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Beneficial Microorganisms in Medical and Health Applications

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Preface

This volume communicates aspects of beneficial microorganisms in relation to medical treatment and health protection. They have been much emphasized on their roles to regulate gut well-being, including the alleviation of lactose intolerance, improvement of diarrhea, and inhibition of pathogenic bacteria in the gut. Recent evidence has also shown great potentials in immunomodulation, atopic applications, and stress management. Sophisticated in vitro tools and omics approaches have translated these potentials to the development of new vaccines and antibiotics, playing major roles in pharmaceutical and up-scaled industrial applications.

Penang, Malaysia — Min-Tze Liong

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Health Effects of Pro- and Prebiotics: Utilization of Sophisticated In Vitro Tools

Koen Venema

Abstract This chapter is a summary of my keynote lecture given during the International Conference on Beneficial Microbes in Penang, Malaysia, from 27 to 29 May 2014. It describes the use of sophisticated dynamic, computer-controlled in vitro models of the gastrointestinal (GI) tract, developed by the Dutch Organization for Applied Scientific Research (TNO), nicknamed TIM. Among others, these have been used for determining and predicting survival of probiotics and the effects of prebiotics on the composition and activity of the gut microbiota. These sophisticated multicompartmental models closely mimic the dynamic conditions in the gastrointestinal (GI) tract and are therefore perfect tools to study mechanistically what happens in the GI tract. They can be used to study and optimize survival of probiotic strains and to screen for, e.g., an efficacious dose of prebiotics. Some examples on their use in optimizing probiotic survival, for instance, by combining them with prebiotics or developing a protective coating, are given. Also, the use of stable isotope-labeled substrates to trace metabolism of the gut microbiota as a tool to decipher what is going on in the colon is highlighted. Specific labeling of members of the microbiota and cross feeding are shown.

1 Introduction

Over the past several decades, the research into the health benefits of probiotics and prebiotics has rocketed sky high. There are several new applications and diseases and disorders for these healthy dietary components that were previously unthinkable. However, the efficacy has not been scientifically substantiated for all these applications yet, and care needs to be taken that pro- and prebiotics are not considered as a cure for everything. For starters, probiotic effects are strain dependent, and hence not all strains are beneficial for all disorders. In fact, some strains may be detrimental when given to certain patients, and it may aggravate the problems that these patients have. Similarly, prebiotics are not identical and will

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stimulate different microorganisms in different individuals, in some case leading to worsening of the disease. Moreover, dose dependency has rarely been studied, and in the case of probiotics, culture conditions may affect their efficacy as well. In addition, although numerous positive results have been obtained with several well-studied probiotic strains, the mechanism of action usually is still completely unclear, let alone what the molecular molecule is that is responsible for the benefit. So, despite several decades of intense research, there is still much to be discovered.

2 Definition of Probiotics and Prebiotics

2.1 Probiotics

The definition of *probiotics* has undergone several revisions over the past two decades. About a decade ago, it has been refined by an expert panel commissioned by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) and is now commonly accepted to be "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2002). This definition is preferred over historical definition as it includes both intestinal and other forms of probiotics, such as vaginal or topical probiotics. This definition requires that the term "probiotic" only be applied to live microbes having a substantiated beneficial effect (Reid et al. 2003), although preparations of dead cells and cell components may also exert some health-promoting physiological effects (e.g., Lau 2014, Adams 2010). These have recently been referred to as *postbiotics*, which have been defined as nonviable bacterial products or metabolic byproducts from probiotic microorganisms that have biological activity in the host (Tsilingiri and Rescigno 2013). For some physiological benefit, it is clear that the cells need to be active. However, for other benefits, e.g., lactose intolerance, it is hypothesized that lysis of the cells within the GI tract is required to increase digestion of lactose in lactose-intolerant individuals (Venema 2012). A new trend that may soon, probably within the next 5 years, be reality is the development of new therapeutic strategies, such as the development of phagebiotics, psychobiotics, and (genetically modified) *pharmabiotics* (Shanahan et al. 2012; Eeckhaut et al. 2014; Paton et al. 2012).

2.2 Prebiotics

Like for probiotics, the original definition for a *prebiotic* was limited to a benefit to the gastrointestinal (GI) tract, and hence it was originally defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the

colon, and thus improves host health" (Gibson and Roberfroid 1995). This definition was later refined by the same authors to include other bodily areas of the host that may benefit from selective targeting of particular microorganisms (Gibson et al. 2004). A more recently adopted definition by the International Scientific Association for Probiotics and Prebiotics (ISAPP) is "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Blatchford et al. 2014), in which the phrase "gastrointestinal" reappeared, indicating that the jury is still out on non-GI prebiotics. Even though other areas of the body are being targeted by prebiotics, this is usually done by giving prebiotics orally, e.g., pro- and prebiotics for skin health (Miyazaki et al. 2014; Foolad and Armstrong 2014).

2.3 Synbiotics

Prebiotics function complementary to, and possibly synergistically with, probiotics. Gibson and Roberfroid first defined synbiotics as "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements (read: probiotics) in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare" (Gibson and Roberfroid 1995). Very few efficacious synbiotic mixtures have been developed (Venema 2015).

3 Health Effect of Pro- and Prebiotics

3.1 Probiotics

Probiotics have been in use for several decades now. These mostly include strains of the genera *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Propionibacterium*, *Enterococcus*, *Escherichia coli*, and *Saccharomyces boulardii*. Still, we hardly know the molecular mechanisms underlying the probiotic effect, which are very likely to be various different modes of actions for different strains. It has been clearly shown that probiotic effects are strain dependent and that one strain that is efficacious for one disorder likely does not have an effect on another unrelated disorder. Probiotics should not be seen as being able to cure or prevent all (modern Western) diseases, although for individual strains, there are promising effects. Two strains, *L. rhamnosus* GG and *L. plantarum* WCFS1, have been studied in great detail by many groups throughout the world, and mutants of these strains have greatly aided in our understanding of the interaction with the host. However, several surprising

results were obtained as well and leave more questions than answers. Recent advances in the molecular understanding of interaction of probiotic lactobacilli with the host show that especially surface molecules are thought to play a crucial role in this interaction.

It is generally accepted that secreted molecules and cell surface molecules interact with the host and aid to probiotic functionality. Most of the probiotic effector molecules are present in the bacterial cell envelope, which is the first contact side to interact with intestinal host cells (Remus et al. 2011; Wells et al. 2011). Probiotic effector molecules include peptidoglycan, lipoteichoic acid, wall teichoic acid, extracellular or cell-wall-associated polysaccharides, glycoproteins, lipoproteins, and other cell surface exposed cell-wall-attached proteins such as pili (Venema and Meijerink 2015).

Probiotics (mostly lactobacilli and bifidobacteria) have been used and studied for several decades now. They beneficially affect a long list of diseases and disorders. These have been described before in excellent reviews and will not be repeated here. It is hard to list good review for probiotics, as nowadays reviews are split into disease areas. But a quick search in PubMed with the phrases "probiotic, review and 2014" will yield some 30+ hits (Dec 2014) with papers describing about as many different disorders and diseases. These vary from general effects on the microbiota (de Moreno de LeBlanc and LeBlanc 2014; Vitetta et al. 2014) and effects on the host immune system (Kemgang et al. 2014) to inflammatory bowel disease (Mardini and Grigorian 2014), eczema in children (Mansfield et al. 2014), necrotizing enterocolitis in neonates (Parker 2014), obesity (Mekkes et al. 2014), and its associated diseases, such as nonalcoholic liver disease (Yasutake et al. 2014).

3.2 Prebiotics

To be classified as a genuine prebiotic, a dietary compound historically had to fulfill three criteria: (1) resistance to gastric acidity, resistance to hydrolysis by mammalian enzymes and gastrointestinal (GI) absorption, (2) fermentation by the intestinal microbiota, and (3) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being. Given the fact that other areas of the host besides the GI tract are now also targeted, these criteria are not all applicable anymore. In fact, they are not applicable at all any more, unless the phrase "intestinal" is deleted from criteria 2 and 3. However, most prebiotics developed up to date are geared toward the GI tract.

Most prebiotics that have been developed so far (and that carry sufficient scientific evidence) are carbohydrates or fibers. However, though most prebiotics (so far) are fibers, not all fibers are prebiotics (Slavin 2013). Although the concept of prebiotics is relatively new, foods high in prebiotics have been consumed since prehistoric times: Archaeological evidence from dry cave deposits in the northern Chihuahuan Desert, which straddles the US-Mexico border, shows intensive utilization of desert plants that were high in what we now consider prebiotic fructans

(Slavin 2013). Dietary intake of fructans was estimated to be about 135 g/day for the typical adult male hunter-forager. The fructans inulin and fructo-oligosaccharides (FOS) occur naturally in foods such as leeks, asparagus, chicory, Jerusalem artichokes, garlic, and onions. Nowadays, consumption in typical US and European diets has been estimated to be only several grams per day (Slavin 2013). Despite the possibility of ingesting high quantities of inulin (135 g/day for hunter-foragers), in our current time, inulin and FOS consumption in large quantities is associated with impaired gastrointestinal tolerance (Lied et al. 2011; Grabitske and Slavin 2009). Other prebiotic fibers (e.g., wheat dextrin, polydextrose) exhibit higher gastrointestinal tolerability (up to 30–45 g per day) (Pasman et al. 2006).

Examples of well-established prebiotics are inulin and FOS, galacto-oligosaccharides (GOS; a.k.a. transgalacto-oligosaccharides or TOS), and lactulose. Less well-studied prebiotics include lactitol, isomalto-oligosaccharides (IMO), gluco-oligosaccharides (Gl-OS), lactosucrose, xylo-oligosaccharides (XOS), arabinoxylo-oligosaccharides (AXOS), resistant starch, raffinose (Gal-Glc-Fru) and stachyose (Gal-Gal-Glc-Fru) from soy, and (acidic) pectic oligosaccharides, although there is accumulating evidence for these.

3.2.1 Chemical Structure and Linkage Types of Common Prebiotics

Inulin and FOS are composed of linear chains of β-2,1-linked fructose units, with or without a terminal glucose. GOS are a mixture of oligosaccharides derived from lactose by enzymatic transglycosylation. The mixture generally consists of oligosaccharides from tri- to pentasaccharides with β-1,6, β-1,3, and β-1,4 linkages (Coulier et al. 2009), which makes it refractive to digestion. Lactulose is manufactured by the isomerization of lactose to generate the disaccharide galactosyl-β-1,4-fructose. Lactitol is a sugar alcohol and is the hydrogenated form of lactose. IMO are manufactured from starch, which is first hydrolyzed by the combined action of α-amylase and pullulanase, and the resultant malto-oligosaccharides are acted upon by α-glucosidase to create IMO. IMO is partly digestible by pancreatic enzymes but is digested slowly. Depending on the individual enzyme activity and food transit, variable amounts of IMO may reach the colon. Gl-OS are synthesized by the action of the enzyme dextran-sucrase on sucrose in the presence of maltose. Due to the presence of glycosidic bonds uncharacteristic of starch, it is not hydrolyzed by pancreatic α-amylase. Lactosucrose is produced from a mixture of lactose and sucrose using the enzyme β-fructofuranosidase. XOS and AXOS are manufactured by the enzymatic hydrolysis of (arabino)xylan from various cereal sources.

3.2.2 Health Effects of Common Prebiotics

The above brief exposé on the chemical nature of several different prebiotics indicates the wide range of different carbohydrates that are being or have been evaluated for their prebiotic potential. This is partly due to the fact that we have

increased our insight in the health beneficial aspects (and at the same time the detrimental activities) of the endogenous gut microbiota. Whereas in the past a prebiotic activity was synonymous with a bifidogenic activity (Gibson and Roberfroid 1995), currently the microbiota has been implicated in numerous diseases and disorders, and hence modifying the microbiota composition beyond a bifidogenic stimulation with different prebiotics is now also considered to be beneficial, although even in recent applications, classic bifidogenic prebiotics seem to function as well, as has been shown, for instance, for FOS in obesity (Geurts et al. 2014).

The majority of the adult world population cannot digest lactose (Venema 2012), as the expression of the lactase enzyme in the gut wall is decreased with increasing age. This phenomenon is called lactase deficiency (no lactase) or hypolactasia (some remaining activity) (Venema 2012). If these individuals would ingest lactose (Gal-Glc), the disaccharide would make it to the colon. As such, it can be considered a prebiotic. However, lactose ingestion is very frequently associated with gastrointestinal complaints, similar to the intake of large amounts of FOS or inulin. This lactose (and FOS/inulin) intolerance is thought to occur due to fermentation of the substrate by the gut microbiota (He et al. 2008). There is a lot of anecdotal evidence and some scientific data that slowly increasing the dose of lactose, FOS, or inulin would diminish the intolerance. Adaptation to long-term lactose ingestion in lactose maldigesters has been thought to be related to adaptation of the colonic microbiota and colonic function (Hertzler and Savaiano 1996; He et al. 2008). The mechanism underlying lactose, FOS, or inulin intolerance is hypothesized to be the very quick fermentation of the di- or oligosaccharides by the microbiota, likely leading to the accumulation of microbial metabolites and gas, which leads to the experience of bloating, rumbling, and diarrhea (He et al. 2008). Adaptation of the microbiota or adaptation of the colonic epithelium to cope with the increased metabolites is thought to relieve the symptoms. Experiments in validated in vitro models of the colon have indicated that the fructans (degree of polymerization (DP) ranging from 3 to 9) are very quickly fermented (van Nuenen et al. 2003) and might thus indeed lead to the accumulation of microbial metabolites and gas, with consequent GI complaints. A very-long-chain inulin (average DP >55, with a maximum DP of ~75) extracted from globe artichoke had pronounced prebiotic effects in human subjects but was well tolerated (Costabile et al. 2010). Besides DP, also the biochemical structure of the oligosaccharides determines (speed of) fermentability. GOS contains many different molecules with different DP and glycosidic linkages (Coulier et al. 2009), which cannot all be degraded by the same microbial species. Similarly, fermentation of linear α-1,6 dextrans and dextrans with α-1,2 branching by the human microbiota was different (Sarbini et al. 2011). In addition, a branched inulin from *Agave* shows different properties than the linear inulin from, e.g., chicory: fermentation of the *Agave* fructan was slower (Koenen, Cruz Rubio, and Venema, personal communication). This may be (part of) the reason why the hunter-foragers in the Chihuahuan Desert could ingest higher quantities of these fructans, as the *Agava* fructans are highly branched (López and Urías-Silvas 2007) and hence more difficult to ferment. It has been

shown that different bifidobacterial strains fall into one of four clusters with reference to their metabolism of inulin and shorter-chain FOS or the monosaccharide fructose. Some strains only metabolized fructose; other strains preferred fructose but could also metabolize FOS but with decreasing affinity as molecular weight (or DP) increased. Some strains preferred FOS with little ability to grow on the monosaccharide or inulin, while the last cluster had equal affinity to grow on fructose and FOS and have some ability to grow on inulin (Falony et al. 2009). Similarly, structurally different wheat-derived arabinoxylo-oligosaccharides with different DP showed different fermentation characteristics (Van Craeyveld et al. 2008). This shows that the structure but also the dose defines the beneficial properties of the prebiotic that is ingested.

While we do not have a clear and comprehensive understanding of the molecular mechanisms involved in the bacterial fermentation of complex carbohydrates in the gut, it is clear that these processes will involve the action of glycosyl hydrolases and transport systems to take up prebiotics or their breakdown products. Our diet contains a lot of carbohydrate structures from, e.g., plants, which our own pancreatic enzymes cannot digest. Our gut bacteria play a pivotal role in the digestion of these dietary polysaccharides by producing a very large number of carbohydrate-active enzymes (Koropatkin et al. 2012). The genus *Bacteroides* alone already produces > 500 different carbohydrate-active enzymes (Martens et al. 2009; McNulty et al. 2013), with as an example > 500 genes in *Bacteroides cellulosilyticus* strain WH2, comprising 373 glycosylhydrolases, 23 pectate lyases, 28 carbohydrate esterases, and 84 glycosyltransferases (McNulty et al. 2013), which are more carbohydrate-active enzymes than any previously sequenced member of the *Bacteroidetes*. Hence it is expected that this genus will thrive on a large number of different carbohydrates, both of dietary and endogenous origin. Indeed, in a study where gnotobiotic mice were colonized with an artificial microbiota comprising 12 sequenced human gut bacterial species, *B. cellulosilyticus* WH2 was shown to be an adaptive forager that tailors its versatile carbohydrate utilization strategy to available dietary polysaccharides, with a strong emphasis on plant-derived xylans abundant in dietary staples like cereal grains. Using transcriptional profiling (RNA-Seq), two highly expressed, diet-specific polysaccharide utilization loci (PULs) were found, one with characteristics of xylan utilization systems, which were highly expressed when triggered by carbohydrates (McNulty et al. 2013). Another tool used to look at carbohydrate-active enzymes was a custom microarray that contained nonredundant DNA probes for more than 6500 genes encoding glycoside hydrolases and lyases selected from 174 reference genomes from distal gut bacteria. Examination of eight stool samples allowed the identification of a core of carbohydrate-active enzymes, containing 46 families of glycoside hydrolases and polysaccharide lyases, which suggests the functional stability of the gut microbiota despite large taxonomical variations between individuals. Using comparative genomics, a regulatory network for carbohydrate utilization in *B. thetaiotaomicron* was created (Ravcheev et al. 2013). The inferred regulatory network contains 308 genes encoding polysaccharide and sugar catabolic enzymes, carbohydrate-binding and transport systems, and transcription factors (Ravcheev et al. 2013).

3.3 How to Study Efficacy of Pro- and Prebiotics?

Despite the numerous reports of effects of pro- and prebiotics on health of the host, we understand very little about the (molecular) mechanisms of action. It is extremely difficult to investigate this in man. Therefore, numerous animal models have been used in the past to screen collections of strains for the "proper" probiotic or to study the mode of action. However, these results can only rarely be translated to man (Meijerink et al. 2013), and predictive selection of probiotics on the basis of animal or in vitro experiments (e.g., with immune cells) has been rare. For instance, recently it has been shown that the effect of probiotics in vitro depends on the presence of an extracellular vesicle (EV) fraction in serum (van Bergenhenegouwen et al. 2014). In the absence of these vesicles, the response is different. For instance, EVs were found to enhance cellular TLR2/1 and TLR4 responses, while TLR2/6 responses were suppressed. It was shown that EVs play a role in bacterial aggregation, suggesting that EVs interact with bacterial surfaces. EVs were found to slightly enhance dendritic cell (DC) phagocytosis of *Bifidobacterium breve* whereas phagocytosis of *Lactobacillus rhamnosus* was virtually absent in the absence of EVs, suggesting that EVs modify the DC-microbe interaction. Depending on the microbe tested, combined effects of EVs on TLR activity and phagocytosis result in a differential proinflammatory DC cytokine release. Overall, these data suggest that EVs play a yet unrecognized role in host-microbe responses, not by interfering in recipient cellular responses but via attachment to, or scavenging of, microbe-associated molecular patterns. EVs can be found in any tissue or bodily fluid; therefore insights into EV-microbe interactions are important in understanding the mechanism of action of potential probiotics and gut immune homeostasis (van Bergenhenegouwen et al. 2014). This means that previous research that was mostly done in the absence of the vesicles may not be predictive for the clinical situation.

It is clear that if we want to screen for efficacious pro- and prebiotics, we need predictive models that properly mimic the in vivo situation.

4 In Vitro Models of the Gastrointestinal Tract

To study the bioactivity of (food-derived) oligosaccharides or the survival of probiotics, several sophisticated in vitro models are available. These include in vitro models of the stomach and small intestine as well as fermentation models that mimic the large intestine and are inoculated with a gut microbiota to study degradation of the oligosaccharides, changes in the composition of the microbiota, and production of short-chain fatty acids (SCFA) by the gut microbes. Changes in composition and especially changes in activity of the gut microbiota may affect human health. Here, SCFA have especially attracted attention, as several studies have shown anti-inflammatory activity of these microbial metabolites. Other

in vitro tools are cell culture assays that study the direct interaction of pro- or prebiotics with pattern recognition receptors of the host. Assays have been set up for epithelial and immune cells using either cells isolated from blood or cell lines. In addition, cell reporter assays for increased throughput have been constructed in such cell lines for immune cells and, e.g., epithelial cells of the gut. These cell culture models are used to study the effect of pro- or prebiotics on gene expression in these cell, usually using chemokine and cytokine production as the readout of bioactivity. Despite all these in vitro tools, it remains difficult to predict the bioactivity of pro- or prebiotics. In the subsequent paragraphs, the use of the TNO in vitro models of the gastrointestinal tract (the TIM systems) to study the efficacy and to decipher the mechanism of action of pro- and prebiotics will be highlighted. Other in vitro models that are used for similar purposes have recently been reviewed (Venema and van den Abbeele 2013) and will not be discussed here. Also the use of cell cultures will not be discussed here, but the interested reader is referred to Venema (2014).

4.1 The TNO In Vitro Models of the GI Tract: A Brief Introduction

Over the past 16 years, I have been working with the TNO in vitro models of the gastrointestinal (GI) tract (nicknamed TIM). These sophisticated, computer-controlled systems accurately mimic the dynamic conditions in the GI tract of man (and of several animals). Two separate models have been developed: one for the stomach and small intestine (TIM-1) and one for the colon (TIM-2) (Minekus et al. 1995, 1999; Minekus 1998). The key features of the models are that they mimic the changing (dynamic) conditions that occur in the GI tract with respect to pH, concentrations of enzymes and bile, etc. The data used to program the system comes from literature. Depending on the host (baby, adult, elderly) and the diet (glass of water, milk, yoghurt, complete breakfast, high-fat meal, etc.), the proper parameters, such as gastric pH decline, amount of pancreatic enzymes excreted, bile concentration, and transit time, are chosen. Both models contain dialysis mechanisms to maintain physiological concentrations of enzymes, bile, microbial metabolites, etc., while at the same time digestion products are removed, simulating uptake by the body. TIM-2 is inoculated with a fecal microbiota originating from healthy volunteers or, e.g., people with inflammatory bowel disease (van Nuenen et al. 2004), obese individuals (Aguirre et al. 2014; Bussolo et al. 2014), etc. The models have been extensively validated with respect to digestion of dietary components in the upper GI tract and the composition and metabolic activity of the microbiota in TIM-2. The latter is based on data from sudden-death individuals that had given consent to be sampled for science. An extensive description of the systems is beyond the scope of this publication. The interested reader is referred to Venema et al. (2009).

5 Examples of the Use of the TNO In Vitro Models to Determine Potential Health Effects of Pro- and Prebiotics

5.1 The Use of TIM-1 for Studying the Survival of Probiotics

The TNO in vitro model of the stomach and small intestine (TIM-1) (Minekus 1998; Minekus et al. 1995) has been used extensively to study the survival of (potential) probiotics but also to develop concepts to increase the survival of certain strains. The model has been validated by comparing survival of the same strains (in the same products) in humans and in the model (Marteau et al. 1997). Samples in volunteers were taken from the end of the ileum using a nasal catheter, and these samples were plated for viable microorganisms. Similarly, at the end of TIM-1, which mimics the same position as that sampled in the human volunteers, samples were taken and plated. For the several strains tested, there was a remarkable match in survival (Marteau et al. 1997), and now these systems are used to predict survival of probiotic preparation in a human population. Moreover, the systems are used to optimize probiotic survival. A number of these examples will be discussed below.

As discussed in Sect. 2.3, synbiotics are combinations of probiotics and prebiotics. The prebiotics present in these mixtures are generally considered to have two functions. First and foremost, they act as prebiotics, in the sense that they stimulate the numbers and activity of beneficial microbes endogenous to the (gut of the) host. Second, they are considered a "lunch box" for the probiotics and should increase their survival while the beneficial microbes travel through the hostile environment of the GI tract. The lunch-box function of prebiotics assumes that the probiotics are able to use the prebiotics as a substrate, and this is certainly not always true. Not all currently commercially available probiotics, for instance, are capable of fermenting fructo-oligosaccharides (FOS) or galacto-oligosaccharides (GOS). A second assumption is that the presence of a fermentable substrate indeed does increase the survival of the probiotic. Although there is evidence that this is the case (Martinez et al. 2011), we also have some yet unpublished data that this depends on the dose of prebiotics added. At relatively low doses, it appears that survival of the probiotic strain may in fact be lower than that without the presence of the prebiotic. We hypothesize that this is caused by the following: Most probiotics when tested for survival have been cultured until the stationary phase of growth before they are harvested. When grown in a product, this harvesting is simply distribution over individual packages and shipping out for retailing. When the strains are to be incorporated into tablets, capsules, or other formulations, they are usually freeze- or spray-dried. When entering the stationary growth phase, bacteria express stress proteins that help them cope with this phase of low substrate availability, accumulation of toxic metabolic products, unfavorable pH, etc. This has been generally shown in lactic acid bacteria and is also true for probiotics (Mills et al. 2011). These stress proteins protect the cells against stresses they can encounter in the production pipeline, such as low or high temperature, low pH,

high pressure, etc. When cells that express these stress proteins encounter the hostile environment in the GI tract, with low gastric acidity and high bile concentrations in the small intestine, the cells are protected to a certain extent by the stress proteins. However, this never is a full protection, and some cells will die when traveling through the GI tract. This has been nicely shown using TIM-1 (Marteau et al. 1997). This model is an excellent tool to study the mechanisms underlying optimal survival of probiotics in the GI tract. For instance, by changing physiological parameters such as gastric transit or bile concentrations (which are not the same for all of human mankind), one can establish what is a crucial physiological parameter that influences probiotic survival. Some strains are more sensitive to acid, and some are more sensitive to bile. Now what happens if these probiotic strains are allowed to have access to a prebiotic during GI transit? When the probiotics start to feed on the prebiotic, they leave the stationary phase and enter the logarithmic growth phase. In this log phase, there is no need to express the stress proteins, and hence cells are less able to cope with the stresses encountered in the GI tract. Hence, survival drops. However, one could argue that since the prebiotic is a substrate for growth, one would get multiplication of the cells as well. One would be right. However, there appears to be a delicate balance between the dose of prebiotics added that destroys protection by the stress proteins (and leads to lower survival) and the dose that allows for sufficient growth to occur that the number of viable cells increases. This increase in viable cells appears to be increased survival, but in reality is the sum of increased death due to loss of protection by stress proteins and increased numbers of viable cells due to growth. As researchers, until today, we have no way of distinguishing this. From this, two things follow: (a) there should be a dose of prebiotics that exactly balances increased death and increased cell numbers and that for the researcher does not seem to affect survival, and (b) apparent increased survival can be obtained by choosing (usually by trial and error) the proper dose of the prebiotic. The TIM-1 system is a perfect tool to study this under standardized conditions.

Since some strains are very sensitive to gastric acid, strategies have been developed to prevent the exposure of probiotic cells to the hostile conditions in the stomach. This can, for instance, be accomplished by protecting the cells with a coating. The composition (polymers used for) and thickness of the coating for a commercial probiotic three-layer tablet have been chosen based on experiments in TIM-1. The three-layer tablet contains a layer of minerals, a layer of vitamins, and a layer of probiotics, consisting of three strains. The tablet is coated with an enteric coating that does not dissolve under acidic conditions, but rather disintegrates when the pH is raised to above approximately pH 5. One would therefore think that such a coating would protect the cells in the stomach, since the pH in the gastric compartment is always low. However, this is not always the case. In fact, when we ingest a meal, the pH in the gastric compartment is almost neutral, due to the high buffering capacity of the meal. Only gradually the pH then declines to a pH around 2 due to gastric acid secretion, but this takes approximately 2.5–3 h. A tablet with a coating that disintegrates at a pH above 5 therefore will also dissolve in the gastric compartment when taken with a meal, and some of the probiotics may therefore become exposed to a lower pH. Therefore, besides protecting the cells with a

coating, it is also important to determine whether or not such a tablet should be taken with a meal, prior to a meal, or after a meal.

Another interesting use of the system has been the search for "personalized probiotics" (Mäkivuokko et al. 2012). Some microbes, such as bifidobacteria, have been shown to be able to utilize blood group antigens or glycans found in ABO and Lewis antigens (Hoskins et al. 1985). The ABO blood group antigens are not present in the mucus of all individuals. These individuals, said to have the "nonsecretor" blood group, do not have the functional FUT2 gene needed in the synthesis of secreted blood group antigens (Henry 1996). Fecal samples from individuals stratified according to the secretor status were pooled and introduced into TIM-1, and at the end of the model, samples were taken for surviving bifidobacteria. Bifidobacteria in the secretor pool survived the transit through TIM-1 system better than those of the nonsecretor pool, and also the number of distinct genotypes (determined by RAPD) was clearly lower in the nonsecretor pool than in the secretor pool. This approach enabled the isolation of secretor/nonsecretor-specific potentially probiotic bifidobacteria (Mäkivuokko et al. 2012).

5.2 The Use of TIM-2 to Study the Effect of Prebiotics

The TNO in vitro model of the colon (TIM-2) (Minekus 1998; Minekus et al. 1999) has been used extensively to screen for the effect of (potential) prebiotics on the modulation of the composition and activity of the colonic microbiota. For instance, it has been used to study fermentation of inulin with different degrees of polymerization (van Nuenen et al. 2003), lactulose (Venema et al. 2003), and tagatose (Venema et al. 2005) and to determine the difference in fermentability of different resistant starch fractions (Fassler et al. 2006), used for the screening of potential novel prebiotics (Maathuis et al. 2009), and to determine the effect of a combination of pro- and prebiotics on the gut microbiota in both a microbiota originating from man and pigs (Martinez et al. 2013).

However, major insights into the metabolism of substrates by the gut microbiota have been obtained using ^{13}C-labeled substrates. These stable isotopes can be used to trace the appearance of the isotope into both microbial biomass and microbial metabolites that are produced from the substrate. Molecular DNA technologies such as the use of microarrays or next-generation sequencing allow for a comprehensive and integrated approach to assessing the structure of microbial communities. However, although these tools have significantly advanced our understanding of the gut microbial diversity, they do not provide functional insight on which microbes are relevant for specific dietary conversions (de Graaf and Venema 2008; Egert et al. 2006). The challenge was to develop and apply methodologies for analyzing the functionality of the microbiome, with the eventual aim to be able to predict its effect on human health. For this it is important to know which species (eventually strains) are responsible for the observed activities and elucidate dominant microbial functionalities in the human gastrointestinal tract and ultimately the

effect on host health. Stable isotopes (primarily ^{13}C has been used) have played an important role in answering these questions.

For instance, by observing the incorporation of label over time into microbial biomass, coupled to the appearance of label into microbial metabolites, it was possible to detect cross feeding on starch between a member of the *Bacteroidetes* (the identity could not be determined to the level of the species by the molecular method used at the time) and *Eubacterium rectale* (Kovatcheva-Datchary et al. 2009). The *Bacteroidetes* member produced acetate (and propionate) which was subsequently taken by *E. rectale* and converted into butyrate. The major butyrate isotopomer that was produced was the isotopomer containing two ^{13}C-atoms, which stems from the coupling of a fully (M + 2) labeled acetate with an unlabeled acetate. This type of cross feeding cannot be observed without the use of stable isotopes.

By measuring the different isotopomers and their concentrations over time, it was possible to model the metabolism of the gut microbiota in silico (Binsl et al. 2010; de Graaf et al. 2010). Although currently this model has all microbes in the gut microbiota incorporated as a single "entity," the use of stable isotope probing, which measures the incorporation of label into the individual microbes, would allow for the deconvolution of the in silico model into signals of individual species that contribute to the metabolism of certain substrates.

Another example is the use of GOS by bifidobacteria (Maathuis et al. 2012). Although it has long been known that upon the administration of GOS bifidobacteria increase in fecal samples, alternative explanations other than a direct stimulation by GOS could not be ruled out before. For instance, in fecal samples, bifidobacteria could be increased due to a better survival during transit from the proximal colon (where fermentation of GOS takes place) to the distal colon (i.e., feces). If bifidobacteria survive this transit (which takes from 24 to 48 h. depending on the individual) better than other bugs, this would seemingly lead to a stimulation by the substrate but rather is the reflection of a greater death of other bugs. We have shown however using stable isotope probing that upon supplementing the gut microbiota with ^{13}C-labeled GOS, the label becomes fairly selectively incorporated into *B. bifidum* (and lower levels of labeling for other bifidobacterial species and *L. gasseri*). This is an irrefutable proof of the fact that *B. bifidum* used the GOS as a substrate, as there is no other way of incorporation of the label into the bacterial biomass. Or in other words, the alternative explanations would not explain this label incorporation.

Other ^{13}C-labeled substrates that have been used include glucose (Egert et al. 2007), inulin (as yet unpublished), lactose (Venema 2012), and 6′-sialyl-lactose (Venema 2014).

6 Future Perspectives

Sophisticated and predictive in vitro models of the GI tract are excellent tools to study and optimize the survival of probiotics. Moreover, they allow to study mutants of probiotic strains that have been created to study which genes are

important in survival. In the colon model, the activity of the gut microbiota can be studied. This has mostly been done with a microbiota originating from healthy volunteers but can also be done with a microbiota from patients or from lean and obese people as described in Sect. 4.1.

Naturally, the in vitro models also have limitations, an important one being that there is no interaction with cells of the host. Ideally, one would couple experiments in the TIM systems to cell culture models that mimic, e.g., the gut epithelium or the underlying immune system. These combinations can already be performed by taking samples from the models and incubating these separately on cultured cells, but a fully integrated system would be optimal.

With the improvement in analytical methods, both chemically and through molecular DNA methods, such as transcriptomics, it is expected that in the not so distant future, a wealth of information will become available on the role of pro- and prebiotics on the health of the host.

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In Vitro and In Vivo Inhibition of Atopic Dermatitis (AD) by a Novel Probiotic Isolate *Lactobacillus sakei* Probio-65

Irfan A. Rather, Vivek K. Bajpai, and Yong-Ha Park

Abstract Atopic dermatitis (AD) represents a severe inflammatory state of skin diseases affecting enormous percentage of the world's population. Probiotics have been known to modulate immune responses on AD effectively and are being used as potential treatment strategies for the allergic conditions. A probiotic strain *Lactobacillus sakei* probio-65, previously isolated from Korean traditional fermented food kimchi, has shown multitude of functional and therapeutic efficacy as well as serves as a potent antimicrobial candidate inhibiting the growth of a number of pathogenic microorganisms including *Staphylococcus aureus* which causes severe skin disorder such as AD. In vitro studies have confirmed that probio-65, in terms of its potent immunostimulating potential, increases the production of nitric oxide and decreases the production of histamine, a potentially harmful biogenic amine. An in vivo study of probio-65 conducted on experimental mice (NC/Nga) revealed that oral administration of live or heat-killed cells of probio-65 had significant ($P < 0.05$) effect on AD and reduced the skin-scratching frequency dramatically. In addition, other tested parameters confirmed that administration of probio-65 cells remarkably decreased the concentration of AD markers including serum levels of IgE, cutaneous T-cell-attracting chemokine (CTACK), interleukin-4 (IL-4), and IL-6. Furthermore, children population associated with AD upon supplementation of probio-65 resulted in an improved reduction in chemokine levels. The results of a double-blind placebo-controlled trial confirmed that "minimum scoring of AD" (mSCORAD score) was lower after the administration of probio-65 than placebo treatment with mean disease activity percentage of 31 % and 13 %, respectively. These findings confirm that probio-65 could be a novel therapeutic candidate for the treatment of AD.

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1 Overview of Atopic Dermatitis

Atopic dermatitis (AD), also referred to be atopic eczema, is a long-lasting chronic inflammatory skin disease which affects around 20 % of children in the developed and developing countries (Johansson et al. 2004; Odhiambo et al. 2009; Shaw et al. 2011). AD is referred as a noncontagious disease unable to infect person to person. In AD, the term "dermatitis" refers to inflammation of the skin, whereas "atopic" stands for a majority of diseases with inherited tendency to develop other allergic conditions including asthma. The major types of AD include extrinsic-type AD associated with IgE-mediated sensitization, affecting 70–80 % of patients, while AD without IgE-mediated sensitization is called intrinsic-type AD affecting 20–30 % of patients (Johansson et al. 2001). It has been estimated that AD symptoms are developed among 65 % of patients in the first year of life and among 90 % of the patients before the age of five (Wahlgren 1992). Symptoms include pruritus and chronic or releasing eczematous lesions typically in children and at the flexural sides in adults (Leung and Bieber 2003). During AD, the skin of a patient reacts abnormally and easily to irritants, food, and environmental allergens and becomes red, flaky, and very itchy as well as shows redness, swelling, cracking, and crusting. It also becomes vulnerable to surface infections caused by bacteria. Skin examinations of AD patients often reveal spongiosis, hyperkeratosis, and parakeratosis in cases of acute lesions, while marked epidermal hyperplasia, acanthosis, and perivascular accumulation of lymphocytes and mast cells are often observed in cases of chronic lesions (Jin et al. 2009). In addition, AD is known as exacerbations during worst condition while referred as remissions when skin improves (NIAMS 2013).

Atopic dermatitis referred to as "eczema" is a type of various skin inflammatory diseases. AD occurs in equal proportion in male and female and about 30 % AD cases are reported each year in the USA (NIAMS 2013). AD can occur at any stage of life span; usual cases are dominated during infant or childhood state. AD is usually caused by exposure of human skin to harsh or wet conditions. AD is the most common of various types of eczema with similar diagnostic symptoms. The following types of eczema or AD can occur as skin inflammation (NIAMS 2013):

1. *Allergic contact eczema*. This type of eczema refers to red, itchy, weepy reaction on the skin in which the skin comes into contact with a substance that the immune system recognizes as foreign, such as poison ivy or certain preservatives in creams and lotions.
2. *Atopic dermatitis*. A kind of prolonged skin disorder due to higher moisture content which is characterized by itchy and inflamed skin.
3. *Contact eczema*. In contact eczema, skin becomes red and itchy with burning sensation during a localized reaction. During this state, the skin comes into contact with an allergen (an allergy-causing substance) or with an irritant such as an acid, cleaning agent, or other chemicals.
4. *Dyshidrotic eczema*. In this skin disorder, the skin becomes irritated specially on palms and hands including feet soles which can be diagnosed by clear, deep blisters that itch and burn.

5. *Neurodermatitis*. During this eczema state, scaly patches of high irritation can be visualized on the skin especially on the forehead, lower legs, wrists, or forearms through a localized reaction.
6. *Nummular eczema*. During this state, irritated patches of coin shape are found most commonly on the arms, back, hips, and lower parts of legs which may be crusted, scaly, and extremely itchy.
7. *Seborrheic eczema*. During this state, yellow-colored patchy skin scalps can be observed especially on the scalp, face, and occasionally other parts of the body.
8. *Stasis dermatitis*. This type of eczema usually occurs on the skin of the lower parts of legs more commonly associated with circulatory problems.

1.1 Symptoms of AD

Symptoms of AD can be varied from person to person with most common diagnostic symptoms of itching and rashes of skin. Itching is considered the most prevalent state of AD. However, rubbing and scratching result from the skin itching leading to increased skin inflammation and making life more complicated during sleep (NIAMS 2013). In addition, inflammation severity during AD is associated with the amount of itching and scratching as well as secondary skin infection. AD can also affect the eye skin, eyelid, and eyebrow and eyelash leading to eye skin redness or swelling. Scratching and rubbing of the eye skin can also result in patchy loss of eyebrows and eyelashes (NIAMS 2013). AD-related researches have confirmed that differences can be notified in human skin during AD that may lead to different symptoms of diseases. The epidermis (outer layer) of human skin is composed of two layers, inner part and outer part. The inner part of the epidermis is composed of living cells while the outer part, also known as horny layer or stratum corneum, is a form of dry, flattened, and dead cells. In general, the outer part behaves as a barrier, protecting the rest of the skin to become dry as well as providing safety to the other layers of the skin from outside detrimental exposure of irritants and infections. Adverse changes to the stratum corneum may lead to hyperactivity of skin which further leads to AD (NIAMS 2013). During AD, the human skin (epidermis) shows deficiency of moisture, leading to skin dryness which may result in the reduction of protecting efficiency of the epidermis. This phenomenon when combined with abnormal skin immune system may cause human skin to become more likely infected by other microbial pathogens (NIAMS 2013).

1.2 Stages of AD

During infancy and childhood, AD affects each individual differently in terms of both onset and severity of symptoms. In infants, AD typically begins around 6–12

weeks of age. It may first appear around the cheeks and chin as a patchy facial rash, which can progress to red, scaling, and oozing skin (NIAMS 2013). The skin may become infected. Once the infant becomes more mobile and begins crawling, exposed areas, such as the inner and outer parts of the arms and legs, may also be affected. An infant with AD may be restless and irritable because of the itching and discomfort of the disease (NIAMS 2013). During childhood, the rash tends to occur behind the knees and inside the elbows; on the sides of the neck; around the mouth; and on the wrists, ankles, and hands (NIAMS 2013). Often, the rash begins with papules that become hard and scaly when scratched. The skin around the lips may be inflamed, and constant licking of the area may lead to small, painful cracks in the skin around the mouth (NIAMS 2013). In some cases, the AD results in remission for a prolong duration, revert back only during the onset of puberty when hormones, stress, and the use of irritating skin care products or cosmetics may cause the disease to flare (NIAMS 2013). AD affects human beings similarly both in childhood and adulthood being widespread or limited to only a few parts of the body. For instance, only the hands or feet may be affected and become dry, itchy, red, and cracked (NIAMS 2013). Sleep patterns and work performance may be affected, and long-term use of medications to treat the AD may cause complications. Adults with AD also have a predisposition toward irritant contact dermatitis, where the skin becomes red and inflamed from contact with detergents, wool, or other potential irritants and friction from clothing. It is more likely to occur in occupations involving frequent handwashing or exposure to chemicals. Some people develop a rash around their nipples. These localized symptoms are difficult to treat. Because adults may also develop cataracts, the doctor may recommend regular eye exams (NIAMS 2013). Cataracts can develop in the cortex of the lens and are structurally different than anterior subcapsular cataracts or posterior subcapsular cataracts seen with AD.

1.3 Diagnosis of AD

Each and every individual suffering from AD may have different combination of disease symptoms from mild to severe over the duration of the AD. It is importantly advisable to visit the specified doctor in order to diagnose the accuracy of the disease (NIAMS 2013). In addition, other microbial infection during AD can cause more severe disease symptoms leading to skin irritation. Usually when a person is infected by AD, the patient is referred to a dermatologist or allergist for further examination. Hereditary of individual family may also help doctor to diagnose accurate symptoms of AD. In general, the diagnosis of AD is based on the family history of allergy or related symptoms. This may also help to diagnose accurate diseases and their possible causes. Diagnosis of AD begins with collecting the following information such as hay fever or asthma and about exposure to irritants, sleep disturbances, any foods that seem to be related to skin flares, previous treatments for skin-related symptoms, and the use of steroids or other medications.

Features of the disease fall into two categories: major features and minor features. Currently, there is no single test to diagnose atopic dermatitis. However, there are some tests that can give the doctor an indication of allergic sensitivity (NIAMS 2013). Pricking the skin with a needle that contains a small amount of a suspected allergen may be helpful in identifying factors that trigger flares of atopic dermatitis. Negative results on skin tests may help rule out the possibility that certain substances cause skin inflammation. Positive skin prick test results are difficult to interpret in people with AD because the skin is very sensitive to many substances, and there can be many positive test sites that are not meaningful to a person's disease at the time. Positive results simply indicate that the individual has immunoglobulin E or IgE (allergic) antibodies to the substance tested. IgE controls the immune system's allergic response and is often high in AD (NIAMS 2013).

1.4 Features of AD

The major features of AD include intense itching, characteristic rash in locations typical of the disease, chronic or repeatedly occurring symptoms, and personal or family history of atopic disorders (eczema, hay fever, and asthma). On the other hand, minor features of AD include the following: early age of onset; dry skin that may also have patchy scales or rough bumps; high levels of immunoglobulin E (IgE), an antibody, in the blood; numerous skin creases on the palms; hand or foot involvement; inflammation around the lips; nipple eczema; susceptibility to skin infection; and positive allergy skin tests. When the concentration of IgE antibodies becomes higher in the blood of patient to a certain extended level, this is the primary diagnosis of food allergy. During food allergy, individual can be instructed to maintain a daily diet plan in order to diagnose the reason of the disease. Restriction of eating certain specific foods may lead to determine the symptomatic food allergy if present. Identifying the food allergen may be difficult when a person is also being exposed to other possible allergens at the same time or symptoms may be triggered by other factors, such as infection, heat, and humidity (NIAMS 2013).

2 Factors Associated with AD

The causes of AD are poorly understood, but the disease seems to result from a combination of genetic (hereditary) and environmental factors, making it a major subject of ongoing research. There is no single proven cause of AD so far. Researches have shown that AD is developed as a result of interaction between the environment, immune system, and genetic inheritance. AD is largely inherited, characterized by an overactive immune response to the environmental factors. The same factors have no effect on the skin of a nonatopic person. Some children who share common genetic background and are from an atopic family never show signs

of AD and children with no family history of AD can suffer from it. However, AD occurs in about 60 % children where one parent has the condition and in about 80 % children in case of both parents having the same condition. The various environmental factors that may play a significant role in AD occurrence include air pollution; exposure to allergens, such as pollen, animal dander, or molds; workplace irritants such as fumes and chemical; and climate factors like winter and low humidity. In addition, emotional stress, exposure to certain foods, and excessive skin washing may also play a role in AD.

Children are more likely to develop this disorder if a parent has had it or another atopic disease like asthma or hay fever. If both parents have an atopic disease, the likelihood increases. Although some people outgrow skin symptoms, approximately half of children with AD go on to develop hay fever or asthma. Environmental factors can bring on symptoms of AD at any time in affected individuals (NIAMS 2013).

Microbial infection is also one of the main factors of AD. In more than 60 % of patients with AD, IgE to Pityrosporum ovale can be detected in the peripheral blood, supporting the hypothesis of the importance of this organism as an allergic trigger factor in AD (Scheynius et al. 2002). *Staphylococcus aureus* is found in more than 90 % of patients with chronic AD skin lesions (Leung 2003). Acute exudative skin lesions can contain over 10 million of these organisms per square centimeter, and increased numbers have been found even in normal skin and the nasal vestibular or intertriginous areas of patients suffering from AD.

Atopic dermatitis is also associated with malfunction of the body's immune system: the system that recognizes and helps fight bacteria and viruses that invade the body. The immune system can become misguided and create inflammation in the skin, even in the absence of a major infection. This can be viewed as a form of autoimmunity, where a body reacts against its own tissues. Many factors or conditions can make symptoms of AD worse, further triggering the already overactive immune system, aggravating the itch–scratch cycle, and increasing damage to the skin. These factors can be broken down into two main categories: irritants and allergens. Emotional factors and some infections and illnesses can also influence AD (NIAMS 2013).

In addition, irritants are substances that directly affect the skin and, when present in high enough concentrations with long enough contact, cause the skin to become red and itchy or to burn. Frequent wetting and drying of the skin may affect the skin barrier function. Also, wool or synthetic fibers and rough or poorly fitting clothing can rub the skin, trigger inflammation, and cause the itch–scratch cycle to begin. Soaps and detergents may have a drying effect and worsen itching, and some perfumes and cosmetics may irritate the skin. Exposure to certain substances, such as solvents, dust, or sand, may also make the condition worse. Cigarette smoke may irritate the eyelids.

Allergens are substances from foods, plants, animals, or the air that inflame the skin because the immune system overreacts to the substance. Inflammation occurs even when the person is exposed to small amounts of the substance for a limited time. Although it is known that allergens in the air, such as dust mites, pollens,

molds, and dander from animal hair or skin, may worsen the symptoms of AD in some people, scientists aren't certain whether inhaling these allergens or their actual penetration of the skin causes the problems.

Children with atopic disease tend to have a higher prevalence of food allergy than those in the general population. An allergic reaction to food can cause skin inflammation (generally an itchy red rash), gastrointestinal symptoms (abdominal pain, vomiting, diarrhea), and/or upper respiratory tract symptoms (congestion, sneezing, and wheezing). The most common allergenic (allergy-causing) foods are eggs, milk, peanuts, wheat, soy, tree nuts, shellfish, and fish. In addition to irritants and allergens, emotional factors, skin infections, and temperature and climate play a role in AD. Although the disease itself is not caused by emotional factors, it can be made worse by stress, anger, and frustration. Interpersonal problems or major life changes can also make the disease worse (NIAMS 2013).

Bathing without proper moisturizing afterward is a common factor that triggers a flare of atopic dermatitis. The low humidity of winter or the dry year-round climate of some geographic areas can make the disease worse, as can overheated indoor areas and long or hot baths and showers. Alternately sweating and chilling can trigger a flare in some people. Bacterial infections can also trigger or increase the severity of atopic dermatitis. If a patient experiences a sudden flare of illness, the doctor may check for infection (NIAMS 2013).

3 Mode of Action of Probiotics (*L. sakei* Probio-65) on AD

3.1 Animal Trials

Although a number of beneficial effects of probiotics to cure AD have been made intensely, very scarce information is available on how the probiotics modulate the immune system to combat against AD. The current scenario of literature on AD-related research confirms that there is only a few amount of information available on curing effects of probiotics in murine or human models of AD as well as skin allergy reactions. This book chapter focuses on the experimental models and clinical studies showing modes of action of probiotics and their possible curative effects in AD and skin allergy (Park et al. 2008; Woo et al. 2010; Kim et al. 2013).

In our previous study, we determined the effect of probiotic strain *L. sakei* probio-65 in mice model to visualize its effect on AD in terms of its potent anti-inflammatory potential (Park et al. 2008; Kim et al. 2013). It was found in this study that levels of IgE and IL-4 were determined from murine serum and splenocytes, respectively. Supplementation of *L. sakei* probio-65 in murine model on IgE levels resulted in 451.71 ± 185.59 ng/mL, which were lower than that (741.67 ± 287.58 ng/mL) of the positive control group as shown in Fig. 1a. This pattern was also observed for IL-4 levels. The IL-4 levels of the test group were 0.37 ± 0.39 pg/mL, which were lower than that (0.80 ± 0.66 pg/mL) of the positive

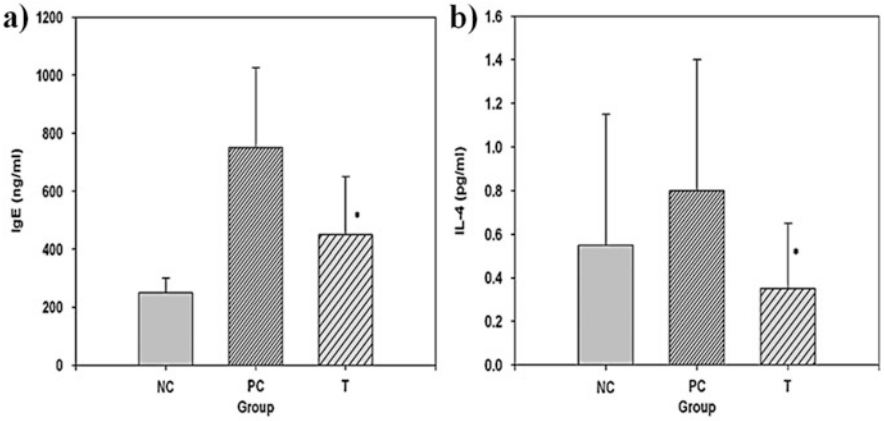

Fig. 1 (**a**) Change in IgE level in serum from DNCB-induced mice. NC, negative control; PC, positive control; T, test group supplemented with *L. sakei* probio-65. $*P < 0.05$ compared with NC and PC by Duncan's multiple range test. (**b**). Changes in IL-4 levels in splenocytes from DNCB-induced mice. NC, negative control; PC, positive control; T, test group supplemented with *L. sakei* probio-65. $*P < .05$ compared with NC and PC by Duncan's multiple range test (Park et al. 2008)

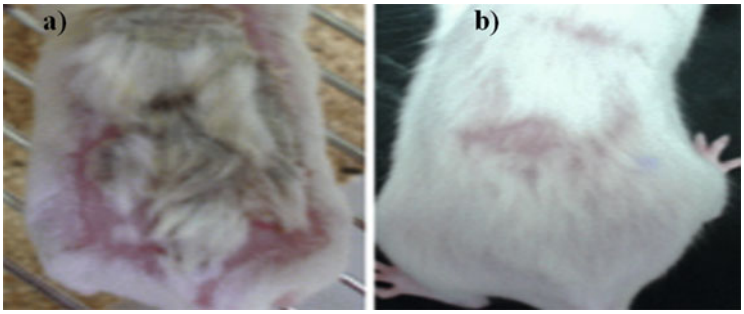

Fig. 2 Improvements in the skin of DNCB-induced mice by oral administration of *L. sakei* probio-65. (**a**) Starting of experiment; (**b**) end of experiment (Park et al. 2008)

control group as shown in Fig. 1b (Park et al. 2008; Kim et al. 2013). The body weights of the test and positive control mice did not differ over the course of the experiment. However, while both groups initially showed severe AD after dinitrochlorobenzene (DNCB) treatment, the skin of the test group improved significantly faster (Fig. 2). Although significant differences were observed in the scores after 12 days, the positive control still had a score of 3.5, whereas the test group score decreased up to 0.83 as shown in Table 1 (Park et al. 2008; Kim et al. 2013).

In the mouse model of skin inflammation, it was observed that the levels of serum IgE and splenocyte IL-4 induced by AD were significantly receded by the administration of probio-65 as shown in Fig. 1 (Park et al. 2008; Kim et al. 2013). The results were statistically significant with more rapid improvement in skin

Table 1 Changes in degree of allergic dermatitis in mice as determined by visual evaluation (Park et al. 2008)

Group	Allergen	Day				
		0	3	6	9	12
NC	–	0	0	0	0	0
PC	DNCB	10.7	11.33	11.17	4.33	3.5
T	DNCB	10	12	10.83	3.67	0.83

NC, negative control; PC, positive control; T, test mouse group supplemented with *L. sakei* probio-65 in feed

inflammation. Similar effect of *L. gasseri* TMC 0356 was observed on IgE serum levels, and it was found that oral administration of TMC 0356 resulted in the reduced IgE serum levels in AD patients as well as in perennial allergic rhinitis (Morita et al. 2006). Moreover, oral administration of *Lactobacillus* GG decreased the serum IgE levels and severity scoring of AD in IgE-sensitized infants (Viljanen et al. 2005). In addition, the *Lactobacillus* strain *L. casei* inhibited allergen-triggered production of IgE in murine splenocytes by promoting IL-12 secretion by macrophages (Isolauri et al. 2000). Apart from this, it has been reported that probiotic strains have ability to downregulate the IL-4 production of Th2-skewed splenocytes in vitro (Fujiwara et al. 2004). Moreover, preincubation of mononuclear cells with different strains of LAB such as *L. plantarum*, *L. lactis*, *L. casei*, and *L. rhamnosus* significantly inhibited the production of Th2 cytokines IL-4 and IL-5 (Pochard et al. 2002; Park et al. 2008; Kim et al. 2013). The latter inhibitory effect of lactic acid bacteria was shown to be mediated by IL-12 and interferon-γ since neutralization of these cytokines restored IL-4 production. In addition, the effect of lactobacilli inducing interferon-γ production in animal models has also been observed (Fig. 1), thus reducing allergen-stimulated production of IL-4 and IL-5 (Sutas et al. 1996; Joo et al. 2009). In addition, AD-like skin lesions were artificially induced in NC/Nga mice via repeated topical application of DNCB (Fig. 3a). Administration of *L. sakei* probio-65 (both live and dead cells) significantly suppressed the development of AD-like skin lesions as compared with the control group for two weeks. Similar findings were also observed in the experimental animals treated with dexamethasone, a well-known anti-inflammatory and immunosuppressant. Based on these findings, it was hypothesized that both live and heat-killed cells of *L. sakei* probio-65 may have potent ability to cure AD-like lesion in mice as did by dexamethasone. In addition, the effect of live and dead cells of probio-65 was also examined on murine scratching behavior. It was observed that DNCB dramatically increased the scratching behavior frequency in murine by 15-folds as shown in Fig. 3a. However, when treated with both live and dead cells of probio-65, the scratching behavior was significantly decreased in experimental mice as shown in Fig. 3b (Park et al. 2008; Kim et al. 2013). As reported previously, certain characteristic symptoms of AD are believed to be due to the strong polarization of the Th2 immune response, thus resulting in IgE overproduction (Park et al. 2008; Kim et al. 2013). Hence, we reported quantified determination of serum

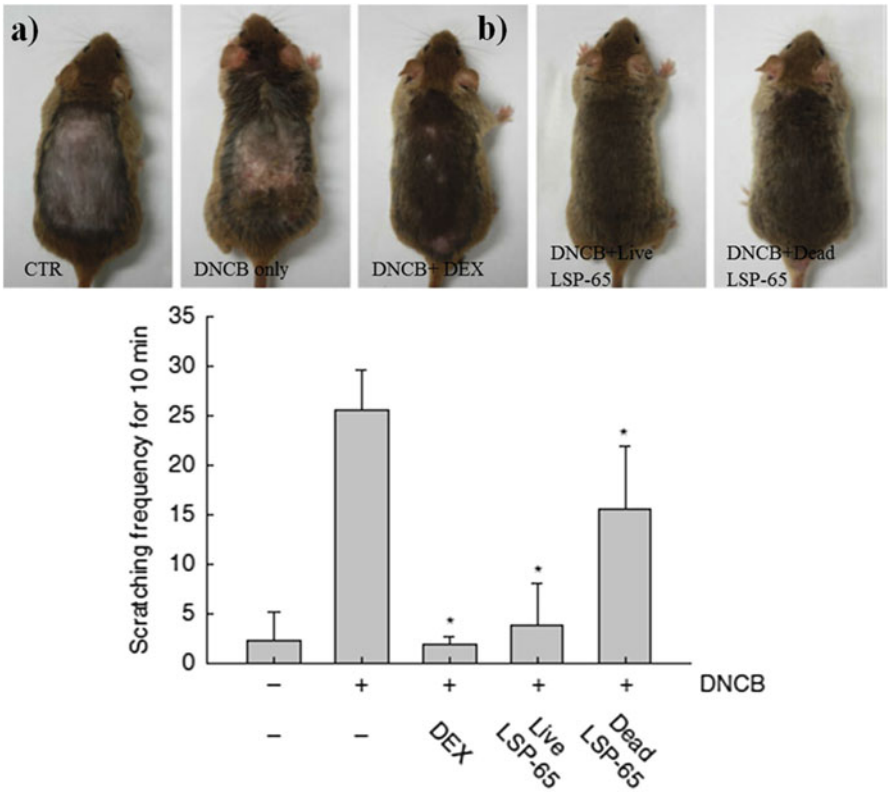

Fig. 3 Atopic dermatitis (AD)-like skin lesions and scratching behaviors in DNCB-induced mice. (**a**) AD-like skin lesions were induced via the topical application of 1-chloro-2,4-dinitrobenzene (DNCB) four times, with oral treatment with *Lactobacillus sakei* probio-65 improving overall skin condition (CTR and DNCB only, fed with distilled water; DNCB + DEX, fed with 0.06 mg ml^{-1} of dexamethasone, DNCB + Live LSP-65, fed with 5×10^9 CFU ml^{-1} of live *L. sakei* probio-65; DNCB + dead LSP-65, fed with 5×109 CFU ml^{-1} of heat-killed *L. sakei* probio-65). Skin features of mice were noted at the end of this experiment. Representative images from five mice. (**b**) Scratching behavior was quantified after the completion of all treatments. In brief, the number of scratching behaviors was counted over 10 min. This measurement was then repeated five times (50 min in total). *Significantly different from DNCB-treated group ($P < 0.05$). CTR, control; DEX, dexamethasone; LSP-65, *L. sakei* probio-65 (Kim et al. 2013)

IgE concentrations in DNCB-induced mice. It was found that serum IgE levels were significantly decreased in groups treated with dexamethasone, live and dead cells probio-65 as compared to DNCB-induced group (Fig. 4a). To evaluate the effect of probio-65 on Th2 cytokine and chemokine production in DNCB-induced mice, serum levels of IL-4, IL-6, and CTACK/CCL27 were also quantified by ELISA (Park et al. 2008). Interestingly, serum concentrations of IL-4 and IL-6 were significantly reduced by treatment with heat-killed *L. sakei* probio-65, but not by live *L. sakei* probio-65 (Fig. 4b, c). Serum CTACK concentrations were markedly

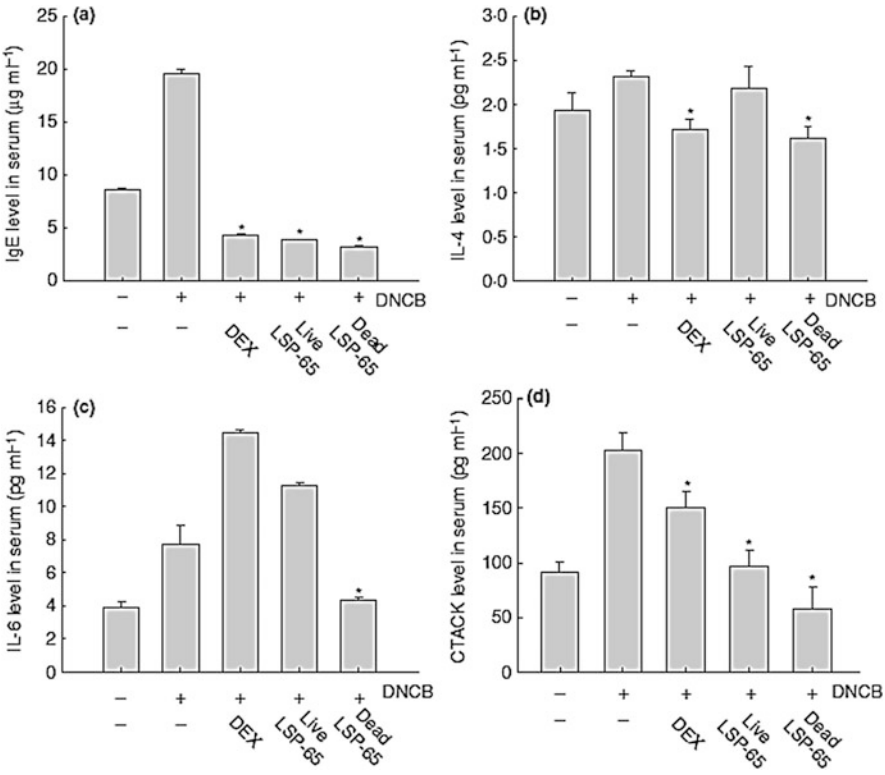

Fig. 4 The effect of *L. sakei* probio-65 on serum levels of IgE, IL-4, IL-6, and cutaneous T-cell-attracting chemokine (CTACK) in DNCB-induced mice. After the completion of all treatments, blood samples were collected from the inferior vena cava, and the sera was separated. The serum concentrations of IgE (**a**), IL-4 (**b**), IL-6 (**c**), and CTACK (**d**) were quantified by ELISA. All data are presented in the form of mean ± SEM. *Significantly different from DNCB-treated group ($P < 0.05$). DEX, dexamethasone; LSP-65, *L. sakei* probio-65 (Kim et al. 2013)

inhibited in animals treated with dexamethasone, live *L. sakei* probio-65, and dead *L. sakei* probio-65 compared with the DNCB-induced control group as shown in Fig. 4d (Park et al. 2008; Kim et al. 2013).

