became adults [159]. Several mechanisms may be involved. Stress causes changes in signalling via the vagus nerve and the enteric nervous system that alter gut mobility and function and, together with stress-induced circulatory changes that redirect blood away from the gut, these mechanisms result in an altered microbiota [160]. Moreover, some of the mediators such as catecholamines released systemically or by cells within the gut as part of the stress response have direct effects on microbial growth [161]. Stress also impacts the immune system and drives inflammatory responses that alter the "farming" of the microbiota [162]. Some external stressors that are particularly associated with low SES are described below.

6.3.1 Noise

A recent study of 504,271 participants from the UK, the Netherlands and Norway found associations between local road traffic noise levels and several markers of obesity [136]. It was difficult to disentangle the role of the noise itself from other factors such as PM2.5 pollution and the area-level markers of low SES, but the finding reinforces the concept of health deficits linked to low SES [136].

6.3.2 Heat

A major review published in 2020 suggested that excessive heat was responsible for 296,000 deaths globally in 2018, accompanied by large reductions in gross national incomes, particularly in India and Indonesia but also in Europe [133]. There are also correlations between even small rises in temperature and increased hospitalisations, the effect size being inversely related to the income level of the cities studied [163]. Similarly, high temperatures during pregnancy are associated with increased risks of preterm birth, low birth weight and stillbirths, especially in low SES contexts [164]. It is clear that individuals of low SES are less likely to benefit from air conditioning, and may also suffer from the heat output from the air conditioners of their wealthier neighbours. In the context of this review, the question is whether any of these detrimental effects on health could have been mediated via changes in the

microbiota. Interestingly, heat stress reduced the biodiversity of the faecal microbiota of cows [165], and even mild heat stress, within the temperature ranges commonly found in poultry facilities, reduced the biodiversity and altered the composition of the microbiota within the cecum and airways of chickens [166]. Could similar effects occur in humans? At this stage we do not know, and it will be extremely difficult to disentangle the effects of heat from the effects of the psychological stress resulting from discomfort.

6.3.3 Sleep Disorders

In humans and in laboratory rodents, sleep disturbance is associated with altered microbiota, including decreased abundance of SCFA-producing strains (discussed in [167]). Recent experiments using faecal microbiota transplantation (FMT) have demonstrated that this altered microbiota plays a central role, and can induce sleep disturbances in recipient rodents [167]. A recent study in the USA has revealed that the prevalence of sleep disturbances such as very short or long sleep correlated with SES in both African Americans and whites [135].

6.4 Smoking

Smoking is increasingly associated with low education and low SES, and the links to cardiovascular disease, periodontitis, chronic obstructive pulmonary diseases (COPD), Crohn's disease and various cancers are well-known [141]. Smoking is also associated with autoimmune disorders such as MS and rheumatoid arthritis, suggesting compromised immunoregulation. Moreover a recent study that used magnetic resonance imaging of the brain confirmed that low SES is associated with a lower brain volume and increased risk of dementia, and identified smoking as the major causal factor [168]. Are some of these effects mediated via changes in the microbiota? Smoking causes clear changes in the oral, nasopharyngeal, airway and gut microbiotas [141]. The oral microbiota of smokers shows a switch from aerobes to strict or facultative anaerobes, and although the microbiome remains diverse it contains more potential pathogens and fewer commensals. These changes also influence the input of microbiota to the gut and airways. Smoking increases the development of biofilm which enhances pathogenicity of many organisms and protects them from defences and antibiotics (see Sect. 3.1.1). Smoking increases the number of phagocytic cells in the airways, alters macrophage and neutrophil function and decreases the number of dendritic cells.

In the gut smoking increases the risk of *Clostridioides difficile* infection, indicating a disturbance of the gut microbial community. The faecal microbiome of smokers is reported to contain increased Bacteroidetes and decreased Firmicutes and Proteobacteria. A loss of Treg-inducing Clostridia has been implicated in susceptibility to MS [25], for which smoking is a risk factor. Smoking also decreases

the abundance of butyrate-producing bifidobacteria that have important anti-inflammatory and anti-cancer roles [103].

Some of these smoking-induced changes in the microbiota could be direct effects on microorganisms of chemicals in smoke, but smoking also has profound effects on both the innate and adaptive immune systems [169], some of which are mediated by epigenetic pathways [170]. I will not review this topic here.

6.5 Sex Hormones and Abnormal Development

Sex hormones are conjugated with sulphate or glucuronide in the liver and secreted into the gut. To be reabsorbed efficiently they must be deconjugated by the gut microbiota, which can also make functionally significant alterations to the steroid [171]. Antibiotics therefore cause a striking reduction in reabsorption, and a large increase in the levels of conjugated steroids in the faeces [171]. So the gut microbiota can regulate the levels and the nature of circulating sex steroids. In the NOD mouse model of type 1 diabetes microbial exposures in early life modulate sex hormone levels and modify progression to autoimmunity [172]. In post-menopausal women the biodiversity of the gut microbiota influences the levels of sex hormone metabolites that are relevant to the risk of breast cancer [173]. In the USA black and Hispanic girls undergo menarche earlier than white girls. Much of this effect appears to be related to low SES [174]. Similarly, earlier appearance of some secondary sexual characteristics was recorded in a cohort of German children of low SES [175]. But early puberty is associated with increased risk of breast cancer [176], so modulation of sex steroids by the microbiota of low SES children might hold the key to this association.

Table 2 also lists three other factors associated with low SES that would be expected to impact the composition of the microbiota, including antibiotic misuse [142], vaccine hesitancy or refusal [143] and overuse of toxic cleaning agents in the confined spaces of small homes [144]. The last point is considered in greater detail below.

6.6 Th2 Adjuvant Effects of Products Used to Clean the Home

It has been known for some years that repeated exposure to cleaning agents as experienced every working day by cleaning personnel, particularly when used as sprays, has detrimental effects on the lungs [177]. Many of these cleaning products are not only toxic to cells [127] but also increase epithelial permeability [128]. Interestingly this notion that local cell damage and increased epithelial permeability might activate the immune system has been suggested in relation to both airway

and gut allergies [127, 178]. For example, antigens in food usually evoke tolerance, but if detected by the immune system in the gut in the context of a cytotoxin, an allergic Th2 response may be generated [127]. The food antigen then becomes a proxy for recognition of the cytotoxic molecule (which might not itself be immunogenic), and will evoke an allergic reaction in the future even if the cytotoxin is not present. It seems possible that something like this occurs in some homes, particularly in low SES contexts, where children in confined spaces are exposed to aerosols, or crawling on floors recently treated with potentially toxic cleaning products. In a UK cohort where personal hygiene was associated with wheeze and atopic eczema [179], it also emerged that intense use of chemical household products was inversely associated with socioeconomic status and correlated with low educational level, smoking and poor, crowded housing [144].

A recent study of eight different commercially available adjuvants has confirmed that mild cytotoxicity can lead to Th2 adjuvant properties. These adjuvants were combined with an influenza vaccine and administered to mice by intranasal injection [180]. Evidence of host cell death in the airways was then sought by measuring levels of double-stranded DNA in bronchoalveolar lavage within 24 h of this challenge. Interestingly, 3 of the vaccines tested (Alum, AddaVax [an oil in water emulsion] and SiO2 nanoparticles) caused release of host DNA and elicited potent Th2 responses but little or no Th1 [180]. Previous work had shown that DNA released by cell death in response to aluminium adjuvant enhances MHC Class II mediated antigen presentation and prolongs interaction of dendritic cells with CD4 T cells [181], suggesting that local cytotoxicity initiated by the adjuvant and release of DNA are an integral part of the Th2 adjuvant's mode of action.

Therefore many of the huge numbers of publications stating that personal hygiene or cleaning the home either do enhance the incidence of allergic disorders [179], or do not do so [182], might be in conflict because two unrelated effects were involved. In some settings cleaning the home could conceivably reduce exposure to essential microorganisms, but there might also have been a Th2-adjuvant effect of exposure to toxic aerosols derived from cleaning agents.

7 Resolving the Hygiene Paradox

The issues described above allow us to suggest a framework for reconciling the need for microbial exposures with the equally important need for hygiene to protect us from pathogens [129]. This framework is outlined in Fig. 3.

In the preceding sections it is made clear that we need exposure to the microbiota of our mothers and other family members, and also to the microbiota and spores of soil and the natural environment. On the other hand we probably do not need exposure to the microbiota of the modern home unless it resembles that of the natural environment (Sect. 4.2) so there is no reason not to keep the home clean and hygienic, particularly when the home is of low SES and might harbour a toxic microbiota.

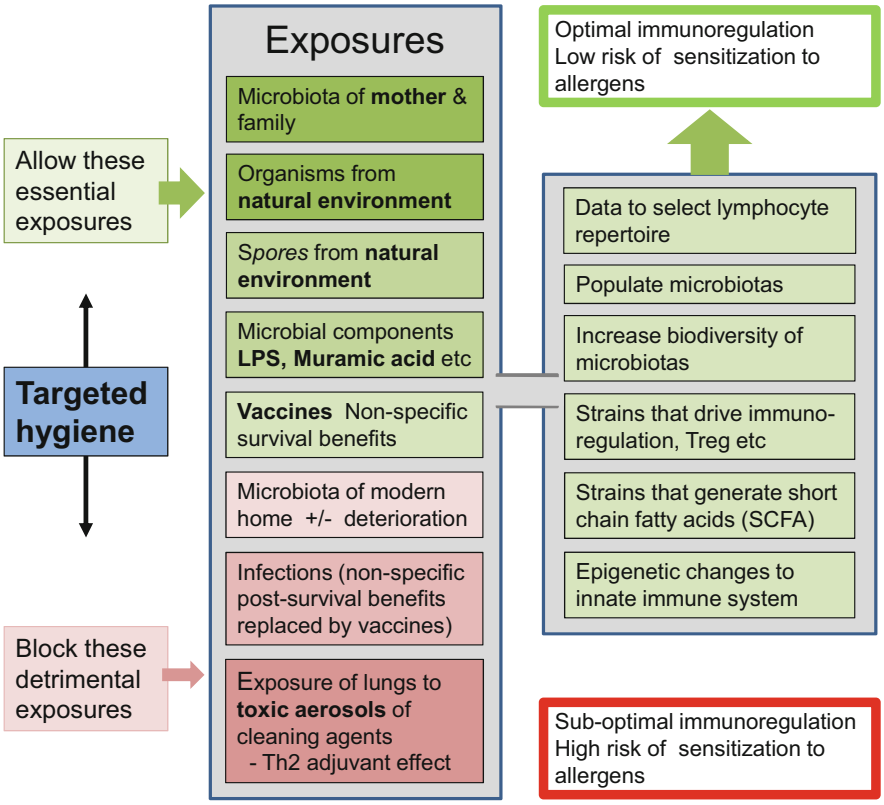

Fig. 3 Essential microbial exposures, and non-essential or detrimental ones. The essential exposures include the microbiota of mothers, family and the natural environment. Some of the benefits derive from molecular signals rather than colonisation. The non-specific immune system-training benefits of infections (if you survive them) can be replaced by vaccines. Targeted hygiene can maintain the essential exposures while protecting from pathogens

Another aspect of the framework outlined here is the shielding of children from aerosols of cleaning agents. This might be considered a part of the concept of "Targeted Hygiene" which suggests that hygiene practices concentrate on the places and moments when disease transmission is most likely, mostly hands and surfaces frequently touched by hands [129, 183, 184]. Targeting large surfaces such as walls and floors is unimportant because they rarely transmit pathogens, and such "hygiene theatre" (useless interventions intended to convince the public that the government is doing something useful) merely increases exposure of the residents, particularly small children, to toxic aerosols. Indeed, at least some of the reports of increased prevalence of allergic disorders in children from homes subjected to intense cleaning are likely to be misleading because they neglect the role of Th2-adjuvanticity of cleaning agents.

Similarly we do not need to take the risk of experiencing diseases caused by pathogens, because the non-specific health benefits derived from such pathogens, if you survive them, can be mimicked by receiving the appropriate vaccines. (It is worth noting that these revelations about the non-specific health benefits of vaccines show that the "anti-vaccine" lobby is even more dramatically wrong than we previously knew). The study of Trained Immunity and of non-specific benefits of vaccines is in its infancy, and we are a long way from knowing precisely how to optimise the timing or sequence in which the vaccines are given, so that we simultaneously maximise both protection from pathogens, and the non-specific survival benefits. It is clear that the sequence of vaccinations is crucial.

Finally, many aspects of the lifestyles and environments of low SES citizens have detrimental effects on essential microbial exposures, and on the composition of the microbiota. Therefore this constitutes yet another reason for demanding that social inequality be reduced.

8 Conclusions

There is no doubt that many of the health problems that increase in rich urban societies are at least partly a consequence of failing microbial exposures, because organisms, data, signals and DNA from these microorganisms are required for the establishment and function of the immune and metabolic systems. An evolutionary approach helps us to identify the necessary exposures and the factors that reduce or distort them. It is striking that many of these factors are closely linked to low SES, suggesting that social reform could lighten much of the burden of illness associated with lack of the essential microbial exposures. Finally, we provide a framework that might enable us to reconcile the need for essential microbial exposures with the equally important need for domestic cleaning and personal hygiene to protect us from pathogens.

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Biodiversity, Microbiomes, and Human Health

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Abstract Humans have evolved in a microbe-rich environment and have become dependent on some of these microbes to colonise us, provide essential chemicals, and prime our immune systems. In many urbanised, western countries, there has been a loss of contact with these biodiverse environmental microbiota, which might be associated with the increased burden from diseases such as asthma, allergies, and autoimmune disorders. Here we summarise our growing understanding of the relationship between exposure to biodiverse environmental microbiota and their potential to provide health benefits. We start by covering the known range of health outcomes associated with green space exposure and then explore the possibility that these benefits are mediated by microbes. We provide evidence to support the notion that environmental microbiota influence the human microbiota and that this in turn leads to a range of health effects. The evidence is strong enough to recommend biodiverse green space exposure both as a clinical and as a public health intervention and discuss what types of environments might be most suitable to recommend. To maximise potential health benefits, we need to improve both the quantity and quality of green spaces and ensure that these are accessible to those communities that stand to benefit most.

Keywords Microbiome · Health · Immune disease · Biodiversity · Green space · Urbanisation · Public health · Environment · Accessibility

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1 Introduction

During human evolution, we were and remain totally dependent on our environment—including the microbiota (collection of microbes [1]) therein—a symbiotic relationship that is also relevant today. We are inextricably linked to our symbiotic microbiota, but also to the environmental microbiota that prime our immune system, and colonise us. Some of these microbes have co-evolved with humans to become 'Old Friends', developing commensal relationships with us, some colonising our bodies, and others priming our immune systems with their antigens, other molecular signals (e.g. lipopolysaccharides, muramic acid), and metabolites (e.g. short-chain fatty acids such as butyrate) [2]. However, that relatively stable microbial exposure has changed dramatically over the last 10,000 years, with the development of agriculture and animal domestication; not only was there a significant reduction in biodiversity exposure, but there was a dramatic increase in regular and close association with animals and their faeces, resulting in 'host jumping' of pathogens—the transfer of one animal's pathogen to a different host—and its subsequent adaptation to completing its reproductive cycle in that new host. The emergence of numerous human infectious diseases (e.g. smallpox from the cowpox of bovines) is the result of such host jumping. The environmental microbiota to which we were exposed changed again with urbanisation and industrialisation, moving us one further step away from exposure to naturally biodiverse environments and opening the door to yet another set of emerging pathogens that thrived on high human population densities (e.g. tuberculosis, diphtheria). To combat this onslaught of emerging infectious diseases, we did what humans do best—we used tools: houses, sewerage, water chlorination, antibiotics, and vaccination. In developed countries, that is where we now are today; with some notable exceptions (e.g. epidemics of human immunodeficiency virus, Ebola virus, and severe acute respiratory syndrome *coronavirus* 2), the disease burden from many of these important pathogens has been removed—however, so have the 'Old Friends', and it is only in the last few years that the adverse health effects of the loss of this environmental microbiome exposure has begun to be understood. The 'biodiversity hypothesis' [3] holds that it is this loss of contact with the biodiverse environmental microbiota with which we evolved, that has resulted in the dramatic increase in asthma, allergies, and autoimmune disorders.

In this chapter, we summarise the state of our growing understanding that healthy ecosystems have more biodiverse microbial communities with a higher relative abundance of microbes that are beneficial for human health, and how contact with healthy environmental microbes changes the human microbiota. The links between these changes in the human microbiome and the associated psychoneuroimmunoendocrinological sequelae for health are covered in other chapters, so we focus here more on public health than pathophysiology.

2 Green Space and Health

Green space exposure is associated with a wide range of health and cognitive outcomes (Box 1), including a positive effect on birth outcomes [4–7], atopic dermatitis [7, 8], all-cause mortality [9, 10], mental health [11, 12], and life satisfaction [13], and potentially chronic pain [14]. Importantly, some results differ between different sub-populations [7, 8], and by the quality of the green space [8, 15]. For example, green space exposure only reduced atopy in children and adolescents in the 6–12 and 13–20 years age groups, not those under 6 years of age [8]. Similarly, the benefits to physiological health appear stronger for children of mothers with lower education levels, and the association between green space exposure and more positive birth outcomes is more prominent for children of mothers of lower education levels and socioeconomic status [7]—suggesting that improving green space exposure for more vulnerable populations may assist in reducing both disease burden and health inequities.

Box 1: Positive Health Associations with Green Space Exposure

Birth outcomes

- Head circumference at birth [7].
- Birth weight [4–7].
- Pre-term births [6, 7].
- Small for gestational age [4, 6, 7].

Allergies and asthma

- Atopic dermatitis [7, 8].
- Wheezing and other respiratory conditions (except where exposed to certain pollens) [7].

Metabolic conditions and cardiovascular disease

- Being overweight/obese [7, 15, 16].
- Diabetes mellitus type 2 [16].
- Cardiovascular disease [9].

Mortality

- Stroke-specific mortality [9].
- All-cause mortality [9, 10].

Cognitive outcomes

- Attentiveness [7, 17].
- Memory [7, 17].

(continued)

Box 1 (continued)
- School performance [17].
- Cognitive development [17].

Sleep

- Sleep [18].

Mental health

- Emotional wellbeing [7].
- Mental health [11, 12].
- Anxiety [19].
- Depressed mood [20].
- Stress [19, 21–23].

The mechanisms linking green space exposure and human health are poorly understood, but the human health benefits of green space exposure are often attributed to increased opportunity for physical and outdoor social activities, and increased exposure to the sights and sounds of nature, negative air ions, sunlight, phytoncides, and the environmental microbiome [24]. But, healthy, functioning ecosystems within green spaces also bring a range of other ecosystem services [25], including reduced exposure to heat [26], noise [26], light [27], and air pollution [26], thus improving health outcome. In this chapter, we focus on the environmental microbiome as a potential linkage mechanism between green space exposure and human health outcomes.

3 Environmental Microbiome–Human Microbiome

The human microbiome has been associated with a wide range of health conditions, many of which are discussed in other chapters of this book. But where do our resident microbiota come from? The microbiota is strongly influenced by birth mode, diet, antibiotic use, age, and the local environment [28, 29]. Local environments—including green spaces—contain rich sources of microbiota that can and do colonise us, which is the focus of the remainder of this chapter.

It is well established that pathogenic environmental microbiota colonise humans, but the role of exposure to environmental microbiota in the development and maintenance of a ‘healthy’ human microbiota is less well understood. Historically, our understanding of microbes was reliant on cell-based methods (i.e. microscopy and culture-based methods) which were limited to those taxa that were culturable [30]. With the development of new methods which utilise DNA (i.e. metabarcoding and metagenomics) and RNA (metatranscriptomics), culture-independent assessment of the microbiota could be undertaken. New methods that characterise proteins

(i.e. metaproteomics) and metabolites (i.e. metabolomics) are now allowing us not only to determine the characteristics of the microbiome and its potential functions, but also its active metabolic pathways [30]. With these technological advances, new research questions can be addressed regarding the influence of microbiota on human, animal and ecosystem health, particularly as analytic costs decrease and the requisite laboratory and bioinformatics skills become more commonplace.

In this section, we follow the development of the evidence-base regarding the transfer of microbiota from the environment to human hosts, focusing on observational human studies, and experimental animal and human experimental studies.

3.1 Observational Human Studies

We know that different environments support the presence of different microbiota. For instance, differences in environmental microbiota have been reported across ecosystem restoration [31, 32] and vegetation biodiversity gradients [33], within households across urbanisation gradients [34, 35], between areas with different types of vegetation [36], agricultural and urban houses [37, 38], the houses of the Amish (who engage in non-mechanised farming) and Hutterites (who engage in mechanised farming) [39], within different healthcare facilities [40], different areas of a manufacturing facility [41], and different areas of animal houses [42]. Similarly, differences are reported in the human microbiome across industrialisation [43], urbanisation, [34] and land use gradients [44] and between those living in rural and urban areas [45–47], with different types of surrounding land use [45, 48, 49], farmers and non-farmers (in the same area) [50, 51], pig and cattle farmers [51], those residing in different healthcare facilities [40], those working in different areas within a manufacturing facility [41], and depending on the vegetation characteristics in the individual's residential yard [45] (Table 1). These findings suggest that the exposures to environmental microbiota likely influence the human microbiome; however the differences identified may also relate to potential confounders such as diet, activity levels, genetics, and historical states differing between populations—including the degree to which humans themselves influence the microbiota of the environments with which they interact. Therefore, to confirm any possible associations between environmental and human microbiota, we look to experimental studies.

3.2 Experimental Animal Studies

Animal studies provide the first experimental evidence to demonstrate the impact of the environment on an animal's microbiome, and allow better control of variables not typically possible in humans (see Table 2 for summary of relevant studies in mammals). Three studies have compared piglets in different environments [53–

Table 1 Characteristics of observational human studies regarding the impact of environmental microbiota exposure on human microbiome

Study Study design	Sample characteristics	Exposures	Samples taken	Key microbiome findings
Fragiadakis et al. [43] Synthesis of several studies	Four populations: Hadza people from Tanzania, people living in rural Malawi, Amazonas living in Venezuela, and people living in metropolitan areas in the USA No other information was reported	Compared: 1. Hadza people (hunter-gatherers), 2. People living in rural Malawi, 3. Amazonas living in rainforest, and 4. People living in metropolitan areas in the USA	Faecal samples	Bacterial composition differed between industrialised and traditional cohorts ↑ Relative abundance of Bacteroidaceae and Verrucomicrobia among those from industrialised populations compared with traditional populations ↓ Relative abundance of Spirochaetaceae, Prevotellaceae, Paraprevotellaceae, and Succinovibrionaceae among those from industrialised populations compared with traditional populations
McCall et al. [34] Cross-sectional	Communities along an urbanisation gradient from the jungle to metropolis, in the Amazon (199–320 samples)	Urbanisation gradient (jungle to metropolis)	Nasal, oral, skin (right arm, right hand, left arm, left hand, right foot, and left foot), anal, and faecal samples	*Bacterial analysis* ↑ Skin Proteobacteria with urbanisation *Fungal analysis* Urbanisation was the strongest differentiating factor for fungal communities Foot fungal composition was clustered by village ↑ Foot fungal alpha diversity with urbanisation ↑ Relative abundance of *Candida* and *Aspergillus* from foot samples with urbanisation ↓ Relative abundance of *Trichosporon*, *Debaryomyces*, and *Saccharomyces* from foot samples with urbanisation

(continued)

Table 1 (continued)

Study Study design	Sample characteristics	Exposures	Samples taken	Key microbiome findings
				↓ Combined faecal and anal samples fungal diversity with urbanisation ↑ Relative abundance of *Candida* and *Aspergillus* from combined faecal and anal samples with urbanisation ↓ Relative abundance of *Debaryomyces*, *Saccharomyces*, *Trichosporon* and *Fusarium* from combined faecal and anal samples with urbanisation ↑ Relative abundance of *Malassezia* from skin samples with urbanisation *Microeukaryotic analysis* ↓ Foot and combined faecal and anal microeukaryotic alpha diversity with urbanisation Parasites were present in the faecal samples from the metropolis, but not the rural areas
Lehtimäki et al. [46] Prospective cohort	Children at age 1 week, 1 month, 3 months, and 1 year (n = 544–657)	Living in urban or rural areas	Airway (hypopharyngeal aspiration, at 1 week, 1 month, and 3 months) and stool samples (1 week, 1 month, and 1 year)	Airway bacterial composition differed at all three time points At 1 month, ↑ Shannon diversity and richness of airway bacteria for those in urban areas Airway bacteria were more homogenous for urban children compared with rural at all three time points *Veillonella*, *Haemophilus*, and *Rothia* were

(continued)

Table 1 (continued)

Study Study design	Sample characteristics	Exposures	Samples taken	Key microbiome findings
				associated with urban living *Moraxella* and *Dolosigranulum* were associated with rural living Gut bacterial composition differed at 1 year, but only in the unadjusted model No differences in relative abundance of gut bacteria after adjusting for multiple testing ↑ *Firmicutes*: *Bacteroidetes* in urban children at 1 year ↓ *Bacteroidetes* richness in urban children at 1 year
Lehtimäki et al. [47] Cross-sectional	Children aged 2 months to 14 years ($n = 275$)	Living in urban, semi-urban, or rural areas	Skin (forearm) swab sample	Skin bacterial composition differed between rural and urban children, particularly those aged 1–4 years, with no difference between rural and urban children at 14 years 51% of the variation of the skin bacteria could be attributed to the land use type
Hanski et al. [49] Cross-sectional	Children aged 14–18 years ($n = 118$)	Land use surrounding place of residence	Skin (wrist) swab samples	Living near more forest and agricultural land was associated with greater genetic diversity of Proteobacteria, compared with built area and water bodies, and the opposite was true for other bacterial classes

(continued)

Table 1 (continued)

Study Study design	Sample characteristics	Exposures	Samples taken	Key microbiome findings
Ruokolainen et al. [48] Cross-sectional	15 to 20 year olds from Russian Karelia ($n = 88$) or Finnish Karelia ($n = 76$)	Russian Karelia v Finnish Karelia	Skin (dominant forearm, volar surface) and nasal samples	*Bacterial analyses* Bacterial community composition in nasal and skin samples differed between Russian and Finnish participants ↑ Bacterial Shannon diversity in nasal and skin samples among Russian participants compared with Finnish *Micrococcus* and *Corynebacterium* were characteristic of Finnish samples *Acinetobacter*, *Aerococcus*, and *Jeotgalicoccus* were characteristic of Russian samples *Fungal analyses* Nasal fungal community composition differed between Russian and Finnish participants Russian fungal flora was more diverse than Finnish *Aspergillus* and *Phoma* were characteristic of Russian Karelia
Parajuli et al. [45][a] Prospective cohort	Retired people aged 65–79 years ($n = 48$)	Living in homes in rural or urban environments Vegetation in their yard Land use within 200 m radius of home	Stool samples (August and November)	*Impact of environmental variables on gut bacterial community composition* August: Stool bacterial community composition was associated with the number of shrub species November: Stool bacterial community composition was not associated with any environmental variable

(continued)

Table 1 (continued)

Study Study design	Sample characteristics	Exposures	Samples taken	Key microbiome findings
				Impact of number of yard shrub species on gut bacterial community composition ↑ Number of yard shrub species was associated with ↑ relative abundance of *Faecalibacterium, Blautia*, and *Prevotella* at the genus level, Ruminococcaceae at the family level, and Firmicutes at the phylum level ↑ Number of yard shrub species was associated with ↓ relative abundance of *Clostridium* sensu stricto at the genus level, Clostridiaceae_1 and Prevotellaceae at the family level ↑ Number of yard shrub species was associated with a ↑ Firmicutes: Bacteroidetes *Non-woody flowering plant diversity on gut bacterial community composition* ↑ Non-woody flowering plant diversity associated with ↑ relative abundance of Prevotellaceae at the family level, Bacteroidia at a class level, and Bacteroidetes at a phylum level *Impact of built area on gut bacterial community composition* ↑ Coverage of built

(continued)

Table 1 (continued)

Study Study design	Sample characteristics	Exposures	Samples taken	Key microbiome findings
				area was associated with ↑ Relative abundance of *Parabacteroides* and *Bacteroides* at the genus level ↑ Coverage of built area was associated with ↓ Relative abundance of *Prevotella* at the genus level *Impact of rural versus urban living on gut bacterial community composition* Urban living was associated with ↑ relative abundance of *Dorea* and *Blautia* at the genus level Urban living was associated with ↓ relative abundance of *Clostridium* sensu stricto and *Prevotella* at the genus level, and Clostridiaceae_1 at the family level
Shukla et al. [50] Cross-sectional	Dairy farmers ($n = 21$) and urban non-farmers ($n = 18$)	Dairy farming v urban living and not farming)	Nasal samples and oral samples (saliva and buccal surfaces)	*Nasal samples* Dairy farmers' nasal samples had ↑ bacterial species richness and Shannon diversity index compared with non-farmers nasal samples. The bacterial community compositions were also significantly different Dairy farmers' nasal samples had ↑ relative abundance of Bacteroidetes and of rare phyla at a phylum level, Carnobacteriaceae, Ruminococcaceae, Lachnospiraceae,

(continued)

Table 1 (continued)

Study Study design	Sample characteristics	Exposures	Samples taken	Key microbiome findings
				Flavobacteriaceae, Sphingobacteriaceae, Micrococcaceae, and Bacteroidaceae at a family level, and *Sporobacter*, *Paraprevotella*, *Bacteroides*, *Psychrobacter*, *Parapedobacter*, and *Acetivibrio* at a genus level Dairy farmers' nasal samples had ↓ relative abundance of Staphylococcaceae, Pseudomonadaceae, and Dietziaceae at a family level and *staphylococcus* and *pseudomonas* at a genus level *Oral samples* No difference between dairy farmers and non-farmers for oral sample bacterial species richness, nor bacterial community compositions No difference in the relative abundance at a phylum nor family level
Kraemer et al. [51] Cross-sectional	Pig farmers ($n = 43$), cow farmers ($n = 17$), and non-animal exposed people ($n = 26$)	Pig farming v cow farming v no animal exposure	Nasal samples	↑ Bacterial richness and Shannon index among pig farmers compared with non-animal exposed people ↑ Bacterial richness and Shannon index among cow farmers compared with non-animal exposed people Bacterial community composition differed

(continued)

Table 1 (continued)

Study Study design	Sample characteristics	Exposures	Samples taken	Key microbiome findings
				between pig farmers, and both cow farmers and non-animal exposed people (cow farmers and non-animal exposed people were not significantly different) ↓ Beta-diversity dispersion among pig farmers, compared with cow farmers and non-animal exposed people ↑ Relative abundance of 82 sequence variants in pig farmers, compared with cow farmers and non-animal exposed people
Chen et al. [40] Cross-sectional	Residents in three different healthcare institutions ($n = 88$)	Three different healthcare institutions	Nasal samples	Significant differences in Chao 1 diversity, bray-Curtis distance, and relative abundance of *Corynebacterium, Dysgonomonas, Neisseria,* unclassified genera into Bacillales and *Propionibacterium* between the three healthcare institutions
Wu et al. [41] Cross-sectional	Workers from a manufacturing facility	Working in the machine shop, assembly, or administration	Skin, nasal, and oral wash samples	↓ Bacterial alpha diversity in nasal samples for those working in assembly Skin and nasal bacterial composition differed between workers from different areas
Lai et al. [42] Prospective cohort	Workers in an academic mouse research facilities	8-h work day in an academic mouse research facility	Nasal, skin (retro-auricular crease), and oral wash samples	Work environmental microbiome accounted for $0.1 \pm 0.1\%$, $3.1 \pm 1.9\%$, and

(continued)

Table 1 (continued)

Study Study design	Sample characteristics	Exposures	Samples taken	Key microbiome findings
			(pre-post) for bacteria	3.0 ± 1.5% of the pre-shift oral, nasal, and skin microbiome, respectively Work environmental microbiome accounted for 0.0 ± 0.0%, 3.7 ± 2.1%, and 14.1 ± 28.5% of the post-shift oral, nasal, and skin microbiome, respectively Change in the proportion of microbiome attributed to work environmental microbiome was not statistically significant
Roslund et al. [52] Cross-sectional (baseline data used)[b]	Children aged 3–5 in day care ($n = 75$)	*Nature-based day care*: Daily forest visits *Standard day care:* No forest visits	Skin and stool samples	↑ Divergent skin bacterial Shannon index for those in the nature-based day care compared with the standard day care ↑ Bacterial diversity of the skin alphaproteobacterial community composition for those in the nature-based day care compared with the standard day care ↑ Bacterial diversity of Ruminococcaceae in the nature-based day care compared with the standard day care

[a]Only findings for those who did not own animals are reported here
[b]Roslund et al. [52] is also reported as an experimental study as the participants in two types of day care centres reported in this table are compared with those at day care centres which have undergone a greening intervention

55]. The first of these compared three groups of piglets: those that were kept indoors, those kept outdoors, and those born indoors and moved into isolators at 24 h of age with antibiotics administered. At 56 days, there were several differences in the ileal

Table 2 Characteristics of the experimental animal studies regarding the impact of environmental microbiota exposure on animal microbiome

Study Study design	Sample characteristics	Interventions	Sample(s) taken	Key microbiome findings
Mulder et al. [54] Controlled experiment	Large white × landrace sows and piglets Involved in the study from birth 6 outdoor sows and 6 indoor sows 18 outdoor piglets, 18 indoor piglets, and 18 indoor piglets that were moved to an isolator at 24 h of age and who had antibiotics	Indoors v outdoors v isolator with antibiotics 6 piglets from each group were humanely killed at days 5, 28, and 56	Piglets: Ileal contents were collected at 56 days ($n = 4$ per group) Sows: Faecal samples	*Piglets:* ↑ Species richness in the indoor and isolator groups ↑ Firmicutes in the outdoor group compared with isolator group ↑ *Lactobacillus reuteri, L. delbrueckii*, and *L. johnsonii NC533* in the outdoor group compared with indoor and isolator ↑ *L. amylovorus* and *L. mucosae* in the outdoor group compared with the isolator group ↑ Uncultured clone BARB _aaa01f06 and *Clostridium beijerinckii* in the indoor group compared with the outdoor and isolator groups ↑ Uncultured clones BARB_aaa0d03 and HH_aai33h06 in the indoor group compared with the outdoor group *Sows:* No significant differences
Mulder et al. [55] Controlled experiment	Large white × landrace piglets Involved in the study from birth 18 piglets in the indoor group and 18 in the outdoor group	18 piglets were born indoors and 18 outdoors. 28 days after birth, all piglets were moved into isolators, until they were humanely killed at day 5, 28, or 56	Ileal contents were collected at 5, 28, and 6 days	For the indoor piglets there was clustering of day 5 and 28 samples For the outdoor piglets there was clustering of day 5 and 56 samples Most indoor piglets had *L. johnsonii* and *Peptostreptococcus*

(continued)

Table 2 (continued)

Study Study design	Sample characteristics	Interventions	Sample(s) taken	Key microbiome findings
				sp. Most outdoor piglets had *L. johnsonii, Actinobacillus porcinus*, and *Peptostreptococcus* sp.
Schmidt et al. [53] Controlled experiment	Large white × landrace piglets Involved in the study from birth 5 piglets in the indoor group and 5 in the outdoor group	5 piglets were born indoors and 5 outdoors. 2 days after birth, all piglets were moved into isolators, until they were humanely killed at day 56	Ileal contents were collected at 56 days	There were no significant differences in the bacterial phylotype comparisons between the groups
Ottman et al. [56] Controlled experiment	Female BALB/c mice Aged 4 weeks at the start of the experiment 16 healthy mice (8 intervention, 8 control) 16 allergen-sensitized (8 intervention, 8 control), induced asthma model protocol after 6 weeks of exposure	Intervention: Soil in bedding (replenished weekly) for 11 weeks Control: Clean bedding for 11 weeks	Fresh faecal samples, and jejunal and ileal tissue samples	Bacterial composition was significantly different between treatments for the faecal and ileal samples, but not the jejunal samples There were no significant differences in bacterial diversity for the three types of samples between treatments ↑ Bacteroidetes: Firmicutes in the intervention group than control ↓ Relative abundance of Lachnospiraceae in the intervention group compared with control ↑ Relative abundance of Bacteroidales family S24–7 and Proteobacteria compared with the control *Alistipes* and two

(continued)

Table 2 (continued)

Study Study design	Sample characteristics	Interventions	Sample(s) taken	Key microbiome findings
				Prevotellaceae operational taxonomic units were exclusively present in the control group
Zhou et al. [57] Randomised controlled experiment	Female and male BALB/c mice Aged 6 weeks at the start of the experiment 24 mice (8 per group)	Intervention 1: Reared in a farmhouse, with bedding composed of farm top soil, and decaying wheat, hay, and fallen leaves Intervention 2: Reared in an animal house, with top soil, decaying fallen leaves and hay, and room dust used as bedding Control: Reared in a specific pathogen-free animal room with sterile bedding	Caecal samples were taken	↑ Number of operational taxonomic units and bacterial species, and bacterial richness and diversity (both phylogenetic and Shannon index) in the two intervention groups, compared with control. There were no significant differences between the two intervention groups ↓ Relative abundance of *Firmicutes, Enterorhabdus, lactobacillus*, and *Acinetobacter* in both intervention groups compared with the control ↑ Relative abundance of *RC9_gut_group* and *S24–7_norank* in both intervention groups compared with the control ↑ Relative abundance of Actinobacteria, *Tenericutes, staphylococcus*, and *Desulfovibrio* for intervention 1, compared with intervention 2 and the control ↑ Relative abundance of *Bacteroidetes* and

(continued)

Table 2 (continued)

Study Study design	Sample characteristics	Interventions	Sample(s) taken	Key microbiome findings
				Bacteroides for intervention 2, compared with intervention 1 and the control ↑ Number of genera in the two intervention groups, compared with control
Liddicoat et al. [58] Randomised controlled experiment	Female and male BALB/c mice Aged 3–5 weeks at the start of the experiment 18 per treatment group (9 females, 9 males)	High diversity soil group (intervention 1): Mice were exposed to trace-level dust from higher diversity soil for 7 weeks Low diversity soil group (intervention 2): Mice were exposed to trace-level dust from lower diversity soil for 7 weeks Control: Mice were not exposed to any dust nor soil	Faecal samples were analysed at weeks 0 and 7, and caecal samples were compared at 7 weeks	The composition for the faecal and caecal samples at week 7 was significantly different between treatment groups The relative abundance of certain taxa changed within treatment group between weeks 0 and 7 for the faecal samples, including an ↑ in the relative abundance of *Kineothrix alysoides*-like operational taxonomic units for mice in the high diversity group No other significant differences were reported *Note:* There was a drop in alpha diversity of environmental microbiome samples by week 7

microbiomes of the piglets. For example, the outdoor piglets had higher relative abundance of Firmicutes, *Lactobacillus reuteri, L delbrueckii*, and *L johnsonii NC533* compared with the isolator group [54] (see Table 2 for further information). When the indoor or outdoor exposure was only 2 days (i.e. piglets were moved into isolators when aged 2 days), there were no significant differences in the ileal microbiome at Day 56 [53]; hence 2 days is unlikely a sufficient exposure to result in colonisation of the gut.

Three mouse studies have examined the effect of direct soil exposure [56, 57] or indirect soil exposure (trace-level dust) [58]. Ottman et al. [56] allocated mice to one

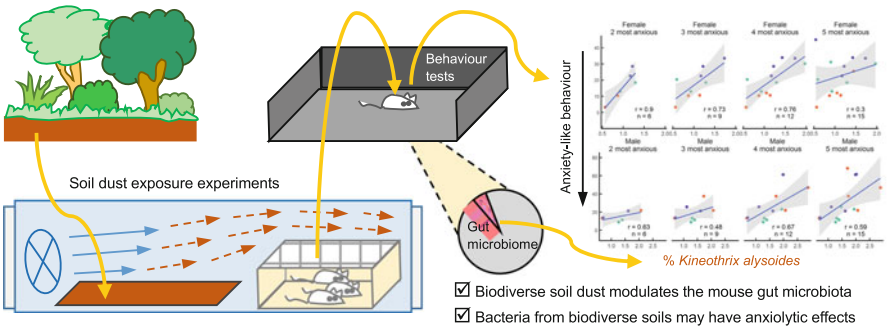

Fig. 1 Graphic abstract of the design and key finding from Liddicoat et al. [56], reproduced with permission from the publisher

of two groups: soil exposure or clean bedding. Fresh soil was introduced every week, and the experiment ran over a 6-week period. There were significant differences between the two groups in the faecal and ileal microbiomes, but not the jejunal microbiome, at the end of the experiment. Differences in bacterial composition were detected, confirming that the environmental exposures had influenced the gut microbiome.

Another mouse study allocated mice to different types of housing environments, and compared their gut microbiomes [57]. In that study, mice were housed in a (1) specific pathogen-free animal room, (2) a general animal room with exposure to house dust, decaying hay, wheat straw, and top soil from the university, or (3) a farmhouse with exposure to house dust, decaying hay and wheat straw, and soil from the farmyard where dogs, hens, ducks, and goats were present. Differences in the gut microbiome of the three groups of mice were detected, with Groups 2 and 3 demonstrating more diverse gut microbiota than Group 1, and with differences also present in the relative abundance of particular taxa. The overall microbial diversity did not differ between Groups 2 and 3; however there were significant differences in composition. This study demonstrated that living in different environments influences the gut microbiome, supporting the notion that observed differences in the human microbiome may be driven by differences in residential environment (e.g. rural versus urban).

These two mouse studies provide important insights into the mechanisms underlying the development of the host microbiome; however they do not offer realistic simulations of potential human public health interventions; not all people can live on farms! Evidence from more realistic environmental exposures is required to inform public health recommendations, and one such study was recently published, using trace-levels (<0.005 g soil mouse^{-1} week^{-1}) of naturally diverse soil exposure to simulate the exposure of people during everyday activities (e.g. commuting to work) [58]. Mice were allocated into one of three groups: (1) exposure to high diversity soil dust, (2) exposure to low diversity soil dust, and (3) no soil dust exposure (Fig. 1). Mice were physically separated from the soil, but fans were used to move the trace-levels of airborne dust into the area with the mice, with the trial conducted over a

7-week period. Female mice in the high diversity exposure group had a different gut microbiome composition at the conclusion of the experiment than those in the low and control groups. The trace-level exposure was also sufficient to influence levels of anxiety-like behaviour in the mice, which was reduced in high-diversity soil dust-exposed female mice. The findings of this study provide promising results to support potential public health interventions that might modulate the gut microbiome in ways that can influence important health outcomes, as well as a possible role of high biodiversity environments in supplying important, health-associated microbiota.

3.3 Human Intervention Studies

We are also starting to see human trials that investigate the transference of environmental microbiota to humans (Table 3). Selway et al. [59] recently reported a case series ($n = 3$, all authors of the study) across Australia, India, and the UK, whereby direct interactions with urban green spaces (i.e. soil, air, and vegetation sampling) resulted in changes to the skin and nasal microbiome; however these changes were not sustained until the next day, and would therefore not be expected to influence the gut microbiome, particularly if more passive exposure occurred. Furthermore, due to the lack of a control, it is unclear to what extent the green space exposure itself led to the changes in the microbiome, further compounded by the validity of a study where the authors were the participants. Fortunately, some of these limitations have been overcome in other human intervention studies.

Very short (20 s), direct exposure to composted soil- and plant-based materials has recently been demonstrated to change the skin microbiome [60]. In this experiment participants rubbed their hands in the materials for 20 s, then washed their hands without soap for 5 s, and dried their hands with paper towelling. In an associated experiment [60], packs of sphagnum moss that had been dried, crushed, and sieved were placed on the anterior surface of the forearm, with a control pack used on the other arm. The packs were left for 3 h and 45 min. The skin microbiome changed with the intervention, with higher bacterial diversity in the intervention group. This finding is consistent with the observational evidence [49] regarding living close to nature and altered skin microbiome. Together these findings suggest a relationship between the exposure to nature and the human microbiome, but we do not yet have a good understanding of the dosage required, the long-term effects, nor the influence on the gut microbiome.

A small non-randomised controlled trial [61] has provided some early evidence that short duration (20 s), direct exposure to a soil- and plant-based composition can change both the skin and gut microbiomes. The intervention group rubbed their hands in a soil- and plant-based composition for 20 s before washing their hands without soap for 5 s and drying their hands with a towel, before breakfast, before dinner/evening snack, and before bed, over a 14-day period. There were no differences in the microbiomes nor demographic characteristics between groups at baseline. At Day 14, those in the intervention group had a higher diversity and relative

Table 3 Characteristics of the human intervention studies regarding the impact of environmental microbiota exposure on human microbiome

Study Study design	Sample characteristics	Intervention(s)	Microbiome outcome (s) investigated	Key significant microbiome findings
Selway et al. [59] Case series	Study authors ($n = 3$), no other information reported	*Case 1:* Visited green spaces in Australia (~1.5 h), the UK, and India (~15 min) *Case 2:* Visited green spaces in Australia (~1.5 h) *Case 3:* Visited green spaces in the UK and India (~15 min) *Note:* It is unclear whether the time reported refers to the total exposure time or the exposure time at each site. Participants visited 15 locations (different types of green space) over 4 days in Australia and 15 locations (different types of green space) in India and the UK. In each location, participants interacted directly with elements of the green space (collecting air, soil, and vegetation samples)	Skin (left anterior wrist) and nasal samples	*Australia:* ↑ Skin and nasal microbial diversity (both observed species and Faith's phylogenetic diversity) Shift in skin beta diversity which became closer to the soil beta diversity Shift in the nasal beta diversity which became closer to the air beta diversity ↓ Relative abundance of *Micrococcus*, *Staphylococcus*, *Tetrasphera*, *Corynebacterium*, *Paracoccus*, *Acinetobacter*, *Brevundimonas*, and *Cutibacterium* decreased in the skin samples ↑ Relative abundance of rare taxa and *Sphingomonas* increased in relative abundance in the skin samples ↓ Relative abundance of *Staphylococcus* and *Lawsonella* decreased in the nasal samples ↑ Relative abundance of rare amplicon sequence variants in the nasal samples The skin and nasal microbiome 'reset' the next day *India:* ↑ Skin and nasal microbiome diversity (observed species and Faith's phylogenetic

(continued)

Table 3 (continued)

Study Study design	Sample characteristics	Intervention(s)	Microbiome outcome (s) investigated	Key significant microbiome findings
				diversity) Shift in skin composition (beta diversity), but no change in nasal composition ↓ Relative abundance of *Staphylococcus*, *Corynebacterium*, *Finegoldia*, and *Sphingomonas* in the skin samples ↑ Relative abundance of *Bacillus* and rare taxa ↓ Relative abundance of *Staphylococcus* in the nasal samples ↑ Relative abundance of *Corynebacterium* and low abundance taxa in the nasal samples For 1 case only, ↑ relative abundance of *Moraxella* in the nasal sample In another case, ↑ relative abundance of *Peptoniphilus* and *Anaerococcus* in the nasal sample *UK:* ↑ Skin microbiome diversity (observed species and Faith's phylogenetic diversity) Shift in skin and nasal composition (beta diversity) ↓ Relative abundance of *Staphylococcus*, *Corynebacterium*, *Finegoldia*, *Lawsonella*, and *Acinetobacter* in the skin samples ↑ Relative abundance of *micrococcus*,

(continued)

Table 3 (continued)

Study Study design	Sample characteristics	Intervention(s)	Microbiome outcome (s) investigated	Key significant microbiome findings
				Moraxella, and rare taxa in the skin samples Little change (<3%) in the relative abundance of dominant genera in the nasal microbiome
Grönroos et al. [60] *Study 1* Case series	Urban volunteers ($n = 2$)	Rubbed hands in composted soil- and plant-based materials for 20 s, then washed hands without soap for 5 s, and dried their hands with paper towelling In total 16 different materials were trialled (8 per person), with a maximum of 2 per day with at least 5 h in between trials	Skin (posterior surface of the right hand) samples (immediately before exposure and immediately after drying hands)	↑ In Shannon index and taxon richness (at all taxonomic levels and all tested taxonomic groups, except the phylum Firmicutes) ↑ Relative abundance of Bacteroidetes, Acidobacteria, and several less common phyla ↑ Total bacterial abundance
Grönroos et al. [60] *Study 2* Case series	Urban volunteers ($n = 2$)	Packs of sphagnum moss that had been dried, crushed, and sieved were placed on the anterior surface of the forearm, and the packs were left on for 3 h and 45 min The other arm served as the control	Skin (anterior surface of the forearm) samples (immediately before and after the intervention)	↑ In Shannon diversity index and bacterial richness Shift in the bacterial composition towards the bacterial composition of the moss material of exposure
Nurminen et al. [61] Comparative study with concurrent controls	Healthy, urban-dwelling adults aged 27–63 years ($n = 14$)	*Intervention group:* Rubbed hands in soil- and plant-based compost for 20 s, before washing their hands without soap for 5 s	Skin and stool samples (baseline, day 14 and day 35)	*Baseline:* No significant differences in skin and gut bacterial diversity *At day 14:* ↑ Alpha diversity and the relative abundance of phylum Bacteroides

(continued)

Table 3 (continued)

Study Study design	Sample characteristics	Intervention(s)	Microbiome outcome (s) investigated	Key significant microbiome findings
		and drying their hands with a towel, 3× a day (before breakfast, before dinner/ evening snack, and before bed) over a 14-day period *Comparison group:* No intervention		in the faecal samples No significant change in alpha diversity in the skin samples (except 2 individuals who only washed their hands superficially and did not dry them with a towel afterwards, who also had an ↑ in the abundance of the phyla Chloroflexi, Actinobacteria, and Bacteroidetes) *At day 35 (intervention stopped at day 14):* No significant difference in gut microbiome alpha diversity between groups
Roslund et al. [52] Non-randomised cluster controlled trial	Children aged 3–5 in day care ($n = 75$)	*Greening intervention:* Greening of day care centres (e.g. planters, peat blocks, sods, and segments of forest floor added to the centres). Children were encouraged to interact with the natural elements *Comparison (nature):* No intervention, but these centres already had daily forests visits which continued during the study *Comparison (standard):* No intervention	Skin and stool samples (baseline and day 28)	*At baseline:* ↑ Divergent skin Shannon index for those in the nature-based day care compared with the standard day care ↑ Diversity of the skin alphaproteobacterial community composition for those in the nature-based day care compared with the standard day care ↑ Diversity of Ruminococcaceae in the nature-based day care compared with the standard day care No significant differences between the standard and intervention day care groups *Day 28:* ↑ Skin proteobacterial and gammaproteobacterial

(continued)

Table 3 (continued)

Study Study design	Sample characteristics	Intervention(s)	Microbiome outcome (s) investigated	Key significant microbiome findings
				diversity in the intervention group compared with the standard group ↑ Gut Ruminococcaceae diversity in the intervention group compared with the standard group *Pre-post (intervention not stopped):* ↑ Skin alphaproteobacterial diversity in the intervention group ↑ Gut Ruminococcaceae diversity in the intervention group ↓ Gut Clostridiales relative abundance in the intervention group

abundance of bacteria in the phylum Bacteroides in their stool samples; however there were no significant differences in their skin microbiomes. By Day 35 (21 days after the intervention) there were no significant differences in the skin nor stool microbiomes between the groups. This study provides promising evidence supporting the modulation of the gut microbiome via direct exposure to soil and plant compositions; however it might be that the dosage and duration of exposure were insufficient for colonisation of the gut to occur.

While direct exposure to composted soil and plant material might prove to be a valuable intervention, direct environmental manipulation and microbiome-inspired management are more realistic public health interventions, for they do not necessarily require changes in behaviour to be made on the part of the individual. The first human trial of the impact of environmental biodiversity manipulation on the human microbiome was published in 2020 [52]. That intervention involved introducing green space into day care centres, with planters, peat blocks, sods, and segments of forest floor added to the intervention centres, which were compared with standard urban day care centres with no intervention. Children in the intervention day care centres were encouraged to interact with the introduced natural elements. After 28 days, differences were reported in the skin and gut microbiomes of the children, as well as in the soil microbiome itself. This study provides the first direct experimental evidence that improving environmental green space exposure can influence

the human microbiome, with associated changes to blood biomarkers that shifted towards immunoregulatory.

Taken together, the current evidence-base supports the 'Old Friends' and Biodiversity hypotheses. While there are still many unknowns and more work required to determine the specifics of human microbiome changes and health outcomes resulting from environmental exposures, there is already sufficient evidence to make public health recommendations for increasing our exposure to biodiverse green space for better health.

4 What Are Good Environments for Beneficial Microbiota?

A detailed understanding of the environmental sources of health-associated or health-promoting microbiomes is lacking. What is well-known is that the human microbiome stems from our environment *sensu lato*, with only very small human genetic effects on its composition [28]. Therefore, a better understanding of the environmental sources of the human microbiome and the exposure pathways to colonisation are of great interest.

A growing literature associates ecological integrity—the absence of degrading ecological processes or ecological impacts—with the presence of potential health-promoting microbiota. This literature aligns with the Old Friends and Biodiversity hypotheses. Indeed, ecological restoration—the repair of degraded or damaged ecosystems—can create soil microbiota that are indicative of more late successional soils (i.e. soil microbiota that are characteristic of mature and more functional ecosystems), sometimes with greater microbial diversity (Fig. 2). These late succession and more diverse microbiota have been shown to be more resilient to introduction of potentially pathogenic taxa [62]. For example, soils from lower ecological integrity sites across Australia have been shown to have greater relative abundance of environmental microbial opportunists (incl. *Bacillus, Clostridium, Enterobacter, Legionella*, and *Pseudomonas*, which are all pathogen-containing genera [32]). These opportunists were associated with soils that were human-impacted and of lower ecological integrity. Interestingly, the relative abundance of these taxa was negatively correlated with the degree of ecological restoration. Furthermore, those microbial taxa most associated with higher ecological integrity included those that were associated with late-successional ecosystems. These taxa included DA101—a very abundant soil bacterium that is associated with grasslands [63]—and with the presence of photoheterotrophs (which use light energy as their energy source and rely on organic compounds from the environment as their carbon source, as opposed to exclusively carbon dioxide). They also included *Mycobacterium*, a bacterial genus that (a) contains the common non-pathogenic soil-dwelling *M. vaccae* which has been associated with reduced inflammatory responses [64, 65], reduced anxiety-like behaviour [65, 66], and prevention of stress-induced sleep impairment [67] in laboratory rodent models; and (b) has been linked to non-specific beneficial effects

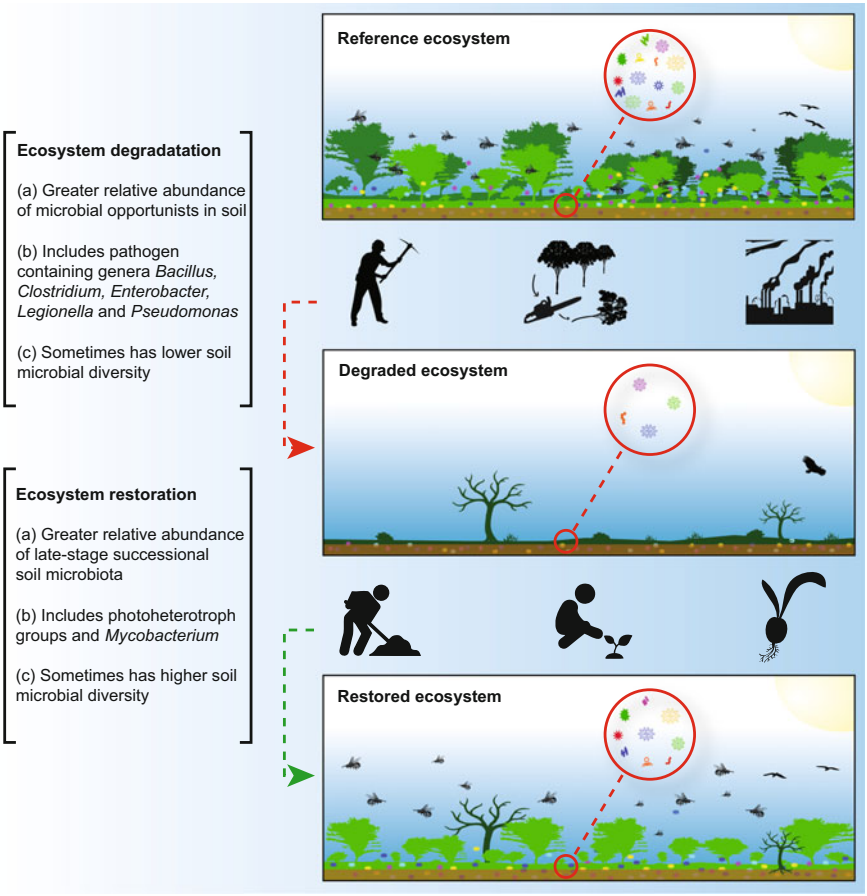

Fig. 2 Ecological degradation and the restoration of these degraded ecosystems impact on the soil microbiota. There is growing evidence that ecosystem degradation leads to greater microbial opportunists (including pathogen-containing groups), whereas ecosystem restoration tends to favour late-stage successional soil microbiota, including some groups that have health supporting associations. (Figure adapted from [70], with assistance from Jake Robinson)

on survival via its use in the *Bacillus* Calmette-Guérin (BCG) vaccine against tuberculosis—a live attenuated strain of *M. bovis* [68, 69].

5 Public Health Recommendations

There are three key, related public health recommendations that flow from the evidence presented above, if we are to capitalise on the health-enhancing aspects of exposure to biodiverse green space: (1) more biodiverse urban green space is

required, (2) urban green spaces should be optimised for human health, and (3) urban green spaces must be accessible, including to those with chronic health conditions.

5.1 More Urban Green Spaces

With increased urbanisation typically comes less urban green space. For example, in Australia the majority of local government areas had a reduction in green space from 2013 to 2020 [71]. There are therefore two aspects to consider: the protection of existing green spaces and the addition of new green spaces, both of which would likely benefit health. It is not only important to increase urban green spaces but also to protect existing 'natural' green spaces (which are likely more biodiverse and therefore more important than the development of new spaces). For example, a recent review investigated how green gentrification influenced human health [72]. For existing residents, green gentrification resulted in a lower sense of community, less utilisation of green spaces, and a lesser sense of belonging in the new green spaces than it did for new residents. These findings are important in exploring how best to increase and optimise urban green spaces, and improved stakeholder collaboration might offer some solutions. Importantly, organisations and households can also increase their green spaces: the abovementioned greening of a day care centre [52] is an excellent example of how this might occur. Indeed, many areas have existing community-groups that tend green spaces already and these groups should be encouraged to expand the extent of green spaces in traditional (e.g. parks) and non-traditional areas (e.g. road verges, median strips, rooftops, pocket parks, tiny forests).

5.2 Enhanced Urban Green Spaces

While we do not know exactly which type of vegetation leads to a health-enhancing or health-promoting microbiome exposure (including aerobiome—the airborne microbiome), increasing biodiversity in urban green spaces will likely provide the most opportunities for increasing exposure to microbiota with health benefits. There is already observational evidence to support this notion [73, 74], further supported by the experimental evidence of Liddicoat et al.'s [58] mouse study investigating the impact of different diversity levels of aerobiome exposure. Soil microbiota have been shown to be more similar to that in pristine ecological sites after ecological restoration [31, 32, 75–77], and across vegetation biodiversity gradients [33]. We therefore recommend that existing urban green spaces be further restored to increase their ecological integrity and/or vegetation diversity, and that new urban green spaces be designed with biodiversity enhancement at the centre of landscaping and architectural decisions [78].

5.3 Accessible Urban Green Spaces

For urban green spaces to be of benefit to human health, they must be either directly (e.g. playing with plants, soil) or indirectly (e.g. exposed to aerobiomes) accessible. Accessibility should therefore influence decision-making around where green spaces should be located, and what amenities they should include, as well as how to attract people to transit and/or engage with the green spaces themselves. One approach to improving green space exposure is by greening areas that are already accessed by people, thereby improving their exposure to green spaces without requiring behaviour change. Examples include the abovementioned day care greening intervention [52], with similar approaches possible in schools [79], healthcare facilities [80], residential aged care settings, commuter corridors, and workplaces. Indeed schoolyard greening interventions already have reported health benefits [79]; however the microbiome as a potential mechanism has not yet been explored. Similarly, public health advice might include enhancement of biodiversity in residential yards. Improving the biodiversity surrounding urban transport corridors is another such valuable approach. As demonstrated by Liddicoat et al.'s [58] mouse study, greening—and increasing the plant diversity—of urban transport corridors may be sufficient to influence the gut microbiome and human health, providing a relatively easy and low-risk approach to increasing the amount of green space, the quality of that green space, and the exposure and accessibility thereto.

Utilisation of green spaces has increased with environmental interventions, behaviour change techniques [81, 82], and community co-design [81]. Promoting the use of green spaces may include consideration for the features that people find desirable and ensuring that the spaces are accessible. Desirable features include natural, specific landscaping, educational, cultural, and recreational features (Box 2). To improve accessibility, both accessibility to and within green spaces must be considered (Box 3). For example, the location of the green spaces should consider proximity to homes and other places of interest, and safety options. Within green spaces, older people desire features that protect against crime, injury, and getting lost [83, 84], while common barriers for those with mobility issues include high contrast paving, narrow paths, slopes, sudden height differences, inappropriate ground surfaces (e.g. stones), and a lack of handrails, shelter, rest facilities, and clear visual access to entries and exits [84], all of which can be overcome with informed design.

Box 2: Desirable Aspects of Green Spaces to Promote Utilisation
Natural features

- Biodiversity [85].
- Natural and wilderness areas [83–85].
- Abundance of trees and flowers [84, 85].

(continued)

Box 2 (continued)
- Abundance of wildlife [83–85].
- Natural sounds [83].

Landscaping features

- Open views [83, 84].
- Water features [83–85].
- Landmarks and distinctive features [83].
- Attractive architecture and statues [83].

Cultural features

- Worship areas [83].
- Cultural heritage [83].
- Features that consider ethnocultural preferences [86].

Recreational features

- Playgrounds [83, 85].
- Features that promote recreational activities (e.g. ball games, exercise equipment, bicycle trails, gardening) [83, 85].
- Features that promote social interactions [83, 85].

Educational features
Information signs and environmental education [85].

Box 3: Considerations for Green Space Accessibility
Location of green spaces

- Close proximity to homes [83, 85].
- Close proximity to city centres [85].
- Close proximity to other places of interest (e.g. shops, cafes) [83, 85].
- Good connectivity [83].

Transportation to/from green spaces

- Public transport [83].
- Active transport [83].

Accessibility within green spaces

- Clear visual access to entries and exits [84].
- Map information [83].
- Paths linking different parts of the green space [83].

(continued)

Box 3 (continued)
- Appropriate paths (e.g. low contrast, wide, no sudden height differences) [84].
- Pavements with anti-slip, water-resistant material [83].
- Slopes of less than 5% [83].
- Handrails [84].
- Shelter [83, 84].
- Toilets [83].
- Seating [83, 84].
- Lights [83].
- No free-running dogs [83].
- Reliable, ongoing maintenance [83, 85].

From both a clinical and a public health perspective, green space and other nature-based interventions may be recommended to improve health outcomes [87], and part of the benefit may derive from modulation of the microbiome [24]. These interventions may include direct contact with soil and plant compost as described above [60, 61], or through indirect or incidental exposure when engaged in other activities, such as exercise [87, 88] (including active transport [89]), bird watching [87], arts and crafts [87], nature play [90], gardening [91, 92], environmental enhancement and conservation activities [93], outdoor experiential activities [84], and rehabilitation activities [84]. Relaxation techniques, as well as cognitive and behavioural therapies conducted in green space, have also been used as a means of increasing green space exposure [88]. As such, new and existing clinicians should be trained in environmental health, nature-based therapies, and sustainable practice [94, 95], to support the development and maintenance of green spaces, and to ensure their utilisation by clients and the broader community.

6 Recommendations for Future Research

We have summarised the state of knowledge about the relevance and importance of the environmental microbiome in influencing the human microbiome, and the burgeoning array of associated health effects that we are only now beginning to understand. In doing so, several deficiencies in our knowledge have also become clear; it will be critical to address these knowledge gaps if we are to provide the strength of evidence required to effect widespread implementation of an arguably novel public health intervention such as increasing the quantity and quality of green space available. Our interpretation of these key research needs follows below:

1. Research that improves the evidence-base used to make public health recommendations. We already know enough to recommend green space exposure as a

public health intervention, but we do not know enough to target that intervention in the most cost-effective way. For example, might the greatest health benefit be achieved by focusing limited resources on increasing opportunities for exposure to biodiverse environmental microbiota in areas with socioeconomically deprived children?
2. Establish the characteristics that determine the optimal environmental microbiome for enhancing human health: is it biodiversity per se, a number of 'magic bullets', a functional trait of the microbiota, or some combination of these? Does the 'optimal' environmental microbiome vary by population, depending on what environment that particular population is evolutionarily adapted to?
3. Quantify the dose-response relationship: what intensity, duration, or frequency of environmental microbiota exposure is required to achieve a detectable health benefit at a population level? Does the relationship vary with age, sex, ethnicity, or other individual factors?
4. Research that advances basic science: there is a need to establish the physiological mechanisms that link green space and its microbial biodiversity to improved health outcomes. What psychoneuroimmunoendocrinological processes are at play? Are the effects of environmental microbiota exposure enhanced by associated 'macro' biodiversity, the sights and sounds of nature, or by accompanying exposures such as to phytoncides, negative air ions, sunlight, or reduced pollution (e.g. light, air, noise, water)?
5. Research that creates entrepreneurial opportunities for industry. Can an optimal environmental microbiota be packaged and sold for domestic aerosolisation, deployed as part of home-use potting mix, or be incorporated into commercial coatings for the insides of submarines and spacecraft that are removed from green space for prolonged periods? Can building designs incorporate biodiversity elements that increase sales prices and competitive advantage?

7 Conclusion

Exposure to green spaces is associated with a wide range of human health benefits; however our understanding of the linkage mechanisms is currently incomplete. There is, however, emerging evidence suggesting that exposure to biodiverse environmental microbiomes may play a role, in turn suggesting that biodiverse green spaces may provide greater human health benefits. This view is consistent with the notion of ecosystem services, whereby healthy, functioning ecosystems are required for human health and wellbeing. We therefore recommend the maintenance and development of biodiverse urban green spaces to protect human health.

Ethical Approval
This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest All authors declare they have no conflict of interest.

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Regulation of Host Immunity by the Gut Microbiota

Hannah Partney and Nissan Yissachar

Abstract Constant exposure to diverse microorganisms has accompanied human evolution and continues to shape immunological development throughout life. In mucosal tissues, both innate and adaptive arms of the immune system are required to support healthy mutualistic interactions with the resident microbiota, while aggressively fighting pathogenic infections. Technological breakthroughs over the past decade facilitated groundbreaking discoveries that transformed our understanding of intestinal immunology and established the gut microbiota as a critical factor that shapes immunological development and function. Indeed, alterations to microbiota composition (dysbiosis) are associated with a wide array of human diseases, including autoimmune diseases, chronic inflammation, the metabolic syndrome, and cancer. In this chapter, we discuss fundamental concepts that underlie microbiota-immune system crosstalks, and their subsequent impact on host immunity, in health and disease. Given the widespread scope of this growing research field, we focus primarily on adaptive immune responses induced by the gut microbiota and mediated by intestinal effector and regulatory T cells. We further highlight non-immune cellular components that facilitate homeostatic host-microbiota communications in the gut. Finally, we discuss how disruptions to microbiota-immune system interactions provoke inflammatory responses in the gut and beyond and propose that rationally designed microbiota-based therapy may serve as an innovative avenue for future therapeutics.

Keywords Gut microbiota · Immune system · Effector and regulatory T cells · Autoimmunity · Inflammation

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1 Introductory Remark: Overview of Intestinal Tissue Structure

From the esophagus to the stomach and the intestine, the gastrointestinal tract functions to digest and absorb nutrients and water and also serves as a one-way exit for waste products formed. More recently, the gut has been discovered as an invaluable environment for immunoregulation and housing specialized intestinal immune cells. For the purposes of this chapter, we will shortly summarize the anatomical makeup of the intestines as their particularities are what offer insight in understanding homeostatic and pathogenic inflammatory responses (for a detailed review, see [1]).

The small intestine is distinguished by its finger-like projections called villi while the large intestine is reflected by a flat epithelial surface more suitable for water absorption. There are three compartments making up the small intestine that, in total, reach 6–7 m in length: the duodenum, the jejunum, and the ileum. The large intestine, while much wider, measures approximately 1.5 m. The mucosa is the innermost layer of the intestines (i.e., adjacent to the lumen of the intestines) and plays an extremely important part in the immune reactions as it is exposed to the luminal environment consisting of symbionts and pathogens. Moving outwards lie the submucosa, muscularis, and serosa layers [1]. Taking a closer look at the mucosa, mucus, made up of glycoproteins called mucins (the most abundant being MUC2), acts as the buffer between the epithelium and lumen; a single, unattached mucus layer is secreted in the small intestine, and a combination of a loose mucus layer and epithelial-attached dense mucus inner layer is formed in the colon. Bacteria, which penetrate the single mucus layer of the small intestine and the outer mucus layer of the large intestine, reside in the mucus, feed on the glycoproteins, and interact with a number of immune mediators that affect the microbiota such as IgA, defensin, and lysozyme [2, 3]. Antibacterial mediators in the small intestine keep the bacteria away from the epithelium, and in the large intestine the inner mucus layer remains mostly impenetrable [3].

Specialized intestinal epithelial cells (IECs) that make up the epithelial layer serve as unconventional immune cells that sense luminal content and maintain homeostasis among the harsh conditions and constant exposure to antigens. Invaginations of the epithelium called crypts of Lieberkühn penetrate the underlying connective tissue (termed lamina propria (LP)) within the mucosa [4]. Transit-amplifying cells, generated by intestinal stem cells (ISCs), residing at the base of these intestinal crypts differentiate into mature cell types known as IECs; the entire process takes only 3–5 days and characterizes the intestines through rapid-cell proliferation. Enterocytes are absorptive columnar epithelial cells and are the most prolific out of all the IECs. Enteroendocrine and goblet cells are also found among the cell types migrating upwards to the tips of the villi that secrete hormones for digestive regulation and mucins with antimicrobial properties, respectively. Paneth cells are another specialized IEC with antimicrobial properties as well as MUC2-producing capabilities [3] that remain beside ISCs at the base of intestinal crypts. Further, tuft

cells are responsible for sensing luminal contents [4, 5]. Identified in the ileum by decreased microvilli, Peyer's patches are lymphoid follicles with a barrier made up of microfold (M) cells that also sense luminal content and transport antigens as well as bacteria into the LP [5]. M cells-mediated sampling of luminal antigens facilitates antigen presentation by cells of the innate immune system (i.e., intestinal dendritic cells) to cells of the adaptive immune system [1].

Throughout this chapter, we will review the main cellular components that underlie microbial regulation of the enteric immune system and promote local and systemic immunological homeostasis.

2 Regulatory T Cells and Gut Microbiota

Regulatory T cells (Tregs) are critical regulators of host immunity. Although they constitute about 10% of total CD4+ T cell populations, they control the activity of most immunological cell types (both innate and adaptive arms) and can be found in every organ in the body. Thus, Tregs are involved in autoimmunity, allergy, inflammation, anti-tumor responses, and more. Tregs express the lineage-specific transcription factor (TF) FoxP3, and co-expression of additional TFs differentiates between major Treg subsets. Along the gastrointestinal (GI) tract, Tregs that co-express Helios and Gata3 are abundant in the small intestine, while Tregs co-expressing the RAR-related orphan receptor RORγ are most abundant in the colon [6].

2.1 Molecular Mechanisms of Microbiota-Mediated Immunoregulation: The Case of Bacteroides fragilis

The importance of the gut microbiota to homeostatic immunological maturation and function has long been proposed, most notably by Elie Metchnikoff by the end of the nineteenth century [7]. In more recent years, fundamental mechanistic studies have pinpointed Myd88-dependent pathways and toll-like receptors (TLRs) as central factors for recognition of gut microbiota by the intestinal epithelium. These findings contribute to our understanding of gut homeostasis and protection from intestinal injury [8].

Until recently, bacterial molecules that facilitate host-microbiota mutualism remained enigmatic. Groundbreaking studies by Dennis Kasper and colleagues revealed that a bacterial polysaccharide A (PSA) expressed by the gut commensal *Bacteroides fragilis* [9, 10] directs proper development and maturation of the intestinal immune system [11]. Monocolonization of germ-free (GF) mice with *B. fragilis* alone (but not PSA-mutant *B. fragilis*) corrected systemic T cell deficiencies, T-helper 1/2 (Th1/Th2) imbalances, and abnormal lymphoid organogenesis

observed in GF mice. Supplementing in vitro co-cultures of dendritic cells (DCs) and T cells with purified PSA molecule resulted in PSA presentation by DCs and triggered dose-dependent expression of the Th1 cytokine interferon gamma (IFNγ). Similarly, in vivo colonization of GF mice with PSA-deficient *B. fragilis* resulted in Th2-mediated pathologies of the thymus, including outgrowth of B cell-like follicles in the thymic medulla. These disorders were probably caused by inability to maintain microbiota-mediated Th1/Th2 balance in GF mice, and it has been proposed that *B. fragilis*-derived PSA protects against immune-mediated pathologies [11].

In a follow-up study [12], Mazmanian and Kasper demonstrated that treating mice with purified PSA protects them from experimentally induced colitis. Purified PSA induced anti-inflammatory interleukin (IL)-10 expression and suppressed pro-inflammatory IL-17 expression in intestinal immune cells. Collectively, these findings illustrate that microbiota-derived molecules possess immunomodulatory capacity that may be harnessed for therapeutic purposes in chronic inflammatory diseases [12].

Deeper mechanistic investigations enhanced our understanding of the immunomodulatory capabilities of *B. fragilis*. This bacterium was shown to induce Treg development and to increase their suppressive capacity (including IL-10 production), an effect mediated by PSA not only when administered prior to the induction of inflammation, but also after the induction of experimental colitis in mice [13]. Furthermore, *B. fragilis* was shown to activate the TLR pathway in order to establish host-microbial symbiosis and to actively suppress immunity, rather than to induce pathogen elimination [14]. PSA was found to signal via TLR2 expressed on intestinal Tregs to promote immunological tolerance; in the absence of PSA, *B. fragilis* was unable to restrict inflammatory T helper 17 (Th17) cell responses. This study suggests that colonization of symbiotic bacteria is accompanied by immunological recognition of the microbiota via specific recognition molecules that discriminate between mutualistic commensals and pathogens [14].

In a different inflammatory model, TLR2-dependent sensing of *B. fragilis*-derived PSA was shown to protect against demyelination and inflammation of the central nervous system (CNS) in animal models for autoimmune multiple sclerosis (MS) (experimental autoimmune encephalomyelitis (EAE) in mice) thus suggesting a systemic anti-inflammatory effect for *B. fragilis* [15].

2.2 PSA Molecule Transportation to Host Cells

How are PSA molecules delivered to host cells? One mechanism of such interkingdom communication is the release of PSA by *B. fragilis* via outer membrane vesicles (OMVs), which are then sensed by TLR2-expressing DCs, resulting in induction of Tregs and production of anti-inflammatory cytokines [16]. *B. fragilis*-derived OMVs were shown to protect the host from experimentally induced colitis [16], yet this OMV-mediated protection requires *Atg16l1* and *Nod2* genes, which are

associated with an increased risk for developing inflammatory bowel disease (IBD) in humans [17]. The expression of *ATG16L1* in CD11c + DCs is required to trigger a non-canonical autophagy pathway in a NOD2-dependent manner, required for Treg priming and protection from colitis [17]. Accordingly, immunocytes derived from human Crohn's disease (CD) patients containing the *ATG16L1* T300A risk variant failed to promote Treg development in response to *B. fragilis* OMVs. Thus, genetic polymorphisms in CD susceptibility genes result in failure to sense microbiota-derived protective signals, collectively suggesting that gene-environment interactions underlie the etiology of IBD [17].

2.3 B. fragilis *Modulates* iNKT *Cells Activity*

In addition to PSA production, *B. fragilis* shapes the host immune system by another thought-provoking mechanism: controlling the activity of invariant natural killer T (iNKT) cells by bacterially derived inhibitory sphingolipids [18]. iNKT cells are critical regulators of both innate and adaptive immunity, due to their ability to quickly release large quantities of cytokines upon activation by lipid antigens presented by non-polymorphic major histocompatibility complex (MHC) class I-like, CD1d protein [19–21]. *B. fragilis*-derived inhibitory sphingolipids were shown to significantly inhibit iNKT cell proliferation in the colon, a process that takes place early in life, during neonatal development. As a result, colonic iNKT cell numbers are constrained throughout development and into adulthood, which subsequently protect the host from experimental iNKT cell-mediated, oxazolone-induced colitis. This novel mechanism may support future development of bacterially derived sphingolipids as a potential therapy for autoimmune and allergic disorders that are mediated by damaging activity of iNKT cells [18].

2.4 Tissue Tregs

In addition to the established function of the immune system in protection from microbial challenges, recent studies highlight another critical function in maintaining local and systemic homeostasis. For instance, distinct populations of Tregs that reside in non-lymphoid tissues, including the visceral adipose tissue, skeletal muscle, and colonic LP, promote tissue functions, repair, and homeostasis in response to local signals [22].

2.5 Colonic Tregs and the Microbiota

Over the past decade, a collection of ground-breaking studies identified specific microbial species within the human microbiome that, when transferred into mice, can induce Treg development in the intestine as well as systemically. Similarly, microbe-derived metabolites (such as short-chain fatty acids (SCFA)) were found to mediate some of these effects and emerge as a new therapeutic approach to immunological diseases, including autoimmunity and allergy.

