The Microbiomes of Humans, Animals, Plants, and the Environment 1

Maria Gazouli
George Theodoropoulos *Editors*

Gut Microbiome-Related Diseases and Therapies

Springer

The Microbiomes of Humans, Animals, Plants, and the Environment

Volume 1

This series covers microbiome topics from all natural habitats. Microbiome research is a vibrant field of science that offers a new perspective on Microbiology with a more comprehensive view on different microorganisms (microbiota) living and working together as a community (microbiome). Even though microbial communities in the environment have long been examined, this scientific movement also follows the increasing interest in microbiomes from humans, animals and plants. First and foremost, microbiome research tries to unravel how individual species within the community influence and communicate with each other. Additionally, scientists explore the delicate relationship between a microbiome and its habitat, as small changes in either, can have a profound impact on the other. With individual research volumes, this series reflects the vast diversity of Microbiomes and highlights the impact of this field in Microbiology.

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Preface

The human digestive tract is colonized by a highly diverse ecosystem of microorganisms that comprise the gut microbiota. Microbiota has been acknowledged to play a crucial role in maintaining a healthy state, as well as in drastically modifying susceptibility and progression of common human diseases. Diverse mechanisms including, but not limited to, inflammation are implicated in this complex bidirectional crosstalk between the gut microbiota and the host. A substantial body of evidence has been progressively accumulated, has enlightened the mechanistic details involved in this crucial interaction, and has opened novel avenues on the ways we will envisage diagnosis and treatment of human pathologies. An in-depth understanding of this relationship will be vital not only to advance the human health but also to enhance our understanding of diseases and to highlight new therapeutic approaches.

The book primarily focuses on the host-gut microbiome interaction and on cause-effect mechanisms. The authors aspire to offer basic researchers and medical professionals a comprehensive insight on the concepts of microbiome-related diseases susceptibility and progression, on the significance of microbiota disturbances in gut dysbiosis, and on the array of interactions between the microbiome and the human genome and epigenome. This collective work, eventually, aims in aiding the reader to acquire profound knowledge on the interplay between the gut microbiota promoting and protective features and the pathogenesis of benign and malignant human diseases and their respective therapies.

Whether you are a clinician, biomedical researcher, student, or patient, or just interested in Gut Microbiome, we hope you enjoy reading this book as much as we have enjoyed researching, writing, and organizing it!

Athens, Greece — Maria Gazouli
Athens, Greece — George Theodoropoulos

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About the Editors

Maria Gazouli is Professor of Biology - Nanomedicine, Medical School, National and Kapodistrian University of Athens, Athens, Greece. She was admitted as a PhD student in the Biology Department and Medical School of National and Kapodistrian University of Athens and was granted a honored Hellenic Pasteur Institute scholarship. She continued her postdoc training in Cell Biology Department, Georgetown University Medical Center, Washington DC, USA. Dr. M. Gazouli's work focuses on the molecular basis of diseases mainly autoimmune diseases and cancer, on the molecular detection of pathogens, and on the investigation of the pathogenesis of the diseases they cause to humans. These activities have produced more than 250 publications in peer-reviewed journals, 11515 citations (*h*-index: 55), more than 150 announcements in scientific congresses that were awarded in 17 cases, 1 granted International Patent, and 3 European Patent Applications. Recently Dr. Gazouli was involved in the incorporation of nanotechnology to targeted cancer detection, imaging, and drug delivery. She was honored with a Fulbright Scholarship for the Development of Nanotechnology-based Biosensor Arrays for the Detection of Circulating Colorectal Cancer Cells at the University of Maryland, College Park, MD, USA. The research has been recognized by distinguished awards and funded by national and international (EU) competitive research grants. Maria Gazouli has been actively involved in undergraduate and postgraduate training, as well as ERASMUS program, and her laboratory has trained a significant number of young scientists.

George Theodoropoulos was graduated from Athens Medical School in 1992. His PhD research was in Tumor Markers in Gastrointestinal Malignancies. He completed a 6-year residency program in General Surgery and a fellowship in Colon and Rectal Surgery in the USA. He is currently holding an academic post as an Professor of Surgery at Athens Medical School, Athens, Greece. He is a Diplomat and a Fellow (FACS) of the American Board of Surgery and of the American Board of Colon and Rectal Surgery (FASCRS). He completed a 6-month research fellowship in the Department of Colorectal Surgery, Cleveland Clinic Florida, Weston, FL, USA. He has set up and coordinated a clinic of Health-Related Quality of Life surveillance of colorectal cancer patients, has been supervising the Colorectal Unit of the Athens Medical School First Department of Propaedeutic Surgery, and has

established a multidisciplinary "Lower Digestive Tract Study Unit" in the hospital he is currently practicing.

He has performed about 3000 general surgery and colorectal surgery procedures. He applies a variety of minimally invasive techniques, and he is skilled at laparoscopic colorectal procedures for cancer and inflammatory bowel diseases, as well as management of common and complex anorectal pathologies. He has delivered presentations in more than 200 meetings and has been an invited speaker for 130 talks in congresses and workshops. He is the author/coauthor of 130 internationally cited peer-reviewed journal publications (5500 citations, *h*-index: 37).

Among other societies, he is a member of the European Association for Endoscopic Surgery (EAES) Research Committee and the International Committee of the American Society of Colon and Rectal Surgeons, while representing Greece as one of the committee members of a European COST (European Cooperation in Science and Technology) research platform on perioperative care of cancer patients.

The Human Microbiome

1

Nick-Panagiotis Andreou and Maria Gazouli

Abstract

Humans have coevolved with the trillions of microorganisms that inhabit their body, namely human microbiome. The human microbiome, especially gut microbiome, has gained an extensive interest over the last decades due to state-of-the--art technology and large-scale metagenomics studies that attempt to unravel the mystery of this complex, heterogenous ecosystem and its repercussions to host physiology. Bacteria have been the center of attention across research literature, but here an overview of the role of fungi, archaea, viruses, and protozoa is addressed as well. The aim of this chapter is to explore the diversity of taxonomic composition of human microbiota and their pivotal role in regulating host metabolism, immune system, and protection against invading pathogens. The chapter also focuses on the potential external factors (initial colonization, diet, lifestyle) prompting variable configurations of human microbiota that lead to imbalance of homeostasis (dysbiosis) and result in a broad spectrum of pathological diseases, such as obesity, inflammatory bowel disease, and *Clostridium difficile*-induced diarrhea.

Keywords

Microbiome · Microbiota · Dysbiosis · Diet · Antibiotics

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1.1 Introduction

The human body is inhabited by a vast number of microorganisms that live in concordance with their host and are commonly referred to as human microbiota of microflora. The human microbiota contains a collection of commensal, symbiotic, and opportunistic pathogenic bacteria, fungi, archaea, viruses, and protozoa (Sekirov et al. 2010). Bacteria are considered the most prominent group in the community, estimated to be approximately 10^{13} to 10^{14} microbial cells, with around 1:1 microbial cells to human cells ratio (Sender et al. 2016). Therefore, microbiome research has been mainly focused on bacteria, whereas fungi and viruses have recently started to gain more attention concerning their pivotal role in homeostatic regulation (Vemuri et al. 2020). The microbiota colonizes various sites of the human body including oral cavity, skin, genital organs, and respiratory and gastrointestinal (GI) tract (Lloyd-Price et al. 2016). The GI tract occupies a major surface, highly enriched in nutrients, creating a preferable environment for microbial growth and colonization. Additionally, the gut microbiota is not homogenous and microbial composition varies between sites or different layers of the same tissue, such as the intestinal epithelium, where the microbes present in the intestinal lumen are significantly distinct from the microbes attached to the epithelium or those entrapped within the mucus layer. The majority of intestinal microbiota is primarily comprised of strict anaerobes that dominate over anaerobes and facultative anaerobes and is classified to the four major phyla of *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*, with minor proportions of species belonging to the phyla of *Fusobacteria*, *Tenericutes*, and *Verrucomicrobia* (Sekirov et al. 2010).

The intestinal microflora is involved in host physiology, regulating digestion, vitamin production, xenobiotic drug metabolism, immunological responses as well as conferring protection against pathogen perturbation (Gouba et al. 2019). Changes in the balance of healthy microbial communities, namely dysbiosis, are often associated with numerous pathological conditions, such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and obesity (Gouba et al. 2019). The gut microbiota community is dynamic (Li et al. 2016), meaning that not all microorganisms can colonize the gut permanently, hence homeostasis relies on maintaining the microbial biodiversity, which is characterized by its species evenness (the different kinds of species) and richness (the number of different species) (Vemuri et al. 2020). This is challenging for studies focusing on humans, since biopsy sampling is infeasible and the majority of data is obtained by fecal specimens, which may contain occasional species (Sam et al. 2017). Consequently, the use of "humanized" gnotobiotic animal models could provide insight into the mechanisms of microbiome regulation, evaluate potential therapeutic treatment in microbiome-related diseases and assess the pharmacological monitoring of the selected treatment (Kho and Lal 2018).

The composition and the properties of human microbiome were formerly poorly characterized due to technology limitations regarding lack of optimized techniques for noncultivable microbial species and curated reference databases (Gouba et al. 2019). Advances in sequencing technology (e.g. NGS) and bioinformatic tools

enabled large-scale sequenced-based microbiome projects such as Human Microbiome Project (HMP) and Metagenomics of Human Intestinal Tract (MetaHIT), funded by the United States National Institutes of Health (NIH) and the European Commission, respectively, that resulted in reference genome mapping, metagenomic assembly, gene cataloging, and metabolic reconstruction of human microbiome (Kho and Lal 2018). Analysis of HMP samples along with lifestyle information has revealed that life history features and microbiome composition are considerably intertwined (Cresci and Bawden 2015). Microbial establishment in the human gut begins promptly after birth, hence delivery and feeding method of the infant determine initial colonization, and it is assumed that this initial colonization sets the ground for the composition of intestinal microbiota throughout adulthood. Dietary habits and use of antibiotics can directly affect the gut microbiome composition, while host genetics is suggested to have an indirect impact, probably by altering host metabolism. Notably, composition of intestinal microflora remains fairly stable at the phylum level and the four dominant groups are highly conserved across individuals, despite their proportional variation. Functional redundancy within those groups allows for interindividual variation of microbial species while preserving the maintenance of proper function (Sekirov et al. 2010).

A remarkable progress has been made to elucidate the relationship between the commensal microbiome and its host, as well as their subsequent impact on dysbiosis--related disease and therapeutic approach. However, human microbiome research is still in its infancy and further investigation is required to unravel the mystery of this field. The aim of this review is to compile information from various studies in order to redefine the composition and the function of the human microflora, depending on colonization site, and exemplify the dysbiotic features that are associated with a particular set of diseases.

1.2 Microbiome Composition

The composition of the human commensal microbiome exhibits a large variety of microorganisms with distinctive characteristics. Researchers were formerly constricted to culture-based methods for classification, performing biochemical tests, using different growth media to select specific populations and staining for phenotypic identification under microscope (e.g. Gram stain for bacteria, lactophenol stain for fungi) (Gouba and Drancourt 2015). These methods have a limited ability in providing sufficient information since more than 80% of the gut microbiome and mycobiome are unculturable under standard laboratory conditions (Eckburg et al. 2005). However, combination of high-throughput cultivation followed by MALDI--TOF-MS and 16S rRNA identification allows for “culturomics” to be still widely used (Gouba et al. 2019; Lagier et al. 2012).

Since the advance of molecular, genomic, and bioinformatic tools, research has been focused on genome sequencing approaches, “fingerprinting” methods, DNA microarrays, FISH, and qPCR to avoid culture bias (Sekirov et al. 2010). These techniques require the use of relatively small genes as markers of genetic diversity,

providing that they maintain balance of conservation and variance (Peterson et al. 2008). Microbial classification is based on the 16S ribosomal RNA (rRNA) sequence, while fungal characterization targets the 18S rRNA or the internal transcribed spacer (ITS) sequence (Suhr and Hallen-Adams 2015). Targeted sequences are then clustered into Operational Taxonomic Units (OTUs), based on their sequence identity and compared with existing databases (Gouba et al. 2019). Each technique has its benefits and its drawbacks and the selection is determined by the application. "Fingerprinting" methods, such as denaturing gradient gel electrophoresis (DGGE), are primarily used for comparative studies, but they are limited by the resolution of fragments on gel. Microarrays, FISH, and qPCR have been proved useful as screening tools for clinical applications, yet are incapable of identifying novel species of microorganisms. Next generation sequencing (NGS) technology has significantly decreased the cost of full-length (Sanger) sequencing and expanded our knowledge in microbiome diversity, though it demands extensive data analysis (Sekirov et al. 2010).

Despite the continuously growing number of identified commensal microbes in the human body, there was inadequate reference regarding their roles in human physiology, and numerous species were still unculturable or uncharacterized. Consequently, the National Institutes of Health (NIH) and the European Commission initiate the Human Microbiome Project (HMP) and the MetaHIT (METAgenomics of Human Intestinal Tract), respectively, to address these issues. Metagenomic analysis provided information from the collective genomes of a community about the organisms' composition and their function in the community. Therefore, both projects established a microbial genes record depending on specific body sites, revealed the implications of microbiome on human diseases, and they developed new tools and reference databases for organization, storage, and comparative analysis (NIH HMP Working Group 2009; Qin et al. 2010; Weinstock 2012).

The human body is inhabited by trillions of microorganisms that symbiotically live and have coevolved with the host, rendering this ecosystem as one of the most important mediators of human health and disease (Lloyd-Price et al. 2016). These commensal microbes are referred as microbiota or microflora and are comprised of bacteria, viruses, archaea, and eukaryotes, mainly fungi and protozoa (Lederberg and McCray 2001). They reside in the gastrointestinal (GI) tract (25%), the oral cavity (25%), the skin (21%), the airways (14%), and urogenital tract (9%) (HMP). The most well-studied microbiota in humans are bacteria, with the majority of them belonging to the phyla of *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Rajilić-Stojanović et al. 2007). Although bacteria were initially thought to predominate, it is now recognized that the healthy human gut is inhabited by 10^{15} bacteriophages, making viruses the most prevalent microorganisms (Lozupone et al. 2012). Less extensive references considering the archaea demonstrate that they are mostly methanogens (methane-producing organisms) and they play an important role in gut function (Gaci et al. 2014; Matijašić et al. 2020). The eukaryotic community is mainly represented by fungi (also referred as mycobiota) which belong to the phyla of Ascomycota, Basidiomycota, and Zygomycota (Sam

et al. 2017; Huseyin et al. 2017), followed by protozoan parasites with *Blastocystis hominis* being the most common (Matijašić et al. 2020).

The human GI tract is extremely colonized by microbes and the gut microbiome has received the greatest attention so far. The GI tract is comprised of esophagus, stomach, small intestine, and large intestine thus providing an enormous surface for microbial colonization. There are 10 to 10^2 CFU/ml of microbes starting from the stomach and duodenum (*Lactobacilli*, *Helicobacter*, *Streptococci*, *Veillonella*, Yeasts), 10^4 to 10^8 CFU/ml moving on to jejunum and ileum (*Bacteroides*, *Bifidobacteria*, Coliform bacteria, *Fusobacteria*, *Lactobacilli*, *Streptococci,* members of Actinomycetaceae and Corynebacteriaceae) and 10^{10} to 10^{12} CFU/ml reaching the colon (*Bacteroides*, *Bifidobacteria*, *Clostridia*, Coliform bacteria, *Eubacteria*, *Fusobacteria*, *Lactobacilli*, *Proteus*, *Pseudomonades*, *Staphylococci*, *Streptococci*, *Veillonella*, members of Enterobacteriaceae, Lachnospiraceae, Prevotellaceae, and Methanobacteriaceae, Yeasts, Protozoa) (Sekirov et al. 2010; Lloyd-Price et al. 2016; Cresci and Bawden 2015). Longitudinal variations can also be observed in the intestine with the epithelium and the intestinal lumen governed by particular species (*Clostridium*, *Enterococcus*, *Lactobacillus* and *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterobacteria*, *Enterococcus*, *Lactobacillus*, *Ruminococcus*) (Sekirov et al. 2010). The composition of the gut mycobiome has been relatively unstable with great interindividual variability, therefore predominant species differ among various studies (Hallen-Adams and Suhr 2017). However, there are some species often encountered in the GI tract, but it is not clear whether they are true inhabitants or they are "passing through" (Sam et al. 2017). These include *Candida* and *Phialemonium* in stomach gastric fluid, *Cladosporium* in ileum and fecal samples, *Galactomyces* and *Geotrichum* in stool samples, *Dothideomycete* sp., *Galactomyces geotrichum*, and *Ustilago* sp. in colon mucosa, as well as species of *Aspergillus*, *Debaryomyces*, *Penicillium*, *Saccharomyces*, and *Trichosporon* (Sam et al. 2017; Hallen-Adams and Suhr 2017; Witherden et al. 2017).

The oral cavity is the second most habituated body part following the gut and most individuals share a common core oral microbiome at the genus level. The microbial communities of the mouth consist of viruses (*Herpes simplex*, Human Papilloma Virus) (Scott et al. 1997), protozoa (*Entamoeba gingivalis*, *Trichomonas tenax*), archaea (*Methanobrevibacter oralis*, *Methanobacterium curvum/congolense*, *Methanosarcina mazeii*) (Matarazzo et al. 2011), fungi (*Aspergillus*, *Aureobasidium*, *Candida*, *Cladosporium*, *Cryptococcus*, *Fusarium*, members of Saccharomycetales) (Ghannoum et al. 2010) and bacteria (Wade 2013). The dominant bacterial phyla are Actinobacteria (*Actinomyces*, *Angustibacter*, *Corynebacterium*, *Kineococcus*, *Rothia*), Firmicutes (Gemella, Paenibacillus, *Selemonas*, *Streptococcus*, *Veillonella*), Proteobacteria (*Aggretibacter*, *Alysiella*, *Kingella*, *Neisseria*), Bacteroidetes (*Capnocytophaga*, *Tannerella*, *Porphyromonas*), Spirochaetes and Fusobacteria (Dewhirst et al. 2010). There are no significant geographical differences suggesting that diet and environment do not affect the oral microbiome composition (Wade 2013; Solbiati and Frias-Lopez 2018).

The skin represents the largest organ of the human body, with each body surface providing various microenvironments for microbe colonization depending on pH, moisture, sebum content, etc. (Segre 2006). It has been observed that the skin microbiota communities retain their stability regardless of environmental changes with the exception of eukaryotic DNA viruses that exhibit high intraindividual variance (Oh et al. 2016). Once again bacterial colonization is enriched in the skin with species of the lipophilic *Propionibacterium* dominating sebaceous sites and species of *Staphylococcus* and *Corynebacterium* thriving in moist areas (Segre 2006). Interestingly, bacteriophages associated with *Propionibacterium* and *Staphylococcus* are persistently present in every skin site studied, whereas no core DNA virome is found to be conserved (Oh et al. 2016; Byrd et al. 2018). The less abundant mycobiome exert great similarity across the body with *Malassezia* being the most prevalent in core body and arm sites. *Malessezia* spp. is prevalent in dandruff-affected scalps (Park et al. 2012) and is implicated in atopic dermatitis (Zhang et al. 2011). Conversely, foot sites are susceptible to transient fungal colonization of diverse species (*Malassezia*, *Trichophyton*, *Aspergillus*, *Cryptococcus*, *Epicoccum*, *Rhodotorula*) and this might also explain the remarked variability of eukaryotic DNA viruses at that site (Byrd et al. 2018). Bacterial communities on hands belong to the phyla of Firmicutes (classes Bacilli and Clostridia, families Staphylococcaceae and Streptococcaceae), Actinobacteria (families Corynebacteriaceae and Propionibacteriaceae), Proteobacteria (classes Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria), Bacteroidetes (classes Bacteroidiia, Flavobacteria, and Sphingobacteria) and Fusobacteria, while fungal communities included *Malassezia*, *Aspergillus*, *Candida*, and *Saccharomyces* (Edmonds-Wilson et al. 2015).

The vagina hosts a dynamic microbial ecosystem that alters its composition in consideration of numerous factors such as age, menstrual cycle, and types of birth control. The main phyla present in the vagina is Firmicutes, with the predominance of the *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus gasseri* and *Lactobacillus jensenii*. These four species are well adjusted to the vaginal environment, have different properties than nonvaginal species (e.g. lower %G + C content, inability to metabolize glycogen), differentiate across ethnicity groups (found in 91% of Caucasian vs. 68% of African women) and depend on estrogen and glycogen levels. Studies during pregnancy reveal that pregnant women have higher abundance of *L. crispatus* and *L. iners* and also confirm that there is a positive correlation between increase of estrogen levels and stability of vaginal communities (Nunn and Forney 2016). Other species that may flourish in the vaginal environment include *Gardenerella vaginalis*, *Atopobium*, *Bifidobacterium*, *Corynebacterium* (Actinobacteria), *Enterococcus*, *Megasphaera*, *Peptostreptococcus*, *Staphylococcus*, *Veillonella* (Firmicutes), *Prevotella* (Bacteroidetes), *Escherichia* (Proteobacteria), and *Candida* spp. Microbial invasion of amniotic cavity is a common cause of intra--amniotic infection and the usual suspects are *Mycoplasma hominis* and *Ureoplasma urealyticum* from the phylum of Tenericutes. Additional species may include *Fusobacterium*, *Leptotrichia*, *Sneathia* (Fusobacteria), *Gardenerella vaginallis*

(Actinobacteria), *Bacteroides* (Bacteroidetes), *Streptococcus* (Firmicutes), and *Candida* spp. (DiGiulio 2012; Zhou et al. 2004).

Bacteria are the prominent members of the human microbiome and therefore extensively studied, yet there is a growing interest about the viruses and the archaea that cohabit the human gut. The human virome includes phages, prophages, eukaryotic viruses, and retroviruses (Vemuri et al. 2020), while it is also considered that a "core-phageome" exists and consists mainly of double-stranded DNA viruses of the order Caudovirales (families Myoviridae, Podoviridae, Siphoviridae) and single--stranded DNA viruses (family Microviridae) (Manrique et al. 2016). The eukaryotic virome contains species of the families Adenoviridae, Anelloviridae, Astroviridae, Parvoviridae (genus *Bocavirus*), Picornaviridae (genus *Enterovirus*), and Picobirnaviridae (Vemuri et al. 2020; Matijašić et al. 2020). Considering the archaea, there are four commensal species with *Methanobrevibacter smithii* as the dominant species of the gut, followed by *Methanosphaera stadtmanae*, *Methanomassiliococcus luminensis* (fecal samples) and *Methanobrevibacter oralis* (oral mucosa). There are also two nonmethanogenic species found namely *Haloferax massiliensis* and *Haloferax assiliense* and several members of the orders Methanosarcinales, Methanobacteriales, Methanococcales, Methanomicrobiales, Methanopyrales, Desulfurococcales, Sulfolobalales, Thermoproteales, Nitrosphaerales, and Halobacteriales (Matijašić et al. 2020).

1.3 Function of the Microbiome

A wide range of microbes reside in the human body, composing a complex and dynamic system that is associated with numerous functions such as vitamin production, metabolic processes, regulation of the immune response, and protection against pathogens perturbation (Li et al. 2016; Kho and Lal 2018). Most of these microorganisms have developed a symbiotic relationship with the host and they are not harmful, yet some of them are potential pathobionts, meaning that under certain conditions or relocation can be responsible for various diseases. At this point it should be noted that even though the terms microbiota and microbiome are interchangeable throughout international literature they are equally distinct. Microbiota refers to the community of microorganisms that live in an individual's body and is composed of bacteria, archaea, viruses, fungi, and other eukaryotes, whereas microbiome refers to the collection of genomes and genes present in the microbiota (Gordon 2012).

The gut microbiota is responsible for the fermentation of complex carbohydrates, indigestible polysaccharides, and insoluble dietary fibers resulting in the production of short chain fatty acids (SCFAs) (Donia and Fischbach 2015; Lee and Hase 2014). SCFAs (acetate, propionate, and butyrate) serve as energy metabolites for colonocytes, as their implication in water and electrolyte absorption contributes to a large extent in the mitochondrial ATP production (Dumas 2011), prevent impairment of intestinal barrier and provide protection against pathogens (e.g. butyrate inhibits yeast to hyphae transition of *C.albicans*) (Swidergall and Ernst 2014). Energy is

also provided from the glycosaminoglycan degradation and is supplied to liposaccharides (LPS) synthesis, which are vital components of the outer membrane of Gram-negative bacteria (Poole 2002).

The gut bacteria are also essential in the metabolism of bile acids, the production of antimicrobial proteins (AMPs), and the synthesis of essential amino acids and vitamins. Primary bile acids are synthesized in the liver, secreted into the intestine tract where they are mostly reabsorbed, while the unabsorbed part is bioconverted to secondary bile acids by bacterial enzymes (e.g. from *Clostridium perfringens*) and the secondary bile acids are then transported back to the liver (Ajouz et al. 2014; Gopal-Srivastava and Hylemon 1988). Epithelial cells of the gut, skin, and respiratory tract produce a group of proteins with antimicrobial properties (AMPs) that act as natural antibiotics. Defensins, cathelicidins, and C-type lectins are among the most common AMPs that aim to the disruption of the microbial cell wall (or membrane). Apart from their direct actions against pathogens, AMPs act as mediators of inflammatory responses through their chemotactic activity on leukocytes and interaction with TLR ligands (Gallo and Hooper 2012).

Vitamins are indispensable for metabolic processes and gut microbiota along with food-supplied lactic acid bacteria help producing them in the human body. Species from the genera of *Lactobacillus*, *Bifidobacterium*, *Bacillus* and *Escherichia* are involved in the synthesis of menaquinone (vitamin K_2), riboflavin (vitamin B_2), pantothenic acid (vitamin B_5), folate (vitamin B_9) and cobalamin (vitamin B_{12}) (LeBlanc et al. 2013). Vitamin K is essential in reducing vascular calcification, increasing HDL and decreasing cholesterol levels thus confining the risk for cardiovascular disorders (Geleijnse et al. 2004; Kawashima et al. 1997). Members of the vitamin B complex act as coenzymes for key metabolic pathways and it is worth mentioning that vitamins B_5 and B_{12} are exclusively synthesized by the gut microbiome (Andrès et al. 2004; Gominak 2016).

Aside from bacteria, archaea participate in the anaerobic fermentation producing SCFAs, CO_2, and H_2 (Samuel and Gordon 2006). Methanogens then use H_2 and CO_2 for methanogenesis, a process that results in improved bacterial fermentation, complete anaerobic degradation of organic substances, and inflammatory responses. It has been recently documented that *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* are implicated in monocyte-derived dendritic cell maturation and their subsequent pro-inflammatory cytokine release (Chaudhary et al. 2018), whereas *Methanomassiliococcus luminyensis* could degrade trimethylamine (TMA) (Borrel et al. 2017) and reduce TMA-N-oxide plasma levels impeding cardiovascular and chronic kidney diseases (Liu et al. 2015; Tang et al. 2015).

Interaction between intestinal microflora and host immune system is being extensively studied since disturbance of this homeostatic relationship could lead to pathogenesis. It has been reported that a key regulator of intestinal homeostasis is the balance between T regulatory cells (T_{reg}) and T helper 17 cells (T_{H17}). Firmicutes as well as *Bacteroides fragilis* and *Bifidobacterium infantis* promote maturation of T_{reg} cells, which suppress aberrant T_{H17}-induced inflammation. Hence T_{reg}/T_{H17} ratio, along with SCFAs, maintain the integrity of the intestinal barrier against immune inflammatory response (Atarashi et al. 2008; Chen et al. 2017; El Aidy et al. 2012;

Lawley and Walker 2013; Paust et al. 2004; Peng et al. 2007). Enteric nervous system (ENS) is comprised of enteric glial cells (EGCs) which are astrocyte-like cells that control exocrine/endocrine secretions, gut motility, blood flow, and inflammation (Ochoa-Cortes et al. 2016; Yu and Li 2014). Malfunction of ENS and EGCs could lead to disruption of intestinal barrier, motility disorders (e.g. constipation), various GI disorders (e.g. IBD, IBS), or infection-induced gut inflammation (Kho and Lal 2018).

Commensal fungi are also involved in the immune system both directly by interacting with the immune cells and indirectly by regulating essential metabolites (Lee and Mazmanian 2010). The role of Candida species is ambiguous as *Candida kefyr* reduces IL-6 production thus attenuating gut inflammation (Takata et al. 2015), whereas *Candida albicans* produces ligands (e.g. β-1,3 glycan) for pattern recognition receptors (PRRs) that stimulate host cells to secrete prostaglandins and inflammatory cytokines (Lee and Mazmanian 2010). *C. albicans*-produced prostaglandin E2 is transferred through the bloodstream to the lungs where it acts on macrophages inducing allergic airway inflammation (Kim et al. 2014). Conversely, *Saccharomyces boulardii* stimulates intestinal anti-toxin IgA (Qamar et al. 2001), IL-10, and EGF production (Thomas et al. 2011) and decreases the secretion of proinflammatory cytokines (e.g. TNFα, IL-6) exerting a protective role against gut inflammation (Thomas et al. 2011).

Intestinal microbiota accounts for the defense of the host against perturbation of pathogenic invaders or overgrowth of pathobionts. This could be achieved through competition of human microbiome and pathogens for common habitats and nutrients ("competitive exclusion") or by activating the host immune system (Kho and Lal 2018; Belzer and de Vos 2012). Competition is often observed between *Lactobacillus* and fungal overgrowth in the gut or vagina (Rizzo et al. 2013). In terms of immune system modulation, *Saccharomyces boulardii* secretes enzymes to inactivate toxins produced by *Clostridium difficile* and *E. coli* (Buts et al. 2006; Castagliuolo et al. 1999) and inhibits proliferation of *C. albicans*, *Salmonella typhimurium*, and *Yersinia enterocolitica* (Enaud et al. 2018). Therefore, trans--kingdom interactions are responsible for maintaining the balance of the healthy human microbiome (Lloyd-Price et al. 2016).

Skin microbiota has been assigned to survive in an acidic environment, with ultraviolet light exposure and minimum nutrients (basic proteins and lipids). Sweat, sebum, and stratum corneum are their main resources and microbes have been adapted to utilize them for their benefit. Keratinocytes are in the first line of defense and occupy PRRs that can sense pathogenic microbial molecules and promote the excretion of AMPs to attack potential invaders. Moreover, recruitment of T cells in response to microorganisms' presence could occur in the absence of classical inflammation ("homeostatic immunity") (Byrd et al. 2018).

Oral cavity is heavily colonized by commensal microbiome and an inquisitive potential of oral bacteria is the reduction of nitrate to nitrite contributing to cardiovascular health. Oral bacteria facilitate the fermentation of dietary carbohydrates, which leads to reduction of pH. Microbial species of oral cavity as units are unable to process complex substrates, so instead they cooperate and combine their

enzymatic activities for food digestion. Streptococci can remove oligosaccharides and glycoproteins, Gram-negative anaerobic species (e.g. Prevotella, Porphyromonas) cleave proteins to peptides, whereas Fusobacterium and Peptostreptococcus ferment amino acids producing SCFAs. Disturbance of the oral cavity microenvironment could cause a shift in the composition of oral microbiome resulting in dental caries or other periodontal diseases. Opportunistic infections by *Candida* and *Staphylococcus* can still be caused, especially following antimicrobial treatment (Wade 2013).

Vagina confers an excellent residence for microorganisms as vaginal secretions are loaded with amino acids, carbohydrates, mucins, proteins, and glycoproteins. However, this content is highly influenced by the host physiology thus directly affecting the composition of vaginal microbiome. Estrogen levels control the accumulation of glycogen and the proliferation rate of *Lactobacillus*. Glycogen is depolymerized by α-amylase into simple sugars which in turn are fermented by vaginal *Lactobacilli* to produce lactic acid. Lactic acid creates an acidic environment which is not favorable for nonindigenous microorganisms. The origin (human or microbial) of α-amylase and whether glycogen is indirectly supplied to *Lactobacilli*, after it is metabolized by other microbes, or is accumulated due to the inability of *Lactobacilli* to directly use it remains uncertain and future studies would elucidate these issues (Nunn and Forney 2016).

State-of-the-art technology has conferred great advantages toward data acquisition, and considering the aforementioned, it is obvious that microbiota is an indispensable part of the human physiology and that several pathologies occur as a consequence of the disturbance in the dynamic equilibrium between host and microbes.

1.4 Microbiome and Dysbiosis

Research in the field of commensal gut microbiome ecology attempted to identify a group of microbial taxa universally present in healthy individuals but this pursuit proved infeasible. Conversely, the alternative hypothesis of a healthy "functional core" was proposed, describing a complement of metabolic and other molecular functions that are performed by the microbiome within a particular habit but are not necessarily provided by the same organisms in different people (Shafquat et al. 2014). In accordance to this statement, a healthy-associated microbiome requires a degree of resistance against external (e.g. dietary, pharmaceutical) or internal (e.g. age) changes and the ability of resilience afterwards. Therefore, microbial health comprises not a single static state but rather a dynamic equilibrium (Lloyd-Price et al. 2016).

Perturbation of this equilibrium exerts imbalance in the composition and regulation of microbial communities, a term which is widely known as dysbiosis. Dysbiosis is more likely to occur in response to insufficient presence of commensal microbes, loss of regular microbial diversity or competition between commensal microbiome and pathogenic species for the same colonization sites and/or nutrients supply

(Tamboli et al. 2004). Other external factors that contribute to the progression of dysbiotic features include malnutrition or lack of dietary fibers and vitamins, certain food additives (e.g. preservatives, emulsifiers), chronic alcohol consumption, use of drugs or pharmaceuticals (antibiotics, anti-inflammatories, contraceptives, chemotherapy), exposure to toxic environmental substances (chemical toxins, heavy metal, radiation), and stress levels (anxiety, depression). Dysbiosis is implicated in diverse pathologies, a number of which are briefly reported in the following sections.

1.4.1 Diet

Consumption of food is related to providing the body with a range of nutrients in order to perform fundamental metabolic processes. Anthelme Brillat-Savarin, in 1826, wrote in his book The Physiology of Taste, "Tell me what you eat and I will tell you what you are," implying that eating what is regarded as being healthy your organism will be healthy as well. Bearing in mind that intestinal microbiota is involved throughout the route of food processing, presuming that gut colonization by beneficial microbial communities is favored by the consumption of healthy nutrients (e.g. plant fibers, complex carbohydrates) supports further this argument. Diet is a complex concept that depends on geographical restrictions, ethnic and cultural customs, or even moral constraints, but irrespective of what lifestyle individuals choose to follow as adults, their gut microbiome is established from the very moment they were born.

Microbes are present in the placenta (DiGiulio 2012), amniotic fluid (Satokari et al. 2009) and umbilical cord blood (Jiménez et al. 2005) and their colonization starts in utero, although the adult-like configuration occurs after the first three years of life (Yatsunenko et al. 2012), therefore delivery mode and feeding methods of infants seems to have higher impact. Vaginally delivered infants acquire their mother's vaginal microbiome, whereas caesarean delivered infants are encountered with the skin microbiota of the mother. Infants born vaginally have higher prevalence of Bacteroidetes over Firmicutes compared to infants delivered thought caesarean section (Dominguez-Bello et al. 2010), while the latter show higher microbial diversity, delayed colonization of Bacteroidetes (Jakobsson et al. 2014) and an enrichment of pathobionts such as *Enterobacter cancerogenus*, *Haemophilus* spp, *Staphylococcus* spp, and *Veillonella dispar* (Dominguez-Bello et al. 2010; Bäckhed et al. 2015).

Breastfeeding favors the growth of Bifidobacterium, Bacteroides, and microbes that are transmitted after contacting the maternal skin (Dominguez-Bello et al. 2010; Zivkovic et al. 2011). Human breast milk is a complex of undigestible oligosaccharides that serve as a resource of prebiotics especially for *Bifidobacterium* species (*B. breve*, *B. adolescentis*, *B. longum*, *B. bifidum*, *B. dentium*) (Martín et al. 2009). Formula-fed infants are often colonized by *E. coli* and *Clostridium difficile* (Penders et al. 2006) and their fecal samples contain more anaerobic or facultative anaerobic microbes compared to that of breast fed infants (Stark and Lee 1982). Early establishment of infant gut with SCFA-producing species, such as *Bacteroides*, *Bifidobacterium*, *Lactobacillus* and *Faecalibacterium*, is indicative of a healthy

microbiome (Byrne et al. 2015). Dietary changes, illness or antibiotic treatment could induce a shift in the microbial composition during infancy which is associated with higher risk of asthma, atopic eczema (Abrahamsson et al. 2012) and allergic rhinitis (Bisgaard et al. 2011).

Bacterial community composition gradually shifts from Bifidobacterium--dominated in infancy to Bacteroidetes and Firmicutes dominance in adulthood and remains relatively stable (Ottman et al. 2012). However, recession of gastrointestinal function over senescence affects gut microbiome, with limited presence of *Bacteroides*, *Bifidobacteria*, and *Clostridium* cluster IV in elderly, yet higher prevalence of Bacteroidetes compared to the abundance of Firmicutes in younger adults (Zwielehner et al. 2009). As opposed to age, nutritional value has a greater influence on microbiome configuration. High protein intake is associated to increased *Bacteroides*, *E. coli*, and *Enterobacteria*, while growth of *Candida* species is positively correlated with carbohydrate consumption and negatively correlated with saturated fatty acids (Hoffmann et al. 2013). Vegetarian or vegan diet is enriched in carbohydrates and insoluble fibers that are fermented into SCFAs, leading to lower luminal pH, which is inhibitive for *E. coli* or *Enterobacteria* (Cresci and Bawden 2015) but favorable for the plant pathogenic *Fusarium*, and the fungal species of *Malassezia*, *Aspergillus*, and *Penicillium* (Hoffmann et al. 2013). Dietary habits are also affected by the availability of food resources. A study comparing European and African children concluded that there are differences in their gut microbiomes, with higher levels of Firmicutes and Proteobacteria in European compared to predominance of Actinobacteria and Bacteroidetes in African (De Filippo et al. 2010). Although SCFA-producing species were found in both groups, African children were exclusively colonized by *Xylanibacter*, *Prevotella*, *Butyrivibrio*, and *Treponema*, which utilize xylene, xylose, and carbomethylcellulose to produce SCFAs, resulting in fourfold increase in levels of butyrate and propionate (Flint et al. 2008).

Obesity is a medical condition where energy intake (food) exceeds the energy expenditure (thermogenesis) resulting in excess body fat accumulation (Maruvada et al. 2017) and is associated with abnormalities in the composition of human microbial communities. Significantly increased abundance in the butyrate-producing Firmicutes and reduction in Bacteroidetes has been observed in distal colonic microbiome of obese patients. Elevated levels of Firmicutes are attributed to higher levels of class Mollicutes (phylum Tenericutes) species (Turnbaugh et al. 2006). Biodiversity of fungal species is also altered, notably decreased in the Zygomycota phylum, with prevalence of *Nakareomyces*, *Candica*, *Penicillium*, and *Pichia* in obese patients compared to *Mucor*, *Candida*, and *Penicillium* in non-obese (Mar Rodríguez et al. 2015).

Type 2 diabetes (T2D) is a metabolic disorder of insulin resistance that is linked to obesity and changes in the gut microbiome are implicated in T2D development (Karlsson et al. 2013; Larsen et al. 2010; Qin et al. 2012). Increased Bateroidetes/Firmicutes ratio, abundancy of Betaproteobacteria species and significantly lower proportion of *Clostridia* have been documented in T2D patients versus nondiabetic controls (Larsen et al. 2010). Higher percentage of butyrate-producing species such

as *Feacalibacterium prausnitzii*, *Roseburia intestinalis*, and *R.inulinivorans* has been also observed in healthy individuals compared to greater colonization of pathobionts including *Eggerthela lenta*, *Clostridium symbiosum*, and *E. coli* in T2D patients (Qin et al. 2012). Significant reduction of Verrucomicrobia has been noticed in prediabetes subjects suggesting that assessment of Verrucomicrobiaceae concentration could be potentially used as a diagnostic biomarker for progression of T2D (Zhang et al. 2013).

1.4.2 Antibiotics

Antibiotics are antimicrobial compounds that either target the bacterial cell wall/membrane or interfere with bacterial essential enzymes thus inhibiting their growth (bacteriostatic agents) or block bacterial protein synthesis and immediately kill them (bactericidal agents). Narrow-spectrum antibiotics affect specific types of bacteria (e.g. Gram positive), whereas broad-spectrum target a wider range of bacteria (Kohanski et al. 2010). Use of broad-spectrum antibiotics that affect anaerobic bacteria is correlated with growth of yeast flora in the gut compared to antibiotics with poor anaerobic activity (Samonis et al. 1993). Treatment with antibiotics could be detrimental not only for the targeted pathogen but also for the hosts' bacterial community resulting in both short- and long-term effects on human microbiome (Jernberg et al. 2010). One approach indicates the introduction of a new species, whereas the other suggests alteration in the bacterial resistance genes (Antonopoulos et al. 2009; Jakobsson et al. 2010; Robinson and Young 2010).

Resistance is categorized as active (e.g. adapting to a counterattack against an antibiotic) or passive (antibiotic-independent adaptations). Active antibiotic resistance is achieved through efflux of the drug from the cell via membrane-associated pumping proteins, modification of the drug target (e.g. mutation of rRNA) or synthesis of modifying enzymes that impede with the drug activity (Wright 2005). Gram-negative bacteria are shielded with a bacterial outer membrane, constituted of porins and liposaccharide (LPS), and that often confers intrinsic resistance to species like *E. coli*, *Pseudomonas aeruginosa*, *Burkolheria* sp., *Stenotrophomonas maltophilia*, and *Acinetobacter* sp. Antibiotic resistance genes are typically found in Firmicutes (52%), Proteobacteria (32%), and Bacteroidetes (15%). Recently, studies have identified 1093 genes that confer resistance to 50 of the total 68 antibiotic groups and most of these genes code for proteins that modify or protect the target of the antibiotic (Quinn 1998).

Clostridium difficile infection (CDI) is a gastrointestinal disease, strongly correlated to antibiotic treatment, caused by the *Clostridium difficile*, with symptoms of diarrhea and pseudo-membranous colitis and is the most common cause of hospital-acquired diarrhea (Kho and Lal 2018; Di Bella et al. 2015). *Clostridium difficile* is a Gram-positive, anaerobic, sporogenic, and toxin-producing bacterium that belongs to the Firmicutes. Under steady state, overgrowth of *C. difficile* is prevented by colonization resistance of commensal gut microbiome, presumably by metabolizing primary bile acids to secondary bile acids. It is proposed that primary

bile acids (cholate derivatives) serve as germinant for *C. difficile* spores, while secondary bile acids (deoxycholate) inhibit its growth (Song et al. 2008). Antibiotic treatment results in lower diversity of secondary bile acids-synthesizing microbes (e.g. C. Scindens) and a subsequent reduction of microbial bioconversion of primary bile acids to secondary bile acids, allowing *C. difficile* overgrowth (Antonopoulos et al. 2009; Theriot et al. 2014). Secretion of toxins A and B (TcdA and TcdB) produced by *C. difficile* causes damage to the cytoskeleton and colonial epithelial barrier integrity (Genth et al. 2006; Pruitt et al. 2012), followed by severe inflammatory response that induce impairment in intestinal ion absorption leading to diarrhea (Kho and Lal 2018).

1.4.3 Lifestyle

Stress is a situation that triggers a biological response to a specific demand or threat. Physiological and psychological stressors activate the hypothalamic-pituitary--adrenal (HPA) axis (Lucassen et al. 2014: 100). The gut microbiota is sensitive to stress mediators responding to the release of stress-related neurotransmitters or acting as carriers of neuroactive compounds (Lyte et al. 2011). Exercise is a physiological stressor that is beneficial for the healthy microbiome, yet high intensity training is extremely stressful for the body and that may prompt alterations in microbial communities or intestinal barrier aggravation (de Oliveira et al. 2014). Professional athletes follow a strict dietary plan of high protein and caloric intake which positively correlates with enhanced gut microbial diversity and interestingly that was reflected by the presence of 22 bacterial phyla compared to 11 and 9 phyla in the low and high Body Mass Index (BMI) controls, respectively. However, prolonged excessive training may lead to intestinal hypoperfusion, increased intestinal permeability, and endotoxin translocation (Gleeson and Williams 2013).

The human GI tract function is governed by millions of neurons that comprise the enteric nervous system (ENS), which is the second largest pool of neurons, outside the brain (Spencer et al. 2018). The ENS propagates and receives signals from the central nervous system (CNS) through the parasympathetic (via the vagus nerve) and sympathetic (via the prevertebral ganglia) nervous systems, but has also the ability to operate independently, therefore it has been characterized as a "second brain" (Li and Owyang 2003). The interplay of biochemical signaling between ENS and CNS along with the association of gut microbiome is commonly described by the term "gut–brain axis" (Mayer et al. 2014). This axis includes neuronal, endocrine, immune and metabolic pathways that are intertwined and collectively regulate the functioning of each other, maintaining homeostasis. Alterations in microbial communities or other physical and psychological stressors that interfere with the proper function of the axis are held responsible for dysbiotic features (Sommer and Bäckhed 2013).

There are numerous mechanisms by which intestinal microflora affects the gut–brain axis contributing to the pathogenesis of functional gastrointestinal disorders (e.g. IBS) (Martinucci et al. 2015) or even CNS diseases (e.g. anxiety, depression)

(Pirbaglou et al. 2016). It is noted that gut microbiota is capable of producing neurotransmitters that can either act locally or cross the mucosal intestinal layer and exert their actions in other systems (Wall et al. 2014). *Lactobacillus* and *Bifidobacterium* synthesize and release GABA; Bacillus, *S. cerevisiae*, and *Penicillium chrysogenum* produce norepinephrine; while serotonin can be synthesized by *Candida*, *Streptococcus*, and *Enterococcus* spp. (Tetel et al. 2018) A study proposed that serotonergic enterochrommafin cells in the gut epithelium act as chemosensors and transduce chemosensory information to the nervous system (Bellono et al. 2017). *C. albicans* is also able to produce histamine, a neurotransmitter involved in appetite regulation, circadian rhythm, and cognitive activity (Voropaeva 2002).

Activity of HPA axis can also be impacted by commensal gut microbiome, probably through microbial secretion of cytokines (e.g. IL-1, IL-6) and subsequent acute release of cortisol by HPA axis stimulation (Dantzer 2006). Persistent activity of HPA axis and increased levels of cortisol are highly correlated with anxiety and depression. Decreased microbial richness and diversity is observed in patients diagnosed with depression along with changes in colonization by specific taxa. Depressed patients are characterized by higher levels of Bacteroidetes, Proteobacteria and Actinobacteria and lower levels of Firmicutes compared to controls. The same study revealed increased levels of Enterobacteriaceae (Proteobacteria) and Alistipes (Bacteroidetes) and reduced proportion of Faecalibacterium (Firmicutes) (Jiang et al. 2015). However, there is a limited number of human studies concerning the effect of gut microbiome in behavioral disorders and further research is required.

The oral microbiota is extensively affected by smoking (Monteiro-da-Silva et al. 2013) and eating disorders (ED) (Back-Brito et al. 2012). Smoking is a causal factor for periodontitis and many species are associated with this disease, such as *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Anaeroglobus germinatus*, *Eubacterium saphenum*, *Filifactor alocis*, *Porphyromonas endodontalis*, and *Prevotella denticola* (Kumar et al. 2003: 80). *Candida* is present in fecal samples of smokers (58%) more frequently than in nonsmokers (29%) (Jobst and Kraft 2006). Opportunistic oral candidiasis is common to ED patients and is attributed to nutritional deficiencies in Zn, Fe, vitamin K, and water-soluble vitamins (Ghannoum et al. 2010; Lo Russo et al. 2008). Although there is a link between alcohol and fungal colonization in gut, there was no association in oral cavity (Hoffmann et al. 2013).

1.4.4 Human Genetics

1.4.4.1 GI Tract

Inflammatory bowel disease (IBD) is a group of gastrointestinal inflammatory conditions, featuring Crohn's disease (CD), in which inflammation can occur anywhere in the GI tract and ulcerative colitis (UC), which affects mainly the colon (Baumgart and Carding 2007). IBD probably emerges as repercussion of the abnormalities in host defense against commensal microbiome of genetically predisposed subjects

(Kho and Lal 2018). Normally GI mucus layer and AMPs, such as human defensins, cooperate to hinder direct interaction between luminal gut microbiota and epithelial cells preventing inflammatory responses. Dysbiotic impairment of the intestinal mucus barrier induces the growth of mucolytic bacterial species (e.g. *Ruminococcus* sp.) (Png et al. 2010) promoting gut inflammation (Johansson et al. 2008: 70; Salzman et al. 2010).

A tendency for higher portions of Actinobacteria and Proteobacteria (family Enterobacteriaceae) with a subsequent decrease in Firmicutes (family Lachnospiraceae) and Bacteroidetes is observed in IBD patients (Frank et al. 2007; Willing et al. 2010). Firmicutes is comprised of important butyrate-producing and anti-inflammatory bacteria that reduce the secretion of pro-inflammatory cytokines (IL-12, IFN-γ) and induce the production of anti-inflammatory IL-10 (Machiels et al. 2014; Sokol et al. 2008). IBD patients have lower proportions of *Feacalibacterium prausnitzii*, *Roseburia* sp., *Dialister invisus* (Firmicutes) and *Bifidobacterium adolescentis* (Actinobacteria) (Willing et al. 2010; Machiels et al. 2014; Joossens et al. 2011). Conversely, colonization is favored for *Ruminococcus gnavus* (Firmicutes), which produces a glucorhamnan recognized by innate immune cells (Henke et al. 2019), *Bacteroides fragilis* (Bacteroidetes) and members of the Enterobacteriaceae family, which have both highly endotoxic LPS on their outer membrane (Darfeuille-Michaud et al. 1998).

Fungal dysbiosis has also been noticed on IBD patients, with higher Basidiomycota/Ascomycota ratio, abundance of *C. albicans*, *Malassezia symbodialis* and reduction in *Saccharomyces cerevisae*. It has been observed that fungal and bacterial interactions are higher in UC patients and lower in CD patients (Sokol et al. 2017). Studies documented that there was greater fungal richness and diversity in inflamed mucosa versus noninflamed mucosa of CD patients and compared to healthy controls (Li et al. 2014; Ott et al. 2008). CD patients had a positive correlation with *C.glaberata* (Liguori et al. 2016) and also anti-*Saccharomyces cerevisiae* antibodies (ASCA) have been detected in their serum (Main et al. 1988). In pediatric IBD patients there is a dominance of Basidiomycota (Mukhopadhya et al. 2015) compared to the prevalence in Candida parapsilopsis and Cladosporium cladosporoides in healthy children (Chehoud et al. 2015).

Archaeal overgrowth results in reduction of butyrate and increased removal of SCFA from biofilms, prompting bacteria to become endoparasitic and invade intestinal epithelial tissue, triggering gut inflammation (Gonçalves et al. 2018; White 2017). *Methanobrevivacter smithii* levels are lower in IBD patients compared to healthy individuals (Ghavami et al. 2018). Virome is also implicated in IBD pathology with higher proportions of phages affecting Bacterial Alteromonadales, Clostridiales (*C. acetobulicum*), and Herpesviridae (increase of HBx protein) (Pérez-Brocal et al. 2015; Ungaro et al. 2019). Decreased Vigaviridae and Polydnaviridae, Tymoviridae are detected in CD and UC patients respectively, whereas in the latter there is increased abundance of Pneumoviridae and Anelloviridae (Pérez-Brocal et al. 2015; Ungaro et al. 2019; Zuo et al. 2019). UC patients are also less colonized by *Blastocystis hominis* and *Dientamoeba fragilis* (Petersen et al. 2013).

Irritable bowel syndrome is a functional gastrointestinal disorder with three subtypes: constipation-subtypes (IBS-C), diarrhea-subtypes (IBD-D), and mixed-type (IBD-M) (Longstreth et al. 2006). IBS and IBD are two distinct conditions, despite sharing similar symptoms, yet they are both associated with gut microbiota dysbiosis. Enrichment of Firmicutes and reduction of Bacteroidetes is observed in IBS patients (Jeffery et al. 2012), with Lachnospiraceae (Krogius-Kurikka et al. 2009) and *Veillonella* (Malinen et al. 2005) expressing higher abundance in IBS-D and IBS-C patients respectively. IBS patients have also higher proportion of *Dorea*, *Ruminococcus*, *Clostridium*, and lower proportion of *Bifidobacterium*, *Faecalibacterium*, and methanogens compared to healthy controls (Rajilić--Stojanović et al. 2011). The pathogens Pseudomonas aeruginosa and Staphylococcus aureus are two possible candidates for IBS pathology (Kerckhoffs et al. 2011; Rinttilä et al. 2011). Moreover, IBS-C patients have greater abundance of methane producer archaea, especially *M. smithii* and *M. stadtmanae*, compared to IBS-D patients.

Individuals with IBD are at increased risk of developing colorectal cancer (CRC), consequently changes in composition of microbial communities are also implicated in this disease (Hu et al. 2015). Non-colitogenic *Fusobacterium nucleatum* and enterotoxigenic strains of *Bacteroides fragilis* are markedly enriched in CRC patients (Toprak et al. 2006; Wang et al. 2012; Wu et al. 2013). Conversely, butyrate-producing *Feacalibacterium* and *Roseburia* are less expressed, which is associated with partial impairment of immunosurveillance and enhancement of tumorigenesis (Wang et al. 2012; Wu et al. 2013). Considering fungal mycobiome, there is an increase of Basidiomycota/Ascomycota ratio, depletion of *S. cerevisae* and enrichment of *Rhodotorula*, *Malassezia*, *Acremonium*, and *Aspergillus flavus* in CRC patients. Mycobiota differentiation has also been noted according to adenoma size and stage. Advanced adenoma biopsy samples have less diversity and increased abundance of Saccaromycetales, while nonadvanced adenoma tissues have lower proportion of Fusarium and Trichoderma, compared to adjacent rectal tissue (Luan et al. 2015).