Further, to determine the efficacy of probio-65 to suppress TARC and CTACK expression in DNCB-induced AD mice, DNCB dorsal skin of treated mice was homogenized, and the levels of chemokine such as TARC and CTACK in the homogenized skin were determined by Western blot analysis (Park et al. 2008; Kim et al. 2013). It was found that the expression of both TARC and CTACK increased remarkably in the DNCB-treated group (Fig. 5a). However, treatment with live and heat-killed cells of probio-65 resulted in a significant decrease in the expression of DNCB-induced TARC and CTACK. To further determine the effects of live and dead cells of probio-65 on Foxp3 expression, murine dorsal skin and ears were

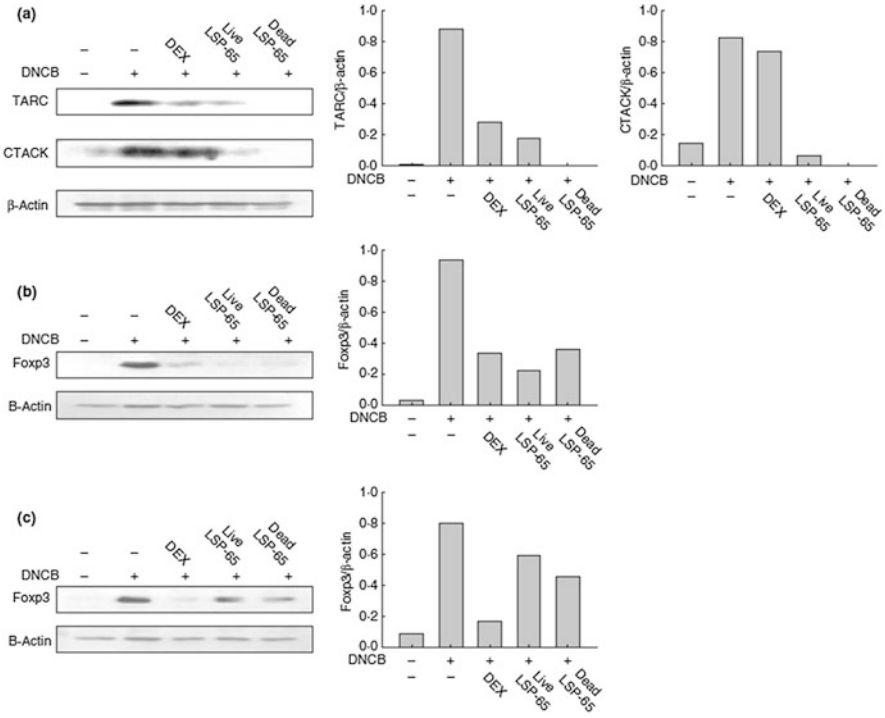

Fig. 5 The effect of *L. sakei* probio-65 on Thymus and activation-regulated chemokine (TARC) and cutaneous T-cell-attracting chemokine (CTACK) expression in dorsal skin and Foxp3 protein expression in the dorsal skin and ears of DNCB-induced mice. After the completion of all treatments, the dorsal skin and ears of mice were homogenized. The chemokines in the homogenized skin (**a**), as well as the protein expression of Foxp3 in the homogenized skin (**b**) and ears (**c**), were measured via Western blot analysis, with the protein expression of β-actin used as an internal control. The results illustrated are from a single experiment and are representative of three separate experiments. The expressions of TARC, CTACK, and Foxp3 are expressed in arbitrary units, and the data are normalized to β-actin protein. DEX, dexamethasone; LSP-65, *L. sakei* probio-65 (Kim et al. 2013)

homogenized, and Foxp3 protein expression was determined by Western blot analysis (Park et al. 2008). Since Foxp3 expression is directly related with AD, it was observed that the protein expression of Foxp3 was remarkably increased in both dorsal skin and the ears of DNCB-induced mice when compared with the control animals (Fig. 5b, c). However, expression of Foxp3 was significantly suppressed in both locations in mice treated with dexamethasone as well as live and dead cells of probio-65 (Park et al. 2008; Kim et al. 2013).

A well-established degranulation marker, β-hexosaminidase secretion, is considered as a hallmark of allergic reactions resulting from exposure of mast cells to allergens. In our previously reported study, it was found that probio-65 showed significant inhibitory effect on AD-like skin lesions in experimental mice (Park et

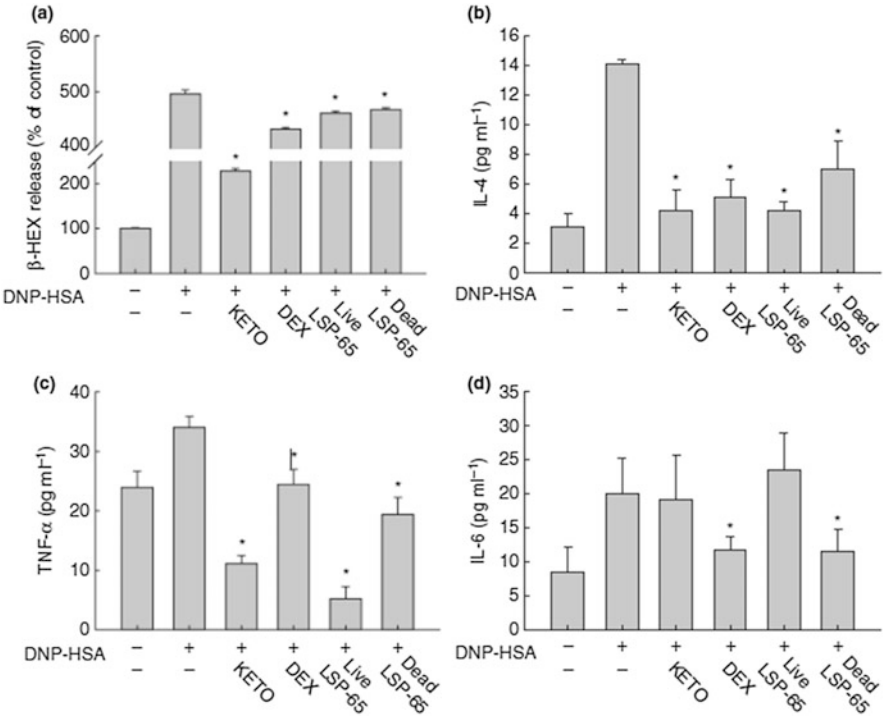

Fig. 6 The effect of *L. sakei* probio-65 on antigen-induced degranulation and cytokine release in RBL-2H3 cells. In brief, RBL-2H3 cells were sensitized overnight with 500 ng ml^{-1} of DNP-specific IgE. These IgE-primed cells were then pretreated with either live or dead *L. sakei* probio-65 (5×10^9 CFU $well^{-1}$), dexamethasone (100 nmol l^{-1}), or ketotifen (100 μmol l^{-1}) for 20 min and then stimulated with 100 ng ml^{-1} of DNP-HSA for 10 min. The release of β-hexosaminidase (**a**) from IgE-sensitized RBL-2H3 cells was quantified using a microplate reader at 405 nm. The concentrations of IL-4 (**b**), TNF-α (**c**), and IL-6 (**d**) in the supernatant were measured by ELISA. The results of three independent experiments are expressed in the form of mean ± SEM. *Significantly different from DNCB-treated group ($P < 0.05$). DEX, dexamethasone; KETO, ketotifen; LSP-65, *L. sakei* probio-65 (Kim et al. 2013)

al. 2008). To further this phenomenon, efficacy of probio-65 was tested in vitro in on mast cells through examining its inhibitory effect on β-hexosaminidase secretion. It was found that DNP-HSA significantly induced degranulation in IgE-sensitized RBL-2H3 cells compared with untreated cells (Park et al. 2008; Kim et al. 2013). Interestingly, it was found that live and heat-killed cells of probio-65 significantly reduced β-hexosaminidase secretion in IgE-sensitized RBL-2H3 cells as shown in Fig. 6a (Park et al. 2008; Kim et al. 2013). Since large number of cytokines such as IL-4, IL-6, and TNF-α play a crucial role in allergic inflammation, it was reported that probio-65 had profound effect on quantitative determination of inflammatory markers (Park et al. 2008; Kim et al. 2013). It has been reported that probio-65 in addition with ketotifen and dexamethasone significantly inhibited the

Table 2 Demographic clinical characteristics of the 75 patients enrolled in the study (Woo et al. 2010)

	Probiotic group ($n = 41$)	Placebo group ($n = 34$)	*P* value
Sex, M/F no.	20/21	13/21	.36
Age, mean (range), years	3.3 (2.3–9.8)	5.8 (2.0–97)	.50
Age group, no. (%)			
2–6 years	25 (61)	22 (65)	.74
7–10 years	16 (39)	12 (35)	.74
Asthma or allergic rhinitis, no. (%)	22 (54)	18 (53)	.95
Sensitization, no. (%)			
Overall	34 (83)	28 (82)	.95
Aeroallergen	27 (66)	24 (71)	.66
Food allergen	22 (54)	19 (59)	.85
Log total IgE, mean 9 range 0, IU/mL	2.37 (0.96–3.38)	2.31 (1.18–3.66)	.69
Log CEP, mean (range), mg/dL	1.37 (0.30–2.23)	1.30 (0.43–2.29)	.51
SCORAD score, mean (range)			
Total	42.6 (26.4–75.7)	40.0 (27.2–76.5)	.50
Extent and intensity	32.4 (17.0–55.8)	31.0 (17.7–56.5)	.66
Pruritus and sleep loss	10.2 (1.0–20.0)	9.0 (3.0–20.0)	.29

production of IL-4 and TNF-α secretion induced by DNP-HAS (Fig. 6b, c). Since heat-killed cells of probio-65 were able to suppress the production of IL-6 but not by live cells, it was hypothesized that probio-65 shows its differential effects on cytokine production as inflammatory markers by diverse modes of action.

3.2 Human Trails

Recent research scenario has confirmed that probiotics have useful potential in children with atopic eczema–dermatitis syndrome (AEDS). Supplementation of *L. sakei* probio-65 in children with AEDS has been associated with a substantial clinical improvement with a significant decrease in chemokine levels, reflecting the severity of AEDS.

As reported previously, a study enrolled 88 children where 45 children were allocated to probiotic therapy (Woo et al. 2010; Kim et al. 2013). Seventy-five children completed the study, with 4 dropouts in the probiotic group and 9 in the placebo group (Table 2). It has been reported that at week 12, SCORAD total scores adjusted by pretreatment values were lower after probiotic treatment than after placebo treatment. Administration of probiotic resulted in 31 % (13.1-point) improvement in mean disease activity as compared with a 13 % (5.2-point) improvement with placebo use (Woo et al. 2010; Kim et al. 2013). In addition,

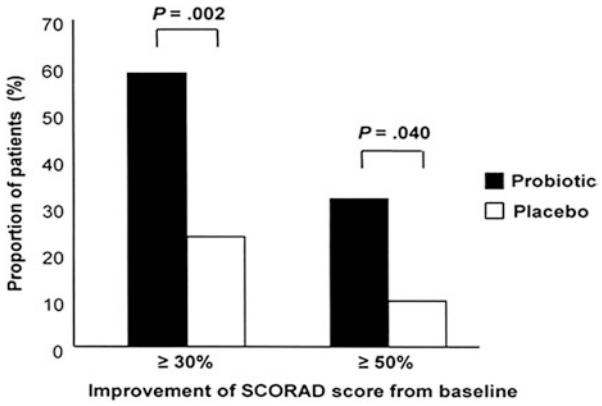

Fig. 7 Patients achieving at least 30 % and 50 % improvement in SCORing Atopic Dermatitis (SCORAD) scores at the study end point (Woo et al. 2010)

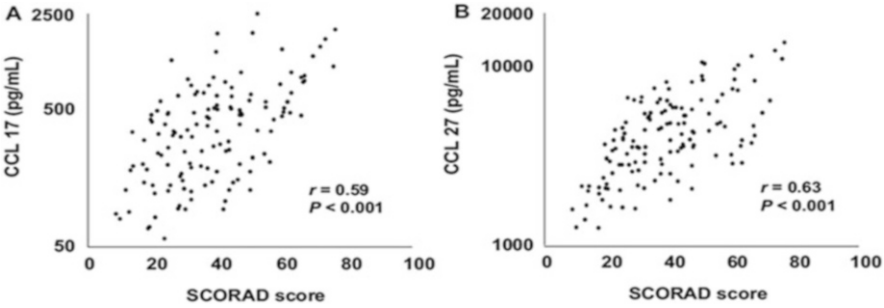

Fig. 8 Correlation of serum logarithmic CCL17 (**a**) and CCL27 (**b**) levels with disease severity assessed by SCORing Atopic Dermatitis (SCORAD) scores in 75 patients with atopic eczema–dermatitis syndrome (Woo et al. 2010)

when the efficacy of probiotic supplementation in AEDS was assessed by comparing the proportion of patients who achieved at least 30 % and 50 % reductions in the SCORAD score, there was a significant difference between the probiotic and placebo groups (Fig. 7). After adjusting for baseline differences, mean objective and subjective scores at week 6 were not lower in the probiotic group compared with those in the placebo group. However, pretreatment-adjusted SCORAD total scores were significantly lower at week 12 in probiotics group compared to placebo group (Woo et al. 2010; Kim et al. 2013). While pretreatment-adjusted subjective scores after 12-week probiotic treatment were lower compared with those after placebo treatment, the difference did not reach the level of significance.

Similarly others reported the levels of serum CCL17 and CCl27 levels which were associated with the severity of AEDS (Hinjnen et al. 2004; Kakinuma et al. 2001) (Fig. 8). Probiotic administration was associated with lower pretreatment-adjusted serum levels of CCL17 and CCL27 as compared to placebo treatment,

Table 3 Serum pretreatment and posttreatment values of CCL17 and CCL27 in the probiotic and placebo group[a] (Woo et al. 2010)

	Probiotic group	Placebo group	*P* value
Log TARC/CCL 17 (pg/ml)			
Baseline	2.60 (2.50–2.70)	2.50 (2.35–2.64)	0.24
Week 12[b]	2.27 (2.14–2.40)	2.48 (2.33–2.62)	0.03
Log CTACK/CCL27 (pg/ml)			
Baseline	3.68 (3.60–3.76)	3.63 (3.53–3.73)	0.46
Week 12[b]	3.52 (3.45–3.59)	3.62 (3.54–3.70)	0.03

CTACK, cutaneous T-cell-attracting chemokines; TARC, thymus and activation-regulated chemokines

[a]Values are presented as mean (95 % confidence interval)

[b]Adjusted by the pretreatment value

Fig. 9 Improvements in atopic dermatitis by the administration of *L. sakei* probio-65

which were significantly correlated with SCORAD total score. Moreover, the changes in serum CCL17 and CCL27 levels during 12 weeks correlated with the changes during the same period in SCORAD total scores (Woo et al. 2010; Kim et al. 2013). In patients treated with the probiotic, serum levels of CCL17 and CCL27 were significantly decreased from baseline at the end of the intervention (Woo et al. 2010; Kim et al. 2013). On the other hand, decreases in serum CCL17 and CCL27 levels in the placebo group did not reach the level of significance. Pretreatment-adjusted serum CCL17 and CCL17 levels were significantly lower in the probiotic group after 2 weeks than in the placebo group (Woo et al. 2010; Kim et al. 2013) as shown in Table 3. Schematic representation on how probio-65 improved AD has been given in Fig. 9.

In our recent publication, we compared the effects of an emollient that contains *L. sakei* probio-65 with a normal emollient on AD. Before human trial, extracts were tested on animals and no irritation was observed from the use as shown in Fig. 10. Furthermore, the double-blind, randomized, split-body clinical trial involving 28 patients with AD proved that *Lactobacillus*-containing emollients may improve the skin permeability of patients with AD as shown in Fig. 11 (Park et al. 2014).

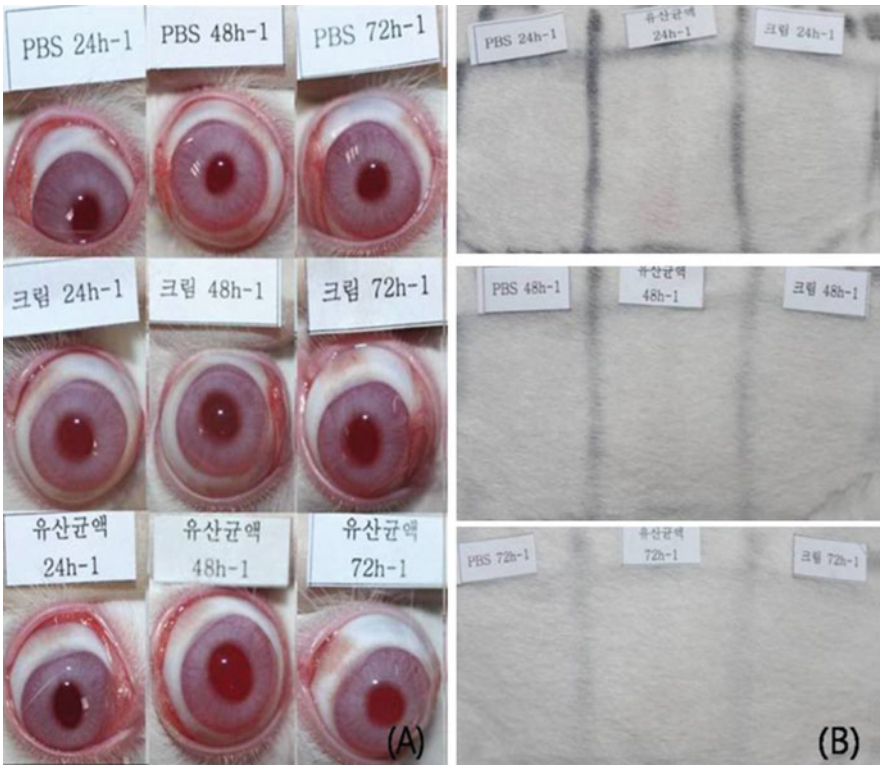

Fig. 10 Three groups showed no responses in eye stimulation test (*top* PBS, *middle Lactobacillus sakei* probio-65 extract containing emollient, *bottom* L. *sakei probio*-65 extract) (**a**) and skin stimulation test (*left* PBS, *middle L. sakei* probio-65 extract, *right L. sakei* probio-65 extract containing emollient) (**b**). PBS, phosphate buffer saline (Park et al. 2014)

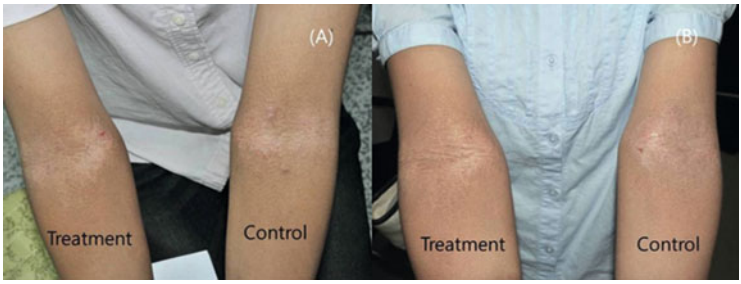

Fig. 11 Effect of emollients containing *Lactobacillus* in atopic dermatitis patients at baseline (**a**) and after 4 weeks (**b**) (Park et al. 2014)

4 Future Prospects

Although the symptoms of AD can be difficult and uncomfortable, the disease can be successfully managed. People with AD can lead healthy, productive lives. As scientists learn more about AD and what causes it, they continue to move closer to effective treatments and perhaps, ultimately, a cure. Researchers supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS 2013) and other institutes of the National Institutes of Health (NIH) are gaining a better understanding of what causes AD and how it can be managed, treated, and, ultimately, prevented. Recently, World Allergy Organization plans to release the first section of the Guidelines for Allergic Disease Prevention, which will recommend the use of probiotics by pregnant and lactating women and their breastfed infants to prevent the development of AD.Discoveries about how the body creates and maintains skin will be helpful in searching for causes and treatments for diseases like AD in which the skin barrier breaks down. Investigators are trying to find out if drugs that are already on the market can help repair the skin barrier. Also, they are looking at melanin, the component of skin that gives it pigment, to see if there is a link between melanin production and regulation of skin function (NIAMS 2013). For babies that may be at high risk for developing atopic dermatitis, investigators are trying to find out what effects emollients may have if applied in infancy before symptoms appear. Others are looking at what cells are triggered by stress and how those cells contribute to skin breakdown. One of the most difficult symptoms of AD, itching, is being studied to determine what mechanisms trigger the sensation of itch. Researchers are studying how the nervous system and the immune system communicate to cause the inflammation, itch, and pain seen in AD. Also, in an attempt to break the itch–scratch cycle, researchers are investigating how the brain and the skin are working together to make scratching and itch more likely to make the skin itch even more (NIAMS 2013). Researchers are trying to understand the role of dendritic cells, which are found in the skin, and other tissues of the body that initiate an immune response. Other researchers are trying to see what other factors play a role in the immune response, causing inflammation like that seen in AD (NIAMS 2013).

5 Conclusion

Nowadays, probiotics are gaining enormous important due to their potential health beneficial effects. It has been encountered that probiotics have shown remarkable efficacy in both in vitro and in vivo to improve the AD in animal and human trials. Administration of *L. sakei* probio-65, a probiotic strain isolated from Korean traditional fermented food kimchi, exhibited fast recovery of the damaged skin-induced allergy by DNCB along with decreased IgE and IL-4 levels in an animal model. Moreover, oral treatment of both viable and heat-killed cells of probio-65

remarkably inhibited skin inflammation and AD-like skin lesions in vivo, as well as mast cell activation in vitro. These findings reinforce the suggestions that application of probiotics may provide fruitful results with potent immunostimulatory potential leading to improve AD-like lesions, considering probiotics a potent, safe, and natural alternative for clinical AD treatment in the future.

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Bifidobacterium for Infants: Essence and Efficacy

Amy Sie-Yik Lau, Jin-Zhong Xiao, and Min-Tze Liong

Abstract Bifidobacteria are the predominant bacteria in the gastrointestinal tract of an infant. The colonization pattern of the bifidobacteria is affected by mode of delivery and types of feeding. Newborns from vaginal delivery are firstly exposed to maternal vaginal, fecal, and skin bacteria, such as *Lactobacillus*, *Prevotella*, and *Atopobium*. In the few days after birth, a reduced environment in the gastrointestinal tract favors the arrival and proliferation of obligate anaerobic bacteria, such as *Bifidobacterium*, and subsequently becomes predominant in the infant's gastrointestinal tract. Also, in breast-fed infants, *Bifidobacterium* concentration is higher than formula-fed infants as human breast milk contains human milk oligosaccharides (HMOs) which are bifidobacterial growth-promoting factors. In addition to HMOs, existing lysozyme in breast milk could be involved in selection of infant-type *Bifidobacterium* as inhabitants of the infant intestines. Bifidobacteria colonization in infants initiates the development and maturation of the infant's naive immune system by stimulating dendritic cells through Toll-like receptors (TLR) and leads to differentiation of naive T lymphocytes into Th1 cells by recognizing the peptidoglycans in bifidobacteria. Hence, immune response, control intestinal inflammation, and mucosal tolerance are initiated. Production of immunoglobulin A (IgA) is also stimulated by the presence of bifidobacteria to stimulate intestinal antigenic ability. Bifidobacteria are able to prevent and treat atopic diseases in infants by restoring Th1/Th2 balance and enhance the production of IFN-γ. In addition, bifidobacteria are also effective in reducing the duration of gastrointestinal diseases by modification and stabilization of GIT microflora, reduction in the duration of retrovirus shedding, reduction of GIT permeability, as well as induction of general immune response by increasing IgA antibodies. In respiratory tract infections, bifidobacteria enhance several immune responses by increasing immune cell activity, modulating signals in epithelial and immune cells, increasing local and

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systemic antibody production, and inducing phenotypic changes in dendritic cells. Considering that *Bifidobacterium* are the natural inhabitants of infant intestines, and much clinical evidence of efficacy to infants have been documented, *Bifidobacterium* strains are highly encouraged for use as probiotics for infants.

1 Introduction

In the human body, it is known that the total number of microbial cells within the gastrointestinal tract lumen is approximately 10 times more than the total number of tissue cells in the human body, contributing to several hundred grams in total (Picard et al. 2005; Mikami et al. 2012; Putignani et al. 2014). With the abundances, these microbial cells colonize every surface of the human body, including the skin, oral cavity, urogenital tract, gastrointestinal tract, and respiratory tract (Gerritsen et al. 2011). Among all these body sites, the gastrointestinal tract is the most densely populated site, with a vast and diverse community of microorganisms consisting of over 500–1000 different species of bacteria (Mikami et al. 2012; Sjögren et al. 2009; Gerritsen et al. 2011; Makino et al. 2013). The colon contains over 70 % of all the microorganisms in the human body (Sekirov et al. 2010). The human gastrointestinal tract is a preferred site for microbial colonization as it is rich in nutrients for microorganisms as well as its large surface area which is equivalent to the size of a tennis court (200 m^2) (Holzapfel 2006; Sekirov et al. 2010). According to Sartor and Mazmanlan (2012), there are four predominant bacteria phyla in the human gut, namely, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* (Lee and O'Sullivan 2010; Glendinning and Free 2014). In addition, a large number of viruses, predominantly bacteriophages, are also present in the gastrointestinal tract, as a part of the intestinal microorganisms (Sekirov et al. 2010; Glendinning and Free 2014). The presence of these various species of bacteria is crucial in maintaining the homeostasis of the intestinal ecosystem (Schell et al. 2002). For example, *Clostridium*, *Lactobacillus*, and *Enterococcus* are found in epithelial surface and the mucus layer in the intestine, whereas *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Enterobacteriaceae*, *Enterococcus*, *Clostridium*, *Lactobacillus*, and *Ruminococcus* are present in the intestinal lumen which will then be secreted in feces (Sekirov et al. 2010).

Homeostasis of the intestinal ecosystem is crucial to maintain health. The intestinal microorganisms possess immense benefits to the human host. They are responsible in metabolizing exfoliated epithelial cells, dietary carbohydrate, and mucus (Sartor and Mazmanlan 2012), production of essential vitamins, detoxification of harmful substances (Glendinning and Free 2014), as well as regulation of host fat storage (Eckburg et al. 2005; Glendinning and Free 2014). They also produce metabolites that affect the function of intestinal epithelial cells and influence host immune regulation and homeostasis, as well as hepatic function

(Schell et al. 2002; Picard et al. 2005; Turroni et al. 2012; Sartor and Mazmanlan 2012). It is well documented that gastrointestinal disorders are closely related to the balance of the intestinal microbiota (Hawrelak and Myers 2004). Dysbiosis is delineated as "qualitative and quantitative changes in the intestinal flora, their metabolic activity and their local distribution" (Hawrelak and Myers 2004). In other words, it refers to the compositional disruption of the intestinal microbiota, which will cause harmful effects on host (Hawrelak and Myers 2004; Glendinning and Free 2014). It is characterized as a reduction in the obligate anaerobes such as *Bacteroidetes* and an increase in the facultatively anaerobic *Proteobacteria* (Glendinning and Free 2014). Patients suffering from gastrointestinal disorders such as inflammatory bowel diseases (IBD), functional dyspepsia, irritable bowel syndrome, alcoholic liver disease, obesity, and diabetes were found to have microbial dysbiosis (Sartor and Mazmanlan 2012; Turroni et al. 2012; Hawrelak and Myers 2004; Schulz et al. 2014). Diarrhea, which is caused by agents such as adenovirus, norovirus, and rotavirus, is related with dysbiosis with the reduction of *Bacteroidetes* levels and increase of *Proteobacteria* (Glendinning and Free 2014). Also, in comparing the composition of intestinal microflora between allergic and nonallergic infants, allergic infants had lower bifidobacterial population but higher in the number of *Staphylococcus aureus* and *Enterobacteria* (Xiao et al. 2006).

Therefore, the formation of a stable gastrointestinal microbial consortium is critical and essential to the development of the infant gastrointestinal tract (Schell et al. 2002). In an unborn fetus, the gastrointestinal tract is initially sterile or known as germ free (Schell et al. 2002; Makino et al. 2011; Grimm et al. 2014; Balamurugan et al. 2010; Vebø et al. 2011; Rigottier-Gois 2013). With the exposure to an external environment which is abundant with various bacterial species, microbial colonization takes place and the gastrointestinal tract will be fully colonized within the first week of birth (Makino et al. 2011; Turroni et al. 2014). The microbial colonization pattern of gastrointestinal tract microbiota in infants is greatly affected by the mode of delivery, which determines microbial exposure at the time of birth (Turroni et al. 2012; Morelli and Patrone 2014). Newborns from vaginal delivery are firstly exposed to maternal vaginal, fecal, and skin bacteria, such as *Lactobacillus*, *Prevotella*, and *Atopobium* (Morelli and Patrone 2014; Grimm et al. 2014).

At birth, the presence of oxygen in the gastrointestinal tract promotes the initial colonization of the aerotolerant bacteria such as *Staphylococcus*, *Enterobacteriaceae*, and *Streptococcus*. These aerotolerant bacteria will dominate for the first hours or days and consume the residual oxygen in the gastrointestinal tract (Makino et al. 2011; Rigottier-Gois 2013). A few days after birth, a reduced environment in the gastrointestinal tract favors the arrival and proliferation of obligate anaerobic bacteria, such as *Bifidobacterium*, *Bacteriocides*, and *Clostridium* (Mikami et al. 2012; Makino et al. 2013; Morelli and Patrone 2014; Grimm et al. 2014). The presence of these aerotolerant predecessors is transient and soon replaced by obligate anaerobic bacteria which eventually become the predominant bacteria remaining in the gastrointestinal tract (Mikami et al. 2012). One month after birth, *Bifidobacterium* appears to be the most predominant bacteria and

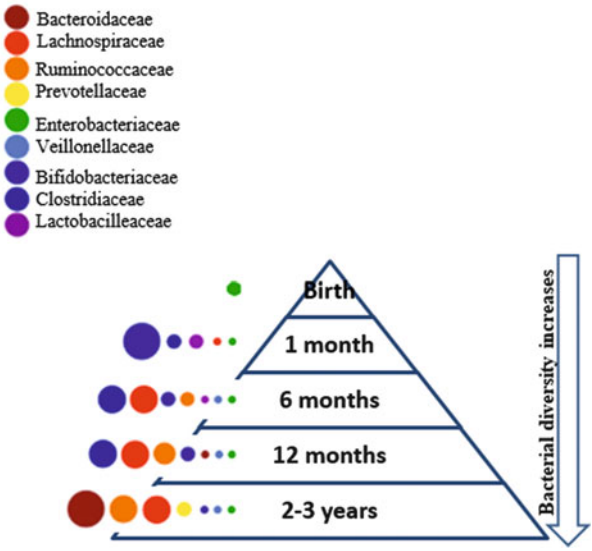

Fig. 1 Changes of intestinal microbiota composition of infants and children. The intestinal microbiota of the newborn is initially colonized by *Enterobacteria* prior to the colonization by bifidobacterial species, followed by an expansion of clostridial species (*Lachnospiraceae*, *Clostridiaceae*, and *Ruminococcaceae*), and finally *Bacteroidaceae*, *Lachnospiraceae*, and *Ruminococcaceae* by 2–3 years of age and remains stable into adulthood

remains in the gastrointestinal tract throughout infancy (Mikami et al. 2009; Balamurugan et al. 2010). Bacterial biodiversity continues to change from infant to children 2–3 years old (Fig. 1), where then microbiota composition consists of mainly *Bacteroidaceae*, *Lachnospiraceae*, and *Ruminococcaceae* that remains stable into adulthood (Arrieta et al. 2014).

2 General Features of Bifidobacteria

Bifidobacteria were firstly isolated from the feces of healthy breast-fed infants in 1899 by Henri Tissier (Kleerebezem and Vaughnan 2009; Lee and O'Sullivan 2010). Bifidobacteria are primarily Gram-positive prokaryotes, non-spore-forming, nonmotile, and catalase-negative anaerobic bacteria (Schell et al. 2002; Martinez et al. 2013). They produce acetic and lactic acids by fermentation of lactose, glucose, galactose, and fructose (Wasilewska et al. 2003). These obligate anaerobes belong to the *Actinomycetales* branch of the high-G + C Gram-positive bacteria, presented as pleomorphic rods which vary in shapes, including curved, short, and bifurcated Y shapes (Schell et al. 2002; Martinez et al. 2013). Differences in their morphology are also due to different culture medium, growth, and culture conditions. Also, their branching nature is also strain dependent (Fig. 2) (Tannock 1999; Olvera et al. 2013).

Up to date, the genus *Bifidobacterium* comprises of 32 species (Schell et al. 2002; Baffoni et al. 2013). Among these, a few were isolated from human vagina and oral cavity, but mammalian gastrointestinal tracts harbor the vast majority of bifidobacteria. They present naturally in the gastrointestinal tract as

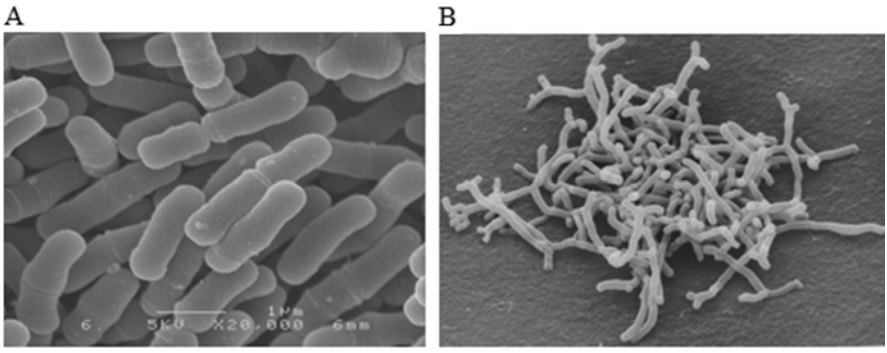

Fig. 2 Morphology of bifidobacteria is strain dependent where (**a**) *Bifidobacterium longum* subsp. *infantis* M-63 appears in uniform rod shape, while (**b**) *Bifidobacterium longum* BB536 appears in bifurcated Y shape. (Photos courtesy of Morinaga Milk Industry Co. Ltd, Japan)

the dominant commensal colonic microbiota and promote a healthy gastrointestinal tract (Schell et al. 2002; Picard et al. 2005; Martinez et al. 2013). Nonetheless, the composition of bifidobacteria will decrease with age (Léké et al. 2007; Turroni et al. 2014). According to Martinez et al. (2013), in adults' microbiota, bifidobacteria are accounted for around 3–7 % while up to 91 % are found in newborns. Nevertheless, Grimm et al. (2014) also reported that in naturally delivered, breast-fed infant, 95 % of all bacteria are bifidobacteria. The abundance of bifidobacteria was confirmed by metagenomic analyses (Turroni et al. 2014).

2.1 Ecology of Bifidobacteria

Bifidobacterial species have been isolated from different ecological niches, such as the human intestines, oral cavity, food, animal gastrointestinal tract (GIT), insect intestine, and sewage (Ventura et al. 2012a, b; Bottacini et al. 2014). Bifidobacteria are currently known to contain 47 taxa, including 38 species and nine subspecies (Milani et al. 2014). Most of the taxa are residents of the intestine, including in warm-blooded mammals and social insects. Some of these bifidobacteria species are typical inhabitants of the human gut and are thus designated as human-residential bifidobacteria (HRB) (Harmsen et al. 2000; Roger et al. 2010; Boesten et al. 2011). In the human gastrointestinal tract, the most abundant species of bifidobacteria are *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium pseudolongum*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium adolescentis*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium catenulatum*, *Bifidobacterium angulatum*, and *Bifidobacterium breve* (Ventura et al. 2012a, b; Grimm et al. 2014; Turroni et al. 2014). All of these bifidobacterial species are associated with the well-being of the host. Bifidobacterial species found in the

human gastrointestinal tract vary according to an individual's age. For instance, *B. breve*, *B. bifidum*, and *B. longum* subsp. *infantis* are known infant-specific bifidobacterial microbiota, while on the other hand, adult-specific bifidobacterial species include *B. adolescentis*, *B. catenulatum*/*pseudocatenulatum*, and *B. longum* subsp. *longum* species (Ventura et al. 2012a, b). In addition, a number of bifidobacterial species have also been isolated from swine feces, including *B. longum* subsp. *suis*, *B. thermophilum*, *B. choerinum*, *B. aerophilum*, *B. psychroaerophilum*, *B. thermacidophilum* subsp. *porcinum*, and *B. boum* (Matteuzzi et al. 1971; Ventura et al. 2012a, b), while *B. animalis* subsp. *animalis*, *B. magnum*, *B. pseudolongum* subsp. *pseudolongum*, *B. pseudolongum* subsp. *globosum*, *B. merycicum*, *B. ruminantium*, *B. saeculare*, and *B. cuniculi* have been isolated from other mammals and birds. *B. asteroides*, *B. coryneforme*, *B. indicum*, and *B. bombi* have been reportedly isolated from the hindgut of honeybee (Ventura et al. 2012a, b; Kawasaki 2011). Another ecological niche where *Bifidobacterial* species have been isolated was from sewage which includes *B. minimum*, *B. subtile*, and *B. thermacidophilum* subsp. *thermacidophilum* (Ventura et al. 2012a, b). In addition, *B. animalis* subsp. *lactis* has been used as a probiotic or yogurt starter in industrial production, but is generally not the natural colonizer of human or animal intestines. These species are thus designated as non-human-residential-bifidobacteria (non-HRB).

2.2 *Bifidobacteria in Infants*

The gastrointestinal tract of breast-fed infants is predominantly colonized by bifidobacteria of species such as *B. breve*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, and *B. bifidum* (designated as infant-type HRB) (Mitsuoka and Kaneuchi 1977; Roger et al. 2010). Colonization of bifidobacteria in the infant's gastrointestinal tract is suggested to be affected by types of feeding, i.e. breast-fed versus formula-fed (Balamurugan et al. 2010; Turroni et al. 2012). Numerous studies have substantiated that the concentration of bifidobacteria in the feces of breast-fed infants was higher than formula-fed infants (Lee and O'Sullivan 2010; Roger et al. 2010; Grimm et al. 2014). On the other hand, insufficient colonization of bifidobacteria in the gastrointestinal tract during early infancy was observed in premature infants, decreased breast-feeding, as well as abuse of antibiotics which caused increasing inflammation, autoimmune, and atopic diseases (Gueimonde et al. 2007; Gill et al. 2012; Dong et al. 2010).

It is thought that higher concentration of bifidobacteria in breast-fed infants is due to two primary reasons: (1) the presence of bifidobacteria in the human milk which are passed down to the infant during feeding and (2) the presence of *Bifidobacterium*-stimulating properties of human breast milk (Lee and O'Sullivan 2010; Ročková et al. 2013). A study by Solis et al. (2010) on microbiota of breast-fed infants at 1, 10, 30, and 90 days after birth revealed that after 90 days, the fecal microorganisms belonging to *Bifidobacterium* were predominant as compared to

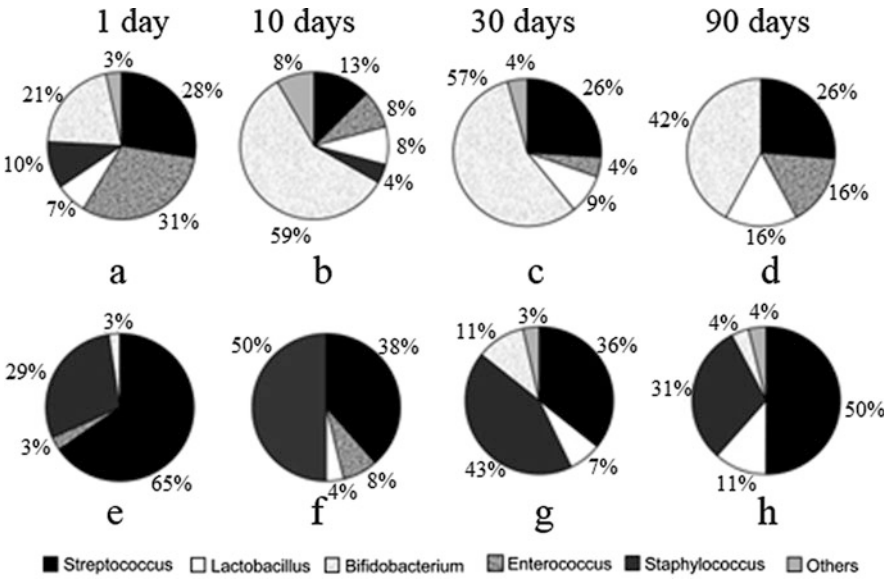

Fig. 3 Fecal microbiota profiles of breast-fed infants at 1, 10, 30, and 90 days after birth (**a**–**d**) and breast milk microbiota profile of breast milk from their respective lactating mothers at 1, 10, 30, and 90 days after birth (**e**–**h**). Reprinted from Solis et al. (2010), with permission from Elsevier (License number: 3605180193715)

day 1, where *Enterococcus* and *Streptococcus* were predominant (Fig. 3a–d). Bacterial genera of breast milk predominantly comprised of *Streptococcus* (mainly *S. salivarius*), *Staphylococcus* (mainly *S. epidermidis*), *Lactobacillus* (mainly *L. gasseri*), and *Bifidobacterium* (mainly *B. longum* and *B. breve*) (Fig. 3e–h). The bifidobacterial strains in both the breast milk and infant feces showed identical RAPD (random amplified polymorphic DNA), suggesting that the breast milk is the supplier of the bifidobacterium in the infants' microbiota and justifying that bifidobacteria were passed on to infants through consumption.

Of the various bioactive components in breast milk, human milk oligosaccharide (HMO) is thought to be an important factor for explaining the residential characteristics of infant-type bifidobacteria (Sela and Mills 2011). HMOs are a major constituent of human milk but not in infant formula. They are unconjugated complex carbohydrates consisting of short-chain trisaccharides, such as sialyllactose or fucosyllactose, and *N*-acetyllactosamine polymers (Lee and O'Sullivan 2010; Goehring et al. 2014). HMOs are present in human milk at 5–15 g/L and are the third largest component after lactose and lipids. Among the different oligosaccharides, type I oligosaccharides are predominant in HMOs, where fucosyllactose, lacto-N-tetraose (LNT), and lacto-N-fucopentaose I are the most abundant components of HMOs (Asakuma et al. 2008; Kobata 2010; Urashima et al. 2012). Previous in vitro studies substantiated that HMOs are able to reach the lower gastrointestinal tract without being digested by the host

hydrolases during transit through the small intestines (Ward et al. 2007; Jantscher-Krenn and Bode 2012; Goehring et al. 2014). This was further justified where significant amounts of HMOs were recovered in the feces of the neonates (Ward et al. 2007; Jantscher-Krenn and Bode 2012). With the presence of the genes involved in breakdown, uptake, and utilization of a wide variety of complex polysaccharides, bifidobacteria have the ability to ferment these complex carbohydrates in HMO and, hence, boost bifidobacterial populations in the gastrointestinal tract (Lee and O'Sullivan 2010; Grimm et al. 2014). This further supports their prevalence and dominance in newborns.

In addition, some reports have demonstrated that the susceptibility of bifidobacteria to lysozyme, a protein with antimicrobial activity that is naturally present in biological fluids, such as tears, saliva, and breast milk (Lönnerdal 2003; Masschalck and Michiels 2003), may play some roles in the selective colonization of bifidobacteria in the intestines of breast-fed infants (Gagnon et al. 2004; Rada et al. 2010). It was reported that strains of human origin were more resistant to lysozyme than animal strains (Rada et al. 2010). In a study investigating the growth of strains belonging to different bifidobacterial species or subspecies in breast milk, most strains of infant-type HRB (*B. longum* subsp. *infantis*, *B. breve*, *B. longum* subsp. *longum*, and *B. bifidum*) were able to grow in breast milk, but adult-type HRB (*B. adolescentis*, *B. catenulatum*, *B. pseudocatenulatum*, etc.) and non-HRB generally failed to grow and were killed upon incubation in breast milk (Minami et al. in submission). Most strains of infant-type HRB were tolerant of high concentrations of lysozyme, while strains of adult-type HRB were susceptible to lysozyme of egg white or human origins (Minami et al. in submission). These findings suggest that, in addition to selection by the capacity to assimilate HMOs, lysozyme is possibly involved in selecting infant-type HRB as inhabitants of infant intestine. The results also imply that infant-type bifidobacterial species in the intestine of infants are the results of selection by breast milk as a function of "carrot and stick," providing HMOs to support the growth of infant-type HRB and using lysozyme to exclude other bifidobacterial species. From a viewpoint of nature's law, strains of infant-type HRB are more suitable candidates as probiotics for infant use.

Establishment of the bifidobacteria is important to protect gut mucosa against pathogenic bacteria during development of the infant's mucosal immune system (Sela et al. 2008; Makino et al. 2011, 2013; Putignani et al. 2014). Bifidobacteria are important to establish and maintain infants' health through modulation of immune system, anti-inflammatory role on the mucosal surface (Mikami et al. 2009), competition with pathogens, relief of atopic disease symptoms, reduction of diarrhea, rotavirus infections, and lactose intolerance (Roger et al. 2010; Khokhlova et al. 2012). Breast-fed infants are less susceptible to gastrointestinal (GI) diseases in their first year as compared to the formula-fed infants (Roger et al. 2010). As compared to formula-fed infants, breast-fed infants have significantly lower rates of respiratory and urinary infections as well as atopic diseases such as food allergies, atopic dermatitis, and asthma (Stone 2003; Grönlund et al. 2007; Greer et al. 2008; Roger et al. 2010). This is contributed to the abundant presence of *Bifidobacterium* in the gastrointestinal tract of infants.

2.3 Metabolic Pathways of Bifidobacteria

Bifidobacteria are the major constituent of the breast-fed infant's gastrointestinal tract, particularly in the lower gastrointestinal tract, which is rich in human milk oligosaccharides (HMOs) (Ward et al. 2007; Fushinobu 2010; Jantscher-Krenn and Bode 2012; de Bruyn et al. 2013; Goehring et al. 2014; EUFIC 2015). Thus, bifidobacteria as saccharolytic bacteria possess different kinds of extracellular glycosidases and unique sugar metabolic pathways to utilize various oligosaccharides such as mucin glycans and the two types of HMO based on their core sugars for their survival and colonization (Fushinobu 2010; Pokusaeva et al. 2011; de Bruyn et al. 2013).

Generally, bifidobacteria possess an exclusive carbohydrate fermentation pattern through fructose-6-phosphate pathway or known as "bifid" shunt with the presence of the enzyme fructose-6-phosphate phosphoketolase (F6PPK) and xylulose-5-phosphate phosphoketolase (X5PPK) which are known as X5P/F6P phosphoketolase (XFPK, EC 4.1.2.9, EC 4.1.2.22) (Palframan et al. 2003; Turroni et al. 2010; Pokusaeva et al. 2011; Fushinobu 2010). The presence of XFPK contributes to the high efficiency of the bifid shunt where xylulose-5-phosphate (X5P) and fructose-6-phosphate (F6P) are directly converted to acetyl phosphate without using ATP. Subsequently, acetyl phosphate is converted by acetate kinase into acetate as well as conversion of glyceraldehyde-3-phosphate into lactate to generate ATP (Fushinobu 2010). This exclusive fermentation pattern in bifidobacteria is able to break down indigestible polysaccharides into readily absorbed short-chain fatty acids (SCFAs), namely, acetate and lactate, to exert beneficial effects to the host (Glendinning and Free 2014). Also, through this bifid shunt, bifidobacteria can produce more energy in the form of ATP from carbohydrates (Pokusaeva et al. 2011; Palframan et al. 2003).

Furthermore, as mentioned earlier, bifidobacteria concentration specifically in breast-fed infants is higher as human milk contains over 200 structurally different HMOs which stimulate the growth of bifidobacteria to enrich beneficial bacteria in the lower gastrointestinal tract in infants (Kitaoka 2012; Pokusaeva et al. 2011; Fushinobu 2010). There are two categories of HMO, such as type 1 lacto-N-biose (LNB)-containing oligosaccharides and type 2 *N*-acetyllactosamine (LacNAc)-containing oligosaccharides (Fushinobu 2010; Asakuma et al. 2011). Interestingly, in HMO, type 1 oligosaccharides are predominant over type 2 oligosaccharides and are an exclusive and unique feature of HMO (Fushinobu 2010; Asakuma et al. 2011; Kitaoka 2012). Bifidobacteria possess a unique metabolic pathway that is specific for LNB and galacto-N-biose (GNB) (Kitaoka et al. 2005). LNB is a building block for the type 1 HMOs, while GNB is a core structure of the mucin sugar that is present in the human intestine and milk (Podolsky 1985; Lloyd et al. 1996). GNB/LNB pathway to metabolize HMO is found to be distributed in infant-type bifidobacterial species such as *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. bifidum*, and *B. breve* (Xiao et al. 2010; Pokusaeva et al. 2011; Kitaoka 2012).

The GNB/LNB pathway, as previously illustrated by Wada et al. (2008), involves proteins/enzymes that are required for the uptake and degradation of

disaccharides such as GNB/LNB transporter (Wada et al. 2007; Suzuki et al. 2008), galacto-N-biose/lacto-N-biose I phosphorylase (GLNBP, LnpA) (Kitaoka et al. 2005; Nishimoto and Kitaoka 2007a), N-acetylhexosamine 1-kinase (NahK) (Nishimoto and Kitaoka 2007b), UDP-glucose-hexose-1-phosphate uridylytransferase (GalT), and UDP-galactose-4-epimerase (GalE). In infant-type bifidobacterial species, there are genes *gltA-C* which encode for the specific GNB/LNB-specific transporter to allow GNB and LNB intakes (Kitaoka 2012) and also the presence of *lnpABCD* operon which codes for GLNBP, NahK, GalT2, and GalE2 (Kitaoka 2012; de Bruyn et al. 2013). Some bifidobacteria have also been demonstrated to be enzymatically equipped to release LNB from HMOs that have a type 1 structure (lacto-N-biosidase, LnbB) (Wada et al. 2008) or GNB from the core 1-type O-glycans in mucin glycoproteins (end-α-N-acetylgalatosaminidase) (Fujita et al. 2005; Katayama et al. 2005, 2008). With the presence of GLNB phosphorylase, phosphorolytic cleavage is catalyzed to cleave galactosyl-beta-1,3-*N*-acetylhexosamine into *N*-acetylhexosamine (HexNAc), and galactose-1-phosphate (gal1P) phosphorylated sugar can be produced without consuming ATP and, therefore, produce higher amount of energy (Kitaoka 2012; de Bruyn et al. 2013). It has been suggested that the presence of the LnbB and GNB/LNB pathways in some bifidobacterial strains could provide a nutritional advantage for these organisms, thereby increasing their populations within the ecosystem of these breast-fed newborns (Wada et al. 2008).

3 Clinical Benefits of Bifidobacteria in Infants

3.1 Effects on Immune System

Immune system consists of cells and organs which have the ability in recognizing foreign substances or infectious microorganism (Riera et al. 2003). An infant has a different immune system as compared to adult, and the proper development of the immune system is vital for the prevention of immune disorders (Clinton 2010; Tregoning and Schwarze 2010; Balamurugan et al. 2010). Infant's immune system remains immature and functionally naïve after birth (Clinton 2010; Yoshida et al. 2010; Tregoning and Schwarze 2010; Gill et al. 2012; Kim and Ji 2012). This is characterized as lack of prior exposure to pathogens which causes lack of immune memory (Tregoning and Schwarze 2010).

3.1.1 Development of T Lymphocyte Cells

Naive T lymphocyte cells in infants will be differentiated into T-helper 1 (Th1) and T-helper 2 (Th2) cells (Yoshida et al. 2010). Infant's immune system is predominantly Th2-driven immune system with less Th1 memory effector function (Clinton

2010; Tregoning and Schwarze 2010). Th1 cells are the predominant cells, which are used against bacterial and viral infections, whereas Th2 cells are responsive to allergic responses and parasites (Clinton 2010). In infant's immune system, there are different levels of Th1 cytokines such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ), and Th2 cytokines include interleukin-4 (IL-4). IL-2 and IL-4 appear in higher levels while less IFN- γ in infants as compared to adults (Clinton 2010). This Th2-skewed environment is essential during pregnancy to avoid rejection of the fetus (Gill et al. 2012). However, on the other hand, this Th2-driven immune system will be extended into early childhood which influences the immune responses to infection. Subsequently, this may cause the development of certain atopic diseases such as asthma and allergy (Tregoning and Schwarze 2010).

Therefore, colonization of gastrointestinal tract microbial is crucial to restore balance immune response (especially Th1/Th2 balance) during infancy (Ménard et al. 2007). Bifidobacteria have the advantage to colonize the gastrointestinal tract due to various reasons, ranging from competitive inhibition to the presence of physical structures needed for attachment. The former has been much documented, while the latter has attracted much attention lately. Most bacterial pathogens possess long filamentous structures which protrude from their cellular surface (pili or fimbria), which are often required for host tissues during colonization. *B. bifidum*, *B. longum* subsp. *longum*, *B. dentium*, and *B. adolescentis* have been documented to possess cell surface-located pilus-like appendages (Fig. 4). Genomic analyses revealed the presence of one to three predicted sortase-dependent pilus gene clusters, while quantitative reverse transcription (qRT)-PCR analysis revealed that the genes encompassing the major and minor pilin subunits of each of these sortase-dependent pilus gene clusters can be differentially expressed depending on the availability of different carbohydrates during growth (Ventura et al. 2012a, b).

Th1 cells are stimulated and produced by Gram-positive bacteria, whereas Th2 cells are stimulated and produced by Gram-negative bacteria (Yoshida et al. 2010). Dendritic cells which are stimulated through Toll-like receptors (TLR) with the presence of virus- or bacteria-derived molecules in the differentiation process are vital in initiating an immune response, control intestinal inflammation, and mucosal tolerance (Yoshida et al. 2010; Weber and Polanco 2012; Szczawinska-Poplonyk 2012; Jungersen et al. 2014). This is further explained that TLR2 receptors are stimulated by the presence of Gram-positive bifidobacteria. Stimulated TLR2 caused differentiation of naive T cells into Th1 cells by recognizing the peptidoglycans in bifidobacteria. During infancy, *B. breve*, the major bacteria in intestinal flora can promote differentiation of naive T cells to Th1 and hence establish the balance of Th1/Th2 (Yoshida et al. 2010). Contrarily, in sterile environments of the newborns, TLR is not stimulated; therefore, the naïve T cells will differentiate into Th2 cells, resulting in Th2-skewed immune system in infants (Yoshida et al. 2010; Szczawinska-Poplonyk 2012). In healthy infants, *B. breve* also stimulates and causes maturation of dendritic cells. Subsequently, anti-inflammatory interleukin-10 (IL-10) was increased through TLR2 (Yoshida et al. 2010).

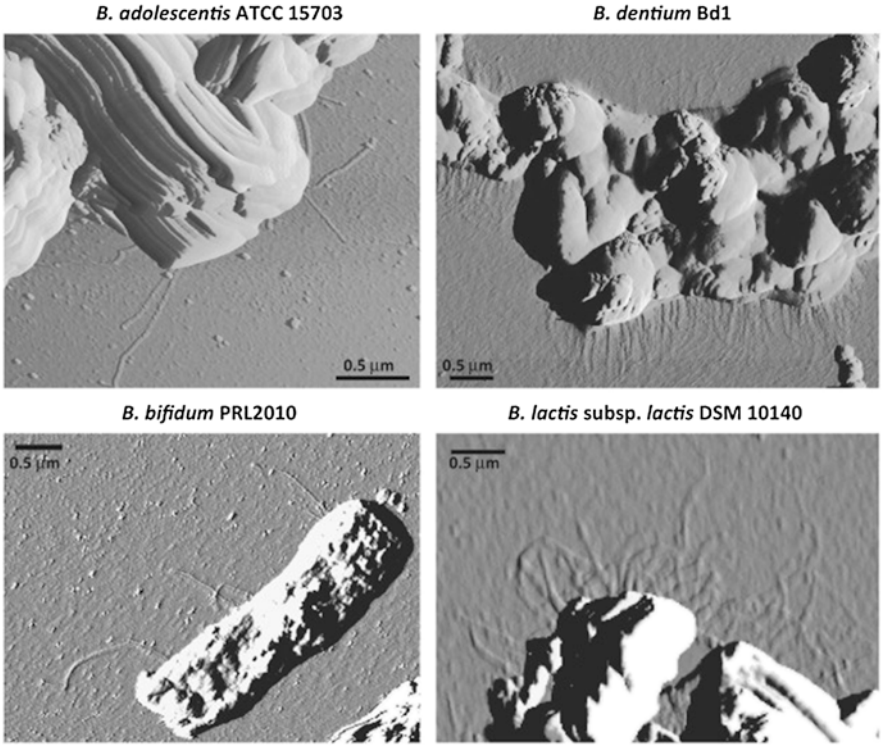

Fig. 4 Atopic force microscopy of bifidobacterial pili; structures that aid in bifidobacterial colonization in the gut and/or other host interactions. Reprinted from Ventura et al. (2012a, b), with permission from Elsevier (License number: 3605201005588)

3.1.2 Stimulation of Immunoglobulin A (IgA)

Nevertheless, due to the abundance of bifidobacteria in breast-fed infants, it can affect the production of immunoglobulin, namely, immunoglobulin A (IgA) which is located on mucosal surfaces to enhance mucosal resistance to infections in infants (Balamurugan et al. 2010; Taipale et al. 2011; Gill et al. 2012; Kelly et al. 2007). For example, *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) stimulated and increased the production of local IgA in healthy infants which enhanced the mucosal resistance to infections (Taipale et al. 2011).

Secretory immunoglobulin A (sIgA) is the most abundant class of antibodies which is found in mucosal surfaces including human intestinal lumen (Mantis et al. 2011; Gill et al. 2012). sIgA acts as the first line of defense by protecting the intestinal epithelium from mucosal antigens, enteric pathogens, and toxins which cause infection and intestinal inflammatory disorders (Mantis et al. 2011; Gill et al. 2012).

As an infant is born with an immature immune system, there are only few IgA-producing cells. Through stimulation of bifidobacteria, the number of IgA-producing cells increases progressively. This is similar to the condition in germ-free mice where IgA inductions are increased through monocolonization with segmented filamentous bacteria which is a major component of commensal Gram-positive bacteria (Forchielli and Walker 2005). With the increase in the IgA-producing cells, infant's intestinal antigenic ability is also being stimulated (Gill et al. 2012). Bifidobacteria can increase the numbers of IgA-producing cells in the lamina propia (LP, tissue site where immune cells recognize bacterial antigen to express the inflammatory response) which promote secretion of sIgA. In immunological activation effect, bifidobacteria cell or cell component can induce specific and nonspecific antibody production. In mice, the administration of single-strain *Bifidobacterium longum* BB536 increased the production of anti-*B. longum* antibodies and total IgA concentration, followed by improved resistance to pathogenic *Escherichia coli* (Ishibashi et al. 1997).

3.2 Atopic Diseases

Atopic diseases can occur throughout the human life span and affect different locations of the human body at different ages (Fig. 5). Despite affecting individuals across all ages, atopic diseases such as atopic dermatitis, allergic rhinitis, asthma, and food allergy are known as one of the most endemic chronic disorders among infants and children (Toh et al. 2012; Yeşilova et al. 2012). The incidence of these atopic diseases has been reported to increase dramatically over the past several decades such that up to 20 % of the infants in some developed populations can have a high risk of being affected thereby resulting in an increase morbidity among infants and children (Greer et al. 2008; Toh et al. 2012). Furthermore, the atopic diseases also diminished the quality of life for the infants and the families as well as increased the burden on healthcare costs (Toh et al. 2012).

It was suggested that the increase in the prevalence of the atopic diseases may be contributed by the environmental factors which is referred to as the "hygiene hypothesis" proposed by Strachan in 1989 (Stone 2003; Toh et al. 2012). The hypothesis suggested that the increases in atopic diseases among infants and children were contributed by a more hygienic environment in developed countries (Stone 2003). For example, improved hygiene, decreased microbial exposure due to industrialization, the use of antibiotics, vaccinations, and reduced household size are the factors in contributing to the rise of atopic diseases (Stone 2003; Toh et al. 2012; Kim and Ji 2012; Szajewska 2012). These conditions subsequently affect the balance of Th1/Th2 (Meneghin et al. 2012).

The occurrence of atopic diseases during infancy can be related to immunologic aspect where it was usually related to the development and production of immunoglobulin E (IgE) through the aggressive Th-2 cell immune response to environmental or antigens (Greer et al. 2008; Toh et al. 2012). The activation of Th2

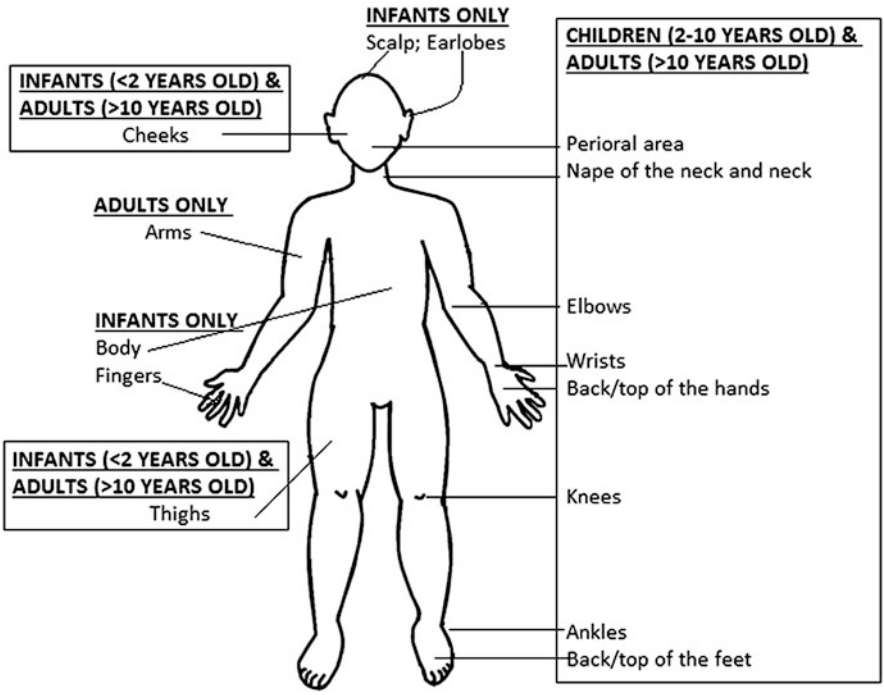

Fig. 5 Age-dependent locations of atopic diseases. Most of the atopic diseases are found on scalp, earlobes, fingers, body, and extensor surfaces such as thighs among infants (<2 years old). Perioral area, nape of the neck and neck, elbows, wrists, knees, ankles, and back/top of the hands and feet are common areas of atopic diseases among children (2–10 years old) and adults (>10 years old). Extensor surfaces such as thighs and arms are also common areas of atopic diseases among adults

cytokine responses also leads to suppression of Th1 activity and causes imbalance of Th1/Th2 which then causes the appearance of atopic diseases (Toh et al. 2012; Meneghin et al. 2012). Th1 is responsible in direct immune responses to fight the infection by the production of cytokines interferon-gamma (IFN-γ) and interleukin-2 (IL-2) (Stone 2003). Contrarily, Th2 mediates the allergic responses to allergens by producing IL-4, IL-5, IL-9, and IL-13 (Stone 2003; Toh et al. 2012).

Among multiple prevention strategies sought by the pediatric community, it has been widely recognized that early infant nutrition including probiotics and the colonization of infant's gastrointestinal microbiota are important to prevent atopic diseases through the development and maturation of the immune system (Toh et al. 2012; Szajewska 2012). There are many studies which have found the benefits of breast-feeding as well as the introduction of probiotics in preventing the development of atopic diseases since the 1930s (Greer et al. 2008; Yeşilova et al. 2012). In some studies related to breast-feeding and atopic diseases, it was found that when comparing breast-fed and non-atopic infants to infants with atopic diseases and formula-fed, the latter has more clostridia but fewer bifidobacteria (Sjögren

et al. 2009; Szajewska 2012; Meneghin et al. 2012; Kim and Ji 2012). As mentioned in the introduction, the gastrointestinal tract of the breast-fed infants is dominated by bifidobacteria as compared to the formula-fed infants due to the presence of human milk oligosaccharides (HMOs).

Bifidobacteria are effective against pathogenesis of atopic diseases (Michail 2009; Yeşilova et al. 2012) through the modulation of Toll-like receptors and the proteoglycan recognition proteins of enterocytes. This in turns leads to activation of dendritic cells and the modulation of Th (Th1/Th2) balance by favoring a Th1 cell response as demonstrated in the studies on efficacy of *B. longum* BB536 in reducing allergy-related symptoms (Michail 2009; Xiao et al. 2006, 2007). Th1 response will then be stimulated while suppressing Th2 allergic response that caused the enhancement of IFN-γ production and reduced the amount of antigen-induced TNF, IL-5, and IL-10 secretion as well as allergen-specific IgE (Xiao et al. 2006; Michail 2009; Yeşilova et al. 2012; Kim and Ji 2012). Such enhancement of IFN-γ production is also affected by the action of bifidobacteria (Namba et al. 2010). From the animal studies using cultured spleen cells from rats fed with *B. longum* BB536, increased in the production of IFN-γ was observed (Namba et al. 2010). The increase of the IFN- γ is important in host defense against infection as well as increasing resistance to bacteria and viruses and hence reducing the prevalence of atopic diseases (Schroder et al. 2004).