Seminal work by Kenya Honda and his colleagues revealed that spore-forming members of the gut microbiome, specifically the *Clostridium* genus (clusters IV and XIVa), induce the development and colonic accumulation of Tregs [23]. Moreover, oral supplementation of these microbes into mice triggered resistance to experimentally induced colitis and allergic immunoglobulin E (IgE) responses [23]. In follow-up work, a rationally selected mixture of 17 bacterial strains (clusters IV, XIVa, and XVIII of *Clostridia*, which lack virulence factors) isolated from a healthy human gut induced colonic Treg development when transferred into mice [24]. In addition to increasing Treg abundance, these microbes also induced the expression of anti-inflammatory mediators such as IL-10 and inducible T cell costimulator (ICOS) in Tregs and ameliorated inflammatory and allergic responses in mouse models of colitis and allergic diarrhea [24].

A unique subset of colonic Tregs was recently identified and thoroughly characterized [25, 26]. Surprisingly, these colonic Tregs co-express the RAR-related orphan receptor RORγ (a lineage marker of pro-inflammatory Th17 cells) in addition to the expression of FoxP3. Yet in the context of colonic Tregs, RORγ controlled the expression of major effector molecules (including *Il23r*, *Cxcr3*, *Tbx21*, and *Havcr2*), and promoted a transcriptional signature that differs from its activity in Th17 cells. Interestingly, the majority of RORγ+Helios-colonic Tregs are induced by the microbiota, and specific bacterial species of the human gut microbiota induced the accumulation of colonic Tregs when transferred into GF mice [25, 26]. The functional role of microbiota-induced colonic Tregs was demonstrated by monocolonization of GF mice with selected bacterial strains that induced different levels of colonic Tregs. In these mice, the colitis score was improved in association with increased levels of colonic Tregs [25]. Another factor that favors the development of FoxP3 + Tregs and of FoxP3 + RORγ+ colonic Tregs over Th17 cells is the vitamin A metabolite, retinoic acid (RA) [27, 28]. In mice, vitamin A deficiency specifically blocked the development of RORγt+ Tregs but not of Helios+ Tregs or Th17 cells [25, 26]. Mice lacking RORγ in Tregs were generated in two independent studies [25, 26], resulting in the absence of colonic Tregs, and subsequently increased frequencies of GATA3+ Helios+ Tregs, and increased production of the type 2 cytokines IL-4 and IL-5 in T cells. Compared with their wild-type littermates, these mice were more sensitive to experimental colitis, as indicated by trinitrobenzenesulfonic acid (TNBS)–induced [25] and oxazolone-induced colitis models [26]. The latter colitis model is dependent on the type 2 cytokines IL-4 and IL-13, suggesting that RORγ+ colonic Tregs regulate type 2 immune responses.

Furthermore, mice lacking RORγt in Tregs were more resistant to helminth infection (*Heligmosomoides polygyrus*) due to the increased production of IL-4, IL-5, and IL-13 throughout the infection [26]. Collectively, these studies identify *RoR*γ as a bacterially modulated factor that directs distinct cellular and transcriptional immunological responses and regulates opposite functional outcomes.

2.6 Commensal Gut Bacteria with Immunomodulatory Abilities

The quest for microbes that naturally inhabit the human gut, and possess unique immunomodulatory abilities, was significantly advanced by a recent systematic in vivo screen [29]. Cohorts of GF mice were monocolonized at 4 weeks of age with 53 individual bacterial species (selected based on the Human Microbiome Project [30]) representing the variety of phyla in the human gut microbiota (phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria*). A comprehensive analysis (microbiologic, immunologic, and transcriptomic) was performed at 2 weeks post-colonization, spanning the small and large intestines, as well as lymphoid organs including draining lymph nodes and spleen. This study generated a broad atlas that systematically dissects immune system-microbiota interactions in the gut. It yielded both anticipated and unanticipated insights, such as the identification of microbial strains capable of driving Th17 cell development in the SI (reaching a similar level as driven by segmented filamentous bacteria (SFB) [31, 32]; the identification of an array of bacterial strains, encompassing a diversity of species, that could induce RORγ+ Tregs in the colon (further investigated at: [25]; and the identification of bacterial strains that modulate the enteric immune system immune in unexpected ways (i.e., expansion of colonic, IL-10-producing CD4+ T cells by *Veillonella* 6.1.27 species, and reduction of plasmacytoid dendritic cell (pDC) numbers by *L. rhamnosus*) [29]. Furthermore, these findings suggest that bacterially induced changes, both immunologic and transcriptional changes, are not related to a particular microbial phylogeny. The experimental approach selected in this study – examination of the immunological effects induced by individual bacterial strains, one by one, in a gnotobiotic environment – simplifies the immense complexity of the intestinal microbiota. Clearly, these important findings (and their underlying mechanisms) should be further evaluated in a "real-world" environment, where complex inter-species interactions (between different microorganisms and the host) take place in the gut.

2.7 Colonic Treg Abundance Is Determined by Maternal Transmission of IgA in Breast Milk

The intestinal immune system maintains a homeostatic balance that tolerates the heavy burden of mutualistic microbiota, while retaining the capacity to eliminate gastrointestinal pathogens. The abundance of intestinal Tregs in the tissue greatly influences this balance, as Tregs limit tissue inflammation but delay pathogen clearance. Diverse inbred mouse strains differ in their colonic RORγ+ Treg proportions, which are stable for life and transmitted through multiple generations. But how is the intestinal setpoint for RORγ+ colonic Tregs determined? A recent study approached this question by a series of cross-fostering experiments between two lines of inbred mice that differ in their colonic proportions of RORγ+ Tregs: the B6 background (in which RORγ+ Treg abundances were ~ 40%–60% of total colonic Tregs) and the BALB/c background in which much lower frequencies were observed (~20%) [33, 34]. In a sequence of elegant experiments, the authors showed that the RORγ+ Treg setpoint is not driven by genetics or maternal microbiota transfer. Instead, colonic RORγ+ Tregs form a double-negative regulatory loop with immunoglobulin A (IgA), in which variable amounts of IgA are transferred by mothers to their offspring. This results in differences in neonatal IgA coating of gut microbes, which in turn condition RORγ+ Treg proportions in adults and regulate levels of intestinal IgA+ plasma cells. This phenotype is transferred through multiple generations because in female offspring, IgA+ plasma cells expand and migrate in late gestation via the entero-mammary axis, resulting in the IgA differences that are reflected in their breast milk. Thus, the abundance of RORγt+ Tregs in mouse offspring and in subsequent generations is dictated by a non-genetic, non-epigenetic mode of maternal inheritance during a critical time window after birth through IgA presence in breast milk [34].

2.8 Regulation of Intestinal Tregs by Cytokines

The cytokine IL-2 plays a major role in the development, maintenance, and function of effector and regulatory T cells [35–37]. A recent study identified IL-2 as a central factor that maintains intestinal Tregs, and thus immunological homeostasis [38]. Interestingly, cellular and transcriptional analysis of intestinal IL-2-expressing immunocytes identified type-3 innate lymphoid cells (ILC3s) as the major source of intestinal IL-2. IL-2 expression by ILC3s was induced by interleukin (IL)-1β produced by macrophages in response to microbiota-derived signals, and in a MYD88- and NOD2-dependent manner. Loss of IL-2 production by intestinal ILC3s led to a significant reduction in peripherally induced small intestinal (but not colonic) Tregs (detected by low neuropilin-1 expression) and an increase in Th1 effector T cells. Induction of small intestinal Tregs specific to the dietary antigen ovalbumin (OVA) was also impaired in mice deficient in IL-2-producing ILC3s, and

consequently OVA feeding did not induce oral tolerance in these mice. Intestinal biopsies collected from Crohn's disease (CD) patients contained reduced frequencies of both Tregs and ILC3s, compared with biopsies collected from healthy individuals. Remarkably ILC3s isolated from inflamed regions of resected small intestinal tissues of CD patients expressed low levels of *IL2* transcript compared with non-inflamed regions, and similarly the number of IL-2+ ILC3s was reduced in the terminal ileum of CD patients compared with healthy controls. Thus, microbiota-derived signals drive IL-1β production in macrophages, which triggers IL-2 secretion by ILC3s, required to maintain intestinal Tregs, oral tolerance to dietary antigens, and small intestinal homeostasis [38].

Another cytokine that regulates colonic Treg functions is the IL-1 family member, IL-33. In response to tissue damage, epithelial IL-33 functions as an intestinal danger signal (alarmin). Indeed, high levels of IL-33 were found in inflamed intestinal lesions of human IBD patients. Interestingly, ST2 (the IL-33 receptor) is highly expressed on colonic Tregs, and ST2 signaling promotes Treg function in the inflammatory environment, by supporting Treg accumulation and maintenance, and by inducing transforming growth factor (TGF)-β1-mediated Treg differentiation [39]. Intriguingly, IL-23, a pro-inflammatory cytokine involved in IBD pathogenesis, was found to confine Treg function by inhibition of IL-33 signaling in Tregs. Thus, the balance between IL-33 and IL-23 controls Treg responses and immunological tolerance in the intestine, in health and in disease [39].

2.9 Microbial Metabolites and Treg Induction: From Bench to Bedside

Despite significant advances in the identification of specific microbial strains within the human microbiota that drive anti-inflammatory (Tregs) or pro-inflammatory (Th17 cells) responses, the underlying molecular signals remain mostly elusive. Three groundbreaking studies revealed that the microbial metabolites, SCFAs, control Treg development and function to maintain homeostasis in the gut [40–42].

Undigestible dietary components, such as complex dietary fibers, are fermented by the microbiota in the colon. The metabolic by-products of this microbial fermentation are the SCFAs propionate, acetate, and butyrate, for which the luminal concentrations range from 50 to 100 mM under physiological conditions. However, in GF mice, SCFA concentrations are significantly reduced to ~10% of their concentrations in mice reared under specific pathogen-free (SPF) conditions [40]. Similarly, Treg abundance within the GF colon is similarly impaired. Strikingly, administering SCFAs to GF mice successfully restored Treg numbers and function (i.e., IL-10 production) in the colon. SCFAs could also augment Treg quantities and suppressive capacity in SPF mice. Moreover, administration of propionate [40] or butyrate [41, 42] alone could induce Treg differentiation and function, in vitro and in vivo. Functionally, SCFA administration to mice

ameliorated experimentally induced T cell–dependent colitis in a Treg-intrinsic, *Ffar2*-dependent manner. How do SCFAs exert their anti-inflammatory effects? Mechanistically, SCFAs signal directly on Tregs via the GPCR43 receptor, encoded by the *Ffar2* gene which is highly expressed by Tregs in response to microbiota-derived signals. SCFA signaling (most notably butyrate and propionate [41, 42]) inhibits histone deacetylase (HDAC) activity and thus triggers epigenetic chromatin modification (acetylation) at the *Foxp3* locus (CNS1, CNS2, and CNS3 enhancers) which promotes *FoxP3* transcription and Treg development. These findings mechanistically link colonic production of SCFAs (primarily butyrate and propionate) by microbiota-mediated fermentation of dietary fibers, with colonic Treg accumulation and the maintenance of anti-inflammatory immune homeostasis in the gut and systemically.

The substantial clinical potential of the use of SCFAs to treat autoimmune and chronic inflammatory diseases was evaluated in several follow-up studies. An impressive example of the effects of dietary fatty acids (FAs) on systemic autoimmunity was demonstrated for multiple sclerosis (MS), a T cell-mediated autoimmune disease of the CNS [43, 44]. Administration of propionic acid (PA) to mice, using the EAE model for MS, resulted in the accumulation of small intestinal Tregs, increased production of anti-inflammatory cytokines (i.e., TGF-β and IL-10), and an improvement in clinical outcome, as reflected by reduced inflammatory cell infiltration in the spinal cord, as well as reduced demyelination, and increased axonal preservation [44]. In sharp contrast, long-chain FAs (the most abundant component of the Western diet) exacerbated disease, by enhancing Th1 and Th17 differentiation in the small intestine via the p38-MAPK pathway in T cells [44]. Collectively, these findings show that dietary-induced changes in the gut modulate pro- and anti-inflammatory T-helper cell responses, leading to exacerbation or amelioration of CNS autoimmunity.

These findings were leveraged for a translational study that examined the effects of PA administration in human MS patients [43]. Metabolite analysis in serum and stool samples revealed that PA levels are reduced in MS patients, compared with healthy individuals. These reduced PA levels were accompanied by a dysbiotic gut microbiome composition, and altered Treg/Th17 cell balance, where MS patients exhibit increased levels of Th17 and a reduced level of Tregs in their serum.

MS patients (both therapy-naïve and as an add-on to already administered therapy) were then supplemented with PA pills (orally, on a daily basis). Remarkably, only 2 weeks following PA therapy, Th1/Th17 proportions were decreased, while Tregs proportions were significantly increased, and their suppressive capacity was augmented. In agreement, analysis of clinical data (including MRI scans) revealed a significant reduction in annual relapse rate (ARR), reduced disease progression, and an increase in volume of subcortical gray matter in MS patients under PA supplementation. These effects lasted at least 3 years after PA intake. Examination of the functional effects of post-PA microbiome was performed by transfer of microbiota samples collected from MS patients before and after PA therapy, into a 3D gut organ culture system. Surprisingly, post-PA microbiota elicited rapid transcriptional responses that correlate with Treg induction

(as determined by [45]), suggesting that PA therapy transforms gut microbiome composition into a Treg-inducing configuration. Collectively, these findings suggest that the anti-inflammatory effects of PA could serve as a potent immunomodulatory treatment that supplements available MS drugs, and may potentially benefit other autoimmune diseases [43].

2.10 Bile Acid Metabolites

In addition to SCFAs, two recent studies reveal another group of microbe-derived bioactive molecules that interact with the host and modulate colonic Treg development and function: these are microbiota bile acid (BA) metabolites [46, 47]. In the liver, hepatocytes synthesize primary BAs that are released into the small intestine (duodenum) to enable the absorption of lipids and fat-soluble vitamins. Although most BAs are utilized in the small intestine, a small portion (~5%) reach the colon, where the gut microbiota converts them into a complex collection of steroid hormones that regulate host metabolism (including energy balance and cholesterol metabolism) via nuclear- and G-protein-coupled receptors. Two independent studies revealed that enteric BA composition is determined by both dietary and microbial factors, which also modulate tissue accumulation and function of colonic RORγ+ Tregs.

In the first study, SPF and GF mice were fed either a nutrient-rich diet or minimal diet, in order to identify microbially modified metabolites that possess Treg-inducing capabilities [47]. Analysis of colonic RORγ+ Treg abundance detected lower numbers of colonic Tregs in both minimal-diet SPF mice and rich-diet GF mice, compared with rich-diet SPF mice. This effect was specific to the colon, as no difference in RORγ+ Treg abundance was detected in other regions of the GI tract or in other lymphoid organs (including mesenteric lymph nodes, Peyer's patches, thymus, or spleen). Colonic RORγ+ Treg frequency was recovered by switching from a minimal to nutrient-rich diet (in a microbiota-dependent manner), suggesting that dietary components modified by bacterial symbionts are probably responsible for colonic Treg induction. Colonizing mice with specific gut microbes that were genetically modified and could not support bacterial BAs metabolism resulted in a significant decrease of this colonic Treg population. Vice versa, restoration of the enteric BAs triggered colonic RORγ+ Treg expansion and ameliorated intestinal inflammation in experimentally induced colitis via BA nuclear receptors [47].

In the second study, major types of deconjugated BAs were screened for their ability to induce colonic Treg differentiation [46]. The secondary BA, 3-β-hydroxydeoxycholic acid (isoDCA), was found to increase Treg development by direct impact on dendritic cells (DCs), resulting in reduced immunostimulatory activity of DCs. In addition, genetic depletion of the farnesoid X receptor in DCs mimicked the effects of isoDCA on cellular transcription and Treg induction. Likewise, colonization of mice with engineered consortia of gut *Bacteroides* strains

that produce isoDCA induced colonic RORγ+ Treg differentiation and tissue accumulation [46].

Collectively, these studies identify a novel class of bioactive molecules that mediate the immunological effects of the gut microbiota, control immunological homeostasis in the host, and may be utilized as future therapeutic interventions (Fig. 1).

3 Effector T Cells and Gut Microbiota

3.1 T Helper 17 Cells: Development and Function

CD4+ T helper 17 (Th17) cells are characterized by the expression of the RAR-related orphan receptor RORγt and by the production of hallmark cytokines: IL-17, IL-22, and IL-21 [48–50]. Th17 cells are most abundant in the intestinal LP, where they control immunological responses to various extracellular bacterial and fungal infections (e.g., by promoting high-affinity secretory IgA humoral responses and antimicrobial proteins secretion by IECs). The cytokines IL-6 and TGF-β are required to induce Th17 differentiation from naïve T cell precursors, while IL-23 promotes the expansion of differentiated Th17 cells [48, 51]. TGF-β is required for differentiation of both Th17 and Treg cells, although development of these two cell types depends on distinct transcription factors (RORγt and FoxP3, respectively). Both TFs are co-expressed in naive CD4+ T cells, and the decision of antigen-stimulated T cells to differentiate into Th17 or Tregs is regulated by TGF-β concentrations, as well as the balance between TGF-β concentrations and the presence of pro-inflammatory cytokines such as IL-6 and IL-21 [52].

Besides their role in mediating immunity against infection, Th17 cells were shown to promote autoimmunity and inflammatory diseases in mouse models and in humans, including MS, IBD, rheumatoid arthritis, psoriasis, and more [53]. In addition to infiltration of pathogenic Th17 cells into the affected tissues during pathology, Th17 cells facilitate tissue accumulation of other immunocytes including other T-helper cells, macrophages, and neutrophils, which foster pathologic inflammation and tissue damage.

Thus, Th17 cells can promote both homeostatic, non-inflammatory roles (i.e., by supporting intestinal barrier integrity) and pathogenic, pro-inflammatory roles in many inflammatory diseases [54]. Interestingly, the gut microbiota regulates differentiation and function of homeostatic and pathogenic Th17 cells, as we will discuss below [55].

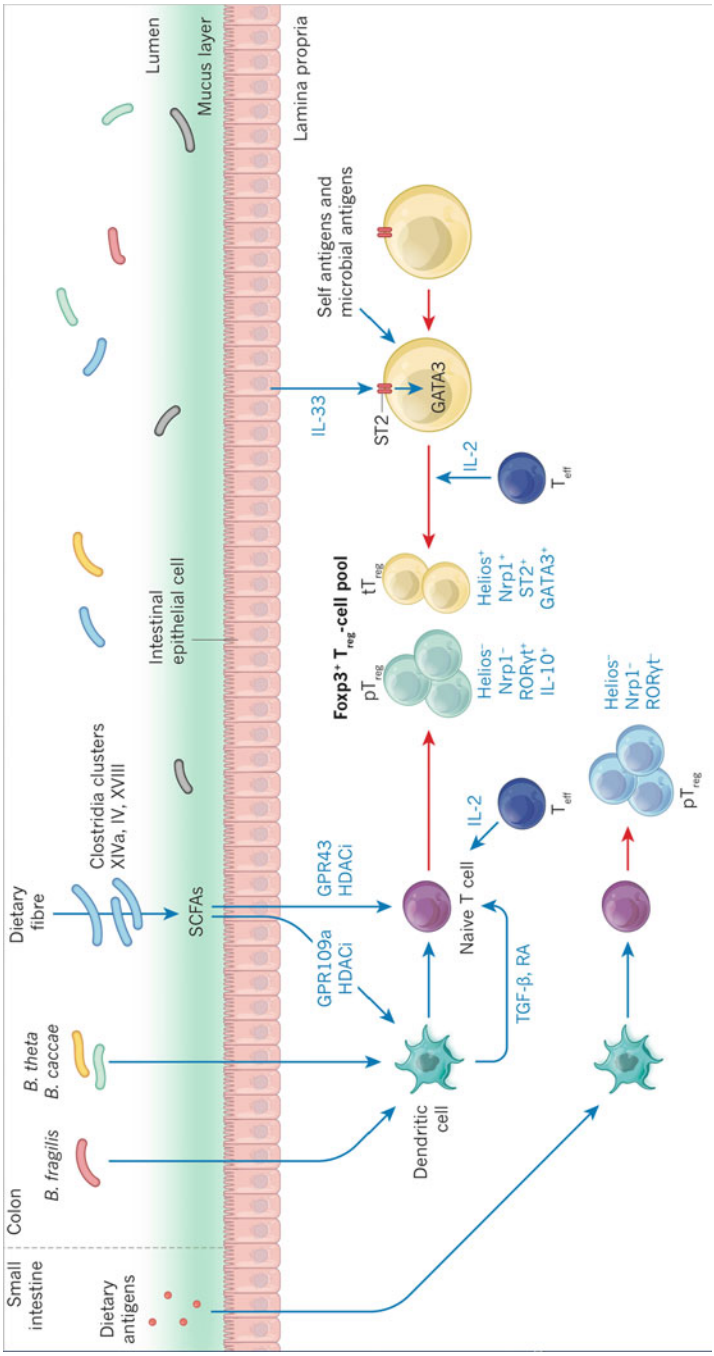

Fig. 1 Influence of the microbiota and diet on subsets of regulatory T cells in the intestine. Foxp3-expressing T_{reg} cells in the intestine can be subdivided into at least three subsets on the basis of their expression of RORγt, GATA3, Helios, and Nrp1. T_{reg} cells that express RORγt but not Nrp1 are induced at peripheral sites by antigens derived from the microbiota. Known as pT_{reg} cells, they are the main producers of IL-10, which suppresses the aberrant activation of myeloid cells, γδ T cells, and T_H17 cells. Dendritic cells produce mediators of pT_{reg}-cell differentiation, including TGF-β and retinoic acid (RA). Short-chain fatty acids (SCFAs), which are produced from dietary fiber by certain members of the microbiota, particularly species of Clostridia, also contribute to the induction of pT_{reg} cells. On binding to the G-protein-coupled receptor (GPR)109a on dendritic cells, SCFAs induce the expression of aldehyde dehydrogenase (ALDH), which

3.2 *Induction of Th17 Cells by Segmented Filamentous Bacteria (SFB)*

The notion that the gut microbiota controls the development and accumulation of intestinal Th17 cells was established by a series of seminal studies by Dan Littman and Ivaylo Ivanov [31, 56], a discovery that was followed up by intensive research efforts that deeply characterized how microbial signals regulate effector T cell development and function.

Littman and colleagues noticed that inbred mice purchased from different vendors markedly differ in their intestinal Th17 numbers, despite an identical genetic background [56]. Moreover, they showed that Th17 cells (which are particularly enriched in the small intestinal LP) do not develop in mice lacking microbiota. However, Th17 development could be induced by co-housing Th17 cell-sufficient mice with Th17-deficient mice through horizontal microbiota transfer.

Strikingly, transfer of a single bacterial species, segmented filamentous bacterium (SFB), is sufficient to induce Th17 development in the small intestinal LP [31, 57]. SFB was found to colonize mainly the small intestinal terminal ileum, and to tightly adhere to the intestinal epithelium. SFB colonization activated inflammatory gene expression as well as antibacterial defenses including antimicrobial peptides and mucosal IgA responses, collectively resulting in increased resistance to pathogenic infection by the murine pathogen *Citrobacter rodentium* [31, 57]. Moreover, SFB colonization induces the production of serum amyloid A (SAA) in the terminal ileum, which acts on DCs to promote Th17 cell differentiation. *Saa* genes (*Saa1/Saa2/Saa3*) encode acute-phase response proteins that function as cytokines to induce pro-inflammatory cytokine secretion (including IL-8, TNFα, IL-1β, and IL-23) during infection or tissue damage.

Of note, SFB-induced Th17 cells produce the Th17 hallmark cytokines IL-17 and IL-22 and are fully capable of promoting pro-inflammatory responses, not only during infection but also in an autoimmune context. This was demonstrated using the K/BxN mouse model for autoimmune arthritis [58]. Inflammatory arthritis in the

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Fig. 1 (continued) metabolizes vitamin A into RA. SCFAs entering dendritic cells act as inhibitors of histone deacetylase (HDACi) to suppress the expression of pro-inflammatory cytokines. They also directly act on naive T cells through GPR43 or the upregulation of Foxp3 expression through HDAC inhibition. IL-2 derived from T_{eff} cells probably helps to stabilize the differentiation of T_{reg} cells. Several species of *Bacteroides* contribute to the induction of pT_{reg} cells that express RORγt but not Nrp1 through dendritic cells. A second pool of pT_{reg} cells expresses neither RORγt nor Nrp1; these T_{reg} cells are induced by, and maintain immune tolerance to, dietary antigens. It should be noted that induction of pT_{reg} cells through dietary antigens occurs largely in the small intestine, whereas the induction of pT_{reg} cells by the microbiota occurs largely in the colon. T_{reg} cells that express both GATA3 and Nrp1 are thought to be generated in the thymus and are known as tT_{reg} cells. $GATA3^+$ T_{reg} cells express ST2 (a component of the IL-33 receptor that is also known as IL1RL1). IL-33, which is probably released from the epithelial cells of the intestine at steady-state, is markedly upregulated under conditions of inflammation. IL-33 acts with IL-2 (from T_{eff} cells) to induce the expression of GATA3 in T_{reg} cells. (Adapted from [55])

K/BxN T cell receptor (TCR) transgenic mouse model is provoked by an initiation phase and an effector phase [59, 60]. During the initiation phase, which relies on the adaptive immune system, a self-peptide derived from glucose-6-phosphate isomerase (GPI) is presented by the major histocompatibility complex class II (MHCII) molecule, Ag7, and recognized by T lymphocytes that display a transgenic TCR. These autoreactive T cells effectively help GPI-specific B cells to produce GPI autoantibodies that drive the effector stage, which is executed primarily by cells of the innate immune system. Here, GPI/anti-GPI immune complexes initiate an inflammatory response that recruits mast cells, neutrophils, the complement pathway, etc. Shortly after (beginning at 4 weeks of age), arthritis develops with almost 100% penetrance. Intriguingly, the effector phase can be mimicked by transfer of serum from K/BxN mice into wild-type healthy mice.

In an elegant study by the Mathis/Benoist lab [58], the authors demonstrated that autoimmune arthritis in the K/BxN mouse model was significantly attenuated under GF conditions, establishing a role for the gut microbiota in disease initiation and progression. Under GF conditions, the anti-GPI serum autoantibody titer was significantly reduced, and similar reduction was observed in splenic autoantibody-secreting B cells, splenic and small intestinal Th17 cells, and in overall ankle thickening. The pathological link to Th17 cells was further demonstrated by treating SPF K/BxN mice with neutralizing anti-IL-17 antibodies which prevented B-cell responses and arthritis development. Remarkably, monocolonizing GF mice with SFB alone restored the intestinal Th17 cell compartment, and autoantibody production, and rapid arthritis developed. These findings highlight the role of microbiota-induced Th17 cells in driving extra-intestinal autoimmune disease [58].

3.3 How Does SFB Trigger Intestinal Th17 Development?

Whole-genome sequencing and annotation analysis reveals that the 1.57 Mb SFB genome contains no virulence genes, despite its resemblance to pathogenic Clostridia [61, 62]. SFB was found to be an auxotroph for most essential cofactors and amino acids, and the SFB genome contains TLR5 agonists that are flagellin proteins, which stimulate Th17 cell production.

Intestinal induction of Th17 cells was shown to be mediated by MHCII molecules expressed by CD11c + intestinal dendritic cells (DCs) that present SFB antigens [63]. Comprehensive analysis of the TCR repertoire demonstrated that intestinal Th17 cells are specific for SFB- and other microbiota-derived antigens [64]. T cells having a TCR specific for SFB-derived peptides were shown to differentiate into Th17 cells. Moreover, T cell differentiation toward Th1 or Th17 lineages was found to depend on bacterial context, or which type of luminal bacteria delivered the antigen [64].

Further studies identified a role for the intestinal epithelium, as well as for type 3 innate lymphoid cells (ILC3), in mediating Th17 induction in the small intestinal ileum [65, 66]. Adherence of SFB to the ileal IECs was found to be a critical signal

for Th17 induction. Indeed, colonizing mice with a non-adherent SFB strain isolated from rats failed to induce Th17 cells. Epithelial adherence of SFB facilitates IL-22 secretion by ILC3 cells, which then trigger the production of serum amyloid A proteins 1 and 2 (SAA1/2) by IEC (in a STAT3-dependent manner). SAA1/2 proteins act directly on intestinal Th17 cells and amplify the production of IL-17A as well as other hallmark effector cytokines. Recently, SAA1/2/3 proteins were shown to function as pro-inflammatory factors that induce pathogenic Th17 cells and promote intestinal inflammation independently of TGF-β [67].

Interestingly, bacterial adhesion to the IEC was required for Th17 induction not only by SFB, but also by a wide array of Th17-inducing microbes, including the murine pathogen *Citrobacter rodentium*, *Escherichia coli O157*, and a mixture of 20 bacterial strains isolated from feces of a human colitis patient, which induced Th17 development in an IEC-adherence dependent manner [66]. The importance of epithelial adhesion was clarified in a recent study by using electron tomography, illustrating that SFB proteins are transferred into IECs by adhesion-directed endocytosis [68]. In this process, which is different from the endocytosis induced by invasive pathogens, proteins associated with bacterial cell wall are delivered into the cytoplasm of the IECs, eventually leading to the induction of Th17 cells. This mechanism illustrates how the host and the microbiota communicate under physiological conditions, and how T cell responses specific to commensal antigens are triggered by IECs [68].

3.4 Th17-Inducing Microbes in Humans

The identification of SFB as a potent inducer of intestinal Th17 cells in rodents motivated a search for microbial symbionts that naturally inhabit the human gut and possess Th17-inducing properties. A systemic approach for in vivo monocolonization of GF mice with a large collection of human symbionts led to the identification of several bacterial species that possess the ability to induce the development and tissue accumulation of Th17 cells in the small intestinal LP [32]. These phylogenetically diverse species provoked Th17 development as efficiently as SFB. The most robust Th17-inducer identified in this screen was *Bifidobacterium adolescentis*, a human symbiont that (together with other *Bifidobacterium* strains) can be found in the healthy human gut microbiota across age and geography. When introduced into GF mice, *B. adolescentis* was closely associated with the gut epithelium – similar to SFB. Moreover, as with SFB, *B. adolescentis* inoculation exacerbated autoimmune arthritis in the K/BxN mouse model. Yet the transcriptional responses induced by *B. adolescentis* in the small intestine were different than SFB-induced gene programs, suggesting that these two microbes elicit Th17 development using distinct molecular mechanisms. Overall, this study proposes that Th17-inducing bacteria exist in the healthy human microbiota, in numbers and diversity yet to be determined [32] (Fig. 2).

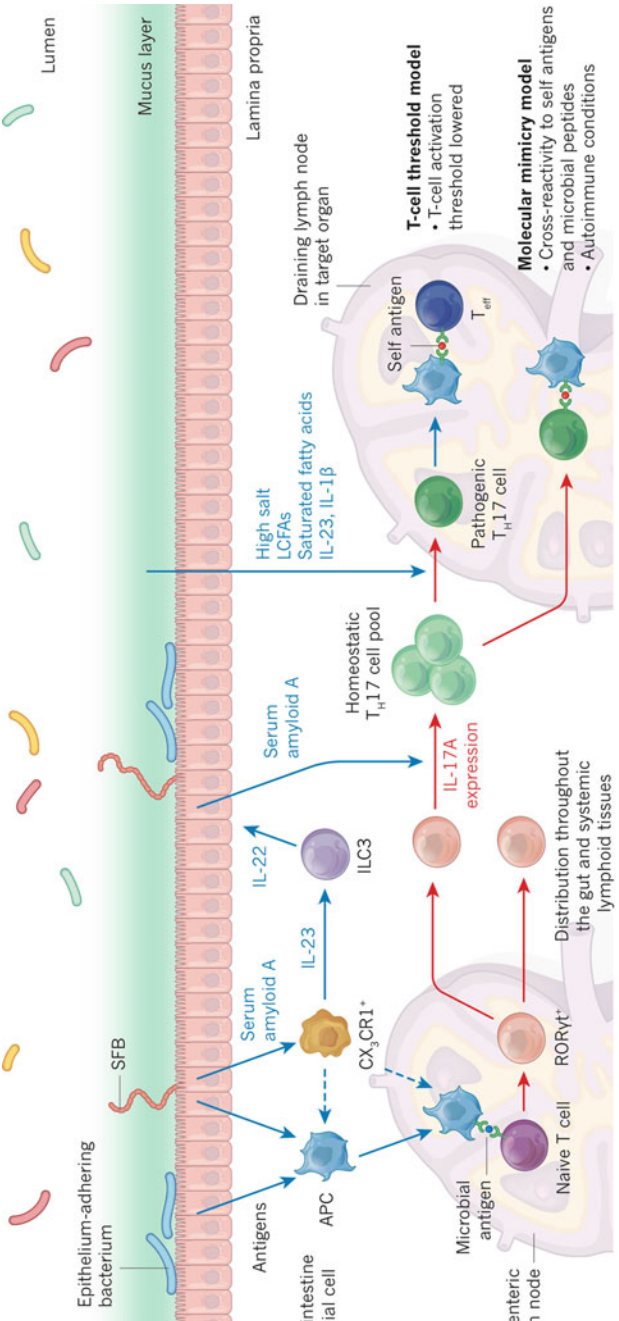

Fig. 2 Microbiota-mediated induction of T_H17 cells and autoimmunity. Epithelium-adhering bacteria initiate the differentiation of naive $CD4^+$ T cells into RORγt-expressing T cells (T_H17 polarized cells) (red) in the mesenteric lymph node through as-yet-undefined antigen-presenting cells (APCs). T_H17 polarized cells then accumulate and further differentiate into IL-17-expressing homeostatic T_H17 cells (green) in the lamina propria of the small intestine. These homeostatic T_H17 cells then stimulate epithelial cells to enhance the integrity of the intestinal mucosal barrier. The adhesion of segmented filamentous bacteria (SFB) elicits a unique program of gene expression in the epithelial cells, including the upregulation of serum amyloid A. Serum amyloid A from epithelial cells of the small intestine seems to function as a cytokine, and it modulates CX_3CR1-expressing cells (that are derived from monocytes) to produce IL-23, which stimulates the production of IL-22 by ILC3s. As well as its effects on CX_3CR1-expressing cells, serum amyloid A can stimulate RORγt-expressing T cells directly to upregulate the expression of IL-17A. Dendritic cells that express the antigens CD11b and CD103 have also been implicated in the expansion and maintenance of T_H17 cells (not shown). T_H17 cells become pathogenic when they are stimulated with IL-23, IL-1β, higher concentrations of salt, long-chain

3.5 Induction of Intestinal Th1 Cells by the Oral Microbiota

The oral cavity contains a large number of oral-resident bacteria which, in heathy individuals, hardly colonize the healthy intestine when ingested [69, 70]. However, transmission of oral bacteria to the gut can be detected under pathological conditions, such as IBD [71], liver cirrhosis [72], and colon cancer [73]. A recent study by Kenya Honda and collogues isolated a group of antibiotic-resistant *Klebsiella* strains from human saliva [74]. These oral bacteria were found to colonize the gut under dysbiotic conditions, where they act as potent inducers of pro-inflammatory, effector T-helper 1 (Th1) cells. Oral administration of isolated *Klebsiella* species to *Il10−/−* GF mice elicited potent induction of colonic Th1 cells, accompanied with severe gut inflammation. In agreement, the relative abundance of these *Klebsiella* species was significantly higher in human IBD patients compared with healthy individuals. These finding propose that the microbiota of the oral cavity may serve as a pool of pathobionts that exacerbate intestinal inflammation in genetically susceptible individuals [74].

Interestingly, periodontal inflammation within the oral cavity (periodontitis) facilitates the expansion of oral pathobionts, such as *Klebsiella* and *Enterobacter* species [75]. When ingested, these oral microbes trigger colonic inflammation by activating the inflammasome in colonic mononuclear phagocytes. Separately, Th17 cells that possess oral pathobiont specificity are generated in the oral cavity during periodontitis and migrate to the inflamed gut, where they are activated by translocated oral pathobionts (but not gut-resident microbiota) and elicit colitis development. Thus, parallel pathways of oral microbiota translocation and migration of orally generated effector T cells mediate and exacerbate gut inflammation [75].

3.6 Effector T Cells, Microbiota, and Disease

The concept that regulation of effector T cell activity by the gut microbiota may control both intestinal and systemic pathological processes was demonstrated in numerous studies over the past decade.

In the gut, a large-scale in vivo analysis of GF mice colonized with gut microbiotas from healthy and IBD individuals followed enteric T cell responses following colonization [76]. Interestingly, GF mice colonization with IBD-related

Fig. 2 (continued) fatty acids (LCFAs), and saturated fatty acids. Pathogenic T_H17 cells can migrate to the draining lymph nodes of target organs, where they contribute to autoimmune disease through cross-reactivity between peptides from microbes and self antigens (the molecular mimicry model). Alternatively, microbiota-specific T_H17 cells migrate to the lymph nodes and lower the threshold of activation of auto-reactive T cells such as T_{eff} cells (the T cell threshold model). (Adapted from [55])

microbiota promoted differentiation and enteric accumulation of Th17 and Th2 cells and decreased RORγt+ Tregs levels, thus altering the balance between Th17 and colonic Treg cells. Moreover, IBD microbiota transfer exacerbated disease in a mouse model of experimentally induced colitis. Strikingly, Th17/Treg proportions induced by each microbiota were predictive of disease status in the human donors, and of disease severity induced in the colitis mouse model. This study illustrates the impact of the gut microbiota in controlling the balance between inflammation and immunological tolerance in the gut, and emphasizes that the gut microbiota is a major factor determining IBD pathogenesis [77].

In the spinal cord, synergistic effects induced by two strains of gut microorganisms, OTU0002 (a newly isolated strain of the *Erysipelotrichaceae* family) and *Lactobacillus reuteri*, were shown to exacerbate spinal cord inflammation in mice by enhancing Th17 responses [78].

In the kidney, intestinal Th17 cells were shown to participate in an aggressive autoimmune kidney disease called crescentic glomerulonephritis (cGN) [79]. cGN is associated with high morbidity and mortality as it destroys the kidneys and leads to end-stage renal failure within weeks, or as little as several days. Th17 immune responses play a major role in cGN pathogenesis. Labeling and tracking intestinal Th17 cells using photoconversion of intestinal cells in Kaede mice upon glomerulonephritis induction led to the identification of Th17 cell migration from the intestine to the kidney. A functional role for intestinal Th17 cells in renal disease pathogenesis was demonstrated by depletion of Th17 cells (in GF mice and in mice treated with broad-spectrum antibiotics) which ameliorated disease, and by expanding Th17 cells (during *Citrobacter rodentium* infection) that exacerbated pathology. Thus, microbiota-induced Th17 cells migrate from the intestine to distal organs, where they participate in pathological immune responses [79].

In the eye, activation of retina-specific T cells was shown to be dependent on the gut microbiota [80]. These cells break through the blood-retinal barrier (which normally sequester retinal antigens to prevent pathogenic T cell activation) and promote the development of autoimmune uveitis, a major cause of blindness in humans. In this study, Caspi and colleagues used mice that express a TCR specific to interphotoreceptor retinoid binding protein (IRBP), a major uveitogenic epitope. These mice spontaneously develop uveitis at weaning age and reach 100% incidence by 2 months. The authors showed that retina-specific Th17 cells are activated by microbiota-derived signals in the intestinal lamina propria, leading to disease onset in the eyes. Interestingly, activation of retina-specific T cells was independent of the endogenous retinal autoantigen, but involved TCR signaling in response to non-cognate antigen in the gut. Thus, commensal microorganisms signal to autoreactive T cells that drive spontaneous uveitis in the eyes – an immune-privileged site. The notion that autoreactive T cells are activated by the gut microbiota might represent an underappreciated mechanism for autoimmunity in general [80].

In the heart, myocarditis is a chronic inflammation of the heart muscle associated with heart failure. It can be developed by chronic stimulation of Th1 and Th17 cells that specifically recognize myosin heavy chain 6 (MYH6)-derived peptides. Using a

mouse model of spontaneous autoimmune myocarditis, a recent study discovered that the progression of autoimmune myocarditis to dilated cardiomyopathy is microbiome-dependent [81]. Peptide mimics from commensal *Bacteroides* species were shown to imprint cardiac myosin-specific Th17 cells in the intestine, a process which could be prevented by antibiotics. In agreement, human myocarditis patients display immune reactivity against *Bacteroides* and cardiac myosin antigens, characterized by significantly elevated *Bacteroides*-specific CD4+ T cell and B cell responses. This study suggests that microbiome manipulation may restrain cardiotoxic T cell responses, and thus turn inflammatory cardiomyopathy into a targetable disease.

3.7 Diet, Microbiome, and Th17 Cells

Diet is one of the most significant factors that shapes human microbiome composition and function [82, 83]. In the past decades, several groundbreaking studies began to unravel the effects of diet on host immunity, and to identify mechanistic links that associate a diet-modified microbiome to immunological development and function.

One example is salt (sodium chloride), a nutritional component that is highly abundant in "western" diets (in which processed foods high in salt, sugar, and fat are abundant, and fresh fruits and vegetables are scarce). In addition to sodium in the plasma (in concentrations of approximately 140 mM), immunocytes are also exposed to sodium in the interstitial fluid and in lymphoid tissues, where sodium concentrations are significantly higher (between 160 mM and up to 250 mM). Two independent studies found that increased salt concentrations enhance Th17 induction in mice and humans [84, 85]. Mechanistically, high-salt conditions during cytokine-induced Th17 polarization activate the TF nuclear factor of activated T cells 5 (NFAT5) as well as serum/glucocorticoid-regulated kinase 1 (SGK1) via the p38/MAPK pathway. In response to increased salt concentrations, SGK1 promotes IL-23R expression by deactivation of mouse Foxo1, resulting in stabilization of the Th17 phenotype. These high-salt-induced Th17 cells present a pathogenic, pro-inflammatory phenotype, characterized by the production of pro-inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α and IL-2. Moreover, these Th17 cells amplified autoimmunity in a mouse model for MS (murine EAE). Thus, increased dietary salt intake induces pathogenic Th17 cell development, and serves as a risk factor that promotes autoimmune inflammation [84, 85].

Another more recent study investigated the effect of high-salt intake on gut microbiome composition and Th17 development [86]. Interestingly, a high-salt diet broadly alters gut microbiome composition in mice, and particularly depletes the symbiont *Lactobacillus murinus*. In two disease models, EAE and salt-sensitive hypertension, supplementing mice with *L. murinus* cultures ameliorated pathological phenotypes by reducing Th17 levels. Moreover, a proof-of-principle study in healthy humans showed that a moderate high-salt challenge increased blood pressure

and Th17 cell abundance and reduced *Lactobacillus* species levels in the gut. Thus, Th17 induction via the diet-microbiome axis may account for the striking increase in the incidence of autoimmune diseases in developed countries, within the past half-century [87].

Another example is the effect of a ketogenic diet on microbiome composition and intestinal Th17 cell abundance. Ketogenic diets are very low in carbohydrate and very high in fat. This triggers a metabolic shift that increases the levels of circulating ketone bodies, and then uses them for energy production. A recent study illustrates that transition to a ketogenic diet modifies gut microbiome composition, including alteration in the main microbial phyla (*Bacteroidetes*, *Firmicutes*, and *Actinobacteria*) in a representative cohort of healthy humans [88]. Shotgun metagenomic analysis revealed that these changes were distinct from changes observed in response to a high-fat diet, and partly resulted from direct inhibition of gut bacteria, especially bifidobacteria species, by ketone bodies. Some of these microbes, such as *B. adolescentis*, were shown to induce Th17 development in the small intestinal LP [32]. Indeed, using monocolonizations and human microbiome transplantations into germ-free mice, the authors revealed that reduced levels of intestinal *B. adolescentis* during a ketogenic diet resulted in deficiency of intestinal Th17 induction, in part due to the production of ketone bodies. This study further demonstrates the importance of diet-microbiota interactions on pro-inflammatory Th17 cell development [88]. Collectively, understanding how dietary changes modulate host immunity via alterations to gut microbiome composition represents a novel and promising frontier that may lead to the development of personalized approaches to prevent and treat human diseases by dietary interventions.

4 Enteric Neuro-Immune-Microbiota Interactions

4.1 The Enteric Nervous System

The enteric nervous system (ENS) is the largest component of the autonomic nervous system with over 100 million neurons (comparable to the amount in the spinal cord) making up the ENS in humans. Intrinsic ENS sensory neurons (whose cell bodies inhabit the gut wall), interneurons, and motor neurons are spatially organized in two layers of interconnected ganglia: the myenteric (or Auerbach) plexus (MP) and the inner, submucosal (or Meissner) plexus (SP). In addition to enteric neurons, the ENS contains enteric glial cells (EGCs) that can be found in the enteric ganglia, the smooth muscle layer, and the LP. These complex neuronal networks synchronize smooth muscle contractions (MP) and control gut secretions and nutrient absorption (SP). Remarkably, the ENS can control gastrointestinal functions independently of CNS input (although normally, bi-directional communication between the CNS and ENS mutually regulate these two systems).

Many anatomical features, neurotransmitters, and signaling pathways are shared between the ENS and CNS. The use of cutting-edge approaches for single-cell RNA

sequencing allowed, only recently, deep characterization of the mouse and human ENS [89, 90]. Besides the generation of detailed cellular atlases of the ENS at single-cell resolution, and the identification of dozens of neuronal subsets, these studies revealed novel intercellular communication modules that connect the ENS to the intestinal epithelium, immune system, and stromal cells, as well as to extraintestinal organs such as the brain.

These studies are in line with emerging research, collectively suggesting that the close interactions of the ENS with cells of the enteric immune system and the microbiota profoundly affect host immunity and homeostasis [91, 92].

4.2 Neuron-Macrophage Interactions with the Microbiota Control Gut Homeostasis

Tissue-residing macrophages are highly abundant in all layers of the small and large intestine [1]. They preserve mucosal integrity by scavenging dead cells, promoting epithelial cell renewal and tissue remodeling, and producing the anti-inflammatory cytokine IL-10, in addition to their phagocytic activity towards pathogenic and commensal bacteria. After decades of simplistic partition of macrophage into "M1-like" and "M2-like" subtypes, they are now perceived as a highly heterogeneous and diverse population that retain specialized, tissue-specific properties [93].

In a seminal study, Bogunovic and colleagues identified a unique subset of muscularis macrophages (MMs) residing along nerve fibers in the colonic muscularis externa, forming close interactions with enteric neurons [94]. Intriguingly, these cells were found to regulate intestinal peristaltic contractions, as specific depletion of MMs resulted in intestinal dysmotility. Mechanistically, MMs were shown to produce high quantities of bone morphogenetic protein 2 (BMP2)—a secreted protein of the TGF- ß superfamily—and perturbations to BMP signaling altered gut peristalsis and accelerated colonic transit time. Remarkably, MM-derived BMP2 stimulated enteric neurons via BMP receptor II (BMPRII), to secrete the growth factor colony-stimulating factor 1 (CSF1), a crucial factor required for MM development and homeostasis. Strikingly, manipulating the gut microbiota (by antibiotic treatment) altered MM-ENS crosstalks and impaired GI motility, however this phenotype could be restored by microbial reconstitution.

A follow-up study by Mucida and colleagues further characterized the interactions between enteric macrophages and neurons using a combination of state-of-the-art transcriptional, genetic, and imaging techniques [95]. Substantial differences in transcriptional profiles were identified by comparing lamina propria (LpM) and muscularis (MM) macrophages, especially in genes related to immunologic and metabolic processes. Gene expression in MMs resembled alternatively activated (M2) macrophages, expressing tissue-protective genes which were upregulated following *Salmonella* infection, via neuron-derived adrenergic signaling that induced tissue-protective gene expression in MMs expressing the adrenergic

receptor ß$_2$AR (*Adrb2*). Taken together, both gut motility and innate responses to bacterial infection are controlled by reciprocal neuro-immune crosstalks. In both cases, microbiota-derived signals influence these interactions and thus control GI motility and tissue-protective responses to luminal perturbations [94, 95].

4.3 Neuronal Interactions with Enteric Innate Lymphoid Cells

The discovery and characterization of ILCs over the past decade greatly expanded our understanding of immunological regulation of tissue homeostasis [96]. ILCs are lymphocytes that do not express the classic, genetically rearranged, adaptive antigen receptors as T and B cells do. These cells sense, and quickly respond to diverse stimuli, and promote immunity by serving as the innate counterparts of T lymphocytes. Several ILC subtypes were identified, which mainly include type-1 ILCs (ILC1) that respond to intracellular pathogens with Th1 cells; ILC2s that respond to extracellular parasites and allergens with Th2 cells; and ILC3s that react to extracellular microbes along with Th17 cells. ILCs are especially abundant at barrier surfaces. In the gut, the activity of ILC2 and ILC3 cells was recently found to be regulated by enteric neurons and glial cells, in response to microbiota-derived signals.

4.4 Modulation of ILC2 Activity by Enteric Neurons

The neuropeptide neuromedin U (NMU) was recently identified as a potent stimulator of ILC2 activity and type 2 immunity in the gut and the lungs [97–99]. Interestingly, NMU is expressed in cholinergic neurons of the myenteric and submucosal plexus, and the receptor for NMU (Nmur1) is specifically expressed on ILC2 cells, which are close in proximity to these neurons. Functionally, neuronally derived NMU triggered potent ILC2 activation and proliferation, as well as extensive expression of the pro-inflammatory type-2 cytokines IL5, IL13, Amphiregulin (Areg), and Colony-stimulating factor 2 (Csf2). Mechanistically, stimulation of Nmur1 on ILC2 cells activated the calcineurin-NFAT signaling pathway and ERK1/2 phosphorylation, which mediated ILC2 responses to NMU. Using multiple in vivo models, neuronally derived NMU was shown to boost ILC2 responses to infection by the gastrointestinal helminth, *Nippostrongylus brasiliensis*, as well as to allergic lung inflammation, where NMU acts together with IL-25 to expand inflammatory ILC2. These findings reveal a novel neuro-immune circuit, in which cholinergic neuron-derived NMU rapidly stimulates ILC2 responses to promote type 2 immunity in mucosal tissues.

In addition to neuronally mediated ILC2 activation, two recent studies identified neuron-derived molecules that suppress intestinal ILC2 activity [100, 101]. In the first study [101], both murine and human ILC2 cells were shown to express high levels of the adrenergic receptor ß$_2$AR gene (*Adrb2*). Additionally, ILC2 cells were located near TH+ adrenergic neurons in the small intestine, suggesting that ILC2 cells may respond to the ß$_2$AR ligand norepinephrine (NE). Indeed, ß$_2$AR-deficiency enhanced ILC2 responses to *N. brasiliensis* infection, including excessive eosinophilia, goblet cells hyperplasia, and reduced parasite burdens. These results and others reveal that the adrenergic nervous system negatively regulates type 2 inflammation at mucosal tissues through ß$_2$AR signaling on ILC2 cells. Another study [100] identified a subset of ILC2 cells in the lungs and in the intestine that express high levels of the neuropeptide calcitonin gene-related peptide (CGRP; encoded by the gene *Calca*) and its receptor components. Here again, CGRP was found to act as a negative regulator of ILC2 activity and type 2 inflammation following parasitic infection.

4.5 Enteric Glial Cells Control ILC3 Responses to Luminal Microbes

Enteric glial cells (EGCs) are a fundamental component of the ENS and, similar to enteric neurons, form extensive cellular networks in the gut mucosa that were shown to be controlled by the microbiota [92, 102, 103]. EGCs are the main producers of neurotrophic factors including the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFL) neurturin (NRTN), artemin (ARTN), and persephin (PSPN), which modulate enteric neuronal function.

In a study by Veiga-Fernandes and colleagues, ILC3 were found to express high levels of RET, a tyrosine kinase receptor activated by GFLs [104]. Gain of function of RET increased the abundance of IL-22-producing ILC3, which acts on the intestinal epithelium, to express reactivity and repair genes, including antimicrobials and mucins. *Ret*-deficient mice were highly sensitive to experimentally induced colitis and to *Citrobacter rodentium* infection. In contrast, *Ret*-gain of function protected mice from developing colonic inflammation. Mechanistically, short-term activation of ILC3 by GFLs triggered ERK, AKT, p38, and STAT3 phosphorylation and increased *Il22* transcription, demonstrating a direct effect of RET signaling on ILC3 activity. Interestingly, both microbiota-derived products and host-derived alarmins (IL-1ß and IL-33) regulated GFL expression in glial cells, in a MYD88-dependent manner. Collectively, these findings reveal that enteric glial cells sense microbiota-derived products and host alarmins and control ILC3 activity and barrier defense in the gut.

4.6 *ENS-Derived Neuropeptides Control Intestinal T Cell Development*

The notion that the gut microbiota induces differentiation of naïve T cells into Tregs or Th17 lineages was well established over the past decade. Yet, despite significant progress, a comprehensive understanding of the cellular and molecular mechanisms by which complex microbial communities promote Tregs and Th17 development is still missing.

Microbiota-induced T cell differentiation occurs in a matter of days upon microbial exposure. Thus, most studies dissect immune responses in animal models, days or weeks after microbial colonization. The recent development of a microfluidic-based, 3D gut organ culture system allowed a zoomed in look at the early events that initiate Tregs and Th17 induction [45]. Monocolonization of intact gut fragments ex vivo with an array of human commensals revealed that Treg- and Th17-inducing microbes elicit rapid and diametrically opposite transcriptional responses from the enteric immune and nervous systems. Remarkably, these findings suggest that Treg/Th17-inducing microbes modulate the same intestinal pathways, but in an opposite direction than during initial encounter, resulting in opposite Treg/Th17 outcomes in the longer term. For example, the gene *Tac1* which encodes a precursor protein to four neuropeptides of the tachykinin family, including neurokinin A and substance P, was rapidly (2 h) downregulated by Treg-inducing microbes and upregulated by the Th17-inducing microbe SFB. In agreement, the frequency of colonic Tregs in *Tac1*−/− mice was higher than in their *Tac1*-proficient littermates. These findings suggest that immediate-early neuronal responses to the microbial colonization promote long-term Treg/Th17 development and balance immunological tolerance and inflammation [45].

A molecular mechanism by which microbial stimulation of enteric neurons controls colonic Treg development was recently discovered [105]. RORγ+ colonic Tregs were found to reside close to nitrergic and peptidergic nerve fibers in the colonic LP. In vitro, enteric neuron and Treg co-culture experiments indicated that enteric neurons prevented Treg induction, independently of cell contact. A search for ENS-secreted factors led to the identification of neuronally derived IL-6 as a major modulator of RORγ+ Treg formation. Conditional ablation of IL-6 in neurons increased total FoxP3 Treg cells but decreased the colonic RORγ+ subset. These findings suggest that microbial signals regulate enteric neuronal density and activation, thus controlling Treg cell generation and immunological tolerance in the gut (Fig. 3).

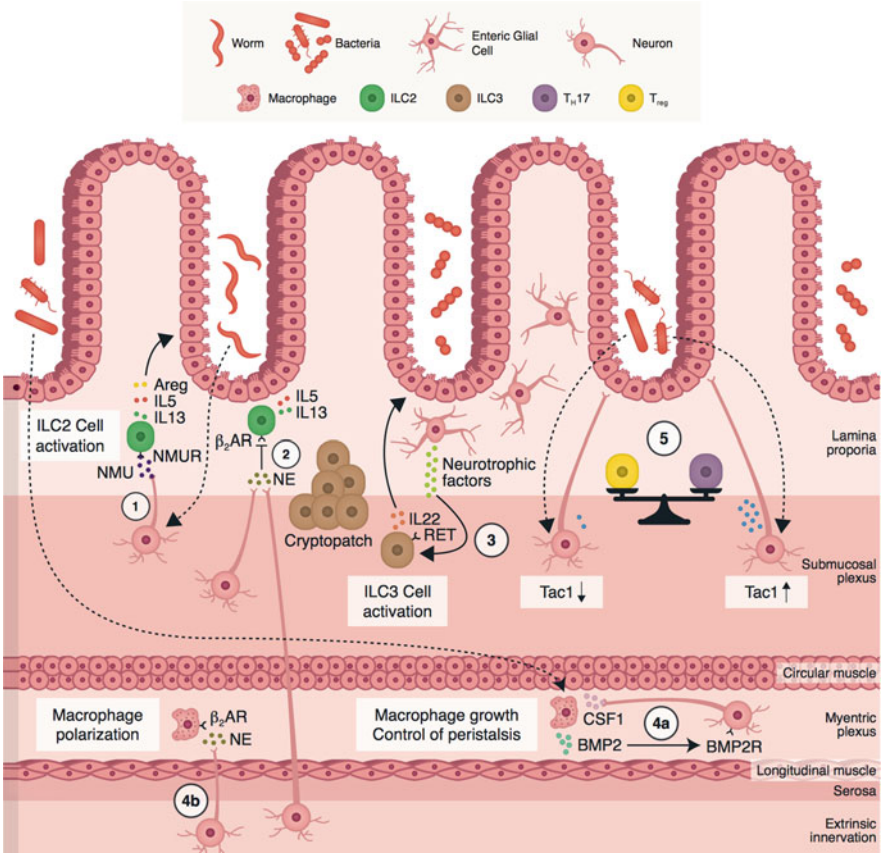

Fig. 3 Summary of enteric neuro-immune interactions, and their regulation by the gut microbiota. (1) Parasitic worm infection triggers neuropeptide neuromedin U (NMU) production by cholinergic neurons which activates NMU receptor (NMUR)-expressing innate lymphoid cells (ILC2s) and leads to type 2 cytokine production and host defense. (2) Adrenergic neuron-derived norepinephrine (NE) inhibits ILC2 proliferation and effector functions via ß2-adrenergic receptor ($ß_2$AR) signaling. (3) Neurotrophic factors secreted by enteric glial cells in response to bacterial infection and tissue damage activate RET-expressing ILC3s and trigger the release of tissue-protective interleukin (IL)-22. (4a) Microbiota-derived signals induce bone morphogenetic protein 2 (BMP2) release from myenteric macrophages. Neuronal signaling via the BMP2 receptor (BMP2R) stimulate enteric neurons to secrete colony stimulating factor 1 (CSF1) that supports macrophage growth and control of gut peristalsis. (4b) Adrenergic neuron-derived NE promotes myenteric macrophage polarization upon bacterial infection, via $ß_2$AR signaling. (5) Differential activation of enteric neurons by immunomodulating microbes rapidly control neuronal gene expression and neuropeptides production, ultimately affecting regulatory and effector T cell balance. (Adapted from [128])

5 Humoral Immunity and the Microbiota

Antibody-mediated immunity shapes gut microbiota composition and tissue localization and thus serves as a critical component in the establishment of life-long host-microbiota mutualism [106]. In the gut, large quantities of immunoglobulin A (IgA) antibodies are produced and secreted from intestinal plasma cells (PCs) in a T cell-dependent or independent manner. Dimerization of IgA antibodies is facilitated by the J chain polypeptide and polymeric immunoglobulin receptors, which facilitate transcytosis-mediated IgA secretion into the gut lumen. IgA antibodies protect from pathogenic infection and regulate symbiotic microbiota growth and diversity by binding to specific microorganisms, subsequently leading to bacterial neutralization and exclusion.

Experiments in GF mice illustrated that the production and secretion of bacteria-specific IgA antibodies is largely regulated by commensal microbiota [107, 108]. However, microbiota-induced IgA is considered to be of relatively low affinity and specificity, compared with pathogen-induced IgA. Thus, a dysbiotic microbiota developed in pathological conditions may drive high-affinity antigen-specific IgA responses, resulting in increased IgA "coating" of the dysbiotic microbiota compared with homeostatic microbiota. A recent study tested this hypothesis by the development of a unique flow cytometry-based method to isolate IgA-coated and uncoated bacteria from human and mouse fecal samples, and to analyze their taxonomic composition (IgA-SEQ) [109]. Remarkably, high IgA coating identified colitogenic bacteria in a colitis mouse model and in human IBD patients. These highly coated gut bacteria conferred significant susceptibility to colitis when transferred to GF mice. These findings propose that targeted elimination of highly IgA-coated gut bacteria may ameliorate or even prevent disease development [109].

In addition to their role in protection from pathogenic infection, antibodies play an important role in facilitating the establishment and stability of the gut microbiota. For example, the common human commensal *Bacteroides fragilis* was found to change its surface capsule architecture in order to induce specific IgA responses [110]. This IgA response enhances its epithelial adherence and allows *B. fragilis* to stably colonize a defined mucosal niche while gaining a competitive edge over exogenous bacterial competitors.

High affinity antibodies to various antigens are developed in B cells undergoing B cell antigen receptor (BCR) selection and affinity maturation in germinal centers, formed in response to infection or immunization. In the gut, germinal centers are chronically present due to constant exposure to antigens derived from diet and the microbiota. Interaction of antigens with cells of the immune system takes place in gut-associated lymphoid tissues (GALT), located in the mucosa and submucosa. Hypermutation of immunoglobulin genes takes place in gut-associated germinal centers (gaGCs) in the gut-draining mesenteric lymph nodes (mLNs) and Peyer's patches. A recent study followed the kinetics of clonal selection in steady-state gaGCs using a combination of cell-fate mapping techniques and single cell

sequencing of immunoglobulin genes [110]. Commensal-binding B cell clones are selected in gaGCs in steady-state, so that 5–10% of gaGCs from mice contain dominant B cell clones at steady-state. These B cell clones produce antibodies with increased binding properties to commensal bacteria. Overall, positive selection of B cells takes place in gaGCs under homeostatic conditions, at a rate that is tunable by microbiota presence and composition. Thus, targeted control of individual bacterial species shapes overall microbiota composition by the generation of specific antibody-mediated responses to the gut microbiota.

The multifaceted composition of the gut microbiota drives constant B cell stimulation and the development of complex and highly individualized immunoglobulin repertoires. In a recent study, Macpherson and colleagues used a unique mouse model that enables transient exposures of GF mice to different microbial taxa, followed by analysis of the B cell pool and immunoglobulin repertoires by deep sequencing [111]. Interestingly, exposing mice to the same microorganism (*Escherichia coli* strain HA107) but in different body sites (intestine or bloodstream) resulted in the generation of distinct immunoglobulin repertoires, characterized by distinct IgA or IgG diversity. Compared with mucosal exposure, the thresholds of systemic exposure are lower and result in responses that diversify the B cell and IgA repertoire. In addition to the effect of differing body sites, the order of exposures to different microbial antigens determines functional B cell immune responses and immunoglobulin repertoire generation. Collectively, these findings highlight the differences in humoral responses to mutualistic microorganisms at mucosal sites (restricted response) and to systemic exposure that elicits a flexible response required to avoid lethal sepsis.

Taken together, antibody-mediated humoral immunity is a crucial component in the establishment of healthy host-microbiota homeostasis, in controlling overall microbial load and bacterial tissue localization, and in shaping global microbiota composition, which in turn, regulate immunological development and function.

6 Perspective

Since the early 2000s, next-generation sequencing technologies have enabled deep characterization of microbiome composition in a culture-independent manner and revolutionized our understanding of host-microbiota interactions [112]. Pioneering studies introduced technical and computational methods to study the microbiome, initiating an explosion of research that analyzed microbiome composition in healthy individuals, throughout development, or in response to environmental and physiological changes associated with changes in diet or disease [30, 113–116].

Further studies established the realization that the gut microbiota is crucial for proper development and function of the immune system [55, 117], and promoted mechanistic dissections of the cells and molecules that mediate immunological

modulation by the gut microbiota. Translational and clinical studies supported the feasibility of microbiota-based therapies in treatment of recurrent *Clostridium difficile* infection using fecal microbiota transplantation (FMT) [118], and in boosting the efficacy of anti-cancer immunotherapy [119–125].

Although significant progress has been made over the past decade, many fundamental questions remain mostly unanswered. In the upcoming years, research efforts will likely address these questions in order to promote translational studies and clinical applications of microbiome-based therapeutics.

First, host-microbiota research will have to advance beyond a general description of changes to microbiome composition, to the identification of functional connections between specific microbial taxa and their physiologic impact on the host. Moreover, the relevance of an individual taxon to a specific host phenotype will have to be validated within the physiological context of the whole microbiota community. This may require the development of new experimental tools for the identification of functional host-microbe communication modules.

Another major issue is the relevance of current immunological research, mostly based on inbred lab mice, to real-life microbiota-immune system interactions in humans. One interesting approach to address this issue is the use of wildling mice—a combination of laboratory inbred mice colonized with natural "wild" microbiota—which stimulates physiological immune maturation, increases bench-to-bedside safety, and promotes more realistic pre-clinical immunological studies [126].

Additional challenges and questions stem from the substantial variability in immunological and microbial configurations between individuals. What is the definition of a "healthy" microbiome in an individual? Should microbiome-based therapeutics (such as FMT or probiotics) be tailored for each patient? Will universal immunomodulatory pathways be identified? Moreover, the available toolbox required for microbiome manipulation (including probiotics, prebiotics, bacterial-derived metabolites, and more) will have to be improved and expanded to support long-term colonization of desired microbial species, or to diminish an undesired microbial colonizer. For example, the use of bacteriophages is expected to enable precise depletion of specific bacterial strains from the microbial milieu [127].

Lastly, there is a critical need to set up an experimentally validated framework that defines guidelines for microbiome "editing" and facilitates rational intervention with microbiome configuration in order to safely shift a particular microbiome from an undesired ecological steady-state (dysbiosis) to a stable heathy state (eubiosis).

Collectively, based on the substantial technological and intellectual advancement over the past two decades, we anticipate that the near future will provide fascinating insights into microbe-immune crosstalks that will significantly expand our scientific knowledge and will revolutionize medicine as we know it.

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Compliance with Ethical Standards
The article does not contain any studies with human participants performed by the authors.

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The Gut Microbiota and Host Metabolism

Björn O. Schröder

Abstract The intestinal tract is colonized by a tremendous number of microorganisms, termed the gut microbiota. This microbial community can be considered as an active bioreactor that converts dietary substrates into metabolites that are sensed or further metabolized by the host.

Early studies in germ-free mice identified that the microbial community is an important contributor to host metabolism. These findings have been confirmed in different mouse models of metabolic diseases and by comparing microbial communities between healthy individuals and patients with metabolic diseases. Microbiota transplantations within or between human and mouse species could transfer at least part of the metabolic profile of the donor, confirming a causative role of the microbial community. Research is currently ongoing to mechanistically understand which and how gut microbiota and their metabolites affect host metabolism.

This chapter will provide an overview of the influence of the gut microbial community on host metabolism. By highlighting selected studies, the crucial function of the gut bacteria will be demonstrated. Current and future options to modulate a dysbiotic microbiota in order to improve host metabolism will be discussed, thereby illustrating that the microbiota has the potential to become a therapeutic target for the treatment of metabolic diseases in the future.

Keywords Bile acids · Gut hormones · Gut microbiota · Intestinal barrier · Microbial translocation · Mucus layer · Obesity · Short-chain fatty acids · Type 2 diabetes · Germ-free mice

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1 Introduction

The term *metabolism* describes the chemical processes in an organism that convert substrates into energy and building blocks for macromolecules and that eliminate waste products from the body. Substrates are largely of dietary origin and enter the organism through food and drinking. Well-defined and strictly regulated metabolic pathways define the fate of individual substrates, and, intertwined in a complex metabolic network, concentrations and the presence or absence of substrates and products determine the path a substrate will take.

Essential metabolic pathways are conserved between organisms, allowing the exchange of metabolites, even across species or kingdoms. As a consequence, different organisms can participate in the same metabolic network, and the outcome can be described in ecological terms: In a mutualistic interaction, two or more species benefit from each other, in a competitive interaction the different species suffer from the presence of each other, and in a parasitic interaction one species benefits at the expense of the other. Finally, in a commensal interaction (*commensalis*: "sharing a table") one species benefits while the other(s) are unaffected from the interaction (Fig. 1).

In the human body, the lumen of the intestine contains a smorgasbord of partly digested substrates that enter from the stomach. Polypeptides derived from proteins are processed into amino acids and dipeptides by exopeptidases, including trypsin and chymotrypsin. Triglycerides derived from animal or plant fat are processed into mono- and diglycerides as well as free fatty acids with the help of lipases.

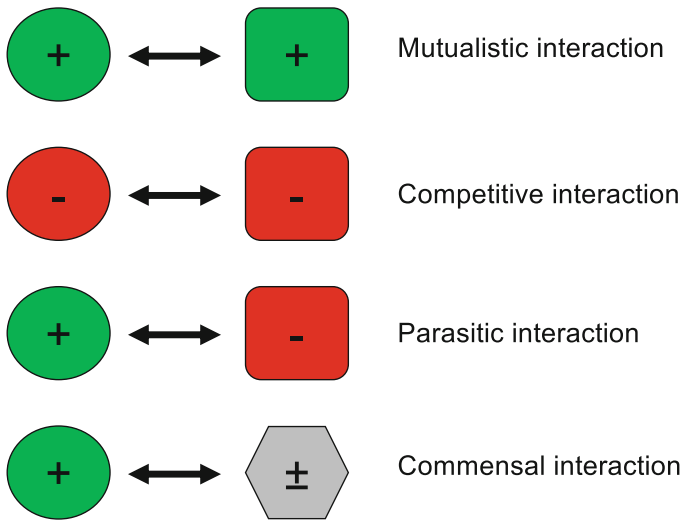

Fig. 1 The relationship between two or more species can be described by ecological terms, in which the participating species benefit (green circle; green square), suffer (red circle; red square), or are unaffected (gray hexagon)

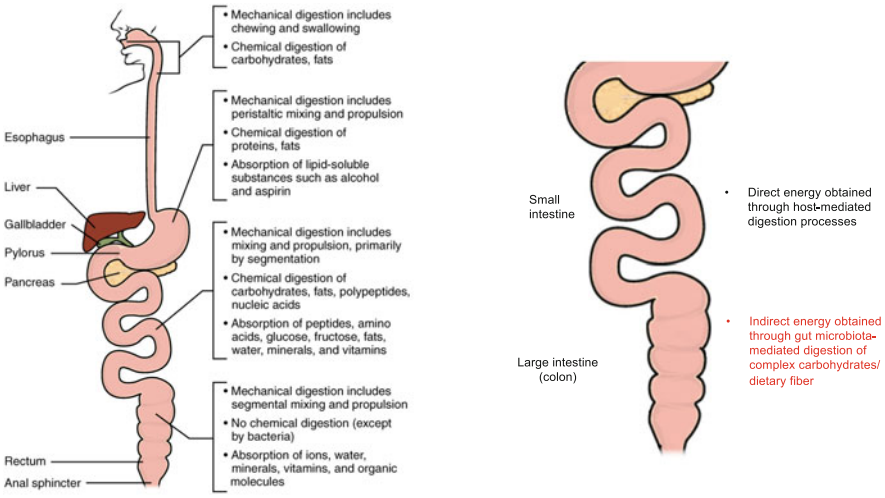

Fig. 2 Overview of the digestion process in the human body. Most dietary substrates have been digested and absorbed by the body before reaching the colon. Undigested material, such as complex carbohydrates (dietary fiber), is chemically digested by the gut microbiota, mostly in the colon. (Image obtained and modified from "Anatomy and Physiology" (https://openstax.org/details/books/anatomy-and-physiology) by OpenStax, licensed under a Creative Commons Attribution License v4.0 (https://creativecommons.org/licenses/by/4.0/))

Furthermore, secretion of bile from the liver into the duodenum facilitates absorption of the fat through emulsification.

Carbohydrates are the main drivers of metabolic reactions, and glucose, the most abundant monosaccharide, can be absorbed in the small intestine after digestion from its dietary polymer-form starch by the pancreatic enzyme amylase. In addition, the disaccharide maltose, consisting of two glucose residues, is a starch-derived degradation product that can be readily absorbed in the small intestine. Besides dietary starch, lactose can be degraded into its monomers glucose and galactose by the secreted enzyme lactase, while sucrose, the common table sugar, is degraded into fructose and glucose by sucrase. All these simple monosaccharides are absorbed in the small intestine through specific hexose transporters in the epithelial brush border, and often a transporter is able to transfer different types of hexoses. An overview of the different digestion processes is illustrated in Fig. 2.

Besides these rather simple and digestible carbohydrates, diet is also a source of more complex carbohydrates, often termed as dietary fiber. This group includes, among others, non-starch polysaccharides, such as cellulose and pectin, resistant oligosaccharides, such as fructo- or galacto-oligosaccharides, and animal-based carbohydrates such as chitin. Due to a limited number of carbohydrate-active enzymes (about 17), this group of carbohydrates cannot be digested by the human body, and the energy preserved in these molecules cannot be extracted. Thus, these molecules escape the chemical and enzymatic challenge in stomach and small intestine and continue their passage through the intestinal tract into the colon.

However, fibers are far from being purely bulking agents that increase the water content of stool and thereby lead to softer and easier passage. Instead, many dietary fibers can be metabolized by the gut microbiota, and with its repertoire of about 11,000 carbohydrate-active enzymes the microbes are able to digest the vast majority of these complex structures, which can vary in type and linkages of monosaccharides, chain length, as well as other physicochemical properties. Remarkably, the microbial degradation of dietary fiber does not only benefit the microbial community but instead also strongly affects the host. Even more, it appears that the metabolites that are produced from gut microbial carbohydrate degradation are crucial for wellness of the host, as will be discussed in this chapter.

2 Metabolism of Germ-Free Mice

The idea that the host-associated microbial community might influence host physiology dates back as far as the nineteenth century, but during that time no experimental approach existed to test this hypothesis. The probably first documented discussion on the concept of germ-free animals is assigned to Louis Pasteur in 1885, in which he reasoned that a life without microbes will be impossible:

> *"Often in our laboratory talks, and for many years, I have spoken to the young scientists around me about the value of feeding a young animal (rabbit, guinea pig, dog, chicken), from birth with pure nutrients. By this last expression, I mean food products that would be artificially and completely deprived of common microbes.*
>
> *Without wishing to assert anything, I do not hide that, if I had the time, I would undertake such a study with the preconceived idea that under these conditions, life would become impossible."* [1] *(translated)*

While it is now clear that Pasteur's preconceived idea was not correct, life is possible without exposure to microbes, but germ-free animals do indeed differ from their microbially colonized ("conventional-raised") counterparts, and since the middle of the twentieth century, germ-free mice have become an essential tool in research. Consequently, the majority of information on how microbial communities or isolated microbial species affect host function is derived from mouse studies. Nevertheless, it can be assumed that microbial colonization of the human body overall follows the same principles, despite the fact that the individual microbes or mechanistic details may differ.

The short-chain fatty acids (SCFAs) butyrate, propionate, and acetate are generated during microbial fermentation of dietary fibers that escape the digestion in the small intestine and consequently can reach the cecum (in mice) and colon (in humans). SCFAs are an important energy source for the human body, and their concentration increases along the length of the gut, thereby following a similar pattern as the number of colonizing microorganisms [2]. Butyrate is the major energy source for colonocytes, and it is estimated that microbial fermentation products provide the body with 6–10% of its total energy requirement [3]. Due to the absence of a microbiota, germ-free mice have strongly reduced concentrations of

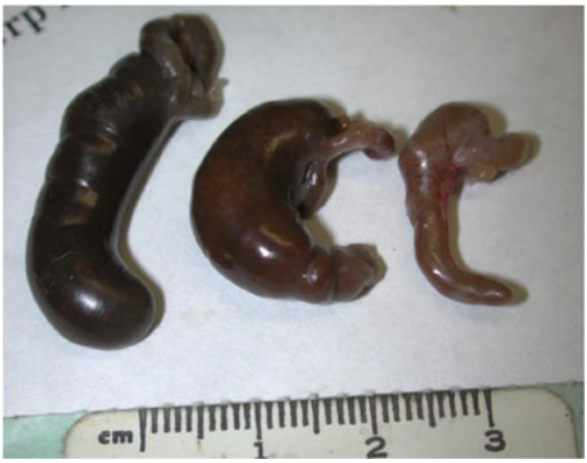

Fig. 3 Morphological cecum changes associated with microbiota status. Shown here are a cecum from a germ-free mouse (left), a mono-associated mouse (center), and a conventional-raised microbiota mouse (right). Note the increased size of the cecum from the GF mouse. (Image obtained from Overstreet et al. [6] under a Creative Commons Attribution 3.0 License (http://creativecommons.org/licenses/by/3.0))

SCFAs in their gut when compared to colonized animals [4]. Moreover, the deficiency in fiber degradation can lead to retention of undigested fiber in the murine cecum. Combined with accumulation of intestinal mucus that is normally degraded by the gut bacteria and the subsequent influx of water, the cecum of germ-free mice can be up to 5 times the volume of the cecum of a conventional mouse, leading to up to 20% of the total body weight of the animal [5] (Fig. 3). Yet, overall germ-free mice are leaner and have lower fasting glucose and insulin levels than conventional mice, despite the fact that germ-free mice are eating more [7]. This initially paradoxical phenotype can be explained by the fact that the gut microbiota is able to extract more energy from the diet and also regulates specific host genes to promote energy storage [7, 8]. An overview of gut bacterial pathways to convert diet-derived monosaccharides into SCFAs is illustrated in Fig. 4.