Celiac disease is a serious autoimmune disease that occurs in genetically predisposed people, where the ingestion of gluten leads to damage in the small intestine. Significant reduction in total Gram+/Gram− bacteria ratio is observed in all phases of celiac disease, with less *Bifidobacteria* and more *Bacteroides*/*Prevotella* groups (De Palma et al. 2010; Marasco et al. 2016; Nadal et al. 2007). Studies in human colon Caco-2 cells demonstrate that gliadin, a component of gluten, induces increased gut permeability and *Bifidobacterium lactis* protects the epithelial junctions from the adverse gliadin-induced effects (Lindfors et al. 2008), whereas *Bifidobacterium longum* and *Lactobacillus casei* can regulate the production of pro--inflammatory cytokines and reduce the risk for gliadin-induced enteropathy in animal models (Laparra et al. 2012).

1.4.4.2 Neurodevelopmental

Autism spectrum disorder (ASD) is a range of neurodevelopmental disorders including autism and Asperger syndrome. ASD is significantly associated with

intestinal dysfunction and microbiome dysbiosis (Wang et al. 2011) and impaired tyrosine kinase MET signaling is potentially implicated (Ieraci et al. 2002; Okunishi et al. 2005). Higher levels of *Clostridium histolyticum*, *Bacteroides*, *Lactobacillus*, and *Desulfovibrio* (a sulfate-reducing bacterial genus) (Finegold et al. 2012) and lower levels of *Bifidobacteria*, carbohydrate-degrading *Prevotella*, *Cryptococcus*, and unclassified Veillonaceae have been reported in ASD children compared to control (Adams et al. 2011; Kang et al. 2013; Parracho et al. 2005; Song et al. 2004). Increased levels of *Sutterella* (Proteobacteria) were solely reported in children experiencing both autism and GI dysfunction but not in children with mere GI dysfunction (Williams et al. 2012).

Intestinal microbiome dysbiosis appears evident in neurodegenerative diseases such as Parkinson's (PD) and Alzheimer's (PD). Changes in SCFA concentration (Unger et al. 2016) and altered levels of species belonging to the families of Bifidobacteriaceae, Lachnospiraceae, Lactobacillaceae, Pasteurellaceae, Christensenellaceae, and Verrucomicrobiaceae are detected in PD patients (Hill-Burns et al. 2017). Likewise, AD patients with brain amyloidosis show low proportion of the anti-inflammatory Eubacterium rectale and higher proportions of the pro-inflammatory *Escherichia/Shigella* (Cattaneo et al. 2017).

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In Silico Metagenomics Analysis

2

Nikolas Dovrolis

Abstract

The field of metagenomics (study of a system's microbiome) comes with various questions researchers are called to answer. Questions about the microbiota's identity, the interactions of the participating bacteria, fungi, and viruses and their associations with health and disease. Nowadays, the answers to these questions are revealed via next-generation sequencing (NGS) and bioinformatics pipelines. NGS has allowed us to study even the unculturable microbiota whereas the development of appropriate in silico methodologies has made analyzing them fast, accurate, and accessible.

Keywords

Metagenomics · 16S rRNA · Next-generation sequencing (NGS) · Bioinformatics · Shotgun · α and β diversity

2.1 Introduction

The field of metagenomics (study of a system's microbiome) comes with various questions researchers are called to answer. Questions about the microbiota's identity, the interactions of the participating bacteria, fungi, and viruses and their associations with health and disease. Nowadays, the answers to these questions are revealed via next-generation sequencing (NGS) and bioinformatics pipelines. NGS

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has allowed us to study even the unculturable microbiota whereas the development of appropriate in silico methodologies has made analyzing them fast, accurate, and accessible. Not only that, but also these technologies have allowed us to focus on the microbiome (the total genomic profile of the microbiota) itself. As it is highlighted in this book, studies of the microbiome have emerged as key components of understanding human health. An accurate profiling of the microbiome and its function can support new diagnostic, prognostic, and personalized therapeutic strategies. This chapter lays the foundation of computational microbiome basics and provides a hands-on tutorial on statistical analysis of microbial data using two publicly available open source online platforms along with their sample data.

2.2 Sequencing Methodologies

Before understanding the functional role of microbiota in human pathophysiology we must be able to identify them with high sensitivity and specificity. Two essential NGS approaches have allowed for this advancement; 16S ribosomal RNA (rRNA) amplicon sequencing and shotgun sequencing.

2.3 16S rRNA Amplicon

As the 16S rRNA gene is the de facto housekeeping gene for identifying bacterial and archaeal populations, this first approach is focused on amplifying and sequencing it. Less commonly, other ribosomal RNAs like the 18S are used to identify fungi but those are underrepresented in literature. 16S rRNA metagenomics are cheaper and easier to conduct in a laboratory setting, than their shotgun metagenomics counterpart, when we want to study cohorts of control and patient samples (fecal matter or tissue biopsy). Library preparation for 16S is usually based on ready-to-use commercial handling and extraction kits. It should be noted here, that our results heavily depend on the sequencing technology or kit that is used for which a global standard does not exist (Salipante et al. 2014; D'Amore et al. 2016; Minich et al. 2018). The amplification steps of hypervariable regions of the 16S rRNA gene via multiplex PCR primers (Rintala et al. 2017) also introduce a burden to the process which needs to be addressed in silico.

2.4 Shotgun Metagenomics

Shotgun metagenomics is a much broader approach targeting both host and microbiota DNA in the samples. Although substantially more expensive, both in monetary terms and bioinformatics effort, it provides a broader understanding of the biological background and interacting mechanisms with higher resolution and accuracy of the results. The results are more complex to analyze but require no amplification correction and can be used to directly identify the functional role of the microbiome.

2.5 Data Pre-processing and Quality Control

For both technological approaches (sequencing of a whole sample or the 16S rRNA amplicons) small reads are produced (25–500 base pairs), enabling the detection of even low abundance or uncharacterized microorganisms. These reads require bioinformatics preprocessing via select pipelines for quality control (denoising, chimera detection, and exclusion), assembly and taxonomical categorization.

2.6 Clustering and OTU Picking

Selecting the sequencing method a researcher wants to employ relies solely on the experimental needs. In both cases we end up with a fastq or ubam (unaligned bam file) which contains the sequencing reads. After being processed for quality control purposes, these sequences will allow us to cluster them by similarity and characterize their origin (also known as binning). Operational Taxonomic Units (OTU) is the term used to describe these clusters of similar sequences which can be assigned a representative one to be used for phylogenetic alignment. Tools for this process usually rely on homology- and prediction-based algorithms.

These algorithms are usually implemented as de novo or reference-based OTU picking (16S rRNA approach) or taxonomy-independent/dependent binning (shotgun metagenomics) tools. When the host environment contains mostly known species, like the gut microbiota, a reference-based strategy (or a taxonomy dependent one respectively for shotgun metagenomics) will produce fairly accurate results with unparalleled speed using algorithms which try to align the sample's sequences to reference databases like GreenGenes (DeSantis et al. 2006), SILVA (Pruesse et al. 2007), NCBI's RefSeq (Pruitt et al. 2007), etc., and just count the aligned hits for calculating abundance. However, each database often follows its own naming scheme and should be carefully examined and cross-matched for experiments using multiples. Current implementations of this method can be found in standalone applications like Taxonomer (Flygare et al. 2016) and SPINGO (Allard et al. 2015) for 16S rRNA amplicons while MetaPhlAn2 (Segata et al. 2012), MEGAN (Huson and Weber 2012), and MGMapper (Petersen et al. 2017) work best for shotgun metagenomics data. For de novo clustering based on similarity, tools like UPARSE (Edgar 2013) and CD-HIT (Fu et al. 2012) try to individually align sequences between them, not based on a known reference, and assign them to specific clusters. The same is done for homology-independent binning in applications CONCOCT (Alneberg et al. 2014), MetaFast (Ulyantsev et al. 2016), and MetaBAT (Kang et al. 2015) (each with its own approach). This method is usually applied when trying to characterize pathogenic microorganisms of unknown origin. De novo pipelines in general are more computationally intensive but can be more extensive due to the fact that no sequences are discarded for not matching to a preexisting reference. The output of these pipelines, regardless of the methodology used, is, most commonly, a table containing all the distinct OTUs found in a sample, their abundance, and their assigned taxonomy along with some user-provided metadata where applicable.

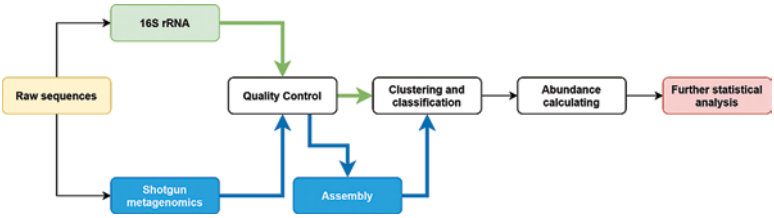

Fig. 2.1 Bioinformatics pipeline based on library preparation for microbiome analysis

Even though the need for specialists like bioinformaticians keeps rising in the research community, some ready to use, albeit with some training on computational skills, pipelines exist, which streamline the aforementioned processes allowing researchers of any discipline to create their own workflows and produce results. Implementations like QIIME2 (Bolyen et al. 2019) and mothur (Schloss et al. 2009) can perform multiple steps of data preparation and rudimentary statistical analysis on the microbiota populations. These provide an easy way for scientists to quantify and analyze their microbiome data while producing standardized reproducible results. It should be noted that bioinformatics analysis of the microbiome is computational power intensive and requires a lot of effort to standardize.

Figure 2.1 showcases a visual representation and provides a graphical summary of the appropriate steps for a bioinformatics microbiome pipeline.

2.7 Downstream Statistical Analysis

As is the case with all –omics approaches metagenomics produces a vast amount of data which need to be analyzed, associated, and understood. Data visualization provides, in most cases, the simplest and most comprehensive way for researchers to infer hypotheses, regarding the condition under which the microbiome is studied, from their results. Once again, bioinformatics and biostatistics provide solutions towards any questions one would have about the data. We will study each category of results a researcher might obtain using two online platforms for statistical microbiome analysis; Calypso (Zakrzewski et al. 2017) and MicrobiomeAnalyst (Dhariwal et al. 2017). This will allow for a more hands-on approach to this chapter. Each of these platforms requires the microbial data to be imported in their own way.

2.8 Taxonomic Analysis

When we want to know which taxa are abundant and their actual hits (raw number or relative percentages of representative sequences), in our samples or based on groupings (e.g. controls vs. patients), we employ taxonomic analysis. Following the biological taxonomy for OTUs of phylum → class → order → family → genus → species, we can visualize the distinct levels for our microbiota's

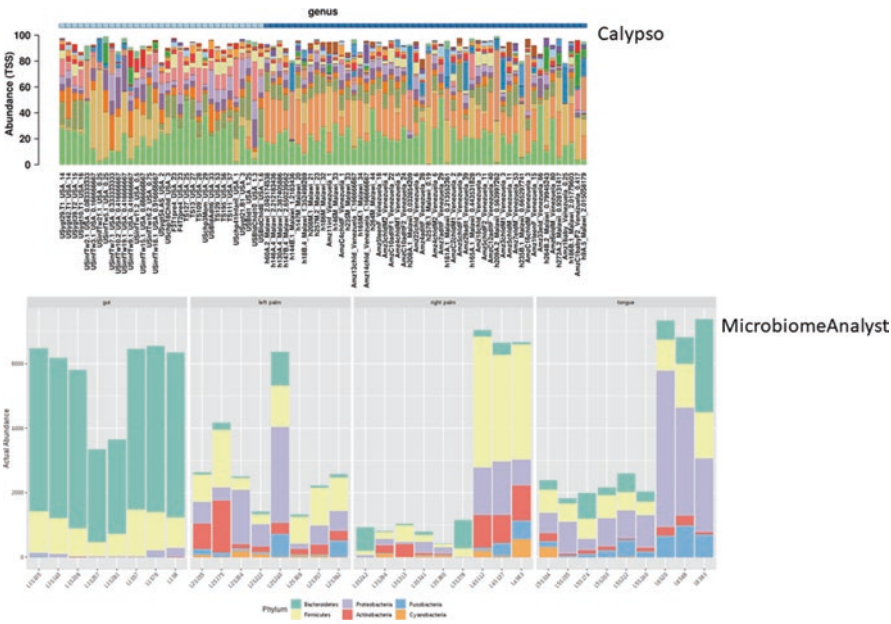

Fig. 2.2 Taxonomical analysis

composition and even their phylogenetic hierarchies using a variety of diagrams like barplots (Fig. 2.2).

For that in Calypso from the main menu we can select "Basic → Quantitative Visualization" and in MicrobiomeAnalyst we can choose "Visual exploration → Stacked Bars".

2.9 Phylogenetic Analysis

Phylogenetic analysis is the means of estimating the evolutionary relationships between our microbiota, which can be achieved by calculating the similarity of distinct sequence clusters. This analysis is relevant when we want to visually represent the ancestry and relationships of the taxa in our samples (Fig. 2.3). In Calypso from the main menu "Advanced → Hierarchy → Dendrogram" and in MicrobiomeAnalyst "Visual Exploration → Phylogenetic tree".

2.10 α and β Diversity Analysis

There are two basic metrics for diversity analysis in microbial communities. α-Diversity represents how rich a sample is in terms of distinct microbial taxa and it is a quantitative metric. On the other hand, β-Diversity is a qualitative metric which characterizes how different the composition of the microbiome is between

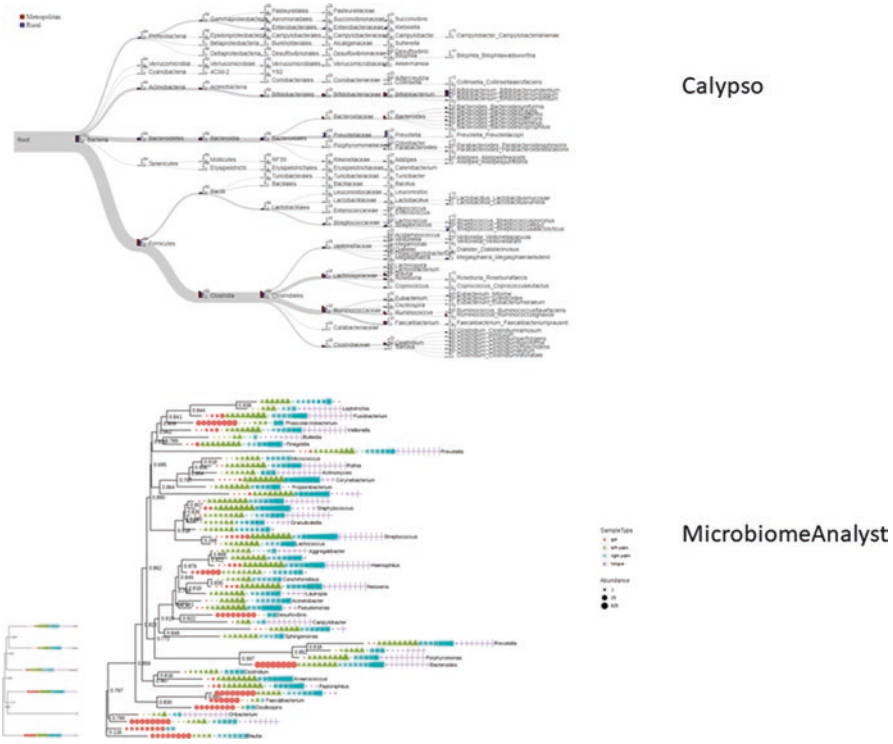

Fig. 2.3 Phylogenetic analysis

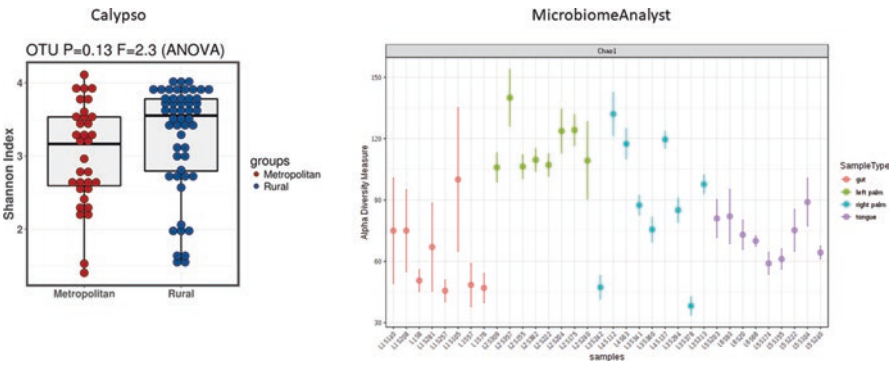

Fig. 2.4 α-Diversity analysis

different sample groupings (e.g., Controls vs. Patients). α-Diversity can be calculated using rarefaction and algorithms like Chao1, Shannon index, and various Evenness metrics and represented via rarefaction, dot or box plots (Fig. 2.4). Regarding α-diversity in Calypso we can do "Diversity" from the main menu and for MicrobiomeAnalyst "Community profiling → alpha diversity."

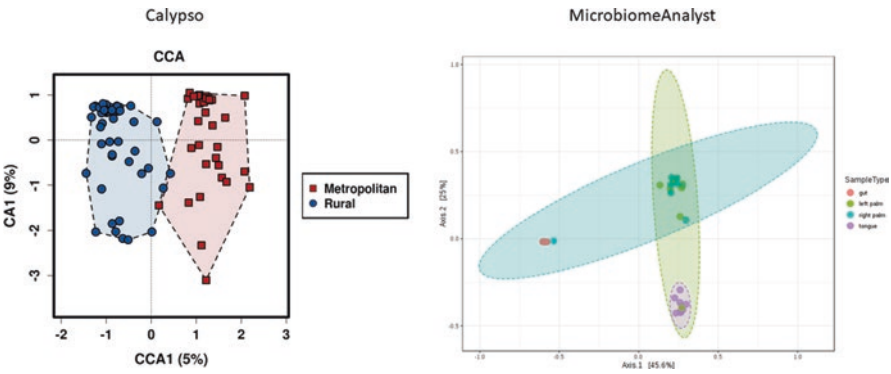

Fig. 2.5 β-Diversity analysis

β-diversity is calculated using distance metrics and illustrated with Principal Coordinates Analysis (PCoA) plots. There are also other ways to calculate β-diversity with more popular and modern methods like Canonical Correspondence Analysis (Ter Braak 1986) and MixMC (Le Cao et al. 2016) (Fig. 2.5).

Regarding β diversity from calypso we can choose any of the different methods in "Multivariate" and for MicrobiomeAnalyst "Community profiling → beta diversity." It is important to note here that when working with human samples from different people, which vary in microbial compositions, β diversity plots will not always provide helpful answers, but that is to be expected.

2.11 Differential Analysis and Biomarker Discovery

When trying to infer meaningful biological associations between microbial taxa and specific sample groupings we rely on biomarker discovery methods which are commonly based on taxa differential abundance between sample groupings. It enables us to highlight which taxa contribute with statistical significance to dysbiosis. Parametric and nonparametric tests, depending on the distributions of our data, like Negative binominal (DeSEQ2), Kruskal-Wallis, Wilcoxon rank test, anova, and t-test, are popular for this purpose. Microbiome analysis requires multiple pairwise tests elevating the need for False Discovery Rate (fdr) correction using algorithms like Bonferroni and Benjamini-Hochberg. In addition, more specialized biomarker discovery algorithms, like LeFSe (Segata et al. 2011), can be used to indicate meaningful associations between microbial taxa and health conditions (Fig. 2.6).

For differential abundance within sample groups in Calypso we suggest using the "Group" and "FeatureSelect" options from the main menu, whereas in MicrobiomeAnalyst any of the "comparison & classification" methods can be used.

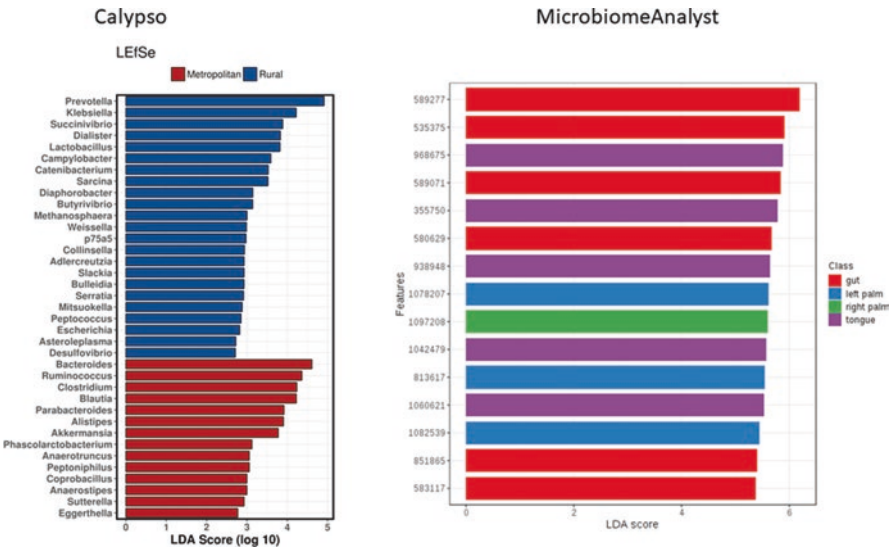

Fig. 2.6 Biomarker discovery

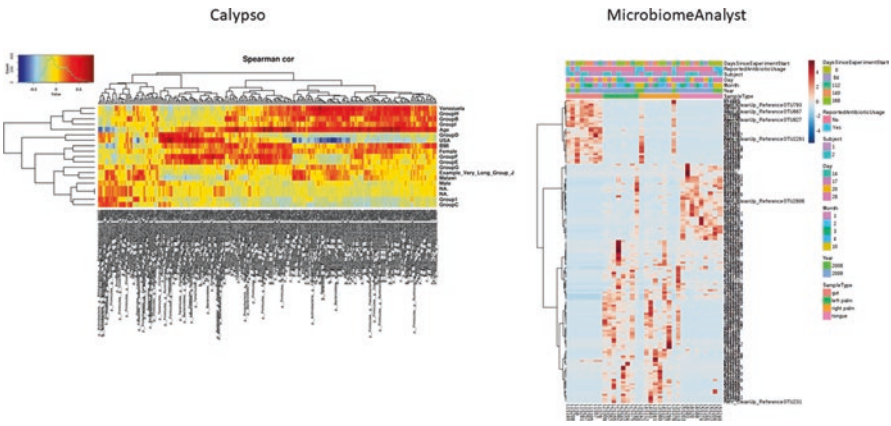

Fig. 2.7 Correlation analysis

2.12 Association Analysis

Association analysis, also known as correlation analysis, enables the identification of principles that have an affinity for each other but also the strength of that relationship. This particular type of analysis is useful when a researcher wants to establish if there are possible connections between continuous numerical variables. In our case our variables are the abundances of specific microbial taxa. The statistical test usually employed for this is Spearman's correlation coefficient, since microbial taxa are considered ordinal, and is visualized using heatmaps (Fig. 2.7).

In Calypso we select "Multivariate" from the main menu and then correlation heatmap from the drop-down menu "Type". For MicrobiomeAnalyst we can select "clustering and correlation → heatmap clustering".

2.13 Network Analysis

Like the association analysis, network metrics are used to detect microbial species that co-exist or are competitive to each other. They can also be used to form a clearer image of taxa–host interactions. By modeling microbial community interactions researchers can infer their effects and taxa that antagonize pathogens or other taxa that contribute to dysbiosis. Networks are visualized by nodes and edges which represent taxa and their interactions. Again, Spearman's rho or newer algorithms like mLDM (Yang et al. 2017) are used to extract these taxon–taxon interactions. Similarly, networks can be constructed to represent and analyze inter-taxa or host–taxa interactions with tools like MMinte (Mendes-Soares et al. 2016) which associates taxa based on predicting their metabolic interactions. Finally, all-purpose network analysis and visualization applications like Cytoscape (Shannon et al. 2003) can provide network analysis statistics (like network centralities) to networks constructed from microbial data. Both Calypso and MicrobiomeAnalyst provide the visualization and export functionality of such networks (Fig. 2.8).

2.14 Functional Analysis and Inference

All the above approaches mainly focus on the composition and quantification of microbial data. They make assumptions based on statistical approaches of how the microbiome can contribute to the host's pathophysiology and the taxonomical

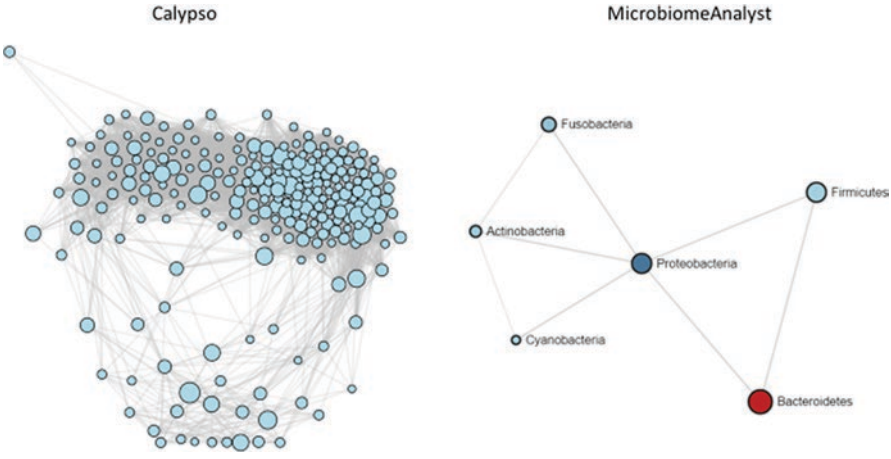

Fig. 2.8 Microbial networks analysis

composition during dysbiosis. What we know nowadays as researchers though is that the microbiome's true power to affect changes comes from its functional role. So, it makes sense that the study of the microbiota's metabolome must ultimately be our goal. It is known that microbes use metabolites as offense or defense primarily for their survival. It is exactly these metabolites that can interact with the host in specific tissues and create or prevent health issues. Of course, the host itself via its own metabolic processes also creates a hostile or nurturing environment for specific microbial taxa. Traditional methods like chromatography, mass spectrometry, and nuclear magnetic resonance can of course be used in microbial studies but bioinformatics provides alternative means.

As we have mentioned earlier the main advantage of shotgun metagenomics is the fact that their specific applications can detect and report on the functional role of the microbes. In 16s rRNA approaches we are forced to use "inference"-based methodologies to deduct the functional load of microbial communities based on their abundance using tools like PICRUST (Davenport et al. 2014) and piphillin (Iwai et al. 2016). Both Calypso and MicrobiomeAnalyst support PICRUST biom files as input but MicrobiomeAnalyst can also run its own PICRUST implementation on the provided microbial data directly.

2.15 Closing Remarks

There is a vast variety of applications and platforms to guide researchers through microbiome analysis. Choosing the right tool usually comes down to our needs of accuracy and speed and also to our hypotheses. Bioinformatics specialists can guide researchers of other disciplines through these processes and together they can interpret the results. Statistical analysis of these complex data, which have many variables especially when we try to associate them with the host's health condition, should be reviewed extensively and perhaps even compared with the output of other similar tools.

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Gut Microbiome and Gastrointestinal Disorders

3

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Abstract

The human gut harbors more than 10^{14} microorganisms, with bacteria being the main population. Gut microbial composition and diversity participates in vital physiologic and immunologic processes maintaining host homeostasis. The disruption of the healthy microbial structure has been associated with various gastrointestinal disorders including inflammatory bowel disease, celiac disease, irritable bowel syndrome and others.

Keywords

Microbiota · Inflammatory bowel disease · Crohn's disease · Ulcerative colitis · Irritable bowel syndrome · Brain–gut–microbiome axis · Celiac · Fecal microbial transplantation

3.1 Introduction

The gastrointestinal tract system colonizes during pregnancy through mother's placenta. The vast majority of commensal microorganisms reside in the colon. The human gut harbors more than 10^{14} microorganisms, comprising more than 500–1000 species with bacteria being the main population (>99%) (Sonnenburg et al. 2004; Qin et al. 2010). Microbial mass of the human colon is estimated about up to 1–2 kg of body weight (Forsythe and Kunze 2013). The human gut microbiome is called our second genome as it accounts for more than five million different genes

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(D'Argenio and Salvatore 2015; Gorkiewicz and Moschen 2018). Gut microbial composition and diversity participates in vital physiologic and immunologic processes endocrine signaling, prevention of enteropathogen colonization, regulation of immune function, and metabolism of xenobiotic compounds maintaining host homeostasis. Various factors appear to influence a person's microbiome as genetics, habits, sex, and location within the gastrointestinal (GI) tract as well as environmental factors including diet since childhood (Marques et al. 2010), the geographical report (Yatsunenko et al. 2012) and the use of antibiotics (Vangay et al. 2015). An aberrant gut microbiota has been described in several disorders recalling the words of Hippocrates: "All disease begins in the gut." (Mohajeri et al. 2018) Research data indicate that alterations in the composition or the balance of the intestinal microbiota, or dysbiosis, are associated with many GI and autoimmune disease susceptibilities, but it is unclear whether the microbiome participates directly in the pathogenesis of these disease states (Chang and Lin 2016). The disruption of the healthy microbial structure has been associated with various gastrointestinal disorders including inflammatory bowel disease, celiac disease, irritable bowel syndrome and others (Quigley 2017). The diversity and the richness of the intestinal microbiota are considered as measures of a "healthy microbiota" as diversity and richness are thought to be important for maintaining the microbiota homeostasis and function, particularly during exposure to microbiota stressors (e.g., a change in diet or exposure to antibiotics).

3.2 Microbiome and Inflammatory Bowel Disease (IBD)

Inflammatory bowel diseases, Crohn's disease (CD), and ulcerative colitis (UC) included, are chronic multifactorial immune-mediated diseases induced by genetic predisposition, environmental changes, abnormal gut microbiota and immune response dysregulation leading to an excessive inflammation of the gut (Geuking et al. 2014). However, it still remains to be elucidated which factors are the initiators or the result of inflammation (Holleran et al. 2017).

Since the beginning of the twenty-first century, IBD has been considered one of the most prevalent gastrointestinal diseases. The highest prevalence of IBD was reported in Europe and North America; over one million residents in the USA and 2.5 million in Europe are estimated to be suffering from IBD. The incidence and prevalence of IBD is increasing latest years in Western countries, and specifically in newly industrialized countries of Asia, Africa, and South America revealing the environmental impact (Ng et al. 2017). The involvement of germs in the etiology of IBD has been widely investigated. There is growing evidence that dysbiosis of the gut microbiota plays a key role in IBD development and treatment. To date, it has not been clarified whether IBD-related changes in intestinal microflora constitute the cause or the effect of inflammation. Both Crohn's and ulcerative colitis usually occur in the large intestine and/or in the final ileum, where the largest concentrations of intestinal microbiome are observed (Sartor 2008).

Genetic factors appear to be associated with the onset of Crohn's disease and ulcerative colitis. Genome-wide association studies have identified >200 IBD

associated-susceptible genes, some of which are known to be involved or implicated in mediating host responses to gut microbiota. In addition these genetic factors seem to affect intestinal microbiome composition leading to dysbiosis (Glassner et al. 2020; Nishida et al. 2018). The discovery of CD susceptibility gene NOD2/CARD15, coding a protein responsible for microbial recognition, induction of antimicrobial genes and stimulation of the host's acquired immunity upon binding to cell wall peptidoglycan muramyl dipeptide, comprised the first sign that host genetics and the microbiome are linked (Cario 2005). Mutations in the NOD2/CARD15 gene block the mechanism of microbial recognition and cancel the normal cytokine inhibition mechanism, resulting in microbial dysbiosis and intestinal inflammation mucosa. The number of Enterobacteriaceae in IBD patients seems to be influenced by specific genetic variants of NOD2 gene (Ogura et al. 2001; Knights et al. 2014). Furthermore, both patients with Crohn's disease or ulcerative colitis in the presence of NOD2 gene mutations presented reduced bowel concentrations of *Clostridium* XIVa and IV and a parallel increase in Actinobacteria and Proteobacteria (Frank et al. 2011). Mice with mutations in NOD2/CARD15 gene also presented alterations in microbial concentrations such an increased amount of Bacteroides, Firmicutes, and Bacilli compared to mice with no mutations (Petnicki-Ocwieja et al. 2009). Furthermore NOD2 mutations in CD patients lead to increased possibility of microbial infections such as *Mycobacterium avium* paratuberculosis, *Listeria monocytogenes*, and *E. coli* (Glasser and Darfeuille-Michaud 2008). Another gene linked to IBD pathogenesis as well as intestinal dysbiosis is the autophagy gene ATG16L1. Patients with mutations in the ATG16L1 gene and in parallel augmented stress indicators at Paneth cells are more likely to have localized disease in the small intestine, present a syringe Crohn's disease and have a greater need to undergo a surgery. Inflamed tissues of CD patients carrying a mutation in the ATG16L1 gene, are characterized by increased concentrations of Bacteroides, Fusobacteria and *Escherichia coli* and lower concentration of Lachnospiraceae (Deuring et al. 2014; Sadaghian Sadabad et al. 2015). Individuals with a high genetic burden in functional variants associated with IBD in genes involved in the bacterial handling such as NOD2, CARD9, ATG16L1, IRGM and FUT2 display a declined number of *Roseburia spp*. (Imhann et al. 2018).

Recent technological advances (metagenomics) have revealed many features of dysbiosis in the microbiome of IBD patients. Multiple studies have shown a change in the composition of the intestinal microbiome in both ulcerative colitis and Crohn's disease. IBD patients present overall reduced microbial diversity in the intestinal microflora compared to healthy controls. The microbiome also presents difference when measured in inflamed compared to non-inflamed tissue even within the same patient (Walker et al. 2011; Sepehri et al. 2007). Disease location is of major importance for gut microbiota composition in IBD; the gut microbiota of colonic CD patients is more contiguous to the microbiota of UC patients than to that of ileal CD patients (Imhann et al. 2018). Comparing the microbes of IBD patients versus healthy control subjects over time, the widest variation is observed in the patient group. Also, the subgroup of patients with ileal CD (especially people who had a previous surgical resection) presented the broadest deviation from healthy subjects. The main changes in microbiome is the reduction of microbes with anti-inflammatory

capacities such as the Firmicutes and Bacteriodetes strains and the increase of microbes attached to the mucus. Firmicutes executives are the major short-chain fatty acid producers (Sartor 2008).

IBD patients display an altered composition of their gut microbiota characterized by a decrease in microbial diversity, in particular a reduction in the predominant populations of a healthy intestine (Gevers et al. 2014; Morgan et al. 2012; Matsuoka and Kanai 2015). The pathogenic microorganisms increase in IBD patients, showing their preference for an inflammatory environment, while bacteria with anti--inflammatory capacities are reduced. The reduced abundance and diversity of Firmicutes phyla are the most consistent changes in patients with CD and UC. *Faecalibacterium prausnitzii*, *Butyricicoccus pullicaecorum,* and *Roseburia hominis*, members of the Firmicutes, are decreased in patients with IBD compared to healthy subjects (Manichanh 2006; Frank et al. 2007; Sokol et al. 2009; Miquel et al. 2013). Bacteriodetes, the other important anaerobic phylum, are found reduced in IBD patients. These bacteria are known for their anti-inflammatory intestinal capacity through the production of short-chain fatty acid metabolites, such as the butyric and acetic acid, and the induction of the Treg (T regulatory) extension regulatory cells that suppress intestinal inflammation (Furusawa et al. 2013; Ohkusa and Koido 2015; Atarashi et al. 2013). Conversely, a higher abundance of Proteobacteria and Actinobacteria characterize the bowel of IBD patients (Frank et al. 2007; Lepage et al. 2011).

High levels of Enterobacteriacae, including *Escherichia coli* and *Klebsiella pneumonia*, Pasteurellacaea, Veillonellaceae, Fusobacteriaceae, and *Ruminococcus gnavus* have been recorded in IBD patients while Ersipelotrichales, Bacteriodales, Clostridales (*Clostridium groups IV and XIVa*), Suterella and Bifidobacterium species are decreased (Gevers et al. 2014; Rolhion and Darfeuille-Michaud 2007; Garrett et al. 2010; Mukhopadhya et al. 2012; Lupp et al. 2007). The microbiome of IBD patients exhibits an instability even during recession. CD, in particular, is associated with a more altered and unstable gut microbial composition than UC (Pascal et al. 2017). Even among patients with CD with inactive disease after small-bowel resection, there were reductions in Parabacteroides species and Clostridiales and increases in Enterobacteriaceae compared with patients with CD who had not undergone prior surgery (Yilmaz et al. 2019). A reduction of microbiome diversity and specifically of certain normal anaerobes bacteria such as *Bacteroides*, *Escherichia*, *Eubacterium*, *Lactobacillus*, and *Ruminococcus has* been observed before a relapse of UC (Ott et al. 2008). A characteristic of CD patients' microbiota is the loss of the beneficial butyrate-producing organisms. In fact, alterations in eight microorganisms—lower abundance of *Faecalibacterium* species, *Peptostreptococcaceae* species, *Anaerostipes* species, *Methanobrevibacter* species, *Christensenellaceae*, and *Collinsella* species and increased abundance of *Fusobacterium* and *Escherichia* species—have been proposed as biomarkers for distinguishing CD subjects from others (Pascal et al. 2017). *F. prausnitzii*, which belongs to *Clostridium* cluster IV, has been reported to have an anti-inflammatory effect by producing butyrate. *F. prausnitzii* stimulate the production IL-10 secretion and downregulate the secretion of inflammatory cytokines, such as IL-12 and IFN-γ (Sokol et al. 2008). Low levels of *F. prausnitzii* predict a higher risk of relapse of

ileal CD after surgery whereas higher levels are associated with maintenance of endoscopic remission.

Patients with both CD and UC demonstrate increased concentration of *E. coli* strains and especially of the category AIEC (adherent-invasive *E. coli*). Studies refer that AIEC population elevates in about 38% of patients with active CD compared to 6% in healthy subjects. AIEC are more enriched in mucosal than in fecal sample and within CD granulomas. AIEC strains show pro-inflammatory properties; they have the potential to attach to, penetrate intestinal epithelial cells and replicate within macrophages, releasing large amounts of pro-inflammatory cytokines. The pathogenic bacteria which are able to adhere to the intestinal epithelium affects the permeability of the intestine, alters the diversity and composition of gut microbiota, and induce inflammatory responses by regulating the expression of inflammatory genes, consequently leading to the induction of intestinal inflammation (Darfeuille--Michaud et al. 1998; Conte et al. 2006; Baumgart et al. 2007; Martinez-Medina et al. 2009; Ahmed et al. 2016). IBD patients especially those with CD present antibodies to microbial antigens such as ASCA (anti-*Saccharomyces cerevisiae* antibodies), and OmpC (*Escherichia coli* external membraneporin C) at a rate of up to 50–60%. A higher incidence of stenosis and fistula in CD patients have been associated with the presence of such antibodies (Mokrowiecka et al. 2009).

An indicative treatment for patients with moderate to severe IBD is anti-TNF agents; however, only 50–70% of patients respond to anti-TNF therapy; the reason of non-responsiveness is still unknown (Yamamoto-Furusho 2017). Lack of response may be related to different immuno-inflammatory mechanisms including differences in the intestinal microbial flora of patients before and/or during anti--TNF therapy (Jones-Hall and Nakatsu 2016). A study using an experimental IBD mice model concluded that microbial synthesis is linked to TNF levels and disease severity (Jones-Hall et al. 2015). Environmental factors, including treatment, promote changes in gut microbiome of IBD patients. Patients who received oral corticosteroids for a disease flare had greater microbiome variations than patients who did not require corticosteroid therapy, suggesting that apart from disease activity, medical therapy could contribute to microbiota changes. Anti-TNFa treatment was likewise followed by an increase in Firmicutes and Clostridium (Morgan et al. 2012). Likewise alterations in microbiota composition are correlated with likelihood of treatment response in patients. Responders and non-responders to anti-TNF therapy had a different expression of antimicrobial peptides suggesting that intestinal antimicrobial/microbial composition may affect the outcome of treatment (Magnusson et al. 2016). Specifically, *Bifidobacterium* species, *Collinsella* species, *Lachnospira* species, Lachnospiraceae, *Roseburia* species, and *Eggerthella* taxa have been linked with responsiveness to anti–TNF-a treatment (Yilmaz et al. 2019).

Changes in microbiota profiles in IBD aren't fully representative for disturbances in gut physiology. After research between regular and germ-free or SPF mice, there was important discrepancy in serum and tissue metabolites. This fact highlights the important role of microbiome in the host metabolic progress. Sundry human studies derived metabolite variation in stool, serum, or mucosa of IBD patients in contrast with controls (Franzosa et al. 2019; Jacobs et al. 2016; Kolho et al. 2017; Scoville et al. 2018). Seeing that metabolite profile commends an immediate tool to measure

functional activity, a more efficacious method to extract putative mechanistic connections between gut microbiota and disease is by quantifying them. More generally active and functionally important metabolites have been shown to be depleted in IBD patients; a loss of "metabolic diversity" is analogous to the loss of taxonomic (ecological) diversity observed in the IBD microbiome. Combined evidence from metabolomics and microbial taxonomic analyses show a potent association between disease-associated microorganisms and metabolites. A scheme with microbial metabolites and metabolites coming from diet may conduce to inflammatory diseases such as IBD (Thorburn et al. 2014).

Short-chain fatty acids (SCFA) producing bacteria such as Bacteroidetes, *F. prausnitzii* and *Clostridium* clusters IV, XIVa, XVIII present a low abundance in IBD patients and as a consequence SCFA have been also found decreased (Schirmer et al. 2018). Similarly, the secondary bile acids lithocholate and deoxycholate, were found to be reduced in patients with IBD. SCFA, acetate, propionate, and butyrate included, are important anti-inflammatory bacterial metabolites which support epithelial cells growth and promote the expansion and differentiation of regulatory T cells in the colon maintaining intestinal homeostasis (Atarashi et al. 2013; Goverse et al. 2017; Parada Venegas et al. 2019). Some metabolites, such as hydrogen sulfide, can block the use of butyrate by colonocytes. Sulfate-reducing bacteria such as *Desulfovibrio*, is higher in IBD patients resulting in the production of hydrogen--sulfate that damages intestinal epithelial cells and induces mucosal inflammation (Roediger et al. 1993; Smith et al. 2005; Loubinoux et al. 2002; Zinkevich and Beech 2000; Rowan et al. 2010). Similar to SCFAs, medium-chain fatty acid (MCFA) such as caprylic acid, may occur in the gut as a breakdown product from anaerobic fermentation of fiber. MCFA have been found decreased in IBD while in non IBD subjects are abundant. Caprylic acid has been positively associated with "good" gut anaerobes microbes, including *Alistipes shahii*, *A. putredinis*, and *A. finegoldii* while a negative association has been revealed with the number of *Ruminococcus gnavus*. Other microbial metabolites like taurine, histamine, and spermine can modulate the intestinal inflammation and clinical response in a DSS colitis mice model (Levy et al. 2015). Moreover, an increasing in taurine and cadaverine is observed in UC patients, when the circumspect by fecal calprotectin levels of carnosine, ribose, and choline relates to inflammation (Kolho et al. 2017). Increased amounts of tryptophan, bile acids, and unsaturated fatty acids have been correlated with ileal CD (Jansson et al. 2009). Two more markers that are really decreased in the gut of IBD patients are vitamin pantothenate (vitamin B5) and nicotinate (vitamin B3), though they aren't usually deficient in patients serum. Especially, nicotinate has been associated with anti-inflammatory and antiapoptotic ability in the gut (Li et al. 2017). Other metabolites remarkable to mention are sphingolipids and carboximidic acids, which are quantitatively excessive in CD patients (Franzosa et al. 2019). The bacterium *B. fragilis* is able to compose sphingolipids with the ability of minimizing the onset of iNKT cell and outspread driven by self and microbial trigger in neonatal mice (An et al. 2014). This ability drives to reduction in the number of iNKT cells as soon as the neonate becomes adult and is defensive to experimentally induced colitis (Glassner et al. 2020).

There is not a straight analogy between functional activity and functional potential of an organism of gut microbiota. The presence at RNA levels of *R. gnavus* is extremely elevated in IBD patients in contrast with healthy control subjects, when it's a little increased at DNA level. This evidence highlights that even a small diversion in the presence of *R. gnavus* at the DNA level can lead to significant effects in IBD patients. On the contrary, *B. fragilis* is lower in terms of DNA, and much lower in RNA levels of UC patients compared to its abundance in healthy control subjects. *F prausnitzii*, *B. vulgatus*, and *Alistipes putredinis* have been shown to have an important contribution to metabolic progress transcription in IBD patients, even when they aren't the plentiest organisms present (Schirmer et al. 2018). Furthermore, abundances of *Clostridium hathewayi*, *Clostridium bolteae*, and *R. gnavus* were found significantly elevated in transcriptional activity of IBD patients in a relation with genomic abundance, proposing that their impact may be more marked than previously thought based only in genomic (Lloyd-Price et al. 2019).

3.2.1 Fungal and Virus Composition

Apart from bacteria, the composition of fungi and viruses in the gut microbiome is also disturbed in IBD patients. Fungi represent only a small percentage of the gut microbiota, approximately <0.1% of the total microbes (Qin et al. 2010). The different body sites present variation in the fungal composition (Underhill and Iliev 2014). The most common fungi found in human GI tract, urogenital tract, and oral cavity belongs to the *Candida* genus (Soll et al. 1991; Huffnagle and Noverr 2013). There is a competitive relation between gut bacteria and fungi which influence their abundance. Studies in mice have shown that environmental factors could affect the stability of gut mycobiota; antibiotics are an important promoter of fungal overgrowth and infection (Noverr et al. 2004; Dollive et al. 2013). Various data indicate the potential importance of mycobiota in IBD pathogenesis; it is biologically plausible as many IBD susceptibility genes are involved in antifungal immune responses (for example, CARD9, CLEC7A and RELA) (Richard et al. 2015). Components of the fungal cell wall such as chitin, β-glucans, and mannans, could trigger host immune responses. These glycoproteins of the fungal wall activate receptors including dectin-1 (a C-type lectin receptor), Toll-like receptors (TLR2 and TLR4), components of the complement system, and members of the scavengers receptor family leading to an immune cascade (Levitz 2010; Sartor and Wu 2017). Experiments in mice have shown that deficiency of dectin-1 (encoded by Clec7a) increase the risk to dextran sodium sulfate (DSS) colitis due to the expansion of opportunistic pathogenic fungi (Iliev et al. 2012). One more proof for the connection between fungi and IBD derives from a colitis mice model, where fungi act like bacteria by permeating the disrupted mucosal barrier, trigger TLRs, Dectin-1 and CARD9 in the lamina propria causing disease embitter. Likewise, in Card9−/− mice the bacterial and fungal microbiota is diverted in a way that it isn't able to metabolize tryptophan into ligands for the aryl hydrocarbon receptor, and so inevitably can't upregulate IL-22, a protein required for recuperation from colitis (Brun et al. 2007; Lamas et al. 2016).

Recent studies have revealed that *S. cerevisiae* colonization boosts the metabolic process of purine in mice, with result an increase in uric acid levels, which is known for its pro-inflammatory properties (Chiaro et al. 2017). On the other hand, *S. cerevisiae* present also an anti-inflammatory potential by inducing IL-10 production, thus may exhibit regulatory effects. On the other hand, normal gut mycobiome (including *Malassezia* spp. and *C. albicans*) is assumed to play a beneficial role. For instance, *Malassezia* species educe the innate immune cells with CARD9 gene mutations usually connected with IBD to product inflammatory cytokines. Also, they sharpen colitis in disease mouse models (Limon et al. 2019). Especially, *M. sympodialis* is able to excrete powerful allergens that in an already inflamed gut of IBD patients boost the inflammation, trigger mast cells to let cysteinyl leukotrienes go and amplify the mast cell IgE response, which also concurs to inflammation. After a protracted treatment of mice with the antifungal factor fluconazole, fungal dysbiosis was presented marked with expanded abundance of opportunistic species including *Aspergillus amstelodami*, *Epicoccum nigrum*, and *Wallemia sebi*. The results for mice enhanced with these fungal organisms were poorer not only in DSS-associated colitis but also in T-cell transfer-mediated colitis, where the IFNγ and IL-17-secreting $CD4^+$ T cells were pullulated in intestine (Wheeler et al. 2016). Studied as a group, animal models research propose that fungi may affect intestinal health and disease by repressing the outgrowth of eventual pathobiotics, urging immunoregulatory processes and modifying host metabolism. However, whether fungal colonization is related to disease pathogenesis or whether it is a consequence of gut inflammation, immune suppressive therapy or a specialized restricted diet is yet to be determined.

Differences in fungal composition between IBD patients and healthy subjects as well as between patients in flare and in remission have been reported. Contrary, pediatric IBD patients present a decreased fungal gut microbiota. In general, CD patients exhibit a relatively increased diversity of fungi, especially those with ileal CD, compared to UC patients. Furthermore variations have been observed between in-flamed and non-inflamed mucosa (Sokol et al. 2017). The Basidiomycota-to--Ascomycota abundance ratio constitute one of the most important discriminative features between IBD and healthy individual. Furthermore, an imbalanced ratio—higher level of Basidiomycota and lower level of Ascomycota—has been observed in patients with in flare IBD compared to patients in remission. Of note, *Candida albicans*, *Candida tropicalis*, *Clavispora lusitaniae*, *Cyberlindnera jadinii*, and *Kluyveromyces marxianus* present a significant increase in IBD patients while *Saccharomyces cerevisiae* is significantly decreased (Knox et al. 2019). The abundance of Saccharomyces has been positively correlated with the number of *Bifidobacterium*, *Blautia*, *Roseburia*, and *Ruminococcus*; bacteria found decreased in IBD as well. *Malassezia* spp. present variation at the species level; *Malassezia sympodialis* is decreased while *Malassezia restricta* is abundant in the intestinal mucosa of CD patients. The genus *Dioszegia* and species *Candida glabrata* are the dominant fungi in flared CD patients, while *Trichosporon* and *Leptosphaeria* genera are reduced (Mukhopadhya et al. 2012; Standaert-Vitse et al. 2009; Schwiertz et al. 2010; Hansen et al. 2013; Liguori et al. 2016). In addition, *Xylariales* were

abundant in CD inflamed mucosa whereas *Filobasidium uniguttulatum* and *Saccharomyces cerevisiae* were elevated in non-inflamed mucosa (Liguori et al. 2016). Antibodies to *Saccharomyces cerevisiae* are also more frequent in CD patients than in healthy controls or in patients with UC (Quinton et al. 1998).

Virobiota, including both eukaryotic viruses and prokaryotic bacteriophages, are assumed to participate in IBD pathogenesis, but their exact role has not yet been elucidated. The dynamics of relationships between bacteriophage and bacteria may determine the composition of complex bacterial communities. A hypothesis is that bacteriophage through their diverse effects on bacteria, such as cell lysis, transfer of genetic material encoding toxins or antibiotic resistance, etc., promotes bacterial dysbiosis suggesting a possible link with IBD pathogenesis. An increased virome diversity and richness has been referred in IBD in contrast to the bacterial diversity which is reduced (Zuo et al. 2019). On the other hand, a reduced variety but richer variability of gut virome was present in CD patients compared to controls (Pérez--Brocal et al. 2013; Norman et al. 2015). *Caudovirales* have been reported as the most abundant bacteriophage in pediatric and adult both CD and UC patients (Norman et al. 2015). Animal studies have shown that an expansion of *Caudovirales* could be triggered by a western diet, suggesting a role for diet in gut virome composition (Kim and Bae 2016). Enteric bacteriophages may interact directly with their host; bacteriophages can translocate from the GI lumen to systemic sites, induce immune responses and inflammation. Contrary, certain viruses present a beneficial role ameliorating intestinal abnormalities in germ-free mice, diminishing susceptibility to intestinal damage caused by chemical injury and bacterial infection and protecting the epithelium against bacteria invasion (Zuo and Ng 2018). A norovirus gut infection in subjects carrying mutation in the ATG16L1 gene lead to CD manifestation, suggesting a synergistic effect of virome and genes in disease pathogenesis and/or progression.

3.3 Microbiome and Irritable Bowel Syndrome (IBS)

Irritable bowel syndrome (IBS) is a common chronic functional gastrointestinal disorder characterized by abdominal discomfort and pain as well as altered bowel habits. The major subtypes depending on the predominant stool pattern are IBS with diarrhea (IBS-D), constipation (IBS-C), or mixed bowel habits with diarrhea and constipation (IBS-M) and unclassified IBS (Lacy et al. 2016). Recently revised Rome criteria (Rome IV) define IBS as "recurrent abdominal pain on average at least one day a week in the last 3 months associated with two or more of the following:

1. related to defecation,
2. associated with a change in frequency of stool, and,
3. associated with a change in form (consistency) of stool; symptoms should have persisted for at least six months." (Simren et al. 2017).

IBS prevalence is estimated to affect approximately 15% of the population worldwide with great variation among countries. The etiopathogenesis of IBS still

remains unknown. Several risk factors have been associated to IBS including dietary, behavioral and lifestyle habits, genetic predisposition, visceral hypersensitivity, altered gut-brain axis, gut dysmotility, and dysfunction of innate immunity implying that gut microbiome alterations may play a major role. However it is not well understood which of these factors trigger IBS or deteriorate the already existing symptoms (Bellini et al. 2014). The prevailing hypothesis is that an imbalance in gut bacterial communities, or "dysbiosis," leads to activation of the gut immune system and potential low-grade inflammation. A key argument supporting the importance and the causal role of the microbiome in IBS is that experimental models of germ-free animals showed changes in intestinal motility, gut barrier function, and intestinal permeability similar to those in IBS when stools transferred from IBS patients (Crouzet et al. 2013). The association between IBS and the intestinal microbiota is also highlighted from the dramatically increased risk of developing IBS after acute gastroenteritis (Halvorson et al. 2006). Microbiological and infectious bases of IBS pathogenesis have been widely described; various infectious triggers combined with other susceptibility factors can activate the immune system (Menees and Chey 2018). Another strong link between IBS and microbiota is that IBS patients appear to have increased expression of intestinal Toll-like receptors (TLRs), which are important mediators of intestinal immune response to gut microbe via their implication in bacterial lipopolysaccharide (TLR4) or fagellin (TLR5) recognition (Brint et al. 2011; McKernan et al. 2011; Ringel 2017).