There are increasing evidences from human and animal studies which further proves that bifidobacteria have the ability to mediate antiallergy and anti-inflammatory effects which helps in lowering the prevalence of allergy (Kim and Ji 2012; Yeşilova et al. 2012). A double-blind, placebo-controlled trial using *B. lactis* Bb12 in infants illustrated the modulation of gastrointestinal microbiota and alleviation in the early onset of allergic inflammation in atopic diseases (Kirjavainen et al. 2002; Kim and Ji 2012), and a decrease of atopic dermatitis scores (SCORAD) from a baseline of 16 to 0 after 2 months of BB12 intervention (Michail 2009). Atopic diseases were reportedly observed in infants with low degree of intestinal bifidobacteria colonization. With the supplementation of lyophilized *Bifidobacterium*, their allergic symptoms were ameliorated (Kim and Ji 2012). Also, infants with hypersensitivity to cow's milk had reduced symptoms of allergy upon oral administration of *B. breve* M-16V (Taniuchi et al. 2005). In a randomized-controlled trial, in combination of galacto-oligosaccharides/fructo-oligosaccharides, *B. breve* M-16V alleviated the allergic symptoms in infants with IgE-mediated atopic dermatitis (van der Aa et al. 2010). Furthermore, one-year follow-up evaluation of these infants who have been administered with a mixture of *B. breve* M-16V and the prebiotics found that the incidence of asthma-like symptoms in infants was significantly reduced in the treated group as compared to the placebo group (van der Aa et al. 2011). Recently, it was reported that supplementation of two bifidobacterial strains, *B. breve* M-16V and *B. longum* BB536 to mother (for 1 month prior to delivery) and their infants (for 6 months after birth), significantly suppressed the risk of eczema development in the infants during the first 18 months after birth (Enomoto et al. 2014).

3.3 Gastrointestinal Diseases

Gastrointestinal diseases include inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), diarrhea/gastroenteritis, constipation, and necrotizing enterocolitis (NEC). These diseases are contributed by environmental, genetic, immunological, as well as the gastrointestinal tract microbiota.

From the previous studies done, probiotics showed the efficacy in treating inflammation-related, diarrhea-related, and IBS symptoms through several mechanisms of action, such as modification and stabilization of GIT microflora, reductions in the duration of retrovirus shedding, reduction of GIT permeability, as well as induction of general immune response by increasing IgA antibodies (Ritchie and Romanuk 2012).

3.3.1 Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is referred to as inflammation and death of intestinal tissue and, in severe cases, perforations of the intestines. Hence, it is the most common life-threatening gastrointestinal medical condition among infants. It is a significant clinical problem where highest incidence was reported in infants with low birth weight less than 1000 g (Weber and Polanco 2012; Szajewska 2012). Though the exact pathogenesis of NEC remains unclear, the possible factors are intestinal hypoxia-ischemia, colonization by pathogenic bacteria, formula feeding, as well as prematurity (Khailova et al. 2009; Weber and Polanco 2012; Szajewska 2012). It is suggested that immaturity of tolerance mechanism which is influenced by microbiota quantity might be related to the occurrence of NEC as there is a higher proportion of *Proteobacteria* found in infants with NEC.

In a neonatal rat model done by Khailova et al. (2009), the results showed that *B. bifidum* OLB6378 reduced the incidence of NEC from 57 % to 17 % and severity of ileal damage in the rat. *B. bifidum* OLB6378 exerted bifidobacterial-mediated reduction through the reduction in cytokine expression in the site of injury on the intestinal lining or wall, accompanied by improved development of cellular junctional proteins in the intestinal epithelium (Khailova et al. 2009). In addition, *B. bifidum* OLB6378 induced strong IgA production and promoted anti-inflammatory properties via secretion of inflammatory mediators such as IL-6. Also, *B. bifidum* decreased colonization by pathogenic bacteria, leading to the reduction of inflammation and trefoil factors 3 (Tff3) (key peptide in mucosal protection and repair, overproduction is observed in a variety of gastrointestinal inflammatory conditions) (Khailova et al. 2009). In a randomized double-blind placebo-controlled trial, the supplementation of *B. breve* M-16V improved bifidobacterial colonization in preterm neonates (Patole et al. 2014). In addition, the administration of *B. breve* M-16V prevented the development of NEC in preterm infants (Satoh et al. 2007) and suppressed TLR4-triggered inflammatory responses in IECs via TLR2 signaling and negative regulators of TLR4 signaling (Tomosada et al. 2013). The regulation of TLR4 signaling is a key factor in the

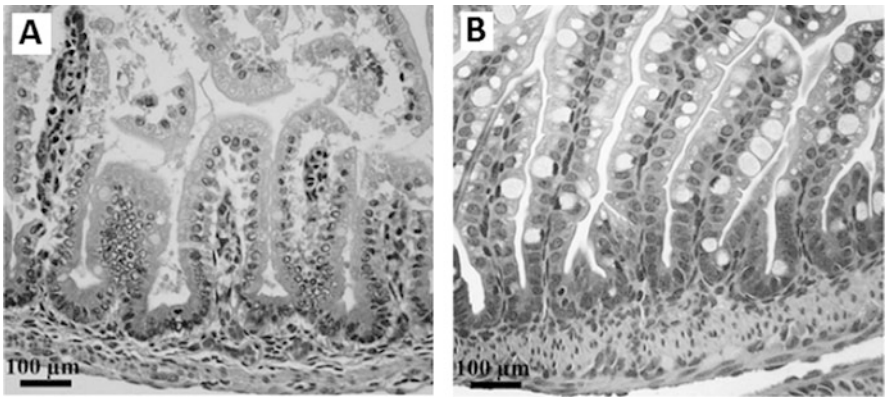

Fig. 6 Histology of intestines from NEC mouse pup model that were left untreated (**a**) and upon administration with *B. infantis* BB-02 (**b**) which showed intact villi. Reprinted from Bergmann et al. (2013), with permission from Elsevier (License number: 3605990476291)

treatment of NEC. Furthermore, using a mouse model, Bergmann et al. (2013) illustrated that *B. infantis* BB-02 decreased the incidence of NEC via maintaining intact villi of the small intestines (Fig. 6) and preserving the localization of claudin-4 and occludin at tight junctions, as compared to non-bifidobacteria-treated rats where occludin and claudin-4 were found throughout the cytoplasm (Fig. 7). Translocation of claudins and occludin is often an indicator of altered tight junctions and gut barrier integrity, a common attribute of NEC.

3.3.2 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) includes Crohn's disease (CD), ulcerative colitis (UC), and pouchitis that often occur due to the disruption of tightly regulated GIT immune response by uncontrolled immune cell activation and production of pro-inflammatory cytokines (Weber and Polanco 2012; Szajewska 2012), leading to intestinal damages. Although bifidobacteria have been much documented to alleviate IBD in animal models (Fig. 8; Philippe et al. 2011) and in adults (Saez-Lara et al. 2015), little information is available on the efficacy of bifidobacteria among infants. Sani et al. (2008), Kappelman and Grand (2008), and Uslu et al. (2009) have reported that the occurrence of IBD among infants is rare and remains unclear whether IBD during infancy is related to immunological disorder. From published data on epidemiological studies and IBD registries, it is suggested that less than 1 % of infants have IBD during the first year of life (Kappelman and Grand 2008).

3.3.3 Diarrhea

Diarrhea is commonly defined as three or more loose or watery stools in the last 24 h. As rotavirus infection is the leading cause of severe gastroenteritis among

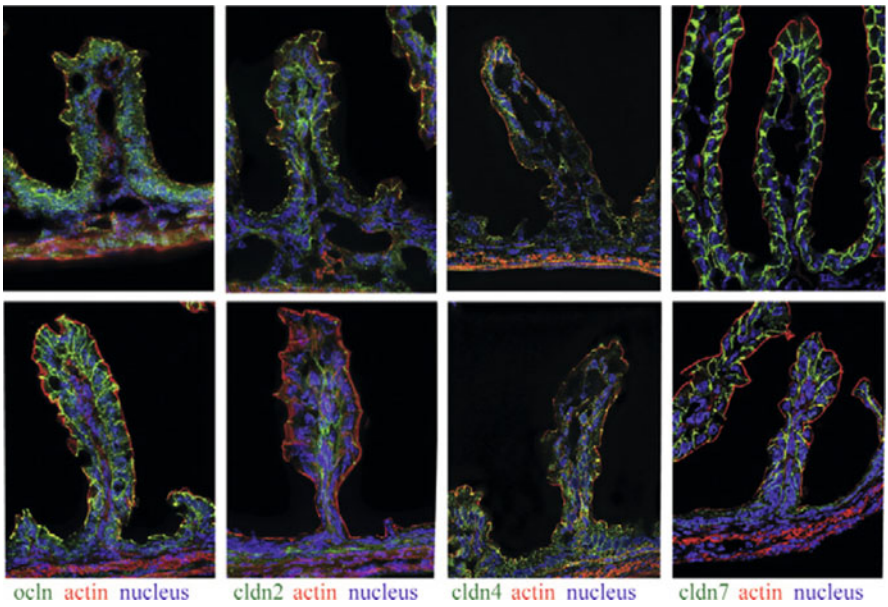

Fig. 7 Frozen sections of small intestines of NEC pups treated with control (*top row*) and *B. infantis* BB-02 (*bottom row*) and examined for claudins-2, claudins-4, and claudins-7 and for occludin by immunofluorescence. B-actin (*red*) and DAPI nuclear staining (*blue*) can also be observed. In control NEC pups, occludin and claudin-4 were found throughout the cytoplasm, whereas in *B. infantis* BB-02-treated NEC mice, claudin-4 remained localized at tight junctions and occludin was found both in the cytoplasm and at the tight junctions. Reprinted from Bergmann et al. (2013), with permission from Elsevier (License number: 3605990476291)

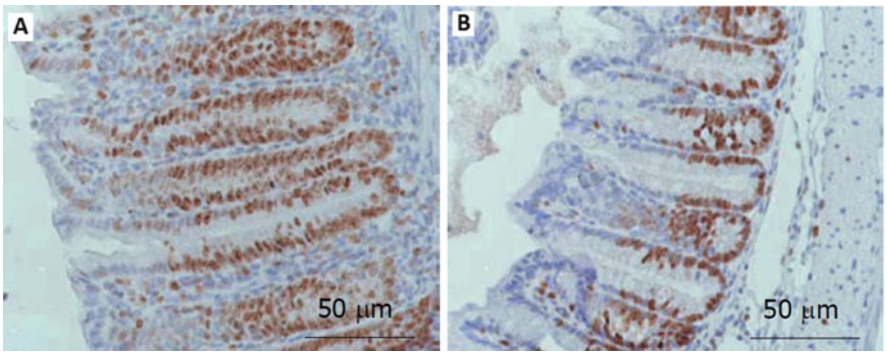

Fig. 8 Histological immunostaining showing proliferation of epithelial cells (*brown* nuclear staining), from colonic longitudinal slices of control colitic (**a**) and *B. animalis* subsp. *lactis* NCC 2818-fed colitic (**b**) mice. Colonic epithelium of *B. animalis* mice showed a high proliferation rate in the lower two-thirds of the colonic crypts as compared to the epithelial hyperproliferation of the control. This indicated that *B. animalis* reduced mucosal thickness and, subsequently, the inflammatory onset of colitis. Adapted from Philippe et al. (2011). Open Access Article

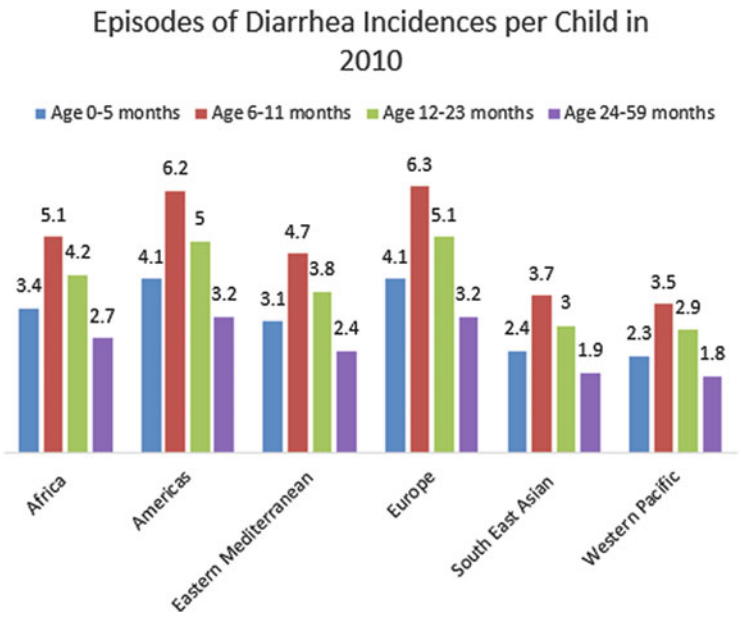

Fig. 9 Episodes of diarrhea incidence per child under 5 years old living in low- and middle-income countries of different regions including Africa (45 countries), the Americas (29 countries), Eastern Mediterranean (15 countries), Europe (23 countries), South East Asia (11 countries), and Western Pacific (16 countries). (Adapted from Walker et al. (2012), Open Access)

infants and young children worldwide, each year, the infection causes 2 million hospitalizations in children under 5 years of age for diarrhea (Walker et al. 2012; Jungersen et al. 2014). In addition, diarrhea incidence rate was also recognized as a leading cause of morbidity and mortality in low- and middle-income countries such as Africa, the Americas, Eastern Mediterranean, Europe, South East Asia, and Western Pacific (Walker et al. 2012) (Fig. 9).

Diarrhea can be caused by, for example, infections, radiation, antibiotic therapy, as well as tube feeding (Narayan et al. 2010). Bifidobacteria have been shown to have a protective effect against both acute and persistent diarrhea (Jungersen et al. 2014). Bifidobacteria reduce diarrhea through modulation of the host immune system and restoration of the composition of gastrointestinal tract microbiota (Fox et al. 2015; de Vrese and Marteau 2007). In a review done by de Vrese and Marteau (2007), administration of probiotic such as bifidobacteria can shorten the duration of diarrhea episodes by 1–1.5 days, promote systemic or local immune response, and also increase the production of rotavirus-specific antibodies in acute diarrhea from rotavirus. Besides, bifidobacteria are able to affect the gastrointestinal tract microbiota by stimulating nonimmune mechanisms through antagonism with potential pathogens. These include the production of bacteriocins and organic acids such as acetic acid and lactic acid to alter the gastrointestinal tract pH to an unfavorable environment for pathogens which subsequently inhibits the

colonization of enteropathogens competitively (World Gastroenterology Organisation 2008; Narayan et al. 2010). Bacteriocins produced by bifidobacteria can act against a broad spectrum of Gram-positive and Gram-negative organisms (Natural Medicines Comprehensive Database 2014).

In 12 clinical studies performed in infants and children, mean duration of diarrhea was reduced by 29.20 h in people taking probiotics (Allen et al. 2009). Additionally, in a study involving probiotic treatment using *Bifidobacterium lactis* HN019 on diarrhea associated with rotavirus, lower concentrations of fecal rotavirus and *E. coli* were reported, while blood leukocyte phagocytic and T-lymphocyte proliferative responses were upregulated (Shu et al. 2001). It is reported that tumor necrosis factor-α (TNF-α) was downregulated, while IL-10, IL-12, and IFN-γ were upregulated in infants who were treated with probiotics (Salari et al. 2012).

3.3.4 Constipation

According to Rome III criteria, chronic constipation is defined as the presence of at least two of the following symptoms for 2 or more months: (1) two or fewer defecations per week, (2) at least one episode of fecal incontinence per week, (3) history of retentive posturing or excessive volitional stool retention, (4) history of painful or hard bowel movements, (5) presence of a large fecal mass in the rectum, and/or (6) history of wide-diameter stools that may obstruct the toilet (Weber and Polanco 2012; Tabbers et al. 2011). Generally, prevalence of constipation among infants ranges widely between different regions of the world (Chang et al. 2013; Horvath et al. 2013) (Fig. 10), with a higher prevalence in South Africa (29.2 %) and South America (26.8 %) and a lower prevalence in Asia (10 %) and North America (16 %) (Mugie et al. (2011).

In general, probiotics treat constipation through the improvement of the intestinal motility, as with the production of lactic acid, acetic acids, and other short-chain fatty acids by bifidobacteria and lactobacilli, pH in the colon can be lowered. Subsequently, there is enhanced peristalsis of the colon and colonic transit time is decreased (Coccorullo et al. 2010).

In infants, as compared to breast-feeding, formula-fed infants have 4.5 times higher risk of constipation (Weber and Polanco 2012). This is because bifidobacteria are predominant in the GIT of breast-fed infants which promotes the reduction of pH and prevent colonization of pathogenic bacteria. In another pilot study which had been carried out in children, the results showed that administration of *B. breve* significantly increased the defecation frequency and mean stool consistency score. Also, *B. breve* significantly decreased the pain during defecation, episodes of fecal incontinence, as well as abdominal pain. This was assumed that the probiotic stimulated water and electrolyte secretion to soften the stools (Tabbers et al. 2011).

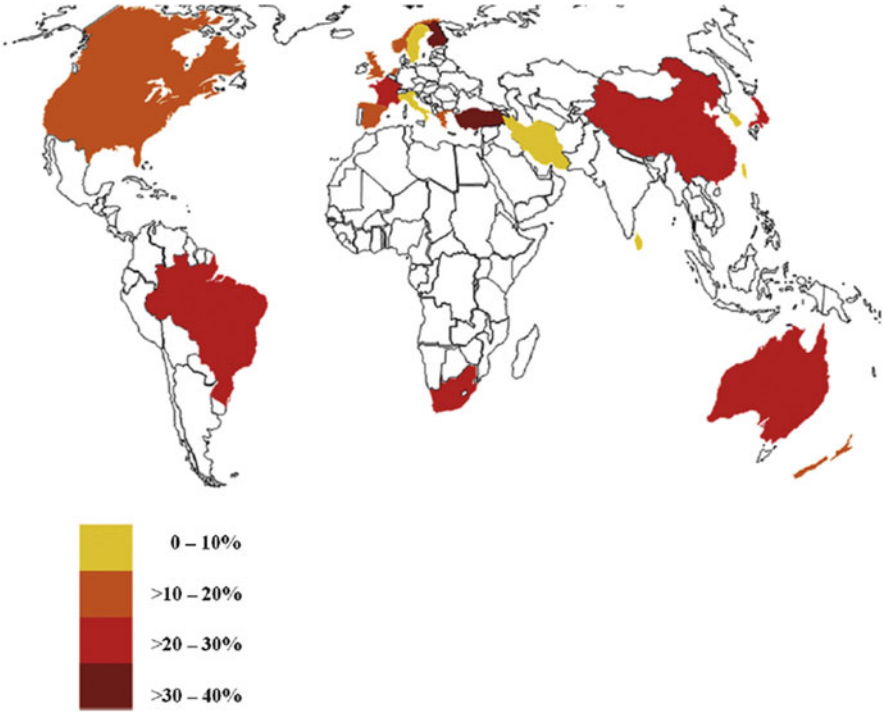

Fig. 10 Geographical distribution of constipation in children and adults shown in median prevalence rates [Reprinted from Mugie et al. (2011)], with permission from Elsevier (License number: 3614611463001)

3.4 Respiratory Tract Infections

Respiratory tract infections (RTIs) affect large populations, especially in infants and children, which contribute to the major cause of the morbidity and mortality globally (Vouloumanou et al. 2009; Lehtoranta et al. 2014). RTIs refer to the infections of the upper and lower respiratory tract. Upper respiratory tract infections affect sinuses and throat and result in common cold, flu, acute otitis media, sinusitis, and tonsillitis, while lower respiratory tract infections affect the airways and lungs, resulting in bronchiolitis and pneumonia (Vouloumanou et al. 2008). During infancy, upper respiratory tract infections are the most common respiratory virus infections in infants (Tregoning and Schwarze 2010).

The occurrence of respiratory tract infections is much dependent on the weather of the region, either during winters in the northern and southern hemisphere or less seasonal periods in the tropical and temperate countries (Simonsen et al. 2011). For example, in Malaysia, most occurrences of RTIs among infants peak in mid year (month of May) and year end (months of November and December) (Sam et al. 2010).

Probiotics have been documented to reduce the incidence, duration, and/or severity of respiratory infections, especially upper respiratory tract infections (Gill et al. 2012; Lehtoranta et al. 2014). Probiotic administrations are believed to exhibit the effects such as inhibition of the growth of pathogenic microorganisms by producing antimicrobial products (Lehtoranta et al. 2014; Esposito et al. 2014), bacterial interference by competing for nutrients and adhesion sites with pathogenic bacteria (Vouloumanou et al. 2009), and enhancement of local and systemic immune responses (Lehtoranta et al. 2014). However, in most respiratory infection cases are viral in origin; therefore, it is suggested that immunostimulation by probiotics is preferred to explain the action of probiotics in respiratory tract infection interventions (Taipale et al. 2011; Gill et al. 2012).

The suggested mechanisms of bifidobacteria in reducing respiratory tract infections are associated with the enhancement of several immune responses which include increasing immune cell activity, modulation of signals in epithelial and immune cells, increasing local and systemic antibody production, and induction of phenotypic changes in dendritic cells (Gill et al. 2012; Jungersen et al. 2014). Interferons-α (IFN-α) and interferons-γ (IFN-γ) are important in host protection, especially against intracellular pathogens/viral infections (Gill et al. 2012). There are increasing evidences which showed that administration of bifidobacteria results in increasing levels of IFN-γ in bloods and the capacity of blood leukocytes to produce IFN-γ ex vivo was also increased. Besides, RTIs affect the production of IFNs. Therefore, bifidobacteria supplementation was effective in restoring IFN-producing capacity of infants with acute RTIs (Gill et al. 2012).

There are several randomized, placebo-controlled studies that have been done to study the effectiveness of bifidobacteria in the prevention and treatment of respiratory infections in healthy infants (Taipale et al. 2011). From the clinical studies done, it was shown that with the administration of the combination of *B. lactis* Bi-07 and *L. acidophilus* NCFM, the incidence and duration of fever, coughing, and rhinorrhea were significantly reduced in Chinese children (Leyer et al. 2009). As reported by Taipale et al. (2011), administration of *B. animalis* subsp. *lactis* BB-12 showed significant reduction in the occurrence of respiratory infections during the first 8 months of life of the infant. There were 65 % of the infants in the BB-12 group who experienced one or more episodes of respiratory infections as compared to the 94 % from the control group (Taipale et al. 2011).

Also, Weizman et al. (2005) compared infant formulas with *B. lactis* BB-12 or *L. reuteri* ATCC 55730 in infants aged 4–10 months old, which resulted in a reduction in the occurrence of respiratory infections which showed significantly fewer febrile episodes, but relative to the control, there is no effect on respiratory illnesses during a 12-week follow-up.

4 Safety Issues

Typically probiotics which are widely used in infants are lactobacilli and bifidobacteria and are generally recognized as safe (Sanders et al. 2010). From several studies done, healthy infants have shown good tolerance toward those probiotics (Borriello et al. 2003; Sanders et al. 2010; Tabbers et al. 2011; Underwood et al. 2013). However, there are still some extremely rare cases of infection resulted from lactobacilli and bifidobacteria (Borriello et al. 2003) which have normally occurred in immunocompromised patients, having a chronic disease or debilitation (Boyle et al. 2006; Gueimonde et al. 2013). These groups of patients demonstrate a higher risk of bacteremia, bacterial translocation, and sepsis (Liong 2008).

The transferable antimicrobial resistant factors of probiotic strains are another major concern for consumption of probiotics (D'Aimmo et al. 2007; Sato and Iino 2010). The European Food Safety Authority (EFSA) (2012) has established a guideline to examine the susceptibility of bacterial strains toward antibiotic resistance and possible transferable traits, through the expression of minimum inhibitory concentration (MIC) of antibiotics that inhibit the growth of bacteria. Types of antibiotic included in the guideline, to detect a wide range of determinants for resistance, are ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol, whereas tylosin, apramycin, nalidixic acid, sulfonamide, and trimethoprim are used in specific cases (Table 1). Certain bacterial strains can be generally accepted for consumption when these strains possess an intrinsic (specific for a bacterial species or genus) or acquired resistance (due to gaining of exogenous DNA or mutation of indigenous genes) to the antibiotics at a low or minimal potential. In addition, the nature of resistance toward certain antibiotics can be assessed genetically within the taxonomical unit, should a higher than cut-off MIC value is detected.

There are some probiotic strains which have been reported to possess antibiotic-resistance genes, for example, *Enterococcus* and *Lactobacillus* species (Moubareck et al. 2005). With the presence of antibiotic-resistance genes, there is possibility where the antibiotic resistance might be transferred from the probiotic strains to the intestinal pathogens. However, the bifidobacterial strains have not yet been reported to have conjugative plasmids (Moubareck et al. 2005). This is further supported where there are five species of *Bifidobacterium* with Qualified Presumption of Safety (QPS) status by European Food Safety Authority (EFSA), namely, *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum* which have not been linked to any infection in healthy individuals (Gueimonde et al. 2013). Also, it has not been experimentally proven that bifidobacteria have the potential to transfer antibiotic resistance genes to other enteric bacteria (Gueimonde et al. 2013).

Table 1 Types of antibiotics, their respective microbiological cut-off values (mg/L), and the different types of probiotic microorganisms as per guidelines from EFSA (2012)

	Ampicillin	Vancomycin	Gentamicin	Kanamycin	Streptomycin	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol
Lactobacillus obligate homofermentative[a]	1	2	16	16	16	1	I	4	4
Lactobacillus acidophilus group	1	2	16	64	16	1	1	4	4
Lactobacillus obligate heterofermentative[b]	2	n.r.	16	32	64	1	1	8	4
Lactobacillus reuteri	2	n.r.	8	64	64	1	1	16	4
Lactobacillus facultative heterofermentative[c]	4	n.r.	16	64	64	1	1	8	4
Lactobacillus plantarum/pentosus	2	n.r.	16	64	n.r.	1	2	32	8
Lactobacillus rhamnosus	4	n.r.	16	64	32	1	1	8	4
Lactobacillus casei/paracasei	4	n.r.	32	64	64	1	1	4	4
Bifidobacterium	2	2	64	n.r.	128	1	1	8	4
Pediococcus	4	n.r.	16	64	64	1	1	8	4
Leuconostoc	2	n.r.	16	16	64	1	1	8	4
Lactococcus lactis	2	4	32	64	32	1	1	4	8
Streptococcus thermophilus	2	4	32	64	64	2	2	4	4

Bacillus spp.	n.r.	4	4	8	8	4	4	8	8	
Propionibacterium	2	4	64	64	64	0.5	0.25	2	2	
Other Gram +	1	2	4	16	8	0.5	0.25	2	2	
	Ampicillin	Vancomycin	Gentamicin	Kanamycin	Streptomycin	Erythromycin	Clindamycin	Tylosin	Tetracycline	Chloramphenicol
Enterococcus faecium	2	4	32	1024	128	4	4	4	4	16
	Ampicillin	Gentamicin	Kanamycin	Streptomycin	Tetracycline	Chloramphenicol	Nalidixic acid	Sulfonamide	Trimethoprim	Apramycin
Escherichia coli	8	2	8	16	8	16	16	256	2	8

n.r. not required.
[a]Including *L. delbrueckii*, *L. helveticus*
[b]Including *L. fermentum*
[c]Including the homofermentative species *L. salivarius*

5 Conclusions

Colonization of bifidobacteria in gastrointestinal tract plays a pivotal role in infant's health. The colonization of the bifidobacteria is influenced by type of feeding and delivery mode where breast-fed infants and vaginal delivery result in bifidobacteria as predominant bacteria, whereas formula-fed infants' GIT microbial environment is heterogenous. Bifidobacteria are essential for the development of infant's immune system and prevention of atopic diseases, gastrointestinal diseases, as well as respiratory tract infections. Bifidobacteria exert health benefits in infants through initiation, stimulation, and modulation of the native immune system by balancing Th1/Th2; prevention of pathogenic bacteria colonization; as well as homeostasis maintenance of the gastrointestinal tract microbiota to prevent dysbiosis. Bifidobacteria are generally safe for consumption for healthy individuals and infants.

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The Role of Integrated Omics in Elucidating the Gut Microbiota Health Potentials

Wanping Aw and Shinji Fukuda

Abstract In recent years, DNA sequencing and mass spectrometry technologies have advanced greatly, enabling the collection of more information on the gut microbiome and its metabolome in order to assess the influence of the gut microbiota on human health at a whole-system level. As the gut microbiota has been likened to a functional and measurable organ consisting of prokaryotic cells, which creates the unique gut ecosystem together with the host eukaryotic cells, metagenome and metabolome technologies have demonstrated that the gut microbiota contributes to host overall health status to a great extent. In this chapter, the detailed relationships between gut microbiota and its metabolites like choline, phenols, bile acids and short-chain fatty acids in host health and etiopathogenesis of various metabolic diseases such as obesity, diabetes, atherosclerosis, non-alcoholic fatty liver disease and extraintestinal diseases like multiple sclerosis, chronic kidney disease and autism will be discussed. In addition, therapeutic interventions like probiotic and prebiotic administrations and faecal microbiota transplantations which are recently used in dysbiosis restoration will be reviewed. This unique biology-wide approach of integrating metagenome and metabolome information would aid in the better understanding of the intricate interplay between gut microbiota and host metabolism. We believe that this novel integration of the microbiome, metatranscriptome and metabolome information will lay the way towards an improved holistic understanding of the complex mammalian superorganism. This modelling of the metabolic interactions between lifestyle, dietary habits and the gut microbiota, otherwise known as the "integrated omics-based understanding of the gut ecosystem", will culminate in the comprehensive interpretation of the role and impact of microbial health potentials, thereby providing exciting novel therapeutic approaches for optimal host health.

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1 The Gut Microbiota and Metabolomics-Based Integrated Omics Approach

1.1 The Gut Microbiota

The gut microbiota refers to all the microorganisms inhabiting the gastrointestinal tract. Four bacterial phyla, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, dominate the gut microbiota in mammals, and these phyla have been reported to characterize the role of the host metabolism and physiology (Qin et al. 2010). The bacteria populating the gut possess extensive metabolic capabilities (Qin et al. 2010) and amount to about 100 trillion cells, which is approximately three times higher than the total number of cells in the human body (Bianconi et al. 2013). As such, the gut microbiota is often associated to be a functional and measurable organ consisting of prokaryotic cells, which forges with host eukaryotic cells to create a unique gut ecosystem (Fukuda and Ohno 2014). Bacterial communities vary in composition along the digestive tract and evolve within and between individuals over time in accordance to the lifestyle and nutritional status of the host (Xu et al. 2007). It is only in recent years that we have started to comprehend the systemic impact of the gut microbiota on the whole host metabolic repertoire. The gut microbiota is involved in other functions of the body like drug metabolism and toxicity (Clayton et al. 2006), dietary calorific bioavailability (Hooper 2001), immune response (Macpherson 2000) and postsurgical recovery (Kinross et al. 2011). However, more importantly, apart from its obvious role in digestion, gut microbiota has been implicated in maintaining optimal host health and the etiopathogenesis of various metabolic diseases such as obesity (Turnbaugh et al. 2006) and diabetes (Wen et al. 2008; Qin et al. 2010; Wang et al. 2012), intestinal diseases like inflammatory bowel diseases (IBD) (Marchesi et al. 2007) and colonic cancer (Scanlan et al. 2008) and extraintestinal diseases like allergy (Kirjavainen et al. 2002), multiple sclerosis (Berer et al. 2011), chronic kidney disease (Wang et al. 2012) and autism (Finegold 2008) (Fig. 1).

1.2 What Is Metabolomics?

Technological breakthroughs have enabled the simultaneous examination of thousands of genes (genomics), transcripts (transcriptomics), proteins (proteomics), metabolites (metabolomics) and gut microbiota (metagenomics) with high-throughput techniques and analytical tools (Ellis et al. 2007). Since the comprehensive understanding of the organ and systemic metabolism is vital in maintaining health and nutritional status (Nicholson et al. 2012), the rapid advances in DNA sequencing and mass spectrometry (MS) technologies in recent years have enabled the extensive collection of data on the gut microbiome and metabolome to comprehensively evaluate the impact of the gut microbiota on human health (Tringe and

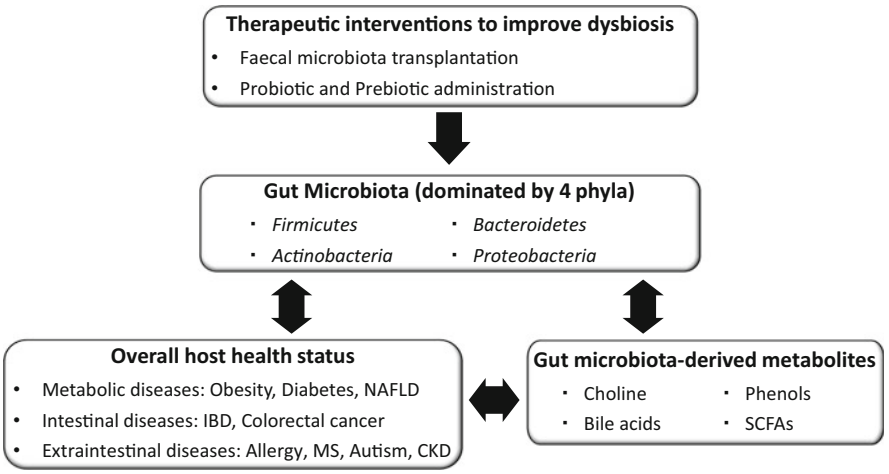

Fig. 1 The role of gut microbiota and its metabolites. Four bacterial phyla, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*, dominate the gut microbiota in mammals, and these phyla have been reported to characterize the role of the host metabolism and physiology. The gut microbiota and its metabolites like choline, bile acids, phenols and short-chain fatty acids are highly implicated in the etiopathogenesis of metabolic diseases, intestinal diseases and extraintestinal diseases, thereby playing a vital role in host health. Therapeutic interventions to improve dysbiosis include faecal microbiota transplantation and probiotic and prebiotic administration. *NAFLD* non-alcoholic fatty liver disease, *IBD* inflammatory bowel disease, *MS* multiple sclerosis, *CKD* chronic kidney disease, *SCFAs* short-chain fatty acids

Hugenholtz 2008). This has led to major advances in metagenome and metabolome technologies to reveal the important role that the gut microbiota play in contributing to host overall health status.

Two of the most commonly used wide-range metabolomic analytical methods include nuclear magnetic resonance (NMR) and MS, and they are commonly used in the identification of disease biomarkers. By using these approaches, we can accurately identify and have a robust understanding of the metabolites produced by microbiota and host cells in faecal, blood, tissue and urine samples (Dettmer et al. 2007). Methodologies ranging from targeted to untargeted approaches have been reported in screening biochemical pathways involved in central carbon metabolism, glycolysis, tricarboxylic acid cycle, amino acid metabolism, lipid metabolism and selected secondary metabolic pathways (Wenk 2005; Moco et al. 2006; Buescher et al. 2010). These tools allow scientists to comprehend the extent of impact of treatments on the host metabolic profile by the simultaneous analysis of the presence and quantity of thousands of metabolites.

1.3 Using Metabolomics to Understand the Gut Microbiota

Evaluation of the metabolome profile is commonly used to directly compare the metabolism of the gut microbiota and the metabolic outcomes in the host. In an investigation on the systemic influence of administering probiotics or prebiotics or a combination of both in initially germ-free mice colonized with a combination of microbes representing that of a human infant (Martin et al. 2008), it was revealed that dietary supplementation significantly modified the relative composition of gut microbiota community, culminating in systemic changes in the metabolic profiles of different tissues. Prebiotics increased proportions of *Bifidobacterium breve*, *Bifidobacterium longum* and *Bacteroides distasonis*; decreased proportions of *Escherichia coli* and *Clostridium perfringens*; and modulated lipid metabolism by decreasing concentrations of glucose and hepatic triglycerides in the plasma (Martin et al. 2008). In another report by Wikoff et al. (2009), the effects of gut microbiota on the host were evaluated via plasma metabolome profile comparison between germ-free and conventionally raised mice. There were many metabolites that were detected only in conventionally raised mice and not in germ-free mice. Additionally, in mice with or without gut microbiota, concentrations of more than a tenth of all metabolites differed by more than 50 % (Wikoff et al. 2009).

The integration between the activities of the gut microbiome and our gene reflects overall human metabolism at the systemic level. Our gastrointestinal tract provides nutrients to cells and tissues via the circulatory system, and likewise, so are the metabolites originating from the gut microbiota. This delicate interplay amongst gut microbiota-derived metabolites, the gut microbiota itself and the host immune system is transmitted through an extensive array of signalling pathways that extend beyond the immune system. The direct chemical interactions between gut microbiota and the host and the immune-mediated signalling mechanisms influence various organs such as the gut, liver, skeletal muscle and the brain, and these complex interrelationships come together mutually to culminate in a series of host–microbe metabolic axes. Within these axes, metabolic reactions can be regulated by gut microbial genomes, resulting in the production of choline, phenols, bile acids and short-chain fatty acids (SCFAs) by both the gut microbiome and host genome that are essential to host health (Aw and Fukuda 2015) (Fig. 2).

2 Metabolites

2.1 Choline

Choline, which plays a vital role in fat metabolism and synthesis of very-low-density lipoproteins in the liver, is primarily metabolized in the liver and is a vital component of cell membranes mostly obtained from foods like eggs and red meat (Vance 2008). The microbial conversion of dietary choline leads to an altered gut

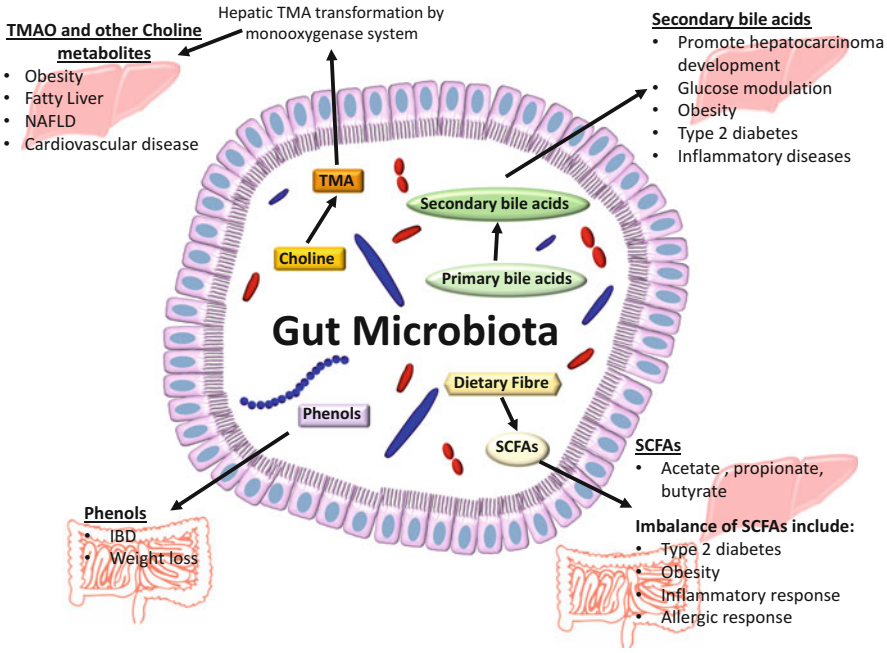

Fig. 2 The influence of gut microbiota-derived metabolites on host physiology. Choline has been implicated in the pathogenesis of obesity, fatty liver, non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases. Bile acids are reported to promote hepatocarcinoma development, glucose modulation, obesity, type 2 diabetes and inflammatory diseases. Phenols are involved in inflammatory bowel disease and weight loss. Short-chain fatty acids (SCFAs) have been reported as health-promoting metabolites, and the imbalance of SCFAs mediates type 2 diabetes, obesity, inflammatory and allergic responses

microbial ecology, which results in obesity and liver steatosis both in mice (Henao-Mejia et al. 2012) and in humans (Spencer et al. 2011), and promotes cardiovascular disease in human subjects (Wang et al. 2011). Importantly, it was reported that low concentrations of γ-proteobacteria and high levels of *Erysipelotrichia* in human faecal microbiota are correlated with hepatic steatosis (Spencer et al. 2011). Dietary choline is converted to trimethylamine (TMA) (Vance 2008) by gut microbial enzymes, which is then further metabolized by the flavin monooxygenase (FMO) system in the liver to produce trimethylamine-*N*-oxide (TMAO) (Dumas et al. 2006; Prentiss et al. 1961). This phenomenon lowers the levels of choline bioavailability and is implied to trigger non-alcoholic fatty liver disease (NAFLD) in mice (Dumas et al. 2006). In a report investigating the relationship between oral intake of phosphatidylcholine and the involvement of the gut microbiota in proatherogenic TMAO production in humans, plasma TMAO levels were significantly lowered after the administration of antibiotics and then reappeared after withdrawal of antibiotics. Elevated plasma TMAO concentrations were correlated with an increased risk of a major adverse cardiovascular event independently of

traditional cardiovascular risk factors, even in low-risk cohorts. As such, the lines of evidence mentioned above support the modulation of gut microbiota as a probable therapeutic approach in relation to these events (Tang et al. 2013). These studies provide evidence behind a potential relationship between the gut microbiota, dietary choline and risk of cardiovascular disease development.

2.2 Phenols

Approximately 50–100 mg of volatile phenols, mainly in the form of 4-cresol and phenol (predominantly as glucuronide and sulfate conjugates), is excreted by humans daily (Bone et al. 1976). The members of the genera *Clostridium*, *Bifidobacterium* and *Bacteroides* are implicated in the production of cresols in mammals. A large diversity of physiological and pathological conditions, ranging from IBD to weight loss, is reported to be correlated with altered levels of urinary 4-cresol metabolites in humans. Additionally, these conditions are also associated with altered gut microbiota composition, particularly, lowered diversity of microbiota due to the loss of *Lactobacillus* and *Bacteroides* species in IBD (Ott 2004) and differences in the ratio of *Firmicutes* and *Bacteroidetes* members due to weight loss (Ley et al. 2005; Turnbaugh et al. 2006).

2.3 Bile Acids

In the human liver, primary bile acids such as cholic acid and chenodeoxycholic acid are synthesized from cholesterol and then secreted in bile. Primary bile acids are mainly involved in facilitating the metabolism of dietary fat and the absorption of fat-soluble vitamins and cholesterol. Primary bile acids undergo an enterohepatic cycle between the gut and the liver eight times per day, where roughly 90–95 % of the bile acids are reabsorbed by the intestine and returned to the liver, where they are conjugated to taurine in mice and to glycine in humans, to form bile salts (Ridlon et al. 2006; Swann et al. 2011). Some of the remaining primary bile acids are deconjugated and further metabolized to secondary bile acids such as deoxycholic acid (DCA) and lithocholic acid by gut microbes, which belong to the genera *Bacteroides*, *Eubacterium* and *Clostridium*, whilst the others are lost in the faeces (Ridlon et al. 2006; Watanabe et al. 2006). Secondary bile acids are then reabsorbed, mainly by both bile acid transporters in the ileum and passive absorption in the intestine (Watanabe et al. 2006). Aerobic bacteria have been reported to be involved in the biotransformation of a minor portion of bile acids (Ridlon et al. 2006). The induction of senescence-associated secretory phenotype (SASP) in hepatic stellate cells by the enterohepatic circulation of DCA (Friedman 2008) secretes various hepatic inflammatory and tumour-promoting factors, thus facilitating murine hepatocarcinoma development after

exposure to chemical carcinogens. More importantly, the suppression of DCA production or reduction in the number of gut microbes efficiently prevents hepatocarcinoma development in obese mice as observed in mice lacking an SASP inducer (Orjalo et al. 2009) or the depletion of senescent hepatic stellate cells. These results indicate that the DCA–SASP axis in hepatic stellate cells is important in obesity-associated hepatocarcinoma development. Moreover, it was also observed that there were signs of SASP in the hepatic stellate cells of hepatocarcinoma arising in patients with non-alcoholic steatohepatitis (Coulouarn et al. 2012), suggesting that there might be a similar pathways responsible for certain aspects of obesity-associated hepatocarcinoma development in humans (Yoshimoto et al. 2013). As the gut microbiota is heavily related to bile acid transformation, conventionally raised rodents have lesser bile acids and a more diverse gut microbiota profile than their germ-free counterparts (Wostmann 1973; Swann et al. 2011). Besides, bile acids also function as signalling molecules and bind to cellular receptors (Watanabe et al. 2006) like the nuclear receptor farnesoid X receptor (FXR) (Prawitt et al. 2011) and the G protein-coupled receptor (GPCR) TGR5 (Thomas et al. 2009). FXR is activated by primary bile acids, whilst TGR5 binds secondary bile acids such as DCA and lithocholic acid. In enteroendocrine L-cells, TGR5 signalling induces secretion of glucagon-like peptide-1 (GLP-1), thereby leading to enhanced hepatic and pancreatic function and improved glucose tolerance in obese mice (Thomas et al. 2009). Both FXR and TGR5 are involved in murine glucose metabolism modulation whereby FXR impairs and TGR5 promotes glucose homeostasis (Prawitt et al. 2011; Thomas et al. 2009). Furthermore, the activation of TGR5 in skeletal muscle and brown adipose tissue has been reported to elevate energy expenditure and protect against diet-induced obesity (Watanabe et al. 2006). As reported by David et al. (2014), in mice that fed on saturated fats from milk and also in humans who consume high-fat diets, the levels of bile-tolerant microorganisms (*Alistipes*, *Bilophila* and *Bacteroides*) were increased and the levels of *Firmicutes* species that metabolize dietary plant polysaccharides (*Roseburia*, *Eubacterium rectale* and *Ruminococcus bromii*) were decreased. The differences between herbivorous and carnivorous mammals were reflected in the differences in microbial activities. In mice that fed on animal-based diet, the levels of *B. wadsworthia* were increased, allowing us to draw the correlations between animal-based diets, changes in bile acid concentrations, gut microbiota composition and the increase in the probability of IBD development (David et al. 2014). In light of the above, it has been demonstrated that all major bacterial groups are involved in bile salt hydrolase activity. As such, gut microbiota modulation may control lipid and glucose metabolism via the composition of bile acid pools and the modulation of FXR and TGR5 signalling, thereby playing a role in effective obesity and type 2 diabetes management (Jones et al. 2008).

2.4 Dietary Fibre Fermentation and Short-Chain Fatty Acid Production

When dietary fibre or complex carbohydrates are consumed, they are digested and then fermented mainly in the colon by gut microbiota into SCFAs such as acetate, propionate and butyrate. These SCFAs are recognized by the GPCRs such as GPR41 and GPR43, which are expressed by gut enteroendocrine cells (Samuel et al. 2008). Gut microbiota induces peptide YY expression by L-cells via a GPR41-dependent mechanism as observed in the study where lowered adiposity was observed in conventional *Gpr41*-deficient mice as compared to conventional wild-type mice, whereas adiposity in germ-free, wild-type and *Gpr41*-deficient mice were comparable (Samuel et al. 2008). Inflammation can be inhibited by SCFAs via GPR43 signalling in neutrophils (Maslowski et al. 2009; Sina et al. 2009) and insulin signalling in adipocytes (Kimura et al. 2013). In addition, SCFAs also modulate the secretion of the hormone GLP-1, which may improve insulin secretion and suppress diabetes (Tolhurst et al. 2012). In a recent report by Trompette et al. (2014), the composition of both gut and lung microbiota were interestingly changed by dietary fibre content via the alteration of the ratio of *Firmicutes* to *Bacteroidetes*. In mice fed with high-fibre diets, SCFA levels were elevated, and they were subsequently protected against allergic inflammation in the lungs, whereas the contrary was observed in mice fed with low-fibre diets. It was observed that in mice that were treated with SCFA propionate, there were increased generation of macrophages and dendritic cell precursors and subsequent seeding of the lungs by dendritic cells of high phagocytic capacity. However, these effects of propionate on allergic inflammation were depended on GPR41 but not GPR43 (Trompette et al. 2014). Other SCFAs such as acetate and propionate are taken up by the liver and used as substrates in gluconeogenesis and lipogenesis (Tremaroli and Backhed 2012). SCFAs can modulate histone deacetylase function by stimulating the sympathetic nervous system, thereby influencing social behaviour in rats (MacFabe et al. 2011). The SCFAs propionate and butyrate, which are generated by the fermentation of soluble fibres, activate intestinal gluconeogenesis that improves glucose and energy homeostasis (De Vadder et al. 2014). Out of all the SCFAs produced from microbial fermentation, butyrate which is mainly produced by species of the order *Clostridiales*, a dominant order in gut microbiota, is particularly vital as an energy substrate for cellular metabolism in the colonic epithelium (Donohoe et al. 2011). Gut microbiota-derived butyrate activates GPR109a niacin receptor and suppresses both colonic inflammation and carcinogenesis (Singh et al. 2014). In addition, it has also been reported that intestinal gluconeogenesis gene expression is activated by butyrate via a cAMP-dependent mechanism, whilst propionate, a substrate of intestinal gluconeogenesis, activates intestinal gluconeogenesis gene expression through the gut–brain neural circuit involving the fatty acid receptor FFAR3 (De Vadder et al. 2014). In a recent metabolomic-based integrated omics study, it was revealed that in germ-free mice fed with high-fibre diet, there was colonization of *Clostridiales* which promoted gut microbial fermentation,

thereby resulting in the accumulation of luminal SCFAs (Furusawa et al. 2013). Amongst all SCFAs, butyrate induces the in vitro differentiation of regulatory T (T_{reg}) cells. Administration of butyrylated starch induces colonic T_{reg} cell differentiation and attenuates the development of colitis in vivo. Treatment of naïve T cells under T_{reg}-polarizing conditions with butyrate enhances histone H3 acetylation particularly in the promoter and CNS 3 enhancer regions of the *Foxp3* gene, the master transcriptional regulator of T_{reg} cells. As such, butyrate derived from dietary fibre fermentation by commensals of the order *Clostridiales* epigenetically induces the differentiation of colonic T_{reg} cells, which play critical roles in the suppression of inflammatory and allergic responses (Atarashi et al. 2011, 2013; Furusawa et al. 2013). In the view of protection from enteropathogenic infection, using an integrated omics in a simplified model of lethal infection with enterohaemorrhagic *Escherichia coli* O157:H7, it was revealed by Fukuda et al. (2011) that acetate produced by probiotic bifidobacteria improves intestinal defence by enhancing gut epithelial barrier function (Fukuda et al. 2011). In a study by Okada et al. (2013), microbiota-derived lactate has been reported to be an important factor in the enterocyte hyperproliferation induction in firstly starved then refed mice. During a 12- to 36-h period of starvation, colonic epithelial cell turnover was halted. It then increased 12–24 h after refeeding. As such, the increase in live *Lactobacillus murinus*, lactate production and dietary fibre content could also enhance epithelial cell proliferation (Okada et al. 2013). Thus, it is evident that SCFAs can influence a range of host processes with significant effects and are thus vital products of microbial fermentation of dietary fibres.

3 Dysbiosis and Metabolic Diseases

The gastrointestinal tract, a complex and well-balanced ecosystem, has commensal microbes and their hosts in a symbiotic relationship under normal conditions. However, qualitative and quantitative changes in the gut microbiota will lead to an imbalance in this equilibrium. The changes in the metabolic activities of the gut microbiota and the changes in their local distribution lead to dysbiosis, which is a condition in which microbial imbalance exerts adverse effects on the host. Various factors like antibiotic consumption, nutritional status and environmental conditions can disrupt microbial stability and are partly responsible for intestinal dysbiosis (Hawrelak and Myers 2004). This leads to various metabolic diseases like obesity, diabetes, chronic kidney disease, atherosclerosis, NAFLD and extraintestinal diseases like multiple sclerosis and autism (Fig. 3).

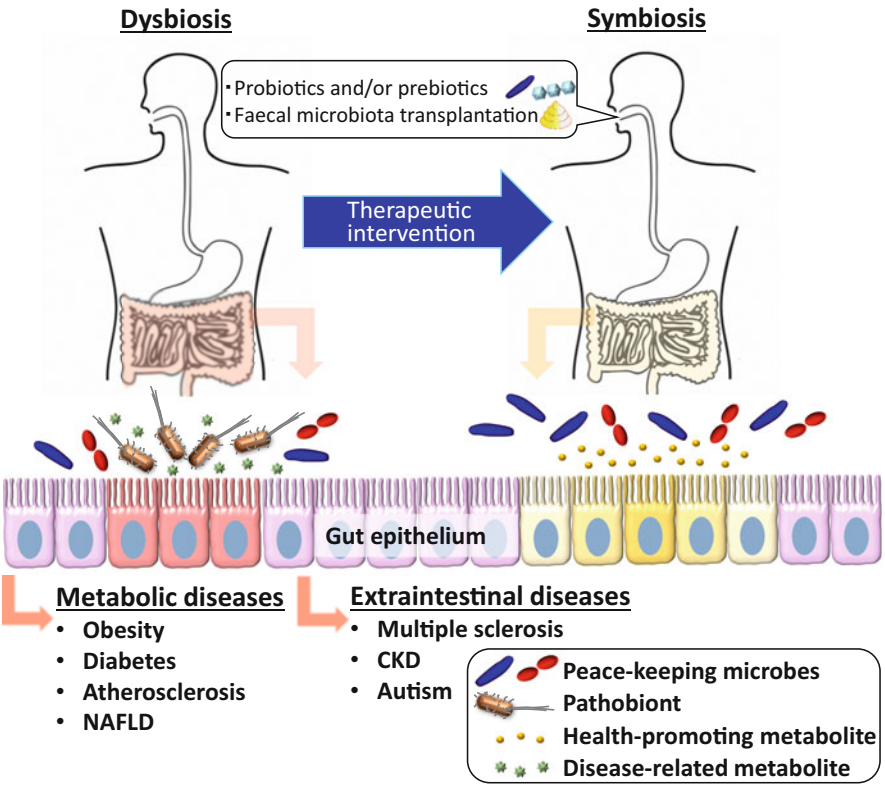

Fig. 3 Dysbiosis-related diseases and therapeutic interventions. Dysbiosis refers to the imbalance between the peacekeeping bacteria and pathobionts, leading to metabolic diseases like obesity, diabetes, atherosclerosis and NAFLD and extraintestinal diseases like multiple sclerosis, chronic kidney disease (CKD) and autism. Ingestion of probiotics and/or prebiotics and faecal microbiota transplantation have been reported to restore symbiosis

3.1 Obesity

It was first suggested in 2005 that obesity could be attributed to gut microbial composition levels as the phylum *Bacteroidetes* was lower and the phylum *Firmicutes* was higher in abundance in obese *ob*/*ob* mice than their lean littermates although both groups were administered similar diets (Ley et al. 2005). Subsequently, in a study with obese human twins, a correlation was observed between an increase in *Firmicutes* proportion and a decrease in *Bacteroidetes* proportion to the enrichment of microbial genes encoding key enzymes involved in carbohydrate metabolism. This is implicated in the host's ability to digest food and supply energy substrates such as SCFAs (Turnbaugh et al. 2006, 2009). Importantly, the transfer of gut microbiota from both obese mice (Vijay-Kumar et al. 2010) and obese humans (Turnbaugh et al. 2006; Ridaura et al. 2013) into germ-free recipient

mice reproduced the obese phenotype. In addition, it has also been reported that bifidobacterial numbers were lower, levels of *Staphylococcus aureus* were higher (Kalliomäki et al. 2008), levels of *Enterobacteriaceae* species were significantly higher and levels of *Desulfovibrio* and *Akkermansia muciniphila*-like bacteria were significantly lower in obese children than in normal-weight children (Karlsson et al. 2012a). Significant decreases in the number of *A. muciniphila*, a novel mucin-degrading bacterium that colonizes the mucosal layer constituting of 3–5 % of the bacterial community (Belzer and de Vos 2012), were observed in genetically and high-fat diet-induced obese mice (Everard et al. 2013). Other studies have also shown that this bacterium is inversely correlated with type 1 (Hansen et al. 2012) and type 2 diabetes (Qin et al. 2012) and body weight (Collado et al. 2008; Santacruz et al. 2010; Everard et al. 2011; Karlsson et al. 2012a). Recently, obesity and glucose homeostasis disorders were induced in germ-free mice fed with a high-fat diet that were colonized with endotoxin-producing *Enterobacter cloacae* B29 strain. This implies that lowering plasma endotoxin levels might be a probable approach in controlling metabolic diseases (Fei and Zhao 2013). In addition, faecal transplantation of gut microbiota from lean healthy donors in human patients presenting with metabolic syndrome resulted in improved insulin sensitivity, which was correlated with an increase in the number of butyrate-producing bacteria, thereby implying that microbial butyrate may be important in promoting this improvement (Vrieze et al. 2012).

3.2 Diabetes

Type 1 and type 2 diabetes development have been correlated with gut microbiota composition. Type 1 diabetes (T1D) is an autoimmune disorder that involves the destruction of pancreatic insulin-producing cells. In studies that were performed in a T1D mouse model, a nonobese diabetic (NOD) mouse, it was revealed that in *Myd88*$^{-/-}$ mice, during antibiotic treatment or under germ-free condition, protection against diabetes was diminished (Wen et al. 2008). Bacterial 16S ribosomal RNA gene sequencing also showed variations in the gut microbiota composition in *Myd88*$^{-/-}$ NOD mice as compared to their normal littermates. The *Firmicutes*/*Bacteroidetes* ratio was lowered, and this indicates that certain bacterial populations were essential in the protection against T1D. However so, it has been recently reported that MYD88 deficiency alone does not remodel gut microbiota composition, and therefore, it is plausible that the genetic background of NOD mice might have influenced the effects of MYD88 (Ubeda et al. 2012). Segmented filamentous bacteria (SFB) have also been reported to protect against T1D (Kriegel et al. 2011) gender specifically as male NOD mice with SFB have lowered incidence of T1D than their female counterparts. Furthermore, the gut microbiota composition in male NOD mice was observed to be different from that in their female counterparts, most probably due to increased testosterone levels in the former, which is associated with T1D protection (Kriegel et al. 2011; Markle

et al. 2013). When gut microbiota was transferred from male NOD mice to their female counterparts, the increase in testosterone concentrations and reduced susceptibility to T1D were observed (Markle et al. 2013). In the *ob*/*ob* diabetic mice model, it was also observed that a 2-week antibiotic treatment significantly reduced the numbers of both aerobic and anaerobic gut microbes, leading to lower levels of hepatic triglycerides, lower plasma lipopolysaccharide (LPS) concentrations, elevated levels of hepatic glycogen and increased plasma adiponectin concentrations than in their non-treated counterparts (Membrez et al. 2008). In type 2 diabetic (T2D) patients, Larsen et al. (2010) reported significantly lower levels of *Firmicutes* and *Clostridia* in male T2D subjects as compared to nondiabetic control subjects. The same study also reported correlations between plasma glucose concentrations to both the ratios of *Bacteroidetes* to *Firmicutes* and of the *Bacteroides–Prevotella* to the *C. coccoides–E. rectale* group. As *Bacteroidetes* and *Proteobacteria* are composed of gram-negative bacteria and have LPS in their outer membranes, these findings suggest that T2D may be promoted via an endotoxin-induced inflammatory response (Larsen et al. 2010). In a metagenome-wide association study of gut microbiota using data from a total of 345 T2D patients and non-T2D control subjects, most of the genes upregulated in the T2D group were mainly from opportunistic pathogens including *Bacteroides caccae*, *Clostridium hathewayi*, *Clostridium ramosum*, *Clostridium symbiosum*, *Eggerthella lenta* and *Escherichia coli* which have been implicated as causes of underlying human infections, and the genes that were upregulated in the control group were from various butyrate-producing bacterial species like *Clostridiales* sp. SS3/4, *E. rectale*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis* and *Roseburia inulinivorans*. The mucin-degrading species *A. muciniphila* and the sulfate-reducing species *Desulfovibrio* sp. were also found in abundance in the T2D group (Qin et al. 2012). In another metagenome study conducted in 145 64-year-old European women with normal, deficient or diabetic glucose control, a mathematical model to identify T2D based on gut microbiota metagenomic profile was developed. The same model was also applied to a similar study but using Chinese subjects, and different metagenomic markers in the European T2D and Chinese T2D cohorts were reported (Karlsson et al. 2013). This suggests that age and geographical location may play a role in the variation of gut microbial metagenomic profiles that influence T2D development.

3.3 Atherosclerosis

Host genetic and environmental factors, such as dietary intake and the presence of commensal gut microbes, play important roles in the pathogenesis of atherosclerosis, a vascular disease with a complex pathologic phenotype. Three phospholipid-associated molecules, choline, betaine and TMAO, which are present in the plasma, could be used as biomarkers for predicting the risk of cardiovascular diseases and seemed to promote atherosclerosis (Wang et al. 2011). In another study, Koeth

et al. (2013) reported that murine gut microbiota metabolism of dietary L-carnitine, a type of TMA present in large amounts in red meat, also produces TMAO and accelerates the rate of atherosclerosis (Koeth et al. 2013). In addition, vegans produced lesser TMAO than omnivorous human subjects following ingestion of L-carnitine via a microbiota-dependent mechanism (Koeth et al. 2013). There were correlations observed between specific bacterial taxa in human faecal samples, plasma TMAO levels and nutritional status (Koeth et al. 2013). In subjects undergoing cardiac evaluation, concentrations of plasma L-carnitine were predicted to be at higher risk for both prevalent cardiovascular disease and major adverse cardiac events, but only in subjects with coexisting elevated TMAO levels (Koeth et al. 2013). Caecal microbial composition in mice was modified by chronic dietary L-carnitine dietary intervention, and this supplementation also significantly elevated the synthesis of TMA and TMAO and increased the rate of atherosclerosis (Koeth et al. 2013). However, this was not observed when gut microbiota was suppressed (Koeth et al. 2013). In mice with intact gut microbiota, dietary supplementation with TMA, carnitine or choline reduced the rate of reverse cholesterol transport in vivo (Koeth et al. 2013). As such, gut microbiota may contribute to the well-established link between high levels of red meat consumption and cardiovascular disease risk (Koeth et al. 2013). In another study, 16S rRNA gene pyrosequencing of caecal microbiota in mice revealed that the family *Prevotellaceae* and the genus *Prevotella* were enriched and positively correlated with the plasma TMAO levels in mice fed with L-carnitine-supplemented diet (Karlsson et al. 2012b). Faecal microbiome metagenomic evaluation also revealed that the genus *Collinsella* was enriched in symptomatic atherosclerosis patients, whereas the genus *Roseburia* and *Eubacterium* were enriched in healthy subjects (Karlsson et al. 2012b). Additionally, metagenomic characterization of the faecal microbiome revealed that genes responsible for peptidoglycan synthesis were upregulated, and phytoene dehydrogenase was depleted in symptomatic atherosclerosis patients (Karlsson et al. 2012b). Accordingly, these patients also presented with lowered serum β-carotene levels (Karlsson et al. 2012b). These findings suggest that characteristic changes in the gut microbiome may be associated with the inflammatory status of symptomatic atherosclerosis patients.