2.1 SCFA Metabolism

In addition to butyrate being a locally consumed energy source, microbially produced SCFA have a far more complex role on host metabolism, as they can enter the systemic circulation by draining into the portal vein and can also act as signaling molecules through activation of G protein-coupled receptors (GPCRs). As such, GPR41 (also called free fatty acid receptor 3 (FFAR3)) and GPR43 (also called FFAR2) have been identified as receptors for acetate, propionate, and butyrate in the intestinal epithelium, yet with differing specificities [10]. Mice lacking the FFAR2 and FFAR3 encoding genes *ffar2* and *ffar3* exhibited impaired glucose tolerance, thereby linking microbial fermentation products to glucose metabolism in the host. This effect was found to depend on the SCFA-triggered secretion of the anorectic incretin hormone (i.e., a metabolic hormone that stimulates a decrease in blood glucose levels by stimulating insulin secretion) glucagon-like peptide 1 (GLP-1)

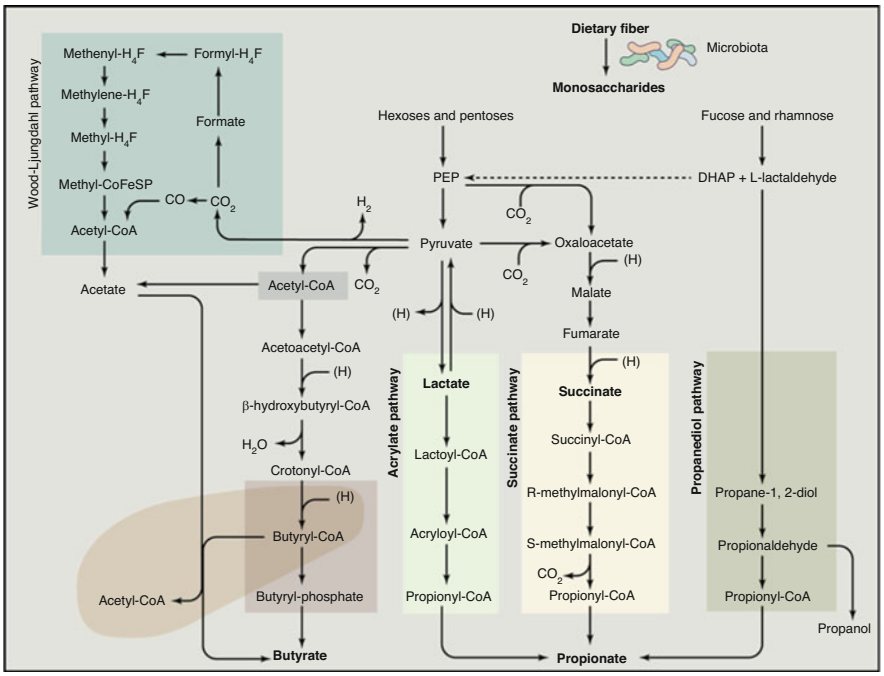

Fig. 4 Selection of pathways for biosynthesis of SCFAs from carbohydrate fermentation and bacterial cross-feeding. The microbial conversion of dietary fiber in the gut results in synthesis of the three major SCFAs, acetate, propionate, and butyrate. Acetate is produced from pyruvate via acetyl-CoA and also via the Wood-Ljungdahl pathway. Butyrate is synthesized from two molecules of acetyl-CoA, yielding acetoacetyl-CoA, which is further converted to butyryl-CoA via β-hydroxybutyryl-CoA and crotonyl-CoA. Propionate can be formed from phosphoenolpyruvate (PEP) through the succinate pathway via succinate or the acrylate pathway via lactate. Microbes can also produce propionate through the propanediol pathway from deoxyhexose sugars, such as fucose and rhamnose. PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate. (Graphic obtained from Koh et al. [8] with permission from Elsevier)

from L-cells, which are specialized enteroendocrine cells that are located along the length of the intestine and have their highest density in the microbially dense colon (see Sect. 2.3). Interestingly, the phenotype seemed to be more strongly dependent on FFAR2 when compared to FFAR3, indicating that the two receptors may either have distinct specificities for different SCFAs, might be expressed differently on the cellular surface, or may signal through different intracellular metabolic pathways [11].

In the systemic circulation microbially derived SCFAs can be measured in the micromolar range, with acetate reaching the highest concentration (70 μM), whereas propionate (5 μM) or butyrate (4 μM) only reach a fraction of this [9] (and references therein). Consequently, the strongest SCFA-dependent effects outside of the gut can be expected for acetate. Indeed, despite similar food intake mice deficient in FFAR2 developed obesity during growth that was characterized by increased weight of the

white adipose tissue [12]. In addition to direct metabolic impairments, *ffar2*-deficient mice developed an increase in insulin resistance and an inflammatory phenotype that was even more pronounced when mice were fed a high-fat diet. As *ffar2*-deficient mice had increased fecal SCFA and plasma acetate concentrations when compared to wild-type mice, an involvement of the gut microbiota in the metabolic impairments, insulin resistance, and inflammatory phenotype of *ffar2*-deficient mice could be assumed. And indeed, treatment with antibiotics led to a significant decrease in (i.e., normalization of) fecal SCFAs and plasma acetate concentrations in *ffar2*-deficient mice, an observation that was also true for wild-type germ-free mice. Remarkably, the differences in body weight and white adipose tissue that have been observed between colonized wild-type and colonized *ffar2*-deficient mice were completely eliminated in germ-free *ffar2*-deficient mice and reappeared after colonization of the mice, confirming a crucial involvement of the gut microbiota [12]. Consequently, microbial metabolism in the gut is an important source for the production of ligands, such as acetate, that can activate FFAR2 on peripheral adipose tissue, thereby modulating the metabolic homoeostasis of the host.

Microbially produced propionate as the second-most-abundant SCFA has also been shown to modulate host metabolism. In a double-blinded placebo-controlled trial with healthy female volunteers, the daily supplementation of propionate over 7 weeks led to improved glucose tolerance and insulin sensitivity and lipid metabolism [13]. Mechanistically, microbially produced propionate can serve as a substrate for glucose synthesis in the intestine, so-called gluconeogenesis. In addition, propionate and butyrate are both able to induce genes of the intestinal gluconeogenesis pathway, leading to increased glucose production by the intestinal epithelium. The increased glucose concentration is subsequently sensed by the vagus nerve in the portal vein, which leads, via a gut-brain neural circuit that also involves signaling through FFAR3, to reduced body weight and adiposity as well as improved glucose control and insulin sensitivity [14].

A selection of SCFA-producing bacteria is shown in Table 1. Of note, some bacteria can utilize the SCFA acetate (A) or other organic acids such as lactate (L) that have been generated by other gut bacteria to produce butyrate. This process is called cross-feeding and is very common in the complex microbial community in the gut. While some bacteria are limited to utilize only A or L as a substrate, other bacteria can use either substrate (A,L) for butyrate production.

2.2 Bile Acid Metabolism

Primary bile acids are produced in the hepatocytes of the liver from cholesterol and comprise the major organic fraction of bile. They are stored in the gallbladder, and after conjugation with glycine (predominant in humans) or taurine (predominant in mice), the formed bile salts are secreted into the small intestine to facilitate the absorption of cholesterol, dietary fats, and fat-soluble vitamins [19]. The major

Table 1 SCFA production by microbes in the gut

SCFAs	Pathways/Reactions	Producers	References
Acetate	from pyruvate via acetyl-CoA	most of the enteric bacteria, e.g., *Akkermansia muciniphila*, *Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp., *Ruminococcus* spp.	(Louis et al. 2014; Rey et al. 2010)
	Wood-Ljungdahl pathway	*Blautia hydrogenotrophica*, *Clostridium* spp., *Streptococcus* spp.	
Propionate	succinate pathway	*Bacteroides* spp., *Phascolarctobacterium succinatutens*, *Dialister* spp., *Veillonella* spp.	(Scott et al. 2006; Louis et al. 2014)
	acrylate pathway	*Megasphaera elsdenii*, *Coprococcus catus*	
	propanediol pathway	*Salmonella* spp., *Roseburia inulinivorans*, *Ruminococcus obeum*	
Butyrate	phosphotransbutyrylase/ butyrate kinase route	*Coprococcus comes*, *Coprococcus eutactus*	(Duncan et al. 2002; Louis et al. 2014)
	butyryl-CoA:acetate CoA-transferase route	*Anaerostipes* spp. (A, L), *Coprococcus catus* (A), *Eubacterium rectale* (A), *Eubacterium hallii* (A, L), *Faecalibacterium prausnitzii* (A), *Roseburia* spp. (A)	

A acetate is the substrate for producing butyrate, *L* lactate is the substrate for producing butyrate. Reprinted from Koh et al. [8] with permission from Elsevier

fraction of the bile acids is reabsorbed, mainly in the distal ileum, and transported back to the liver, thereby closing the enterohepatic circulation.

Yet, in the distal part of the small intestine as well as in the colon, the gut microbiota further modifies bile salts through initial deconjugation, followed by dehydroxylation, epimerization, and oxidation, leading to a diverse pool of secondary bile acids. These secondary bile acids are generally reabsorbed to a lower extent and thus support the elimination of cholesterol through their excretion with the feces [5]. Consequently, bile acids in germ-free rodents remain conjugated, and a higher proportion of bile acids is reabsorbed from the intestine, leading to about three times higher concentrations of bile acids in the entire enterohepatic recirculation [20, 21]. However, the increased concentration is differentially distributed over the enterohepatic system, with higher bile acid levels in the gallbladder and small intestine and lower concentrations in cecum, colon, feces, and serum of germ-free

mice [22]. This uneven distribution thus confirms previous observation of reduced elimination of cholesterol from the body through the feces in germ-free mice [23].

In addition to an overall increase in bile acid levels, microbial modifications of bile acids are specific for individual bile acids. While taurine-conjugated cholic acid and beta-muricholic acid are the most common species in the small intestines and livers of both germ-free and colonized mice, microbial metabolism leads to reduced levels of taurine-conjugated beta-muricholic acid as well as increased concentrations of taurocholic acid and tauro-alpha-muricholic acid when compared to microbiota-deficient mice. Moreover, tauro-omega-muricholic acid and tauro- deoxycholic acid could only be detected in colonized animals [22]. These differences in bile acid composition are probably of minor importance for the absorption of lipids, but in addition to their emulsifying activity bile acids have recently been identified as ligands for distinct receptors, including the nuclear farnesoid X receptor (FXR) and the G-protein-coupled receptor TGR5. As a consequence, chemical modifications of bile acids by the microbiota can affect the signaling through both receptors. For example, binding of tauro-beta-muricholic acid to FXR is antagonizing FXR signaling in mice, and consequently, microbial activity on this bile acid releases FXR inhibition and thus re-activates its signaling [22]. The modulation of FXR signaling will then have an influence on bile acid synthesis via a gut microbiota-liver feedback loop.

2.3 Gut Hormones

The complex surveillance and regulation of the metabolic processes in the host require efficient communication between the intestine and distant organs, including the liver, adipose tissue, pancreas, and brain. As means of communication, enteroendocrine peptide hormones can be secreted from specialized cells of the gut epithelium to convey nutritional status of the gut to the body to coordinate food digestion, absorption, insulin secretion, and appetite [24]. L-cells are one type of enteroendocrine cells that are found throughout the intestinal tract with highest numbers in the ileum and especially in the colon [25].

While individual enteroendocrine cells can secrete a mixture of peptide hormones, including cholecystokinin (CCK), secretin, glucagon-like peptide 1 (GLP1), gastric inhibitory polypeptide (GIP), peptide YY (PYY), or neurotensin [26, 27], L-cells predominantly secrete GLP1. Together with GIP that is secreted from K-cells, another type of enteroendocrine cell, GLP1 is released postprandially in the gut and induces glucose-dependent insulin secretion from the pancreas. This so-called incretin effect is the explanation why orally provided glucose results in higher insulin secretion when compared to intravenously administered glucose, despite the same blood glucose levels [28]. However, while GLP1 secretion from L-cells can be detected upon a stimulus with sugars, amino acids, and long-chain fatty acids, these meal-derived nutrients commonly do not reach the colon, where L-cell numbers are highest [29, 30]. Consequently, other stimuli that are not directly

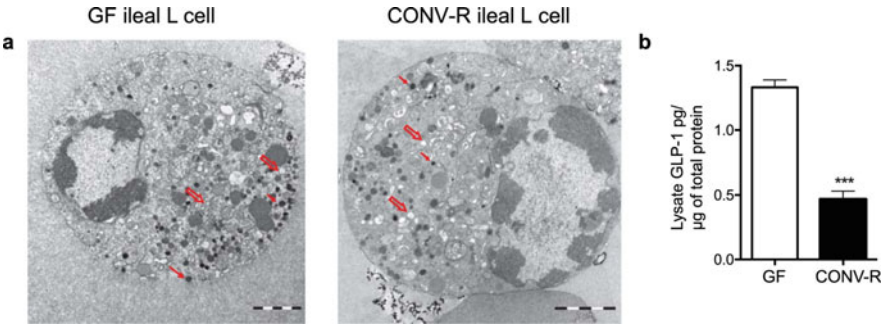

Fig. 5 The gut microbiota regulates glucagon-like peptide 1 (GLP-1) content in ileal L-cells. (**a**) Electron microscope images of ileal L-cells from germ-free (GF) and conventional-raised (CONV-R) mice. Red arrows indicate the densely packed vesicles and open arrows indicate the open (empty) type vesicles, scale 2 µm. (**b**) Intracellular GLP-1 content in the lysate from primary crypt cultures of ileum of GF and CONV-R mice. Data are mean + SEM; $***p < 0.001$ indicates significance in CONV-R versus GF comparison. (Image obtained from Arora et al. [26] under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/))

derived from ingested meals are likely to be present in the colon, and as an active bioreactor the gut microbiota is an obvious guess.

Analyses of germ-free mice revealed that the absence of a microbiota led to an increased number of intestinal L-cells and consequently higher plasma levels of GLP1 (Fig. 5). These alterations increased the intestinal transit time, which is thought to be a compensatory response by the host to allow more time for the extraction of energy from the diet in the absence of microbial metabolism. Indeed, colonization of germ-free mice reduced the number of L-cells and GLP1 levels and resulted in the normalization of transit time [30]. Interestingly, the transcriptional response of L-cells toward the presence of gut microbiota was stronger in the ileum than in the colon, indicating a compartmentalized regulation [31].

The observation that a supplement of dietary fibers resulted in increased GLP1 levels in rodents and humans led to the conclusion that the colonic gut microbiota can modulate GLP1 secretion through production of SCFAs [32–34]. This has been confirmed in vitro [11, 34], but in addition, other microbial metabolites have also been identified that affect L-cell function. These include indole, a metabolite that is produced during microbial catabolism of aromatic amino acids [35], and deconjugated secondary bile acids [36], but even lipopolysaccharide (LPS) following intestinal epithelial barrier damage [37]. Thus, it is obvious that the gut microbiota can modulate the hormonal response of the host in a rather specific manner. Remarkably, however, short-term disturbances of the gut microbiota through a 4 to 7 day course of antibiotic treatment in humans did not affect tissue-specific glucose-dependent insulin secretion or insulin sensitivity [38, 39]. Thus, when comparing these results with the findings from germ-free mice, it indicates that the microbiota-mediated influence on gut hormones is rather relevant for long-term

development and appears to be resilient toward short-term perturbations or reductions in microbial numbers and metabolites.

2.4 Summary

The contribution of the gut microbial community to host metabolism can best be studied in animals that are completely deficient of microbiota. Despite being an artificial system that is not physiologic in humans, relevant findings can be obtained from such germ-free models. Based on those studies, the gut microbiota is tightly linked to essential metabolic functions of the host. Mainly, it assists in the degradation of complex carbohydrates to extract energy that would otherwise be lost. Moreover, the microbial community modulates digestion and absorption processes to fine-tune energy metabolism even in organs distant from the gut (Fig. 6). Consequently, the contribution of the gut microbiota to host physiology underlines the concept of a meta-organism, in which the host and the gut microbiota strongly depend on each other and both sides benefit.

Microbial composition in the gut is strongly dependent on the host diet, and depending on the diet, specific metabolites will be produced with varying effects on

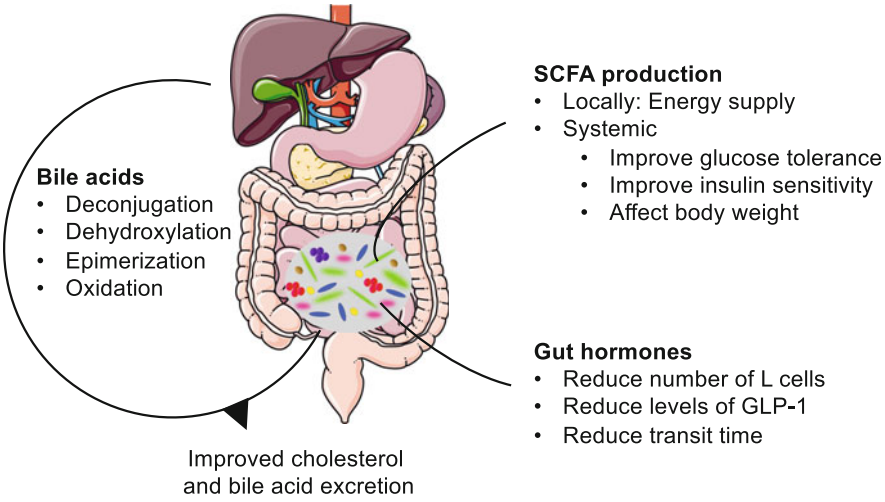

Fig. 6 The gut microbiota is tightly linked to essential metabolic functions of the host. Degradation of complex carbohydrates leads to production of short-chain fatty acids (SCFAs), which provide energy to the colonocytes and regulate glucose metabolism. Through metabolization of bile acids and regulation of gut hormones, such as glucagon-like peptide 1 (GLP-1), the microbial community modulates digestion and absorption processes to fine-tune energy metabolism even in organs distant from the gut. (Image contains material from Servier Medical Art (https://smart.servier.com) that has been modified under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/))

host physiology. As different microbial members in the gut contain distinct metabolic pathways for substrate utilization, the metabolic output will unquestionably depend on the present microbial composition. In the next section the relation between microbial composition and host metabolism will be discussed in more detail.

3 Gut Microbiota and Metabolic Diseases

3.1 Obesity

3.1.1 Microbial Alterations

Based on the findings that the gut microbiota modulates host metabolism, the interest in identifying the role of the individual taxa increased. Early studies by Ley et al. compared the microbiota composition between lean wild-type mice and their genetically obese (*ob/ob*) siblings [40]. These obese mice carry a mutation in the gene encoding the satiety hormone leptin, which leads to hyperphagia and consequently to morbid obesity due to an overfeeding on their diet [41]. Microbiota analyses in these mice revealed a clear difference in the intestinal community: the obese mice had a 50% reduction in the phylum Bacteroidetes and a corresponding increase in the phylum Firmicutes [40]. Subsequently similar microbial differences were also reported from obese humans in which the abundance of Bacteroidetes correlated with the loss of body weight, but not with changes in dietary calorie content over time [42]. The ratio between these two predominant bacterial phyla, Bacteroidetes and Firmicutes, which dominate the mouse and human gut microbiota with up to 90% [40, 42, 43], has since then been used to characterize intestinal microbial communities. However, both the Bacteroidetes and Firmicutes phyla comprise a high number of different species with various metabolic functions and pathways, so this ratio is only a superficial characterization that requires deeper analysis to draw any meaningful conclusions.

While the general cause of obesity is often an excess of caloric intake compared to caloric expenditure, the microbial differences between obese and lean mice and humans suggest that the microbial community could also be a potential contributing factor. And indeed, it was shown that the microbiome of obese *ob/ob* mice is enriched in genes encoding for glycoside hydrolase families, including α-glucosidases, α-galactosidases, and β-galactosidases, that are capable of degrading dietary polysaccharides, including starch, sucrose, and galactose [44]. Moreover, to further facilitate the energy extraction from the provided food, the microbiome of *ob/ob* mice is also enriched in genes that encode for transporters and metabolic enzymes that convert these carbohydrates into fermentation end products, such as butyrate and acetate, which can be detected in higher concentrations in the cecum of *ob/ob* mice when compared to their lean littermates. The increased capacity of the "obese" microbiota to extract more energy from the diet was further corroborated by the

finding that the feces of *ob/ob* mice has significantly less remaining energy than the feces of the lean mice [44].

Microbiota studies by using mouse models allow tight control of genetic background, host physiology, diet, and environmental conditions, which all have an influence on the microbiota composition [45–47]. These factors are much more difficult to control for humans, making it challenging to disentangle the contribution of these factors on the human microbiota composition. Monozygotic twins, however, have the same genome and often experience the same environmental exposure early in life. Comparing monozygotic twin pairs with dizygotic pairs thus allows one to study the influence of genetics on human phenotypes.

Gut microbiota analyses in adult female monozygotic and dizygotic twin pairs concordant for leanness or obesity revealed that a "core microbiome" existed on the gene level, rather than the organismal level, and that deviations from this core microbiome could be linked to obesity or leanness [48]. Moreover, reduced bacterial diversity and altered metabolic pathways were detected in the obese participants, indicating that an obesity-associated microbiota cannot only be detected in mice but also in humans, thereby suggesting a more generalizable concept of the link between host obesity and its associated microbiota.

3.1.2 Causal Role of the Microbiota

To further confirm the contribution of the gut microbiota on host obesity, germ-free mice were colonized with microbiota samples obtained from twins discordant for obesity. Confirming previous transplantations from *ob/ob* mice [44], mice obtaining the microbiota from the obese twins displayed a significantly greater increase in body mass and adiposity than mice that were colonized with microbiota obtained from the lean twins [49] (Fig. 7). The increase in body weight of the transplanted mice correlated with decreased SCFA production and an increase in the metabolism of branched-chain amino acids as well as a decrease in microbial bile acid transformation, leading to increased FXR signaling [49].

Interestingly, when the mice transplanted with "lean" and "obese" microbiota were co-housed and consequently gut microbiota could be exchanged due to the coprophagic behavior of mice, the increase in body fat and body mass was prevented. This prevention correlated with an increase of Bacteroidetes in the "obese"-transplanted mice and only occurred when the mice were fed a fiber-rich diet [49].

Further support for an active contribution of the gut microbiota on host obesity has been obtained by depleting conventionally raised mice of their microbiota by antibiotic treatment. Similar to germ-free mice, antibiotic-treated mice had improved glucose tolerance and insulin sensitivity while their white fat and adipocyte size decreased [50]. In addition, the microbiota-depleted mice had increased browning and thermogenic capacity, independent of whether *ob/ob* or high-fat diet-fed mice were analyzed. Consequently, these observations not only demonstrate that interactions between specific gut microbial taxa and diet can modulate host metabolism, but

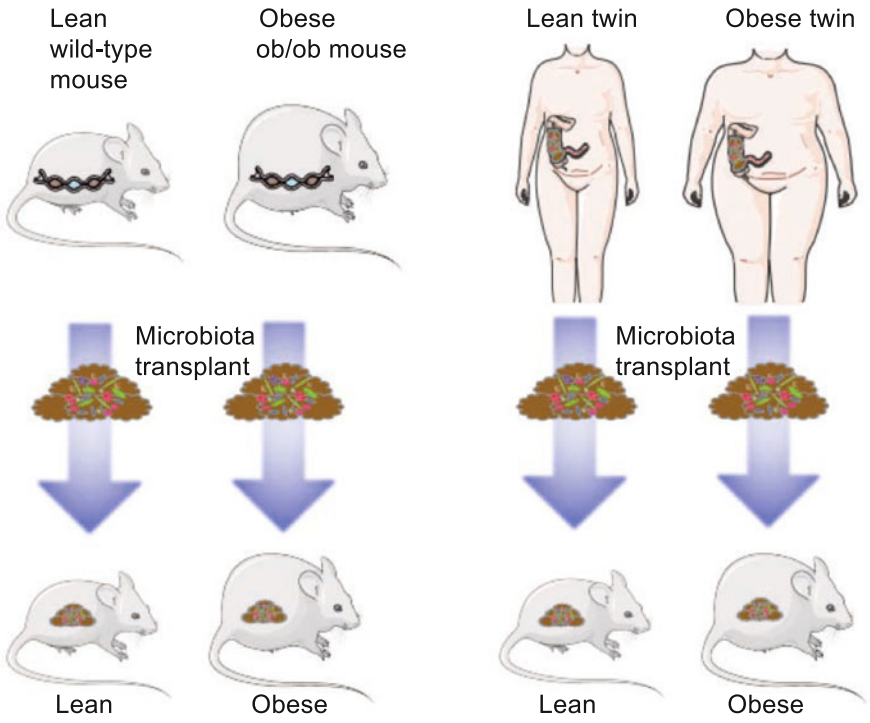

Fig. 7 Gut microbiota transplantations reproduce metabolic phenotypes in the recipient. Microbiota transplantations from obese mice (left) and obese humans (right) to germ-free mice lead to increased obesity when compared to mice that receive gut microbiota from lean donors. (Image contains material from Servier Medical Art (https://smart.servier.com) that has been modified under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/))

also that the presence or absence of distinct microbial taxa can be a crucial factor for physiological outcomes.

3.2 Type 2 Diabetes

3.2.1 Microbial Alterations

The findings that the gut microbiota interacts with the host endocrine system and modulates host glucose metabolism, as discussed in Sect. 2.3, suggests that the microbial community may be a relevant contributing factor in type 2 diabetes, the most prevalent endocrine disease worldwide. Indeed, a metagenome-wide association study in 345 Chinese individuals could identify a distinct gut microbial profile in patients with diabetes that differed from the microbial composition in non-diabetic controls [51]. In this cohort, the non-diabetic controls had higher

abundances of known butyrate-producing bacteria, including *Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Roseburia inulinivorans* as well as *Clostridiales* sp. *SS3/4*. In contrast, the microbial community of individuals with type 2 diabetes was enriched with opportunistic pathogens, such as *Bacteroides caccae*, *Clostridium hathewayi*, *Clostridium ramosum*, *Clostridium symbiosum*, *Eggerthella lenta*, and *Escherichia coli* [51]. Moreover, the mucus-associated species *Akkermansia muciniphila* and a sulfate-reducing *Desulfovibrio* species were also increased in persons with type 2 diabetes. Yet, while the presence or absence of individual taxa has the potential to be used as a biomarker, the reduction in the beneficial butyrate producers (see Sect. 2.1) can also lead to a "functional dysbiosis" [51], which may destabilize the microbial community and facilitate colonization with opportunistic pathogens in individuals with type 2 diabetes.

The gut microbial composition is influenced by age, sex, and geography [52, 53], and it is therefore not surprising that a second study investigating the fecal metagenome of 145 European women with normal, impaired, or diabetic glucose control identified different discriminatory metagenomic markers for individuals with type 2 diabetes when compared to the Chinese cohort [54]. In the European study, women with type 2 diabetes had increased metagenomic clusters of a Clostridiales order, two *Clostridium clostridioforme*, *Lactobacillus gasseri*, and *Streptococcus mutans*. Of those, *C. clostridioforme* correlated positively with the levels of C-peptide, a pancreas-produced and insulin-associated clinical marker for type 2 diabetes, while *L. gasseri* had a positive correlation with fasting blood glucose levels as well as with glycation levels of hemoglobin (HbA1c), which indicates excessive sugar in the blood. In contrast, the non-diabetes group had lower levels of several Clostridiales, Coriobacteriaceae, *Roseburia*, *Eubacterium eligens*, and *Bacteroides intestinalis* [54].

Of note, a mathematical model was developed which could identify the diabetic phenotype with high accuracy based on metagenomic clusters. Yet, by using this model for the previously discussed Chinese cohort [51], it became evident that the metagenomic clusters that were discriminatory for type 2 diabetes were different from the European cohort. Thus, while it is likely that similar microbiota-encoded functions are associated with type 2 diabetes on the different continents, it is important to identify microbial disease associations or biomarkers in their relevant geographical and environmental context [54].

Taking this observation a step further, microbial modulation of host glucose metabolism differs even on an individual level. In a cohort of 800 Israelis representative of the adult non-diabetic Israeli population, the post-meal spike in blood glucose concentration (postprandial glycemic response, a risk factor for type 2 diabetes [55]) was measured after habitual and standardized meals [56]. The glycemic response to specific meals was found to not only correlate with clinical biomarkers, such as HbA1c level, body-mass index (BMI), and systolic blood pressure, but also with distinct microbiota profiles and functions. Even more, based on a combination of these clinical markers and the microbiota profile, the individual glucose response to a distinct meal could be well predicted [56]. It may thus be possible in the future to

utilize microbiota profiles in a personalized prediction to select diets with low glucose response for the individual to prevent the development of type 2 diabetes.

3.2.2 The Anti-Diabetic Drug Metformin

For the time being, the most prescribed drug for the treatment of individuals with type 2 diabetes is metformin [57], and due to its extensive usage it is likely that the microbiota profiles in persons with type 2 diabetes might be confounded by this medication [58]. Indeed, studies in high-fat diet-fed and metformin-treated mice found that metformin-treated rodents had an increased abundance of *Akkermansia muciniphila* when compared to a control group, and this increase in *A. muciniphila* could at least in part explain the enhanced glucose tolerance of these mice [59]. Similarly in rats fed a high-fat diet, metformin treatment was associated with a shift in the microbiota composition that was characterized by an increase in potentially beneficial butyrate-producers [60].

In humans, metformin-treated individuals with type 2 diabetes differed in their gut metagenome from metformin-naïve patients with type 2 diabetes when analyzing 784 available human gut metagenomes from Denmark, Sweden, and China [58]. Comparing these two groups, the authors found that metformin treatment was associated with an increase in *Escherichia* spp. and reduced abundance of *Intestinibacter* spp. and on a functional level with significantly enhanced butyrate and propionate production potential [58].

However, since metformin treatment is more effective in lowering blood glucose levels when given orally as compared to intravenously [61], it is possible that the changed gut microbiota in treated individuals with diabetes is not only an association but part of the mode of action of metformin. This hypothesis was consequently tested by transplanting fecal samples that were obtained from three participants before and after 4 months of metformin treatment [62]. While body weight, body fat, or fasting insulin levels were not different in the transplanted mice, glucose tolerance improved in those mice that received microbial transplants from the participants after metformin treatment. Furthermore, by incubating stool samples from the participants in the presence of metformin, clear microbiota changes and alterations in gene expression were observed [62]. Thus, this study strongly suggests that the mode of action of metformin can at least in part be explained by its modulation of the gut bacterial community.

3.3 Undernutrition

In parallel to the increase in metabolic diseases linked to overnutrition in the industrialized world, undernutrition is the opposite extreme and a leading cause of child mortality worldwide.

Kwashiorkor is a virulent form of severe acute malnutrition and is characterized by edema, hepatic steatosis, skin rashes, and ulcerations as well as anorexia [63, 64].

Studies of fecal microbiomes from twin pairs in Malawi who became discordant for kwashiorkor found that the microbiota of healthy twin pairs and healthy co-twins displayed a steady maturation of the microbiome, which was not observed in the twins with kwashiorkor [63]. Despite the fact that no consistent taxonomic "kwashiorkor microbiota" profile was observed, transplanting kwashiorkor co-twin's microbiota into germ-free mice resulted in two out of three cases in more extreme weight loss in the recipient mice when compared to the microbiota transplants of the heathy twin pairs. Interestingly, this weight-loss phenotype only occurred when the kwashiorkor-transplanted mice were fed a diet that was similar to the Malawian diet, but not when fed a common chow diet. Mechanistically, the authors proposed that the kwashiorkor microbiota may disturb sulfur metabolism and produce metabolites that interfere with the energy-generating tricarboxylic acid cycle, which would result in less effective energy conversion. Consequently, the microbiota appears to be a causal factor for the development of kwashiorkor, but for this development a specific dietary environment is required [63].

In addition to the twin cohort in Malawi, similar results were obtained from children living in Bangladesh, confirming the importance of an immature microbiota profile in children with severe acute malnutrition [65]. Most importantly, identifying that the interaction between diet and microbiota is crucial for the development of severe undernutrition allows new therapeutic approaches that include both contributing factors. As such, a pilot study with 118 children in Bangladesh could indeed show that providing a microbiota-directed complementary food prototype (MDCF-2) for 3 months was successful in altering distinct plasma proteins and associated microbial taxa which were linked to bone growth and neurodevelopment [66]. It is thus possible to support growth of undernourished children by targeted manipulation of the gut microbiota.

3.4 Evolutionary Perspective

3.4.1 Example 1: The Hibernating Brown Bear

Obesity and type 2 diabetes are metabolic diseases in humans and develop due to a changed lifestyle in the industrialized Western society. However, from an evolutionary perspective when fluctuations between food availability and food scarcity were more common, the capacity of the microbiota to extract as much energy as possible during times of plenty might have been a key factor for survival.

An enlightening observation in that regard has been obtained from the wild-living and hibernating brown bear, which has a seasonal lifestyle that cycles between intense eating during the summer months and prolonged fasting during the winter months [67]. During the hyperphagic phase in the summer, the brown bear accumulates fat and body weight, but without developing metabolic impairments,

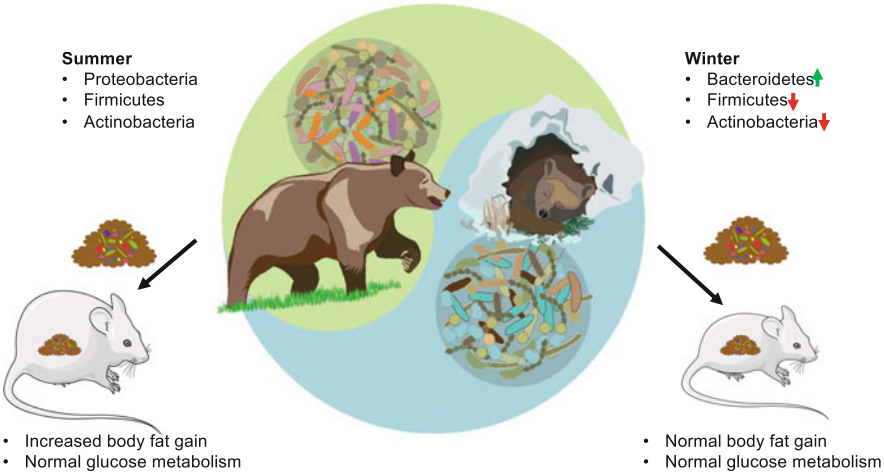

Fig. 8 The gut microbiota modulates energy metabolism in the hibernating brown bear. The microbiota during the active summer phase of the brown bear is more diverse and has increased levels of Firmicutes and Actinobacteria, and decreased levels of Bacteroidetes when compared to the microbiota during the hibernation period. Colonization of germ-free mice with the brown bear microbiota led to increased body fat in the mice colonized with "summer microbiota" when compared to "winter microbiota" while glucose metabolism remained unaffected. (Graphic contains item from Sommer et al. [64] (with permission from Elsevier) and material from Servier Medical Art (https://smart.servier.com) that has been modified under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/))

thereby representing a healthy obese phenotype [68]. Moreover, the bear microbiota differed between the summer and winter, and was dominated by Proteobacteria, Firmicutes, and Actinobacteria during the summer while the abundance of Bacteroidetes increased relative to Firmicutes and Actinobacteria during the winter [69]. These microbial changes are likely due to the diverse seasonal diet in the summer and the scarce/absent diet in the winter months.

Interestingly, however, colonization of germ-free mice with the brown bear summer or winter microbiota reproduced some of the metabolic phenotypes, resulting in increased body fat gain in the mice colonized with "summer microbiota" when compared to "winter microbiota." Yet, despite the increase in body fat, glucose metabolism did not differ between the two colonized mouse groups [69]. Thus, the seasonal bear microbiota is contributing to the extraction of an excess of energy during the summer to survive the nutrient-scarce winter without leading to metabolic impairments (Fig. 8). While the mechanistic details of these findings remain to be elucidated, this observation demonstrates that a mammalian gut microbiota community could evolve with its host to serve the fluctuating metabolic needs and food supply without the development of metabolic dysfunction. In our industrialized society in which energy-dense food is omnipresent and periods of hunger or fasting are rare, this evolutionary beneficial mechanism may thus contribute to the development of metabolic diseases.

3.4.2 Example 2: Fight-or-Flight

Opposed to a sedentary lifestyle that is characterized by an excess of energy intake is the active metabolism during acute or prolonged activity, in which energy expenditure equals or exceeds the supply. While in evolutionary terms such a situation could occur during a chase or flight, in our modern society such energy demand would occur during athletic long-endurance exercise, such as a marathon race. Athletes in general have been shown to differ in their gut microbiota composition from less active groups by having increased abundances of Veillonellaceae, *Bacteroides*, *Prevotella*, *Methanobrevibacter*, or *Akkermansia* [70, 71] and in the case of *Veillonella*, this bacterium was specifically enriched after a marathon race [72]. Intriguingly, when isolating a *Veillonella atypica* strain from an athletes' stool and inoculating it into mice, these mice showed better performance during exhaustive treadmill run times than the control group that was inoculated with *Lactobacillus bulgaricus*. Mechanistically, the authors found that *Veillonella* utilizes lactate as its only carbon source to produce acetate and propionate. As the systemic lactate that is generated during exercise can translocate into the gut lumen, *Veillonella* can metabolize this SCFA to generate propionate, which in turn can lead to improved performance of the host [72]. Consequently, the increased abundance of this specific member of the gut microbiota can thus lead to a metabolic advantage that can lead to prolonged performance in mice. However, despite the fact that the tested *Veillonella* strain has been isolated from the human gut, it is unclear whether "microbiota doping" will work equally well in humans.

3.5 Summary

Associations between the gut microbial composition and host metabolism have been identified in animal models and humans. In contrast to many other host phenotypes that have been correlated to microbial alterations, for metabolic diseases even a causal role of the gut microbiota is suggested and strongly supported by findings from different animal studies. However, such a definite causative "proof" is difficult to obtain for the human situation, even though data are available that also support this hypothesis. For example, transplantation of microbiota from lean donors into recipients with metabolic syndrome led to increased insulin sensitivity and an increase in butyrate-producing microbiota after 6 weeks [73]. However, the microbial community returned to its baseline status 3 months after the transplantation [74], indicating that such a transplant is rather transient when the inherent microbiota of the recipient is present. In a different case study, a woman that received a microbiota transplant to treat a recurrent *Clostridioides difficile* infection developed new-onset obesity after receiving a microbiota transplant from an overweight donor [75]. Hence, this rather unintended result further supports the idea that a causative gut microbial effect on host metabolism in humans is likely.

Of note, despite the fact that microbial alterations are linked to metabolic diseases, identification of individual responsible taxa is often difficult, especially in humans. A loss of microbial diversity appears to be a consistent observation, as is the loss of butyrate-producing bacteria. Due to strongly interrelated microbial networks and cross-feeding, it is likely that not the presence or absence of individual taxa, but rather the disturbance of a fine-tuned microbial network contributes to the development of metabolic impairments of the host. It has to be realized, however, that the energy-providing function of the microbiota has been an evolutionary beneficial trait, and that only through the modern lifestyle, characterized by omnipresence of energy-dense food and reduced movements, this beneficial feature is now a contributing factor to metabolic diseases.

4 Mechanistic Aspects

4.1 Early Life Influence

Infants are born without a microbiota, and the development of the microbial community begins during and after birth. Yet, this assembly is not random but follows predictable dynamics that involves multi-kingdom interactions, changes in relative and absolute abundances, as well as distinct temporal patterns of bacterial species [76, 77]. However, during this time the establishing microbial community is unstable and thus more susceptible to disturbances [78], which may have consequences that even persist during adulthood. As the modulation of host metabolism by the gut microbiota is becoming increasingly clear, an evident aspect to focus on is the influence of the gut microbiota early in life, and whether a disturbed microbial community in this labile phase can contribute to metabolic phenotypes later in life.

4.1.1 Mode of Birth

The process of birth is the first significant event of microbial exposure that determines the primary colonizers of the infant. While infants delivered vaginally are primary colonized with taxa that resemble their mothers' vaginal microbiota, including *Lactobacillus* or *Prevotella*, children delivered by caesarian section are dominated by a more skin-like microbiota, including *Staphylococcus*, *Streptococcus*, *Corynebacterium*, and *Propionibacterium* spp. [79, 80]. These differences between mode of birth, which were also characterized by lower microbial diversity, gradually decrease over time, but even after up to 5 years of age a caesarian section-associated microbial profile can be detected [77, 79, 81]. Of note, a study in preschool age children found that delivery by caesarean section increases the risk for the development of obesity [82]. These findings were corroborated by a later study that identified that caesarian section-born infants of overweight mothers had a 5 times increased risk of developing overweight at the age of 1 year, when compared to

children born vaginally to a mother of normal weight [83]. Yet, infants that were delivered vaginally to obese mothers had already a three-times increased risk, indicating that obesity from the mother and delivery mode affect childhood obesity. While the abundance of microbial taxa belonging to the Lachnospiraceae family was found to be more abundant in children of overweight mothers when compared to lean mothers in this study [83], a causative proof of this or other gut microbial taxa on childhood obesity is difficult to obtain in humans. However, it appears that seeding the gut of the newborn child with a dysbiotic microbiota might increase the risk for metabolic (and other) diseases later in life.

4.1.2 Antibiotics

Significant disruptions of the instable developing microbial community in neonates are caused by the application of antibiotics [84, 85]. In an American cohort consisting of 64,580 children, repeated courses of antibiotic treatment increased the rate ratio to develop early childhood obesity, with broad-spectrum antibiotics and early exposure having the strongest effect [86]. While narrow-spectrum antibiotics in this cohort did not increase the risk to develop obesity, analysis of data from population-based Danish National Registries revealed that narrow-spectrum and bactericidal antibiotics increased the odds ratios for type 2 diabetes in an adult cohort, and this association was found up to 15 years before the diagnosis of type 2 diabetes [87]. In a smaller American cohort, children with the exposure to antibiotics had lower abundance of Clostridiales and *Ruminococcus.* Moreover, antibiotic treatments initially reduced gut bacterial diversity, which then recovered within the first 12 months, however, with a delay in the trajectory that was observed in children without any exposure to antibiotics [84].

While a direct causal relationship between antibiotic-mediated microbial disturbances during early-life and metabolic impairments is difficult to obtain in humans, support for this hypothesis is again obtained from mouse experiments. When low doses of the antibiotic penicillin were given to newborn mice, this treatment led to microbiota perturbations, including lower levels of *Lactobacillus*, *Allobaculum*, Rikenellaceae, and *Candidatus Arthromitus* (SFB), and induced metabolic alterations, such as the amplification of diet-induced obesity [88]. Interestingly, even short-term microbiota perturbations led to long-term metabolic impairments in the mice, and this obesity phenotype could be transferred to germ-free mice by microbiota transplantations [88]. Accordingly, having the “appropriate” microbial community composition during the different phases of microbial maturation in the infant gut appears to be an important factor to prevent the development of obesity and other metabolic disorders even in adulthood (Fig. 9).

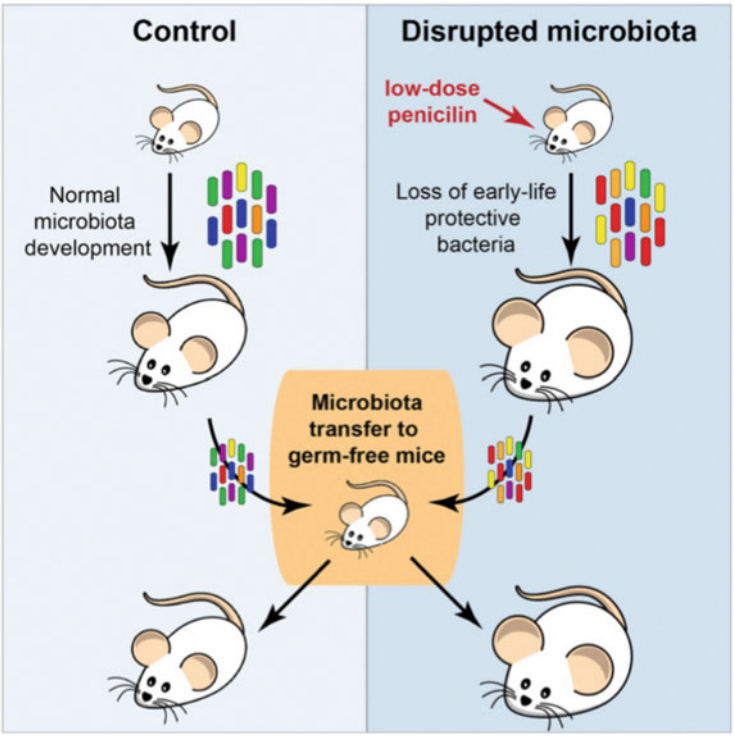

Fig. 9 Altering the gut microbiota early in life has lasting metabolic consequences. Low-dose penicillin treatment directly after birth amplifies diet-induced obesity and leads to long-term increased adiposity in mice. Transplantation of the penicillin-altered microbiota reproduced the obesity phenotype when transplanted into germ-free mice, demonstrating a causal role of the microbiota in inducing metabolic changes. (Image obtained from Cox et al. [83] with permission)

4.2 Intestinal Barrier Function

The interface with the strongest interaction between the gut microbiota and the host is the intestinal mucosal barrier. This protective internal surface comprises physical, biochemical, and immunological mechanisms to protect the host from the tremendous number of microbial organisms in the gut. As the outermost defense system, a secreted mucus gel is a reservoir and energy source for selected gut bacteria, but at the same time forms an impenetrable physical barrier that inhibits microbial access to the epithelium [89]. The second layer is comprised of a single epithelial layer, in which neighboring cells are sealed by tight junctions. The epithelial layer includes specialized secretory cells, including goblet cells that secrete the aforementioned mucus and Paneth cells that secrete antimicrobial peptides as a biochemical defense. Below the epithelial lining, a network of numerous immune cells that secrete effector molecules such as cytokines and IgA is present to surveil the integrity and status of the intestinal barrier and to elicit an immune response upon pathogen encounter or a

barrier breach [90]. Due to its prominent position between the microbial community and the host, the intestinal mucosal barrier is discussed in the context of metabolic diseases.

4.2.1 Microbial Translocation

The observation that the plasma concentration of bacterially produced LPS fluctuated between periods of feeding and fasting in mice and that a high-fat diet intervention for 4 weeks caused a two- to three-times increase in systemic LPS levels [91] seeded the hypothesis that this microbial cell membrane product could play a role in metabolic disease. Supporting this theory, continuous infusion of LPS through implantation of an osmotic pump in mice resulted in metabolic impairments, including increased body weight and liver insulin resistance [91]. Similarly, monocolonization of germ-free mice with an LPS-bearing *E. coli* strain for 4 weeks resulted in impaired glucose and insulin tolerance as well as recruitment of proinflammatory macrophages to white adipose tissue. However, by testing an *E. coli* strain that produces LPS with low immunogenicity, similar impairments on glucose and insulin tolerance were observed, indicating that other bacterial products than LPS are required to exacerbate host metabolism [92].

Also in humans, dietary fat and energy intake correlated with plasma LPS concentrations, which was thought to be caused by fat being more efficient in translocating luminal LPS across the mucosal barrier [93]. Moreover, feeding a high-fat diet to mice increased intestinal permeability by reducing the expression of genes encoding for tight junction proteins while antibiotic treatment of high-fat-fed and *ob*/*ob* mice was sufficient to reduce plasma LPS levels and to prevent glucose intolerance, body weight gain, and fat mass development [94]. Incited by the detection of bacterial fragments and DNA in adipose tissue of mice and humans [95, 96], the authors generated the *tissue microbiota hypothesis*, stating that the origin of metabolic disease could be due to a dysbiosis of microbiota that have translocated to peripheral tissues [96].

Of note, mice that were fed saturated lipid-containing lard had an altered gut microbiota profile and developed obesity and white adipose tissue inflammation, whereas mice fed a fish-oil diet enriched in polyunsaturated fatty acids did not develop these metabolic impairments [97]. Gut microbiota transplantations from fish-oil fed mice to germ-free mice could further demonstrate that the microbial community was even able to protect against the detrimental effect of the lard diet, but that the overall effect of the gut microbiota on host metabolism was rather through the stimulation of inflammation than by an actual increase in bacterial translocation from the intestine [97].

That bacterial translocation to adipose tissue does occur even under natural conditions has recently been demonstrated in a human cohort consisting of healthy volunteers as well as patients with Crohn's disease (CD) and ulcerative colitis (UC), the two major forms of inflammatory bowel disease [98]. Alive and cultivable bacteria could be isolated from the mesenteric adipose tissue, which is located

adjacent to the intestine. Remarkably, the microbial composition in mesenteric adipose tissue from patients with CD differed from the healthy volunteers, and specifically the bacterium *Clostridium innocuum* was identified in the so-called creeping fat, which is hyperplastic mesenteric adipose tissue and an extra-intestinal manifestation of CD.

CD is characterized by intestinal barrier defects [99], and oral gavage of germ-free mice with *C. innocuum* could confirm that this bacterium can indeed translocate from the intestine to the mesenteric fat. Most strikingly, this translocation resulted in expansion and restructuring of the mesenteric adipose tissue and thereby in prevention of further microbial translocation from the intestine to the systemic circulation [98]. Consequently, while the translocation of gut microbiota to white adipose tissue appears to contribute to metabolic dysfunction in the host, the specific translocation of microbes to other fat tissues, such as the mesenteric adipose tissue, may rather have a beneficial role.

4.2.2 The Intestinal Mucus Layer

To be able to translocate across the mucosal barrier bacterial cells and their metabolites first need to penetrate the mucus layer. While smaller metabolites commonly penetrate rather undisturbed, the mucus layer is impenetrable for the majority of the gut microbiota. Interestingly, however, the presence of gut bacteria is required for proper functioning of the mucus layer, as the colonic mucus layer in germ-free mice is penetrable and microbial colonization of up to 8 weeks is required in order to become impenetrable and thus protective [100].

Impairments of the mucus layer have been linked to metabolic diseases. As such, ZG16 (zymogen granule protein 16) is a lectin-like protein in mice that aggregates Gram-positive bacteria in the mucus and thereby prevents their motility. Mice that are deficient in ZG16 had more bacteria translocating to lymph nodes and spleen and developed an increase in abdominal fat pad mass [101]. Treatment of the ZG16-deficent mice with antibiotics prevented the development of the increased fat pad size, indicating that the gut microbial translocation may be causative for this metabolic effect.

Similar observations have been obtained from mice that consumed the common dietary emulsifiers carboxymethylcellulose and polysorbate-80, which are detergent-like molecules often found in processed food [102]. Mice that were fed low concentrations of these emulsifiers had more bacteria in their mucus layer, gained higher body weight and adiposity, and developed impaired glycemic control as well as low-grade inflammation. As the gut microbiota composition in these mice was also altered when compared to untreated mice, the authors transplanted gut microbiota from the emulsifier-fed mice to germ-free mice and could reproduce the metabolic impairments in the treatment mice. Hence, dietary components that induce microbial community disturbances appear to contribute to the development of metabolic impairments of the host [102].

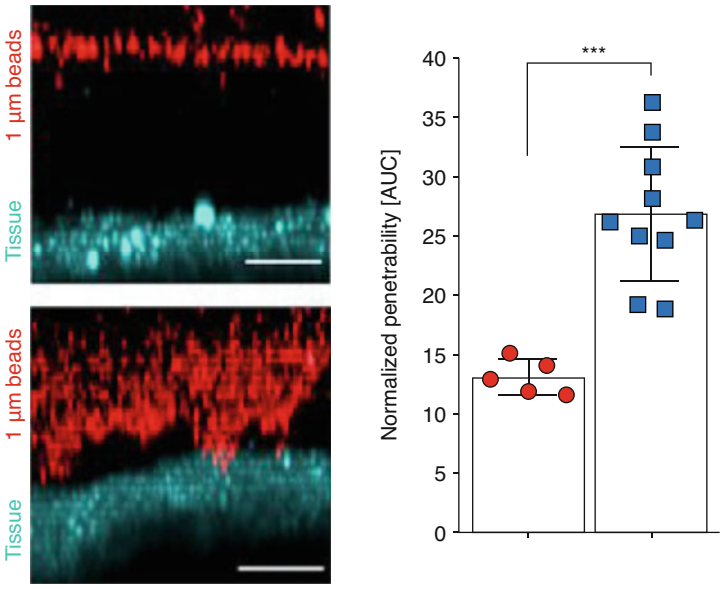

Fig. 10 Intestinal mucus in the colon is penetrable in genetically obese (*ob/ob*) mice. Confocal imaging of the intestinal mucosal barrier function by providing red, 1 µm bacteria-sized beads on top of the inner colonic mucus of lean (top) and *ob/ob* mice (bottom) to determine its normalized penetrability (right). Turquoise staining indicated intestinal tissue. Scale bar = 50 µm. Data are presented as mean ± S.D.; *** $p \leq 0.001$ indicates statistically significant difference in lean versus obese comparison. (Graphic obtained from Schroeder et al. [100] under the terms of the Creative Commons CC-BY license (https://creativecommons.org/licenses/by/4.0/))

In addition to containing emulsifiers, modern dietary habits are characterized by high amounts of simple carbohydrates and saturated fats but being low in dietary fiber. This so-called Western-style diet has been shown to lead to microbiota-mediated defects in the mucus layer [103, 104]. Of note, similar defects of the mucus layer have also been observed in genetically obese *ob/ob* mice that overfeed on a fiber-rich chow diet [105]. Even in these mice the mucus defect appeared to be due to an altered gut microbiota composition that in this case is rather linked to the obese phenotype (see Sect. 3.1.1) than to the quality of the food (Fig. 10).

In humans, increased microbial penetration into the mucus layer has been observed in patients with insulin resistance–associated dysglycemia [106]. This bacterial penetration correlated with BMI, fasting glucose, and HbA1c level but was driven by patients with hyperglycemia. However, penetration of microbiota into the mucus layer was not observed in two different mouse models of type 1 diabetes, suggesting that high blood glucose levels per se do not cause a mucus defect [105, 106].

Following translocation into the mucus, bacteria will be detected by the intestinal mucosa through pattern recognition receptors. As such, Toll-like receptor 5 (TLR5) is expressed by the intestinal epithelium and recognizes bacterial flagellin. Mice

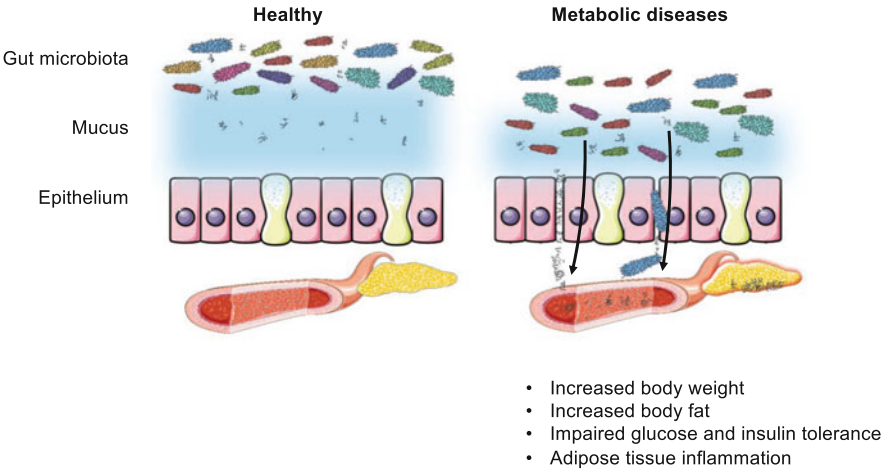

Fig. 11 The intestinal mucosal barrier in metabolic disease. Under healthy conditions the gut microbiota is separated from the host through an impermeable epithelial barrier. Bacterial penetration into the mucus layer is observed in metabolic diseases; translocation of bacterial products, such as lipopolysaccharide (LPS), across the epithelial barrier leads to metabolic impairments. (Graphic contains material from Servier Medical Art (https://smart.servier.com) that has been modified under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/))

lacking TLR5 displayed a 20% increase in body weight as well as higher body fat, impaired glucose tolerance, and decreased insulin sensitivity when compared to wild-type littermates [107]. This effect was strongly reduced after treating the TLR5-deficient mice with antibiotics, indicating that the gut microbial community indeed contributes to the observed metabolic effects. This hypothesis was further strengthened by microbial transplantation from TLR5-deficient mice into germ-free mice, in which many of the metabolic impairments were reproduced [107]. Consequently, prevention of microbial translocation through the mucus layer as well as bacterial recognition by the host epithelium are essential mechanisms of the body to not only ward off infections but also to control microbial modulation of host metabolism (Fig. 11).

4.3 Examples of Novel Microbial Metabolites

Besides SCFAs that have been discussed before (Sect. 2.1), the gut microbiota produces numerous additional molecules that are either metabolized by other bacteria or that can signal to the host and modulate physiology in different organs [9, 108]. Most of these metabolites and how they affect host function are still unknown, but an increasing number of microbial signals are being identified. Two

of the recently identified microbial products that affect host metabolism in different directions will be discussed shortly.

4.3.1 Imidazole Propionate

Untargeted metabolomics analysis of a small cohort of obese patients revealed that four amino-acid derived metabolites were increased in the portal blood of patients with type 2 diabetes [109]. Additional metabolomics analyses of germ-free, antibiotic-treated, and conventionally raised mice further detected that out of these, only imidazole propionate was microbially produced and therefore of interest to investigate further. Imidazole propionate is a histidine-derived metabolite that is generated during the microbial degradation of aromatic amino acids.

Elevated imidazole propionate levels were confirmed in a larger cohort of patients with type 2 diabetes when compared to non-diabetic controls, and interestingly, microbial communities from patients with type 2 diabetes produced higher amounts of this metabolite in an in vitro continuous fermentation model when compared to samples obtained from the control group [109]. Injection of imidazole propionate into germ-free mice induced glucose intolerance, while body weight, insulin levels, food intake, or weight of white adipose tissue remained unaffected. This indicates that the effect of imidazole propionate is interfering with a specific metabolic pathway and not leading to overall metabolic impairments. Indeed, the authors identified that imidazole propionate inhibits insulin signaling through activation of the mammalian target of rapamycin complex 1 (mTORC1) pathway, which was even more active in patients with type 2 diabetes than in matched controls [109]. Several microbial producers of imidazole propionate could be identified, including *Streptococcus mutans* and *Eggerthella lenta*, which emphasizes that distinct members of the gut microbial community can have very specific effects on host metabolism, and that the relative and/or absolute abundance of such microbes in a complex community may determine the metabolic consequences for the host.

4.3.2 *Akkermansia muciniphila* Proteins

A bacterium that has been in the focus for positively modulating host metabolism is *Akkermansia muciniphila*. This mucus-residing bacterium has been shown to be decreased in obese and type 2 diabetic mice while enrichment of this bacterium by oligofructose feeding correlated with an improved metabolic profile in mice fed a high-fat diet [110].

While this initial study found that *A. muciniphila* needs to be viable to exert its beneficial effect, later follow-up research could demonstrate that pasteurized *A. muciniphila* was even more efficient than the viable bacterium to reduce fat mass development, insulin resistance, and dyslipidemia in mice [111]. The authors could identify a protein in the outer bacteria membrane, termed Amuc_1100, which through binding to TLR2 could partly reproduce the beneficial effects of the whole

bacterium. Despite the fact that the downstream mechanism of Amuc_1100 after TLR2 activation is not yet identified, a pilot study investigating the supplementation of alive or pasteurized *A. muciniphila* to obese human volunteers for 3 months could confirm its tolerability and safety and already observed improvements of several metabolic parameters [112].

Recently, another protein from *A. muciniphila* has been shown to improve glucose homeostasis and metabolic disease in mice [113]. This secreted protein, termed P9, was able to induce GLP-1 secretion in L-cells and activate thermogenesis in brown adipose tissue of mice fed a high-fat diet. Mechanistically, P9 interacts with the intercellular adhesion molecule 2 (ICAM-2), and its beneficial metabolic effect was dependent on interleukin 6 [113]. Consequently, *A. muciniphila* produces at least two different proteins in its membrane or as a secreted molecule that interact with distinct host proteins to beneficially modulate host metabolism.

4.4 Summary

Gut microbial modulation of host-metabolism functions through various mechanisms that we are only beginning to understand. It appears that the presence of the "correct" microbial composition during a critical time window of up to 6 months after birth is important for long-term metabolic health. During this period, mode of birth, medication, diet, and environmental exposure are the main factors that affect the developing microbial community. Furthermore, microbial localization and containment within the intestinal lumen appear to be important to prevent the development of metabolic impairments, as translocation of bacteria or their products across the mucosal barrier has been found to cause metabolic defects, mainly in different types of adipose tissue. While more and more microbial metabolites are being identified that alone can have an effect on host physiology, the overall effect on the host will be determined by the output that is generated through complex interactions and cross-feeding of the whole microbial ecosystem.

5 Translational Perspectives and Conclusion

The understanding of how the gut microbiota modulates host physiology and specifically metabolism has advanced from observational studies in germ-free mice to mechanistic insight in mice and humans. The identification of relevant individual microbial members, their metabolites, and their reactions under specific dietary conditions thus allows targeting of the microbiota as a therapeutic strategy to correct impaired metabolism of the host, or even to prevent the development of metabolic disorders at an early stage.

Several options exist to achieve "microbial corrections." The simplest—yet most difficult—is to change dietary habits and switch from an industrialized Western-style

diet to a more fiber-rich diet. Providing more complex carbohydrates to the microbial community would increase its diversity and its production of SCFAs and other molecules that are beneficial for host metabolism [114, 115]. A similar outcome, however, can be achieved by supplementing the habitual diet with prebiotics, which often include dietary fibers that specifically enrich beneficial bacteria. Moreover, pioneering clinical trials could already demonstrate that it is possible to predict a metabolic host response toward a dietary substrate based on the individual microbiota composition [56]. In the future, it is thus likely that personalized dietary recommendations can be generated, which are based on the individual's microbiota composition and with the aim to correct specific metabolic phenotypes [116].

Besides microbial modulation through diet, microbial supplementation and complete replacement of the microbiota are more direct approaches. Novel next-generation probiotics can be developed with the aim to mitigate specific metabolic disorders, and the promising findings of the *A. muciniphila* supplementation [112] are probably only the beginning. Still, the utilization of currently innovative probiotics may just be an intermediate step until the active molecules or metabolites are identified, as exploitation of such "postbiotics" [117] would simplify regulatory and production processes.

Lastly, a complete replacement of the microbial community by fecal microbiota transplantation is a current research focus to treat metabolic disorders. Clinical trials are ongoing, and results have shown that in some cases metabolic phenotypes of the donor can be transferred [73, 75, 118]. However, no long-term success in the treatment of metabolic disorders has yet been obtained, and a better understanding of why the engraftment of samples from some donors is more efficient than from others is required before fecal microbiota transplantation can be considered as an alternative treatment for metabolic diseases [119].

In conclusion, there is strong evidence that the gut microbial community is an important contributor to the metabolism of the host. The microbiota is essential to extract energy from the food and convert it into fuel and signals that can be utilized and sensed by the body. While the evolutionary function of this symbiosis was likely to increase the chances of survival when food was scarce, our modern dietary habits and lifestyle turned this microbial benefit into a situation in which the microbiota generates an excess of energy that eventually leads to metabolic diseases. While exposing the body to the "correct" type and "correct" number of microbes during the "correct" time window might stop and reverse the epidemic of metabolic diseases, a better mechanistic understanding of what the term "correct" means is required. However, already now being good microbial farmers and nourishing our microbes adequately, we will be able to utilize and strengthen the relationship with our gut microbiota for the benefit of metabolic well-being.

Compliance with Ethical Standards

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The Impacts of Microbiota on Animal Development and Physiology

Kathleen T. Walsh and Karen Guillemin

Abstract Animals evolved in a world dominated by microbes. While pathogenic microbes have long been appreciated as the cause of infectious diseases, only more recently have we understood that diseases can be caused by a lack of beneficial microbes. Microbial genomic sequencing can provide insights into the vast diversity of microbiomes associated with human health and disease, but experimental animal models are required to test hypotheses about the beneficial or detrimental effects of these microbes and their molecular products. Studies in gnotobiotic animal model systems reveal the aspects of animal biology shaped by our microbial associates and shed light on new possible mechanisms underlying human diseases. Here, we survey insights from the widely used animal model systems in microbiome research. We explore emerging shared themes across these diverse animal hosts about the interconnected impacts of microbiota on immune system maturation, intestinal epithelial homeostasis, nervous system development, endocrine signaling, and metabolic regulation. Research in animal models can provide both the basis for uncovering microbial influences on human health and disease, and also the starting point for developing treatment strategies to correct dysregulation of animal-microbe interactions in disease.

Keywords Microbiome · Gnotobiology · *Drosophila* · Zebrafish · Mouse · Immune system · Metabolism · Nervous system

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1 Introduction

Not only oxygen and water but also microorganisms have been a fixture of the world in which animals evolved. Microorganisms inhabited the earth for some three billion years prior to the emergence of the first simple animals, shaping the earth's atmosphere and generating the oxygenated environment that would allow for the existence of aerobically respiring multicellular organisms [1]. The coexistence of animals and microbes is evident in the genomes of single-celled eukaryotes such as amoebae, which encode extensive repertoires of genes with signatures of innate immune sensing and antimicrobial defenses [2]. The biological properties of extant animals, including humans, are shaped by both their evolutionary history with microbes and by their lifelong associations with microbes in and on their bodies [3]. Understanding the normal functioning of animal-microbial interactions is critical for diagnosing diseases in which these associations go awry.

In this chapter, we consider the experimental frameworks necessary for establishing causative relationships in host-microbe systems. We discuss the nature of the molecules produced by microbes and perceived by animal cells and tissues to modulate developmental decisions and physiological programs. We describe several prominent animal models that have been instrumental in revealing the molecular nature of these relationships. We then discuss lessons learned from these animal models about the roles resident microbes play in the development and function of different animal tissues and systems. Studies in these model animal systems reveal the many facets of animal biology that are shaped by our microbial world and highlight new possible mechanisms that can underlie human diseases.

2 Establishing Causation in Host-Microbe Systems

The traditional focus of medical microbiology has been on diseases caused by the presence of a single specific microbe, which we term a pathogen. Studying the action of pathogens in order to develop strategies to treat and prevent infection requires animal models of those infectious diseases. In the late nineteenth century, Robert Koch developed a rigorous experimental framework for using laboratory animals to test the causative role of specific microbes in infectious diseases. Fulfillment of Koch's postulates in an animal model has become accepted as proof that a specific pathogen is both necessary and sufficient to cause a specific disease in humans. In the late twentieth century, with the advent of microbial molecular genetics, this framework was updated in what was termed "molecular Koch's postulates" [4] to establish the causal role of a specific pathogen toxin or effector molecule in a specific disease.

More elusive has been the understanding of diseases caused not by a single microbe but by disturbances in normal associations with microbes. The concept of disease predisposition due to the absence rather than the presence of specific

microbes was first introduced by David Strachan in 1989 as the "hygiene hypothesis," which posited that allergic diseases could be due to an insufficient stimulation of the immune system in the absence of childhood exposure to infections [5]. This idea was further refined by Martin Blaser and Stanley Falkow as the "disappearing microbiota hypothesis" that proposed the loss of certain ancient members of human-associated microbial communities, or microbiota, as the basis for increased incidence of diseases of immune dysregulation in high income countries [6].

The technological capacity to test these ideas about missing microbes and modern diseases followed in subsequent decades with advances in high throughput sequencing that enabled the comprehensive cataloguing of microbial community genomic sequences, or microbiomes, from human tissues. At first the complexity of these communities was overwhelming, with inter-individual differences dwarfing anticipated signatures of health and disease. As cataloguing efforts have become more comprehensive, with studies of people across the globe and longitudinal studies of individuals over time, patterns have emerged that corroborate the hypotheses that lifestyles of high income countries are associated with reduced human-associated microbial diversity. In addition, signatures have started to emerge of microbiomes associated with different diseases. The term "dysbiosis" was coined to describe disease-associated microbial communities that deviate from normal patterns.

Testing causal relationships between a particular microbial community and a specific disease required new experimental frameworks using animal models that are amendable to microbiota manipulations. For this, researchers turned to the field of gnotobiology in which eukaryotic organisms are grown in the absence of any microbial associations ("germ-free" or "axenic") and then following this sterile derivation, associated with single microbes or defined microbial consortia [7]. Using gnotobiotic animals, researchers could test whether a dysbiotic microbiota was necessary and sufficient for a disease phenotype, using a framework termed "ecological Koch's postulates." [8] Starting with an animal model of a disease with a suspected microbiota etiology, they could test whether deriving the animals germ-free would eliminate the disease symptoms. Conversely, they could test whether a microbial community, harvested from a diseased donor animal or human subject, and transferred to a healthy germ-free recipient animal would confer the disease phenotype in this new host. Such microbiota transplantation experiments have become the standard in the field for establishing the causal relationships between dysbiosis and disease [9].

Evidence that a perturbed microbiota causes a disease does not immediately provide an explanation for how the disease arises. Investigating the mechanistic basis for microbiota-associated disease requires understanding normal microbiota-host interactions in the healthy state. Here gnotobiotic animal models have proved to be invaluable. By studying the properties of animals reared in the absence of their microbial associates, researchers can infer the normal functions these microbes play in animal development and physiology. The same experimental manipulations described in the frameworks of ecological Koch's postulates and molecular Koch's postulates can be employed to test the role of specific microbial communities, microbes, and microbial products in animal development and health.

3 Microbiota-Derived Molecules Perceived by Animals

A major question in the field of microbe-host interactions is the nature of the molecules that modulate animal developmental and physiological programs. The answers now emerging from different experimental models, examples of which are listed in Table 1, provide fundamental insights into animal biology and also suggest new molecular approaches for treatment of human diseases with microbial etiologies. From the studies of bacterial pathogens, using the framework of molecular Koch's postulates, we know of a diversity of microbial molecules that impact animal cells and induce the pathologies of infectious diseases. On one end of the spectrum are generic microbial molecules such as lipopolysaccharide (LPS), the cell wall component of all Gram-negative bacteria that was first discovered as endotoxin based on its capacity to induce many symptoms of infections [10]. On the other end of the spectrum are toxins produced by specific bacterial species or strains that determine their infectious disease pathology, such as the flaccid paralysis caused by Botulinum toxin that cleaves SNARE proteins and inhibits neurotransmitter release [11]. Studies of bioactive molecules from microbiota reveal a similar spectrum of effectors and allow us to understand the nature of the informational exchange between animals and their microbes [3]. On one hand, microbial effectors can be classified as molecular cues, produced for other purposes and perceived by animals to inform them about their microbial residents. On the other hand, these molecules may function as signals specifically produced to communicate with animal cells and elicit responses that are beneficial to the microbial producer.

Clear examples of microbial cues are the generic, microbial-specific molecules like LPS that Charles Janeway and colleagues classified as "Pathogen Associated Molecular Patterns" or PAMPs [12]. Their cognate receptors, such as the LPS binding Toll-Like Receptor 4 (TLR4), were termed "Pattern Recognition Receptors," or PRRs, to describe the innate immune receptors that recognize common microbial molecules. PRRs are ancient and widespread across eukaryotes [2], in contrast to the receptors of the adaptive immune system that are exclusive to the vertebrate lineage of animals. Although these concepts of conserved microbial

Table 1 Different classes of microbial molecules affect host cells

Bioactive microbial molecules Classes and examples	
Microbial molecule	Animal cell receptor or target
Generic microbial associated molecular patterns (MAMPs)	
Lipopolysaccharide (LPS)	TLR4
Generic microbial metabolites	
Short-chain fatty acids (SCFAs)	G protein-coupled receptors (GPCRs)
Species-specific microbial toxins or molecules	
Botulinum toxin	SNARE proteins
Polysaccharide A (PSA)	TRL2/1 heterodimer and Dectin-1

Examples from the classes of microbial molecules and the corresponding host cell receptor or target

detection were transformative, the term PAMPs was a misnomer because molecules such as LPS are not exclusive to pathogens but rather define basic features of microbial cell biology. Identification of bacterial cell wall molecules functioning in beneficial symbioses prompted a rename of these molecules as "Microbial Associated Molecular Patterns" or MAMPs [13]. As discussed below, innate immune reception of such molecules plays important roles in host responses to resident microbiota.

Another example of microbial cues is metabolites that are the products of specific microbial physiologies. These molecules are less generic than MAMPs, and thus metabolite perception can confer information about the identity of the producing microbes, although some metabolites are made by phylogenetically unrelated microbial lineages and through different enzymatic processes. The best studied of these types of molecules are the short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate that are the byproducts of the fermentation reactions of many anaerobic bacteria [14]. The absolute and relative abundances of SCFAs can modulate properties ranging from intestinal barrier function to nervous system activity. These molecules are perceived by G protein-coupled receptors (GPCRs) expressed on many cell types throughout the body. More generally, animal genomes encode large numbers of orphan GPCRs, hormone receptors, and other receptors that are likely involved in detecting microbial metabolites [15].

Fewer examples exist in the microbiome literature that resemble the specificity of bacterial toxins which target particular host receptors or signaling pathways. One such effector molecule, the *Bacteroides fragilis* polysaccharide A (PSA), has potent immunomodulatory activity that can correct immune system immaturity in germ-free mice [16] and behavioral abnormalities in a maternal immune activation mouse model of autism spectrum disorder [17]. Although potent and specific in its effects, PSA is a cue rather than a signal that is produced by *B. fragilis* as a component of its protective capsule and is perceived by receptors of the innate immune system: TLR2/1 heterodimers signaling in parallel with the C-type lectin carbohydrate receptor Dectin-1 [18]. The challenge of identifying microbiota-derived signals, similar to pathogen toxins, may come from the complexity of animal-associated microbial communities. Another possibility is that our perception of bacterial toxins' specificity for animals has been warped by a focus on human infections without considering the ecology of the producing bacteria [19]. For example, the fitness benefit of Botulinum toxin production conferred on the soil bacterium *Clostridium botulinum* is unknown, but more plausibly involves competition with other soil bacteria than intoxication of animals. Similar selective pressures that drive *C. botulinum* to produce its toxin may induce members of animal-associated microbiota to produce specific molecules that happen to have potent and specific collateral effects on their hosts.

4 Gnotobiotic Animal Models

Exploring the impacts of resident microbes and their associated molecules on animal biology requires experimentally tractable gnotobiotic models. Here we provide brief descriptions of several of the prominent gnotobiotic animal systems that have advanced our understanding of the impacts of microbiota (Fig. 1). Each system has its unique strengths. Studies of different animal models complement each other and advance our understanding of the common ways in which microbiota shape animal tissues and the common mechanisms through which animals perceive and respond to their microbial inhabitants.

4.1 The Bobtail Squid Model

The bobtail squid, *Euprymna scolopes*, forms an exclusive symbiosis with the luminescent marine bacterium *Vibrio fischeri*. The squid, a night-active predator, harbors an active culture of light-producing bacteria in a specialized tissue called the light organ, which allows it to evade detection while moonlit hunting. Pioneered as a

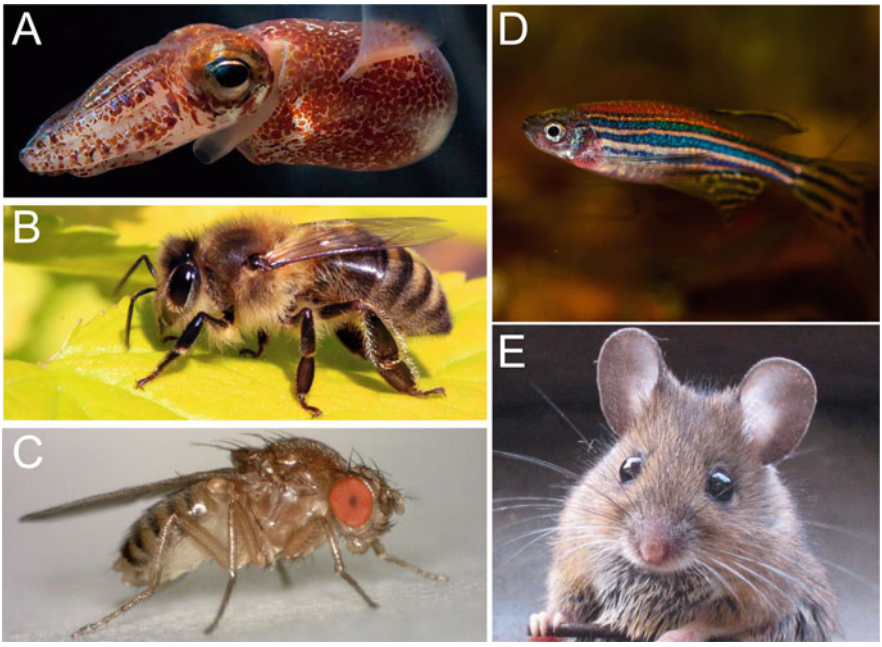

Fig. 1 Gnotobiotic animal model systems. Animal models used in research of host and microbiota interactions include (**a**) *Euprymna scolopes*, the Hawaiian bobtail squid; (**b**) *Drosophila melanogaster*, the fruit fly; (**c**) *Apis mellifera*, the Western honey bee; (**d**) *Danio rerio*, the zebrafish; and (**e**) *Mus musculus*, the laboratory mouse

model system by Margaret McFall-Ngai, Edward Ruby, and colleagues, the squid-*Vibrio fischeri* symbiosis features a simple binary association between an animal host and a bacterium [20, 21]. Importantly, the two partners can be grown in isolation from each other, making the system tractable to experimental manipulations of the symbiosis.

The squid-*Vibrio fischeri* symbiosis has been an especially powerful model for understanding the impact of microbes on animal development [22]. In the absence of their luminescent symbiont, juvenile squid will fail to undergo a normal developmental transformation of the epithelial tissue surrounding their symbiont-harboring light organ. Elegant studies exposing aposymbiotic squid to bacterial products showed that the normal developmental events of tissue remodeling around this organ could be triggered by a combination of LPS and a specific fragment of the bacterial cell wall polymer peptidoglycan [13]. The model has also been powerful for understanding how animals maintain long-term associations with resident microbes. Within the squid, *Vibrio fischeri* undergoes diurnal cycles of growth and expulsion, which maximizes light production for the squid during its nightly hunting. Transcriptional and metabolomic profiling reveals dramatic daily cycles of changes in the genetic regulation and chemical environment of the partnership, reminiscent of circadian cycling in humans [23, 24].