Several studies aiming to characterize and map the microbiome signature of IBS have shown controversial results. The attempts to identify IBS-specific alterations of the gut microbiome conclude that in general overall microbial diversity and stability of the intestinal microbiota of IBS patients is reduced when compared to healthy individuals (Rajilić-Stojanović et al. 2015; Carroll et al. 2011, 2012; Chong et al. 2019). In addition, alterations in bacterial taxa have been demonstrated between IBS and healthy controls and between clinically relevant subtypes of IBS on the basis of bowel characteristics and the presence of bloating symptoms (Öhman et al. 2015; Bennet et al. 2015). Different subtypes of IBS present different gut microbiota composition. Thus, the dysbiosis of its intestinal microbiota has been recognized by the Rome Foundation Working Team as one reasonable responsible causal factor for IBS (Simrén et al. 2013).

An altered Firmicutes/Bacteroidetes ratio which is a possible indicator of bacterial population shifts has been mentioned to IBS patients (Jeffery et al. 2012; Salonen et al. 2010; Rajilić et al. 2011); however, it is not clear if high or low ratios of Firmicutes/Bacteroidetes characterize the disease (Jeffery et al. 2012; Tap et al. 2017; Jalanka-Tuovinen et al. 2014; Lozupone et al. 2013). There are evidence suggesting a relative richness of pro-inflammatory bacterial species including Enterobacteriaceae, with a parallel decline in *Lactobacillus* and *Bifidobacterium* both in mucosal and fecal samples (Rodiño-Janeiro et al. 2018; Johnsen et al. 2018). Conversely, a certain subtype of IBS (IBS-D) presents an increase in the *Lactobacillus* genus (Tana et al. 2009; Rigsbee et al. 2012; Labus et al. 2017). *Bifidobacterium* can interact with other bacterial species or the host resulting to a modulation of microbiota. Several species of *Lactobacillus* and *Bifidobacterium* genera can secrete

bacteriocins, compounds that in vitro cause a bactericidal effect against pathogens such as the *Salmonella* genus or *Listeria monocytogenes* species. Moreover, *Lactobacillus* and *Bifidobacterium* genera can also modulate the host immune system through the development of a tolerogenic response via dendritic cells by interacting with CD209 (Angelakis et al. 2013; Pace et al. 2015). So the decreased amount of *Lactobacillus* and *Bifidobacterium* leads to disturbances in short-chain fatty acid production and in immunologic and bactericidal activity, with a negative effect on microbiota function and stability (Rajilić et al. 2011; Zhuang et al. 2017; Balsari et al. 1982; Malinen et al. 2005; Carroll et al. 2010; Kerckhoffs et al. 2009; Duboc et al. 2012). The main biomarkers in IBS come from uncultivated bacteria. Two non-cultivated Clostridiales species are significantly reduced to IBS, while members of the Ruminococcus spp. such as phylotypes of *Clostridium* Group XIVa related to *R. gnavus* and *R. torques* (mucin degraders) seems to be significantly increased in patients with IBS and their levels are positively associated with intestinal symptoms (Rajilić et al. 2011; Jalanka-Tuovinen et al. 2014; Saulnier et al. 2011; Scully et al. 2010; Malinen 2010). Furthermore increased level of *Veillonella* (Tana et al. 2009; Rigsbee et al. 2012) and lower levels of *Faecalibacterium* (Carroll et al. 2012; Rajilić et al. 2011) and *Erysipelotrichaceae* family have been observed (Pozuelo et al. 2015; Załęski et al. 2013).

Recent data suggest that the community of fungi known as "mycobiome" is also altered in patients with IBS and may be associated with the development of visceral hypersensitivity Botschuijver and his colleagues firstly reported the associations between the gut mycobiome and visceral hypersensitivity in IBS patients and animal models. Ingredients with antifungal properties like peppermint and caraway oils reversed visceral hypersensitivity and changed the composition of gut mycobiome in these animal models. *Saccharomyces cerevisiae* and *Candida albicans* were revealed to be the dominant species in both healthy and IBS group, whereas the proportion of the two species in IBS patients was much higher than in the healthy. Moreover, the mycobiome signature of hypersensitive IBS patients was distinct from patients with normal sensation. Additionally, the study demonstrated that the hypersensitivity of rats, which separated from their mothers, could be reduced to normal levels after being administered with fungicides. More interestingly, transplanting the fecal mycobiome from hypersensitive rats to those normosensitive rats could restore the hypersensitivity of colonic distension. In short, fungal dysbiosis was confirmed existent in IBS patients, and the elimination of fungi could recover the visceral hypersensitivity to normal levels (Botschuijver et al. 2017, 2018). In addition with this finding, some earlier studies similarly reported yeast-free diets and antifungal treatments to be helpful for IBS subjects (Costabile et al. 2014).

IBS is associated with increased gas and this phenomenon could be responsible for flatulence and abdominal pain (King et al. 1998). Hydrogen accumulation due to fermentation of dietary components by bacteria in the gut does supply nutrients and energy, but also hinders the efficiency of the gut. The excessive gas production can cause faster fecal passage in patients with IBS-D, as the large intestine of these individuals is more sensitive to an increase in intestinal volume in healthy individuals (Pritchard et al. 2014). Intestinal gases are effectively removed by

methane-producer microorganisms. Methane production is limited to methanogens from the Archaea kingdom that convert H2 to produce methane. Methanobacteriales, specifically the *Methanobrevibacter smithii*, are the most common methane producers in the human gut microbiota (Pimentel et al. 2012). Lower methane producers as well as lower methane secretions have been mentioned in IBS-D (Tap et al. 2017; Pimentel et al. 2003; Kim et al. 2012). Contrary, IBS-C patients have increased amount of *Methanobrevibacter smithii* and therefore higher levels of methane, concluding that there is a positive correlation of methane levels and constipation. Methane has been related to slower intestinal transit and also to anti-inflammatory effects. It has been demonstrated in animal models that methane gas can slow gut transit and increase gut contractions bidirectionally. The increased production of methane in constipated patients could be related to microbial overgrowth because Methanobacteriales detection is associated with microbial richness within the enterotype Clostridiales, which is further associated with slower transit (Kim et al. 2012; Dridi et al. 2011; Pimentel et al. 2006; Jahng et al. 2012). The degree of methane production could also be associated with the severity of constipation in IBS-C patients (Chatterjee et al. 2007). In fact, IBS symptom severity correlates with all microbial richness, exhaled methane, presence of methanogens and enterotypes enriched with Clostridiales or *Prevotella* species (Rodiño-Janeiro et al. 2018).

An important role of the microbiome is the decomposition of the indigestible food ingredients (Cummings and Macfarlane 1997). A possible pathway for the involvement of the microbiome in IBS is the protein degradation. The intravascular content of IBS patients contains elevated proteases levels (Buhner et al. 2009), may be due to increased secretion of endogenous and microbial proteases as a response to a Western—rich in protein—diet, but also due to insufficient decomposition of endogenous proteases by the disturbed intestinal microflora (Tooth et al. 2014). Serine protease inhibitors are produced by many bacteria, such as bifidobacteria, and their activity could prevent increased proteolytic activity of intestinal contents; a decrease in their numbers have been recorded in IBS patients (Rajilić et al. 2011; Kerckhoffs et al. 2009; Ivanov et al. 2006). Protein fermentation produces innumerable substances dangerous to health. Among them, hydrogen sulfide is a toxin which damages epithelial metabolism and can be converted to tetraethyone, which stimulates the growth of microbes that use tetraethyone from Gammaproteobacteria (Rajilić-Stojanović 2013; Jørgensen and Mortensen 2001; Thiennimitr et al. 2011; Weissfeld and Sonnenwirth 1982). The abundance of some Gammaproteobacteria species is significantly linked to intestinal symptoms in IBS patients and the levels of inflammatory markers such as interleukin 6 (IL-6) and interleukin 8 (IL-8) 53 which are typically increased in IBS (Rajilić et al. 2011; Jalanka-Tuovinen et al. 2014).

It is generally accepted that the fermentation of carbohydrates is desirable because of the beneficial effects of the main fermentation products like short-chain fatty acids (SCFAs)—in the energy supply of gastrointestinal epithelial cells, in reducing inflammation and improving bowel function (Hamer et al. 2007). In patients with IBS, the presence of resistant carbohydrates FODMAPs may cause IBS symptoms (Shepherd et al. 2008). This can be the result of either increased or

decreased production of relevant metabolites. The quality and composition of SCFAs in the gut varies among IBS patients and healthy individuals, although there is no consensus in the literature on this (Treem et al. 1996; Mortensen et al. 1987). IBS has been associated with increased colonic SCFA production that might contribute to changes in visceral pain responses and motility which characterize IBS (Salem et al. 2018). An altered gut microbiota community producing less SCFA has been described in IBS-D subjects, in an in vitro fermentation system after the consumption of with various carbohydrates and fibers (Treem et al. 1996). It has also been shown that *Lactobacillus paracasei* metabolites modulate contractility of intestinal smooth cells, and *E. coli* Nissle secretions modulate contractility of human muscle strips. Moreover, *Lactobacillus acidophilus* and *L. paracasei* have been reported to modulate pain and visceral hypersensitivity perception, respectively (Verdú et al. 2004; Baer et al. 2009; Eutamene et al. 2007; Rousseaux et al. 2007). Interestingly, an increased sulfate-reducing microbiota population in the gut of IBS-C patients has been reported, which could lead to enhancement in toxic sulfide production, which in turn could influence gut physiology and contribute to IBS pathogenesis (Chassard et al. 2012).

Gut microbiota and its metabolites can influence GI motility by affecting one of several pathways involving enteric neurons, glia, or enteric muscularis macrophages. A known example is the promotion of enteric neuronal survival by gut microbiota-derived lipopolysaccharide (LPS) and SCFAs (SCFAs) (Anitha et al. 2012; Soret et al. 2010). In addition SCFAs also affect neurotransmitter release and influence the cross talk between enteric neurons, smooth muscles and muscularis macrophages to regulate GI motility (Kashyap et al. 2013; Muller et al. 2014). Alteration in GI motility is also a basic characteristic of IBS. Microbiota and their products also affect the development, maturation, and generation of mucosal enteric glial cells, which might play a role in regulating GI motility (Bassotti et al. 2007; Kabouridis et al. 2015). Recently, gut microbiota bile acid metabolism has been implicated in GI motility (Duboc et al. 2012) and their interaction with the enteric nervous system (Dey et al. 2015). The role of gut microbiota in regulating GI motility in IBS is further supported by interventional studies using probiotics.

SCFAs of bacterial origin promote intestinal barrier integrity and function (Kim and Bae 2016; Zuo and Ng 2018; Angelakis et al. 2013). Butyrate which is also a bacterial SCFA inhibits bacterial translocation via boosting the expression of tight junction proteins including claudin, occludin, and zonula occludes proteins (Peng et al. 2007, 2009; Plöger et al. 2012; Suzuki et al. 2008). Intestinal barrier's structure is crucial for the nutrient transport, but it also functions as a barrier for pathogens inside the lumen. Both gut microbiota and their respective metabolites are also important for the integrity of the barrier's integrity but at the same time any alterations in their populations can be harmful (Kelly et al. 2015). IBS-D is characterized by higher intestinal permeability, a clinical visible manifestation (Camilleri and Gorman 2007; Dunlop et al. 2006; Zhou et al. 2009). Of note, a decline in the number of butyrate-producing bacteria have been observed in IBS patients (Pozuelo et al. 2015). Moreover, *Lactobacillus rhamnosus* GG, which is a probiotic stain, induces claudin expression in newborn mice a finding that suggests that early life

bacterial exposure promotes the epithelial barrier's maturation (Kajander et al. 2005; Patel et al. 2012). Additionally, gut microbiota and their by-products can regulate the mucus layer (Dohrman et al. 1998; Smirnova et al. 2003). This layer is formed between the lumen and the epithelium and its role is the prevention of pathogen access to the epithelial surface (Tlaskalová-Hogenová et al. 2011). Inflammatory responses can be triggered of the mucus composition; *Ruminococcus toques* and *R. gnavus* are linked with severe bowel symptoms in IBS (Malinen 2010; Taverniti and Guglielmetti 2014; Tailford et al. 2015; Lyra et al. 2009). Moreover, a formulation of multispecies probiotic that includes *L. rhamnosus GG*, *L. rhamnosus Lc705*, *Propionibacterium freudenreichii* spp., *Shermanii JS*, and *Bifidobacterium breve Bb99* seem to decrease the levels of mucolytic *R. torques* in IBS. This is possible mediated via the upregulation of cell-surface mucin secretion and limiting its adherence to the epithelial layer (Lyra et al. 2010; Mack 2003; Mack et al. 1999; Ohland and MacNaughton 2010).

3.3.1 Brain–Gut–Microbiome Axis

Changes in gut motility are usually triggered by stress via gut–brain axis (GBA). The brain–gut axis (GBA) is a bidirectional communication system between the gut and the brain. Along this conduit, the brain interacts with the gut through neural components (CNS and ANS), endocrine system (hypothalamic-pituitary-adrenal axis), immune components (cytokines and metabolic) and gastrointestinal components (microbiota, intestinal barrier and intestinal immune response) (Oświęcimska et al. 2017). IBS patients frequently present comorbid psychological disorders, such as anxiety and depression, and those with psychological stress are more likely to develop post-infectious (PI)-IBS. Various studies have related these diseases with gut microbiome, intestinal inflammation and immune response suggesting the concept that the gut microbiota drives brain alterations (Liebregts et al. 2007; Mayer et al. 2014). The microbiota in the gut can be altered by brain function, and microbial alteration can, in turn, influence brain function. Nevertheless, literature is not clear whether brain which drives these psychiatric comorbidities seen in IBS patients is involved in manifesting the gastrointestinal symptoms or the gut is driving the brain manifestations. It has been suggested that alteration in the gut microbiota as part of brain gut axis, activates mucosal immunity which leads to loss of epithelial layer which functions as a protective barrier leading to dysmotility and hypersensitivity in IBS patients. Formation of host-derived immune mediators by gut microbiota effects enteric nerve plexus. In general, GBA dysregulation is a common feature in the pathogenesis of IBS and recent data evidence suggests that gut microbiota and their products can alter brain connectivity and function confirming the effect of gut microbiota on the GBA (Cryan and Dinan 2012). Intestinal microbiota have the ability to produce many neurotransmitters and free fatty acid (FAA) affecting brain function; this fact implies the participation of endocrine pathways in microbiota–gut–brain axis. FAA produced by gut bacteria, for example propionic acid, readily crosses the blood–brain barrier and influences brain function and

behavior in animals (Schreiber et al. 2012; Van Oudenhove et al. 2011). *Lactobacillus* and *Bifidobacterium* species generate γ-amino butyric acid (GABA), an inhibitory neurotransmitter in the human brain. *Escherichia*, *Bacillus*, and *Saccharomyces* spp. produce norepinephrine, *Candida* produces dopamine, *Streptococcus*, *Escherichia*, and *Enterococcus* spp. produce 5HT, *Bacillus* and *Lactobacillus* also produce acetylcholine (Dinan et al. 2015).

Another potential mechanism by which gut microbes may affect the gut–brain axis leading to IBS symptoms is the modulation of serotonin(5-HT) production. Serotonin has been indicated to affect inflammation and intestinal barrier integrity, as well as visceral hypersensitivity. The availability of tryptophan, an essential amino acid and precursor for 5-HT, seems to be coordinated by gut microbiota via an alternative metabolic pathway. An increase in the enzymes that participate in tryptophan's degradation has been recorded, in IBS patients (Clarke et al. 2009, 2012; Fitzgerald et al. 2008). Likewise, the intestinal microbiota can be affected by signals from the central nervous system produced in response to stress or psychological disturbances. Stress can change GI motility and secretions, which alter the microbial habitat. The microbial habitat may also be altered by changes in gene expression of some microbial species.

Animal studies have demonstrated the influence of the intestinal microbiota on brain development. Brain dysfunction in germ-free (GF) mice was reported, including an exaggerated hypothalamic-pituitary response to mild stress (Sudo et al. 2004), more exploratory and risk-taking behavior (Neufeld et al. 2011) and altered brain chemistry and memory, indicative of impaired hippocampal development (Gareau et al. 2011). Brain chemistry and behavior were also influenced by altered microbiota; a study showed that transient alteration of the microbial composition by diet provoked exploratory behavior, accompanied by changes of in the levels of brain-derived neurotrophic factor in the specific regions of the brain such as hippocampus and amygdala (Bercik et al. 2011a). The gut microbiota and the brain may be communicated by neural, metabolic (bacterial and host), immunologic, or endocrine pathways (Collins et al. 2012). The neural pathways were first suggested in animal models; anxiety-related behavior was reduced after probiotic treatment, provided vagus nerve integrity was maintained (Bercik et al. 2011b). Certain psychological disorders were associated with pro-inflammatory cytokines, whose levels had been altered by manipulating the composition of the microbiota showing a role of immunologic pathways (O'Mahony et al. 2005; Lotrich et al. 2011; Desbonnet et al. 2010). Altered signaling by muscle-residing macrophages and secretion of cytokines, both of which may be influenced by the gut microbiota, have also been suggested to affect inflammatory responses and gut motility, possibly via effects on the interstitial cells of Cajalt hat again are mediated by TLR signaling (Anitha et al. 2012; Mikkelsen 2010).

Small intestinal bacterial overgrowth (SIBO) could possibly have an effect on GI motility visceral sensation, immune activation, carbohydrate digestion and absorption, bile acid metabolism, and intestinal epithelial permeability which are the major pathophysiological mechanisms of IBS (Vantrappen et al. 1977; Coelho et al. 2000; Giannella et al. 1974; Hofmann and Poley 1972; Hajjar et al. 1975; Deitch et al.

1991; Riordan et al. 1997). It is biologically plausible that SIBO plays a role in IBS and could provoke the onset of a wide range of IBS symptoms, however, it still remains a controversial issue if IBS patients present SIBO (Simrén et al. 2013; Pimentel et al. 2000, 2003; Simren 2006; Vanner 2008; Posserud et al. 2007; Walters and Vanner 2005). There are several studies evaluating frequency of SIBO among IBS patients when compared to healthy individuals using different diagnostic methods such as GHBT, LHBT, and quantitative upper gut aspirate culture. Variations in prevalence of SIBO in patients with IBS and controls in several studies might be attributed to difference in geographical origin of studied population, different criteria for diagnosis of IBS (such as Manning, Rome I, II, and III), and methods for diagnosis of SIBO using different breath tests which lack reliability(such as nature of substrates, gases analyzed, instrument). For example SIBO hypothesis has been supported by results of the lactulose hydrogen breath test which has poor sensitivity and specificity (Simren 2006; Ghoshal et al. 2010). Recently, one study based on after the cultivation of jejunal aspirates, *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter lwoffii*, *Staphylococcus* species, *Klebsiella pneumoniae*, *Streptococcus* species, *Acinetobacter baumannii*, *Enterococcus faecalis*, and *Enterococcus faecium* were the most common bacteria among patients with SIBO (Ghoshal et al. 2014). It has been mentioned that almost 40% of IBS have SIBO, *E. coli*, with *Enterococcus* species and *K. pneumoniae* to predominate (Pyleris et al. 2012).

SIBO is more often associated with diarrhea than constipation IBS.7 Mechanism of diarrhea in patients with SIBO include de-conjugation of bile salts, enterotoxic effect of bacterial metabolites, increased small intestinal permeability, deficiency of vitamin B12 and low-grade inflammation resulting from immune activation in the small intestinal mucosa (Bures 2010; Nucera et al. 2005; Fan and Sellin 2009).

3.4 Microbiome and Celiac Disease

Celiac disease (CeD) is an immune-mediated enteropathy triggered by ingestion of gluten in genetically predisposed individuals. CeD is a disorder with a complex non-Mendelian pattern of inheritance, involving major histocompatibility complex (MHC) and non-MHC genes. The main genetic risk factor for CeD falls within the MHC regions, a region located on 6p21 responsible for the strongest association signals observed in most immune-mediated diseases. The alleles encoding human leukocyte antigen (HLA)-DQ2/8 have been identified as a key modulator in the genetic risk associated with the MHC region in CeD and is found in patients with CD much more frequently than the general population. The main function of the MHC II molecules is to present bacterial antigens to T cells and to activate the immune system (Spurkland et al. 1992; Cenit et al. 2015).

HLA-DQ2/8 genotype as well as the type of infant feeding were shown to influence the intestinal microbial composition. However, regardless the type of feeding, changes in the abundance of some beneficial species, *Bifidobacterium* spp., *B. longum,* and *Staphylococcus* spp. were observed (De Palma et al. 2012a): suggesting that the HLA-DQ genotype itself influenced the microbial composition. The

high-risk infants, those carrying the HLA-DQ2 genotype, were shown to carry an increased proportion of "harmful bacteria" species belonging to the Firmicutes and Proteobacteria phyla (Olivares et al. 2015). Several environmental triggers involving intestinal viral, bacterial, and parasitic infections are capable of initiating or expanding gut mucosal responses to gluten thus may play a role in the pathogenic mechanism of celiac disease.

In Western countries, the cause of the well documented increase in the overall prevalence of CeD has not yet fully explained. The combination of epidemiological, clinical, and animal studies suggests that wide exposure to various commensal, non--pathogenic microorganisms early in life are associated with protection against CeD and that pre-, peri-, and post-natal environmental factors may strongly influence the gut ecosystem (Verdu et al. 2015). Several studies have shown an association between CeD and a change in the microbiome composition (Olivares et al. 2018; Chander et al. 2018). Many environmental factors known to influence the composition of the intestinal microbiota are also thought to play a role in the development of CeD (Lionetti et al. 2014; Vriezinga et al. 2014). Current data are based on associative-descriptive studies, which do not necessarily imply causation between microbiota composition and CeD pathogenesis. Therefore, to fill the gap between cause and effect, further longitudinal studies are necessary to define if and how gut microbiota composition and metabolomic profiles may influence the loss of gluten tolerance and subsequent onset of CeD in genetically susceptible subjects.

The first microbiome data comes from pediatric CeD patients, despite the prevalence in both adults and children. It has been reported that, compared to control infants, neonates with increased family risk of CeD had a decreased representation of Bacteriodetes and a higher abundance of Firmicutes. Furthermore infants who developed autoimmunity had decreased lactate signals in their stools coincident with a diminished representation in Lactobacillus species in their microbiome, which preceded the first detection of positive antibodies (Sellitto et al. 2012). Early microbiota alterations in infants were also suggested in a recent study comparing microbial communities between DQ2+ and DQ2− infants (Olivares et al. 2015). The Firmicutes are the most abundant bacteria in adults with CeD, whereas Proteobacteria are present mainly in children with CeD. Initially, increased levels of rod-shaped bacteria, *Clostridium* spp., *Prevotella* spp., and *Actinomyces* spp. included, was reported in small-bowel mucosa of active and inactive CeD patients, reinforcing the concept of dysbiosis (Ou et al. 2009). Both stool cultures and duodenal biopsies present an increased abundance of gram-negative organisms such *Bacteroides*, *Clostridium*, *E. coli* in CeD patients (Collado et al. 2009; De Palma et al. 2010a; Nadal et al. 2007).

Currently, there are several studies on fecal samples and duodenal mucosa using various techniques including 16SrRNA gene sequencing reporting similar results (Bascuñán et al. 2019; Caminero et al. 2019; Bonder et al. 2016; Di Cagno et al. 2011). Some differences have been indicated in the intestinal microbiota between children and adults with celiac disease; Bacteroidetes and Actinobacteria phyla are shared between adults with CeD and children with CeD (Rostami Nejad et al. 2015). Overall most of the duodenal biopsies from adults CeD patients compared to healthy subjects showed dysbiosis and revealed an increased number of Gram-negative

bacteria, *Bacteroides*, *Firmicutes*, *E. coli*, *Enterobacteriaceae*, *Staphylococcus*, and a decrease in *Bifidobacterium*, *Streptococcus*, *Prevotella* and *Lactobacillus* spp. Moreover, adults with CeD harbor larger numbers of *Mycobacterium* spp and *Methylobacterium* spp. Otherwise, in pediatric patients with active celiac disease *Proteobacteria*, *Enterobacteriaceae* and *Staphylococcaceae* were the most common while, the phyla *Firmicutes* and *Streptococcaceae* were less common compared to non-active celiac disease and controls. An abundance of *Neisseria* spp and *Haemophilus* spp are more abundant in children with CD have been described in pediatric CeD patients (Nistal et al. 2012a).

It is difficult to determine whether an altered gut microbiota is a cause or consequence of CeD, as the type of diet (gluter or gluten-free) can also modulate gut microbiota. The studies of fecal samples and duodenal biopsies in CeD patients on gluten-free diet (GFD) versus gluten diet (GD) and normal healthy population also showed an alteration of gut microbiota. CeD patients on GD showed an abundance in Bacteroides-Prevotella, *Clostridium leptum*, *Histolitycum*, *Eubacterium*, *Atopobium* and lower number of *Bifidobacterium* spp., *B. longum*, *Lactobacillus* spp., *Leuconostoc*, *E. coli* and *Staphylococcus* compared to the normal population (Di Cagno et al. 2011; Nistal et al. 2012a, b; Sánchez et al. 2013; Bodkhe et al. 2019; Golfetto et al. 2014).

After treatment with GFD, the increased microbial concentration was reduced to that in the normal population, suggesting that diet influenced gut microbiota. In particular, a decrease in *Clostridium lituseburense*, *Lactobacillus*, and *Faecalibacterium prausnitzii*, and an increase in Enterobacteriaceae and *E. coli* strains were revealed. However, most studies showed only partial restoration of the microbiota when CeD patients were put on a GFD. Event after GFD less abundant bacterial richness were recorded compared to healthy and untreated subjects, with a persistent imbalance of the ratio of potentially harmful/beneficial bacteria. In addition, some patients continued to present CeD symptoms even on GFD presenting a high number of Proteobacteria and decreased Firmicutes and Bacteroides; thus dysbiosis could be the cause of persistent GI symptoms even on GFD (Collado et al. 2009; Bascuñán et al. 2019; Caminero et al. 2019; Bonder et al. 2016). Changes in the fecal and duodenal microbiota structure of celiac patients on a gluten-free diet have shown that some commensal bacteria, such as *E. coli* and *Bifidobacteria* stimulated the initiation of innate immune cells by gliadin and have inhibitory effects, respectively (Collado et al. 2009; De Palma et al. 2010b).

Although no cause or effect relationship can be deduced from these studies, the consensus is that dysbiosis may contribute to CeD. The precise reason for the inability of GFD to restore the microbiota similar to healthy subjects is not well understood, but it can be speculated that this may be due to individual genetics or prebiotic effect of GFD (Wacklin et al. 2014; Tjellstrom et al. 2005; de Meij et al. 2013). Evidence that gut microbiota may play a role in disease clinical manifestation comes from a study in which patients with Dermatitis Herpeteformis (DH) presented a characteristic gut microbiota, with increased Firmicutes and Bacteriodes (Sterptococcus and Prevotella) (Wacklin et al. 2013).

The possible pathway that several bacterial species and specific strains affect CeD pathogenesis remains to be elucidated. Bacteroides fragilis strains, which are increased patients with CeD, carrying metalloprotease genes may lead to increased intestinal permeability and production of gliadin immunogenic peptides. In addition, these peptides are able not only to keep but also to strain their capacity of stimulating TNF-alpha-mediated inflammatory response. These increases in TNF--alfa production by epithelial cells could have deleterious effects that fuel both innate and adaptive immunity in CeD onset (Sánchez et al. 2012). Some *Prevotella* species, *Lachnoanaerobaculum umeaense* and *Actinomyces graevenitzii*, were isolated from CeD jejunal biopsies. It is possible for the aforementioned species to cause an IL-17A-driven immune response (Sjöberg et al. 2013). This emphasizes the possibility that the increased IL-17A response seen in active CD could be in part attributable to host-microbiota interactions, and this may additionally justify why the IL-17A membrane response in CD isn't consistent in some CD patients (La Scaleia et al. 2012). Neisseria flavescens is the cause of inflammation and disruptions in the mitochondrial chain processes of Caco-2 epithelial cells. This latter metabolic alteration seems to be partly corrected once Lactobacillus paracasei CBA is run (Labruna et al. 2019). Another study involving N. flavescens showed that five different strains isolated from adults with untreated CD led to an inflammatory activation of both human and murine dendritic cells (DC) (D'Argenio et al. 2016). Nevertheless, it is not clear whether N. flavescens causes inflammation, or the inflammatory process occurring in the gut of CD patients may favor its colonization, which then simply maintains an activated pro-inflammatory response. Moreover, it has been demonstrated by Galipeau et al. that gut microbiota can either reduce or exacerbate gliadin--induced damage in a mouse model of CD (Galipeau et al. 2015). In this study, the expansion of the Proteobacteria phylum caused more severe intestinal damage induced by gluten. This could possibly be explained by the fact that the intestinal mucus layer is more penetrable to bacteria and toxins where Proteobacteria prevail (Jakobsson et al. 2015). A Spanish research presents similar evidence about Caco-2 cells. Enterobacteriaceae (belonging to the Proteobacteria phylum) were found to act similarly to gliadins concerning DC maturation, i.e., attachment, spreading, and pro-inflammatory cytokine polarization. On the other hand, *Bifidobacterium longum CECT 7347* counterbalanced IFN-production as a consequence of gliadin stimulation and increased IL-10 release (De Palma et al. 2012b). Taken together, the above evidence highlight the important role of the biological milieu of the intestinal lumen for disease progress.

3.5 Microbiome and Microbiome-Targeted Therapies

3.5.1 Antibiotics

Antibiotics, probiotics, and prebiotics have been utilized to treat gastrointestinal disorders with contradictory results. Each antibiotic has a unique spectrum against bacteria but also can favor beneficial bacteria. Antibiotics are significant factors for

modulating bacterial metabolites like SCFAs and other beneficial products and present immunomodulatory effects (Maccaferri et al. 2010; Rafii et al. 1999; Sartor 2016; Morikawa et al. 1996; Wan et al. 2015; Garrido-Mesa et al. 2011). The concept of dysbiosis in both IBD and IBS patients who present increased number of pathobionts support the antibiotic therapeutic strategy. Long-term metronidazole is effective against Bacteroides, with bacterial concentrations correlated with disease activity (Krook et al. 1981). Ciprofloxacin eliminates enteric pathogens such as Gram-negative Enterobacteriaceae. Rifamycin reduces bacterial attachment increases *Lactobacillus*, *Bifidobacterium*, and *F. prausnitzii*; however it has an effect on overall bacterial diversity (Maccaferri et al. 2010; Sartor 2016; Gao et al. 2014). On the other hand, the long-term use of broad-spectrum antibiotics have been shown to negatively impact the gut microbiota by reducing diversity and may cause antibiotic resistance. IBD patients present a higher rate of methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, and extended-spectrum beta-lactamases (ESBL)-producing E. coli (Leung et al. 2012). Furthermore, most antibiotics inhibit also the protective bacteria among others leading to overgrowth of pathogenic bacteria (C. difficile), fungi (candida), and bacteriophages (Dethlefsen et al. 2008; Dethlefsen and Relman 2011; Lewis et al. 2015). Antibiotics are widely used for the treatment of IBD. Combinations of antibiotics could be more effective but also the single antibiotics could diminish disease complications and prevent post-resection recurrence (Ohkusa et al. 2010; Turner et al. 2014). Rifamycin, ciprofoxacin, and metronidazole alone or in combination, show improved remission rates in IBD. Notwithstanding, the benefit of antibiotics in CD patients has not been confirmed by meta-analysis studies. Their beneficial effect is weak and decline over time (Wang et al. 2012; Khan et al. 2011; Townsend et al. 2019; Su et al. 2015; Holubar et al. 2010). Of note, anti-*Mycobacterium* agents demonstrate some benefit for inducing remission (Khan et al. 2011; Patton et al. 2016; Prantera et al. 2006; Selby et al. 2007). Additionally, there are evidence for significant improvement of IBS symptoms after consumption of nonabsorbable antibiotics. A meta-analysis indicated rifaximin as an effective treatment for ameliorating IBS symptoms. Furthermore an efficacy of rifaximin has been shown in IBS-D subtype; however a great proportion of patients appeared recurrent symptoms (Menees et al. 2012; Lembo et al. 2016).

3.5.2 Probiotics

Probiotics are living microorganisms, which are given in sufficient quantities to provide a beneficial effect on the host's health (Gueimonde and Collado 2012). Most of the germs currently used as probiotics have been isolated from the intestinal microflora of healthy individuals and belong mainly to the genera *Lactobacillus* and *Bifidobacterium*. Probiotic bacteria can potentially provide various health benefits through modifying the intestinal microflora and its metabolite such the SCFA production. Probiotics action lies on the production of antimicrobial agents like defensins that inhibit pathogen colonization, on the enhancement of the integrity of the

intestinal barrier by upregulation of tight junction proteins, and stimulation of IgA secretion, resulting in reduced microbial transmission and modification of immune mechanisms (Dimidi et al. 2017; Plaza-Diaz et al. 2019; Gallo et al. 2016). The most commonly used probiotic organisms include *Lactobacillus* and *Bifidobacterium*; other bacteria scarcely used include *Bacillus* and *Streptococcus* as well as the yeast, *Saccharomyces boulardii*. The indispensable and vital characteristic of probiotics is the survival in the acidic environment of the stomach and bile acid to colonize the intestines (Barko et al. 2018). There are many different probiotic preparations with varying formulations, some containing single organisms, others multiple organisms. Single strain probiotics appear to be more effective in improving overall IBS symptoms, but not quality of life (Zhang et al. 2016). Therefore, the major number of studies has focused on a probiotic mixture called VSL#3 of lyophilized bacteria Lactobacillus (*L. casei*, *L. plantarum*, *L. acidophilus*, *L. bulgaricus*), Bifidobacterium (*B. longum*, *B. breve*, *B. infantis*), and *Streptococcus salivaris*.

The first indications of a therapeutic effect of probiotics come from the early twentieth century when treatment with *Streptococcus lacticus* and *Bacillus bulgaricus* lead to improvement of autoimmune arthritis (Warden 1909). To date, some beneficial effects have been demonstrated in experiments in animals or humans; in general, there is clinical evidence to support the use of probiotics for treating acute infectious diarrhea, antibiotic-associated diarrhea, *C. difficile*-associated diarrhea, ulcerative colitis, and irritable bowel syndrome, but not for acute pancreatitis or Crohn's disease (Shen et al. 2014; Wilkins and Sequoia 2017; Tojo et al. 2014; Sánchez et al. 2017; Allen et al. 2010; Goldenberg et al. 2017). Different probiotic species have been studied for ameliorating GI symptoms, though it is not always clear which species or strains are most beneficial (Ford et al. 2014).

The modulation of the gut microbiota by probiotics in IBS is well studied; over 30 randomized controlled trials (RCTs) have been performed. One of the first crossover studies concluded that treatment with *Lactobacillus acidophilus* offers a significant therapeutic benefit in 50% of the patients (Halpern et al. 1996). *Lactobacillus plantarum* (299 V) supplementation has been also evaluated; IBS patients demonstrated limited abdominal pain and flatulence and an overall improvement of IBS symptom but no alteration in colonic fermentation (Niedzielin et al. 2001; Nobaek et al. 2000; Sen et al. 2002). More recent trials of probiotics in IBS have been of better quality than the earlier studies. A trial investigating *Bacillus coagulans MTCC 5856* found improvements in IBS symptoms including abdominal pain, diarrhea and bloating in patients with the diarrheal form of the condition (Majeed et al. 2015). IBS patients followed a 4–8 week therapy with *Bifidobacterium infantis 35,624* experienced some improvements from baseline symptoms. Although an investigation into the use of *Escherichia coli* Nissle 1917 failed to improve symptoms in an IBS population as a whole, differing responses were found when patients were sub grouped according to their bowel habit (Faghihi et al. 2015). Similarly, an efficacy in *Bifidobacterium lactis DN-173010* supplementation was revealed in a female IBS-C population and a healthy population with digestive symptoms (Agrawal et al. 2009; Guyonnet et al. 2009a, b). Two further trials investigating the effect of *Lactobacillus casei* Shirota were conducted concluding to non-significant effect.

Although no alterations in gut microbiota were recorded, some GI related symptoms and SIBO were improved (Thijssen et al. 2016). The administration of the multi-species probiotic mixture VSL#3 as well as another mixture containing *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium breve*, *Bifidobacterium actis*, *Bifidobacterium longum*, and *Streptococcus thermophilus* seems to alter mucosal and fecal bacterial profile and to improve diarrhea-symptom scores in IBS patients (Yoon et al. 2015).

In addition to bacteria, the gut microbiota contains a variety of other organisms such as viruses, mainly bacteriophages, fungi, and yeasts. A meta-analysis of two randomized controlled studies of *Saccharomyces cerevisiae* showed that abdominal pain/discomfort and bloating were significantly improved with probiotic therapy in a certain subgroup of IBS-C patients but no other significant effect was observed. Data are currently lacking to demonstrate a direct effect of yeast on the gut microbiota of patients with IBS (Cayzeele-Decherf et al. 2017).

A meta-analysis of 21 RCTs involving 1639 adults with IBS found that probiotics significantly improved overall symptom response and quality of life compared with placebo (Zhang et al. 2016). Another meta-analysis of 35 randomized controlled trials revealed a beneficial effect on abdominal pain, bloating, and flatulence scores indicating combinations of probiotics as more advantageous than individual species or strains (Ford et al. 2014). A meta-analysis of children with IBS or functional abdominal pain found that probiotics increased the likelihood of treatment success compared with placebo and decreased abdominal pain intensity; however, there was no effect on abdominal pain frequency (Korterink et al. 2014). Altogether, meta-analyses have demonstrated a positive effect for patients with IBS; however, this type of analysis should include probiotic containing the same organisms or group of organisms for more accurate results.

As regards probiotics supplementation in ulcerative colitis, data suggest an efficacy in increasing remission rates but not in maintenance of remission. A mixed product containing *B. breve*, *B. bifidum*, and *L. acidophilus YIT 0168* has been examined as a dietary adjunct in the treatment of ulcerative colitis but the colonoscopic results showed no difference (Ishikawa et al. 2003). The combination of mesalazine plus *E. coli* (Nissle 1917) did not reveal any difference in the maintenance of remission in two studies (Kruis et al. 1997; Rembacken et al. 1999). A Cochrane review of four studies involving 587 participants found no significant difference between probiotics and mesalamine for the maintenance of remission in ulcerative colitis (Naidoo et al. 2011). A meta-analysis of 23 RCTs with 1763 adults found that probiotics significantly increased the remission rates in patients with active ulcerative colitis compared with placebo (Shen et al. 2014).

The efficacy of probiotics administration on the induction and maintenance of remission in CD has not fully unraveled as a small number of patients are involved in most trials and the results are contradictory. Sometimes the determination of the extent of inflammation is unclear; thus, the efficacy of probiotics is not easy to be estimated. Additionally, the possible effect of probiotics on active CD have not been broadly studied. A placebo-controlled study has been performed in order to evaluate the preventive effect of on appearance of recurrent lesions of Crohn's disease after

surgical intervention (Prantera et al. 2002). Similarly, in other studies probiotics failed to prevent a relapse following surgery (Chermesh et al. 2007; Marteau 2006; Van Gossum et al. 2007). Patients who were administered with the antibiotic rifaximin and a combination of probiotics (VSL#3) presented a significantly lower rate of severe endoscopic recurrence (Gionchetti et al. 2003). There are some positive signs but not with statistical significance that treatment of active CD with prednisolone plus *E. coli* (Nissle 1917) or mesalamine plus *S. boulardii* lead to fewer or retarded relapse (Guslandi et al. 2000; Malchow 1997). The simultaneous supplementation of a mixture of probiotics (*B. breve*, *B. longum*, and *L. casei*) and a prebiotic (psyllium) lead to a complete response in six out of ten patients (Fujimori et al. 2007). Lactobacillus GG administration for 1 year have not shown any statistically significant differences on appearance or severity of recurrent lesions of Crohn's disease after surgery (Prantera et al. 2002). In one study with only 11 patients, probiotics provided no additional benefit to steroids and antibiotics in inducing remission. More controlled studies have been performed on the maintenance of remission in adults with CD but in general these studies fail to show any benefit of probiotic administration (Guslandi et al. 2000; Malchow 1997; Schultz et al. 2004). Several meta-analyses and systematic reviews have shown that probiotics were ineffective in maintenance of remission in CD (Rahimi et al. 2008; Rolfe et al. 2006).

3.5.3 Prebiotics

Prebiotics have been used to regulate microorganisms in the host in order to improve measurable health outcomes from the middle 1990s. Twelve years later, prebiotics have been defined as a "nonviable food component that confers a health benefit on the host associated with modulation of the microbiota" (Pineiro et al. 2008). Recently, an update to the definition of prebiotics was published as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al. 2017). Prebiotics are basically classified as disaccharides or oligosaccharides, such as lactulose, oligosaccharides including fructo-oligosaccharides (FOS), galacto oligosaccharides (GOS), isomalto-oligosaccharides, xylo-oligosaccharides, transgalacto-oligosaccharides (TGOS) and soybean oligosaccharides, and polysaccharides, such as the fructan inulin, reflux starch, cellulose, hemicellulose, or pectin (Markowiak and Śliżewska 2017). Apart from the artificial prebiotics, cereals, fruit, green vegetables and plants including bananas, asparagus, artichokes, berries, tomatoes, garlic, onions, legumes, chicory, linseed, oats, barley, and wheat are natural sources of prebiotics (Lee and Salminen 2009). The use of prebiotics is based on the concept of providing dietary substrates, such as oligosaccharides and fiber in order to selectively increase the abundance of SCFA and SCFA producing microbes (Sartor and Wu 2017). Their characteristic is the resistance to enzymatic and chemical digestion before reaching the colon. After fermentation by non-pathogenic colonic bacteria, prebiotics have the potential to stimulate the generation of microbial metabolic products such as short-chain fatty acids (acetate, butyrate, and propionate) which offer direct benefits to colonocytes (provide energy, improve blood flow, etc.) (Alvarez-Curto and Milligan 2016; Roberfroid et al. 2010).

Notably, prebiotics may also induce other microbiota indirect benefits for the host promoting health, such as potent immunomodulatory effects (Franzosa et al. 2019), promotion of barrier integrity, reduction in visceral hypersensitivity, regulation of GI motility and total restoration of intestinal dysbiosis (Jacobs et al. 2016); therefore prebiotics may play mechanistic role of in managing gastrointestinal disorders symptoms. Prebiotics have great potential for modifying individual strains and species of the gut microbiota, favoring some beneficial bacteria and decreasing some harmful. For example, *Bifidobacteria* can specifically ferment prebiotic GOS and water-insoluble cocoa fraction, a polyphenol substance, promoting the growth of *Bacteroides*, *Lactobacilli* and especially *Bifidobacterium* (Roberfroid et al. 2010; Hunter et al. 1999). Current prebiotics are predominantly carbohydrate-based, but other substances, including polyphenols and polyunsaturated fatty acids, are used to such maximize prebiotic effects. Bifidobacteria have the ability to efficiently metabolize low-molecular-weight via various cell-associated and extracellular glycosidases while *Bacteroides* genus are able to cleave high molecular weight polysaccharides. Furthermore Ruminococcus spp. can facilitate the breakdown of resistant starch (Rivière et al. 2018; Flint et al. 2012; Hamaker and Tuncil 2014; Ze et al. 2012, 2013).

Several clinical studies have examined prebiotics' efficiency in improving symptoms of bowel disorders; however, the results of prebiotics use are not satisfying.

Data shown the efficacy of prebiotics in ameliorating IBD symptoms are limited; however, there are a few human and animal studies with controversial results (Langlands 2004; Videla et al. 2001; Winkler et al. 2007; Cherbut et al. 2003; Camuesco et al. 2005). The efficacy of FOS in CD was firstly examined in ten CD patients receiving 15 g of this prebiotic; patients presented improved disease activity index and increased mucosal *Bifidobacteria* (Lindsay 2006). A latest study, involving a larger number of participants, have shown that patients receiving FOS had neither clinical improvement nor alterations in *Bifidobacteria* levels, but they had reduced proportions of interleukin (IL)-6-positive lamina propria dendritic cells (DC) and increased DC IL-10 staining (Benjamin et al. 2011). In another study, fecal metabolome and microbiome were assessed after treatment with oligofructose--enriched inulin in patients with active CD; a significant increase in fecal SCFA was revealed as well as a decrease in fecal Ruminococcus gnavus and increase in B. longum leading to clinical improvement (De Preter et al. 2013; Joossens et al. 2012; Zimmerman et al. 2012).

Inulin have been also shown to increase other microbes including *F. prausnitzii* (Ramirez-Farias et al. 2009), a firmicute found to be decreased in the gut of patients with higher relapse rates in CD (Sokol et al. 2008). Notwithstanding the positive evidence, one-third of the subjects received oligofructose-enriched inulin presented side effects (De Preter et al. 2013).

Similarly, to CD there have been few prebiotic studies in UC. Many studies focus on QOL, symptoms, and bacterial metabolites in UC treated with various prebiotics. Psyllium, germinated barley foodstuff (GBF), lactulose, and oligofructose-enriched inulin significantly improve QOL and symptoms in UC patients (Fujimori et al. 2009; Hafer et al. 2007; Casellas et al. 2007; Hanai et al. 2004). UC patients

supplemented with oligofructose-enriched inulin had a lower fecal calprotectin, an inflammatory marker, than controls (Casellas et al. 2007). Another evidence of inulin efficacy in bowel disorders comes from a study in pouchitis; inulin supplementation was linked to an increased level of butyrate, a lower concentration of *Bacteroides fragilis* and secondary bile acids in feces as well as a reduced endoscopic inflammation (Welters et al. 2002).

A potential role of the prebiotics germinated barley foodstuff (GBF) and Ispaghula husk, in inducing remission in patients with mild-to-moderate active ulcerative colitis have been demonstrated. GBF contains low-lignified hemicellulose that is efficiently fermented by colonic microbiota (Kanauchi et al. 1999). GBF reduced CRP and improved clinical and endoscopic scores in active UC (Kanauchi et al. 2002, 2003; Bamba et al. 2002; Hallert et al. 1991; Faghfoori et al. 2014). Intake of psyllium and wheat bran significantly increased fecal butyrate. A large RCT with psyllium demonstrated equivalent effectiveness to 5-ASA to maintain remission in UC (Hallert et al. 2003; Fernández-Bañares et al. 1999). A promising prebiotic may be curcumin, the biologically active component of turmeric, as it exhibits anti-inflammatory and antioxidant properties and can promote the growth of protective bacteria (Ghiamati Yazdi et al. 2019). A large randomized control trial in UC revealed that curcumin improved remission rates with clinical and endoscopic scores compared to controls (Hanai et al. 2006). Restricted dietary fiber did not improve symptoms need for surgery or hospitalization in CD patients (Levenstein et al. 1985). On the contrary, fiber-rich diets significantly reduced surgery in active CD (Heaton et al. 1979) and prevented relapse during remission (Jones et al. 1985).

Few studies have investigated the effect of prebiotics on IBS symptoms; overall, data show no benefit in symptom management or improve QoL in IBS or other functional gastrointestinal disorders. Meta-analysis showed that prebiotics did not significantly impact integrative symptom scores, severity of abdominal pain, bloating, or flatulence but the do increase *Bifidobacteria* (Wilson et al. 2019). Early work demonstrated that selected prebiotics promoted the growth of potentially beneficial *Bifidobacteria* while inhibiting the growth of potentially harmful *Bacteroides*, *Clostrida*, or Coliforms. Two controlled studies observed no effect of treatment with inulin on IBS (Hunter et al. 1999; Olesen and Gudmand-Hoyer 2000). On the contrary, when IBS subjects supplemented with a short-chain inulin-type fructan, the frequency and intensity of digestive symptoms as well as the quality of life were improved (Paineau et al. 2008). Other studies investigating the effect of trans-GOS and β-GOS supplementation in IBS patients indicated improved stool consistency, flatulence, and bloating as well as total symptom score and, significantly increased *Bifidobacteria* and *Lactobacilli* abundance (Silk et al. 2009; Vulevic et al. 2018; Marteau and Seksik 2004).

Prebiotic use in either IBD or IBS patients have generated mixed results. Based on available evidence, general use cannot be recommended in patients with gastrointestinal disorders; more controlled studies are needed to decide their beneficial or harmful role.

3.5.4 Synbiotics

The term synbiotics refers to mixtures of probiotics and prebiotics that can confer a synergistic beneficial effect to the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract through the selective stimulation and/or the activation of the metabolism of one or a few health--promoting bacteria (Wasilewski et al. 2015; Pandey et al. 2015). Probiotics frequently used for the symbiotic formulae include *Lactobacilli*, *Bifidobacteria* spp., *S. boulardii*, *B. coagulans*, while the most common prebiotics are oligosaccharides like FOS, GOS xylose oligosaccharide (XOS) and inulin. A systematic review that examined the role of synbiotics in patients with IBD, suggested that synbiotics could be an effective treatment modality for acute and active CD. Regarding UC patients, the use of synbiotics appears to have a positive outcome in maintenance of remission, with a concomitant reduction of pro-inflammatory cytokines expression and induction of anti-inflammatory cytokines expression (Saez-Lara et al. 2015).

Synbiotics containing Bifidobacterial strains and GOS appeared to improve endoscopic scores and minimize inflammatory markers in treated UC patients. The combination of *Bifidobacterium longum* and inulin-oligofructose as well as *B. longum* and psyllium presented a synergistic effect more impressive than probiotic or prebiotic alone suggesting synbiotics as a supplement to conventional therapy in UC patients. Additionally, the *B. breve* Yakult strain and GOS mixture showed a significant anti-inflammatory effect in mild-to-moderate UC patients (Saez-Lara et al. 2015; Ishikawa et al. 2011; Laake et al. 2003). Similarly, administration of *B. longum* plus Synergy1 synbiotic to patients with active UC increased the abundance of Bifidobacteria on the mucosal surface in active UC and reduced inflammatory markers such as TNF-a and IL-1b levels. The efficiency of this symbiotic have also been indicated in CD (Furrie et al. 2005; Steed et al. 2010). Similarly, short bowel syndrome was relieved upon administration of a supplement containing *B. breve*, *Lactobacillus casei*, and galactooligosaccharides (Kanamori et al. 2001).

Overall, prebiotic therapy appears safe and promising, but RCTs are needed to assess the efficacy of dietary/prebiotic interventions. However, clinical studies of synbiotics are limited. Therefore, more human and animal studies are needed to collect convincing data and provide a better understanding of their direct effects on health, particularly in IBD.

3.5.5 Fecal Microbial Transplantation

Stool transfer from healthy donors to the sick in order to treat disease has been described very early in history. In particular, in China the fourth century a fecal suspension was tested as a treatment for food poisoning or severe diarrhea. Since 1985, fecal clysters have been used for the treatment of "pseudomembranous colitis" (Sbahi and Di Palma 2016). In modern medicine, transfer stool is known as fecal microbiota transplantation (FMT) and include the process of replacing or

reinforcing the "dysbiotic" gut microbiota of a patient with the microbiota from a healthy donor (Lee et al. 2017; König et al. 2017). The first step of the procedure is the selection of a donor without a family history of autoimmune, metabolic, and malignant diseases and screening for any potential pathogens. Afterwards, the feces are mixed with water or normal saline, and then filtered to remove any particulate matter. The mixture is most commonly administered as a fecal retention enema, but alternative methods such as infusion via a nasogastric tube, nasojejunal tube, esophagogastroduodenoscopy, colonoscopy have been developed. The most effective route seem to be the colonic, however, all modalities have been shown overall comparable efficacy (Cammarota et al. 2017; van Nood et al. 2013; Aas et al. 2003; Persky and Brandt 2000; Silverman et al. 2010).

FMT has been increasingly used for the treatment of different disorders; Data from healthy subjects have shown that even a small stool mass (11–22 g) induces profound alterations in microbiota composition, due to engraftment of donor bacteria. The potential mechanisms of their action include the horizontal gene transfer, effects of the non-bacterial stool components, and functional interactions between microbial communities (Goloshchapov et al. 2019).

Despite the increasing use of FMT, most clinical experience on this intervention has been derived from recurrent or refractory *Clostridium difficile* infection presenting a considerable therapeutic potential with an efficacy greater than 90%. The European Consensus Conference on FMT in Clinical Practice, strongly proposed the implementation of FMT for the treatment of refractory or recurrent *Clostridium difficile* infection, as well as in severe or fulminant *C. difficile* induced colitis (Cammarota et al. 2017; Austin et al. 2014; Kassam et al. 2013; Kelly et al. 2016).

As FMT is an inexpensive and easy treatment, it gains popularity for the management of gastrointestinal disorders including IBS and IBD (Distrutti et al. 2016). Several studies have used FMT as a therapeutic option for IBS patients, but data are based on open-label trials and small cohorts of IBS patients. These studies involved all of the subtypes of IBS, have concluded to considerable relief in IBS symptoms and improvements in patients' quality of life. The short-term response rate was higher than the long term suggesting repeat of treatment at regular intervals (Distrutti et al. 2016; Holvoet et al. 2017; Pinn et al. 2014; Mazzawi et al. 2018). A study which included patients, diagnosed with IBS based on Rome III Diagnostic Criteria, who received fecal materials via colonoscopy, showed that FMT administration in IBS patients is safe, and relatively effective method, which improved the psychological status of IBS patients (Mizuno et al. 2017). A review of six FMT studies found that more than half IBS patients treated with FMT were in benefit (Halkjær et al. 2017). Two other controlled studies have shown that FMT treatment either via oral administration or via colonoscopy led to increased enteric biodiversity and overall improvement of IBS symptoms (Johnsen et al. 2018; Halkjær et al. 2017). Furthermore, a total of 70% of patients reported overall symptomatic improvements after FMT administration via esophagogastroduodenoscopy (EGD). In particular, 72% patients reported relief of pain, 67% of dyspepsia, 56% alterations in bowel habits, 50% improvement in bloating, and 45% in flatus (Pinn et al. 2014). The success of FMT treatment depends on the donors' intestinal microbiome; a donor's

microbiome enriched to Bifidobacterium efficiently induce symbiosis in IBS patients (Mizuno et al. 2017). Nevertheless, more randomized controlled trials with greater number of patients are needed to determine the efficacy of FMT in IBS and to standardize the procedure including the amount of feces used, the form of feces (fresh or frozen), the route of administration, and donor selection and screening (El-Salhy et al. 2020).