3.4 Non-alcoholic Fatty Liver Disease

NAFLD, one of the most prevalent liver diseases worldwide, is characterized by fat deposition (steatosis) in the liver that is unrelated to excessive alcohol consumption and is usually observed in states of insulin resistance and metabolic syndrome. Non-alcoholic steatohepatitis (NASH), which is the most severe form of NAFLD, affects approximately 10–20 % of all NAFLD patients and is a major cause of hepatic cirrhosis (Moschen et al. 2013). The prevalence of NASH is increasing, and it is often assumed that various genetic, metabolic, inflammatory and environmental factors contribute to its pathogenesis; however, the exact underlying mechanisms

remain unknown (Abu-Shanab and Quigley 2010). The progression of NAFLD usually starts from fatty liver to liver cirrhosis and then to hepatocellular carcinoma (Torres et al. 2012; Satapathy and Sanyal 2010). There have been numerous human and animal studies documented to investigate the relationships between the gut microbiota and NAFLD (Cani et al. 2007, 2008; Rivera et al. 2007; Swann et al. 2011; Sabate et al. 2008; Miele et al. 2009; Verdam et al. 2011; Backhed et al. 2004; Cope et al. 2000). Backhed et al. (2004) have reported that in conventional mice, the concentrations of hepatic triglycerides were higher than those in germ-free mice, although the amount of food intake was lower in the former. Colonization of germ-free mice with gut microbiota was associated with a higher rate of monosaccharide absorption from the gut lumen, which promotes de novo fatty acid synthesis and triglyceride production in the liver, which was confirmed by elevated acetyl-CoA carboxylase and fatty acid synthase activities (Backhed et al. 2004). In addition, Cope et al. (2000) have found that microbial fermentation-derived metabolites in the gut, which include ethanol, are key factors in the induction of obesity in mice and may be related to the pathogenesis of fatty liver disease (Cope et al. 2000). As with obesity, Cani et al. (2007, 2008) reported that hepatic Kupffer cell activation in mice contributes to NAFLD progression, and microbial endotoxin-related chronic inflammation involves CD14–TLR4 signalling (Cani et al. 2007, 2008; Rivera et al. 2007). Bile acid metabolism is also regulated by gut microbiota. Swann et al. (2011) have reported that the murine gut microbiota indirectly promotes hepatic steatosis and lipoperoxidation via farnesoid X receptor-mediated signalling, thereby affecting bile acid secretion These animal studies implicated that the gut microbiota, via the production of hepatotoxic ethanol, can induce fatty liver, resulting in an increase in monosaccharide absorption (Backhed et al. 2004), microbial endotoxin-induced low-grade chronic inflammation (Cani et al. 2007, 2008; Rivera et al. 2007), as well as bile acid metabolism (Swann et al. 2011). In human subjects, Sabate et al. (2008) have reported that gut microbial overgrowth in obese patients may be linked to hepatic steatosis, and correspondingly, Wigg et al. (2001) have also reported that half of NASH patients have microbial overgrowth and elevated serum TNF-α levels, suggesting that NASH might be related to imbalance in gut microbial composition and systemic inflammation, despite unaffected intestinal permeability. Miele et al. (2009) have reported that NAFLD development in human subjects is associated with increased intestinal permeability due to microbial overgrowth in the small intestine and disruption of intestinal mucosal tight junctions; small intestinal microbial overgrowth in NAFLD patients might contribute to hepatic fat deposition. Furthermore, Verdam et al. (2011) have shown that chronic endotoxemia in patients is correlated with NAFLD severity, which was also similarly observed in murine models. In addition, NAFLD development in humans might be correlated with dietary choline depletion (Spencer et al. 2011). In studies conducted by Turnbaugh et al., 15 female subjects were placed on well-controlled diets with adjusted choline levels. It was reported that dietary choline deficiency modified gut microbial composition and that the levels of the bacterial classes *Gammaproteobacteria* and *Erysipelotrichi* were positively correlated with changes in the liver fat content. The same group also

reported that as compared to both healthy controls and steatotic patients, NASH patients had a lower percentage of species of the phylum *Bacteroidetes*, which was similar to the gut microbial profiles in obese human subjects (Turnbaugh et al. 2006, 2009). Systemic ethanol concentrations were also significantly higher in NASH patients than in the controls, indicating that ethanol-producing microbes might be correlated with NASH pathogenesis (Zhu et al. 2013). Taken together, the results of the above-mentioned findings implicate that the differences in gut microbial profile amongst NAFLD, NASH, obese and healthy controls might serve as a viable diagnostic marker, as well as acting as a target for preventive or therapeutic medicine.

4 Dysbiosis and Extraintestinal Diseases

4.1 Multiple Sclerosis

Numerous reports have implicated the role of intestinal microbiota in the development of various autoimmune central nervous system (CNS) disorders. In mice that exhibited symptoms of experimental autoimmune encephalomyelitis (EAE), broad-spectrum antibiotics were administered orally (Ochoa-Reparaz et al. 2009). In another report documenting a murine model of multiple sclerosis, mice that were immunized with the self-antigen myelin oligodendrocyte glycoprotein (MOG) in complete Freund's adjuvant (CFA), under germ-free conditions, disease symptoms in both spontaneous EAE and MOG–CFA-induced EAE were diminished (Lee et al. 2011; Berer et al. 2011). Mice monocolonized with SFB related to the genus *Clostridium* were reported to demonstrate elevated levels of interleukin-17-producing helper T (T_H17) cells in both the intestinal lamina propria and the CNS, resulting in severe EAE (Lee et al. 2011). However, it has yet to be confirmed if EAE is a result of the migration of SFB-specific T_H17 cells into the CNS or by the expansion of pathogenic autoantigen-specific T cells that are promoted by T_H17 cell responses. Conversely, certain populations of commensal bacteria like polysaccharide A $(PSA)^+$ *Bacteroides fragilis* can attenuate CAS inflammation via the differentiation of $Foxp3^+$ T_{reg} cells, thereby preventing EAE symptoms. As such, the pathogenesis of CNS disorders like multiple sclerosis may be dependent on the balance of the levels of different bacterial strains in the gut microbiota.

4.2 Chronic Kidney Disease

Due to the fact that the intestinal immunity can be affected to the extent that it is no longer possible to maintain physiological control over the gut microbiota, some extraintestinal non-communicable diseases like chronic kidney disease are

associated with gut dysbiosis (Chow and Mazmanian 2010). In chronic kidney disease patients with impaired renal functions, they often present with not only metabolic derangements but also systemic inflammation, and the intestinal microbiota has been increasingly identified as a trigger for immune dysregulation (Schepers et al. 2010; Kotanko et al. 2006). Vaziri et al. (2013) characterized the gut microbiota of uraemic versus non-uraemic rats and in patients and reported that uraemia was associated with an increase in intestinal pathobionts, indicating that the metabolic and haemodynamic alterations in chronic kidney disease influence the composition and role of gut microbiota. Colonic bacteria also generate uraemic toxins like α-phenylacetyl-L-glutamine, 5-hydroxyindole, indoxyl glucuronide, *p*-cresol sulfate and indoxyl sulfate, all of which contribute to end-stage renal disease (Ranganathan et al. 2006; Aronov et al. 2011). As chronic kidney disease progresses, circulating bacterial endotoxin/LPS levels increase and have been observed to be the highest in patients on kidney dialysis. These LPS levels were comparable to that in patients with liver disease, gut irradiation and decompensated heart failure. As LPS originates from the cell wall component of gram-negative bacteria, microbiota enriched in γ-proteobacteria may be a source of circulating LPS (McIntyre et al. 2011). In a recent report by Wang et al. (2012), rats with experimentally induced uraemia had increased bacterial translocation from the gut into the mesenteric lymph nodes, liver and spleen, and this was associated with elevated levels of serum interleukin-6 and C-reactive protein. In another report, after oral supplementation of nonpathogenic *Sporosarcina pasteurii* to uraemic rats, this gut microbiota-directed intervention improved uraemic state as exemplified in improved renal function and prolonged lifespan (Ranganathan et al. 2006). In addition, the neutralization of the bacteria-derived uraemic toxin indoxyl sulfate in the gut delayed the progression of chronic kidney disease and cardiovascular disease in uraemic rats. Lastly, when the same indoxyl sulfate-binding agent was administered to pre-dialysis patients, the 5-year survival rate in patients was improved (Niwa 2011). In a recent study by Mishima et al. (2014), the effects of the ClC-2 chloride channel activator lubiprostone (commonly used for the treatment of constipation) on chronic kidney disease were evaluated using an adenine-induced renal failure mouse model. Oral administration of lubiprostone (500 μg/kg per day for 12 days) lowered elevated blood urea nitrogen levels and attenuated tubulointerstitial damage, renal fibrosis and inflammation. Gut microbiome analysis revealed that lubiprostone treatment altered the murine gut microbial composition by recovering the levels of species of the family *Lactobacillaceae* and genus *Prevotella*, which were significantly decreased in the renal failure mice. Furthermore, CE-TOFMS-based metabolomics showed that lubiprostone treatment lowered the plasma levels of uraemic toxins, such as indoxyl sulfate, hippurate and *trans*-aconitate, which are mainly derived from gut microbiota. These results indicate that lubiprostone administration ameliorates chronic kidney disease progression and uraemic toxin accumulation by improvement of the gut ecosystem (Mishima et al. 2014). Although the relationship between gut microbiota and chronic kidney disease is not completely understood, the gut should no longer be overlooked as a potential trigger for chronic kidney disease, and future studies

should focus on unravelling the pathogenetic role of gut microbiota in kidney disease and to discover appropriate therapeutic interventions to manipulate the gut microbiota to correct chronic kidney disease-related immune dysregulation and to prevent further health complications.

4.3 Autism

One of the several reported comorbidities in autism spectrum disorder (ASD), a serious neurodevelopmental condition, is gastrointestinal distress (Buie et al. 2010; Coury et al. 2012). It is also correlated with symptom severity (Finegold et al. 2010). There have been numerous studies reporting the altered composition of intestinal microbiota in ASD subjects (Finegold et al. 2010, 2012; Kang et al. 2013; Parracho et al. 2005; Williams et al. 2011, 2012). *Bacteroidetes* was found at high levels in the severely autistic group, whilst *Firmicutes* were more predominant in the control group. Small but significant differences in the levels of *Actinobacteria* and *Proteobacteria* were also observed. In faecal samples obtained from severely autistic children, both *Desulfovibrio* species and *Bacteroides vulgatus* were present in significantly larger numbers than in control subjects (Finegold et al. 2010). In another study by the same author, autistic children were also reported to have higher levels of *Lactobacillus* species, lower levels *Bifidobacterium* species and lower levels of total SCFAs including acetate, propionate and valerate (Finegold et al. 2010). In a separate study with autistic children, significantly lower abundances of the genera *Prevotella*, *Coprococcus* and unclassified *Veillonellaceae* were observed (Kang et al. 2013). In addition, it was also reported that there was a correlation between the presence of members belonging to the family *Alcaligenaceae* in autistic children with autism and who suffered from gastrointestinal dysfunction (Kang et al. 2013). Hsiao et al. (2013) has also recently reported that the oral administration of PSA^+ *B. fragilis* in the offspring of maternal immune activated (MIA) mice corrected gut permeability. This altered gut microbial composition and attenuated defects in ASD associated behavioural and physiological abnormalities in the offspring of MIA mice. Further investigation showed that this treatment with PSA^+ *B. fragilis* also corrected the levels of MIA-induced serum metabolites by restoring the levels of 4-ethylphenylsulfate, which in naïve mice results in certain behaviour abnormalities (Hsiao et al. 2013). These findings implicate relationships between the gut microbiota, metabolome and the brain, providing novel alternative therapeutic opportunities in human neurodevelopmental disorders.

5 Microbial Health Potentials

5.1 Therapeutic Interventions and Dysbiosis

Commonly used approaches in gut microbiota remodelling for maintaining optimal health status in humans include therapeutic interventions such as bacteriotherapy (Borody et al. 2004) and bioecological control (Gibson and Roberfroid 1995; Holmes et al. 2012). The modulation of intestinal microbiota populations by prebiotics, probiotics or synbiotics may also be beneficial to human health (Gibson and Roberfroid 1995; Holmes et al. 2012). Prebiotics are certain food materials such as oligosaccharides that promote the proliferation of probiotics whereas probiotics, typically contained in dairy fermented products such as yogurt, are well known as healthy microbes that, when orally administered in adequate amounts, yields benefits to host health. Probiotics and prebiotics are marketed as health-promoting supplements, and there are numerous publications highlighting the beneficial effects of probiotics and prebiotics in the improvement of the gut environment (Gareau et al. 2010), the prevention of pathogenic infections (Fukuda et al. 2011) and the regulation of immune functions (Round and Mazmanian 2009).

5.2 Therapeutic Implications of Gut Microbiota Modulation

When human baby microbiota-associated mice were treated with the probiotics *Lactobacillus paracasei* or *Lactobacillus rhamnosus* and two galactosyl–oligosaccharide prebiotics, the numbers of *B. longum* and *B. breve* were increased, whereas the numbers of *C. perfringens* were lowered. This gut microbiota composition remodelling has resulted in changes in various host metabolic pathways like gluconeogenesis, lipid profiles and amino acid metabolism (Guarner 2009). Conjugated linoleic acid is a naturally occurring isomer of linoleic acid found in ruminant-derived meat and dairy products (Fukuda et al. 2005) and has been reported to protect against colon carcinogenesis, atherosclerosis as well as obesity in mice (West et al. 1998; Fukuda et al. 2006). Lowered relative abundances of both *Firmicutes* and *Clostridium* cluster XIVa in the small intestine were observed in mice that were supplemented with *L. rhamnosus* GG and *Lactobacillus sakei* NR28. This led to lower body weight gain, decreased fat mass and downregulation of genes involved in lipogenesis (Ji et al. 2012). However, *L. acidophilus* NCDC13 supplementation in high-fat diet-induced obese mice resulted in the increase in the total number of *Bifidobacterium* in caecal and faecal content without reducing adiposity (Arora et al. 2012). As reported by Parnell and Reimer (2012), prebiotic fibres have also been implicated in the reduction of the ratio of *Firmicutes* to *Bacteroidetes* in obese rats and to ameliorate NAFLD by lowering de novo hepatic lipogenesis. Chitin–glucan supplementation increased the number of bacteria closely related to the *Clostridium* cluster XIVa including *Roseburia* spp. Chitin

dietary intervention also attenuated fat fain and fat mass development (Neyrinck et al. 2012). Inulin supplementation in obese women increased the levels of *Bifidobacterium* spp. and *F. prausnitzii* and decreased the levels of *Bacteroides intestinalis*, *Bacteroides vulgates* and *Propionibacterium* (Dewulf et al. 2013). In another study involving healthy human subjects, the consumption of galactooligosaccharides for 12 weeks increased the levels of several types of *Bifidobacterium* spp. and decreased in the number of *Bacteroides* (Davis et al. 2011). The numbers of *Bacteroides–Prevotella* spp. and *Roseburia* spp. were restored, and the numbers of *Bifidobacterium* spp., in particular *Bifidobacterium animalis* subsp. *lactis*, were increased in mice that were fed on high-fat diet supplemented with wheat-derived arabinoxylans (Neyrinck et al. 2011). In healthy overweight humans, after oral administration of *Lactobacillus gasseri* SBT2055, abdominal visceral and subcutaneous fat were lowered (Arora et al. 2012). Besides, infants who were supplemented with *L. rhamnosus* GG in formula for half a year reported better growth and larger weight gain (Vendt et al. 2006). In a follow-up study, pre- and postnatal administration of *L. rhamnosus* GG protected against excessive weight gain in children (Luoto et al. 2010). In addition to the prebiotic and/or probiotic treatment, faecal microbiota transplantation has been reported to be beneficial in antibiotic-associated diarrhoea or *Clostridium difficile* infection (Borody 2000; Khoruts et al. 2010; van Nood et al. 2013). Faecal microbiota transplantation can also potentially modulate gut microbiota composition in order to improve pathogenesis of various diseases such as chronic gastrointestinal infections, IBD, insulin resistance, multiple sclerosis and idiopathic thrombocytopenic purpura (Smits et al. 2013). Collectively, the improvement of gut microbiota composition by faecal microbiota transplantation or treatment with probiotics and/or prebiotics may be beneficial in the prevention and medical treatment of several dysbiosis-associated disorders (Fig. 3).

6 Conclusions

We strongly advocate for the integration of metagenomic and metabolomic information on a system biology-wide approach as we believe that it is a valuable methodology that would enable us to understand this intricate interplay between gut microbiota and host metabolism to a greater extent. The integration of information derived from microbiomic, metatranscriptomic and metabolomic platforms will lead to an improved comprehensive understanding of the complex mammalian superorganism. The application to integrated omics-based understanding of the metabolic interactions between lifestyle, nutritional habits and gut microbiota promises to provide intriguing novel therapeutic avenues to maintain and promote optimal host health.

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Immune Modulation by Probiotics

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Abstract Probiotics are defined as live microorganisms, which when administered in adequate amounts confer health benefits on the host. Lactic acid bacteria and *Bifidobacterium* strains are the most common groups of bacteria with claimed probiotic properties. These bacterial strains have been conventionally incorporated into food and beverage products as dietary adjuncts, aimed at promoting gastrointestinal health. Meanwhile, a growing number of studies have also revealed that probiotic strains could exert beneficial health effects beyond the gut, mainly attributed to their peculiar immunomodulatory properties. Probiotic strains are capable of modulating the innate and adaptive immune response through both immunostimulation and immunoregulation and can thereby exert prophylactic and therapeutic effects on the host. Indeed, experimental evidences have demonstrated that administration of live probiotics and/or probiotic-derived products can be potentially applied in the prevention and/or treatment of a wide range of non-gastrointestinal diseases, such as metabolic disorders, allergic and inflammatory skin disorders, respiratory diseases, osteoporosis, male hypogonadism, and rheumatoid arthritis. However, more clinical trials on the efficacy of different probiotic strains in the prevention and treatment of these health conditions are needed to generate more definitive results. The exact mechanisms by which specific probiotic strains can stimulate and/or regulate immune functions remain to be elucidated. Nonetheless, recent advances in biotechnology have provided rapid ways to explore possible immunomodulatory mechanisms of probiotics. Altogether, this chapter provides a succinct summary of the updated evidence on the immunomodulatory effects of probiotics and discusses their possible mechanisms of action. This chapter also presents the future directions to promote a better understanding of the underlying immunomodulatory actions of probiotics.

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1 Introduction

In 1953, Werner Kollath introduced the term "probiotika" for "active substances that are essential for a healthy development of life" (Guarner et al. 2005). This term evolved to "probiotic," which is derived from the Greek word "biotikos," meaning "for life." The term probiotic was first used by Lilly and Stillwell (1965) to describe "substances produced by one microorganism that stimulate the growth of another." However, Parker (1974) applied the word probiotic to describe animal feed supplements that improved animal health and eventually introduced a new definition as "organisms and substances that contribute to intestinal microbial balance." Since then, probiotics have received an increasing interest for the promotion of human and animal gastrointestinal health. Thereafter, Fuller (1989) revised Park's definition by focusing on microorganisms and redefined probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." The definition of probiotics has been re-modified throughout the years until 2001, when the joint Food and Agriculture Organization (FAO) and the World Health Organization (WHO) recommended the adoption of the definition of probiotics as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). This definition has been continuously retained and reinforced by an expert panel from the International Scientific Association for Probiotics and Prebiotics recently, as it is still relevant and capable of accommodating current and anticipated applications (Hill et al. 2014).

In order for a microorganism to be classified as a probiotic, it must fulfill the following criteria: (1) must be able to survive in the intestinal transit, (2) must be able to adhere to mucosal surfaces and colonize the intestine, (3) must be resistant to technological processes, (4) must be safe for consumption, (5) must possess documented beneficial health effects, and (6) must be taxonomically identified at the genus, species, and strain level (Borchers et al. 2009). The most common microorganisms with claimed probiotic properties are bacteria, *Bifidobacterium*, and lactic acid bacteria (LAB) strains, including the genera of *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Pediococcus*. Additionally, *Saccharomyces*, a yeast genus, has also been identified as a microorganism associated with probiotic properties. Meanwhile, it is extremely important to note that the health-promoting effects of probiotics are strain-specific.

Lactobacillus and *Bifidobacterium* strains are among the primary candidates of probiotic bacteria inhabiting the gastrointestinal tract and are widely used as dietary adjuncts owing to their long history of safe use. There is unequivocal evidence that administration of specific strains of probiotic bacteria can help in the maintenance of a balanced intestinal microbiota and therefore potentially promote overall host health (Butel 2014). Dairy products have been considered as a desirable delivery vehicle for delivering probiotic bacteria to the human gastrointestinal tract (Xiao et al. 2014). Hence, probiotic strains are most often incorporated in dairy-based food and beverages globally, including fermented milk, yogurt, ice cream, frozen yogurt, cheeses, pasteurized milk, and kefir. Nevertheless, non-dairy probiotic products are also increasingly being developed due to the increase in vegetarianism

as a dietary habit, lactose intolerance, and the cholesterol content of dairy products (Céspedes et al. 2013).

Among the many purported health benefits attributed to the administration of probiotics, the ability of probiotics to modulate the gut immune system has attracted more attention from the probiotic community. The intestinal microbiome plays an important role in the maintenance of the host immune homeostasis. It has been reported that probiotics may induce the production of intestinal defensin and immunoglobulin that inhibit the growth of pathogens, thereby maintaining a balanced intestinal microbiome (Thomas and Versalovic 2010). Probiotics have also been demonstrated to stimulate the production of cytokines, which can promote the activation of epithelial cells and both innate and adaptive immune cells, thus leading to the maintenance of a healthier intestinal microbiome and immune homeostasis (Hemarajata and Versalovic 2013). On the other hand, Furness et al. (1999) have mentioned that 70–80 % of the host's immune cells are located in the gut. Therefore, in addition to the local gut immunomodulatory effects, probiotics may also extend its immunomodulatory activity to the systemic levels. Increasing interest has been focused on proving the previously proposed hypothesis that probiotics may stimulate and/or regulate the immune system beyond the gut, such as on the gut-liver axis and the gut-brain-skin axis (Arck et al. 2010; Smith et al. 2014). Indeed, compelling evidence has demonstrated that live probiotics and/or probiotic-derived bioactives extend its beneficial roles by modulating the immune system beyond the gut (Bowe and Logan 2011; Saulnier et al. 2013). This chapter documents up-to-date in vitro and in vivo evidences of immunomodulatory effects of probiotics in health and non-gastrointestinal diseases and discusses their possible molecular and cellular mechanisms of action.

2 Probiotics and Immune Modulation

Innate and adaptive arms of the immune system work together to maintain the host immune homeostasis and to protect against external insults and undesirable inflammation (Yang and Polk 2011). However, imbalance of innate and adaptive immune responses can result in cell and tissue damage that further contribute to diseases. Experimental studies have proposed that probiotics can modulate humoral components and hematopoietic cells (natural killer cells, mast cells, neutrophils, macrophages, T cells, B cells, and dendritic cells) in both the innate (aspecific) and adaptive (specific) immune systems (Fig. 1), thereby promoting host defense.

Dong et al. (2012) have evaluated the immunomodulatory potential of live *Lactobacillus* and *Bifidobacterium* strains in human peripheral blood mononuclear cells in vitro. The authors found that *L. casei* Shirota, *L. rhamnosus* GG, *L. plantarum* NCIMB 8826, *L. reuteri* NCIMB 11951, *B. longum* SP 07/3, and *B. bifidum* MF 20/5 not only induced the expressions of $CD69^+$ on T lymphocytes, T helper (Th) cells, cytotoxic T (Tc) cells, and natural killer cells but also $CD25^+$ on T lymphocytes and NK cells, in a strain-dependent manner. These strains also

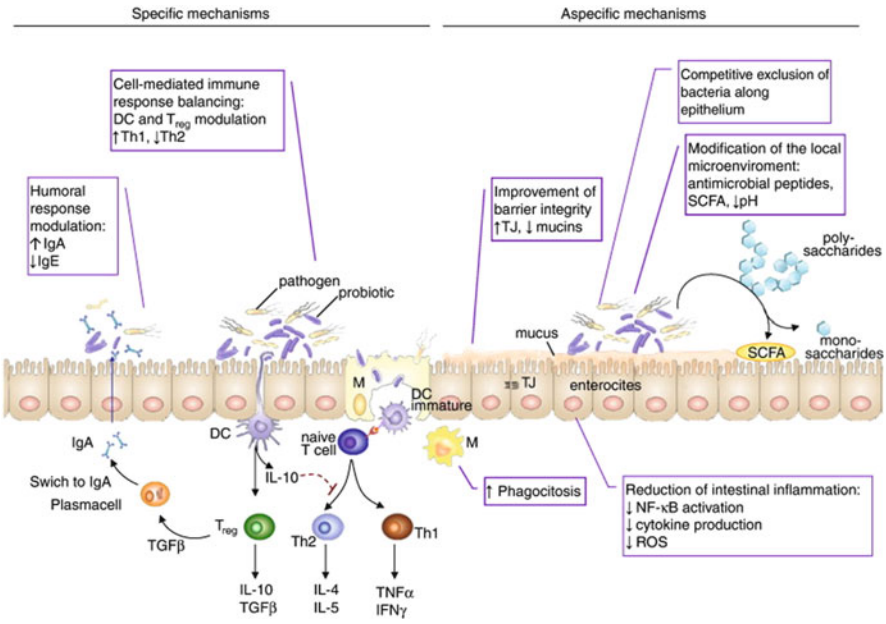

Fig. 1 Immunomodulatory actions of probiotics. Specific mechanisms: involvement of probiotics in cell-mediated and humoral immune responses. Aspecific mechanisms: enhancement of epithelial barrier function, competitive exclusion of bacteria along epithelium, modification of local microenvironment, and reduction of intestinal inflammation. Reprinted from Iacano et al. (2011) with permission from Elsevier (License number: 3537870510625)

increased the production of cytokines (IL-1ß, IL-6, IL-10, TNF-α), granulocyte-macrophage colony-stimulating factor, and macrophage inflammatory protein-1α (MIP-1α). It has also been reported that 16-month-old male Swiss mice orally administered with milk fermented with *L. fermentum* MTCC 5898 (3×10^9 CFU/day) for a period of 2 months showed significantly higher neutrophil phagocytosis and lower humoral antibodies (IgG_1/IgG_{2a} ratio and IgE levels) compared to the control, thereby demonstrating the potential of probiotics to improve immune functions in aging subjects (Sharma et al. 2014).

Macrophages serve as one of the immune sentinel cells with both protective and pathogenic roles in host defense (Galli et al. 2011). They express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domains (NODs) that recognize pathogen-associated molecular patterns (PAMPs) expressed by pathogens, which subsequently lead to the activation of a range of immune responses, including phagocytosis and cytokine production. It has been reported that peptidoglycans of *L. johnsonii* JCM 2012 and *L. plantarum* ATCC 14917 inhibited IL-12 production by macrophages that was induced by *L. casei* through PRRs such as TLR2 and NOD2, as well as suppressed IL-12p40 mRNA (Shida et al. 2009). The overproduction of IL-12, which subsequently results in the overstimulation of Th_1 cells, is considered as one of the main factors that contribute to autoimmune and inflammatory diseases. In addition, IL-12p40 is a subunit of IL-23

that regulates the proliferation and maintenance of Th_{17} cells, which plays a major role in the development of inflammatory diseases. Hence, findings in this study suggest that the ability of peptidoglycans to downregulate the production of IL-12 and IL-23 may be useful for the treatment of inflammatory diseases. On the other hand, cell wall extracts of *B. adolescentis* BBMN23, *B. longum* BBMN68, and *L. saliva* Ren were shown to increase phagocytic activity and production of nitric oxide, IL-6, and TNF-α, thereby enhancing the overall activities of macrophage RAW264.7 cells (Zhu et al. 2011). Oral administration of dahi containing *L. acidophilus* LaVK2 ($2–20 \times 10^7$ CFU) and *B. bifidum* BbVK3($2–20 \times 10^7$ CFU/g) for 4 months was also found to improve production of reactive oxygen intermediates (ROI), phagocytic activity, and adherence indices of peritoneal macrophages in aged mice, thus potentially improving immune functions in aging individuals (Kaushal and Kansal 2014).

Probiotics also modulate the function of mast cells, which specialize in stimulating or suppressing homeostatic or pathophysiological responses. Mast cells are well known for their role in allergic inflammation due to the ability of allergen-specific IgE to activate high-affinity IgE receptor (FcεR1) on the cell surface, which leads to the release of mediators and cytokines (Forsythe et al. 2012). Using global microarray analysis (Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array), Oksaharju et al. (2011) have found that *L. rhamnosus* LGG and Lc705 downregulated genes encoding mast cell activation and mediator release [high-affinity IgE receptor subunits α (*Fcer1a*) and γ (*Fcer1g*) and histamine H4 receptor (*Hrh4*)] and immune responses (IL-8, TNF-α, CCL2, and IL-10) and thus could be applied in the cases of allergy mediated by mast cell activation. Oral administration of *L. rhamnosus* JB-1 (1×10^9 CFU/daily) for 9 days in healthy male Sprague-Dawley rats have also revealed that this probiotic strain systemically inhibited mast cell membrane potassium current and degranulation (enhanced mast cell stabilization) by inhibiting ß-hexosaminidase (mast cell mediator release) in response to the IgE-mediated activation, thus reducing passive cutaneous anaphylaxis and peritoneal mast cell responses to the KCa3.1 opener, a potent therapeutic target in a wide range of autoimmune diseases (Forsythe et al. 2012).

Dendritic cells are critical antigen-presenting cells for naïve T cells and generation of the T cell response. In vitro studies have demonstrated that *L. rhamnosus* Lcr35 can modulate the immunological functions of human dendritic cells. High dose of *L. rhamnosus* Lcr35 (MOI, 100) has been shown to upregulate nearly 1700 genes mainly involved in the immune response, with threefold changes (Evrard et al. 2011). Flow cytometry and ELISA analyses have also further revealed that *L. rhamnosus* Lcr35 upregulated the maturation of dendritic cell membrane phenotypes (CD86, CD83, HLA-DR, and TLR4) and increased the pro-Th_1/Th_{17} cytokine levels in a dose-dependent manner. Despite pro-Th_1/Th_{17} cytokines mediating inflammation and autoimmune diseases, in vivo studies have proposed that upregulation of Th_1/Th_{17} responses could alleviate certain inflammation or allergic diseases (Lin et al. 2009). IgE-dependent allergies are diseases characterized by imbalance of Th_1/Th_2 responses. Yu et al. (2010) have demonstrated that *L. rhamnosus* Lcr35 is capable of regulating the impaired Th_1/Th_2 cytokine profile

by modulating the Th_1/Th_2 responses toward the Th_1 ($/Th_{17}$) balance in dendritic cells. These findings suggest that *L. rhamnosus* Lcr35-treated dendritic cells have a higher potential of antigen presentation and co-stimulation and therefore may induce immune responses as required during inflammation and/or allergies. Till date, this is the first report of probiotics found to have a pro-Th_1/Th_{17} effect on dendritic cell maturation that merits further investigation. On the other hand, You et al. (2014) have conducted a study to evaluate the effect of *B. longum* bv. *infantis* CCUG 52486, *B. longum* SP 07/3, *L. rhamnosus* GG, and *L. casei* Shirota on dendritic cell function in an allogeneic mixed leukocyte reaction model, using peripheral blood obtained from healthy young (20–30 years) and old (55–75 years) subjects. The authors found that these strains increased the expression of CD40, CD80, and CCR7 in both young and old dendritic cells and only enhanced cytokine production (TNF-α and TGF-ß) in old dendritic cells. Additionally, probiotic treatment was also found to stimulate the activation of young T cells activation by old dendritic cells, suggesting that probiotics may be able to modulate dendritic cell function in aging individuals.

Since probiotics are mainly used to maintain healthy intestines, a large proportion of research attention has focused on exploring the mechanisms of intestinal immunomodulation by probiotics. Probiotics have been found to exhibit their immunomodulatory effects in a strain-dependent and cell subset-specific manner. Suda et al. (2014) have evaluated the potential of *L. jensenii* TL2937 in modulating the immune response in cocultures of porcine intestinal epithelial cells and antigen-presenting cells from porcine Peyer's patches. The authors found that *L. jensenii* TL2937 not only stimulated the expression of IL-10, MHC class II, Bcl-3, and CD80/CD86 in $CD172a^+CD11R1^-$ and $CD172a^+CD11R1^{high-}$ antigen-presenting cells but also suppressed TLR4 activation and subsequent inflammatory response by upregulating negative regulators (MKP1, Bcl3, and A20) of TLR4 in porcine intestinal epithelial cells. Smelt et al. (2013) have also demonstrated that specific probiotic strains exhibited specific immune responses in specific sites of the intestine in vivo. Although *L. plantarum* WCFS1, *L. lactis* MG1363, and *L. salivarius* UC118 increased the numbers of $CD11c^+$MHC class II^+ dendritic cells in the mice Peyer's patches, *L. salivarius* UCC118 was the only strain which exhibited immunoregulatory effects by decreasing the number of effector T cells and increasing the number of regulatory T cells (Treg cells; $CD4^+CD25^+Foxp3^+$ T cells) in the small intestine lamina propria. IgA is an important intestinal humoral component, which is secreted from B cells in gut-associated lymphoid tissues (Mora et al. 2006). Sakai et al. (2014) have revealed that oral administration of diet containing *L. gasseri* SBT 2055 (1.0×10^9 CFU/g/daily) for 5 weeks activated the TLR2 signaling pathway and increased the rate of IgA^+ cells and IgA production in both the lamina propria and Peyer's patch of the small intestine in healthy mice, as well as stimulated the expression of TGF-β in bone marrow-derived dendritic cells. These findings suggest that *L. gasseri* SBT 2055 could enhance host immune responses and protect against intestinal inflammation. In addition to animal studies, a number of clinical trials have also been conducted to elucidate the immunomodulatory actions and to evaluate the efficacy of probiotics in healthy populations (Table 1).

Table 1 Clinical evidences on the immunomodulatory activity of probiotic strains in healthy populations

Treatment	Probiotic strain	Subject	Dose and duration (intervention)	Immune response elicited	References
Enhancement of humoral responses in preschool children	*L. plantarum* IS-10506	12 healthy Indonesian children (aged 12–24 months)	Diet containing *L. plantarum* IS-10506 at a dose of 2.3×10^{10} CFU/g; daily for 90 days (randomized, double-blind, placebo-controlled, pre-post trial)	– Fecal sIgA excretion ↑	Surono et al. (2014)
Enhancement of immunomodulatory factors in breast milk	*B. lactis* HN019 *L. rhamnosus* HN 001	69 pregnant women; *L. rhamnosus* HN001 ($n = 34$) and *B. lactis* HN019 ($n = 35$)	Capsule powder containing *B. lactis* HN019 at a dose of 9×10^9 CFU/capsule or *L. rhamnosus* HN001 at a dose of 6×10^9 CFU/capsule; daily for 2–5 weeks before delivery and 6 months in lactation period	– Cord blood IFN-γ levels ↑ – Detectable blood IFN-γ levels↑ – TGF-β1 levels↑ in early breast milk (week 1) – IgA ↑ in breast milk	Prescott et al. (2008)
Maintenance of intestinal immune homeostasis in adult	*L. rhamnosus* CNCMI-4036	20 healthy volunteers enrolled in three cities in Spain (average age of 28 years)	Capsule containing *L. rhamnosus* CNCM I-4036 at a dose of 9×10^9 CFU; daily for 30 days (multicenter, randomized, double-blind, placebo-controlled trial)	– IL-4 ↑ – IL-10↑ – IL-12 ↓ – IL-10/IL-12 ↑ – TNF-α/IL-10 ↓	Plaza-diaz et al. (2013)
Modulation of anti-inflammatory cytokine profile in elderly	*L. casei* Shirota	Sixteen healthy volunteers, 10 females and 6 males (aged 55–74 years)	Probiotic drink containing *L. casei* Shirota at a dose of 1.3×10^{10} CFU; daily for 4 weeks (randomized placebo-controlled, single-blind cross-over trial)	– NK cell activity ↑ – CD25 T cells ↓ – IL-10/ IL-12 ↑	Dong et al. (2013)
Modulation of pro- and anti-inflammatory	*L. rhamnosus* GG ATCC 53103	Fifteen elderly volunteers (aged 66–80 years)	Capsule containing *L. rhamnosus* GG ATCC 53103 at a dose of 1×10^{10} CFU/	– IL-8 ↓, and returning to baseline 1 month after discontinuation	Hibberd et al. (2014)

(continued)

Table 1 (continued)

Treatment	Probiotic strain	Subject	Dose and duration (intervention)	Immune response elicited	References
cytokine profile in elderly			capsule; twice daily for 28 days and followed through day 56 (Phase 1 open-label clinical trial)	− No significant effects on IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, TNF-α, and IL-6	
Modulation of NK cell activity in healthy men	*L. casei* Shirota	35 healthy male Caucasian nonsmokers (aged 18–60 years)	Probiotic drink containing *L. casei* Shirota at a dose of 1.95×10^{10} CFU; daily for 4 weeks	− No significant effects on NK cell numbers, cytokine profile, and peripheral blood mononuclear cells	Seifert et al. (2011)

3 Non-gastrointestinal Diseases Modulated by Probiotics

The potential immunomodulatory roles of probiotics in non-gastrointestinal diseases are still emerging and might offer promising novel applications in the near future. Results from preliminary studies have proposed that probiotics may modulate immune responses in diseases such as metabolic syndrome, respiratory diseases, allergic and inflammatory skin disorders, osteoporosis, rheumatoid arthritis, and male hypogonadism, which will be further discussed in later sections (3.1–3.6). Although immunomodulatory effects of probiotics represent a new avenue for discovering the potential use of probiotics in non-gastrointestinal health, a number of clinical trials have already been performed. However, more clinical studies are needed in order to establish the immunomodulatory role of probiotics in the treatment of non-gastrointestinal diseases.

3.1 Metabolic Syndrome

Metabolic syndrome is a constellation of multiplex factors, such as abdominal obesity, hyperlipidemia, hypertension, and nonalcoholic fatty liver disease that raises the risk of type 2 diabetes mellitus and cardiovascular diseases (Okubo et al. 2014; Fig. 2). It has been estimated that 25 % of the global population is suffering from metabolic syndrome (Shivakumar et al. 2014). Accumulating

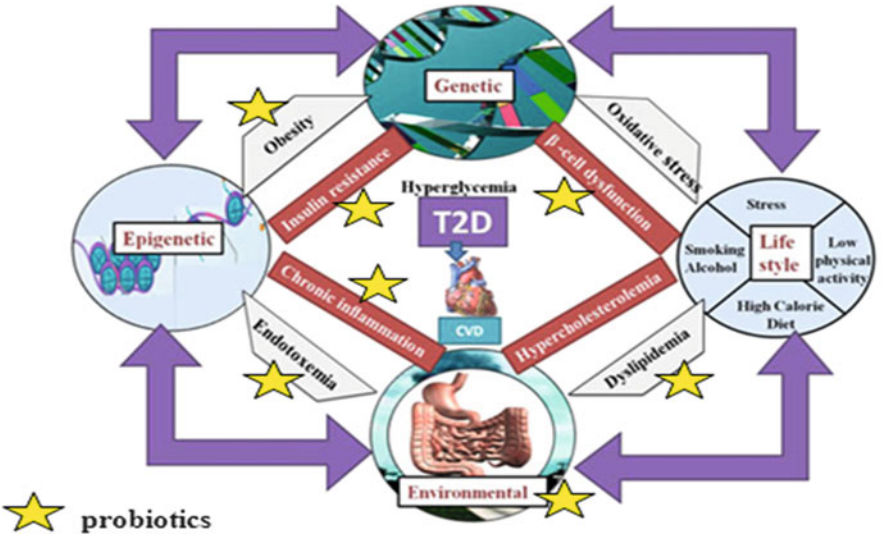

Fig. 2 Probable immunological actions of probiotics to reduce the risk factors that contribute to type 2 diabetes mellitus and cardiovascular diseases. Reprinted from Panwar et al. (2013) with permission from John Wiley and Sons (License number: 3522340228488)

evidence has shown that altered gut microbiota mediated low-grade inflammation which contributed to the onset of metabolic syndrome and related diseases, via mechanisms responsible for intestinal barrier dysfunction (Cani et al. 2012; Zhang et al. 2014). Thus, the administration of probiotics appears to be a promising approach to treat metabolic syndrome owing to their ability to modulate gut microbiota and to restore impaired intestinal barrier function. Indeed, to date, probiotic approaches that target the gut microbiota have shown the most positive results in the prevention and treatment of abdominal obesity, hypertension, hyperlipidemia, and nonalcoholic fatty liver disease via immune-mediated mechanisms. Preliminary findings have shown that probiotics are capable of stimulating and regulating local and systemic immune responses in a diet-induced metabolic syndrome murine model. However, their potential warrants further investigation and clinical trials.

3.1.1 Abdominal Obesity

An increased high-fat diet has been linked to the development of abdominal obesity and increased risk of type 2 diabetes mellitus (Kaur 2014). Recently, Núñez et al. (2014) have conducted a study evaluating the effect of *L. casei* CRL 431 on intestinal and humoral responses using a high-fat-induced mouse model. The mice were orally administered a balanced diet or high-fat diet supplemented with milk, fermented milk containing *L. casei* CRL 431 ($8 \pm 2 \times 10^8$ CFU/mL/daily), water suspension containing *L. casei* CRL 431 ($2 \pm 1 \times 10^8$ CFU/mL/daily), or water for 60 days. The authors found that while both the administration of fermented milk or water suspension containing *L. casei* CRL 431 enhanced the phagocytic activity of macrophages, only fermented milk containing *L. casei* CRL 431 increased the number of macrophages and IgA^+ cells in the small intestine. These results indicate that fermented milk containing *L. casei* CRL 431 can attenuate high-fat-diet-induced obesity by modulating mucosal immunity altered by obesity.

TNF-α is known as a cytokine that can stimulate the apoptosis of adipocytes and inhibit the synthesis of fatty acid synthase (Cawthorn and Sethi 2008). It has been reported that oral administration of *L. rhamnosus* PL 60 (1×10^7 or 1×10^9 CFU/mouse; daily) for 8 weeks significantly downregulated both TNF-α and fatty acid synthase mRNA expression levels in epididymal white adipose tissues, leading to an antiobesity effect in high-fat-diet-induced mice (Lee et al. 2006). Meanwhile, Park et al. (2013a, b) have also demonstrated that the mRNA expression of TNF-α, IL-1β, IL-6, and MCP1 (pro-inflammatory genes) in adipose tissues was downregulated in high-fat-diet-induced mice upon receiving *L. curvatus* HY 7601 (5×10^9 CFU/day) or *L. plantarum* KY 032 (5×10^9 CFU/day) treatment for 8 weeks. Recently, it has also been reported that oral administration of *L. gasseri* SBT 2055 (5×10^8 CFU/g; daily) for 24 weeks downregulated mRNA expression of CCL2 and CCR2 (pro-inflammatory genes) in epididymal adipose tissues of high-fat-induced mice (Miyoshi et al. 2014). These findings collectively suggest

that probiotic strains may also attenuate inflammation of adipose tissues in high-fat-induced obesity by suppressing pro-inflammatory immune responses (TNF-α, IL-1β, IL-6, MCP1, CCL2, and CCR2). On the other hand, Yoo et al. (2013) have conducted an animal study involving 50 C57BL/6J mice that were fed with high-fat high-cholesterol diet (HFCD) supplemented with *L. plantarum* KY 1032 (10^{10} CFU/day), HFCD supplemented with *L. curvatus* HY 7601 (10^{10} CFU/day), or HFCD supplemented with *L. plantarum* KY 1032 and *L. curvatus* HY 7601 (10^{10} CFU/day) for 9 weeks. The authors found that only mice orally administered with *L. plantarum* KY 1032 were capable of suppressing plasma TNF-α and IL-1β (pro-inflammatory cytokine), indicating that probiotics may reduce the incidence of systemic low-grade inflammation in high-fat-diet-induced metabolic syndrome.

A reduction in the number of adipose tissue macrophage and macrophage infiltration has been shown to attenuate adipose inflammation and other obesity complications (Cani et al. 2008). Recently, Wang et al. (2015) have conducted an animal study involving 40 male high-fat diet-induced C57BL/6J mice fed with *L. paracasei* CNLM I-4270, *L. rhamnosus* I-3690, or *B. animalis* subsp. *lactis* I-2494 (10^8 CFU/day) for 12 weeks. The authors found that all three probiotic strains significantly reduced the number of adipose tissue macrophages and infiltration of pro-inflammatory macrophages ($CD11c^+$ and $MMP\text{-}12^+$) and may therefore attenuate inflammation in adipose tissues.

In addition to abdominal obesity, several studies have also determined the immune modulating potential of probiotics in the prevention of type 2 diabetes mellitus using an obesity-induced diabetes murine model. Inflammatory cytokines, such as TNF-α, IL-6, and IL-8, can cause insulin resistance and inflammation and consequently onset type 2 diabetes mellitus (King 2008). Hence, suppression of these inflammatory cytokines is a promising therapeutic approach to combat type 2 diabetes mellitus. Interestingly, several studies have demonstrated that probiotics exhibit antidiabetic effects by suppressing Th_1 immune responses. Amar et al. (2011) have found that high-fat-diet-induced diabetic mice orally treated with *B. lactis* 420 (10^9 CFU/day) downregulated inflammatory cytokine (TNF-α, IL-1β, IL-6, and PAI-1) mRNA in mesenteric adipose tissues, the muscle, and the liver, thereby resulting in improved insulin sensitivity and overall inflammatory status. Another recent study also showed that oral administration of *L. casei* (4×10^9 CFU/day) inhibited the development of type 2 diabetes mellitus by suppressing plasma Th_1-associated pro-inflammatory cytokines (IFN-γ and TNF-α) and downregulating Th_1 responses related to *Tbet* gene mRNA levels in high-fat high-sucrose diet-induced pre-insulin-resistant rats (Zhang et al. 2014). Recently, a randomized, double-blind, controlled clinical trial was conducted on 44 patients (22 for placebo group and 22 for intervention group), BMI $\geq$ 25, over a period of 8 weeks to evaluate the effect of probiotic yogurt (3.7×10^6 CFU/g of *L. acidophilus* A5 and *B. lactis* BB12) consumption (300 g/day) on inflammatory biomarkers in patients with type 2 diabetes mellitus. The consumption of probiotic yogurt in the intervention group was found to reduce plasma TNF-α, thus ameliorating the onset of type 2 diabetes (Mohamadshahi et al. 2014).

On the other hand, probiotics can also improve type 2 diabetes by stimulating Th_2 immune responses. Chen et al. (2014) have reported that oral administration of *L. rhamnosus* CCFM 0528 (10^9 CFU/day) for 12 weeks upregulated IL-4 and IL-10 (Th_2 immune responses) expression and downregulated IL-6, IL-8, and TNF-α (Th_1 immune responses) expression in the spleen, leading to improved glucose tolerance in high-fat-diet, streptozotocin-induced type 2 diabetic mice. Dendritic cells from nonobese diabetic mice have been shown to produce elevated levels of IL-12 that activate IFN-γ-producing T cells which can promote the development of diabetes (Trembleau et al. 1995). Meanwhile, nonobese diabetic dendritic cells stimulated with *L. casei* enhanced IL-10 over IL-12 in vitro, and their transfer reduced the incidence of diabetes in vivo, thus proposing another potential immunological role of probiotics for the treatment of type 2 diabetes mellitus (Manirarora et al. 2011).

3.1.2 Hypertension

Hypertension is one of the central metabolic syndromes that is increasing worldwide at an alarming rate. It has been estimated that the number of adult with hypertension will reach a total of 1.56 billion in the year 2025 (Kearney et al. 2005). Although the exact mechanisms underlying the immunomodulatory potentials of probiotics for hypertension still remain poorly understood, findings in animal studies have proposed that probiotics may attenuate pathogenesis in hypertension by targeting immune responses in the kidney and aorta.

Angiotensin type 1 receptors (AT_1R) specifically activate immune and non-hematopoietic cells to promote pathogenesis in angiotensin II-dependent hypertension mice (Ryan 2013). A mutational analysis has revealed that $CD3^+$ T cells and renal macrophages were increased in bone marrow-specific AT_1R knockout mice associated with exaggerated hypertensive responses (Crowley et al. 2010). Mycophenolate mofetil is a drug that is used to improve hypertension (Herrera et al. 2006). It has been demonstrated that high-protein-diet-induced hypertension in Dahl salt-sensitive rats treated with mycophenolate mofetil reduced renal cortical T cell infiltration, thereby attenuating hypertension (De Miguel et al. 2011). Additionally, Barhoumi et al. (2011) have demonstrated that angiotensin II-induced hypertension is associated with the reduction of Treg cells in the renal cortex, and adoptive transfer to increase Treg cells suppressed TNF-α expression. On the other hand, inhibition of IL-6 has also been shown to downregulate IL-6 expression, T cell, and macrophage cell infiltration in cold-induced hypertension (Crosswhite and Sun 2010). Nonetheless, Chan et al. (2012) have also further revealed that deoxycorticosterone acetate-salt-induced hypertension was accompanied with an increase in the number of macrophage and expression levels of chemokine (CCR2, CC12, CCL7, and CC8) in the aorta. Altogether, these results propose that probiotics may attenuate hypertension by modulating immune cells (T cells, macrophages, Treg cells) and pro-inflammatory cytokines (TNF-α and IL-6) in the kidney, as well as chemokines (CCR2, CCL12, CCL7, and CC8) in the aorta.

3.1.3 Hyperlipidemia

Hyperlipidemia is the term used to describe the increased concentration of any or all lipids in the plasma (Stocks et al. 2005). Elevated very-low-density lipoproteins in the plasma can contribute to the risk of atherosclerosis. Apolipoprotein E-deficient mice ($ApoE^{-/-}$) are characterized by elevated very-low-density lipoproteins and are widely used as murine models of atherosclerosis, steatosis, and hyperlipidemia (King et al. 2010). Interestingly, Mencarelli et al. (2012) have used this model to evaluate the potential of a probiotic cocktail (VSL#3) consisting of eight probiotic strains (*L. acidophilus* MB 443, *L. delbrueckii* subsp. *bulgaricus* MB 453, *L. casei* MB 451, *L. plantarum* MB 452, *B. longum* Y10, B. *infantis* Y1, *B. breve* Y8, and *Streptococcus salivarius* subsp. *thermophilus* MB 455) in protecting against low-grade intestinal inflammation in the development of arthrosclerosis. The $ApoE^{-/-}$ mice were orally administered with VSL#3 (20×10^9 CFU/kg/day) or 0.2 % dextran sulfate sodium (DSS) filtered drinking water containing VSL#3 (20×10^9 CFU/kg/day) six days a week for 12 weeks. The authors found that VSL#3 downregulated the expression of TNF-α and RANTES (inflammatory mediators) in both the colonic mucosa and mesenteric adipose tissues of $ApoE^{-/-}$ mice challenged with DSS. VSL#3 treatment was also shown to stimulate the expression of nuclear receptors (PPAR-γ and VDR) of positive regulators in the intestine and to increase IL-10 (anti-inflammatory cytokine) production from $CD5^+$T cells in the spleen. Another set of results in this study also highlighted that the administration of VSL#3 downregulated the expression of inflammatory mediators (ICAM-1, VCAM, and RANTES) in the aorta, as well as decreased the percentage of $CD35^+$ cells in circulating macrophages of $ApoE^{-/-}$ mice challenged with DSS, thereby preventing the generation of new plaques in the thoracoabdominal aortas and inhibiting the extension of atherosclerotic plaques (Fig. 3). Altogether, these results demonstrate that VSL#3 can potentially protect subjects with low-grade inflammation of hyperlipidemia against atherosclerosis by decreasing local and systemic inflammatory immune responses (aorta, intestine, adipose tissue, and plasma) while stimulating the expression of nuclear receptors of positive regulators and IL-10-producing T lymphocytes.

3.1.4 Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease is the common cause of chronic liver disease worldwide that increases with the prevalence of obesity, dyslipidemia, and insulin resistance (Kneeman 2012). Numerous studies have highlighted a causative relationship between liver (local) and systemic inflammation and nonalcoholic fatty liver disease. The invariant natural killer T (*i*NKT) cells are hepatic lymphocytes that play a role in the pathogenesis of nonalcoholic fatty liver disease (steatosis and nonalcoholic steatohepatitis). Although the exact mechanisms of NKT cells in liver diseases remain unclear, *i*NKT cells are capable of balancing the production of

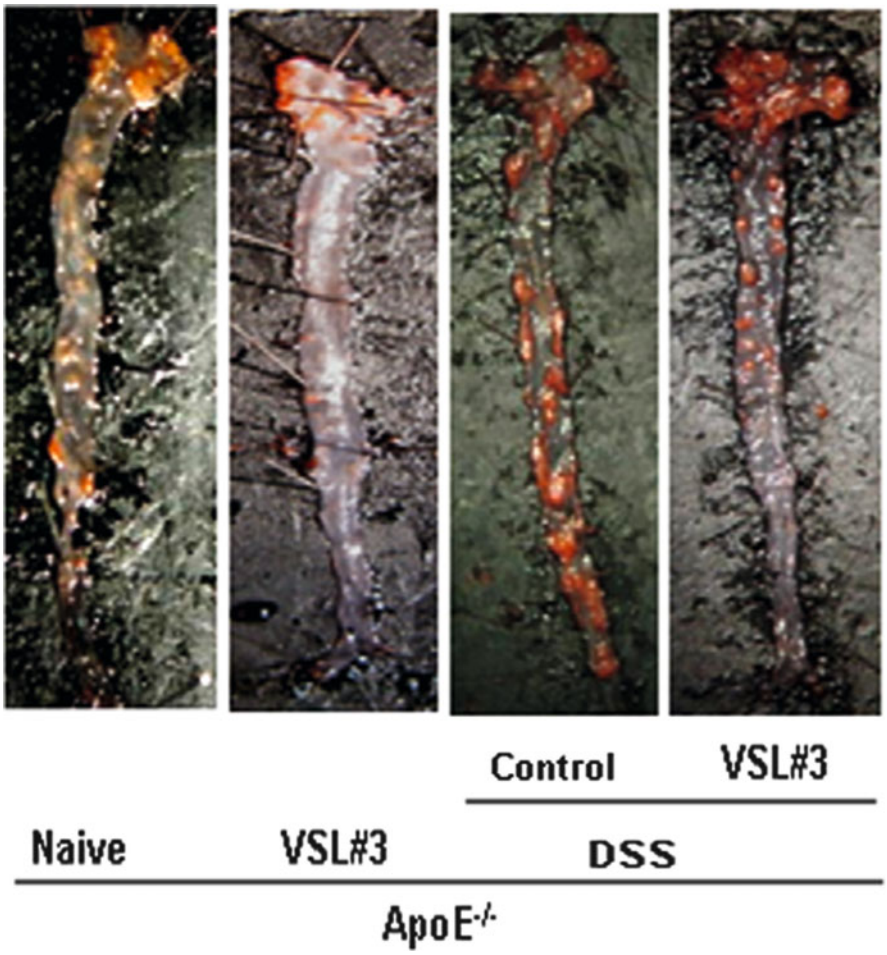

Fig. 3 Effect of VSL# 3 administration on aortic plaques in ApoE$^{-/-}$ mice. The lipids in the vessel wall were stained with Sudan IV. ApoE$^{-/-}$mice administered with VSL#3 for 12 weeks decreased the number of new plaques in thoracoabdominal aorta and inhibited the extension of atherosclerotic plaques. Reprinted from Mencarelli et al. (2012); free access from PLOS

pro-inflammatory and anti-inflammatory cytokines (Seki et al. 2000). On the other hand, it has been reported that chronic liver inflammation is mediated by the Iκk-β/NF-κB signaling pathway, where increased NF-κB activity was associated with upregulated expression of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the liver of high-fat-diet-induced steatosis mouse model (Cai et al. 2005). Therefore, *i*NKT cells and Iκk-β/NF-κB signaling pathway could be promising targets in treating nonalcoholic fatty liver damage and inflammation.

Ma et al. (2008) have evaluated the potential of VSL#3 (a mixture of lyophilized *Bifidobacterium*, *Lactobacillus*, and *Streptococcus thermophilus*) to improve

high-fat-diet-induced hepatic steatosis mice. The authors found that oral administration of VSL#3 (1.5×10^9 CFU/day) daily for 4 weeks significantly improved high-fat-diet-induced steatosis by stimulating IL-4 that contributes to an improvement in hepatic NKT cell depletion. Elevated TNF-α expression was found to activate Iκk-β activity (represented by ratio of phosphor-IKB-α/total IKB-α) in high-fat-induced hepatic steatosis mice. Nevertheless, VSL#3 treatment and adoptive transfer of NKT cells suppressed TNF-α expression associated with reduced Iκκ-β activity and downstream signal for IKB-α (NF-KB p65 activity). Similarly, Liang et al. (2013) have also found that high-fat-diet mice orally administered with lipid extracts of VSL#3 for 4 weeks stimulated hepatic *i*NKT cells, suggesting that probiotic strains in VSL#3 may contain glycolipid antigens that directly confer effector functions to hepatic *i*NKT cells. Matrix metalloproteinases (MMPs) play an important role in the pathogenesis of liver diseases (Kurzepa et al. 2014). According to Esposito et al. (2009), VSL#3 treatment also suppressed the hepatic gelatinase activity of MMP-2 and MMP-9, thereby inhibiting liver inflammatory damage in rats receiving high-fat diet.

Several clinical studies have also been performed to evaluate the potential of probiotics for the treatment of nonalcoholic fatty liver disease, ranging from children to adult populations. Malaguarnera et al. (2012) have conducted a double-blind and randomized study to determine the effects of oral administration of *B. longum* with fructo-oligosaccharides in 66 patients (aged of 30–65 years) with abnormal hepatic steatosis. The authors found that patients who received *B. longum* with fructo-oligosaccharides and lifestyle modification (diet and exercise) had significantly decreased serum TNF-α and nonalcoholic steatohepatitis activity index compared to patients who received lifestyle modification alone. Furthermore, Eslamparast et al. (2014) have evaluated the effects of synbiotic capsules (Protexin) containing 2×10^8 CFU of seven probiotic strains (*L. casei*, *L. rhamnosus*, *S. thermophilus*, *B. breve*, *L. acidophilus*, *B. longum*, and *L. bulgaricus*) in a 28-week randomized, double-blind, placebo-controlled trial involving 52 patients with nonalcoholic fatty liver diseases. Synbiotic intervention significantly decreased TNF-α in plasma and NF-KB p65 in peripheral blood mononuclear cells. However, Vajro et al. (2011) reported that consumption of *L. rhamnosus* GG (12×10^9 CFU/day) for 8 weeks showed no significant effect on TNF-α production in twenty obese children (aged of 10.7 ± 2.1 years) with persisting hypertransaminasemia and ultrasonographic bright liver. Altogether, these findings warrant further investigation.

3.2 Respiratory Diseases

Respiratory diseases are a major leading cause of mortality and morbidity throughout the world, while respiratory infections have been reported to account for over four million deaths per year in low- or middle-income countries (World Health Organization 2008; Zar and Ferkol 2014). Thus, effective strategies are needed to

Table 2 Clinical trials on the immunological potential of probiotics for the treatment of respiratory diseases

Treatment	Probiotic strain	Subject	Dose and duration (intervention)	Immune response elicited	References
Prevention of asthma	*L. rhamnosus* GG ATCC 53103	131 children (aged 6–24 months old) with at least two wheezing episodes and family history of atopic disease	*L. rhamnosus* LGG (10^{10} CFU/day; 6 months) (prospective double-blind, randomized study)	– No significant effect on IgE levels	Wickens et al. (2008)
Breathing illness	*L. fermentum* VRI-003	20 healthy elite male distance runners	Freeze-dried powder in gelatin capsules containing *L. fermentum*-VRI-003 (1.2×10^{10} CFU; twice a day; 72 days)	– No significant effect on IgA, IgA1, IL-4, and IL-12 levels – Plasma IFN-γ↑	Cox et al. (2010)
Prevention of influenza	*L. fermentum* CECT 5716	50 volunteers (aged 22–56 years)	Capsule containing *L. fermentum* CECT5716 (1×10^{10} CFU/day; 28 days), 2 weeks before and after vaccination, respectively	– No significant effect on IFN-γ, IL-10 – Specific anti-influenza IgA ↑	Olivares et al. (2007)

properly tackle the burden of respiratory diseases. In brief, there are a growing number of in vitro and animal studies suggesting that probiotics may modulate pulmonary immune responses, thereby attenuating respiratory diseases, in particular asthma and influenza viral infections. However, clinical trials addressing the immunomodulatory potentials of probiotics in subjects with asthma and influenza are still very limited (Table 2).

3.2.1 Asthma

Asthma is a chronic inflammatory disease of the airways, with an estimated 300 million people of all ages and all ethnic backgrounds worldwide suffering from asthma (Masoli et al. 2004). Several studies have highlighted that Th_2 lymphocytes, Th_{17} cells, as well as their cytokines (IL-4, IL-5, IL-12, IL-17A,

IL-17F, and IL-23) are key inflammatory mediators that play critical roles in orchestrating the asthma pathogenesis (Li and Hua 2014). Thus, targeting these components may be a promising immunotherapeutic approach for the treatment of asthma. Interestingly, Jan et al. (2011) have demonstrated that oral administration of *L. gasseri* A5 (4×10^6 CFU/daily) starting from 2 weeks before sensitization for 4 weeks not only significantly decreased IL-17A production but also reduced TNF-α production and activation-regulated chemokines in bronchoalveolar lavage fluids of *Dermatophagoides pteronyssinus*-sensitized and *Dermatophagoides pteronyssinus*-challenged mice. Furthermore, the number of IL-17-producing alveolar macrophages was also decreased in *L. gasseri* A5-treated mice compared to untreated mice, suggesting that *L. gasseri* A5 treatment is capable of downregulating Th_{17} pro-inflammatory responses and improving asthma.

In addition to immunosuppressive effects on Th_{17} pro-inflammatory responses, probiotics (*L. rhamnosus* Lcr35, *L. rhamnosus* GG, *B. lactis* BB12, and *L. reuteri* strains) may attenuate asthmatic responses via a Treg cell-mediated mechanism (Jang et al. 2012; Karimi et al. 2009; Feleszko et al. 2007). The daily oral administration of *L. rhamnosus* Lcr35 at a dose of 1×10^9 CFU/mL for 7 days before the first sensitization was found to increase the percentage of Treg cells in the mouse spleen and also decrease Th_2 (IL-4, IL-13, and IL-5) and Th_1 (IFN-γ) cytokines in the mouse serum (Jang et al. 2012). Administration of anti-CD25 monoclonal antibody was also shown to inhibit the protective effects of *L. rhamnosus* Lcr35, confirming that Treg cells are essential targets in mediating the immune response of *L. rhamnosus* Lcr35 in asthma. Additionally, oral administration of *L. rhamnosus* Lcr35 (1×10^9 CFU/mL/daily; 1 week) prior to sensitization was also found to significantly decrease ovalbumin-specific IgE production in ovalbumin-induced murine model of asthma, to suppress Th_2 responses (CCL11, Th2-related chemokines) and to increase Treg cells following the adoptive transfer of *L. rhamnosus* Lcr35-treated dendritic cells (Yu et al. 2010; Kim et al. 2013). These studies collectively suggest that *L. rhamnosus* Lcr35 can stimulate dendritic cell activation and suppress the Th_2 responses by upregulating Treg cells in the murine model of asthma. However, the efficacy of *L. rhamnosus* Lcr35 in subjects with asthma still remains unclear. Similar to *L. rhamnosus* Lcr35, *L. reuteri* strains have also been shown to increase the number of Treg cells in the spleen and to suppress Th_2 responses (TNF, MCP-1/CCL2, IL-5) in an ovalbumin-induced murine model (Karimi et al. 2009).

3.2.2 Influenza

Influenza is a respiratory viral infectious disease caused by RNA viruses, belonging to the family of *Orthomyxoviridae* (influenza viruses). In vivo studies have proposed that probiotics may attenuate influenza virus infections by stimulating NK cells, which are major cellular components of the innate immunity that can recognize and control a broad spectrum of pathogens, including viruses. Intranasal administration of heat-killed *L. pentosus* S-PT84 (200 μg/day) for 3 days

significantly increased IFN-γ and IL-12 (Th1 antiviral cytokines) production in mediastinal lymph node cells and bronchoalveolar lavage fluids, subsequently stimulating NK cell activity in the lung of mice infected with influenza virus PR8 (mouse-adapted H1N1 strain) (Izumo et al. 2010). A similar conclusion has also been drawn in another study by Harata et al. (2010), where daily intranasal administration of *L. rhamnosus* GG (200 μg/day) for three consecutive days upregulated mRNA expression of IL-1β, monocyte chemotactic protein (MCP), and TNF-α following activation of lung NK cells in mice infected with influenza virus A (H1N1). On the other hand, Park et al. (2013a, b) have also demonstrated that intranasal (10^9 CFU/mouse) or oral (10^9 CFU/daily for 10 days) administration of *L. plantarum* DK119 2055 can protect mice from influenza A/PR8 virus infection by increasing $CD11c^+$ dendritic and macrophage cells and stimulating IFN-γ and IL-12 production. Altogether, these studies suggest that probiotics most probably exhibit their antiviral effects against influenza virus A by stimulating macrophage- and dendritic cell-mediated immune responses and NK cell activation.

3.3 Allergic and Inflammatory Skin Disorders

Contemporary studies have focused on the possible deployment of probiotics for alleviating allergic and inflammatory skin disorders due to their ability to balance intestinal microbiota, which ameliorates the immune system at both the local and systemic levels. Preliminary data suggest that probiotics could produce dermal bioactives such as bacteriocins and may thereby enhance the skin immune defense (Tan et al. 2014). A bacteriocin, CBT-SL5 produced from *E. faecalis* at a concentration of 100 ng/mL, was found to suppress *P. acnes*-induced $NF\text{-}_KB$ translocation, mRNA expression, and protein production of IL-8 in vitro, highlighting its potential as a topical agent for acne vulgaris (Jin et al. 2008). On the other hand, Pinto et al. (2011) have also demonstrated that plantaricin A, synthesized by *L. plantarum*, significantly induced proliferation and migration of human keratinocytes and increased expression of TGF-β1, VEGF-A, and IL-8, indicating a function in accelerating wound healing (Pinto et al. 2011). At a low concentration (10 μg/mL), plantaricin A was also found to increase antioxidant defenses of human keratinocytes and mRNA expression levels of filaggrin, involucrin, β-defensin 2, and TNF-α, which can promote antioxidant defenses, barrier functions, and antimicrobial activity of the skin.