4.2 The Fruit Fly Model

The fruit fly *Drosophila melanogaster* has a long history as a model organism, and research with this simple animal has resulted in six Nobel Prizes to date. It was first studied in depth by Thomas Hunt Morgan and colleagues in the early twentieth century to elucidate basic principles of genetic inheritance. The genetic tools developed by these studies enabled the whole genome, forward genetic screens of Christiane Nüsslein-Volhard, Eric Wieschaus, and colleagues that uncovered the genetic basis for patterning of the basic animal body plan during development. These foundation screens uncovered many genes whose protein products are important in signal transduction pathways employed across the animal kingdom. One example is the Toll receptor, discovered in a subsequent screen by Kathryn Anderson as being required for dorsal-ventral patterning, and subsequently found by Jules Hoffmann and colleagues to be one of a family of innate immune sensors critical for protection against infectious diseases [25, 26].

More recently, *Drosophila* has emerged as a model for studying animal interactions with their resident microbes [27]. The foundational knowledge of developmental biology and innate immunity, the sophisticated genetics tools, and the ease of fruit fly husbandry have fueled this field. In addition, a strong history of *Drosophila* population genetics and ecology research propelled analyses of the microbes associated with fruit flies in the wild. In comparison to vertebrate animals, the gut microbiomes of both wild caught and laboratory fruit flies proved to be relatively simple in composition, typically consisting of less than a dozen distinct strains.

Drosophila melanogaster gut microbiomes are similar to the communities found associated with rotting fruit, dominated by bacteria belonging to the genera *Lactobacillus*, *Acetobacter*, *Gluconobacter*, and *Enterococcus*, and yeasts belonging to the genera *Kloeckera*, *Pichia*, and *Saccharomycodes* [28].

The phenotypes of germ-free *Drosophila* reveal that the microbiota is required for aspects of normal growth and metabolism, intestinal development, insulin signaling, and behavior. The low cost and ease of rearing *Drosophila*, which enabled the large screens described above, have advanced the field of microbiome research by allowing unbiased discovery of host and bacterial factors that determine these traits. Researchers have conducted genome-wide association studies to identify host genes that modulate microbiota composition [29] and host metabolic responses to microbiota [30]. Additionally, the ability to screen large populations of hosts has allowed studies in which *Drosophila* are mono-associated with individual isolates from collections of bacterial mutants to identify bacterial determinants of host-microbe interactions, for example, with *Acetobacter pomorum* [31] and *Lactobacillus plantarum* [32]. Large population sizes and experimental tractability have also enabled comprehensive studies of the interactions between host diet, microbiota, and physiology.

4.3 The Honey Bee Model

The Western honey bee, *Apis mellifera*, is a newer insect gnotobiotic model [33]. Developed independently by Nancy Moran and Irene Newton to study microbiota assembly and function, the model builds on extensive research about honey bee social behavior and ecology as a pollinator for economically important crops. Similar to fruit flies, honey bees have relatively low complexity microbiomes. Although the model lacks as extensive a history of genetic manipulations, recent innovations in genome engineering enable transgenesis and disruption of honey bee genes [34]. In addition, members of the honey bee microbiota can be genetically manipulated [35], which has been used to study the microbiota-host association and to engineer beneficial microbiota-dependent properties such as increased immune activation and protection against pathogens [36].

The complex social structure of the honey bee hive contributes to the assembly of distinct intestinal microbiomes of workers and queens [37]. The queens are the most important individuals in the hives, and as such they are protected from pathogens both by being isolated from the foraging workers who are at greatest risk of acquiring infections and by being provisioned with microbiota members that prevent infection [38]. The properties of honey bee microbiota members that confer protection against different pathogens are beginning to emerge [38, 39], and this knowledge will have immediate applications for protecting honey bee populations used in agriculture.

Studies of germ-free honey bees have revealed important roles for the microbiota in growth and metabolism. Similar to germ-free fruit flies, honey bees derived

without their microbiota exhibit decreased growth [40]. In addition, expression of key genes involved in the insulin pathway are decreased in expression in germ-free honey bees, demonstrating critical roles for the honey bee microbiota in regulating endocrine signaling.

4.4 The Zebrafish Model

The zebrafish, *Danio rerio*, was established as a model system by George Streisinger at the University of Oregon in the late 1970s with the hope of applying powerful forward genetic screening approaches to a vertebrate animal [41]. The model had many important attributes that would attract prominent researchers from other systems, including Christiane Nüsslein-Volhard from *Drosophila*, and propel the zebrafish to become a major platform for biomedical research. Its high fecundity and relative ease of husbandry enables forward genetic screens and other experiments with large population sizes. The embryos' optical transparency and rapid, ex-utero development have shed new light onto processes of vertebrate embryogenesis. In the last few decades, an explosion of genetic engineering approaches has opened up new avenues of investigation with zebrafish. These include transgenic expression of fluorescent reporters of cell types and processes and CRISPR/Cas9 mediated site-specific mutagenesis.

All of these attributes make zebrafish a powerful model for microbiome studies [42]. Foundational work for this model profiled the gut microbiome composition of lab reared and wild-caught zebrafish [43] and surveyed gut microbiomes across development [44]. This work revealed high complexity microbial communities, with hundreds of bacterial species belonging to similar bacterial domains as represented in the mammalian intestine, but with different proportional representation and species membership. The larval stages, when the animals are first colonized after hatching, are dominated by facultative aerobes of the *Gammaproteobacteria*, similar to the early neonatal stages of human microbiome assembly. Cultivation and genomic engineering of representative gut bacteria from larval zebrafish [45] has generated a collection of fluorescent protein expressing strains, allowing visualization of processes of bacterial colonization dynamics in living animals [46]. This work reveals how bacterial behaviors, such as swimming motility versus biofilm formation, influence the biogeography of the microbiota [47] and host immune responses to resident bacteria [48].

4.5 The Mouse Model

The laboratory mouse, *Mus musculus*, has a long history of use in gnotobiology, dating back to the 1940s [49]. These small mammals are well-accepted animals for preclinical studies modeling human diseases and responses to therapeutics. Decades

of mouse research have yielded sophisticated genetic resources such as large collections of mutants and tools for cell type specific gene manipulations. The field of immunology is built on mouse research, which has generated deep molecular insights into innate and adaptive immune responses to microbes. All of these tools have been invaluable for characterizing host-microbiota interactions in the mouse.

Of the standard laboratory models for microbiome research, the mouse harbors a gut microbial community that is most similar in composition to that of humans, dominated by the same phyla of *Bacteroidetes* and *Firmicutes*. The mouse is also the only standard gnotobiotic animal model that can be efficiently transplanted with human microbiota samples, creating "humanized" microbiome mice [50]. Transplantation of human microbiomes has become a standard approach for evaluating the potential functional properties of human microbiota samples. For example, fecal samples from twins discordant for obesity were shown to differentially impact the metabolism of transplanted germ-free mice, with the murine recipients of microbiota from an obese twin gaining more weight than those that received microbiota from the corresponding lean twin [51]. However, others have argued that the conclusions drawn from such humanized mouse experiments are subject to investigator bias and over-interpretation [52].

5 Impacts of Resident Microbes and Lessons for Human Health

Collectively, these major gnotobiotic animal models are teaching us about the impacts of resident microbes on various animal tissues and organ systems (Fig. 2). Comparisons across animal systems reveal generalities about the nature of microbial factors and activities that influence animal biology.

5.1 Intestinal Epithelial Renewal

The epithelium that lines the intestinal tract is the tissue in the closest contact with the densest and most populous microbial community of the animal body. A conserved response of this epithelium to the presence of resident microbes is an elevated rate of epithelial renewal. Across the model systems of flies, zebrafish, and mice, the absence of a microbiota results in reduced numbers of proliferating cells as compared to conventionally reared counterparts [53]. It is not yet known whether the molecular mechanism that stimulates intestinal epithelial cell proliferation in response to microbiota is conserved across these animal hosts. However, the intestinal epithelium in all of these organisms responds to inflammatory insults and physical injury by upregulating programs of epithelial renewal through conserved pathways such as the Jak/Stat, EGF, and Wnt pathways [54]. Additionally,

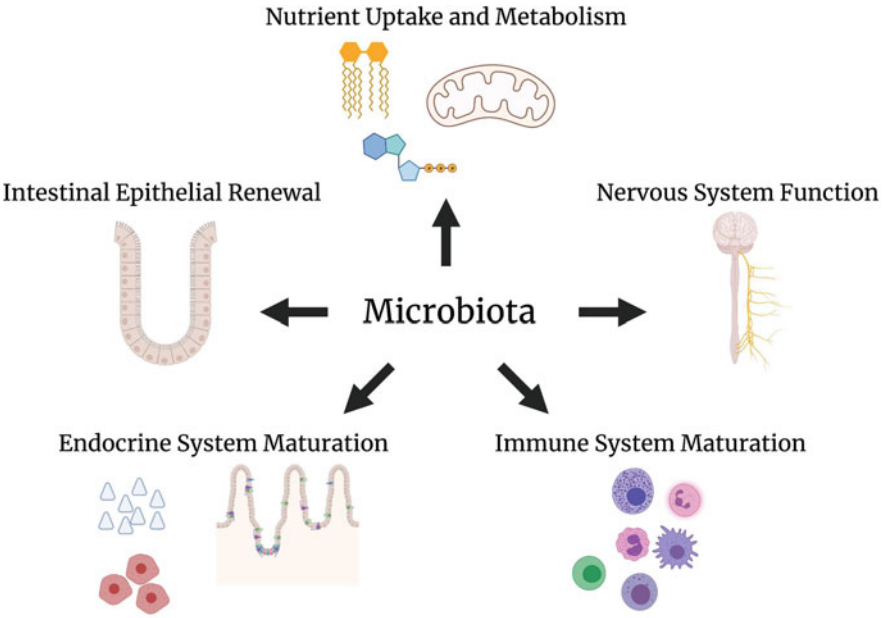

Fig. 2 Summary of the impact of resident microbes on animal tissues and organ systems. Research using gnotobiotic animal models has revealed microbiota influences on diverse host aspects including *(clockwise from top)* nutrient uptake and metabolism functions, such as mitochondria, lipid metabolism, and ATP synthesis; nervous system function and development; maturation of the immune system; endocrine function including insulin signaling (triangles), beta cell development, and intestinal enteroendocrine cell number; and the proliferation rate of intestinal epithelium. See text for details

conserved innate immune signaling pathways are required for sensing and responding to microbiota derived cues that stimulate intestinal epithelial proliferation. For example, zebrafish deficient for the common TLR adaptor protein, Myd88, have low rates of intestinal epithelial proliferation, resembling germ-free rates [55]. Both the presence of microbiota and Myd88 is also required for colonic epithelial proliferation in a mouse model of intestinal injury with the chemical irritant dextran sodium sulfate [56]. Thus, it is plausible that homeostasis of the intestinal epithelium in response to the presence of colonizing gut microbes is a result of a subtle triggering of inflammatory and tissue repair programs by generic microbial stimulants perceived through innate immune pathways.

5.2 Nutrient Uptake and Metabolism

The primary function of the intestine is to absorb nutrients that are subsequently metabolized and disseminated throughout the body. There is a complex interplay

between gut microbiota, diet, and metabolism [57]. Dietary changes have profound impacts on gut microbiota composition. Reciprocally, microbiota influence the processes of nutrient absorption and utilization. A general feature of germ-free animals is that they typically have the metabolic traits of an undernourished state even when given unlimited access to food. Germ-free *Drosophila* are delayed in their development in the transition from their larval to pupal stages, and under nutrient-limited conditions they will fail to pupate and die as larvae. Germ-free zebrafish and mice share conserved programs of microbiota-regulated nutrient acquisition gene expression [58], and both exhibit nutrient acquisition defects such as reduced lipid absorption [54, 59].

Dissecting the complex interactions between microbiota, diet, and metabolism requires not just gnotobiology but also experimental control of nutrient intake, which can be challenging even with laboratory animals. The *Drosophila* field has developed elementally defined diets for fruit flies, allowing them to systematically eliminate macro- and micronutrient components of the diet and study the impact of these dietary manipulations in the context of colonization with different gut bacteria [60]. These studies show that gut bacteria provision certain essential nutrients to their hosts including essential amino acids and trace metals. Similar provisioning, for example, of sphingolipids, also occurs in the mammalian intestine [61].

The fact that germ-free animals generally exhibit reduced metabolic rates may reflect a requirement for additional factors normally provisioned by resident microbes. Recent whole body transcriptomic and metabolomic profiling of conventionally reared versus germ-free *Drosophila* reveal that the lack of bacteria causes an overall reduction of host mitochondrial function and ATP production [62]. This deficit could be reversed by supplementation of bacterial riboflavins, which are precursors of the universal mitochondrial co-enzymes FAD and FMN, suggesting that bacteria normally are sources of these molecules. Limited metabolic capacity could then impact developmental programs throughout the body, similar to developmental alterations associated with nutrient deprivation [63]. Indeed, early childhood deprivation of nutrients can impair normal programs of microbiome maturation in humans and result in developmental defects such as growth stunting and neurological deficits reminiscent of developmental defects in germ-free animals [64]. Mechanistic studies in model systems are critical for providing molecular insights into the diversity of human metabolic diseases of both under- and overnutrition, such as environmental enteropathy, diabetes, and cardiovascular disease, which are linked, based on epidemiological studies, to interactions between diet and the gut microbiota.

5.3 Endocrine System Maturation

Critical for nutrient utilization is the regulation of cellular metabolism by endocrine hormones. Across multiple animal models, endocrine signaling is impacted by the

microbiota. This occurs both at the level of signaling regulation and through impacts on the development of endocrine cells and tissues.

The quintessential endocrine signaling pathways is the insulin pathway. Insulin signaling is reduced in germ-free fruit flies [31] and honey bees [40], resulting in their reduced growth in the absence of their microbiota. Forward genetic screening in the fruit fly commensal *Acetobacter pomorum* identified a metabolic pathway involved in acetic acid production as a critical cue for promoting normal insulin signaling [31]. Additionally in fruit flies, the immune deficiency (IMD) innate immune signaling pathway was found to be critical for sensing acetate, the bacterial fermentation SCFA, and regulating insulin signaling [65].

In germ-free zebrafish, insulin levels are reduced, and circulating glucose is elevated because the larvae fail to develop the normal number of insulin-producing beta cells in their pancreas [66]. This defect can be rescued by supplementation with a single commensal bacterial secreted protein, Beta Cell Expansion Factor (BefA), of novel sequence and function [66]. BefA homologues are found in the genomes of human intestinal microbiota members, raising the possibility that lack of this protein during early postnatal development could predispose individuals to the development of type 1 diabetes, a disease of beta cell paucity.

Within the intestinal epithelium, specialized enteroendocrine cells secrete hormones that regulate metabolism and intestinal function. The specification of these cells is dependent on the presence of microbiota and innate immune signaling. Germ-free zebrafish have fewer enteroendocrine cells in their intestines, a trait that is recapitulated in conventionally reared animals lacking the TLR adaptor Myd88 [67]. Germ-free mice have reduced numbers of an enteroendocrine cell type, enterochromaffin cells, which are also reduced in mutants lacking Tlr2 and Myd88 and restored by addition of the commensal bacterium *Clostridium ramosum* [68] or by the enteric parasite *Trichuris muris* [69].

In addition to their impacts on enteroendocrine cell development, microbiota also play key roles in regulating the functions of these cells. For example, serotonin secretion is reduced from germ-free mouse enterochromaffin cells and restored by addition of certain spore-forming bacteria [70]. In zebrafish, signaling from enteroendocrine cells was found to be silenced by high fat diet, but this silencing required the presence of the microbiota or monoassociation with a commensal *Acinetobacter* strain [71]. The ability of enteroendocrine cells to sense both microbial and nutrient information makes them important cells to consider in the etiology of human metabolic disorders with a microbial component.

5.4 Immune System Maturation

Another common feature of germ-free fruit flies, zebrafish, and mice is an immature immune system. In fruit flies, this has been studied as the lack of antimicrobial peptide expression [72], in zebrafish as a lack of neutrophil immune cells recruited to the intestine [73], and in mice as T cell deficiencies [16]. In-depth studies of

immunological deficiencies in each of these models reveal both local and systemic effects of the presence or absence of microbes. For example, the skin microbiota of mice has been shown to modulate the maturation of local innate immune cells, as well as to educate adaptive T cell populations with systemic functions [74]. One emerging theme is that the context of microbial exposure, both as a function of the cell type and the developmental timing, influences host responses. The importance of timing may explain why early childhood experiences, such as infections or repeated courses of antibiotics, have been linked to adult diseases of immune dysregulation. Another important theme is the connection between immunity and metabolism. Immune cells have been found to play key roles in sensing endogenous perturbations in tissue metabolism, such as drops in nutrient availability [75]. This has prompted a new appreciation for the same diseases discussed above, such as environmental enteropathy, diabetes, and cardiovascular disease, as being immunometabolic disorders, with immune and metabolic dysfunction being inextricably linked in the disease pathologies.

A limitation of laboratory animals for investigating human immunological diseases is their capacity to model human immune system function. Much of immunological research is based on using "specific pathogen free" (SPF) mice, reared in clean, barrier facilities, as the normal reference against which to compare other treatment groups, such as germ-free mice. However, recent work has called into question the normalcy of the immune development of SPF mice. Analysis of the immune systems of wild caught mice or even pet store mice has shown them to have immune cell populations more similar to adult humans, whereas the SPF mouse immune system resembled that of human neonates [76]. Similar immune system maturation can be induced by "wilding" laboratory mice through co-housing or fecal exposures, demonstrating that the microbiomes of the donor mice are responsible for the immune maturation. Although the procedures for generating "dirty" mice pose experimental challenges that SPF mice were designed to overcome, such as lack of reproducibility and the introduction of new pathogens [77], they demonstrate the extent to which microbial exposures mediate immune system development and function, and provide further experimental evidence for associations between human diseases of immune dysregulation and microbiome dysbiosis.

5.5 Nervous System

Of the many impacts of the microbiota on animal biology, one of the most fascinating is its impacts on the nervous system, with implications for regulation of behavior and cognition. These impacts have been studied at different levels, from behavior to neuroanatomy [78]. Across different model systems, deprivation of microbiota is associated with alterations in behavior. For example, germ-free adult *Drosophila* exhibit increased walking activity, which was normalized by colonization with certain bacterial residents, exposure to a bacterial enzyme xylose isomerase that modules host sugar metabolism, or by modulating octopaminergic neuronal

signaling [79]. Similarly, germ-free zebrafish exhibit hyperactivity, which could be reversed by colonization with certain gut bacteria but not by exposure to heat-killed products [80]. Germ-free mice exhibit a number of aberrant behaviors, which vary across genetic backgrounds and settings, but generally can be categorized as increased baseline exploration behaviors and impairments in social behaviors [81]. Modulations of hypothalamic-pituitary-adrenal responses to stress appear to underlie many of these behaviors [82]. The molecular nature of the microbial cues that impact nervous system function is still being uncovered, but conserved microbial molecules and products of metabolism seem to be critical for mediating many of the responses to complex microbiotas [83]. As with the immune system, microbiota impacts on the nervous system are influenced by location, developmental timing, and metabolism.

The microbiome is emerging as an important feature of many human neurodevelopmental disorders, such as autism spectrum disorder (ASD) and schizophrenia, and of neurodegenerative diseases, such as Parkinson's and Alzheimer's disease [84]. There is an urgent desire to deploy knowledge about microbiome dysbiosis in these diseases for therapeutic purposes, but major challenges remain. One hurdle is the complexity of many of these neurological disorders, which cannot easily be modeled in laboratory animals. For example, the social and communication deficits that define ASD cannot be recapitulated in animals that lack the capacity for complex language acquisition. Better understanding of the cellular and molecular bases of these neurological disorders will be needed to reveal the potential for microbial interventions as therapeutics.

6 Conclusions

When viewed through the lens of any specific human disease, microbiota-host interactions can appear intractably complex. Yet when examined through the lens of the common responses to microbiomes shared across well-studied gnotobiotic animal models, certain themes emerge that help provide context for individual human diseases. The importance of resident microbes as sources of limiting nutrients explains their profound impacts on the metabolic states of tissues and organs, setting rates of tissue homeostasis to match metabolic capacities. Resident microbes are also important immunological stimulants of tissue homeostasis, defense, and repair programs. The molecular cues that trigger these programs are likely to be diverse but highly redundant. Collectively, the metabolic state and immune activation of an organism, as determined by its microbiota, will impact the developmental trajectories of many tissues and organ systems, each of which may have different critical windows of sensitivity. Thus, a productive starting point for understanding microbiome-associated human diseases is to uncover the earliest manifestations of metabolic and immunological dysregulations in the affected tissues. Such metabolic and immunological processes may be amenable to experimental modeling in gnotobiotic animal models, providing a path forward for uncovering molecular

mechanisms of disease and developing effective microbial prophylactic and therapeutic treatment strategies.

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Compliance with Ethical Standards
For this article no studies with human participants or animals have been performed by the author.

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The Development of the Gut Microbiota in Childhood and Its Distortion by Lifestyle Changes

John Penders and Niels van Best

Abstract The gut microbiota is established in the newborn period and plays a pivotal role in the development of the mucosal tissue, immune maturation, and host metabolism. Distortions in the assembly and maturation of the microbiota during this critical time-window can therefore have profound effects on future health and the susceptibility to non-communicable diseases.

In this chapter, we provide an overview of the ecological processes involved in the establishment of the indigenous microbial communities during infancy and childhood. Moreover, we summarize the current knowledge on the disruptive effects of lifestyle changes on gut microbiota assembly and maturation. Finally, we highlight important areas for further research in order to identify approaches to revert the deprivation of our microbiota.

Keywords Ecological theory · Microbiota assembly · Birth mode · Breastfeeding · Complementary food · Social interactions · Natural environment · Antibiotics

1 Introduction

The indigenous microbiota of the human gut has long been recognized to contribute to health and disease by influencing gut and immune maturation, host nutrition, and protection against pathogen invasion [1].

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In line with this, perturbations in the development and composition of the gut microbiota have been associated with the development of obesity [2–4], allergies [5–7], inflammatory bowel diseases [8–10], and many other non-communicable diseases [11, 12].

Infancy sets the stage for intestinal microbial assembly, diversification, and maturation. Moreover, this period is a critical time-window during which the microbiota provides a stimulus for the ontogeny of the enteric mucosal tissue and immune system with persistent local as well as systemic effects [13, 14]. Insights into the processes that drive the first inoculum to differentiate into a highly individualized [6, 15–18] and stable microbial ecosystem, as established after the first years of life, will therefore have a direct impact on our ability to manage and maintain human health [19, 20]. To develop successful strategies to restore or maintain a healthy microbiota, we thus need to refine our understanding of the processes driving the inter-individual variation in microbial composition and assembly. It is increasingly being recognized that the principles of ecological theory can help us to understand and predict community variations in the human microbiota [19].

In this chapter we will discuss the development of the gut microbiota during infancy and childhood from an ecological theoretical perspective, and describe how lifestyle changes may distort the natural development of microbial assembly and maturation.

2 Ecological Principles of Microbial Community Assembly

The ecologist Mark Vellend has synthesized various concepts of community assembly by categorizing the underlying processes into four groups: dispersal, selection, drift, and diversification [21, 22].

Dispersal of bacteria from the meta-community is an important process to seed the initially sterile infant gut but is a process that also plays an important role thereafter. The meta-community consists of numerous local communities, some of which are host-related (e.g., maternal skin, gut, and vaginal microbiota), while others are not (e.g., soil, food-born, building environment microbial communities) [23].

In this respect, priority effects describe how the order and timing of dispersal from the meta-community might alter how diversification, drift, and selection affect the assembly of the infant gut microbiota. In other words, the impact that particular species can have on the community assembly in the infant gut would depend on the timing and order in which they arrive (history) [23, 24].

Environmental selection or niche-based interaction is the deterministic process that, based upon the conditions in a given habitat, drives differences in growth and death rates among microbial taxa based upon their fitness and niche-differences [23]. Diet, host metabolites, and the immune system are primary sources that drive environmental selection. From the perspective of environmental selection, the human body can, for example, be seen as a “habitat filter”—a collection of

conditions and resources that nourish the growth of some microbes, but not others. This view implies that the interaction is unidirectional; the host shapes the microbiota. However, in many circumstances of environmental filtering, the host-microbe interactions are bi-directional [19]. The interactions between the host immune system and the microbiota are a classical example, but also the interactions between host metabolites such as bile acids and the microbiota [25] exemplify such bi-directional selection processes. Also, the abrupt shifts in the composition and structure of the microbiota that are commonly observed during an infectious episode or a course of antibiotics are examples of environmental selection processes.

Ecological drift is a completely stochastic (chance-driven) event in which changes in species population size occur regardless of species identity [23, 26]. The effect of drift is expected to be stronger on low abundant species as slight negative changes in their abundance could already push them stochastically to local extinction, unless they have (or gain, e.g., via diversification) a competitive advantage or become replenished by dispersal from outside the local community [19, 27].

It is however almost impossible to distinguish the effect of drift from the effect of other ecological processes except when studied in an experimentally controlled setting [26].

Diversification is the process of the generation of new genetic variants, often as a result of a persistent selective pressure. Highly abundant and dense microbial populations, rapid growth rates, and strong selective pressures, conditions that are all met in the adult human gastrointestinal tract, can fuel microbial adaptation via mutation or recombination (e.g., via horizontal gene transfer) [19]. However, as the selective regimes during infancy frequently shift as a result of, among other factors, a developing host and alterations in feeding regimes, the degree to which diversification is involved in the infant gut during assembly remains largely unknown [23].

Neutral Community Assembly

A theoretical framework of community assembly which assumes that dispersal, diversification, and ecological drift are completely stochastic processes is the **neutral community model** (NCM). According to this model, neither environmental selection nor inherent species differences in their ability to disperse or diversify play a role in the community assembly. Although such models do not account for deterministic factors and make many simplifying assumptions, they have among others successfully been applied to predict the structures of aquatic and respiratory microbial communities [28, 29]. Such models moreover are important for gaining insight into the importance of neutral dispersal in shaping the structure of microbial communities, to identify conditions that lead to divergence from neutral dynamics or to identify microbial taxa that do not assemble in a neutral manner [30]. NCM has also been applied to the assembly of gut microbial communities. In a study on the

(continued)

zebrafish intestinal microbial communities from larvae to adulthood, the importance of non-neutral processes increased as the host matured [31]. A recent study among the Tsimane, an indigenous Bolivian population, also underscored the importance of neutral forces in shaping microbiota assembly in early life and to a lesser extent in adulthood [30]. These observations suggest that a significant amount of diversity in microbial community structures between individuals could be explained by neutral processes of drift and dispersal [31].

Glossary of Terms and Definitions Used in This Review

Terminology	Description	Reference
Community	A group of potentially interacting species that live together in a specified place and time	[21, 27]
Dispersal	Movement of microorganisms across space	[19, 21]
Ecological drift	Stochastic changes in the relative abundance of different microbial taxa within a community throughout time as a result of birth, death, and reproduction	[19, 21, 32]
Environmental selection	Changes in the microbial community structure as a result of deterministic fitness differences between microbial taxa	[19, 21]
In situ diversification	Generation of new genetic variants by mutation or recombination (e.g., via horizontal gene transfer)	[19, 21]
Meta-community	A set of local communities that are linked by dispersal of multiple potentially interacting species	[33]
Neutral assembly theory/ neutral community model	Theory/model assuming that dispersal, diversification, and ecological drift are completely stochastic processes and that neither environmental selection nor species traits play a role in community assembly	[19, 34]
Perturbation	An external event/stressor that causes a distinct selective pressure on the ecosystem, also called disturbance	[35, 36]
Priority effects/historical contingency	The order in which species arrive at local sites (e.g., in the infant gut) dictates the effect of species on one another	[19, 21, 32]
Resilience	The property of a microbial ecosystem that defines how fast, and to what extent it will recover its initial functional or taxonomical composition following perturbation	[35, 36]
Resistance	The power of an ecosystem to remain unchanged upon a perturbation	[35, 36]

3 Establishment of the Microbiome

As compared to the microbiome of adults, which has been suggested to be relatively stable and resilient [37–39], the microbiome in infants is highly dynamic. The microbial richness and diversity gradually increase from early infancy to childhood, while the variation in microbial composition between children decreases [6, 39].

The first colonizers of the infant gut microbiota are typically facultative anaerobes, particularly *Enterobacteriaceae*, but also facultative anaerobic genera within the Firmicutes phylum including staphylococci, streptococci, lactobacilli, and enterococci [6, 40–43]. Although members of the *Enterobacteriaceae* were shown to be specific for the infant gut, most of the initial colonizers are homogeneously distributed across different body sites [44]. During the following months, obligate anaerobes, including *Bifidobacterium*, *Bacteroides*, *Veillonella*, and *Clostridium*, start to dominate [6, 43]. While *Enterobacteriaceae* wane, they still have an important share during the first half year of life after which their abundance further decreases [6, 40, 41, 43]. Under the impact of weaning and transition to an adult-like diet, *Veillonella* and *Bifidobacterium* start to decrease, *Bacteroides* further increase, and members of the *Lachnospiraceae* (e.g., *Blautia* and Roseburia) and *Ruminococcaceae* (e.g., *Faecalibacterium*, *Ruminococcus*) rise to become dominant members of the gut microbiota [6, 41–43]. Many of these bacteria are butyrate-producers, and a concomitant increase in butyrate levels has indeed been observed [45, 46].

Finally, some prokaryotes, including the archaeal genus *Methanobrevibacter* and the bacterial genera *Desulfovibrio*, *Bilophila*, and members of the *Christensenellaceae* family, only colonize after infancy and keep rising in prevalence and abundance beyond the age of 5 years [39]. This indicates that the adequate niche has first to be created before these genera can settle. These niche formations are partly driven by the prior arrival of other microbial taxa for necessary cross-feeding interactions, but also on a fully reduced environment and the availability of complex dietary carbohydrates.

Several studies have aimed to identify distinct phases of microbiome progression [6, 39, 43, 47]. Although the exact period differed between studies, it is evident that most rapid changes in microbiome development occur within the first 6–12 months of life. Microbiome maturation thereafter continues in a less profound manner, but the exact age at which a stable adult-like microbial community structure is reached is still a matter of debate. While it has previously been suggested that stabilization of the microbiome occurs around the age of 2–3 years [47, 48], differences could still be observed in the microbiome of Swedish 5-year-old [39] and Dutch 6–9-year-old children [49] as compared to Swedish and Dutch adults, respectively. A recent meta-analysis on metagenomic data from over 1900 fecal samples from nine studies confirmed that the microbiome could predict a child's chronological age well beyond the first 3 years of life [42]. In part, this controversy can be attributed to the sparseness of data on the microbiome of children beyond the age of 3 years [37].

4 Determinants of Infant Gut Microbiome Assembly and the Impact of Lifestyle Changes (Fig. 1)

4.1 Seeding of Newborn Ecosystem

The microbial species that colonize first play a crucial role in the development of the ecosystem in the gut, potentially influencing the ultimate composition and functionality of the microbiota for life. However, at what stage the first microbes start to seed and colonize the gut is still a matter of debate. In 2014, Agaard and colleagues reported the existence of a microbiome in the placenta of healthy women [50]. Since then, many other human studies detected microbial signals in amniotic fluid, fetal intestine, cord blood, placenta, or meconium samples, thereby suggesting in utero colonization and challenging the concept that seeding of the gut ecosystem initiates at birth [51–54]. In utero dispersal of microbes would have tremendous implications for fetal and neonatal health and development, but objections were raised against

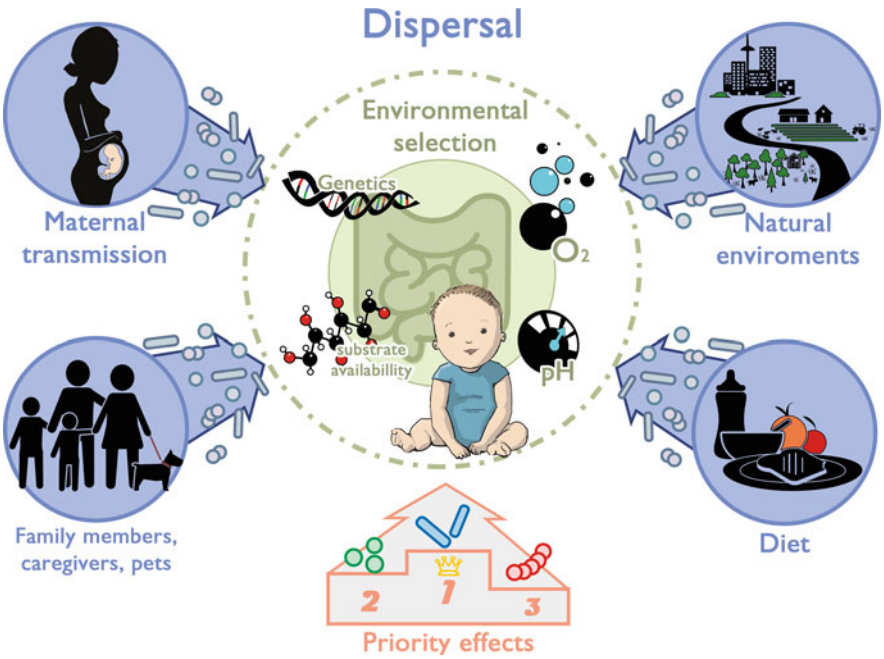

Fig. 1 Infographic depicting the main ecological processes and deterministic factors shaping the microbiota throughout infancy and childhood. Main sources of microbial dispersal to the infant gut comprise the infant's mother and other family members, pets and peers, food sources, and the natural environment (depicted in blue). The order at which microbial taxa arrive in the infant gut may dictate the colonization success of subsequent incoming bacteria (depicted in orange). Additionally, the environment of the infant gastrointestinal tract may select for or exclude specific microbial taxa (depicted in green). This environmental selection is driven by among others the substrate availability, oxygen concentration, pH, and host genetics

most of these studies (see review [52]). First, the majority of studies relied on the detection of bacterial DNA which does not confirm the existence of a living microbial community [55]. Second, no consistent microbial profile was detected across the different studies (i.e., the dominant bacterial taxa varied widely between studies *Micrococcus*, *Lactobacillus*, *Staphylococcus*). Lastly, it appeared that results were biased by contamination, i.e., the detected bacteria in low-biomass samples were also identified in negative controls [56, 57]. As a result, many other studies that appropriately controlled for contamination could not provide evidence of microbial taxa in utero [58–62]. On the other hand, recent studies demonstrated the existence of viable bacteria-like morphology in the fetal intestine and placenta by culture and microscopy [53, 63] albeit debated as well [64, 65]. In addition, oral administration of trackable bacteria (e.g., genetically labeled *Enterococcus faecium*) to pregnant female mice could be recovered from amniotic fluid and meconium of the offspring [66, 67]. Today, the debate still continues, and it is still questionable whether a prenatal intrauterine microbiome exists.

Birth is likely the first step in seeding the newborn gut and definitely the most dramatic one, given the amount and diversity of microbes to which a newborn gets exposed. The passage through the birth canal has long been recognized as the major transmission of first microbes, i.e., *Lactobacillus* and *Prevotella* [68, 69]. However, studies comparing microbial strain profiles of infant fecal to both maternal vaginal and rectal samples revealed that mother-to-child transmission mainly occurs for rectal rather than vaginal strains [42, 70]. In particular, as consistently shown in numerous studies, maternal *Bacteroides* strains are most frequently transferred to the intestine of neonates born via a natural delivery [6, 70–72].

The mode of delivery evidently impacts the dispersal of microorganisms from the mother and results in distinct microbial profiles between infants born vaginally and via Cesarean section (C-section). The microbiota of C-section delivered infants is characterized by delayed colonization of mainly *Bacteroides* and *Bifidobacterium* compared to vaginally delivered infants [40, 43, 47, 70, 71, 73–75]. In a recent study, we longitudinally monitored the establishment of the human infant gut microbiota and further supported that birth mode most strongly affects members of the genus *Bacteroides* [6]. The decreased levels of *Bacteroides* in infants born by C-section (Cesarean section) remained significant after careful adjustment for other confounders and persisted for at least 31 weeks [6]. In addition, other studies demonstrated that this founder effect could even last until 4-years postpartum [47, 76]. Although C-section is accompanied by prophylactic antibiotic administration to the mother, the effect of delivery mode on the infant gut microbiota has recently been demonstrated to be independent of intrapartum antibiotic prophylaxis (IAP) [77]. In this study, IAP was administered only after clamping of the umbilical cord in mothers undergoing C-section, enabling the researchers to examine the impact of the birth mode on the neonatal microbiota in the absence of antibiotic exposure to the baby. The latter study confirmed the lower levels of *Bacteroides* spp. and *Bifidobacterium* spp. in C-section delivered infants. Altogether, this suggests that dispersal of maternal microbes during vaginal delivery is crucial to acquire certain microbial species in early life.

Cesarean section delivery is perhaps one of the factors most commonly linked to distortions in the microbiota establishment in early life [40, 71]. C-section numbers are rising worldwide [78]. Nowadays one out of five babies is delivered by C-section with numbers having almost doubled since 2000, while in Brazil rates have risen up to 45%. Notably, the Netherlands has one of the lowest C-section rates (17%) among developed countries, especially when compared to neighboring countries such as Germany (30%) [78]. The latter might be because pregnant women at low risk of birth complications get unique midwife-led care in the Netherlands which is associated with lower intervention rates [79, 80]. Although in some cases it might be a life-saving procedure, the high numbers of C-sections without medical reason (i.e., elective C-section) are worrisome.

Many efforts have recently been made to reverse the dispersal limitation in C-section delivered neonates by exposing them to the maternal vaginal microbiota immediately upon delivery, a procedure termed "vaginal seeding" or "bacterial baptism" [81]. The recent evidence that maternal fecal rather than vaginal bacteria are depleted in the intestinal microbiota of C-section delivered neonates [42, 70], however, challenges the efficacy of vaginal seeding to abolish the dispersal limitation during C-section. In a recent proof-of-principle study by Korpela et al., fecal microbiota transplantation (FMT) was therefore used to restore the microbiota in infants born by C-section [82]. An enhancement of *Bacteroidaceae* and *Bifidobacteriaceae* in FMT-treated as compared to untreated infants was observed without any short-term adverse effects. Although FMT might be a more successful strategy to restore maternal transfer in C-section delivered infants, concerns remain on the risks and uncertainties of this treatment (i.e., dispersal of pathogens or overstimulation). It is therefore crucial to obtain further insight into the maternal microbial species that C-section delivered infants fail to achieve and the corresponding health implications in order to move towards more controlled attempts to restore the initial microbial colonization of newborns, e.g., in the form of synthetic microbial transplants.

4.2 Infant Diet—Environmental Selection and Dispersal of Milk Microbes

The World Health Organization (WHO) and the United Nations International Children's Emergency Fund (UNICEF) advocate exclusive breastfeeding (EBF) for the first 6 months of life [83]. Despite these global recommendations, breastfeeding prevalence, particularly EBF, is low in Europe and the Americas with dramatic disparities between countries [84].

Low rates and early cessation of breastfeeding have important adverse public health impacts. Besides nutrients, breast milk contains a wide variety of bioactive factors (e.g., lysozyme, lactoferrin, and immunoglobulins) that support the

development and maturation of the infant gut, the innate and adaptive immunity, and systemic metabolism [85].

Moreover, breastfeeding plays a crucial role in the establishment of the infant gut microbiome both directly, by the dispersal of living bacteria in breast milk, and indirectly, by environmental selection in the form of prebiotic nutrient substrates and bioactive components.

Breastfeeding directly impacts the infant microbiome by dispersal of viable microorganisms in human milk. An exclusively breastfed infant will daily consume the significant amount of approximately 10^5–10^7 commensal bacteria while suckling [86]. Facultative anaerobic skin and throat bacteria, such as members of the genera *Staphylococcus*, *Streptococcus*, and *Cutibacterium*, are the predominant bacteria detected in breast milk by traditional culturing approaches [86, 87]. At lower concentrations lactic acid bacteria, including *Lactobacillus* and *Enterococcus*, members of the gram-negative *Enterobacteriaceae*, as well as obligate anaerobes, such as *Bifidobacterium* and *Veillonella* have been frequently isolated from breast milk [86–88]. The application of culture-independent molecular approaches has revealed a human milk microbial diversity beyond expectancy, including major gut-associated obligate anaerobes such as *Bacteroides* and several members of *Clostridia*, including the butyrate-producing *Faecalibacterium* and *Roseburia*, which are important for colonic health [87]. The origin of the microbes in breast milk is not fully understood, but likely involved the maternal skin, the infant's oral cavity during suckling, and potentially even the mother's gut via the entero-mammary pathway [89]. The exact composition of the human milk microbiome is highly variable and potentially influenced by geography, birth mode, lactation stage, as well as diet, health status, medication use, and genotype of the mother [89, 90]. Numerous studies have shown the importance of breast milk microbiota as an important source of successfully seeding the infant gut. Identical strains of *Bifidobacterium*, *Lactobacillus*, and *Staphylococcus* have been isolated from breast milk and infant feces in mother-infant dyads [91–93]. Transmission of bifidobacterial strains from breastmilk to the infant gut has additionally been demonstrated by a combination of gene marker-based amplicon sequencing, metagenomic shotgun sequencing, and strain isolation followed by genomic analysis [91, 94].

The dispersal of milk microbes to the infant gut obviously contributes to the profound differences in the microbial communities in breastfed as compared to formula-fed infants. The indigenous microbiota of exclusively breastfed infants is low in diversity as a result of the predominance of bifidobacteria, which can account for up to 70–80% of all bacteria in the stools of breastfed infants [6, 95]. In addition, lactobacillus species that are commonly used as probiotics have also been found to be enriched among breastfed infants [73]. Although the absolute bifidobacterium counts in infants receiving formula feeding tend to be as high as in breastfed infants [96–100], the microbiota of formula-fed infants is characterized by a much higher diversity and increased numbers of *Bacteroides*, *Clostridium*, and *Enterobacteriaceae*, including opportunistic pathogens such as *Clostridioides difficile* and *E. coli* [6, 99, 101–103]. Moreover, the *Bifidobacterium* composition at the species level differs according to feeding type with *B. catenulatum*, and

B. adolescentis, species that are commonly found in adults, being relatively more abundant in formula-fed infants [73, 104]. In breastfed infants, bifidobacterial species that thrive on human milk oligosaccharides (HMOs), including *B. longum*, *B. bifidum*, and *B. breve*, prevail [37, 47, 73]. Over 200 different types of HMOs have been known to be present in breastmilk, and the composition is affected by genetic factors, such as secretor genotype, suggesting that the maternal genome can affect the infant microbiota [37]. Indeed, Lacto-*N*-fucopentaose I and 2-′-fucosyllactose, which are dominant HMOs in secretor women, but absent in non-secretors, are associated with the infant microbiota composition [105]. Next to the important selective pressure of HMOs, other bioactive compounds, including lysozyme, lactoferrin, and immunoglobulins, may also impact the infant microbiota [106–108].

4.3 Introduction of Complementary Foods

Weaning is a critical next step in microbiota maturation as the indigenous microbiota becomes exposed to a variety of food components, including plant- and animal-derived glycans. Upon weaning the microbial diversity increases as a result of the complementation and gradual replacement of HMO-utilizing bacteria, such as bifidobacteria, by a more complex ecosystem consisting of specialists such as members of *Bacteroides*, *Lachnospiraceae*, and *Ruminococcaceae* which are more capable of degrading complex plant-derived carbohydrates and starch [37, 73, 109]. The major pectin-degrading enzyme, pectinesterase, has, for example, been shown to be enriched in infants by the age of 1 year, most likely resulting from the increased intake of pectin-rich foods as compared to younger infants [73]. In line with the increased capacity to ferment complex dietary carbohydrates, levels of fecal short-chain fatty acids increase [37, 45]. Additionally, the microbiome shows an increased capacity to produce amino acids and vitamins and metabolize xenobiotics following the introduction of solid foods [37, 73, 110].

The particular effects of complementary foods, however, strongly depend on the geography in line with the major differences in dietary habits around the world. The intestinal microbiota of non-Western populations, known for their high consumption of dietary fibers, has consistently been shown to be more diverse, enriched in *Prevotella* and depleted in *Bacteroides* when compared to populations in Western countries consuming a diet high in simple sugars, starch, and animal fat and protein [111]. Along with other profound differences, this trade-off between *Prevotella* and *Bacteroides* was also reported in a study comparing the microbiota of children living in rural Africa and Europe [112]. However, among children that were still being breastfed, these differences between both populations were not yet apparent, highlighting that both cessation of breastfeeding in combination with the subsequent dietary pattern drive the geographical differences in the intestinal microbiota composition.

Next to the selective pressure of dietary substrate availability, food is also a major source of allochthonous bacteria, ranging from 10^4 to 10^9 bacteria per gram of food with fermented food having the highest bacterial counts [113]. Interestingly, a diet meal plan as recommended by the US Department of Agriculture (emphasizing fruits and vegetables, lean meat, dairy, and whole grains) was found to contain a thousandfold higher numbers of viable bacteria than an average American diet [113]. Reduced consumption of fresh fruits and vegetables and increased consumption of ultra-processed foods (e.g., preserved meats, refined grains, hydrogenated oils) as observed in typical western diets thus substantially reduces the ingestion of food-borne microbes.

Knowledge on the fate of food-borne microbes is largely limited to probiotic bacteria from the genera *Lactobacillus* and *Bifidobacterium* and shows only a transient integration of such bacteria in the intestinal microbiome [113]. However, this may be different for microbial specialists that can colonize intestinal niches that emerge in later infancy or childhood. The archaeon *Methanobrevibacter smithii*, which only colonizes after infancy [39], has, for example, been shown to be more prevalent among school-aged children consuming organic dairy products [114]. Molecular analysis confirmed the presence of *M. smithii* in milk products suggesting that this may be a source of archaea colonization in children.

4.4 Dispersal from Siblings, Peers, and Pets

Social interactions with siblings and peers (e.g., during daycare attendance) may result in dispersal of microbes. Singletons have indeed been shown to have a distinct colonization pattern when compared to infants that grow up together with older siblings. The Canadian Healthy Infant Longitudinal Development (CHILD) cohort showed a lower abundance of *Clostridioides difficile* and its family *Peptostreptococcaceae* in 4-month-old infants with older siblings as compared to singletons [115]. A longitudinal German study reported that exposure to older siblings was associated with an increased diversity as well as increased levels in several genera within the phylum of Actinobacteria (*Bifidobacterium* and *Corynebacterium* at 5 weeks and *Eggerthella* at 21 weeks) and a higher microbial diversity at 31 weeks of age [6]. Several other studies also observed a higher abundance of bifidobacteria among children with older siblings [99, 116]. A higher richness and diversity in children with siblings have been observed in some [117] but not all studies [47, 115]. Within the large TEDDY study, infants with older siblings had a different microbial community structure and accelerated microbiome maturation as compared to infants growing up in the absence of older siblings. Both species level microbial community structure as well as microbiota maturity, however, only started to significantly differ between children with and without siblings after the first months of life [47]. This delayed effect of older siblings might reflect more close interactions and dispersal when infants grow older or the opening of a niche that allows colonization by specific strains from household members. Alternative

explanations for this sibling-effect, such as an altered vaginal microbiota or breastmilk composition in multiparous as compared to primiparous women, appear less likely as the strongest effect would in such cases be expected in earliest infancy.

Companion animals might also have an impact on the infant microbiome development as an altered microbial environment, indicated by different microbial composition of household dust or surfaces in the homes, has consistently been observed among households with indoor pets [118–122]. The influence of pets on the environmental microbiome might subsequently impact both the immune and microbiome development of infants [120]. In particular the intestinal microbial gene content of dogs shows striking similarities with the human gut microbiome [123]. Dogs were the first animals to be domesticated in modern human history and frequently shared food resources with humans, which has likely contributed to the co-evolution of the human and canine gut microbiome. However, the limited studies on the impact of pet exposure on the infant gut microbiome have so far not differentiated between the effect of different pet species. Results from the TEDDY study revealed that infants living with furry pets had an altered microbial community structure and accelerated maturation of the microbiome when compared to infants growing up in the absence of furry pets [47]. In another study, the animal-specific *Bifidobacterium pseudolongum* was detected in a significantly larger proportion of 1-month-old pet-exposed as compared to non-exposed infants [124]. Moreover, increased levels of *Ruminococcus* and *Oscillospira* were observed among infants with furry pets in their households in the CHILD-study [125]. However, not all studies did observe an impact of furry pets on the establishment and maturation of the infant gut microbiota [115], likely due to the more subtle effects as compared to the effects of siblings [47].

Large studies on the identification of determinants of infant gut microbiota development have so far not observed an association between daycare attendance and gut microbial diversity and community structure [47, 115]. A recent study for the first time compared the microbiota of infants before and 4 weeks after entering center-based childcare to that of infants being fully cared for by the parents at home. In line with the previous studies, this study also found the infant gut microbiota not to be affected in a uniform way by center-based childcare [126]. These studies do however not rule out an impact of daycare attendance on an individual level, e.g., by dispersal of microbial taxa between peers. In fact, studies showing the spread of fecal multi-drug resistant *E. coli* strains in daycare centers prove the existence of such dispersal events and underscore the importance of further investigation. Dispersal between socially interacting individuals is further supported by results from the Wisconsin Longitudinal Study in which spouses had a more similar microbiota and shared more bacterial taxa than siblings (adult siblings not living together) and unrelated pairs. Moreover, married individuals, especially those reporting close relationships, harbor microbial communities of greater diversity and richness relative to those living alone [127].

4.5 Dispersal of Microbes from the Natural Environment

The intestinal microbial ecology also differs between infants from various geographical regions. Even within different Western-European countries, differences in the infant microbiota have been observed. Infants from Northern European countries were characterized by an overrepresentation of *Bifidobacterium*, while an increased microbial diversity was found in infants born in Southern European countries [103]. More pronounced differences are observed when comparing the microbiota of infants living in western countries to that of children born in low- and middle-income countries, with the latter group being characterized by a higher diversity and enhanced levels of *Prevotella* and decreased abundance of *Bacteroides* in early life [18, 128–130]. Diet, with a typical Western diet being low in plant-derived carbohydrates and high in animal protein, sugar, starch, and fat as compared to more agrarian societies, is likely the most important driver for geographical variations in microbiota composition via environmental selection of microbes that can benefit from the substrate availability [111]. However, besides selective pressures such as diet and host genetics, the unique natural environment might at least also partly explain geographic variations in the indigenous microbial community structures [131]. According to the biodiversity hypothesis [132], reduced contact with diverse microbiota and macrobiota in our natural environments adversely affects the assembly and composition of the human indigenous microbiota and in turn to inadequate immune stimulation and ultimately increased susceptibility to non-communicable diseases. Several recent studies do suggest that these natural and human microbial ecosystems are indeed interrelated. Most evidence so far stems from studies that show associations between the skin microbiota and living near natural environments [133–135], but the first studies linking living in proximity to natural environments to the gut microbiota also start to emerge. Preliminary results from the Wisconsin Infant Study Cohort (WISC) showed that the microbiota of infants raised in farming versus non-farming environments differed modestly, yet significantly from each other [136]. Within the context of the Canadian CHILD-study, the association between living near to natural environments in the urban context and the gut microbiota in 4-month-old infants was examined. Although proximity to a natural environment was associated with an altered microbiota composition, this could only be observed for formula-fed infants who were exposed to pets in their homes [137]. This suggests that both environmental selection (e.g., breastfeeding prevents the colonization of environmental microbes) and indirect dispersal of environmental microbes via pets as vectors may be involved. In the PASTURE birth cohort, growing up on a family-run farm, and particular farm exposures such as visits to animal sheds and consumption of eggs or milk directly from the farm, influenced the maturation of the gut microbiota during the time window from 2 to 12 months. This accelerated microbiota maturation could moreover explain a substantial proportion of the well-known protective farm effect on asthma. Interestingly, growing up close to green areas (forest and agriculture land) has also been shown to reduce the risk of atopic sensitization, supporting the hypothesis of a strong environmental effect on

the commensal microbiota [138]. The most direct and causal evidence in support of the biodiversity hypothesis, however, comes from intervention studies in which exposure to natural environments is stimulated. In a Finish trial, the environmental biodiversity of urban daycare centers was enriched by covering their yards with forest floor and sod [139]. Not only did the skin microbial (Proteobacteria) diversity increase among participating 3–5-year-old children, also the gut microbiota composition changed with a decreased relative abundance of Clostridiales and an increased diversity after as compared to before the intervention. Another example is the "Play & Grow" program from the University of Hong Kong, a family-oriented early environmental education program aimed to reconnect preschoolers to nature and induce changes in health behaviors and outcomes by having outdoor activities that promote exposure to nature [140]. In a proof-of-principle study of this "Play & Grow" program exposure to nature activities resulted in a decreased Bacteroidetes richness and increased Proteobacteria richness in the gut microbiota of children in the intervention group [141].

Together these results demonstrate the interrelatedness of the microbial ecosystems of our natural environments and our indigenous microbiota. Reduced contact of people with the natural environment as well as biodiversity loss of our wider environment will thus inevitably impact the biodiversity and composition of our intestinal microbiota.

4.6 Perturbations by Antibiotic Exposure

Broad-spectrum antibiotics, particularly beta-lactam antibiotics (e.g., amoxicillin, amoxicillin/clavulanate, cephalosporins) and macrolides (e.g., azithromycin), are by far the most commonly administered drugs during infancy and early childhood. Prescription rates, however, vary widely across the globe, reflecting differences in medication policies, preferences of health-care providers and mothers, infection rates, access to care, as well as over-the-counter sales of antibiotics [142, 143]. A significant portion of broad-spectrum antibiotics are still being prescribed for upper respiratory infections, which are mostly self-limiting and of viral origin with little evidence for clinical benefit [144, 145]. The negative impact of such misuse and overuse of antibiotics is substantial as it not only drives the development and dissemination of antimicrobial resistance but also results in profound perturbations on the indigenous microbiota [40, 43, 75, 99, 146]. A reduction in microbial diversity and decrease in obligate anaerobes, including *Bifidobacterium* and *Bacteroides*, and increase in Proteobacteria are commonly observed in the fecal microbiota of infants exposed to antibiotics, although perturbations vary according to type of antibiotics administered [147]. While in adults the microbiota shows a high level of resilience upon antibiotic exposure, the infant microbiota is far less resistant and resilient. In previously antibiotic-naive infants, a single course of amoxicillin was found to profoundly disrupt the microbiota composition. Rather than returning to the original composition after the antibiotic course, an accelerated

maturation towards a depletion of bifidobacteria and enrichment of clostridia was observed [147]. This demonstrates that even an antibiotic pulse has a lasting effect on the maturation process of the infant microbial ecology.

Maternally administered antibiotics could potentially also impact the infant microbiota assembly either by altering the maternal vaginal and intestinal microbiota and thus dispersal (limitation) of maternal microbes [148], or by placental transfer of antibiotics [149]. The first route is likely most important for antibiotics administered in the third trimester, while the second route might come into play for intrapartum antibiotic prophylaxis (IAP) to prevent maternal wound and neonatal Group B *Streptococcus* (GBS) infections. IAP is not routinely practiced across the globe, but recent adjustments of international guidelines will lead to a further increase of prophylactic antibiotic administration during delivery and consequently increased antibiotic exposure to the infant [150].

A profound impact of IAP on the infant gut microbiota has been consistently observed across studies, with a decreased diversity and relative abundances of Actinobacteria and Bacteroidetes and concomitant increased levels of Proteobacteria belonging to the most robust findings [150–155]. Far fewer studies have been examining the impact of antibiotic exposure earlier during pregnancy and results are so far contradictory, likely due to the heterogeneity in study designs and potential confounding factors [150].

5 Conclusions

Human lifestyle changes have profoundly affected our indigenous microbiome and depleted microbial diversity at an unprecedented pace [156, 157]. Given the pivotal role of the infant microbiota as a stimulus for the ontogeny of the enteric mucosal tissue and immune system [13, 14], lifestyle changes that distort the assembly of the microbiota in early life can particularly impact future health and disease susceptibility. From an ecological perspective, lifestyle changes could both affect priority effects and other types of dispersal (limitation) as well as environmental selection. Delayed or limited exposure to maternal vaginal or fecal microbes during C-section delivery is a key example of dispersal limitation. Microbial dispersal is further limited as a result of reduced family sizes, reduced contact with the natural environment, and loss of biodiversity in our natural ecosystems. Reduced breastfeeding and increased consumption of ultra-processed foods reduce the dispersal of human milk and food-borne microorganisms. Moreover, changed dietary habits (e.g., more animal fat, simple sugars, and reduced dietary fibers) cause a distinct selective pressure on the indigenous microbial ecosystem of Western populations. These perturbating external events can be classified as pulses which are short-term perturbations (e.g., a course of antibiotics), or as presses which are long-term or continuous perturbations (e.g. dietary habits) [35]. As such, besides our western dietary habits, even relatively discrete, short-term perturbations (pulses), such as antibiotics, can

profoundly and persistently alter the assembly and maturation of the dynamic and low resilient microbiota in early life [147].

Despite the considerable advances in our understanding of the microbiota maturation during infancy over the past decades, a large part of inter-individual variation in microbiota composition remains unexplained. This suggests that either important determinants have so far been overlooked or that other ecological processes such as historical contingency or stochastics effects play a major role [158]. Further research should take these ecological phenomena into account, but also focus on unravelling other so far unknown deterministic factors that might contribute to the variation and acquisition of the microbiota, and thereby the vital biological processes in life.

Many studies are ongoing to examine the impact of targeted treatments, including specific probiotics, prebiotics, and post-biotics (e.g., products of bacterial metabolism) to strengthen the natural development of the intestinal microbiota or restore its disruption. In addition, maternal FMT has recently been shown to be able to overcome the limited dispersal of maternal microbes and to postnatally restore the microbiota of C-section delivered infants. Although promising, data are still scarce and FMT requires careful screening of donor stools. It should therefore not yet be offered as standard care and only be used within the context of well-controlled experimental settings. Ultimately, synthetic multi-microbial substitutes of FMT will likely be an inevitable further development to make this a viable treatment strategy [159].

Next to the abovementioned dietary or clinical interventions, re-connecting to nature might be another approach to stimulate a healthy microbiome maturation. The rapid urbanization of our living environment and reduced exposure to natural environments might have impeded the beneficial health effects including the dispersal of bacteria and the maturation and homeostasis of immunological responses. In contrast, outdoor activity in a natural biodiverse environment may improve the microbial colonization, and in turn decrease the risk of non-communicable diseases and improve children's general well-being. Human intervention studies tackling this concept of "microbiota re-wilding" are scarce, but the few initiatives that have been undertaken so far are promising [139, 141]. To further increase our understanding on the impact of our (natural) environment, observational studies embedding the infant gut microbiota development into a broader framework of environmental exposure are highly warranted [131]. Moreover, additional human interventional strategies should be explored to examine if strengthening connections to nature as part of everyday life indeed positively influences microbiota development during infancy.

Finally, education of the general public, healthcare professionals, and policy makers on the importance of our gut microbiota and the damaging health consequences of distortion of microbiota assembly in early life should be one of the top priorities. Refraining from unnecessary antibiotic use, informing on the negative consequences of elective C-section delivery, stimulating breastfeeding rates, and a diet high in fiber and low in animal fat and protein are all key to prevent the impoverishment of the indigenous microbiota during this critical period of life.

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Distortion of the Microbiota of the Natural Environment by Human Activities

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Abstract Natural environmental microbiota is extremely abundant and diverse in environments traditionally occupied by humans. Humans, like other animals, cause shifts in the microbiota in their living environment. The exceptional scale and longevity of these shifts pose a risk to natural and seminatural ecosystems and human health. Environmental pollution, non-native invasive plant species, and vegetation control by humans distort seasonal fluctuation and directly alter natural microbiota. They also reduce the accessibility of natural environmental microbiota in urbanized societies. The removal of organic surface soil and its substitution with man-made surfaces is the most extreme example of the distortion of natural microbiota; it cuts the number of microbial cells per gram soil to one thousandth or one hundred thousandth of the original level. Since humans evolved in continuous contact with environmental microbiota, efforts to rewild urban microbiota are being developed to reintroduce diverse contacts with microbiota of the natural environment to everyday life of urban dwellers. Recent findings suggest that these efforts may lead to enhanced immune modulation. Further research is needed to understand whether this eventually results in a lower incidence of immune-mediated diseases in urbanized societies.

Keywords Environmental microbiota · Biodiversity · Built environment · Dirt · Green infrastructure · Immune response · Plant invasions · Pollution · Urban rewilding · Vegetation control

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1 Natural Environmental Microbiota

1.1 Environmental Microbes Are Ubiquitous

Microbes are ubiquitous and extremely abundant in natural environments. A tiny gram of organic surface soil typically contains one to ten billion bacterial 16S rRNA gene sequences [1]; millions to billions of viruses, including bacteriophages and plant viruses; Archaea; fungal cells; algae; and hundreds to thousands of microscopic multicellular Eukaryotes, like soil animals [2–4]. The exact taxonomic composition of the soil microbiome ranges considerably at all spatial scales, from large-scale variation between geographic regions to differences between nearby field plots within a single forest stand and even between neighboring soil samples at the centimeter scale [5–7]. In the so far largest meta-analysis to map Earth's bacteriome, the predicted average gene copy number of a bacterial strain was less than ten per 1 g soil [6]. In the same study, bacterial 16S rRNA gene copies in plant rhizosphere were slightly more abundant compared to bulk soil samples. Although the number of 16S rRNA gene copies per sample can be ten times lower on plant leaves than in soil [6], microbes occupy virtually every organic surface on Earth. Providing that environmental conditions are not too dry, cold, hot, or toxic, biofilms, i.e., surface-attached clusters of bacteria, often cover inorganic soil particles and bedrock as well [8].

1.2 Patterns in Natural Microbial Diversity

According to current understanding, the taxonomic diversity and the abundance of natural microbial communities tend to follow certain general patterns. At the global scale, Thompson et al. [6] found evidence that low latitudes have richer soil and plant bacterial communities compared to higher latitudes, a pattern known to exist among multicellular organisms, including plants and most fungi [4, 9]. Recent studies indicate a second general pattern: the overall microbial diversity seems to decline with soil depth. Liang et al. [2] sampled agricultural red clay soils from the depth 0–120 cm in Alabama and observed that the diversity of bacterial communities and the abundance of viruses decreased with increasing sampling depth. They also observed an association between viruslike particles and bacterial diversity. Upton et al. [10] observed that grassland fungal diversity declines with soil depth, the richness being 50% lower between 60 and 100 cm than between 0 and 10 cm below soil surface.

The third pattern is related to the utilization of resources: although microbial diversity and the abundance of different taxa vary considerably within small spatial scales, Spain et al. [11] stated *Proteobacteria* to be the dominant phylum in surface soils in a study that included samples from natural tall prairie soils and data from earlier studies covering different continents and ecosystems [12]. The high abundance of soil *Proteobacteria* seems to be limited to the uppermost soil layers where

plant-based organic material is degraded and where the microbial co-occurrence network is complex [11]. As the majority of soil bacteria are still unclassified [6], it is unsure whether the phylum *Proteobacteria* will retain its position as the most common phylum in surface soils. Despite this, the high abundance of *Proteobacteria* in natural surface soils is a fact that may have played a role in human evolution (see other chapters in this book).

While soil microbial diversity seems to follow certain relatively universal patterns, the factors modulating microbial diversity on and inside plant leaves are complex. Experimental evidence suggests that the main determinants of leaf bacterial diversity and community structure are the host species and its functional traits, such as leaf mass per area, leaf longevity and maximum photosynthetic capacity, leaf nitrogen content, and wood density [13]. In a field study, fungal assemblages on beech (*Fagus sylvatica*) leaves were largely dominated by cosmopolitan and generalist species, and the largest variation was found between individual leaves within the same canopy [14]. Between trees, the major determinant of community dissimilarities was tree genotype, instead of geographical distance [14]. In addition, leaf mineral content has been found to be crucial for leaf microbial community composition [15]. Since fertilization, irrigation, and urbanization affect nitrogen availability, natural microbial communities of plant leaves are governed by several factors that are modified and even distorted by humans.

1.3 Seasonal Variation in Natural Microbial Communities

Complex interactions between soil abiotic conditions, like temperature, frost, moisture, porosity, and biotic factors, such as the composition and coverage of vegetation, affect microbial community structure and activity in natural soils. Drought during dry seasons distorts soil invertebrate communities [16] and halts microbial respiration when microscale hydrologic connectivity is poor [17]. Seasonal variation, particularly permanent snow cover, narrows the options humans have to interact with natural microbial communities.

In Mediterranean and warmer climates, the cycling between dry and rainy seasons shapes microbial activity and phylogenetic diversity in natural surface soils [17, 18]. In temperate and boreal climates, fall colors, leaf fall, and the subsequent litter decay are the brightest example of seasonal variation in native plant and microbial communities [19–22]. As seasonal variation does not destroy natural microbial communities but instead is an essential part of most ecosystems on Earth, humans must have adapted to seasonal changes in microbial environment. Thus, the importance of seasonal variation in the context of immune system function is limited to cases where a hostile season, like winter, reduces contacts with environmental microbiota [23].

1.4 Environmental Bacteria Are Lazy Survivors

One of the greatest challenges in environmental microbial ecology has been to understand why microbial activity in surface soils does not rapidly metabolize and recycle all organic carbon that enters soil ecosystems [24, 25]. Recent experimental and modelling approaches have offered at least two complementary scenarios. Firstly, as large biofilms, e.g., clusters of bacteria, may partly prevent the flow of resources in microscopic pores, competition in porous media seems to favor steadily slow-growing biofilm-forming bacteria over fast-growing strains [25]. In parallel, decomposers need to produce extracellular enzymes to utilize organic carbon sources [24]. The resources released by extracellular enzymes can be exploited by any non-decomposing coexisting species; recent findings indicate that the presence of the free riders increases organic matter buildup and bacterial biomass in surface soils [24]. Owing to these two phenomena and the functional complexity of soil organic carbon [26], a successful strategy in natural surface soils is opportunistic laziness. Notably, in addition to soils, many other organic surfaces, such as plant leaves and human skin, can be regarded as partially porous systems where the utilization of resources requires the release of extracellular enzymes. The inevitable conclusion is that humans evolved in the overwhelming presence of lazy microbial survivors that arrive on the skin and mucous membranes in billions each day.

1.5 Lazy Survivors and Modern Medicine

The opportunistic laziness is in striking contrast with the tradition of studying microbes in medical sciences. The modern success of medicine is based on Koch's postulates [27]. The postulates require (1) that a pathogen is distinguished in each patient; (2) that the pathogen, e.g., a bacterial species, is isolated from the host with the disease; and (3) that it is grown in pure culture. Finally, (4) the disease should be reproduced by inserting the cultured pathogen into a healthy host. Koch's postulates are followed also in the prevention of diseases, but in a reverse order. To pass the postulates, the cure, e.g., a probiotic bacterial strain, must be (1) exactly identified and (2) found in former patient, and (3) the strain has to heal new patients. As the human immune system coevolved to continuously cope with an extremely diverse, seasonally and locally changing network of lazy survivors, the characteristics required by Koch's postulates are hardly found in environmental microbiota. In the context of the hygiene hypothesis, if the postulates are followed, over 99% of environmental bacteria drop out simply because they cannot be cultivated. This is unfortunate as recent findings indicate that the whole spectrum of environmental microbiota may be needed for optimal prevention of immune-mediated diseases [28].

2 Man-Made Variation in Environmental Microbiota

2.1 Many Species Shape the Microbiota of Their Surroundings

Humans are not the only species whose activity results in considerable alteration in microbial communities. Tree-fungal interactions largely control microbial environment and are indeed the key to ecosystem productivity in large parts of the world [29]. Many animals, like ants and large herbivores, drastically affect the microbiota in their surroundings [30, 31]. Just like humans, several vertebrates clean their nests to reduce microbial load on the offspring. Termites build mounds and form large colonies where the number of individuals is comparable to human cities. In the mounds, microbial diversity and the seasonal variation of community composition are smaller than in surrounding soils [32]. The main difference between humans and other species is the scale: when cultural evolution proceeded in humans, our species became the first one that is able wipe off natural vegetation and substitute natural ground surface with artificial materials that lack coevolved microbial communities.

2.2 Invasive Species

Invasive species distort native microbial communities and are one of the major reasons for biodiversity decline on this planet. Humans typically control, e.g., weed and substitute, native vegetation in urban environments, which results in low plant diversity. The poor diversity and the short evolutionary history of urban plant communities leave the door open for successful invasion by exotic plant species [33, 34]. Outside developed societies, invasive species destroy entire ecosystems utilized by locals [35]. An example can be found from coastal South-East Asia where sand dunes host a diverse community of native woody species [36, 37]. Many of these plants have traditionally belonged to the local diet [38]. When the native plant community on nutrient-poor sand dunes is invaded and replaced by alien nitrogen-fixing acacias (Fig. 1), the entire lifestyle of locals is under threat, including the fiber- and microbe-rich traditional diet.

Mechanisms behind plant invasiveness are numerous [39]. In the context of soil microbiota, many invasive species release compounds or produce litter that either slows down or accelerates litter decomposition by soil microorganisms [39–42]. This often leads to poor regeneration of native vegetation [39, 43, 44], which plausibly results in shifts in microbial community composition. Some of the mechanisms of the distortion of natural microbial communities are complex; within introduced range, legumes are known to host root symbionts that produce compounds that bind micronutrients [45–47]. As a result of micronutrient binding, soil dominated by invasive garden lupine (Fig. 1; [48], see also Vetter et al. [49]) contains less nematode root feeders than soil dominated by a native legume

Fig. 1 Two examples on how invasive species distort soil natural microbial communities. (**a**) In temporal and boreal Europe, root symbionts of North American garden lupine (*Lupinus polyphyllus*) reduce the abundance of root feeders, which leads to monocultures that shine in early summer and are thereafter less attractive for human recreation. (**b**) In South-East Asia, native dune forests host a diverse plant community adapted to flourish on nutrient-poor sand, but fast-growing Australian acacia grows successfully on the dunes. The pictured, mature tree is 6 years old. (**c**) Acacia litter forms a suitable habitat for acacia seedlings. (**d**) The root system of acacia includes a network of nitrogen-fixing root nodules in the uppermost sand layer, which leads to the distortion of the microbiota of the natural environment and aids in replacement of native vegetation. (Photos taken by the author)

[46]. As root feeding by nematodes reduces plant growth and reproduction [50], the decreased herbivory (i.e., decreased feeding on plants by the nematode root feeders) then provides a competitive advantage for the legume over native plants. This advances the invasion and distorts the original microbial community in surface soil [51]. In addition to direct effects on soil microbiota, invasive plants may reduce the attractiveness of nearby natural and semiwild areas. In Europe, lupine invasion is often accompanied by native but sticky nettles that benefit from the increased nutrient content of lupine-dominated soils. As a result, the recreational value and possibilities for direct contacts with rich microbiota in green areas are diminished.

In well-replicated field experiments, exotic plant species have increased above-ground microbial diversity as a part of diverse plant communities [13, 52]. Despite this, monocultures of alien plants simplify molecular diversity of organic compounds in soil [26]. This is crucial as according to recent models, molecular diversity controls decomposition and thus eventually litter formation [26]. To put it shortly, cumulative evidence supports the view that invasive alien species distort natural microbial communities and the interaction of humans with the surrounding nature.

2.3 Environmental Pollution

Environmental pollution occurs virtually everywhere where human population density is high. In the context of natural microbial communities, contaminants can be divided into organic compounds and elemental contaminants. Nutrients released by human societies often act like organic contaminants [53]. Elemental contaminants are nondegradable, but they may be extracted by plants, bound to organic molecules, and their toxicity can be adjusted by manipulating solubility [54, 55]. While organic contaminants can be recalcitrant, they are usually degradable. Whether or not they are degraded depends on the contaminant and the local microbial community, like the presence of degrader genes [56, 57]. If nutrient availability or oxygen content is low, degradation is delayed [58]. This can be corrected by adding nutrients, which further distorts the original microbial community [59, 60]. The most common organic contaminants are aliphatic and polyaromatic hydrocarbons, pesticides, and chlorinated compounds. All these typically serve as a carbon and sometimes also nitrogen source for indigenous soil microbes. The utilization of a novel carbon or energy source obviously causes shifts in microbial community composition. The distortion changes the abundance and diversity of bacteria associated with human health, particularly immune system disorders [61, 62].

As the release of environmental contaminants has been a part of industrialized, urbanized lifestyle from the very beginning, low contaminant levels are found in urban surface soils [63]. Even low contaminant levels have the potential to distort plant and microbial growth [63–66]. Although some of the most common organic pollutants, like oil hydrocarbons, are often degraded rapidly in nature, the lack of organic topsoil prolongs the time needed for degradation [67, 68]. In urban areas, organic topsoil is regularly removed (see below). Recently, low levels of polyaromatic hydrocarbons in ambient air were observed to be associated with the high endocrine disruption potential of individual gut microbial communities among daycare children [69]. While the method used does not allow conclusions about a causal effect, earlier experimental work with cell models supports the finding by Roslund et al. [69–72]. Later, Vari et al. [73] found a similar but inverse association between the endocrine disruption potential of the gut microbial community and the coverage of broadleaved and mixed forests in urban environment. These findings support the view that environmental pollution causes functional shifts in microbial communities. More detailed studies are needed to confirm or reject the hypothesis about the connection between endocrine disruption potential of the gut metagenome and environmental pollution in urban areas.

In summary, environmental pollution has been shown to change environmental and commensal microbial community composition, and there is indirect evidence that the microbial changes are related to health, including immune-mediated diseases.

2.4 *Vegetation Control and the Network of Lazy Survivors*

In developed countries, rural areas are largely utilized for agriculture and forestry. The resulting land use changes are a threat to biodiversity. In the context of environmental microbial communities, monocultures have different and often poorer microbial networks compared to diverse vegetation [74, 75]. Interestingly, some monocultures decrease the relative abundance of *Proteobacteria* [74]. From the perspective of immune modulation, however, the traditional agricultural lifestyle (Fig. 2) evidently provides protection against allergic disorders [76]. Compared to agricultural and forestry systems that are characterized by rich weed populations and abundant patches of pristine and idle land, vegetation control can be extreme in urban areas. At least the following practices have a major impact on the composition, function, dynamics, and diversity of microbial communities in urban areas:

1. Removal of organic surface soil and plant debris (Fig. 2). In rural areas, plant litter is typically left aside. In urban green spaces, dead plant parts, stumps, fallen

Fig. 2 Distortion of natural microbiota in urban areas. Upper row: Urban playgrounds and other built environment comprising of man-made surfaces have poor possibilities for physical contact with rich environmental microbiota. In traditional agricultural societies (below), lifestyle-facilitated unintentional, rich, and daily contacts with environmental microbiota. Left: Farmer's child in front of cattle and hut in Kenya. Right: Under the Yoke (Burning the Brushwood) by Eero Järnefelt (1893). Birch forest burning for agricultural fields. (Photos by the author except painting: Finnish National Gallery/Yehia Eweis. Creative commons CCO)

autumn leaves, and even thatch are systemically removed. This disturbs the nutrient cycle, eradicates niches suitable for decomposers, and affects water retention on the soil surface. In nongreen urban space, artificial surface materials, like asphalt, concrete, and buildings, prevail. When these surfaces are built, the native microbial community, the rich network of lazy survivors, is removed. The complete elimination of the original microbiota, combined with poor abundance of environmental microbiota on the artificial surfaces, is one of the main reasons for poor microbial exposure among urbanites [77, 78].