Concerning IBD, a total of eight meta-analyses have evaluated the efficacy of FMT. Nevertheless, the majority of them include only UC patients and just three of them examined the role of FMT in both CD and UC (Fang et al. 2018; Jeon et al. 2018; Colman and Rubin 2014; Shi et al. 2016; Sun et al. 2016; Costello et al. 2017; Paramsothy et al. 2017; Narula et al. 2017; Cao et al. 2018). In general, meta--analysis have suggested FMT as an effective and safe treatment particularly in UC patients; however the need of more randomized controlled studies of FMT in IBD and especially in CD is highlighted (Fang et al. 2018; Colman and Rubin 2014; Paramsothy et al. 2017). A meta-analysis of 53 studies, 41 in UC, 11 in CD, and 4 in pouchitis, comprising 661 IBD patients showed that 36% of UC patients (201/555), 50.5% of CD patients (42/83), and 21.5% (5/23) of pouchitis patients undergoing FMT achieved clinical remission (Paramsothy et al. 2017). Another review of the factors that may influence the outcome of the treatment in IBD patients conclude that FMT efficacy is independent from the sort of donor stools (fresh or frozen), the delivery route, and previous treatment with antibiotic. In a recent review, the variable response of IBD patients was highlighted compared to robust clinical outcomes in *C. difficile* infections concluding that FMT may be considered as an adjuvant treatment, for example in combination with immunomodulatory drugs (Basso et al. 2019).

As mentioned above, the effect of FMT has been widely investigated in ulcerative colitis patients. A randomized controlled trials involving patients with active ulcerative colitis have shown that after treatment with fecal enema patients presented higher remission rates than those administered with placebo enema (Moayyedi et al. 2015). Contrary another study examined FMT efficacy via nasoduodenal tube administration concluded that FMT cause no difference in clinical and endoscopic remission suggesting that routes of administration may play a major role in FMT efficacy (Rossen et al. 2015). In ulcerative colitis, the underlying pathophysiology may favor distal as opposed to proximal FMT administration (Lopez and Grinspan 2016). Studies including only a small number of patients with refractory UC to conventional therapy indicated that FMT administration could completely relief from UC symptoms and remission maintenance could last for up to 13 years (Borody et al. 1989, 2001, 2003).

A Cochrane review of four studies including 277 participants concluded that FMT indeed increase rates of clinical remission of UC patients by twofold compared to controls; almost 37% of participants presented symptoms relief. However, an equally large proportion of patients displayed serious side effects included worsening of ulcerative colitis with increased abdominal pain, nausea, flatulence and

bloating, infections such as *Clostridium difficile* and cytomegalovirus or upper respiratory tract infection, headaches, dizziness and small-bowel perforation (Imdad et al. 2018). It is supposed that certain bacteria and metabolites influence FMT responses. An abundance of *Eubacterium hallii*, *Roseburia inulivorans*, SCFAs, and secondary bile acids has been recorded in patients who experience UC remission after FMT. On the contrary non- responders presented increased elevated levels of *Fusobacterium gonidiaformans*, *Sutterella wadsworthensis*, and *Escherichia coli*, as well as enhanced heme and lipopolysaccharide biosynthesis (Paramsothy et al. 2019). The effectiveness of FMT therapy is influenced by various factors such as diversity and abundance of the colonized microflora, similarity of metabolomics and virus omics profiles to those of the donor, and concentration of fecal metabolites (Nusbaum et al. 2018). UC patients with low viral richness had more favorable responses to FMT compared with the patients with higher virome concentrations suggesting that the concentration of colonic viruses is another determinant factor for treatment outcome (Conceição-Neto et al. 2018). Undoubtedly, there is lack of data on the long-term maintenance of remission in UC or CD.

In parallel, there are scarce data on the efficacy of FMT for induction of remission in CD patients (Imdad et al. 2018; Wang et al. 2016; Sunkara et al. 2018). Many promising case reports describe induction of CD remission after FMT. Meta--analysis of six prospective and uncontrolled trials shows 52% clinical remission rate with publication bias. For adult CD, a higher clinical responses, about 58–87% rate, was reported (Paramsothy et al. 2017; Vaughn et al. 2016; Cui et al. 2015). Responders to FMT showed improvement in microbial diversity resembling their donor's microbial profile and increased lamina propria Tregs (Vaughn et al. 2016). The efficacy of FMT has been also indicated in pediatric CD patients; 77.8% of pediatric subjects displayed remission after FMT via nasogastric tube. Nevertheless, there are signs of short-lasting outcome on symptoms and clinical activity after FMT (Vaughn et al. 2016; Cui et al. 2015; Suskind et al. 2015; Goyal et al. 2018). The interval between two courses of FMT is proposed to be less than 4 months in order to maintain the clinical benefits (Li et al. 2019). Responders tended to be those with lower diversity, suggesting that FMT may provide symptomatic improvement for CD patients with more perturbed microbiota at baseline. Although bacterial communities of responders did not become more like donors in all cases, FMT increased the relative abundance of some bacteria observed frequently in donor microbiota and reduced those commonly associated with CD (Cui et al. 2015).

It was only recently that the first randomized controlled study was performed evaluating FMT in maintaining remission achieved with systemic corticosteroids in CD. A higher rate of steroid-free clinical remission as well as improved CDEIS and CRP level was noticed in the FMT than in placebo group, but with no statistical significance. Donor microbiota engraftment was not observed thus single FMT might not be enough to induce significant microbial changes (Sokol et al. 2020). Regarding safety, the rate of adverse effects has been estimated to a rate of 13.6% for patients with refractory CD undergoing FMT indicating this kind of intervention safe enough (Cui et al. 2015; Wang et al. 2018).

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Gut Microbiome and Cancer

4

George E. Theodoropoulos

Abstract

Cancer is a composite disease subjected to a complex interplay between host genetic and environmental factors, such as microorganisms. Microbiota is an ecological community of microorganisms, which, among other important roles, seem to interfere in cancer biology. The alpha-bug hypothesis, the driver--passenger hypothesis, and the bystander hypothesis have been proposed to explain microbiota-driven mechanisms of carcinogenesis. Genetics of the host, diet, infection, or medical interventions, such as antibiotics, may influence the structure of the microbial community, leading to dysbiosis.

Dysbiosis is defined as any change to the composition of resident commensal microbial communities relative to the community found in healthy individuals. Primary interactions between microbiota and immunocytes, or parenchymal cells and local interactions producing distant effects, are considered as dysbiosis--related mechanisms of carcinogenesis. Inflammation, with its complex set of mediators, may contribute to a milieu that favors the outgrowth of specific bacteria, favoring carcinogenesis. Interaction between microbes and epithelial cells can lead not only to DNA damage but also to specific gene mutations that contribute to colorectal cancer development.

Functional studies suggested that several bacteria, including enterotoxigenic *Bacteroides fragilis*, genotoxic *Escherichia coli* and *Peptostreptococcus anaerobius*, may promote colorectal carcinogenesis. Microbiome in colorectal cancer patients is often enriched in proinflammatory opportunistic pathogens and microbes associated with metabolic disorders and depleted in butyrate-producing bacteria, which have been shown to be pivotal for the preservation of intestinal

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homeostasis. Among the putative bacteria, *Fusobacterium nucleatum* is one that has been extensively studied in colorectal cancer; independent studies have identified *Fusobacterium nucleatum* to be more abundant in cancer tissues. Known as a Class I risk factor, infection by *Helicobacter pylori* can stimulate immune responses and inflammation, regulate many signaling pathways, and induce gastric achlorhydria, dysplasia, and cancer.

Gut microbiota can modulate the host response to chemotherapy through numerous mechanisms, including immune interactions. Gut microbiota has been shown to affect cancer response to immunotherapy checkpoint inhibitors including those that aim at the programmed cell death protein ligand 1 (PD-L1) axis. A number of studies have claimed the benefits of probiotics on the suppression of colorectal cancer, notably through participating in the innate immune system and apoptosis, decreasing oxidative stress and improving the community of gut microbiota.

Keywords

Dysbiosis · Cancer · Genotoxicity · Inflammation · Immunity · Autophagy

4.1 Introduction

Cancer remains one of the leading causes of mortality in the Western world (Torre et al. 2015). Lifestyle habits, aging, diets rich in red and processed meat, alcohol consumption, smoking, and genetic factors have been implicated in human carcinogenesis. Both genetic alterations and oncogenic pathways governing the susceptibility to cancer and the carcinogenesis progression have been clearly identified and studied in detail. In the context of this intricate interplay between host genetic and environmental factors, microbiota has emerged as a critical determinant interfering in cancer biology and influencing the malignant development and progression (Rajagopala et al. 2017; Helmink et al. 2019; Gopalakrishnan et al. 2018a; Schwabe and Jobin 2013; Picardo et al. 2019; Li et al. 2019; Wong et al. 2019; Rea et al. 2018; Scott et al. 2019) (Fig. 4.1). Microbiota inhabits the epithelial barrier of human body, such as the skin, respiratory tract, and the gastrointestinal (GI) tract. The GI tract harbors approximately 3×10^{13} bacteria and is lined by an epithelium which is characterized by a constant crosstalk between the gut microbiota, immunological cells, and the mucosal barrier (Gopalakrishnan et al. 2018a; Wong et al. 2019; Rea et al. 2018; Scott et al. 2019). The entire microbial genome is about 150 times larger than the human one (Rea et al. 2018). According to the human gut microbial gene catalog established by metagenomic sequencing, common bacterial phyla, such as *Bacteroides, Firmicutes, Proteobacteria, Actinobacteria, Proteobacteria, Fusobacteria, Verrucomicrobia, Tenericutes*, and *Lentisphaerae*, are included in gut microbiota, while main genera incorporate *Bacteroides, Clostridium, Faecalibacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Lactobacillus, Streptococcus, Streptomyces*, and

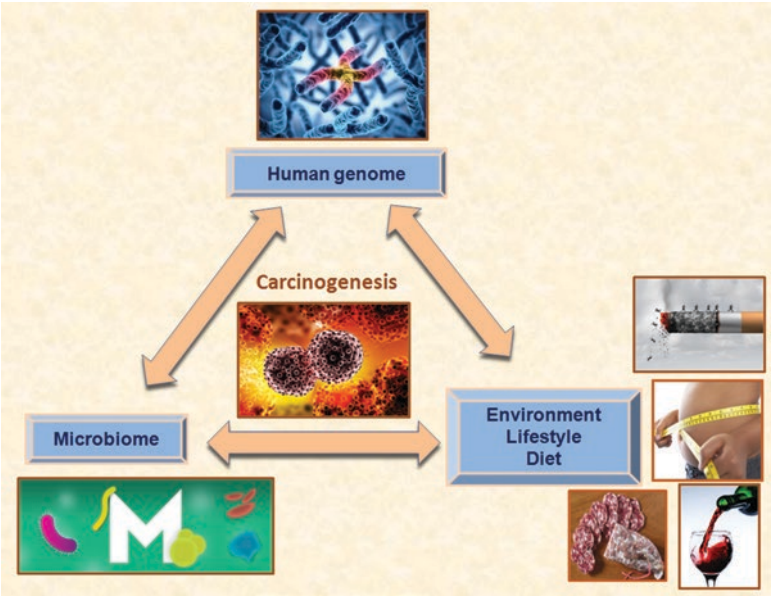


Fig. 4.1 The interplay between host genetic and environmental factors with microbiota critically determines cancer development and progression

Bifidobacterium (Rea et al. 2018). Most microbes residing within the human GI tract are bacteria, viruses, and fungi, and the combined genetic material of all those microorganisms make up the human microbiome (bacteriome, virome, and fungome). Human microbiota should be considered as a group of dynamic symbionts, which may function in a wide spectrum, varying from commensalism to pathogenicity or oncogenicity (Scott et al. 2019).

Many common human cancers are at least partly attributed to infection. Estimates range from 20% in lymphomas and leukemias to almost 100% in cervical cancer (Gilbert et al. 2018). The first report suggesting the importance of microbiota in large bowel cancer was published in 1969 (Aries et al. 1969). *Helicobacter pylori (H. pylori*), hepatitis B and C viruses, and human papilloma virus have been recognized as carcinogenic agents by the International Agency for Research on Cancer, and they have been estimated to account for about 20% of all cancers (Martel et al. 2012). Other types of cancer that are less obviously related to infections might also be triggered or promoted by dysfunctional bacterial growth.

4.2 Gut Microbiome and Carcinogenesis

4.2.1 Dysbiosis and Carcinogenesis

Dysbiosis refers to perturbations to the structure of complex commensal communities, which can lead to deficient or altered host-microbiota interactions and subsequent development of diseases (Petersen and Round 2014). Perturbations of normal

human microbiota may occur through changes in diet, innate immune and inflammatory responses, or infections, and may affect microbial composition, richness, and the metagenome. According to the Human Microbiome Project, dysbiosis can be defined as an abnormality, in composition and/or function, of the host symbiotic microbial ecosystem that exceeds its restitutive capacity and has negative effects on the host (Human Microbiome Project C 2012). Dysbiosis can be categorized into three types: (1) loss of beneficial microbial organisms, (2) expansion of pathobionts or potentially harmful microorganisms, and (3) loss of overall microbial diversity. These three types of dysbiosis are not mutually exclusively and may all occur concurrently (Petersen and Round 2014). Three types of relationships can be considered between the microbiome and immune-mediated carcinogenetic mechanisms. In Class A, the primary interactions involve immunocytes; in Class B, the primary interactions involve local parenchymal cells; and in Class C, the local interactions produce distant effects (Petersen and Round 2014). According to these proposed mechanisms, some types of bacteria are able to stimulate mediators of inflammation, producing toxins that disrupt cell cycle control or contribute to the tumorigenic process through metabolites, respectively (Petersen and Round 2014).

An international cancer microbiome consortium consensus statement on the role of the human microbiome in carcinogenesis has recently been published (Scott et al. 2019). The panel stressed that dysbiosis is likely host-specific and disease-specific; a microbiome may be dysbiotic in one individual but not in another and/or may promote one pathology but not another. With respect to the etiopathogenesis of cancer, they proposed that "dysbiosis should be considered a persistent departure of the host microbiome from the health-associated homeostatic state (consisting of mutualists and commensals), towards a cancer promoting and/or sustaining phenotype (parasitism or amensalism)." The health-associated microbiome should synergize with the host to drive beneficial immune responses and metabolic mutualism. In addition the microbiome should have a tumor-suppressant effect on the host. Loss of these "normal" microbiota properties is considered dysbiotic and may have the potential to incite or sustain cancer (Scott et al. 2019). Firmly establishing causality between the human microbiome and common malignancies remains a challenge. Since in vitro animal and cross-sectional human studies have provided data to support an intimate relationship of microbiome and carcinogenesis, large human cohort studies to amplify suggested theories are lacking. Therefore, a causative role for the human microbiome in the etiopathogenesis of cancer remains largely unproven and the microbiome should be envisaged as one aspect of an interactome with an epigenetically/genetically vulnerable host and the environment (Scott et al. 2019). Progression of a neoplasm may depend on continued exposure to environmental stimuli, maladaptive or adaptive changes in microbiome function and host response.

4.2.2 Gut Microbiome and Carcinogenesis Hypotheses

Hypotheses proposed to explain mechanisms of carcinogenesis through microbiome--host interplay are the following: the "alpha-bug" hypothesis, the "driver-passenger" hypothesis, and the "bystander" hypothesis (Scott et al. 2019). According to the "alpha-bug" hypothesis, as initially proposed by Sears and Pardoll, specific pathogenic bacteria induce colorectal cancer (CRC) (Sears and Pardoll 2011). *Enterotoxinogenic Bacteroides fragilis (ETBF),* which is considered the main candidate "alpha bug," acquires oncogenic traits, causing colonic epithelial damage, primarily by secreting its *Bacteroides fragilis* toxin (BFT), which decreases E-cadherin levels. This loosens the attachments between intestinal epithelial cells and results in exposure to many antigens (Wu et al. 2007). Moreover, decreased E-cadherin promotes intracellular migration of β-catenin and accelerates carcinogenic-related signaling such as the Wnt signaling. High abundance of *ETBF* in colonic tissues is associated with early-stage carcinogenesis. However, the observed lack of consistent overabundance of putative "alpha bugs" in carcinoma tissues led Tjalsma et al. to suggest the "driver-passenger" model (Tjalsma et al. 2012). They proposed that, following the initial epithelial damage caused by the "driver" microbes, proliferating opportunistic "passenger" bacteria thrive at a unique tumor microenvironment and gradually outcompete the driver species, a process which is accentuated with the advancement of tumor stage. *Fusobacterium nucleatum* (*F. nucleatum*) represents the archetypal "passenger" bacterium and has been consistently found to colonize CRC tissue (Castellarin et al. 2011; Kostic et al. 2013; Rubinstein et al. 2013). It easily adapts to the tumor environment which is rich in amino acids, an essential substrate for *F. nucleatum,* while tumor cells express ligands for bacterial cell surface receptors and *F. nucleatum,* itself, interacts with intracellular signaling pathways and immune cells, promoting tumor progression. On the other hand, bacterial "driver" functions, such as toxin secretions, increase hydrogen sulfide (H_2S) and reduce butyrate production, which are key transducers of environmental effects that can stimulate carcinogenesis in a genetically susceptible host. In this framework, the "bystander" hypothesis supports that gut microbiota-produced metabolites induce CRC carcinogenesis (Fig. 4.2). The microbial community's metabolome has a pivotal role; short-chain fatty acids (SCFAs) acetate, propionate, and butyrate function in the suppression of inflammation and cancer, whereas other microbial metabolites, such as secondary bile acids, promote carcinogenesis (Louis et al. 2014). The secondary bile acids, i.e., deoxycholic acid and lithocholic acid, are produced from bile acids by intestinal bacteria, induce DNA damage, and contribute to carcinogenesis. Apart from secondary bile acids, several bacterial metabolites including H_2S, reactive nitrogen species (RNS), and reactive oxygen species (ROS) have the potential to cause direct DNA damage or to provoke inflammation [via interleukin 6 (IL-6) and tumor necrosis factor (TNF) production], which, thus, promotes carcinogenesis. N-nitroso compounds (NOCs) can also promote cancer by generating mutations owing to DNA alkylation.

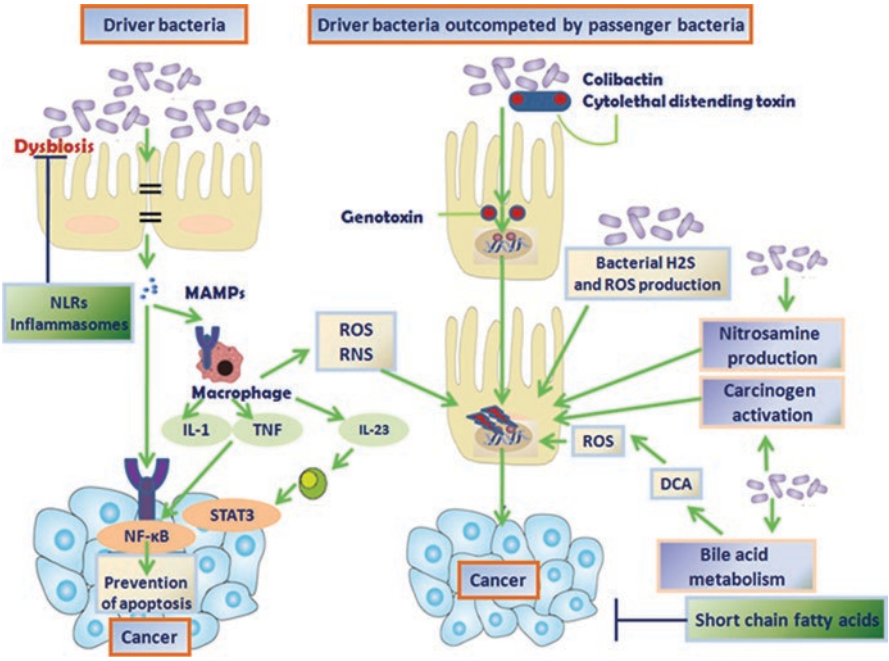


Fig. 4.2 Dysbiosis-related mechanisms linked to carcinogenesis

4.2.3 Gut Microbiome and Carcinogenesis Mechanisms

Prolonged host cell survival, enhanced replicative capacity, and dedifferentiation are indigenous elements of carcinogenesis potentially interrelated to microbiota--induced mechanisms involving its genotoxicity and effects on host inflammation, immunity and metabolism, as well as disturbance of cellular hemostasis through autophagy (Fig. 4.2).

4.2.3.1 Genotoxicity

DNA damage: Structural DNA damage may cause cell death or may affect tumor suppressor genes or oncogenes leading to carcinogenesis. Several dysbiosis-related microbiota exert their genocidal properties via the production of well-characterized genotoxins. *Escherichia coli* (*E. coli*) secretes several virulence toxins, called cyclomodulins, which are genotoxic and may modulate cellular differentiation, apoptosis, and proliferation (Nešić et al. 2004; Buc et al. 2013). Cytotoxic necrotizing factor (CNF) activates Rho GTPases, leading to cytoskeletal alterations and affecting the cell cycle (Collins et al. 2011). The genotoxin colibactin is a hybrid polyketide-non-ribosomal peptide compound (Nešić et al. 2004; Buc et al. 2013; Collins et al. 2011). Colibactin is encoded by the *polyketide synthase (pks)* genomic island and causes DNA double-strand breaks, chromosomal aberrations, and cell cycle arrest in cells in vitro (Nešić et al. 2004; Buc et al. 2013; Collins et al. 2011).

Recent studies have shown that, upon exposure to cells, this genotoxin induces intra-strand DNA cross-linking (Bossuet-Greif et al. 2018). This cross-linking is accompanied by a robust ATR-dependent replication stress response, in which ATR phosphorylates many proteins that regulate origin of replication firing, cell cycle transitions, and replication fork progression (Bossuet-Greif et al. 2018). This response prevents cells with damaged DNA from entering mitosis. Serine/threonine--protein kinase ATR also known as ataxia telangiectasia and Rad3-related protein (ATR) or FRAP-related protein 1 (FRP1) is an enzyme that, in humans, is encoded by the ATR gene and belongs to the phosphatidylinositol 3-kinase-related kinase protein family; ATR is activated in response to single-strand breaks.

In studies conducted by Dejea et al., *pks + E. coli* were found to work synergistically with *ETBF* to cause increased DNA damage and increased tumor formation in a mouse model of CRC (Dejea et al. 2018). This DNA damage was accompanied by a heightened inflammatory response that was necessary, but not sufficient, for increased colon tumor formation. The increased tumorigenesis was also highly dependent on the presence of both colibactin and BFT. This evidence empowers the direct correlation between these bacterial toxins, an increased inflammatory response, DNA damage, and tumor formation (Dejea et al. 2018).

In one study, Maddocks et al. showed that *enteropathogenic E. coli (EPEC)* depleted the mismatch repair proteins of host cells, leading to an increased mutation frequency, as measured using an artificially inserted microsatellite (Maddocks et al. 2013). The effect was mediated by an EPEC-secreted protein (EspF) that targeted the mitochondria of epithelial cells and induced post-translational modifications of mismatch repair proteins.

The *E. coli* produced cytolethal distending toxin (CDT) also induces DNA damage via its DNAse activity (Collins et al. 2011). *Campylobacter jejuni* produces the genotoxin CDT as well. In recent animal experiments colonization of germ-free (GF) $Apc^{Min/+}$ mice with human clinical isolate *Campylobacter jejuni 81–176* proved the CDT promotion of CRC via the induction of changes in microbial composition and transcriptomic responses (Hassane et al. 2003). Aside from specific toxins, bacterial metabolites may also exert genotoxic effects. ROS (produced by *Porphyromonas* sp.) and H_2S (produced by *Bilophila* and *F. nucleatum*) are two examples that have been associated with CRC (Scott et al. 2019).

DNA methylation: The effects of DNA methylation on cancer development have been examined extensively. Both hypomethylation and hypermethylation have been linked to CRC development, but the mechanisms by which they contribute to cancer development differ. Using a porcine model, Pan et al. found more than 80 differentially methylated region (DMR) microbes on gene methylation status (Pan et al. 2018). This study showed that treatment of these cells with probiotic species (*Lactobacillus acidophilus* and *Bifidobacterium infantis*) or *Klebsiella* species resulted in methylation changes in several hundred genes of interest (Cortese et al. 2016). In mice models, Yu et al. demonstrated that the presence of gut microbes led to an increase in the 3′ CpG island methylation of specific genes, which correlated with increased gene expression, suggesting a functional role for these changes (Yu et al. 2015a). In another study, Maiuri et al. showed that, when inoculated with

ETBF, *Apc*$^{min/+}$*/Msh2*$^{-/-}$ *mice* produced more tumors than *Apc*$^{min/+}$ mice with intact Msh2 mismatch repair proteins. The increase in tumor burden was not seen in the absence of *ETBF* inoculation, suggesting that mismatch repair proteins play an important role in preventing tumorigenesis after *ETBF* colonization (Maiuri et al. 2017).

Microbial DNA integration: Bacterial DNA integrations into host genomes through RNA intermediates occur more frequently in tumors than in normal samples (Riley et al. 2013). Random integration of *Acinetobacter*-like DNA in human mitochondrial genome in acute myeloid leukemia samples and specific integration of *Pseudomonas*-like DNA in the 5′-UTR and 3′-UTR of four proto-oncogenes that are upregulated in their transcription, consistent with conversion to an oncogene, at stomach cancer support the hypothesis that bacterial integrations occur in the human somatic genome and may play a role in carcinogenesis.

Chromatin structure: The location of histones in the DNA-histone complex, referred to as a nucleosome, is tightly regulated by a number of proteins and enzymes that modify the histones or serve as docking sites for other histone modifications-recognizing proteins. Histone modifications include the methylation, acetylation, or phosphorylation of various residues. Histone acetylation and deacetylation are regulated by histone acetyltransferases and histone deacetylases (HDACs). Mutations in enzymes that belong to each of these groups have been found in cancer. HDAC inhibitors have already been approved for the treatment of hematologic malignancies, and growing evidence suggests they might be useful in CRC as well. Major bacterial fermentation products, such as the SCFAs butyrate, propionate, and acetate, can be recognized by receptors [i.e., the G protein-coupled receptors (GPCRs) GPR41, GPR43, and GPR109A] on the surface of colonocytes and immune cells. SCFAs are also transported into host cells, which results in the subsequent inhibition of histone deacetylase (HDAC) activity by butyrate and propionate, causing hyperacetylation of histones. Several studies have shown that the interactions between SCFAs and GPCRs, as well as SCFA inhibition of HDACs, also occur in cell types other than colonocytes, including macrophages and T cells. HDAC inhibition and GPCR signaling result in an increase in total colonic regulatory T cell (TReg) numbers and the production of the anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGF-β). HDAC inhibition is also thought to promote apoptosis of CRC cells (Meng et al. 2018).

In an effort to expand our understanding of the effects of gut microbes on chromatin structure, two studies on intestinal epithelial cells isolated from the jejunum of GF and conventional reared mice identified an upregulation in the accessibility of histone binding sites for transcription factors in the signal transducer and activator of transcription factor (STAT), the interferon regulatory factor (IRF), and the E26 transformation specific (ETS) families, each of which has been implicated in CRC progression (Davison et al. 2017; Friedrich et al. 2017). Furthermore, many of these transcription factors were also identified by another research group as being differentially expressed after co-culture of colonic epithelial cells with gut bacteria (Yanai et al. 2012). Taken together, these studies suggest that microbes alter the chromatin structure in specific regions, and that these changes impact on CRC genes' deregulated expression.

Novel histone modifications have also been associated with gut microbiota. Histone crotonylation is the addition of crotonyl groups to a lysine residue of a histone subunit. Crotonylation on lysine 18 of the histone subunit H3 (H3K18cr) is a common histone mark in the colon. Moreover, increased crotonylation at H3K18 is associated with the increased expression of genes that are linked to multiple cancers, including CRC (Fellows et al. 2018). H3K18 crotonylation in the colon decreased in mice treated with antibiotics for three days. This decrease was associated with a concomitant decrease in SCFAs and HDAC2 protein expression. Subsequent experiments showed that the SCFAs butyrate and crotonate promoted H3K18 crotonylation by inhibiting HDACs (Fellows et al. 2018).

MicroRNAs (*miRNAs*): Noncoding RNAs (ncRNAs) are RNA molecules that are transcribed from DNA but not translated into protein. The most commonly studied ncRNAs are the microRNAs (miRNAs), which are approximately 22 nucleotides long. Deregulation of miRNAs has been associated with CRC (Luo et al. 2017). Using NanoString technology to examine the fecal miRNA profile of GF, conventional, and antibiotic-treated mice, Liu et al. showed that the presence of gut microbes was associated with decreased fecal miRNA expression (Liu et al. 2016). Moloney et al. showed that conventional mice produced higher levels of three of the four examined miRNAs (miR-7b, miR-141, and miR-200a) than GF mice. When they utilized an antibiotic-treated rat model, all four miRNAs showed lower levels of expression after 6 weeks of antibiotic treatment. The potential functional consequences of these changes were not examined and are difficult to predict as miR-7b functions as an anti-onco-miRNA (miRNA that inhibits proto-oncogenes) and miR-141 and miR-200a function as onco-miRNA in CRC (Moloney et al. 2018). In Nakata et al.'s study, heat-killed *Bacteroides acidifaciens type A43* and *Lactobacillus johnsonii 129* resulted in an upregulation of a well-studied onco-miRNA, the miR-21-5p. Therefore, molecules derived from these bacteria can directly regulate the expression of this onco-miRNA (Nakata et al. 2017). Paradoxically, both of these bacteria are regarded as probiotic bacteria and not oncogenic, again indicating the need for studies focused on functional outcomes. Yu et al. used global miRNA expression profiling to identify several miRNAs that were downregulated in *F. nucleatum*-rich tumor samples from patients with recurrent colorectal cancer (Yu et al. 2017). A CRC xenograft model has also been used to demonstrate that *F. nucleatum* causes resistance to oxaliplatin and 5-FU by downregulating miR-4802 and miR-18a* (Yu et al. 2017). Gut microbes might interact with colonic epithelial cells miRNAs to modulate CRC progression and that might be used as a model for future investigations.

4.2.3.2 Inflammation

Inflammation has been recognized as a principal oncogenic mechanism (Elinav et al. 2019; Chen et al. 2017; Lucas et al. 2017). More than 150 years ago Virchow made the first connection between inflammation and cancer by observing leukocytes in neoplastic tissues (Virchow 1881). Failure of apoptosis and malignant phenotype may be the end stage of a multistep process initiated by microbial-induced host tissue inflammatory alterations and subsequent cellular proliferation

stimulation. The linkage between chronic inflammation and cancer is underpinned by the relation of 20% of all human cancers to premalignant inflammation (Elinav et al. 2019; Chen et al. 2017; Lucas et al. 2017). *H. Pylori* is a type of bacterium found in the stomach of about two thirds of the world's population and has long been associated with gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma. The *H. pylori*-derived virulence factor CagA (cytotoxin-associated gene A) interacts with host proteins to activate downstream signaling pathways, including the MEK/ERK pathway, the NF-κB pathway, and the β-catenin pathway, activating host inflammatory responses and cell proliferation (Elinav et al. 2019). Striking examples are also patients suffering from inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), which have a high risk of developing colitis-associated CRC with poor prognosis (Elinav et al. 2019; Chen et al. 2017; Lucas et al. 2017). Moreover, inflammatory signatures implicated in colorectal carcinogenesis studies include inflammasome activation and activation of the NF-κB pathway, both of which can occur by changes in the mutational landscape or in response to either microbial stimuli or cytokines (Brennan and Garrett 2016). NF-κB pathway activation mediates production of proinflammatory cytokines like IL-6, which has a pathogenic role in CRC by allowing survival and proliferation of intestinal epithelial cells, especially in colitis-associated cancer. The NF-κB pathway also serves as an important regulator of the genes encoding tumor necrosis factor (TNF) and cyclooxygenase 2 (COX-2), both of which are often highly overexpressed in IBD as well as in colorectal adenomas and adenocarcinomas (Brennan and Garrett 2016). TNF can also promote activation of the NF-κB pathway, driving a feed-forward loop that promotes cell proliferation and survival. COX-2 is an enzyme catalyzing the production of prostaglandins and bio-reactive lipids, which influence both colonic inflammatory state and tumor progression through multiple mechanisms (Brennan and Garrett 2016). Aspirin prevents CRC by inhibiting COX-2, presumably by limiting tumor-promoting inflammation (Elinav et al. 2019). Indeed, a recent long-term study, with 20 years of follow-up data, revealed that people who took aspirin (at least 75 mg) regularly had 40–50% reduction in CRC risk, while a 70% reduction of CRC risk was observed if taken for 5 or more years (Thigpen 2012).

The concept of tumor-elicited inflammation (TEI) supports the fact that even seemingly "non-inflammatory" solid tumors possess the ability to recruit immune cells and upregulate proinflammatory cytokines and growth factors, which further influence tumor progression and metastasis (Elinav et al. 2019). This process may be important for further malignant progression and spread of tumors, as well as for regulation of resistance to anticancer therapies. The inflammatory mediator granulocyte macrophage colony-stimulating factor (GM-CSF) has particularly been demonstrated to be critical in the acceleration of tumor development and in the acquisition of metastatic potential via recruitment of macrophages to premalignant areas (Elinav et al. 2019). Moreover, tumor expression of oncogenic ras gene is thought to be responsible for the upregulation of the proinflammatory cytokine IL-8, which leads to increased tumor size, immune cell infiltration, and angiogenesis in nude mouse models (Elinav et al. 2019). Tumor production of cytokines

recruits myeloid cells to the tumor, which secretes IL-6, activating STAT3 and its subsequent downstream pro-oncogenic signaling in tumor cells. Damaged epithelial junctions in CRC, due to lack of mucin production and decreased cadherin expression, result in a robust "Th17-like" inflammatory response (IL-23 and its downstream cytokines IL-17, IL-22, and IL-6), exacerbating tumor growth and progression. Loss of tumor suppressor p120-catenin, vital to E-cadherin stability and, thus, to epithelial junctional integrity, is linked to disrupted barrier homeostasis and to induction of an influx of immature myeloid cells and activated fibroblasts, which continue to support tumor growth (Elinav et al. 2019). Both oncogenic *F. nucleatum* and *B. fragilis* possess virulence factors, which negatively regulate E-cadherin, activating WNT/β-catenin signaling and driving cell proliferation (Scott et al. 2019).

One commonality across many microbiota interfering with chronic diseases is the mucosal barriers of organs, allowing bacterial metabolites to enter compartments that are not normally in close proximity to microbes. This can trigger a local chronic inflammatory response, due to perpetually injured tissue. So, in IBD and CRC, the underlying mucosal barrier is disrupted, either by genetic defect or by rapidly expanding tumor cells, exposing the colon tissue and local immune cells to large amounts of microbial antigens and their products (Chen et al. 2017). Commensal microbiota induces IL-23, IL-17, IL-22, and IL-6 signaling in colon adenoma mouse models, due to defects in colon barrier integrity, and antibiotic treatment or genetic ablation of IL-23 abrogates tumorigenesis (Grivennikov et al. 2012). IL-18 was shown to downregulate IL-22 during injury to the colon, which allowed an increase in IL-22 signaling, which, if left unchecked, promoted tumorigenesis (Huber et al. 2012). Similarly, inhibition of IL-22 signaling was shown to reduce inflammation and tumor burden in a microbial-driven CRC model (Kirchberger et al. 2013). Antibiotic depletion of commensals results in normalization of colon morphology, increased mucin production, and reduction of infiltrating inflammatory cells (Kosa et al. 2011).

In addition to direct, niche-organ-specific effects, evidence exists that microbe--associated molecular patterns (MAMPs) can induce proinflammatory effects in remote organs via their interactions with host pattern recognition receptors (PRRs), such as toll-like (TLR) and nucleotide-binding oligomerization domain-like (NOD) receptors (NLRs) (Scott et al. 2019). MAMPs are molecular signatures that are highly conserved in whole classes of microbes but are absent from the host. Recognition of each MAMP is performed by specific surface-localized receptors, which are termed as pattern recognition receptors (PRRs). NLRs recognize pathogen-derived molecules and host-derived damage signals. The mammalian NLR family contains a C-terminal leucine-rich repeat domain, a central nucleotide--binding domain, and a N-terminal protein–protein interaction domain composed of a caspase activation and recruitment domain (CARD) or Pyrin domain (Karan 2018; Levy et al. 2014). The group of Jurg Tschopp first demonstrated that NLR family members can form, upon certain stimuli and under tightly regulated conditions, a multi-protein complex termed inflammasome (Karan 2018). The large amount of different endogenous and exogenous stimuli that have subsequently been described

to activate the inflammasome has led Jurg Tschopp to propose a function for inflammasomes as "guardians of the body" (Karan 2018). TLRs are single-pass membrane-spanning receptors usually expressed on macrophages and dendritic cells and recognize structurally conserved molecules derived from microbes. Pertinent to their interaction with gut microbiota and the subsequent cancer progression, Dapito et al. demonstrated that hepatocarcinogenesis depended on the intestinal microbiota and the TLR4 activation in non-bone-marrow-derived resident liver cells for promoting liver cancer, through the expression of the hepatomitogen epiregulin, as well as the prevention of apoptosis, while gut sterilization acted reversely (Dapito et al. 2012).

4.2.3.3 Immunity

Microbiota plays a significant, albeit incompletely defined, role in determining innate and acquired immunity (Rajagopala et al. 2017). Immune system maturation and tolerance development start with microbiota organization at birth and continue at later life through signaling by immune cells receptors and by the acquired immune response guided by microbial flora and its metabolites (Rajagopala et al. 2017). In this context, upregulation of TLRs by microbial lipopolysaccharides (LPs) and other by-products can activate the NF-kB, c-Jun/JNK, and JAK/STAT3 pathways, all of which play a principal role at cell proliferation and immunosuppression (Rajagopala et al. 2017). Among the innate immune cells, macrophages are the most abundant. In the intestine, macrophages, the predominant cells at the innate immunity system express their phagocytic activity via the antibacterial phagocytic receptor TREMC2 (triggering receptor expressed on myeloid cells 2) and produce the anti-inflammatory cytokine IL-10 which contributes to the maintenance of intestinal homeostasis (Lucas et al. 2017). Neutrophils also play a major role in innate immunity by stimulating the adaptive immune responses via the production of immunoglobulin-A (IgA) (Lucas et al. 2017). Activated by locally acting cytokines innate lymphoid cells, and specifically the type 3 innate lymphoid cells (ILC3), which are activated by IL-1, IL-6, and IL-23, are producers of effector cytokines such as IL-17 and IL-22, and require the presence of commensal bacteria for their development (Lucas et al. 2017). When being activated, ILC3 have also the ability to induce the production of mucus and antimicrobial peptides (AMPs) by the epithelium. Moreover, ILC3 have a direct impact on adaptive immune response through the production of GM-CSF production, which, as a consequence of the detection of commensal bacteria and the production of IL-1 by stimulated macrophages, leads to the generation of Tregs (Elinav et al. 2019). Dendritic cells are key regulators of adaptive immune responses by recruiting and activating naïve T cells by inducing T cell receptors (Gopalakrishnan et al. 2018a; Wong et al. 2019; Lucas et al. 2017). One subpopulation of dendritic cells is predominant in Peyer's patches, key site of microbiota-induced immune responses, and can promote Tregs production, while the other subpopulation seems to have proinflammatory properties by promoting T cell repertoire (Lucas et al. 2017).

Peyer's patches and isolated lymphoid follicles are the major sites for adaptive immune responses. These two sites are enriched in microfold cells (M cells), which allow the translocation of bacteria that can be captured by dendritic cells and

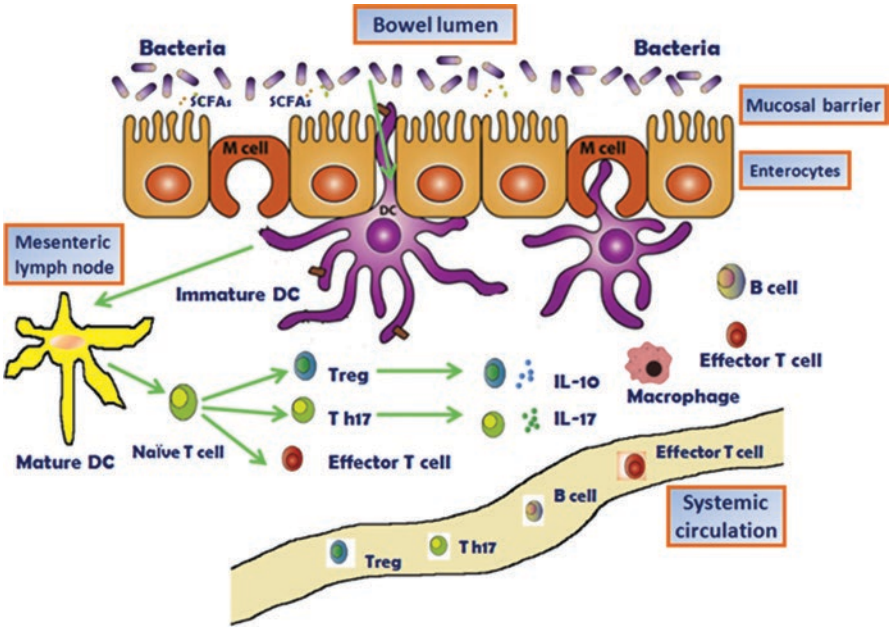


Fig. 4.3 Gut microbiota-driven immunomodulatory mechanisms linked to carcinogenesis

presented to naïve T cells (Lucas et al. 2017). So, activation of local dendritic cells by bacterial metabolites, like SCFAs, or bacteria, themselves, leads to their maturation and their migration to mesenteric lymph nodes (Gopalakrishnan et al. 2018a; Wong et al. 2019). Mature dendritic cells activate naive T cells to differentiate into effector T cells, Tregs or Th17 cells, which can migrate back into the intestinal mucosa or into the systemic circulation. For local immune responses, Tregs secrete IL-10 and act to produce a local anti-inflammatory cytokine environment. Cytokine secretion from Th17 cells including IL-17 induces intraepithelial cells to develop tight junctions and secrete antimicrobial proteins, while IL-17 can further lead to the release of other inflammatory cytokines. Systemic immune responses can also be shaped by microbiome-mediated immune cell priming. When dendritic cells present antigens from commensal bacteria in the lymph nodes of the intestine, Ig-A producing B cells and T cells, including Tregs and Th17 cells, can enter the systemic circulation and promote immune responses against distant identical antigens, or against other antigens by cross-reacting with similar epitopes (Gopalakrishnan et al. 2018a; Wong et al. 2019) (Fig. 4.3). It can be presumably assumed that this complex microbiota-immune system crosstalk facilitates the maintenance of a basic health-associated anticancer immune-surveillance, which is deregulated at cancer--associated dysbiotic states.

4.2.3.4 Metabolism

The metabolism of dietary vitamins and nutrients as well as host-derived compounds is largely influenced by genes that abound the human microbiome. There is

extensive experimental evidence that the products of fiber fermentation, in particular butyrate, have anti-inflammatory and anti-neoplastic properties, while the products of bacterial bile acid conjugation, i.e., the secondary bile acids, have carcinogenic role (O'Keefe et al. 2015; Ríos-Covián et al. 2016). In line with this evidence, suggested counter-competing mechanisms for diet-associated cancer risk may include the protective effect of dietary fiber in increasing butyrogenesis, and, on the other hand, the promotional effect of dietary fat on stimulating bile acid synthesis by the liver (O'Keefe et al. 2015; Ríos-Covián et al. 2016). Indeed, O'Keefe et al. showed the anticipated increase in saccharolytic fermentation and butyrogenesis, the suppression of secondary bile acid synthesis, and the associated significant reduction in colonic mucosal inflammation and proliferation biomarkers of cancer risk by switching African Americans to a high-fiber, low-fat diet; the opposite effect was observed when rural African's diet was switched to a high-fat, low-fiber diet (O'Keefe et al. 2015). The substantial xenometabolic role of gut microbiota is stressed by their capability to form ultimately carcinogenic end products, such as acetaldehyde from alcohol (Seitz and Stickel 2007). Although alcohol is not carcinogenic, the first metabolite of ethanol oxidation, i.e., acetaldehyde, is highly toxic, mutagenic, and carcinogenic. In addition to somatic cells, normal human microbial flora is also able to produce acetaldehyde from ethanol. Ingestion of alcoholic beverages results in high local acetaldehyde concentrations in the saliva, gastric juice, and the contents of the large intestine. In addition, microbes may produce acetaldehyde endogenously without alcohol administration. The first findings of microbial ethanol metabolism were reported as early as 1940 when Still showed that *E. coli* possesses alcohol dehydrogenase (ADH) activity (Still 1940). Later on, it was established that there are considerable differences in the ADH activity and acetaldehyde-producing capacities of the aerobic and facultative anaerobic bacteria representing the normal human colonic flora (Jokelainen et al. 1996). There is a clear association between chronic alcohol consumption and the development of cancers of the upper gastrointestinal tract, the liver, the large bowel, and the female breast (Seitz and Stickel 2007). Acetaldehyde is mainly responsible for the carcinogenic effect of ethanol on the upper aerodigestive tract owing to its multiple mutagenic effects on DNA (Seitz and Stickel 2007).

4.2.3.5 Autophagy

Autophagy is a lysosome-dependent degradative process that targets intracellular components, such as damaged organelles, misfolded proteins, toxic aggregates, and intracellular pathogens, into double-membraned vesicles known as autophagosomes, which fuse with lysosomes to form auto-lysosomes, where the contents are degraded (Rajagopala et al. 2017). Autophagy has a complex and tissue-dependent role in carcinogenesis. Autophagy serves as a surveillance mechanism that protects normal cells from the transformation to malignancy by removing damaged organelles and aggregated proteins and by reducing damaged mitochondria, ROS, and DNA damage. Many bacteria have evolved mechanisms to prevent degradation by autophagy, including *H. pylori*. Prolonged exposure to *H. pylori* protein VacA prevents autophagosome maturation, and the bacteria are able to persist in these

compartments (Greenfield and Jones 2013). This promotes an environment that favors carcinogenesis by the accumulation of damaged organelles and protein aggregates, persistent *H. pylori* infection, and chronic inflammation. The effect of autophagy on carcinogenesis appears to be mediated through the microbiome. In the pancreas and lung, inhibition of autophagy predisposes the tissue to lesions (Lévy et al. 2015). However, in models of CRS, the inhibition of autophagy prevents the development of precancerous lesions (Lévy et al. 2015). Lucas et al. recently showed that infection of human intestinal epithelial cells (IECs) and susceptible mice with colibactin-producing *E. coli* promotes autophagy, which is required to prevent colorectal tumorigenesis. Loss of ATG16L1 (a marker of autophagy) from IECs increased markers of inflammation, DNA damage, and cell proliferation as well as colorectal tumorigenesis in the mice (Lucas et al. 2020).

4.3 Gut Microbiota and CRC

4.3.1 Normal Colon Microbiota

The large intestine is the main colonization niche in the human body. It is estimated that the colon houses about 10^{14} microbial cells, most of them are bacteria. *Bacteroidetes* and *Firmicutes* are the dominant phyla in the large intestine, followed by *Actinobacteria* and *Verrucomicrobia.* The phylum *Proteobacteria* is also present, but to a lesser extent (Nistal et al. 2015; Hollister et al. 2014; Tlaskalová--Hogenová et al. 2004). Factors that facilitate bacterial growth in the colon are the increased, almost neutral, pH and the slow colonic transit time, which provides microorganisms with the opportunity to proliferate and ferment available substrates derived from diet or endogenous secretions (Nistal et al. 2015). Due to the reductive, devoid of oxygen, colonic environment, most microbiota are strictly anaerobic, such as the ones of the *Bacteroides* genus, which is one of the most abundant (Tlaskalová-Hogenová et al. 2004). Gram-positive non-spore-forming microorganisms such as *Eubacterium, Bifidobacterium, Peptostreptococcus,* and *Ruminococcus* are also dominant (Tlaskalová-Hogenová et al. 2004). Spore-forming Gram-positive bacilli are mainly represented by the genus *Clostridium*. To a lesser extent, facultative anaerobes ones such as enterobacteria, enterococci, lactobacilli, and streptococci appear in the large intestine. Differences were observed in the composition between the microbiota that is present in the intestinal lumen and the one associated with the mucosa, but their biological significance is still unclear (Nistal et al. 2015).

4.3.2 Gut Dysbiosis and CRC Pathogenesis

CRC is the third most common cancer in both males and females with about 1.36 million of new cases per year and the fourth leading cause of cancer-related deaths worldwide with 700,000 deaths per year (Stimpfel and Virant-Klun 2016). Progression to CRC is a multistep process following the adenoma-carcinoma

sequence, which has a background of genomic instability. Several molecular features are common to sporadic colorectal cancers, including microsatellite instability (MSI), chromosomal instability (CIN), and epigenetic silencing through the CpG island methylator phenotype (CIMP) (Chen et al. 2017; Beaugerie and Itzkowitz 2015). The initial formation of regions of polyps occurs in response to the loss of tumor suppressor genes like APC (adenomatous polyposis coli), a component of the Wnt/β-catenin pathway that is important for controlling cell proliferation. In addition, mutations in genes that encode the machinery for DNA repair, such as hMSH2, lead to MSI and can also contribute to colorectal tumorigenesis. Apart from their common occurrence at sporadic CRC, the APC loss and the mismatch repair genes (MMR) mutations can be inherited, as in familial adenomatous polyposis (FAP) and the Lynch syndrome, respectively. Hereditary CRC types account for approximately 5–10% of all cases of CRC. Furthermore, the development of dysplasia and CRC is strongly influenced by the inflammatory state of the colon. In patients with IBD, chronic, severe inflammation of the colon increases the likelihood of developing CRC (Beaugerie and Itzkowitz 2015). More subtle inflammation in otherwise healthy colonic tissues plays a major role in the conversion of a healthy colon to a dysplastic colon, as well. As crypts become dysplastic, the barriers between the epithelium and the microbiota begin to break down. Barrier disruption facilitates the bacterial translocation and, ultimately, already described in detail, exposure of immunogenic microbial compounds to both epithelial cells and antigen-presenting cells (see Sects. 4.2.3.2 and 4.2.3.3 under the Sect. 4.2.3).

An abundance of experimental and clinical human studies pinpoints the strong relationship of gut microbiota to CRC development and progression (Collins et al. 2011; Lucas et al. 2017; Brennan and Garrett 2016; Nistal et al. 2015; Saus et al. 2019). Animal experimental models used to empower that intimate association include the $Apc^{Min/+}$ model, in which mice bear a point mutation in one copy of the APC tumor suppressor gene, spontaneously forms adenomas along the intestinal tract and the IL10-deficient mice, which develop spontaneous colitis and when treated with the carcinogen azoxymethane (AOM), they develop tumors that resemble the pathology seen in colitis-associated CRC (Brennan and Garrett 2016). Indeed, early data coming from the $APC^{Min/+}$ genetic mouse model showed that when mice were housed in GF conditions developed less intestinal tumor compared with those in conventional conditions (Dove et al. 1997). Furthermore, transfer of stool from CRC patients to two different mice models promoted carcinogenesis (Wong et al. 2017a). Wong et al. fed stool samples from patients with CRC and heathy individuals to GF mice and conventional mice with AOM; they found that stool from patients with CRC increased the numbers of polyps, levels of intestinal dysplasia and proliferation, markers of inflammation, and proportions of Th1 and Th17 cells in colon, compared with stool from individuals without CRC (Wong et al. 2017a). As a result, fecal microbiota from patients with CRC can promote tumorigenesis in GF mice and mice given a carcinogen. Recently, it was also shown that GF $APC^{Min/+}$ $/IL10^{-/-}$ mice exhibit almost no tumor compared to conventionalized $APC^{Min/+}$ $/IL10^{-/-}$mice, indicating the primordial role of the gut microbiota in inflammation-induced CRC (Tomkovich et al. 2017). Gnotobiotic studies revealed

that while *F. nucleatum* clinical isolates with FadA and Fap2 adhesins failed to induce inflammation and tumorigenesis, *pks*$^{+}$ *Escherichia coli* promoted tumorigenesis in the *Apc*$^{Min/+}$ */IL10*$^{-/-}$model in a colibactin-dependent manner, suggesting colibactin is a driver of carcinogenesis (Tomkovich et al. 2017). Using the AOM/Dextran Sodium Sulfate (DSS) mouse model of colitis-induced CRC, Zackular et al. showed a shift in fecal microbiota composition with a significant decrease in the diversity following the first round of DSS treatment, which was expressed with increment of *Bacteroides* and decrement of *Prevotella* (Zackular et al. 2013). However, following the third round of DSS treatment, a significant decrease in *Bacteroides* and *Porphyromonadaceae* was found, which had also been observed in IBD patients (Zackular et al. 2013). The authors proposed that these species could have a protective role as the anti-inflammatory mediators in the gut. When they conventionalized GF mice with either the healthy microbiota of untreated mice or the microbiota of tumor-bearing AOM/DSS-treated mice, the mice that had been conventionalized with tumor-bearing mice-associated microbiota exhibited more tumors and decreased gut microbiota diversity compared to those conventionalized with the healthy microbiota (Zackular et al. 2013). Analyses of the diversity and richness of the intestinal lumen microbiota have also been performed via the analysis of the feces in an animal model of CRC induced by the carcinogenic agent 1,2-dimethylhydrazine (Zhu et al. 2014). An increase in *Bacteroides* and *Proteobacteria* in the lumen of CRC rats was observed compared to healthy rats. A reduction of butyrate-producing bacteria such as *Roseburia* and *Eubacterium* in the gut microbiota of CRC rats was also detected (Zhu et al. 2014).