The possible immunomodulatory actions of probiotics have also been investigated using animal models. Hacini-Rachinel et al. (2009) have revealed that oral administration of *L. casei* (DN-114 001) reduced the number of $CD8^+$ effector T cells and increased the recruitment of $CD4^+$ effector T cells and Treg cells in the skin of mice with antigen-specific-induced skin inflammation. *L. ca*sei (DN-114 001) treatment also enhanced the IL-10 production from Treg cells in skin-draining lymph nodes of hapten-sensitized mice, thus suggesting its potential to treat allergic skin diseases in human. It has also been further reported that oral administration of

L. rhamnosus Lcr35 increased Treg cells, leading to the suppression of IL-4 production, and thymic stromal lymphopoietin (TSLP), which can promote Th_1 cytokines that initiate the inflammation cascade in ovalbumin-induced atopic dermatitis SK-1 hairless mice via a mechanism that may be associated with Treg cells (Kim et al. 2012). Moreover, *L. rhamnosus* 1.3724 treatment was also found to prevent the development of atopic dermatitis in NC/NgaTnd mice by stimulating IFN-γ in skin, while *L. sakei* probio 65 treatment reduced plasma IgE level and IL-4 production in the spleen of sensitized mice (Tanaka et al. 2009; Park et al. 2008). On the other hand, several studies have also reported that probiotics can attenuate skin inflammation via the gut-skin axis. Inoue et al. (2007) have revealed that *L. johnsonii* NCC533 treatment can prevent the development of atopic dermatitis by stimulating intestinal IgA production, whereas Sawada et al. (2007) have suggested that oral administration of heat-treated *L. rhamnosus* LGG upregulated IL-10 mRNA expression levels in both Peyer's patches and mesenteric lymph nodes in maternal and infant NC/Nga mice (a model of human atopic dermatitis) and may thus delay the onset and prevent the development of atopic dermatitis in human.

In addition to animal studies, Guéniche et al. (2010) have performed an ex vivo study to evaluate the immunomodulatory potential of *L. paracasei* CNCM I-2116 on substance P-induced skin inflammation. The authors found that *L. paracasei* CNCM I-2116 inhibited mast cell degranulation and TNF-α production induced by substance P ex vivo, thereby accelerating skin barrier recovery. Moreover, clinical trials have been performed in patients with other skin disorders, particularly on atopic dermatitis (Table 3). However, several clinical trials showed that probiotic strains were incapable of modulating immune responses in subjects with atopic dermatitis. Guéniche et al. (2009) have also conducted a randomized, double-blind, placebo-controlled trial to determine the immunomodulatory effects of probiotics in 57 volunteers upon exposure to ultraviolet (2×1.5 minimal erythema dose). The authors reported that volunteers who ingested *L. johnsonii* NCC 533 daily for 8 weeks had significantly increased production of regulating cytokines and growth factors such as TGF-β, which lead to the preservation of cutaneous immune homeostasis.

3.4 Osteoporosis

Estrogen deficiency is the primary risk factor of developing osteoporosis, a degenerative skeletal disease in postmenopausal women. In osteoporosis, osteoclastic bone resorption exceeds osteoblastic bone formation, thus leading to bone loss. Interestingly, probiotic treatment may attenuate osteoporosis in a postmenopausal woman by suppressing inflammatory cytokines (TNF-α and IL-1β) and T cell subsets (Treg and $CD4^+$ T lymphocytes) involved in osteoclastogenesis. Recently, Ohlsson et al. (2014) have demonstrated that oral administration of either *L. paracasei* DSM 13434 or a cocktail of three strains (*L. paracasei* DSM 13434, *L. plantarum* DSM 15312, and DSM 15313) for 6 weeks, starting 2 weeks before

Table 3 Clinical trials on the immunological potential of probiotics for the treatment of allergic and inflammatory skin disorders

Treatment	Probiotic strain	Subject	Dose and duration (intervention)	Immune response elicited	References
Prevention of eczema and atopic dermatitis in infants	*L. rhamnosus* HN001 *B. animalis* subsp. *lactis* HN019	512 pregnant women (mother or infant's father had a history of treated asthma, eczema, or hay fever)	*L. rhamnosus* HN001 (6×10^9 CFU/day) *B. animalis* subsp. *lactis* HN019 (9×10^9 CFU/day) Mother: 35-week gestation until 6 months if breastfeeding Infant: birth until 2 years (double-blind, randomized placebo-controlled trial of infants)	– IgE ↓	Wickens et al. (2008)
Atopic dermatitis in infants	*L. rhamnosus GG* ATCC 53103	131 children with at least two wheezing episodes and a first-degree family history of atopic disease (aged 6–24 months old)	*L. rhamnosus* LGG (10^{10} CFU/day for 6 months) (prospective double-blind, randomized study)	– No significant effect on TGF-β and IgE levels	Rose et al. (2010)
Children with mild-to-moderate atopic dermatitis	*L. casei* *L. rhamnosus* *L. plantarum* *B. lactis*	100 children with mild-to-moderate atopic dermatitis (aged 2–9 years)	Juice containing each strain (1×10^9 CFU; twice a day; 6 weeks) (randomized, double-blind, placebo-controlled, parallel trial)	– No significant effects on IL-4; IL-10 and TNF-α in serum	Yang et al. (2014)
Atopic dermatitis in infants	*B. breve* M-16V	90 full-term infants with atopic dermatitis (aged 0–7 months)	Galacto- and fructo-oligosaccharide mixture containing *B. breve* M-16V (1.3×10^9 CFU/day; 12 weeks) (double-blind, placebo-controlled multicenter trial)	– No significant effect on IgE-associated atopic dermatitis	van der Aa et al. (2010)
Incidence of eczema in children	*B. bifidum* W23 *B. lactis* W52 *L. lactis* W58	102 pregnant mothers during the last 6 weeks of pregnancy and postnatally for 12 months to their offspring	Freeze-dried powder containing three probiotic strains (3×10^9 CFU/day; 3 months) (double-blind, randomized, placebo-controlled trial)	– No significant effect on IgE level – No significant effect on IL-10 production – IL-5 ↓ – IL-13 ↓	Niers et al. (2009)
Chronic infected leg ulcers	*L. plantarum* ATCC 10241	34 patients (14 patients with type 2 diabetes) (aged 40–70 years)	Gauze pad containing *L. plantarum* ATCC 10241 (1×10^5 CFU/day for 10 days)	– IL-8 ↓	Peral et al. (2010)

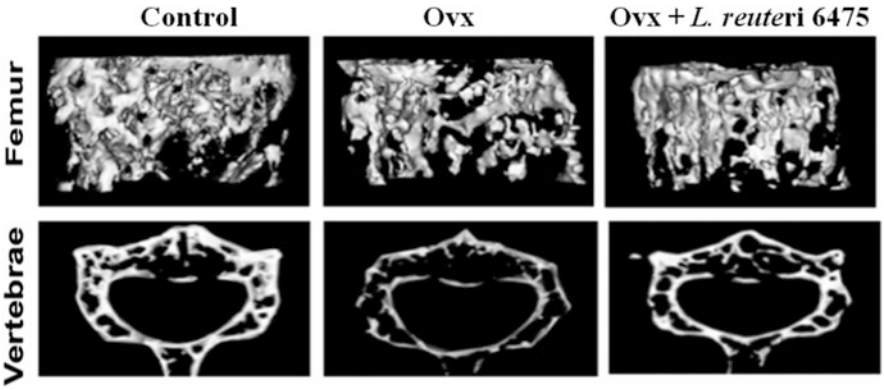

Fig. 4 Micro-computed tomography isosurface images of femur and vertebral trabecular bone of mice. Ovariectomized (ovx) mice treated with *L. reuteri* 6475 for 4 weeks display femur and vertebral trabecular bone volumes that are similar to ovary-intact control mice. Reprinted from Britton et al. (2014) with permission from John Wiley and Sons (License number: 3534800317389)

ovariectomy, downregulated gene expression of TNF-α and IL-1β, maintained the number of Treg cells, and increased TGF-β1 production in ovariectomized mice. TGF-β1 has been shown to stimulate Treg cells, thereby inhibiting osteoclast differentiation and functions. Meanwhile, Britton et al. (2014) have also reported that ovariectomized mice fed with *L. reuteri* 6475 (1×10^9 CFU/mL; three times per week) for 4 weeks reduced the number of ovariectomized-induced $CD4^+$ T lymphocytes, which prevented femur and vertebral trabecular bone loss (Fig. 4).

3.5 Rheumatoid Arthritis

Rheumatoid arthritis is a chronic autoimmune inflammatory disease in which the Th_1/Th_2 balance shifts toward Th_1 (pro-inflammatory responses), leading to joints deformities with destruction of cartilage and bone (Geusens et al. 2006). Owing to the immunosuppressive effect of probiotics, their potential use in reducing the pro-inflammatory responses were investigated in rats with type II collagen-induced rheumatoid arthritis. So et al. (2008a) have demonstrated that oral administration of *L. casei* (5×10^9 CFU/dose; 3 times/week) at day 7 after induction and continued for 11 weeks suppressed $CD4^+$ T cells from releasing type II collagen reactive pro-inflammatory components, including IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IL-12, IL-17, and Cox-2. Meanwhile, *L. casei* treatment also upregulated IL-10 expression and subsequently reduced the translocation of NF-KB, which is a key transcription factor that stimulates the production of certain pro-inflammatory cytokines (Driessler et al. 2004). Hence, *L. casei* may suppress pro-inflammatory cytokines by stimulating IL-10 production from $CD4^+$ T cells. In another study,

coadministration of *L. casei* (2×10^{10} CFU, 500 mg/kg) and collagen type II (250 mg/kg; glucosamine, 250 mg/kg) three times a week for 12 weeks has also been shown to suppress pro-inflammatory cytokines (IL-12, IL-17, IFN-γ, TNF-α, IL-1β, IL-2, and IL-6) and stimulate anti-inflammatory cytokines (TGF-β and IL-10) in mice with type II collagen-induced rheumatoid arthritis (So et al. 2008b). Meanwhile, this treatment also increased the numbers of Treg cells, upregulated Foxp3 expression, and suppressed collagen type II-reactive Th_1-type IgG isotypes, IgG2a, and IgG2b. Although the ability of probiotics to suppress Th_1-type cellular and humoral immune responses seems promising for the treatment of rheumatoid arthritis, more studies are needed to justify their potential.

3.6 Male Hypogonadism

High-energy and high-fat-diet-induced obesity has been shown to cause an increased risk for male hypogonadism (Cebler et al. 2010). Recently, Poutahidis et al. (2013) demonstrated that the pro-inflammatory cytokine IL-17 alone is capable of implicating a chronic inflammatory pathway in male hypogonadism, while probiotics can attenuate the incidence of male hypogonadism. Oral administration of *L. reuteri* ATCC PTA 6475 inhibited diet-induced obesity by restoring the Treg/Th_{17} balance and suppressing the systemic pro-inflammatory cytokine IL-17, thus demonstrating its potential in preventing male hypogonadism (Poutahidis et al. 2013). Poutahidis et al. (2014) then evaluated the anti-inflammatory effect of *L. reuteri* ATCC PTA 6475 in sustaining reproductive fitness in aging mice. Interestingly, the authors found that oral administration of *L. reuteri* ATCC PTA 6475 (3.5×10^5 CFU/mouse/day) at 2 months of age significantly increased the seminiferous tubule cross-sectional area, conspicuous Leydig cell area, and testicular weight of aging mice (aged of 5 months old).The beneficial effect of *L. reuteri* in the testicular health of 12-month-old mice was also recapitulated by blocking IL-17 signaling, indicating that systemic pro-inflammatory cytokine IL-17 could be a potential immunological target of probiotics in male hypogonadism. Recently, Al-Asmakh et al. (2014) have also reported that the gut microbiota plays a role in testicular health by modulating the permeability of the blood-testis barrier. Considering that probiotics can promote healthy gut microbiota to maintain immune homeostasis, probiotics may also be able to modulate the permeability of the blood-testis barrier and contribute to testicular health.

4 Future Trends

Despite the extensive use of probiotics as immune modulators in the treatment of gastrointestinal disorders, recent trends in probiotic research have clearly indicated a tendency toward discovering the potential applications of probiotics in

non-gastrointestinal diseases. There are a growing number of studies suggesting that probiotics may stimulate and/or regulate gastrointestinal immune responses at both the local and systemic levels, thereby exerting beneficial effects beyond the gut (Vandenplas et al. 2014). Until today, the precise mechanisms of local and/or systemic immunomodulation by probiotics remain largely unknown. The turning point came in year 2009, when Ventura et al. (2009) suggested that probiogenomics could pave the way for the identification of genes and/or cell constituents in probiotics responsible for immune responses. Since then, omics high-throughput techniques, such as transcriptomics, proteomics, and metabolomics are continuously being introduced in the field of probiotics. Specifically, probiogenomics and these high-throughput analyses can be combined and used to bridge the mechanistic gap between genotype and phenotype, which could provide valuable insights into the way probiotics modulate the gastrointestinal and non-gastrointestinal immune responses in the human host (Sánchez et al. 2013). Meanwhile, we are approaching a new frontier, where computational (in silico) tools can be applied to screen and predict the immunological targets of probiotics through the discipline of immunoinformatics (Flower 2007). By using this approach, the immunogenicity of immunogenic substances of probiotics can be predicted based on the sum of predicted binding energies. On the other hand, Wendelsdorf et al. (2012) have also developed an in silico gut, known as ENteric Immunity Simulator (ENISI) for the studies of the inflammatory and regulatory immune responses in the gastrointestinal tract. With ENISI, we can now better understand immunological mechanisms of gastrointestinal pathogens in the gut and facilitate subsequent screening of probiotics that can be potentially deployed in the treatment of gastroenteric infections. Altogether, these prerequisites could not only facilitate and hasten the discovery of plausible and new immunity mechanisms of probiotic strains but also lead to a boost in the development of immunobiotic products in the near future. Nonetheless, a large number of clinical trials are still needed to determine the immunological efficacy and safety of beneficial probiotic strains for specific health claims.

5 Conclusions

In addition to regulating the gastrointestinal mucosal immunity, probiotics have the ability to modulate the activity of immune cells in both the innate and adaptive immune systems. Hence, probiotics represent a potential breakthrough therapeutic approach for other organs and systemic autoimmune disorders. It appears that immunostimulatory and/or immunoregulatory effects of probiotics on the human host are strain-specific and operate through a specific mechanism. Certainly, recent advances in experimental and computational tools, as well as animal models, can now be applied to unravel the mechanisms underpinning the immunomodulation effect of probiotic strains and to accelerate future development of probiotic-based products.

Conflict of Interest No.

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Efficacy of Probiotics in Prevention of Influenza

Tadaaki Miyazaki

Abstract The human gut microbiota is critical for regulation of whole body metabolism and the immune system. Probiotics provide many benefits on human health by regulating this microbiota. In the prevention of infectious diseases, several probiotic *Lactobacillus* strains are shown to be effective to enhance the effects of influenza vaccine. These effects are mediated by increment of NK cell population, T-helper type 1 response, and production of virus-specific IgA, IgG, and IgM to inhibit the viral infection. Recently, we have shown that *Lactobacillus gasseri* LG2055 is effective to prevent influenza by induction of IFNβ production and expression of antiviral genes to inhibit the viral replication. In addition, the inflammatory response is inhibited, and cytokine production to stimulate B cells is induced by the administration of LG2055. Especially, IFNβ inhibits the replication of influenza virus and IFNβ production signal is mediated by IPS-1 through the activation of IRF3 and NF-κB. Therefore, in the future, the regulatory effects on the function or expression of these signaling molecules by the administration of probiotics should be studied. In addition, since the inhibition of FasL-induced apoptosis is effective to protect mice from death by the viral infection, the function of other death receptors, TNFR, DR4, and DR5, or signaling molecules, Siva-1, DAP3, and DELE, should be clarified in the viral replication and apoptosis induction. Further investigation for the regulatory effects of probiotics on the function of these molecules is required and might lead to the development of novel influenza drugs or vaccine adjuvants.

1 Introduction

Gut microbiota is an important environmental factor for the regulation of hepatic lipogenesis and energy homeostasis in the host (Bäckhed et al. 2004; Nicholson et al. 2005). It is demonstrated that the functional members of the microbiome affect host metabolism regulating human health condition (Nicholson et al. 2005; Ley et al. 2006; Li et al. 2008). Gut microbiota is also critical for regulation of the

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immune system, to prevent immune disorders such as a chronic inflammatory bowel disease (Mazmanian et al. 2008), and contributes to the control of inflammatory responses in obesity or type II diabetes (Cani et al. 2008) and development of type I diabetes (Wen et al. 2008). Probiotics, live microorganisms, when administered in adequate amounts, provide many potential benefits on human health. Especially, intestinal and dairy species of *lactobacilli* and *bifidobacteria* are well known to be health-promoting microbes in the human gastrointestinal tract (Kleerebezem and Vaughan 2009). In fact, clinical trials show that certain microorganisms including these bacterial strains can prevent the intestinal infections of pathogens by reducing the duration of diarrhea, inflammatory bowel syndromes (Reid et al. 2003), and even the early atopic disease in children (Kalliomäki et al. 2001). Some probiotics are also demonstrated to be effective for enhancement of the effects of vaccination against influenza viruses (Olivares et al. 2007; Davidson et al. 2011).

Influenza A viruses are well known to cause highly contagious respiratory illness, influenza (Taubenberger and Morens 2010), and belong to the *Orthomyxoviridae* viruses, negative sense, single-stranded RNA viruses carrying eight segmented RNA genome (Fig. 1) (Medina & García-Sastre 2011). Infection of influenza A virus caused several pandemic diseases in the past and is also the cause of almost annual epidemics "seasonal flu." In addition, influenza A virus is a cause of lethal infectious diseases culminating in severe pneumonia. Older adults, very young children, pregnant women, and patients with chronic diseases are higher risk groups to be exposed to serious illness or death by the viral infection. Therefore, development of drugs or vaccines for prevention or treatment of influenza is very important to protect these people from diseases that cause serious illness. Importantly, identifying the viral and host molecules, critical for the viral replication and pathogenesis, is very useful for the development of the effective drugs or vaccines. In addition to drugs or vaccines, probiotics are also good candidates to regulate the function of these molecules and that of immune cells to prevent or treat influenza.

Recently, we found that oral administration of a probiotic strain, *Lactobacillus gasseri* SBT2055 (LG2055) (1.6×10^9 cfu/mouse, administrated once a day for 21 days), was effective to prevent influenza by the inhibition of viral replication through upregulation of the expression of antiviral genes in mice (Nakayama et al. 2014). LG2055 is a probiotic lactic acid bacterium, shown to improve the intestinal environment, and has preventive effects on abdominal adiposity in humans (receiving 1.0×10^8 cfu /g/day of LG2055 in 200 g fermented milk for 12 weeks) (Sato et al. 2008). In addition, its oral administration to mouse dams was shown to prevent rotavirus infection in their pups (Kadooka et al. 2012). Interestingly, it was demonstrated that LG2055 induces TGF-β expression in dendritic cells and subsequently TGF-β induced IgA production by B cells in the small intestine (Sakai et al. 2014). Therefore, LG2055 as a probiotic strain plays a pivotal role in the prevention of rotavirus infection by produced IgA. In the lumen of the intestinal tract, secretory IgA is very important to constitute significant barriers to exclude pathogens from mucosal surfaces (Corthésy 2013). There is a possibility that IgA

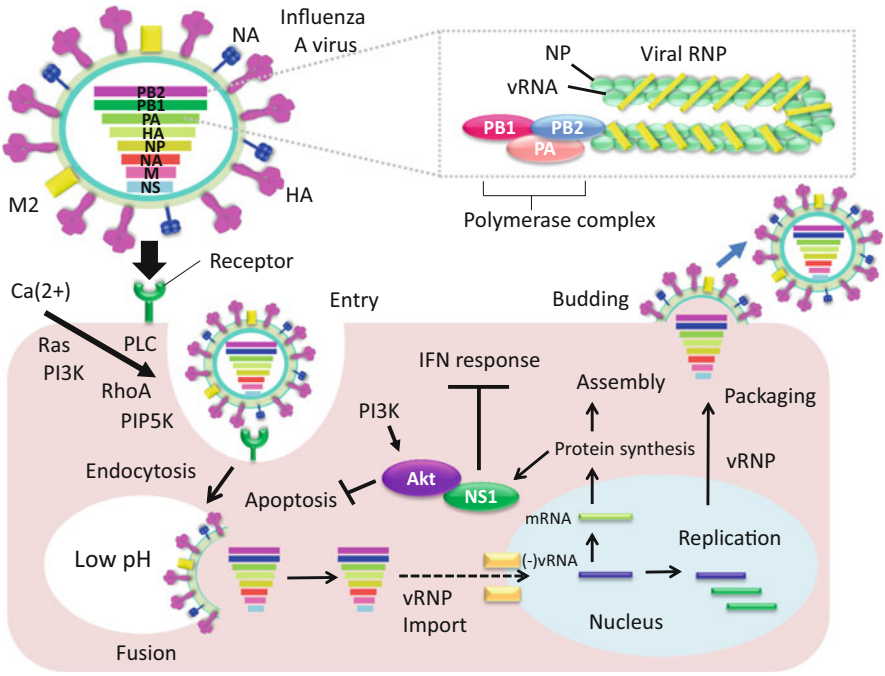

Fig. 1 Structure of influenza A virus and life cycle of the virus. Influenza A virus has eight RNA genomic segments, encoding the viral RNA polymerases (PB1, PB2, PA), nucleoproteins (NPs), hemagglutinin (HA), neuraminidase (NA), matrix protein (M1), and nonstructural protein (NS1). HA binds to the host cell surface receptors, and the virus is transported intracellularly by endocytosis. Low pH in the endosome induces fusion of the viral and endosomal membranes and also triggers releasing the viral RNP (vRNP) into the cytoplasm and transporting it into the nucleus. In the nucleus, the viral polymerase initiates viral mRNA synthesis. The mRNA is transported to the cytoplasm and is translated into proteins. NS1 has a critical role to inhibit the production of host mRNAs, including interferon (IFN) β mRNAs. The vRNP is transported to the cytoplasm, by a M1–NS2 complex. After the vRNP is translocated to the cell membrane, it is incorporated into new viruses and released after the budding on the host cell surface

production is induced in the serum and bronchoalveolar lavage fluid (BALF) by the administration of LG2055 and secreted IgA protects the lung from the infection of influenza A virus.

In this chapter, the molecular mechanisms of influenza virus infection and host defense system against the viral replication are demonstrated, relating to the immunoregulatory effects of probiotics on the prevention of the viral infection. Based on these mechanisms, accumulated results by the functional analysis of the probiotics to regulate the host defense system could lead to the development of effective adjuvants of the influenza vaccine and drugs for the prevention and treatment of influenza.

2 Function of Cellular Signaling Molecules for the Infection of Influenza A Virus

Recently, it is demonstrated that the Ras–phosphoinositide 3-kinase (PI3K) signaling pathway mediates the entry of influenza A virus into the infected cells (Fig. 1) (Fujioka et al. 2011). Binding of Ras with PI3K is specifically involved in clathrin-independent endocytosis, endosomal maturation, and intracellular transport of the viruses. Influenza A virus infection activates Ras and subsequently PI3K in the early endosomes. Furthermore, it is shown that influenza A viruses enter cells via redundant pathways of clathrin-mediated and clathrin-independent endocytosis, and intracellular Ca (2+) increase is required for both signaling pathways (Fujioka et al. 2013). In addition, RhoA, Rho-kinase, phosphatidylinositol 4-phosphate 5-kinase (PIP5K), and phospholipase C (PLC) regulate this Ca (2+) signaling. The viral infection induces oscillations in the cytosolic Ca (2+) concentration of host cells, and this event is crucial for viral internalization and infection (Fig. 1). RhoA is activated at downstream of the virus-induced Ca (2+) response and induces Ca (2+) oscillations in a manner dependent on Rho-kinase and subsequent PIP5K-PLC signaling. This signaling circuit regulates both clathrin-mediated and clathrin-independent endocytoses and constitutes a key mechanism for regulation of the viral internalization and infection.

Influenza A virus consists of eight segmented minus stranded RNA, and among the proteins encoded by these segments, nonstructural protein 1 (NS1, encoded on segment 8) regulates suppression of apoptosis induction in host cells (Fig. 1), inhibition of nuclear export of mRNA, and splicing of mRNA by binding U6 small nuclear RNA (Hale et al. 2008). NS1 also binds to double-stranded RNA and, as a consequence, inhibits PKR kinase activity (Chen et al. 1998) and regulates apoptosis induction in host cells not through PI3K (Jackson et al. 2010). Recently, the functional interaction of NS1 with serine threonine kinase Akt, a core intracellular survival regulator, is reported (Fig. 1) (Matsuda et al. 2010). Akt is activated in response to PI3K by a wide variety of growth factors, antigens, and inflammatory stimuli. Activation of PI3K produces PtdIns (3,4,5)P_3 (PIP_3), which binds to the lipid-binding module of the PH domain of Akt, and induces activation of its downstream signals. It is demonstrated that NS1 directly interacts with Akt and the interaction is mediated primarily through the Akt-PH (pleckstrin homology) domain and the RNA-binding domain of NS1. NS1 preferentially interacts with phosphorylated Akt, but not with non-phosphorylated Akt. Interaction of NS1 with Akt enhances the kinase activity and phosphorylated Akt interacts with NS1 during the interphase of the cell cycle predominantly within the nucleus. Functionally, NS1 activates the signaling pathway to ensure efficient viral replication by enhancing anti-apoptotic responses (Fig. 1).

3 Significance of IFN and Apoptosis Signals During Influenza A Virus Infection

Viral proteins are translated from viral mRNA transcribed by the RNA-dependent RNA polymerase of influenza A virus. Viral polymerase complex is a heterotrimer consisting of polymerase acidic protein (PA), polymerase basic protein 1 (PB1), and PB2, and each component is crucial for the replication of the virus (Fig. 1) (Neumann et al. 2004). In addition to the function of NS1, it is also shown that the viral polymerase plays an important role for regulating host antiviral response through inhibition of interferon (IFN) β production by the binding to IFNβ promoter stimulator 1 (IPS-1; also called MAVS, Cardif, or VISA) (Fig. 2) (Iwai et al. 2010). IFNβ is an important factor in the activation of host defensive mechanisms against influenza A virus. IPS-1 is a downstream mitochondrial adapter protein that transmits the signal to induce type I IFN through the activation of transcription factors IFN regulatory factor 3 (IRF3), IRF7, and nuclear factor κB (NF-κB) (Seth et al. 2005; Xu et al. 2005; Kawai et al. 2005; Meylan et al. 2005). PB2 or

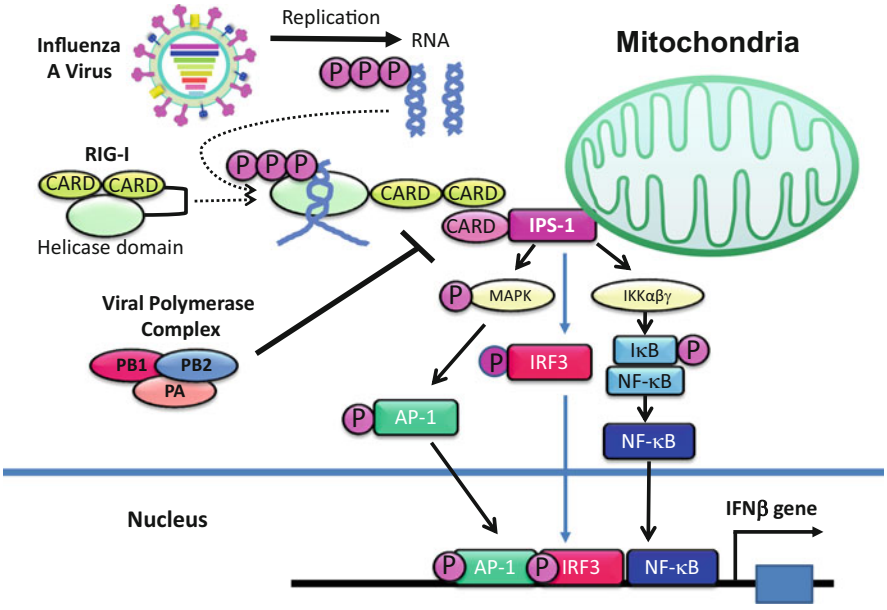

Fig. 2 Inhibition of IPS-1-mediated IFN production by influenza A virus polymerase. The RNA helicase, retinoic acid-inducible gene 1 (RIG-I) is a RNA virus sensor for influenza A virus. Viral RNA is recognized by the helicase domain of RIG-I and interaction through homotypic caspase activation and recruitment domain (CARD) with the interferon promoter-stimulating factor 1 (IPS-1) induces the activation of transcriptional factors, IRF3, AP-1, and NF-kB, by phosphorylation. These activated transcription factors are translocated into the nucleus to induce IFNβ gene expression. Functions of IPS-1 to induce the gene expression are inhibited by the viral polymerase complex of influenza A virus by the direct interaction with IPS-1

PB2-containing complex inhibits IPS-1-induced activation of IFNβ promoter after influenza A virus infection (Fig. 2) and this function is not competitive with the inhibitory action of NS1 (Pichlmair et al. 2006; Mibayashi et al. 2007; Guo et al. 2007; Opitz et al. 2007).

Recently, regulation of cellular apoptosis is shown to be significantly important for the host defense system against influenza A virus infection (Iwai et al. 2013). For instance, apoptosis induction is critical for the virus elimination through the removal of the virus-infected cells, and tissue damage during the course of viral infection including multiple organ dysfunction is caused by apoptosis (Hinshaw et al. 1994; Chan 2002). Furthermore, abnormal apoptosis induction of alveolar epithelial cells and lymphocytes is related to the influenza disease symptoms.

Apoptosis is known as a programmed cell death in which the morphological features of apoptotic cells are associated with cytoplasmic shrinkage, plasma membrane blebbing, DNA fragmentation, and chromatin condensation. Finally, the cells form cell fragments, termed apoptotic bodies, and are engulfed by phagocytic cells. Two major apoptosis signals are known as death receptor-mediated signal and mitochondria-mediated signal (Gupta 2001). Death receptors, such as tumor necrosis factor α receptor 1 (TNF-α R1), Fas, death receptor 4 (DR4, TRAILR1), and death receptor 5 (DR5, TRAILR2), are defined by the death domain in their cytoplasmic region and mediate signals to activate caspase-8 (Fig. 3). On the other hand, mitochondrion is an important organelle for the host cells to determine the cell destiny, and generally the mitochondrial membrane potential is disrupted during apoptosis induction. After disruption of the mitochondrial membrane potential, the cytochrome c in the mitochondrial inner membrane is released into the cytoplasm, and caspase-9 is activated. Importantly, these two major apoptosis pathways are crucial for the elimination of influenza A virus by the removal of the virus-infected cells from the body. However, there is a possibility that excessive induction of apoptosis in lung tissue or other organs by influenza A virus infection induces severe illness often leading to death. In fact, we had shown that a variety of types of cells in the lung express FasL, a specific ligand of Fas, after the viral infection and the induction level of *FasL* gene expression was correlated with the severity of influenza (Fig. 3) (Fujikura et al. 2013). Furthermore, inhibition of Fas/FasL signal by treatment with a recombinant decoy receptor for FasL increases the survival rate of mice after the lethal viral infection. In the future, it might be an effective treatment method of influenza to inhibit the excessive apoptosis signal induced by the viral infection.

We also had shown that Siva-1, a proapoptotic protein, is crucial for effective replication of influenza A virus (Fig. 3) (Shiozaki et al. 2011). Since Siva-1 function in the viral replication completely disappears after treatment with a pan-caspase inhibitor, Z-VAD fmk, Siva-1 appears to modulate the viral replication by regulation of caspases activation. These results suggest that Siva-1 and caspases are critical targets for the inhibition of the viral propagation.

In addition, death-associated protein 3 (DAP3) is known to be crucial for apoptosis induced by the extrinsic death receptor stimulation (Fig. 3) (Miyazaki and Reed 2001; Kim et al. 2007) and is also related to other IPS-1 functions. A

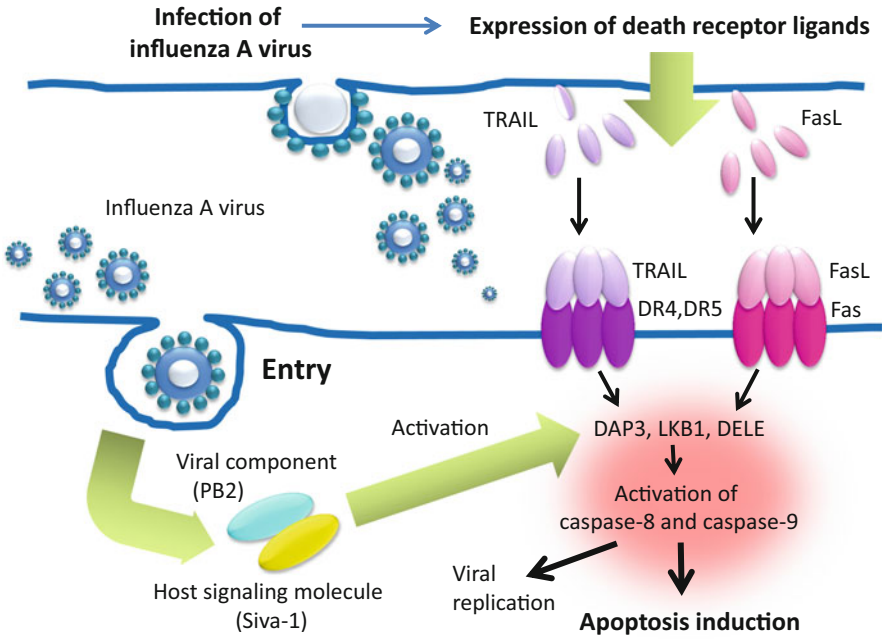

Fig. 3 Apoptotic signaling pathways induced by influenza A virus infection. Infection of influenza A virus induces the expression of death receptor ligand, TRAIL or FasL on macrophages, DC cells, NK cells, or CD4 T cells, and these ligands stimulate their receptors, DR4/DR5 or Fas, to induce apoptosis in the alveolar epithelial cells. On the other hand, PB2 of influenza A virus binds to the host cellular molecule, Siva-1, to induce the activation of caspases 8 and 9. Siva-1 is involved in the effective viral replication through the activation of these caspases. In addition, DAP3 is essential for death receptor-mediated apoptosis induction and LKB1 or DELE and DAP3 associating protein also might be critical to regulate apoptosis induced by the viral infection

previous report has demonstrated that IPS-1 is also involved in the induction of anoikis (Li et al. 2009), known to be a form of apoptosis induced by anchorage-dependent cells detaching from the surrounding extracellular matrix. In anoikis induction, DAP3 binds to IPS-1 and recruits FADD for activation of caspase-8, and then apoptosis is executed. The DAP3 function in anoikis induction is inhibited by Akt-dependent phosphorylation (Miyazaki et al. 2004), and AKT is activated by the NS1 protein of influenza A virus. Therefore, the DAP3 function inducing anoikis is thought to be inhibited in the virus-infected cells. DAP3 is also crucial for apoptosis induced by TNF-α, FasL, and TRAIL stimulation (Miyazaki and Reed 2001; Kim et al. 2007). Therefore, DAP3 and signaling molecules to mediate the function of DAP3, such as liver kinase B1 (LKB1) (Takeda et al. 2007) and death ligand signal enhancer (DELE) (Harada et al. 2010), are thought to be involved in the viral replication and apoptosis induction caused by the viral infection (Fig. 3). Further investigation of the molecular mechanism of apoptosis induction mediated by these molecules during influenza A virus infection is required and might provide a strategy for the development of novel influenza drugs.

4 Beneficial Effects of Probiotics as Adjuvants of Influenza Vaccine

Oral consumption of *Lactobacillus fermentum* CECT5716 for co-adjuvant capability was performed to address the immunologic effects of vaccination in human clinical trial (50 volunteers aged 22–56 years, received an oral daily dose of probiotic bacteria 1.0×10^{10} cfu/day for 2 weeks before vaccination and 2 weeks after vaccination) (Olivares et al. 2007). Two weeks after vaccination, the proportion of natural killer cells was increased by the administration of CECT5716. The responses of T-helper type 1, antigen-specific immunoglobulin A and total immunoglobulin M were increased. The incidence of an influenza-like illness during 5 months after the vaccination was lower in the group consuming the probiotic bacteria. *Lactobacillus rhamnosus GG* (LGG) is also shown to be an important adjuvant to improve influenza vaccine immunogenicity by randomized double-blind placebo-controlled pilot study (42 healthy subjects aged 18–49 years, receiving 1.0×10^{10} cfu of LGG and 295 mg inulin in gelatin capsule, twice daily for 28 days after the vaccination) (Davidson et al. 2011). In the case of the H3N2 influenza A virus, 84 % subjects receiving LGG versus 55 % subjects receiving placebo had Hemagglutinin inhibition titers at day 28 after vaccination.

Infection of influenza A virus leads to lethal diseases, and especially, older adults are higher risk groups to cause serious illness or death by infection. Therefore, to develop drugs or vaccines for prevention or treatment of influenza in older people is very important. To investigate the effect of a probiotic dairy drink on the immune response to the vaccination against influenza viruses in an elderly population over 70 years of age, two randomized, multicenter, double-blind, controlled studies were performed (Boge et al. 2009). A fermented dairy drink, containing the probiotic strain *Lactobacillus casei* DN-114 001 was consumed twice daily for a period of 7 weeks for the pilot study or 13 weeks for the confirmatory study. These subjects took a vaccine after 4 weeks of the consumption of fermented dairy drink. Geometric mean antibody titers (GMT) against the three viral strains, influenza viruses A(H1N1), A(H3N2), and B, of the vaccine were measured at several time intervals post-vaccination by hemagglutination inhibition test.

The virus-specific antibody titers increased after vaccination, being consistently higher in the probiotic product group compared to the control group in the pilot study. Similarly, in the confirmatory study, antibody titers against the influenza virus B strain increased significantly more in the probiotic group than in the control group. Even 5 months after vaccination, significant differences in seroconversion between the groups were still found and also similar GMT results were observed for the influenza A virus H3N2 and H1N1 strains. These results demonstrate that in older people daily consumption of the probiotic product is effective for the increment of specific antibody responses to influenza vaccination.

Recently, *Lactobacillus plantarum* CECT7315/7316 is reported to have an immunostimulating effect and could be used to improve the response to the vaccination in elderly person (60 institutionalized volunteers aged 65–85 years, receiving 5.0×10^9 cfu/day of CECT 7315/7316 in 20 g powdered skim milk for 3 months after the vaccination) (Bosch et al. 2012). For the volunteers aged 65–85 years, a randomized, double-blind, placebo-controlled human trial was performed. The consumption of the probiotics strains during 3 months after the vaccination of trivalent influenza vaccine (A/Wisconsin/67/2005 NYMC X-161B (H3N2), A/Solomon Islands/3/2006 (H1N1), and B/Malaysia/2506/2004) increased the levels of virus-specific IgA and IgG antibodies. Moreover, a trend toward an increase in the virus-specific IgM antibodies was also observed. These results suggest that probiotics are good candidates for the effective adjuvants of influenza vaccine. In the future, effects of the probiotics should be examined as effective adjuvants of vaccines against a variety of pathogens.

5 Beneficial Effects of Probiotics on the Prevention of Influenza

In addition to the utility as an adjuvant of vaccine, the consumption of only probiotics is effective for the prevention of influenza virus infection. Heat-killed *Lactobacillus plantarum* L-137 (HK-LP) stimulates macrophage/dendritic cells to produce T-helper (Th) 1-related cytokines. The effect of oral administration of HK-LP on protection against H1N1 influenza virus A/FM/1/47 infection was examined in mice (Maeda et al. 2009). The survival time was significantly prolonged and the viral titers in the lung were significantly lower in mice treated with HK-LP administration. An appreciable level of IFNβ was detected in the serum of mice treated with HK-LP, suggesting that HK-LP is a potent IFNβ inducer.

Recently, from the screening of 158 bacterial strains, including a majority of lactic acid bacteria, using two different cellular models of tumor necrosis factor alpha (TNF-α)-activated human colon adenocarcinoma grade II cell, HT-29, and peripheral blood mononuclear cell (PBMC), novel candidate probiotics having an immunomodulatory function were identified (Kechaou et al. 2013). Different strains responsive to both models were selected, and their protective effects were tested against influenza virus infection in mice. With daily intragastric administrations after viral infection (H1N1 influenza virus A/Puerto Rico/8/1934 [A/PR8/34]), *Lactobacillus plantarum* CNRZ1997 was demonstrated to have functions reducing body weight loss, alleviating clinical symptoms, and inhibiting significantly viral replication in the lungs (daily administered intragastrically 1.0×10^9 cfu of each strain suspended in 200 μl of PBS, for 10 days before and 10 or 14 days after virus challenge). This screening method for the probiotic strains to have an

immunomodulatory function might be useful strategy to identify the strain, effective for the prevention of influenza.

Since influenza A (H1N1) pdm virus caused the first human pandemic of the twenty-first century, to prevent the infection of this virus strain is very critical to save many people from the next influenza pandemic. The prophylactic efficacy of heat-killed *Lactobacillus pentosus* b240 against lethal influenza A (H1N1) pdm virus infection was examined in a mouse model. Mice were orally administered heat-killed b240 every day at a dose of 10 mg/mouse, which corresponds to 1.0×10^{10} cell counts in 200 μl of buffered saline for 5 weeks (Kiso et al. 2013). Expression of three acyl-CoA thioesterase (Acots) genes was significantly downregulated in mouse lungs by oral administration of b240. Acots play an important role in the generation of arachidonic acid, the precursor to eicosanoids, responsible for manifestations of inflammation. It suggests that the regulation of Acots expression by b240 might contribute to the recovery of mice from the viral infection by the regulation of inflammatory response. And the expression of the FBJ osteosarcoma oncogene (Fos), early growth response 1 (Egr1), and cysteine-rich, angiogenic inducer, 61 (Cyr61) genes was downregulated by b240 administration. Cyr61 activates IL-6 production, resulting in the progression of inflammation, and the transcription factors Egr1 and Fos are early responders during influenza virus infection. Egr1 is a critical regulator of host inflammatory chemokines and is associated with $CD8^+$ T cell-mediated lung injury in the influenza virus infection. The downregulation of these genes induced by oral administration of b240 may play a role in alleviating pulmonary injury caused by the inflammatory response. In addition, expression of Rsad2 (radical S-adenosyl methionine domain-containing protein 2) gene was upregulated in the lungs of uninfected mice after oral administration of b240. Rsad2 is an interferon-stimulated gene (ISG), induced by IFNs after viral infection. These results suggest that inflammatory responses and ISG expression are regulated by the administration of heat-killed *Lactobacillus pentosus* b240.

We investigated the effect of lysozyme-treated *Enterococcus faecalis* FK-23 (LFK), isolated from human intestinal tract, to prevent influenza in the virus-infected mice (Fukada et al. 2013). Mice were orally administered LFK and infected with influenza virus A/PR8/34 at lethal doses. After the viral infection, the survival rate of the LFK-administered mice was significantly higher than that of control mice. Oral administration of LFK suppressed the excessive infiltration of leukocytes into the lung after viral infection. The arrest was mediated by modulation of pulmonary alveolar–capillary permeability. In fact, expression levels of genes involved in matrix degradation, correlated with vascular permeability, were downregulated in LFK-administered mice. These results suggest that stabilizing the integrity of the alveolar–capillary barrier by the administration of LFK protects mice from death after the viral infection. Furthermore, we examined the efficacy of the water-soluble fraction (SLFK) of LFK against a lethal influenza A virus challenge (Kondoh et al. 2012). Mice were orally administered SLFK and intranasally infected with A/PR8/34 virus. The survival rate of SLFK-administered mice after the viral infection was significantly improved compared with that of control

mice. In addition, the mRNA expression level of the anti-inflammatory cytokine interleukin-10 (IL-10) in the lung tissues was enhanced by the administration of SLFK. These observations suggest that the oral administration of SLFK exerts a protective effect against influenza virus infection through the activation of the anti-inflammatory response. Taken together, each component of probiotics has a different function to regulate the expression of anti-inflammatory cytokine or genes, critical for stabilizing the integrity of alveolar–capillary barrier. In the future, identification of the substances of these components is required for the development of influenza drugs.

Recently, we studied the immunomodulatory function of *Lactobacillus gasseri* LG2055 and demonstrated that oral administration of LG2055 increases in the survival rate of mice infected with the A/PR8/34 virus and decreases the ratio of body weight losses by the viral infection (Nakayama et al. 2014). The survival rate of mice tended to improve by the administration in a dose-dependent manner. The oral administration of LG2055 is effective to protect the mice from lethal virus infection, and it might be as a result of that the virus titer in the bronchoalveolar lavage fluid is significantly decreased by LG2055 administration after the virus infection. LG2055 administration induces the mRNA expression of the antiviral ISGs, myxovirus resistance 1 (Mx1), and 2′–5′ oligoadenylate synthetase 1A (Oas1a) in the lung tissues. In addition, Mx1 and IFNβ mRNA were strongly induced in macrophage-like cell, RAW264.7 after LG2055 treatment (Fig. 4). The intestinal cells were stimulated by LG2055 components, and type I IFN produced by these cells including macrophages in the intestine may secondarily stimulate the lung cells or macrophages in the lung for ISG production (Fig. 4). These results indicate that the oral administration of LG2055 is effective on the prevention of influenza by the inhibition of virus replication through upregulation of the expression of antiviral genes.

On the other hand, in mucosal immunity, IgA antibodies are very critical for preventing influenza virus transmission and the production of secretory IgA depends on the commensal bacteria and viral antigens in the gastrointestinal tract. IgA is produced by IgA(+) plasma cells, differentiated from IgA(+) B cells, and this differentiation is induced by the intestinal dendritic cells (DCs), stimulated by their antigens incorporated through M cells (Fig. 5). Recently, we have found that oral administration of LG2055 induced IgA production and increased the rate of IgA(+) cell population in Peyer's patch lamina propria of the mouse small intestine (Fig. 6) (Sakai et al. 2014). This effect of LG2055 was significantly stronger than that of the *L. gasseri* type strain or of other *Lactobacillus* species. LG2055 administration markedly increased IgA amount in a co-culture of B cells and bone marrow-derived dendritic cells (BMDCs), and TLR2 signal was critical for this induction of IgA production. In addition, LG2055 stimulated BMDC to promote the production of TGF-β, BAFF, and IL-6, all critical for IgA production from B cells. B cells stimulated with both BAFF and LG2055 enhanced the induction of IgA production (Fig. 6). In these processes, TGF-β signal was critical for LG2055-induced IgA production in the B cell and BMDC co-culture system, but TGF-β did not induce IgA production by only B cells stimulated with LG2055. Furthermore, TGF-β was

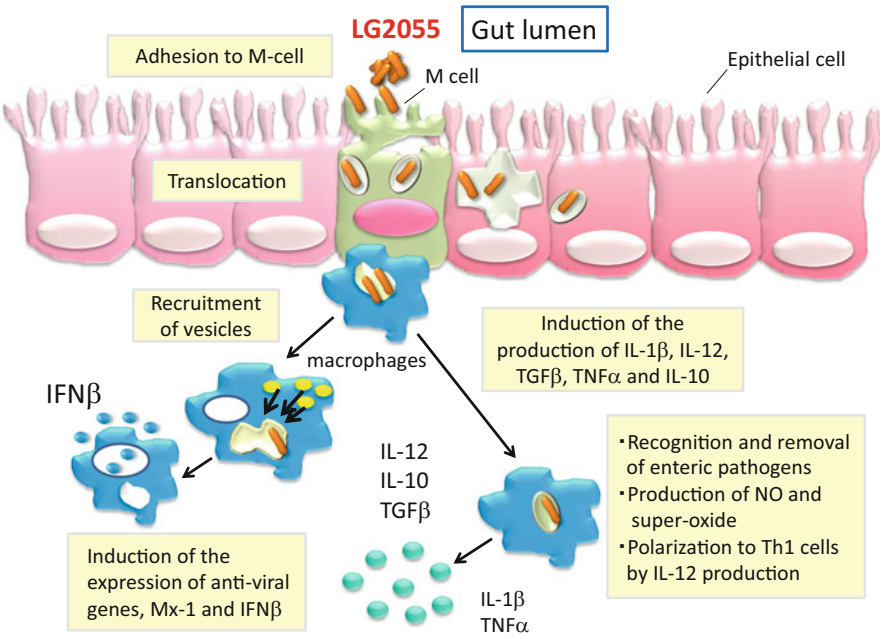

Fig. 4 Induction of the antiviral responses in macrophages by LG2055. Phagocytosis of LG2055 by macrophages is mediated through the M (microfold) cells in the Peyer's patches of the small intestine. Macrophages secrete pro-inflammatory mediators such as tumor necrosis factor (TNF)α, nitric oxide (NO), and IL-1β, critical for the activation of various antimicrobial mechanisms, including oxidative processes to kill the invading microorganisms. Activated macrophages produce IL-12 to induce the polarization to Th1 cells and NO or superoxide, highly toxic for microorganisms. In addition, transcription of mRNA of antiviral genes, Mx1 and IFNβ, is induced in macrophages by LG2055 stimulation. IFNβ produced by macrophages in the intestine may secondarily stimulate the lung epithelial cells or alveolar macrophages to induce ISG production in the lung

critical for the production of BAFF, IL-6, IL-10, and TGF-β itself from LG2055-stimulated BMDC. Therefore, TGF-β is produced by BMDC stimulated with LG2055 and it is essential for BMDC to induce the production of BAFF and IL-6 (Fig. 6). LG2055 is effective to induce IgA production by regulation of these cytokines. Induction of IgA production by LG2055 should play an important role in the control of the intestinal microflora and prevention of the infection of pathogenic bacteria and viruses. Other group also identified other probiotic strain, named as *Lactobacillus plantarum* AYA, stimulating induction of IgA production by murine Peyer's patch cells (Kikuchi et al. 2014). IL-6 production was induced by this strain in Peyer's patch dendritic cells, promoting IgA(+) B cells to differentiate into IgA-secreting plasma cells. Oral administration of this probiotic strain increased IgA production in the small intestine and lung in mice. The function of the induction of IgA production was strongly correlated with the activity to protect mice from death after lethal influenza virus infection.

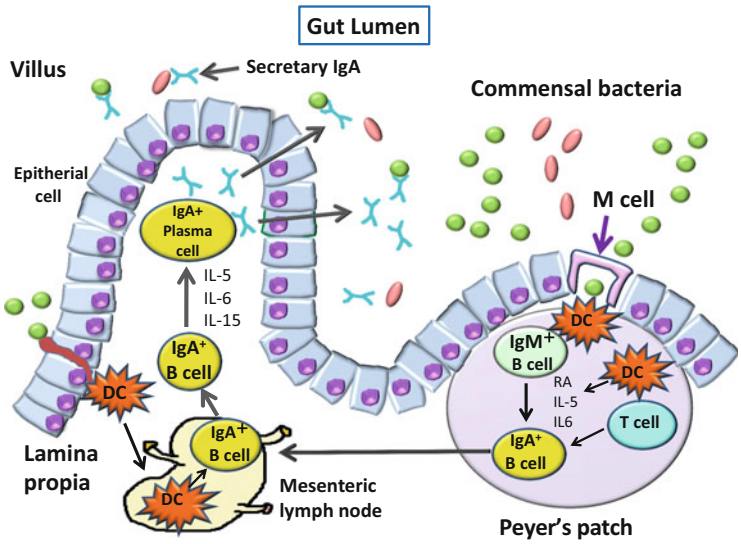

Fig. 5 Mechanism of the IgA secretion into the intestinal lumen. In mucosal immunity, IgA antibodies are very critical for preventing the infection of pathogens and maintaining the intestinal homeostasis. The production of secretory IgA by IgA(+) plasma cells and the differentiation from IgM(+) B cells to IgA(+) B cells are induced in the secondary lymphoid tissues (Peyer's patches and mesenteric lymph nodes). This differentiation is activated by the intestinal DCs, stimulated by their antigens incorporated through M cells. The recruitment of DCs, T cells, and B cells is regulated in the process of immune responses to the microbiota. Stimulated DCs interact with T cells to induce T cell differentiation and T cell-dependent B cell maturation. Differentiated IgA(+) B cells migrate to the draining mesenteric lymph node. Subsequently, the development of IgA(+) plasma cells is induced and these cells leave the mesenteric lymph node and migrate to the blood circulation. IgA(+) plasma cells secrete IgA into the intestinal lumen. Secreted IgA has a function to control the intestinal homeostasis and to protect against the invasive pathogens

In summary, probiotic *Lactobacillus* strains are effective to prevent influenza as adjuvants of the vaccine, by different mechanisms depending on the strain such as increment of the population of natural killer cells, the response of T-helper type 1, and the amounts of virus-specific IgA, IgG, and IgM. Furthermore, administration of these probiotic strains in mice induced IFNβ production, expression of ISG and antiviral genes to inhibit the viral replication, inhibition of the inflammatory responses, and induction of BAFF, IL-6, and TGF-β to produce IgA by B cells.

Especially, IFNβ is an important factor against the replication of influenza virus. IPS-1 transmits the IFNβ induction through the activation of transcription factors IRF3, IRF7, and NF-κB. In contrast, viral polymerase complex inhibits IPS-1-induced activation of IFNβ promoter. Therefore, the effect and mechanism for the regulation of these signaling molecules by probiotics should be studied in the future. In addition, since inhibition of excessive apoptosis induction by Fas/FasL was effective to protect mice from death by the viral infection, significance of the function of other death receptors, such as TNFαR1, DR4, and DR5, should be

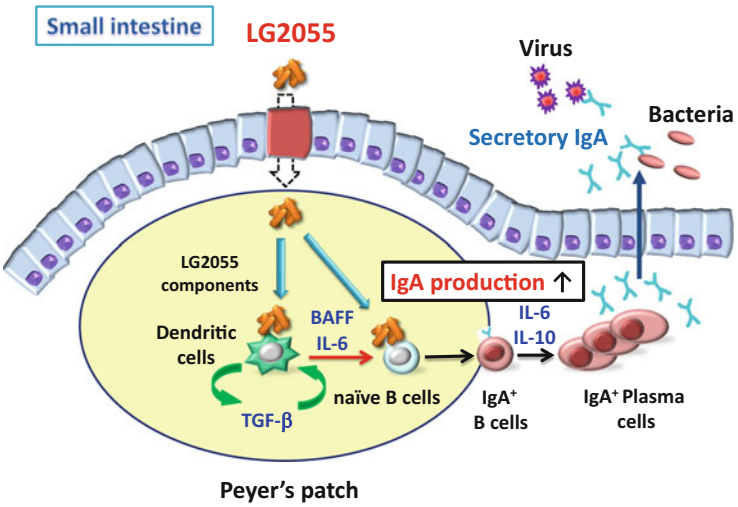

Fig. 6 Mechanism of IgA production induced by LG2055 in the small intestine. LG2055 induces IgA production and increases the rate of IgA(+) B cell population in Peyer's patch and in the lamina propria of the small intestine. The production of BAFF, TGF-β, IL-6, and IL-10 is upregulated in LG2055-stimulated dendritic cells (DCs). TGF-β and BAFF induce an IgA class-switch recombination. IL-6 and IL-10 induce the differentiation of IgA-producing plasma cells in IgA(+) B cells. TGF-β signal contributes to the production of IL-6, IL-10, BAFF, and TGF-β itself by LG2055-stimulated DCs through the TLR2 signaling pathway. In addition, combined stimulation of B cells with BAFF and LG2055 enhanced the induction of IgA production

clarified. Furthermore, signaling molecules, Siva-1, DAP3, and DELE, might be involved in the viral replication and apoptosis induction caused by the viral infection. Further investigation of the effects on the regulation of the function or expression of these molecules by the administration of probiotics during influenza virus infection is required and might lead to the development of novel influenza drugs or vaccine adjuvants.

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Gut Commensal Microbes and the Gut Immune System

Hiroshi Ohno

Abstract An enormous number of bacteria, the gut commensal microbiota, inhabit in the intestinal tract of animals. The microbiota forms a unique and complex gut ecosystem by interacting with the host, and this affects the health and diseases of the host in various ways, including the modification of the immune system. To protect from the gut commensal microbiota, animals have evolved a unique gut immune system, which is by far the largest component of the peripheral immune system, harboring ~70 % of peripheral immune cells. The gut immune system has evolved to sense the quality and quantity of the gut microbiota to contain as well as maintain them and has several distinguishing features such as unique antigen-sampling epithelial M cells and a preponderance of secretory immunoglobulin A in the gut. On the other hand, certain gut commensal microbes promote the development of particular subsets of CD4+ T cells, such as Th17 and regulatory T cells. As such, the host gut immune system and gut commensal microbiota have coevolved and influence each other to maintain gut ecosystem homeostasis.

1 Introduction

An enormous number of bacteria inhabit the intestinal tract of animals including humans, and our ancestors have coevolved with them since the most ancient times (Blaser and Falkow 2009). Collectively termed the gut commensal microbiota, in the human colon, it consists of as many as hundreds of trillions of cells, made up of ~500–1000 species (Peterson et al. 2015), and easily outnumbers the ~40 trillion somatic cells that make up the human body (Bianconi et al. 2013). As such, the gut

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commensal microbiota forms a unique and complex gut ecosystem by interacting with the host and affects our health and diseases in various ways (Lozupone et al. 2012). The modification of the gut immune system is one such example. The relationship of the gut microbiota and the mucosal immune system is not one way but rather reciprocal; while commensal microbes change the quality and quantity of the gut immune cells, the gut immune system controls the composition of the gut microbiota. As a result, gut immune homeostasis is normally maintained. In this chapter, the features of the gut immune system will first be introduced, followed by a discussion of how it interacts with the microbiota and then how gut microbes shape the host immune system.

2 The Gut Immune System

2.1 Immune Effector Sites in the Gut

The mucosal immune system is by far the largest peripheral immune tissue in the body, with the majority of immune cells accumulated in the gut (Neutra et al. 1996; Macpherson et al. 2008). The gut immune system is both anatomically and functionally separated into two compartments, inductive and effector sites (Brandtzaeg et al. 2008). In the immune effector sites, effector cells are diffusely situated in the intestinal lamina propria. These cells include adaptive immune lymphocytes (e.g., effector T cells and immunoglobulin (Ig) A (IgA)-producing B cells or plasma cells) as well as innate immune cells such as dendritic cells (DCs) and macrophages, in addition to the recently identified innate lymphoid cells. There are also cells of a unique T-cell subset, the intraepithelial lymphocytes. As their name suggests, these cells are found scattered between the epithelial cells lining the gut.

In contrast to the blood where IgG is the most abundant class of Ig, IgA is predominant in the mucosal tissues, including the gut. Indeed, IgA is estimated to account for at least 70 % of all Ig produced in mammals (Kraehenbuhl and Neutra 2000; Macpherson et al. 2008; Neutra et al. 1996), a fact that is often unappreciated, since most measurements of Ig levels are performed on serum, where IgG predominates. Human serum IgA is primarily monomeric, but, in striking contrast, IgA produced in mucosal tissues is usually in the form of dimers, or polymers. This polymeric IgA (pIgA) contains joining (J) chain, allowing for its capture by the polymeric immunoglobulin receptor (pIgR) expressed on the basal surface of intestinal epithelial cells (Fig. 1a). The pIgA–pIgR complex is transcytosed across the epithelial cytoplasm to reach the apical surface. The extracellular domain of the pIgR [also called "secretory component" (SC)] is cleaved before reaching the apical surface, resulting in the release of SC into the intestinal lumen with bound pIgA. The SC–pIgA complex is called secretory IgA (SIgA) and is more resistant than free pIgA to degradation by gut microbial proteases and more stable in the harsh intestinal luminal environment (Macpherson et al. 2008).

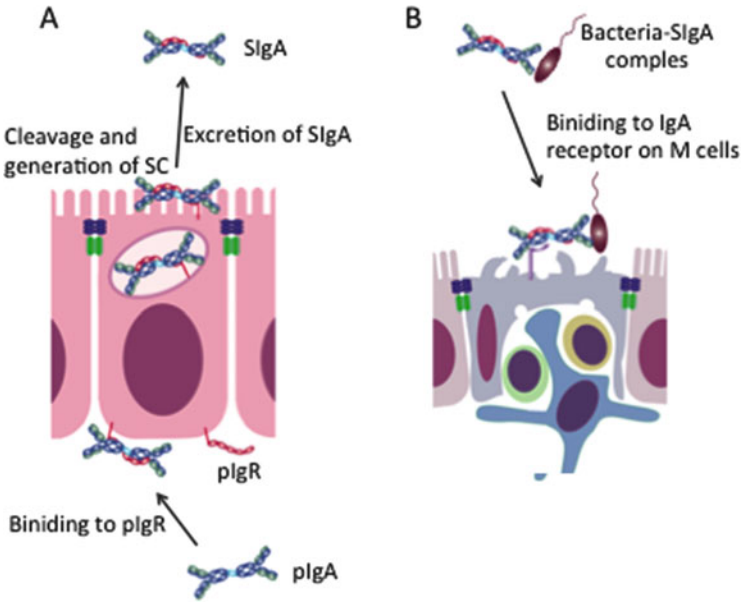

Fig. 1 Schematic view of IgA secretion and uptake. (**a**) Secretion of IgA by the polymeric Ig receptor (pIgR) expressed on intestinal epithelial cells. In mucosal sites, polymeric IgA (pIgA) composed of two or three IgA molecules covalently bound at their C-terminal constant region by joining chain (J chain) is secreted by subepithelial plasma cells. The pIgA is captured in a J chain-dependent manner by pIgR expressed on the basal surface of epithelial cells. The pIgA–pIgR complex is transcytosed through the cytoplasm of epithelial cells via vesicular transport to reach the apical surface. The extracellular domain of the pIgR (also called secretory component, SC) is endoproteolytically cleaved in the vesicle and pIgA is excreted attached to SC and the complex is called secretory IgA (SIgA). (**b**) Uptake of IgA–bacteria complexes by M cells. The bacteria–SIgA complex is bound to and taken up via a putative IgA-specific receptor expressed by M cells. This is thought to serve as the underlying mechanism for immunosurveillance of luminal microbiomes. Note that the basal plasma membrane of the M cell is deeply invaginated to form the "M-cell pocket" into which immune cells migrate and remain closely associated with M cells

2.2 The Immune Inductive Site: Gut-Associated Lymphoid Tissue

In contrast to the rather diffuse nature of immune effector sites, the immune inductive sites in the intestine consist of organized lymphoid structures in the form of aggregated lymphoid nodules (or follicles) such as Peyer's patches (PPs), cecal patches and colonic patches, and isolated lymphoid follicles. These structures consist of B-cell follicles with germinal centers (GCs) in most cases, and the follicles are surrounded by a T-cell rich area (Fig. 2). The immune inductive sites in the intestine are collectively called gut-associated lymphoid tissue (GALT) (Brandtzaeg et al. 2008). The intestinal immune system somehow senses the quality

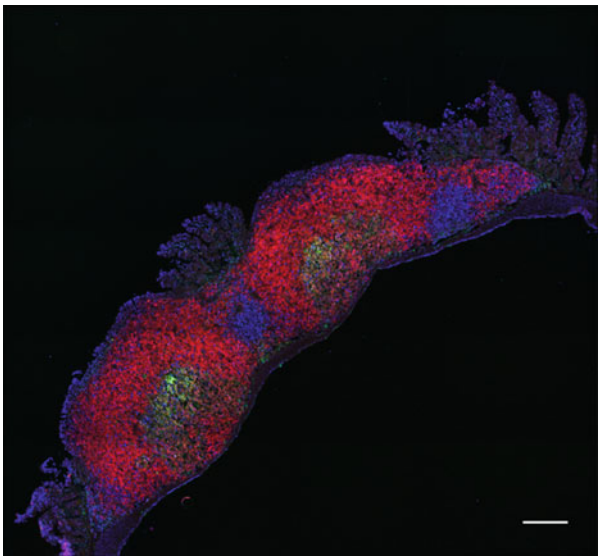

Fig. 2 Immunofluorescence staining of a Peyer's patch. Two B-cell follicles (stained for the B-cell marker B220 in *red*) are surrounded by a T-cell-rich "interfollicular region" (stained for the T-cell marker CD3 in *blue*). Germinal centers are depicted in *green* by staining with the activation marker GL7. Scale bar, 100 μm

and quantity of microbes in the gut and evokes immune responses to produce microbe-specific IgA to both contain pathogens and to maintain homeostasis of the gut microbiota.