2. Intensive fertilization and irrigation. While agricultural fields are regularly treated with nitrogen-fixing legumes or slowly soluble or mineral fertilizers, the rest is usually left outside severe disturbance in rural areas. In contrast, most urban lawns and ornamentals receive intensive fertilization and—depending on climate—irrigation to meet recreational needs [79, 80]. Since fertilization and natural variation in soil moisture are crucial determinants of microbial community composition, the man-made modification of urban soils will evidently distort natural microbial communities [81].
3. Removal of dust particles from city centers. In nature, wind blows dust and tiny organic particles to nearby rocky and otherwise bare patches. Little by little, this leads to accumulation of organic matter suitable for plant and microbial growth. In urban areas, streets, sidewalks, cycle paths, and city centers are cleaned, brushed, and even washed to keep dirt away. As dirt-free mineral soils and artificial surfaces have low microbial abundance [3, 82, 83], dirt removal severely limits the network of lazy survivors, i.e., environmental microbial communities in urban areas. The low abundance and likely patchiness of urban environmental microbiota is reflected on skin microbiota of urban dwellers: in a study by Grönroos et al. [84], touching organic gardening soils or moss for less than a minute multiplied the number of 16S rRNA gene sequences on the skin of urban volunteers, even though bacterial abundance was measured after washing hands with tap water.
4. Green space design. Touching is beneficial for efficient transfer of environmental microbiota onto the skin and mucosal membranes [83–86]. Typical urban public green spaces, parks, and playgrounds do not particularly encourage physical contact with organic soil, herbs, perennials, and woody plants. On the contrary, greenery is typically a decorative element that promotes recreation but discourages active interaction, like touching and tasting (Fig. 3). Related to this, high durability is a main target in playground design. Because natural materials wear out, artificial materials like gum crumb, asphalt, concrete, gravel, and sand are preferred (Fig. 2). All these are hostile environments for most environmental microbes, which keep the microbial network of lazy survivors out of reach of many urban children.
5. City design. Most cities were largely planned before urban principles for ecological landscape design were thought about [88]. Nowadays, new evidence supporting the link between immune-mediated diseases and urban land cover is being published regularly (see [23, 89]). This new knowledge has not yet been fully considered in urban planning, e.g., in the placement of small green spaces

Fig. 3 Upper left: Urban green is often for decoration, not for active interaction. Combined with dirt removal from city centers and sidewalks, intentional rewilding of urban areas is needed. Lower left: A hole in multispecies lawn hosts a rich network of environmental microbiota and attracts skin contact. Lower middle: Woody plant parts, like sticks, provide close contact with environmental microbiota in winter. Right: High-biodiversity green space has diverse vegetation [77] and dead wood [87], and it encourages engaging with natural elements [3]. (Figures by the author, except: Right permission by Maria Hyvönen)

that enrich nearby microbial communities and allow abundant contacts with natural microbiota. Taken together, two parallel phenomena affect to the same direction in urban areas. These are the rare contacts with rich sources of environmental microbiota in urban green areas and the reduction of natural microbial abundance and diversity within built areas per se. The five factors mentioned above lead to severe distortion of ground surface microbiota in urban centers and neighborhoods, which according to Parajuli et al. [90] limit contacts with diverse and abundant environmental microbiota also indoors. In detail, Parajuli et al. [90] studied standardized doormats that were kept in rural and urban households for 2 weeks and analyzed doormat bacterial communities. The results revealed that the high coverage of built environment reduces the transfer of environmental microbial communities indoors. Later, the reduced transfer was found to exist in both summertime and winter samples [91]. Alarmingly, the authors also realized that the winter minimum in rural areas was at the same level as the summer maximum within urban, heavily built neighborhoods [91]. Later, Parajuli et al. [77] realized that low yard vegetation diversity is associated with dysbiosis in stool microbiota among urban dwellers. The distortion of microbiota within built areas has been observed in indoor dust and air samples as well [92–94]. The conclusion is that environmental microbiota, the rich network of lazy survivors that humans coevolved with, is deprived in urban settings, which severely distorts and limits microbial exposure among urbanites.

3 Biodiversity Intervention, i.e., How to Cure the Consequences of the Distortion

3.1 Why Bother?

Changes in land use typically lead to biodiversity loss. Hanski et al. [89] were the first to find that the high coverage of built environment is related to immune-mediated diseases. Recently, Nurminen et al. [23] found that a high coverage of built environment next to infant's homes increases the probability of type 1 diabetes among genetically vulnerable individuals. As an opposite, the abundance of agricultural environment—comprising nonirrigated arable land, fruit trees and berry plantations, pastures, natural pastures, land principally occupied by agriculture with significant areas of natural vegetation, and agroforestry areas—was inversely associated with the probability of the disease. The study by Nurminen et al. [23] took place in three hospital districts in Finland. When the districts were analyzed separately, the inverse association of the agricultural environment with type 1 diabetes was found to be pronounced in the southernmost study district. The annual snow-free period decreases from north to south in Finland. Nurminen et al. [23] collected doormat debris to study seasonal variation in biodiversity carried inside by families participating in the study. The results revealed that indoor exposure to environmental biodiversity was low when snow covers the ground, compared to snowless samples. The authors explain that when snow covers the ground for several months, the benefits of high microbial diversity in the agricultural environment cannot be accessed as easily and unintentionally as during the rest of the year. When the results by Nurminen et al. [23] are considered in the context of intentionally increasing exposure to biodiversity among urbanites, any potential solutions should consider winter and other natural factors that may prevent contacts with microbially diverse soil and vegetation. In warmer regions, seasonal patterns in precipitation need to be considered when planning biodiversity interventions and urban rewilding.

3.2 Outdoor Vegetation Interventions

While the global network of protected areas has been built to cover natural and seminatural ecosystems [95], the need to protect and rewild urban ecosystems has been recognized recently [96]. In addition to the global network of the most valuable natural ecosystems, the biodiversity in urban forests in some countries is increasing due to modern management practices, e.g., saving dead wood [87]. As a striking contrast, dominant management practices in parks and small green spaces inside built areas result in low exposure to biodiversity among urban dwellers (see Sect. 2.3. and [83, 90]). Since the low exposure to rich environmental microbiota within built areas is often accompanied with a lifestyle that actively or passively avoids visits to forests and other natural and seminatural areas, only nature-oriented

urbanites are likely to receive the benefit of the rich microbial network of lazy survivors that lurks in dirt of urban nature in developed countries [97–99]. Since efforts to change human behavior typically have limited value, there is a good reason to concentrate on the opposite approach, i.e., biodiversity interventions within built areas [78]. These would rewild urban ecosystems by adding biodiversity to areas where urbanites usually spend time in their everyday lives.

Roslund et al. [78] were the first to test if the transfer of green elements known to contain rich and abundant environmental microbial communities affects immune modulation among urban dwellers. They selected daycare children aged 3–5 years as the target group. Three types of daycare centers were included: the so-called nature daycare centers where children spend time in nearby forests on a daily basis, regular urban daycare centers that have a yard dominated by artificial and mineral soil materials, and the so-called intervention daycare centers that received green yards overnight. The intervention daycare centers were randomly selected from the participating regular urban daycare centers. Intervention materials consisted of readily vegetated, boreal forest floor; sod, i.e., transferable lawn; peat blocks; and planting boxes that daycare personnel were advised to fill with microbially rich gardening soil, instead of bulk gardening soil that is a microbially poor mixture of peat, sand, and fertilizers. The intervention lasted for 4 weeks, and the children were actively guided to be in contact with the green materials on a daily basis, 5 days a week. Since children were fascinated by the green elements, the guidance was not as crucial as originally assumed [3]. Skin swabs and stool and blood samples were collected before the intervention and after it on Day 28. The results showed that skin microbiota among the intervention children shifted during the intervention and became similar with skin microbiota among children in nature daycare centers. The shifts were particularly evident within proteobacterial classes *Alpha-* and *Gammaproteobacteria*. These shifts were associated with enhanced immune regulation [78].

The study by Roslund et al. [78] had a relatively small sample size of altogether 75 children. The sampling size was further limited due to unwillingness of study participants to donate blood. A parallel difficulty was to keep the interest of families participating in the trial at standard daycare centers without the green intervention. Ideally, the study by Roslund et al. [78] will be repeated as a randomized block design with the participation of tens of daycare centers and hundreds of children that would be followed for years to understand potential effects on disease incidence. Despite these difficulties, the work by Roslund and collaborators paved the way for intervention trials that will be needed to reach a sound basis for microbially oriented rewilding of urban neighborhoods.

Hui et al. [83] described an alternative strategy for rewilding the urban microbiome, i.e., enriching mineral soils with biologically rich, standardized dirt. The authors allowed volunteers to touch pure commercial sand materials or the same materials mixed with biodiverse dirt. The results showed how skin biodiversity increased in dirt-enriched sand. The authors monitored how the dirt intervention affected the relative abundance of bacterial genera containing opportunistic pathogens on the skin [83]. They concluded touching dirt that contains the rich network of

lazy environmental microbiota does decrease the relative abundance of genera containing opportunistic pathogens on the skin, compared to touching pure sand materials. An interesting revelation was that touching any soil decreased the relative abundance of the genera containing opportunistic pathogens, compared to samples taken before the intervention started [83].

Today, city-level, regional, and even national practices and guidelines have been created to advance urban greening. Children are usually a special target group in those guidelines. Preference for wooden jungle gyms and other playground structures, use of sod, and urban revegetation are likely to add to urban biodiversity. However, to optimize the microbial benefit of urban greening, it is necessary to understand the factors that regulate microbial diversity, abundance, and community composition within urban green space. Today, as described above, it is known that monocultures, no matter how beautiful, may not be optimal. Urban design should also allow and encourage active physical contacts with green elements (Fig. 3).

3.3 Indoor Interventions

In preindustrial and traditional agricultural societies, human settlements contained vast microbial diversity also indoors (Fig. 2). Today, due to vacuum cleaners and disinfectants, indoor microbial abundance and the proportion of microbiota originating in soil and vegetation are low. Since urbanites spend most of their time indoors, it is unsure whether outdoor interventions can reach the entire urban population. Therefore, it has been hypothesized that the insertion of rich environmental microbiota indoors can enhance immune regulation and decrease the incidence of immune-mediated diseases. So far, only a single study has been reported. Nurminen et al. [85] manufactured a microbiologically rich powder from soil and plant-based organic materials. They instructed volunteers to touch the biodiverse organic soil-like material three times a day (before breakfast, dinner, and going to sleep) for 20 s for 2 weeks. After the exposure period, Nurminen et al. [85] observed increased stool bacterial diversity and a positive association between the increasing diversity of commensal microbiota and immune regulation. Similar studies and at least one large intervention trial in which infants are exposed to rich environmental microbiota for the first year of their life are going on, but so far the results have not been reported in the scientific literature [100]. As the use of biodiversity elements may require a change in living habits, willingness to test and use health-enhancing innovations has been recently explored [101, 102]. The results indicate the crucial role of scientific evidence and give advice on how to design potential future intervention trials [102, 103].

4 Future Perspectives

Nature and greenness are associated with mental well-being, low incidence of immune-mediated diseases, and several other health benefits [23, 97, 104] (other chapters in this book). Despite this, a short distance to coniferous forests predisposed urban dwellers to asthma and allergic rhinitis in a large study that combined several cohorts from various Central and Northern European countries [105]. A potential reason lies in human behavior, i.e., the benefits of nature are received by the minority that visits frequently urban seminatural ecosystems, particularly forests, while those who always prefer urban built areas receive mainly allergens, such as pollen [106]. The current chapter has reviewed research showing the multiple ways how natural microbial communities are distorted in the urban living environment. While it is hard to separate the importance of any single factor, the combined effect of human activities has led to distortion and eradication of original microbial communities in urban neighborhoods.

The crucial question is how to integrate attractive biodiversity hotspots to urban milieu and how to plan vegetation and decomposer networks that flourish under intense use. Weeds are enemies in agricultural fields, but in urban green space, weeds forming runners and root weeds and woody plants resprouting quickly, e.g., willows, dandelions, goatweeds, and their nontoxic colleagues, may provide an option to increase biodiversity in places used frequently by children. Dead wood, sticks, and cones contain a rich degrader community [87], are attractive, and have traditionally been available for children to play and exercise with. Fallen leaves and turfgrass thatch contribute to natural seasonal variation in soil surface microbial communities. These and other ordinary possibilities may turn out to be the easiest and economically sound options for urban rewilding. In addition to these, research is needed to find out how important it is to insert planting boxes and potted trees and bushes, i.e., sources of plant debris and dirt, outside green spaces. In other words, there is no knowledge how dense the green urban network should be to beget health benefits.

An interesting future research direction is the role of *Proteobacteria*, particularly *Gammaproteobacteria* and *Acinetobacter* spp., in immune modulation [107]. Certain *Acinetobacter* strains are known to produce biosurfactants [108]. Biosurfactants allow bacteria to stick tightly on the skin. Studies by Hanski et al. [89], Roslund et al. [78], and several others identify associations between *Proteobacteria*, *Gammaproteobacteria*, or *Acinetobacter* and immune modulation or immune system problems. It is currently not known whether the reason for the associations is related to the ability to stick to the skin, which may have clinical significance, or whether it is a consequence of the dominance of *Proteobacteria* in organic surface soils (see above). Interestingly, proteobacterial microbes did not have associations in the study by Nurminen et al. [85] that used a homogenized mixture of commercially available gardening soils and plant materials, instead of surface soil transferred from the field.

While high hygiene level is mandatory in welfare states, the distortion and destruction of the natural microbial environment, i.e., the ever-changing network of lazy microbial survivors, are not. On the contrary, the need to control biodiversity loss, invasive species, and environmental pollution has been internationally recognized. Sustainable cities and communities are one of the UN sustainability goals, and sustainability in cities is based on welfare of ecosystems. Although the first intervention trials showing the immunomodulatory role of biodiversity have been published [78, 85], there is an urgent need for research on how biodiversity interventions change microbial and vegetation diversity in urban living environments, particularly at playgrounds, daycare units and schools, yards, and parks. The goal of the upcoming research should be to find practical solutions to enrich taxonomic and functional microbiota and to allow the entire urban population to reach the positive health and welfare effects associated with green, biodiverse environments. Notably, as natural ecosystems are diverse, any potential solutions must consider local needs and conditions. Local sources of biodiversity may facilitate successful rewilding of the current low-biodiversity urban settlements.

5 Conclusions

The distortion of the microbiota of the natural environment by humans results from several parallel factors. In addition to direct devastation of greenery and organic ground surface, environmental pollution and exotic, invasive species distort the original microbiota, including the diversity of bacteria associated with health impacts. The distortion of the microbiota of the natural environment is a likely core reason for the high incidence of immune-mediated diseases in urbanized societies. Attempts to rewild urban microbiota and reintroduce natural microbiota to urban areas have shown promising results. The reintroduction must not be limited to existing green areas. Instead, to reach the health benefits, the reintroduction needs to encourage repeated close contacts with dirt, vegetation, and any elements hosting diverse and abundant microbiota. The implementation of this is likely to require large-scale development, production, and dissemination of elements containing diverse microbiota. Optimally, urban dwellers will have plenty of options for safe interaction with diverse environmental microbiota in everyday life.

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Compliance with Ethical Standards International and local laws and the ethical standards of the publisher were always followed. When a study by the author's research group recruited human volunteers, the ethical approval was obtained from the ethical committee of the local hospital district (Tampereen yliopistollisen sairaalan erityisvastuualueen alueellinen eettinen toimikunta, Pirkanmaa, Finland). All participants received oral and written information about the study, and they or the parents/guardians of the children provided a written informed consent that was in accordance with the Declaration of Helsinki.

Conflict of Interest Statement The author has been named as an inventor in two patent applications submitted by the University of Helsinki (patent application number 20165932 "Immunomodulatory compositions" and patent application number 20175196 "Immunomodulatory gardening and landscaping material" at the Finnish Patent and Registration Office). The author has not received royalties from the patent applications. The author, jointly with University of Helsinki and other key investigators in the application number 20165932, is a founder and member of the board of Uute Scientific Ltd, which develops biodiversity-based interventions for the prevention of immune-mediated diseases.

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The Nature and Functions of Vertebrate Skin Microbiota

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Abstract The skin is the first layer of protection from the environment, preventing pathogens from entering the body. Although the skin is often considered to be a hostile microenvironment for microbes, numerous microbes have adapted and thrived as colonizers of the skin in different animal species. Several intrinsic and extrinsic factors can contribute to the diversity and composition of the skin microbiome including skin biology, the environment, health status, and lifestyle. Despite its highly variable morphology across different animal species, the skin microbiome plays important roles that are conserved across the vertebrate phylogenetic tree. Along the evolutionary process, the microbial communities evolved with the host, building a symbiotic relationship that allowed the survival of both microbes and the host. This intricate balanced relationship between microbes inhabiting the skin and the host may easily be disrupted by damage to the skin barrier leading to microbial dysbiosis and often times development of skin lesions in the host. We are now recognizing the need to use these symbiotic microbes colonizing the skin to recover dysbiosis and improve skin health. These different aspects that can influence the cutaneous microbiome in humans and animals will be covered within this chapter.

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1 Introduction

Within the subphylum of Vertebrata are several incredibly diverse classes of animals; depending on the source, this may include up to seven classes which broadly include amphibians, birds, fish, reptiles, and mammals. Breaking these groups down to an even larger number of orders still does not fully capture the diversity of animals. The skin microbiome across the body of even a single animal can be different based on the body site; therefore, considering animals which have vastly different environments, lifestyles, anatomy, and physiology reveals an incomprehensible range of microbial communities that may be present on the skin. Within this chapter, we hope to familiarize readers with the unique communities present on the groups but also consider the conserved nature and functions of the skin microbiota across different animal species.

2 Factors Influencing the Skin Microbiome

Several factors are known to influence the skin microbiome, which we have divided between five categories: microenvironment, biology, environment, health status, and lifestyle (Fig. 1). While we attempted to divide the factors known to influence the

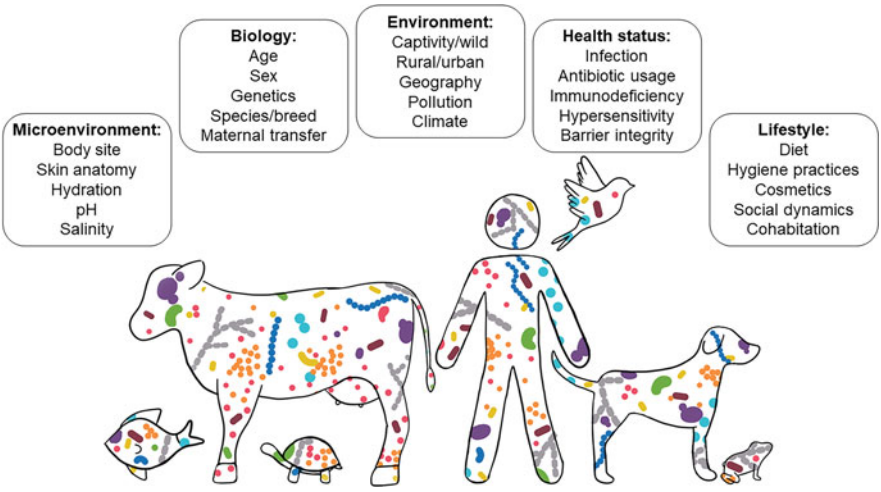

Fig. 1 Factors influencing the skin microbiome

skin microbiome in a single category, it is important to note that many of these factors are connected to each other. For example, a host's genetic makeup can inform some of the factors which we have classified as "skin physiology," including the natural pH of the skin and hydration; a host's genetics also has obvious influences on health status. Prior to describing some of the many factors that may dictate what the skin microbiome looks like on animals, it is important to first understand the diversity of environment that these microbes inhabit.

2.1 Microenvironment

While what is considered to be the "skin" of an animal may appear dramatically different depending on the animal, all animals do have an exterior organ which plays important roles that are conserved across the phylogenetic tree. The skin is the first layer of protection from the environment, preventing pathogens from entering the body and keeping internal tissues safe from the sometimes harsh environmental conditions. Each animal's skin has adapted to the environment where it lives, with some mammals having large amounts of fur to better insulate critical organs in extremely cold climates and with some fish having scales that act as a hard armor to protect against predators.

Features of the skin that are not obvious to the naked eye also exist to protect the body from microbial threats; for example, on human skin, antimicrobial peptides [1] and a low pH create a hostile microenvironment for microbes [2], directly having an influence on the composition of communities. Fish skin has a mucus layer that acts as a physical barrier to trap pathogens and prevent them from entering the skin. This mucus layer also has several molecules that act as a biological barrier, including antimicrobial peptides, proteases, and immunoglobulins [3].

2.2 Biology

In addition to the diversity of the anatomy and physiology of the skin that is seen across different animal species, there can also be numerous distinct microenvironments across the skin of a single animal. In humans, body sites that are less exposed to the external environment are usually more humid, for example, the axilla, which creates a different environment for microbes to live in compared to a more exposed body site such as the arm [2, 4]. Newborns are colonized with homogeneous microbes across different body sites, with these colonizer microbes briefly varying depending on mode of delivery. Despite the changes in the microbes colonizing infants at delivery, within a few weeks after birth, infants will start to change their cutaneous microbiome with variable community composition across different body sites and with mode of delivery no longer playing a role in the microbes colonizing their skin [5]. As infants grow, their skin microbiome continues to change as well,

resulting in significant differences from the cutaneous microbiome they were initially colonized with [6].

2.3 Environment

When thinking about the constant exposure the skin has to the exterior, it becomes easy to see that environment can play a significant role in altering the landscape and composition of the skin microbiome. The effect of environment has been well documented in humans, where individuals living in urbanized areas are much more likely to have lower skin microbial diversity compared with secluded indigenous populations that have not previously had contact with Western civilizations [7]. Furthermore, individuals living in rural areas and with exposure to diverse environments, as well as contact with animals, are much more likely to have higher skin microbiome diversity and be colonized with certain bacterial taxa, such as *Acinetobacter* sp., compared to those living in urban areas [8, 9]. Many individuals living in urbanized areas are exposed to high levels of air pollution, which can significantly affect the skin microbiome resulting in increased richness and diversity, as well as alterations in the functional capacity of the microbiome [10]. Perhaps one of the most compelling pieces of evidences that supports that the skin microbiome is affected by the environment occurs in astronauts within the international space station. These individuals can present alterations in the structure of their skin during space flight [11] which may make them more prone to develop skin lesions and infections. Remarkably, their skin microbiome can change significantly with reduction of Gammaproteobacteria and Betaproteobacteria, at the expense of increased Firmicutes. It is perhaps the constant filtration of the air within the space station or the lack of contact with natural environments that leads to these microbial changes [12].

Research has indicated that exposing individuals to green environments is a method that can be used to increase the diversity of the microbiome, which could potentially have a favorable impact on cutaneous health status [13, 14]. This has been shown by a study performed in adults [13], as well as a biodiversity intervention study, where children kept in a nature-oriented daycare facility versus an urban facility had more diverse bacterial communities, with increases in regulatory T cells and TGF-β1 levels. Similar interventions could be implemented long term aiming to increase microbiome diversity in infants and children and potentially reducing the development of immune-mediated disorders [14].

While the environment plays a role in influencing the skin microbiome of all animals, the relationship between the environment and the skin microbiome of aquatic animals is unique since the water they spend the majority of their time in has its own microbial populations [15, 16]. The microbiome of water influences the cutaneous microbiome; however interspecies variation does exist [17] and individuals of the same species living in different environments have some of the same core microbiota [18], indicating the distinct microbiome that exists on their skin. Studies

evaluating the skin microbiome of aquatic animals and the surrounding waters indicate that these microbiomes are distinct from each other [15, 17]. Remarkably, the microbial composition of human skin is also affected by swimming in the ocean, and this altered microbial composition is associated with increases in antibiotic resistance genes, with changes that can persist for at least 6 h after swimming in the ocean [19].

Seasonality is another important factor contributing to alterations of the skin microbiome. Dogs [20] and horses have been shown to have variable composition in different seasons. In horses, winter and summer were characterized by higher alpha diversity compared to spring and fall. During the winter and summer, horses were primarily colonized by Firmicutes, whereas during the autumn and spring, their skin was predominantly colonized with Proteobacteria [21].

2.4 Health Status

Health status factors are likely some of the most studied influences on the skin microbiome, given the direct implications of the skin microbiome on cutaneous health and vice versa. Antibiotics have often been the first choice for bacteria-driven disease; however clinicians and patients are becoming more concerned of the effect these drugs may have on nonpathogenic microbes [22]. In addition to antibiotic usage, the immune system and microbiome are closely linked; microbes are important for training the immune system in early life to be tolerant of commensals, and immune dysfunction can have important implications. In terms of the skin microbiome, immune abnormalities may result in inherent dysbiosis [23, 24], further putting individuals at risk for infection and exacerbation of disease. Given the wealth of knowledge with respect to health and the skin microbiome, more information is included in later sections in this chapter.

2.5 Lifestyle

Interestingly, but not unexpectedly, cohabitation is another factor that plays a significant role in the skin microbiome. Individuals that cohabit together are much more likely to share their skin microbiomes, compared to those that live in different households. Pet ownership, and in particular dog ownership, can increase the diversity of the human skin microbiome, and owners and their dogs tend to share their skin microbiomes [25]. Cohabitation also changes the skin microbiome of our pets, with strictly indoor cats that cohabit with humans presenting several bacterial taxa that predominate within human skin [26] and with dogs cohabiting together being one of the strongest effects on their cutaneous microbiomes [27]. For humans, hygiene practices, in particular the use of cosmetics and antiseptics, are important factors influencing the skin microbiome. The topical application of hygiene products

to the skin can significantly alter not only the composition of the microbiome but also the metabolites that are synthesized in the different body locations [28, 29]. The common use of hand sanitizers, in particular, within health-care workers [30], has been demonstrated to be an excellent way to reduce transmission of pathogens between hospitalized patients. These products became a necessity within the general population during the COVID-19 pandemic, and despite their benefits, these products may lead to alterations in the skin barrier and the cutaneous landscape, resulting in significant reduction in hand microbial diversity and lower production of antimicrobial peptides [31].

Hygiene products to reduce axillary malodor, including deodorants and antiperspirants, which are two of the most common cosmetic products used around the world, are associated with increased diversity, selection for bacteria that cause bad axillary odor, and selection of increased proportions of *Staphylococcus* spp. and the malodorous bacteria in the genus *Corynebacterium* spp. [32] Microbiome axillary transplantation [33] and microbially converted plant-derived products [34] have been successfully used to counter bad axillary odor, although the effects were just transient and after a few days individuals returned back to their own microbiomes. These "alternative" treatment options are likely to become potential less harmful options to reduce body malodor.

In addition to hygiene products, certain types of clothing, such as polyester, have also been associated with increase in bad body odor and overgrowth of certain bacterial types, including micrococci [35]. Since clothing can lead to changes in odor and cutaneous bacteria, why not create clothing that could actually reduce bad odor bacterial composition? Well, some researchers have begun investigating the potential of using clothing to modulate the skin microbiome to reduce malodor, as well as for other purposes such as wound healing, and it is likely that in the upcoming future we may see many clothing items that will be used to augment a "favorable" skin microbiome [36].

Strong body odor in pets is another topic in the realm of hygiene products and a concern for individuals that cohabit with indoor pets. It has been found that certain bacterial taxa, including *Psychrobacter* spp., which can be found in spoiled food and predominates in aquatic animals, and to a lesser extent *Pseudomonas* spp., have been associated with malodor in a colony of bloodhound dogs. The microbial diversity was reduced in dogs with malodor. Interestingly, the use of essential oils reduced the skin odor, as well as the bacteria that were associated with the odor [37].

Despite its constant external exposure and influence from so many extrinsic factors, the skin microbiome tends to be fairly stable within an individual, especially the facial microbiome, most likely due to recolonization from the follicles and pores, which act as special microbial reservoirs [38, 39]. Changes that occur are often transient, and healthy individuals are very likely to return to their own microbiomes after being influenced and altered by different external factors.

3 Composition of Microbial Communities in Humans and Across Different Animal Species

In humans, the skin microbiome composition varies across the different body sites which have been divided as dry, sebaceous, and moist microenvironments [2]. Each of these niches are characterized by core microbial communities. Overall, the predominant bacterial phyla on human skin include Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes [2, 4, 40, 41]. Sebaceous regions have lower diversity and tend to be colonized with the Actinobacteria Cutibacterium acnes (formerly known as *Propionibacterium acnes*), whereas *Corynebacterium* spp. and *Staphylococcus* spp. dominate moist regions. Dry areas are the most rich microenvironment, with more even distribution of the predominant phyla [2].

Animal species tend to have much higher diversity of their microbiomes, compared to humans (Fig. 2). Host taxonomic order is the most significant factor influencing skin microbiota of animals, followed by their geographic location [26]. Studies in several animal species have found a more similar microbiome across the different body sites covered with hair, although the ear and mucocutaneous junctions are more likely to be colonized with different microbes. In dogs, the individual and to a lesser extent the body site are some of the factors playing a role in the composition of the cutaneous microbiome. Some of the most common phyla found in canine skin include Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Fusobacteria [42]. In cats, similar phyla were identified; interestingly, Bacteroidetes, a phylum that predominates in the oral cavity, was one of the most common phyla found on the haired feline skin, which is likely related to their grooming behaviors [43]. Equine skin is highly diverse and influenced by the different body sites, with some of the most common genera including *Psychrobacter*, *Macrococcus*, *Pseudomonas*, *Acinetobacter*, *Planomicrobium*, *Arthrobacter*, *Carnobacterium*, *Desemzia*, and *Corynebacterium* [21]. Bovine skin studies have mostly focused on the udder and feet, due to health issues related to the mammary gland [44] and high rates of development of pododermatitis in this species [45]. The udder is primarily colonized by high abundances of Corynebacteriaceae and Staphylococcaceae, with significant differences seen between cows and between milk samples collected from the different quarters within the same individual [44]. Even-toed and odd-toed ungulates presented congruence of their skin microbiota, which supports phylosymbiosis in skin microbial communities and their hosts [26].

Avian skin is covered with feathers, which harbors high abundances of diverse bacterial communities. Their microbiota are highly influenced by their social groups, with finches in the same family having a very similar microbiota compared to individuals in other families. Some of the most common families colonizing their skin included Planococcaceae, Carnobacteriaceae, Rhodobacteraceae, Moraxellaceae, and Bacillaceae. It is well known that bacteria can secrete volatiles that may alter odor, and in these birds, it is speculated that volatiles secreted by

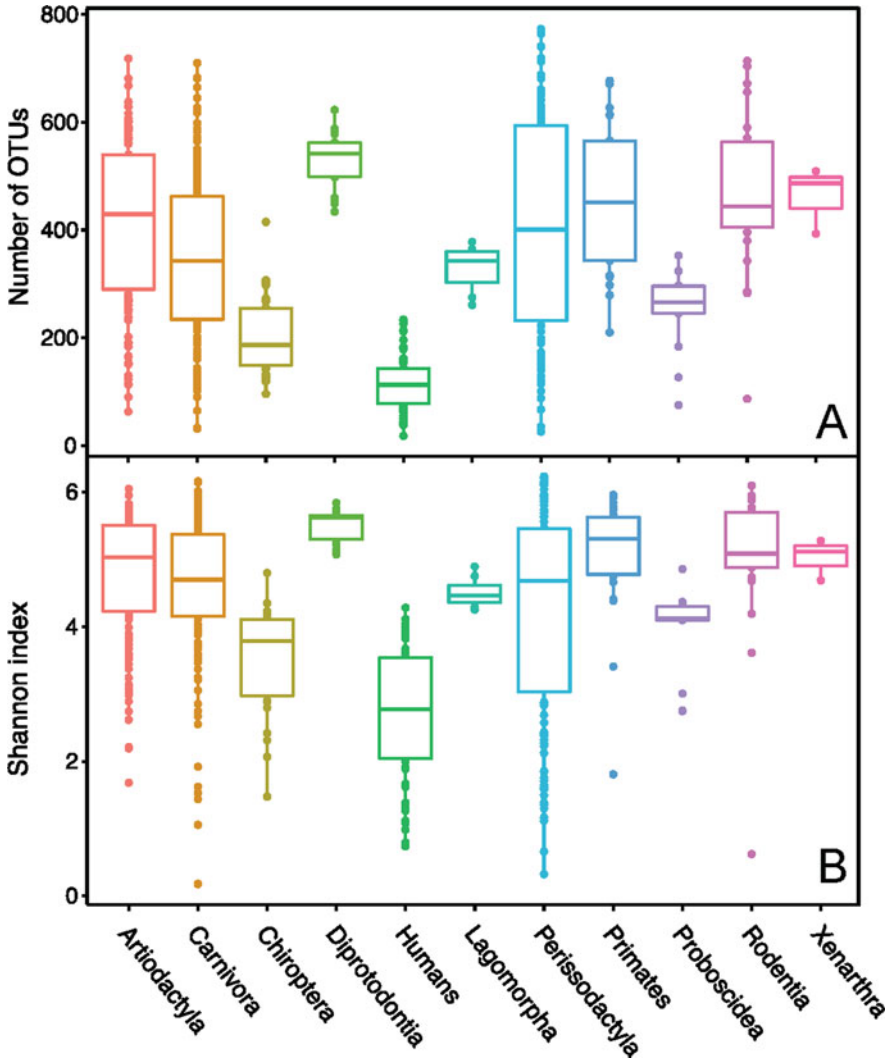

Fig. 2 Boxplots of diversity indices for 10 mammalian orders and humans, including both number of OTUs (**a**) and Shannon indices (**b**). (Reprinted with permission from Ross, A. A.; Muller, K. M.; Weese, J. S.; and Neufeld, J. D. Comprehensive skin microbiome analysis reveals the uniqueness of human skin and evidence for phylosymbiosis within the class Mammalia. *Proc Natl Acad Sci U S A* **115**, E5786-E5795, doi: 10.1073/pnas.1801302115 (2018). Copyright © 2018 the Author(s). Published by PNAS)

cutaneous bacteria may play a significant role in social communication in these birds [46].

The skin of amphibians harbors Bacteroidetes, Proteobacteria, Firmicutes, and Sphingobacteria. In one study, the host species was a strong predictor of microbial

community composition. Within the same species, wetland site is considered a significant factor related to the composition of the microbiota [47]. Since the beginning of the chytridiomycosis outbreaks, which have decimated several amphibian populations across the world, significant attention has been paid to the composition of the skin microbiota of these animals [48].

Aquatic vertebrates encompass a large number of species which inhabit incredibly diverse environments. This group includes completely aquatic mammals, which include whales and dolphins; semiaquatic mammals, such as seals and otters; and fish. Most of the skin microbiome research that has been on aquatic animals has focused on fish and cetaceans (e.g., whales and dolphins); few studies have described the skin microbiome on semiaquatic animals. Among the cetaceans that have been studied are humpback whales [18, 49, 50], killer whales [51], and bottlenose dolphins [52]. The fish species that have been studied so far are mostly those of economic importance in the aquaculture industry, including salmon [53–55] and catfish [56, 57], in addition to many wild species [17] (see Gomez et al. 2020 for a comprehensive review of fish skin microbiome) [58]. Some of the few semiaquatic animals to have their skin microbiome studied thus far are the Antarctic fur seal [59] and harbor seal [60]. Regardless of host species, Proteobacteria appears to be the most prevalent bacterial phylum found on the skin of aquatic animals, with the genus *Psychrobacter* identified on many fish species [16, 59, 60]. Besides Proteobacteria, the phyla Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria are also typically present [15, 16, 59].

Despite the range of animals described here, there are some consistencies in the skin microbiome composition. Most of the skin microbiota on animals appears to be composed primarily of bacteria within the phyla Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Fusobacteria. As mentioned previously, animals tend to harbor diverse cutaneous communities compared to humans. While humans do appear to have unique microbiomes, comparison between animals also indicates that host taxonomy is an important modulator of the skin microbiome [26].

4 Functions of Skin Microbiome

In addition to the intrinsic and extrinsic factors that have been previously described to influence the cutaneous microbial communities, the microbes present on the skin are also important determinants of the composition of communities. Resident microbes can influence the skin microbiota directly, through interspecies interactions, or indirectly through activating the host immune system to partake in community surveillance [1, 61–63].

Some microbes are able to impair the skin barrier, through the production of superantigens or exfoliative toxins [64, 65]. This method is particularly useful on the skin of a compromised individual, where they are already able to gain deeper access into the body. In certain diseases where immune dysfunction is a key characteristic,

such as atopic dermatitis, this can cause further inflammation and destruction of the skin barrier [66].

This ability of the skin microbiome to activate the immune system in a way that is harmful to host highlights the importance of training the immune system to appropriately react to microbes. Immune training is a systemic process, with some of it occurring on the skin, but with much of it occurring through the gastrointestinal tract. The critical period for training the immune system to be tolerant to commensal microbes is early in an individual's life. The importance of immune tolerance has been demonstrated through several mouse studies evaluating the influence of a germ-free environment, particularly on the gastrointestinal microbiota [67]. However, studies using murine models have demonstrated that interactions between commensal microbes and regulatory T cells in the skin are vital in the development of tolerance to commensals [68, 69]. This developmental interaction is specific to commensals; colonization of the neonatal skin by pathogenic *S. aureus*, as opposed to the commensal *S. epidermidis*, did not confer the same tolerance [70]. This study as well as evidence from other studies [71–73] supports the hypothesis that many chronic skin disorders may be due to an exaggerated immune response to commensal microbes. Perhaps, excessive cleanliness during early life may lead to augmentation of immune responses later in life and development of hypersensitivities.

The importance of the skin microbiome modulating the host immune system extends past the period of immune tolerance training. As described further below, microbes can alert the host to pathogens and induce production of antimicrobial peptides [74–76]. Commensals can also contribute to what has been termed "homeostatic immunity," which refers to the development and establishment of adaptive immune responses to the microbiota, but without inflammation [77]. In the skin, some commensals have been found to be important in recruiting Th17 cells to the epidermis; the presence of these T cells serves as a layer of protection by enhancing epidermal barrier function and inducing antimicrobial peptide production [78].

5 Host Health and Pathogen Resistance

Microbial communities have an intimate relationship with the host and have direct influences on host health. Along the evolutionary process, the microbial communities evolved with the host, building a symbiotic relationship that allowed the survival of both microbes and the host. One example of this symbiotic relationship is the microbial community on the face of vultures [79]. Vultures are scavenger animals and therefore are in contact with several microorganisms that would normally cause disease in non-scavenger species, such as tuberculosis, anthrax-like disease, pneumonia, gas gangrene, and gastroenteritis. A study of the facial skin and gut microbiome of these birds revealed a microbial core that contains *Hylemonella gracilis* and *Lactobacillus sakei*. *H. gracilis* has been shown to prevent long-term *Yersinia pestis* colonization in experiments performed in freshwater samples [80], whereas *L. sakei* has inhibitory effect against *L. monocytogenes* and certain *E. coli*

strains [81]. In addition, microbial genes involved in the biosynthesis of antibiotics, fungicides, and parasiticides were identified, indicating functional capacity of the microbiome that would benefit the host. The bacterium *Arthrobacter phenanthrenivorans* was found to be highly abundant in the skin of vultures and is capable of degrading phenanthrene, a polycyclic aromatic hydrocarbon that has skin-irritating effect, emitted from animal carcasses.

In the context of human skin, the skin commensal *Staphylococcus hominis* has shown antimicrobial activity against *Staphylococcus aureus*, an important skin pathogen in patients with atopic dermatitis [76], while *Corynebacterium accolens*, present in the nostril, inhibits the growth of *Streptococcus pneumoniae*, a pathogen of the respiratory tract [82]. Some commensal bacteria help the host by promoting wound healing, such as *S. epidermidis* which limits inflammation post-injury and whose bacterial products can prevent pathogen invasion [83]. All of these examples illustrate different pathways that the microbial population can contribute to the host health and resistance against pathogens.

Furthermore, a minor change in the microbial communities does not necessarily reflect disease to the host due to functional overlap among different taxa [79, 84]. An example of this functional redundancy is the genera *Pseudomonas*, *Acinetobacter*, and *Janthinobacterium*, all of which have shown some degree of antifungal activity against the fungus *Pseudogymnoascus destructans* [85–87], known for causing white-nose syndrome (WNS), that has caused the death of millions of bats in North America. All three genera can be highly abundant in WNS-positive bat colonies [88, 89]. A similar pattern is also observed in amphibian colonies positive for the chytrid fungus *Batrachochytrium dendrobatidis*, in which antifungal bacteria such as *Janthinobacterium lividum*, *Bacillus cereus*, *Pseudomonas fluorescens*, and *Flavobacterium* spp. are highly prevalent [90]. Additionally, in rainbow trout, *Arthrobacter* sp. and *Psychrobacter* sp. showed inhibitory activity against the aquatic fungal pathogens *Saprolegnia australis* and *Mucor hiemalis* [91]. This pattern raises the possibility of an adaptive mechanism of the microbiome to induce pathogen resistance or tolerance by the host [84].

While the primary function of the pre-disease microbial community may be altered, the post-disease microbial community may be selectively modified to respond to this new event. These changes in the microbial communities after disturbances may be temporary or permanent, depending on how resilient the microbe is and how strong the disturbance is. However, the selective pressure of adapted microbial communities that allow the coexistence with the pathogen may present as herd immunity, if enough individuals from the colony have an adapted microbiome [92]. This effect was observed in a population of frog species, *Rana muscosa*, in an area with endemic chytrid. This particular population was naïve to the chytrid fungus and thus thought to be at high risk for extinction. Two years after the initial observation of this population and despite neighboring populations being affected by chytrid outbreaks, the population survived. Researchers suspect this was likely due to a high proportion of individuals with antifungal bacteria on their skin [93].

6 Antimicrobial Peptide Production and Their Role in Maintaining a Stable Microbiome

Microbes are in constant battle with each other to maintain their position in an environment. To establish themselves as residents, rather than simply transient microbes, they need to ensure their survival at the cost of others. Some microbes will naturally be more suited to inhabit the skin than others, being able to survive even in the nutrient-poor environment that is the skin. However, oftentimes, microbes will need to take it upon themselves to adapt novel methods to thrive over their competitors, for example, through the production of metabolites that interfere with others' ability to grow and establish themselves.

Lipid metabolism by microbes can decrease the pH of the skin and thus create an even more hostile environment for many microbes; the products of this metabolism can even be directly antimicrobial [82]. Several bacteria that are known commensals of the human skin microbiome, including *C. acnes*, *S. epidermidis* [94], and *Malassezia* spp. [95], are known to perform lipid metabolism, which has likely allowed them to establish themselves as permanent residents.

Some microbes also produce molecules, including bacteriocins and antimicrobial peptides, which are likely not necessary for their own existence on the skin in the absence of competition but are produced to enhance their chance of survival. Microbes can also induce antimicrobial peptide production by the host, which often not only benefits the microbes modulating the host immune system but also the host. Many of these interspecies interactions and interactions with the host have been demonstrated on human skin with respect to staphylococcal populations.

On healthy skin, staphylococci usually represent a relatively small fraction of the bacteria that are present; several species may be present including two coagulase-negative *Staphylococcus* species (CoNS), *S. epidermidis* and *S. hominis*. One of the primary targets of healthy cutaneous staphylococcal populations is *S. aureus*; this staphylococcal species is typically present in very low abundances, if at all on healthy skin, but dramatically increases its abundance and often becomes the most abundant staphylococcal and bacterial species on the skin of patients with atopic dermatitis [24]. Both *S. epidermidis* [96] and *S. hominis* are able to produce antimicrobial compounds that target and inhibit *S. aureus* [74–76]. Some antimicrobial peptides produced by CoNS can also activate host production of AMPs and act synergistically, mounting an even more effective response [74–76].

7 Skin Disorders Affect the Structure and Composition of the Skin Microbiome

Individuals with skin disorders, such as atopic dermatitis in humans (as well as pets), acne, and psoriasis, are often presented with microbial dysbiosis, which either lead to or are a result of damage to the skin barrier. In atopic dermatitis, cutaneous dysbiosis

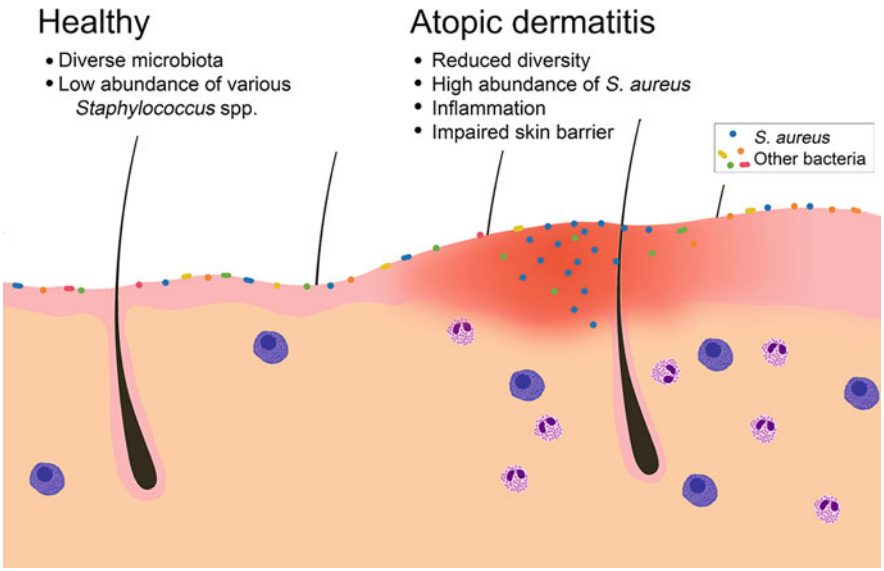

Fig. 3 The skin microbiome in human atopic dermatitis

is often characterized by increases in *Staphylococcus aureus* with loss of microbial diversity (Fig. 3). The reduction in diversity often occurs at the expense of increased relative and absolute abundances of *S. aureus*, with dysbiosis in children being described even prior to flare-up and presentation of cutaneous lesions [24]. In experimental mouse models of atopic dermatitis, it has been demonstrated that dysbiosis in the cutaneous microbiome can be responsible for the development of skin lesions [97]. High *S. aureus* abundances are also implicated in perpetuation of skin lesions [98]. Although *S. epidermidis* is often referred to as a commensal and beneficial microbe, some strains of *S. epidermidis* can have proteolytic activity on corneocytes, in a similar fashion as *S. aureus*, which can result in damage to the epidermal barrier in AD patients [99].

Loss of cutaneous microbial diversity is not only affecting the human population but also pets that often cohabit within the same household. The urbanization lifestyle of many individuals with less exposure to diverse microbial communities is leading to development of cutaneous disorders in pet populations across the world. In particular, dogs and cats are now mostly kept indoors, and in addition to their genetic susceptibility to development of allergic skin disorders, these changes in behavior and environment have been significantly associated with increases in cutaneous allergic disorders in these animal species. In some regions, development of atopic dermatitis, the most common skin disorder in dogs, can affect more than 10% of the canine population. These individuals are likely to present lower richness and/or diversity of their microbiomes, which often coincides with increases in *Staphylococcus pseudintermedius* [42].

Psoriasis, an inflammatory skin disorder, affects approximately 2% of humans worldwide. This disease is characterized by epidermal hyperplasia and hyperkeratosis and inflammatory cell infiltration. Both genetic and environmental factors are thought to play a role in the development of psoriasis lesions. The microbiome is also thought to play a role in psoriatic lesions, although its role is still not well defined and a core microbiome in these patients has not yet been identified [100]. Research studies investigating this disorder have had conflicting findings, which can either show increased or decreased microbial diversity and/or richness. Significant increases in the phylum Firmicutes, at the cost of reductions in Actinobacteria, have been found in those with higher diversity [101], whereas patients with lower richness and diversity of their bacterial microbiota were primarily colonized by four major bacterial genera: *Corynebacterium*, *Propionibacterium*, *Staphylococcus*, and *Streptococcus* [102].

Patients with acne vulgaris have inflammation of their pilosebaceous units which occurs in association with the bacterium *C. acnes*. *C. acnes* colonizes microcomedones formed within hair follicles, and the anaerobic and lipid-rich environment allows proliferation of this commensal organism. Microbiome studies have demonstrated that *C. acnes* is actually one of the most common bacteria found on human skin, especially in sebaceous microenvironments in both healthy and individuals presented with acne [100]. Different *C. acnes* phylotypes are identified in sebaceous follicles in skin biopsies, and macrocolonies are observed in approximately 37% of patients with acne versus 13% with healthy skin [103]. Similar *C. acnes*-relative abundances have been found in both healthy skin and acne lesions. However, certain strains are more common in individuals with acne lesions, with strong association with development of acne [104].

Impaired wound healing with development of chronic skin ulcers is a common chronic problem involving the skin, especially in diabetic patients. Given its severity and impaired wound healing, characterization of the core microbiomes in chronic ulcers in diabetic patients is crucial. Some studies have presented conflicting data. In a study that included almost 3000 patients with chronic ulcers, these lesions often presented high proportions of *Staphylococcus* and *Pseudomonas* species, with these bacteria accounting for approximately 63% and 25% of the composition of all wounds [105]. There were no differences in the composition of the chronic wound microbiome, regardless if a patient presented with diabetic foot ulcers, venous leg ulcers, decubitus ulcers, or nonhealing surgical wounds. Remarkably, the resident microbiota in patients that formed pustules versus those that were able to resolve skin lesions were different, with the former being composed by increased relative abundances of the phyla Proteobacteria and Bacteroidetes and the genus *Micrococcus*, *Corynebacterium*, *Paracoccus*, and *Staphylococcus*, whereas Actinobacteria and *Propionibacterium* spp. were more abundant in the latter [106].

8 Skin Microbiome Modulation

Since the discovery of the first antimicrobial drug, antibiotics have been the most available, reliable, and pragmatic choice for bacterial infections in both human and veterinary medicine. Even though still largely successful and available, the last decades were marked by an alarming increase in antimicrobial resistance, caused by the indiscriminate use of broad-spectrum antibiotics, voluntary treatment interruption, and selective pressure due to use of antibiotics as growth promoters in meat production. The surge of multidrug-resistant microbes has urged the scientific community to discover new alternatives for antibiotic use.

On healthy skin, many microbes live in a balanced interaction, where both microbe and host profit from each other. For microbes, the host provides nutrients and a stable environment. For the host, the microbes can compete against pathogens and protect the host. As discussed previously, microbes have the capacity to modulate microbial populations and the host's immune system and, therefore, the general health status of an individual. Studying the methods by which they are able to do this can provide useful insights into the development of new therapies and strategies to reduce the likelihood of developing antimicrobial resistance.

The skin microbiome has also been found to take part in skin regeneration. Bacteria using the IL1β pathway can stimulate epidermal regeneration, promoting wound healing. These findings support the need to reduce use of topical antibiotics in superficial lesions, as these products have been shown to delay wound healing by impairing the microbiota [107].

Two important ways we have exploited the microbiome to improve host health are prebiotics and probiotics, which are currently being used in the development of therapeutics and cosmetics. On the cosmetic side, several bacterial species, individually or in combination with prebiotics, are being studied for their antiaging properties [108]. On the therapeutic side, for example, the strain *Staphylococcus hominis* A9 is being tested as a new probiotic against *S. aureus* in humans with atopic dermatitis [109]. Additionally, a nasal strain of *Staphylococcus lugdunensis* has been shown to inhibit colonization of *S. aureus* by producing lugdunin, a novel thiazolidine-containing cyclic peptide antibiotic [110]. In frogs, administration of *Janthinobacterium lividum* prior to exposure to the chytrid fungus *Batrachochytrium dendrobatidis* mitigated morbidity and mortality, and the microbe persisted in the population after several months of administration [111].

Another strategy is the transplantation of a "healthy" microbiome to the skin of an individual with microbiome dysbiosis [112]. This method depends on the donor and recipient microbial composition and the load of transplant [108]. This strategy has been studied in atopic dermatitis patients who received creams with CoNS strains isolated from donors. The donor strains were capable of secreting antimicrobial peptides, properties that were lacking in the AD patients and that significantly reduced the burden of *S. aureus* [76]. A different approach to this technique is the use of autologous application of CoNS from the patient's non-lesioned skin in lesional areas [109]. Beyond its therapeutic applications, skin microbiome

transplantation can also be used as a method to mitigate the detrimental effects captivity has on the animal microbiome. Some examples are the parental contact with the offspring and the inclusion of natural subtracts, such as soil, sand, and water, to allow a more diverse microbiome [113].

Phage therapy is another method that can be used as an alternative to antibiotics, particularly for infections with antibiotic-resistant pathogens, given its high specificity against pathogenic microorganisms, while sparing nonpathogenic microbes. This therapy is based on bacterial viruses (phages), which penetrate the target bacteria, replicate, lyse the host prokaryote, and release to continue infecting and killing other bacterial cells [112, 114]. In nature, vulture skin contains the bacteriophage BPP-1, which attacks pathogenic *Bordetella* bacteria, as well as anti-*Clostridium* phages [79]. As a clinical therapy, phages have been used to treat cutaneous infections caused by several bacteria including *Propionibacterium acnes*, *Klebsiella pneumoniae*, *Staphylococcus*, *Pseudomonas*, *Proteus*, and *Escherichia* [115–117]. However, its use has been limited, given the complexity of the technique, which requires purification, characterization, and regulation. Additionally, the targeted bacterium may become resistant to the phage infection and lysis in the long term, due to evolutionary dynamics [112].

9 Conclusions

The skin represents a unique environment for microbes to live in. It is the outermost layer to the body and the first layer of protection for the host; thus it is often a harsh environment to exist on. Skin physiology is variable across vertebrates and even across the body of individuals. Despite striking anatomical and physiological differences across animal species, consistencies exist in the nature and function of the microbiome. Regardless of animal species, the skin microbiome is affected by many factors related to the skin microenvironment, host biology, environment, health status, and lifestyle. All animals have microbes that are pathogens and symbiotics living on their skin, and we now recognize the ability of skin microbes to interact with each other and with the host in many ways to keep a balanced microenvironment. Determining what interactions are occurring and how they are regulated is crucial to understand many aspects of diseases that not only affect the human and animal health but also affect the conservation of many endangered species.

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The Influence of the Microbiota on Brain Structure and Function: Implications for Stress-Related Neuropsychiatric Disorders

John D. Sterrett, Nathan D. Andersen, and Christopher A. Lowry

Abstract Based on research conducted during the last decade, it is becoming clear that the human microbiota plays an important role in the maintenance of human health. Recently, it has become clear that the human microbiota plays a role not only in physical health but also in mental health, which will be the focus of this chapter. Data suggest that, depending on the diversity and community composition of the human microbiota, the microbiota can either contribute to negative mental health outcomes or promote stress resilience. Here we will focus on the mechanisms through which the human microbiota influences mental health outcomes, with a focus on impacts on brain structure and function. In the context of these mechanisms, we will consider the consequences in humans of the large-scale transition from a hunter-gatherer existence or rural lifestyle to an urban lifestyle and the implications for functioning of the microbiota-gut-brain axis, brain structure and function, and mental health. Finally, we will consider the role of the human microbiota in vulnerability and resilience to stress-related psychiatric disorders, including anxiety disorders, affective disorders, and trauma- and stressor-related disorders, including posttraumatic stress disorder, and the mechanisms involved.

Keywords Anxiety · Depression · Gut-brain axis · Microbiota · Microbiota-gut-brain axis · Posttraumatic stress disorder

1 Introduction

The human body harbors communities of microorganisms at many locations including all mucosal and epithelial linings that cover the body's internal and external surfaces [1, 2]. These communities of organisms have been termed microbiota, and they are known to play a role in regulating many facets of host health. Where the

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term microbiota is used to describe the organisms making up the community, the term microbiome refers to the entire "theater of activity" from microorganisms, including genetic material and metabolites [3]. Due to the inability of many microorganisms to be cultured, many microbiota are typically assessed via the microbiome through whole genome shotgun sequencing or sequencing of the 16S ribosomal RNA gene region, while the molecular products of these microorganisms are assessed using metabolomics, proteomics, and transcriptomics. Mammals have historically coexisted symbiotically with their microbiota, forming the "holobiont," or the combination of a eukaryotic organism with its microbial colonies [4, 5]. However, due to increased sanitization and urbanization, altered dietary patterns, use/overuse of antibiotics, and lifestyle changes, human microbiota have experienced disruptions characterized by decreased biodiversity and a loss of contact with specific immunoregulatory organisms with which humans coevolved [6–10]. These immunoregulatory organisms, such as the saprophytic soil bacterium *Mycobacterium vaccae* NCTC 11659, the unique human milk oligosaccharide degrader *Bifidobacterium longum* subspecies *infantis* (*B. infantis*), and even the parasitic helminth *Schistosoma mansoni*, modulate the host immune system in order to coexist, which is proposed to be important for the maintenance of health under the Old Friends hypothesis [11–14].

With reduced exposure to immunoregulatory organisms, we have seen an increased prevalence of immune, allergic, and inflammatory disorders, and an increasing body of research suggests a causal link [12, 15]. Importantly, the heightened prevalence of chronic low-grade immune activation, as well as immune and inflammatory disorders, has contributed to increased rates of psychiatric conditions, as the physiological state of the body impacts brain neurophysiology, ultimately affecting behavior [7, 16]. The altered risk of psychiatric conditions is evident when studying stress responses from rural versus urban participants, as individuals who grow up in urban environments without daily close contact with animals have exaggerated immune and autonomic nervous system responses to psychosocial stressors, relative to the rural participants [17]. Microbiota-mediated modulation of psychiatric states occurs through a number of distinct mechanisms, including (1) afferent neural signaling; (2) altered immune signaling from the periphery to the brain; (3) humoral mechanisms involving effects of microbially derived metabolites, altered host metabolism, or altered host endocrine signaling; and (4) influencing the gut-blood and blood-brain barriers. Here we will discuss each of these mechanisms in turn, as well as our rapidly increasing understanding of their role in determining mental health outcomes. Figure 1 outlines mechanisms covered in this review.

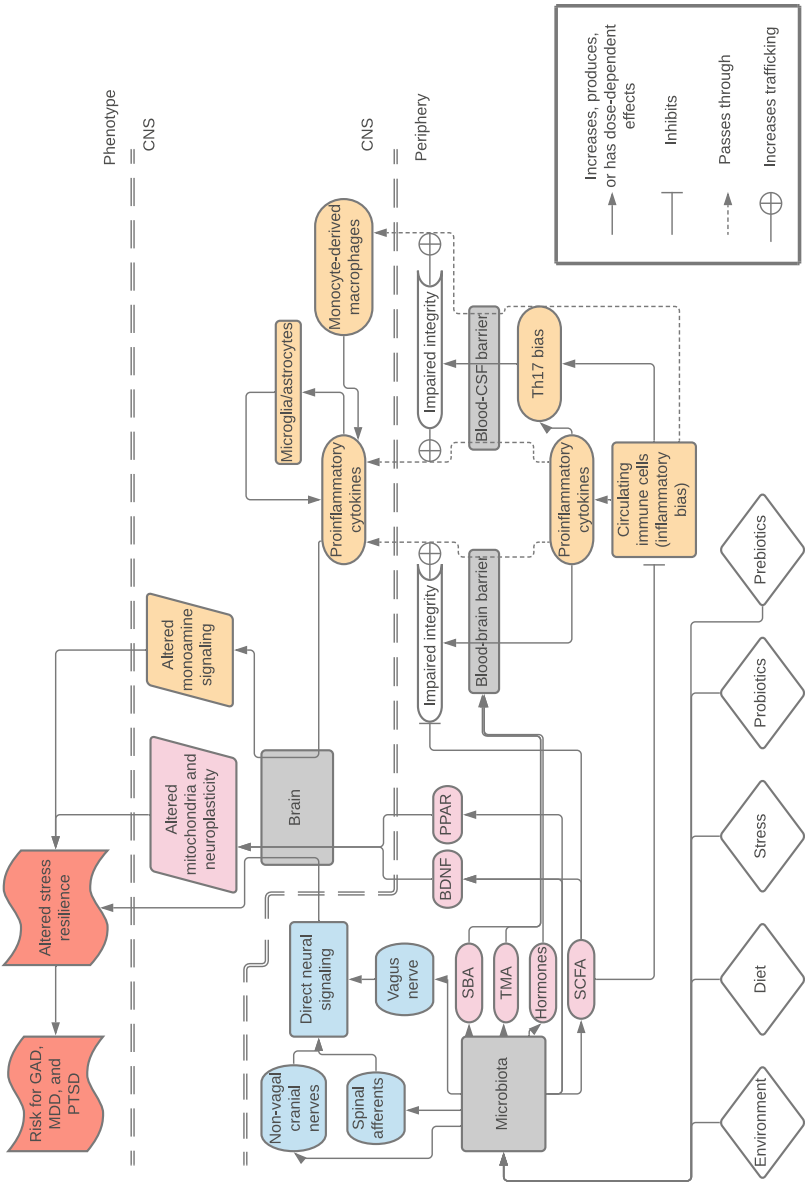

Fig. 1 Mechanisms contributing to modulation of neuropsychiatric outcomes by the microbiota. *BDNF* brain-derived neurotrophic factor, *CNS* central nervous system, *CSF* cerebrospinal fluid, *GAD* generalized anxiety disorder, *MDD* major depressive disorder, *PPAR* peroxisome proliferator-activated receptor, *PTSD* posttraumatic stress disorder, *SBA* secondary bile acids, *SCFA* short-chain fatty acids, *Th17* T helper 17 cells, *TMA* trimethylamine

2 Neural Signaling

The gut microbiota has been heavily implicated in the modulation of the central nervous system (CNS) structure and function. Given the speed of neural transmission, direct signaling to the CNS by nerves innervating mucosal surfaces that are in direct contact with microbiomes is the fastest means of microbiota-brain signaling. Though much research has focused on mediation of the gut-brain axis by the vagus nerve, methods for studying vagal signaling have unaddressed drawbacks, and other understudied neural pathways are also potentially important for microbiota-CNS signaling. Examples of non-vagal neural signaling include spinal afferents from areas such as the skin, gut, airways, and lungs and cranial nerve afferents from nasal and oral microbiota.

2.1 Vagal Afferents

The vagus nerve has long been implicated in communication from the gut microbiota to the brain [18]. The efferent arm of the vagus nerve, as a portion of the autonomic nervous system, controls heart rate, respiration, digestive tract function, as well as immune function [19]. Importantly, however, over 80% of vagus nerve fibers are afferent, transmitting information to the brain, whereas 10–20% are efferent [20]. Neurons from the vagal afferent pathway innervate much of the digestive system, including a large portion of the enteric nervous system (ENS) [21]. Additionally, they have receptors for many gut peptides and microbial metabolites. A prime example is the expression of toll-like receptor (TLR) 4 on vagal afferent neurons, allowing them to detect the common bacterial antigen lipopolysaccharide (LPS) [22]. Moreover, vagal afferent neurons also express TLR2 (which detects components of gram-positive bacteria such as acylated lipopeptides, peptidoglycan, and lipoteichoic acids) and TLRs 3 and 7 (which detect viral mRNA) [23–25]. Afferent vagal fibers terminate almost exclusively in the brainstem nucleus of the solitary tract, which can relay signals to neural systems within the brain. The afferent vagal fibers originating in different organ systems innervate different subregions of the nucleus of the solitary tract, suggesting that different organ systems, i.e., the large intestine versus the bronchopulmonary system, can have different effects on brain structure and function [26].

2.2 What Have We Learned from Vagotomies?

Vagotomies, or surgical procedures that cut or remove portions of the vagus nerve, date back to 1814, when Benjamin Brodie observed that a vagotomy prevented mucous secretion in the stomach after arsenic insertion into a thigh wound of a dog

[27]. In the years since, vagotomies have seen widespread use in clinical practice and are presently being phased out due to the creation of therapeutic interventions with fewer side effects [28]. Currently, vagotomies are often used in animal models to study vagus-mediated aspects of the periphery-brain axis signaling [28].

Notably, Konsman et al. [29] demonstrated that vagotomy blocks behavioral depression in response to peripheral inflammation in rats. Vagotomies in mice prevented a broad spectrum of neurophysiological, endocrine, and behavioral responses following 28 days of chronic oral *Lactobacillus rhamnosus* JB-1 supplementation, including (a) decreased gamma aminobutyric acid $(GABA)_{B1b}$ mRNA expression in the cingulate cortex and prelimbic cortex; (b) increased $GABA_{B1b}$ mRNA expression in the hippocampus, amygdala, and locus coeruleus; (c) reduced $GABA_{A\alpha2}$ mRNA expression in the prefrontal cortex and amygdala; (d) increased $GABA_{A\alpha2}$ mRNA expression in the hippocampus; (e) blunted stress-induced increases in plasma corticosterone concentrations; and (f) reduced anxious and depressive behavior [30]. Similarly, Sgritta et al. [31] showed that vagotomies prevented the stress resilience effects of 28 days of oral *L. reuteri* MM4-1A (ATCC-PTA-6475) in mice.

Vagotomies also have been shown to blunt neuroactive cytokine signaling and alter behavior following experimentally induced peripheral inflammation. For example, Laye et al. [32] demonstrated that a vagotomy blocks interleukin (IL)-1β mRNA expression in the hypothalamus and hippocampus (but not the pituitary gland) in mice in response to peripheral LPS injection. Luheshi et al. [33] also demonstrated that vagotomy in mice blocks decreased social exploration but does not prevent fever following intraperitoneal IL-1β injection. Wieczorek et al. [34] showed that the effects of peripheral IL-1β and LPS injection in mice (including decreased appetite and locomotor activity, increased plasma adrenocorticotropic hormone and corticosterone concentrations, and altered serotonin and tryptophan metabolism in the brain) were somewhat attenuated by vagotomy. However, the attenuation was "marginally significant," leaving room for other mechanisms, such as immune activity. This is supported by Van Dam et al. [35], who showed that vagotomy in rats did not block the LPS injection-induced increase of IL-1β-immunoreactive cells in areas where the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) are weak, such as the circumventricular organs and choroid plexus, respectively. Ji et al. [36] additionally demonstrated that vagotomy in rats increased monocyte chemoattractant protein 1 (MCP-1; also known as C-C motif chemokine ligand 2 [CCL2]) in the dorsal motor nucleus of the vagus nerve, suggesting that the vagotomies also impact monocyte chemotaxis. Overall, vagotomies have demonstrated that the vagus nerve is involved in signaling from the peripheral nervous system (PNS) to the brain, and it is involved in altering behavior and neuroinflammation, but it does not fully control all relevant immune responses.

2.3 What Have We Learned from Vagal Stimulation Studies?

Vagal stimulation methods (vagal nerve stimulation, VNS), in contrast to vagotomies, have initially been studied as tools for altering brain structure and function in the context of neurological disorders such as epilepsy [37]. The observed effects of VNS on monoamines in the brains of individuals and animals with epilepsy prompted more research on the effects of VNS on anxiety, affective disorders, and trauma- and stressor-related disorders. Overall, it has been found that in animal models VNS decreases anxious and depressive behavior and increases extinction of conditioned fear (a hallmark of resilience to trauma and stress) partially via peripheral muscarinic receptor activity. Vagal activity can be modulated by certain microbes; for example, intestinal injection of *Lactobacillus johnsonii* La1 in rats increases gastric vagal nerve activity [38]. Given that the vagus nerve innervates the gut and the vagus nerve can be stimulated by microbes, it follows that stimulation of the vagus nerve by the microbiota could modulate physiological and behavioral responses relevant to psychiatric disorders.

Noble et al. [39] demonstrated that VNS generally reduces anxious behavior in rats exposed to 2 days of auditory fear conditioning, as evaluated by elevated plus-maze behavior. Furmaga et al. [40] found that the anxiolytic effects of VNS in rats require activation of serotonergic and noradrenergic neurons, as administration of 5,7-dihydroxytryptamine and 6-hydroxydopamine (serotonergic and noradrenergic neuron neurotoxins, respectively) to the lateral ventricles blocked the anxiolytic effects of 2 weeks of VNS. Additionally, Noble et al. [41] demonstrated that blocking peripheral muscarinic receptors (of the parasympathetic nervous system) via intraperitoneal administration of the muscarinic receptor antagonist methyl scopolamine reverses the anxiolytic effects of 2 weeks of VNS in rats, indicating a role of peripheral signaling via the parasympathetic nervous system in VNS's anxiolytic effects. When combined with the facts that VNS attenuates the systemic inflammatory response to endotoxin in rats and that VNS attenuates neuroinflammation in response to LPS in mice, the necessity of parasympathetic nervous system activation for anxiolytic effects demonstrates that VNS's effects are at least partially dependent on peripheral inflammatory responses, not solely direct afferent signaling [42, 43].

VNS has also been found to exhibit antidepressant-like effects in rats undergoing chronic stress. Two weeks of VNS in rats increased the expression of 5-hydroxytryptamine (5-HT) receptor 1A in the dorsal raphe nucleus and nucleus tractus solitarius, along with the expression of 5-HT_{1B} receptor and brain-derived neurotrophic factor (BDNF) in the hippocampus, and it prevented decreases in expression of hippocampal 5-HT_{1B} receptor and BDNF induced by 2 weeks of chronic restraint stress [44, 45]. Notably, the modulation of hippocampal 5-HT_{1B} receptor and BDNF expression by VNS was accompanied by decreased depressive behavior in the rats who underwent chronic restraint stress [44, 45]. Moreover, the increase in hippocampal BDNF expression was blocked by injection of 5,7-dihydroxytryptamine into the dorsal raphe nucleus, demonstrating that effects

of VNS on BDNF expression are dependent on 5-HT signaling in the dorsal raphe nucleus [44, 45]. Furmaga [40] also found that 5,7-dihydroxytryptamine administration to the lateral ventricles blocked the antidepressant effects of VNS, but 6-hydroxydopamine administration did not, indicating involvement of serotonergic but not noradrenergic neurons in VNS's antidepressant-like behavioral responses, as assessed by forced swim test performance.

In addition to the ability of VNS to decrease anxiety-like behaviors and induce antidepressant-like behavioral responses, VNS has been shown to enhance fear extinction in mice and rats. For example, Noble et al. [46] found that VNS every other day for 12 days during the extinction phase of a posttraumatic stress disorder (PTSD) model (involving a single prolonged stressor followed by auditory fear conditioning) enhanced fear extinction and decreased PTSD-like symptoms. Furthermore, Souza et al. [47] showed that the effects of 5 days of VNS in rats follow an inverse U-shaped curve, where 0.4 and 0.8 mA VNS enhance fear extinction, but efficacy declines at 1.6 mA. Moreover, Noble et al. [41] demonstrated that 2 weeks of VNS in rats enhances fear extinction, and this was not blocked by intraperitoneal administration of the muscarinic receptor antagonist methyl scopolamine, indicating that peripheral signaling of the parasympathetic nervous system is involved in VNS's anxiolytic effects, as described above [39–41], but not its effects on fear extinction. Overall, VNS in animal models has shown to affect anxious, depressive, and PTSD-like behavior in a dose-dependent manner, via both serotonergic signaling and modulation of peripheral muscarinic receptor activity.

2.4 Epistemology of Vagal Signaling in the Microbiota-Brain Axis

Though vagotomies and vagal stimulation studies inform researchers about the relevance of the vagus nerve in the gut-brain axis, we must be critical of how they actually affect host physiology. Importantly, the vagus nerve is not solely composed of afferent fibers: up to 20% of vagal nerve fibers are efferent [20]. Thus, cutting the vagus nerve will indisputably have effects on non-CNS host physiology via altered efferent signaling. For example, Kessler et al. [48] demonstrated that a vagotomy modulates the immune system of septic mice, increasing the risk of death and elevating serum concentrations of tumor necrosis factor (TNF) and IL-6. Moreover, Di Giovangiulio et al. [49] demonstrated that vagotomized mice have increased susceptibility to dextran sulfate sodium (DSS)-induced colitis, along with decreased colonic lamina propria and mesenteric lymph node regulatory T cell (Treg) populations, indicating that vagotomies decrease peripheral immune regulation in mice. This suggests that previously mentioned immune changes in neural tissue are not purely a result of afferent signaling; vagal efferent modulation of the peripheral immune system is involved in these changes as well. Previously discussed research on vagal stimulation complements this, as it was demonstrated that VNS's anxiolytic

effects are dependent on peripheral signaling of the parasympathetic nervous system, and VNS suppresses immune and inflammatory responses to endotoxin and LPS exposure.

Due to the technological limitations of vagotomies (which affect both afferent and efferent fibers), it cannot be concluded from vagotomy or VNS studies that the results are solely due to afferent vagal signaling. To elucidate the roles of afferent and efferent vagal signaling, techniques such as selective optogenetic stimulation of afferent vagal fibers as demonstrated by Booth et al. [50] and efferent vagal fibers as demonstrated by Fontaine et al. [51] must be utilized in mechanistic studies. In conclusion, one should consider that the vagus nerve contains both afferent and efferent fibers before deriving causality from vagotomy- and vagal stimulation-based studies.

2.5 Non-vagal Afferents

Though the vagus nerve is perhaps the most studied direct afferent pathway relaying signals from the microbiota to the brain, it is not the only one. Other pathways include cutaneous spinal afferents, the remaining cranial nerves, and interoceptive afferent signals that travel in sympathetic nerve bundles. More thorough discussion of the effects of interoceptive signaling can be found in the human research section of this chapter.

2.5.1 Spinal Afferents from the Skin and Bronchopulmonary System

Emerging research in preclinical and human studies supports the hypothesis that activation of afferent spinoparabrachial and spinothalamic pathways from the skin and bronchopulmonary system, including activation by microbial inputs, modulates serotonergic signaling in the brain [11, 52]. For example, subcutaneous injection of *M. vaccae* NCTC 11659, which has been shown to alter serotonergic signaling in the dorsal raphe nucleus of the brainstem and to prevent stress-induced anxiety-like defensive behavioral responses, is hypothesized to exert these effects via spinoparabrachial and spinothalamic pathways, though the direct neural mechanisms involved have yet to be determined [11, 53–55]. Kim and Yosipovitch [56] review the ability of the skin microbiota to contribute to interoceptive stimuli (particularly itch) that are likely relayed to the brain via spinoparabrachial and spinothalamic pathways and are also modulated by the amygdala, which is sensitized by chronic stress and hyperactive in germ-free (GF) mice. However, direct mechanistic studies on these pathways from the skin microbiota to the amygdala are lacking.

Additionally, Hale et al. [26] identified that bronchopulmonary inflammation in mice (which is linked to the lung microbiome [57]) activated both spinal and vagal pathways. Given that bronchopulmonary microbiome literature is still in a nascent stage, it's not yet possible to draw a clear link between bronchopulmonary

microbiome and psychiatric outcomes via interoceptive signaling, but this should be considered a target for future research.

2.5.2 Cranial Nerve Signaling

Cranial nerves innervating the oral, nasal, and skin microbiota have the ability to impact neuropsychiatric outcomes. However, like other afferent signaling, the mechanisms of non-vagal cranial nerve afferents' effects on brain structure and function are understudied in animal models. This is despite the fact that some microbial taxa that are thought to be relevant to mental health, e.g., mycobacteria, appear to be restricted, or at least highly overrepresented, in oral and nasal compartments, relative to the gut microbiota [58, 59]. Although trigeminal nerve stimulation has been studied as a treatment modality for reducing major depressive disorder (MDD) symptoms in humans, the mechanisms through which trigeminal nerve stimulation affects behavior have not been thoroughly evaluated in animal models [60]. Additionally, studying trigeminal nerve activity and stimulation faces similar challenges as studying the vagus nerve since it is a combination of afferent and efferent fibers. Studies in animals have shown that microbes can traffic to the brain via the trigeminal nerve, as Riviere et al. [61] demonstrated trafficking of *Treponema*, a spirochaete bacterium with various subspecies that cause the diseases syphilis, bejel, and yaws, to the brain via the trigeminal nerve in mice.

The trigeminal nerve is not the only direct microbial trafficking pathway to the CNS; the olfactory nerves also allow the spread of herpes simplex virus 1 from the nasal mucosa to the CNS in rodent models [62]. Olfactory bulbectomy is a mouse model of depression that results in similar immunologic changes seen in MDD-, PTSD-, and anxiety-vulnerable populations, along with alterations to neuronal signaling [63]. Importantly, Ozcan et al. [64] demonstrated that olfactory bulbectomy in mice causes neuronal loss and morphological changes in the dorsal raphe nucleus, a major source of serotonergic innervation of forebrain circuits controlling stress-related behaviors and stress resilience. Of note, microbes can activate painful stimuli via olfactory sensory neurons. For example, mouse olfactory sensory neurons express the formyl peptide receptors (FPRs) that detect *n*-formyl peptides, which are produced by bacteria such as *Escherichia coli* [65, 66]. Importantly, FPRs activate nociceptive neurons during infection with *E. coli* or *Staphylococcus aureus* in mice [67]. Microfold cells in the nasal epithelium and upper respiratory tract sense microbial antigens and could also be responsible for triggering immune responses that activate cranial or spinal afferents [68]. Overall, the convergence of multiple pathways by which microorganisms in the skin, mouth, lungs, and gut activate spinal afferents with integration in somatosensory and affective CNS regions suggests that multiple microbiota act on non-vagal cranial or spinal afferent nerves to impact neuropsychiatric outcomes. The paucity of research in these areas, however, must be addressed by future studies.

3 The Microbiota-Immune Axis Modulates Brain Structure and Function

3.1 The Microbiota Modulates the Immune System

Multiple microbial ecosystems such as the gut and skin microbiota are known to modulate the immune system, which, in turn, plays a role in stress resilience and the risk of development and persistence of symptoms of stress-related psychiatric disorders, including anxiety disorders, affective disorders, and trauma- and stressor-related disorders such as PTSD [15, 69–75]. Immune-mediated effects on brain structure and function can occur in various ways, such as by cytokines from the periphery passing into the CNS; by immune cells passing through the BBB, BCSFB, or circumventricular organs into the CNS; or by neural afferents in the periphery relaying signals to the CNS [76]. It should also be noted that the effects of microbial exposure on the immune system do not require the microbes to be alive or to colonize the microbiota. Pseudocommensals, as they have been termed, are organisms that pass through the gut without colonizing it and exhibit strong immunomodulatory effects [77]. Exposure to living, dead, and even partial microbes has strong roles for regulation of the immune-brain axis in the context of mental health.

3.2 Sickness Behavior: An Insight into the Immune-Behavior Axis

Most who have dealt with infections, vaccines, broken bones, or other physical trauma are familiar with the associated psychological symptoms, such as reduced appetite, malaise, increased pain sensitivity, social withdrawal, and difficulty concentrating. These symptoms are collectively known as sickness behavior and, interestingly, overlap heavily with the symptoms of MDD [71, 78]. Generally, sickness behavior is induced by physiological or psychological stressors, ranging from chronic psychosocial stress to broken bones or signals of infection, such as elevated LPS [55, 79–81]. The stressors trigger a systemic immune response in both humans and rodents, notably including the systemic release of the proinflammatory milieu, IL-6, IL-1β, and TNF, along with interferons (IFN) such as IFN-γ [79, 82]. A portion of these proinflammatory cytokines and the immune cells they prime pass into the CNS and activate microglia (the brain's resident immune cells) and astrocytes to alter tryptophan-serotonin pathways, increase reactive oxygen species/reactive nitrogen species ROS/RNS concentrations, decrease BDNF, and contribute to excitotoxicity via altered glutamate signaling [76]. Additionally, increased BBB permeability by the proinflammatory state further allows the trafficking to occur. Together, these changes elicit increased anxious or depressive symptoms and decreased neuroplasticity and stress resilience, providing a window into how cytokines impact mental health and behavior [76]. Research suggests that sickness

behavior via the outlined immune response was evolutionarily advantageous to prevent the spread of diseases and to support healing [83]. However, in modern societies where psychological stressors are much more common than predator attacks, we may often be at odds with sickness behavior, with chronic low-grade inflammation and immune activation likely contributing to the increase in mental health disorders seen globally [84].