Several human studies have demonstrated a link between alterations of gut microbiota and CRC. In a pioneering study in 1995, fifteen bacterial taxa from the human fecal flora were significantly associated with a high risk of colon cancer, and five were significantly associated with a low risk of colon cancer (Moore and Moore 1995). Total concentrations of *Bacteroides* species and, surprisingly, *Bifidobacterium* species were generally positively associated with an increased risk of colon cancer. Some *Lactobacillus species* and *Eubacterium aerofaciens*, which also produce major amounts of lactic acid, showed closest associations with low risk of colon cancer (Moore and Moore 1995). Chen et al. utilized pyrosequencing-based analysis of 16S rRNA genes to determine the overall structure of microbiota in patients with CRC and healthy controls; their findings indicated that the microbial structure of the intestinal lumen and cancerous tissue differed significantly (Chen et al. 2012). Phylotypes that enhance energy harvest from diets or perform metabolic exchange with the host were more abundant in the lumen, with more abundant *Firmicutes* and less abundant *Bacteroidetes* and *Proteobacteria* to be revealed. Moreover, tumor microbiota exhibited lower diversity and the structures of the intestinal lumen microbiota and mucosa-adherent microbiota were different in CRC patients compared to matched microbiota in healthy individuals. *Lactobacillales* were enriched in cancerous tissue, whereas *Faecalibacterium* was reduced (Chen et al. 2012). In the mucosa-adherent microbiota, *Bifidobacterium, Faecalibacterium,* and *Blautia* were reduced in CRC patients, whereas *Fusobacterium, Porphyromonas, Peptostreptococcus,* and *Mogibacterium* were enriched. In the lumen, predominant

phylotypes related to metabolic disorders or metabolic exchange with the host, *Erysipelotrichaceae, Prevotellaceae,* and *Coriobacteriaceae,* were increased in CRC patients (Chen et al. 2012). More recently, Goa et al. showed that the predominant phylum in CRC patients is the *Firmicutes*, whereas the *Proteobacteria* is the leading phylum in healthy individuals. In addition, a relatively higher abundance of *Lactococcus* and *Fusobacterium* and lower abundance of *Pseudomonas* and *Escherichia* and *Shigella* was observed in cancerous tissues compared to adjacent noncancerous tissues (Gao et al. 2015). Additional pyrosequencing data of CRC-associated gut microbiota revealed over-representation of some bacteria such as *Bacteroides, Prevotella, Faecalibacterium,* and *Fusobacterium* (Wang et al. 2011). However, varying results have been derived depending on the analysis techniques and sample localization. Indeed, Sobhani et al. showed that *Bacteroides* are over-represented in CRC patients' tissues compared to normal tissues from control subjects. In the stool samples, though, the same researchers showed a significant increase of *Bacteroides* and *Prevotella* in CRC samples compared to healthy subjects' samples (Sobhani et al. 2011). When focusing on early-stage CRC, studies have shown an increase of *Proteobacteria* and *Fusobacteria* and a decrease of *Bacteroides* in normal mucosa from CRC patients compared to control subjects (Shen et al. 2010; Mccoy et al. 2013). At species levels, *B. fragilis, E. coli, Streptococcus bovis/gallolyticus, Enterococcus faecalis,* and *F. nucleatum* are increased in the fecal samples from CRC patients, while *Bacteroides vulgatus* and *Faecalibacterium prausnitzii* are decreased when compared to fecal samples from healthy volunteers (Wu et al. 2013). Viljoen et al. reported a significant increase in *Fusobacterium* in tumor samples compared to nontumoral adjacent mucosa, as well as the association of this phenomenon with the late stages of CRC (Viljoen et al. 2015). Gut microbiota over- and under-represented in CRC are demonstrated in Figs. 4.4 and 4.5, respectively.

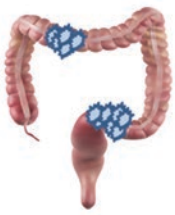
4.3.3 Role of Specific Bacteria in CRC Progression

4.3.3.1 *E. coli*

E. coli is a Gram-negative, aero-anaerobic, commensal bacterium that colonizes the human gut soon after birth (Lucas et al. 2017). Particular strains belonging to *E. coli* have been identified as a potential risk factor for CRC. The species *E. coli* can be divided into four phylotypes (A, B1, B2, and D) (Lucas et al. 2017; Wassenaar 2018). Commensal *E. coli* strains frequently belong to phylotype A, while phylotype B2 strains are more frequent carriers of virulence genes compared to the other phylotypes, and often cause extraintestinal infections (Wassenaar 2018). Cancer-inducing properties of *E. coli* strains belonging to B2 have been demonstrated, and observations providing mechanistic evidence have been accumulated and analyzed in the following paragraphs. Nevertheless, there is not an absolute agreement between researchers that *E. coli* is implicated in causing CRC, when considering this particular microbiota in the ecological environment of the human gut where they reside. In a holistic view, *E. coli* strains may not be responsible for CRC cases

PHYLA Genus	Species	Mechanism
ACTINOBACTERIA		
Collinsella	---	
Slackia	---	Anti-oxidant potential
BACTEROIDETES		
Alistipes	*A. finegoldii*	Inflammatory
Bacteroides	*B. fragilis*	Inflammatory, enterotoxigenic (fragilisin)
Porphyromonas	*P. asaccharolytica*	Inflammatory
Prevotella	*P. intermedia*	Inflammatory
EURYARCHAEOTA		
Methanobrevibacter		Methane producer

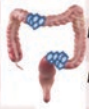
PHYLA Genus	Species	Mechanism
PROTEOBACTERIA		
Escherichia	---	Genotoxin (colibactin), DNA mismatch repair, DNA damage checkpoint
Helicobacter	*H. pylori*	Inflammatory
Klebsiella	---	---
SYNERGISTETES		
Thermanaerovibrio	*T. acidaminovorans*	---
VERRUCOMICROBIA		---
Akkermansia	*A. muciniphila*	Immune modulatory (involved in PD-1 blockade efficacy)



PHYLA Genus	Species	Mechanism
FIRMICUITES		
Enterococcus	*E. faecalis*	Inflammatory, oxidative stress
Gemella	---	---
Mogibacterium	---	---
Parvimonas	*P. micra*	Inflammatory, Immune response
Peptostreptococcus	*P. Stomatis* *P. Anaerobious*	Oxidative stress
Solobacterium	*S. moorei*	---
Streptococcus	*S. gallolyticus*	Inflammatory
FUSOBACTERIA		
Fusobacterium	*F. nucleatum*	Inflammatory, butyrate producer

Fig. 4.4 Microbiota over-represented at colorectal cancer

PHYLA Genus	Species	Mechanism
ACTINOBACTERIA		
Bifidobacterium	Several	Immune modulatory, anti-inflammatory, butyrate production
BACTEROIDETES		
Bacteroides	*B. Vulgatus* *B. uniformin*	Inflammatory
FIRMICUITES		
Anaerostipes	---	Butyrate producer
Clostridium	*C. butyricum*	Secondary bile acids producer, apoptosis of CRC cells, inhibition of tumorigenesis
Eubacterium	*E. ventriosum*	Inflammatory, butyrate producer, DNA damage
Faecalibacterium	*F. prausnitzii*	Anti-inflammatory, butyrate producer



PHYLA Genus	Species	Mechanism
FIRMICUITES		
Lactobacillus	---	Immune modulatory (activation T- cells), mucus barrier maintenance
Roseburia	---	Anti-inflammatory, butyrate producer
Ruminococcus	*R. gnavus*	SCFA producer, secondary bile acid producer
PROTEOBACTERIA		
Citrobacter	---	Inflammatory
Cronobacter	---	Inflammatory
Kluyvera	---	Inflammatory
Salmonella	---	Inflammatory
Serratia	---	Inflammatory

Fig. 4.5 Microbiota depleted at colorectal cancer

in which their presence is observed and their isolation may be just co-incidental and not necessarily strictly causative (Wassenaar 2018).

Repeated observations that *E. coli* are frequently found to colonize CRC lesions and neighboring epithelium, often in large numbers and, sometimes, as the only cultivable microbiota, were derived from previous studies (Swidsinski et al. 1998;

Martin et al. 2004; Raisch 2014). In the first polymerase chain reaction (PCR) study, performed more than twenty years ago, Swidsinski et al. demonstrated *E. coli* strains in 90% and 93% of patients with adenomas and carcinomas respectively, whereas only 3% of colonic biopsies from asymptomatic control subjects were positive for *E. coli*. Subsequent investigations proved the presence of invasive *E. coli* in biopsies from 71% patients with Crohn's disease, 57% with CRC, 48% with UC, and 29% controls, while its detection rate was at least 3 times higher in CRC compared to diverticulosis cases (Martin et al. 2004; Raisch 2014). When particular *E. coli* strains of the B2 phylotype are incubated in vitro with various epithelial cell lines, they cause cell elongation, cell cycle arrest, and they render them to a state of senescence (Wassenaar 2018). These effects are due to a group of compounds collectively named cyclomodulins, which introduce double-strand DNA breaks in the target cells (Wassenaar 2018; Nougayrede 2006). The following cyclomodulins are produced by *E. coli*: (1) the cytolethal distending toxin (CDT), which is encoded by the cdtA, cdtB, and cdtC genes, was first identified in 1988 in the culture of *E. coli* strains isolated from patients with diarrhea and acts via its DNAse activity inducing DNA double-strand breaks, cell cycle arrest, and cell apoptosis if the DNA double--strand breaks exceed the repair capacity of the cell, (2) the cycle-inhibiting factor (CIF) encoded by the cif gene, (3) the cytotoxic necrotizing factor (CNF), which is encoded by the cnf1 gene and acts via deamination of Rho-GTPase resulting in actin cytoskeleton activation and multinucleation, (4) the intimin-dependent attachment, which is encoded by the eae and the type III secretion system and it downregulates the DNA mismatch repair system, resulting in DNA strand breaks, and (5) the colibactin, which is first described in 2006 by Nougayrede et al. and is a hybrid polyketide-non ribosomal peptide compound produced by a complex biosynthetic machinery encoded by the pks island (Wassenaar 2018; Nougayrede 2006) (see Sect. 4.2.3.1 under the Sect. 4.2.3). The cytotoxic phenotype is overrepresented in *E. coli* isolated from CRC patients. For instance, 26 cyclomodulin-positive *E. coli* strains, as defined by PCR detection of pks-specific genes, were isolated from 50 biopsies from CRC patients, compared to 17 cyclomodulin-negative stains in the study of Bonnet et al. (2013).

Colibactin was shown to induce double-strand DNA breaks in mammalian cells. In the first publication describing the cyclomodulin effect of colibactin, it was shown that direct contact between bacteria and the target cells was required and that the bacteria need to be alive for the toxic effect (Nougayrede 2006). The required contact between bacteria and cells was confirmed in a second publication by Buc et al. (2013). Colibactin is most likely a combination of hybrid molecules containing both a peptide and a polyketide. The pks locus responsible for its biosynthesis was first characterized in 2007 from probiotic *E. coli Nissle 1917 (EcN)* (Homburg et al. 2007). This pks locus is present on a 54-kb long genomic island that contains at least 18 genes; all genes, except one, i.e., the clbM, are required for the active expression of colibactin, while the genes clbB and clbN can be used as markers for presence of the complete pks island (Wassenaar 2018; Nougayrede 2006). In 2015, Vizcaino and Crawford were successful in purifying a pre-colibactin compound and showed that the pre-colibactin is able to induce in vitro DNA crosslink but not DNA

double-strand breaks (Vizcaino and Crawford 2015). The authors, thus, hypothesized that DNA double-strand breaks may not be induced directly by colibactin but rather as a response of infected mammalian cells to repair their DNA (Vizcaino and Crawford 2015). Since colibactin had not been isolated or structurally characterized, until recently, studying the physiological effects of colibactin-producing bacteria in the human gut had been difficult. Xue et al. used a combination of genetics, isotope labeling, tandem mass spectrometry, and chemical synthesis to deduce the structure of colibactin (Xue et al. 2019). Their structural assignment accounted for all known biosynthetic and cell biology data and suggested roles for the final unaccounted enzymes in the colibactin gene cluster.

In vivo models show that *pks* + *E. coli* strains can induce CRC (Arthur et al. 2014; Cougnoux et al. 2014; Dalmasso et al. 2014). So, the carcinogenic effect of the *pks locus-harboring E. coli strain NC101* was demonstrated in an AOM-treated IL-10 double knockout (*IL10−/−*) mouse colitis-induced CRC model (Arthur et al. 2014). Another mouse model was used to test the carcinogenic properties of strain *E. coli 11G5*, a B2 strain obtained from a human CRC biopsy (Bonnet et al. 2013). A percentage of 92% of *APC−/−* animals colonized with the *E. coli strain 11G5* developed colonic polyps in contrast to the wild-type mice that did not develop neoplasia, despite being colonized with high levels of *E. coli 11G5* (Arthur et al. 2014).

In the murine model of Cougnoux et al., the subcutaneous injection of tumor cells infected with *E. coli* expressing colibactin from a pks-containing bacterial artificial chromosome (pBAC) caused the development of tumors in both the control group (*E. coli* without pks) and in the treatment group, but in the latter the tumors were larger (Cougnoux et al. 2014). Reversely, at high multiplicity of infection (MOI), *E. coli* strains expressing pks can actually suppress the proliferation of tumor cells, at least in a murine model using xenografts of *E. coli*-infected HC116 cells, as the one used by Dalmasso et al. (2014). This tumor-suppressing effect was observed with an MOI of 100, while at an MOI of 20, tumor growth was accelerated by *pks* + *E. coli*. That cells treated with *pks* + *E. coli* produced a variety of cytokines or growth factors in vitro, as was demonstrated by intramuscular injection of the cell culture supernatant in mice (Dalmasso et al. 2014).

4.3.3.2 *B. fragilis*

B. fragilis is a strict anaerobe commonly colonizing the human colon (Lucas et al. 2017). Among the two known subtypes of *E. fragilis*, i.e., the nontoxigenic *B. fragilis (NTBF)* and the enterotoxigenic *B. fragilis (ETBF)*, the *ETBF* contains a pathogenic island, called the *B. fragilis* pathogenicity island (BFPAI), which allows the production of an enterotoxin called "fragilysin" or BFT, encoded by the bft gene (Sears 2001). The first study demonstrating an increased prevalence of *ETBF* in CRC patients was the one by Toprak et al. in 2006, when the enterotoxin gene (bft) was detected by PCR in 38% of the isolates from CRC patients, compared to 12% of the ones from the control group (Wexler 2009). BFT has proteolytic activity and is responsible for the degradation of tight junction proteins, such as zonula occludens-1, leading to intestinal epithelial barrier failure and enhanced epithelial

permeability (Riegler et al. 1999). Additionally, BFT rapidly cleaves the extracellular domain of E-cadherin, leading to the complete degradation of the E-cadherin protein. E-cadherin is the primary intercellular adhesion protein of the zonula adherens, and its cytoplasmic domain associates with the nuclear signaling protein beta--catenin. Loss of the membrane-associated E-cadherin after BFT treatment of human colonic epithelial cells triggered beta-catenin nuclear localization with subsequent c-myc transcription and translation, inducing persistent cellular proliferation which was mediated in part by beta-catenin/T cell factor-dependent transcriptional activation (Wu et al. 2003). These results suggest that genetic evolution of this common colonic commensal bacterium has rendered an organism with the potential to contribute to oncogenic transformation of the colon. A consequential study showed that *ETBF* triggers colitis and strongly induced colonic tumors in multiple intestinal neoplasia (Min) mice. *ETBF* provoked a robust, selective colonic signal transducer and activator of transcription-3 (STAT-3) activation with the colitis to be characterized by a selective Th17 response (Wu et al. 2009). Antibody-mediated blockade of IL-17, as well as the receptor for IL-23, a key cytokine amplifying Th17 responses, inhibited *ETBF*-induced colitis, colonic hyperplasia, and tumor formation. These results show a Stat3- and Th17-dependent pathway for inflammation-induced cancer by a common human commensal bacterium, i.e., *ETBF*, providing mechanistic insights into human colon carcinogenesis (Wu et al. 2009). In a recently published retrospective analysis of more than 13,000 patients from Hong-Kong hospitalized for bacteremia, the authors associated later diagnosis of CRC with *B. fragilis* and *S. gallolyticus* and other intestinal microbes. These bacteria might have had entered the bloodstream from intestinal dysbiosis and perturbed barrier function. These findings further supported the model in which specific members of the intestinal microbiota promote colorectal carcinogenesis (Kwong et al. 2018).

4.3.3.3 *F. nucleatum*

Among the putative bacteria, *F. nucleatum* is one that has been extensively studied in CRC (Tjalsma et al. 2012; Castellarin et al. 2011; Kostic et al. 2013; Rubinstein et al. 2013; Yu et al. 2017; Lucas et al. 2017; Brennan and Garrett 2018). *F. nucleatum* is a Gram-negative, strictly anaerobic oral commensal and periodontal pathogen associated with diverse diseases (Lucas et al. 2017; Brennan and Garrett 2018). Independent studies have identified *F. nucleatum* to be more abundant in cancer tissues. Its prevalence is enhanced in mucosa from patients with CRC compared to control subjects (Mccoy et al. 2013). It is also detected in higher proportion in CRC tumors compared to adjacent normal tissues (Abed et al. 2016). Moreover, Castellarin et al. verified overabundance of *Fusobacterium* sequences in tumor versus matched normal control tissue by quantitative PCR analysis from a total of 99 subjects (Castellarin et al. 2011). A replication study by Repass et al., though, questioned those results; when measuring *F. nucleatum* DNA by qPCR in CRC, adjacent normal tissue, and separate matched control tissue, they did not detect a signal for *F. nucleatum* in most samples and only 25% of CRCs, 15% of adjacent normal, and 0% of matched control tissue were positive (Pepper 2008). In addition, when only samples with detectable *F. nucleatum* in CRC and adjacent normal tissue were

compared, the difference was not statistically significant, as had noted by Castellarin et al. (Pepper 2008).

In the study by Yang et al., *F. nucleatum* increased proliferation and invasive activities of CRC cell lines compared with control cells (Balamurugan et al. 2008). CRC cell lines infected with *F. nucleatum* formed larger tumors, more rapidly, in nude mice than uninfected cells. $APC^{min/+}$ mice gavaged with *F. nucleatum* developed significantly more colorectal tumors and had shorter survival times. Several inflammatory factors were significantly increased in serum from mice given *F. nucleatum*, while 50 miRNAs were upregulated and 52 miRNAs were downregulated in CRCs incubated with *F. nucleatum*. Infection of cells with *F. nucleatum* increased expression of miR21 by activating Toll-like receptor (TLR) 4 signaling, leading to activation of the NF-κB. Levels of *F. nucleatum* DNA and miR21 were increased in tumor tissues compared with nontumor colon tissues from patients. Patients whose tumors had high amounts of *F. nucleatum* DNA and miR21 had shorter survival times than patients whose tumors had lower amounts (Balamurugan et al. 2008).

In a study conducted by Kostic et al., $APC^{min/+}$ mice infected with *F. nucleatum* exhibited enhanced proportion of myeloid-derived suppressor cells, which had a tumor permissive role, an increased tumor-associated neutrophils, and an enrichment of tumor-associated carcinogenesis-promoting macrophages, and an increase in antitumor dendritic cells (Kostic et al. 2013). *F. nucleatum* may not only impact the tumor microenvironment but has also a more direct impact on the tumor. Accumulating evidence suggests that *F. nucleatum* can increase cell proliferation in cancer cells themselves. First, the binding of FadA, which is a specific *F. nucleatum* adhesin, to E-cadherin drives activation of the β-catenin and Wnt pathway (Rubinstein et al. 2013). Once the tumor has developed, *F. nucleatum* can localize to the Gal-GalNAc-expressing tumor cells through binding of its Fap2 lectin, which results in enrichment of *F. nucleatum* (Abed et al. 2016). Actually, if it is assumed that tumoral *F. nucleatum* originates in the oral cavity, then *F. nucleatum* must first migrate to dysplastic tissues to exert its effect on tumorigenesis. CRC tissues over-express a specific sugar residue, Gal-GalNAc67, which can be recognized by the fusobacterial adhesin Fap2, which also mediates co-aggregation and hemagglutination functions. A study using an orthotopic graft model showed that *F. nucleatum* localized to colorectal tumors in an Fap2-dependent manner via a hematogenous route, which mimics the transient bacteremia that can occur after flossing or dental procedures (Pepper 2008). However, *F. nucleatum* has been found in CRC tissues at early stages of tumorigenesis before Gal-GalNAc over-expression, suggesting that there may be multiple routes by which localization to the developing tumor microenvironment occurs.

4.3.3.4 *Enterococcus faecalis*

Enterococcus faecalis (E. faecalis), a Gram-positive facultative anaerobic bacterium, is normally found in the human colonic ecosystem (Lucas et al. 2017). Real-time polymerase chain reaction using primers aimed at 16S rDNA to quantitate bacterial species demonstrates that extracellular superoxide-producing *E. faecalis*

populations were considerably higher in CRC patients compared to healthy volunteers, while the butyrate-producing *Eubacterium rectale* and *Faecalibacterium prausnitzii* were decreased approximately fourfold (Balamurugan et al. 2008). Moreover, GF IL-10 knockout mice developed IBD after they were colonized with a pure culture of *E. faecalis*, which not only induced IBD, but also rectal dysplasia and CRC (Balish and Warner 2002). Additionally, *E. faecalis*-monoassociated *IL-10−/−* but not wild-type mice lack the protective TGF-beta/Smad signaling and fail to inhibit TLR2-mediated proinflammatory gene expression in the intestinal epithelium (Ruiz et al. 2005). Except for inducing chronic inflammation, *E. faecalis* produces extracellular superoxide and hydrogen peroxide, which leads to luminal colonic cells' DNA damage of rats (Huycke et al. 2002). Wang et al. proved that macrophage COX-2 is induced by superoxide from *E. faecalis* and promotes chromosomal instability in mammalian cells through diffusible factors (Wang and Huycke 2007).

4.3.3.5 *Streptococcus bovis/gallolyticus*

Streptococcus bovis (S. bovis) was first associated with CRC in 1951 (Khan et al. 2018). It has long before been suggested that all patients with *S. bovis* septicemia need aggressive evaluation of the gastrointestinal tract, especially the colon for exclusion of neoplastic lesions (Klein 1979). In 1977, Klein et al. found that the prevalence of *S. bovis* in fecal cultures from patients with CRC was significantly increased compared to that in controls (Klein et al. 1977). The study of Abdulamir et al. indicated that CRC is remarkably associated with *Streptococcus gallolyticus* member bacteria (SGMB); moreover, molecular detection of SGMB in CRC was superior to link SGMB with CRC tumors highlighting a possible direct and active role of SGMB in CRC development through most probably inflammation-based tumor propagation via IL-1, cyclo-oxygenase-2 (COX-2), and IL-8 (Abdulamir et al. 2010).

4.3.3.6 *Clostridium septicum*

Clostridium septicum (*C. septicum*) is an aerotolerant, Gram-positive, spore--forming bacillus not usually present in the normal intestinal flora of humans. *C. septicum* produces a hemolytic α-toxin, which is lethal (Lucas et al. 2017). *C. septicum* infections have a strong association with malignancy (Klein et al. 1977). When this infection occurs without an obvious underlying etiology, there should be a high index of suspicion about associated malignancy. In the absence of hematological malignancy a colonoscopy is warranted (Klein et al. 1977). A recent study showed the ability of the α-toxin-producing *C. septicum* to induce activation of mitogen--activated protein kinase (MAPK) signaling, which has been shown to be deregulated in various diseases including cancers, and causes release of the proinflammatory cytokine TNF-a (Chakravorty et al. 2015). Nevertheless, a firm relationship to CRC development has not been proved yet.

4.3.3.7 *H. pylori*

H. pylori is a Gram-negative bacterium that colonizes the gastric epithelium of more than 50% of the population and is related to chronic inflammation, gastric ulcers, and development of gastric malignancies (Lucas et al. 2017). Despite its gastric colonization, it may be associated with extragastric adverse occurrences, as well (Lucas et al. 2017). Even if initial studies had perpetuated controversies on the potential link between *H. pylori* and CRC, more recent investigations underlined the significant association between *H. pylori* infection and the increased CRC risk (Kim et al. 2017). In a large-scale study, carefully controlled for confounding factors, involving asymptomatic participants, *H. pylori* infection was significantly associated with the risk of any colorectal adenoma (OR:1.3) and advanced colorectal neoplasm (OR: 1.9) (Nam et al. 2016). Several hypotheses have been proposed to explain the possible link between *H. pylori* infection and CRC (Kapetanakis 2013; Shmuely et al. 2001). Hypergastrinemia in the setting of *H. pylori*-associated atrophic gastritis may promote colorectal tumorigenesis (Kapetanakis 2013). This hypothesis was supported by both experimental models, in which gastrin gene knockout mice showed decreased proliferation of the colonic mucosa, and by clinical case-control studies, which indicated elevated serum gastrin levels in patients with colorectal adenomatous polyps and adenocarcinoma (Kapetanakis 2013). Atrophic gastritis secondary to *H. pylori* infection is associated with reduced acid production, which permits a greater number and variety of microbial species to enter and colonize the intestinal tract. It has been proposed that shifts in the composition of colorectal microflora resulted from *H. pylori* atrophic gastritis may facilitate selective growth of bacteria such as *B. fragilis and E. faecalis*, which are linked to the development of CRC (Kapetanakis 2013). Moreover, based on the observation that patients infected with *H. pylori* that expresses CagA gene are more likely to develop gastric cancer, Shmuely et al. tested patients with various malignancies for serum antibodies against *H. pylori* and CagA protein and found that CagA seropositivity was associated with an increased risk not only for gastric adenocarcinoma but also for CRC, when compared with CagA seronegative controls (Shmuely et al. 2001).

Finally, assuming that chronic mucosal inflammation may be a predisposing factor for CRC development, as occurs in IBD cases, *H. pylori's* well-established proinflammatory and carcinogenic effect may also appear in the colon following a likely direct *H. pylori* colonization. However, there have been no reports of chronic or active colitis resulted from direct *H. pylori* infection in the colon (Kapetanakis 2013). Nevertheless, one should always keep in mind that simply identifying *H. pylori* organisms in CRC samples does not necessarily prove a causal relationship.

4.3.3.8 *Bifidobacterium*

Bifidobacterium is the most common type of Gram-positive lactic acid bacteria (along with the *Lactobacillus* bacterium) which has beneficial effects on the host. These bacteria are called probiotics. Several studies have confirmed the prophylactic and therapeutic effect of probiotics in patients with IBD or CRC (see Sect. 4.9).

For example, increased level of *E. coli* and decreased level of *Bifidobacterium* was observed in CRC (Wieczorska et al. 2020).

4.3.3.9 *Lactobacillus*

Lactobacillus is a Gram-positive, facultatively anaerobic bacterium. Its strain *L. rhamnosus* has a documented anti-inflammatory activity by modulating the cytokine-producing dendritic cells, by reducing the expression of β-catenin, and NF-kB, and by inducing the expression of tumor-suppressing p53 and Bcl-2--associated proteins. Research evidence underlines the regulating effects of *L. rhamnosus* on TLR expression, thereby increasing the function of the TLR2- and COX2-dependent intestinal epithelial barrier (Wieczorska et al. 2020).

4.3.4 Gut Microbiota Metabolites and CRC Development

Gut bacteria contribute to nutrient metabolism and produce small molecules termed the "metabolome," which may contribute to the development of neoplasia in the large bowel (Nistal et al. 2015; Nugent et al. 2014). Nugent et al. assessed, by chromatography and spectrometry, the metabolome in normal rectal mucosal biopsies of 15 subjects with colorectal adenomas and 15 nonadenoma controls, and identified a total of 274 metabolites (Nugent et al. 2014). Twenty-three metabolites contributed to the separation of metabolomic profiles between adenoma cases and nonadenoma controls; an increase of the inflammatory metabolite prostaglandin E2 and a decrease in antioxidant-related metabolites 5-oxoproline and diketogulonic acid were observed in adenoma cases (Nugent et al. 2014). Those differential metabolites demonstrated correlations with six bacterial taxa that were different between cases and controls (Nugent et al. 2014).

Microbial metabolites, such as secondary bile acids, have been identified as potential carcinogens and have been detected at high levels in fecal samples from CRC patients (Rubin et al. 2012). Cholic acid and chenodeoxycholic acid are converted by intestinal microbiota, via the 7α-hydroxylation process, to the secondary bile acids, deoxycholic acid and lithocholic acid, respectively (Nistal et al. 2015). Deoxycholic acid damages the mucosa contributing to an increase of ROS, insults DNA generating genomic instability, and benefits tumor growth (Nistal et al. 2015). Secondary bile acids may also influence CRC by supporting of apoptosis-resistant cells or by interacting with important secondary messengers of the signaling system that are activated in CRC (Nistal et al. 2015).

Protein fermentation-derived microbial products, especially with increased protein intake diets, lead to an increase of waste in the colon, such as sulfide, nitrate, ammonium, amines, branched-chain amino acids, and H2S (Nistal et al. 2015). As a result the growth of sulfate-reducing bacteria, such as *Desulfovibrio* and *Desulfomonas* spp., is stimulated (Nistal et al. 2015). CRC patients have a higher concentration of H2S compared to healthy subjects, and their colons have decreased ability to detoxify, thus promoting genotoxic effects (Ramasamy et al. 2006). Several species of *Bacteroides* and *Firmicutes* genus ferment aromatic amino acids

leading to potentially bioactive products, such as phenylacetic acid, phenols, indoles, and p-cresol. Some of these nitrogen products, particularly NOCs, exert their carcinogenic effect by alkylating DNA, leading to mutations (Nistal et al. 2015). Protein-rich diets are associated with an increase of NOCs and higher consumption of red or processed meat is associated with an outgrowth of bacteria that might contribute to CRC (Nistal et al. 2015; Larsson and Wolk 2006).

4.3.5 Relation of Gut Microbiota to CRC Phenotype and Prognosis

Certain studies have addressed the question whether the CRC-related microbiota are associated with the tumors' behavior and the patients' prognosis (Lauka et al. 2019). Boleij et al. compared the *ETBF*-related bft gene presence in mucosal samples from CRC patients and an outpatient colonoscopy healthy control group (Boleij et al. 2014). They found that the mucosa of cases was significantly more often bft--positive on left (85.7%) and right (91.7%) tumor compared with left (53%) and right (55.5%) control biopsies ($p = 0.04$), while there was a trend towards increased bft positivity in mucosa from late- vs early-stage CRC patients (100% vs 72.7%, respectively) (Boleij et al. 2014). On the other hand, Purcell et al. found an association of *ETBF* positivity and increased abundance with early-stage carcinogenic lesions, such as dysplastic adenomas and actually more pronounced in left-sided biopsies, compared to those from the right side of the colon (Tahara et al. 2014). The authors suggested that detection of *ETBF* may be a potential marker of early colorectal carcinogenesis.

F. nucleatum abundance has been linked to specific tumor phenotypes and such evidence may ultimately be exploited to shape CRC treatment (Brennan and Garrett 2018). *F. nucleatum*-high (pre-)malignant colonic lesions (either malignant or premalignant) have been subtyped according to MSI, CIMP status, BRAF, Kras, and p53 mutations-bearing status and localization to the proximal vs left colon (Brennan and Garrett 2018; Tahara et al. 2014; Ito et al. 2015; Mima et al. 2016; Dejea et al. 2014; Dienstmann et al. 2017; Purcell et al. 2017; Bullman et al. 2017).

Tahara et al. detected *F. nucleatum* in 74% of CRC cases, and, although the microbiome was also detected in cancer-free healthy subjects, that was 250 times less in quantity (Tahara et al. 2014). The same group demonstrated that the *F. nucleatum*-high CRC group was significantly associated with CIMP positivity, p53-wild type, hMLH1 methylation positivity, MSI and CHD7/8 mutation positivity (Tahara et al. 2014). Ito et al. investigated the presence of *F. nucleatum* in premalignant colorectal lesions (Ito et al. 2015). In total, 465 premalignant lesions (343 serrated lesions and 122 non-serrated adenomas) and 511 CRCs were studied. *F. nucleatum* was detected in 24% of hyperplastic polyps, 35% of sessile serrated adenomas (SSAs), 30% of traditional serrated adenomas (TSAs), and 33% of non--serrated adenomas. *F. nucleatum* was more frequently detected in CIMP-high premalignant lesions than in CIMP-low/zero lesions ($p = 0.0023$). In SSAs, *F. nucleatum* positivity increased gradually from sigmoid colon to cecum ($p = 0.042$). *F.*

nucleatum positivity was significantly higher in CRCs (56%) than in premalignant lesions of any histological type ($p < 0.0001$). Their data indicated that *F. nucleatum* positivity in SSAs may support the "colorectal continuum" concept (Ito et al. 2015). Mima et al. also showed that the proportion of *F. nucleatum*-high colorectal cancers gradually increased from rectal cancers (2.5%) to cecal cancers (11%) and that the percentage of *F. nucleatum*-low cancers was higher in rectal, ascending colon, and cecal cancers than in cancers of middle segments (Mima et al. 2016). Their results challenge the prevailing two-colon (proximal vs. distal) dichotomy paradigm.

Dejea et al. showed that the mucosal microbiota organization is a critical factor associated with a subset of CRC. They identified invasive polymicrobial bacterial biofilms, structures previously associated with nonmalignant intestinal pathology, nearly universally (89%) on right-sided tumors but on only 12% of left-sided tumors (Dejea et al. 2014). Patients with biofilm-positive cancers or adenomas had biofilms on their tumor-free mucosa far distant from the neoplastic lesions. Bacterial biofilms were associated with diminished colonic epithelial cell E-cadherin and enhanced epithelial cell IL-6 and Stat3 activation, as well as increased crypt epithelial cell proliferation in normal colon mucosa (Dejea et al. 2014).

The advent of large-scale sequencing technologies has recently facilitated the development of a Consensus Molecular Subtyping (CMS) system for CRC based solely on tumor gene expression: CMS1 (microsatellite instability immune, 14%), hypermutated, microsatellite unstable and strong immune activation; CMS2 (canonical, 37%), epithelial, marked WNT and MYC signaling activation; CMS3 (metabolic, 13%), epithelial and evident metabolic dysregulation; and CMS4 (mesenchymal, 23%), prominent transforming growth factor-β activation, stromal invasion, and angiogenesis (Dienstmann et al. 2017). For the first time, Purcell et al. have recently associated individual bacterial species to those CRC subtypes (Purcell et al. 2017). They showed enrichment of *Fusobacteria* and *Bacteroidetes* and decreased levels of *Firmicutes* and *Proteobacteria* in CMS1. The most highly enriched species associated with CMS1 included *Fusobacterium hwasookii* and *Porphyromonas gingivalis.* CMS2 was enriched for *Selenomas* and *Prevotella* species, while CMS3 had few significant associations. Targeted quantitative PCR also showed an enrichment of *F. nucleatum, Parvimonas micra,* and *Peptostreptococcus stomatis* in CMS1 (Purcell et al. 2017). Bullman et al. showed that colonization of human CRC with *Fusobacterium* and its associated microbiome-including *Bacteroides, Selenomonas,* and *Prevotella* species was maintained in distal metastases, demonstrating microbiome stability between paired primary and metastatic tumors (Bullman et al. 2017). With in situ hybridization they revealed that *Fusobacterium* was predominantly associated with cancer cells in the metastatic lesions. Mouse xenografts of human primary CRCs were found to retain viable *Fusobacterium* and its associated microbiome through successive passages. Treatment of mice bearing a CRC xenograft with the antibiotic metronidazole reduced *Fusobacterium* load, cancer cell proliferation, and overall tumor growth. These observations argue for further investigation of antimicrobial interventions as a potential treatment for patients with *Fusobacterium*-associated CRC (Bullman et al. 2017).

Accumulating literature data, collectively depicted in Table 4.1, support the relationship of specific gut microbiota with CRC pathologic features and patients'

Table 4.1 Published series on the relation between gut microbiota and CRC pathologic features and prognosis

Author (year)	Microbiota	Association with pathologic features	Association with prognosis
Flanagan et al. (2014)	*F. nucleatum.*	No association with stage	Low fold increase *F. nucleatum* survival > high fold increase *F. nucleatum* survival (significant difference) Low fold increase *F. nucleatum* median survival: >3 years vs high fold increase *F. nucleatum* survival: <2 years (HR = 19.96, 95% CI = 1.42–281.42, $p = 0.0266$)
Flemer et al. (2018)	Pathogen CAG Prevotella CAG Bacteroides CAG Firmicutes CAG	Not reported	Pathogen CAG-type microbiota was associated with longer survival (HR = 0.8, CI = 0.6–1.06; $p = 0.12$) Prevotella CAG-type microbiota was associated with longer survival (HR = 0.36, CI = 0.12–1.1; $p = 0.075$) Bacteroidetes CAG was associated with longer survival (HR = 0.75, CI = 0.58–1.03; $p = 0.078$) Firmicutes CAG 2 was associated with shorter survival (HR = 1.52, CI = 0.84–2.75; $p = 0.17$)
Kosumi et al. (2018)	*Bifidobacterium*	No association with stage	No association with survival
Mima et al. (2015)	*F. nucleatum*	Association with T stage ($p = 0.0007$) No association with N or M stage	*F. nucleatum*-high cases cancer-specific mortality > *F. nucleatum* negative cases (HR = 1.58, 95% CI = 1.04–2.39)
Wei et al. (2016)	*B. fragilis* *F. nucleatum* *F. prausnitzii*	High abundance of *F. nucleatum* significantly correlated with positive lymph node metastasis High abundance of *F. prausnitzii* and *F. nucleatum* significantly correlated with depth of invasion	Nonsurvival vs survival group: Higher levels of *B. fragilis*, *F. prausnitzii*, *F. nucleatum,* and *Methylobacterium suomiense* Greater abundance of *F. nucleatum* at recurrence group High abundance *B. fragilis* and *F. nucleatum* 3-year overall survival < low abundance *B. fragilis* and *F. nucleatum* 3-year overall survival ($p = 0.001$, $p = 0.003$) Low abundance *F. prausnitzii* 3-year overall survival < high abundance *F. prausnitzii* 3-year overall survival ($p = 0.06$) *B. fragilis* (HR = 2.01; 95% CI = 1.02–3.96; $p = 0.044$) and *F. nucleatum* (HR = 1.99; 95% CI = 1.02–3.87; $p = 0.042$): independent predictors of the 3-year overall survival *B. fragilis* (HR = 2.04; 95% CI = 1.11–3.73; $p = 0.021$) and *F. nucleatum* (HR = 1.82; 95% CI = 1–3.34; $p = 0.05$) associated with worse 3-year disease-free survival

(continued)

Table 4.1 (continued)

Author (year)	Microbiota	Association with pathologic features	Association with prognosis
Yan et al. (2017)	*F. nucleatum*	*F. nucleatum* level significantly associated with T stage ($p = 0.015$), N status ($p = 0.008$), and distant metastasis ($p = 0.020$)	High *F. nucleatum* level significantly associated with worse cancer-specific and disease-free survival in stage IIIB and IV patients High *F. nucleatum* level: significantly worse cancer-specific (HR = 2.22; 95% CI = 1.48–3.32; $p < 0.001$) and disease-free survival (HR = 2.0; 95% CI = 1.39–2.86; $p < 0.0010$)
Yu et al. (2017)	*F. nucleatum*	*F. nucleatum* positively associated with AJCC stage and tumor size	*F. nucleatum*-high group 5-year recurrence-free survival < *F. nucleatum*-low group *F. nucleatum* was an independent predictor of worse oncologic outcome

CRC colorectal cancer, *F. nucleatum Fusobacterium nucleatum*, *CAG* co-abundance group, *HR* hazard ratio, *CI* confidence interval

prognosis (Yu et al. 2017; Lauka et al. 2019; Flanagan et al. 2014; Flemer et al. 2018; Kosumi et al. 2018; Mima et al. 2015; Wei et al. 2016; Yan et al. 2017). As easily appreciated from the reported series, *F. nucleatum* increase is associated with worse patients' prognosis.

4.3.6 Gut Microbiome and CRC Screening and Early Diagnosis

In the context of the availability of a perfect CRC screening tool, the procedural risks of conventional colonoscopy cannot counter-compete the limited sensitivities of stool-based occult blood tests (Li et al. 2019). Basic research and clinical scientists remain at an incessant quest for the development of an accurate, noninvasive and highly sensitive test that could be applied at CRC screening. Fecal microbial detection may be a useful metagenomic marker for both early disease diagnosis and CRC screening (Li et al. 2019). Zackular et al. proved that, combined with known clinical risk factors of CRC (e.g., BMI, age, race), data from the gut microbiome significantly improved the ability to differentiate between healthy, adenoma, and carcinoma clinical groups relative to risk factors alone (Zackular et al. 2014). Using Bayesian methods, they determined that using gut microbiome data as a screening tool improved the pretest to posttest probability of adenoma more than 50-fold. Microbial genomic DNA sequences were clustered into operational taxonomic units (OTU) and they found that the addition of 6 OTUs [e.g., OTUs associated with *Fusobacterium* (OTU 2458), *Porphyromonas* (OTU 1905), etc.] to age, gender, race, and BMI (body-mass index) significantly improved the ability to distinguish between the healthy and CRC groups [area under the receiver-operating curve (AUC) = 0.922; 95% CI, 0.858–0.986; p = 0.012]. Because guaiac fecal occult blood test (gFOBT) is the most common, noninvasive screening tool for colorectal cancer, they evaluated whether the microbiome-based models could be improved by including gFOBT results. The model combining BMI, gFOBT, and the microbiome data (OTUs 1905, 2395, 2458, and 3235) provided excellent discriminatory ability (AUC = 0.969; 95% CI, 0.935–1.000) (Zackular et al. 2014). The results of their study demonstrated the feasibility of using the composition of the gut microbiome to detect the presence of precancerous and cancerous lesions. Subsequently, Zeller et al. used metagenomic sequencing of fecal samples to identify taxonomic markers that distinguished CRC patients from tumor-free controls in a study population of 156 participants (Zeller et al. 2014). Accuracy of metagenomic CRC detection was similar to the standard FOBT and when both approaches were combined, sensitivity improved >45% relative to the FOBT, while maintaining its specificity. Accuracy of metagenomic CRC detection did not differ significantly between early- and late--stage cancer and could be validated in independent patient and control populations from different countries (Zeller et al. 2014).

Baxter et al. demonstrated the potential for microbiota analysis to complement existing screening methods to improve detection of colonic lesions (Baxter et al. 2016). They sequenced the 16S rRNA genes from the stool samples of 490 patients and they used the relative abundances of the bacterial populations within each

sample to develop a random forest classification model using the relative abundance of gut microbiota and the concentration of hemoglobin in stool to detect colonic lesions.

The microbiota-based random forest model detected 91.7% of cancers and 45.5% of adenomas while fecal immunohistochemical test (FIT) alone detected 75% and 15.7%, respectively (Baxter et al. 2016). They also found that the loss of potentially beneficial organisms, such as members of the *Lachnospiraceae*, was more predictive for identifying patients with adenomas when used in combination with FIT (Baxter et al. 2016).

Yu et al. reported the first successful cross-ethnic validation of metagenomic gene markers for CRC, including data from four countries (Yu et al. 2015b). They discovered significant enrichment of novel species, including *Parvimonas micra* and *Solobacterium moorei,* and a strong co-occurrence network between them in the fecal microbiomes of patients with CRC. They also identified 20 gene markers that significantly differentiate CRC-associated and control microbiomes in the initial Chinese cohort and succeeded the trans-continental validation of four of them in a Danish cohort. Further validation of the four gene markers in published cohorts from the French and Austrian cohorts was found to have AUCs of 0.72 and 0.77 (Yu et al. 2015b). Quantitative PCR abundance of two gene markers (butyryl-CoA dehydrogenase from *F. nucleatum*, and RNA polymerase subunit β, rpoB, from *Parvimonas micra*) clearly separated CRC microbiomes from controls (AUC = 0.84, OR: 23) (Yu et al. 2015b). The four microbial gene markers shared between the Chinese, Danish, Austrian, and French cohorts suggested that, even though different populations may have different gut microbial community structures, signatures of CRC-associated microbial dysbiosis could have universal features. This study took a step further towards affordable early diagnosis of CRC by targeted analysis of metagenomic biomarkers in fecal samples (Yu et al. 2015b).

4.3.7 Gut Microbiota and CRC Surgery

Our understanding of gut microbiota role in surgical treatment and outcomes remains rather limited. The routine of intestinal antisepsis before gastrointestinal surgery may not be beneficial to the microbiome's role in immune function and wound repair (Gershuni and Friedman 2019). Peri-operative medications can also alter microbiome composition. For instance, antacids disrupt the balance of acid--sensitive organisms, vasoactive medications decrease perfusion and oxygen delivery and may induce a shift in bacterial virulence and opioids impair gut motility resulting in ileus, dysbiosis, and bacterial overgrowth (Gershuni and Friedman 2019).

Based on the shortage of relevant data, Lin et al. recently investigated the changes of microbiota status and related metabolic profiles after partial colectomy for curable CRC (Lin et al. 2019). Compared with control group, the right hemicolectomy (RH) group showed lower bacterial diversity ($p = 0.007$), whereas the low anterior resection (LAR) group showed significantly higher bacterial diversity at the genera level ($p = 0.016$). Compared with the control group, the principal component

analysis revealed significant differences in bacterial genera abundance after RH and LAR ($p < 0.001$). Furthermore, the *Firmicutes* to *Bacteroidetes* ratio was significantly lower in the RH group than the control group (22.0% versus 49.4%, $p < 0.05$) (Lin et al. 2019). The occurrence of metabolic syndrome was significantly higher in patients after RH, but not LAR, when compared with the controls over the long--term (>5 years) follow-up ($p = 0.020$). In parallel with metabolic change, patients with RH showed dysbiosis with a tendency to decreased richness and a significant decrease in the diversity of gut microbiota (Lin et al. 2019). Comparing fecal samples before and 7 days after CRC surgery, Ohigashi et al. observed that total bacterial counts and the numbers of six groups of obligate anaerobes were significantly decreased after surgery (Ohigashi et al. 2013). In contrast, the populations of *Enterobacteriaceae, Enterococcus, Staphylococcus,* and *Pseudomonas* were significantly increased. The postoperative concentration of total organic acids was lower than preoperatively, whereas a significant reduction of SCFAs was observed postoperatively (Ohigashi et al. 2013).

Additional data suggest that the normal dynamic response to surgery might lead to increased microbial virulence. Analyzing the changes in luminal versus tissue-associated microbiota at anastomotic sites created in the colon of rats, Shogan et al. indicated that anastomotic injury induced significant changes in the anastomotic tissue-associated microbiota with minimal differences in the luminal microbiota (Shogan et al. 2014). The most striking difference was a 500-fold and 200-fold increase in the relative abundance of *Enterococcus* and *Escherichia/Shigella*, respectively. Functional profiling predicted the predominance of bacterial virulence--associated pathways in post-anastomotic tissues, including production of hemolysin, cytolethal toxins, fimbriae, invasins, cytotoxic necrotizing factors, and coccolysin (Shogan et al. 2014). Moreover, intestinal *Pseudomonas aeruginosa* (*P. aeruginosa*) is capable of responding to host signals released during stress (Gershuni and Friedman 2019). In mice, morphine exposure led to a shift to a more virulent phenotype of *P. aeruginosa* that expressed greater biofilm formation, increased antibiotic resistance, and the ability to cause lethal gut-derived sepsis, while the emergent mucus-suppressing phenotype of the bacteria disrupted the mucus layer and degraded the gut epithelial integrity (Babrowski et al. 2012). The increased virulence in *P. aeruginosa* has been attributed to a single nucleotide polymorphic mutation in the mexT gene that displays increased tissue destruction and collagenase expression (Olivas et al. 2012).

The creation of an anastomosis for re-establishing bowel continuity is an integral part of CRC surgical procedures. In up to almost 20% of cases, a non-well-healed anastomosis leads to a frequently catastrophic anastomotic leak (AL), which has a substantially negative impact on patients' morbidity and mortality as well as other sequelae, such as increased hospital costs and length of stay and delay or even omission of adjuvant chemotherapy (Gaines et al. 2018). Shogan et al. has demonstrated that *E. faecalis* contributes to the pathogenesis of AL in an animal model and that the anastomotic tissues of human subjects undergoing colon surgery are colonized with *E. faecalis* (Shogan et al. 2015). *E. faecalis* has been found to be highly prevalent in anastomotic tissues, likely due to its high adherence affinity to extracellular

matrix proteins, including collagen. *E. faecalis* is capable of producing gelatinase (GelE), which contributes to the development of AL by breaking down collagen and activating intestinal matrix metalloproteinases (MMP), which are capable of degrading collagen. So, the researchers concluded that incidence of AL is associated with microbiota (i.e., *E. faecalis*) that has both increased production of collagenase (aka gelatinase) and increased capacity to activate host intestinal MMP (Shogan et al. 2015). They were also able to suppress MMP9 activation via direct application of topical antibiotics to intestinal tissues, but this protective effect was not replicated with intravenous antibiotics (Shogan et al. 2015). Additionally, the unique environmental context created across the continuum of CRC care (i.e., surgery, antibiotics, and adjuvant oncologic treatments) promotes colonization by collagenase-producing microbes, such as *E. faecalis*, followed by implantation of cancer cells, which are shed continuously both during and after surgery. High collagenase-producing microbes may activate local macrophages such that anastomotic healing is impaired in a manner that promotes cancer cells implantation and migration to extra-mucosal sites, leading to local tumor recurrence (Gaines et al. 2018).

In a recently published Dutch study, bacterial DNA was isolated from 123 "donuts" of patients where a stapled colorectal anastomosis was made and was analyzed using 16S MiSeq sequencing (Praagh et al. 2019). In 63 patients, this anastomosis was covered with a C-seal, a bioresorbable sheath stapled to the anastomosis. In non-C-seal patients, AL development was associated with low microbial diversity ($p = 0.002$) and correspondingly with a high abundance of the dominant *Bacteroidaceae* and *Lachnospiraceae* families ($p = 0.008$ and $p = 0.01$, respectively). In C-seal samples, where AL rates were slightly higher (25% vs 17%), an association with the gut microbiota composition was almost undetectable. The researchers concluded that AL in patients without a C-seal can be linked to the intestinal microbiota, in particular with a low microbial diversity and a higher abundance of especially mucin-degrading members of the *Bacteroidaceae* and *Lachnospiraceae* families. In C-seal patients, however, it seems that any potential protective benefits or harmful consequences of the gut microbiota composition in regard to wound healing are negated, as progression to AL is independent of the initially dominant bacterial composition (Praagh et al. 2019).

Ileus and adhesion formation remain important concerns for surgeons. The extent to which the intestinal microbiome contributes to these complications is unknown. However, there is compelling evidence to suggest that the intestinal microbiome plays a key and contributory role in their pathogenesis (Alverdy et al. 2017). This assumption is based on experimental and clinical observations in which GF conditions or antibiotic use, such as oral nonabsorbable antibiotics, reduces or eliminates the incidence of these complications (Oncel et al. 2001). Today it is still not known which of the intestinal microbes should be preserved and which should be eliminated. Furthermore, the pathogens that drive surgical complications within the microbiome cannot be eliminated selectively while at the same time preserving the health-promoting microbiota (Alverdy et al. 2017).

Bowel preparation, including the use of oral and intravenous antibiotics, is a topic of much debate in general and colorectal surgery. Historically, the goal was

extensive decontamination with mechanical bowel preparation (MBP), which includes mechanical cleansing and oral nonabsorbable antibiotics, to prevent anastomotic complications and surgical-site infections (Gaines et al. 2018). Nevertheless, high level evidence data stated that MBP is unnecessary and does not decrease postoperative infectious complications (Oncel et al. 2001). In 2015, large databases--derived clinical evidence validated the original practice of MBP combined with oral antibiotics, demonstrating a decrease in AL and surgical-site infection rates (Cao et al. 2011).

The inherent flaw of a broad-based intestinal decontamination approach to prepare the bowel for surgery is the lack of recognition that a diverse gut microbiome actually serves to suppress the development of potentially harmful pathobiota and promotes intestinal healing. Indeed, a distinct subpopulation of the normal mucosa--associated gut microbiota expands and preferentially colonizes sites of damaged murine mucosa in response to local environmental cues (Kiran et al. 2015). Alam et al.'s results demonstrated that formyl peptide receptor 1 (FPR1) and neutrophilic NADPH oxidase (NOX2) are required for the rapid depletion of microenvironmental oxygen and compensatory responses, resulting in a dramatic enrichment of an anaerobic bacterial consortium (Alam et al. 2016). The dominant member of this wound-mucosa-associated microbiota *Akkermansia muciniphila* (an anaerobic, mucinophilic gut symbiont) stimulated proliferation and migration of enterocytes adjacent to the colonic wounds in a process involving FPR1 and intestinal epithelial-cell-specific NOX1-dependent redox signaling (Alam et al. 2016). These findings demonstrate how wound microenvironments induce the rapid emergence of "probiont" species that contribute to enhanced repair of mucosal wounds. Such microorganisms could be exploited as potential therapeutics (Alam et al. 2016). Instead of mass destruction of gut microbiota, a more gentle cleansing of the bowel in combination with nutritional supplements and non-microbicidal antivirulence agents has been proposed (Reddy et al. 2007). Reddy et al. found that the combination of synbiotics with neomycin and MBP led to a significant reduction of the harmful *Enterobacteriaceae* in fecal samples and in bacterial translocation, apparently due to a better intestinal barrier preservation, without, though, this selective decontamination regimen to be clinically translated to a decreased rate of septic complications (Reddy et al. 2007).