2.3 Follicle-Associated Epithelium

Intestinal epithelial cells in the area covering the GALT lymphoid follicles, called follicle-associated epithelium (FAE) (Fig. 3), have different features from those overlying the villi (Kraehenbuhl and Neutra 2000; Neutra et al. 1996). Mucin-producing goblet cells account for 10 % (in the small intestine) to 20 % (in the colon) of intestinal epithelial cells; in FAE, however, few goblet cells are observed. In addition, not many antimicrobial peptide-secreting Paneth cells are found in the crypts adjacent to PP FAE (Giannasca et al. 1994; Kraehenbuhl and Neutra 2000). These observed exclusions are probably due to the fact that the interaction of epithelial stem cells with immune cells in the follicles inhibits their differentiation into these types of progeny cells. Furthermore, the FAE is devoid of the pIgR, resulting in impaired IgA translocation in this region (Kraehenbuhl and Neutra 2000; Neutra et al. 1996; Pappo and Owen 1988). All of these FAE features are conducive for unimpeded local contact of intact gut microbes and pathogens with the FAE surface. However, the most distinguishing feature of the FAE is the presence of "microfold" or "membranous" cells (M cells), as described in the next section.

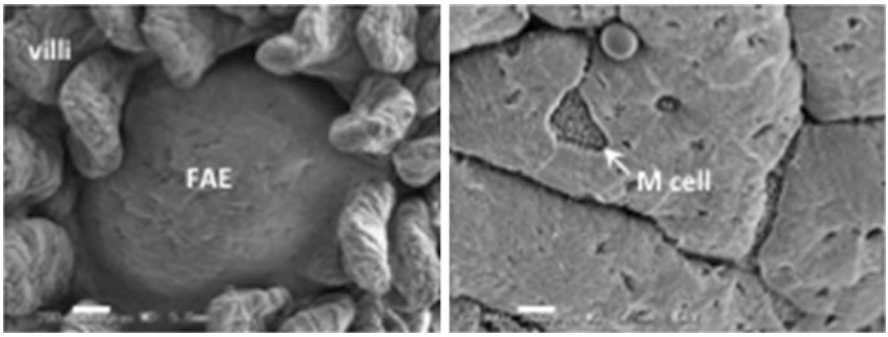

Fig. 3 Scanning electron micrograph of murine Peyer's patch follicle-associated epithelium (FAE) and M cells. *Left*, FAE is seen as a dome-like region in the center, surrounded by villi, the tonguelike protrusions. Scale bar, 50 μm. *Right*, A higher magnification of the FAE. Lacking microvilli, the M-cell surface appears lower compared to the surrounding epithelium. Scale bar, 3.3 μm. Adapted from Ohno and Hase (2006)

2.4 M Cells, a Unique Epithelial Cell Subset Specialized for Transcytosis of Particulate Antigen

In 1922, FAE were discovered as the only region throughout the intestinal tract where particulate materials such as bacteria are taken up across the epithelial layer (Kumagai 1922). However, it took some 50 years to identify the cells in FAE responsible for this uptake. Thanks to ultrastructural analysis made possible by the development of the electron microscope, Bockman and Cooper (1973) found cells vigorously taking up particulate antigens in the FAE of rabbit cecal patches as well as in the chicken bursa of Fabricius, and Owen and Jones (1974) found equivalent cells in human PP FAE (Fig. 3). The cells are called M cells because of their distinguishing morphological feature—small microfolds over their luminal surface instead of microvilli, structures especially prominent in humans. These cells also have a very thin cytoplasm and appear membranous by electron microscopy (illustrated in Fig. 1b) (Owen 1999). This characteristic appearance is due to the fact that the basal plasma membrane of M cells is often deeply invaginated to form a large pocket-like structure (the so-called M-cell pocket) into which PP myeloid cells and lymphocytes migrate (Fig. 1b) and is thought to be essential for M cells to quickly transcytose and transfer the macromolecules and particulates they have taken up to the underlying immune cells (Kanaya and Ohno 2014; Mabbott et al. 2013; Owen 1999). Even though M cells are highly phagocytic, they are definitely of the epithelial lineage, and the expression of Spi-B transcription factor in immature epithelial progenitor cells is prerequisite for M-cell differentiation (i.e., there is virtually no M cell in Spi-B knockout mice) (de Lau et al. 2012; Kanaya et al. 2012; Sato et al. 2013).

M cells uniquely express uptake receptors for various viruses and bacteria, both pathogenic and commensal, on their apical plasma membrane facing the gut lumen

(Chiba et al. 2012; Hase et al. 2009; Iwasaki et al. 2002; Nakato et al. 2012; Tyrer et al. 2006), and the M-cell-mediated delivery of microbes to PP immune cells can initiate efficient antigen-specific mucosal immune responses (Hase et al. 2009; Kanaya and Ohno 2014; Mabbott et al. 2013; Nakato et al. 2012). In the absence of Spi-B in mice, virtually no M-cell maturation is observed, and uptake of *Salmonella Typhimurium* and subsequent immune responses to the bacteria are hampered (Kanaya et al. 2012).

M cells also express IgA receptors on their apical plasma membrane and transport SIgA-bound antigens to the PP (Mantis et al. 2002; Rey et al. 2004). This is also true for the uptake of commensal bacteria bound to SIgA (Fig. 1b) (Rol et al. 2012). Consistent with this notion, the composition of the gut commensal microbiota seems altered in M-cell-deficient compared to wild-type mice (our unpublished observation), suggesting that M-cell deficiency causes dysbiosis. Taken together, M cells play an important role in immunosurveillance, not only of pathogens but also of gut commensal microbiota, at the interface between the intestinal lumen and the intestinal immune system in the gut ecosystem to evoke efficient intestinal immune responses that contain these microbes.

3 Maintenance of Gut Ecosystem Homeostasis by IgA

3.1 Importance of IgA in the Maintenance of Gut Microbiota Homeostasis

The intestinal immune system senses the quality and quantity of gut commensal microbiota and tries to contain it to maintain gut ecosystem homeostasis. The importance of IgA in this process is confirmed by the fact that mice completely devoid of B cells or SIgA have altered gut microbiota (Sutherland and Fagarasan 2012). One such example is mice deficient in the enzyme activation-induced cytidine deaminase (AID). AID is essential for class-switch recombination required for switching from IgM to the other class of Ig, as well as for somatic hypermutation of Ig genes, a process that can contribute to the diversification of antibodies in both humans (Revy et al. 2000) and mice (Muramatsu et al. 2000). As a result, AID-deficient mice lack IgA (as well as IgG and IgE) and instead only possess IgM antibodies with intermediate affinities for antigens. AID-deficient mice display enlarged GALT lymphoid follicles with B-cell hyperplasia, concomitant with a 100-fold expansion of anaerobes in their microbiota. Reduction in the number of gut bacteria with antibiotic treatment normalizes the size of lymphoid follicles and the number of B cells (Fagarasan et al. 2002). AID with the G23S point mutation loses somatic hypermutation activity while maintaining class-switch recombination in mice, resulting in the production of normal amounts of IgA but with less diversity. These mice still suffer from B-cell hyperplasia with gut microbiota

expansion (Wei et al. 2011). Together, these studies suggest that the intestinal immune system can sense abnormalities of the gut microbiota, or dysbiosis, and try to contain them by secreting diversified IgA.

3.2 *IgAs and Gut Commensal Microbiota Reciprocally Regulate Each Other's Diversity to Maintain Homeostasis of the Gut Ecosystem*

The gut commensal microbiota is normally fairly diverse and maintains its homeostasis or symbiosis; once homeostasis is disrupted, however, the diversity of the gut microbiota tends to disappear, and this dysbiosis is thought to contribute to pathogenesis and/or persistence and progression of diseases rather than merely being the result of diseases (Blaser and Falkow 2009; Lozupone et al. 2012). In addition to their well-characterized regulatory T-cell function to be described later, $Foxp3^+$ T cells are also required to generate follicular helper T cells and follicular regulatory T cells in PP GCs. These cells are responsible for diversification and affinity maturation of IgAs, which in turn are required for the maintenance of gut microbiota diversity (Chung et al. 2011; Cong et al. 2009; Kawamoto et al. 2014; Linterman et al. 2011; Tsuji et al. 2009). Reciprocally, gut microbial diversity is required for the expansion of $Foxp3^+$ T cells and subsequent induction of GC reactions and IgA responses in the gut (Kawamoto et al. 2014).

3.3 *IgA Binding Could Discriminate Harmless and Potentially Harmful Microbes (Pathobionts) in the Gut Microbiota*

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, are among the diseases where host susceptibility seems to correlate with gut microbiota composition (Blaser and Falkow 2009; Chow et al. 2011; Lozupone et al. 2012). However, it has been difficult to identify specific members of the intestinal microbiota with disease-driving potential (pathobionts), especially in humans. Palm et al. (2014) have shown that gut microbial species highly coated with IgA are colitogenic and are enriched in the gut microbiota from IBD patients. This observation suggests that IgA could discriminate potential pathobionts and harmless microbes in the gut and that targeted elimination of such species could be disease preventive or therapeutic.

4 Shaping of the Gut Immune System by Commensal Microbiota

The importance of the commensal microbiota for normal development of the host immune system has been suggested from observations made in human infants as well as in germ-free animals. The development of the immune system is impaired in germ-free mice; they have smaller PPs with poor GC formation and decreased numbers of T cells and IgA-producing plasma cells in the lamina propria (hence a low level of fecal IgA) compared to normal gut microbiota-bearing mice (Chinen and Rudensky 2012; Troy and Kasper 2010). Not only the intestinal immune system but also the systemic immune system is affected by the gut microbiota. The normal tissue organization of the spleen is lost in germ-free mice, which have fewer $CD4^+$ helper T (Th) cells, although in contrast, the numbers of $CD8^+$ T cells and B cells are unaffected (Troy and Kasper 2010). $CD4^+$ naïve, i.e., having never encountered specific antigen, T cells have been shown to differentiate into Th1 and Th2 subsets upon antigen stimulation (Mosmann and Coffman 1989); Th1 cells are involved in the eradication of viruses and intracellular bacteria via activation of macrophages and $CD8^+$ cytotoxic T cells, and their unregulated responses can lead to autoimmune disorders, whereas Th2 cells play a role in regulating antibody-mediated humoral immunity and their over activation can cause allergic diseases such as asthma. The balance of Th subsets is skewed to Th2 in the spleen of germ-free mice or in the peripheral blood of human newborns, whose exposure to the gut microbiota is absent or limited, respectively, compared to gut microbiota-bearing mice or adult humans fully exposed to the gut microbiota (Chinen and Rudensky 2012; Troy and Kasper 2010).

Application of recent advances in technology to gnotobiotic mice has revealed some of the mechanisms by which gut microbes shape host systemic and intestinal immune systems.

4.1 Bacteroides fragilis *Polysaccharide A Normalizes the Th1/Th2 Balance of Germ-Free Mice*

Bacteroides are Gram-negative anaerobes and are one of the most predominant bacterial genera in the feces of mammals including humans. *Bacteroides fragilis*, a minor component of the genus, possesses eight gene isoforms encoding capsular polysaccharide, PSA ~ PSH, and each bacterium randomly expresses one of them (Krinos et al. 2001). Kasper and colleagues have been studying the role of PS in the host immune system and have shown that PSA specifically normalizes tissue architecture as well as Th balance of the spleen upon oral administration to germ-free mice (Mazmanian et al. 2005; Troy and Kasper 2010). PSA is reportedly processed in antigen-presenting cells and presented on class II MHC to T cells, leading to the increase in Th cell number (Troy and Kasper 2010). PSA is also shown to interact

with the microbial pattern recognition receptor Toll-like receptor (TLR) 2 on DCs to stimulate these cells to secrete interleukin (IL)-12, which then promotes Th1 differentiation to correct the Th1/Th2 balance (Mazmanian et al. 2005)

4.2 *Small Intestinal Th17 Cells Are Induced by Certain Gut Microbes*

Th17 cells are a relatively newly identified Th subset that secretes interleukin (IL)-17, a strong chemoattractant for neutrophils; hence Th17 cells induce strong inflammatory responses and play important roles in containment of bacterial and fungal infections. On the other hand, excessive Th17 responses can result in autoimmune and inflammatory diseases (Bettelli et al. 2008). Th17 cells accumulate in the intestinal lamina propria, especially in the small intestine.

It has been reported that the small intestinal Th17 cells are induced by so-called segmented filamentous bacteria (SFB) (Ivanov et al. 2009). SFB have been found in the intestine of many animals, including mammals, birds, and fish; however, no SFB-homologous sequence has so far been identified by metagenome analysis of the human gut microbiota (Prakash et al. 2011), suggesting the presence of Th17-inducible microbes other than SFB in the human gut. SFB antigens presented by MHCII on DCs are crucial for the differentiation of Th17 cells, which takes place in the intestinal lamina propria—secondary lymphoid organs are not required (Goto et al. 2014a). SFB-induced Th17 cells are SFB specific, and T cells with TCR specific for SFB antigens become skewed to differentiate into Th17 and not other $CD4^+$ T subsets (Goto et al. 2014a; Yang et al. 2014).

4.3 *Enhancement of Regulatory T-Cell Differentiation by Gut Microbiota-Derived Butyrate*

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells that play an important role in immune tolerance and homeostasis by negatively regulating adverse immune responses, such as autoimmunity, allergy, or excessive inflammation, through their immunosuppressive ability (Shevach 2011). The transcription factor Foxp3 is a specific marker for Tregs and is the master regulator for their differentiation. There are two distinct differentiation pathways for Tregs (Lee et al. 2011). In the thymus, Treg lineage commitment of thymocytes appears to be determined by the expression of Foxp3 upon specific recognition of self-antigen by their T-cell receptor (TCR). By contrast, differentiation of Tregs from $Foxp3^-$ naïve T cells also occurs in peripheral tissues. These two Treg subsets are classified as thymus-derived Treg (tTreg) and peripherally derived Treg (pTreg), respectively (Abbas et al. 2013).

pTregs are especially abundant in the intestinal lamina propria. Differentiation of pTregs appears to be enhanced by the presence of gut microbiota, since germ-free

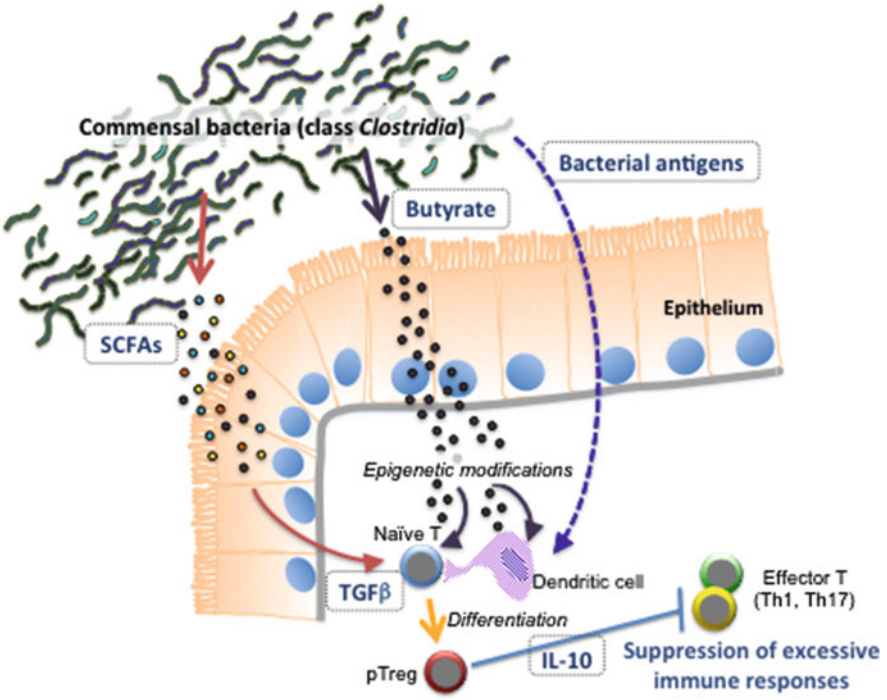

Fig. 4 Schematic view of colonic pTreg induction by gut microbe-derived butyrate. Refer to the text for a detailed explanation [Adapted from the webpage of the Laboratory for Intestinal Ecosystem, RIKEN Center for Integrative Medical Sciences (http://leib.rcai.riken.jp/riken/index.html)]

mice have fewer pTregs in the colonic lamina propria compared to normal gut microbiota-bearing specific pathogen-free (SPF) mice (Atarashi et al. 2011). Honda's group has isolated gut microbes from mice and humans capable of increasing the number of colonic pTregs and has found that these microbes belong to a predominant gut microbial phylum *Firmicutes*, more specifically order *Clostridiales*, with most of them classified into clusters IV and XIVa (Atarashi et al. 2011, 2013).

Metabolome analysis has revealed that colonic pTreg induction in *Clostridiales*-associated mice depends on the bacterial metabolic products of dietary fibers, short-chain fatty acids, especially butyrate (Fig. 4) (Furusawa et al. 2013). Dietary butyrate, for example, in the form of butyrate covalently conjugated with starch (Annison et al. 2003), can induce colonic pTregs in mice via its inhibitory effect on histone deacetylase (HDAC) (Arpaia et al. 2013; Furusawa et al. 2013). Butyrate has long been known as a potent HDAC inhibitor (Candido et al. 1978; Davie 2003). Histone acetylation is regulated by the balance of acetylation by histone acetyltransferases and deacetylation by HDACs, functioning as a molecular switch for epigenetic transcriptional regulation. Chromatin DNA is wrapped around deacetylated histones more tightly than acetylated ones, resulting in easier access of transcriptional regulators to the latter. Indeed, butyrate can upregulate histone

acetylation of promoter and enhancer regions of the *Foxp3* gene locus in naïve T cells to enhance Treg differentiation (Furusawa et al. 2013). As described later in this chapter, G-protein-coupled receptors have also been reported to sense SCFAs and to exert immunomodulatory functions. Nonetheless, based on the studies on mice deficient in these receptors, they are not likely involved in butyrate-mediated colonic pTreg differentiation (Arpaia et al. 2013; Park et al. 2015).

HDACs can act not only on histones but also non-histone proteins to catalyze deacetylation (Seto and Yoshida 2014). Foxp3 is one such protein in that it has been reported to be stabilized and exert enhanced function upon acetylation (Zhang et al. 2012). Consistent with this notion, butyrate can upregulate Foxp3 protein levels per cell in $CD4^+$ T cells (Arpaia et al. 2013).

In addition to its direct effect on $CD4^+$ T cells, butyrate modulates DC function to enhance Treg differentiation, also likely through epigenetic modification via HDAC inhibition (Arpaia et al. 2013). One possible mechanism for this DC effect may be augmentation of antigen presentation. Butyrate alone cannot induce colonic pTregs, and the bacterial cells themselves are also required; butyrylated starch can increase the amount of fecal butyrate in germ-free mice without increasing the colonic pTregs (Furusawa et al. 2013). Bacterial pattern recognition through Toll-like receptors is not involved in the pTreg induction by butyrate, suggesting that bacterial antigens themselves are required.

In addition to TCR signaling, TGF-β is required for Treg differentiation (Chen et al. 2003). A potential source of TGF-β is the epithelium, since treatment of intestinal epithelial cell lines with a mixture of SCFAs can induce TGF-β production (Atarashi et al. 2013).

Butyrate-induced colonic pTregs are important for the maintenance of gut ecosystem homeostasis (Atarashi et al. 2011, 2013; Furusawa et al. 2013). They produce the anti-inflammatory cytokine IL-10 and ameliorate symptoms in disease models of colitis and allergic diarrhea in mice (Fig. 4).

Apart from the above, *B. fragilis* PSA has also been reported to induce colonic Tregs via TLR2 signaling (Round and Mazmanian 2010).

4.4 Anti-inflammatory Property of Colonic Macrophages

Among immune cells in the colonic lamina propria, macrophages are the most abundant (Smith et al. 2005). Different from macrophages in the other tissues, those in the colonic lamina propria show a lower sensitivity to TLR ligands, are more anti-inflammatory, and rather play a regulatory function (Smythies et al. 2005). The low sensitivity to TLR ligands is explained by epigenetic regulation of colonic macrophages by gut microbial butyrate-mediated HDAC inhibition (Chang et al. 2014). Distinct from the above-mentioned pTreg induction, in this case, Mi-2β, a component of the Mi-2/NuRD repressor complex, is recruited to the promoter region of genes encoding pro-inflammatory cytokines IL-6 and IL-12 upon lysine acetylation of histone H3, resulting in the decreased expression of these cytokines.

4.5 *Immunomodulatory Effect of Gut Microbial SCFAs Through G Protein-Coupled Receptors*

SCFAs are also thought to modulate immune responses locally in the intestine as well as systemically through G protein-coupled receptors recognizing SCFAs. GPR109A, a receptor for nicotinic acid (niacin) that also recognizes butyrate with low affinity, is expressed on the luminal surface of intestinal epithelial cells and is suggested to mediate the tumor-suppressive effects of the bacterial fermentation product butyrate in the colon (Thangaraju et al. 2009). GPR109A is also reported to promote anti-inflammatory activity in colonic macrophages and DCs for the induction of Tregs and IL-10-producing T-cell differentiation (Singh et al. 2014). As a result, GPR109A signaling suppresses colitis and colon cancer in mice.

SCFAs have been reported to activate intestinal epithelial cells through their specific receptors, GPR41 and GPR43, for the production of chemokines and cytokines, which mediate protective immunity against bacterial infection and tissue inflammation in mice (Kim et al. 2013a). A study including germ-free and GPR43-deficient mice indicates that gut microbial SCFAs, especially acetate, attenuate inflammation in a mouse experimental colitis model, concomitant with reduced levels of the pro-inflammatory cytokine TNF-α and the neutrophil inflammation mediator myeloperoxidase in the colonic tissue (Maslowski et al. 2009). Mechanistically, systemically absorbed acetate transduces apoptotic and chemotactic signals to neutrophils through GPR43 expressed on these cells, thus suppressing their migration/infiltration to peripheral tissues for exaggerated inflammatory responses. Of note, oral acetate administration ameliorated not only colitis but also asthma (pulmonary inflammation) and arthritis (joint inflammation) disease models in mice (Maslowski et al. 2009). Trompette et al. (2014) have also shown the importance of circulating SCFAs produced by the gut microbiota in protection of mice from allergic inflammation in the lung. In this case, propionate in the drinking water alters hematopoiesis in the bone marrow to enhance generation of macrophage and DC precursors and subsequent seeding in the lung by DCs with impaired ability to induce allergy-promoting Th2 cells, in a GPR41-dependent, but not GPR43-dependent, manner.

GPR43 is also expressed on tTregs, and the oral administration of acetate and propionate, but not butyrate, causes migration of these cells to the intestine through GPR43 signaling to upregulate the expression of GPR15 (Smith et al. 2013), an orphan receptor that is involved in gut homing (Kim et al. 2013b). These results are consistent with the fact that orally administered SCFAs are mostly absorbed in the small intestine to exert a systemic effect rather than acting on the colonic tissue (Pomare et al. 1985) and that butyrate is a weaker agonist than acetate and propionate for GPR43 (Brown et al. 2003).

4.6 Type 3 Innate Lymphoid Cells Regulate Epithelial Glycosylation Important for Host-Microbiota Symbiosis

Fucosylated carbohydrate moieties on intestinal epithelial cells provide an environmental niche for commensal bacteria as a dietary carbohydrate in humans and mice (Bry et al. 1996; Coyne et al. 2005). It has been shown that type 3 innate lymphoid cells (ILC3) promote intestinal epithelial fucosylation by inducing expression of the fucosylation enzyme gene Fut2 (Goto et al. 2014b). This activity requires commensal microbiota-dependent IL-22 and microbiota-independent lymphotoxin production by ILC3. Impaired epithelial fucosylation makes mice more susceptible to *Salmonella* infection, suggesting that ILC3 plays a role in host defense by regulating glycosylation-mediated host–gut microbiota symbiosis. ILCs are recently identified cell types in the immune system, and further studies are required for understanding of their roles in the physiology and pathology of the mucosal immunity (as well as systemic immunity).

5 Conclusion and Perspective

In the gut ecosystem, as described in this chapter, gut commensal microbiota and the host immune system affect each other in various ways, which ultimately is reflected in the host health and disease conditions. Structural components of bacteria sometimes play a role in host immunomodulation, and bacterial metabolites mediate the functions in other cases. A powerful and promising strategy to comprehensively analyze and understand gut ecosystem is the integrated omics approach, where different levels of cyclopedic analyses such as (meta) transcriptomics and metabolomics are combined with the metagenomics. Our knowledge on the functions of gut commensal microbiota is still quite limited, but comprehensive understanding of their roles in our physiology and pathology should allow us to utilize a combination of microbes themselves and their metabolites for preventive and therapeutic use in the future.

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Production of Hepatitis B Vaccines by Beneficial Microorganisms

Chean Yeah Yong and Wen Siang Tan

Abstract Hepatitis B virus (HBV) has infected billions of people worldwide, and currently about 370 million people serve as chronic HBV carriers. Chronic infection by HBV may result in severe liver damage, which may eventually progress to liver cirrhosis and liver cancer. To date, an effective treatment for chronic HBV infection has yet to be established. Microorganisms have been used widely to combat HBV. Since the 1980s, recombinant HBV vaccines based upon HBV surface antigen (HBsAg) produced in yeasts have been used to prevent HBV infection. Large HBsAg (L-HBsAg) has also been produced in yeasts, which is believed to be a solution to nonresponders of the commercial vaccines. Apart from yeasts, bacteria, particularly *Escherichia coli*, have been extensively exploited for the production of HBV vaccines. Although the HBsAg produced in *E. coli* has many limitations as a vaccine, the potential of bacteria-produced HBV core antigen (HBcAg) as a therapeutic vaccine is promising. In addition, bacteriophages which can be used to display foreign epitopes and encapsidate foreign DNA make them excellent tools for developing multicomponent and DNA vaccines. Current recombinant vaccines, although considerably effective, are facing a big challenge to compete with rapid viral mutations, thus justifying a continuous need in the development of HBV vaccines.

1 The Impact of Hepatitis B Virus

Hepatitis B virus (HBV) has a narrow host range and possesses high affinity toward human liver cells. This virus is a hundredfold more infectious than the human immunodeficiency virus (HIV; Centers for Disease Control and Prevention 2015).

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Approximately two billion people worldwide have been infected by HBV, which correspond to one third of the world population. About 370 million people worldwide are chronically infected by HBV (Ward and Byrd 2012), of which about one million die each year (Yan et al. 2012). In other words, about 2 people die of HBV infection every minute. Majority of healthy adults (90 %) infected by HBV undergo acute infection, in which the body immune system responses well to HBV, clearing the virus effectively within weeks to months (World Health Organization 2014). Individuals who recovered from acute HBV infection thus receive protection against future HBV infection. However, about 5–10 % of healthy adults, 50 % of young children, and 90 % of infants infected by HBV developed chronic infection (Hepatitis B Foundation 2014). This happens when the body immune system fails to respond efficiently to the virus, thus allowing HBV persistence.

HBV persistence in the liver can result in severe liver damage, which may eventually progress to liver cirrhosis and hepatocellular carcinoma. About 60–80 % of hepatocellular carcinoma cases were caused by HBV infection (Lavanchy 2005). Current approved treatments for chronic HBV infection include interferon alpha, pegylated interferon, and nucleoside analogs: lamivudine, dipivoxil, adefovir, telbivudine, entecavir, and tenofovir. Interferon alpha and pegylated interferon are cytokines that regulate immune systems against viral infections, while the nucleoside analogs inhibit HBV genome replication. However, all of these treatments only delay or stop the viral replication in order to allow enough time for liver cell recoveries, but do not eradicate the virus totally from individuals chronically infected by HBV. Other approaches aiming to eradicate HBV are still under development and have not been clinically proven effective. Therefore, mass vaccination is still the best strategy to prevent the spread of HBV.

2 Hepatitis B Virus Structure and Replication

HBV belongs to the family of *Hepadnaviridae* and more specifically is grouped under genus *Orthohepadnavirus*. The virus consists of a partially double-stranded DNA genome of approximately 3200 bases, which encodes HBV core antigen (HBcAg), HBV surface antigen (HBsAg), DNA polymerase (reverse transcriptase), and HBV e antigen (HBeAg). The negative-sense strand of the genome is bound to the viral DNA polymerase. The genome and DNA polymerase are encapsidated inside an icosahedral particle made from many copies of HBcAg monomers: $T = 3$, 180 monomers; $T = 4$, 240 monomers (Lee and Tan 2008; Lee et al. 2012a, b; Tang et al. 2007; Yoon et al. 2013). This particle is then enveloped in a lipid bilayer membranous component derived from an infected host cell. Many copies of HBsAg are embedded inside the membrane. The whole virus particle, also known as the Dane particle (Dane et al. 1970), is 42 nm in diameter and is highly infectious to susceptible individuals.

Studies on the replication of HBV had been heavily dependent on the closely related animal cell cultures, and most of the available data have been obtained by genetic approaches using transfection of cells with HBV DNA. Binding of HBV

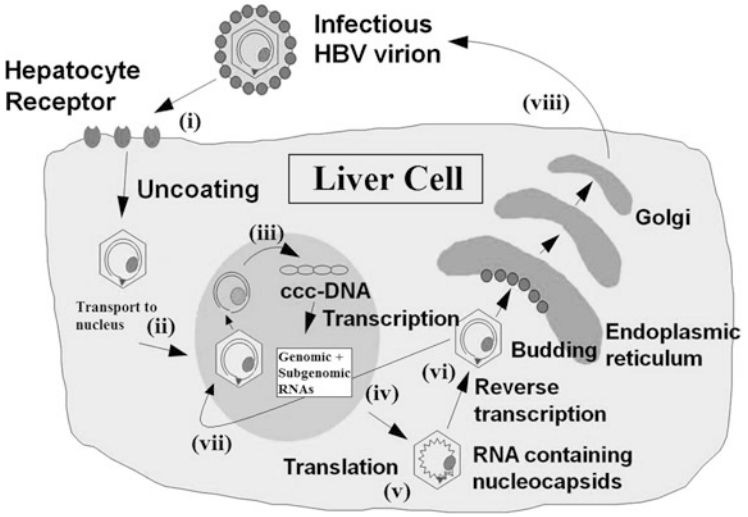

Fig. 8.1 Steps involved in hepatitis B virus (HBV) replication: (i) attachment of HBV virion to hepatocyte receptors and penetration into the liver cell, (ii) removal of envelope and transport of nucleocapsid containing the viral genome into the nucleus, (iii) conversion of viral genome into cccDNA as the template for transcription, (iv) genomic and subgenomic RNA synthesis and transport to the cytoplasm, (v) translation of core protein (HBcAg) and assembly of RNA-containing nucleocapsid, (vi) reverse transcription of RNA, (vii) return of assembled nucleocapsid to the nucleus for the cccDNA amplification, and (viii) envelopment of surface antigens (HBsAg) which are translated and processed in the endoplasmic reticulum and release of HBV progenies from the cell

toward the host cell through the preS1 region of the large HBsAg (L-HBsAg) had been proposed by Neurath et al. (1986) and Pontisso et al. (1989). Receptors on the surface of liver cells that have been reported to interact with HBV include asialoglycoprotein receptor (Treichel et al. 1994), interleukin-6 receptor (Neurath et al. 1992), transferrin receptor (Franco et al. 1992; Gagliardi et al. 1994), and sodium-taurocholate cotransporting polypeptide (NTCP) (Yan et al. 2012). Cell lines expressing NTCP are susceptible to HBV infection, allowing replication of the virus (Ni et al. 2014).

Following uptake of HBV virion, the viral nucleocapsid is transported to the cell nucleus, in which the genome is converted to a covalently closed circular (ccc) form, which serves as a template for transcription of genomic and subgenomic RNAs by using the host RNA polymerase. The mRNAs are then transported to the cytoplasm and translated into their respective proteins. HBcAg assembles into a capsid, packing the genomic RNA along with the viral reverse transcriptase. The partially double-stranded DNA genome is then generated inside the capsid through reverse transcription, as the RNA template gets degraded by RNase. The assembled nucleocapsid is either exported as enveloped virion through the Golgi apparatus or returned to the nucleus to amplify the intranuclear copies of cccDNA. A brief overview of HBV replication is shown in Fig. 8.1.

3 Hepatitis B Virus Surface Antigens

HBsAgs are transmembrane glycoproteins that exist in three different forms (Tan et al. 1999). The full-length *HBsAg* gene has three start codons and one common stop codon; therefore, it produces three polypeptides with different lengths (Bruss and Ganem 1991). If the third start codon is used in the translation, the small HBsAg (S-HBsAg) of 226 amino acids (aa) is produced. When the second start codon is recognized, the middle HBsAg (M-HBsAg) containing 281 aa with an additional 55 aa known as the preS2 region at the N-terminus of the S-HBsAg is produced. If the first start codon is utilized, the L-HBsAg is synthesized. The L-HBsAg has the preS1 region of 108 or 119 aa (depending on HBV genotype) at the N-terminus of the M-HBsAg. All the three polypeptides (S-, M- and L-HBsAgs) have been demonstrated to elicit virus-neutralizing and protective antibodies against HBV infection (Chisari and Ferrari 1995; Neurath et al. 1986; Murray et al. 1984).

In hepatitis B patients, noninfectious viruslike particles (VLPs) of approximately 22 nm (diameter) in spherical and filamentous forms (Fig. 8.2) coexist with HBV infectious particles of 42 nm. These noninfectious particles are mainly composed of S-HBsAg, with only a small proportion of M- and L-HBsAgs. In the 1980s, these

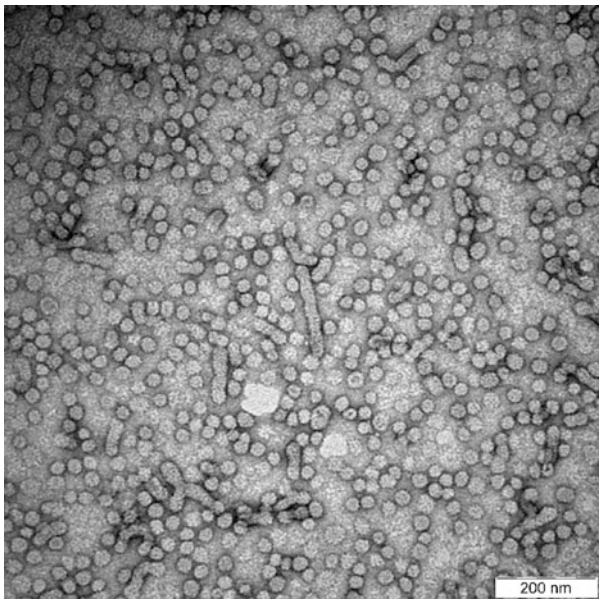

Fig. 8.2 Transmission electron micrograph of noninfectious spherical and filamentous viruslike particles (VLPs). These particles were purified from human plasma and stained negatively with uranyl acetate and viewed under a transmission electron microscope (TEM)

noninfectious VLPs were purified from the sera of infected patients and used as a vaccine. Although this vaccine has been shown to be effective, it involves tedious purification processes and requires large amount of human plasma. To overcome these limitations, recombinant DNA technology was employed to produce the S-HBsAg in *Saccharomyces cerevisiae* as an alternative vaccine (McAleer et al. 1984; Murray et al. 1984). Since then, recombinant S-HBsAg has been expressed in various prokaryotic and eukaryotic systems (Tan and Ho 2014).

The S-, M-, and L-HBsAgs share a common immunodominant region which is located in the S-HBsAg, known as the "a" determinant (Howard and Allison 1995). This immunodominant region induces the production of neutralizing antibodies against HBV infection. It is located in between amino acid residues 121–149 of S-HBsAg (Tan et al. 2005). Although many subtypes of HBV were discovered, the "a" determinant was found to be conserved and highly immunogenic, which often induces cross-protective antibodies against HBV infection.

4 Production of Hepatitis B Vaccines with Yeast Expression Systems

Saccharomyces cerevisiae was the first yeast expression system used in the production of recombinant HBV vaccine based upon S-HBsAg (Hitzeman et al. 1983; McAleer et al. 1984; Miyanohara et al. 1983; Murray et al. 1984; Valenzuela et al. 1982). Studies showed that the recombinant S-HBsAg assembled into 22 nm particles resembling the noninfectious HBsAg particles present in the sera of infected individuals. This recombinant S-HBsAg was demonstrated to protect chimpanzees against HBV challenge through induction of virus-neutralizing antibodies (McAleer et al. 1984; Murray et al. 1984). Since 1986, HBV vaccine based upon recombinant S-HBsAg produced in *S. cerevisiae* has been commercialized as a safe and effective vaccine against HBV infection (Zanetti et al. 1993). To date, the most widely used HBV vaccines in the market include Engerix B, Recombivax HB, Elovac-B, Gene Vac-B, and Shanvac-B. All of these vaccines utilize the yeast expression systems (*S. cerevisiae*, *Pichia pastoris*, and *Hansenula polymorpha*) for the production of the recombinant S-HBsAg.

The commercial HBV vaccines consist of S-HBsAg, although considerably effective, yet 5–10 % of healthy individuals do not or inadequately respond to these vaccines upon immunization (Ottone et al. 2007; Tolosa Martinez et al. 1998). The preS regions of the L-HBsAg are believed to be highly immunogenic (Milich et al. 1985; Neurath et al. 1985) and thus would be of potential to enhance the immune responses of vaccine recipients. When several epitopes have been identified in the preS regions (Sylvan et al. 2009), studies have focused to incorporate these regions into the recombinant S-HBsAg vaccine (Han et al. 2006; Lanford

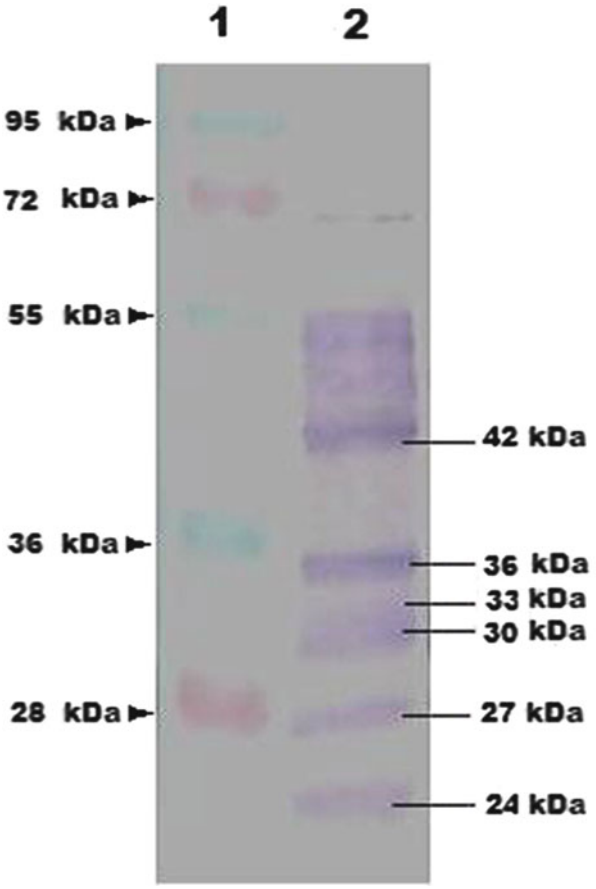

Fig. 8.3 Western blot analysis of HBV surface antigen (HBsAg) produced in *Pichia pastoris*. Cell lysate (~50 µg) was separated on 12 % SDS-PAGE, electro-transferred to a PVDF membrane, and probed with anti-*myc* antibody (lane 2). Lane 1 is the molecular mass markers in kDa

et al. 1989; Shu et al. 2006; Lou et al. 2007). Ferrari et al. (1989) showed that the preS1 region stimulated the cellular and humoral immune responses. In addition, Neurath et al. (1986) demonstrated that residues between 120 and 145 of the preS2 region are the recognition sites for neutralizing antibody production.

L-HBsAg has been expressed in a variety of heterologous systems, including *S. cerevisiae* (Dehoux et al. 1986; Kuroda et al. 1992), baby hamster kidney cells (BHK-21; Patient et al. 2007), Chinese hamster ovary cells (CHO; Diminsky et al. 1997; Yum et al. 2012), insect cells (Sf9; Lanford et al. 1989), and tomato plants (Lou et al. 2007). In these systems, L-HBsAg is able to assemble into particles in the absence of other viral proteins. The L-HBsAg was expressed extracellularly by *P. pastoris*, and two translated products of 48 and 27 kDa were obtained (Han et al. 2006). However, this does not correspond well with the number of translational and posttranslational products produced in eukaryotic systems. We have cloned and expressed L-HBsAg intracellularly in *P. pastoris* (Fig. 8.3). Both the non-glycosylated (24 kDa) and glycosylated (27 kDa) S-HBsAgs were detected,

followed by non-glycosylated (30 kDa), mono-glycosylated (33 kDa), and double-glycosylated (36 kDa) M-HBsAgs. As for the L-HBsAg, the non-glycosylated (39 kDa) form was not detected, but the mono-glycosylated (42 kDa) form was observed clearly. In between 42 and 55 kDa, there are HBsAgs of various sizes, which could be a mixture of double-glycosylated, multiple-glycosylated, and HBsAg dimer (Fig. 8.3). Despite the potential of the L-HBsAg in enhancing the immune responses of its recipients, it has yet to be applied to the vaccine industry. We strongly believe that a relatively higher yield of L-HBsAg in yeast and an efficient purification system will benefit the vaccine industry in the future.

5 Production of Hepatitis B Vaccines with Bacterial Expression Systems

The HBV DNA was first cloned into an *Escherichia coli* plasmid pBR322 by Burrell et al. (1979), where the expressed proteins were demonstrated to be antigenic toward anti-HBV sera. Although currently there are no available HBV vaccines based upon bacterial expression system in the market, the bacterial expression system remains useful in search of an alternative HBV vaccine. S-HBsAg was expressed as a fusion protein with β-lactamase by Edman et al. (1981) in *E. coli*. It was later shown by Fujisawa et al. (1983) that the expression of whole S-HBsAg (without fusion) in *E. coli* retarded the growth of the bacteria and that truncation of the N-terminal hydrophobic region resolved this issue. Expression of L-HBsAg in *E. coli* was also reported as a fusion protein with the anthrax lethal factor, where the fusion protein induced a cell-mediated T cell cytotoxicity (Shu et al. 2006), which is vital for HBV clearance (Bertoletti et al. 2003; Guidotti et al. 1996; Phillips et al. 2010; Thimme et al. 2003). Elghanam et al. (2012) reported that the S-HBsAg with a glutathione S-transferase (GST) tag expressed in *E. coli* was not soluble and thus extraction and renaturation steps were required for solubilization of the HBsAg. In addition, HBsAg expressed in *E. coli* was not found to assemble into VLPs. Despite the difficulties in direct expression of HBsAg in *E. coli*, the common "a" determinant of HBsAg has been successfully displayed on *Macrobrachium rosenbergii* nodavirus capsid particle, which successfully induced both humoral and cellular immune responses in BALB/c mice (Yong et al. 2015).

Although HBsAg expression is less successful in bacteria, HBcAg has achieved great success in bacterial expression system, particularly *E. coli* (Tan et al. 2003; Tey et al. 2006; Yap et al. 2009). The HBcAg produced in *E. coli* self-assembles into VLPs, and many methods have been developed to release the VLPs from the host cell (Ho et al. 2006, 2008a, b, c). The HBcAg VLPs can be purified easily by using sucrose density gradient ultracentrifugation (Tan et al. 1999, 2003; Tan 2002), chromatography (Ng et al. 2006, 2007, 2008, 2012; Tang et al. 2007; Ho et al. 2009; Yap et al. 2010), or electrophoresis (Yoon et al. 2013).

Pasek et al. (1979) expressed HBcAg in *E. coli*, where the HBcAg is highly immunogenic in rabbits, which induced antibodies reactive toward human-derived HBcAg. Murray et al. (1984) showed that the bacteria-produced HBcAg renders chimpanzee partial protection against HBV challenge. It has been demonstrated that HBcAg induces Type 1 T helper (T_h1) responses, which are important for HBV clearance (Aguilar et al. 2004; Chen et al. 2009). HBcAg has also been studied immensely as a vaccine carrier. Additional epitopes, be it from HBV (Lee et al. 2012a, b; Stahl and Murray 1989) and other viruses [human immunodeficiency virus (HIV; Stahl and Murray 1989), Nipah virus (Yap et al. 2012), influenza A virus (De Filette et al. 2005)], have been successfully fused to HBcAg and displayed on the surface of the VLPs formed.

HBcAg, instead of its use to prevent HBV infection, is widely studied for its potentials as a therapeutic vaccine for chronic HBV-infected patients. It has been shown that the HBcAg induced better immune responses than the HBsAg in HBV transgenic mice, which represent chronic HBV carriers (Akbar et al. 2012), and that HBcAg used in conjunction with the yeast-derived HBsAg has demonstrated antiviral and liver-protecting capacities in chronic HBV patients (Akbar et al. 2013).

6 Production of Hepatitis B Vaccines with Phage Display

Current HBV vaccines based on recombinant HBsAg produced in yeast require refrigeration during transportation, which greatly increases the cost of these vaccines, thereby limiting mass vaccination programs in some developing countries. Bacteriophages, commonly known as phages, are viruses which infect bacteria. In addition to yeast and bacteria, recombinant phages have also been used for displaying foreign epitopes, which include those of HBV, for enhanced immune responses (Tan and Ho 2014). The robust structure of phages and their high stability at room temperature provide a solution toward eradication of HBV, as these reduce the need of refrigeration, allowing better access of the phage-based vaccines to the developing countries. The well-established phage display systems include the filamentous phage M13, phage T4, phage T7, and phage lambda.

Phage M13 is an excellent phage display system, as it is a non-lytic phage which does not lyse the host cells during its propagation. This allows the phage particles to be purified easily in the absence of or minimal host proteins, including proteases. Phage M13 also allows the display of longer peptides in its minor coat proteins (pIII) at low copy number (5 per phage) or shorter peptides in its major coat proteins (pVIII) at very high copy number (2700 per phage). Different regions of HBV proteins have been cloned to M13 vectors to demonstrate the potential of this phage display system. These regions include S-$HBsAg_{28–39}$ fused to pVIII protein (Wan et al. 2001), where the recombinant phage induced cytotoxic T cell responses in mice without the need of an adjuvant. Kok et al. (2002) fused the preS region of the

L-HBsAg$_{1–163}$ to pIII in low copy number, where the preS was shown to be antigenic.

Phage T7 encapsidates its double-stranded DNA genome in an icosahedral head formed from 415 copies of 10B proteins (Wong et al. 2013). It is a lytic phage. Unlike M13 phage, the peptide fused to phage T7 does not need to be secreted across host cell membrane, as the phage assembly takes place in the host cytoplasm. This prevents possible misfolding of certain fusion proteins during transportation across the host membrane. The T7 phage particle is also extremely robust, allowing the phage to withstand harsh conditions that would inactivate other phages (Tang et al. 2009). The "a" determinant of S-HBsAg$_{111–156}$ was displayed on phage T7 (Tan et al. 2005). This recombinant phage, when used to immunize rabbit, induced antibodies specific against the "a" determinant of HBsAg, which is believed to be capable of neutralizing HBV (Howard and Allison 1995; Ottone et al. 2007; Pride et al. 1992).

Apart from displaying epitopes, phages such as phage λ are capable of carrying up to 15 kilobase pair (kbp) of foreign genes, allowing it to be used as a carrier for DNA vaccines. A DNA vaccine employing a plasmid containing an eukaryotic promoter such as pCMV (cytomegalovirus promoter) which facilitates the expression of the desired immunogen inside a host cell would thereby induce immune responses. This has been demonstrated by introducing a plasmid-based eukaryotic expression vector harboring *S-HBsAg* gene in mice (Davis et al. 1993). The S-HBsAg translated in cells induced both cellular and humoral immune responses specific to HBsAg (Davis et al. 1993). Plasmids expressing S-HBsAg were packaged into phage λ, which was shown to mount a better immune response compared to the use of a naked plasmid. This is due to a better uptake of the phage by antigen-presenting cells (APC), as well as protection from nucleases (Clark and March 2004; March et al. 2004). It was reported by Clark et al. (2011) that the "phage vaccine" in fact performed better than a commercially available HBV vaccine when tested in rabbits.

7 The Need for Continuing Development of Hepatitis B Vaccines

Up till now, there are still approximately 370 million people worldwide suffering from chronic HBV infection. Currently available yeast-derived S-HBsAg vaccines protected 90–95 % healthy adults. However, 5–10 % of recipients did not or inadequately respond to these vaccines (Ottone et al. 2007; Tolosa Martinez et al. 1998). In addition, 30–80 % of people infected by HIV did not respond to these vaccines (Laurence 2005). Thus, vaccines of higher efficacy, as well as therapeutic vaccines for chronic HBV patients, are in demand.

Mutations of the HBV "a" determinant have also been reported, of which the mutants are infectious even toward HBV-vaccinated individuals (Coleman 2006;

Pawlotsky 2005; Zanetti et al. 1988; Zuckerman and Zuckerman 2003). Prolonged usage of nucleoside analogs in the treatments of HBV chronic carriers generates vaccine escape mutants, of which their *polymerase* and *HBsAg* genes would mutate easily, causing treatments and vaccines ineffective. The nonresponders among HBV vaccine recipients, persistence of chronic HBV infection, as well as the existence of life-threatening mutants justify strongly a continuing need for the development of new HBV vaccines.

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SCFA Producing Gut Microbiota and its Effects on the Epigenetic Regulation of Inflammation

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Abstract Complex carbohydrates are microbial fermented by saccharolytic bacteria in the human gastrointestinal tract, resulting in the production of short-chain fatty acids (SCFAs). SCFAs are considered to affect inflammation and chronic diseases. SCFA-producing bacteria are the link between microbiota functions and epigenetic regulation of inflammatory mechanisms, markers, and personalized preventive healthcare. Even minor changes in single bacterial strains have been shown to affect health or disease, depending on a complex cascade of regulatory immune responses.

1 Introduction

The human gut microbiota (GI MB), our lifestyle, our diet, and our health are clearly associated as exemplified in Fig. 1. Several studies provided detailed insight into host–microbiota interactions, for example, with the metabolic system, immune system, or central nervous system. Culture-independent studies have revealed that a typical gut ecosystem harbors thousands of phylotypes from less than ten bacterial phyla dominated by *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (Salonen et al. 2012). The collective genome of the intestinal microbes has 100 times more genes than the human genome (Qin et al. 2010). All humans have an individual microbiome, so the discovery of the high-level clusters, such as enterotypes (Arumugam et al.), or microbial markers, such as the butyryl-CoA–acetate CoA-transferase gene (Louis and Flint 2007), is of huge interest for routine and research analysis. A high microbial diversity is associated with human health and is observed to be less suggestible to dietary changes. Long-term diet as high meat consumption is associated with a microbiota composition high in *Bacteroides* or a diet high in dietary fibers is associated with *Prevotella*.

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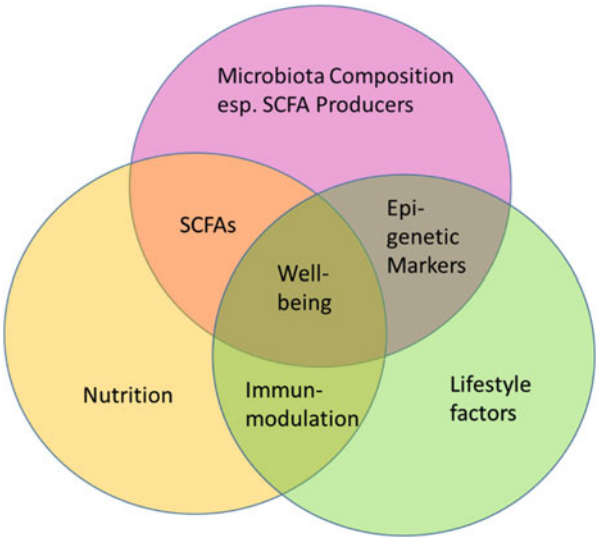

Fig. 1 Exemplified overview of well-being modulating factors including gut microbiota composition, SCFA's nutrition, immune modulation, epigenetic markers, and lifestyle factors, as physical activity or smoking

Modifications in diet can improve diet-microbiota-induced disorders (Zoetendal and de Vos 2014). A diverse and abundant microbiota can provide many functions far beyond nutrient degradation, vitamin synthesis, and resistance against pathogens (Neish 2009). Microbiota degrades complex food ingredients to short-chain fatty acids which are linked to impact the metabolic health of humans due to regulating pathways as satiety, gut permeability, and immune functions.

Complex carbohydrates are microbial fermented by saccharolytic bacteria in the human gastrointestinal tract, resulting in the production of short-chain fatty acids (SCFAs) (as acetate, propionate, and butyrate) (Wong et al. 2006), H_2, and CO_2 (Macfarlane and Macfarlane 2003); proteins and amino acids can also be fermented in the gut by proteolytic bacteria to yield branched-chain fatty acids (BCFA), H_2, CO_2, CH_4, phenols, and amines (Roberfroid 2005). Many products of protein fermentation have toxic effects (Roberfroid 2005).

Metabolized SCFAs are estimated to provide an additional 10 % of total energy intake per day. SCFA may be used for de novo hepatic triglyceride and glucose synthesis (Flint et al. 2008). Due to an effective microbiota, an energy increase of 1 % means an additional 20 kcal per day, resulting in a weight gain of 1 kg after one year (Payne et al. 2011).

Changes in diet are directly associated with changes in GI microbiota and its metabolites (Walker et al. 2011). David et al. (2014) showed that even short-term consumption (3 days) of a plant-based diet alters microbial community structure and overwhelms interindividual differences in microbial gene expression (David et al. 2014). Recent findings have identified specific microbiota profiles and metabolites as predictors of disease risk, e.g., abundance of *Akkermansia muciniphila* for type 2 diabetes (Joyce and Gahan 2014).

Depending on the location in the gastrointestinal tract, the levels of SCFAs are diverse. The highest levels of SCFAs are found in the proximal colon, where they are used locally by enterocytes or transported across the gut epithelium into the bloodstream. Two major SCFA signaling mechanisms are due to inhibition of histone deacetylases (HDACs) and activation of G-protein-coupled receptors (GPCRs).

Inhibition of HDACs has a vast array of downstream consequences (Tan et al. 2014) due to the regulation of gene expression. The main SCFA receptors are the G-coupled receptors GPR43, GPR41, and GPR109A. These receptors are involved in the regulation of inflammation, metabolism, and disease. Modulation of the expression of SCFA receptors has been shown to alter chemotaxis and phagocytosis, has anti-inflammatory effects, change cell proliferation and function, can have antitumorigenic effects, affect reactive oxygen species (ROS) production, has antimicrobial effects, and alters gut integrity. SCFAs are so a major player in the maintenance of gut and immune homeostasis.

Microbiota and SCFAs have been shown to affect the gut barrier function, where a decreased function of the barrier (leaky gut) is believed to contribute in many inflammatory diseases. An increased colonization of *B. thetaiotaomicron* can lead to an increase in villous epithelial expression, which is important for the intestinal epithelial barrier function (Maslowski and Mackay 2011). The barrier depends mainly upon the states of tight junctions (TJs) (Turner 2006). Wang et al. (2012) showed that butyrate at physiological levels (20 mM) was able to stimulate expression of TJ protein claudin-1 and to enhance barrier function. High levels of butyrate could even lower the barrier function due to an increase of the apoptosis rate. A loss of the HDAC3 expression decreases the intestinal barrier function.

2 SCFA Producers Over Lifetime

After birth a fast development of the gut microbiota occurs which is influenced by multiple factors, including the neonate's mother; prenatal exposure; gestational age; mode of delivery; feeding type; pre-, pro-, and antibiotic use; and host genetics. It develops into a complex ecosystem through dramatic changes primarily in the first years of life (Favier et al. 2002; Palmer et al. 2007). An overview of the relative abundance of key phyla of the human microbiota composition in different stages of life is shown in Fig. 2. In genetically susceptible individuals, changes in the gut microbiota induced by environmental factors may contribute to the development of immune-related disorders in childhood, including atopic diseases, inflammatory bowel disease, irritable bowel syndrome, and necrotizing enterocolitis (Li et al. 2014). The adult-like profile of the human intestinal microbiota is stably maintained over time in healthy adults (Lozupone et al. 2012; Faith et al. 2011). Although the mechanisms for the selection of the intestinal microbes are not fully understood, the resulting ecosystem is unique for each subject and is influenced by both genetics and environment (Zoetendal et al. 2001; Turnbaugh and Gordon

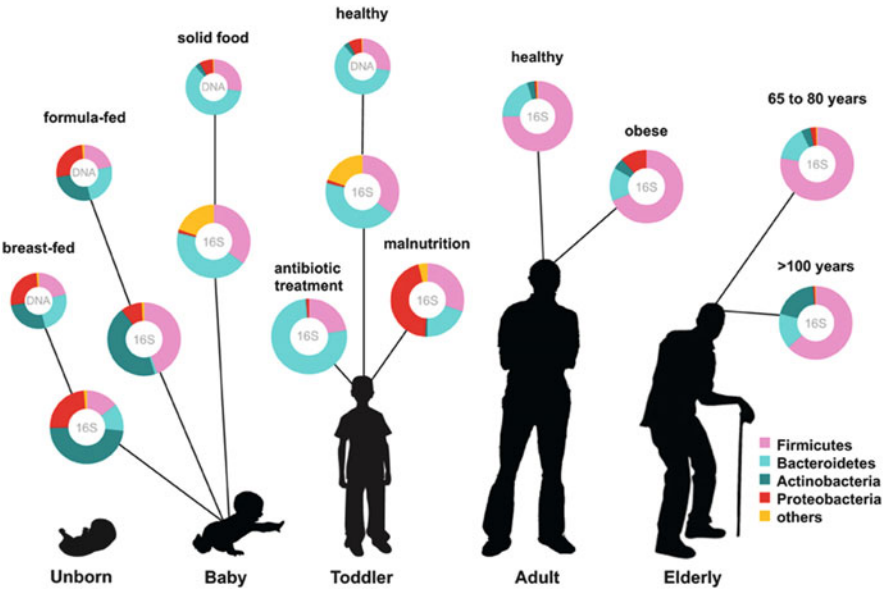

Fig. 2 Human microbiota shift of the four most abundant phyla, over lifetime. Measured by either 16S RNA or metagenomic approaches (DNA). Data arriving from breastfed and formula fed babies (Schwartz et al. 2012), baby solid food (Koenig et al. 2011), toddler antibiotic treatment (Koenig et al. 2011), healthy or malnourished toddler (Monira et al. 2011), adult, elderly, healthy centenarian (Biagi et al. 2010), and obese adult (Zhang et al. 2009). Source of figure: Ottman et al. (2012)

2009). This subject-specific microbiota consists of several hundred microbial species and remains remarkably stable over short-term intervals (Jalanka-Tuovinen et al. 2011; Rajilic-Stojanovic et al. 2012).

The microbiota in children is predominantly composed of *Firmicutes* and *Bacteroides*. *Roseburia*/*E. rectale*, a member of *Clostridia cluster* XIVa, represents a larger *Firmicutes* subgroup in comparison with cluster IV of which *F. prausnitzii* is a member. A higher proportion of *Roseburia*/*E. rectale*/*F. prausnitzii* is detected in children (Payne et al. 2011). In obese children higher levels of butyrate could be observed (Payne et al. 2011). In normal weight children, higher levels of lactate could be observed compared to higher concentrations of butyrate in obese children, suggesting the presence of lactate-utilizing butyrate-producing species in obese children. Acetate/butyrate levels are comparable between lean and obese children (Payne et al. 2011).

In life periods of high energy needs of the host, microbiota adaption has been observed while comparable with eating behavior. Additional proteolytic pathways support the host with additional energy but also with an increase of branched-chain fatty acids as isobutyrate and isovalerate, which are linked to low-grade inflammation and an increase of gut permeability. For example, in pregnancy, Jost et al. (2014) demonstrate that the maternal gut microbiota remains stable over the

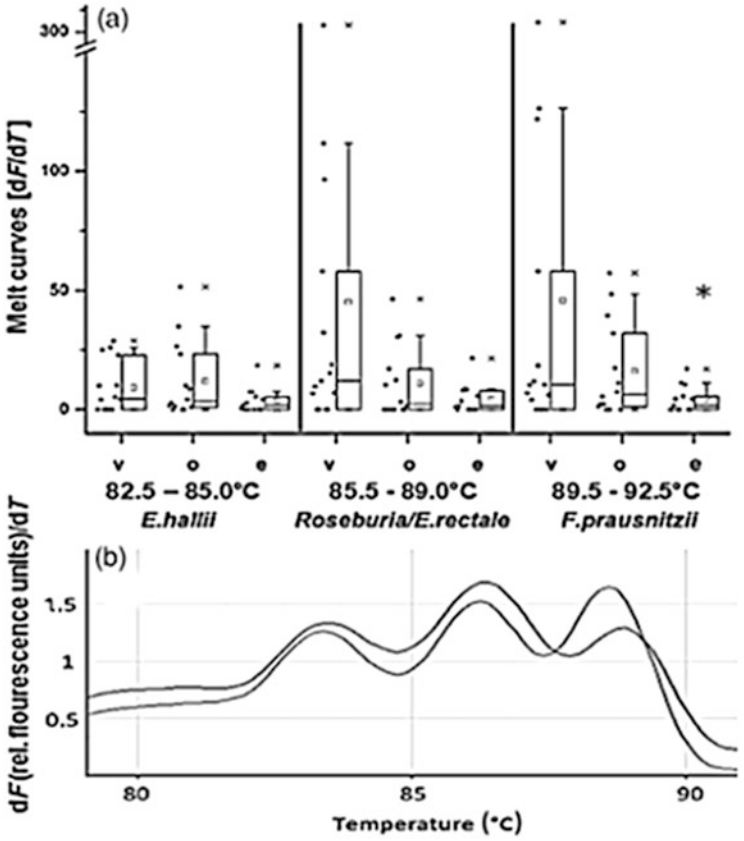

Fig. 3 Significant lower abundance of SCFA producers in elderly subjects (age, 86 ± 8 years; BMI, 21.75 ± 5.08) compared to a young control group (age, 24 ± 2.5 years; BMI, 22.68 ± 3.4). Melt curves are related to *Eubacterium hallii* and *Anaerostipes coli* (82.5–85.0 °C), *Roseburia/E. rectale* spp. (85.5–89.0 °C), and *F. prausnitzii* (89.5–92.5 °C), the main SCFA-producing bacteria in the human gut. Figure source: Hippe et al. (2011)

perinatal period despite altered metabolic activity and low-grade inflammation. Despite its individual gastrointestinal stability, the microbiota seems to change with age as significant differences between the composition of the microbiota of healthy middle-aged adults, elderly, and centenarian subjects have been reported (Rajilic-Stojanovic et al. 2012; Claesson et al. 2012). Hippe et al. showed that especially SCFA producers were significantly reduced in elderly subjects compared to a young control group as shown in Fig. 3 (Hippe et al. 2011).

Aging is linked to a decreased microbiota diversity and abundance; these changes have been connected to immunosenescence and inflammaging (Biagi et al. 2013; Cheng et al. 2013; Cevenini et al. 2013; Franceschi et al. 2000). Due to the reduction of flavor and chewiness, the diet changes over time (Yatsunenko et al. 2012). The reduction in the abundance of genes in pathways involved in SCFA

production is often described in elderly (Hippe et al. 2011; Zwielehner et al. 2009). The aged microbiota shifts from a saccharolytic to a putrefactive metabolism, involving more genes belonging to pathobionts. Due to the loss of commensal bacteria, pathobionts, opportunistic pro-inflammatory bacteria, are able to thrive in inflamed conditions, sustaining and nurturing physiological inflammation (Biagi et al. 2013). The rearrangement of the core metabolic potential of the centenarian intestinal microbial ecosystem is presented by 116 genes (Qin et al. 2010; Turnbaugh et al. 2007; Kurokawa et al. 2007) which significantly correlate with aging. Rampelli et al. highlight the relationship between intestinal bacteria and human metabolism and resume that the microbiota shifts modify or promote physiological changes over lifetime (Rampelli et al. 2013).