3.3 *Cytokines and Brain Structure and Function*

A large body of research demonstrates associations between altered proinflammatory cytokines, including elevated circulating IL-6, C-reactive protein (CRP), TNF, and IL-1β, and impaired stress resilience, as reviewed by Raison et al. [83] and by Maier and Watkins [85]. In addition to being able to alter BBB permeability, IL-6, IL-1β, and TNF can pass into the CNS through saturable transport mechanisms or through gaps in the BBB [86]. Once in the brain, IFNs, IL-1β, and TNF affect monoamine signaling, including serotonin, noradrenaline, and dopamine, as well as glutamate in humans and rodents (for review, see [76]). Serotonin signaling is altered by induction of indoleamine 2,3-dioxygenase (IDO), which is upregulated by IFN-γ, IL-1β, and TNF [87]. In both humans and rodents, IDO diverts the metabolism of tryptophan to kynurenine, decreasing the production of serotonin and potentially increasing the production of neurotoxic quinolinic acid [76, 88]. In rats, quinolinic acid activates *N*-methyl-D-aspartate (NMDA) receptors (a subset of glutamate receptor) while also stimulating astrocyte glutamate release and inhibiting reuptake [89]. These effects are further amplified by proinflammatory cytokines directly decreasing astrocyte glutamate reuptake and increasing glutamate release, which contributes to excitotoxicity in human cell lines and in vivo in rats [76, 90, 91].

IL-1β and TNF additionally activate p38 mitogen-activated protein kinase (p38 MAPK) in mice, increasing expression and function of serotonin reuptake transporters [92]. Furthermore, elevated proinflammatory cytokines can decrease serotonin, norepinephrine, and dopamine synthesis via destruction of tetrahydrobiopterin, a cofactor for tryptophan hydroxylase and tyrosine hydroxylase, by ROS [93, 94]. Under the monoamine hypothesis of MDD, increased serotonin reuptake and decreased serotonin, norepinephrine, and dopamine synthesis contribute to MDD. Overall, elevated proinflammatory cytokine concentrations in the periphery and CNS alter monoamine signaling and contribute to excitotoxicity, altering brain structure and function, which modulates stress resilience. Given that microbial exposure can alter circulating cytokine concentrations (see Sect. 3.5) and chronic low-grade inflammation is a risk factor for stress-related psychiatric disorders, it is evident that cytokines are a potential mechanism by which microbiota modulate stress resilience [16].

3.4 Cellular Access to the Brain

3.4.1 Stress Creates a Proinflammatory Repertoire of Circulating Immune Cells

Upon being exposed to a psychological or physiological stressor, the immune cell profile of the body is shifted toward a proinflammatory state, generally increasing the quantities of proinflammatory cytokines produced in response to exposure to proinflammatory microbial antigens such as LPS. Additionally, chronic, lower-grade stress also pushes the immune cell repertoire toward a proinflammatory state characterized by resistance to glucocorticoids (GCs). Of note, repeated social defeat in mice increases CD14 and CD86 expression on macrophages [95], and the chronic subordinate colony housing model induces GC resistance of Th2 lymphocytes and a decrease in Tregs [55, 81, 96].

Psychosocial stressors also lead to upregulated expression of TLR4 in mice, increasing the likelihood of nuclear factor-kappa B (NF-κB) priming of peripheral immune cells, including macrophages and monocytes [97, 98]. Wan et al. [99] demonstrated a positive feedback cycle, where NF-κB increases TLR4 expression, increasing sensitivity to LPS and further upregulating NF-κB in THP-1 cells (a human monocytic cell line). Activation of TLR4 and NF-κB during this cycle primes monocytes to a proinflammatory state, characterized by increased IL-6, pro-IL-1β, and TNF production [76]. Additionally, in mice, repeated sympathetic nervous system activation by stressors increases systemic norepinephrine and encourages myelopoiesis, resulting in a less mature and more inflammatory population of immune cells (particularly bone marrow-derived monocytes) in circulation [100, 101]. Due to the shift of the circulating immune cell repertoire to a proinflammatory state that is induced by stress, chronically stressed individuals may exhibit heightened sensitivity to disrupted microbial communities and increased epithelial permeability at locations such as the gut mucosa. Thus, interventions focused on increasing microbiota community health to improve the functioning of the microbiota host-epithelia barrier may prevent or attenuate activation of the immune system that can contribute to impaired stress resilience.

3.4.2 Stress and Microbes Modulate the Inflammatory State of Microglia in the Central Nervous System

Thion et al. [102] showed that absence of a microbiota during development or disrupted microbiota community structure by antibiotic exposure results in altered microglia transcriptome in a sex-specific manner in mice. Notably, mice with GF mothers have altered expression of microglial immune response genes indicative of immaturity beginning in utero, with an increased expression of the genes *Ly86* and *Aoah*, which are involved in the response to LPS [102]. These changes affected males more strongly in utero but had more lasting effects into adulthood in female

mice, highlighting sex-specific modulation of behavior-relevant immune activity [102]. Moreover, treatment with an antibiotic cocktail containing ampicillin, streptomycin, colistin, and amphotericin for 1 week induced changes in microglial gene expression, including decreased concentrations of the anti-inflammatory and immunosuppressive genes *Nfkbia* (NF-κB inhibitor 1 alpha), *Tsd22d3* (glucocorticoid-induced leucine zipper, GILZ), and *Ddit4* (DNA damage inducible transcript 4) in both male and female adult mice [102]. Moreover, Boehme et al. [103] found that 12 weeks of consumption of a fructooligosaccharide-enriched inulin prebiotic alters microbiome composition and prevents an age-related increase in the fraction of activated microglia in mice. Together, these data demonstrate that (1) the lack of a microbiota impairs microglia development; (2) disruption of the microbiota alters the inflammatory reactivity of microglia; and (3) microbiota-bolstering techniques such as prebiotic administration are able to attenuate microglial reactivity.

Additionally, psychosocial stress can alter microglial gene expression; Wohleb et al. [95] demonstrated that repeated social defeat in mice increases CD14, CD86, and TLR4 expression on microglia. Moreover, Frank et al. [104] demonstrated that inescapable shock (an acute stress model) induces microglial priming in rats. Rats exposed to inescapable shock had increased concentrations of major histocompatibility complex II (MHCII) and decreased neuronal glycoprotein CD200 in vivo, along with heightened production of IL-1β in response to stimulation with LPS ex vivo 24 h after inescapable shock exposure [104]. Increased MHCII and decreased CD200 contributing to microglial reactivity and increased IL-1β after LPS challenge demonstrate this ex vivo. Stress-induced microglial priming and stress-induced increases in anxiety-like defensive behavioral responses, assessed 24 h following stress exposure, are prevented by prior immunization with *M. vaccae* NCTC 11659 [104], demonstrating that microbial exposures have the potential to increase stress resilience. Overall, microbial exposure modulates the state of microglia in the murine brain, conferring resilience against stress-induced microglial changes and resulting neuroinflammation and altered behavior.

3.4.3 Stress Causes Immune Cell Trafficking into the Brain

Multiple lines of evidence suggest that exposure to chronic stressors can increase immune cell trafficking into the brain. Repeated social defeat stress causes an increase in brain chemoattractant production in mice, causing GC-insensitive monocytes from the bone marrow to traffic to the brain [83, 105]. To elaborate, repeated social defeat causes the release of C-C motif chemokine ligand (CCL) 2 from cytokine-stimulated astrocytes in the brain, attracting CCL2 receptor $(CCR2)^{+}$/ $CX3CR1^{+}$ monocytes; consistent with a significant role for CCL2 signaling, this monocyte trafficking to the brain is largely blocked in CCR2 knockout mice [76, 106]. Furthermore, monocytes trafficking into the brain due to peripheral inflammation produce TNF upon arrival, increasing the proinflammatory cytokine load in the brain [107, 108]. Upon arrival in the brain, monocytes differentiate into brain resident macrophages, which are capable of proinflammatory responses

stronger than those from microglia [105, 109]. Overall, systemic inflammation from psychosocial or physiological stressors primes the circulating immune cell repertoire to a proinflammatory state and induces trafficking to the brain, resulting in impaired stress resilience and anxious behavior via cytokine release.

3.4.4 The Choroid Plexus Is a Gatekeeper of Immune Cell Access to the Brain

The brain is enveloped by three layers of meninges, the dura mater, arachnoid mater, and the pia mater. The choroid plexus resides in the innermost layer of the meninges (pia mater), which is in close contact with the cerebral cortex and spinal cord. Within the choroid plexus (CP), the blood-cerebrospinal fluid barrier (BCSFB) is characterized by fenestrated capillaries [110]. Upon passing through the fenestrated capillaries into the parenchyma of the CP, circulating lymphocytes, accompanied by (antigen-presenting) dendritic cells (DCs), await translocation into the CSF [110]. This exposure of lymphocytes to DCs immediately before crossing into the CSF can be critical for encouraging a proinflammatory lymphocyte bias if the DCs are presenting antigens that promote proinflammatory responses [111]. In cases of infection or hyperpermeable host-microbiota epithelia (at any location harboring a microbiota), high relative abundances of microbial antigens presented by DC could prime lymphocytes to a proinflammatory state prior to entering the CNS. Notably, Th17 lymphocytes, increased by IL-1β, are a chink in the BCSFB's armor, which is particularly important given that microbial exposure alters Th17 lymphocyte concentrations through multiple mechanisms [84, 112]. Even in an uninflamed brain, $CCR6^+$ Th17 lymphocytes can cross the BCSFB at the CP [113]. After crossing, their interactions with DC in the subarachnoid space activate a proinflammatory cascade that can damage BCSFB tight junction integrity [113]. This proinflammatory cascade is associated with the release of vascular cell adhesion molecule (VCAM) 1, a driver of lymphocyte trafficking [114, 115]. Thus, a Th17 lymphocyte bias from systemic or peripheral inflammation characterized by increased IL-1β can result in a permeabilized BCSFB at the CP and further lymphocyte trafficking into the CNS. Moreover, Kertser et al. [116, 117] demonstrated that severe psychological stress in mice impairs CP BCSFB function, allowing increased leukocyte trafficking in a manner dependent on GC signaling. Blocking GC receptors restores BCSFB immune surveillance by increasing Treg trafficking and attenuates posttraumatic behavioral deficits. When combined with Baruch and Schwartz's [118] review of how CNS-specific $CD4^+$ T cells shape brain function via the CP, this research suggests a role of the Th17/Treg balance (an identified therapeutic target in autoimmune conditions) in maintaining the BCSFB for proper stress resilience [119]. Notably, exposure to microbial old friends, such as the helminth *S. mansoni*, regulates the Th17/Treg balance, highlighting the importance of microorganisms in protecting the BCSFB to prevent proinflammatory lymphocyte trafficking, which can impair stress resilience downstream [84].

Pathogens (naked or attached to or inside immune cells) can trigger cells in the CP to relay inflammatory signals to the brain or even cross the CP and enter the CNS. For example, *Listeria monocytogenes* enters the CNS via a "Trojan horse" method, passing across the BCSFB inside peripheral mononuclear phagocytes [120]. Likewise, *Streptococcus suis* can enter the CNS via a "Trojan horse" method inside polymorphonuclear neutrophils [121]. Another example is that death following infection with SARS-CoV-2 is associated with CP inflammation, increased CCL2 and CXCL2 expression in the brain, and increased CP to cortex proinflammatory signaling associated with microglial activation [122]. These proinflammatory responses occur via SARS-CoV-2 binding at the CP but without SARS-CoV-2 actually entering the brain [122], but antigens including the M1 spike protein from SARS-CoV-2 have been shown to cross the BBB in mice, outlining a potential mechanism by which proinflammatory cascades could be triggered from within the CNS [123]. Though it is impossible to know how SARS-CoV-2 infection alters CP inflammation and CCL2 and CXCL2 expression in individuals who survive the infection, this suggests that viral exposure can modulate the inflammatory state of the CP and that infection may confer long-term risk for impaired cognition and depression. Schwerk et al. [124] have reviewed the evidence that because some pathogens can cross the BCSFB at the CP, the CP responds to pathogen challenge by increasing cytokine and chemokine production and BCSFB permeability to encourage leukocyte trafficking into the brain. In the case of pathogens in the brain, the response of the CP to increase leukocyte trafficking is protective against the pathogens, but it also has the unfortunate "side effects" of impairing cognition and decreasing stress resilience by encouraging proinflammatory cytokine production in the CNS [124]. Notably, exposure to dysbiotic microbiota with overgrowth of pathogens or pathobionts such as *Neisseria meningitidis* or *E. coli* or disruption of the host-microbiota epithelial barriers has the potential to trigger these "side effects," highlighting the importance of maintaining diverse microbiota that are resilient to pathogen overgrowth and microbiota that support healthy epithelial barriers [124]. The CP serves as a gatekeeper of immune access to the brain, but modulation of immunophenotypes by a microbiota encouraging inflammation and a Th17-dominant lymphocyte repertoire as well as pathogen infection (which could be somewhat prevented by a diverse microbiota) can impair the BCSFB, resulting in decreased stress resilience.

3.5 Impacts of Microbial Exposure on the Immune-Brain Axis

The ability of stressors to modulate the immune-brain axis raises the question of what can be done to intervene. One potential means of regulating the immune system to confer stress resilience is through microbial exposure. It's important to note that effects of microbe-immune system interactions on brain structure and function do

not rely on microbe colonization or even live/whole microbes. Prime examples of this include the ability of immune stimulation by LPS injection or by subcutaneous or intratracheal administration of heat-killed *M. vaccae* to activate serotonergic neurons in the dorsal raphe nucleus, conferring stress resilience in mice [125]. Initial research demonstrated that subcutaneous injection with heat-killed *M. vaccae* NCTC 11659 activated a subset of serotonergic neurons in the dorsal raphe nucleus in mice, improving performance in the forced swim test [11]. Since then, a series of follow-up studies has demonstrated immunoregulatory effects of *M. vaccae* NCTC 11659. For example, Reber et al. [55] demonstrated that *M. vaccae* NCTC 11659 immunization prevents stress-induced colitis and anxiety in response to the chronic subordinate colony (CSC) housing model, a validated model of PTSD [81]. Additionally, Amoroso et al. [58] demonstrated that *M. vaccae* NCTC 11659 prevents stress-induced aggravation of dextran sulfate sodium-induced colitis in mice, likely through the induction of Tregs [126]. Moreover, *M. vaccae* NCTC 11659 improved stress resilience, stabilized the gut microbiome, and attenuated proinflammatory physiological responses to a "two-hit" stress exposure mouse model of circadian disruption followed by acute social defeat [54]. Further research demonstrated the ability of a novel lipid derived from *M. vaccae* NCTC 11659, 10(*Z*)-hexadecenoic acid, to act on peroxisome proliferator-activated receptor alpha (PPARα) to decrease IL-6 mRNA and protein expression following LPS challenge in freshly isolated murine peritoneal macrophages [127]. In this research, 10(*Z*)-hexadecenoic acid also attenuated LPS activation of TLR4, resulting in less NF-κB downstream signaling.

Similarly, exposure to other microbe-derived lipids, such as conjugated linoleic acids (CLAs) from *Lactobacillus* spp. and *Bifidobacterium* spp., can be immunomodulatory. For example, Miyamoto et al. [128] demonstrated that 10-hydroxy-cis-12-octadecenoic acid prevents TNF-induced gut epithelial dysfunction. Additionally, oral supplementation of CLA has been shown to prevent age-related deficits in BDNF and synaptic function in an aged mouse model of depression risk [129]. The attenuation of hallmarks of age-related depression pathophysiology was found to be mediated by nuclear erythroid-related factor 2 (NRF2), a transcription factor important for anti-inflammatory response regulation [130]. Due to NRF2's roles, including inhibition of NF-κB, NRF2 and NRF2-modulating phytochemicals have been identified as a potential pharmacological target for inflammatory disorders [130]. Hashimoto [131] reviews the role of NRF2 in affective disorders, including evidence such as (a) lower NRF2 expression in the prefrontal cortex (PFC) and CA3 and dentate gyrus (DG) regions of the hippocampus in mouse models of depression, (b) depressive-like behavior in NRF2 knockout mice, and (c) decreased BDNF in the PFC, CA3, and DG. Overall, a variety of living and dead microbes (i.e., postbiotics, see Salminen et al. [132] for elaboration) as well as their metabolites can activate the host immune system to confer stress resilience.

4 The Microbiome, the Blood-Brain Barrier, and Neuropsychiatric Outcomes

4.1 Blood-Brain Barrier Integrity Influences Neuropsychiatric Outcomes

The BBB is an important component of the CNS in maintaining proper cognitive and behavioral function. The BBB functions as a primary gatekeeper, controlling which molecules pass between the circulatory system and the CNS [133]. Though the BBB was initially described as a static barrier, current research has characterized it as a highly dynamic and sensitive system of inter-woven brain microvascular endothelial cells (BMECs), neurons, pericytes, astrocytes, and smooth muscle cells stitched together by protein complexes [134]. These components, combined with circulating blood cells, comprise neurovascular units (NVU), which are responsible for maintaining hemodynamic homeostasis in response to cerebral hypo- or hyperemia and for the regulation of molecular and cellular transport into the brain [135].

It is becoming clear that the gut microbiota influences BBB structure and function. Although not all of the underlying mechanisms are fully understood, evidence suggests a number of distinct mechanisms are involved. For example, there are many microbial metabolites that can affect BBB permeability including bacterial metabolites such as short-chain fatty acids (SCFAs), trimethylamine *n*-oxide (TMAO), and modified bile acids, along with host-derived signaling molecules induced by the microbiota, such as cytokines, hormones, and ROS. Notably, there is complex interplay between the host and microbiota for the production of these molecules, as some (e.g., SCFA) are purely microbe-derived; some (e.g., TMAO) are microbe-derived and host-altered, meaning the host modifies the structure of the molecule to convert them to a bioactive form (e.g., oxidizing TMA to form TMAO). Some (e.g., secondary bile acids) are host-derived but microbe-altered, meaning that the microbiota is involved in converting them to their bioactive form; and others (e.g., cytokines, hormones, ROS) are host-derived and structurally unaltered by microbes, but their quantities in the host are altered by microbes.

Allostatic load placed on the BBB by a dysbiotic microbiota, trauma, or sickness across a lifetime can lead to BBB dysfunction, which is associated with increased risk for affective and stress-related disorders in humans or anxiety-like/depressive-like behavior in murine models [136, 137]. Additionally, chronic psychosocial stress can cause BBB disruption in mice, and the resulting molecular changes to the BBB further contribute to decreased stress resilience [136, 138]. Upon BBB disruption by stress and/or peripheral inflammation, macrophages and monocytes primed to a proinflammatory state by microbial antigens and proinflammatory cytokines in circulation can more easily traffic into the CNS, contributing to anxious and depressive-like behavior [76, 105, 139]. Thus, maintenance of the BBB by a variety of host- and microbe-derived metabolites is important for maintaining stress resilience.

4.2 *Bacterial Metabolites Influence Blood-Brain Barrier Integrity*

4.2.1 Short-Chain Fatty Acids (SCFA)

The human digestive system lacks many enzymes that are required to break down complex plant fibers, and transit time in the gastrointestinal tract is too short to allow the complete breakdown of resistant starches. These fibers and resistant starches pass through the small intestine into the colon (or large intestine), where they are fermented by the members of the gut microbiota. One major product of this fermentation is a class of molecules known as short-chain fatty acids: fatty acids up to six carbons (C) in length. Ninety-five percent of SCFAs produced are acetate (2C), propionate (3C), and *n*-butyrate (4C), which generally exist in a ratio of 60:20:20, respectively, in the stool [140, 141].

As the major energy substrate for the cecocolonic epithelium, butyrate has been the subject of much research, which has uncovered important roles in maintaining host health [142]. One important mechanism by which butyrate maintains host health is through regulating epithelial function, which has historically been primarily studied at the gut epithelium. Decreased butyrate concentration in the gut results in changes to intermediary metabolism (decreased NADH/NAD(+), oxidative phosphorylation, and ATP) within colonocytes that confer catabolic processes, leading to poor colonocyte health [142]. Furthermore, butyrate's mechanisms for modulating epithelial function include non-energetic mechanisms. For example, it acts as a histone deacetylase (HDAC) inhibitor throughout the body, regulating cell proliferation and resistance to oxidative stress, and also acts through its binding to immunomodulatory G protein-coupled receptor (GPR) 41 and GPR43 expressed on enteroendocrine cells in the gut [143–145]. GPR41 and GPR43 are also referred to as free fatty acid receptor (FFAR) 2 and FFAR3, respectively. They have high affinity for butyrate and propionate but low affinity for acetate [145].

The benefits of SCFAs for epithelial function are not localized exclusively to the gut. FFAR3, found on vascular endothelial cells in the brain, responds to physiologically relevant quantities of propionate to protect the BBB from lipopolysaccharide (LPS)-induced tight junction disruption and damage from oxidative stress in human cell lines in vitro [146]. Braniste et al. [147] demonstrated that oral butyrate administration in GF mice decreased BBB permeability to the same extent that exposure to a pathogen-free microbiota did. Additionally, this decrease in BBB permeability was thought to be mediated by increased expression of occludin proteins, which also mediate the effects of the microbiota on epithelial function in the gut and testis and are known as key modulators of tight junction function in the BBB [148–150]. Moreover, butyrate exerts protective effects on the BBB via the immune system, as it induces Treg proliferation and inhibits NF-κB production [151, 152]. Tregs are associated with protection against BBB damage following stroke and traumatic brain injury in mice [153, 154], and inhibition of NF-κB blocks

a proinflammatory cascade of cytokines that disrupts BBB integrity (discussed in cytokine section below).

Although acetate is known to readily cross the BBB in humans, not much is known about its direct actions on the BBB in humans or mice [155]. Additionally, the effects of other less abundant SCFAs on the BBB are not well characterized, though they are known to have effects in other areas of the body. For example, similar to butyrate, valerate (5C, also referred to as pentanoate) has demonstrated activity as a HDAC inhibitor in lymphocytes in mice, assessed both in vivo and in vitro, yet its direct impacts on the BBB remain unknown [156]. Future studies should further evaluate the mechanisms through which other SCFAs act on immune and BBB function.

4.2.2 Trimethylamine N-Oxide

Another class of molecule known to modulate the BBB is TMAO. TMAOs are derived from quaternary amines such as choline, carnitine, and lecithin sourced from the diet [157]. Such amines are converted to trimethylamine (TMA) in the gut by *Anaerococcus*, *Clostridium*, *Escherichia*, *Proteus*, *Providencia*, and *Edwardsiella* and then absorbed and oxidized to form TMAO in the liver [158, 159]. TMAO has been studied for its impact on endothelial function in humans and animal models, as reviewed by Naghipour et al. [160] and Tang et al. [161], and recently some studies have uncovered roles of TMAO in modifying the BBB. Hoyles et al. [162] and McArthur et al. [163] have shown that low doses of TMAO exert protective effects on the BBB in in vitro human cell culture and in vivo animal models, likely through effects on actin cytoskeletons and tight junctions. However, Liu and Huang [164] demonstrated that chronically elevated TMAO concentrations in the plasma of poststroke patients were associated with the development of impaired cerebrovascular function, and their follow-up rat model demonstrated an impaired BBB following high TMAO diets. Current research on TMAO's effects on the BBB cannot draw a full story of dose responsiveness but, to date, suggests the potential of a U-shaped dose response curve of TMAO-BBB interactions.

4.2.3 Secondary Bile Acids

For years, bile acids, synthesized from cholesterol in the liver, were primarily considered as facilitators of lipid digestion and absorption in the gut. However, research emerging over the past two decades has demonstrated their function as signaling molecules throughout the body, with receptors in endocrine glands, adipocytes, skeletal muscles, immune organs, and the nervous system [165]. Additionally, when passing through the digestive tract, bile acids can be deconjugated and decarboxylated by specific gut bacteria to form secondary bile acids, increasing the diversity of the bile acid repertoire [166]. These unconjugated and uncharged bile acids can be passively absorbed in the colon, where they are directed toward hepatic

portal circulation [166]. In humans, less than 10% of absorbed bile acids make it past enterohepatic circulation to systemic circulation, resulting in a plasma concentration between 5 and 15μmol/L [166]. Highly elevated bile acid concentrations in the blood can result in disruptions of the BBB in rats and guinea pigs, likely due to cell membrane damage from the same detergent properties that make bile acids useful in digestion [167, 168]. The effects of lower concentrations of bile acids, however, may not be generalizable across all types of bile acids. For example, in rats, the unconjugated secondary bile acids chenodeoxycholic acid and deoxycholic acid at low relative abundances increase phosphorylation of occludin tight junction proteins, disrupting barrier function, whereas other secondary bile acids, ursodeoxycholic acid and glycol-ursodeoxycholic acid, exert protective effects on the cerebrovascular epithelium in human cell lines [166, 169]. It is important to consider that the beneficial effects of certain secondary bile acids on the BBB could be mediated by a hormetic response. That is, secondary bile acids that improve BBB integrity could do so by causing acute physiological damage that induces BBB proliferation in response. Secondary bile acids that have been shown to exert protective effects at low concentrations may not be protective when chronically elevated or at high concentrations, but research to date has not fully elucidated these effects.

4.3 Host Signaling Molecules Whose Quantities Are Altered by the Microbiome Influence Blood-Brain Barrier Integrity

4.3.1 Cytokines

The gut, skin, and oral microbiota are well known to regulate immune function (as reviewed in Lowry et al. [7], Kau et al. [170], Park and Lee [171], and Idris et al. [172]), which affects the integrity of the BBB (as reviewed by Banks and Erickson [173]). While proper regulation of immune function can lead to maintenance of BBB integrity, immune dysregulation can lead to BBB disruption via increased proinflammatory cytokine production. Of note, dysbiotic gut microbiota states associated with inflamed gut mucosa can upregulate production of the cytokines TNF, IL-6, and IL-1β, leading to increased BMEC permeability [174, 175]. Likewise, dysbiotic states or the presence of extracellular RNA from pathogens in the oral mucosa can increase TNF, IL-6, and IL-1β abundances in mice and human macrophages (in vitro), widening tight junctions of the BBB via decreasing claudin-5 protein expression [176, 177]. Moreover, TNF production in mice encourages neutrophil trafficking to the CNS, encouraging BBB permeability by releasing chemokine ligands (CXCL) 1, 2, 3, and 8 and other metabolites such as ROS [178, 179]. This breach further enables proinflammatory cytokine and immune cell trafficking into the brain [179]. However, the master regulator of proinflammatory cytokine production NF-κB, which upregulates IL-6, IL-1β, and TNF production, is

inhibited by butyrate, blunting the inflammatory milieu mentioned above [180]. Overall, the milieu of proinflammatory cytokines triggered by gut, oral, and skin inflammation impairs BBB integrity, but a diverse gut microbiota capable of promoting immunoregulation and producing SCFA can exert protective effects on the BBB, conferring stress resilience [181].

4.3.2 Hormones

In addition to cytokines, hormones also play a role in maintaining BBB integrity. Interest in the impacts of estrogen and testosterone on BBB integrity was sparked after a study showed sex differences in lateral striatal artery vulnerability mediated by estrogen and testosterone in mice [182]. Since then, research has shown that estrogen is a strong regulator of BBB integrity, protecting against tight junction disruption by inducing estrogen receptor α and nuclear receptor corepressor to downregulate matrix metalloproteinase (MMP) transcription in rats in vivo and in vitro [183, 184]. Thoroughly reviewed in Baker et al. [185], the gut microbiota is a primary modulator of circulating estrogen in animals and humans. Bacteria in the mammalian gut secrete β-glucuronidase, which deconjugates estrogens and phytoestrogens, conjugated in bile, to their active and absorbable forms [185]. Dysbiotic states of the gut microbiota with low richness and bacterial biomass decrease β-glucuronidase production, altering the estrobolome, which can exert direct effects on the BBB [185]. Wilson et al. [186] demonstrated that these effects may also be modulated by serum gonadotropins, which are dysregulated in GF mice [148].

Altered estrogen concentrations could also exert indirect effects on the BBB via the vaginal microbiome. Increased estrogen at puberty is associated with enhanced glycogen deposition at the vaginal mucosa, shifting the vaginal microbiome toward a *Lactobacillus*-dominated community [187]. As could occur with low estrogen concentrations, a non-*Lactobacillus*-dominated vaginal microbiome is associated with production of the previously mentioned proinflammatory milieu of IL-1β, IL-6, and TNF in humans, but this has not been studied thoroughly in murine models [188]. Notably, though diverse microbial exposure is important for training the immune system and protecting against infection in skin, oral, and gut microbiota, high vaginal microbiome diversity is associated with high pH and resultant pathogen susceptibility in humans [189, 190].

In addition to estrogen, testosterone is also modulated by the microbiota and has effects on the BBB. Chronically low testosterone concentrations in gonadectomized mice result in increased BBB permeability when compared to testosterone-supplemented gonadectomized mice roughly 2 months after castration [191]. The increase in BBB permeability was associated with astrocyte and microglia activation, along with increased hypothalamic expression of IL-1β and TNF, which were almost completely attenuated in testosterone-supplemented mice, suggesting indirect effects of testosterone on BBB function [191]. Notably, though testosterone often decreases with age, Poutahidis et al. [192] demonstrated that 3–9 months of

daily *Lactobacillus reuteri* ATCC PTA 6475 consumption prevented age-related decline of testosterone concentrations and testicular size in mice in an IL-17-dependent manner. Moreover, early life antibiotic exposure decreases Leydig cell testosterone function through both microbiome- and non-microbiome-mediated mechanisms [193, 194]. This is mirrored in humans as well, where microbiome diversity positively correlates with testosterone concentrations [44, 45].

Another hormone known to exert protective effects on the BBB and to be influenced by the microbiota is vitamin D (also known as 1,25-OH-cholecalciferol or calcitriol in its active form). This is important for BBB integrity because human BMECs express vitamin D receptors with detectable abundance of both mRNA and protein [195]. In vitro treatment with activated vitamin D prevents the decrease in occludens-1 and claudin-5 and the increase of intercellular adhesion molecule-1 and NF-κB caused by TNF exposure [195]. Direct binding to vitamin D receptors associated with the BBB is postulated to be a mechanism for this, as human BMECs express vitamin D receptors at both the mRNA and protein levels [195]. These findings suggest that vitamin D is another hormone mediating microbiota-BBB interactions.

4.3.3 Reactive Oxygen/Nitrogen Species

ROS and RNS are present in moderate concentration across most cells, acting as signaling molecules via oxidative modification of biological molecules [196]. However, high concentrations of ROS and RNS are associated with increased oxidative damage to tissues including the BBB [196, 197]. Specifically, MMPs, which act as proteolytic enzymes degrading extracellular proteins, are activated by high ROS and RNS concentrations in humans and animal models [197]. This is achieved directly via oxidation or S-nitrosylation of MMPs and indirectly by upregulation of the proinflammatory cytokine milieu IL-1β, IL-6, and TNF [197].

Mitochondria are major sources of ROS in the human body, as they produce ROS in the electron transport chain [198]. Microbial metabolites impact host mitochondrial function, resulting in altered ROS production, as reviewed by Ballard and Towarnicki [198]. A particular example from Wikoff et al. [199] demonstrated that GF mice have many dysregulated metabolic pathways, such as indole metabolism, which affect mitochondrial membrane potential, conferring altered organism-wide ROS concentrations [198].

Furthermore, SCFAs can alter ROS concentrations. Hoyles et al. [146] demonstrated that ROS production in response to proinflammatory stimuli in human BMECs in vitro was ameliorated by propionate treatment. Butyrate also exerts neuroprotective effects in vitro in human cell lines and in vivo in mice by stimulating mitochondrial biogenesis, which is widely associated with improved mitochondrial function, often defined as more efficient electron transport chain production of adenosine-5′-triphosphate and less aggressive production of ROS [200, 201]. Overall, since systemic ROS can lead to BBB damage, modulating mitochondrial biogenesis

may be another mechanism by which butyrate exerts protective effects on the BBB. Mitochondrial biogenesis will be revisited in more detail later in this chapter.

4.4 Circumventricular Organs

The third and fourth ventricles of the brain are associated with circumventricular organs (CVOs), including the subfornical organ, the area postrema, the organum vasculum of lamina terminalis (OVLT), the median eminence, the posterior pituitary, and the pineal gland, all of which lack a BBB. Because of their lack of a BBB, CVOs are particularly sensitive to and points of entry into the brain for contents of the circulatory system. This includes cells, cytokines, microorganisms, prions, and autoantibodies [202]. CVOs and disrupted (or "leaky") sections of the BBB allow humoral access for immune cells and cytokines to the CNS [76]. As a result of their access and sensitivity to circulatory system contents, CVOs play critical roles in regulation of immune access to the CNS and other processes that can affect mental health [7].

5 The Microbiota, Neuroplasticity, and Mitochondrial Function

It is important to note that neural architecture in the brain is not static; dynamic restructuring of neural connections throughout life occurs in normal, healthy humans [203]. The processes surrounding neuronal growth and restructuring are referred to as neuroplasticity and include neurogenesis, neuronal death, synapse formation and synaptic pruning, dendritic remodeling, and axonal sprouting and pruning [203]. Though most prevalent during early stages of life, neurogenesis occurs in healthy adults and is associated with learning and adaptation to new stimuli [203, 204]. In humans, neurogenesis is widely accepted to occur in two areas: the subgranular zone of the dentate gyrus with incorporation into the hippocampus and the subventricular zone with incorporation into the olfactory bulb [205–207].

Though olfactory bulb neurogenesis is not directly linked to psychiatric outcomes, one can reference the fact that olfactory bulb deficits, such as through olfactory bulbectomy (previously mentioned as an animal model for depression), downregulate hippocampal neurogenesis [208]. This is likely at least partially mediated by altered serotonin signaling, given that (a) neuronal death in the dorsal raphe nucleus following olfactory bulbectomy permanently impairs hippocampal serotonin signaling, (b) serotonin signaling encourages hippocampal neurogenesis, and (c) selective serotonin reuptake inhibitor (SSRI) treatment restores hippocampal neurogenesis following olfactory bulbectomy [209–212]. Activation of serotonergic neurons in the dorsal raphe nucleus via microbial exposure (e.g., as shown by

M. vaccae NCTC 11695 exposure [11]) may reduce stress susceptibility, but the effects of microbial exposure on neurogenesis via the dorsal raphe nucleus have not been studied directly [213].

5.1 Brain-Derived Neurotropic Factor as a Microbiota-Mediated Modulator of Neuroplasticity

BDNF is a key modulator of neuroplasticity in human and rodent brains, with roles in neuronal cell growth, survival, and function, conferring emotion and cognitive behavioral roles [214, 215]. Importantly, BDNF concentrations in regions of the brain including the hippocampus and brainstem can be altered by the gut microbiota. To establish a baseline, Sudo et al. [216] demonstrated that GF mice have decreased hippocampal BDNF receptor expression when evaluated following stressor exposure. Furthermore, Gareau et al. [217] showed that GF mice experience a reduction in BDNF and deficits in nonspatial and working memory after being stressed, which was mirrored in mice infected with *Citrobacter rodentium* and ameliorated upon 17 days of daily treatment with *L. rhamnosus* (R0011) and *L. helveticus* (R0052). In contrast to other GF studies, Neufeld et al. [218] found increased BDNF in the granule cell layer of dentate gyrus of the hippocampus of GF female mice, which was associated with anxiolytic behavior. Bercik et al. [219] showed that oral treatment with broad-spectrum antibiotics in nonstressed mice increased hippocampal BDNF protein expression and exploratory behavior, along with decreasing amygdala BDNF protein expression, changes that are associated with altered fear learning [220]. Notably, given that BDNF is released during and plays a critical role in the response to stressors, and given that the effects of BDNF are site-specific, a decrease in BDNF in stressed, GF mice does not necessarily contradict increased abundances of BDNF in the hippocampus of unstressed, GF or antibiotic-treated mice; there appears to be a complex interaction between microbial exposure and site-specific BDNF release in response to stress, and mechanisms have not been fully elucidated [221].

Gut mucosal infection from *Trichuris muris* was shown to increase peripheral inflammation, decrease hippocampal BDNF mRNA, and increase anxiety-like behaviors [222]. Notably, the decrease in BDNF was not attenuated by administration of anti-inflammatory agents; however, treatment with the probiotic *B. longum* NCC3001 (ATCC BAA-999) did attenuate BDNF expression and behavioral alterations without altering concentrations of proinflammatory cytokines. This suggests that BDNF expression is largely controlled by mechanisms unrelated to inflammation. Likewise, Savignac et al. [223] demonstrated that prebiotic feeding increases BDNF in central regions of the brain via gut hormones such as peptide YY in rats. Notably, the SCFA butyrate is another trigger for BDNF release, which has been shown to occur via both HDAC inhibition and decreased methylation of the *Bdnf*

gene [224, 225]. Overall, BDNF concentrations in the murine brain are altered by the microbiota through mechanisms separate from inflammatory cytokines.

5.2 *Microbiota-Immune Mediation of Neuroplasticity*

As previously discussed, microbiota alter the host immune system, which is important, as neuroplasticity is also regulated by immune mechanisms [226]. For example, in mice, low (physiological) concentrations of IL-1β are critical for long-term potentiation and memory formation, but excess IL-1β leads to impaired memory [227, 228]. Similar to the U-shaped effect of IL-1β, varying concentrations of IL-4, IL-6, and TNF appear to have differential effects on neuroplasticity under different conditions [226, 229]. Though chronically elevated IL-6 inhibits adult hippocampal neurogenesis, acute IL-6 responses are important for neuroplasticity in response to stressors, such as brain injury and ischemia in mice and gerbils [230, 231]. Likewise, TNF is involved in neurogenesis, but chronically elevated concentrations are not typically associated with increased cognitive function in animal models or humans [226, 232]. In addition to their direct effects on neurogenesis, IL-6 and TNF may play stronger roles by regulating inflammation in the CNS. For example, Cheng et al. [233] demonstrated that though chronic unpredictable mild stress decreases hippocampal BDNF and 5-hydroxytryptamine receptor 1 alpha, which is associated with increased hypothalamic IL-1β, IL-6, and TNF, along with depressive behavior, administration of Amuc_1100 (an outer membrane protein of the mucin degrader *Akkermansia muciniphila*) attenuates these changes. Amuc_1100 has been shown by Wang et al. [234] to act on TLR2, which Cheng et al. [233] postulate to be the mechanism of its effects on immune, serotonin, and BDNF signaling in the brain. Generally speaking, chronic elevation of proinflammatory cytokines in the CNS—which can be caused by microbiota-induced immunodysregulation discussed in the immune section of this chapter—is associated with decreased neuroplasticity, as the proinflammatory cytokines downregulate BDNF production in both animal models and humans [83, 235].

5.3 *Mitochondrial Health and Neuroplasticity*

It should be noted that the microbiota alters mitochondrial biogenesis, structure, and function [236–238], that mitochondria are involved in neuroplasticity, and that mitochondrial dysfunction is seen in multiple psychiatric disorders, including anxiety disorders, MDD, bipolar disorder, and PTSD as well as in rodent models designed to model endophenotypes of these conditions [239–243]. Regulation of key transcription factors for mitochondrial biogenesis by the gut microbiota (reviewed by Clark and Mach [236]) can modulate cellular differentiation in the CNS as well as axon outgrowth and synaptic plasticity. Undifferentiated human

senescent-induced pluripotent stem cells and embryonic stem cells exhibit an anaerobic state characterized by oxidative damage, low mitochondrial ATP abundance, and low mitochondrial biomass [244]. However, in these human cell lines, as the cells differentiate, mitochondrial biomass increases, and the cells shift toward a more aerobic state [244].

Increased mitochondrial mass not only supports neuron growth and cell differentiation via higher ATP concentration in the cell but also through the production of mitochondrial uncoupling protein 4, which decreases ROS production and mitochondrial calcium accumulation in rats [245]. Moreover, mitochondria are necessary for axon outgrowth. In rat hippocampal cell lines, depletion of mitochondria prevents axon growth even when ATP concentrations are maintained, suggesting an importance of mitochondrial function and mitochondrial biogenesis in neural remodeling [246].

Additionally, BDNF stimulates mitochondrial mobilization in neurons, which is crucial for synaptic plasticity and axon growth in rat hippocampal cell lines [247]. BDNF is stimulated by peroxisomal proliferator-activating receptor (PPAR) α and γ [248, 249]. Moreover, PPARs are postulated to have a role in the prevention of anxious and depressive behaviors through neuroplasticity-, mitochondria-, and inflammation-mediated mechanisms, as PPARs are major negative regulators of NF-κB expression [250, 251]. Notably, PPARγ has been identified as a therapeutic target for neurological diseases in which mitochondrial dysfunction is implicated, but much of the research to date has focused on animal models, and in humans, it has focused on other diseases [252].

Intriguingly, Loupy et al. [253] demonstrated that subcutaneous injection of *M. vaccae* NCTC 11659 in rats prevents stress-induced downregulation of PPARγ in the liver, which can potentially attenuate negative downstream impacts of stress exposure on BDNF and neuroplasticity subsequent to induction of proinflammatory cascades. Furthermore, Smith et al. [127] demonstrated that 10(*Z*)-hexadecenoic acid activates PPARα signaling in vitro, repressing the proinflammatory cascade that can prevent downstream neurogenesis and mitochondrial biogenesis. Additionally, in mice, intestinal PPAR signaling is also activated by SCFA produced by the gut microbiota, and it is upregulated upon 8 weeks of consumption of a prebiotic blend containing fructooligosaccharide, galactooligosaccharide, inulin, and anthocyanins in mice [254]. Moreover, *Lactobacillus* probiotics (8 weeks of *L. casei* Shirota in mice and 14 weeks of *L. reuteri* GMNL-263 in rats) attenuate the decreased PPAR expression seen in extremely high fructose-containing, nonalcoholic fatty liver disease-inducing diets in mice, highlighting another mechanism by which microbial exposure decreases risk of psychiatric conditions via inflammation, mitochondrial health, and neuroplasticity [255, 256]. Overall, the microbiota modulates mitochondrial structure and function via regulation of transcription factors, BDNF, and PPAR, conferring modulation of stress resilience via neuroplasticity.

6 Meningeal Immunity

Research suggests involvement of the meninges for maintenance of well-being and modulation of CNS inflammation, psychiatric diseases, and neurodegeneration. For in-depth reviews, see the studies by Kipnis and colleagues, including Norris and Kipnis [257]; Alves de Lima et al. [258]; and Kipnis [259]. Overall, the meninges contain a vast repertoire of CNS-privileged immune cells that participate in the neuroimmune response to injury as well as neurodegeneration and brain function. However, much research on meningeal immunity has focused on brain injury and neurodegenerative diseases, though some emerging research has connected meningeal immunity to social behavior in mice [260]. Thus, more research is needed on the interactions between meningeal immunity and anxiety disorders, affective disorders, and trauma- and stressor-related disorders.

7 Human Clinical Research

There is strong evidence for the impact of microbial exposure on psychiatric outcomes in human clinical studies. Many of these studies have demonstrated disrupted microbiota-brain axes (including neural and immune mechanisms), along with altered BBB integrity, brain structure, and neuroplasticity in individuals with psychiatric conditions. Additionally, they have found that microbiota-targeted interventions through modalities such as prebiotic/probiotic/postbiotic administration are feasible, tolerable, and safe, and many of these trials show that microbial exposure interventions are effective for ameliorating changes seen to the microbiota-brain pathways and for decreasing symptoms of psychiatric conditions. Studies investigating the microbiomes of persons with these disorders are outlined in Table 1.

7.1 Microbiota-Brain Signaling in Humans: Neural Signaling

Evidence suggests that interoceptive signals (including vagal and spinal afferents), to which microbes can contribute, play an important role in determining mental health outcomes in humans [274]. To note, interoceptive dysfunction is implicated in anxiety disorders; affective disorders, including MDD; and PTSD, and it is both an outcome of and a contributor to mental health conditions [274]. Additionally, the contributions of interoception to mental health conditions are not limited to painful interoception. Even non-painful interoception can contribute to behavior via vagal and spinal afferents with integration occurring in CNS regions including the autonomic ganglia, spinal cord, brainstem (including nucleus of the solitary tract, parabrachial nucleus, and periaqueductal gray), thalamus, hypothalamus, and

Table 1 Characterization of the gut microbiome in humans with generalized anxiety disorder, major depressive disorder, and posttraumatic stress disorder

Study	Participants and study design	Alpha diversity	Composition (beta diversity)	Altered taxa
Generalized anxiety disorder				
Jiang et al. [261]	$N = 76$	↓ (GAD)	Significant difference in unweighted UniFrac distance	**Phylum level**
	GAD ($n = 40$)			↑ Bacteroides
	vs.			↑ Fusobacteria
	healthy controls ($n = 36$)			↓ Firmicutes
				Genus level
				↑ *Bacteroidetes*
				↑ *Fusobacterium*
				↑ *Ruminococcus gnavus*
				↓ *Faecalibacterium*
				↓ *Eubacterium rectale*
				↓ *Sutterella*
				↓ *Lachnospira*
				↓ *Butyricicoccus*
Chen et al. [262]	$N = 60$	↓ (GAD)	Significant difference in unweighted and weighted UniFrac distances	**Phylum level**
	GAD ($n = 36$)			↑ Tenericutes
	vs.			↓ Firmicutes
	healthy controls ($n = 24$)			**Family level**
				↑ Bacteroidaceae
				↑ Enterobacteriaceae
				↑ Burkholderiaceae
				↓ Prevotellaceae
				Genus level
				↑ *Bacteroides*
				↑ *Escherichia-Shigella*
				↓ *Prevotella 9*
				↓ *Dialister*
				↓ *Subdoligranulum*
Major depressive disorder				
Mason et al. [263]	$N = 70$	No difference seen across any psychiatric conditions	No difference seen across any psychiatric conditions	↓ *Clostridium leptum* (MDD compared to healthy controls)
	Comorbid MDD + GAD ($n = 38$)			↓ Total bacterial load per gram of stool (comorbid MDD + GAD compared to healthy controls)
	vs.			
	MDD ($n = 14$)			
	vs.			
	GAD ($n = 8$)			
	vs.			
	healthy controls ($n = 10$)			

(continued)

Table 1 (continued)

Study	Participants and study design	Alpha diversity	Composition (beta diversity)	Altered taxa
Jiang et al. [264]	$N = 76$	↑ (active MDD)	No difference (both groups)	**Phylum level**
	Active MDD ($n = 29$)	No difference (responded MDD)		Active MDD:
	vs.			↑ Bacteroides
	treatment-responded MDD ($n = 17$)			↑ Proteobacteria
	vs.			↑ Fusobacteria
	healthy controls ($n = 30$)			↓ Firmicutes
				↓ Actinobacteria
				Responded MDD:
				↑ Bacteroides
				↑ Proteobacteria
				↓ Firmicutes
				↓ Fusobacteria
				↓ Actinobacteria
				Family level
				Active MDD:
				↑ Acidaminococcaceae
				↑ Enterobacteriaceae
				↑ Fusobacteriaceae
				↑ Porphyromonadaceae
				↑ Rikenellaceae
				↓ Bacteroidaceae
				↓ Erysipelotrichaceae
				↓ Lachnospiraceae
				↓ Prevotella
				↓ Ruminococcaceae
				↓ Veillonellaceae
				Responded MDD:
				↑ Acidaminococcaceae
				↑ Enterobacteriaceae
				↑ Porphyromonadaceae
				↑ Rikenellaceae
				↑ Bacteroidaceae
				↓ Ruminococcaceae
				↓ Veillonellaceae

(continued)

Table 1 (continued)

Study	Participants and study design	Alpha diversity	Composition (beta diversity)	Altered taxa
Huang et al. [265]	$N = 54$ MDD ($n = 27$) vs. healthy controls ($n = 27$)	↓ (MDD)	Difference evident from weighted UniFrac PCoA, no statistical testing performed	**Phylum level** ↓ Firmicutes
Lin et al. [266]	$N = 20$ 1 timepoint from drug-naïve MDD participants ($n = 10$) prior to receiving escitalopram followed by 2 timepoints while receiving escitalopram vs. healthy controls ($n = 10$)	Mentioned in methods, no results reported	Difference evident from weighted UniFrac PCoA, no statistical testing performed	**Phylum level** ↑ Firmicutes ↓ Bacteroides **Genus level** ↑ *Prevotella* ↑ *Klebsiella* ↑ *Streptococcus*
Aizawa et al. [267]	$N = 100$ MDD ($n = 43$) vs. healthy controls ($n = 57$)	N/A	N/A	↓ *Bifidobacterium* ↓ *Lactobacillus* (absolute cell counts, not relative abundances)
Kelly et al. [268]	$N = 77$ MDD ($n = 34$) vs. healthy controls ($n = 33$)	↓ (MDD)	Significant difference in Bray-Curtis, unweighted UniFrac, and weighted UniFrac distances	**Family level** ↑ Thermoanaerobacteraceae ↓ Prevotellaceae **Genus level** ↑ *Paraprevotella* ↓ *Prevotella* ↓ *Dialister*
Zheng et al. [269]	$N = 121$ MDD ($n = 58$; drug-naïve $n = 39$) vs. healthy controls ($n = 63$)	No difference	Difference evident from weighted and unweighted UniFrac PCoA, no statistical testing performed	**Phylum level** ↑ Actinobacteria ↓ Bacteroidetes

(continued)

Table 1 (continued)

Study	Participants and study design	Alpha diversity	Composition (beta diversity)	Altered taxa
Naseribafrouei et al. [270]	$N = 55$	No difference	N/A	**Order level**
	MDD ($n = 37$)			↓ *Bacteroidales*
	vs.			
	healthy controls ($n = 18$)			
Yang et al. [271]	$N = 311$	Bacteria	Bacteria	**Bacteria (phylum level)**
	MDD ($n = 156$)	No difference	Significant difference in bacterial Bray-Curtis distance	↑ Bacteroidetes
	vs.			↓ Firmicutes
	healthy controls ($n = 155$)	Viruses	Viruses	**Viruses**
		↓ (MDD)	No significant difference in viral Bray-Curtis distance	↑ *Escherichia* phage ECBP5
				↓ *Clostridium* phage phi8074-B1
				↓ *Klebsiella* phage vB KpnP SU552A
Posttraumatic stress disorder				
Hemmings et al. [272]	$N = 30$	No difference	No difference	**Phylum level**
	PTSD ($n = 18$)			↓ Actinobacteria
	vs.			↓ Lentisphaerae
	trauma-exposed controls ($n = 12$)			↓ Verrucomicrobia
	Population: South African citizens			
Bajaj et al. [273]	$N = 93$	↓ (PTSD)	N/A	**Family level**
	PTSD ($n = 29$)			↓ Ruminococcaceae
	vs.			↓ Lachnospiraceae
	controls ($n = 64$)			
	Population: military Veterans with cirrhosis			

Notes: Due to the likelihood of false positives, taxa identified below the family level were not included in this table if authors did not correct for multiple testing or if those taxa made up <1% of the microbiome. Studies were only included if participants were grouped based on clinical diagnosis of the psychiatric condition. If studies included multiple timepoints during treatment, results reported in this table only include those from the timepoint(s) prior to treatment. "N/A" indicates that the study did not mention assessing the outcome

GAD generalized anxiety disorder, *MDD* major depressive disorder, *PTSD* posttraumatic stress disorder

somatosensory cortex [275, 276]. There is a strong overlap of interoceptive neural integration regions with affective regions, and, importantly, interoceptive feedback may confer psychological alterations to vigilant behavior, the magnitude of reactions to stressors, and perception of stress magnitude [274]. Over time, interoceptive overstimulation leads to altered physiological stress axes with effects such as hypersecretion of cortisol, reduced sensitivity of negative feedback by GC, and a sympathetic bias of the autonomic nervous system resulting in impaired stress resilience through constant activation of "fight or flight" systems [277]. Given that microbial organisms shape the host's interactions with the "outside" in locations including the skin, nasal cavity, mouth, lungs, and gut, microbiota surely impact interoceptive stimuli, conferring potential to alter mental health outcomes through this mechanism.

A prime example of interoceptive overstimulation from a microbiota is irritable bowel syndrome (IBS). In the case of dysbiotic microbiota associated with IBS, increased sensory input from the gut mucosa alters CNS structure. Labus et al. [278] found altered volume of somatosensory brain regions in participants with IBS. Of particular interest, they demonstrated that increased volumes of the somatosensory regions evaluated were observed with higher relative abundances of Clostridia and lower relative abundances of Bacteroidia, characteristic of the subgroup of IBS participants who experienced early life trauma. Additionally, Mayer et al. [279] characterized an increased viscerosensory input to the brain and sensitization of the dorsal horn of the spinal cord as contributors to altered brain structure in IBS patients. Together, the microbial alterations associated with dysbiosis contribute to decreased gray matter volume in the insula and prefrontal cortex and to altered white matter tracts in the thalamus and basal ganglia [280, 281]. These changes to brain structure confer increased risk of neurodegeneration and chronic pain, and they are associated with both childhood and adult onset of MDD [282–284].

Additionally, spinal afferents from mucosal and cutaneous surfaces, such as the gut, lungs, and skin, regulated by local microbiota, contribute to psychiatric disorder risk. Evidence suggests [285–288] that activation of spinothalamic and spinoparabrachial pathways from the skin in humans may have antidepressant effects [282, 283, 284, 285], but these pathways have not been studied in the context of the skin microbiota and should be considered a target for future research.

Vagal afferents, which can come from multiple locations including the gut and lungs, have also been shown to modulate brain structure and psychiatric symptoms in humans. For example, Tillisch et al. [289] demonstrated that consumption of a probiotic fermented milk product modifies resting state networks, likely via vagal afferents signaling to the nucleus tractus solitarius and spinal afferents ascending to the periaqueductal gray. This resulted in alterations to brain connectivity associated with improved responses to emotional stimuli and decreased chronic pain signaling, thus conferring stress resilience and decreased symptoms associated with MDD. This is complemented by multiple clinical trials demonstrating efficacy and tolerability of transcutaneous vagal stimulation in patients with MDD, as reviewed by Kong et al. [290].

Other cranial nerves, such as the trigeminal and olfactory nerves, have additionally been shown to modulate psychiatric outcomes in humans. Notably, trigeminal nerve stimulation has been demonstrated as a potential treatment modality for reducing MDD symptoms, and olfactory nerve dysfunction is implicated in MDD [60, 291, 292]. Given that these nerves innervate the oral and nasal mucosa and can be stimulated by microbes, it follows that the oral and nasal microbiota have the potential to modulate mental health outcomes via non-vagal cranial nerves as well. However, these pathways are understudied but could be a focus of future research and could be targeted for development of novel alternative treatments for psychiatric conditions.

Emerging research suggests that metabolites from the gut microbiome can activate neural circuits in the brain in humans. For example, Osadchiy et al. [293] showed that gut microbial indole metabolites (produced from tryptophan by genera such as *Clostridium*, *Burkholderia*, *Streptomyces*, *Pseudomonas*, and *Bacillus*), including indole, indoleacetic acid, and skatole, correlate with activity and connectivity in the extended reward network of the brain in healthy humans. They were notably associated with activation of and connections in the amygdala-nucleus accumbens and amygdala-anterior insula circuits, which are known to be altered in humans with treatment-resistant depression and PTSD [294, 295]. However, research on the ability of microbiome-derived indole metabolites to act on monoamine and reward circuit signaling in the brain is sparse, especially in the context of psychiatric disorders in humans, and this merits future research.

7.2 Microbiota-Brain Signaling in Humans: Immune-Brain Interactions

Similar to microbiota-neural signaling, microbiota-immune system signaling has also been shown to be involved in modulating brain structure and neuropsychiatric outcomes in humans. Again, immune modulation has been shown to be a contributor to development of the psychiatric conditions, an outcome of stressors, and a potential therapeutic for psychiatric conditions.

7.2.1 Cytokines

It has been demonstrated (and extensively covered in the preclinical section of this chapter) that microbial exposure can alter circulating cytokine concentrations. Modulation of cytokines is particularly notable, as circulating concentrations of proinflammatory cytokines are elevated in anxiety disorders, affective disorders, and PTSD in humans. Hou et al. [296] found that individuals with generalized anxiety disorder (GAD) have elevated serum concentrations of proinflammatory cytokines TNF and IFN-γ, as well as decreased IL-10. Additionally, Hou et al.

[193, 194] found that treatment with SSRIs lowers serum CRP, IL-1α, IL-6, IL-8, IL-12, and IFN-γ and that elevated baseline CRP and IL-6 are positive predictors of SSRI treatment responsivity.

Moreover, cytokine concentrations are disrupted in MDD. Zou et al. [297] found that antidepressant drug-naïve individuals with MDD have elevated serum IL-1β, IL-10, and TNF compared to nondepressed individuals and that IL-1β and TNF abundances positively correlate with the severity of depressive symptoms. Alesci et al. [298] found disruption of the circadian rhythm of plasma IL-6 in MDD patients. Furthermore, a genetic link can be drawn between proinflammatory cytokines and psychiatric outcomes, as polymorphisms of the IL-1β gene are associated with symptomatology and responsiveness to antidepressant treatment [299]. Additionally, immune reactivity is attenuated during MDD treatment, as Kéri et al. [300] found that decreasing symptoms during cognitive behavioral therapy were associated with decreased TLR4-dependent priming of peripheral blood mononuclear cells in depressed patients. On a predictive level, elevated serum concentrations of proinflammatory cytokines including IL-6 and CRP are predictive of development of depressive or common mental health symptoms over the course of 12 years in adults [301, 302] and over the course of 9 years (from age 9 to 18) in children [303, 304].

Individuals with PTSD also exhibit elevated concentrations of proinflammatory cytokines. Wang et al. [305] found that individuals with PTSD from a deadly earthquake event have elevated serum IL-1β and TNF concentrations, along with elevated total proinflammatory cytokine scores (based on serum concentrations of IL-1β, IL-2, IL-6, IL-8, IFN-γ, and TNF). Likewise, Lindqvist et al. [306] found that the proinflammatory cytokine milieu (including IFN-γ, TNF, and the sum of IL-1β, IL-6, CRP, IFN-γ, and TNF concentrations) is elevated in individuals with combat-related PTSD independent of depression symptoms and early life stress. Moreover, Gola et al. [307] demonstrated that ex vivo cultured peripheral blood mononuclear cells (PBMCs) of study participants with PTSD had increased spontaneous production of IL-1β, IL-6, and TNF.

Altered cytokine concentrations have been shown to change neurotransmitter activity in the brain, conferring behavioral deficits and impaired neuroplasticity. Magnetic resonance spectroscopy showed increased glutamate in the basal ganglia and dorsal anterior cingulate cortex (dACC) in individuals receiving IFN-α treatment and in depressed individuals; however, these changes are not conserved across all depressed individuals, likely due to the heterogeneity of the diagnosis [308–310]. Altered glutamate signaling in individuals diagnosed with MDD is well supported by preclinical studies and is thought to contribute to excitotoxicity and decreased BDNF, impairing neuroplasticity and neurogenesis [76]. Additionally, concentrations of plasma proinflammatory cytokines can be predictive of PTSD development. Prime examples include Schultebraucks et al. [75] demonstrating that blood CRP concentration prior to military deployment is one of the top predictors of PTSD development following deployment and Pervanidou et al. [311] demonstrating that elevated serum IL-6 concentrations the morning following a motor vehicle accident are predictive of PTSD development 6 months later. Overall, it is evident

that cytokine concentrations are altered in anxiety disorders, MDD, and PTSD, that proinflammatory cytokines can alter neural signaling, that higher concentrations of proinflammatory cytokines subside during treatment for anxiety disorders and MDD, and that levels of proinflammatory cytokines are predictive of the development of psychiatric symptoms and disorders.

7.2.2 Leukocyte Populations

Similar to cytokine concentrations, populations of circulating leukocytes can be altered by microbial exposure in humans, as has been thoroughly characterized through the study of the "farm effect," reviewed by Vercelli and colleagues [312, 313]. As can be seen in the meta-analysis by Segerstrom and Miller [314], acute psychological stressors additionally induce a plethora of changes to immune cell populations in humans. Many of these changes, such as increased neutrophils, natural killer cells (along with increased natural killer cell function), and large granular lymphocytes and T helper cells as a percentage of leukocytes, correlate with the duration of the acute stressor [314].

To complement knowledge of the impacts of stressors on leukocyte populations in humans and the associations between leukocyte populations and anxious behaviors in stressed animal models, military Veterans with anxiety (according to DSM-III criteria) have elevated lymphocyte and T cell counts [315]. Additionally, individuals with panic disorder have increased abundances of natural killer cells, B lymphocytes, human leukocyte antigen DR isotype-presenting cells, and B lymphocytes presenting human leukocyte antigen DR surface markers [316]. Leukocyte populations are also altered in individuals with MDD. Ekinci and Ekinci [317] found an elevated neutrophil to lymphocyte ratio in depressed individuals who had attempted suicide, compared to healthy controls. Likewise, Schleifer et al. [318] found a decreased number of lymphocytes, along with decreased reactivity of the lymphocytes, in hospitalized depressed individuals.

Schultebraucks et al. [75] demonstrated that plasma basophil (referred to as large granular lymphocytes by Segerstrom and Miller) and monocyte abundances prior to military deployment to Afghanistan are predictive of PTSD development. Additionally, Schultebraucks et al. [75] also found that eicosanoids, which promote neutrophil stimulation and chemotaxis, are significant predictors of PTSD development. Human research, however, has yet to establish a causal link between leukocyte populations and altered risk for the development of PTSD. Altered leukocyte populations could co-occur with a past history of psychological trauma and stressors (as demonstrated by Segerstrom and Miller [314]) modifying neural circuitry independent of the immune system. Due to the lack of human studies that assess the ability of immunoregulatory interventions to prevent development of PTSD in traumatized or stressed individuals, we must rely partially on preclinical research for our knowledge in this area. Given the alterations in leukocyte populations following acute stress and the ability of leukocyte abundances to predict PTSD development, along with the preclinical evidence that immunoregulation via

microbial exposure attenuates immune responses to stress and the resulting impaired stress resilience, it should be noted that leukocyte populations have potential to play a strong role in modulating neuropsychiatric outcomes. Future research should investigate the long-term effects of immunoregulatory interventions, such as probiotic trials or nature exposure, in preventing the development of psychiatric disorders.

7.2.3 Brain Barriers and Leukocyte Trafficking

The CP, a key point of cellular trafficking into the CNS, has been demonstrated to be disrupted in individuals with psychiatric disorders. This is relevant because, as discussed above, a proinflammatory state of the immune system has been shown to alter BCSFB integrity at the CP in preclinical models. Such proinflammatory immune states (such as an increased population of Th17 cells that impairs BCSFB integrity, encouraging lymphocyte trafficking to the brain) can be modulated by microbial exposure. Additionally, in individuals with poor BCSFB integrity, microbiota with low diversity and therefore low resistance to pathogen overgrowth could allow pathogen translocation across epithelial barriers (e.g., the gut mucosa) and invasion of the CNS, triggering neuroinflammation that impairs stress resilience.

Turner et al. [319] demonstrated a downregulation in mRNA transcripts related to cytoskeleton and extracellular matrix maintenance in the CP of individuals with MDD postmortem, suggesting impaired BCSFB function. Lizano et al. [320] found consistent enlargement of the CP across a spectrum of psychiatric illnesses. These changes to the CP are complemented by the findings of Schiweck et al. [321], who found elevated T helper cells, particularly Th17, in peripheral blood mononuclear cell suspensions from individuals with MDD and high suicide risk, demonstrating the Th17 bias that has been shown to drive BCSFB permeability in preclinical studies.

Additionally, BBB dysfunction is associated with increased risk for affective and stress-related disorders in humans [136, 137]. Reviewed by Patel and Frey [322], BBB disruption is implicated in multiple clinical studies of psychiatric conditions, and many metabolites altered by the microbiota can modulate BBB integrity.

Although preclinical studies have firmly established that disruptions to the BCSFB and BBB integrity allow leukocytes and cytokines to traffic into the CNS, this has not been studied in vivo in humans. Though the disruptions are associated with affective and stress-related disorders, no human interventions have investigated modulation of the BCSFB/BBB to decrease symptoms or risk of anxiety disorders, MDD, or PTSD. In fact, to date, no human trials have investigated microbiota-targeted interventions for improving the integrity of the BCSFB and BBB to modulate mental health outcomes.

Microbiota-mediated modulation of vitamin D, which exerts protective effects on the BBB in preclinical models, could prove a promising intervention in humans. Circulating vitamin D concentration is associated with gut microbiota composition and has been clearly demonstrated to affect microbiome composition via vitamin D receptors in the human gut [323, 324]. Interestingly, 9 weeks of daily *L. reuteri*

NCIMB 30242 supplementation increased circulating vitamin D concentrations, indicating a bidirectional relationship between the microbiome and vitamin D [325]. However, the effects of increased vitamin D concentration from probiotic supplementation on the BBB with implications for mental health outcomes have not been studied. Overall, studies investigating the microbiota-BBB axis as a modulator of mental health in humans are sparse and could be a direction for future research.

7.2.4 Probiotic Interventions

To date, many probiotic trials have been carried out to assess impacts on psychiatric outcomes, often involving strains of *Lactobacillus* or *Bifidobacterium* [326]. Based on the meta-analysis by Amirani et al. [326], these studies typically show decreased depressive symptoms and decreased markers of systemic inflammation, such as CRP. Additionally, probiotics have been shown to modulate immune activity and chemotaxis proteins in humans; a randomized control trial of 12 weeks of supplementation with *L. rhamnosus* strain GG and *Bifidobacterium animalis* subsp. *lactis* strain Bb12 found decreased acute-phase reactant protein von Willebrand factor (vWF) and increased abundances of monocyte chemotactic protein-1 (MCP-1; also known as CCL2) and BDNF, suggesting immunomodulatory properties [327].

Of note, immunomodulatory probiotic trials targeting stress resilience have been repeatedly shown to be safe, feasible, and tolerable. Probiotic supplementation with *L. reuteri* DSM 17938, a gut microbe capable of CLA biosynthesis, was demonstrated to be a safe, feasible, and potentially effective intervention for military Veterans with co-occurring PTSD and mild traumatic brain injury [328, 329]. Results from the pilot study by Brenner et al. [328] showed a trend for decreased CRP, along with attenuated autonomic nervous system responses to the Trier Social Stress Test after 8 weeks of supplementation with *L. reuteri* DSM 17938. Moreover, Browne et al. [330] showed that 4 weeks of daily supplementation with a multispecies probiotic with multiple immunomodulatory taxa (*B. bifidum* W23, *Bifidobacterium lactis* W51, *B. lactis* W52, *Lactobacillus acidophilus* W7, *Lactobacillus brevis* W63, *L. casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19, and *L. lactis* W58) geared toward reducing maternal anxiety symptoms was safe and tolerable in pregnant women. Wallace and Milev [331] also demonstrated that 8 weeks of daily supplementation with *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 in treatment-naïve individuals with MDD was safe, tolerable, and able to improve affective symptoms. However, most randomized, controlled clinical trials targeting affective and stress-related conditions in human participants to date (including the three above) have only been pilot studies. Though there will be more pilot studies in the future to determine the safety of new probiotics in humans, other studies need to build off of existing pilot studies to create clinical guidelines for microbe-based interventions in humans with psychiatric disorders. Table 2 outlines the outcomes of probiotic interventions in individuals with GAD, MDD, and PTSD.

Table 2 Outcomes of probiotic interventions for individuals with clinically diagnosed generalized anxiety disorder, major depressive disorder, and posttraumatic stress disorder

Study (design)	Participants	Probiotic and methods	Major outcomes assessed	Findings	Limitations
Generalized anxiety disorder					
Eskandarzadeh et al. [332]	48 antidepressant- and anxiolytic-free participants diagnosed with GAD	8 weeks of daily:	Anxiety severity	↓ Anxiety severity (Hamilton Rating Scale for Anxiety)	Probiotic strain designations are not given
(double-blinded, randomized, placebo-controlled)	Probiotic $n = 24$	*Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*	Quality of life	No significant decrease in Beck Anxiety Inventory	Participants were drug-free but not drug-naïve
	Placebo $n = 24$	Both groups received 25 mg sertraline daily		↓ State Anxiety Inventory	
				No significant decrease in Trait Anxiety Inventory	
				No significant change in quality of life	
Major depressive disorder					
Romijn et al. [333]	79 antidepressant-free participants scoring ≥11 on the Quick Inventory of Depressive Symptomatology or ≥14 on the depression scale of the Depression, Anxiety, and Stress Scale, characteristic of MDD	8 weeks of daily:	Mood, stress, and anxiety symptoms	No difference found between probiotic and placebo groups for any psychological outcomes or blood-based biomarkers	Participants were drug-free but not drug-naïve
(double-blinded,	Probiotic $n = 40$	*Lactobacillus helveticus* R0052	Irritable bowel syndrome symptoms	High baseline vitamin D was significantly	

randomized, placebo-controlled)				associated with greater improvement in self- and clinician-reported mood and functioning in the probiotic group but not the placebo group	
	Placebo $n = 39$	*Bifidobacterium longum* R0175	Blood concentration of proinflammatory cytokines and brain-derived neurotropic factor		
Rudzki et al. [334]	79 participants with clinically diagnosed DSM-IV MDD	8 weeks of twice daily:	Severity of depressive symptoms	↑ cognitive function (attention and perceptivity test, California Verbal Learning Test)	Some participants were taking SSRIs prior to the start of the study, though they were evenly distributed between groups
(double-blinded, randomized, placebo-controlled)	Probiotic $n = 40$	*Lactobacillus plantarum 299v* (DSM 9843)	Cognitive function	↓ serum kynurenine	Not all participants received the same SSRIs, and authors did not identify reasons for the differing SSRIs
	Placebo $n = 39$	Both groups received daily selective serotonin reuptake inhibitors (SSRIs) to be taken twice daily with the probiotic/placebo. Most participants received the SSRI escitalopram, and there were no significant differences in SSRI type during treatment	Blood concentration of biochemical markers of tryptophan metabolism and proinflammatory cytokines	↑ serum 3-hydroxykynurenine to kynurenine ratio	
				No significant changes in severity of depressive symptoms or proinflammatory cytokines	
Akkasheh et al. [335]	40 participants with clinically diagnosed DSM-IV MDD	8 weeks of daily:	Severity of depressive symptoms	↓ Beck Depression Inventory total scores	Probiotic strain designations are not given
(double-blinded, randomized, placebo-controlled)	Probiotic $n = 20$	*Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*	Serum metabolome, C-reactive protein, and markers of oxidative stress	↓ serum insulin and markers of insulin resistance	
	Placebo $n = 20$			↓ serum C-reactive protein	

(continued)

Table 2 (continued)

Study (design)	Participants	Probiotic and methods	Major outcomes assessed	Findings	Limitations
				No significant change in serum lipid profiles or serum antioxidant capacity	
Kazemi et al. [336]	81 participants with MDD	8 weeks of daily:	Severity of depressive symptoms	↓ Beck Depression Inventory total scores (probiotic vs. placebo; probiotic vs. prebiotic)	Authors do not provide justification for adjusting for isoleucine concentration, and the difference in kynurenine to tryptophan ratio was not significant when not adjusting
(three-arm, double-blinded, randomized, placebo-controlled)	Probiotic $n = 28$	*Lactobacillus helveticus* R0052, *Bifidobacterium longum* R0175 (CNCM strain I-3470)	Serum tryptophan, kynurenine, and branch chain amino acids	↓ kynurenine to tryptophan ratio when adjusting for serum isoleucine concentration (probiotic vs. placebo)	
	Prebiotic $n = 27$	*or*		↑ tryptophan to isoleucine ratio (probiotic vs. placebo)	
	Placebo $n = 26$	8 weeks of daily:			
		5 grams galactooligosaccharide			
Miyaoka et al. [337]	40 participants with treatment-resistant MDD who had already been taking antidepressants for at least 1 month	8 weeks of daily:	Severity of depressive symptoms	↓ depressive symptoms (Beck Depression Inventory, Hamilton Depression Rating Scale)	Study was open-label. Treatment was determined to be safe and tolerable
(open-label, randomized, placebo-controlled)	Probiotic $n = 20$	*Clostridium butyricum* MIYAIRI 588 (CBM588)	Severity of anxiety symptoms	↓ anxiety symptoms (Beck Anxiety Inventory)	
	Placebo $n = 20$		Safety and tolerability of intervention	70% of participants responded to probiotic supplementation treatment	

Reininghaus et al. [338]	61 participants receiving inpatient care for MDD	4 weeks of daily:	Depressive symptom severity	No significant difference in depressive symptoms between groups, though both groups improved over time	Authors did not assess microbiome beta diversity from timepoint 0 to timepoints 1 and 2 within the probiotic group. Thus, it cannot be determined whether the differences between groups at 1 and 4 weeks are due to the probiotic group changing or the placebo group changing, or to both groups changing
(double-blinded, randomized, placebo-controlled)	Probiotic $n = 28$	*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W22, *Lactobacillus casei* W56, *Lactobacillus paracasei* W20, *Lactobacillus plantarum* W62, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W29	Intestinal barrier function, assessed via plasma zonulin concentration	No significant change in plasma zonulin concentration	
	Placebo $n = 33$	+<30 mg (exact quantity not specified) fructooligosaccharide	Gut microbiome diversity and composition	No significant change in alpha diversity	
		Both groups received daily vitamin B7 (biotin)		Significant difference in beta diversity between probiotic and placebo after 1 and 4 weeks but no significant difference prior to the start of the study	

(continued)

Table 2 (continued)

Study (design)	Participants	Probiotic and methods	Major outcomes assessed	Findings	Limitations
Posttraumatic stress disorder					
Brenner et al. [328]	31 US military Veterans with comorbid PTSD and persistent post-concussive (PPC) symptoms from mild traumatic brain injuries	8 weeks of daily: *Lactobacillus reuteri* DSM 17938	Reactivity to the Trier Social Stress Test (TSST)	No significant difference in perceived stress during the TSST	Study did not assess changes to PPC symptoms or PTSD symptoms with the probiotic intervention
(double-blinded, randomized, placebo-controlled)	Probiotic $n = 16$		Plasma concentration of C-reactive protein, biomarkers of inflammation, and biomarkers of intestinal permeability	↓ in TSST-induced increase in heart rate	
	Placebo $n = 15$		Gut microbiome diversity and composition	↓ C-reactive protein (approaching significance)	
			Feasibility, safety, and acceptability of intervention	No significant difference in biomarkers of inflammation or intestinal permeability	
				No significant differences in microbiome diversity or composition	
				Treatment was determined to be feasible, safe, and acceptable	

GAD generalized anxiety disorder, *MDD* major depressive disorder, *DSM-IV* Diagnostic and Statistical Manual of Mental Disorders 4th edition, *PPC* persistent post-concussive, *PTSD* posttraumatic stress disorder, *TSST* Trier Social Stress Test

7.3 *Neurogenesis and Mitochondrial Function in Humans*

As discussed in Sect. 5 of this chapter, microbes can modulate many host signaling molecules involved in neuroplasticity, such as cytokines, BDNF, and PPARs. This is particularly relevant for psychiatric disorders in humans, as decreased hippocampal neuroplasticity (typically evaluated under the assumption that increases in hippocampal volume indicate increases in neurogenesis) is generally associated with anxiety disorders, MDD, and PTSD [339, 340]. Particularly, though decreased neurogenesis does not have an incredibly strong relationship with the development of psychiatric disorders, it is strongly associated with the maintenance of psychiatric disorders in humans [340–342]. To elaborate, the ability to increase hippocampal volume via neurogenesis appears important in recovery from MDD and PTSD, as individuals who recover experience increases in hippocampal volume compared to those who do not recover.

Mitochondrial dysfunction, which can be modulated by the microbiota, is also seen in multiple psychiatric disorders, including anxiety disorders, MDD, bipolar disorder, and PTSD [239, 241–243]. Moreover, Shapira-Lichter et al. [343] demonstrated that IL-6 is responsible for changes in mood and memory following surgery, suggesting that cytokines, which are altered by microbial exposure, play roles in mood and memory formation.

However, few studies have investigated the impacts of probiotics on neurogenesis and brain mitochondrial function in humans in the context of stress-related psychiatric disorders. One randomized, double-blinded clinical trial by Haghighat et al. [344] did show that 12 weeks of consumption of a synbiotic (prebiotic/probiotic blend) containing fructooligosaccharides, galactooligosaccharides, inulin, *L. acidophilus* T16, *B. bifidum* BIA-6, *B. lactis* BIA-7, and *B. longum* BIA-8 decreased anxiety and depressive symptoms and increased BDNF in a subgroup of individuals with MDD, but further human research is lacking. Overall, neurogenesis and mitochondrial function have been found to be impaired in individuals with stress-related psychiatric disorders, but human research on microbiota modulation of these issues is sparse, and more research is highly warranted.

8 The Case for Non-probiotic Interventions Under the Old Friends and Biodiversity Hypotheses

Moving toward a larger-scale focus, Lowry et al. [7] outlined a case for reduced microbial exposure and environmental microbial diversity across modernized societies contributing to the increased global mental health burden via impaired immunoregulation. Throughout history, mammals have been in close contact with dirt and mud (and thus the microbes contained in them), but urban "concrete jungles" are far from ideal for growth of the microbes with which we evolved, such as environmental mycobacteria [345]. However, soil bacteria aren't the only important microbes.