4.4 Gut Microbiome and Gastric Cancer

In the second half of the nineteenth century Louis Pasteur and Robert Koch introduced and popularized the germ theory of disease (Engstrand and Graham 2020). At that time, gastric cancer was the most common cause of cancer deaths in most countries making the stomach an early site of microbial research with a focus on gastric luminal and mucosal bacteria. In 1895, Izmar Isidor Boas and Bruno Oppler reported the association of gastric cancer with the presence of both lactic acid and a large amount of bacteria in the stomach and in 1916 Heinemann and Ecker confirmed that the Boas-Oppler bacillus was a Lactobacillus, or several types of *Lactobacilli* that

were able to overgrow in states associated with hypo- or achlorhydria (Engstrand and Graham 2020). They concluded that the Boas-Oppler bacillus was neither causative nor diagnostic of gastric cancer (Heinemann and Ecker 1916). Interest in the gastric microbiome resurged in the last quarter of the twentieth century based on the premise that intestinal and gastric bacteria might be a potential source of carcinogens. The nitrosamine hypothesis was most popular in the pre-*H. pylori* era and suggested that reduction in dietary nitrates to nitrite could convert dietary amines into carcinogenic N-nitroso compounds (Heinemann and Ecker 1916). Even though animal and some epidemiologic human studies supported this hypothesis, data from epidemiologic studies relating nitrate ingestion and gastric cancer were eventually proven inconclusive (Loh et al. 2011). Carcinogenic N-nitroso compounds produced could cause progressive genetic instability resulting in gastric cancer development. This hypothesis, though, was haunted by the fact that this conversion requires acid which is lacking in the precancerous achlorhydric stomach (Engstrand and Graham 2020). The *H. pylori*-infected hypochlorhydric stomach typically contains both acute and chronic inflammation and very low levels of ascorbic acid which favors formation of N-nitrosamine rather than S-nitrosothiol, but, when such patients were directly examined by Sobala et al., it was reported that total levels of N-nitroso compounds were not increased (Sobala et al. 1991). *H. pylori* is one of the primary infectious agents deemed a class I carcinogen and 325 of the two million new cancer cases attributed to infections worldwide are related by this bacterium (Plummer et al. 2014).

H. pylori is the only bacterium that is recognized as causally being associated with malignant neoplasia in humans and it confers a risk of approximately 89% for non-cardia gastric carcinoma which translates to around 780,000 new gastric cancer cases (Plummer et al. 2014). The incidence and mortality rates of gastric adenocarcinoma in developed countries have declined significantly over the past century. This is primarily connected to a decline in intestinal-type distal gastric adenocarcinomas and may be related to decreased transmission of *H. pylori* in childhood due to improved hygiene and smaller family units (Howson et al. 1986). Distal gastric adenocarcinomas are strongly associated with *H. pylori* infection, but the causal relationship between *H. pylori* and gastric cardia adenocarcinomas, which have been increasing, along with the Barrett's esophagus-related gastroesophageal junction adenocarcinomas, is less well defined. Infection with *H. pylori* was associated with 6.2% of all gastric cancers (Plummer et al. 2014). However, the combined incidence of intestinal and diffuse-type gastric cancer in *H. pylori*-infected individuals was reported to be approximately 3%, compared with 0% in uninfected subjects (Uemura et al. 2001).

H. pylori is an epsilon proteobacterium and a member of the Helicobacteraceae family that selectively colonizes gastric epithelium. *H. pylori* virulence factors play a key role in determining the risk of developing gastric cancer. One *H. pylori* pathogenic constituent that is linked to carcinogenicity is the Cag pathogenicity island (CagPAI), which contains a cluster of genes encoding proteins that form a type IV bacterial secretion system (T4SS). The Cag T4SS translocates CagA from adherent *H. pylori* across the bacterial and epithelial membranes into host cells. Around 60%

of *H. pylori* isolates from Western countries and almost all from East Asia are positive for CagPAI (Shaffer et al. 2011). Infection with CagA-positive *H. pylori* strains increases the risk by two to three times compared to the CagA-negative ones for the development of intestinal and diffuse gastric cancers (Azuma et al. 2004).

CagA exists in alternative structures and contains different glutamate-proline--isoleucine-tyrosine-alanine (EPIYA) repeat polymorphisms, which may be used as indicators of pathologic outcome (Basso et al. 2008). Four different EPIYA motifs (EPIYA-A, -B, -C, or -D) have been identified. EPIYA-A and EPIYA-B motifs are found in most strains, while the EPIYA-C motif is predominately found in Western strains and is associated with an elevated risk of developing gastric cancer (Basso et al. 2008). EPIYA-D strains are typically East Asian strains and carry more increased cancer risk than the EPIYA-C (Basso et al. 2008). Following translocation, CagA is tyrosine phosphorylated at EPIYA motifs and has carcinogenic potential. The activity of oncogenic pathways containing ERK/MAPK, PI3K/Akt, NF-κB, Wnt/β-catenin, Ras, sonic hedgehog, as well as STAT3 is upregulated with the infection of Cag + *H. pylori* strains. Conversely, tumor suppressor pathways are inactivated with induced p53 mutations. Other sequelae involve proinflammatory and mitogenic responses, disruption of cell-cell junctions, and loss of cellular polarity (Murata-Kamiya et al. 2007; Saadat et al. 2007). Independent of CagA, *H. pylori* can also induce mislocalization of the tight junction proteins occludin and claudin-7 and alter barrier function (Wroblewski et al. 2014).

Another *H. pylori* virulence factor is the multifunctional cytotoxin VacA which causes vacuolation, altered plasma and mitochondrial membrane permeability, autophagy, and apoptosis (Boquet and Ricci 2012). The VacA gene is found in all strains of *H. pylori* and contains a number of variable loci in the 5′ region of the gene termed s, i, and m regions. This 5′ terminus encodes the signal sequence and amino-terminus of the secreted toxin (allele types s1a, s1b, s1c, or s2), an intermediate region (allele types i1 or i2), and a mid-region (allele types m1 or m2) (Rhead et al. 2007). Strains containing type s1, i1, or m1 alleles are highly associated with gastric cancer and are associated with a greater risk of developing gastric cancer than Cag status (Rhead et al. 2007).

Blood group antigen binding adhesin (BabA) and sialic acid-binding adhesion (SabA) are two other important *H. pylori* constituents that have been linked to the development of gastric cancer (Yu 2002). BabA is an outer membrane protein that binds to fucosylated Lewis b antigen (Leb) on the surface of gastric epithelial cells. The presence of babA2, the gene encoding BabA, is associated with gastric cancer, and BabA expression is linked with adenocarcinoma of the gastric cardia (Yu 2002). The combined effect of BabA with cagA and vacA s1 alleles is strongly linked to a more severe gastric disease outcome (Yu 2002). Sialyl-Lewis x is expressed in the gastric epithelium and expression is increased by chronic inflammation (Yamaoka 2006). SabA binds to sialyl-Lewis x antigen, suggesting that *H. pylori* may modulate sialyl-Lewis x in the host to enhance attachment and colonization (Mahdavi 2002).

Epstein-Barr virus (EBV) infection is another pathogen that is associated with gastric cancers. EBV-positive tumors comprise almost 10% of gastric cancers, are associated with extensive gene methylation, predominately affect males, and are

generally located in the cardia or corpus (Murphy et al. 2009). EBV and *H. pylori* may act synergistically in the gastric epithelium to promote the progression towards gastric cancer, and the majority of EBV-positive individuals are also positive for *H. pylori* (Camargo et al. 2015). A case-control study has shown that the combination of EBV and *H. pylori* induces severe inflammation and augments the risk of developing intestinal-type gastric cancer (Cárdenas-Mondragón et al. 2015).

When *H. pylori* is present it dominates in the gastric niche such as in patients with gastritis and ulcers. Positive *H. pylori* status has been associated with increased relative abundance of non-*Helicobacter* bacteria from the *Proteobacteria, Spirochetes,* and *Acidobacteria*, and with decreased abundance of *Actinobacteria, Bacteroidetes,* and *Firmicutes* (Maldonado-Contreras et al. 2010). *H. pylori*--negative subjects also contain a diverse microbiota ecosystem. Ferreira et al. studied the microbiota composition differences between chronic gastritis and gastric cancer (Ferreira et al. 2017). The gastric carcinoma microbiota was characterized by reduced microbial diversity, by decreased abundance of Helicobacter, and by the enrichment of other bacterial genera, mostly represented by intestinal commensals. Overall, the gastric microbiota was dominated by five phyla: *Proteobacteria* (69.3%), *Firmicutes* (14.7%), *Bacteroidetes* (9%), *Actinobacteria* (4.3%), and *Fusobacteria* (1.3%). Although these phyla were present in the two patient groups in the same order of relative abundance, the gastric carcinoma microbiota had a statistically significant over-representation of *Actinobacteria* and Firmicutes and a lower abundance of *Bacteroidetes* and *Fusobacteria* (Castaño-Rodríguez et al. 2017). A significant reduction in the abundance of *Helicobacter* and an over--representation of non-*Helicobacter Proteobacteria* were detected in gastric carcinoma, as well. In gastric carcinoma, an enrichment in *Proteobacteria* taxa was observed, including the genera *Phyllobacterium* and Achromobacter and the families *Xanthomonadaceae* and *Enterobacteriaceae*. Although no specific genus could be identified within the *Xanthomonadaceae*, in the *Enterobacteriaceae*, the genus *Citrobacter* was identified as being significantly enriched in gastric carcinoma. Additionally, *Lactobacillus, Clostridium,* and *Rhodococc*us were also significantly more abundant in gastric carcinoma. *Helicobacter, Neisseria, Prevotella,* and *Streptococcus* were most abundant in the microbiota of patients with chronic gastritis (Ferreira et al. 2017). The presence of a significant mucosa microbial dysbiosis in intestinal metaplasia and gastric carcinoma patients was confirmed by Coker et al. (Castaño-Rodríguez et al. 2017). Five gastric cancer-enriched bacterial taxa whose species identifications corresponded to *Peptostreptococcus stomatis, Streptococcus anginosus, Parvimonas micra, Slackia exigua,* and *Dialister pneumosintes* had significant centralities in the gastric cancer ecological network and distinguished gastric cancer from superficial gastritis (Castaño-Rodríguez et al. 2017). Moreover, stronger interactions among gastric microbes were observed in *H. pylori*-negative samples compared with *H. pylori*-positive samples in superficial gastritis and intestinal metaplasia (Castaño-Rodríguez et al. 2017). However, there is currently no solid evidence that the non-*H. pylori* bacterial community in the stomach is directly involved in gastric carcinogenesis (Castaño-Rodríguez et al. 2017).

4.5 Gut Microbiome and Esophageal Cancer

Common human-infecting viruses, such as the human papilloma and Epstein-Barr viruses, have been recognized to play a pathogenetic role on the esophageal squamous cell carcinoma (ESCC) (Baba et al. 2017). On the other hand, bacterial infections may contribute to esophageal adenocarcinoma (EAC) development. The premalignant component of the latter malignancy, known as the Barrett's esophagus, which, in turn, is directly related to the gastroesophageal reflux disease (GERD) and the subsequent chronic esophagitis, has been found to be accompanied by a relative abundance of *Enterobacteriaceae* in the stomach, whereas antibiotics may modify the GERD's patients esophageal microbiome (Neto et al. 2016). In the meantime, parietal cells-suppressing and acid-reducing *H. pylori* infections may be related to GERD-associated esophageal carcinoma (Meng et al. 2018). Significant differences in the composition of gastric fluid bacteria have been found between patients with normal esophageal tissue versus patients with esophagitis or Barrett's esophagus, but relatively subtle microbiota differences were observed in the esophagus-associated microbiota (Amir et al. 2013). The same investigators found that treatment with proton pump inhibitors (PPIs) had dramatic effects on microbial communities both in the gastric fluids and the esophageal tissue (Amir et al. 2013). Nevertheless, no dysplasia or cancer-protective effects of PPIs usage in patients with Barrett's esophagus were identified by a recent meta-analysis (Hu et al. 2017).

Gagliardi et al. revealed that *Streptococcus viridans*, a member of the phylum *Firmicutes*, is the most frequent microorganism in both the normal esophagus and the oropharynx (Gagliardi et al. 1998). These findings were consolidated by Norder Grusell et al. who reported the occurrence rate of *Streptococcus viridans* as 95–98% (Grusell et al. 2012). Pei et al. examined the normal esophagus by 16S rRNA sequencing technology and identified 95 species in six phyla: *Firmicutes* (e.g., *Streptococcus*), Bacteroides (e.g., Prevotella), *Actinobacteria* (e.g., *Rothia*), *Proteobacteria* (e.g., *Haemophilus*), *Fusobacteria* (e.g., *Fusobacterium*), and *TM7* (Pei 2005). Remarkably, the findings were similar across specimens, suggesting a stable esophageal biota that is distinct from the flora of the oropharynx and stomach. Microscopic examination of the tissue confirmed a close association between the bacteria and the cell surfaces of the mucosal epithelium in situ, suggesting a residential, rather than a transient, microbiota (Pei 2005).

Several studies have documented microbiome status in esophagitis and Barrett's esophagus. Yang et al. analyzed microbiomes from biopsy samples by bacterial 16S rRNA gene survey and classified them into types using unsupervised cluster analysis and phenotype-guided analyses (Yang et al. 2012). Esophageal microbiomes can be classified into two types. The type I microbiome was dominated by the genus Streptococcus and concentrated in the phenotypically normal esophagus. Conversely, the type II microbiome contained a greater proportion of Gram-negative anaerobes/microaerophiles (phyla: *Bacteroidetes, Proteobacteria, Fusobacteria,* and Spirochaetes) and primarily correlated with esophagitis (OR: 15.4) and Barrett's esophagus (OR: 16.5) (Yang et al. 2012). It is uniformly accepted that the esophageal bacteria differ among normal esophagus, GERD and Barrett's esophagus,

supporting that esophageal disease is related to the bacterial community profile, possibly through the innate immune system. Gram-negative organisms, which predominate in GERD and Barrett's esophagus, produce specific constituents such as lipopolysaccharide (LPS) that stimulate the innate immune system's TLR4 in the epithelial or inflammatory cells, leading to NF-κB activation and elevated levels of inflammatory cytokines (IL-1b, IL-6, IL-8, TNF-a) (Abdel-Latif et al. 2009). So, the increased Gram-negative bacteria in GERD and Barrett's esophagus may induce chronic inflammation and trigger a cascade that leads to EAC (Abdel-Latif et al. 2009).

Blackett et al. isolated a total of 111 species belonging to 26 genera in GERD and Barrett's esophagus (Blackett et al. 2013). Campylobacter was significantly more enriched in GERD and Barrett's esophagus than in the controls and esophageal adenocarcinoma, with the *Campylobacter concisus* being the dominant species (Blackett et al. 2013). Significant increases in carcinogenesis-associated IL-18 were seen in GERD and Barrett's esophagus colonized by *Campylobacter*. The role of *Campylobacter* in EAC progression might mimic that of *H. pylori* in gastric cancer (Man 2011). Zaidi et al. revealed a prevalence of *Escherichia coli* in Barrett's esophagus and EAC (Zaidi et al. 2016). TLR 1–3, 6, 7, and 9 were significantly upregulated in EAC compared with normal epithelium. This suggests an association between the TLR signaling pathway and *E. coli*, hinting that early molecular changes are mediated by microbes in the rat model of EAC carcinogenesis. Studies on human clinical samples also corroborated those results to some extent (Zaidi et al. 2016).

As far as ESCC is concerned, the gastric corpus microbiota of patients affected by esophageal squamous dysplasia and ESCC are enriched in *Clostridiales* and *Erysipelotrichales,* suggesting that gastric dysbiosis is involved in the progression from esophageal squamous dysplasia to ESCC (Nasrollahzadeh et al. 2015). Gao et al. revealed that *Porphyromonas gingivalis* infects the cancerous and adjacent esophageal mucosa of ESCC patients but not the healthy mucosa of controls, supporting a pathogenesis role of this organism in ESCC (Gao et al. 2016). The presence of *Porphyromonas gingivalis* was also positively correlated with the aggressiveness of ESCC and with poor clinical outcome. Therefore, *Porphyromonas gingivalis* may serve as a biomarker of ESCC. According to Chen et al., altered bacterial microbiota in the saliva is related to a higher risk of ESCC (Chen et al. 2016). The carriage of genera *Lautropia, Bulleidia, Catonella, Corynebacterium, Moryella, Peptococcus,* and *Cardiobacterium* is lower in ESCC patients than in individuals without this cancer.

Yamamura et al. revealed that the prognosis of ESCC relates to the presence of *F. nucleatum*, which primarily inhabits the oral cavity and causes periodontal disease (Yamamura et al. 2016). Given the close proximity of the esophagus to the oral cavity, they suspected that *F. nucleatum* also plays an important role in esophageal cancer. They assessed DNA in the cancer tissues of 325 patients who underwent surgical removal of esophageal cancer and 74 out of 325 patients (23%) contained *F. nucleatum* in their cancer tissues. The presence of *F. nucleatum* in cancer tissue was associated with significantly shorter survival time (Yamamura et al. 2016).

4.6 Gut Microbiome and Hepatocellular Carcinoma

Chronic viral hepatitis, especially hepatitis B virus (HBV) and hepatitis C virus (HCV), is the leading cause of the pathophysiological progression of hepatocellular carcinoma (HCC) (Wong et al. 2017b). Other etiologies, such as drug abuse, autoimmunity, intake of liver toxins, alcohol, and nonalcoholic fatty liver disease (NAFLD), are also correlated with a high risk of HCC (Marrero 2009). The role of the microbiota in hepatocarcinogenesis is mostly driven by inflammatory pathways, which are initiated by crosstalk between the intestinal bacteria, immune system, and liver. The process involves the interplay of macrophages, Kupffer cells, damage--associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs) populations in the liver. Macrophages and Kupffer cells react to PAMPs, endotoxins, or LPS via the activation of NF-κB by binding to TLRs, especially TLR-4, TLR-9, and NOD-like receptor, and this process generates an inflammatory chain reaction that promotes inflammation and cytokine release (Wong et al. 2017b). Gut microbiota dysbiosis boosts the secretion of inflammatory cytokines, such as TNF-a, IL-8, and IL-1β, which stimulates lipid accumulation and cell death in hepatocytes, causing steatosis, induction, and progression of nonalcoholic fatty liver disease (NAFLD) to nonalcoholic steatohepatitis and cirrhosis (Wong et al. 2017b). Dysbiosis may lead to increased deoxycholic acid, which provokes the senescence-associated secretory phenotype of the hepatic stellate cells, resulting in the secretion of various inflammatory and tumor promoting factors (Yoshimoto et al. 2013). Animal studies have demonstrated the key role of the microbiome in NASH aggravation and potentially in the development of NASH-associated HCC, as well as the reduction of such risk by antibiotics' administration (Henao-Mejia et al. 2012). Yu et al. found that the circulating levels of LPS were elevated in animal models of carcinogen-induced hepatocarcinogenesis. Reduction of LPS using antibiotics regimen in rats or genetic ablation of its receptor TLR4 in mice prevented excessive tumor growth and multiplicity. Additional investigation revealed that TLR4 ablation sensitizes the liver to carcinogen-induced toxicity via blocking NF-κB activation and sensitizing the liver to ROS-induced toxicity (Yu et al. 2010). The class *Clostridia* particularly *Clostridium cluster XIVa* and the phylum *Proteobacteria* have been closely linked to HCC (Singh et al. 2018).

In clinical trials, the profile of the gut microbiota associated with the presence of HCC in cirrhotic patients is characterized by increased fecal counts of *E. coli* (Grat et al. 2016). Liu et al. recently investigated the differences between the gut microbiota of HBV-related HCC (B-HCC) and non-HBV non-HCV-related HCC (NBNC--HCC) patients (Liu et al. 2019). They found that the species richness of fecal microbiota of B-HCC patients was much higher than other two groups. The feces of NBNC-HCC patients harbored more potential proinflammatory bacteria (*Escherichia-Shigella, Enterococcus*) and reduced levels of *Faecalibacterium, Ruminococcus,* and *Ruminoclostridium* which resulted in decreased potential of anti-inflammatory short-chain fatty acids. The feces of NBNC-HCC patients had relatively fewer abundance of multiple biological pathways related to amino acid and glucose metabolism, but higher level of pathways related to their transport and

secretion. However, the B-HCC patients had opposite results of bacterial composition and associated multiple biological pathways than the NBNC-HCC patients (Liu et al. 2019). Ren et al. demonstrated that the microbial diversity was increased from cirrhosis to early HCC with cirrhosis (Seok and Suk 2020). Phylum *Actinobacteria* was increased in early HCC versus cirrhosis. Correspondingly, 13 genera including *Gemmiger* and *Parabacteroides* were enriched in early HCC versus cirrhosis. Butyrate-producing genera were decreased, while LPS-producing genera were increased in early HCC versus controls (Seok and Suk 2020). The authors suggested that gut microbiota-targeted biomarkers may represent potential noninvasive tools for early diagnosis of HCC (Seok and Suk 2020). According to Ponziani et al., the fecal microbiota of patients with NAFLD-related cirrhosis is characterized by higher abundance of *Enterobacteriaceae* and *Streptococcus a*nd reduction of *Akkermansia* (Ponziani et al. 2018). *Bacteroides* and *Ruminococcaceae* are increased in HCC, while *Bifidobacterium* is reduced. *Akkermansia* and *Bifidobacterium* are inversely correlated with calprotectin concentration, which is associated with humoral and cellular inflammatory markers (Ponziani et al. 2018).

4.7 Gut Microbiome and Pancreatic Cancer

Pancreatic adenocarcinoma (PDAC) is one of the most lethal cancers worldwide, and only 30% of patients survive 1 year after the diagnosis (Michaud and Izard 2014; Zambirinis et al. 2014). Based on the assumption that *H. pylori* infection may exert its extragastric manifestations on pancreatic physiology alteration, its presence has been reported to be associated with acute, chronic, and autoimmune pancreatitis, as well as the PDAC itself (Rabelo-Gonçalves 2015; Warzecha et al. 2002; Kountouras et al. 2005). Directly pathogenic substances, such as ammonia, as well as inflammatory cytokines and *H. pylori*-driven deregulatory pathways, such as NF-κB and AP-1, may lead to pancreatic carcinogenesis (Meng et al. 2018; Bulajic 2014; Abadi 2019). K-RAS gene's mutations and STAY-3 activation, both stimulated by *H. pylori*-produced LPS, may further promote PDAC progression, via upregulation of anti-apoptotic pathways (Meng et al. 2018; Huang et al. 2013; Fukuda et al. 2011). Additionally, TLR4 initiates a complex signaling pathway when it interacts with LPS, which ultimately results in a proinflammatory response (Wörmann et al. 2013). Shariff et al. showed that the severity of acute pancreatitis was ameliorated in mice that lacked either TLR4 or CD14 receptors and their results reinforced the concept that TLR4 plays a significant proinflammatory role in the progression of acute pancreatitis (Sharif et al. 2009). Furthermore, in a mouse model of pancreatic cancer, TLR7 ligation accelerated tumor progression and induced STAT3 activation, whereas mice lacking TLR7 exclusively within their inflammatory cells were protected from neoplasia (Ochi et al. 2012).

Taste receptor 2 member 38 (T2R38) belongs to the family of bitter receptors and was initially detected in cells of the oral cavity. T2R38 is also expressed in pancreatic cancer cells and a quorum sensing molecule of *Pseudomonas aeruginosa* is the only known natural ligand for T2R38 (Gaida et al. 2016). Activation of T2R38 has

been linked to phosphorylation of the MAP kinases p38 and ERK1/2 and increased expression of the multi-drug resistance protein 1 (also known as ABCB1), a transmembrane transporter molecule, participating in shuttling of a plethora of drugs, such as chemotherapeutics or antibiotics. T2R38 can be stimulated by a bacteria--derived signaling molecule and that could represent another pattern of linkage between microbiota and PDAC (Gaida et al. 2016).

Japanese results derived from a database of 283 patients with PDAC revealed an 8.8% detection rate of *Fusobacterium* species in pancreatic cancers (Mitsuhashi et al. 2015). Tumor *Fusobacterium* status was not associated with any clinical and molecular features but with significantly higher cancer-specific mortality rates. Therefore, tumor *Fusobacterium* species status was independently associated with a worse prognosis of PDAC, suggesting that *Fusobacterium* species may be a prognostic biomarker (Mitsuhashi et al. 2015). Riquelme et al. found higher alpha diversity in the tumor microbiome of long-term surviving PDAC patients and identified an intra-tumoral microbiome signature (*Pseudoxanthomonas-Streptomyces--Saccharopolyspora-Bacillus clausii*) highly predictive of long-term survivorship (Riquelme et al. 2019). They were also able to differentially modulate the tumor microbiome via human-into-mice fecal microbiota transplantation (FMT) and affect tumor growth as well as tumor immune infiltration. Their study demonstrated that PDAC microbiome composition, which cross-talks to the gut microbiome, influences the host immune response and natural history of the disease (Riquelme et al. 2019).

4.8 Nondigestive System Cancers

4.8.1 Breast Cancer

Several studies have demonstrated that the gut microbiome of patients with breast cancer is altered relative to that of healthy matched controls (Chen et al. 2019; Goedert et al. 2015). An increasing amount of evidence also implicates involvement of the microbiome environment in the metabolism of estrogen, which has a strong correlation with breast cancer development. One study showed that patients that received ampicillin had increased fecal excretion of conjugated estrogens, emphasizing the active involvement of the gut microbiota in estrogen metabolism (Adlercreutz et al. 1976). This suggests gut microbes may be involved in the metabolism of estrogen; thus microbiome modification may affect breast cancer pathogenesis. In addition, sex hormones can also impact the gut microbiome composition (Org et al. 2016).

A population-based case-control study showed that postmenopausal women with breast cancer had altered composition and estrogen-independent low diversity of their gut microbiota (Goedert et al. 2015). Xuan et al. reported that the bacterium *Methylobacterium radiotolerans* is relatively enriched in tumor tissue, while the bacterium *Sphingomonas yanoikuyae* is relatively enriched in paired normal tissue. The relative abundances of these two bacterial species were inversely correlated in

paired normal breast tissue but not in tumor tissue, indicating that dysbiosis is associated with breast cancer (Xuan et al. 2014). Furthermore, the total bacterial DNA load was reduced in tumor versus paired normal and healthy breast tissue and the bacterial DNA load correlated inversely with advanced disease, a finding that could have broad implications in diagnosis and staging of breast cancer. Those data indicate that microbial DNA is present in the breast and that bacteria or their components may influence the local immune microenvironment (Xuan et al. 2014).

A study comparing the microbial composition of nipple aspirate fluid in women with a history of breast cancer versus normal controls demonstrated a relatively higher incidence of the genus *Alistipes* and lower incidence of a genus from the *Sphingomonadaceae* family (Chan et al. 2016). Other studies demonstrate the microbiome of breast skin swabs and breast tissue from patients with breast cancer relative to health controls is enriched in particular microbes, including *Fusobacterium, Atopobium, Gluconacetobacter, Hydrogenophaga, Bacillus, Enterobacteriaceae, Staphylococcus, Comamonadaceae,* and *Bacteroidetes* (Urbaniak et al. 2016).

4.8.2 Lung Cancer

Lung cancer (LC) is one of the most serious malignant tumors, which has the fastest growing morbidity and mortality worldwide. A role of the lung microbiota in LC pathogenesis has been analyzed, but a comparable role of the gut microbiota has not yet been investigated. So, a recent study has determined that the oral microorganisms *Veillonella* and *Capnocytophaga* were found to be significantly higher in the saliva samples of lung cancer patients and that this may be used as a biomarker for early detection of lung cancer (Yan et al. 2015). Another study by Greathouse et al. examined the presence of a lung tissue microbiome in 33 patients without lung cancer and 142 patients with lung cancer and found a distinct lung microbiome in patients with lung cancer (Zhang et al. 2008).

In regard to gut microbiome, Zhuang et al. found that there was no decrease in significant microbial diversity (alpha diversity) in LC patients compared to controls, while the composition (beta diversity) differed significantly between patients and controls (Zhuang et al. 2019). Controls had a higher abundance of the bacterial phylum *Actinobacteria* and genus *Bifidobacterium*, while patients with LC showed elevated levels of *Enterococcus.* These bacteria were found as possible biomarkers for LC. A decline of normal function of the gut microbiome in LC patients was also observed (Zhuang et al. 2019). Zheng et al. also found that LC patients displayed a significant shift of microbiota composition in contrast to the healthy population. In order to identify an optimal microbiota signature for noninvasive diagnosis purpose, they came up with a predictive model with 13 OTU-based biomarkers, which achieved a high accuracy in LC prediction (AUC = 97.6%) (Zheng et al. 2020). Their study uncovered the microbiota spectrum of lung cancer patients and established the specific gut microbial signature for the potential prediction of the early--stage lung cancer (Zheng et al. 2020).

4.9 Gut Microbiota and Therapeutic Implications

4.9.1 Chemotherapy

Gut microbiota may influence responses to chemotherapy and may also affect treatment-associated toxicity (Helmink et al. 2019). Chemotherapy-induced gastrointestinal toxicity (CIGT) involves a constellation of cancer treatment-related adverse events and occurs in up to 80% of all patients undergoing cancer treatment (Secombe et al. 2018). It is believed the gut microbiome and its interactions with the host's innate immune system plays a key role in the development of this toxicity and potentially other cancer-related toxicities. The immune system controls composition and compartmentalization of the microbiome, the microbiome affects development of antigen-presenting cells, and finally, the NLRP6 inflammasome orchestrates the colonic host-microbiome interface. These processes even call into question the role of pretreatment risk factors in the development of CIGT (Secombe et al. 2018).

Rigby et al. showed the role of gastrointestinal bacteria in mediating doxorubicin--induced gastrointestinal damage by showing that GF mice did not display the changes in crypt depth and proliferative cell numbers that conventional mice treated with doxorubicin showed (Rigby et al. 2016). Preclinical studies on animal models have shown a decrease in commensal species after chemotherapy, which may lead to reduced protective effects and decreased resistance to pathogenic colonization, a phenomenon mainly attributed to an increase of the inflammation-provoking, LPS--producing Gram-negative species (Secombe et al. 2018).

Irinotecan is linked with severe mucositis and diarrhea, the mechanisms of which remain poorly understood. Bacterial beta-glucuronidase is thought to be involved in the metabolism of irinotecan, implicating the intestinal flora, while intestinal mucins may also be implicated in the development of chemotherapy-induced diarrhea (Stringer et al. 2009). In an animal model with rats treated with irinotecan, among other observations, Stringer et al. detected modifications of the intestinal flora profile, especially *E. coli*, and an increase in the expression of beta-glucuronidase. They concluded that irinotecan-induced diarrhea may be caused by an increase in some beta-glucuronidase-producing bacteria, especially *E. coli*, exacerbating the toxicity of active metabolites (Stringer et al. 2009). In a subsequent rat study with intraperitoneal chemotherapy agents injection, Forsgård et al. found that irinotecan increased the relative abundance of *Fusobacteria* and *Proteobacteria*, while 5-FU and oxaliplatin caused only minor changes in the composition of fecal microbiota (Forsgård et al. 2017). All chemotherapeutics increased the levels of serum fatty acids and $N(CH_3)_3$ moieties and decreased the levels of Krebs cycle metabolites and free amino acids. They concluded that chemotherapy induced several microbial and metabolic changes, which may play a role in the pathophysiology of CIGT (Forsgård et al. 2017).

A number of clinical studies on chemotherapy-treated patients have replicated the adverse results shown in animal studies (Stringer et al. 2013; Vliet et al. 2009; Zwielehner et al. 2011; Montassier et al. 2014; Nam et al. 2013; Flórez et al. 2016; Kong et al. 2019) and are summarized in Table 4.2. Human studies have supported

Table 4.2 Human studies investigating the effects of chemotherapy on gut microbiota composition

Author (year)	No of patients	Chemotherapy	Microbiota increases	Microbiota decreases
Stringer et al. (2013)	16	Various chemotherapies	*E. coli* and *Staphylococcus* spp.	*Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides* spp., and *Enterococcus* spp.
Vliet et al. (2009)	9	Various chemotherapies	Enterococci (100-fold)	Anaerobic bacteria (10,000-fold). Commensal species (*Bacteroides* spp., *Clostridium* cluster XIVa, *Faecalibacterium prausnitzii* and *Bifidobacterium* spp., 3000–6000-fold)
Zwielehner et al. (2011)	17	Various chemotherapies	Bacteroides (2%), *Clostridium* cluster IV (2%)	Bifidobacteria (0.9%) and *Clostridium* cluster XIVa (22% to 19%)
Montassier et al. (2014)	8	Carmustine, etoposide, aracytine, and melphalan	Bacteroidetes (32%), Proteobacteria (14%) ($p = 0.008$)	Firmicutes (56%) and Actinobacteria (5%) ($p = 0.008$)
Nam et al. (2013)	9	Pelvic radiotherapy (concurrent chemotherapy in subset of patients)	*Fusobacteriaceae* (sixfold) and *Streptococcaceae* ($p < 0.05$)	Firmicutes (10%)
Flórez et al. (2016)	NR	Doxorubicin, afatinib, 5-fluorouracil, gemcitabine, and pemetrexed		*Lactobacillus* spp., *Bifidobacterium* spp.
Kong et al. (2019)	43	Capecitabine, oxaliplatine	Bacteroidetes, Bilophila, Comamonas, Collinsella, Butyricimonas, Eggerthella, Anaerostipes, Sellimonas (*Lachnospiraceae* genus)	Firmicutes, Morganella, Pyramidobacter, Proteus, Escherichia-Shigella

the decrease in total bacteria counts and their diversity after chemotherapy. Whether the patient's gut microbiome profile precancer treatment could also predict toxicity severity is largely unknown, particularly in the setting of chemotherapy-induced damage. Although no study has investigated this after chemotherapy, one study on pelvic radiotherapy patients indicated that patients who suffered diarrhea had lower bacterial diversity and a higher *Firmicutes/Bacteroidetes* ratio (Wang et al. 2015).

Another study used the novel method of an electronic nose and the Field Asymmetric Ion Mobility Spectrometry method for analysis of pre-radiotherapy stool samples, gases, and microbiota fermentation by-products (Covington et al. 2012). Patients who suffered from gastrointestinal toxicity were successfully separated from those who did not and, by this way, a clinically applicable test was proposed for future cancer treatment planning (Covington et al. 2012).

A β-glucuronidase inhibitor may be particularly useful for patients undergoing irinotecan treatment. SN-38, the active form of irinotecan, is conjugated in the liver to a less toxic metabolite, SN-38G, which is excreted to the gastrointestinal tract via bile and is hydrolyzed back to the toxic SN-38 form by microbe-derived β-glucuronidase (Secombe et al. 2018).

Although dysbiosis of gut microbes is often linked to aberrant immune responses and abnormal production of inflammatory cytokines, commensal bacteria may also have protective effects on the integrity of the gastrointestinal mucosal barrier, including interactions with tight junctions and regulation of mucous layer. On the other hand, the concept that bacteria or their products have a therapeutic part to play in cancer is not novel. In 1891, Coley used the toxins from *Streptococcus erysipelas* and *Bacillus prodigiosus* (now referred to as *Serratia marcescens*) to treat sarcoma, and mycobacteria are still used in the treatment of bladder cancer (Coley 1906; Lamm et al. 2014). Nowadays, though, "pharmacomicrobiomics" opens new avenues to an age in which the entire ecology of the gut could be targeted to influence therapeutic efficacy, in such a way that the gut microbiota will be central to the future of personalized cancer treatment strategies (Alexander et al. 2017). In this context, Alexander et al. proposed the "TIMER" mechanistic framework to explain how gut bacteria influence chemotherapy effects on the host: Translocation, Immunomodulation, Metabolism, Enzymatic degradation, and Reduced diversity and ecological variation (Alexander et al. 2017). Dietary modifications, probiotics, and synthetically engineered bacteria are anticipated as potential gut microbiota manipulating tools (Alexander et al. 2017).

Commensal bacteria are able to induce CD4+ T cell differentiation. *B. fragilis* can induce the development of a systemic Th1 response through polysaccharide A molecules and is decreased by chemotherapy (Lin et al. 2012). The post-chemotherapy decreased ability to mount a Th1 response may affect the severity of CIGT. Treatment with cyclophosphamide was found to trigger the translocation of several Gram-positive bacteria to the secondary lymphoid organs; this translocation was required for the promotion of antitumoral Th1 and Th17 responses via a MyD88-dependent pathway, as GF mice and mice co-treated with antibiotics in addition to cyclophosphamide displayed larger tumors than mice with intact microbiota (Viaud et al. 2013). Likewise, the antitumoral response following treatment with the ROS-inducing oxaliplatin was similarly reliant on functional MyD88 signaling triggered by microbes, as GF mice, mice treated with antibiotics, and MyD88$^{-/-}$ mice did not demonstrate successful tumor regression (Poutahidis and Erdman 2016). A critical point of these studies is that antibiotics, which may be required during the course of a cancer patient treatment, should be administered with caution due to the dependence of various cancer drugs on a functioning microbiota.

4.9.2 Immunotherapy

Cancer immunotherapy has become an emerging promising anticancer treatment modality (Elkrief et al. 2018). Immune checkpoint inhibitors (ICI) function by suppressing the interaction of T lymphocyte inhibitory receptors with their ligands on malignant or myeloid cells, blocking the cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death protein 1/programmed cell death 1 ligand 1 (PD-1/PD-L1) and by this way they re-stimulate the T lymphocyte-mediated immune response against tumor-associated antigens (TAAs). ICIs have been successfully used to treat both solid cancers, such as melanoma, renal cell carcinoma, non-small cell lung cancer (NSCLC), and mismatch repair deficient CRC and hematological malignancies. Since gut microbiome plays an irreplaceable role in immunity, it may also have an important role in cancer immunotherapy. Two cardinal studies, both published in *Science* in 2015, triggered further investigative attempts to clarify the role of gut microbiota in regulating the response to immunotherapy (Sivan et al. 2015; Vetizou et al. 2015). Sivan et al. compared melanoma growth in mice harboring distinct commensal microbiota and observed differences in spontaneous antitumor immunity, which were eliminated upon cohousing or after fecal transfer. They identified *Bifidobacterium* to be associated with the antitumor effects and, actually, oral administration of *Bifidobacterium* alone improved tumor control to the same degree as programmed cell death protein 1 ligand 1 (PD-L1)-specific antibody therapy while combination treatment nearly abolished tumor growth. Augmented dendritic cell function leading to enhanced CD8(+) T cell priming and accumulation in the tumor microenvironment mediated the effect (Sivan et al. 2015). Likewise, Vétizou et al. found that the antitumor effects of CTLA-4 blockade depended on distinct *Bacteroides* species (Vetizou et al. 2015). T cell responses specific for *B. thetaiotaomicron* or *B. fragilis* were associated with the efficacy of CTLA-4 blockade. Tumors in antibiotic-treated or GF mice did not respond to CTLA blockade. This defect was overcome by gavage with *B. fragilis*, by immunization with *B. fragilis* polysaccharides, or by adoptive transfer of *B. fragilis*-specific T cells (Vetizou et al. 2015).

Studying 26 patients with metastatic melanoma, treated with ipilimumab, an ICI targeting CTLA-4, Chaput et al. concluded that a distinct baseline gut microbiota composition was associated with both clinical response and ipilimumab-related colitis (Chaput et al. 2017). Specifically, baseline gut microbiota enriched with *Faecalibacterium* and other *Firmicutes* was associated with beneficial clinical response to ipilimumab (i.e., longer progression-free and overall survival) and more frequent occurrence of ipilimumab-induced colitis (Chaput et al. 2017). Routy et al. have also suggested that the primary resistance to ICIs targeting the PD-1/PD-L1 axis may be attributed to abnormal gut microbiome composition (Routy et al. 2017). Antibiotics inhibited the clinical benefit of ICIs in patients with advanced cancer. Fecal microbiota transplantation (FMT) from cancer patients who responded to ICIs into GF or antibiotic-treated mice ameliorated the antitumor effects of PD-1 blockade, whereas FMT from nonresponding patients failed to do so (Routy et al. 2017). Correlations between clinical responses to ICIs and the relative abundance of

Akkermansia muciniphila (A. muciniphila) were also revealed, whereas oral supplementation with *A. muciniphila* after FMT with non-responder feces restored the efficacy of PD-1 blockade in an interleukin-12-dependent manner by increasing the recruitment of CCR9 + CXCR3 + CD4+ T lymphocytes into mouse tumor beds (Routy et al. 2017). Gopalakrishnan et al. further confirmed the important role of gut microbiota in anti-PD-1 immunotherapy by showing significantly higher alpha diversity and relative abundance of bacteria of the *Ruminococcaceae* family in responding patients (Gopalakrishnan et al. 2018b). In metagenomic studies, they revealed functional differences in gut bacteria in responders, including enrichment of anabolic pathways. Immune profiling suggested enhanced systemic and antitumor immunity in responding patients with a favorable gut microbiome as well as in GF mice receiving FMT from responding patients (Gopalakrishnan et al. 2018b). Jin et al. studied 37 patients with advanced NSCLC receiving treatment with nivolumab, an anti-PD-1 ICI, and found that responding patients harbored higher diversity of gut microbiome at the starting point with stable composition during the treatment (Jin et al. 2019). Patients with high microbiome diversity had significantly prolonged progression-free survival when compared to those with low diversity. Compositional difference was also observed with enrichment of *Alistipes putredinis, Bifidobacterium longum,* and *Prevotella copri* in responders and *Ruminococcus* to be enriched in nonresponding patients (Jin et al. 2019). Analysis of systemic immune responses revealed that patients with a high abundance of microbiome diversity in the gut had a greater frequency of unique memory CD8+ T cell and natural killer cell subsets in the periphery in response to anti-PD-1 therapy (Jin et al. 2019). Knowledge derived from published series on the link between gut microbiota diversity and composition and ICIs with potential implicated mechanisms is summarized in Table 4.3.

Table 4.3 Published series on the link between gut microbiota diversity and composition and immune checkpoint inhibitors with potential implicated mechanisms

Deleterious microbiota			Favorable microbiota	
Low diversity			High diversity	
Cancer		Mechanism		Mechanism
NSCLC RCC	• Parabacteroides distasonis • Clostridiales bacterium VE202-14	Unknown	• Akkermansia muciniphila • Alistipes indistinctus • Ruminococcus spp	• Tumor: increased CD4+ CCR9+, decreased Tregs • Peripheral blood: increased INFγ production of CD4+ and CD8+
Melanoma	• Roseburia intestinalis • Bacteroidales	Decreased intratumor CD8+	• Collinsella aerofaciens • Bifidobacterium longum • Faecalibacterium	• Tumor: increased CD8+ Peripheral blood: increased INFγ, increased CD8+, decreased Tregs

4.9.3 Prebiotics, Probiotics, And Synbiotics

The World Health Organization (WHO) defines "prebiotics" as "a non-viable food component that confers health benefit(s) on the host associated with modulation of the microbiota" (Meng et al. 2018; Pandey et al. 2015; Peitsidou et al. 2012). An ideal prebiotic should be resistant to the acids of the stomach, bile salts and other intestinal hydrolyzing enzymes in the intestine, should not be absorbed in the upper gastrointestinal tract, and be easily fermentable by the beneficial intestinal microbiota (Pandey et al. 2015). Prebiotics form a group of diverse carbohydrate ingredients potentially acquiring positive health effects, derived from breast milk, soybeans, inulin sources (like Jerusalem artichoke, chicory roots etc.), raw oats, unrefined wheat, non-digestible carbohydrates, and nondigestible oligosaccharides (Pandey et al. 2015; Peitsidou et al. 2012; Pattananandecha et al. 2016; Nuñez-Sánchez et al. 2014; Allsopp et al. 2013; Higashimura et al. 2016; Li et al. 2015; Schlörmann et al. 2015; Miene et al. 2011; Piazzi et al. 2014; Costabile et al. 2011). Context Inulin, a nondigestible carbohydrate isolated from *Helianthus tuberosus* L. (Asteraceae), has been shown to alter the gut beneficial bacteria including *Lactobacillus* spp. and *Bifidobacteria*. Inulin also influences the activities of intestinal microbiota that could prevent the CRC development (Pattananandecha et al. 2016). Inulins significantly decrease the colonic concentration of phenol, p-cresol, and indole. In addition, reduction in the activity of microbial enzymes such as β-glucuronidase, azoreductase, and nitroreductase was observed in inulin-treated animals (Pattananandecha et al. 2016). Agaro-oligosaccharides (AGO) from seaweed show a positive effect on high-fat diet-induced gut dysbiosis. Data from the serum bile acid profile showed that the level of the gut bacteria-produced carcinogenic deoxycholic acid was increased in high-fat diet-receiving mice, but this upregulation tended to be suppressed by AGO supplementation. AGO supplementation also suppressed the azoxymethane-induced generation of aberrant crypt foci in the colon derived from high fat diet-treated mice. So, AGO appears to prevent high-fat diet--induced gut dysbiosis and may inhibit colon carcinogenesis (Higashimura et al. 2016). Polydextrose (PDX) is a complex glucose oligomer used as a sugar replacer. In a placebo-controlled, double-blind, human study, PDX was shown to significantly increase the known butyrate producer *Ruminococcus intestinalis* and bacteria of the *Clostridium clusters I, II,* and *IV* (Costabile et al. 2011). PDX was shown to be slowly degraded in the colon, and the fermentation significantly reduced the genotoxicity of the fecal water. PDX also affected bowel habits of the subjects, as less abdominal discomfort was recorded and there was a trend for less hard and more formed stools during PDX consumption. Therefore, PDX may have potential for reducing the risk factors that may be associated with colon cancer initiation (Costabile et al. 2011).

Probiotics are defined by the Food and Drug Administration (FDA) and WHO as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host." Synbiotics are combinations of prebiotics and probiotics (Meng et al. 2018; Pandey et al. 2015; Peitsidou et al. 2012). Postbiotics are functional bioactive compounds, generated as microbial fermentation components and

include many different constituents including metabolites, SCFAs, microbial cell fractions, functional proteins, extracellular polysaccharides (EPS), cell lysates, teichoic acid, peptidoglycan-derived muropeptides, and pili-type structures (Wegh et al. 2019).

Probiotic strains, such as *Bifidobacterium* and *Lactobacillus,* are present in common fermented milk products and have beneficial effects on health (Górska et al. 2019). Numerous in vitro cancer cell and in vivo animal model studies have firmly established probiotics' modulating effect on suppressing proliferation and inducing apoptosis in cancer cells (Górska et al. 2019). Probiotics exert their antitumor properties via various mechanisms. *L. acidophilus* and *B. bifidum* counteract the cytotoxic bile acid-related reduced intracolonic pH and may hold a promising role as a cancer prevention tool (Lidbeck et al. 1991). Putrefactive bacteria, such as *E. coli* and *Clostridium perfringens,* commonly inhabit the gut and produce putatively carcinogenic compounds using enzymes like b-glucuronidase, azoreductase, and nitroreductase. Since the late 1970s, Goldin and Gorbach have proven that consumption of fermented milk products had a beneficial effect on the increase in the number of *L. acidophilus* in rat's gut, resulting in a reduction of putrefactive bacteria and the deleterious enzymes (Goldin and Gorbach 1980). Ingestion of *Lactobacillus* strain by human volunteers abolished the mutagenic effect on a cooked meat-rich diet, resulting in decreased urinary and fecal excretion of heterocyclic aromatic amines (HAAs) (Hayatsu and Hayatsu 1993). A plethora of studies have demonstrated the ability of probiotic strains to bind or metabolize mutagenic compounds, such as HAAs, nitrosamines, aflatoxin B1, mycotoxins, polycyclic aromatic hydrocarbons (PAHs), and phthalic acid esters (PAEs), and others (Górska et al. 2019; Stidl et al. 2008; Duangjitcharoen et al. 2014).

As previously mentioned, SCFAs, except for their principal function as an energy source for colonocytes, act as signaling molecules affecting the immune system cell proliferation and apoptosis, are involved in the intestinal hormone production and lipogenesis, and play a crucial role in the maintenance of epithelial integrity (Requena et al. 2018). Lactic acid bacteria are not directly involved in SCFA production, but probiotic strains of *Bifidobacteria* and *Lactobacilli* can modulate the gut microbiota composition and consequently affect the production of SCFA. Butyrate, produced by species belonging to the *Firmicutes* families (*Ruminococcaceae, Lachnospiraceae,* and *Clostridiaceae*), promoted apoptosis and inhibited proliferation in cancer cells cultured in vitro (Fotiadis et al. 2008). Administration of the bacterial strain *Butyrivibrio fibrisolvens MDT-1*, which is known for their high production of butyrate in a CRC mouse model, inhibited progression of tumor development, affecting also the reduction of β-glucuronidase and increasing the immune response (Ohkawara et al. 2005). An AZO-induced CRC mice model treated by the probiotic mix composed of seven different strains of *Lactobacilli, Bifidobacteria*, and streptococcus colon carcinogenesis was suppressed due to modulation of mucosal CD4+ T polarization and changes in the genes' expression (Bassaganya-Riera et al. 2012). *B. infantis* administration in a CRC rat model demonstrated a considerable attenuation of chemotherapy-induced intestinal mucositis correlated with decreased level on proinflammatory cytokines

(IL-6, IL-1β, TNF-α) and increased CD4+ CD25+ Foxp3+ Treg cell response (Mi et al. 2017). A probiotic cocktail, comprising *Lactobacillus acidophilus, Bifidobacteria bifidum, and Bifidobacteria infantum (LBB),* enriched with oligo--fructose and maltodextrin, decreases the counts of the species of *Pseudomonas, Congregibacter, Clostridium, Escherichia,* and *Helicobacter,* while increasing the counts of *Lactobacillus* in CRC (Kuugbee et al. 2016). Probiotic Prohep [a mixture of *Lactobacillus rhamnosus GG [LGG], E. coli Nissle 1917 [EcN],* and heat inactivated *VSL#3* (probiotic medical food [1:1:1])] decreases the growth of HCC significantly by inhibiting angiogenesis and inflammation. It has been shown that the population of gut microbiota shifts to specific bacteria, such as *Prevotella* and *Oscillibacter,* creating favorable anti-inflammatory products. Prohep administration helps downregulate the proinflammatory Th17 frequency and the production of IL-1, inhibits angiogenesis, and promotes the differentiation of anti-inflammatory Treg cells in the gut (Li et al. 2016). Several human studies are under way scoping to elucidate the role of probiotics and synbiotics supplementation in cancer patients (Helmink et al. 2019; Vivarelli et al. 2019). Nevertheless, already published studies have investigated their efficacy in human malignancies and are summarized in Table 4.4 (Österlund et al. 2007; Wada et al. 2009; Chitapanarux et al. 2010; Giralt et al. 2008; Gianotti 2010; Demers et al. 2014; Mego et al. 2015; Theodoropoulos et al. 2016; Consoli et al. 2016; Hibberd et al. 2017; Flesch et al. 2017; Tian et al. 2019).

4.9.4 Fecal Microbiota Transplantation

Fecal microbiota transplantation (FMT) was originally used almost 2000 years ago, when Chinese researchers orally administered "yellow soup," a slurry of stool from a healthy individual, to patients to cure them of severe diarrhea (Helmink et al. 2019; Chen et al. 2018). This approach was also used in Africa during World War II, when German soldiers and nomads in the region reportedly used camel stool as treatment for severe dysentery (Helmink et al. 2019). FMT was firstly reported to treat severe pseudomembranous enterocolitis by Eiseman in 1958 (Strada et al. 1983). Nevertheless, this practice was less used until the first documented case of *Clostridium difficile* infection (CDI) treated with FMT was reported in 1983 by Schwan and, currently, FMT has been approved as a clinical method for treating recurrent CDI by 2013 guidelines, with its clinical effectiveness to reach 90% (Schwan 1983; Surawicz et al. 2013). FMT may be beneficial for the treatment of IBD and functional bowel disorders (Chen et al. 2018). Based on the intestinal dysbiosis role on carcinogenesis, FMT may prove beneficial in the management of cancer (Chen et al. 2018). FMT may be delivered via a number of different routes, such as through colonoscopy, enema, or oral administration, either via nasogastric or oral capsules.