3 *Faecalibacterium prausnitzii* Phylotypes in Obesity and Its Consequences in Metabolic Disorders

F. prausnitzii is one of the most common species in the gastrointestinal tract (GIT) of adults consuming a western diet (Scott et al. 2013). *F. prausnitzii* is more common in subjects with high intestinal gut microbiota gene content, and (Le Chatelier et al. 2013) lower concentrations have consistently been observed in patients with Crohn's disease or colorectal cancer. *F. prausnitzii* has been discussed for its anti-inflammatory activity, regulating IL-12, IL-10, and IFN-γ (interferon-γ) in the gut (Furet et al. 2010), and as a marker for a high bacterial gene content and gut well-being (Remely et al. 2013). The two bacterial species *B. thetaiotaomicron* and *F. prausnitzii* are described to colonize the human gut only in coexistence. *B. thetaiotaomicron* is thought to pre-shape the conditions for *F. prausnitzii* colonization (Fig. 4).

F. prausnitzii can convert indigestible fibers into propionate and butyrate (Remely et al. 2013). The colonization of *B. thetaiotaomicron* has been linked to an animal-based diet, high in fat and protein. A balanced diet is associated with the production of SCFA, whereby a high protein intake leads to high amounts of pro-inflammatory branched-chain fatty acids (BCFA), like isobutyrate, and ammonia. In the study of Yatsunenko et al. (2012), metagenomic sequences revealed that enzyme classifications associated with protein degradation and bile salt metabolism were enriched in samples from population where protein and fat consumption is high (Yatsunenko et al. 2012). A diet high in protein and fructose was described to increase TLR 4 expression, leading to activation of innate immune responses. An inulin/oligofructose or resistant starch diet was observed to reduce *Bacteroides* abundance in humans and is known to stimulate the growth of *F. prausnitzii* (Duncan et al. 2002). *F. prausnitzii* has anti-inflammatory activity involved in immune responses: regulating interleukin-2, interleukin-12, and interleukin-10 and IFN-γ (interferon-γ) in the gut (Furet et al. 2010), regulating regulatory T cells and anti-inflammatory cytokines (Qiu et al. 2013).

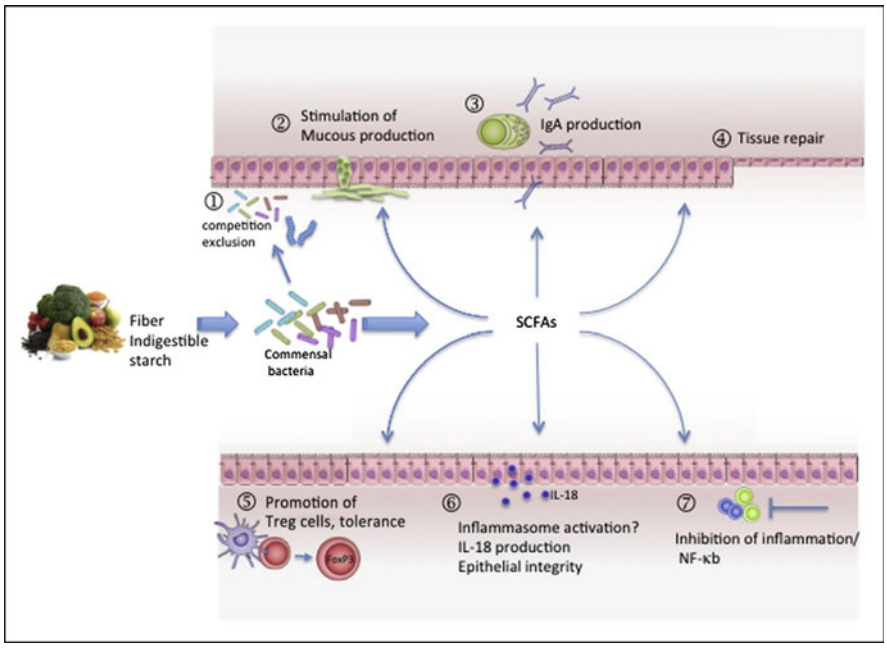

Fig. 4 Graphic simulating the important role for diet and bacterial metabolites such as SCFAs in controlling various immune pathways, including gut and immune homeostasis, regulatory T cell biology, and inflammation. Source of figure: Thorburn et al. (2014)

F. prausnitzii has been suggested as a marker for gut health, but this may only be valid for a western diet, where a *Bacteroides* overgrowth is combined with an efficient butyrate producer to buffer the disadvantages of a high-protein and low-fiber diet (Wrzosek et al. 2013). An increased fiber intake could promote the SCFA production of *B. thetaiotaomicron* and *F. prausnitzii* and benefit from the anti-inflammatory mechanism of butyrate. Butyrate prevents high-fat diet-induced insulin insensitivity due to epigenetic regulation [inhibition of histonedeacetylase (HDAC)], increasing the mitochondrial beta oxidation, and acts so to improve glucose sensitivity and adiposity. *F. prausnitzii* has the ability to switch between diet- and host-derived substrates (Lopez-Siles et al. 2012) and can convert sugars, dietary fibers, proteins, and peptides to butyrate (Remely et al. 2013) using mainly the butyryl-CoA–acetate CoA-transferase route (Louis et al. 2004).

Butyrate is thought to affect diet-induced obesity (Lin et al. 2012) and chronic diseases. It provides additional energy (5–15 % of the total caloric intake) for gut epithelial cells, where the majority of butyrate is metabolized by the colonic epithelium and the remaining is most likely extracted by the liver. It has anticarcinogenic and anti-inflammatory potential (Hamer et al. 2008) and plays a role in adipose tissue expansion (Cani and Delzenne 2011). Concentrations in the venous systemic serum range from 0.5 to 3.3 μmol (Hamer et al. 2008), where

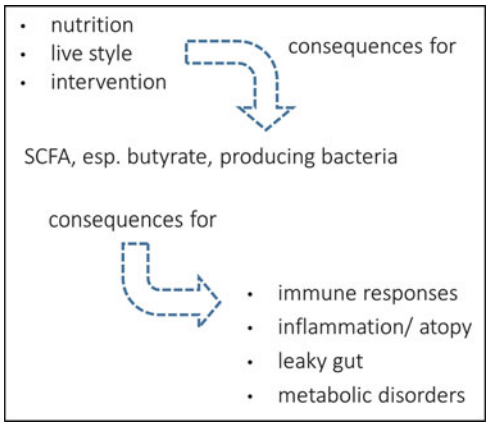

Fig. 5 SCFA producers influence the inflammatory responses, leaky gut, and underlying various complex diseases, including epigenetic regulatory pathways

butyrate acts as a signaling molecule (Brown et al. 2003) via G-protein-coupled receptors (GPRs). *F. prausnitzii* belongs to the *Clostridium leptum* cluster, which has often been associated with type 2 diabetes (Gerritsen et al. 2011) and Karlsson et al. (2013) observed a negative correlation between *Clostridium leptum* and HbA1c values (Karlsson et al. 2013).

Lopez-Siles et al. report that 97 % of *F. prausnitzii* cluster in two phylogroups (Lopez-Siles et al.). The observed strains differed in substrate utilization, pH tolerance, and/or bile sensitivity. Phylogroup I and II strains revealed significant systemic differences in obese subjects compared to diabetic type 2 subjects. *F. prausnitzii* may protect obese subjects from developing type 2 diabetes (Fig. 5).

4 Effects of Short-Chain Fatty Acid-Producing Bacteria on Epigenetic Regulation of Inflammatory Mediators Such As TNF-α, IL-6, FFAR3, TLRs, and NF-κB in Obesity and Related Metabolic Disorders

Since the familiar aggregation of obesity cannot only be explained by the shared environmental conditions, it is widely accepted that the heritability of obesity is between 40 and 70 %. However, risk loci associated with BMI revealed in genome-wide association studies can only explain approximately 16 % of this heritability even if their additive effects are considered (Yang et al. 2011). Thus, epigenetic mechanisms came into the focus of obesity research during the last years to explain the missing heritability. Epigenetic mechanisms describe processes in the surrounding of the DNA that influence the transcription of genes without changes in the DNA sequence itself. These processes include the addition of a methyl group to a cytosine which is followed by a guanosine in the sequence (CpG site). Other mechanisms are structural changes of the chromatin through modifications such

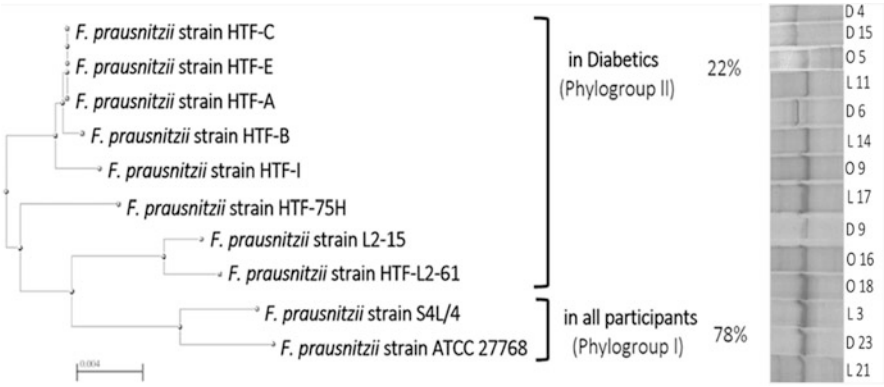

Fig. 6 (**a**) Neighbor-joining phylogenetic tree showing the 16S rRNA sequences matching with sequences from clones of obese, lean, and diabetic individuals. 22 % of sequenced clones match with phylogroup ll, 78 % match with phylogroup I. (**b**) PCR-DGGE fingerprints from *F. prausnitzii* clones of obese, lean, and diabetic individuals. Isolates are distributed in two separate bands that correlate with the phylogroup designation as described by Lopez-Siles et al. (2012). Copy right: Berit Hippe

as, for example, methylation, acetylation, or phosphorylation of the histones. Further, microRNAs which do not code for a protein transcript are involved in epigenetic gene regulation. Several studies have already observed differences in the methylation levels on distinct CpG sites between obese and lean individuals (Feinberg et al. 2010; Soubry et al. 2013; Dick et al. 2014) and after a weight loss intervention (Moleres et al. 2013; Barres et al. 2012; Ronn et al. 2013) (Fig. 6).

Epigenetic mechanisms were also shown to play important roles in the regulation of genes involved in inflammatory processes. Since overweight and obesity are associated with a systemic low-grade inflammation which triggers comorbidities like diabetes mellitus type 2 or cardiovascular events, inflammation is of special interest in the obesity research. The question which still has to be answered is whether the low-grade inflammation is a reason or a consequence of excessive weight gain.

SCFA-producing bacteria are the link between microbiota functions and epigenetic regulation of inflammatory mechanisms, markers, and personalized preventive healthcare (Fig. 7). Even minor changes in single bacterial strains have been shown to affect health or disease, depending on a complex cascade of regulatory immune responses.

Propionate and butyrate are considered to affect glucose regulation, due to regulation of gut peptides (GLP1, PYY), resulting in satiety and weight loss. The pathway is due to inhibiting the insulin signal transduction. Butyrate acts as signaling molecule (Brown et al. 2003) via G-protein-coupled receptors (GPRs). Butyrate can regulate gene expression by inhibiting HDACs (Fig. 7), which has been reported to suppress Treg cell expansion. Butyrate and the less potent propionate induce the differentiation

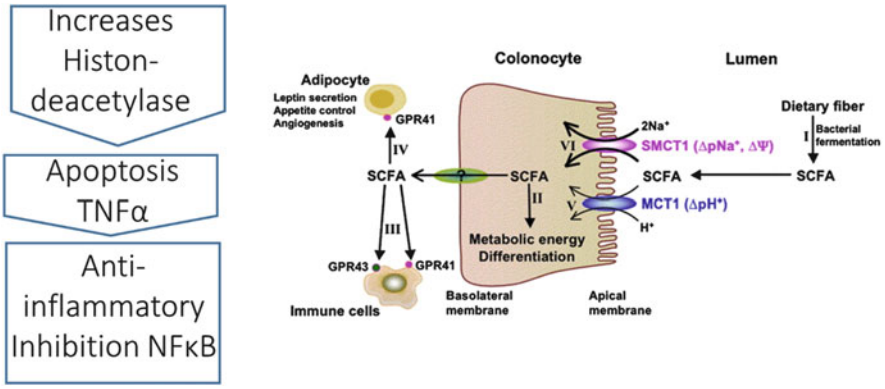

Fig. 7 Intestinal SCFA receptors and transporters. Dietary fibers are fermented by commensal gut bacteria and produce, e.g., SCFAs. SCFAs are taken up by the epithelial cells by diffusion, H+-coupled transport by monocarboxylate transporters (MCT) or by Na+-coupled transport by SLC5A8. Other receptors that are activated by SCFA are localized on colonocytes, peptide YY expressing enteroendocrine cells, or different immune cells. Receptors GPR41 and GPR43 are involved in neutrophil chemotaxis toward sources of SCFA

of colonic regulatory T cells, which affect the suppression of inflammatory and allergic responses in the gut (Furusawa et al. 2013). SCFAs are considered to affect inflammation and chronic diseases (Lin et al. 2012). G-protein-coupled receptor 43 binding regulates activation of B cells, increasing fecal secretory IgA. GPR43 and 41 triggered the inhibition of HDAC 1, enhancing the apoptosis of activated T cells leading to anti-inflammatory potential.

Obesity-associated pro-inflammatory signals like high levels of glucose or lipid intermediates trigger the expression of inactive interleukin-1β (IL-1β). After a repeated signal, IL-1β is activated in an inflammasome complex (Schroder et al. 2010) and subsequently induces the nuclear factor kappa B (NF-κB) pathway leading to an increase of inflammatory mediators (Dinarello 2009).

Adipose tissue produces and secretes several molecules such as cytokines (TNF-α, IL-6, MCP-1, IL-1), leptin, resistin, adiponectin, and visfatin (Kang 2013). Various studies have shown that interleukin-6 (IL-6) is an upstream inflammatory cytokine that plays a central role in propagating the downstream inflammatory response. IL-6 release is stimulated by acute infections, chronic inflammatory conditions, obesity, and physiologic stress (Hartman and Frishman 2014). Studies showed that the circulating levels of IL-6 are elevated in obese individuals up to two- to threefold compared to nonobese (Mohamed-Ali et al. 1997). Further, IL-6 levels were directly correlated with adiposity and insulin resistance (Shoelson et al. 2006).

Intestinal epithelial cells (IECs) contribute especially to the symbiotic system by partially tolerating commensals and regulating mucosal inflammation (Takahashi et al. 2009, 2011). The higher expression of pro-inflammatory cytokines is

coordinated mainly through distribution patterns of toll-like receptor (TLR) and NF (nuclear factor)-κB, a key transcription factor in the inflammatory cascade. Toll-like receptors are common transmembrane proteins in the immune system, recognizing structural molecules derived from microbes (Creely et al. 2007). Each TLR recognizes specific ligands, activating NF-κB (Himes and Smith 2010). LPS (lipopolysaccharide) promotes the transcription of pro-inflammatory molecules and thus induces an inflammatory signaling cascade dependent on the stimulated TLR and TIR (toll/IL-1R homology) domain. Activation of intracellular kinases mediates the activation of the transcription factor NF-κB, which induces expression of pro-inflammatory genes (TNF-α (tumor necrosis factor-α), IL (interleukin)-1, IL-12) in the cell nucleus. These inflammatory markers initialize Th17 (CD4+ T) and Th1 cells and promote an increased generation of chemokines and cytokines (Lee et al. 2010; Sommer and Backhed 2012).

While optimal TLR signaling is required for protection against microorganisms, inappropriate TLR expression and signaling can lead to hyperresponsiveness to bacterial ligands and thus enhance susceptibility to chronic inflammation (Furuta et al. 2008). It can also lead to host destructive pathology, for example, inflammatory bowel disease, diabetes, and multiple sclerosis (Lee et al. 2010; Sommer and Backhed 2012). Metabolic endotoxemia correlates significantly with oxidative stress, macrophage infiltration markers, and all inflammatory markers triggering insulin resistance (PAI-1, IL-1, IL-6, and TNF-α) (Caesar et al. 2010; Cani et al. 2008; Musso et al. 2010; Cani and Delzenne 2009).

TLR4 senses bacterial LPS and saturated fatty acids (SFA) and also endogenous lipids that may contribute to the pathogenesis of lipid-induced insulin resistance (Shi et al. 2006). In vivo, a high-fat diet induced the expression of TNF-α, Il-6, SOC3, and MCP-1. Lauric acid is thought to initiate TLR4 signaling. It is hypothesized that increased fatty acid levels in obese individuals promote TLR4 signaling in adipocytes and macrophages to induce inflammatory signaling and suppress insulin-mediated regulation of glucose metabolism and insulin resistance (Shi et al. 2006). Mice lacking TLR 4 or under antibiotic treatment show a reduction of LPS and are protected from systemic lipid infusion and relieved of metabolic syndrome symptoms (Diamant et al. 2011). A repression of TLR4 gene transcription is mediated by epigenetic mechanisms including DNA methylation and histone deacetylation (Takahashi et al. 2011).

TLR2 senses lipoteichoic acid and FFAs (free fatty acids) (Bayarsaihan). Cell line studies demonstrated that a high CpG methylation of the TLR2 promoter correlates with a lower TLR2 expression (Haehnel et al. 2002). Mice lacking the receptor are associated with lower body weight, lower serum glucose, and improved insulin sensitivity (Kellermayer et al. 2011; Caricilli et al. 2008). Furthermore, an involvement of TLR2 in the regulation of serum lipids is indicated (Himes and Smith 2010).

Given the variety of effects of SCFAs, and that their levels are regulated by diet, they provide a new basis to explain the increased prevalence of inflammatory disease in westernized countries (Tan et al. 2014).

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Bacteriocin from LAB for Medical and Health Applications

Asma Ansari

Abstract The emergence of serious issues of multidrug resistance in the past few years forced the consideration of bacteriocins for combating infections. Numerous concerns have been raised against increased bacterial resistance toward effective drugs and become a debated issue all over the world. Alongside, there is an increase in consumer demand for the antimicrobial compounds isolated or derived from natural sources. Production of antimicrobial compounds is considered as a ubiquitous anti-competitor strategy in microbial ecosystem. Research on antimicrobial compounds with a special interest on bacteriocins is opening a door of a new age. Bacteriocins play an immense role in different industries to overcome various unrestrained environmental issues. Many researchers are now focusing on the bacteriocins of lactic acid bacteria (LAB) with plenty of applications not only in food industries but also in medical and health applications. Their infrequent and targeted use leads to the reduction in the emergence of drug resistance by microbes. Currently, bacteriocins produced by LAB are extensively studied due to their generally recognized as safe (GRAS) status. Various species of LAB are reported to have therapeutic properties that confer beneficial effects on human and animal health. The public health dilemma of drug resistance can be resolved by the discovery of new antimicrobial compounds having target-specific inhibition especially against multidrug-resistant organisms. Consequently, the pool of effective drugs could be available all the time to control newly emerging drug resistance in bacteria.

1 Introduction

Microorganisms produce an astonishing range of defense system and invest substantial vigor for the production and establishment of antagonistic agents. Although the defense system is not as well established as that of vertebrates, these

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microorganisms can protect themselves from foreign invaders by different physical and biochemical defense mechanisms. These arrays of defense mechanisms play a key role in the microbial community and essential for their survival (Riley and Wertz 2002). However, production of antimicrobial substance is an important factor in microbial ecology. Many substances play a key role in bacterial interactions, including enzymes, antitoxins, lytic agents, metabolic products, defective bacteriophages, and bacteriocins (Rice and Bayles 2008). Among them bacteriocins are highly specific and efficient antagonists (Sahl 1994). Undoubtedly, bacteriocins play a crucial role in microbial communities as it is produced by almost all lineages of prokaryotes. By definition, bacteriocins are heterologous antimicrobial peptides produced by microorganisms and have antimicrobial activity against other species. Formerly, it was considered that they are active only against closely related species and have narrow spectrum (Cladera-Olivera et al. 2004), but now bacteriocins have become more imperative because of its broad spectrum of inhibition against both Gram-negative and Gram-positive bacteria. Bacteriocins are nonreplicating peptides synthesized by ribosome. Bacteriocins also inhibit competitors, fighting for the same resources in the similar environment (Whitford et al. 2001). Several kinds of bacteriocins are produced within same species and this property of bacteria makes it more competitive from other species. The bacteriocin producer strain possesses a specific self-protection mechanism against its own bacteriocin (Sahl 1994; Motta and Brandelli 2008).

1.1 Biology and Evolution of Bacteriocins

Although lots of studies have been undertaken on bacteriocins, the detailed evolutionary investigations were done mainly on the bacteriocins of Enterobacteriaceae. Bacteriocins were first identified about 100 years ago in a medium containing culture of *Escherichia coli* and were given the name colicin (Gratia 1925). It was demonstrated that colicins are the narrow spectrum proteins and have antibacterial activity only against the strains, which have receptors on the surface for colicins called sensitive strains (Cascales et al. 2007). Bacteriocins are almost produced by all major groups of bacteria and hence comprising an abundant and diverse family of potent antimicrobials (Gillor et al. 2005). A single species of bacteria can produce multiple types of bacteriocins (Bravo et al. 2009; O'Shea et al. 2011). In microbial ecosystem, 99 % bacteria are capable of producing at least one type of bacteriocin (Riley and Wertz 2002). Bacteriocins are also produced by some members of the Archaea (O'Connor and Shand 2002). They are heterogeneous and diversified proteins in terms of their mode of action, molecular weight, activity spectra, immunity mechanism, release, and target sites. Another feature of bacteriocin is that no specific media and cultural conditions are required for the production and screening of bacteriocins.

The term bacteriocin was often confused by antibiotic. Some specific properties of bacteriocins distinguish it from antibiotics. Bacteriocin is a ribosomally synthesized natural class of antibiotic. Antibiotics are secondary metabolites that are synthesized by multienzyme complexes and cause inhibition in small concentrations. Antibiotics possess no host cell immunity and causes toxicity to host cells (Jack et al. 1995). On the other hand, bacteriocins are nontoxic and have no side effects. Mode of action of bacteriocins also differs from antibiotics as they kill host cells by forming pores and generally affects cell wall synthesis, whereas antibiotics have an effect on cell membrane and targets intracellular structures (Podila and Varma 2005).

1.2 Pharmacodynamics of Bacteriocins

The mode of action of any bacteriocin is species specific. The far and widely accepted hypothesis suggests that generally bacteriocins trigger their effect after binding to the specific receptors which are present on the surface of targeted cells in a reversible manner. Then bacteriocin exerts either morphological and/or biological changes in the metabolic processes of the targeted cells in an irreversible manner. Finally, this change results in the complete eradication of the targeted cells. Burlanek and Yousef (2000) demonstrated that the hydrophobic regions present in the bacteriocin molecules were responsible for the interaction between the bacterial cells and the bacteriocin. However, in some cases, bacteriocins produce a pore in the membrane that causes essential macromolecules to come out and decreases the ATP level of sensitive cell. This mechanism is mostly seen when colicin E1 was studied. Another bacteriocin known as colicin E3 can inhibit protein synthesis while colicin E2 causes denaturation of DNA. Some of the bacteriocins are also known to trigger sensitive cells by the activation of the suicidal enzymes called autolysins. The killing action is varied from species to species. As compared to classical antibiotics, bacteriocins are stronger in action as they exhibit quantal killing process. In this killing mechanism single bacteriocin particle can kill a sensitive cell with specific single hit killing (Bull and Regoes 2006). There are two types of bacteriocins mode of action: bactericidal and bacteriostatic. Some bacteriocins are bactericidal (kill the targeted cells) whereas others act as bacteriostatic (inhibit the multiplication of the targeted cells). One bacteriocin can be bactericidal and/or bacteriostatic for sensitive organisms depending on the species (Pankey and Sabath 2004). Some other factors may also influence this distinction between modes of action. These factors include dose of the bacteriocin used, grade of purification of bacteriocin and conditions, and the state of targeted cells (Cintas et al. 2001). Clinically, the choice of using bactericidal and/or bacteriostatic drug depends on the type of infection. Sometimes combinations of both drugs are used to treat specific infections.

1.3 Genetics and Immunity Against Bacteriocins

The genes responsible for the production and immunity of bacteriocins are generally organized in operon clusters and mostly exist in an autosomal state and associated with plasmids (Ennahar et al. 2000). Many non-lanthionine containing bacteriocins are also encoded by plasmid borne genes (Jack et al. 1995). Bacteriocinogenic organisms also possess a specific self-protection mechanism that confers resistant to their own bacteriocins. There are several ways for the development of immunity. In some cases certain immunity proteins are synthesized after adsorption of bacteriocin to cell surface and protect the producer strain by forming firm complexes between bacteriocin and producer cell. Genes encoding immunity proteins are in close genetic proximity to other structural and processing genes of bacteriocin. Sometimes producer strains can also form protoplasts which provide immunity against bacteriocin. Some bacteriocinogenic organisms are also known to produce lipoteichoic acids due to which the producer strain becomes more positive and repel positively charged bacteriocins (Héchard and Sahl 2002).

Two types of immunity systems have been depicted for the bacteriocins of LAB. In case of lantibiotics (class I) bacteriocins gene cluster consists of a structural gene, genes encoding accessory proteins, transport genes that code for an ABC-superfamily of transport proteins, regulatory genes, and immunity genes (Kolter and Moreno 1992; de Vos et al. 1995; Jack et al. 1995; Sahl et al. 1995). The biologically active molecule of bacteriocins is formed as a result of posttranslational modifications of biologically inactive pre-peptides containing an N-terminal leader. This modification is due to the production of accessory proteins which are involved in the proteolytic processing of the leader peptide. Whereas in class II bacteriocins there is no posttranslational modifications responsible for processing and export of biologically active bacteriocin molecule (Deegan et al. 2006). Gene clusters of class II bacteriocins include a structural gene, an immunity gene, and two genes encoding a membrane-associated ATP-dependent binding cassette (ABC) transporter, and an accessory protein (Klaenhammer 1993; Nes et al. 1996; Eijsink et al. 1998; Ennahar et al. 2000). Bacteriocins are usually synthesized as an inactive molecule that contains N-terminal leader peptide. This leader peptide protects the producer strain from inactive bacteriocin and after the proteolytic cleavage of leader peptide the biologically active molecule of bacteriocin is released extracellular (Nissen-Meyer and Nes 1997; Nes and Holo 2000; Eijsink et al. 2002).

With the advancement in genomics and proteomics, the bacteriocinogenic factor can be transferred to the recipient strains through conjugation. Beside conjugation, transduction and transformation are two other methods of plasmid transfer. By these methods, specific genes encoding different bacteriocins harbored in a single strain can be transfer to non-bacteriocinogenic strains and therefore it became capable of producing bacteriocin (Ross et al. 1999). There are very few bacteriocins were reported which have their genetic determinants located on chromosome such as mesenterocin produced by *Leuconostoc mesenteroides* (Osmanagaoglu and

Kiran 2011), bacteriocin 28b (Guasch et al. 1995), and two other bacteriocins produced by *Lactobacillus brevis* NM 24 and *Lactobacillus fermentum* NM 332 (Mojgani et al. 2009).

1.4 Activity Spectrum of Bacteriocins

Studies on the activity spectrum of bacteriocins against homologous and phylogenetically different organisms are important in the characterization and classifications of bacteriocins. This criterion also helps in the development of bacteriocin typing procedures. Spectrum of inhibition is also an important criterion to differentiate between bacteriocins of Gram-negative bacteria and Gram-positive bacteria. One of the main reasons of escalating the importance of bacteriocins produced by Gram-positive bacteria is their broad inhibitory spectrum. Generally bacteriocins produced by Gram-negative bacteria exhibited narrow spectrum of inhibition only against closely related species, whereas the effect of Gram-positive bacteriocin is not restricted only to their phylogenetically close species. There have been several reports showing inhibition of Gram-negative bacteria by bacteriocins or bacteriocin-like inhibitory substances (BLIS) produced by Gram-positive bacteria (Torkar and Matijasic 2003; Saeed et al. 2004; He et al. 2006; Xie et al. 2009). Bacteriocins can be divided into two main groups on the basis of their production: those produced by Gram-negative bacteria and those produced by Gram-positive bacteria (Heng et al. 2007).

1.4.1 Bacteriocins of Gram-Negative Bacteria

Enterobacteriaceae is a large genus of Gram-negative bacteria mainly involved in the production of bacteriocins. In this genus Col plasmid is responsible for the production of bacteriocin. One of the members of this genus is *E. coli* involved in the production of bacteriocin known as colicin. Colicin is a high molecular weight protein and has been largely studied among various Gram-negative bacteria. After several years of study, colicin is used as a model to determine the mechanism and mode of action of other bacteriocins. Colicins act as a virulence factor and infect the targeted cells by inhibiting one or more essential metabolic mechanisms of targeted cell. Colicin affects DNA replication, arrests cell metabolism, induces pore formation, and halts protein synthesis at transcription and translation level (Cascales et al. 2007). *E. coli* also produces another bacteriocin called microcins, which are similar to the bacteriocins produced by Gram-positive bacteria in terms of thermal stability and pH stability and are resistant to proteases. Beside *E. coli*, other Gram-negative bacteria are also involved in the production of bacteriocins. Pyocins or aeruginocins are naturally occurring bacteriocins produced by *Pseudomonas aeruginosa* (Michel-Briand and Baysse 2002). Similarly, Klebicin is the bacteriocin produced by *Klebsiella pneumonia* which is also a Gram-negative bacteria (Riley

et al. 2001). Along with bacteria, species of Rhizobium were also reported for the production of bacteriocins (Prabhavati and Anthony 2012). The limitation of bacteriocins produced by Gram-negative bacteria is that they are active only against species that are phylogenetically close to them and thus they have narrow spectrum of inhibition.

1.4.2 Bacteriocins of Gram-Positive Bacteria

Bacteriocins produced by Gram-positive bacteria have been largely studied and are biochemically and genetically characterized in detail (Navaratna et al. 1998). They are much diversified and more abundant as compared to the bacteriocins of Gram-negative bacteria. A large number of Gram-positive bacteria are involved in the production of broad inhibitory spectrum bacteriocins (Ansari et al. 2012). Bacteriocins produced by Gram-positive bacteria have been evolved in different ways in terms of their specificity and size due to the difference in cell wall composition of both the bacteria. Gram-positive bacteria also possesses broad spectrum of inhibition in contrast to Gram-negative bacteria. The reason is that in Gram-negative bacteria mostly the outer membrane is involved in receptor-mediated activities of specific proteins, whereas in case of Gram-positive bacteria thick multilayered peptidoglycan wall is present instead of outer membrane which facilitates the penetration of small peptides into the murein network without any receptor binding (Jack et al. 1995).

Production of Bacteriocins by Lactic Acid Bacteria

LAB constitute a clade of ubiquitous Gram-positive cocci and bacilli. They are acid tolerant, non-aerobic but aero tolerant and nonspore-forming bacteria. LAB naturally occupies a wide range of ecological niche and comprises a large number of species from different genera including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, *Carnobacterium*, *Oenococcus*, *Pediococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. Historically, LAB is well known for the production of several fermented products. Production of antimicrobial substances from lactic acid bacteria along with different metabolites is an additional advantage for food industry. The use of LAB as a probiotic in the food industry is emerging because it helps to restore the natural flora of the host (Ljungh and Wadstrom 2006). LAB is considered a beneficial organism as it is nonpathogenic and nontoxigenic to humans, animals, and plants and it does not possess any traits that cause disease. Bacteriocins of LAB are studied extensively due to their generally recognized as safe (GRAS) status by the U.S. Food and Drug Administration (FDA). Large numbers of species from this genus are involved in the production of bacteriocins, which are effective mostly against Gram-positive bacteria (Eijsink et al. 2002; Klaenhammer 1993). Lantibiotics is a class of antibiotics produced by *Lactococcus* species. Nisin is one of the lantibiotic, which is

significant and commercially used as a food preservative in almost 50 countries. Nisin was the first bacteriocin approved by the FDA to use in foods specifically against spores of *Clostridium botulinum*. Nisin is accepted as a safe biopreservative for a narrow range of foods because it is only stable at acidic pH and becomes unstable in alkaline food formulations (Delves-Broughton et al. 1996). Consequently, it becomes a challenge for researchers to investigate new bacteriocins that are stable at wide range of pH, temperatures, and enzymes. Many bacteriocins and bacteriocin-like inhibitory substances have been classified for LAB but still limited classification scheme has been devised for other bacteriocins of Gram-positive genera (Abriouel et al. 2011).

1.5 Classification of Bacteriocins

The understanding of the biochemical nature and the kinetic behavior of any bacteriocin will significantly assist in its classification. There is a drawback in the classification of bacteriocins produced by species other than LAB due to the lack of information of complete sequence and its heterogeneous nature. Hence, there is no complete classification scheme available and to date the classification scheme of LAB bacteriocins is used with slight modifications. The main classification scheme devised by Klaenhammer (1993) was exclusively for bacteriocins of LAB only and was further recategorized by a number of researchers. The antimicrobial proteins and peptides which have been completely sequenced and well characterized are known as bacteriocins, whereas other proteins and peptides which are not well characterized are recognized as bacteriocin-like inhibitory substance (BLIS). Lantibiotics are well-characterized posttranslationally modified peptides produced by LAB. Most of the bacteriocins produced by *Bacillus* species belong to the group of lantibiotics due to analogous characteristics (Asaduzzaman and Sonomoto 2009; Bierbaum and Sahl 2009). Nevertheless, according to Nes et al. (2007), it is impossible to characterize newly emerging diversified bacteriocins in a unifying classification scheme. So, there is a need to design a new classification scheme for bacteriocins of new species using LAB classification scheme as a model.

1.5.1 Classification of LAB Bacteriocins

LAB bacteriocins are small molecular weight, cationic, heat-stable, amphiphilic, and membrane permeabilizing peptides. They are divided into four classes on the basis of their structural, genetic, and biochemical characteristics. The classes are Lantibiotics (Class I), non-lantibiotics include small heat-stable peptides (Class II), large heat-labile proteins (Class III), and large circular proteins (Class IV) (Fig. 10.1). Most of the low molecular weight lantibiotics and non-lantibiotics are found to be highly cationic at physiological pH (Cintas et al. 2001).

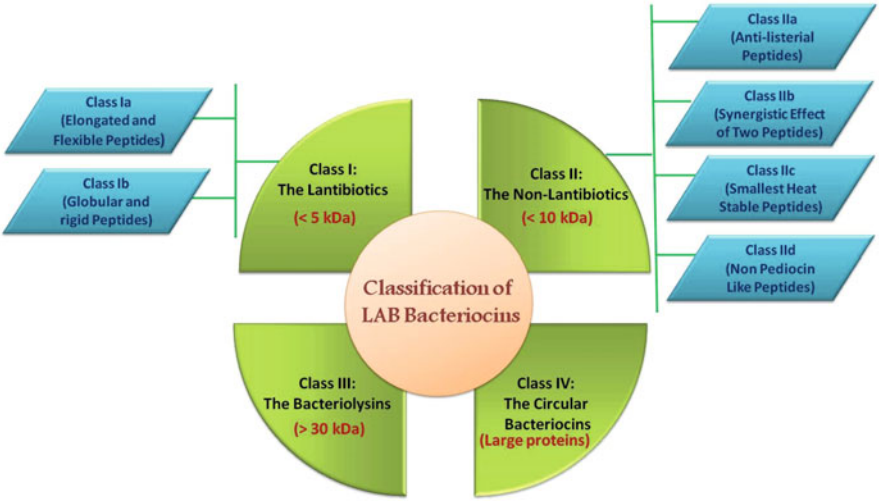

Fig. 10.1 Proposed and modified classification scheme for LAB bacteriocins

Class I: The Lantibiotics

The class I (lantibiotics) is a class of LAB bacteriocins that are modified into distinctive polycyclic thioether amino acids lanthionine or methyllanthionine after translation. It is a two-step process, in the first step unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid were formed after enzymatic dehydration of serine and threonine and then attachment of thiol groups from neighboring cysteins resulting in the formation of unusual amino acids (Sahl and Bierbaum 1998). They are small (<5 kDa), heat-stable peptides effecting on membrane structures. Lantibiotics are the highly studied class of the bacteriocins. On the basis of structural similarities, class I was divided into two subclasses.

Class Ia includes relatively elongated, positively charged, amphipathic, and flexible molecules. Their molecular mass varies between 2 and 4 kDa and they usually induce their antibacterial effect by forming pores through depolarization of the cytoplasmic membrane of the sensitive bacteria. Nisin, epidermin, and subtilin are screw-shaped lantibiotics and representative of the class Ia.

Class Ib is globular in structure and more rigid as compare to class Ia. Their molecular mass lies between 2 and 3 kDa and either have no net charge or a net negative charge. Members of class Ib (mersacidin) generally targets specific components of the bacterial membrane (Brotz et al. 1998). They exert their cytotoxic effect by interfering essential enzymatic reactions of the sensitive strains such as synthesis of cell wall (Deegan et al. 2006).

Class II: The Non-lantibiotics

The distinguished feature of Class II bacteriocins is the absence of lanthionine. They are also small (<10 kDa), relatively heat stable and have three subclasses.

Class IIa constitutes antilisterial peptides with a conserved motif near N-terminal (YGNGVXC). This conserved motif facilitates nonspecific binding to the target surface (Drider et al. 2006; Oppegard et al. 2007). They are synthesized after proteolytic hydrolysis of leader peptide (Venema et al. 1997). Pediocin and pediocin-like bacteriocins, sakacin A, and leucocin A are the well-known representatives of this class.

Class IIb includes two dissimilar peptides that function synergistically to exert their antibacterial effect. One peptide depolarizes the membrane while the other forms pore in the membrane. Lacticin F (Abee et al. 1994), lacticin 3147 (Martin et al. 2004), and lactococcin G (Kjos et al. 2014) are the examples representing this subclass.

Class IIc refers for the smallest heat-stable peptides transported by leader peptides. Some cyclic peptides in which N- and C-terminal are covalently linked with each other are also included in this subclass (Cotter et al. 2005).

Class IId is a new subclass of class II in which nonpediocin-like single peptides are classified by Cotter et al. (2005).

Class III: Bacteriolysins

This class consists of heat-labile, high molecular weight proteins (>30 kDa) that have phospholipase activity. Helveticins J (Joerger and Klaenhammer 1986) and V (Vaugham et al 1992) by *Lactobacillus helveticus* are the distinguished peptides representing this class. Cotter et al. (2005) modified Klaenhmamer classification scheme and renamed class III as bacteriolysins.

Class IV: The Circular Bacteriocins

Class IV constitutes of large proteins. Generally a mixture of lipids and carbohydrates are present with the bacteriocin molecule. This class represents circular bacteriocins. AS-48 (a circular bacteriocin) produced by *Enterococcus faecalis* is classified into this new class (Sanchez-Hidalgo et al. 2011). Some authors also considered cyclic peptides into class IIc of the LAB (Nissen-Meyer et al. 2009).

1.6 Applications of Bacteriocins

In recent years due to the emergence of several health risk factors of chemicals, the consumer's demand for natural food additives and preservatives are extensively increasing. Natural foods containing no chemical preservatives are more fascinated by consumers due to their health benefits. One of the alternatives to fulfill the requirement of food industries is the use of bacteriocins. Bacteriocins can be used in biopreservation, shelf-life extension, and as an antimicrobial agent (Galvez et al. 2007). In the last few decades, multidrug-resistant organisms have received great clinical attention because of increasingly reported cases of drug resistance around the globe (Carlet et al. 2014). Especially in third world countries most of the effective drugs have now turn out to be virtually useless against large number of the organisms. The microorganisms involved in severe infections have developed resistance against one or more than one commonly used broad-spectrum antibiotics (Alanis 2005). The problem is not only due to the microbes that developed different ways to resist effective antibiotics, but also due to the increase over prescribing and inadequate use of the drugs. Previously, several new antimicrobial drugs were either discovered or designed for the control of severe infections. A discovery of new drugs against multidrug-resistant organisms is still in full swing. There are several bacteria in our biome that are capable of producing antimicrobial compounds for combating infections. Among them, bacteriocins are target specific, broad spectrum, and more efficient antagonist as compared to other antimicrobial compounds. Bacteriocins can be used as an alternative therapeutic agent against drug-resistant pathogens and have several promising applications in controlling potential health risk factors (Papagianni 2003). Bacteriocins also play an immense role in different industries to overcome various unrestrained environmental issues (Balciunas et al. 2013).

1.6.1 Medical and Health Applications of LAB Bacteriocins

LAB Bacteriocins in Food Industries

The use of LAB bacteriocins in food industries as a natural preservative can help to reduce the risk of chemical preservatives as well as physical procedures involving intensive heat treatments. Bacteriocin-based strategies for food preservations will result in more naturally preserved foods with rich nutritional properties (Galvez et al. 2007). Bacteriocins can be used in several food formulations to avoid spoilage and deterioration of food, additives or ingredients and also to increase the shelf life of the food (Anthony et al. 2009). Several LAB bacteriocins can be used as an alternative to gratify the increasing consumer's demands for healthy, fresh, safe, minimally processed and ready-to-eat novel food products. Settanni and Corsetti (2008) have reported that bacteriocin producing bacteria can be used to control the flavor and quality of the fermented food products. Nisin is a first licensed

bacteriocin from LAB and is used as a biopreservative (Delves-Broughton et al. 1996). Another broad-spectrum bacteriocin, AS-48, produced by different *Enterococcus* species has been successfully used as a preservative in different products. AS-48 is stable and soluble at wide range of pH and temperatures. Therefore, these characteristics make it an appropriate candidate for food industry (Sanchez-Hidalgo et al. 2011). Bacteriocins produced from *Bacillus* also have applications in food industries. Most of them are active in the presence of food substrates at a wide range of pH and temperatures. Bacillocin 490 fulfills all these features and can be used as a complementary to nisin in processing of acidic, neutral, and alkaline foods processed at high temperatures (Martirani et al. 2002). Along with available commercial preparations of nisin, pediocin PA-1/AcH, enterocin AS-48 and lacticin 3147 also have the potential for food industry.

Besides dairy products, desserts, fruit juices, rice-based foods, sausages, and sauces, bacteriocins also have applications in poultry meat. The contamination of raw poultry and meat products by LAB is a significant public health concern. Specifically *Listeria monocytogenes* is predominantly associated with these products (Chasseignaux et al. 2002). Applications of LAB bacteriocins in poultry and beef meat processing have been explored from decades. The common feature of class IIa bacteriocins is their anti-listerial activity. Several bacteriocins are produced by *Lactobacillus sakei* (Sakacin P, A, G, and Q) and *Lactobacillus curvatus* (Curvacin A) with anti-listerial activity (Dortu et al. 2008). These LAB strains offer promising perspectives as a protective culture and shelf life extenders in meats.

LAB Bacteriocins as Therapeutics

LAB are health-promoting bacteria and very well known as a probiotic in food from decades with the history of safe use. Moreover, everyone expects to see bacteriocins of LAB in food industry as a natural preservative to increase the shelf life of the foods (Anthony et al. 2009). In tandem with all the benefits, various species of LAB are also reported to have therapeutic properties that confer beneficial effects on human and animal health. Though, the emergence of serious issues of multidrug resistance forced the consideration of bacteriocins for combating infections (Lawton et al. 2007). LAB is not only used as a probiotic, but also antimicrobial compounds produced by this genus can be employed for the competitive exclusion of the pathogens (Fig. 10.2). Bacteriocins are target specific and now becoming more imperative because of their wide range of antimicrobial activity against multidrug-resistant pathogens. Broad inhibitory antibiotics are usually nonspecific for target which consequently resulted in the increase in drug resistance by nontargeted microbes. A large number of bacteria from genera LAB are involved in the production of narrow spectrum bacteriocins. This latter feature of LAB bacteriocins makes quite a bit of sense for the eradication of multi drug resistance. Their infrequent and targeted use leads to the reduction in the emergence of new drug resistance by microbes. Since for an effective therapeutics, bacteriocins must

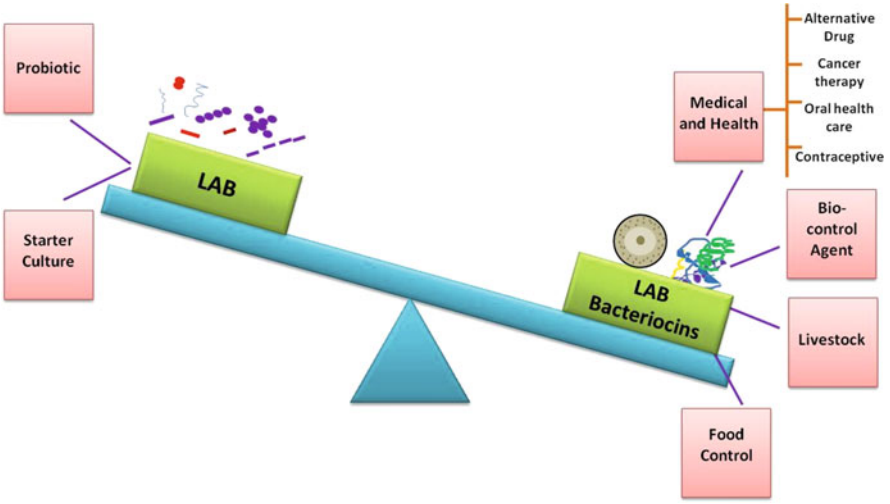

Fig. 10.2 An overview of the applications of LAB and their bacteriocins, emphasizing the role of LAB bacteriocins in medicines and other industries

be active against targeted cells and stable in the intended environment. With the advancement of these target specific drugs the antibiotic resistance will expand more slowly among bacteria. The continuous research and universal abundance of diversified LAB bacteriocins will increase the use of these natural isolates in the health control. Several bioengineered strategies will also be employed to further enhance the commercial potential of LAB and their metabolites in medical and health (O'Shea et al. 2013).

Microorganisms are becoming superbugs by developing new ways to resist multiple drugs. Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococci* (VRE) are multidrug-resistant organisms. MRSA is a more prominent organism in skin infections and involved in several life threatening diseases (Guggenheim et al. 2009; Jensen and Lyon 2009; Kuo et al. 2012). Majority of MRSA infections are classified as community acquired (CA-MRSA) and hospital or health-care-acquired infections (HA-MRSA). This organism is resistant to almost all β-lactam antibiotics and is considered a potential threat to immuno-compromised patients that are at high risk of symptomatic secondary infections (Tacconelli et al. 2008). This organism is considered difficult to treat with a single antibacterial agent because it can frequently develop resistance toward commonly used drugs (Grundmann et al. 2006; Schmitz et al. 1998). Lacticin 3147, a two-peptide lantibiotic produced by *Lactococcus lactis* was reported against MRSA and vancomycin-resistant *Enterococcus faecalis* (Galvin et al. 1999). Another broad-spectrum lantibiotic Mutacin B-Ny266 produced by *Streptococcus mutans* was found to be active against several multiresistant strains of *Staphylococci*, *Streptococci*, and *Neisseria* (Mota-Meira et al. 1997). It was also

active in vivo against the infection of the methicillin-resistant *Staphylococcus aureus* in mice (Mota-Meira et al. 2005).

As discussed earlier that LAB are ubiquitous in nature and comprise not only the surface of animals and plants but also the gastrointestinal (GI) and urogenital tracts of humans and animals. In human body an insubstantial balance exists between the microbial flora and the host which shapes the human health. This microbial flora provides a first stimulus for the development of defense mechanism. The intestinal flora of both human and animals is highly dominated with the species of LAB. Recent studies on LAB have documented their role in the prevention and treatment of gastrointestinal disorders. Lacticin 3147 was found to be inhibitory against *Clostridium difficile* in very low concentrations without affecting the normal resident microflora (Rea et al. 2007). This lantibiotic could be used as an effective oral therapeutic to overcome diarrhea and gastric acidity caused by *C. difficile*. Above all, some reports also highlighted the antagonistic activity of probiotic *Lactobacilli* against human pathogens (Bernet et al. 1994). Predominantly, the *Lactobacillus* and *Bifidobacterium* harbors the GI tract with huge number of species involved in the production of bacteriocins. Some researchers also confirmed an in vivo production of these bacteriocins against food borne pathogens. Candela et al. (2008) reported that several strains of *Lactobacillus* and *Bifidobacterium* were able to protect intestinal lining from acute inflammatory responses caused by the adhesion of *S. typhi* and *E. coli*. As probiotics, these bacteria can confer various health benefits to the host such as reduction of gastrointestinal infections and inflammatory bowel disease by depleting nutrients for pathogens, modulation of the immune system, competition against colonization by pathogenic microorganisms for gut receptor site, and secretion of acids and antimicrobial compounds (Fooks and Gibson 2002). In an in vivo experiment of mice, a broad-spectrum bacteriocin Abp118 produced by *Lactobacillus salivarius* strain UCC118 was found to be potent against food-borne *Listeria monocytogenes* (Dunne et al. 2001).

Alongside other GIT disorders including functional bowel diseases, peptic ulcers are also very frequent. *Helicobacter pylori* is one of the causative agents of peptic ulcers, type B gastritis, and gastric cancer (Cover and Blaser 1995; McGowan et al. 1996; Dunn et al. 1997). It is a Gram-negative organism with a distinguished feature of acid tolerance. Kim et al. (2003) reported that the bacteriocins of LAB such as nisin and lacticins A164 and BH5 can be used for the treatment of peptic ulcers as they found to be inhibitory against *H. pylori*. *Lactobacillus johnsonii* LA1and *Lactobacillus acidophilus* LB were reported for the production of bacteriocin against *Helicobacter pylori* attached with the epithelial cells of intestine. The oral administration of the *L. johnsonii* supernatant (80 ml containing live La1 at concentrations $>10^7$ colony-forming units/mL) to 295 asymptomatic adult patients significantly decreases the infections of *H. pylori* (Gotteland et al. 2008). In another study, *L. acidophilus* strains LB isolated from human stool produce an antimicrobial substance, active *in vitro* and *in vivo* against *S. typhimurium* (Coconnier et al. 1997). *Lactobacillus casei* GG was also screened for the production of antibacterial compound inhibitory against *S. typhimurium* in an in vitro and in vivo experiment (Hudault et al. 1997). Class II nonlantibiotics were also reported

for the control of human diseases. OR-7, a low molecular weight bacteriocin isolated from *Lactobacillus salivarius* strain NRRLB-30514 was inhibited the growth of human gastroenteritis pathogen *Campylobacter jejuni* (Stern et al. 2006). Antagonistic activity of some *Lactobacilli* strains was also reported against the pathogens involved in vaginal infections. A probiotic *Lactobacillus pentosus* strain NCIMB 41114 was patented and used not only to inhibit the GIT pathogens but also for the competitive exclusion of various species of *Candida* associated with vaginal diseases (Wynne et al. 2006). Bacteriocins were also responsible for the inhibition of some important enzymes. Phospholipase A2, an enzyme involved in the production of mediators of inflammation and allergy. Lantibiotics which are able to inhibit the activity of phospholipase A2 can be used as an anti-inflammatory drug (Marki et al. 1991).

LAB Bacteriocins in Livestock

A significant link always exists between the health of human beings and live stock. The use of antibiotics in farm animals was approved by FDA for the treatment and control of diseases and to promote nutritional efficiency. However, the widespread and heavy use of antibiotics is of great concern in the potential transfer of drug resistance from animals to human beings. Research on multidrug resistance in bacteria has proven that the food chain is the major cause of transmission of resistance. Over time, the health threat in this dilemma is the use of same antibiotics of human cure in live stock. Due to this affliction, resistance is more built up and the colonization of human gut with resistant bacteria makes antibiotics less effective. In this context, *Salmonella* and *E. coli* are the two main organisms. Some *Enterococci* were also reported for the direct transfer from animals to the farmers who work with them. Outbreaks of MRSA have also been reported in livestock and their caretakers as well (Ogata et al. 2012).

Some studies on bacteriocins illustrated that bacteriocins can be used to replace antibiotics in live stocks to promote health. In particular, LAB bacteriocins have proven to be induced less antibiotic resistant than other broad inhibitory spectrum bacteriocins. Bacteriocins can play a role to control animal and food-borne pathogens in veterinary industry. Several bacteriocins are reported to promote the animal growth in live stock by improving animal health and controlling pathogens (Diez-Gonzalez 2007). Some of the *Bacillus* strains are also commercially available as a probiotic for farm animal's applications. BioPlus2B is a commercially available mixture of *B. subtilis* and *B. licheniformis* used to improve the health and body weight of live stocks (Mutus et al. 2006). Bacteriocins are also used as a digestive aid in the fermentation of rumen (Pattnaik et al. 2001). Some broad-spectrum bacteriocins reduce mortality rate of farm animals by inhibiting the pathogenic organisms. Mastitis is the major endemic disease in dairy cattle caused by number of bacteria especially *Staphylococci*. Bacteriocins which are active against *Staphylococci* could be applicable to control this inflammation. Stern et al. (2006) were

also reported that purified OR-7 was found to be highly effective in controlling infection of *C. jejuni* in chickens and suggested that nisin, OR-7, and other LAB bacteriocins can be used in replacement of antibiotics in animal feeds.

LAB Bacteriocin in Oral Health Care

Dental caries or tooth decay is a worldwide problem caused mostly by the *Streptococcus mutans* that ferments food carbohydrates into acid. Bacteriocins active against food fermenting organisms can help in controlling tooth decay and gingivitis. A large number of studies declare that LAB bacteriocins are effective in the prevention of this disaster (Howell et al. 1993; Blackburn and Goldstein 1995; Pepperney and Chikindas 2011). Nisin has been formulated in tooth health care products for controlling dental caries and other tooth diseases as it exhibits antimicrobial potential against pathogens causing plaque and gingivitis (van Kraaij and de Vos 1999). Some studies reported the in vivo applications of LAB bacteriocins in various animal models (Howell et al. 1993). In a research, lacticin 3147 was reported to inhibit the growth of *Streptococcus mutans* which is responsible for dental decay (Galvin et al. 1999). Probiotics were available not only for live stocks but also some products were sold in the market for dental diseases. A commercial oral probiotic BLIS K12 contains a strain of *Streptococcus salivarius* which is used to reduce microbes associated with bad breath and also for immune support. *S. salivarius* was reported to produce bacteriocin-like inhibitory substance salivaricin A2 and B responsible for its antibacterial potential (Burton et al. 2006). BLIS M18 by *S. salivarius* is also a new generation of advanced probiotic which goes above and beyond the activity of regular probiotics. It showed wide range of activity against dental pathogens especially against *S. mutans* (Burton et al. 2013). Sometimes due to compromised immune system normal habitants of respiratory tract become pathogen. *S. salivarius* also possesses antibacterial potential against these types of commensals.

LAB Bacteriocins as Biocontrol Agent

The growing knowledge of agrochemicals and their hazards in many facet of life highlighted the need of their substitutes. Agricultural pesticides are an important subset of chemicals and pose unambiguous challenges to humans, animals, and environment (Igbedioh 1991; Forget 1993). These pesticides are nonspecific and in addition to killing insects or weeds they can be toxic to a host of other organisms and nontarget plants. The most effectual way to protect the environment from the pesticides is by reducing their use. Beside these pesticides, traditional procedures were also applied to preserve fruits and vegetables. Arising problems of allergic reactions and carcinogenicity due to these preservatives have diverted the attention of consumers toward natural preservatives. Several overwhelming researches are in the favor of the use of natural compounds for the treatment of plant diseases.

Bacteriocin producing strains as well as their products could be applicable to control phytopathogenic bacteria (Jabeen et al. 2009).

There are number of bacterial destructive diseases affecting both field and greenhouse grown crops. Annually, a great loss occurs due to localized epidemics caused by bacteria to young developing fruits and vegetables. Frequently occurring diseases of crops are bacterial canker, bacterial speck, and bacterial spot. Bacterial canker is a most severe disease of tomato seedlings and tomato plants caused by *Clavibacter michiganensis*. This bacterium also infects sweet pepper, eggplant, and other crops too. It is important to control this disease after the appearance of early signs and symptoms since it is very difficult to control as time passes (Agrios 1997). Ericin S is a bacteriocin active against *Clavibacter michiganensis* and is used as a bioprotectant on crops to control early signs of diseases. Several other BLIS produced by *Bacillus* species also used to control infections caused by *Agrobacterium tumefaciens* (Kamoun et al. 2011). *Bacillus thuringiensis* strain NEB17 produces antibacterial peptide known as thuricin 17 which promote disease resistance in plants (Gray et al. 2006; Lee et al. 2009). LAB and their bacteriocins were also reported as a biocontrol agent against phytopathogenic bacteria and fungi. In vivo efficacy of LAB was detected against phytopathogenic fungi that infect tomato plants (Hamed et al. 2011). In another study bioprotective strains of *Leuconostoc mesenteroides* capable of producing thermostable bacteriocin were found to be effective against infection of *Listeria monocytogenes* in golden apples and iceberg lettuce leaf (Trias et al. 2008). Moreover, some of the BLIS also have a potential to be used as a biocide to resolve problems in petroleum industries. *Bacillus* strains that can tolerate harsh conditions during oil drilling and produce antibacterial compounds against other bacteria involved in biofilm formation can be used to reduce biocorrosion during drilling (Korenblum et al. 2008).

LAB Bacteriocins as Oncolytic Agent

Over the past few years, cancer has become a serious threat to human health. In 2012, 14.1 million new cases of cancer were reported by World Health Organization. One of the most astonishing effects of bacteriocin is its use in the cancer therapy. Bacteriocin due to their microbial cytotoxicity is a new face in the queue of cancer treatment. Some of the bacteriocins produced by Gram-negative bacteria showed cytotoxic effects on malignant human cell lines. Microcin E492 is a bacteriocin with antitumor activity (Lagos et al. 2009). Generally, bacteriocins produced pores in the cytoplasmic membrane which leads to the cell death therefore; it can be used to exert cytotoxic effect on mammalian cells through apoptosis by forming pores. Apoptosis is a general mechanism in cancer therapy which reduces the induction of inflammatory responses. Chumchalova and Smarda (2003) also reported that pore-forming colicin A and E1 inhibited the growth of one human standard fibroblast line MRC5 and 11 human tumor cell lines. Previously, some other pore-forming bacteriocins were also used as an anticancerous agent. Colicin D, E2, E3, and pore-forming colicin A could inhibit the viability of

murine leukemia cells P388. In another case, colicin E1 and colicin E3 suppressed transformed chicken mono blasts (Lancaster et al. 2007). Colicins produced by *E. coli* were also reported as one of the factor to reduce human colorectal carcinoma. Some data also suggested that the bacteriocin producing LAB can also be used to suppress various cancers. Nisin was reported to increase DNA fragmentation or apoptosis and reduced cell proliferation by arresting cell cycle in cancerous cells. In vivo experiments of nisin indicated that this antimicrobial agent also provides a secure and novel therapy for treating Head and Neck Squamous Cell Carcinoma (HNSCC) (Joo et al. 2012). Moreover, nonpathogenic bacteria can be used as a source for the production of anticancerous bacteriocins in specific tumors. The use of bacteriocin for the treatment of cancer could also reduce the risk of secondary infections because during therapy several nonpathogenic bacteria become pathogenic for patients due to suppressed immune system.

LAB Bacteriocins as Contraceptive

Bacteriocins that are active against vaginal pathogens are not only used in feminine health care but also attractive for contraceptive purposes. In 2004, Aranha et al. developed a contraceptive model in rats to evaluate the spermicidal activity of LAB nisin. Results revealed that nisin not only rescued the treated females from pregnancy but also protected them from other side effects. After treatment, fertility was also restored in female animals. This attention grabbing finding came up to the conclusion that 1 mg of nisin completely altered the sperm motility. Subtilosin A is also another safe natural antimicrobial peptide produced by *Bacillus amyloliquefaciens*. One unique property of this bacteriocin is that it is not only antibacterial but also spermicidal (active against spermatozoa) (Sutyak et al. 2008). This feature of bacteriocins may allow the innovative opportunities as natural contraceptives. Further research in this aspect will support the use of bacteriocins as natural contraceptive since many commonly used contraceptive products are unsafe as they contain a compound Nonoxynol-9 (N-9) which is injurious for epithelium.

2 Conclusion

In a nut shell, a large number of bacteria from genera LAB are involved in the production of narrow spectrum bacteriocins. This feature of LAB bacteriocins emphasizes their role in the eradication of multidrug resistance. Undoubtedly, with the progression of target-specific drugs, the antibiotic resistance will be eliminated from the community. Moreover, the continuous research on diversified LAB bacteriocins will increase their applications in the health control. Several strategies can be employed to improve the bacteriocin-mediated protection by health-promoting LAB. Indeed, successful production of genetically engineered

bacteriocins will facilitate the improvement in the bacteriocin potential for medical and health applications. Nevertheless, these bioengineered strategies are only compelling for the generation of bacteriocins for pharmaceutical or fundamental purposes and not for the food. Novel bacteriocins can also be generated by mutating or fusing genes from different bacteriocinogenic species. Besides these recombinant approaches, screening of new antimicrobial agents through highly advanced techniques would also be beneficial for human health. Regardless of all, in many cases, very simple approaches resulted in the production of highly active bacteriocins that may aid to overcome the problems regarding health. After reviewing so many potential applications of LAB bacteriocins in health, it is undeniably to say that these proteins may serve as the next generation of antibiotics.

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Gut Microbiome and Stress

Winnie-Pui-Pui Liew, Jia-Sin Ong, Chee-Yuan Gan, Sawibah Yahaya, Boon-Yin Khoo, and Min-Tze Liong

Abstract The roles of intestinal microorganisms in communication between the gut and brain are gaining increasing recognition. Microbiome driven gut-to-brain communication has been shown to influence stress-related responses in both human and animal models. Sufficient preclinical data are supporting the view that probiotic microorganisms have antidepression potentials. Preclinical evaluations in animal and human models suggested that certain probiotic strains possess anxiolytic activity, similar to antidepressants. The antidepressive effect may be mediated via the vagus nerve, spinal cord, immune systems, or neuroendocrine systems. Such bacterial interactions which cross the interdisciplinary field of microbiology and neurobiology have raised possible alternatives of microbial endocrinology as a natural way to combat stress and/or depression. This current review will address some of the current evidence, possible pathways, and targets of postulated mechanisms.

1 Introduction

A growing recognition has been observed within the fields of microbiology and neurobiology where microorganisms can both produce and recognize neuroendocrine hormones that otherwise have only been associated with a vertebrate nervous

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system (Lyte and Freestone 2010). Studies in the early 1990s have demonstrated that bacterial growth was associated with the presence of neurochemicals, where a log-fold increase in growth of Gram-negative bacteria was observed upon supplementation of stress-related neurochemical norepinephrine. This was supported by subsequent studies which demonstrated that a wide range of bacteria recognized and responded to neuroendocrine hormones leading to changes in growth rates, production of virulence-related factors and aspects of cell physiology in both in vitro and in vivo systems (Oneal et al. 2008). These interactions have since gained much interest, yielding an interdisciplinary field on its own, termed microbial endocrinology or interkingdom crosstalks (Lyte 2013).

2 Stress and the Gut–Brain Axis

Stress arises from physiologically and/or emotionally challenging experiences and is initiated by life stressors. Prolonged stresses often impact negatively on the immunological states. Generally, stressful events are thought to influence the pathogenesis of physical diseases by causing negative affective states such as anxiety and depression. Consequently, this in turn exerts direct effects on stress responses or overall behavior that enhance disease risks. Exposures to chronic stress are considered the most toxic, leading to long-term or permanent changes in the emotional, physiological, and behavioral responses that influence susceptibility to diseases (Cohen et al. 1995).

Stress causes disruptions in homeostasis, placing demands on the body, and activation of two systems, the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS) (Fig. 1). Stressor-induced activation of the HPA axis and the SNS will result in a series of neural and endocrine adaptations known as the "stress response" or "stress cascade". The stress cascade is responsible for allowing the body to make necessary physiological and metabolic changes required to cope with the demands of a homeostatic challenges (Ljung et al. 2000). However, prolonged or repeated activation of the HPA and SNS systems can interfere with their control of other physiological systems, resulting in increased risk for physical and psychiatric disorders (VanItallie 2002). Experimental evidences from animals as well as human studies showed a wide variety of stressful stimuli that can provoke the activation of HPA and SNS systems, subsequently mediating the effects of stress on disease. The primary effector of HPA activation, known as glucocorticoid, regulates a broad range of physiological processes, including anti-inflammatory responses and metabolism. Similarly, catecholamines (noradrenaline and adrenaline), which are released in response to SNS activation, together with the autonomic nervous system act in concert to regulate the cardiovascular, pulmonary, hepatic, skeletal muscle, and immune systems. In addition, stress may also influence disease risk through its effects on the vagal pathway. Psychological stress has been found to impair vagal tone, which may increase the risk of cardiovascular disease (Thayer and Brosschot 2005).