Commensal microbes with immunoregulatory properties, such as *B. infantis*, *B. longus*, and *B. brevis*, have decreased in both urban and wealthy populations due to behavioral and dietary changes including cesarean section delivery, early-life antibiotic use, and increased formula feeding, resulting in increased immune-mediated inflammatory diseases [346–348]. Though probiotic-driven recolonization with these bacteria is possible under the proper conditions, such as colonization of *B. infantis* EVC001 probiotic colonization during breastfeeding as demonstrated by O'Brien et al. [349], most probiotics do not colonize and should not be treated as a permanent way to reintroduce bacteria [350]. This is not to say that probiotics are useless—in fact, as previously outlined, they have demonstrated therapeutic potential in psychiatric disorders. It is important to note, though, that they do not serve a permanent role replacing the taxa that have been lost due to urbanization.

Additionally, it should be noted that supplementation with one or even multiple microbes will not "solve" every host's dysbiotic microbiota and improve host health. Consistent with the Anna Karenina principle, which states that while "happy families are all alike," in this case, perhaps through high diversity and functional redundancy, "every unhappy family is unhappy in its own way," there are many ways that microbial community health can fall apart, resulting in immune dysregulation and impaired stress resilience [351, 352]. Unfortunately, the multitude of microbiota changes seen in disease and the difficulty of predicting community changes from interventions make individualized microbiome-targeted approaches largely prohibitive at present [353]. Thus, perhaps the best microbial approach to decrease immune-mediated psychiatric disorders is not through probiotic cocktails but through using environmental and lifestyle interventions to improve key components of microbiota stability, functional diversity, and functional redundancy [354]. Functional diversity (having microbes that perform many functions) and redundancy (multiple taxa perform the same function) make communities resilient to perturbations that would otherwise lead to disruption [354]. Establishing functional diversity and redundancy at an early age through increasing environmental microbe exposure, social interaction, and dietary diversity could create long-lasting benefits to microbiome stability with conferred enhancement of psychosocial stress resilience [355]. Interestingly, Bastiaanssen et al. [356] demonstrated that microbiome volatility, quantified as the magnitude of changes in community composition (beta diversity) over multiple sampling timepoints within the same individual, is associated with stress and altered behavior in both humans and murine models. Ten days of chronic social defeat stress increased microbiome volatility in mice, and in humans, microbiome volatility correlated with perceived stress. Though no causal link has yet been identified for microbiome volatility impairing psychosocial stress resilience, it could be hypothesized that stress-induced volatility puts microbiota at risk of community disruption, increasing likelihood of pathogen colonization or pathobiont overgrowth, such as the increase in *Helicobacter* spp. caused by the CSC paradigm in mice [96]. These changes could confer immunodysregulation, whereas increased community resilience (through functional diversity and redundancy) has the ability to prevent this disruption.

Over time, individuals' microbiota homogenize with the built environment and undergo microbial transfer via social interaction, emphasizing the importance of diverse environmental microbes and socialization [357, 358]. Additionally, nature exposure can play an important role in microbe-mediated immunoregulation, as Roslund et al. [359] demonstrated that transferring forest floor soil to a preschool playground increased microbiome diversity, Treg, and plasma IL-10:IL-17A ratios (see chapter on "Distortion of the Microbiota of the Natural Environment by Human Activities" in this volume). Moreover, diversity of plants in the diet is associated with gut microbiome richness and composition, as demonstrated by the American Gut Project [360]. Approaches to increase microbial diversity, and therefore stability, can result in sustained diverse microbial exposure and prevent pathogen-mediated immune disruption.

9 Nutritional Psychiatry

Nutritional psychiatry, or the field of modulating psychiatric disorder symptoms through dietary changes, is accumulating evidence supporting its use in clinical settings. Evidence supporting the link between diet and mental health has been found in both epidemiological studies and clinical interventions. Due to the ability of diet to modulate the gut microbiota and the ability of the gut microbiota to modulate mental health symptoms, the diet-mental health link may be at least partially mediated by the microbiome.

9.1 Epidemiological Data Link Poor Diet Quality to Poor Mental Health

Epidemiological studies have shown associations of anxiety and depression with proinflammatory diets, such as diets high in added sugar and saturated fats, and some evidence suggests associations between diet quality and PTSD. Masana et al. [361] demonstrated an association between high consumption of saturated fats and added sugars and anxiety symptoms in adults over 50 years of age with no underlying cardiovascular or chronic diseases. Likewise, Jacka et al. [362] found that low diet quality (constructed from dietary quantities of fried foods, refined grains, sugary products, and beer) was associated with low psychological well-being based on General Health Questionnaire-12 scores across 20- to 93-year-old women. Additionally, Jacka et al. [362] found that consumption of more traditional diets characterized by high consumption of fruits, vegetables, meats, fish, and whole grains was associated with lower rates of anxiety and depression. Westover and Marangell [363] found a strong and significant cross-nation correlation between kilocalories of sugar consumption per capita per day and annual rates of MDD (with a Pearson

correlation of 0.948). Moreover, the meta-analysis by Psaltopoulou et al. [364] showed an association between adherence to a Mediterranean diet (high in fruits, vegetables, whole grains, nuts, and unsaturated fatty acids) and lower risk of depression. High adherence to the Mediterranean diet touted a strong association with lower depression risk independent of age, but moderate adherence was associated with a decreased depression risk that was slightly attenuated as age increased. This is also supported by the systematic review and meta-analysis by Lassale et al. [365], which showed that Mediterranean diet adherence was associated with decreased risk of depression across four longitudinal studies, that a low Dietary Inflammatory Index was associated with decreased risk of depression across four longitudinal studies, and that higher Healthy Eating Index and Alternative Healthy Eating Index scores were associated with lower risk of depression. Additionally, a systematic review demonstrated an association between PTSD and lower diet quality, where individuals with PTSD were more likely to have low diet quality than individuals without PTSD [366].

However, epidemiological studies do have limitations and cannot be used to establish causality in the development of psychiatric disorders. This is highlighted by Kim et al. [367] demonstrating that in 51,965 female participants in the Nurses' Health Study II PTSD sub-study, after the onset of PTSD, participants had a lower improvement in dietary quality over a 20-year follow-up period compared to participants without PTSD symptoms. These changes to diet quality occurred after the onset of PTSD, suggesting that behavioral changes from PTSD symptoms, which overlap with the symptoms of GAD and MDD, may be impacting diet quality.

9.2 Whole Dietary Interventions Alter the Microbiome and Decrease Depressive Symptoms

Whole dietary patterns are associated with microbiome composition, which can impact risk for anxiety, depression, and PTSD through mechanisms previously outlined. Whole dietary interventions can improve symptoms of MDD as well. However, little clinical research has shown an effect of whole dietary interventions on anxiety, and few studies have evaluated the effects of whole dietary interventions on PTSD.

Cotillard et al. [368] found that unsupervised clustering of dietary data revealed wide-scale dietary patterns associated with large differences in microbiome composition, highlighting that dietary patterns may be more relevant for microbiome composition than quantities of individual foods in the diet. For example, following a flexitarian diet (with a flexible dietary pattern rich in various plants and occasionally including animal products) rather than a standard Western diet (with high consumption of processed and fried foods along with saturated fats and added sugars, with low diversity of plants consumed) may have larger effects on microbiome composition than meeting certain quantities of fiber. Likewise, Johnson

et al. [369] found that dietary choices, but not the individual quantities of conventional nutrients, were associated with microbiome composition. Additionally, the American Gut Project identified that individuals who consume more than 30 species of plants per week have altered gut microbiome composition, along with increased microbiome diversity and CLA (independent of estimated dietary CLA consumption) compared to those who consume less than 10 unique plant species per week, highlighting the importance of diversity in dietary patterns [360]. Across multiple other studies, habitual diet and vegetable intake is associated with gut microbiome composition, which was found to mediate changes to host leukocyte profiles [370–372] Thus, whole dietary interventions instead of specific nutrient-based interventions may be an effective microbiome-mediated means of improving mental health symptoms.

To complement the existing research on whole dietary patterns associating with microbiome composition, a randomized controlled trial of a 1-year Mediterranean diet intervention in individuals 65–79 years of age across multiple nations resulted in modified gut microbiome composition and metabolites such as SCFAs, and it decreased serum concentrations of CRP and IL-17 [373]. On a shorter scale, David et al. [374] demonstrated that just 4 days of whole dietary interventions (plant-based diets or animal-based diets) rapidly and reproducibly altered the gut microbiome, including its gene expression and production of metabolites such as SCFAs and the secondary bile acid deoxycholic acid.

In a randomized controlled trial of young adults with depressive symptoms and poor diet quality, Francis et al. [375] demonstrated that just a 3-week wide-scale dietary intervention developed by a dietitian (to encourage adherence to a Mediterranean-style diet; increase dietary consumption of anti-inflammatory dietary components such as omega-3 fatty acids, turmeric, and cinnamon; and to decrease consumption of refined carbohydrates, processed meats, and soft drinks) decreased self-reported depression symptoms compared to controls who did not receive the intervention. Notably, the decrease in self-reported depression symptoms was maintained 3 months after the intervention ended, suggesting long-term effects of short-term, whole dietary interventions. Moreover, the meta-analysis of 16 whole dietary interventions by Firth et al. [376] found that dietary interventions reduce depressive symptoms even in individuals who are not clinically depressed. These effects were conserved across studies that used active and inactive controls, and females tended to experience stronger improvements in depressive symptoms. However, no studies to date have evaluated microbiome changes that are associated with improved depressive symptoms during whole dietary interventions, which should be considered an important objective for future research.

The meta-analysis by Firth et al. [376] additionally investigated symptoms of anxiety, and they concluded that there was no significant effect across 11 studies, potentially due to the heterogeneity of studies and dietary interventions. No studies to date have evaluated the effects of whole dietary interventions on PTSD symptoms, suggesting a target for future research.

Overall, dietary patterns alter the microbiome and decrease symptoms of depression, but existing evidence does not support their efficacy for reducing symptoms of

anxiety and PTSD. Aside from any potential effects on the microbiome-gut-brain axis, at the very least, dietary interventions should be investigated for their ability to improve quality of life via decreasing the risk of chronic diseases such as cardiovascular disease in individuals with psychiatric conditions, given that individuals with psychiatric disorders have poorer diet quality than individuals without psychiatric disorders.

10 Clinical Implications and Conclusions

There is a plethora of ways that microbial exposures impact mental health, including (but not limited to) modulation of the gut mucosa, direct activation of neural afferents, and modulation of immune signaling, metabolic signaling, blood-brain barrier integrity, leukocyte trafficking, cytokine production, neuroplasticity, and neural circuits. These effects can be induced by host exposure to live microorganisms, dead microorganisms, and even metabolites of microorganisms at sites such as the lungs, mouth, nasal cavity, skin, and digestive tract. The list of mechanisms linking microbial exposures and neuropsychiatric outcomes is vast, and many studies to date have demonstrated portions of these mechanisms. The variety of mechanisms is complemented by a large number of studies showing altered microbiome-host pathways in individuals with mental health conditions.

Despite the strong promise of this field, due to study limitations evidence does not currently allow many of these mechanistic pathways to be traced from the point of microbial exposure to behavioral outcomes. Thus, an emphasis on study design that will inform mechanisms involved is an important objective for future studies. Given that the relationship between the microbiota and CNS is bidirectional, researchers should be cautious about implying causality, and it is essential that they design studies with the ability to assess vertical mechanisms from microbes to behavior, rather than a horizontal approach of identifying all altered microbes associated with a disease or all altered host metabolites associated with microbiome disruption.

Moreover, given the high dimensionality, multicollinearity, and compositionality of microbiome data, researchers should be cautious performing and interpreting single taxa hypothesis tests and differential abundance tests, which are often built upon unverifiable assumptions [377–379]. With these limitations, bias toward positive result reporting, and the speed at which microbiome data are being generated, it is likely that microbiota-gut-brain axis research is destined for a similar fate as nutritional epidemiology, where almost every identified food is associated with strong alterations to cancer risk in single studies but effect sizes shrink in meta-analyses [380]. Ratio-based biomarker use (see [379]) is a promising method, but researchers should also develop a thorough understanding (or work with researchers with a thorough understanding) of ecological theory to assess subcommunities and networks of microbes. That being said, network analyses based solely on co-occurrence pose their own issues, as spurious correlations do not imply relationships between microbes. Thus, multi-omics approaches using tools that incorporate

previous mechanistic knowledge, such as those outlined in Vehlow et al. [381], should be used to increase the number of lines of evidence supporting network-based analyses. Additionally, artificial gastric digestive systems are being developed and provide a means for larger system microbiome study in vivo. As this synthetic microbiome research (i.e., in vitro research utilizing artificial digestive systems, for review see Mabwi et al. [382]) improves, investigating direct relationships between previously unculturable microbes will improve our working knowledge of microbial community ecology. Finally, low-hanging fruit for improving study design includes working with biostatisticians to determine adequate sample size for clinical trials (despite challenges associated with conducting human subject research) and proper negative and positive controls.

Notably, many studies, including Wallace and Milev [331], Brenner et al. [328], and Browne et al. [330], have demonstrated that probiotic interventions targeting psychiatric outcomes are safe, feasible, and acceptable. Additionally, meta-analyses have concluded that randomized controlled trials of pre- and probiotic interventions targeting psychiatric outcomes are generally effective to a degree in patients both with and without psychiatric conditions [383–388]. However, results are inconsistent, likely due to low power, a lack of standardized methodology, and inconsistent reporting across studies to such a degree that some meta-analyses have concluded no effect from probiotic interventions [384, 389]. Some incongruencies include differential effects of probiotics in healthy individuals versus individuals with a diagnosis of anxiety disorder, affective disorder, or trauma- and stressor-related disorder, the ability of studies to improve mental health scores without crossing clinical cutoffs for diagnosis, and the presence of comorbid conditions (such as IBS) in participants [387, 388]. Though a great body of research supports their use, the field is not yet at a point where clinical protocols can be outlined for pre-/probiotic interventions targeting psychiatric outcomes.

Additionally, though probiotics increase exposure to specific microbes that have been identified as beneficial, they may not influence all factors that contribute to healthy microbiota. Alternative options such as nature exposure and dietary interventions increase microbiome diversity, alter microbiome composition, and are safe and feasible [359, 360, 373, 390]. Moreover, these changes are associated with increased microbiome stability and immunoregulation, both of which confer stress resilience. Furthermore, there are costs (money and time) associated with individual dietary and probiotic interventions. These costs pose barriers for marginalized groups, furthering public health disparities in underrepresented communities where trauma often already runs rampant [391]. Thus, environmental interventions should be framed as a necessary approach to improving public mental health and stress resilience; they would particularly benefit historically oppressed communities, who already experience higher rates of psychiatric conditions, immune dysregulation, and impaired microbial exposure [7, 391]. On a wide scale, public health interventions aimed at increasing exposure to nature and its abundance of microorganisms serve a role for improving population-wide stress resilience that consumer-available probiotics cannot fill.

The unseen cost of paving paradise is that modern housing, sanitization, and work environments have alienated a large portion of the population from the native biodiversity of microorganisms with which they would have historically had symbiotic contact. Given the evidence that microbial exposure heavily impacts psychiatric disorder risk through neural, immune, and metabolic mechanisms, interventions are necessary. Promising interventions include pre-/probiotics, dietary interventions, and nature exposure, and current research supports the strong promise of this field. However, the field is not yet at a point to establish clinical guidelines, and more research must be performed with the goal of translating the already outlined mechanisms to humans with the aim of prevention or treatment of stress-related psychiatric disorders.

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Neurodegenerative Diseases and the Gut Microbiota

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Abstract Neurodegenerative diseases are characterised by a progressive loss of neurons that leads to a range of cognitive and/or motor dysfunctions. During recent decades, some common pathways leading to neurodegeneration have been identified, such as protein misfolding, neuroinflammation, and the dysfunction of mitochondria and protein clearance systems. More recently, an altered gut microbiota has been identified as another potential feature seen in neurodegenerative disorders, which has been shown to play a central role in health and disease. The gut microbiota communicates with the central nervous system along the microbiota-gut-brain axis modulating host health and disease. Although the specific role of gut microbiota on the pathogenesis of these diseases is still under investigation, therapeutic approaches focusing on the modification of gut microbiota could bring novel therapeutics for neurodegenerative diseases.

Keywords Neurodegeneration · Alzheimer · Parkinson · Huntington · Inflammation · Protein misfolding · Mitochondria · Gut microbiota

1 Introduction

It's been over a century since James Parkinson and Alois Alzheimer first published their observations of the neurodegenerative diseases that bear their names [1, 2]. Today, neurodegenerative diseases are one of the main causes of comorbidity and mortality in older adult populations, and these numbers will likely increase with the proportion (or number) of aged individuals increasing day by day. These debilitating diseases have immense emotional and financial tolls on all societies worldwide.

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In broad terms, neurodegenerative diseases are conditions where neurons in the central or peripheral nervous system progressively degenerate, leading to central nervous system dysfunction. Several neurodegenerative diseases can be identified depending on the neuronal population affected, its localisation in the brain, and the clinical features observed (see Table 1). Most neurodegenerative diseases are characterised by depositions of misfolded native proteins and a widespread clinical symptomatology. The traditional method of classifying neurodegenerative disease is based on clinicopathological features from the anatomical area affected with the neuronal dysfunction supported by molecular pathology patterns of the misfolded proteins [14]. Nevertheless, classifying these diseases is a challenging subject as specific symptoms and protein aggregates can be found in multiple diseases, hampering the diagnosis of the disease [15]. Thus, the idea that neurodegenerative diseases are overlapping or even a continuum has been raised [14].

To date, there is no cure for any single neurodegenerative disease, where diagnosis usually leads to debilitating symptoms and ultimately death, due in part to the lack of complete knowledge of the aetiological factors involved and a limited understanding of their pathological progression. While degenerative mechanisms are not yet fully elucidated, some common mechanisms leading to neurodegeneration have been identified, such as protein aggregation, neuroinflammation, oxidative stress, mitochondrial dysfunction, and impairments in autophagy.

Accumulating evidence indicating that intestinal microbiota influences brain function and behaviour across the lifespan has sparked interest in the role the gut microbiome plays in neurodegenerative disease. The gut microbiota of individuals with neurodegenerative disease differs from healthy people, suggesting a connection between the brain pathology and the gut microbiota. The microbiota-gut-brain axis is a bidirectional communication pathway where gut microbes signal to the brain and the brain signals to the gut. The mechanisms of communication are not fully understood due to large interconnected complex networks but include immune, neural, endocrine, and metabolic pathways [16]. A growing body of evidence now points towards a role for the gut microbiota in age-related disorders including neurodegenerative disease; however, the level of involvement of the gut microbiota in the pathology is still under debate. Our knowledge about the implications of gut microbiota in the pathogenesis of these diseases, as well as the number of therapeutic interventions targeting the gut microbiota for these diseases, are however increasing.

2 Neurodegenerative Diseases

Neurodegeneration is the generic term that describes the loss of neurons leading to a progressive dysfunction of the central nervous system. Neurodegenerative diseases are usually of unknown origin, and the pathogenesis is considered to be driven by a combination of genetic and environmental factors (with the possible exception of

Table 1 Overview of neurodegenerative diseases

Disease	Estimated incidence and prevalence per 100,000 people	Origin	Clinical features	Brain region affected	Aggregating proteins involved	Location of aggregates
Alzheimer's disease	Incidence: 2300 Prevalence: 14,500 in the USA in 2011 [4]	10% genetic, 90% sporadic [3]	Dementia	Hippocampus, cerebral cortex	Aβ, tau	Cytoplasmic, extracellular
Parkinson's disease	Incidence: 5–346 Prevalence: 65–12,500 in Europe in 2005 [6]	5–15% genetic and 85–95% sporadic [5]	Motor disorder	Substantia nigra	α-Synuclein	Cytoplasmic
Huntington's disease	Incidence: 0.4 in Europe in 2005 [7] Prevalence: 10.6–13.7 in Western populations in 2017 [8]	100% genetic	Motor, cognitive, and psychiatric disorder	Striatum, cerebral cortex	Huntingtin	Nuclear
Amyotrophic lateral sclerosis	Incidence: 2.5 Prevalence: 4–6 worldwide in 2012 [9]	10% genetic, 90% sporadic	Motor disorder	Motor cortex, brainstem	Superoxide dismutase, TPD-43	Cytoplasmic
Multiple sclerosis	Incidence: 1–10 in Europe [10] Prevalence: 50–300 worldwide in 2013 [13]	A combination of genetic and environmental interactions [11]	Autoimmune disease	White matter	Basson [12]	Cytoplasmic

Huntington's disease—HD—which is inherited in an autosomal dominant manner).[1] The knowledge around these conditions has increased notably in recent decades thanks to improvements in neuronal imaging and molecular techniques. However, this knowledge varies greatly amongst diseases, and Alzheimer's disease (AD) and Parkinson's disease (PD) are by far the most studied neurodegenerative diseases to date. A brief description of the most common and studied neurodegenerative diseases is given below as they will be mentioned throughout this chapter.

AD is the most common neurodegenerative disease affecting at least 43.8 million people worldwide [17], a number that is growing year after year and expected to double by 2050 [18]. AD is the most common diagnosis of dementia. The main neuropathological hallmarks of AD are the accumulation of extracellular amyloid-beta (Aβ) plaques and intraneuronal deposits of tau protein, a microtubule-associated protein, which constitutes the primary component of neurofibrillary tangles [19]. These pathological features lead to neuronal cell death in the hippocampus and the cerebral cortex and a decline in memory, thinking, and language abilities.

PD is the second most common neurodegenerative disease, and more than six million individuals worldwide were living with PD in 2016 [20]. It is characterised by a slow and progressive degeneration of dopaminergic neurons in the substantia nigra. This neuronal decay in the nigrostriatal pathway generates motor (bradykinesia, rigidity, resting tremor, and postural instability) and non-motor symptoms (constipation, anosmia, and sleep disturbances amongst others). In addition to neuronal loss, PD is also characterised by the presence of intraneuronal protein inclusions of α-synuclein, called Lewy bodies [21]. α-Synuclein is a small protein, mostly present in the presynaptic terminals of neurons, and its function, although not fully understood, is associated with synaptic function modulation and vesicle trafficking [22].

HD is a neurodegenerative disease caused by an inherited autosomal dominant CAG trinucleotide repeat in the huntingtin gene that causes neuronal dysfunction [8]. Hence, unlike other neurodegenerative diseases, HD pathogenesis is triggered by a genetic mutation. This neurodegeneration causes a wide spectrum of movement, cognitive, and psychiatric symptoms that appear in the first decades of adult life [8].

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease and affects motor neurons and causes muscular atrophy and weakness leading to difficulties in speaking and breathing and ultimately death [23]. In ALS, neurodegeneration and neuronal cell death are associated with excess synaptic glutamate and mitochondrial alterations [24]. A blockade of the neurotransmitter γ-aminobutyric acid (GABA) receptor in ALS causes muscle hyperexcitability and a moderate loss of motor neurons [25]. Thus, distortions in the fine balance between the excitatory glutamate activity and inhibitory GABA activity can severely

[1] Autosomal dominant inheritance pattern refers to how a mutation is inherited. In autosomal dominant inheritance, the mutation gene is located in a non-sex chromosome, and only one copy of the mutated gene is needed to be affected.

compromise motor neuron viability. Significant particularities of ALS versus other neurodegenerative diseases include a lower age of onset, fast decay of motor neurons, and quick mortality rate [26].

Multiple sclerosis (MS) is a neurodegenerative and inflammatory autoimmune disease where T cells in the immune system react against oligodendrocytes in the CNS, resulting in neuroinflammation, demyelination, and axonal loss [27]. The consequences of demyelination depend on the area affected but include ataxia, loss of coordination, visual and sensory impairment, and fatigue. The autoimmune profile of MS makes it different from the other neurodegenerative diseases as it is characterised by an earlier onset and episodic manifestations.

The conditions mentioned above do not represent a complete list of neurodegenerative diseases characterised thus far but represent the conditions where the role of the gut microbiome has been mostly investigated. There is a plethora of proteinopathies described in the literature that include infectious acquired neurodegeneration (such as that seen in prion-like disease, transmissible spongiform encephalopathies, or Creutzfeldt-Jakob disease) and conditions with mixed clinicopathological features such as dementia with Lewy bodies.

Although each neurodegenerative disease has a clearly defined set of diagnostic symptoms, most patients present with mixed clinical symptomatology, hampering an accurate diagnosis that in most cases cannot be confirmed until post-mortem. The rising complexity of neuropathological findings demands that all aspects of the clinical picture are analysed and not limited to the cardinal symptoms. Secondary symptomatology, including gastrointestinal, cognitive, and psychiatric manifestations, are increasingly being considered as a prodromal diagnostic tool for neurodegenerative disease, especially given that they can significantly impact quality of life. For example, symptoms of gastrointestinal dysfunction such as constipation are a common symptom of the prodromal phase in PD and can arise several years before any motor function deficits.

Hence, it is becoming increasingly evident that not only do disease-specific brain-related symptoms need to be considered. Gastrointestinal microbes were initially examined for GI-related conditions such as Crohn's disease and irritable bowel syndrome but are now under serious consideration in the field of brain neuropathies.

3 Gut Microbiota

The human microbiota is an entity that includes trillions of microorganisms (bacteria, viruses, fungi, phages, yeasts, archaea) that live in and on our bodies [28]. The gut microbiota specifically consists of a broad community of bacterial species that live in symbiosis in the human gastrointestinal tract and is essential for the digestion of non-digestible substrates in the host, such as dietary fibres [29]. The gut microbiota is also involved in many other functions including host metabolism, immune system regulation, and neuronal development.

The technological advancement of genetic sequencing techniques lately has dramatically improved our knowledge of the gut microbiota composition and abundance; it is estimated that our gastrointestinal tracts are populated with more than 500–1000 different bacterial species [30]. The human gut microbiota is mostly composed of bacterial strains belonging to the phyla[2] *Firmicutes* and *Bacteroidetes*, but other minority phyla that are commonly found include *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* [31]. The gut microbiota is a dynamic entity that experiences many shifts during the host lifespan and can be modulated by many external factors such as lifestyle choices and environmental inputs [32]. However, each individual harbours radically different microbial compositions, even in conserved taxa, and our knowledge about what regulates that variability is limited. For this reason, what constitutes the best microbiome composition is unknown.

Although microbial community composition in the gut has a great inter-individual variability, the maintenance or disruption of the host gut homeostasis, per individual, is key in health and disease. The overall composition ratios of gut microbiota in any one individual are very well conserved once adulthood is reached. In fact, changes in the microbiome and the subsequent relationship with biological systems such as the immune, endocrine, and central nervous system correlate with a broad range of diseases. In particular, the relative abundance between the two major phyla, expressed as the *Firmicutes*/*Bacteroidetes* ratio, has been linked with many pathological conditions [33], and it is currently used as an estimation of gut microbiota alterations. In the context of neurodegenerative diseases, intestinal microbiota dysbiosis[3] seems to contribute to neurodegeneration [35].

The implication of the gut microbiota in disease has sparked interest in the scientific community that foresees gut microbiota modulation as a potential target for therapeutic prevention and intervention. Many therapeutic interventions focused on the enhancement of beneficial bacteria have been described, such as the oral administration of probiotics, prebiotics, or faecal microbiota transplantation (FMT). In probiotic administration, live bacteria and/or yeasts, thought to be beneficial to health, are ingested, whereas prebiotic administration consists of the ingestion of non-digestible fibres that promote the growth of beneficial bacteria. FMT is the transplantation of gut microbiota from a donor individual into the GI tract of a recipient individual. In animal models, FMT has been used to investigate the pathogenic mechanisms of neurodegenerative diseases by transplanting the gut microbiota of healthy donors into a diseased recipient, or vice versa. These approaches are under investigation for neurodegenerative diseases and will be described later in this chapter.

[2] In taxonomy, living organisms are classified into eight ranks ranging from more general to more specific characteristics (domain, kingdom, phylum, class, order, family, genus, and species).

[3] Dysbiosis is an ambiguous term frequently used to describe disruptions of the gut microbial populations, and it is commonly associated to disease [34].

3.1 Gut Microbiota and Ageing

Ageing is an inevitable and progressive deterioration of physiological functions of the host that correlate with increased risk of disease and death. Ageing comes with modifications in life habits (such as diet or exercise) and physiological changes. The ageing process brings changes in gut physiology, as well as gut microbial composition and function [36]. The process of ageing has been classified as a sensitive period for gut microbiota, where it is susceptible to environmental triggers and intrinsic factors in the host [37]. However, the relationship between ageing and gut microbiota is thought to be bilateral, as the gut microbiota can also contribute to normal ageing. Ageing-associated gut microbiota changes result in increased gut permeability, modifications in the production of gut microbiota-derived metabolites, and alterations in the host immune system [38]. Age-associated shifts in the gut microbiota are linked to increased susceptibility to many diseases, including neurodegenerative diseases [39, 40].

The composition of the gut microbiota is markedly different between young and elderly populations [41–43] the latter being characterised by a lower microbial diversity [44, 45]. For instance, in some cohort studies, elderly populations had a reduced *Firmicutes*/*Bacteroidetes* ratio [46, 47], but not in others [48]. Also, a reduction in beneficial commensal bacteria such as *Bifidobacterium* and *Lactobacillus* and an increase in harmful bacteria such as *Enterobacteriaceae* are reported in aged individuals [49]. These microbial changes are linked to changes in physiology attributed to ageing but also to lifestyle changes such as modifications in diet, reduction of physical activity, and an increase in medications [46]. In ageing populations, a lower microbial diversity correlated with diseased conditions [46]. For example, loss of diversity in the core microbiota[4] groups is associated with increased frailty and reduced cognitive performance [50]. Although the mechanisms linking gut microbiota and brain are not fully understood, it is clear that microbial dysbiosis in the gut is associated with a higher risk of brain dysfunction.

4 The Microbiota-Gut-Brain Axis

The concept of a microbiota-gut-brain axis is relatively new but is increasingly accepted due to the mounting evidence that the gut microbiota can regulate brain-specific processes such as host behaviour [37]. The microbiota-gut-brain axis is comprised of bidirectional complex communication networks between the brain and the gastrointestinal tract. This bidirectional communication between the two organs involves many signalling pathways such as the enteric nervous system (ENS), the hypothalamic-pituitary-adrenal axis, the immune and endocrine systems, as well as the gut microbiota and its metabolites [16, 51]. Despite interest in and knowledge of

[4]The core microbiota refers to the taxa that are present in the vast majority of the subjects [41].

the microbiota-gut-brain axis increasing daily, there is still a lack of full understanding of the underlying mechanisms involved in these networks.

The relevance of the gut-brain axis in neurodegenerative diseases became evident 20 years ago when Dr. Braak and colleagues proposed an intriguing hypothesis that PD spread from the gut to the brain as a result of an infection [52]. They presented evidence of Lewy bodies outside the nigrostriatal pathway, in locations such as the olfactory bulb, the ENS, and the vagus nerve. According to Braak and colleagues, the first inclusions of α-synuclein occur in the vagus nerve and olfactory bulbs and then the pathology spreads in an ascending manner to the brainstem and forebrain [52]. Interestingly, the vagus nerve is one of the best characterised communication pathways between the brain and the gut [53]. Animal models were then developed to assess the gut-to-brain spread hypothesis. Now we know that α-synuclein can spread in a prion-like manner both in vitro and in vivo [54]. Moreover, the transport of different forms of α-synuclein from the gut to the brain through the vagus nerve has been reported in rats [55]. However in a recent animal study, although the expected brain-to-gut spread of α-synuclein could not be confirmed, alterations in the ENS and the gut microbiome were apparent [56]. Interestingly, gut-seeded α-synuclein fibrils promoted gut dysfunction and brain pathology in aged mice but not in young mice [57]. Despite the point of origin of PD in the body still being a matter of debate, these investigations have highlighted the importance of the role that the gut-brain axis plays in PD.

Most neurodegenerative diseases were initially viewed as neuronal brain-exclusive diseases, but recent findings have challenged this idea and neurodegenerative diseases are now viewed as a multisystemic disease. Although ageing, genetics, and the environmental are important risk factors for neurodegeneration, as we will see later on, the involvement of the gut microbiome through the bidirectional gut-brain axis cannot be diminished. As a result, much research now focuses on the potential implications of the microbiome in these diseases.

5 Towards Neurodegeneration

For decades, neuroscientists were focused on the specifics of the neuronal decay in each neurodegenerative disease. However, it is now more evident that there are clinical, cellular, and molecular differences that neurodegenerative diseases share, which contribute to the development of neurodegeneration. Furthermore, they share common pathogenic pathways that lead to neurodegeneration. Below, we discuss the main factors contributing to neurodegenerative disease (see Fig. 1).

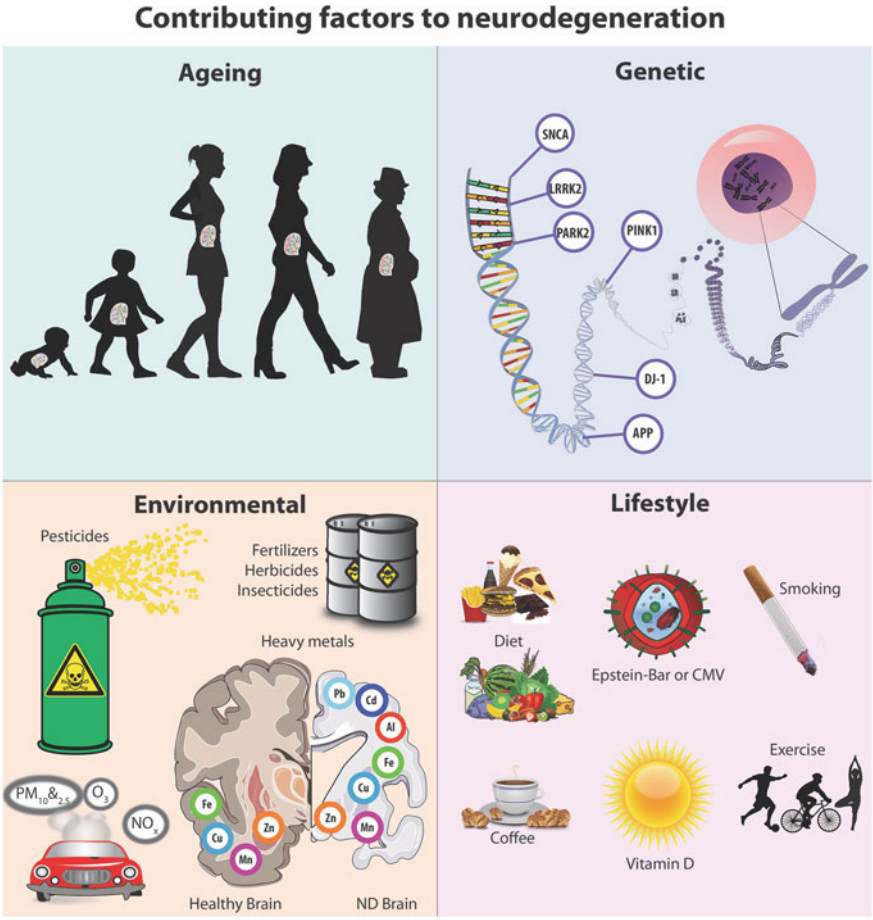

Fig. 1 Known factors that contribute to neurodegenerative disease. The aetiology of neurodegenerative diseases is mostly unknown, but several factors such as ageing, genetics, environmental, and lifestyle choices contribute to the pathogenesis of neurodegenerative diseases. SNCA, synuclein alpha; LRRK2, leucine-rich repeat kinase 2; PARK2, Parkinson disease-2; PINK1, PTEN [phosphatase and tensin homolog]-induced kinase 1; DJ-1, protein deglycase; APP, β-amyloid precursor protein; PM (particulate matter) 10 (<10 μm) 2.5 (<2.5 μm); O_3 (ozone/trioxygen); NO_X, nitrogen oxides; Fe, iron; Cu, copper; Zn, zinc; Mn, manganese; Al, aluminium; Cd, cadmium; Pb, lead; CMV, cytomegalovirus; ND, neurodegenerative disease

5.1 Factors that Contribute to the Development of Neurodegeneration

5.1.1 Ageing

The growth of an ageing population worldwide is increasing rapidly, and with it, the incidence of neurodegenerative diseases. From a cellular and molecular perspective,

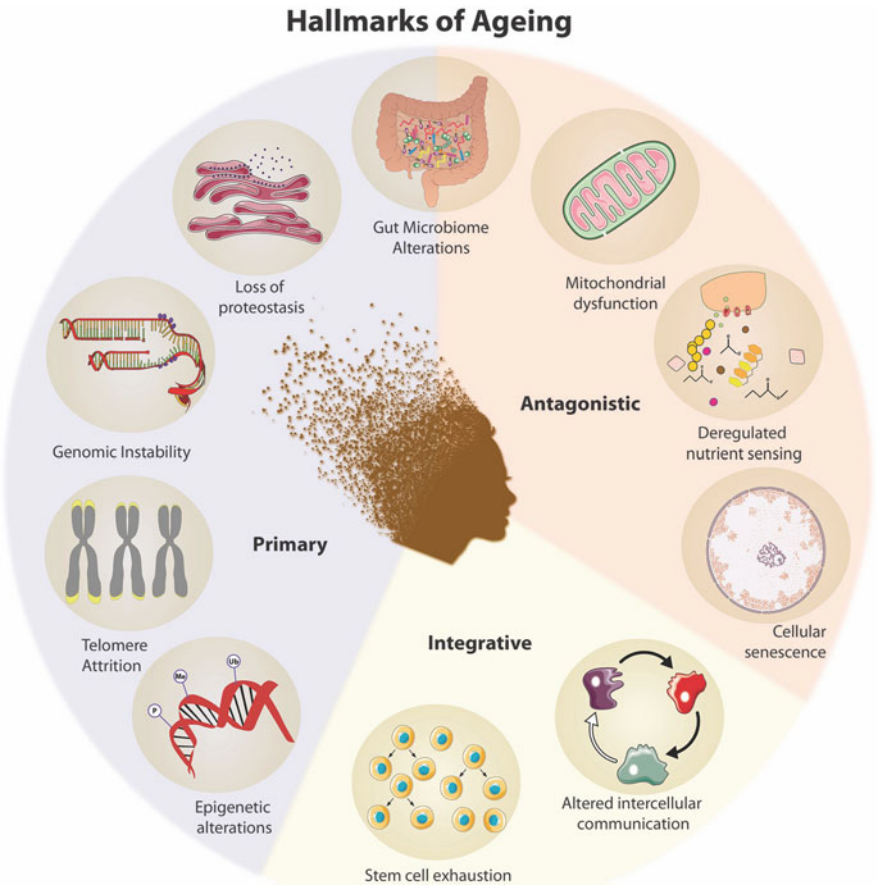

Fig. 2 The main hallmarks of ageing. From a cellular and molecular perspective, the nine hallmarks of ageing have been identified and grouped into primary, antagonistic, or integrative hallmarks

the nine hallmarks of ageing have been identified and grouped into primary, antagonistic, or integrative hallmarks (see Fig. 2) [58]. The primary hallmarks of ageing include genomic instability, epigenetic alterations, telomere attrition, and loss of proteostasis,[5] which are considered to be unequivocally negative processes. The antagonistic hallmarks—mitochondrial dysfunction, cellular senescence, and deregulated nutrient sensing—unlike the primary hallmarks, can have beneficial or deleterious effects depending on their intensity. The integrative hallmarks—stem cell exhaustion and altered intercellular communication—arise as the culprit of the accumulative damage induced by primary and antagonistic hallmarks. These central

[5] Regulatory processes that involve synthesis or degradation of proteins to maintain cell health.

biological mechanisms of ageing and their relationship with neurodegeneration have been reviewed elsewhere [59].

The absence of disease-free brains in the oldest population suggests that brain ageing and neurodegeneration are a continuum rather than a simplistic cause-effect relationship [60]. Thus, not only ageing but congenital predisposition and environmental factors will determine the lesions that will lead to specific diseases.

5.1.2 Congenital Factors

Genetic studies have shown that genetic predisposition plays an important role in the development of neurodegenerative disorders such as AD or PD, especially in young adult onset cases where specific mutations have been identified [61, 62]. In early onset familial AD, mutations in three genes (amyloid precursor protein (APP), presenilin-1, and presenilin-2) involved in the Aβ plaque formation have been identified as inherited in an autosomal dominant pattern [61]. In the familial forms of PD, genes (*SNCA*, *LRRK2*, *PARK2*, *PINK1*, *DJ-1*, and *ATP13A2*) have been identified to be hereditable monogenic PD [63]. In contrast, HD is an exception to neurodegenerative diseases, as the genetic component is needed for the development of the disease.

However, most chronic neurodegenerative diseases are considered to have a multifactorial aetiology since most of the cases are sporadic and cannot be attributed solely to genetic factors.

5.1.3 Environmental Factors

There is mounting evidence that environmental factors play a crucial role in the development of neurodegenerative diseases, in particular in AD and PD. Pesticides, herbicides, fertilisers, and particulate matter (PM), ozone (O_3), nitric oxide, and heavy metals have been demonstrated as having an increased risk of developing AD [64, 65], PD [66, 67], and ALS [68] (see Fig. 1). Pesticides and heavy metals can be neurotoxic leading ultimately to neurodegeneration [69]. Interestingly, some of these environmental factors can directly affect gut microbiota. It is believed that a complex combination of genetic and environmental interactions is key for disease pathogenesis and that the microbiome is a participant in this intricate network of factors. However, how these environmental factors interact is not fully understood, and more investigations on these interactions are needed as they could elucidate mechanisms of pathogenesis and improve prevention and personalised therapy for these diseases [70].

5.1.4 Lifestyle

Lifestyle choices and experiences have also been linked to the development of these diseases. For example, sport-related traumatic brain injury has been reported to increase the risk of developing both AD and PD [71, 72], whereas coffee consumption, smoking [73], and vigorous exercise [74] correlated with a decreased risk for PD and the Mediterranean diet for AD [75]. Lifestyle risk factors for MS include smoking, vitamin D deficiency or low sun exposure, and infections of Epstein-Bar virus or cytomegalovirus [76]. These lifestyle factors can impact gut microbiota, once more highlighting the complexity of factors and systems involved in neurodegeneration diseases.

5.2 Common Pathways to Neurodegeneration

Although each neurodegenerative disease has its own singular pathogenic mechanisms, some commonalities arise in the pathways leading to neurodegeneration.

5.2.1 Protein Misfolding

The most common neurodegenerative disorders are proteinopathies, characterised by the misfolding, aggregation, and accumulation of disease-specific proteins. These proteins change their conformation resulting in a loss of their biological function and can become toxic. AD, characterised by the formation of Aβ deposits as amyloid plaques, as well as neuronal tau inclusions, is probably the most salient proteinopathy, although many others exist such as PD, HD, frontotemporal dementia, and spinocerebellar ataxia type 1. The molecular mechanisms, causing a normal protein with a physiological function to transform into an abnormal conformation, are not well understood [77].

Protein homeostasis is a tightly regulated process essential for cellular integrity. Misfolded or aggregated proteins are quickly targeted by molecular chaperones that repair or degrade faulty proteins to maintain cellular homeostasis [78]. Molecular chaperones use the ubiquitin proteasome system and autophagy pathways to degrade these proteins [79]. However, these systems are altered in many neurodegenerative diseases, facilitating the accumulation of these aberrant proteins. Moreover, cellular ageing, proteotoxic stress, or genetic mutations can interfere with this process, resulting in proteins that escape the cell's quality control system and aggregate into non-native structures [78]. Consequently, although misfolded proteins in neurodegenerative diseases (α-synuclein, Aβ, huntingtin) have very different biological function and location, they share a β-sheet-rich tertiary structure in their pathological form, which facilitates their aggregation into oligomeric fibrillar formations [80].

Thus, despite having differential molecular agents implicated, neurodegenerative diseases share many common altered hallmarks that explain this accumulation of aberrant proteins.

5.2.2 Glial Cells and Neuroinflammation

The brain is not only populated by neurons, in fact, it's estimated that glial cells are at least as abundant as neurons [81]. Neurodegenerative diseases are multicellular in nature, and the implication of both neuronal and non-neuronal populations is now being investigated. This shift was based on extensive research data, showing first the presence of neuroinflammation in neurodegenerative disease, and second, the involvement of glial cells in disease progression. There is compelling evidence that neurodegenerative diseases such as AD, PD, and MS, are strongly associated with immune activation and neuroinflammation [82, 83].

Most neurodegenerative diseases display increased levels of neuroinflammation, which is the inflammatory response in the brain. This permeabilisation leads to lymphocyte infiltration. Neuroinflammation involves the activation of microglia and astrocytes, which secrete inflammatory molecules such as cytokines and chemokines. Evidence from both individuals and animal models reports that there is a recruitment of glial cells into the afflicted areas. For example, activated microglia and/or astrocytes are found in the substantia nigra of PD patients [84, 85] and AD patients, respectively [86]. Not only are innate immune cells found in neurodegenerative processes, cytotoxic T lymphocytes—major cell components of the adaptive immune system—were also reported to be higher in blood and in the affected PD brain regions, than in healthy subjects [87, 88]. The selective location of these T cells indicates an infiltration to the brain parenchyma, suggesting a disruption of the blood-brain barrier (BBB). Altered permeability in the BBB is a common pathological feature in many neurodegenerative diseases [89, 90].

At a molecular level, neuroinflammation has also been confirmed, where increased levels of pro-inflammatory molecules such as tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interferon γ (IFN-γ) were present in the serum and cerebrospinal fluid of PD patients and in the nigrostriatal pathway at *post-mortem* analysis [91]. Thus, although the identification of neuroinflammation during the progression of neurodegeneration is largely understood, these data do not confirm the involvement of neuroinflammation in the degenerative process. However, genetic and epidemiological studies have shown that polymorphisms in neuroinflammation-related genes increase the susceptibility for PD [91] and mutations in APOE and TREM2 genes, mainly expressed in glial cells, increase the risk for AD [92]. Further, genes linked to major histocompatibility complex (MHC) class II have been found to be a risk factor for MS [93].

This evidence together suggests that neuroinflammation is linked to the neurodegenerative process, playing a crucial role in disease progression. First, protein aggregates cause a direct inflammatory reaction that eventually leads to neuronal cell death. Immune responses are a double-edged sword however, with beneficial or

deleterious consequences depending on the specific situation. Furthermore, these cellular and molecular changes seen in patients have been observed in animal models too.

Independent of the origin of neuroinflammation, immunotherapies targeting the neuroinflammation in neurodegenerative diseases could help halt or modify the course of disease. Many immune-based therapeutic interventions are under investigation, including targeting the clearance of protein aggregates, with the inhibition of inflammation and apoptosis amongst other mechanisms of action [94].

5.2.3 Mitochondrial Dysfunction and ROS Generation

Neurons consume high amounts of energy to perform [95]; hence, they rely on mitochondria to fulfil these high metabolic demands. Mitochondria not only produce high quantities of ATP, but they also regulate calcium concentration and generate reactive oxygen species (ROS) from the respiratory chain [96].

Misfolded proteins can negatively affect mitochondria by several mechanisms including direct damage to mitochondrial DNA, trafficking impairment, or promoting mitochondria-dependent cell death pathways [97]. This mitochondrial dysfunction is well known in AD, where Aβ and tau proteins disrupt mitochondrial DNA maintenance, protein import, electron transport chain activity, and reduction-oxidation (redox) balance [97]. Similarly, α-synuclein accumulation in mitochondria reduced mitochondrial complex I activity and increased free radical production in dopaminergic neurons [98]. Mitochondria in motor neurons of ALS patients have an altered structure and appear swollen and vacuolated under histological analysis [99]. *Post-mortem* analysis of ALS brains showed alterations in respiratory chain complexes within mitochondria [100, 101].

Gene mutations are another factor linked to mitochondrial dysfunction. In PD, mutations in PINK1, Parkin, and DJ-1 are closely associated with mitochondrial dysfunction [102, 103]. Parkin and PINK-1 are known regulators of mitophagy, the autophagic process responsible for clearing the cell of defective mitochondria [104, 105].

If mitochondrial dysfunction in neurodegenerative diseases is a cause or a consequence, it's still under consideration, but it seems plausible that a reciprocal toxic cycle exists. Mitochondria are the main source of ROS production, including superoxide (O_2^-), hydroxyl (HO), and hydrogen peroxide (H_2O_2) radicals, which are a by-product of oxidative phosphorylation in cellular respiration. In neurodegeneration, the implication of oxidative damage as a pathogenic factor is well known, and as a result, has often been a target of potential therapeutic treatments; however, clinical trials assessing the benefits of antioxidants in neurodegenerative diseases have been generally negative [106].

5.2.4 Protein Clearance Systems: UPS and Lysosome Dysfunction

The ubiquitin-proteasome system (UPS) is a crucial protein degradation process in cells; briefly, proteins tagged with ubiquitin are targeted for proteasome degradation. Proteasomal turnover is particularly challenging for neurons due to their distinctive morphology (long axons and complex dendritic ramifications) [107]. Lysosomes—the organelle responsible for clearance of cellular debris—become dysfunctional in the pathogenesis of neurodegenerative disease. Lysosomes may be one of the key mechanisms underlying the accumulation of aberrant proteins in neuronal cells.

Interestingly, α-synuclein is degraded via UPS and autophagy-lysosome pathways [108, 109], leading to UPS regulation and lysosomal modification as potential methods of ND therapies.

5.2.5 Microbial Metabolites

Gut microbiota alterations have been observed in neurodegenerative diseases. Short-chain fatty acids (SCFAs)—which include acetate, propionate, and butyrate—are metabolites produced by bacterial fermentation of dietary fibres in the colon and are thought to be key mediators in the gut microbiota-brain axis crosstalk. However, most of the mechanisms by which SCFAs exert these effects remain yet unknown and need to be investigated further.

Tryptophan metabolism is one of the most important signalling pathways of the gut microbiota. Tryptophan is an essential amino acid which serves as a precursor to biosynthetic compounds, such as serotonin, melatonin, and nicotinamide adenine dinucleotide (NAD^+). The tryptophan-kynurenine metabolic pathway degrades tryptophan into several metabolites with inflammatory, oxidative, and neuronal modulatory properties [110]. Moreover, kynurenine enzymes further influence inflammatory processes [111]. Thus, this complex balance between neuroprotective and neurotoxic agents is crucial for the brain, and disturbances in the gut microbiota or other relevant processes such as inflammation could destabilise this equilibrium. In fact, in a systematic review, neurotoxic kynurenines were invariably increased in all major neurodegenerative diseases, while neuromodulatory kynurenines were decreased in AD, PD, and HD [110].

6 The Role of Gut Microbiota in Neurodegenerative Disorders

Through the microbiota-gut-brain axis, gut microbiota can modulate brain function and behaviour across the lifespan, both in health and disease [37]. However, the microbiota-gut-brain axis is bidirectional, as neurodegenerative brain dysfunction can also impact on the gut microbiota [112]. It encompasses a tentative relationship

that is somewhat sensitive to neuronal cell death and generalised inflammation. Furthermore, as mentioned earlier, neurodegenerative disorders are most frequent in aged populations, and ageing directly modifies the gut microbiota. Consequently, the gut microbiota composition of patients suffering from neurodegenerative diseases differs significantly from healthy subjects. In most cases, the relationship between the gut microbiota and neurodegeneration has been reported recently, but if dysbiosis is the cause or the consequence of the pathogenesis is still being investigated.

The gut microbiota and its metabolites interact with many of the pathways leading to neurodegeneration. Thus, it comes as no surprise that many studies have linked microbial dysbiosis to the pathology of neurodegenerative diseases [113, 114]. For instance, reduced diversity of gut microbiota during ageing and neuroinflammation are two common features of gut dysbiosis and neurodegeneration. Gut microbiota are constantly regulating microglial activation [115], and this could have great implications in neurodegeneration. These findings suggest that this process could be manipulated by microbiome-targeted strategies (see Fig. 3). It has been suggested that SCFA-producing bacteria could modulate immune activation in the brain [35]. Similarly, the porousness of the BBB could also be targeted via gut microbiome, as BBB permeability depends on microbiota composition [116].

Next we will summarise the main gut microbiota alterations observed in neurodegenerative diseases (see Table 2).

6.1 PD and Microbiome

Evidence for a role of the microbiome in PD comes from both animal and human studies. Regarding mouse models, PD pathophysiology is greatly reduced in the germ-free mouse—mice lacking any gut microbes—models of induced PD, and this effect can be reversed with oral administration of bacterial metabolites or an FMT [154]. Accordingly, antibiotic treatment ameliorated, while microbial recolonisation promoted, pathophysiology in mice overexpressing α-synuclein. Recently, a preclinical study reported that a gut bacterial amyloid promoted α-synuclein aggregation and motor impairment in mice [155]. These investigations suggest that gut microbiota is required for motor deficits, microglia activation, and α-synuclein pathology, at least in mice.

Gastrointestinal symptoms such as constipation are known to appear in PD patients well before the onset of the motor symptoms [156]. These gastrointestinal comorbidities—constipation, diarrhoea, and microbial dysbiosis—are common in most neurodegenerative diseases, which implicate the gut microbiome in neurodegenerative processes. Interestingly, the full removal of the vagus nerve—a surgical procedure called vagotomy—reduced the risk of developing PD in a clinical cohort [157].

Recently, the gut microbiota of PD patients has been investigated and compared to healthy controls (HCs) in a growing number of studies. Gut microbiota in PD

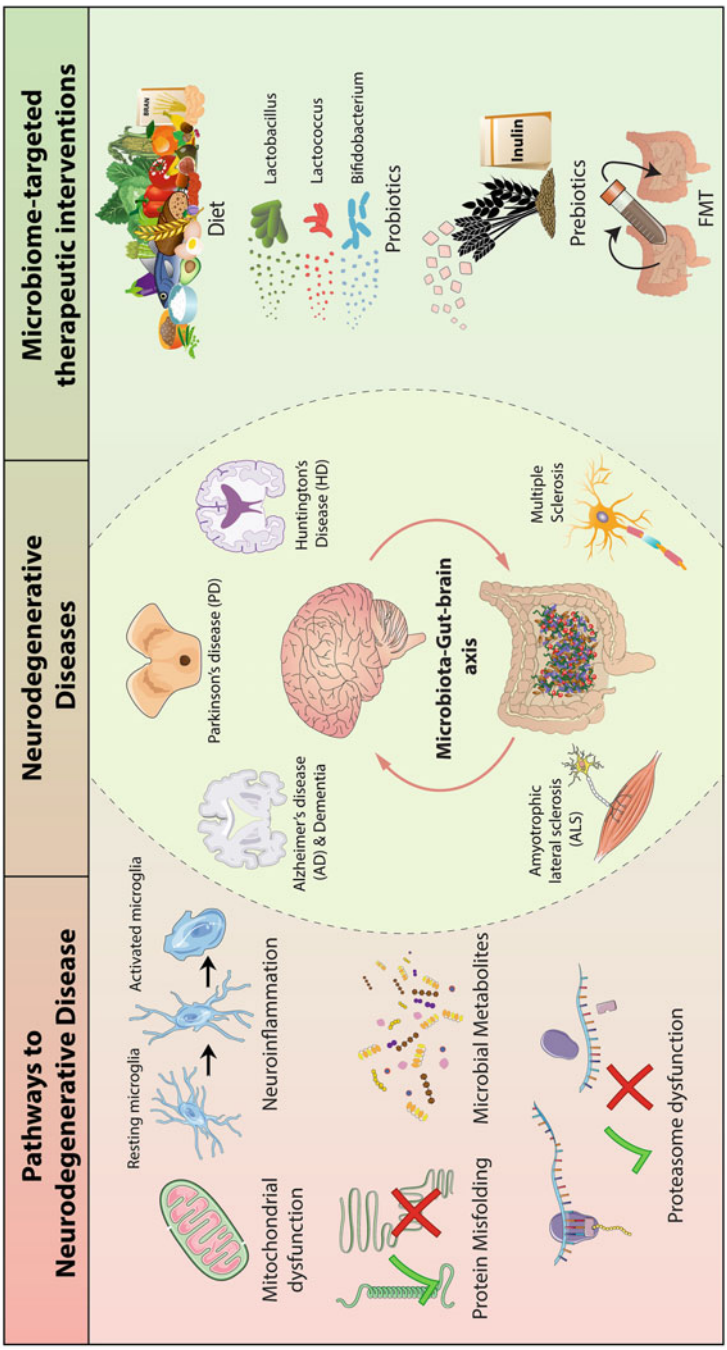

Fig. 3 Microbiota-targeted therapeutic interventions for neurodegenerative disease. The microbiota-gut-brain axis is linked to neurodegenerative diseases and interacts with many factors involved with neurodegeneration. Thus, new therapeutic approaches targeted to the gut microbiota are emerging for neurodegenerative diseases. ALS, amyotrophic lateral sclerosis; AD, Alzheimer's disease; PD, Parkinson's disease; HD, Huntington's disease; FMT: faecal microbiota transplantation

Table 2 Microbiota alterations in individuals with neurodegenerative disease versus healthy controls (HCs)

Type of study	Participants	Results	Conclusions
Alzheimer's disease			
Cross-sectional, observational [117]	Individuals with AD ($n = 24$), with other dementias ($n = 33$), or no dementia ($n = 51$)	Microbiome diversity differs between elders with AD and those with no dementia or other types of dementia. Individuals with AD had increased proportions of *Bacteroides* spp., *Alistipes* spp., *Odoribacter* spp., and *Barnesiella* spp. and decreased proportions of *Lachnoclostridium* spp. Individuals with other dementias had increased proportions of *Odoribacter* spp. and *Barnesiella* spp. and decreased proportions of *Eubacterium* spp., *Roseburia* spp., *Lachnoclostridium* spp., and *Collinsella* spp.	The gut microbiota composition differed in individuals with AD dementia in comparison to elders without dementia or with other dementia types
Cross-sectional, observational [118]	Individuals with AD ($n = 43$) and sex- and age-matched healthy controls (HCs) ($n = 43$)	Individuals with AD had decreased abundance of *Bacteroidetes*, *Verrucomicrobia*, *Negativicutes*, *Bacteroidia*, *Lachnospiraceae*, *Bacteroidaceae*, and *Veillonellaceae* and increased abundance of *Actinobacteria*, *Bacilli*, *Ruminococcaceae*, *Enterococcaceae*, and *Lactobacillaceae* compared with HCs	The gut microbiota composition and diversity of AD individuals were found altered compared with cognitively normal controls
Blind, cross-sectional, observational [119]	Cognitively impaired and amyloid-positive individuals ($n = 40$), cognitively impaired and amyloid-negative individuals ($n = 34$), and cognitively healthy and amyloid-negative individuals ($n = 10$)	Cognitively impaired amyloid-positive individuals had decreased abundance of *Bacteroides fragilis* and *Eubacterium rectale* and increased abundance of *Escherichia* and *Shigella*; significant upregulation of NLRP3,	Cognitively impaired individuals exhibited an increase in the abundance of pro-inflammatory gut microbial groups and a reduction in the abundance of anti-inflammatory microbial groups.

(continued)

Table 2 (continued)

Type of study	Participants	Results	Conclusions
		CXCL2, IL-6, and IL-1β, and downregulation of IL-10	Pro-inflammatory cytokines were also upregulated in these individuals
Blind, cross-sectional, observational [120]	Individuals with AD ($n = 25$) and sex- and age-matched HCs ($n = 25$)	Individuals with AD had reduced α-diversity and β-diversity and decreased abundance of *Firmicutes*. At a genus level, individuals with AD had increased abundance of *Alistipes*, *Bilophila*, *Blautia*, *Gemella*, and *Shigella* and reduced abundance of genus *Adlercreutzia* and *Bifidobacterium* compared with HCs	Individuals with AD had decreased microbial richness and diversity and a distinct microbial composition compared to control individuals
Parkinson's disease			
Cross-sectional, observational [121]	Individuals with PD ($n = 95$) and HCs ($n = 57$)	Individuals with PD had lower fungal DNA relative to bacterial DNA amongst PD patients. No fungi differed in abundance between groups nor were any associations with motor, cognitive, or gastrointestinal features	The gut mycobiome did not differ between individuals with PD and healthy individuals
Cross-sectional, observational [122]	Individuals with PD ($n = 197$) and age-matched HCs ($n = 103$)	No difference in α-diversity was observed between groups; α-diversity positively correlated with stool firmness. Individuals with PD had a higher abundance of *Christensenellaceae* and *Desulfovibrionaceae* at a family level and *Bifidobacterium*, *Collinsella*, *Bilophila*, and *Akkermansia* at a genus level. PD microbiota was enriched for pathways related to nucleic acid degradation and amino acid metabolism	Microbiota of PD individuals was characterised by reduced carbohydrate fermentation and butyrate synthesis capacity and increased production of harmful amino acid metabolites

(continued)

Table 2 (continued)

Type of study	Participants	Results	Conclusions
Cross-sectional, observational [123]	Individuals with PD ($n = 9$) and HCs ($n = 13$)	No differences in α-diversity or β-diversity between groups. Individuals with PD had increased abundance of *Akkermansia*, and a trend towards increased *Bifidobacterium* and decreased *Prevotella* was observed	PD individuals have a specific gut microbiota composition that could be used as biomarkers
Cross-sectional, observational [124]	Individuals with PD and mild cognitive impairment ($n = 13$), individuals with PD and normal cognition ($n = 13$), and HCs ($n = 13$)	In individuals with PD and mild cognitive impairment, the families *Rikenellaceae* and *Ruminococcaceae* and the genera *Alistipes*, *Barnesiella*, *Butyricimonas*, and *Odoribacter* were in higher abundance compared with the other two groups. Genera *Blautia* and *Ruminococcus* were decreased in the PD mild cognitive impairment group compared with the PD normal cognition group. The abundance of genera *Butyricimonas*, *Barnesiella*, *Alistipes*, *Odoribacter*, and *Ruminococcus* negatively correlated with cognition ability	Microbiota of individuals with PD and mild cognitive impairment substantially differed from individuals with PD and normal cognition, indicating a correlation between microbial communities and cognition
Cross-sectional, longitudinal, observational [125]	Individuals with PD ($n = 64$) and sex- and age-matched HCs ($n = 64$), 2 years apart	β-diversity was reduced in PD individuals versus HCs. No differences were observed in the *Firmicutes*/*Bacteroidetes* ratio between groups, but *Prevotella*/*Bacteroides* was higher in HCs. Differences in abundances in *Bifidobacterium*, *Prevotella*, *Lactobacillus*, and *Roseburia* were seen in both groups. Progressed PD individuals were overrepresented in the *Firmicutes*-	Alterations in gut microbiota in individuals with PD persisted after 2 years

(continued)

Table 2 (continued)

Type of study	Participants	Results	Conclusions
		dominated enterotype. Prevotella was less abundant in PD individuals compared to controls and also appears less abundant in individuals with faster disease progression	
Cross-sectional, observational [126]	Individuals with sporadic PD ($n = 75$) and age-matched HCs ($n = 45$)	Individuals with PD had decreased abundances of *Tenericutes*, *Euryarchaeota*, and *Firmicutes* at a phylum level and *Lachnospiraceae* at a family level. *Rikenellaceae* and *Deferribacteraceae* were more abundant in individuals who had suffered from PD for more than 5 years. *Leptotrichia* (*Fusobacteria*) was increased in individuals with PD and tremor, whereas *Roseburia* (*Lachnospiraceae*) was more abundant in individuals with PD and without tremor	Alterations in microbiota seen in individuals with PD were closely related to PD clinical characteristics
Cross-sectional, observational [127]	Individuals with sporadic PD ($n = 10$) and age-matched HCs ($n = 10$)	In individuals with PD, the abundance of the family of *Bacteroides* and *Prevotellaceae* was decreased, while the abundance of *Ruminococcaceae*, *Verrucomicrobiaceae*, *Porphyromonadaceae*, and *Lachnospiraceae* NK4A was enriched in north-eastern Han Chinese individuals with PD	The microbiota alterations in Chinese individuals with PD showed some similarities and disparities to individuals with PD from another region
Cross-sectional, observational [128]	Individuals with PD ($n = 58$) and HCs ($n = 45$)	In individuals with PD, the genera *Clostridium IV*, *Aquabacterium*, *Holdemania*, *Sphingomonas*, *Clostridium XVIII*,	Alterations in microbiota seen in individuals with PD were closely related to PD clinical characteristics

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Table 2 (continued)

Type of study	Participants	Results	Conclusions
		Butyricicoccus, and *Anaerotruncus* were enriched in comparison to HCs. *Escherichia/Shigella* abundance was negatively associated with disease duration, and genus *Dorea* and *Phascolarctobacterium* were negatively associated with levodopa administration. *Butyricicoccus* and *Clostridium XIVb* were associated with cognitive impairment	
Cross-sectional, observational [129]	Individuals with PD ($n = 76$), individuals with idiopathic rapid eye movement sleep behaviour disorder ($n = 21$), and HCs ($n = 78$)	Individuals with PD had a differential abundance of *Anaerotruncus*, *Clostridium XIVb*, several *Bacteroidetes*, *Akkermansia*, and *Verrucomicrobiaceae* compared with HCs. Similar trends in these alterations were also observed in idiopathic rapid eye movement sleep behaviour disorder versus HCs for example, *Anaerotruncus* and several *Bacteroides* spp., correlated with nonmotor symptoms	Individuals with PD or its prodrome idiopathic rapid eye movement sleep behaviour disorder had differential abundances of gut microbial taxa in comparison with HCs
Cross-sectional, observational [130]	Individuals with PD ($n = 29$) and HCs ($n = 29$)	β-diversity analyses, but not α-diversity, differed between groups. Individuals with PD had higher abundance of *Lactobacillaceae*, *Barnesiellaceae*, and *Enterococcaceae* in comparison with HCs	Gut microbiota in PD individuals was characterised by a decrease in taxonomic diversity and significant differences in the bacterial community
Cross-sectional, observational [131]	Individuals with PD ($n = 24$) and HCs ($n = 14$)	Individuals with PD had decreased abundances of *Blautia*, *Faecalibacterium*, and *Ruminococcus* and increased abundances of	The unbalance in beneficial and harmful bacteria in individuals with PD may help explain PD pathogenesis

(continued)

Table 2 (continued)

Type of study	Participants	Results	Conclusions
		Escherichia-Shigella, *Streptococcus*, *Proteus*, and *Enterococcus* in comparison with HCs. Disease severity and PD duration negatively correlated with beneficial bacterial strains and positively correlated with pathobionts	
Cross-sectional, observational [132]	Individuals with PD ($n = 89$) and age-matched HCs ($n = 66$)	Individuals with PD had reduced α-diversity and abundance of *Dorea*, *Bacteroides*, *Prevotella*, *Faecalibacterium*, *Bacteroides massiliensis*, *Stoquefichus massiliensis*, *Bacteroides coprocola*, *Blautia glucerasea*, *Dorea longicatena*, *Bacteroides dorei*, *Bacteroides plebeus*, *Prevotella copri*, *Coprococcus eutactus*, and *Ruminococcus callidus* and increased abundance of *Christensenella*, *Catabacter*, *Lactobacillus*, *Oscillospira*, *Bifidobacterium*, *Christensenella minuta*, *Catabacter hongkongensis*, *Lactobacillus mucosae*, *Ruminococcus bromii*, and *Papillibacter cinnamivorans*	Gut microbiota in PD individuals was characterised by a decrease in taxonomic diversity and significant differences in the bacterial community
Cross-sectional, observational [133]	Individuals with PD ($n = 197$) and HCs ($n = 130$)	Individuals with PD had decreases in abundance of *Bifidobacterium*, *Lachnospiraceae*, and *Blautia* in comparison with HCs. The abundance of *Ruminococcaceae* significantly increased with length of PD symptoms in individuals with PD	PD individuals had an altered abundance of several taxa, and there were independent effects of PD medications on the microbiome

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Table 2 (continued)

Type of study	Participants	Results	Conclusions
Cross-sectional, observational [134]	31 male individuals with PD ($n = 31$) and male age-matched HCs ($n = 28$)	Individuals with PD had increased *Verrucomicrobiaceae* (*Akkermansia muciniphila*) and unclassified *Firmicutes*, whereas *Prevotellaceae* (*Prevotella copri*) and *Erysipelotrichaceae* (*Eubacterium biforme*) were markedly lowered in PD individuals. The intake of either a MAO inhibitor, amantadine, or a dopamine agonist had no overall influence on taxa abundance or microbial functions	Perturbations in the gut microbiome composition of individuals with PD were observed
Cross-sectional, observational [135]	Individuals with PD ($n = 34$) and HCs ($n = 34$)	Faecal SCFA concentrations were significantly reduced in PD individuals compared to HCs. The bacterial phylum *Bacteroidetes* and the bacterial family *Prevotellaceae* were reduced, whereas *Enterobacteriaceae* were more abundant in faecal samples from PD individuals compared to matched controls	An altered microbiota with lower levels of SCFA-producing bacteria was associated with PD
Cross-sectional, observational [136]	Individuals with PD ($n = 38$) and HCs ($n = 34$)	Individuals with PD had increased abundance of *Bacteroidetes*, *Proteobacteria*, and *Verrucomicrobia*. At a genus level, PD individuals had lower abundances of butyrate-producing bacteria from the genera *Blautia*, *Coprococcus*, *Roseburia*, *Faecalibacterium*, and *Ralstonia* compared with HCs	Individuals with PD had a pro-inflammatory gut dysbiosis in comparison to healthy individuals
Randomised, cross-sectional,	Individuals with PD ($n = 52$) and HCs ($n = 36$)	In PD individuals, the abundance of hydrogen-producing bacteria was lower compared with	Intestinal permeability was increased in PD individuals, while the

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Table 2 (continued)

Type of study	Participants	Results	Conclusions
observational [137]		HCs. In these individuals, there was a significant increase in *Lactobacillus* and decrease in *Clostridium coccoides* group, *Clostridium leptum* subgroup, and *Bacteroides fragilis* compared to HCs. Linear regression models revealed that the increased count of *L. gasseri* subgroup was associated with disease duration. In PD individuals, serum lipopolysaccharide (LPS)-binding protein levels were lower than healthy controls, while the levels of serum diamine oxidase remained unchanged in both groups	intestinal mucosal integrity was preserved
Case-controlled, observational [138]	Individuals with PD ($n = 72$) and HCs ($n = 72$)	Individuals with PD had a significant decrease in *Prevotellaceae* compared with control individuals. The abundance of *Enterobacteriaceae* was positively associated with the severity of motor symptoms	The intestinal microbiome was altered in PD and was related to motor phenotype
Huntington's disease			
Case-controlled, observational [139]	Individuals with HD ($n = 33$, 9 pre-manifest stage and 24 diagnosed HD) and sex- and age-matched HCs ($n = 33$)	Increased α-diversity, β-diversity, and altered relative abundances of several taxa compared to those in HCs. *Intestinimonas* and *Bilophila* correlated with concentrations of pro-inflammatory cytokines in HD patients. HD patients had higher abundances of *Intestinimonas*, *Bilophila*, *Lactobacillus*, *Oscillibacter*, *Gemmiger*, and *Dialister* at the genus	Gut microbiota of HD patients correlated with HD clinical characteristics and cytokine levels

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Table 2 (continued)

Type of study	Participants	Results	Conclusions
		level and lower abundance of *Clostridium XVIII* at the genus level	
Case-controlled, observational [140]	Individuals with HD ($n = 42$, 23 pre-manifest stage and 19 diagnosed HD) and sex- and age-matched HCs ($n = 36$)	Reduced α-diversity, β-diversity, and altered relative abundances of several taxa compared to those in HCs. Lower abundances of *Firmicutes*, *Lachnospiraceae*, and *Akkermansiaceae* in male HD patients	HD patients had increased abundances of gut bacterial families linked to pro-inflammatory processes
Multiple sclerosis			
Case-controlled, observational [141]	Individuals with relapsing-remitting MS ($n = 19$) and sex- and age-matched HCs ($n = 17$)	MS patients with high disease activity and increased intestinal TH17 cell frequency showed a higher Firmicutes/*Bacteroidetes* ratio, increased relative abundance of *Streptococcus*, and decreased *Prevotella* strains compared to HCs and MS patients with no disease activity. The relative abundance of *Prevotella* strains was inversely related to the intestinal TH17 cell frequency	Specific microbiota modifications were associated with excessive TH17 cell expansion in the human intestine in individuals with MS
Case-controlled, cross-sectional, observational [142]	Individuals with relapsing-remitting MS who had not received treatment for at least 3 months before sample collection ($n = 71$) and HCs ($n = 70$)	No differences in α-diversity or β-diversity between groups. Individuals with MS had increases in *Acinetobacter* and *Akkermansia* and decreases in *Parabacteroides*. MS individuals showed an impaired ability to differentiate or expand CD25-positive, FoxP3-positive regulatory T cell populations	Specific bacteria were associated with MS, and these bacteria regulated T lymphocyte-mediated adaptive immune responses and contributed to the pro-inflammatory environment of MS
Case-controlled, cross-sectional,	Individuals with relapsing-remitting MS ($n = 31$) and sex- and	β-diversity differed as a function of relapse status. Individuals with MS had	Perturbations in the gut microbiome

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Table 2 (continued)

Type of study	Participants	Results	Conclusions
observational [143]	age-matched HCs ($n = 50$)	increases in abundance of *Pseudomonas*, *Mycoplana*, *Haemophilus*, *Blautia*, and *Dorea* and lower abundance of *Parabacteroides* and *Adlercreutzia* compared to HC	composition were observed in relapsed-remitting MS
Case-controlled, cross-sectional, observational [144]	Children with MS ($n = 18$, within 2 years of relapsing-remitting MS onset) and age-matched HCs ($n = 17$)	β-diversity significantly differed by immunomodulatory drug exposure. Children with MS had increased relative abundance of *Bilophila*, *Desulfovibrio*, and *Christensenellaceae* and decreased relative abundance of *Lachnospiraceae* and *Ruminococcaceae* in children with relapsing-remitting MS compared with HC	Perturbations in the gut microbiome composition were observed in paediatric MS onset
Case-controlled, cross-sectional, observational [145]	Individuals with relapsing-remitting MS ($n = 60$) and HCs ($n = 43$)	MS individuals have increases in *Methanobrevibacter* and *Akkermansia* and decreases in *Butyricimonas* and correlated with variations in the expression of genes involved in dendritic cell maturation, interferon signalling, and NF-kB signalling pathways in circulating T cells and monocytes. Individuals on disease-modifying treatment showed increased abundances of *Prevotella* and *Sutterella* and decreased *Sarcina*, compared with untreated patients	Individuals with MS had alterations in the gut microbiota associated with inflammation
Case-controlled, cross-sectional,	Individuals with relapsing-remitting MS ($n = 20$) and HCs ($n = 40$)	No differences in α-diversity between groups. Individuals with MS had lower abundance	Individuals with MS had lower abundances of SCFA-producing bacteria

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Table 2 (continued)

Type of study	Participants	Results	Conclusions
observational [146]		of *Bacteroides*, *Faecalibacterium*, *Prevotella*, *Anaerostipes*, *Clostridia* clusters *XIVa* and *IV* and increases in abundance of *Eggerthella lenta*	
Case-controlled, cross-sectional, observational [147]	MS individuals (five treated with glatiramer acetate and two with untreated MS) and HCs ($n = 8$)	Individuals with MS had increases in abundance of *Ruminococcus* and decreased abundances of *Faecalibacterium* and *Bacteroidaceae*. Vitamin D3 supplementation in untreated patients with MS increased abundance of *Akkermansia* and *Coprococcus*	Glatiramer acetate and vitamin D supplementation were associated with differences or changes in the microbiota
Amyotrophic lateral sclerosis			
Case-controlled, observational [148]	Individuals with ALS ($n = 66$) and sex- and age-matched HCs ($n = 61$) and neurodegenerative controls ($n = 12$)	The relative abundance of the dominant butyrate-producing bacteria *Eubacterium rectale* and *Roseburia intestinalis* and other species was lower in individuals with ALS	Individuals with ALS had lower abundance of SCFA-producing bacteria, associated with gut integrity and regulation of inflammation
Case-controlled, observational [149]	Individuals with ALS ($n = 50$) and sex- and age-matched HCs ($n = 50$)	Reduced α-diversity and altered relative abundances of several taxa compared to those in HCs. *Cyanobacteria*, at phylum level, and *Lactobacillus*, *Bifidobacterium*, and *Odoribacter* at genus level were more abundant in ALS individuals	Individuals with ALS exhibited an increase of potential neurotoxic or pro-inflammatory activity microbial groups such as *Cyanobacteria*
Case-controlled, observational [150]	Individuals with ALS ($n = 20$) and sex- and age-matched HCs ($n = 20$)	Increased α-diversity in ALS patients. In individuals with ALS, *Bacteroidetes* at the phylum level and several microbes at the genus level were upregulated, *Firmicutes* at the phylum level and *Megamonas* at the genus level were downregulated compared	Individuals with ALS has an altered composition of gut microbiota and metabolic products

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Table 2 (continued)

Type of study	Participants	Results	Conclusions
		to HCs. Decreased gene function associated with metabolic pathways was observed in ALS patients	
Case-controlled, observational [151]	Individuals with ALS ($n = 8$) and sex- and age-matched HCs ($n = 8$)	Individuals with ALS had increased levels of *Firmicutes*/*Bacteroidetes* ratio, genus *Methanobrevibacter*, whereas the relative abundance of beneficial microorganisms (genera *Faecalibacterium* and *Bacteroides*) were decreased. No differences between the two groups were observed in host plasma endotoxin, SCFA, NO_2-N/NO_3-N, and γ-aminobutyric acid	Individuals with ALS had an imbalance in intestinal microflora, with reduced abundance of beneficial bacteria, and increased abundance of harmful bacteria
Case-controlled, observational [152]	Individuals with ALS ($n = 25$, 2 familial, 23 sporadic) and sex- and age-matched HCs ($n = 32$)	No differences in α-diversity, β-diversity, and *Bacteroidetes*/*Firmicutes* ratio between groups. Individuals with ALS had lower abundances of *Ruminococcaceae* at genus level	The gut microbiota of individuals with ALS did not differ from healthy individuals
Case-controlled, observational [153]	Individuals with ALS ($n = 6$) and HCs ($n = 5$)	Individuals with ALS had a decreased *Firmicutes*/*Bacteroidetes* ratio, increased genus *Dorea* (harmful microorganisms), and reduced genus *Oscillibacter*, *Anaerostipes*, and *Lachnospiraceae* (beneficial microorganisms)	Individuals with ALS had an imbalance in intestinal microflora, with reduced abundance of beneficial bacteria and increased abundance of harmful bacteria

individuals is characterised by a decrease in taxonomic diversity and significant differences in the bacterial community. Overall, PD patients showed reduced levels of anti-inflammatory-associated butyrate-producing bacteria such as *Blautia* and *Roseburia* [136, 138], lower concentrations of SCFAs in faeces [135], and increased levels of pro-inflammatory-associated bacteria *Ralstonia* in the mucosa [136]. Moreover, non-significant reductions were observed for *Prevotellaceae* in PD patients, which may contribute to increased gut permeability in PD [136, 137].