Cao et al. identified the role of intestinal dysbiosis induced by deoxycholic acid in the development of CRC, and they demonstrated that the transfer of feces from deoxycholic acid-treated mice increased intestinal tumor development compared to

Table 4.4 Published studies on the effects of pro- and synbiotics on human malignancies

Author (year)	Cancer	Adjuvant treatment	No of patients	Pro-/synbiotics regimens	Objectives of study	Results
Österlund et al. (2007)	CRC	5-FU-based regimens	150	Lactobacillus rhamnosus GG supplementation ($1–2•10^{10}$ per day) and fiber (11 g guar gum per day) during chemotherapy	To compare two 5-FU-based regimens and the effect of Lactobacillus and fiber supplementation on treatment tolerability	Lactobacillus GG supplementation is well tolerated and may reduce the frequency of severe diarrhea and abdominal discomfort related to 5-FU-based chemotherapy
Wada et al. (2009)	Pediatric malignancies	Various	42	Bifidobacterium breve strain Yakult (BBG-01), 10^9 freeze-dried, living BBG-01, cornstarch, and hydroxypropyl cellulose in a 1-g preparation, starting 2 weeks prior to the first day of chemotherapy and continued for 6 weeks	To evaluate the effects of administration of the probiotic on its ability to prevent infection, fecal microflora, and intestinal environments in pediatric cancer patients on chemotherapy	Fever, use of intravenous antibiotics, habitation of anaerobes, disruption of intestinal microbiota after chemotherapy, and the increase in the population levels of Enterobacteriaceae were lower in the probiotic group than the placebo group. The concentrations of total organic acids were maintained at the normal level, which constantly maintained the pH below 7.0 only in the probiotic group
Chitapanarux et al. (2010)	Cervical cancer	External beam whole pelvis radiotherapy and brachytherapy plus weekly cisplatin 40 mg/m^2	63	2×10^9 units of a Lactobacillus acidophilus plus Bifidobacterium bifidum (equivalent to 2 capsules) two times a day, beginning 7 days before starting radiotherapy and continuing every day during radiotherapy	To reduce the incidence of diarrhea and the need for antidiarrheal medication	Grade 2–3 diarrhea was observed in 45% of the placebo group ($n = 31$) and 9% of the study drug group ($n = 32$) ($p = 0.002$). Antidiarrheal medication use was significantly reduced in the placebo group ($p = 0.03$). The patients in the study drug group had a significantly improved stool consistency ($p < 0.001$)

(continued)

Table 4.4 (continued)

Author (year)	Cancer	Adjuvant treatment	No of patients	Pro-/synbiotics regimens	Objectives of study	Results
Giralt et al. (2008)	Cervical cancer, endometrial cancer	Pelvic radiotherapy (45–50 Gy, conventional fractionation)for cervical carcinoma (radiotherapy and weekly cisplatin) or endometrial adenocarcinoma (postoperative radiotherapy)	85	96 mL three times daily of a fermented liquid yogurt containing approximately 10^8 CFU/g of Lactobacillus casei DN-114001, in addition to Streptococcus thermophilus and Lactobacillus delbrueckii, subsp. bulgaricus	To reduce the incidence of diarrhea and the need for antidiarrheal medication	No significant differences between probiotic and placebo groups in terms of diarrhea incidence. Probiotic intervention had a significant effect on stool consistency ($p = 0.04$)
Gianotti (2010)	Colorectal cancer	No	31	Placebo (group A, $n = 10$), or a dose of 107 of a mixture of Bifidobacterium longum (BB536) and Lactobacillus johnsonii (La1) (group B, $n = 11$), or the same mixture at a concentration of 109 (group C, $n = 10$). Treatment continuation from day 2 to day 4	Stools were collected before treatment, during surgery (day 0) and 5 days after operation. During the operation, colonic mucosa samples were harvested to evaluate bacterial adherence and to assess the phenotype of dendritic cells (DCs), lymphocyte subsets by surface antigen, and the presence of BB536 and La1 expression	At day 0, La1 was present in 60% patients in group C, in 27.2% in group B, and none in the placebo group ($p = 0.02$, C vs A). The rate of mucosal colonization by enterobacteriaceae was 30% in C, 81.8% in B, and 70% in A ($p = 0.03$, C vs B). Greater expression of CD3, CD4, CD8, and naive and memory lymphocyte subsets in group C than in group A with a dose response trend (C > B > A). Treatment did not affect DCs phenotype or activation

Demers et al. (2014)	Prostatic, gynecology, rectal cancers	Pelvic radiotherapy for a minimum of 40 Gy, with or without chemotherapy	229	Patients were randomized between a placebo and either of two regimens of double strain Bifilact probiotics (Lactobacillus acidophilus LAC-361 and Bifidobacterium longum BB-536): a standard dose twice a day (1.3 billion CFU) or a high dose three times a day (10 billion CFU), starting on the first day and ended on the last day of radiation	To compare the times to first appearance of grade > 2 diarrhea	The difference between the groups for overall grade > 2 diarrhea was not statistically significant. At 60 days, the proportion of patients without moderate and severe diarrhea in the standard dose group (35%) was more than twice as high as that of the placebo group (17%) with a hazard ratio of 0.69 ($p = 0.04$). In patients who had surgery, the standard probiotics dose group had a better proportion of patients without very severe diarrhea than the placebo group (97% vs 74%, $p = 0.03$)

(continued)

Table 4.4 (continued)

Author (year)	Cancer	Adjuvant treatment	No of patients	Pro-/synbiotics regimens	Objectives of study	Results
Mego et al. (2015)	Colorectal cancer	Irinotecan-based chemotherapy	46	Probiotic formula Colon Dophilus™ containing 10 × 10 CFU of bacteria (Bifidobacterium breve HA-129, Bifidobacterium bifidum HA-132 HA, Bifidobacterium longum HA-135, Lactobacillus rhamnosus HA-111, Lactobacillus acidophilus HA-122, Lactobacillus casei HA-108, Lactobacillus plantarum HA-119, Streptococcus thermophilus HA-110, Lactobacillus brevis HA-112, Bifidobacterium infantis HA-116, inulin, maltodextrin, magnesium stearate, ascorbic acid) for 12 weeks	To determine the effectiveness of the probiotics in the prevention of irinotecan-induced diarrhea	Probiotics compared to placebo led to a reduction in the incidence of severe diarrhea (0% vs 17.4%, $p = 0.11$), reduction of the overall incidence of diarrhea (39.1% vs 60.9%, $p = 0.24$), and incidence of enterocolitis (0% vs 8.7%). Patients on probiotics used less antidiarrheal drugs

Theodoropoulos et al. (2016)	Colorectal cancer	Not specified	75	Synbiotic Forte™, containing 4 lactic acid bacteria: Pediococcus pentosaceus 5-33:3, Leuconostoc mesenteroides 32-77:1, Lactobacillus paracasei ssp. paracasei 19, and Lactobacillus plantarum 2362, and 2.5 g of each of the 4 fermentable fibers (prebiotics): b-glucan, inulin, pectin, and resistant starch, starting at day 2–4 and for 15 days after surgery	Primary endpoints were Gastro-Intestinal Quality of Life Index (GIQLI) questionnaire assessments at 1, 3, and 6 months postoperatively	Patients under synbiotics had a better GIQLI "Global score" compared with those who received placebo at 1, 3, and 6 months ($p = 0.01$) and less diarrhea ($p = 0.04$)
Consoli et al. (2016)	Colorectal cancer	No	33	Once-daily oral lyophilized yeast capsule with 100 mg (0.5×10^9 CFU/g) of S boulardii, starting 7 days prior to surgery to the day of surgery	To measure levels of inflammatory cytokine messenger RNA (mRNA) in surgical samples of intestinal mucosal tissues	Patients who received probiotics had significantly lower mucosal IL-1β, IL-10, and IL-23A mRNA levels than the control group, but no significant differences at postoperative infections
Hibberd et al. (2017)	Colorectal cancer	No	15	Two ProBion Clinica tablets, yielding a daily dose of 1.4×10^{10} CFUs Bifidobacterium lactis Bl-04, 7×10^9 CFUs Lactobacillus acidophilus NCFM, and 0.63 g inulin, for an average of 31 ± 28 days	To investigate the potential to modify the colonic microbiota with probiotics	Patients that received probiotics had an increased abundance of butyrate-producing bacteria, especially Faecalibacterium and Clostridiales spp. in the tumor, nontumor mucosa, and fecal microbiota. CRC-associated genera such as Fusobacterium and Peptostreptococcus tended to be reduced in the fecal microbiota of patients that received probiotics

(continued)

Table 4.4 (continued)

Author (year)	Cancer	Adjuvant treatment	No of patients	Pro-/synbiotics regimens	Objectives of study	Results
Flesch et al. (2017)	Colorectal cancer	No	91	Sachets containing Lactobacillus acidophilus NCFM (10^9), Lactobacillus rhamnosus HN001 (10^9), Lactobacillus paracasei LPC-37 (10^9), Bifidobacterium lactis HN019 (10^9), and fructo--oligosaccharides (FOS) 6 g, for 5 days before surgery and for 14 days after surgery	To evaluate the effect of perioperative administration of synbiotics on the incidence of surgical wound infection	Surgical site infection occurred in 1 (2%) patient in the synbiotics group and in nine (21.4%) patients in the control group ($p = 0.002$). There were three cases of intra-abdominal abscess and four cases of pneumonia in the control group, and no infections in patients receiving synbiotics ($p = 0.001$)
Tian et al. (2019)	Lung cancer	Not specified	41	Three Clostridium butyricum (420 mg/tablet) tablets, 3 times per day, for 3 weeks	To investigate the role of Clostridium butyricum in patients undergoing chemotherapy	Chemotherapy-induced diarrhea was lower in the Clostridium butyricum (CB) group compared with the placebo group. Neutrophil/lymphocyte ratio (NLR) and platelet/lymphocyte (PLR) decreased within the CB group. At week 3, the lymphocyte/monocyte ratio (LMR) was higher in the CB group compared with the placebo group. CB group had notable increase in beneficial flora, including the Clostridium and Lactobacillus genera

untreated donor (Cao et al. 2017a). Moreover, Rosshart et al. reported that laboratory mice transplanted with intestinal microbiomes from wild mice showed better resistance to CRC and amelioration of inflammation, compared to control mice of their own bacteria, supporting the assumption that FMT could harbor a potential therapeutic ability for CRC (Rosshart et al. 2017). FMT improved high-fat diet--induced liver injury and lipid metabolism along with increased gut microbiota diversity in mice, and FMT from donor mice resistant to alcoholic liver disease could prevent alcohol-induced liver injury (Minicis et al. 2014). A recent study of patients with severe alcoholic hepatitis showed that FMT was associated with increased survival and ascites resolution (Llopis et al. 2015).

The transfer of feces harvested from ICI-responding melanoma patients into mice established that FMT could enhance the effectiveness of immunotherapy to optimize the current therapies (Gopalakrishnan et al. 2018b). A clinical study is currently investigating the effect of FMT from PD-1 responders into intestinal tracts of non-responders in melanoma (Mullard 2018). Thus, FMT seems to be promising in enhancing antitumor immunity in melanoma patients by transferring a favorable gut microbiota (Strada et al. 1983). FMT from irradiated mice to GF mice exposed to radiation resulted in more severe radiation damage, compared to mice transplanted with naïve microbiota (Gerassy-Vainberg et al. 2017). Interestingly, transplantation of fecal microbiota from healthy mice significantly alleviated radiation-induced gastrointestinal syndrome and improved the survival rate of irradiated mice (Cui et al. 2017). Therefore, FMT might be employed as a radioprotector in tumor radiotherapy to improve the prognosis (Chen et al. 2018).

Potential risks of FMT include transmission of pathogens, particularly to immunocompromised patients, transmission of recessive elements silent in healthy donors, and transmission of other factors accounting for chronic diseases, i.e., although controversial, a case report suggested transmission of obesity to a patient (Chen et al. 2018). Among future desirable developments it is the combination of FMT with fecal DNA testing for accuracy in CRC screening, as well as a transition from whole microbiome transplant to more precise combinations of microbes.

4.9.5 Antibiotics

There is conflicting data about the association between antibiotics and risk of cancer. Couturier-Maillard et al. showed that NOD2-mediated dysbiosis, predisposing mice to transmissible colitis and CRC, was improved by treatment with antibiotics or an anti-interleukin-6 receptor-neutralizing antibody (Couturier-Maillard et al. 2013). Antibiotic administration during the primary inflammation stage can inhibit the initiation of carcinogenesis in an animal colonic cancer model (Zackular et al. 2013). Oral administration of metronidazole could reduce Fusobacterium load and colorectal tumor growth in mice bearing a colon cancer xenograft (Bullman et al. 2017). Moreover, antibiotic use could clear biofilms and eliminate microbial sulfide, and thereby protect the colon mucous barrier and prevent epithelial hyperproliferation (Ijssennagger et al. 2015). Assuming that ETBF promotes the development

of IBD as well as IL-17A-dependent CRC, DeStefano Shields et al. established an ETBF clearance mouse model by cefoxitin administration (Shields et al. 2016). They found that the expression of the mucosal IL-17A was inhibited with cefoxitin treatment and the ETBF clearance prohibited colon adenoma formation and IL-17A--dependent tumorigenesis (Shields et al. 2016).

However, the effects of antibiotics are two-sided, and antibiotic exposure may induce cancers as well. Long-term antibiotic use was highly correlated with increased colorectal tumor progression in the genetic mouse model for human adenomatous polyposis $Apc^{Min/+}$ (Kaur et al. 2018). Long-term antibiotic use in early--to-middle adulthood was associated with increased risk of colorectal adenoma in a large population study (Cao et al. 2017b). A nested case-control investigation has demonstrated a link between the exposure of penicillin and the high risks of esophageal, gastric, and pancreatic cancers (Boursi et al. 2015). Another recent nested case-control study on liver cancer has also shown a trend to increased risk of liver cancer in cases receiving antibiotic therapy, compared to the cases without antibiotic therapy. However, it was uncertain whether the dose of antibiotics was correlated to the risk of liver cancer (Yang et al. 2016). Further investigations are required to elucidate the impact of antibiotic exposures on outcomes in cancer patients and the underlying mechanisms.

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Gut Microbiome, Diabetes, and Obesity: Complex Interplay of Physiology

5

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Abstract

Diabesity is a common denominator of mortality and morbidity, due to noncommunicable diseases, and it affects populations of different ages, race, and sex. Low-grade, subclinical inflammation derives from diabesity, resulting in deterioration of health status. Human gut microbiome, consisting of bacteriome, virome, and mycobiome, is a complex and significant human organ, participating in dynamic operations of immunity, metabolism, and, thus, inflammation of its host. According to a decade of various studies, human gut microbiome composition disruption (gut dysbiosis) is a major contributor to the onset of metabolic disorders. In this chapter, we gathered evidence to shed light on the complicated interrelations of gut microbiome, diabetes, and obesity, assessing the current literature and suggesting novel concepts and methodologies for future studies.

Keywords

Diabetes · Obesity · Dysbiosis · Human gut microbiome · Subclinical inflammation · Normobiosis · Virome · Mycobiome

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5.1 Introduction

Diabetes and obesity represent interrelated disorders, entailing dysfunction of glucose metabolism, associated with either hyper- or hypoinsulinemia. These disorders affect, over the human lifespan, a significant percentage of the human population in a worldwide range, including patients of all ages. They are two of the most prevalent health problems and leading causes of death globally. Their physiopathologic interconnection is expressed by the term *diabesity* (Bluher 2019; Bhupathiraju and Hu 2016).

Body mass index (BMI) was developed in the nineteenth century to reflect body fat percentage. It is a simple and widely used method for estimating body fat mass. Obesity is defined as the increase of BMI, a state, in which accumulated excess body fat may have adverse effects on health. In pediatrics, the presence of obesity is determined via an age- and sex-specific percentile for BMI, after the age of 2 years of life, rather than via BMI categories employed in adulthood. BMI during childhood changes dramatically with age. In full detail, during childhood and adolescence, a BMI equal, or above the 85th percentile for children and adolescents of the same age and sex is considered to be overweight, and a BMI equal, or above the 95th percentile is considered to be obese. Therefore, BMI levels among children, and adolescents need to be expressed relatively to children of the same age, race and sex (Cote et al. 2013; Greydanus et al. 2018; Division of Nutrition PA, and Obesity, National Center for Chronic Disease Prevention and Health Promotion n.d.; Cole et al. 2000).

For adults, a BMI within the range of 25–29 represents the overweight spectrum, while a BMI $\geq$ 30 represents the obesity spectrum. The latter is subdivided into Class 1: BMI between 30 and < 34; Class 2: BMI between 35 and < 39; and Class 3: BMI of 40 or higher. Class 3 obesity is further categorized as "extreme" or "severe" obesity (Engin 2017).

Diabetic disorders represent a great variety of derangements in glucose metabolism, accompanied by increased serum glucose concentrations, in relation, either to hyperinsulinemia (associated mainly with insulin resistance), or to insulinopenia (associated with β-cell destruction). Type 1 diabetes (T1D) involves β-cell destruction, leading to absolute insulin deficiency, mostly *via* autoimmune mechanisms, while patients with Type 2 diabetes (T2D) presents ultimately with insulin resistance and subsequent insulin deficiency. Other types of diabetes emanate from exocrine pancreatic diseases, endocrinopathies, activity of chemicals and therapeutic modalities, or genetic predisposition, such as Maturity Onset Diabetes of the Young (MODY) (Petersmann et al. 2019). Notably, the type of diet is a common denominator between diabetes and obesity, regarding both causality, and treatment.

Human diet seems to affect human health *via* the gut microbiome, a newly described and fully functional organ, consisted of bacteriome, virome, and mycobiome. It was not until a C. Arthur Scheunert's paper in 1920 (only a couple of decades after E. Metchnikoff suggested the concept of gut microbiome) referred to *Dysbiose der Darmflora* (dysbiosis of intestinal flora) as the cause of bone inflammation in horses (Scheunert 1920; Thursby and Juge 2017) that many researchers started

associating gut dysbiosis with several diseases, usually of metabolic and autoimmune origin (De Luca and Shoenfeld 2019; Li et al. 2018; Siljander et al. 2019).

Gut microbiome harbors a complex ecosystem of over 100 trillion microbial cells, when its function is disrupted, as in case of gut/intestinal dysbiosis, it seems to lead to diabesity (Thursby and Juge 2017). In this chapter, we review the liaisons between obesity, diabetes of metabolic origin, and gut microbiome.

5.2 Healthy Gut Microbiome: "Gut Normobiosis/Dysbiosis"

5.2.1 Physiology and Key Definitions

Gut microbiome describes the genome of the microorganisms, bacteria, viruses, and fungi, residing in the gastrointestinal (GI) tract and contributing to essential functions in its host. It affects immunity, metabolism, homeostasis of the gut, and host physiology, in general (Heintz-Buschart and Wilmes 2018). It represents a beneficial evolutionary symbiotic relationship that has changed significantly throughout the human history (Schnorr et al. 2014).

Official definitions about the status of microbiome in health do not exist, as yet (Thursby and Juge 2017). Several notable researchers have attempted to define the healthy gut microbiome composition, resulting in sketchy definitions (Hooks and O'Malley 2017; Olesen and Alm 2016). It is generally accepted that gut *normobiosis* generally refers to a dynamic state of healthy gut microbiome, when the host is in a state of complete physical, mental, and social well-being, while gut *dysbiosis* is described as a state of loss of beneficial bacteria, increase in the populations of pathobionts, or loss of ecologic diversity, simultaneously exerting grave effects on the health of the host (Dinan and Cryan 2013; Petersen and Round 2014).

Data from recent studies showcased the somber effects of the dysbiotic status of the gut on gut metabolites acting as pro- and anti-inflammatory factors, depending on the host health status (Roberfroid et al. 2010; Kolida et al. 2002; Bosscher et al. 2009). Short chain fatty acids (SCFAs—organic fatty acids with 2–6 carbon atoms) are produced in the colon and caecum of the host by the gut microbiome, after the fermentation of nondigestible dietary proteins, glycoproteins, and fibers. Butyrate, acetate, and propionate are the most studied microbial SCFAs. Except for their role in the modulation of the colonic function, integrity of the gut mucus layer (Schroeder 2019), motility, and microbial SCFAs are absorbed and may affect the metabolism of several organs (Rastelli et al. 2018).

SCFAs and their derivatives induce satiety in different ways: butyrate increases the concentrations of glucagon-like peptide-1 (GLP-1) by acting on intestinal cells to induce its production, while propionate seems to affect intestinal gluconeogenesis (Chambers et al. 2018). Thus, the actions of both metabolites lead to the improvement of glucose homeostasis and satiety control (den Besten et al. 2015). SCFAs seem to act as stimulants for the secretion of the anorexigenic peptide YY (PYY) and the adipose tissue hormone of satiety, leptin. As recently shown, they can

increase the concentrations of serum PYY in overweight patients, when administered as SCFA mixtures by acute rectal infusion (Canfora et al. 2019).

Primary bile acids (cholic and chenodeoxycholic acids) derive of cholesterol molecules in the liver, and they are discharged into the small intestine, where they are conjugated to molecules of glycine, or taurine. In the ileum, these bile acids are deconjugated by the actions of gut bacteriome and converted into secondary bile acids, acting as molecular signals. Also, secondary bile acids modulate various processes, such as energy expenditure, insulin sensitivity, and cholesterol synthesis by binding to cellular receptors such as the farnesoid X receptor (FXR) and the G protein-coupled bile acid receptor 1 (also known as TGR5) (Zietek and Daniel 2015). The contribution of gut microbiome in the metabolism of bile acids underlines its impact to the control of glucose and of lipid metabolism (Moran-Ramos et al. 2017). Remarkably, one of the effects of bariatric surgery is the detoxification of the intraluminal bile acids that act as bactericidals, while it results to liberation of glycine/taurine conjugate that can be used for bacterial metabolic needs. Thus, bile acids are essential for lipid and glucose homeostasis, as they regulate energy expenditure (de Aguiar Vallim et al. 2013; Albaugh et al. 2017).

5.2.2 Gut Bacteriome and Obesity

Several studies have evaluated the way gut bacteria may affect host energy balance, or storage capacity, and have suggested a significant number of mechanisms of action. The "energy harvest" hypothesis postulates that gut bacteriome contributes to the progression to obesity *via* extraction of energy from otherwise indigestible dietary fibers, and thus production of digestible SCFAs (Turnbaugh et al. 2006). The "metabolic endotoxemia" hypothesis suggests that plasma lipopolysaccharide (LPS, or endotoxin, originating from the cell wall of gram-negative bacteria) elicits subclinical, low-grade inflammation, boosting thus adiposity *via* induction of insulin resistance (Cani et al. 2008; Zhao 2013). On the other hand, metabolites of the bacteria, or their metabolic derivatives seem to modify energy balance (Harley and Karp 2012). Of note, SCFAs, in addition to being energy sources to the host, are significant molecular signals with beneficial effects for host energy metabolism (Kimura et al. 2014) and essential protectors against diet-induced obesity in animal models (den Besten et al. 2015; Lin et al. 2012). Other bacterial metabolites, such as methane (Mathur et al. 2013) and secondary bile acids (Parseus et al. 2017), may also affect the host's energy balance.

In an interesting animal study, the fecal transplant from the lean donor mice prevented obesity in the prone to obesity genetically engineered mice, underlining the important role of gut microbiome in obesity (Ridaura et al. 2013). Animal studies have demonstrated a straight link of the gut microbiome to obesity. In addition, a significant number of contemporary human studies have employed comparisons of gut microbiome and bacterial metabolite compositions in obese patients with metabolic disorders and lean healthy controls. The aforementioned studies have associated low diversity and richness in the composition of the gut microbiome, with

elevated relative risk of obesity (Human Microbiome Project C 2012; Koenig et al. 2011; Damms-Machado et al. 2015).

One specific microbial signature associated with obesity was identified in a recent, large study in American adults. Obesity was characterized by increased populations of *Bacilli* and its families, *Streptococcaceae*, and *Lactobacillaceae* and decreased populations of several groups, within the class of *Clostridia*, including *Christensenellaceae*, *Clostridiaceae*, and *Dehalobacteriaceae* (Peters et al. 2018). Comparable microbiome signatures were also found in other two studies (Yun et al. 2017; Beaumont et al. 2016). In these two studies, absence of the populations of *Christensenellaceae* characterized the obese patients, indicating that the family of *Christensenellaceae* is important for promoting leanness and for producing SCFAs, primarily acetate and butyrate (Beaumont et al. 2016; Waters and Ley 2019).

Many factors, influencing gut microbiome, account for the discrepancies between the numerous studies. These are environmental factors differences, such as race, geographic area, diet type, or medication, technologies used, such as qPCR, 16S rRNA sequencing, 16S microarrays, metagenomics, sample size, and bioinformatics approaches. Several patterns of microbial diversity have been linked to various metabolic functions of gut bacteriome and to the presence of bacterial dysbiosis. Overall, most studies until today have demonstrated reductions in the diversity of gut microbiome in obese patients, but there is still much debate on the specific microbial composition in normobiosis and dysbiosis, linked definitely to the gut microbiome of obese patients (Vallianou et al. 2019).

Since, alterations in the populations of bacteria in the human gut can be considered as a factor involved in obesity onset in humans, clinical trials have been performed, involving probiotics, prebiotics, and synbiotics, including dietary interventions in a variety of obese patients (Borgeraas et al. 2018). Administration of probiotics, in a recent meta-analysis, resulted in a significantly large reduction in body weight, BMI, and fat percentage compared with placebo; however, the effect sizes were small (Borgeraas et al. 2018).

Regarding prebiotics, inclusive evidence suggested that prebiotic products did not decrease adiposity parameters (BMI, body weight, and body fat mass), but they could decrease the levels of systemic inflammatory biomarkers, implying that adherence to prebiotic products might be a promising adjunct approach to the management of inflammatory states in overweight and obese patients (Qu et al. 2019).

5.2.3 Gut Bacteriome and Diabetes

A decade ago, it has been suggested that alterations in gut bacteriome composition resulting from obesity could contribute to the pathogenesis of diabetes (Lyte 2010). Several microbial signatures have been identified in the gut microbiome of diabetic patients, with either T1D or T2D, such as low diversity and reduced populations of starch-fermenting bacteria (Kim et al. 2018), along with increased populations of bacteria promoting LPS-driven inflammation.

Recent studies revealed that gut microbiome affects the antidiabetic pharmacologic therapies, while in return, the metabolic products of these therapies altered the structure of gut microbiome. One recent study revealed that hypoglycemic agents contributed to the modification of specific species in gut bacteriome, rather than its bacterial diversity. Metformin increased the populations of *Spirochaete*, *Turicibacter*, and *Fusobacterium*. Insulin, also, increased the populations of *Fusobacterium*, while α-glucosidase inhibitors (α-GIs) contributed to the plentitude of *Bifidobacterium* and *Lactobacillus* populations. Medications that act on glucose absorption in the gut or enhancing gut hormone activity are extensively employed in the therapeutic modalities of diabetes. Metformin and insulin seem to improve taurine and hypotaurine metabolism, while α-GI promoted several amino acid pathways. Although gut bacteriome, in patients treated with metformin or insulin, were similar, significant differences were noticed in the gut bacteriome of these patients, while being in a hypoglycemic state (Zhang et al. 2019).

Several case-control studies have shown statistically significant differences between diabetic patients and healthy controls regarding gut bacteriome, such as decrease in the populations of *Bifidobacteria* and *Faecalibacterium prausnitzii,* increase in the population of Lactobacilli (Sedighi et al. 2017; Navab-Moghadam et al. 2017) in T2D patients, and absence of difference in T1D patients with optimal glycemic control (Stewart et al. 2017).

Lactobacillus spp. secrete catalase, an enzyme with antioxidative capacity. In synthetic media, *Lactobacillus* spp. select and salvage external sources of purine/pyrimidine nucleosides/bases, as precursors for nucleotide synthesis for its growth. In presence of the biochemical substrates of xanthine oxidase (hypoxanthine or xanthine), microbiocidal superoxide and hydrogen peroxide (H_2O_2) are produced. In T1D patients, *Lactobacillus* is presented in decreased populations. Thus, *Lactobacillus* spp. seem to play a "supervisory" role in intestinal integrity and ecology. Contrariwise, growth of *Staphylococcus* spp., a pathobiont, is inhibited in the presence of xanthine oxidase. Also, increases in *Bacteroides* spp., a species, containing sphingolipids and meso-diaminopimelic acid in its peptidoglycan layer, provide continuous stimuli to the immune system, probably contributing to the autoimmunity of the T1D pathogenesis, via the dysregulation of intestinal lumen and mucus integrity.

In healthy subjects, a balanced interrelation between *Bacteroides vulgatus* and *Clostridia* exists, acting as a counter-inflammatory mechanism. This balance is disrupted in T2D patients, resulting to ineffective control of inflammation, leading, thus, to a profile of profound gut dysbiosis. This imbalance is aggravated by the decreased populations of *Bifidobacterium*, possibly contributing to T2D onset. LPS originate from the outer membrane of gram-negative bacteria, such as *Betaproteobacteria*, bind to Toll-like receptor 4 (TLR4), activating proinflammatory signaling pathways, and resulting in low-grade inflammation, thus, decreasing insulin sensitivity. In T2D patients, the ratios of *Bacteroides/Prevotella* and *Clostridia/C. Coccoides-E. rectale* populations are increased, as compared with healthy controls. Similarly, T2D has been, also, associated with high *Bacteroides* and *Clostridium* populations. In the study of Qin et al., *Akkermansia muciniphila,*

Desulfovibrio, and *Eggerthella* populations were increased along with those of *Bacteroides* and *Clostridium* spp. in T2D patients, when compared with healthy controls.

Alternatively, increased populations of *Bacteroides*, *Blautia*, and *Serratia* spp. and decreased populations of *Prevotella* spp. and *Verrucomicrobia* phylum were described in prediabetic patients, when compared with healthy controls. *Blautia* spp. are acetogenic, while certain members of this species ferment hydrolysis--resistant starches. These species have also been associated with beneficial effects. Reduction of populations of *Verrucomicrobia* spp., such as *Akkermansia muciniphila*, has been linked with reduced production of GLP-1. The compensatory mechanism to the bacterial reduction is the binding of lactoferrin, given that iron is essential for the growth of most bacteria (Stefanaki et al. 2017). Remarkably, several randomized controlled trials employing probiotics, prebiotics, or symbionts demonstrated promising but modest results regarding improvement of glycemic control in diabetic or prediabetic patients (Stefanaki et al. 2018; Stefanaki et al. 2019; Barengolts et al. 2019).

5.3 Gut Virome and Mycobiome in Obesity and Diabetes

The community of bacteriophages in the human gut is a combination of three classes: a set of core bacteriophages shared among more than one-half of all people, a common set of bacteriophages found in 20–50% of individuals, and a set of bacteriophages that are either rarely shared, or unique to a person (Manrique et al. 2016).

Mimicry is a common evolutionary phenomenon that occurs when an organism or cell mimics another to gain an advantage in competing for resources, protection, or survival. Viral mimicry is a mechanism employed by viruses to generate molecules that resemble host growth factors, or immune response regulators, such as cytokines, chemokines, and their receptors for the benefit of the virus (Huang et al. 2019).

In some cases, this may, either appoint, or disrupt host immune function to gain an advantage, but it is not known whether this is always true. Bacteria and bacteriophages (phages) are the most abundant biological entities in the gastrointestinal tract, where their coexistence is dynamic and connected. Phages guide and keep bacterial diversity by perpetuating the coevolutionary relations with their microbial kill (De Sordi et al. 2019).

The most frequently detected human viral triggers of islet autoimmunity in T1D patients are members of the *Picornaviridae* family (*Parechovirus* and *Enterovirus*). One recent study reported significant changes in the intestinal virome (*Circoviridae*, *Enterovirus*, *Kobuvirus*, *Parechovirus*, *Parvovirus*, and *Rotavirus*) that preceded autoimmunity in a cohort of T1D patients. Specific components of the virome were, both, directly and inversely associated with the development of human autoimmune disease (Zhao et al. 2017).

Compared to bacterial communities, the human gut mycobiome is poor in diversity, and basically dominated by yeast, including *Saccharomyces*, *Malassezia*, and

Candida. Both inter- and intra-volunteer variability in the Human Microbiome Project (HMP) cohort were high, revealing that unlike bacterial and viral communities, an individual's mycobiome exhibits more variation over time alike the variation it presents, compared to that of another individual (Nash et al. 2017). The human gut mycobiome receives increased research attention due to its potential involvement in the etiology of numerous gut-associated diseases (Kramna and Cinek 2018). This increasing interest is largely led by recent findings, indicating that specific fungi seem to alter the host immune response, and consequently may be a risk factor in immunological disorders in genetically susceptible individuals. Human mycobiome may act as a reservoir for opportunistic pathogens in immunocompromised hosts and may play a role in many disorders not obviously related to or influenced by the gut. Conversely, the potential health benefits, or probiotic effects of some fungal species are well-known, but have yet to be fully explored (Carding 2019).

In 2015, Rodriguez et al. showed that obese patients could be discriminated by their specific fungal composition, which also distinguished metabolically "healthy" from "unhealthy" obesity. A first link to metabolites such as hexadecanedioic acid, caproic acid, and N-acetyl-L-glutamic acid was also found. *Mucor racemosus* and *Mucor fuscus* were the species more represented in nonobese subjects compared to obese counterparts (Mar Rodriguez et al. 2015). Obesity usually entails subclinical atherosclerosis. In obese patients with increased Framingham score and carotid intima-media thickness, it was found that relative abundance in the gut of *Mucor racemosus*, a fungus belonging to the phylum *Zygomycota,* may be a relevant biomarker for cardiovascular risk (Chacon et al. 2018).

Recently, it was reported that the *Candida* spp. populations were greater in T1D and T2D patients with poor glycemic control than in healthy controls, while no difference was found between the two diabetic groups (Gosiewski et al. 2014). Later in 2016, other studies revealed increased diversity of *Candida* spp. along with increased prevalence in T1D patients (Kowalewska et al. 2016; Soyucen et al. 2014). A contemporary study recognized other fungal species in abundance in children with autoimmunity for T1D, along with severe gut dysbiosis. Fungal dysbiosis, characterized by high abundance of fecal *Saccharomyces* and *Candida*, was found in children with β-cell autoimmunity, who progressed to clinical T1D. These children showed, also, bacterial dysbiosis (increased *Bacteroidale*s and *Clostridiales* ratio) (Honkanen et al. 2020).

5.4 Conclusions

The link between gut dysbiosis, bacterial, viral, or fungal, and obesity–diabetes spectrum is irrevocable. Obesity and diabetes are disorders, thoroughly studied with regard to gut microbiome, but there is need for changes in the operational approach. First, the concept of microbial endocrinology should be taken into consideration when exploring the interconnections between the members of bacteriome and the gut environment (Watters et al. 2013). The study of the ability of microorganisms to produce and respond to hormones that originate, either within the gut microbiome

or within the host, serves only as a basis for an evolutionarily derived method of communication between a host and its gut microbiome. Mechanisms elucidated by microbial endocrinology might give new insight into the ways gut microbiome can affect the stress levels, the metabolic efficiency, the resistance to disease, and other factors that may prove relevant to the health status of the host (Huang et al. 2019; Lyte 2014; Lyte et al. 2018).

Second, gut dysbiosis is not a cure-all. And most definitely, gut dysbiosis is not the answer. The possible mechanisms by which gut dysbiosis could be the cause or the trigger of the onset of a disease are still under investigation. The relevance of most microbiome compositions to disease remains hypothetical. A recent paper by prominent researchers suggested the term "dysbiosis" as elusive, being the result of disease, rather than the cause. Indeed, gut dysbiosis has such varying definitions in the literature that the term could apply to either cause or effect (Olesen and Alm 2016). Thus, the challenge is to discern the definite presence of a causative relationship between obesity, diabetes, and the gut microbiome, as a whole.

Last but not least, larger, randomized controlled trials with more sophisticated designs should be performed, analyzing the effects of probiotics, prebiotics, or synbiotics to the various disorders, including evaluation of the gut mucosa and the metabolites of the gut microbiome (Zmora et al. 2018).

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Gut Microbiota in Obesity and Bariatric Surgery: Where Do We Stand?

6

Konstantinos Georgiou

Abstract

The prevalence of obesity is exploding worldwide in our postindustrial era, with increasing morbidity and mortality.

The human gut microbiome exhibits a cardinal role in metabolic, nutritional, physiological, and immunological functions of the human body, and due to this multiplexity some authors consider it as an independent virtual organ by itself. Due to the big progress in phylogenetic investigation and quantification of gut microbiome through modern high-throughput sequencing, our understanding of the gut microbiome in health and diseases is rapidly advancing, and several studies have examined its role in obesity and its changes that occur following bariatric surgery.

There is growing evidence that obesity is associated to a specific gut microbiome profile which confers the host with an augmented ability for calories extraction and reduced gut microbial diversity. However, the mechanism through which the gut microbes and their by-products affect obesity remains mainly undiscovered and therefore more research is required to better comprehend the empirically observed connection between gut microbiome alterations and obesity.

On the other hand, bariatric surgery procedures, such as Roux-en-Y gastric bypass and vertical sleeve gastrectomy, are the most effective interventions for achieving pronounced and sustained weight loss and normalize glucose metabolism in obese patients. Bariatric surgery seems to restore a healthier microbiome with a leaner metabolic profile, and this microbiome rearrangement potentially contributes to the reduced fat mass, increase in lean mass, and resolution of

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comorbidities such as those observed following bariatric surgery. The exact mechanism is not certain, but it could be mediated by altering the enterohepatic bile acid circulation as well as altering the bile acid structure. Moreover, the bile acid activated farnesoid X transcription factor (FXR) is crucial for the positive effects of bariatric surgery on weight loss and glycemic control improvement. However, recent data showed that the gut microbiota is not fully restored after bariatric surgery. Additionally, unidentified downstream targets such as the gut--derived peptide FGF15/19 may potentially explain the positive metabolic effects of bariatric surgery.

More randomized controlled trials and larger prospective studies including well-defined cohorts are necessary to better identify the associations between the gut microbiome, obesity, and bariatric surgery.

Keywords

Bariatric surgery · Obesity · Gut microbiota · Micronutrient deficiency · Probiotics

6.1 Introduction

Obesity is an enormous health problem in our modern society as it is associated with increased morbidity and mortality (Blüher 2019). Recently, research produced a vast amount of evidence of a bidirectional interplay between gut microbiota (GM) and obesity, with the latter considered as both a cause and/or a consequence of gut microbiota disorders (Cătoi et al. 2019). In the healthy human, GM is involved in energy intake, adjustment of glucose and lipid homeostasis, as well as in the micronutrients and vitamins composition (Pascale et al. 2018). This GM balance is disrupted in obesity thus presenting with a series of pathological pathways, such as altered insulin resistance, chronic inflammation, and metabolic disturbances (Cătoi et al. 2019; Pascale et al. 2018). Furthermore, obesity is accompanied by important deficiencies in vitamins and minerals, which aggravate gut microbiota synthesis and function (Astrup and Bügel 2019; Mohajeri et al. 2018).

Bariatric surgery (BS) is, for the time being, the sole long-term successful therapeutic option treatment of morbid obesity (Buchwald 2014). Several studies report a significant change in the structure and diversity of GM after BS. Additionally, subjects who underwent BS, present some micronutrient deficiencies which could result to serious deficiency-related syndromes (Lupoli et al. 2017; Neylan et al. 2016), the most common being anemia (10–74%) and neurological disfunctions (5–9%) (Xanthakos 2009).

However, except the substantial GM alteration after BS, several other factors coexist impairing the postoperative nutritional status of the bariatric patients: the significantly energy-restricted higher protein intake and adequate nutritional supplementation diet, and the anatomical and physiology impairment of the gastrointestinal tract (GIT) with explicit alterations in food digestion and absorption induced by the type of procedure performed (Buchwald 2014; Lupoli et al. 2017). Therefore,

after BS, these patients require a consistent follow-up focused on the prevention of the above side effects, by modulating gut microbiota and prescribing appropriate nutritional supplementation.

The complicated interaction between obesity and GM phylae and the modulation of the gut microbiota and of their by-products balance produced in obese patients that undertake BS as a therapeutic measure represent the main areas of focus in this chapter.

6.2 Obesity

Recent research is showing that each human body hosts a unique set of associated microorganisms which contribute essentially to maintain health and metabolic balance of the subject.

Due to the contemporary modern living style providing easy access to high energy foods and low demanding of physical activity, the prevalence of obesity has exploded. Obesity due to an imbalance of calories ingestion, basal metabolism, and energy expenditure (Wang and Liao 2012). Obesity can be broadly defined as being the result of the discrepancy between calories consumption and energy expenditure. Numerous genetic, behavioral, and environmental factors have been suggested as obesogenic (Cani 2013). Furthermore, obesity is associated with type 2 diabetes (T2DM), hypertension, dyslipidemia, and cardiovascular disease, as well as sleep apnea, musculoskeletal disorders, some forms of cancer, impaired fertility, and with increased incidence of mood disturbances, anxiety, and other psychiatric disorders (Colquitt et al. 2014). Obesity increases mortality and its associated comorbidities, so that today in our modern societies, overweight and obesity associated diseases kill more individuals than undernourishment and starvation (Björklund and Fändriks 2019). Thus, except the burden that obesity provokes to the individual, it also represents a major health and economic load on the healthcare systems into both developed and developing countries (Tremmel et al. 2017).

Commonly, the term Body Mass Index (BMI) is used for classifying obesity and is calculated as body weight (kg) per the square of height (m^2). In adults, a "normal" BMI is 18.5–25 kg m^{-2}; overweight is BMI 25–30, while obesity is defined as BMI over 30 kg m^{-2}. The WHO have classified obesity into three classes where class I relates to a BMI 30.00 to 34.99; class II is between 35.00 and 39.99, and BMI >40.00 kg m^{-2} is regarded as class III obesity (Colquitt et al. 2014). In addition, BMI >50 kg m^{-2} is sometimes termed superobesity.

Regarding obesity treatment, although substantial weight loss can be achieved by lifestyle interventions such as diet and increased physical activity, it has been shown that those lifestyle changes are hampered on the long term (Stefan et al. 2018). Indeed, the main issue is to keep the reduced body weight on the long term, as it has been reported that within 1–2 years most subjects reclaim the weight lost, and furthermore, they usually exceed the pretreatment levels. Additionally, the antiobesity drugs have several limitations due to adverse events and contraindications especially in cardiac and cerebrovascular diseases. Therefore, for morbidly obese

patients, BS is the unique, effective in the long-term procedure to lose weight and to reestablish metabolic health (Miras and le Roux 2014). The term bariatric surgery is introduced and can be defined as a surgical intervention in the GIT for a weight reducing purpose.

6.3 Gut Microbiota in Healthy Subjects

6.3.1 Glossary of Microbiome-Related Terms

Microbes are found in every surface of the body that is exposed to the external environment, including the skin, genitourinary, gastrointestinal, and respiratory tracts (Chen et al. 2018).

The ecological community of symbiotic (promoting the health of the host), commensal (neutral to the host health, without benefit nor negative effects), and pathogenic microorganisms that share our body consists the microbiome (Thomas et al. 2017). The term microbiota comprises the sum of all species which form microbial communities, such as bacteria, archaea, fungi, and protists. When it refers to a specific environment, the term is preceded by the said location, for example, "the gut microbiota" refers to the intestinal tract (Knight et al. 2017).

The term "microbiome" is also commonly referring to the microbiota (i.e., the microorganisms themselves). The study of all microbial DNA of a sample (i.e., the genetic material) directly recovered from a sample such as the gut is called metagenomics. The metagenome, i.e., the collective genome of the microbiota encompasses over 100 times the number of genes of the human genome, thus containing approximately ten-fold more genes in each microbiome (Thomas et al. 2017). The term "shotgun metagenomics" describes the process during which the total DNA of a sample is fragmented in a random manner and thereafter subjected to next--generation sequencing. This process generates primer-independent and unbiased sequencing data which can then be analyzed by means of various reference-based and/or reference-free methods. Thus, shotgun metagenomics targets all DNA material in a sample and produce relative abundance information for all genes, functions, and organisms (Chen et al. 2018).

In a healthy state, the GM is in a stable equilibrium while any imbalance of the gut bacterial ecosystem is called dysbiosis (Aron-Wisnewsky et al. 2012).

6.3.2 Gut Microbiota Under Normal Conditions

Under healthy conditions in adult humans, the microbial composition appears to remain constant (Li et al. 2016). The human microbiota incorporates all the microorganisms that reside in every surface of the body that is exposed to the external environment, including the skin, genitourinary, gastrointestinal, and respiratory tracts. The largest concentrations of microbes are found in the intestine, the skin, and in the oral cavity (Sender et al. 2016). Among those body sites, the gastrointestinal tract is the most densely colonized organ. It is reported that the gut of a healthy

subject contains approximately 1–1.5 kg of microbes, corresponding to about 10^{14} bacteria, i.e., about 10 times more the number of body cells (Fändriks 2017). There are approximately 1000 species of microbes colonizing the gut, with microbial density increasing along the GI tract from 10^{1} to 10^{4} microbes in the stomach and the duodenum, 10^{4} to 10^{8} cells in the jejunum and ileum, to 10^{10} to 10^{12} cells per gram in the colon and feces (Thomas et al. 2017).

Due to the antimicrobial action of hydrochloric acid and nitric oxide, the stomach and the small intestine contain just a small amount of microbes (Lundberg and Weitzberg 2013; Nardone and Compare 2015). On the contrary, the large intestine is presenting better milieu for symbiotic microbes, achieving better conditions to extract energy as well as essential elements from the lumen bulk after digestion/absorption occurring in the small intestine (Mowat and Agace 2014; Woting and Blaut 2016). The bigger number of living microbes is located in the colon but due to the impermeable adherent mucus layer, the direct contact with the epithelium is prevented (Johansson et al. 2008).

The microbiome includes bacteria, fungi, and archaea (Savage 1977). It is estimated that in the gut there are about a 1000 bacterial species which have about 2000 genes per species, yielding to approximately two million genes, which is 100 times the number of nearly 20,000 human genes. The number above is in line with the actual extent of microbial gene catalogues found in MetaHIT and the Human Microbiome Project (Gilbert et al. 2018).

During the whole life, the structure and the function of GM are influenced to a different degree from many factors starting from birth (such as the delivery method) to the diet followed during childhood and adult age as well as the use of antibiotics (Compare et al. 2016). An analysis of the LifeLines DEEP cohort using metagenomic shotgun sequencing of the GM demonstrated a multifactorial involvement among the microbiome and a plethora of extrinsic and intrinsic parameters, including 60 dietary factors, 31 intrinsic factors, 19 drug categories, 12 diseases, and 4 smoking categories, all together accounting for 18.7% of the interindividual variation in the GM. It was also found that diet plays a significant role that alters GM (Zhernakova et al. 2016). It is estimated that about 4.5% of BMI is attributable to the GM (Mohajeri et al. 2018).

The majority of all microorganisms in the human GIT is a diverse community of bacteria, viruses, archaea, fungi, and eukaria (Ejtahed et al. 2018). Gut microbiota are bacteria and belong to two phyla, the Firmicutes (64% encompassing gram--positive genera, e.g., *Clostridium*, *Ruminococcus*, *Lactobacillus*, *Butyrivibrio*, *Anaerostipes*, *Roseburia*, and *Faecalibacterium* and the Bacteroidetes 23% containing gram-negative genera, e.g., *Bacteroides*, *Porphyromonas*, and *Prevotella*) (Mariat et al. 2009). The other phyla occupying the digestive tract include *Proteobacteria (*8% including gram-negative genera, e.g., *Helicobacter and Escherichia*), *Actinobacteria* (3% encompassing gram-negative genera, e.g., *Bifidobacterium*), and less of the phyla Fusobacteria, Spirochaetes, Verrucomicrobia (gram-negative species Akkermansia muciniphila), and *Lentisphaerae* (Zoetendal et al. 2008). The methanogens, *Methanobrevibacter* and *Methanosphaera* are the most dominant archaeal groups (Gill et al. 2006; Mihajlovski et al. 2008). Finally,

fungi and archaea account for less than 1% of the GM. The two common fungal phyla in the gut include Ascomycota (which includes the genera *Candida* and *Saccharomyces*) and Basidiomycota (Scanlan and Marchesi 2008; Ott et al. 2008). Overall, the highest density is located into the colon with the majority of bacteria are anaerobes such as *Bacteroides*, *Porphyromonas*, *Bifidobacterium*, *Lactobacillus*, and *Clostridium* (genera that belong to the most abundant phyla: *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*) (Villanueva-Millán et al. 2015). The GM has also its own energy demands and consumes energy from the luminal contents thereby enhancing energy utilization (Tremaroli and Bäckhed 2012). Collectively, the gut microorganisms are considered to constitute a powerful "organ" capable to influence most physiological functions of the human body (Gill et al. 2006; Tremaroli and Bäckhed 2012).

GI microbiota are of crucial importance in the metabolic, nutritional, physiological, and immunological procedures of the entire human body. The GM encompasses different genes involved in carbohydrates metabolism (glucose, galactose, fructose, arabinose, mannose, xylose, starch, and sucrose), thus producing important nutrients which could not be synthesized otherwise, such as short-chain fatty acids (SCFA) (Macfarlane and Macfarlane 2012), vitamins (vitamin K, vitamin B_{12}, folic acid), certain amino acids (Gerritsen et al. 2011; Hamer et al. 2009), neurotransmitters (Cryan and Dinan 2012), and regulation of gastrointestinal hormones (Dockray 2014; Holzer et al. 2012). The above properties of the GM have pushed some authors to regard it as an independent virtual organ by itself (Al-Najim et al. 2018). The microbiome encodes specific enzymes capable to provoke fermentation of the indigestible carbohydrates mentioned above, that is 10–30% approximately of the ingested energy as well as the main fermentation products, i.e., SCFAs (e.g., acetate, propionate, and butyrate), which are at about 90–95% absorbed in the colon representing approximately about 6–10% of the energy needs of the human body (Young 2017).

Between 2013 and 2017, more than 12,900 publications were published studying the GM, a number highlighting that this field of research is blossoming and that a necessity for advancement is underway (Cani 2018). Human microbiome investigations are focusing to understand the underlying mechanisms and to develop novel clinical interventions (Gilbert et al. 2016).

The human microbiome is not constant, but rather changes with age, diet, and health status. It has been reported that the GM interacts in several ways in health and disease with the host, including:

1. Modulating the inflammatory host response to the gut.
2. Synthesizing small molecules and proteins that are absorbed by the host.
3. Changing the amount of available energy in the diet.

The research of GI microbiota has blossomed enormously recently. This is due to the big progress in phylogenetic investigation and quantification of GM through modern high-throughput sequencing. The recent use of cost-effective, culture--independent molecular techniques (i.e., 16 s rDNA sequencing or whole-genome sequencing/metagenomics) on fecal samples enabled for the first time to study

accurately and reliably the dynamics of the host–GM interactions. In whole-genome shotgun sequencing, the entire DNA in a given sample is fragmented, sequenced, and then remapped into the original genome (Sweeney and Morton 2013). This information is then compared with preexisting databases to identify species and genes. This method has the advantage of identifying all species and all genes present. The method is computationally intense, requiring a considerable amount of bioinformatic mapping (MetaHIT Consortium et al. 2010; The Human Microbiome Project Consortium 2012). One such freely available knowledge base for systematic analysis of gene functions in terms of the networks of genes and molecules is the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg/). It uses different databases to assign functional meanings to genes and genomes and thus predicts the higher level functional changes as KEGG pathway maps (Ogata et al. 1999). However, these studies are valuable since they may provide the most clinically relevant data because they are able to identify gene networks that may be overexpressed in a particular microbiome, for instance vitamin synthesis or decomposition, giving important clues to the physiology changes of the host. However, basic scientific research is based mainly on rodent models and cell cultures, but their relevance for human physiology and clinical conditions remains unknown as very few studies have validated the translation of rodent-based data to a human context in a "head-to-head" fashion.

In contrast to human genetics which have been unsuccessful to explain the obesity epidemic, the GM can classify individuals as lean or obese with over 90% accuracy, although this result depends on using the correct methods (Sze and Schloss 2016). Also, it is worth to note that recent findings support that GM could be implemented as a new marker of cardiovascular disease (Garcia-Rios et al. 2017).

Additionally, the GM exhibits a significant role in the defense against pathogens as the high microbial content found in the large bowel poses a major challenge to the mucosal immune system. In fact, the intestinal mucosa must tolerate commensal microbiota as well as dietary antigens and eliminate pathogens successfully. The GM products are crucial in order to protect the host from various diseases (Zaneveld et al. 2008) as well as shaping systemic immune homeostasis (Dzutsev et al. 2015). In a healthy state, GM, by producing antimicrobial compounds, keeps the barrier intact and it presents anti-inflammatory action which protects the epithelial cells against pathogens (Compare et al. 2016; Villanueva-Millán et al. 2015). This action is intermediated through Toll-like receptors which can induce the synthesis and delivery of pro-inflammatory factors such as tumor necrosis factor alpha (TNFα) and interleukins 1 and 6 (IL1 and IL6) (Villanueva-Millán et al. 2015). The development of this peripheral production requires the presence of GM in the colon. Although the exact mechanism of this anti-inflammatory action is not well clarified, several microbe components have been detected to increase their expansion and function, including SCFAs (especially butyrate) and polysaccharide A of *Bacteroides fragilis* (Hoeppli et al. 2015).

The mechanism on how the beneficial bacteria prevent dysbiosis and maintain balance in healthy state is not known. An example is *Clostridium difficile* which under normal conditions is present in the large intestine in a commensal state not

causing any disease. *Clostridium difficile* colonize and release the exotoxins TcdA and TcdB which can trigger colitis appearance in susceptible subjects (Leffler and Lamont 2015). Recently, a study showed that microcins, which are small size proteins released by numerous favorable bacteria, could restrict the expansion of competing *Enterobacteriaceae* and thus avoid inflammatory bowel disease (Sassone-Corsi et al. 2016).

GM is both a producer and a consumer of vitamins; Prototrophs ("producers") are microbes which are able to synthesize vitamins de novo, in contrast to other microbes that require exogenous vitamins provision called auxotroph ("consumers") (Kim et al. 2017). Some common microbes (i.e., *Bacteroides*, *Enterococcus*, *Bifidobacterium*) have an auxotrophic behavior although they can produce most of the soluble vitamins of the B complex (cobalamin, thiamine, pyridoxine, biotin, folate, nicotinic acid, pantothenic acid) and vitamin K_2 (Das et al. 2019). However, it must be noted that the de novo biosynthesis of small micronutrient molecules is demanding a high consumption of energy and that bacteria prefer to uptake these molecules from the environment when they are available (LeBlanc et al. 2013).

As mentioned before, calorie restriction is causing rapid changes in microbial diversity and function. It has been documented in animal studies that diet develops bacterial phylotypes which are positively correlated with longevity. Moreover, it has been shown that bacteria of the Lactobacillus phyla increase in animals on low-fat diet, and this reduces phylotypes which are negatively correlated with life span (Zhang et al. 2013). It has been shown that the GM quickly responds to both directions of weight alterations (gain/reduction) as the structure of the food consumed is of fundamental importance for the composition of GM (David et al. 2014). Notably, it has been shown that short-term consumption of an entirely animal-based diet increased the abundance of bile-tolerant microorganisms, including *Alistipes*, *Bilophila*, and *Bacteroides* while it decreased the levels of *Firmicutes* that metabolize dietary plant polysaccharides (*Roseburia spp*, *Eubacterium rectale*, and *Ruminococcus bromii*) (David et al. 2014).

In summary, the GM has the capacity to cover the human metabolic needs acting as an energy supplier and as a provider of certain vitamins and micronutrients to the host (Kim et al. 2017). Our understanding of the gut microbiota in health and diseases is advancing rapidly, and several studies have examined the role of the GM in obesity and their change that occurs following BS, although the differences in GM found in obesity and after BS, so far, have been mostly limited to simple comparisons (Sweeney and Morton 2013).

6.4 Gut Microbiota in Obese Subjects

It has been found that the gut microbiome together with host genotype and lifestyle contribute to the pathophysiology of obesity, and therefore, there is an increasing research interest exploring possible associations between obesity and GM (Maruvada et al. 2017; Castaner et al. 2018).