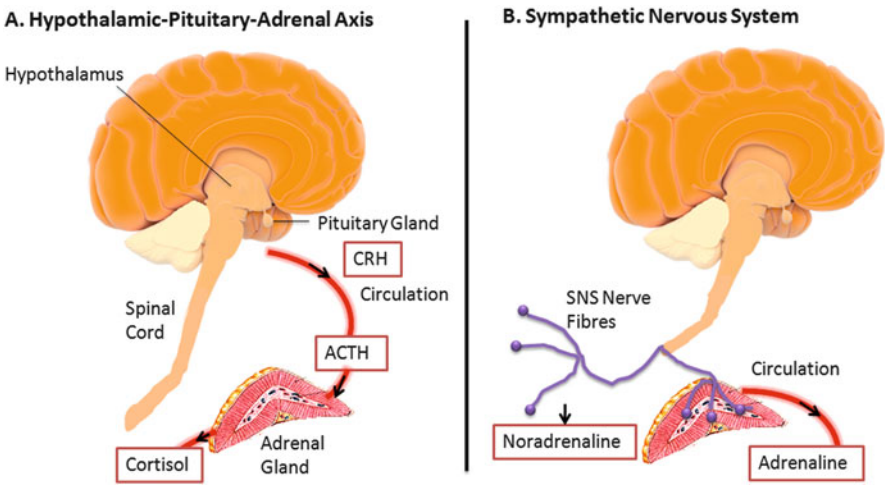

Fig. 1 Chronic stress leads to various diseases, through the activation of HPA axis (**a**) and SNS axis (**b**), with upregulation of glucocorticoid (GCs) (cortisol in human) and catecholamines (CAs) (noradrenaline and adrenaline), respectively. Activation of cytokine receptors in the hypothalamus triggers the production of glucocorticoids by the HPA axis. The anterior lobe of the pituitary gland produces CRH which stimulates the secretion of ACTH, which in turn stimulate the adrenal cortex to produce glucocorticoid hormones. The HPA axis distributes glucocorticoid hormones through the blood to regulate every cell of the body. During fight-or-flight responses and acute injury, nerve fibers from the SNS release neurotransmitter noradrenaline into primary, secondary lymphoid organs and all other major organ systems in which pro-inflammatory reactions occur. SNS nerve fibers can also stimulate the adrenal glands to release stored adrenaline into the systemic circulation. *ACTH*, adrenocorticotropic hormone; and *CRH*, corticotropin-releasing hormone

The "brain–gut axis" is a bidirectional communication system comprising of neural pathways, cytokines, hormones, and neuropeptides as signaling molecules. The bidirectional signaling between the gastrointestinal tract and the brain is dynamic for maintaining homeostasis and is regulated at the neural (both central and enteric nervous systems), hormonal, and immunological levels (Prins 2011). Involvement of these systems in altering both the biological processes and behavioral patterns has gathered much attention lately, typically with a high rate of comorbidity between stress-related psychiatric symptoms and gastrointestinal disorders including irritable bowel disorder (IBS) and inflammatory bowel disorder (IBD). Recent scientific advancement has removed the perception that IBS is an unreal psychological disorder. In addition to the well documented discomfort and irregular bowel patterns associated with IBS, emerging data has shown altered brain structures in IBS patients; brain cortical thinning which may cause impairment in pain inhibition, and increased gray matter density in the hypothalamus which may lead to catastrophizing of pain (Fig. 2) (Blankstein et al. 2010). All these substantiate the crucial roles of the gut–brain axis and the detrimental impacts arising from its dysregulations.

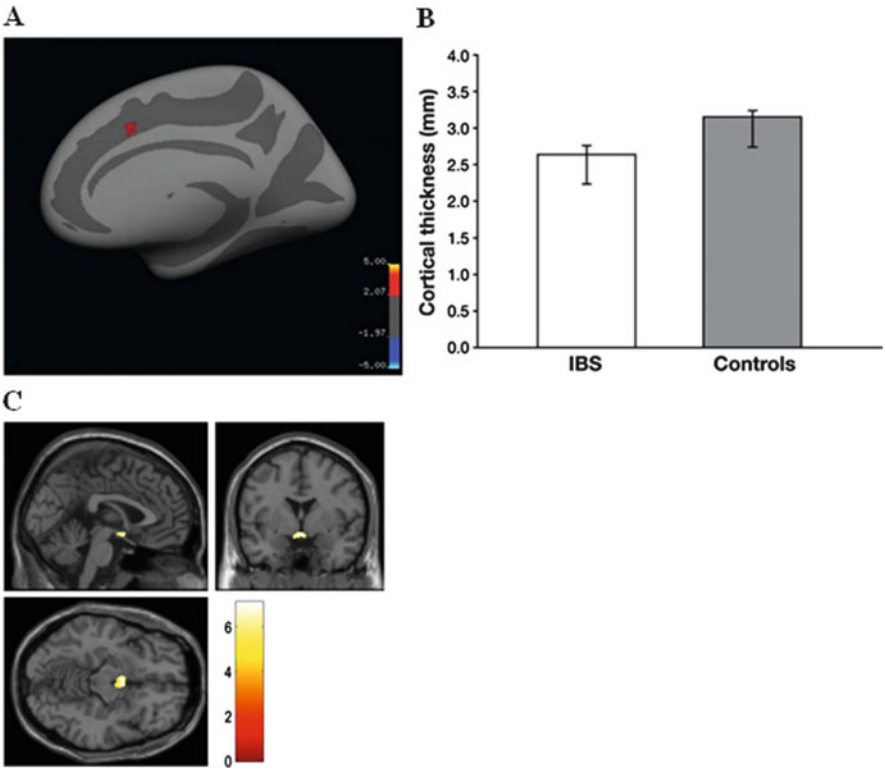

Fig. 2 (**a**) Corticol thinning in IBS patients (*red region*); thickness of cortical in IBS patients which were 16.2 % less than healthy controls (**b**); increased gray matter density (*yellow region*) in the hypothalamus of IBS patients (**c**). Reprinted from Blankstein et al. (2010), with permission from Elsevier (License number: 3603520465613)

Emerging evidences from animal models have supported the role of enteric microbiome in gut–brain communications as signaling components in the gut–brain axis. The microbial gut–brain axis has been recognized to play an important role in mental health-related issues, where these "mind-altering bugs" has been the subject of intensive study since the 1990s (Sajadinejad et al. 2012).

The influence of stress on the intestinal microflora has been investigated in both animals and humans. Various stressors (restraint conditions, acoustic stress, food deprivation, and environmental stress) have been documented to negatively alter gut microflora in the hosts leading to impaired immunity. To simulate stress-associated social disruption, an aggressive male mouse would be placed into the home cage of nonaggressive resident mice. Aggressive mice are often excluded from previous colonies upon observation of aggressiveness toward cagemates. Using such a model, Bailey et al. (2011) have shown that upon six 2-h cycles of social disruption, mice had a higher population of detrimental Firmicutes, accompanied by a lower population of beneficial Bacteroidetes in the cecum (Fig. 3a),

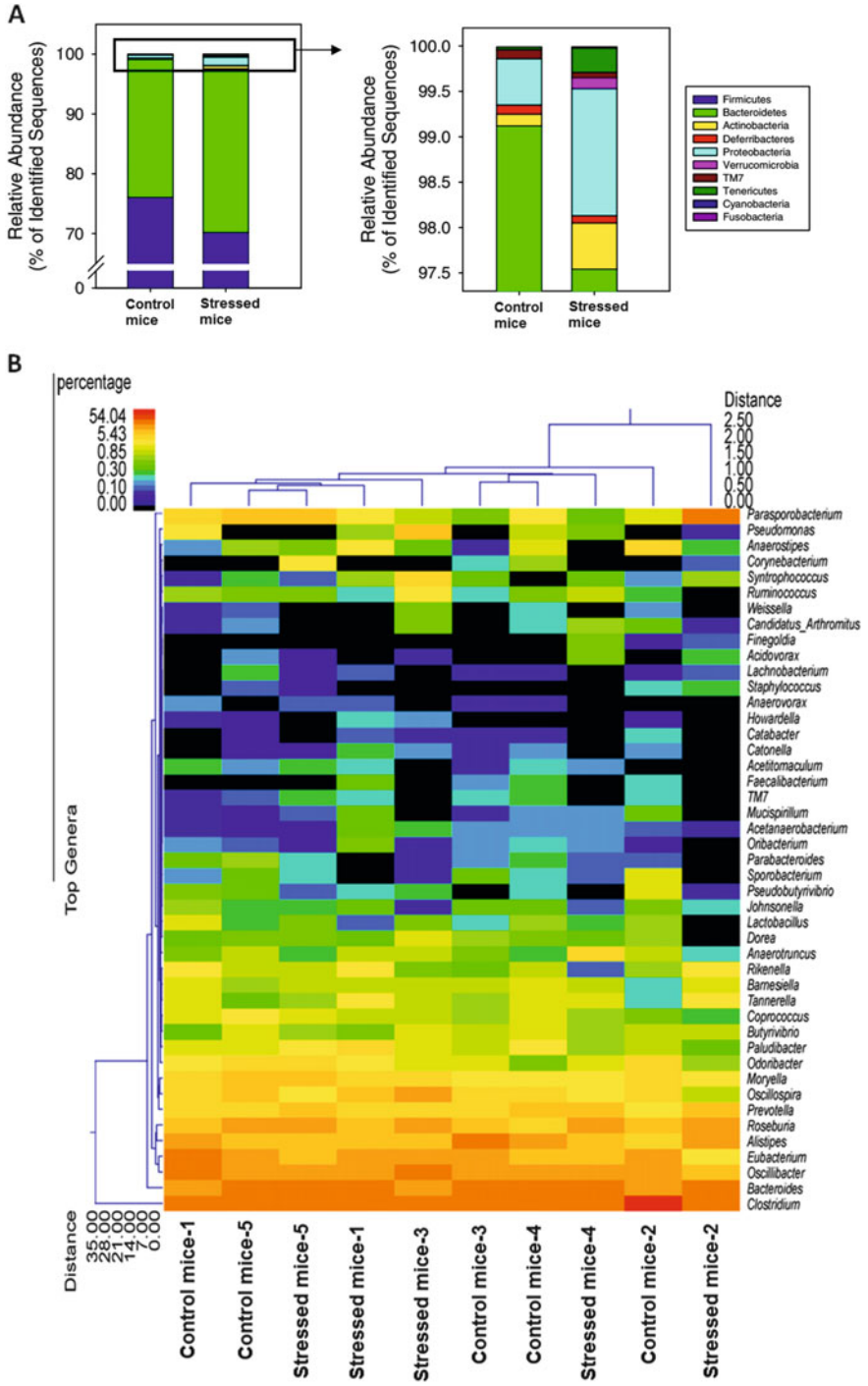

Fig. 3 (**a**) Cecal microbiota of control and stressed mice, upon social disruption via an aggressive male mouse model experiment. Microbiota composition comprised of bacteria from 10 divisions,

although the estimates of diversity and richness at the phylum level of taxonomic analysis revealed similar hierarchical clustering of bacterial genera (Fig. 3b). Stressed mice also had higher circulating levels of interleukin-6 (IL-6), accompanied by lower ($P < 0.05$) population of bacteria in the genus *Coprococcus*, *Pseudobutyrivibrio*, and *Dorea*; and the same time higher circulating levels of chemokine monocyte chemotactic protein-1 (MCP-1), accompanied by lower ($P < 0.05$) population of bacteria in the genus *Coprococcus*. With these suppressions, a bloom in other species such as clostridia may be initiated, which subsequently lead to gut inflammation, as indicated by the increase in pro-inflammatory cytokine IL-6 and chemokine MCP-1 levels.

3 Effect of Gut Microbiome on Stress Responses

In vivo studies have indicated that the enteric microbiota often modify the host's neural functions, leading to altered emotional, and cognitive functions during adulthood. This is supported by the first evidence from studies comparing germ-free (GF) and gnotobiotic animals (Sudo et al. 2004) revealing the importance of microbiota in the development of central nervous system (CNS). The close relationship between gut microbiota and CNS has led to great interest in using probiotics to modulate the gut microbiota to prevent or to treat certain CNS related diseases. Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO 2006). The most common genera of probiotic microorganisms include *Lactobacillus* and *Bifidobacterium*. Although predominantly reported for their beneficial effects on gut health, probiotics have now emerged as beneficial for a wider range of health aspects including, stress management.

A lower gut population of lactobacilli has been associated with stress-indicative behaviors in hosts (Rhee et al. 2009). Maternal stress during pregnancy was seen to be in tandem with a reduced population of both lactobacilli and bifidobacteria in offspring, while a strong connection was observed between prenatal stressors and subsequent depression in offspring (Bailey et al. 2004). In addition, the total population of anaerobes, lactobacilli, and bifidobacteria in guts of infants are positively correlated with independent traits of infants (Pärtty et al. 2012).

From the opposite angles, clinical studies have demonstrated that stress can negatively affect gut microbiota. Emotional and restraint stress, as well as excess

Fig. 3 (continued) n = 5 per group. (**b**) Heat map of control and stressed mice indicating the relative abundance of the different genus. Clustering indicates that 3 of the 5 stressed mice were clustered together with, and thus are similar to 2 of the 5 control mice. One stressed mice clustered with the remaining 3 control mice, and one stressed mouse was found to be unique from the others. Reprinted from Bailey et al. (2011), with permission from Elsevier (License number: 3604670870876)

physical demands have been reported to cause acute and long-term reductions in populations of gut lactobacilli and bifidobacteria (Moore et al. 1978). Understanding the complex relationship between the host and symbiotic microbes will provide important insights for the rational design of therapeutics strategies. Information on the effects of normal gut microbiota and probiotics on stress responses, as well as compounds produced by symbiotic bacteria to reverse the alteration of stress responses implemented by life stressors, will be discussed below. Table **1** provides a detailed summary of the behavioral changes upon manipulation of microbiota profile, while Table **2** tabulates the biochemical and molecular alterations.

3.1 Behavioral Changes

The ability of pathogens to influence host behavior has been well recognized for decades. Among the most dramatic examples is the resulting profound decrease in anxiety-like behavior in *Toxoplasma gondii* infected rodents to a point that they no longer show fear to feline predators (Bisson et al. 2010). In humans, individuals suffering from inflammatory bowel diseases, which are characterized by altered microbial diversity, have demonstrated poorer emotional functions such as having anxiety and depression (Kurina et al. 2001). Many studies have demonstrated that alterations in gut microbiome and pathogens can lead to the release of host immune factors, such as cytokines and other inflammatory mediators that have known neuronal targets both within the CNS and the enteric nervous system (ENS). It is perhaps somewhat surprising that there are an increasing number of studies which discovered the nonimmune, noninfectious, and direct ability of microbes to influence the host behavior. The ability of a gut bacterium to influence host behavior was first shown in a study utilizing *Campylobacter jejuni* in mice. *C. jejuni* was able to induce anxiety-like behavior in mice through vagal-mediated pathway without activating host immune system (Foster and McVey Neufeld 2013). These studies exposed another possible prospect between microbiome and their host.

The stress-induced hyperthermia (SIH) and elevated plus maze (EPM) tests are widely used paradigms for assessing anxiety related behavior. Recolonization of GF mice with specific pathogen-free (SPF) bacteria in early life reversed the anxiety-like locomotor and behaviors in the elevated plus maze (Heijtz et al. 2011). Neufeld and colleagues also demonstrated the evidence of gut microbiota in regulating the set point for HPA axis activity. Results showed that GF mice spent significantly more time in the open arms of the EPM and significantly less time in the closed arms compared to SPF mice. GF mice also showed increased open arm entries but no differences in closed arm entries (activity) compared to SPF mice. However, when comparing both GF mice and SPF mice, GF mice exhibited basal behavior in the EPM that can be interpreted as anxiolytic (Neufeld et al. 2011). Moreover, studies on GF mice have indicated the importance of normal gut microbiome in shaping the behavior of host.

Table 1 Effect of gut microbiota onset and distribution on stress responses (behavioral pattern)

Initial microbial composition	Animals/ subjects	Sex	Treatment; dosage; duration	Behavioral test	Main findings	References
N	Carworth Farms (CF-1) mice; 5-week-old ($N = 38$)	M	*Campylobacter jejuni*; 2×10^7 CFU/ day; 2 days	Hole-board test	Increased anxiety	Foster and McVey Neufeld (2013)
GF	National Medical Research Institute (NMRI) mice; 8–10-week-old ($N = 14$)	M	–	Elevated plus maze	Compared to SPF • Increased anxiety	Heijtz et al. (2011)
GF	NMRI mice; 8–10-week-old ($N = 14$)	M	SPF bacteria	Elevated plus maze	Reduced anxiety	Heijtz et al. (2011)
GF	Swiss Webster mice; 8-week-old ($N = 12$)	F	–	Elevated plus maze	Compared to SPF • Increased anxiety	Neufeld et al. (2011)
N	BALB/c mice ($N = 36$)	M	*L. rhamnosus* (JB-1); 28 days	• Stress-induced hyperthermia • Forced swim test • Elevated plus maze	Reduced anxiety	Bravo et al. (2011)
N	Sprague–Dawley rat; 100 g ($N = 32$)	M	*L. plantarum*/ *L. fermentum*; 1×10^9 CFU twice/day; 2–4 months	Open field test	Decreased locomotor activity	Ushakova et al. (2009)
N	Sprague–Dawley ($N = 40$)/ Wistar rat; 8-week-old ($N = 40$)	M	*B. infantis* 35624 and UCC118; 5×10^9 CFU/ day; 14 days	Open field test	Reduced anxiety	McKernan et al. (2010)

(continued)

Table 1 (continued)

Initial microbial composition	Animals/ subjects	Sex	Treatment; dosage; duration	Behavioral test	Main findings	References
N	Sprague–Dawley; 200–250 g ($N = 20$)	M	*B. infantis* 35624; 1×10^{10} CFU/ day; 14 days	Forced swim test	No significant differences	Desbonnet et al. (2008)

F: female, *M*: male, *N*: normal microbiota, *GF*: germ free, and *SPF*: specific pathogen free

Increasing studies have proved the role and the ability of microbiome in adjusting host behavior. Chronic administration of *L. rhamnosus* (JB-1) showed a reduction in SIH with a larger number of entries and percentage of time spent in the open arms of EPM suggesting an anxiolytic effect. This effect is also reflected in the percentage of time spent in the open arms, although this observation did not reach statistical significance. Regarding depression-related behavior, forced swim test (FST) analysis revealed that *L. rhamnosus* (JB-1)-fed animals spent significantly less time immobile. Bravo and colleagues showed that *L. rhamnosus* (JB-1) is able to reduce anxiety- and depression-related behavior (Bravo et al. 2011). Another study showed that daily stomach feeding of 10^9 cfu of lactic acid bacteria (LAB) for 2–4 months decreased locomotor activity effectively in normal growing rats (Ushakova et al. 2009). From the results presented here, it could be postulated that LAB treatment might play a role in preventing neurological diseases by decreasing locomotor activity and normalizing altered behavior. Similar observations were obtained when rats treated with *B. infantis* 35624 and UCC118 showed increased time spent in the inner zone during open field test (McKernan et al. 2010). Although, another probiotic treatment using *B. infantis* did not elicit the characteristic behavioral patterns of the classic antidepressants in the FST (Desbonnet et al. 2008), there is evidence to suggests that bifidobacteria can affect central neurochemical function and may induce beneficial changes to systems involved in gut–brain communication.

3.2 Stress Biomarkers (Corticosteroids and Catecholamines)

Alterations on the HPA axis have been linked to the development of mood disorders and have been shown to affect the composition of the microbiota in rodents. A study on germ-free (GF) mice showed that confinement stress produces higher levels of plasma corticosterone compared to those where the gut was inhabited by *B. infantis* (Sudo et al. 2004).

Table 2 Effect of gut microbiota onset and distribution on stress responses (Biochemical and molecular alterations)

Parameter	Intial microbial composition	Animals/Subjects	Sex	Treatment; Dosage; Duration	Main findings	References
Stress Biomarker	GF	BALB/c mice; 9-week-old ($N = 12$)	M	*B. infantis*; 9 weeks	Reduced plasma adrenocorticotropic hormone and corticosterone level	Sudo et al. (2004)
Stress Biomarker	N	BALB/c mice ($N = 36$)	M	*L. rhamnosus* (JB-1); 28 days	Reduced plasma corticosterone level	Bravo et al. (2011)
Stress Biomarker	N	Subject; Hospital Anxiety and Depression (HADS) ≤ 20; 30–60 years old ($N = 55$)	M/F	*L. helveticus* R0052 and *B. longum* R0175; 3×10^9 CFU/day; 30 days	Reduced urinary free cortisol level	Messaoudi et al. (2011)
Stress Biomarker	N	C57Bl6 mice; 6–8-week-old ($N = 64$)	M	*L. helveticus* R0052 and *B. longum* R0175; 1×10^9 CFU/day; 2 weeks	Reduced plasma corticosterone, adrenaline, and noradrenaline level	Ait-Belgnaoui et al. (2014)
Cytokine	N	Sprague–Dawley rat; pregnant ($N = 33$)	F	*B. infantis* 35624; 1×10^{10} CFU/day	Decreased IL-10 level	Desbonnet et al. (2010)
Cytokine	N	C57BL/6-and IL-10-deficient mice ($N = 15$)	F	*L. paracasei* DSM 13434, *L. plantarum* DSM 15312, and *L. plantarum* DSM 15313; 1×10^9 CFU/day	Increased serum anti-inflammatory cytokines (IL-4, IL-10, and tumor growth factor-β1) level	Lavasani et al. (2010)
Cytokine	N	Wistar rat ($N = 30$)	M	*L. helveticus* R0052, *B. longum* R0175, and *L. rhamnosus* R0011; 8×10^8 CFU/day	Reduced serum pro-inflammatory cytokines IL-1α, IL-6, IL-γ, and tumor necrosis factor-α level	Bisson et al. (2010)
BDNF	SPF	AKR mice; 6–8 weeks ($N = 12$)	M	*B. longum* NCC3001 + Dextran sodium sulfate (DSS, 3 %) [thrice/day]; 6–8 weeks	Increased in BDNF expression	Bercik et al. (2011b)
BDNF	GF	BALB/c mice; 9-week-old ($N = 12$)	M	9 weeks	Compared to SPF • Decreased BDNF receptors • Decreased BDNF level in the hippocampus and cortical	Sudo et al. (2004)

BDNF	GF	Swiss Webster mice; 8-week-old ($N = 24$)	F	–	Compared to SPF • Increased BDNF mRNA expression in the dentate gyrus of the hippocampus	Neufeld et al. (2011)
BDNF	N	Sprague–Dawley rats ($N = 40$)	M	*B. breve* 6330; 50 days	• Increased BDNF total levels • Decreased BDNF IV expression	O'Sullivan et al. (2011)
BDNF	N	Sprague–Dawley rats; Maternal separation ($N = 40$)	M	*B. breve* 6330; 50 days	No significant difference	O'Sullivan et al. (2011)
BDNF	N	Sprague–Dawley rats; 225–250 g ($N = 24$)	M	Prebiotics galacto-oligosaccharide (GOS)/fructo-oligosaccharides (FOS); 5 weeks	• Increased bifidobacteria • Increased BDNF mRNA expression in the dentate gyrus of the hippocampus.	Savignac et al. (2013)
5-HT	N	Swiss Webster mice; 8–10-week-old ($N = 20$)	M	–	Compared to GF • Microbiota increased 5-HT and its metabolites level	Wikoff et al. (2009)
5-HT	GF	Swiss Webster mice ($N = 20$)	M	–	Compared to SPF • Decreased in the ratio of kynurenine:tryptophan	Clarke et al. (2013)
5-HT	GF	Swiss Webster mice ($N = 20$)	M	Reconstitution of normal flora	Normalized ratio of kynurenine: tryptophan	Clarke et al. (2013)
5-HT	N	BioBreeding diabetes-prone (BBDP) rats ($N = 18$)	–	*L. johnsonii*; 1×10^8 CFU/day; 120 days	Decreased IDO mRNA levels and IDO activity	Valladares et al. (2013)
5-HT	N	AKR mice ($N = 142$)	M	*Trichuris Muris*; 300 eggs/day; 10 days	Increased IDO activity	Bercik et al. (2010)
5-HT	SPF	Sprague–Dawley rats; 180–200 g ($N = 18$)	M	2.5 mmol kg^{-1} ammonium acetate/day	Increased 5-HIAA level in the cerebellum, hippocampus, and prefrontal cortex	Luo et al. (2014)
5-HT	SPF	Sprague–Dawley rats; 180–200 g ($N = 18$)	M	*L. helveticus* NS8 + 2.5 mmol kg^{-1} ammonium acetate/day	Decreased 5-HT level in the cerebellum and hippocampus	Luo et al. (2014)

(continued)

Table 2 (continued)

Parameter	Intial microbial composition	Animals/Subjects	Sex	Treatment; Dosage; Duration	Main findings	References
5-HT	N	Sprague–Dawley rats; 200–250 g ($N = 20$)	M	*B. infantis* 35624; 1×10^{10} CFU/day; 14 days	Decreased 5-HIAA concentrations in the frontal cortex	Desbonnet et al. (2010)
5-HT	N	Sprague–Dawley rats; 200–250 g ($N = 20$)	M/F	*B. infantis* 35624; 1×10^{10} CFU/day	No significant difference	Desbonnet et al. (2010)
DA	GF	F344 rats ($N = 24$)	M	–	Decreased in HVA concentration in the frontal cortex, hippocampus, and striatum	Crumeyrolle-Arias et al. (2014)
DA	N	Sprague–Dawley rats; 200–250 g ($N = 20$)	M	*B. infantis* 35624; 10 log CFU/day; 14 days	Decreased DOPAC in the amygdaloid cortex	Desbonnet et al. (2010)
GABA receptor	N	BALB/c mice ($N = 36$)	M	*L. rhamnosus* (JB-1); 28 days	• Increased mRNA expression of the GABA-B1b subunit in the prefrontal cortex • Reduced GABA-B1b mRNA expression in the hippocampus, amygdala, and locus ceruleus • Reduced $GABA_{A\alpha 2}$ mRNA expression in the prefrontal cortex and amygdala • Increased $GABA_{A\alpha 2}$ in the hippocampus	Bravo et al. (2011)
NMDA receptor	GF	Swiss Webster; 8-week-old ($N = 24$)	F	–	Compared to SPF • Decreased NR1 in the hippocampus • Decreased NR2A in the hippocampus and cortex • Decreased NR2B in the amygdale	Neufeld et al. (2011)

NMDA receptor	N	Sprague Dawley rats; 225–250 g ($N = 24$)	M	Prebiotics galacto-oligosaccharide (GOS); 5 weeks	• Increased bifidobacteria • Increased NR1 and NR2A subunits in the hippocampus • Increased NR1 subunits in the frontal cortex	Savignac et al. (2013)
5-HT receptor	GF	Swiss Webster; 8 week old ($N = 24$)	F	–	Compared to SPF • Decreased 5HT1A mRNA expression in the hippocampus	Neufeld et al. (2011)

N, normal microbiota; GF, germ free; SPF, specific pathogen free; IL, interleukin; BDNF, brain-derived neurotrophic factor; 5-HT, serotonin; IDO, indoleamine-2,3-dioxygenase; DA, dopamine; 5-HIAA, 5-hyroxyindole acetic acid; HVA, homovanillic acid; DOPAC, dihydroxyphenylacetic acid; GABA, ϒ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate

A study demonstrated that *L. rhamnosus* (JB-1) administration reduced the stress-induced elevation in corticosterone, suggesting the impact of the *Lactobacillus* on the CNS at a physiological level (Bravo et al. 2011). The data were in line with previous studies which showed that subchronic or chronic treatment with antidepressants can prevent elevation of stress-induced plasma corticosterone in both mice and rats (Conti et al. 2002). Moreover, it showed that alterations in HPA axis modulation can be reversed by treatment with lactobacilli and bifidobacteria. The test had been elaborated on human subjects, where test subjects were prescribed with two probiotics, *L. helveticus* R0052 and *B. longum* R0175. After a couple of weeks, standardized psychological surveys indicated that the subjects were less stressed, less anxious, and less depressed. These results were confirmed with enzyme-linked immuno assay (ELISA) assays of their 24-h urinary free cortisol levels (Messaoudi et al. 2011). Nonetheless, these data clearly indicated that a bidirectional communication exists between the brain and the gut and highlighted that the HPA axis can be affected by changing the enteric microbiota. Interestingly, a recent study indicated that a 2-week treatment with probiotic formulation Probio'Stick® (*L. helveticus* R0052 and *B. longum* R0175) attenuated both HPA axis and SNS response to chronic stress as reflected by a decrease in plasmatic levels of corticosterone, adrenaline, and noradrenaline in stressed mice (Ait-Belgnaoui et al. 2014). These data supported the role of probiotic in SNS by reducing catecholamines. Taken together, findings showed that probiotics possess potential in lowering stress biomarkers in both rodent and human subjects.

3.3 Cytokines

It is well known that pro-inflammatory cytokines are involved in the inflammation in depressive disorders; they also can act as mediators to induce anxious behavior in mice in case of gut infection. Various cytokine receptors found on peripheral nerves, including the vagus nerve and spinal nerves may initiate inflammation in the brain and potentially evoking anxiety and depression (Goehler 2008). However, at times vagotomy does not prevent anxiogenic and depressive effects from inflammation due to cytokines being able to enter the brain via circumventricular organs (by diffusion) or via cytokine transporters located at the blood brain barrier. Another pathway may involve the activation of interleukin (IL)-1 receptors, located on perivascular macrophages and endothelial cells of brain venules, by circulating cytokines inducing local production of prostaglandin E2 (Vitkovic et al. 2000). Prostaglandin E2 is a bioactive lipid that elicits a wide range of biological effects associated with inflammation (Nakanishi and Rosenberg 2013).

Several studies demonstrated that inflammatory responses in rodents were attenuated by *Lactobacillus* and *Bifidobacterium* strains. Interestingly, oral administration of *B. infantis* 35624 demonstrated antidepressant-like properties in stressed animals. The stressed animals were also associated with anti-inflammatory effects (Desbonnet et al. 2010). This result can be explained by the ability of stress

hormones in manipulating the balance of IL-10/IL-12. Both cortisol and catecholamine reduce IL-12 production; however, catecholamine also increases IL-10 production in a dose-dependent manner. The balance of IL-10/IL-12 production is crucial in the regulation of the immune response, which is responsible for the stress-induced susceptibility of the organism to certain autoimmune, allergic, infectious, or neoplastic diseases (Elenkov and Chrousos 1999).

In addition, probiotic (*L. paracasei* DSM 13434, *L. plantarum* DSM 15312 and *L. plantarum* DSM 15313) treated rats showed a significant increase in serum levels of anti-inflammatory cytokines IL-4, IL-10, and tumor growth factor-β1 in comparison with nontreated controls (Lavasani et al. 2010). Recently, a formulation with a combination of *L. helveticus* R0052, *B. longum* R0175 and *L. rhamnosus* R0011; reduced serum levels of pro-inflammatory cytokines IL-1α, IL-6, IL-γ, and tumor necrosis factor-α in *Escherichia coli* infected rats (Bisson et al. 2010). Thus, the beneficial psychological effects of oral treatment with probiotic may be explained, at least in part, by the anti-inflammatory properties of these bacteria.

3.4 Changes in Level of Neurochemicals

3.4.1 Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is of interest because of its putative role in stress and psychiatric disorders. BDNF is a small dimeric neuroprotective protein and a member of neurotrophin family, which is widely expressed in the adult mammalian brain. It plays critical role in the development and maintenance of peripheral nervous system and CNS, neuronal survival, and neuronal proliferation. These findings are supported by the fact that BDNF-deficient mice showed significant neuronal deficits and defects in postnatal development and maturation of forebrain neurons (Tsai et al. 2003). Cortisol, a stress hormone produced in response to HPA activation acts as an antagonist to BDNF by blocking its effects (Kumamaru et al. 2008).

Both acute and chronic stresses have been shown to decrease the BDNF expression and cause cell loss in limbic brain structures in various animal models (Murakami et al. 2005). The changes in BDNF protein levels appeared to be a compensatory measure, maintaining normal hippocampal neurogenesis, leading to the idea that BDNF protein may counteract stress-induced decreases in neurogenesis. Recently, it has been shown that perturbation of microbiota in SPF rat with antibiotic administration, resulted in an increase in BDNF expression in the hippocampus, as well as a less anxious phenotype (Bercik et al. 2011a). Sudo and colleagues demonstrated that male GF mice have an increased stress response (although no basal changes in HPA axis function were noted) coupled with decreased hippocampal and cortical BDNF, and decreased *N*-methyl-D-aspartate (NMDA) receptors (Sudo et al. 2004). The role of NMDA receptor will be further discussed in Sect. 3.5.2.

However, as opposed to the observations obtained in male GF mice, female GF mice absent with conventional microbiota demonstrated upregulated BDNF mRNA expression in the dentate gyrus of the hippocampus (Neufeld et al. 2011). In fact, neurochemical and endocrine effects of growing up in a GF environment are only evident in male animals. O'Sullivan and colleagues demonstrated that orally administered probiotic can influence hippocampal BDNF protein level. Noteworthy, *B. breve* 6330 influenced BDNF in normal animals, although it had no significant effect on BDNF in those which were maternally separated (O'Sullivan et al. 2011). Indeed, studies have shown that the probiotic *B. longum* NCC3001 reversed colitis-induced anxiety and caused alterations in hippocampal BDNF mRNA expression without impacting gut inflammation or circulating cytokines. However, the anxiolytic effect of *B. longum* NCC3001 was absent in vagotomized mice, suggesting a neural mechanism which was confirmed with ex vivo electrophysiological studies in enteric neurons (Bercik et al. 2011b). Interestingly, prebiotics-mediated proliferation of gut microbiota in rats also resulted in an increased hippocampal BDNF mRNA expression (Savignac et al. 2013).

3.4.2 Serotonin (5-HT) and Metabolites

A dysfunctional in 5-HT system has long been implicated in the pathogenesis of anxiety disorders. Patients with major depression showed fewer platelet serotonin transporters (5-HTT) and lower cerebrospinal fluid 5-hydroxyindole acetic acid (5-HIAA) (Redmond et al. 1986), as well as a reduced growth hormone (GH) response to 5-hydroxytryptophan (5-HTP) (Upadhyaya et al. 1991). The antidepressant efficacy of selective serotonin reuptake inhibitors (SSRIs) suggests that the abnormality in depression is due to impaired serotonergic function. This is because selective enhancement of serotonergic activity is therapeutic. At present, evidence suggests that 5-HT function is reduced in untreated depressed patients but increased in treated depressed patients (Bell et al. 2001). Despite this knowledge, the phenomenon of 5-HT dysfunction in depression and mechanism of antidepressant on HT system has not been established.

Tryptophan is an essential amino acid and has two main metabolic pathways: serotonin (5-HT) pathway and kynurenine (KYN) pathway, as shown in Fig. 4. Bacterial colonization of GF mice results in a >2-fold increase in 5-HT and its

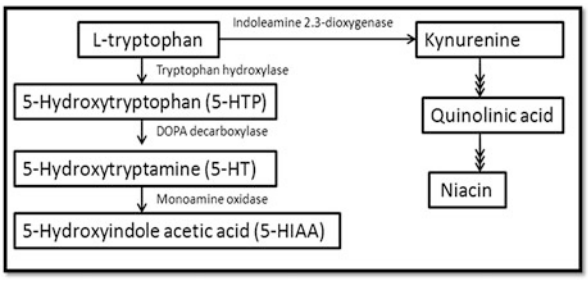

Fig. 4 Schematic diagram of tryptophan metabolism via the kynurenine pathways or through the synthesis of serotonin. Note that the figure is not representative of all intermediates and enzymes

metabolites (due to bacterial metabolism of tryptophan), which in turn influence the brain and behavior (Wikoff et al. 2009). The tryptophan metabolite kynurenic acid acts as an antagonist at excitatory amino acid receptors and has been implicated in major psychiatric illnesses. Alterations in the gut microbial composition result in changes in serum kynurenic acid levels and could thus modify CNS excitation and behavior (Myint 2012).

Indoleamine-2,3-dioxygenase (IDO) is the first enzyme in the KYN pathway, which converts tryptophan to KYN. It has been reported that depression is associated with increased pro-inflammatory cytokines, which lead to the activation of IDO. Studies in GF mice have implicated this pathway with a decrease in the ratio of kynurenine:tryptophan (an index of IDO activity). The IDO activity normalized following introduction of gut microbiota to GF mice immediately postweaning (Clarke et al. 2013). Interestingly, in vitro study showed that *L. johnsonii* is able to reduce IDO activity in HT-29 intestinal epithelial cells. Administration of *L. johnsonii* in rats resulted in a reduction in serum kynurenine concentrations as well. The effect is due to the H_2O_2 produced by *L. johnsonii*, which abolished IDO by inducing protein oxidation and inhibition in enzyme activity (Valladares et al. 2013). Moreover, an increase in IDO activity is observed after infection with *Trichuris Muris* (Bercik et al. 2010). A study using HA (hyperammonemic) rats showed that anxiety behavior resulted from changes in the central 5-HT system. HA rats had enhanced 5-HT metabolism in the cerebellum, hippocampus, and prefrontal cortex, as reflected by increased levels of 5-hyroxyindole acetic acid (5-HIAA), but unchanged levels of 5-HT. Feeding of *L. helveticus* NS8 in HA rats resulted in the reduction of 5-HT levels in the cerebellum and hippocampus, as well as improved anxiety-like behavior (Luo et al. 2014).

On the other hand, chronic oral administration of *B. infantis* 35624 in Sprague–Dawley rats induced an increase in peripheral concentrations of the 5-HT precursor tryptophan. Besides, *B. infantis* 35624 altered 5-HIAA concentrations in the brain accompanied by normalization of behavior compared to control. Following this, antidepressant properties of *B. infantis* 35624 was investigated on maternal separated (MS) rats. Compared to chronic citalopram treatment, *B. infantis* 35624 did not affect basal 5-HT levels in amygdaloid cortex. In general, both citalopram and bifidobacteria treatments have negligible effects on basal serotonergic activity in the brain and on peripheral levels of the 5-HT precursor protein, tryptophan. It is possible that postnatal MS compromises biological systems in these rats such that the capacity for probiotic bacteria to alter tryptophan metabolism is diminished (Desbonnet et al. 2010).

3.4.3 Dopamine and Metabolites

In the CNS, Dopamine (DA) is involved in the control of locomotion, cognition, affect, and neuroendocrine secretion (Jaber et al. 1997). DA can be metabolized into 3,4-dihydroxyphenylacetic acid (DOPAC) or homovanillic acid (HVA) as shown in Fig. 5.

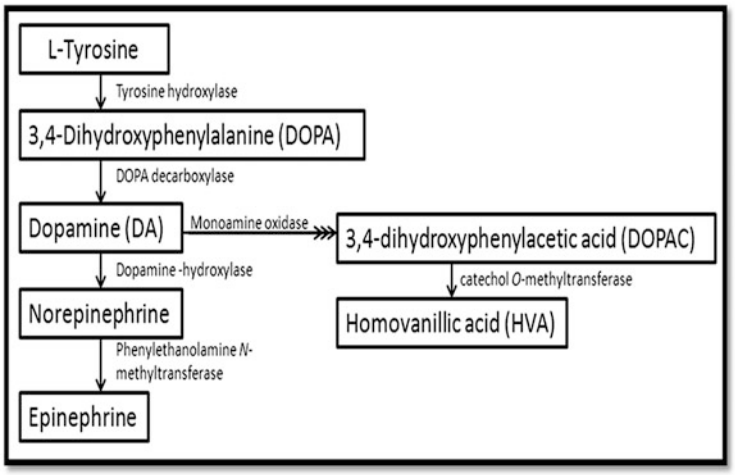

Fig. 5 Schematic diagram of synthesis of dopamine from tyrosine as well as dopamine metabolism into end product: epinephrine and homovanillic acid. Note that, the figure is not representative of all intermediates and enzymes

DA is less studied in stress studies; however, there is reliable evidence on decreasing level of HVA and its metabolites in cerebrospinal fluid of stressed subjects. This has been linked to few clinical stress disorder features, such as motor retardation and melancholia (Roy et al. 1985). In stress-sensitive rats, the absence of gut microbiota exacerbates the neuroendocrine and behavioral responses to acute stress and the results coexist with alterations of the dopaminergic turnover rate in brain upper structures that are known to regulate reactivity to stress and anxiety-like behavior. GF rats displayed a marked (threefold) and specific decrease in HVA concentration in the frontal cortex, hippocampus, and striatum, which led to a lower HVA/DA ratio that can be interpreted as a reduction in dopaminergic turnover rate (Crumeyrolle-Arias et al. 2014).

Abnormalities in mesolimbic dopaminergic activity have been shown in depression. A study reported that there is an alteration on monoamine activity in stress-relevant brain areas in the bifido-treated group, as shown by the decreased DOPAC in the amygdaloid cortex (Desbonnet et al. 2008). Effect of *B. infantis* on DOPAC in this particular brain region is therefore way more important.

3.5 Receptors Expression

3.5.1 γ-Aminobutyric Acid (GABA) Receptors

GABAergic system has complex interactions with other neurotransmitter systems and acts through ionotropic A receptors and metabotropic B type receptors.

GABAergic system plays important roles in the brain and is the target for a variety of endogenous and exogenous modulators (Cryan and Kaupmann 2005). GABA-A receptors inhibit neuronal excitability. GABA-A receptors are ligand-gated ion channels composed of five subunits from eight families: a (1–6), b (1–3), g (1–3), d, e, p, y, and r (1–3) (Neto et al. 2005). Binding of GABA to GABA-A receptors opens up a Cl channel, which is part of the protein structure. Though less well understood, GABA-B receptors are thought to modulate the generation of excitatory postsynaptic potentials and long-term potentiation. They are made up from two subunits, B1 and B2, each with seven transmembrane-spanning elements that come together to form heterodimers. Both B1 and B2 subunits are necessary for the GABA-B receptor to be functional. Accumulating evidence suggests that GABA-B receptors are crucial to maintain normal behavior (Jacobson et al. 2007). Indeed, genetic and pharmacological studies have implicated that GABA-B receptors play a key role in mood and anxiety disorders (Cryan and Kaupmann 2005). Both GABA-A and-B receptors have been shown to regulate many normal and pathological brain mechanisms including sleep, memory, epilepsy, and various emotions.

Studies showed that animal models with depression have reduced GABA-B receptor expression, especially in the frontal cortices (Martin et al. 1989). In a study by Bravo and colleagues, the mRNA expression of the GABA-B1b subunit (main isoform of the GABA-B1 receptor) in the prefrontal cortex (brain region involved in decision-making) was found to be elevated in *L. rhamnosus* (JB-1)-fed animals. These were consistent with behavioral and neuroendocrine responses. In other analyzed areas, *L. rhamnosus* (JB-1)-fed animals have lower-than-normal levels of GABA-B1b mRNA expression in the hippocampus (regulates learning, and memory), amygdale (regulates fear responses), and locus ceruleus (implicated in the connection between autism and fever). Bravo and colleagues also showed that *L. rhamnosus* (JB-1) reduced $GABA_{A\alpha 2}$ mRNA expression in the prefrontal cortex and amygdala, but increased in the hippocampus (Bravo et al. 2011). It is somehow difficult to attribute a causal relationship between changes in brain chemistry and behavioral effects observed. However, reduced mRNA expression of GABA-B1b in the amygdale, hippocampus, and locus coeruleus is consistent with the antidepressant-like effect of GABA-B receptor antagonists. Alterations in these receptors are associated with anxious and depressive-like behaviors in animal models (Cryan and Slattery 2010). Together, these studies showed an association between microbiota and GABAergic pathways which imply the significance of probiotic in stress alleviation. Surprisingly, vagotomized animals prevented the anxiolytic and antidepressant effects of probiotic as well as the effects on the central GABA receptors. Such findings suggest that parasympathetic innervation is necessary for microbiota-brain interaction, particularly in the GABAergic system (Cryan and O'Mahony 2011).

The GABAergic system is emerging rapidly as a target in developing medications for anxiety and mood disorders. Chronic stress exposure, the most common cause of depression, has been shown to activate the GABAergic system in forebrain areas, including dorsomedial hypothalamus, hippocampus, and important parts of depression circuits (Cullinan et al. 2008). Several studies have found significant

reductions in hippocampal volume in depressed subjects, indicating the role of the hippocampus in depression pathogenesis. This may be attributed to the abundance of GABAergic neurons particularly in the hippocampus (Bremner et al. 2000). Hippocampus affects memory, whereas memory affects depression and anxiety. Thus, this implies that depression and anxiety pathogenesis are linked to memory-related GABAergic neurons in the hippocampus (Sala et al. 2004). Therefore, the ability of probiotic in modulating GABAergic system can be employed in therapeutic adjuncts in stress-related diseases.

3.5.2 *N*-methyl-D-aspartate Receptor

NMDA receptor is known to play an important role in synaptic development and plasticity as well as in learning and memory. In the amygdale, NMDA receptor is involved in central expression of anxiety. The NMDA receptor is a heterotetramer composed of two NR1 subunit and two other subunits (NR2A, NR2B, NR2C, or NR2D) (Sala et al. 2004). Alterations in hippocampal NMDA receptor is known to influence corticotropin-releasing hormone released from the hypothalamus; thus changes in NMDA receptor expression explained the altered HPA function in the animals (Holsboer 1999).

Depression is a common manifestation of neuropsychiatric systemic lupus erythematosus (SLE) due to the presence of anti-NMDA antibodies in patients (Lapteva et al. 2006). Similarly, several conventional antidepressive agents (monoamine oxidase inhibitors, tricyclic antidepressants, and selective serotonin reuptake inhibitors) were observed to induce reductions in NMDA mRNA levels (Skolnick 2002).

Changes in NMDA receptor subunit expression have been demonstrated in GF mice compared to specific pathogen-free mice, with decreased NR1 in the hippocampus, decreased NR2A in the hippocampus and cortex, and decreased NR2B in the amygdale (Neufeld et al. 2011). In addition, rats fed with prebiotics such as galacto-oligosaccharide (GOS) demonstrated an elevation of bifidobacteria compared to controls, following an increase of NR1 and NR2A subunits in the hippocampus and NR1 subunits in the frontal cortex (Savignac et al. 2013). Undeniably, microflora such as bifidobacteria and lactobacilli has modulating effect on NMDA receptors which led to changes in behavior development.

3.5.3 5-HT Receptors

5-HT receptors are divided into seven distinct classes (5-HT1 to 5-HT7) on the basis of their structural and operational characteristics (Hoyer et al. 2002). However, only 5-HT1A receptors will be reviewed here. Involvement of 5-HT1A receptors in a number of physiological and behavioral effects has been well established in both human (Akimova et al. 2009) and animals (Ramboz et al. 1998). 5-HT1A receptors have been implicated in the neuroendocrine

regulation of adrenocorticotrophic hormone (ACTH), which causes the adrenal cortex to produce and release glucocorticoids (Jørgensen et al. 2001). The role of 5-HT1A receptors in modulating anxiety-related behaviors is supported by studies utilizing 5-HT1A receptor knockout mice. These animals demonstrated increased anxiety in a number of experimental paradigms (such as in the elevated plus maze, elevated zero maze, and open field). Moreover, these animals demonstrated decreased baseline immobility in the forced swimming tests and tail suspension tests when comparing to normal rats (Grippo et al. 2005). Notably, increased expression of 5HT1A has been observed in GF mice compared to SPF mice in a few studies (Neufeld et al. 2011). Nevertheless, the connections between microbiota and serotonergic pathways remain to be fully elucidated.

4 Compounds Involved in Stress Management Via Gut–Brain Axis

Most studies on the microbial gut–brain axis have highlighted the ability of certain bacteria, whether commensal, pathogen, or probiotic has an effect on a myriad of neural substrates both within the CNS and ENS. While the ability of bacteria to synthesize as well as recognize a wealth of neuroactive substances in the host suggests a bidirectional environment, in which the microbiome and the host shared a common evolutionary pathway of intercellular signaling. This communication implies that both host and microbiome are not empowering or following, but influencing one another. The microbiome produces neuroactive compounds which act via direct interaction with receptors within the gastrointestinal tract or through passive diffusion through the gut wall into the blood circulation. The gut is a highly innervated organ that possesses its own nervous system known as the ENS that is linked with the CNS, which directly connects portions of the gut to the brain. One of the approaches on encompasses the gathering of information of neuroactive compounds contained within the luminal space, lies on the luminal epithelial chemosensors and enteroendocrine cells along the entire length of the microvilli. There is much evidence linking microbiome to gut–brain axis (Fig. 6).

4.1 Neurotransmitters

The “living” environment of a human includes microorganisms, plants, and animals as well as other human beings. The relationship between living organisms occurs via irritation events. For every cell, the mechanism of irritability has uniform chemicals which include acetylcholine, 5-HT, DA, norepinephrine, epinephrine, and histamine, generally known as neurotransmitters. Neurotransmitters have been found not only in animals but also in organisms lacking a nervous system such as in

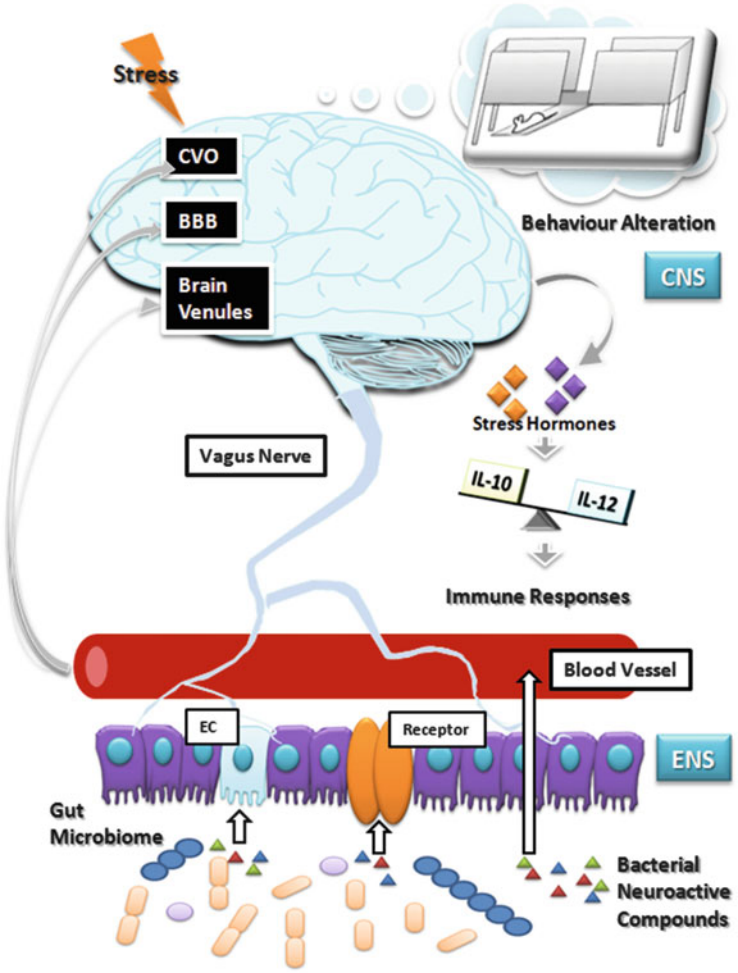

Fig. 6 There are a variety of mechanisms, either via direct or indirect pathways proposed to facilitate gut microbiota to participate in the gut–brain axis. Gut microbiome produces neuroactive compounds and exerts their neuro-modulating effect via receptors that lies within gut epithelial cells or via passive diffusion into the blood circulation. Meanwhile, enteroendrocrine cells serve as an agent to link ENS and CNS, mainly through the vagus nerve. Neuroactive compounds can enter the brain via CVO by diffusion, via transporters at the BBB to gain access to the brain, or via another pathway that occurs at brain venules (perivascular macrophages and endothelial cells). When stress is induced, stress hormones such as glucocorticoids and catecholamines will be released. Neuroactive compounds will act upon in reducing release of stress hormones. Stress hormones in turn control the balance of IL-10 and IL-12 which eventually modulate immune responses of host. At the same time, bacterial neuroactive compounds modulate neuronal signaling in brain and influence host's behavior. *CVO*: circumventricular organs, *BBB*: blood brain–barrier, *CNS*: central nervous system, *ENS*: enteric nervous system, *IL*: interleukin, and *EC*: enteroendocrine

plants and in unicellular organisms (Roshchina 2010). Today, there is growing evidence that neurotransmitters which participate in synaptic neurotransmission are multifunctional substances participating in developmental processes of all living organisms. Moreover, their universal roles as signal and regulatory compounds are supported by studies that examine their role in and across biological kingdoms. All organisms may release neurotransmitters to influence other inhabitant of biocoenosis, thus determining relationships between organisms (Roshchina 2010).

4.1.1 GABA

GABA is one of the chief inhibitory neurotransmitters in the human CNS. GABA is a nonprotein amino acid which induces hypotensive, diuretic, and tranquilizer effect. It also functions as pain regulating and some pain relieving drugs currently on the market act by targeting GABA receptors (Jasmin et al. 2004). The important role of GABA in mood disorders was first postulated back in 1980, and over the last decades much data has emerged to support the hypothesis. The role on GABAergic system had been discussed above. In addition to receptor actions, the role of GABAergic system in anxiety and depression pathogenesis attributes to factors affecting GABA metabolism and uptake. Glutamate decarboxylase (GAD) is a key enzyme in GABA biosynthesis from glutamate (Tappaz et al. 1977). Reduced GABA levels and increased anxiety were both found in mice lacking a small 65-kDa isoform of GAD (GAD65). GAD65 is responsible for GABAergic neurotransmission and its expression was increased by antidepressant reboxetine in septum of stressed rats. Other studies have found decreased expression of GAD65 and GAD67 (another isoform) in mood disorders (Asada et al. 1996).

The most interesting and practical group of bacteria for GABA production is lactic acid bacteria (LAB), in fact GABA is also the most abundant and well-studied bioactive metabolites produced by probiotics (Higuchi et al. 1997). GABA is made by many bacteria, especially lactobacilli, and this property may well serve to protect the organism from the acidic environment encountered in the stomach, since its synthesis involves proton exchange for the uptake of glutamate. The first bifidobacteria strain, *B. dentium* that is capable of secreting large amount of GABA had been identified (Barrett et al. 2012). In Human Microbiome Project, lab analysis of metagenomic DNA sequencing data demonstrates that microbial glutamate decarboxylase encoding gene is very abundant in the intestinal microbiota as compared to other body sites.

4.1.2 Nitride Oxide (NO)

Nitride Oxide (NO) is produced from amino acid l-arginine by NO synthase (NOS) and is involved in several cellular functions, including neurotransmission, regulation of blood-vessel tone, and in the immune response (Sanders and Ward 1992). The contribution of NO to the regulation of stress has been long debated. Early

studies showed that NO did not have an effect on basal CRH release but it did inhibit the release of CRH from rat hypothalamic explants (Costa et al. 1993). Meanwhile, basal NO concentrations have been shown to reduce the release of GABA, whereas high levels of NO increase GABA release (Ohkuma et al. 1996). Certain organisms including lactobacilli are able to convert nitrate to NO, a potent regulator of both the immune and nervous systems (Ranjit et al. 2002). The conversion of nitrate to NO by *Lactobacillus* will increase the concentration of NO and thus able to normalize stress-related disorder changes.

Neural mechanisms that involve direct bacterial activation or inhibition of neurons may account for antinociceptive effects of probiotics. Stress-induced hypersensitivity to colorectal distension (CRD) via epithelial cell cytoskeleton contraction which leads to increased colonic paracellular permeability. In a study, *L. farciminis* ameliorated acute stress-induced hypersensitivity to CRD, mediated by inhibition of colonic epithelial contraction and NO-related mechanisms produced by this probiotic (Ait-Belgnaoui et al. 2005). Intestinal barrier impairment is implicated in the pathophysiology of intestinal gut disorders associated with psychiatric comorbidity. Using the same strain, a 2-week probiotic treatment (*L. farciminis*) attenuates the HPA axis response in acute stressed rats, as reflected both by a decrease in plasmatic ACTH and corticosterone concentration, and reduced hypothalamic corticotropin-releasing factor expression (Ait-Belgnaoui et al. 2012).

Markedly, *L. rhamnosus* GG induces NO production in J774 macrophages and in human T84 colon epithelial cells. The synthesis of NO, although in low level, may contribute to the protective actions of *L. rhamnosus* GG in the gastrointestinal tract (Korhonen et al. 2001). Protection of gut plays a crucial role in the attenuation of the HPA axis response to stress. On the other hand, NO level released by enteroglial cells is different between pathogen and probiotic bacteria and suggests that human enteroglial cells participate in host–bacteria interaction via a different NO release (Turco et al. 2014).

4.1.3 5-HT and Metabolites

5-HT is a metabolite of the amino acid tryptophan and plays an important role in the regulation of a number of bodily functions. It has been shown that plasma 5-HT levels in conventional mice are significantly higher than in GF mice, demonstrating the capacity of the microbiota to influence 5-HT levels (Husebye et al. 1994).

Probiotics have been shown to improve carbohydrate malabsorption, which in turn has been associated with both the early signs of depression and reduced serum tryptophan levels (Adameova et al. 2009). It is possible that treatment with probiotics may elicit its beneficial effect on mood by increasing levels of the serotonin-precursor, tryptophan, and consequently elevating 5-HT availability. The hypothesis is supported by a study which shows that oral ingestion of *B. infantis* increased the levels of 5-HT precursor, tryptophan in the plasma of rats (Desbonnet et al. 2008).

On the other hand, kynurenic acid, another 5-HT metabolite produced by commensal intestinal microbiota is readily absorbed from the GI tract and has been reported to have anxiolytic activity when administered in the periphery (Lapin 1998).

4.2 Short Chain Fatty Acids

Short Chain Fatty Acids (SCFAs) mainly acetate, propionate, and butyrate are the end product of anaerobic bacteria fermentation along a variety of pathways in the GI tract. SCFAs are produced by fermentation from carbohydrate. The major source of SCFA in the human colon is thought to be plant cell wall polysaccharides such as cellulose, pectins, and hemicelluloses, currently referred as dietary fiber in human nutrition (Cummings 1981). Surprisingly, their production is dependent on commensal bacteria when there is almost no SCFA found in GF mice (Høverstad and Midtvedt 1986).

Butyrate produced by obligate anaerobic bacteria is a potential candidate to histone deacetylase inhibitors, an emerging class of epigenetic drugs. Epigenic modification has been implicated in the etiology of stress disorders (Krishnan and Nestler 2008). Systemic injection of butyrate can lead to histone hyperacetylation in some brain regions and exhibit antidepressant effects in mice. Furthermore, a study showed that butyrate has significant effects on the ENS and could modulate brain physiology through indirect control of BDNF transcripts in the frontal cortex (Schroeder et al. 2007).

4.3 Fatty Acids

A growing number of studies support the role of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in promoting antidepressive effects through multiple mechanisms (Pandya et al. 2013). These fatty acids are known to assist in nerve cell signaling and neurodevelopment. Low dietary intake of beneficial omega-3 fatty acids is linked to depressed mood, hostility, and impulsive behavior; conversely, high intake of EPA and DHA is associated with increase in gray matter volume in brain regions modulating depression and mood (Bourre 2005). Some bacteria of marine origin are also known to synthesize EPA and DHA de novo through the actions of polyunsaturated fatty acid synthase genes, which explains the abundance of EPA and DHA in fish and fish oil (Metz et al. 2001). Furthermore, administration of probiotics (*L. rhamnosus* GG and *B. animalis* Bb12) by pregnant women has been shown to affect placental fatty acid composition (Kaplas et al. 2007). It has also been demonstrated that administration of probiotics formula (*B. animalis* Bb12 and *L. rhamnosus* GG) by infants caused changes in serum fatty acid composition (Kankaanpää et al. 2002). Intestinal bacteria of human origin such as bifidobacteria

and lactobacilli can convert linoleic and linolenic acids to bioactive isomers of conjugated linoleic acid and conjugated a-linolenic acid, respectively. In contrast, mammalian cells cannot synthesize these fatty acids, but they can metabolize them into more physiologically active compounds, in which linoleic acid is converted to arachidonic acid and a-linolenic acid is metabolized to eicosapentaenoic acid (Simopoulos 2002). Recent studies have shown that fatty acid concentrations in the brain (including arachidonic acid and docosahexaenoic acid) are elevated in mice whose diets were supplemented with *B. breve* NCIMB 702258 (Wall et al. 2012). Arachadonic acid and docosahexaenoic acid are known to play important roles in neurodevelopmental processes including neurogenesis promotion, alteration in neurotransmission and provide protection against oxidative stress (Balanzá-Martínez et al. 2011).

4.4 Antimicrobial Compounds

Resident bacteria in gut are a crucial resistance barrier to colonization by exogenous microbes and therefore, are highly relevant in the prevention of pathogenic invasion of tissues. Nonpathogenic adherent bacteria can prevent attachment and subsequent entry of pathogen bacteria into the epithelial cells. The bacterial lipopolysaccharide (LPS) is a structural portion of the external membrane of Gram-negative bacteria, and it is estimated that humans harbor at least a gram of LPS in the intestinal lumen (Takeuchi et al. 1999). Even at low levels (e.g. 0.4 ng/kg), LPS administration has been shown to cause acute anxiety, depressive symptoms, cognitive deficits, and increased visceral pain sensitivity. Experimental investigations showed that LPS can induce cytokine production in the CNS and alter behavior without the necessity of systemic cytokine involvement (Miller et al. 2009). Indeed, systemic LPS can compromise the integrity of the normal blood–brain barrier (BBB) and thus facilitate the passage of potential agents and disable the ability of BBB to remove potentially harmful neurotoxins from the brain (Wang et al. 2010).

In addition, LPS-induced inflammation can increase the activity of indoleamine-2,3-dioxyegenase (IDO), an enzyme that breaks down tryptophan in the kynurenine pathway. IDO activity has been positively correlated with depressive symptoms (Wang et al. 2010).

Normal microbiota or probiotic maintain the integrity of intestine barrier thus preventing the effects of LPS on host (Isolauri et al. 2002). Antimicrobial compounds normally secreted by probiotics are SCFA (lactic acid and acetic acid), bacteriocins, and others. The stress alleviation actions of probiotic may due to its ability in maintaining balance of normal gut microbiota and preventing the invasion of pathogen into CNS of host.

4.5 *Folate*

Folate is an essential nutrient involved in numerous biochemical pathways, including neurotransmitter synthesis, DNA biosynthesis, regulation of gene expression, amino acid synthesis, and metabolism as well as in myelin synthesis and repair. Recently, investigations on the closely connection between high homocysteine levels and brain dysfunction, including cognitive function, dementia, Alzheimer's disease, and depression have been reported (Stanger 2002).

Lactobacilli and bifidobacteria have been reported to produce folic acid, niacin, thiamine, riboflavin, pyridoxine, and vitamin K. In a pilot human study, strains of *B. adolescentis* and *B. pseudocatenulatum* given to 23 healthy volunteers significantly increased folate concentration in the feces of the subjects (Strozzi and Mogna 2008). The levels of commensal bifidobacteria in the large intestine correlated with the vitamin availability, suggesting that bifidobacteria are capable of producing folate in the gut and that the folate synthesized in the large intestine can be absorbed and utilized by the host. Meanwhile, the administration of diets containing bifidogenic ingredients (e.g., human milk solids or prebiotics) in folate-depleted rats also increased the folate concentration in the cecum, colon, plasma, and colonic tissue (Rossi et al. 2011). These results support evidence that folate-producing probiotic strains may represent an endogenous source of vitamin folate. Therefore, the trophic effects on colonocytes of folate-producing strains deserve to be evaluated and can be employed as psychotropic probiotic.

5 Conclusions

Bacteria possess an apparent ability to produce neuroendocrine hormones suggesting that the interaction of microbiome with the host go far beyond the earliest role of bacterial–host interactions in infectious diseases. Probiotic treatment in maintaining balance of gut microbiome may provide potential treatment and preventative measure for CNS disorders, particularly in depression and anxiety. Accumulated data have indicated that the gut microbiota communicates with the CNS through neural, endocrine, and immune pathways, which leads to stress regulation and CNS development at critical stages. The emerging concept of gut–brain axis suggests that the modulation of the gut microbiota may provide a novel therapeutic target for the treatment as well as prevention of CNS disorders. Undoubtedly, there is a wealth of neural information that can be detected by the host from bacteria in the gut, which eventually be interpreted and responded by the CNS. However, at this point it is critical to note that the mechanisms by which intestinal bacteria may impact the ENS and hence communicate with the brain and in turn influence stress-related disorders such as anxiety and depression have not been fully understood.

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