Interestingly, gut microbiota alterations were linked with clinical characteristics [126, 128]. For instance, an increase in *Enterobacteriaceae* found in these patients [135, 138] positively correlated with postural instability [138] and disease severity [131]. These alterations in the microbiota of individuals with PD persisted over disease progression [125].

Together, these investigations suggest a pro-inflammatory environment in the gut of PD individuals. A bacterial metabolite, which is a marker of gut dysbiosis, was found in higher concentrations in individuals with PD [158]. On top of that, individuals suffering from PD have increased intestinal permeability that correlates with intestinal α-synuclein [159]. These findings in the gut microbiota of PD patients could be microbial biomarkers for PD used as supplemental evidence for PD diagnosis [123, 135–138].

When other facets of PD have been studied, such as the prodromal phase of PD or PD associated with mild cognitive impairment, the gut microbiota has been reported to be differently altered in comparison to individuals with PD and HCs [124], indicating that the microbiota changes alongside the progression of the disease. Thus, more studies are needed that target these phases of the disease.

6.2 AD and Microbiome

The evidence linking AD and gut dysbiosis is less pronounced than in PD. The modulation of the gut microbiome using germ-free mice, or conventional mice treated with antibiotics or probiotic administration, has shown that changes in the gut microbiota correlate with changes in host cognitive behaviours. For instance, germ-free mice exhibited impairments in spatial and working memory [160]. Temporary depletion of gut microbiota using antibiotics in rats also led to increased anxiety-like behaviours and deficits in spatial memory [161]. The administration of *Lactobacillus fermentum* NS9 reduced the alterations in behaviour induced by antibiotic treatment [161]. Moreover, administration of SCFAs promoted Aβ deposition in germ-free mice and exacerbated Aβ deposition in colonised mice via modulation of microglial phenotype [162]. As well, faecal transplantation of healthy microbiota reduced the formation of Aβ plaques and neurofibrillary tangles, glial reactivity, and cognitive impairment [163]. Thus, the role of gut microbiota in Aβ pathology and cognitive behaviour suggests that they could have a role in the pathogenesis of AD and therefore act as a novel avenue for therapeutic intervention.

Diet has long been considered linked with AD pathogenesis through the modulation of the immune system [164]. A Mediterranean diet is characterised by abundant plant-based foods such as fruits and vegetables, olive oil, and nuts as the main fat component, with a moderate intake of dairy products, fish, poultry, and eggs. Epidemiological investigations have shown that higher adherence to the Mediterranean diet correlated with a reduced risk of AD [165]. For example, omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are essential for normal brain function [166], and abundant ω-3 PUFAs in the diet of elderly populations correlated

negatively with cognitive decline [167]. Furthermore, frequent intake of fruits and vegetables, naturally high in antioxidants and vitamins, can also lower the risk of dementia and cognitive impairment [168]. Diets that are high in fats and sugars, such as the Western diet, can lead to cognitive impairment, memory decay, and increase the risk of AD [169]. High fat diets can induce changes in the gut microbiota and promote intestinal permeability, ultimately increasing inflammation, promoting disease. This suggests that gut microbiota play an important role in the increase/decrease of diet-associated AD risk. Nevertheless, the mechanisms driving the effects of gut microbiota in AD need further study.

Evidence from human studies assessing the role of the microbiome in the pathogenesis of AD is still very recent and limited. Some small case-control studies have indirectly evaluated the oral microbiome of AD individuals, as AD has long been linked to poor dental hygiene. When the gut microbiome of AD individuals was compared to healthy age- and sex-matched controls, AD individuals exhibited lower microbial diversity, decreased abundances of *Firmicutes* and bifidobacteria; moreover, levels of differentially abundant genera correlated with cerebrospinal fluid biomarkers of AD pathology [120].

Calprotectin is a protein biomarker used to assess intestinal inflammation. In a small study, AD individuals presented with a high calprotectin level in their faeces, indicating a disturbed intestinal barrier function in AD [170]. Another study comparing cognitively impaired individuals with and without amyloidosis to HCs found that the group with cognitive impairment and amyloidosis showed a lower abundance of *Eubacterium rectale* and a higher abundance of *Escherichia/Shigella*, which correlated with pro-inflammatory cytokines in blood, suggesting that those patients suffered from peripheral inflammation [119]. Another study found differences in abundance of *Bacteroides*, *Actinobacteria*, *Ruminococcus*, and *Lachnospiraceae* between AD patients and HCs [118].

Moreover, alterations in the GABAergic system are linked to cognitive impairment [171], and the evidence of GABA dysregulation in AD and ageing is substantial [172]. Interestingly, *Lactobacillus* and *Bifidobacterium* can produce GABA in the gut that can influence GABA in the CNS [173].

Although less direct, other evidence also supports the role of gut microbiota in the pathogenesis of AD. The hygiene hypothesis states that reduced microbial exposure due to improved sanitation and lifestyle changes in modern societies induces a malfunction of immunoregulation processes, contributing to autoimmune and inflammatory diseases. AD has many similarities with autoimmune diseases as it is an inflammatory disease with elevated Th1-mediated inflammation [23, 174]. When the relationship between the incidence of AD and environmental microbial diversity were investigated, countries with a greater degree of sanitation, and a lower extent of microbial diversity, had higher incidence of AD [175].

As summarised here, there are multiple connections that link AD and gut microbiome alterations, making microbiome-targeted interventions worth investigating further.

6.3 HD and Microbiome

Gut dysbiosis and increased intestinal permeability in HD are frequently reported together in both clinical and preclinical studies [140, 176, 177]. In a rodent model, although substantial changes in bacterial species abundance in the gut microbiota were not found in a longitudinal study of HD and wild-type mice, the HD gut microbiome was perturbed in the premotor symptom phase, suggesting the occurrence of gut dysbiosis in HD [178]. The sequencing data of another rodent study reinforces this subtle change in the gut microbiome of HD since they observed similar bacterial populations in HD and wild-type mice but differences in abundances [178]. In humans, it is still unclear whether individuals with HD present greater bacterial diversity in the gut, but alterations in bacterial abundances have been reported [139, 140]. For example, the abundance of *Intestinimonas* was higher in individuals with HD and correlated with HD clinical characteristics [139]; here, a correlation was established between altered gut microbiota and the occurrence of chronic inflammation [139]. Nevertheless, the alterations of the gut microbiome in HD are only recently being investigated, and a better understanding of this should become clearer in the future.

6.4 MS and Microbiome

Autoimmune diseases such as MS are characterised by a dysregulation of the immune system, and recently it was shown that the gut microbiota can modulate the immune system. For example, germ-free animals develop an attenuated experimental autoimmune encephalomyelitis (EAE) response, which is a rodent model of MS, unless they are transplanted with gut microbes from colonised mice [179], suggesting that the gut microbiota are key for disease progression. Interventional preclinical studies showed that probiotic administration can ameliorate disease severity in EAE by reducing inflammation and inhibiting Th17 cell differentiation [180, 181]. Similarly, oral administration of broad-spectrum antibiotics ameliorated EAE development in mice [182]. These data together suggest that the gut microbiome is implicated in MS pathogenic severity.

In terms of gut microbial community composition, the level of diversity between patients with MS and HCs was similar, but the relative abundances of specific bacteria differed significantly. In contrast to AD/PD, MS is not presented exclusively in an adult or elderly population, and consequently the microbiome does not necessarily have the particularities seen in an aged microbiome. Nevertheless, MS patients exhibited reduced levels of *Clostridia* family and *Bacteroidetes*, known to produce SCFAs and induce Treg cells [146], potentially facilitating autoimmune processes. Furthermore, *Methanobrevibacter smithii* and *Akkermansia muciniphila* are increased in stool samples of MS individuals, which can affect T cell differentiation [142, 145]. Moreover, excessive expansion of intestinal Th17 cells correlated

with microbiota alterations and disease activity [141]. There is strong evidence surrounding the involvement of the microbiome in MS; however, is it still unclear if the microbiome is the trigger or a driver of the neuroimmune pathogenesis.

6.5 ALS and Microbiome

ALS pathology is intricately linked to alterations in glutamate, GABA, and serotonin, and some strains of our gut microbiota can modulate the production of these neurotransmitters. Moreover, in a transgenic mouse model of ALS, the disruption of the junction structure in the intestine led to increased gut permeability, abnormal Paneth cells in the intestine [183], and reduced levels of *Butyrivibrio* (butyrate-producing bacteria) *fibrisolvens*, *Escherichia coli*, and *Firmicutes* bacteria, in comparison to wild type mice [183], suggesting microbial dysbiosis.

The gut microbiota in individuals with ALS exhibits a reduction of the ratio *Firmicutes*/*Bacteroidetes* phyla [153], which has been associated with detrimental health outcomes. In particular, butyrate-producing bacteria are reduced at early stages of the disease [183, 184]. Furthermore, ALS disease was associated with reduced levels of beneficial bacteria from the genera *Oscillibacter* and *Anaerostipes* and the family *Lachnospiraceae* and increased levels of harmful bacteria such as genus *Dorea* [153]. More evidence supports these changes in the abundance of microbial species between individuals with ALS and healthy subjects. In a randomised controlled trial, ALS patients had higher abundance of *Escherichia coli* and enterobacteria [185]. Furthermore, gut microbiota composition in ALS changes over the course of the disease; significant fluctuations of certain microbial strains were observed in a longitudinal study [149]. Overall, recent studies support the idea that relative abundance of beneficial microorganisms is decreased in ALS [151, 183, 185, 186]. On top of that, a higher vegetable fibre intake was shown to be associated with a slower-progression ALS disease [187].

Microbiota signatures as an element in the aetiology or pathogenesis of the disease have been broadly discussed [188, 189] and have led to investigative approaches towards modulating the gut microbiota in ALS patients as a novel therapeutic. Furthermore, these human studies have not only helped characterise the gut microbiota of ALS patients during the progression of the disease, but they are also the basis for a characterisation of these microbiota changes into an ALS biomarker.

In summary, host gut microbiota of neurodegenerative disease patients is markedly different than healthy subjects and is characterised by an overgrowth of pathogenic and reduction of commensal microbial strains, leading to altered production of beneficial metabolites such as SCFAs, which eventually increases the pathogenic milieu, setting up a vicious cycle. The development of gut microbiota-targeted interventions could help disrupt this endless loop of pro-inflammation and ameliorate at least some of the pathophysiological events, benefiting the patient.

7 Microbiota-Targeted Therapeutic Interventions for Neurodegenerative Diseases

In the search for therapeutic interventions for neurodegenerative disease, much effort has gone into trying to halt or reduce the aggregation of the aberrant protein involved, as well as directly targeting the pathways that lead to neurodegeneration, such as neuroinflammation. Microbiome modulation is an innovative approach that could address those targets indirectly and provide novel microbiome-targeted interventions for these diseases. Directly targeting neuroinflammation through the gut microbiota is one of the most common objectives of these therapeutic investigations. Evidence highlighting the role of gut microbiome in neurodegeneration has uncovered new insights in potential microbiome-based therapeutic approaches, interventions not only targeted to the direct modulation of the gut microbiota but also to their metabolites such as SCFAs.

Therapies include the use of antibiotics, probiotics, prebiotics, and FMT. Below we summarise some of the investigations carried out to date, regarding the potential of the gastrointestinal microbiota and metabolites, to ameliorate some facets of neurodegenerative diseases.

7.1 Microbial Metabolite-Based Interventions

SCFAs are neuroactive biomolecules and as such have a potential interest as a therapeutic agent for neurodegenerative disease. Although the specific signalling around neuroprotective and anti-inflammatory effects of SCFAs are not completely understood, it has thus far been attributed, at least in part, to their histone deacetylase (HDAC) inhibitory action. First, SCFAs are well known to have anti-inflammatory effects [190] and to be involved in the modulation of microglial function [115]. For example, butyrate can decrease microglial activation and pro-inflammatory cytokines in vivo [191, 192]. Second, SCFAs are able to modulate neurotransmitter synthesis and expression. For example, butyrate and propionate can control catecholamine synthesis by regulating tyrosine hydroxylase gene expression [193]. This is very interesting for PD research in particular, as tyrosine hydroxylase is an enzyme involved in dopamine synthesis. Moreover, SCFAs can modulate the concentrations of other neurotransmitters such as glutamate, glutamine, and GABA [194].

These promising neuroprotective and anti-inflammatory properties make SCFAs a good candidate for a potential therapeutic agent in neurodegenerative diseases; for example, HDAC dysregulation has been implicated in memory impairment, and levels of SCFAs are reduced in preclinical models of AD [195]. In a rodent model of AD, sodium butyrate was able to improve associative memory and increase expression of genes associated with learning even at advanced stages of pathology [196]. Butyrate has also shown promising beneficial effects in improving cognitive and motor impairments while reducing dopamine neurodegeneration in several

animal models [197, 198]. If we look at MS, oral administration of SCFAs ameliorated the disease severity in an EAE model [199], and butyrate in particular was able to suppress demyelination and enhance remyelination [200].

SCFAs can also exert anti-inflammatory effects via astrocytes, as SCFAs downregulate the astrocytic production of IL-1β and TNF-α [201]. Further, SCFAs can also contribute to reduce inflammation by inhibition of NF-kB on peripheral blood mononuclear cells, which further reduces the production of pro-inflammatory cytokines [202].

These results provide strong evidence that SCFAs can regulate several CNS processes related to neurodegeneration as well as modulate cognitive and motor behaviours, especially when administered in advanced stages of neurodegeneration. However, caution has to be taken when extrapolating these results to humans as these were mainly observed in animal models.

Ferulic acid (FA) is a gut-derived compound found in fruits and vegetables that can also be synthetised by gut bacteria. FA can prevent Aβ toxicity by inhibiting Aβ aggregation both in vitro and in vivo [203]. Two long-term studies assessed the benefits of oral administration of FA in transgenic mouse models of amyloidosis and found that FA reversed spatial memory deficits, reduced Aβ aggregates in the brain, attenuated neuroinflammation, and stabilised oxidative stress [203, 204].

Dysregulation of the kynurenine pathway is associated with neurodegenerative and other neurological disorders. Targeted interventions with metabolites from the kynurenine pathway could potentially be used to modulate brain physiology and normalise imbalances in this pathway in pathological conditions. For example, indoleamine 2,3-dioxygenase (IDO) inhibitors are being investigated for their protective role against oxidative damage [205]. Since gut microbiota are a key regulator of the kynurenine pathway, probiotic products may be potentially beneficial in regulating kynurenine/tryptophan dynamics [205].

Recently, researchers manipulated disease severity in a rodent model of ALS via supplementation with gut microbial strains; where *Ruminococcus torques* and *Parabacteroides distasonis* increased severity of the disease, *Akkermansia muciniphila* ameliorated it [206]. Interestingly, this reduction of the pathogenesis by *Akkermansia muciniphila* was attributed to increasing levels of nicotinamide, together with changes in mitochondrial function and oxidative stress pathways. Moreover, nicotinamide was associated with functional improvements in ALS patients [207]. Also the therapeutic potential of hydrogen sulphide and molecular hydrogen was tested in mice; it was shown that hydrogen-rich saline administration could preserve mitochondrial function and reduce ROS production [208].

7.2 Probiotic-Based Interventions

Probiotic interventions are being screened in several contexts including neuroprotection, neurodegeneration, and inflammation. These studies inform us of the potential of these probiotic products (see Table 3). For example, a combined

Table 3 Clinical studies assessing microbiota-based interventions for neurodegenerative diseases

Type of study	Intervention	Results
Alzheimer's disease		
Double-blind, randomised, controlled [209]	Patients were given milk ($n = 30$) or a probiotic ($n = 30$) containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum* for 12 weeks	Probiotic treatment resulted in improvements in cognitive function scores and a significant change in metabolic profile
Double-blind, randomised, placebo-controlled [210]	Patients were given either selenium (200 μg/day) plus probiotic containing *L. acidophilus*, *B. bifidum*, and *Bifidobacterium longum* ($n = 27$), selenium (200 μg/day) ($n = 26$), or placebo ($n = 26$) for 12 weeks	Co-supplementation of selenium and probiotic treatment resulted in improvements in cognitive function scores and improvements in some metabolic profiles
Double-blind, randomised, placebo-controlled [211]	Patients were given placebo ($n = 23$) or a probiotic ($n = 25$) containing *L. fermentum*, *Lactobacillus plantarum*, *L. acidophilus*, *Bifidobacterium lactis*, *B. longum*, and *B. bifidum* for 12 weeks	No significant changes in cognitive deficit scales or biochemical measurements between probiotic and placebo groups
Parkinson's disease		
Double-blind, randomised, placebo-controlled [212]	Patients were given a placebo ($n = 60$) or a fermented milk ($n = 80$), containing multiple probiotic strains and prebiotic fibre for 4 weeks.	Probiotic and prebiotic treatment resulted in a significant increase in complete bowel movements in patients with PD compared with patients in the placebo group
Double-blind, randomised, placebo-controlled [213]	Patients were given placebo ($n = 38$) or probiotic capsules ($n = 34$) containing *L. acidophilus*, *L. reuteri*, *Lactobacillus gasseri*, *Lactobacillus rhamnosus*, *B. bifidum*, *B. longum*, *Enterococcus faecalis*, and *Enterococcus faecium* for 4 weeks	Probiotic treatment resulted in the reduction of constipation (increased spontaneous bowel movements)
Double-blind, randomised, placebo-controlled [214]	Patients were given placebo ($n = 30$) or probiotic capsules ($n = 30$) containing *L. acidophilus*, *B. bifidum*, *L. reuteri*, and *L. fermentum* for 12 weeks	Probiotic treatment resulted in improvements in motor function scores and significant improvements on metabolic profiles
Double-blind, randomised, placebo-controlled [215]	Patients were given placebo ($n = 25$) or probiotic capsules ($n = 25$) containing *L. acidophilus*, *B. bifidum*, *L. reuteri*, and *L. fermentum* for 12 weeks	Probiotic supplementation improved gene expression of some pro-inflammatory markers (IL-1, IL-8, TNF-α, TGF-β, and PPAR-γ) but did not change biomarkers of inflammation and oxidative stress
Double-blind, randomised, placebo-controlled [216]	Patients were given fermented milk as placebo ($n = 26$) or probiotic capsules ($n = 22$) containing *L. acidophilus*, *L. casei*, *L. lactis*,	Probiotic treatment resulted in the reduction of constipation (increased gastrointestinal time). No changes

(continued)

Table 3 (continued)

Type of study	Intervention	Results
	B. infantis, and *B. longum* plus 2% fructooligosaccharide and lactose for 8 weeks	were observed in motor function scores
Multiple sclerosis		
Double-blind, randomised, placebo-controlled [217]	Patients were given placebo ($n = 30$) or a probiotic capsule ($n = 30$) containing *L. acidophilus*, *Lactobacillus casei*, *B. bifidum*, and *L. fermentum* for 12 weeks	Probiotic intake improved disability, general health, and depression scales as well as parameters of inflammatory factors and markers of insulin resistance
Double-blind, randomised, placebo-controlled [218]	Patients were given placebo ($n = 30$) or a probiotic capsule ($n = 30$) containing *L. acidophilus*, *L. casei*, *B. bifidum*, and *L. fermentum* for 12 weeks	Probiotic supplementation improved gene expression of some pro-inflammatory markers (IL-8, TNF-α) but not others
Controlled [219]	Patients ($n = 9$) and controls ($n = 13$) were orally administered with a probiotic containing *Lactobacillus paracasei*, *L. plantarum*, *L. acidophilus*, *Lactobacillus delbrueckii bulgaricus*, *B. longum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, and *Streptococcus thermophilus* twice daily for 2 months	Probiotic administration increased the abundance of taxa known to be reduced in patients with MS such as *Lactobacillus* and decreased abundance of taxa associated with dysbiosis such as *Akkermansia*, *Dorea*, and *Blautia*
Double-blind, randomised, placebo-controlled [220]	Patients were given placebo ($n = 24$) or a probiotic capsule ($n = 24$) containing *B. infantis*, *B. lactis*, *Lactobacillus reuteri*, *L. casei*, *L. plantarum*, and *L. fermentum* for 12 weeks	Probiotic intake improved disability, general health, and depression scales as well as parameters of inflammatory factors

administration of *Lactobacillus helveticus* and *Bifidobacterium longum* in a myocardial infarction model reduced pro-apoptotic pathways (caspase-3 and Bax/Bcl-2) and increased anti-apoptotic pathways (Akt phosphorylation), suggesting a role in neuroprotection [221]. *Clostridium butyricum* was also reported to have neuroprotective effects in a vascular dementia model in rats by increasing brain-derived neurotrophic factor (BDNF), Bcl-2, and Akt phosphorylation [222].

Moreover, probiotic administration can modulate long-term memory. In a rodent AD model, administration of *Lactobacillus* and *Bifidobacterium* strains improved memory and learning outcomes and reduced oxidative stress in the hippocampus [223]. In a similar study, along with behavioural recovery and a reduction of Aβ plaques, a probiotic mix formulated of lactic acid bacteria and bifidobacteria was able to partially restore proteasome and autophagy functionality [224]. In a recent study using APP/PS1 transgenic mice, exercise training and probiotic administration reduced Aβ plaques in the hippocampus and improved cognitive performance in a

spatial learning task [225]. Such investigations demonstrate that disease progression could potentially be ameliorated by microbiota-targeted approaches.

For instance, probiotics could reduce duration of the clinical symptoms or reduce their severity, while also reducing levels of pro-inflammatory cytokines in the EAE rodent model of MS [226]. Similarly, a combination of *Lactobacillus* strains produced these same effects by inhibiting pro-inflammatory activation of Th17 cells, while enhancing IL-10^{+} producing regulatory T cells [227].

Hence, preclinical investigations have shown the potential use of probiotics as a therapeutic strategy against neurodegeneration. However, few clinical investigations have been carried out to date investigating the potential benefits of probiotic products in neurodegenerative diseases (see Table 3). Overall, these clinical trials have shown that probiotic products can modulate gut microbiota composition, ameliorate comorbidities such as constipation, and even improve cognitive and motor deficits.

However, there are few clinical trials available to date, with low numbers of patients, and they only assessed the effects of short-term use of probiotics. Moreover, the results of these clinical trials are based on limited analysis of the microbiome and the cognitive and motor functions. Nevertheless, they are a first step into the evaluation of probiotics as a potential therapeutic avenue for neurodegenerative diseases and their comorbidities.

7.3 Antibiotic-Based Interventions

Antibiotic administration is another effective means of gut microbiome modulation. In vitro, many antibiotics can prevent or reduce protein aggregation in the context of neurodegenerative disease. Further, antibiotics such as ceftriaxone—which is a β-lactam antibiotic—have been shown to have neuroprotective and anti-inflammatory effects in many neurodegenerative diseases. For instance, in an AD transgenic mouse model, ceftriaxone reduced the increased levels of glutamate present in the vicinity of Aβ plaques and restored neuronal activity via glutamate transporter 1 [228]. There are several mechanisms by which ceftriaxone could act, including upregulation of glutamate transporter 1 expression, as well as the amelioration of oxidative stress and neuroinflammation [229]. Preventive and therapeutic treatment of ceftriaxone in an EAE mouse model indirectly hampered T cell proliferation and pro-inflammatory cytokine secretion [230]. Thus, antibiotic treatment can attenuate disease course and severity in a rodent model of MS.

Rifampicin inhibited the aggregation and fibril formation of synthetic Aβ peptides [231]. Similarly, doxycycline induced remodelling of α-synuclein oligomers into non-toxic species in vitro [232] and prevented Aβ fibrillisation both in vitro and in vivo [233]. Moreover, a combination of long-term broad-spectrum antibiotics decreased Aβ plaque deposition in a rodent model [234]. However, when doxycycline and rifampicin (alone or in combination) were tested in clinical trials, they had no beneficial effects on cognition in AD patients [235]. However, eradication of

Helicobacter pylori resulted in improvement of cognition outcomes in AD patients [236] and in motor improvements in PD patients [237]. These results indicate that antibiotic administration could be an interesting therapeutic option for particular cases dealing with detrimental bacteria, instead of using of antibiotics as a universal therapy for all neurodegenerative diseases.

AD might be the best candidate to test antibacterial drugs on since it has been postulated that it could have an infectious aetiology. Thus, testing antibiotics in clinical trials could shed some light onto this issue and verify if antibacterial therapy could be beneficial for a subset of (or all) AD patients [238].

7.4 Faecal Microbiota Transplantation

FMT can reconstruct the healthy gut microenvironment and alleviate clinical symptoms of many metabolic, autoimmune, and neuropsychiatric diseases. Recently, FMT has been postulated as a potential therapeutic intervention to restore the microbiome in neurodegenerative disease. Despite limited information about its long-term benefits and risks, some case reports have confirmed the efficacy of FMT for use in the treatment of neurological disorders [239]. In MS, two case reports have shown amelioration or stabilisation of MS symptoms several years after the transplant [240, 241]. In PD, one report stated that a PD patient observed improvements in constipation until the end of the follow-up 3 months after FMT, but no long-term motor improvements [242]. In a more recent study, 15 PD patients were exposed to a colonic or nasointestinal FMT and concluded that although both procedures were safe, colonic FMT achieved significant improvement and longer maintenance of efficacy than nasointestinal FMT [243]. In this study, two patients reported self-satisfying outcomes that lasted for more than 2 years [243]. In AD, there is only one case reported of a patient with rapid reversal of AD symptoms following FMT for recurrent *Clostridioides difficile* infection [244].

Currently, there are two randomised double-blind clinical trials assessing the safety and efficacy of FMT for PD patients with or without constipation (NCT04854291, NCT03808389) and other minor clinical pilot studies with the same aim (NCT03876327, NCT04837313). In parallel, other FMT clinical trials are ongoing at the moment, evaluating the safety, feasibility, and efficacy of FMT in AD patients (NCT03998423) and in ALS patients (NCT03766321). We will have to wait for their findings. However, a clinical trial of FMT for MS (NCT03183869) was finalised recently, reporting that FMT did not have any serious adverse effects, but no measures of efficacy have been reported yet.

The benefits of FMT as a therapeutic intervention in neurodegenerative disease are mostly based on animal studies and only a few case reports. Despite promising results, large-scale clinical trials are needed to evaluate the efficacy of this treatment option. Numerous trials of FMT in neurodegenerative diseases are currently ongoing, and it is expected that evidence on the efficacy of FMT will increase in the near future. Furthermore, these ongoing clinical trials will improve the logistics of FMT

that still need to be refined, such as best donor selection or mode of delivery of the microbiota.

Even if microbiota-targeted interventions prove not to be successful in the goal of stopping or ameliorating the progression of neurodegenerative diseases, they could still be very beneficial in treating gastrointestinal comorbidities.

7.5 Microbiota Modulation Through Diet

Diet has a major impact on gut microbiota. Hence, it has been postulated that diet could be a beneficial avenue for treatment of neurodegenerative diseases, as they are usually characterised by a prevalent and strong microbiota dysbiosis. For example, antioxidants can directly act on gut microbiota to reduce pathogenic bacteria and increase beneficial bacteria [245]. Consequently, these beneficial bacteria can produce beneficial metabolites for brain health, conferring neuroprotection.

Many dietary compounds such as PUFAs, vitamins B and D, or resveratrol have been found to be beneficial with anti-inflammatory properties [246]. In ALS, many preclinical investigations have shown that polyphenols such as resveratrol or curcumin could improve the prognosis of the disease [247]. Some compounds such as vitamin C have largely been investigated in preclinical studies as a treatment option for neurodegenerative disease, but clinical data in humans are limited [248].

However, a growing body of evidence points to the combination of these compounds as a more efficient way to fight neurodegeneration. Clinical and preclinical data assessing dietary interventions for neurodegenerative disease is extensive and has mostly been studied in AD. For example, the ketogenic (high-fat and low-carbohydrate) diet forces the brain to use fatty acids as the main source of energy and alter energy metabolism mechanisms. These metabolic changes reduce the usage of impaired glucose metabolism in neurodegenerative pathologies and neuroinflammation, while improving mitochondrial function, thus conferring neuroprotection to ageing brain cells [249]. In addition, this diet could help to reduce the accumulation of amyloid plaques. Two clinical trials assessed the effects of triglyceride administration on AD patients, resulting in improved cognitive outcomes [250, 251].

Further, adherence to a Mediterranean-styled diet could be a potential preventive therapy as it confers a reduced risk of developing AD and cognitive impairment [252, 253]. Moreover, it has been hypothesised that the Mediterranean diet—abundant in antioxidants, vitamins, flavonoids, polyphenols, and probiotics—could attenuate neuroinflammation via the gut microbiome. The Mediterranean dietary approach to systolic hypertension (DASH) diet intervention for neurodegenerative delay (MIND) diet (that combines Mediterranean and DASH diets) is specific for dementia prevention and can slow cognitive decline [254]. Although clinical trials have shown interesting results, there is a paucity of data surrounding the long-term benefits of these interventions in patients with neurodegenerative disease.

8 Conclusions

Neurodegenerative diseases are a heterogeneous group of disorders where neurons degenerate and ultimately die. These diseases have an unknown cause and include many complex pathological processes that have frustrated the development of a remedy or cure to stop neurodegeneration. However, the scientific knowledge gathered has greatly expanded our general understanding and treatment of neurodegenerative disease. The expert view has shifted from being neuron centric to a more global disease where even entities such as the gut microbiota are now considered.

Gut microbiota have been shown to be implicated in the pathogenesis of neurodegenerative disease, although to what extent remains to be elucidated. It is quite likely that single bacterial perturbations will not be adequate, and perhaps there will not even be a disease-specific bacterial signature but rather an overall alteration of the microbial gut environment. Nevertheless, a new era of potential microbiota-targeted interventions has emerged.

Despite numerous failures in developing therapeutic interventions that can effectively modify the course of the disease, researchers have now new molecular tools to investigate the underlying pathogenic mechanisms involved and assess the efficacy of new compounds or therapeutic interventions. Currently, the evidence supporting a beneficial impact on neurodegeneration due to microbiome modification is limited.

It will be interesting to observe if psychobiotics are assessed in neurodegenerative diseases in the future. Psychobiotics are live organisms that can produce health benefits in patients suffering from psychiatric illnesses through the microbiota-gut-brain axis [255].

The evidence that gut microbiota may be involved in the onset or progression of many neurodegenerative diseases is increasing rapidly, but causality has not been proven. However, gut microbiota could be used as a clinical biomarker for the diagnosis of many neurodegenerative diseases. Furthermore, there is an opportunity to establish potential microbiome-targeted therapies to treat particular aspects of neurodegenerative disease, such as common comorbidities, resulting in improvements of host health.

Nevertheless, adequately powered longitudinal studies are needed to investigate the complex relationship between neurodegenerative disease and the microbiome and should be studied at the onset, the initial progression, and the establishment of the neurodegenerative processes. Of high importance would be the appropriate selection of patients and adequate management of confounding variables. Many factors that were overlooked in traditional neurological studies could have confounding effects in the microbiome field. This could present opportunities for interdisciplinary approaches for the treatment of neurodegenerative disease.

Compliance with Ethical Standards

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Clinical Application of the Biodiversity Hypothesis in the Management of Allergic Disorders

Tari Haahtela

Abstract Advances in understanding the environmental and lifestyle factors explaining the rise of allergic disorders in modern, urban societies have paved the way for change of management and disease prevention. The *biodiversity hypothesis* states that contact with the natural environment enriches the human microbiome, promotes immune balance, and protects from disease. The evidence is still mainly associative, but prevention practices are changing. Based on the new ideas, Finland is the first country to implement a nationwide, systematic programme (*the Finnish Allergy Programme 2008–2018*) to mitigate the overall allergy burden. The prevention strategy was turned from avoidance of allergens to promotion of immunological and psychological resilience. Allergy health and nature relatedness were emphasized. Medicalization, especially in food allergy was reduced and allergy diagnostics certified. The 10-year results are promising; patients are less disabled, attitudes have changed, and major cost savings have been obtained. In asthma, the first-line anti-inflammatory treatment was a paradigm shift in the 1990s and resulted in a major change for the better. The second paradigm shift, in the 2000s, was the *Nature Step* from treatment to prevention for all allergic disorders. The Finnish experience shows the power of implementing new knowledge and the utility of real-world data in outcome evaluation.

Keywords Allergic disorders · Allergy prevention · Allergy programme · Asthma · Biodiversity · Immunological resilience · Implementation · Public health · Real-world data

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1 Introduction

The present chapter describes two paradigm shifts of the management and prevention of asthma and allergic disorders and their nationwide implementation into practice during the last 25 years. The experience is from a Nordic country, Finland, with a population of 5.5 million, which joined the European Union in 1995. The background and the process of planning and implementation of the latest public health action, *The Finnish Allergy Programme 2008–2018*, are described. The main results, including the health outcomes with direct (healthcare) and indirect costs (disability), are also briefly reviewed. The midterm and full results as well as some information about the implementation have been published in detail elsewhere [1–3].

1.1 The First Paradigm Shift to Reduce the Asthma Burden in the Short Term

Asthma has been increasing for decades in Finland and elsewhere [4, 5]. *The Finnish Asthma Programme* (1994–2004) was implemented to improve early diagnostics and treatment of the disease [6]. The programme was based on new evidence of asthma as an *inflammatory disorder* from the very beginning [7]. Accordingly, the anti-inflammatory treatment should be commenced as a first-line therapy immediately after reaching the diagnosis [8]. An ambitious but realistic action plan was implemented in the whole country. A change for the better was recorded in a relatively short period of time [6]. The burden of asthma both to individuals and society started to decline (mortality, hospitalizations, disability). Cost savings were also significant [9]. The patients needed fewer sick leave days from work and disability pensions; they had less symptoms and lived relatively normal lives in spite of the disease. This was the *first paradigm shift*, which was implemented nationwide, without a delay, with favourable results.

1.2 The Second Paradigm Shift to Reduce the Allergic Disease Burden in the Long Term

While the treatment and medication had improved, the challenge and dilemma remained. How to tackle the ‘epidemic’ in modern society, to turn down the occurrence and long-term burden for citizens and society? How to take steps from treatment to primary and secondary prevention?

From the 1950s, the *Great Acceleration* of human activity coincides with the *Anthropocene*, a title suggested for a geological epoch of human impact on Earth’s ecosystems [10]. Health and life expectancy have improved in high-income countries but much at the expense of environment. Population explosion, escalating

urbanization, and overuse of natural resources have become the rule. The increase in emissions of greenhouse gases, global warming, massive extinction of species, and pollution are all part of the Anthropocene. We might be losing resilience as individuals and communities, and we face epidemics of both communicable (fast) and non-communicable (slow) diseases, with unpredictable outcomes [11].

The dawn of non-communicable diseases was evident in the 1960s, when an increase of allergic diseases and asthma also became obvious in most developed countries. Indeed, they are good indicators of the modern health hazards, as shown, for example, in Finnish and Russian Karelia [12]. In a relatively short period of time, after the Second World War, two geoclimatically and genetically close populations have developed contrasting immunological expression. Currently, close to 40% of Finnish schoolchildren and young adults appeared to be sensitized to one or more common environmental allergens [13, 14]. In Russian Karelia, sensitization rate was much less, hay fever was rare, food allergies were few, and peanut allergy was unknown. The contrast was neither explained by hereditary factors nor by air pollution or common chemicals but rather by changes in lifestyle and environment. Understanding the underlying reasons of this disparity would enable measures for prevention.

The *biodiversity hypothesis* emerged from the *Karelia Allergy Study* and stated that contact with the natural environment enriches the human microbiome, promotes immune balance, and protects from disease [15–19]. This was the *second paradigm shift*.

Microbe-immune system interplay is decisive for resilience and the immune homeostasis. If the crosstalk is not versatile enough, dysregulation arises. Reduced contact to environmental microbial diversity is probably the main reason for the compromised immunological resilience of populations living in the modern, urban environment [20, 21]. In logistic regression models, risk factors of the disease in question are evaluated, but the models seldom identify protective factors as mediators of reduced risk or confounders. Indeed, revisiting the allergy paradigm was needed as loss of protective factors, i.e. loss of immune tolerance/resilience, seemed to be a more important determinant of the 'allergy epidemic' than any possible new risk factor.

Allergy is not an isolated case but concurrent with the increase of both type I and II diabetes, cardiovascular diseases, obesity, inflammatory bowel diseases, and even mental disorders and cancer [22, 23] (Fig. 1).

1.3 Incentive for the Nationwide Action Plan

Signs of this problem were discernible already in the 1980s, when the first allergy management guideline in Finland was published [24], and in 1998, when an expert consensus report was prepared [25]. In the 1980s and 1990s, the number of allergic and asthmatic patients grew to a point that neither children nor adults with allergies were able to receive satisfactory medical care in the public sector. Medicalization

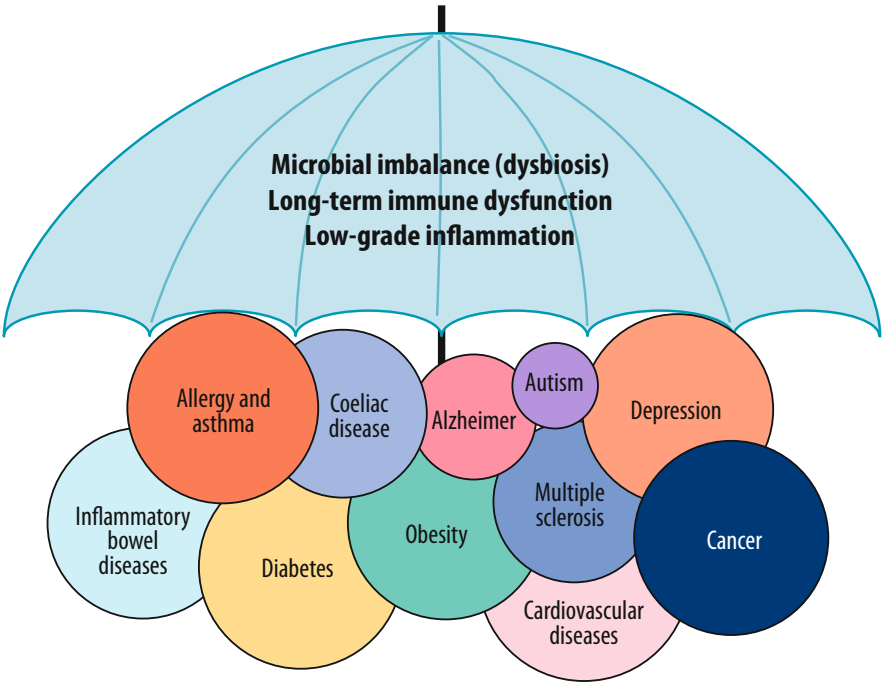

Fig. 1 Allergic disorders and asthma are examples of a large group of chronic diseases, which have been on the rise in modern, urbanizing societies. They all share common features. Individual genetic and epigenetic regulatory factors influence the manifestation of any clinical disease. ([23], with permission https://creativecommons.org/licenses/by/4.0/)

became visible as new 'allergy day-care centres', 'allergy schools', and 'allergen-free environments' in workplaces were demanded. At the same time, new evidence from large trials indicated that avoidance of allergens to prevent clinical symptoms is not a feasible strategy at the population level.

A Finnish expert group agreed that the focus should be turned from avoidance to endorsing both immunological and psychological resilience at the individual as well as at the population level. Children, adolescents, and families needed special attention [26]. A major change of attitudes among healthcare professionals, patients, and lay public was called for. *The Finnish Allergy Programme 2008–2018* was initiated to answer the concerns and to reduce both the short- and long-term burden of allergic conditions and asthma among individuals and in the society.

2 Implementation of the Programme

2.1 Planning

In 2006, the National Institute for Health and Welfare nominated a multidisciplinary counselling group to evaluate the most recent scientific data on allergy management and prevention. A smaller working group prepared the 10-year programme, which was launched in April 2008 [27–29]. The body continued as a programme steering group. The organization was kept simple (Fig. 2).

Allergic disorders are multifaceted and a more complex entity than asthma. The goals and foci of the novel programme had to target the central problems and had to be plausible, pragmatic, and achievable. The programme was based not only on the most recent scientific data but also on long clinical experience, which is important in pursuing change in attitudes and management. When planning prospectively, not all predictions are based on strong scientific evidence. Strategies were chosen, goals set, and tools and evaluation methods defined (Fig. 3).

Allergic diseases cause symptoms, but in the programme the concept of allergy was also linked to health. The idea of *allergy health*, i.e. the idea that one could enjoy a good life even with allergies, was promoted. For example, mild symptoms in childhood are often part of normal immune development and not a reason for any special guidance or intervention [26].

The Allergy Programme revisited old dogmas and attitudes. In prevention and management at the population level, avoidance and fear of all allergen exposure are not the most useful strategies. It can lead to medicalization, isolation, actions that

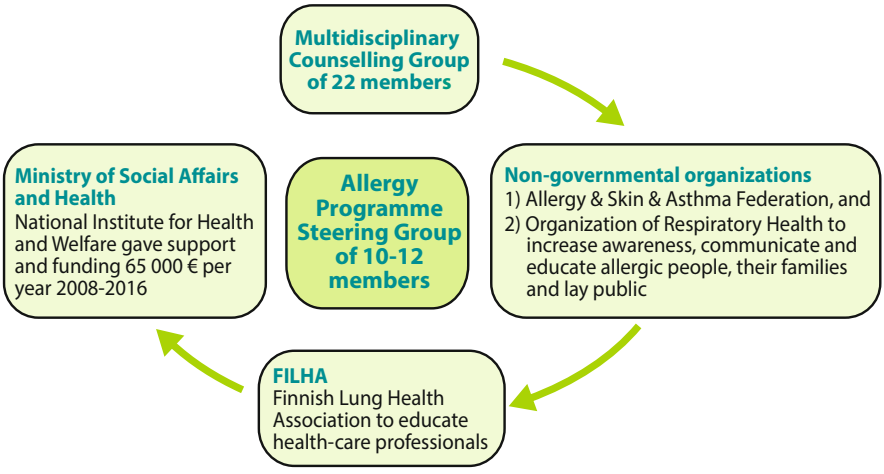

Fig. 2 The programme steering group supervised the programme implementation. The Finnish Lung Health Association (FILHA), a professional non-governmental organization (NGO), implemented the education for healthcare. Patient NGOs ran a campaign to inform patients, their families, and lay public. ([3], with permission)

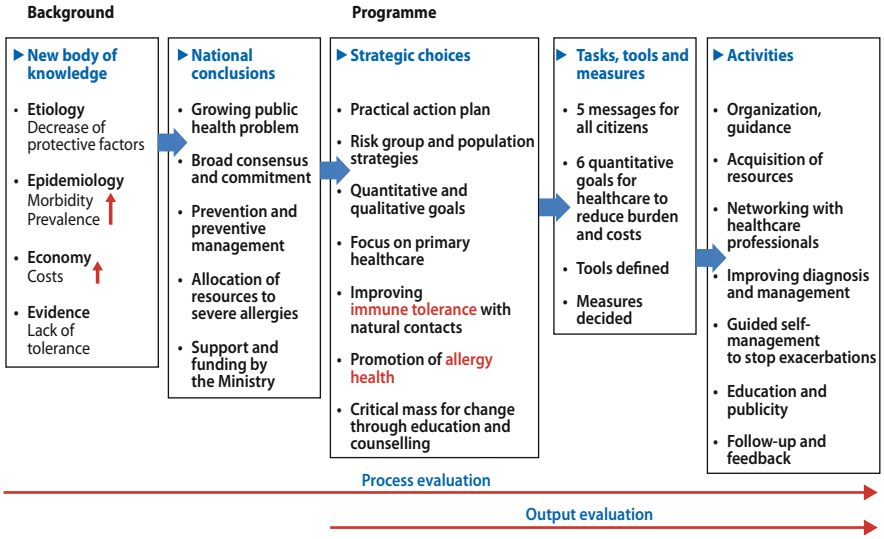

Fig. 3 The strategic planning of the Finnish Allergy Programme 2008–2018. (Adapted from [2] and [27] with permission)

deteriorate daily living, and, in the worst case, serious reactions if exposure occurs unexpectedly (e.g. food allergen exposure). Avoidance of allergens will remain good clinical practice for individual patients, e.g. in severe food allergy. But avoidance must be justified and have precise grounds and defined time limits. Psychosocial factors should also be addressed as they play an important role in patient decisions and adherence to treatment. Tolerance/resilience is both immunological and psychological.

2.2 National and International Collaborators

National collaborators in the Allergy Programme were (1) the Ministry of Social Affairs and Health; (2) the National Institute for Health and Welfare; (3) the Social Insurance Institution; (4) the Finnish Institute of Occupational Health; (5) the Association of Finnish Pharmacies; (6) medical specialist associations, related to allergy and asthma; (7) the Finnish Lung Health Association FILHA (non-governmental organization, NGO for professionals); and (8) the Allergy, Skin and Asthma Federation as well as the Organization for Respiratory Health (NGOs for patients). The NGOs (7, 8) were responsible for the implementation of the programme.

The Finnish initiative was supported by the Global Alliance Against Chronic Respiratory Diseases (GARD), the World Health Organization (WHO), a voluntary alliance of national and international organizations, and institutions and agencies focused on improving global lung health [30]. The Allergy Programme also

Fig. 4 The Allergy Programme was implemented by the Finnish non-governmental organizations (NGOs) for healthcare professionals, patients, and the lay public. It was supported by several international organizations

benefited from support of the European Academy of Allergy and Clinical Immunology (EAACI), the Global Allergy and Asthma European Network (GA2LEN), the Global Initiative for Asthma (GINA), the Allergic Rhinitis and its Impact on Asthma (ARIA) [31], and the World Allergy Organization (WAO) [17]. Importantly, the European Federation of Allergy and Airways Diseases Patients' Associations (EFA) promoted the programme from the beginning.

The international dimensions of the programme may help others to create better models, while learning from the successes and failures of the Finnish initiative. Preventing allergies and asthma will be particularly important outside Europe, in areas with developing national economies [32] (Fig. 4).

2.3 Implementation

2.3.1 Messages and Goals

The key messages targeted all citizens, the lay public, patients with allergic diseases and asthma and their families, public health and patient organizations, experts, and authorities (Table 1).

Table 1 The key messages of *the Finnish Allergy Programme 2008–2018* both for healthcare professionals and the lay public. Allergy health was promoted [27]

Key messages
• Endorse health, not allergy
• Strengthen tolerance/resilience
• Adopt a new attitude to allergy; avoid allergens only if mandatory
• Recognize and treat severe allergies early; prevent exacerbations
• Improve air quality; stop smoking

Table 2 The six goals and their indicators for *the Finnish Allergy Programme 2008–2018* [27]

1. Prevent allergy
Indicator: asthma, rhinitis, and atopic eczema prevalence reduce by 20%
2. Improve tolerance
Indicator: food allergy diets reduce by 50%
3. Improve allergy diagnostics
Indicator: patients are skin prick tested in certified testing centres
4. Reduce work-related allergies
Indicator: occupational allergies reduce by 50%
5. Focus on severe allergies and treat in time
Indicator: good allergy practice works; asthma emergency visits reduce by 40%
6. Reduce allergy and asthma costs
Indicator: allergy costs reduce by 20%

The more specific goals and indicators for healthcare professionals were quantitative, such as allergy diets should drop by 50% and asthma emergency visits by 40% within 10 years (Table 2). Each of the six goals had its specific tasks, tools, and evaluation methods. Tasks were the activities or targets in pursuing the goal. Tools were means by which the tasks were carried out. Evaluation methods were the verification of outcomes [27]. The specific goal was reached if the indicator actualized.

The relevance and acceptance of the programme messages were tested in 2008 in an email survey among 744 asthma contact persons [1]. The messages were well received. For example, GPs scored *strengthen tolerance* as 9.1 on a scale from 4 to 10. Allergy management practice left, however, much room for improvement, e.g. availability of *allergen immunotherapy* was poor (score 5.4).

2.3.2 Contact Person Network

The *Finnish Allergy Programme 2008–2018* was a systematic educational action plan, which took advantage of the contact person network created during the previous Asthma Programme 1994–2004. In each municipal health centre, there were asthma contact persons (in 2008, 200 physicians and 580 nurses specifically trained in asthma). Similarly, in pharmacies, 695 pharmacists had been educated as asthma contact persons (94% coverage of the pharmacies in Finland). These networks were strengthened for the allergy campaign, and a new allergy contact network, with some 200 nurses, was created for the local maternity and child health clinics and schools.

The Finnish Lung Health Association (FILHA), a non-governmental organization for professionals, was responsible for educating healthcare workers (doctors, nurses, pharmacists, medical and nursing students, dieticians). The key issue was improving allergen tolerance/resilience, and simple guidance was provided (Table 3). Allergy

Table 3 Practical advice for building and improving tolerance/resilience (primary prevention) as well as preventing symptoms and exacerbations (secondary and tertiary prevention) [1]

Primary prevention
• Support breastfeeding, with solid foods from 4 to 6 months
• No avoidance of environmental allergen exposure (foods, pets), if not proven necessary
• Strengthen immunity by increasing contact with natural environments (e.g. regular physical exercise, healthy diet such as a traditional Mediterranean or Baltic diet, local food)
• Antibiotics only for true need (majority of microbes are useful and build a healthy immune function)
• Probiotic bacteria in fermented food or other preparations may strengthen immune function
• No smoking (parental smoking increases the risk of asthma in children)
Secondary and tertiary prevention
• Regular physical exercise is anti-inflammatory
• Healthy diets are anti-inflammatory (traditional Mediterranean or Baltic diet may improve asthma control)
• Probiotic bacteria in fermented food or other preparations may be anti-inflammatory
• Respiratory/skin inflammation treated early and effectively. Maintenance treatment titrated for long-term control
• Allergen immunotherapy promoted for more severe symptoms
– Allergens as such (for foods)
– Sublingual tablets or drops (SLIT) (for pollens, house dust mites)
– Subcutaneous injections (for pollens, pets, mites, insect stings)
• Smoking strictly avoided (asthma and allergy drugs do not have full effect in smokers)

treatment practices and guided self-management were also a focus of this educational effort.

The lay public was targeted by two NGOs for patients: (1) the Allergy, Skin and Asthma Federation and (2) the Organization for Respiratory Health. They arranged regional education for their personnel and peer workers, which had a major impact upon direct patient counselling and distribution of educational material.

3 Education, Communication

3.1 Healthcare Professionals

In 11 years, 376 educational sessions for healthcare professionals gathered approximately 24,000 participants all over the country (Fig. 5).

Educational meetings covered a variety of topics essential to prevent and treat allergic disorders (allergy health, allergy-healthy child, allergic rhinitis, anaphylaxis, asthma, atopic dermatitis, food allergy, guided self-management, immunotherapy, indoor air, more tolerance, take care of allergy and asthma, allergy in military service). The activities were organized by a project coordinator at *FILHA*, in collaboration with local healthcare professionals. In practice, two people at *FILHA*

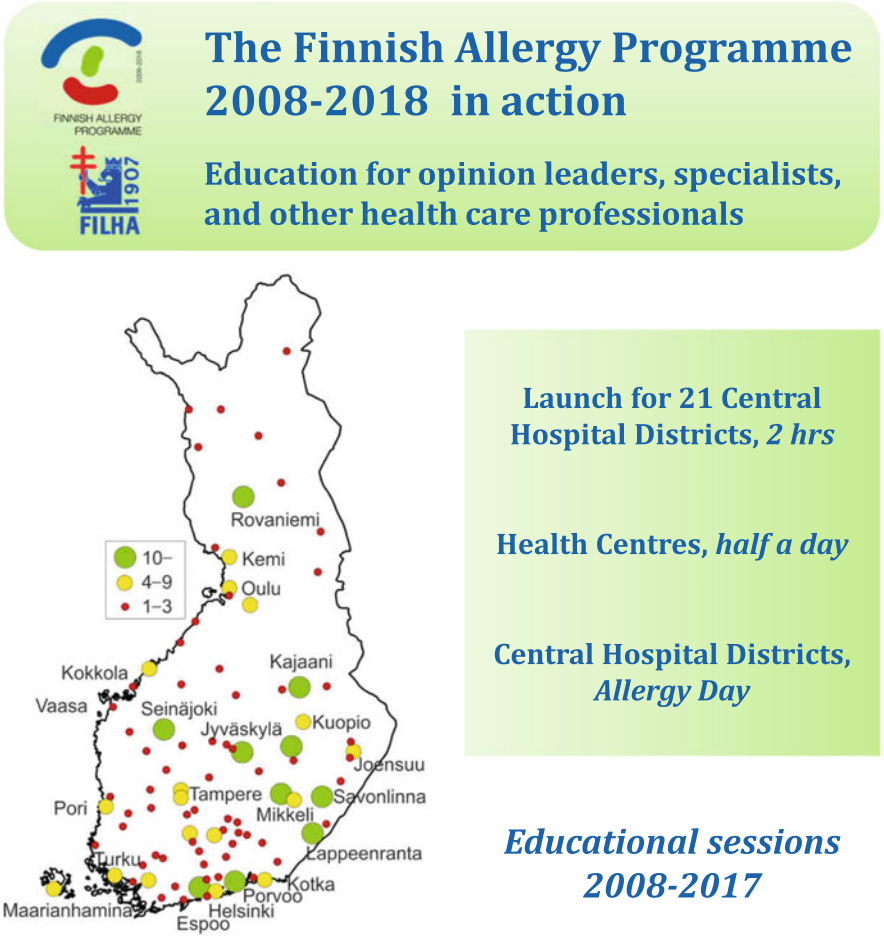

Fig. 5 The Finnish Allergy Programme in action. Green circles indicate cities and municipalities hosting 10 or more, yellow circles 4–9, and red circles 1–3 educational sessions during the period from 2008 to 2019

coordinated and implemented the educational activities. The meetings were free of charge for the participants and arranged mainly at the workplaces during working hours (whole day meetings from 8 am until 4 pm, half day meetings from noon until 4 pm). Meetings were multidisciplinary. Half of them were targeted only for primary care taking place in the primary care settings. Meetings for all healthcare were arranged in the central and university hospitals.

The role of local professionals and experts was most valuable. Topics of the meetings were planned according to the goals and key messages of the programme, but they were tailored with local people to meet their needs. Presentations at the meetings were not only given by the project coordinator and medical advisor from *FILHA* but also by local doctors, nurses, pharmacists, and dieticians. Educational

meetings included plenty of time for questions and discussion. Local implementation of the new strategies is the key to improvements and needs time to change ideas and take up challenges, doubts, and unmet needs. From the very beginning, empowerment and counselling of local people and champions were in focus. They implement or resist the new ideas in everyday practice.

Educational material for professionals was developed by the programme steering group, project coordinator, medical advisor, or the other key opinion leaders. Educational material produced by the pharmaceutical industry supplemented this material if the steering group checked and confirmed its objectivity. Industry could have an information stand for their products. Educational material and the presentations and lectures in the meetings were available in the *FILHA* website (www.filha.fi) if the speakers agreed.

To stop exacerbations of asthma and other allergic disorders, which cause most of the disability and costs, ten simple *self-management guides* were created (asthma, childhood asthma, rhinitis, conjunctivitis, atopic eczema, childhood eczema, urticaria, angioedema, hand eczema, and anaphylaxis). These guides were designed for patients and parents of allergic children. Patients were able to access the guides using their smart phones, but true interactive applications have not yet been developed. These guidelines help the patient and the parents to act proactively when they recognize the first signs and symptoms of an exacerbation or worsening. Guided self-management is an evidence-based tool for patients who have doctor-diagnosed asthma or an allergic condition. It was first proved effective in asthma [33, 34].

To continue the multidisciplinary education also after 2018, regional expert groups began to coordinate local activities. In 2021, 14 groups are working, and the goal is to have a multidisciplinary group in each of the 21 hospital districts in Finland.

Education has been organized also for the personnel of pharmacies, day-care centres, and schools. *The Association of the Finnish Pharmacies* produced material for and ran campaigns for education of these personnel on the topics of allergic rhinitis and atopic eczema during 2009–2016.

The key messages, the educational activities, and the support for everyday clinical work were well received, and criticism was constructive. Positive feedback was received from healthcare professionals in relation to the clear message (from avoidance to tolerance) and the practical action of allocating the existing resources more effectively. The change of focus also from treatment to prevention and promoting allergy health (good life even with allergies) were widely accepted. Importantly, the workload of doctors and nurses did not increase; it even tended to decrease with better practices.

3.2 Lay Public

People with severe allergic symptoms received new recommendations and guidelines from the healthcare professionals updated by the Allergy Programme

educators, but the majority of people with mild or suspected allergy had not previously had much contact with medical care. Their attitude and search for advice and treatment were typically based on media—websites, social media, and magazines—and peer support by Internet groups, friends, and patient organizations. Misinformation, poorly justified avoidance, and fear of all exposure are common elements of these discussions and especially prominent in social media.

In 2011, Allergy, Skin and Asthma Federation and the Organization of Respiratory Health with approximately 60,000 members organized a joint project to produce media material to support the Allergy Programme. In practice, two persons were responsible for the planning, implementation, and evaluation of the project in collaboration with the programme steering group. In the first phase (2011–2013), the focus was the electronic media, and a new website (www.allergiaterveys.fi) was developed. The new website was optimized for search engines and appeared among the first pages of *Google search* when the widely competitive keyword 'allergy' was used.

Several banner campaigns were put into practice, including the biggest social media service (www.Suomi24.fi) and the largest health and welfare online service in Finland (www.terve.fi). These also included the *question and answer section* for allergic people. Several clips and expert interviews were produced for the YouTube channel. The largest single campaign was executed through the national radio channel.

In the second phase (2014–2015), actions were more targeted, e.g. for the Finnish day-care units, the maternity and child health clinics, and the personnel and peer workers of the patient organizations. In 2015, a website (*Healthy at work*) supporting young allergic people selecting education and occupation was launched in collaboration with the Finnish Institute of Occupational Health.

During the 5-year period, altogether 12 media campaigns were executed in the Internet and radio, and 11 bulletins of different topics, three guidebooks, and four posters were released. One hundred and fifteen articles were published, and 119 lectures and 53 interviews were given. The coverage of the information was followed, and 2.3 million Finns (42% of the population) were reached. Since 2016, informing of the lay public has continued via the programme website and through patient organizations.

The main outcomes were measured in a population survey with more than 1000 respondents, carried out in 2011 and repeated in 2015. Although some favourable changes were seen in understanding causes and treatment of allergy, the limitations caused by allergies seem to be on increase rather than on decrease among the Finnish people. Half of the allergic population controlled their food selection because of allergy, and every fourth person of the allergic population took allergy into account throughout the year. Allergy played a role in outdoor activities, having or not having a pet, eating in restaurants, and shopping for daily consumer goods [35].

Besides allergy risk groups, the information project targeted the lay public in general. The main idea was to change the public attitude to how to prevent allergies by turning avoidance more into tolerance/resilience. A healthy contact with diverse

Fig. 6 Information campaign for the lay public during the period 2011–2016 by the patient organizations. Here, some of the titles have been translated from Finnish to English

natural environment was the key element of campaigns, posters, leaflets, interviews, and lectures (Fig. 6).

Nature relatedness was also the basis for the co-operation with the Association of Kindergarten Teachers in Finland. A pilot campaign called *Go to nature!* was carried out in 2014–2015 in Southern Karelia, incorporating various outdoor activities into the day-care routine. New guidelines for early childhood education, with emphasis of outdoor activities and contacts with the natural environment, have been introduced to the whole country (Fig. 7).

4 Measuring Outcomes

For outcome evaluation, the Finnish healthcare registers provided invaluable data sources: especially, the hospital admission register of the National Institute for Health and Welfare and the registers of the Social Insurance Institution (drug reimbursements, sick leave allowances, disability pensions). For occupational diseases, Finland has a strict legislation, and verified cases are registered by the National Institute of Occupational Health. For outcome evaluation the baseline was 2007–2010, depending on survey, source or method.

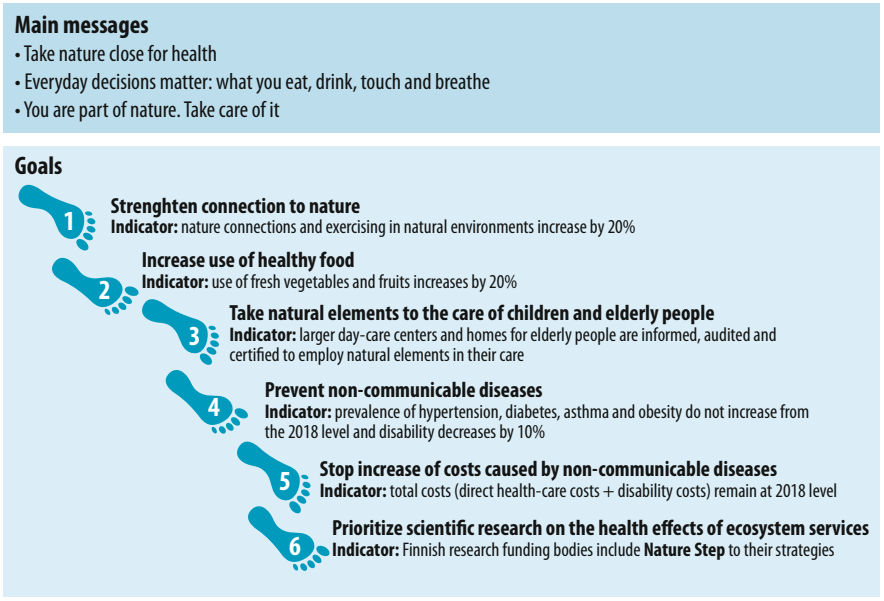

Fig. 7 A Nature Step to prevent chronic non-communicable diseases, allergic disorders among them, was presented in Finland in 2017. ([36], with permission)

The Finnish anaphylaxis register was established in 2000 at the Skin and Allergy Hospital of the Helsinki University Central Hospital [37]. Physicians (mostly allergists) from the whole country voluntarily report cases of severe allergic reactions independent of the causative agent. A one-page questionnaire for medical professionals is available on the Internet.

Allergy and asthma costs were analysed from all data sources in collaboration with government officials [38].

5 Achieving the Goals

In the 10 years that *the Finnish Allergy Programme* was operating from 2008 to 2018, the prevalence of allergic diseases and asthma levelled off. Asthma caused less symptoms and disability and 50% less hospital days. Food allergy diets in day-care facilities and schools halved. Occupational allergies reduced by 45% ([2], Fig. 8). In 2018, the direct and indirect costs of allergic diseases and asthma were €1.5–-1.8 billion. With comparative figures, the costs were €201 million (30%) less in 2018 than at the beginning of the programme [39]. The theoretical, cumulative cost savings were €1.2 billion during the period from 2007 to 2018.

For allergic conditions, there are no reports of coordinated action plans to mitigate the burden nationwide. *The Finnish Allergy Programme 2008–2018* was undertaken

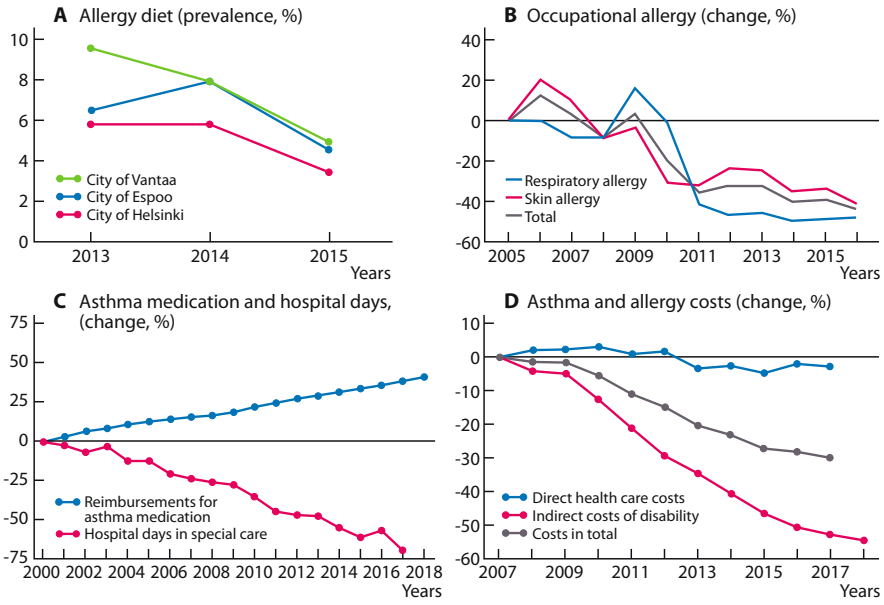

Fig. 8 (**a**–**d**) The main outcomes of the Finnish Allergy Programme (2008–2018). (**a**) Reduction of allergy diets in the day-care centres of three cities [29]. (**b**) Reduction of occupational respiratory and skin allergies. (**c**) Increase in the number of patients entitled to reimbursement for regular asthma medication, and decrease of hospital days in specialist care. (**d**) Direct healthcare and indirect disability costs 2007–2018. ([2], with permission)

to emphasize prevention, nature relatedness, and protective factors for immunological tolerance/resilience. Indeed, revisiting the allergy paradigm and systematic education mitigated the burden in the Finnish society [2, 39].

As this real-world intervention lacked controls, the true impact of the programme on primary prevention remains to be verified. Nevertheless, the Finnish experience differs from the global trend of asthma and allergy, the burden of which has remained high or is even increasing [40–42]. This indicates that the current global prevention strategies are ineffective and new approaches are required.

6 Concluding Remarks

New ideas especially from epidemiological, ecological, microbiological, and immunological studies, systematic planning, and coordinated implementation have changed the allergy landscape in Finland. The overall burden of allergic disorders and asthma has been significantly mitigated, both for the citizens and for the society. Medicalization has also been markedly reduced; the best example are food allergy diets, which halved in day-care centres [43].

Globally, allergy management is divided into many specialities in the medical discipline. In Finland, allergology is recognized as a speciality connected with dermatology (dermatology and allergology) and pulmonary medicine (pulmonary medicine and allergology). Allergy training is also organized in paediatrics and otorhinolaryngology. The position of allergology as a main speciality, subspeciality, or additional training varies a lot in Europe. In most countries, a coordinated public health approach is lacking. However, allergy is a systemic immunological disorder with variable organ manifestations, which also change during the lifespan. It is not self-evident that paediatricians and adult physicians work effectively together. For example, teenagers are often neglected. Are specialists truly giving their support to general practitioners and to those working at the grass-root level? Do the private sector and public health professionals play to the same goals?

Finland is not much of an exception; coordination has been problematic, both between primary and secondary care and between different specialities. The small population (5.5 million) with a relatively high level of education and organized public healthcare allowed us, however, to create a population management model and take steps from treatment to prevention. Thinking and actions were stimulated by the new information of environment, lifestyle, nature relatedness, immune-microbiota interaction, and epigenetic regulation in the 'allergy epidemic'. The *biodiversity hypothesis* collected the wire heads together and gave impetus to the Allergy Programme [44].

The barriers to progress are always there. Adopting new knowledge in real-life and everyday practice is constrained by rigidity and path dependence (we have always done things our way!). The educators must be committed and dedicated experts to convince others. The task becomes easier when the 'ball gets rolling' and critical mass for change accumulates. Then, the regional experts and champions are in key position to spread out the gospel: allergy can be prevented and symptoms proactively stopped!

7 Future Local and Global Allergy Plans

7.1 Lahti Plan

In Finland, the Allergy Programme has inspired a local action in the city of Lahti (120,000 inhabitants) and the surrounding county of Päijät-Häme. The city of Lahti is the *green capital* of the European Union in 2021 [45]. It is preparing a 10-year local health and environment programme, the *Lahti Plan 2022–2032*—an educational and communicational programme, which implements the best practices of public health and environmental care in the spirit of *Helsinki alert* and *planetary health* [46–49]. It constructs a model for everyday life that considers both human health and the planetary boundaries (Table 4).

The goals are defined to more specific *targets* (what to do), *tools* (how to do it), and *outcomes* (what to measure). The Lahti Plan also supports the already existing

Table 4 The tentative goals for the *Lahti Plan 2022–2032* (county of Päijät-Häme, Southern Finland)

1. Reduce disability caused by non-communicable disorders; asthma, diabetes, overweight, and depression:
(a) By implementing practices of *planetary diet*
(b) By increasing nature relatedness, especially of children and senior citizens
(c) By increasing non-motorized mobility and physical activity
2. Preserve environmental biodiversity, stop nature loss
3. Mitigate climate change/global warming
4. Combine public health and environmental science for common education and research
5. Aim to a cost-effective process, where savings in healthcare compensate environmental investments

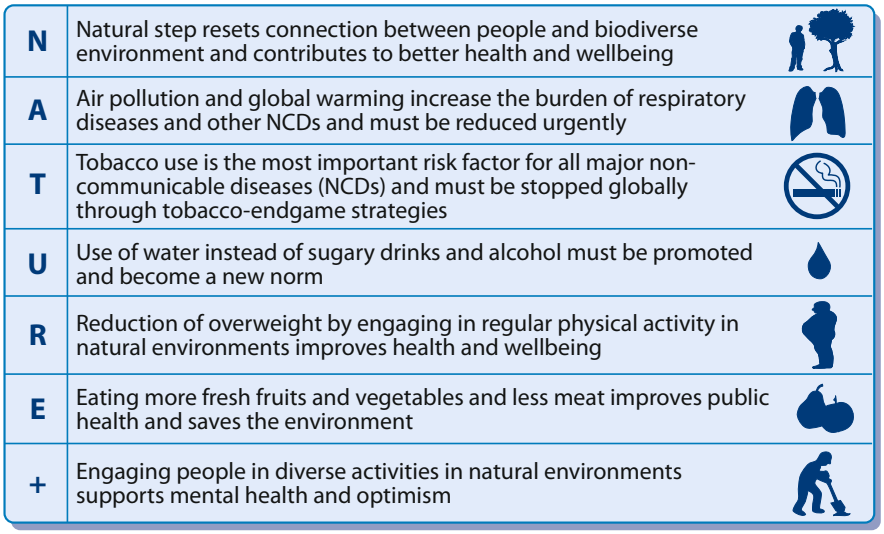

Fig. 9 Imperative actions to promote human health and conserve nature. ([23], with permission. The figure was originally designed by N.E. Billo and T. Haahtela)

public health and environmental projects (energy, water, mobility) of the city of Lahti.

7.2 Global Allergy Plan and the Nature Step

In 2013, the WAO published a position paper on the biodiversity hypothesis and allergic disease [17]. *The Finnish Allergy Programme 2008–2018* was already on going, and the first experience indicated success. Thus, the position paper presented the idea of a *global allergy plan*, which 'could be a powerful tool to increase

Table 5 Planning an *allergy prevention programme*. In practice, implementation means education and dissemination of the new knowledge for (1) prevention, (2) immune tolerance/resilience, and (3) allergy health (support of health instead of disease)

1. Practical steps to start:
(a) Define the *community* (population) for which the programme will be addressed (e.g. city or hospital district, region, province, national level)
(b) Organize a local *consensus meeting* to agree on action to reduce allergy and asthma burden and improve management. Contact local administration. Find support from opinion leaders, decision-makers, and politicians
(c) Set up a *steering group* of experts and opinion leaders (9–12 members) to plan and implement the *campaign* in detail
(d) Apply funding to implement the campaign. Raise some public funding, which can be supplemented with private funding. Funding for the first year means that you get started
(e) Start the campaign. Seek for support also on political and administrative level
2. Set up key messages for all citizens. Set up goals for healthcare. Each goal has specific tasks, tools, and evaluation methods. Goals (indicators) should preferably be quantitative
3. Set up a plan for an educational process:
(a) Education of the healthcare professionals is the key for success. Decide the organization implementing the education. It can be hospital or healthcare based or a non-governmental organization (NGO)
(b) The education is integrated to *everyday work* of professionals. A part-time educator contributing to the field work and a part-time assistant/secretary are employed. The local experts are consulted to set the content for the educational sessions
(c) Information of the lay public and communication via the Internet and social media is planned and needs a part-time worker (at least at the beginning of the programme)
4. Explore public healthcare registers to measure outcomes:
(a) For example, emergency visits, hospitalizations, drug use, days off work, pensions, cost estimates, etc.
(b) Important! Integrate practical actions and systematic follow-up. Is the programme reaching the goals? Motivate actions for research and follow-up surveys
5. Set up timelines:
(a) Planning the campaign takes a year. Two key words: motivate and organize!

awareness of the global public health problem and combat the high burden of allergies. It may also have a preventive effect on other non-communicable diseases'. Since then, a lot of scientific and real-world evidence has accumulated to support the idea.

In 2017, a Finnish group of scientists suggested a *Nature Step* to prevent chronic non-communicable diseases [36] (Fig. 7). It was highlighted in the Helsinki GARD Meeting 2018 (Global Alliance Against Chronic Respiratory Diseases) and developed further [23] (Fig. 9).

Whether a programme/plan/campaign is to be implemented locally, nationwide, or globally, much of the same principles prevail, although the scale varies (Table 5).

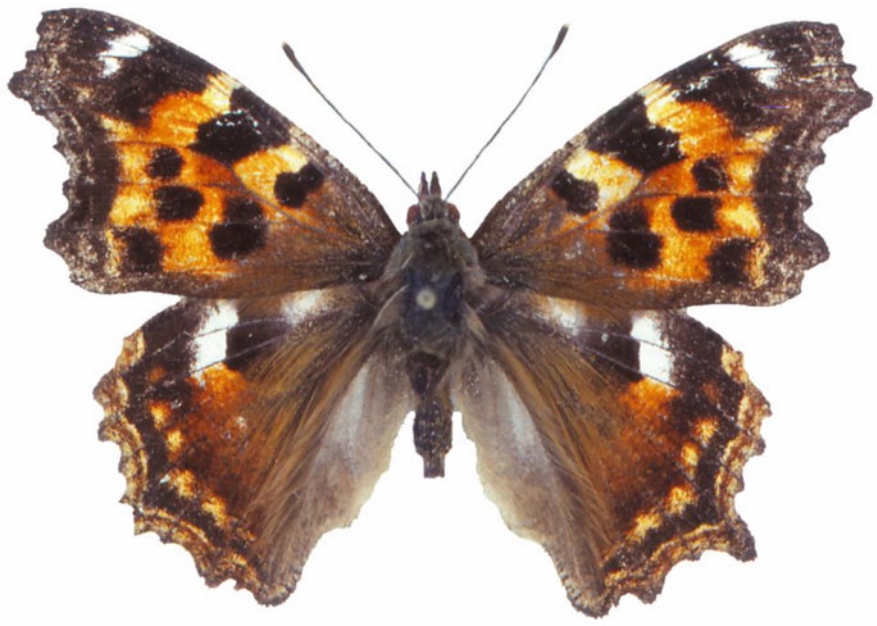

Fig. 10 False comma or Compton tortoiseshell (*Nymphalis vaualbum*). (Hämeenkoski, Finland 27.7.1960 leg. T. Haahtela)

8 A Personal Memory

One incidence leads to another. In 1960, July 27th, a 14-year-old boy got a nice present from his father. A butterfly net and a guidebook for identification, although father did not know anything of butterflies. The family was spending the summer in an old farm in Southern Finland. Almost the first catch hit an extremely rare eastern migrant, a false comma or Compton tortoiseshell (*Nymphalis vaualbum*) (Fig. 10). The young boy got interested in butterflies, biology, medicine, research, and curious for new things. Forty years later, the Karelia Allergy Study commenced to hunt for the origin of allergy. The observations led to the *biodiversity hypothesis*. In 2009, a commentary paper in *allergy* visioned the butterflies and allergic disorders in the same equation [50]. A few years later, a photographic guide of butterflies of Britain and Europe was completed [51], and *the Finnish Allergy Programme 2008–2018* speeded up. Sixty years have passed, *vaualbum* has not changed. It is alive and unites all living things for the young boy and old man. Maybe the *false comma* was not that false, after all.

The Lesson to Learn Getting children to study and take a deeper interest in the natural world would be a way to expose them more to nature—to support health, to enjoy, and to take care.

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Compliance with Ethical Standards This article describes implementation of a public health programme in Finland 2008–2018. The article does not contain any studies with human participants performed by the author.

Conflict of Interest The author (TH) of the present chapter has received personal lecturing fees from GSK, Mundipharma, Orion Pharma, and Sanofi during the last 5 years.

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