A lot of scientific evidence has been presented during the last decade on the role of GM in obesity. It seems that an amphidromous interrelation exists between obesity and gut microbiota, and obesity being considered as both a cause and a consequence of the gut microbiota shift. However, the question still remains on what comes first, the microbiota shift or the obesity, as well as the magnitude of this bidirectional correlation (Cătoi et al. 2019). Several studies performed in mice have shown an interplay between body weight and gut microbiota. It has been demonstrated that this "obese microbiota" pattern is a transferable element, at least in rodents. Thus in a study, a significant increase in body fat of germ-free (GF) mice implanted with microbiota harvested from the cecum of ob/ob mice has been shown, when compared to mice transplanted with a GM from lean rodents (Ley et al. 2006). Specifically, transferring GM from genetically obese mice provoked within 2 weeks a 47% increase of fat mass, while the inoculation from lean mice augmented fat mass just by 26% (Turnbaugh et al. 2006).

It has been reported that GF mice, i.e., mice born and raised in sterile environment without any commensal bacteria, comprise 42% less total body fat when compared to mice with normal GM, although the GF mice daily diet was 29% more than their counterparts. Moreover, GM transfer from conventionally raised mice to GF ones resulted in 60% increase of body fat and insulin resistance despite being on a low food diet (Backhed et al. 2004). Furthermore, the same group reported that the GM of obese mice showed an increased abundance of sensing and digestion of carbohydrate genes, as well as increased SCFA levels. These findings are suggesting that GM is an added factor contributing to the obesity onset (Turnbaugh et al. 2008). The importance of GM composition in the induction of obesity has been proven as a high-fat/high-carbohydrate diet leading to weight/fat gain, induce a GM shift when compared to rodents on a low-fat/high-polysaccharide diet. Additionally, the same authors reported that a low in carbohydrate and fat diet which limits weight gain and reduces obesity can increase *Bacteroidetes* abundance and reduce fat deposition (Turnbaugh et al. 2008). However, those findings are questioned by Fleissner et al. who found that the absence of GM is not protecting against diet-induced obesity (Fleissner et al. 2010).

Additionally, apart the composition, it is the diversity of GM that has been related to obesity. Comparing obese and normal-weighted Danish subjects, those who had reduced GM diversity, with microbial gene size less than 480,000 (median 600,000), had more adipose tissue, insulin and leptin resistance, and dyslipidemia compared to their counterparts which had huge gene numbers. Also, obese subjects with low gene counts had the tendency to gain more weight over time as compared to those with high gene counts, indicating that a low GM diversity identifies a subset of patients at bigger risk for obesity and related comorbidities (Le Chatelier et al. 2013).

There are still unknown mechanisms of how some factors can influence GM and its association to obesity. For instance we still don't know the effect of gender (Haro et al. 2016; Santos-Marcos et al. 2019). In addition, sometimes we only have empirical observations: In children before reaching the age of 2 years, the administration of three or more courses of antibiotic therapy that disrupt GM composition, is linked to an augmented risk of early childhood obesity (Scott et al. 2016).

The disruption of the gut microbiota balance observed in obesity is correlated with insulin resistance, chronic inflammation, and metabolic disturbances which further alter GM structure and are increased by the concomitant shift in GM production of vitamins (Astrup and Bügel 2019). For instance, it has been shown that metformin (used for type II diabetes management) changes the rodents' GM and restore the diminished quantities of *Akkermansia muciniphila* which decreases the negative effect of the diet on the gut barrier, and therefore reduces metabolic endotoxemia, and improves insulin sensitivity (Compare et al. 2016). It has been shown *Akkermansia muciniphila* is decreased in obese subjects and administration of those bacteria is beneficial to the host. It is worth to note that for exercising its beneficial effects only the membrane protein Amuc_1100 of the bacterium is needed (Plovier et al. 2017). Moreover, metformin changes several SCFA producing microbiota including *Butyrivibrio*, *Bifidobacterium bifidum*, *Megasphaera*, and *Prevotella* (de la Cuesta-Zuluaga et al. 2017).

Another beneficial bacterium for weight loss is *Christensenella* as it has been shown that its abundance into the human intestine reduces BMI, and it can induce weight loss when administered to mice (Goodrich et al. 2014).

It has been reported that 75% of patients with severe obesity have low microbial gene richness (MGR), a finding which is related with increased BMI, inflammation, and insulin resistance (Debédat et al. 2019). It has been show that in these patients MGR is improved after a short-term energy-restricted diet (Cotillard et al. 2013).

Phylogenetic analysis of GM of three groups (normal weight, obese, and post-RYGB subjects) revealed the presence of six main bacterial phyla. Most of the bacteria were Firmicutes and Bacteroidetes, while the remaining dispersed among Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia. The distribution of these bacteria in the intestines of the study groups differs greatly. More specifically, *Prevotellaceae* from the *Bacteroidetes* family and *Erysipelotrichaceae from* Firmicutes phyla are mostly abundant in obese subjects. As *Prevotellaceae* is only found in obese individuals, it is considered "obese specific" while, in contrast, *Fusobacteria* and the family *Enterobacteriaceae* within Proteobacteria were found only in the RYGB group (Zhang et al. 2009).

All these data provide evidence that obesity is related to a change of the GM structure and to a disorder deviating from the normal function, with both leading to an augmented energy production from the ingested food. Since this GM dysbiosis is involved from the onset of obesity, it is reasonable to expect that restoring the disturbed GM could result to a metabolic state improvement (Cătoi et al. 2019).

Regarding humans, a milestone study showed that 12 obese subjects were initially exhibiting less *Bacteroidetes* and more *Firmicutes* than their lean counterparts (Ley et al. 2005). When the subjects assigned to caloric-restricted diet (fat- or carbohydrate-restricted), an increase of *Bacteroidetes* and a concomitant decrease of *Firmicutes* occurred, regardless of the type of diet implied. Most importantly, the increased richness of *Bacteroidete*s correlated with the observed percentage of weight loss and not with the diet switch (Ciobârcă et al. 2020). A recently published study showed that 75% of the candidates to BS displayed a low GM gene abundance and this finding correlated with increased fat mass of the trunk and related comorbidities (T2DM, hypertension, etc.) (Aron-Wisnewsky et al. 2019).

Apart from the decreased diversity of GM observed in obese subjects, it seems that they carry more aerotolerant bacteria, which are capable to produce products which can be easily converted to SCFAs. An imbalanced GM is capable to result in weight gain through its potential to extract calories from nondigestible nutrients which escape from ingestion into the small bowel and can then be transformed to digestible compounds that are finally either excreted in feces or reabsorbed and subsequently transferred and stored to the liver until needed (Cani 2013; Jacobs et al. 2009). Bacterial fermentation of carbohydrate and proteins within the large bowel produces SCFAs mainly butyrate, propionate, and acetate (Krajmalnik-Brown et al. 2012; Rowland et al. 2018). Both butyrate and propionate are used as energy sources of the epithelial cells, and furthermore, they can both activate intestinal gluconeogenesis (IGN) (De Vadder et al. 2014). Additionally, acetate plays a role for the growth of other bacteria which are involved in cholesterol metabolism and lipogenesis. Furthermore, acetate may be engaged in central regulation of appetite (Frost et al. 2014). Therefore, although in normal conditions the involvement of GM in energy supply is small (Turnbaugh et al. 2006), it seems that through SCFA production, it can provide additional energy to the host, thus resulting in the expansion of adipose tissue mass (Cani 2013).

Several studies in obese rodents support the above GM mechanism leading to augmented fermentation and increased SCFA production and therefore to the development of obesity (Turnbaugh et al. 2009). However, the hypothesis of bigger SCFA production acting as a trigger for the onset of obesity is still on debate as some studies showed the opposite, i.e., the increased fermentation produced by the GM plays a protecting role against fat mass increase and obesity appearance (Cătoi et al. 2019).

Obesity is also characterized from a low-grade chronic inflammation. It has been found that a high-fat diet for 4 weeks, increased up to two to three times the systemic lipopolysaccharide (LPS) levels and the LPS-containing GM, leading to a condition called as "metabolic endotoxemia." Thus, the circulating high LPS levels may trigger inflammation which could then be the contributing factor for obesity and T2DM (Villanueva-Millán et al. 2015; Cani et al. 2007).

In obesity and in high-fat diet, because of GM disturbance due to a *Bifidobacteria* decrease, a markedly increased gut permeability is installed. Due to the break in the intestinal barrier, at first a mucosal inflammation is observed and then follows a migration of bacteria and/or their by-products from the gut lumen to the mesenteric lymph nodes (Compare et al. 2016; Festi et al. 2014). Consequently, the leakage of LPS and bacteria metabolites, as SCFA, and trimethylamine N-oxide (TMAO) result to the induction of "metabolic endotoxemia" followed by further cellular inflammatory responses. Lastly, this produces systemic low-grade inflammation, insulin resistance, and adipocyte hyperplasia (de Punder and Pruimboom 2015). Lately, two more mechanisms have been suggested to be implicated in gut permeability and bacterial translocation: The first implies that the glucagon-like peptide-2 (GLP-2), an anti-inflammatory as well as an intestinal growth factor, is inhibited by the altered GM. The other one refers to the endocannabinoid system, associated in both maintenance of epithelial barrier integrity and the permeability of the intestine (Compare et al. 2016; Moreira et al. 2012).

Both these mechanisms reveal the link that exists between dysbiotic GM, disruption of the gut barrier function, and "bacterial translocation" associated to a state of low-grade gut inflammation, i.e., "metabolic endotoxemia," finally leading to systemic inflammation and consequently to the pathogenesis of obesity (de Kort et al. 2011).

Opposite to the previous findings, it has been shown that the *Firmicutes/Bacteroidetes* ratio changed in favor of *Bacteroidetes* in overweight and obese subjects (Kasai et al. 2015). Furthermore, other studies reported that the Bacteroidetes and Firmicutes amounts are substantially augmented in the obesity group when compared to the normal-weight one (Ismail et al. 2011). Interestingly enough, some researchers were unable to detect any differences between obese and normal--weighted individuals in the proportion of *Bacteroidetes* abundance (Duncan et al. 2008). Furthermore, they did not discover any association between BMI and the main phyla population (Finucane et al. 2014).

BS candidate obese patients have impaired nutritional status characterized by poor-quality food choices with a diet with low diversity and essential nutrients intake, thus contributing to intestinal dysbiosis (Al-Mutawa et al. 2018). The most common nutritional deficiencies and their prevalence before BS are Vitamin D (65–93%), Iron (13–47%), and Vitamin B_{12} (4–13%) (Frame-Peterson et al. 2017). Those results are indicating that diet might be the main contributor in shaping the GM. Some studies reported that diet change accounts for 57% of the total structural shift of GM, while genetic mutation accounts for less than 12%.

Finally, up to now, it is still challenging to answer whether the GM changes are a cause or a consequence of obesity. However, given that obese phenotype can be installed after obese microbiota inoculation, it is logical to assume that GM alterations could be one reason in inducing obesity (Cătoi et al. 2019). In summary, there is growing evidence that obesity is attributed to a specific GM profile which confers the host with an increased ability for calories extraction. It seems that GM imbalance contributes to the onset of obesity in tandem with an unhealthy diet. Therefore, the GM should be considered as a set of genetic factors that together with host genotype and lifestyle contribute to the pathophysiology of obesity.

6.5 Bariatric Surgery

6.5.1 Bariatric Surgery Modalities

When the lifestyle and/or medication-based approaches for losing weight in obese patients have proven ineffective, then bariatric surgery is an option, as it has been shown to be a highly effective therapeutic procedure for treating obesity (Tuomi and Logomarsino 2016). Thanks to its capability to encourage substantial and sustainable weight loss, bariatric surgery became an increasingly prevalent intervention for obesity treatment (Al-Najim et al. 2018).

Bariatric surgery (BS) interventions have been developed over the years and can be classified as either being restrictive or malabsorptive, both reducing food intake

and promoting weight loss (Andari Sawaya et al. 2012). The different bariatric procedures started from the 1950s with radical small bowel operations such as the jejunal–ileal bypass, to the gastric bypass in the 1960s (Alden 1977; Griffen et al. 1977; Mason and Ito 1967), gastric banding in the 1990s (Kuzmak et al. 1990), and the more recently widely spread vertical sleeve gastrectomy (Almogy et al. 2004). Lately, the whole spectrum of bariatric procedures but especially gastric bypass and sleeve gastrectomy are referred as metabolic surgery procedures, thus emphasizing the health benefits associated with weight loss rather than simply weight loss itself (Santoro 2015).

The armamentarium of metabolic surgery procedures includes laparoscopic adjustable gastric band (LAGB), vertical sleeve gastrectomy (VSG), Roux-en-Y gastric bypass (RYGB), biliopancreatic diversion (BPD), and BPD with duodenal switch (BPD/DS) (Andari Sawaya et al. 2012; Fontana and Wohlgemuth 2010).

From all the abovementioned procedures, the most commonly performed worldwide are RYGB and VSG (Angrisani et al. 2015). Currently, about 50% of the bariatric procedures are VSG and around 40% are RYGB (Angrisani et al. 2017). However, although VSG became more popular during recent years, RYGB has been performed over decades, and therefore it is estimated that millions of RYGB patients are residing worldwide in the general population (Björklund and Fändriks 2019).

Table 6.1 presents a comparison among those two common bariatric procedures.

Today, BS is considered as the only effective treatment for achieving a pronounced and sustained weight loss (Björklund and Fändriks 2019). The Swedish Obese Subject (SOS) trial reports a weight loss following RYGB of 27 ± 12% after 15 years, whereas nonsurgical interventions (lifestyle changes and/or pharmacological treatment) have principally no effect over this time span. Controlled long--term studies (>5–8 years) on the effects of VSG are still few, but weight loss up to 5 years is similar to that occurring after RYGB (Björklund and Fändriks 2019).

Additionally, many studies have reported improvements in obesity-related comorbidities like T2DM, hypertension, metabolic syndrome, sleep apnea, and overall mortality after weight loss (Björklund and Fändriks 2019). It is worth to note that some of these metabolic improvements manifest well before body weight becomes reduced, indicating a direct action on metabolic control by the modified gastrointestinal anatomy and functions (Santoro 2015). As an example, it has been shown that after both RYGB and VSG, glucose levels decrease significantly, well before any considerable weight loss is achieved, due to weight-independent mechanisms (Pucci and Batterham 2019) such as the faster gastric emptying occurring following RYGB and VSG (Melissas et al. 2007; Thaler and Cummings 2009).

In 2016, a joint statement by several international diabetes organizations stated that metabolic surgery should be recommended in patients with class II and III obesity and considered as an option in patients with class I obesity with poor glycemic control (Rubino et al. 2016).

Additionally, after BS, total cholesterol, triglycerides, and LDL were significantly lower, along with increased HDL, implying a normalization of the lipoprotein profile, possibly due to the weight loss (Magouliotis et al. 2017). In a comparison study among RYGB and VSG patients, glucose, triglycerides, and HDL levels were

Table 6.1 Comparison of the two main bariatric surgery procedures

	Roux-en-Y gastric bypass (RYGB)	Vertical sleeve gastrectomy (VLS)
Technique	• 15–30 mL gastric pouch, • Gastrojejunostomy (GJ). • Jejunojejunal anastomosis (Roux-en-Y). – 30–50 cm distal to ligament of Treitz • Remnant disconnected but left in situ.	• Excision of lateral 70–80% of stomach along the great curvature. • ~100 mL gastric reservoir (sleeve)
Mechanism of action	• Instantaneous food transfer to small intestine, altering: – Gut hormones. – Bile acids. – Neural signaling. – Gut microbiota. – Gut–brain–endocrine. – Adipocyte-brain axes. • Results in reduced food intake, increased satiety, and altered food preferences	• Alterations in: – Gut hormones. – Bile acids. – Neural signaling. Gut microbiota. Gut–brain–endocrine. Adipocyte-brain axes. • Results in reduced food intake, hunger, increased satiety, and altered food preferences
Advantages	• Significant long-term weight loss. • Glycemic control improvement in 90% of cases. • Maintain percent EWL in the long term. • Hunger reduction and satiety. • Food preferences changes. • Increases energy expenditure.	• Significant long-term weight loss (~10% less than RYGB). • Glycemic control as effective as RYBG. • Maintain percent EWL in the long term. • Hunger reduction and satiety. • Food preferences change. • No anatomical rerouting of food. • Short length of stay (<2 days). • Technically simpler than RYGB. • Lower complication rate than RYGB.
Disadvantages	• Technically complex (two anastomoses) compared with AGB or VSG). • Higher complication rate than AGB or LSG; for example, anastomotic leak or dumping syndrome can occur • Longer length of stay. • Long-term vitamin and/or mineral deficiencies (for example, vitamin B12, iron, calcium, or folate) • Requires lifelong vitamin and/or mineral supplementation. • Lifelong dietary changes. • Increases alcohol addiction and suicide rates. • Postprandial hypoglycemia.	• Anastomotic leak can be difficult to manage. • Susceptible to long-term vitamin and/or mineral deficiencies (less common than with RYGB) • Precautionary lifelong vitamin and/or mineral supplementation • Lifelong dietary changes. • Irreversible. • Potential risk of Barrett esophagus.

EWL excess weight loss

comparable between the two groups, while insulin levels were significantly greater in the VSG group. Therefore, it is evident that both BS procedures are metabolically efficient, a finding parallel with their similar efficiency in weight loss (Magouliotis et al. 2017).

All the above data demonstrate the significant amelioration of metabolic and lipidemic profiles of patients undergoing bariatric surgeries.

6.5.2 The Mechanisms of Gastric Bypass

Gastric bypass procedures are considered as an artificial condition where the intestinal mucosal energy outflow is a physiological variable which can impact both body weight and glycose levels.

Contrary to an old assumption, the weight loss after a BS procedure is not achieved neither by malabsorption nor by a mechanical restriction of food intake. Instead, the main driving force for weight loss is rather a modified eating behavior which reduces energy intake (Makaronidis and Batterham 2016). Also, regarding the old belief that reduced meal size is due to the limited size of the gastric pouch is not valid anymore, as the current surgical procedure leaves a minimum gastric pouch (20–30 mL) but followed by a large caliber gastroenteroanastomosis (GEA) without any outflow restriction. Therefore, the small pouch together with the Roux limb should be considered as a common cavity, so any possibility for the GEA to act as a restriction site can be excluded. Using high-resolution manometry, it has been confirmed that during eating there is no intraluminal pressure gradient between the pouch and the Roux limb (Björklund et al. 2015). However, it has been reported that RYGB exhibits a restrictive element with the restriction site situated to the Roux limb (Björklund et al. 2010). Until now, the actual clearance rate of Roux limb has not been assessed and therefore to what extent such a dynamic flow restriction of the Roux limb plays a food intake regulating significance remains to be investigated.

In addition to regulating energy intake, different studies revealed an expanded energy expenditure in RYGB patients. Interestingly, it appears not to be the basal metabolic rate (BMR) that becomes upregulated, but rather the thermogenesis associated to meal intake is the causative process (Werling et al. 2015). The exact mechanism involved is unknown, but according to experiments in rodents, it might be due to a reprogrammed mucosal metabolism in the Roux limb.

Another two mechanisms of RYGB effect are the changes of circulating bile acids and these of the intestinal microbiota; More specifically, it is hypothesized that bile acids regulate glucose metabolism through the TGR5 receptor acting on L cells, causing release of GLP-1, and also provoke synthesis and secretion of fibroblast growth factor 19 (FGF19) which improves insulin sensitivity, leading to an improved glycemic control (Madsbad et al. 2014).

It has been reported that transferring feces from RYGB-treated to GF mice caused significantly bigger loss of weight as compared to mice receiving feces from sham-surgery treated mice (Makaronidis and Batterham 2016). Additionally, GF mice inoculated with fecal microbiota from BS patients added less fat than mice

transplanted with microbiota originating from obese patients (Tremaroli et al. 2015). Theoretically, it is expected that the jejunal mucosa into the Roux limb becomes inflamed by the new intraluminal milieu and, in turn, responds starting an antiingestive signaling. Nevertheless, a thorough examination of the postoperative mucosa did not support this hypothesis, and although some pro-inflammatory signs were present, the Roux limb mucosa did not manifest any inflammation (Spak et al. 2010).

In summary, it seems that the biomechanic properties of the Roux limb wall regulate both food intake and intestinal sensing. Thus, the proposed hypothesis that "big mealers" have a low-threshold for inducing Roux limb clearance motility awaits confirmation (Björklund and Fändriks 2019).

6.5.3 Side Effects of Bariatric Surgery

Bariatric surgery has some unwanted consequences, thus requiring a cost-benefit analysis for every individual candidate. About 4% of patients after BS manifest surgical complications within the first 30 postoperative days (Schulman and Thompson 2017; Sjöström et al. 2004). Typical postoperative complications include anastomotic leakages, bleeding, perforation, and infections, as well as inner herniations (Schulman and Thompson 2017), although the herniation incidence has been dramatically lowered after the closure of any mesenteric defect became a standard routine practice during the BS operation (Stenberg et al. 2016). Late surgical complications are also detected in 15–20% of patients, and they include obstruction of the small bowel, anastomotic stenosis, or marginal ulceration (Franco et al. 2011). Both early and late surgical complications can be diagnosed and treated by means of a surgical or endoscopic intervention. Additionally, except typical surgical complications, there are also procedure-dependent side effects, like excess skin requiring additional cosmetic surgery, dumping symptoms and postprandial hypoglycemia, as well as micronutrients deficiency (Björklund and Fändriks 2019).

Unexplained chronic abdominal pain is a common negative side effect seen in patients after RYGB (Cho et al. 2008). It is reported that 54% of RYGB patients suffer from abdominal pain and in a 5-year follow-up, 34% of these patients still experience abdominal pain (Gribsholt et al. 2016; Høgestøl et al. 2017). It is of paramount importance to elucidate the underlying pathology of chronic abdominal pain following BS but its etiology remains still obscure (Greenstein and O'Rourke 2011). The long-term consumption of morphine or its analogs for pain relief in RYBG patients may provoke to opioid-induced bowel dysfunction which presents with constipation, nausea and vomiting, and to the narcotic bowel syndrome (King et al. 2017a). Furthermore, it is estimated that 4% of patients who were not on opioids before became chronic opioid users after BS (Raebel et al. 2014), and therefore the physician of a RYGB patient with chronic postprandial nausea and pain must be aware of the risk for iatrogenic opioid-associated symptom aggravations.

Hypoglycemia in patients without diabetes appears in 64–82% of patients during the first 5 years of BS (Schauer et al. 2017). The underlying mechanism is not clear,

and several theories have been proposed including enhanced B cells mass and function, reduced ghrelin levels, improved insulin sensitivity, and failure of counter regulation (Abdeen and le Roux 2016). The consequent side effects of hypoglycemia often persist throughout the years and can thus worsen the quality of life.

6.6 Gut Microbiota After Bariatric Surgery

Many surgical diseases are related to gut microbiota alterations. So far, obesity, nonalcoholic fatty liver disease, colorectal cancer, intestinal anastomotic leaks, inflammatory bowel disease, and atherosclerosis have been reported (Chen et al. 2018).

As mentioned previously, BS is the treatment of choice to accomplish and maintain in the long term a normal weight to morbidly obese patients. Those patients who undergo BS are losing weight significantly, and they restore their metabolic health regarding T2DM, dyslipidemia, hypertension, and cardiovascular risk (Buchwald et al. 2004; Sjöström et al. 2007).

It has been shown that BS plays a cardinal role by altering the abundance of several microbial species of the GM. However, the available data regarding the changes of GM after BS are highly heterogeneous and insufficient to be included in quantitative analysis (Magouliotis et al. 2017).

The exact mechanisms underlying the postsurgical restructuring of the GM have not yet been elucidated and must yet to be explained. However, it is certain that the dramatic anatomical alterations induced by BS contribute significantly to the substantial metabolic changes observed following BS (Medina et al. 2017). Additionally, several factors coexist that can alter the postoperative status of the BS patients: Caloric restriction (substantially energy-restricted diet with higher protein intake), alterations in the secretion of gut hormones and bile acids, and changes of the GM composition have been proposed as possible mechanisms (Heneghan et al. 2012). Thus, due to the multiple metabolic and hormonal changes which coincide during the early postoperative period, it is rather difficult to establish underlying relationships between factors related to BS and changes in GM composition and function after performing BS (Lakhani et al. 2008).

Several studies have shown that bariatric surgery provokes alterations to the GM which can be installed as early as the first week after surgery and in any case as soon as the first 3 months postoperatively (Tremaroli et al. 2015; Liou et al. 2013; Palleja et al. 2016), and this effect is sustained up to 9 years (Tremaroli et al. 2015).

Additionally, late complications include severe deficiency-related disorders, such as anemia (10–74%) and neurological dysfunctions (5–9%) (Xanthakos 2009). Therefore, the patients who underwent BS are in need of a rigorous follow-up aiming to prevent those side effects through GM modulation and adequate nutritional supplementation (Ciobârcă et al. 2020).

It has been observed that a major alteration in the structure and diversity of GM is taking place after BS. A recent meta-analysis reviewed 22 studies and 562 patients who underwent different types of BS. Despite that different studies reported a

considerable variation in the bacterial species, the overall findings support a postoperative shift of the GM (Makaronidis et al. 2016). Therefore, this GM change might not be the result but rather the reason of weight loss after BS, as it has been recently suggested that metabolic regulation is starting from the gut which then is signaling to the brain and other endocrine organs to adapt to this change (Fetissov 2017).

The most common change observed after BS procedures is a decrease of *Firmicutes* and an increase of *Bacteroidetes*, *Proteobacteria*, especially of *Gammaproteobacteria* (genus *Escherichia*) abundance (Zmora et al. 2019). In another study, a decrease of the *Firmicutes/Bacteroidetes* ratio was reported following BS in subjects with morbid obesity, accompanied with a substantial change of the structure and function morbidly of the GM. However, the whole subject is still under debate (Tremaroli et al. 2015). It is also worthwhile to note that additional GM changes following BS have been reported in a study: An increase in the phyla Verrucomicrobia and Fusobacteria and a diminished amount of Actinobacteria (Ulker and Yildiran 2019).

Some articles focused on fecal microbiota transfer experiments. A well-planned study showed that both RYGB and VBG have similar long-term effects on the composition and functional capacity of the gut microbiome. It is worth to note that the GM changes were independent from BMI or from the magnitude of weight and fat mass loss, thus suggesting that BS can cause specific shifts in the GM. In the same study, feces from BS patients were transplanted to GF mice; 2 weeks after transplantation, the mice gained less fat as compared to reciprocal mice transplanted with GM from obese subjects. Those findings suggest a causal relationship between GM and to BS-induced weight loss (Tremaroli et al. 2015). The same results are reported in another study which showed that GM transplantation from mice which underwent RYGB to sham-surgery germ-free mice provoked weight loss and decrease of adipose tissue when compared to recipients of GM from nonoperated mice (Liou et al. 2013).

A similar GM transplantation study was done in a group of females who, 9 years previously, were randomly assigned to undertake RYGB or VSG: Both types of surgery recipients showed similar GM profiles of their fecal samples (as assessed by means of 16S rRNA amplicon sequencing analysis) and furthermore, they were substantially different from the profiles of nonoperated obese women. When feces from BS patients were inoculated to GF mice, the recipients had decreased fat mass as compared to reciprocal mice that received GM from obese, nonoperated subjects. Additionally, the recipient mice which were transplanted with human post-RYGB GM showed the bigger increase of lean body mass. Therefore, it seems that the human GM can directly trigger the reduction of adipose tissue seen after BS (Tremaroli et al. 2015).

In another longitudinal study of obese individuals, it was found that *Bacteroidetes* were reduced prior to surgery, but 3 months post-RYGB, the *Bacteroidetes* abundance was returned to presurgery levels, being remarkably similar to that of lean control group. Additionally, the observed abundance in *Bacteroidetes* following RYGB correlated with a substantial decrease of adipose tissue and an increased serum leptin levels (Furet et al. 2010).

Methanogenesis facilitates the fermentation of dietary fibers through the consumption of hydrogen and acetate, and methanogenic archaea are found in abundance in obese subjects. In a study comparing the 16S rRNA sequences in the feces of three groups, namely normal weight, morbidly obese, and post-RYGB subjects, distinct differences were found in the GM between the three cohorts; Methanogenic archaea were found in abundance in the obese group, but they were found below detection levels in normal weighted or all-but-one post-RYGB patient (Zhang et al. 2009).

The same changes in the GM are also observed after sleeve gastrectomy: In diet-induced obese mice that underwent VSG, a substantial and sustained increase of *Bacteroidetes* and a relative decrease in *Firmicutes* is reported. Additionally, GM metabolism is related to that of the host. Thus, 3 months after VSG, several metabolic processes of the patients, such as carbohydrate fermentation, citrate cycle, and amino acids production, as determined by shotgun metagenomic sequencing, became more analogous to those of normally weighted control group (Jahansouz et al. 2017). However, regarding the metabolic improvement or the degree of weight loss, it seems that BS itself is more important factor relatively to the feces transplantation, indicating that apart from GM, BS and other pathways are involved in those positive results (Aron-Wisnewsky et al. 2019).

Several other gut bacteria are proliferating after BS; Due to the increased pH into the lumen and high levels of dissolved oxygen, both been observed after BS, the growth of facultative aerobic microorganisms (such as Proteobacteria) and inhibition of anaerobic microbes is observed (Medina et al. 2017). In tandem, the diminished gastric volume resulting after BS increases the pH of both the stomach and distal intestine, and the resulting gastrointestinal acidity leads to microbial overgrowth and promotes the abundance of *Akkermansia muciniphila*, *E. coli*, and *Bacteroides* spp. or of the oral microbiota bacteria (Anhê et al. 2017).

However, there is a couple of studies using sequencing methods, described a high MGR and bigger GM diversity following both RYGB and VSG as well as a change from "obese" to a "lesser obese" microbial species profile (Debédat et al. 2019; Aron-Wisnewsky et al. 2019). Nevertheless, despite profound weight loss and improvement of metabolic markers after both surgeries, the MGR may not be fully restored 1 year after RYGB and remain unchanged even after 5 years (Aron-Wisnewsky et al. 2019; Anhê et al. 2017). The absence of complete repair of GM after BS could explain the observed delayed regain of weight and the recurrence of obesity related comorbidities observed in some patients after BS. The fact that BS alone cannot reestablish MGR indicates that other contributing mechanisms (i.e., metabolic and inflammatory amelioration, weight loss, or diet) are also involved (Debédat et al. 2019).

However, the two BS surgeries might exhibit different functionality due to the different surgical techniques as well as to resulting different intestinal environmental conditions. With that in mind, one would anticipate more profound changes in the intestine after RYGB as contrasted to VSG, as besides caloric restriction, it involves more radical and complex anatomical changes and more functional modifications of the GI tract (Cătoi et al. 2019).

Below are listed some studies exploring the GM-related outcomes of the different surgical BS procedures.

Administration and/or abundance of *Akkermansia muciniphila* is related to enhanced gut barrier function and diminished metabolic endotoxemia as a result of decrease of the circulating levels of systemic lipopolysaccharide (Everard et al. 2013). Also, the administration of *Akkermansia muciniphila* rose L cells numbers which, when stimulated, induce GLP-1 release which is involved in glucose homeostasis (Yan et al. 2016) and GLP-2, an important intestinal growth factor (Everard et al. 2011). It has been reported that after RYGB, the *Akkermansia muciniphila* increases (Graessler et al. 2013) which has been negatively correlated with body mass (Anhê et al. 2015).

Furthermore, following RYGB, *Escherichia coli* abundance is enhanced and, independently of food intake changes, it is inversely correlated with fat mass and leptin levels, in contrast to *Faecalibacterium prausnitzii*, which is found to decrease after RYGB (Furet et al. 2010).

Several factors have been advocated to play a role for the vast GM restructuring observed after RYGB as the disrupted anatomy (small gastric remnant and shortened small intestine) results in decreased food ingestion. Additionally those severe anatomic changes also have some physiological consequences like changes in pH, transit time, and input of dissolved oxygen which promotes the relocation of some of the typically residing in the small bowel microbiota, to the large intestine (Zhang et al. 2009). Additionally, the observed GM change after RYGB could also be attributed to altered bile acid metabolism which is regulated by BS as well (Peck and Seeley 2018).

Two recent meta-analyses reported that although after BS the diversity and richness of GM greatly fluctuated across studies, certain bacterial phylae such as Bifidobacteria was strongly correlated with BMI (Magouliotis et al. 2017; Guo et al. 2018).

A study investigated whether the GM changes after RYGB are preserved and whether inoculation of RYGB modified microbiota can provide a transferable weight loss effect on other recipients. Using a mouse RYGB model which resembles many of the metabolic outcomes seen in humans, fecal samples of three groups were collected for 16*S* ribosomal RNA gene sequencing: after RYGB surgery, sham surgery, or sham surgery coupled to caloric restriction. The sequential analysis showed that distal gastric, ileal, cecal, and colonic microbiota were strongly altered after RYGB. A rapid and sustained increase in the relative abundance of *Enterobacteriales* and *Verrucomicrobiales* was found. Three phyla increases are prevailed: In Bacteroidetes, Verrucomicrobia, and Proteobacteria, with resolution to the genus level of *Alistipes*, *Akkermansia*, and *Escherichia*. The observed GM alterations were unbiased of weight alteration and calories restriction and were found along the entire length of the GIT but mostly evident distally from the surgical manipulation site. The recipient lean GF mice transplanted with feces from RYGB--operated rodents had reduction of fat mass which was not observed after inoculation of GM from mice that had lost weight due to food restriction. The above findings provide evidence to the assumption that GM changes contribute to reduced host weight and fat mass following RYGB surgery (Liou et al. 2013).

A study performed in morbidly obese individuals within 3 months after they underwent RYGB found that their GM featured an increased relative abundance of 31 species, including *Escherichia coli*, *Klebsiella pneumoniae*, *Veillonella* spp., *Streptococcus* spp., and *Alistipes* spp., while *Akkermansia muciniphila and Faecalibacterium prausnitzii* decreased in their relative abundance. Furthermore, an augmented potential for oxygen tolerance as well as for microbial utilization of macro- and micronutrients was reported and those changes were still present 1 year after RYGB (Palleja et al. 2016).

The phylogenetic analysis of GM of three groups (healthy, obese, and post--RYGB subjects) showed six main bacterial phyla to be present but distributed differently in the GI of the study groups. Interestingly enough, *Prevotellaceae* was explicitly detected only in obese subjects, and therefore it is considered as obesity specific bacteria. To the contrary, *Fusobacteria* and the *Enterobacteriaceae* within *Proteobacteria* family were found only in the RYGB group (Al-Najim et al. 2018).

Tremaroli et al. (2015) performed shotgun sequencing of the fecal metagenome to analyze the GM of weight-stable women 9 years post-RYGB. Furthermore, they conducted human-to-mouse GM inoculation. After RYGB, an increased abundance of Gammaproteobacteria was detected, while in contrast lower levels within the Firmicutes phylum of *Clostridium difficile*, *Clostridium hiranonis*, and *Gemella sanguinis* were detected. In contrast, facultative anaerobes within *Proteobacteria* (*Escherichia*, *Klebsiella*, and *Pseudomonas*) family were found augmented in the RYGB recipient mice. The metabolomic comparisons performed after BS showed an inhibited SCFA/branched-chain fatty acid ratio, a finding suggesting an increased amino acid fermentation. The genetic signatures for microbial enzymes participating in the synthesis of secondary bile acids were enhanced in parallel to a shift of secondary to primary bile acid profiles ratio, suggesting that altered bile acid profiles may participate in reductions in fat mass following BS (Al-Najim et al. 2018).

In a study comparing the impact of both RYGB and VSG on GM, an important increase of *Proteobacteria* was found. The same altered pattern (a *Roseburia* abundance) was also shown in T2DM patients who underwent RYGB or VSG when a T2DM remission was achieved. In contrast, 6 months postoperatively, despite similar weight loss, the *Bacteroidetes* increased in RYGB group of patients, while it decreased in the VSG group (Davies et al. 2019).

Additionally, as RYGB provokes greater rearrangements of the digestive tract than VSG, a significantly lower body weight and a greater shift on GM were produced from RYGB as compared to VSG, 9 weeks postoperatively (Shao et al. 2017). It is postulated that the differences observed between the two techniques could be due to the fact that VSG involves much less intestinal manipulations than RYBG. The above results were also confirmed by a study which revealed that RYGB provoked increased *Firmicutes* and *Actinobacteria* but decreased *Bacteroidetes*, but the later been found increased after VSG. Thus, 1 year following RYGB surgery, more significant functional GM alterations were found as compared to VSG, despite similar diet, weight loss, or remission of T2DM (Murphy et al. 2017).

It has been reported that sleeve gastrectomy provokes both early (1 week after surgery) and prolonged (1 month after surgery) changes of the GM. Furthermore,

the same article demonstrated that the altered microbial composition of VSG operated rodents is persisting and does not change even when reexposure to obesity associated GM occurs (Jahansouz et al. 2017). The same findings are also reported regarding the functional capacity of GM after VSG in 23 obese patients. It was found that 3 months post-VSG, the microbial activity was similar to that of lean subjects and a marked increase of *B. thetaiotaomicron*, an anti-obesogenic substance, was observed (Liu et al. 2017).

In a recent systematic review, Davies et al. summarized 14 clinical studies, with a total of 222 subjects (RYGB = 146, VSG = 25, biliointestinal bypass = 30, vertical banded gastroplasty = 7, and adjustable gastric band = 14). Major switches comprise a reduction of the relative abundance of *Faecalibacterium prausnitzii* and an increase of *E. coli*. After VSG, a decrease in the relative abundance of *Firmicutes* while following RYBG an increase in *Bacteroidetes* and *Proteobacteria* was also noticed (Davies et al. 2019).

Their findings are summarized in Table 6.2. It was found that the different types of BS result in dramatic changes of gut bacteria, but the contribution of those alterations to the metabolic benefits achieved is still unclear (Davies et al. 2019).

A systematic review and meta-analysis reviewed the impact of BS in metabolic and GM profiles, of 22 articles published between 2008 and 2016. However, they found that only two studies were randomized, the rest being prospective ones (Tremaroli et al. 2015; Kong et al. 2013). The total sample size was 562; 411 patients had RYGB and 97 underwent VSG (Magouliotis et al. 2017).

As shown in Table 6.3, several microbes are affected by BS. As can be seen from this table, some authors found increased Bacteroides while Firmicutes and Bifidobacterium had lower abundance in the post-RYGB subjects (Graessler et al. 2013; Lips et al. 2014).

More specifically, regarding RYGB, two studies found lower *Firmicutes* abundance after RYGB (Graessler et al. 2013; Lips et al. 2014) while two other studies showed the opposite (Narath et al. 2016; Trøseid et al. 2016). Additionally, another study showed that *Lactobacillus*, been part of the *Firmicutes* family, was in higher abundance after biliointestinal bypass (Papamargaritis et al. 2013). The discrepancies observed among the results of those studies can be explained from the different clinical protocols applied using varying levels of calorie restriction. Furthermore, another couple of studies showed an increased Bacteroides abundance in RYGB patients and the higher was the Bacteroides increase after RYGB, the bigger the decrease in body fat mass and leptin (Graessler et al. 2013; Lips et al. 2014). It is worth to note that the same findings were also reported in less obese subjects (Quercia et al. 2014).

In another study, an increased Bacteroidetes abundance was found after VSG, while after RYGB a decrease for the same phylum was observed (Narath et al. 2016). Regarding *E. coli* population, it was found enhanced in five studies (Graessler et al. 2013; Lips et al. 2014; Trøseid et al. 2016; Papamargaritis et al. 2013; Gralka et al. 2015). The increase in abundance of *Escherichia coli* could be due to anatomical readjustments causing higher oxygen concentrations in the distal intestine (Gralka et al. 2015).

Table 6.2 Gut microbiota changes described after bariatric surgery in human studies

Reference, design of the study	Design of the study	Number of patients with GM analyses	Surgery type (*n* of patients)	DNA extraction	Sequencing technique	Time points sequenced	Changes in GM after BS	Impact of BS on fecal richness	Comments
Zhang et al. (2009)	BS VS obese VS lean individuals	6 MO patients and 3 lean individuals	RYGB ($n = 3$)	QIAamp DNA Stool Kit (Qiagen)	Sanger and 16S rRNA pyrosequencing	8 to 15 months post-BS	↑ *Gammaproteobacteria*, *Verrucomicrobia*, *Fusobacteria* ↓ *Clostridia*	–	–
Furet et al. (2010)	BS VS lean individuals	30 MO (7 with T2D) patients and 13 lean individuals	RYGB ($n = 30$)	Godon et al. (1997)	16S rRNA qPCR	Before, 3 and 6 months post-BS	↑ *Bacteroides/Prevotella ratio*. *Faecalibacterium prausnitzii*, *E. Coli* ↓ *Bifidobacterium Lactobacillus*, *Leuconostoc*, *Pediococcus*	–	GM changes correlated with body weight, BMI, fat mass, leptin levels, and food intake changes after BS
Patil et al. (2012)	BS VS obese VS lean individuals	5 thin, 5 lean, 5 obese, and 5 obese-operated individuals	SG ($n = 3$) and AGB ($n = 2$)	QIAamp DNA Stool Mini Kit (Qiagen)	Sanger	–	↓ *Bacteroides* and Archaea	No changes	–
Kong et al. (2013)	BS	30 MO patients	RYGB ($n = 30$)	Godon et al. (1997)	16S RNA (V3–V4) pyrosequencing	Before, 3 and 6 months post-BS	↑ *Bacteroides*, *Escherichia (proteobacteria)*, *Alistipes* ↓ *Lactobacillus*, *Dorea*, *Blautia*, *Bifidobacterium*	Number of – genera and Chao1 Index	Increased richness of GM after RYGB. Most of the genera modulated by BS were correlated to clinical variables

(continued)

Table 6.2 (continued)

Reference, design of the study	Design of the study	Number of patients with GM analyses	Surgery type (*n* of patients)	DNA extraction	Sequencing technique	Time points sequenced	Changes in GM after BS	Impact of BS on fecal richness	Comments
Graessler et al. (2013)	BS	6 MO patients (*n* = 5 T2D)	RYGB (*n* = 6)	Nycodenz density gradient centrifugation, bacterial lysis, and DNA digestion[7]	Shotgun metagenomic sequencing (Illumina)	Before and 3 months post-BS	↑ *Enterobacter*, *Citrobacter*, *Neurospora*, *Veillonella*, *Salmonella*, *Shigella*, *E. coli*, *Klebsiella (Proteobacteria)*, *Bacteroidetes/Firmicute* ratio, *Verrucomicrobia* ↓ *Firmicutes*, *Cyanobacteria*, *Faecalibacterium*, *Coprococcus*, *Helicobacter*, *Dictyostelium*, *Epidinium*, *Anaerostipes*, *Nakamurella*, *Methanospirillum*, *Thermomicrobium*	–	Several bacteria were correlated to both BMI and CRP post-BS
Ward et al. (2014)	BS	8 MO patients	RYGB (*n* = 8)	Ultra Clean Fecal DNA Kit (MO BIO, Inc.)	16S rRNA (V4) pyrosequencing	Before and 6 months post-BS	↑ *Bacteroidetes*, *Bacteroidetes/ Firmicutes* ratio, *Proteobacteria* (PPI users), *Verrucomicrobia* ↓ *Firmicutes*, *Proteobacteria* (PPI nonusers)	–	–

Damms--Machado et al. (2015)	BS VS VLCD	6 MO patients	SG ($n = 3$)	PSP Spin Stool DNA Plus Kit with lyses enhancer (STRATEC Molecular, Berlin, Germany)	Shotgun metagenomic sequencing (SOLiD)	Before, 3 and 6 months post-BS	↑ *Bacteroidetes*, *Faecalibacterium prausnitzii* ↓ Several *Firmicutes* (*Eubacterium*, *Faecalibacterium*, *Dorea*, and *Coprococcus*), *Bacteroides vulgatus*, *Bacteroidetes/Firmicutes ratio*	–	High interindividual variability regarding Bacteroidetes/Firmicutes ratio at baseline, despite relatively similar BMI
Tremaroli et al. (2015)	RYGB vs VBG vs MO patients	21 MO patients	RYGB ($n = 7$) and VBG ($n = 7$)	QIAamp DNA Stool Mini Kit columns	Shotgun metagenomic sequencing (Illumina)	About 10 years post-BS	↑ *Gammaproteobacteria*, *Proteobacteria (Escherichia, Klebsiella, and Pseudomonas). Not statistically significant increase of E. coli* ↓ 3 species of *Firmicutes (Clostridium difficile*, *Clostridium hiranonis*, *Gemella sanguinis*), *Eubacterium rectale* (VBG), *Roseburia intestinalis* (VBG)	–	Similar microbiota profiles between RYGB and VBG. Differences in GM composition and genetic content mostly due to the intervention and not BMI

(continued)

Table 6.2 (continued)

Reference, design of the study	Design of the study	Number of patients with GM analyses	Surgery type (*n* of patients)	DNA extraction	Sequencing technique	Time points sequenced	Changes in GM after BS	Impact of BS on fecal richness	Comments
Federico et al. (2016)	BS	11 MO patients	BIP ($n = 11$)	Maxwell® 16 DNA Purification Kit (Promega)	qPCR-DGGE	Before and 6 months post-BS	↑ *Lactobacillus crispatus*, *Megasphaera elsdenii*, *Streptococcus* spp. ↓ *Butyrivibrio fibrisolvens*, *Roseburia hominis/faecis*, *Dorea longicatena*, *Blautia* spp., *Ruminococcus* spp., and *Ruminococcus obeum*	–	Highly different bacteria profiles, (50–65% presurgery and 30–65% postsurgery)
Palleja et al. (2016)	BS	13 MO patients ($n = 7$ with T2D and $n = 1$ with IGT)	RYGB ($n = 13$)	IHMS 07 V2	Shotgun metagenomic sequencing (Illumina)	Before, 3 months and 1-year post-BS	↑ *Proteobacteria* (including *Escherichia coli* and *Klebsiella pneumoniae*), *Streptococcus salivarius*, *plus 9* species belonging to the genus *Streptococcus*, 4 from *Veillonella*, 2 from *Alistipes*, *Bifidobacterium dentium*, *Enterococcus faecalis*, *F. nucleatum*, and *Akkermansia muciniphila* ↓ *Faecalibacterium prausnitzii*, *Anaerotruncus colihominis*, *Megasphaera micronuciformis*	↑ Gene richness and Shannon diversity index during the first 3 months and stable afterwards	Surgery, baseline T2D status, metformin usage, GLP-1 levels (at each time point), and BMI (at each time point) explained most of the variation in terms of species composition

Patrone et al. (2016)	BS	11 MO patients (n = 6 T2D)	BIB (n = 11)	Maxwell® 16 DNA Purification Kit (Promega)	Shotgun metagenomic sequencing (Illumina)	Before and 6 months post-BS	↑ *Selenomonadales*, *Acidaminococcus*, *Megasphaera*, *Lactobacillus*, *Enterobacteriaceae*, *Gammaproteobacteria*, *Proteobacteria* ↓ *Lachnospiraceae*, *Ruminococcus*, *Faecalibacterium*, *Clostridiaceae*, *Blautia*	↓ Chao1, Shannon and Simpson indexes	31 bacterial groups were differentially abundant. Decreased fecal pH after BS. *Proteobacteria* correlated to glucose
Murphy et al. (2017)	BS	14 MO patients	RYGB (n = 7) and SG (n = 7)	Qiagen QIAamp DNA Stool Mini Kit	Shotgun metagenomic sequencing (Illumina)	Before and 1-year post-BS	↑ RYGB: *Roseburia intestinalis* (*Firmicutes*), *Actinobacteria*; SG: *Bacteroidetes*, *Roseburia intestinalis* (*Firmicutes*) ↓ RYGB: *Bacteroidetes*	↑ Number of species (RYGB)	*Roseburia intestinalis* is associated with T2D remission both after SG and RYGB
	BS	23 MO patients	SG (n = 23)						
Liu et al. (2017)				Nycodenz Density Gradient centrifugation, bacterial lysis, and DNA digestion (Manichanh et al. 2006)	Shotgun metagenomic sequencing (Illumina)	Before, 1 and 3 months post-BS	↑ *Bacteroides thetaiotaomicron*, *Akkermansia muciniphila*, *Clostridiales bacterium* ↓ *Coprococcus comes* and *Dorea longicatena*	↑ Gene count, alpha diversity	The GM composition of BS-operated obese patients shifted towards those of lean individuals

(continued)

Table 6.2 (continued)

Reference, design of the study	Design of the study	Number of patients with GM analyses	Surgery type (*n* of patients)	DNA extraction	Sequencing technique	Time points sequenced	Changes in GM after BS	Impact of BS on fecal richness	Comments
Aron-Wisnewsky et al. (2019)	BS	34 MO patients	RYGB (n = 14+ 10) and AGB (n = 10)	Godon et al. (1997)	Shotgun metagenomic sequencing (SOLiD)	1, 3, 12 months and up to 5 years post-BS	↑ *GU:99 Roseburia*, *GU:225 Butyricimonas virosa*, *GU:359 Butyricimonas*	↑ Gene richness 3 mo after BS. The ↑ similar proportion for both AGB and RYGB, and remained stable up to 5 years post-op	Higher GM impact of RYGB than AGB. BMI and fat mass correlations: Positive with: *Bacteroides finegoldii*, *Coprobacillus* spp., *Anaerostipes hadrus*: *Fusobacterium nucleatum*, *Dialister* spp., and *Hungatella hathewayi*
Paganelli et al. (2019)	BS	45 MO patients	RYGB (n = 23) and VSG (n = 22)	Godon et al. (1997)	16S rRNA (V3–V4) shotgun sequencing (Illumina)	Before, 3 and 6 months post-BS	↑ *Streptococcaceae*, *Enterobacteriaceae* ↓ *Bifidobacteriaceae*	No changes	

Table 6.3 Postoperative GM changes

Author, year	Postoperative GM changes		
	Increased abundance	Decreased abundance	Comments
Federico et al. (2016)	*Lactobacillus crispatus*	*Butyrivibrio fibrisolvens*, *Roseburia hominis/faecis*, *Dorea longicatena*, *Blautia spp./Ruminococcus spp.*, *Ruminococcus obeum*	Highly heterogenous fecal bacteria profiles, with similarity ranging between 50–65% in presurgery and 30–65% in postsurgery patients
Furet et al. (2010)	*Bacteroides/Prevotella* *E. coli*	*Bifidobacterium* *Lactobacillus/Leuconostoc/Pediococcus*	–
Graessler et al. (2013)	*Enterobacter*, *Citrobacter*, *Neurospora*, *Veillonella*, *Salmonella*, *Shigella* *E. coli* tended to increase	*Faecalibacterium*, *Coprococcus*, *Helicobacter*, *Dictyostelium*, *Epidinium*, *Anaerostipes*, *Nakamurella*, *Methanospirillum*, *Thermomicrobium*	–
Ishida et al. (2014)	–	–	Increased bacterial counts were registered in the gastric pouch
Kong et al. (2013)	*Bacteroides* *Alistipes* *Escherichia*	Firmicutes (*Lactobacillus*, *Dorea*, *Blautia*) *Bifidobacterium*	Increased richness of GM after RYGB
Murphy et al. (2017)	Firmicutes post-RYGB Actinobacteria post-RYGB Bacteroidetes post-SG	Bacteroidetes post-RYGB	–
Palleja et al. (2016)	*Escherichia coli*, *Klebsiella pneumonia*, 10 species belonging to the genus *Streptococcus*, 4 from *Veillonella*, 2 from *Alistipes*, *Bifidobacterium dentium*, *Enterococcus faecalis*, *F. nucleatum*, and *Akkermansia muciniphila*	*E. prausnitzii*	–
Patrone et al. (2016)	*Lactobacillus Megasphaera* *Acidaminococcus* *Enterobacteriaceae*	*Lachnospiraceae* *Clostridiaceae* *Ruminococcaceae* *Eubacteriaceae* *Coriobacteriaceae*	31 bacterial groups were differentially abundant
Tremaroli et al. (2015)	Gammaproteobacteria Several Proteobacteria (*Escherichia*, *Klebsiella*, *Pseudomonas*) *E. coli* tended to increase but not statistically significant	3 species of Firmicutes (*Clostridium difficile*, *Clostridium hiranonis*, *Gemella sanguinis*)	–

In summary, BS seems to restore a healthier microbiome with a leaner metabolic profile, and this realignment of the microbiome potentially contributes to reduced fat mass, increased lean mass, and resolution of BS associated comorbidities. However, the mechanisms by which gut microbes and their by-products affect obesity remain poorly understood and microbiome manipulations that exploit the host–bacteria interaction for the treatment or prevention of obesity still need to be developed (Chen et al. 2018).

6.6.1 Bariatric Surgery–Related Diet on Gut Microbiota

The rearrangement of the gastrointestinal tract following BS leads to alteration of the gut microbial ecology. The postsurgery food intake of patients submitted to RYGB or VSG has major quantitative and qualitative changes; In a matter of days, the calories restriction alters the bacterial structure of the bacterial community (Zmora et al. 2019).

It has been postulated that the observed GM shift after VSG (i.e., the reduction of the *Firmicutes/Bacteroidetes* ratio) might be the adaptive response of bacteria to the caloric constraint imposed by surgery. More precisely, the *Firmicutes* decrease results to diminished fermentation, to subsequent reduced energy intake, and, finally, to concomitant SCFAs production, the latter being substrates for gluconeogenesis and lipogenesis. A study showed that VSG, but not a strict dietary regimen with low calories, enhanced the obesity related GM synthesis towards a lean microbiome phenotype (Damms-Machado et al. 2015). Moreover, it has been shown that, in a mouse model, when only food restriction is applied there are no early changes in GM after RYGB, and therefore, weight loss seems to be one among the least important factors involved in the GM shift (Anhê et al. 2017).

Thus, in 45 subjects submitted to either RYGB ($n = 23$) or VSG ($n = 22$), GM composition and diversity changes were assessed before following a 2-week crash diet (baseline), by the end of it, as well as 1 week, 3 months, and 6 months postoperatively. A substantial but temporary alteration in GM was noticed after the baseline crash diet, but BS provoked more persistent changes in GM composition and to restoration of microbial diversity well before any significant weight loss, irrespectively of the type of BS performed. Both RYGB and VSG groups exhibited the same magnitude GM changes in all phases of the study (Paganelli et al. 2019).

6.6.2 Bariatric Surgery Effect on Small Intestine Bacteria

Obese patients after bariatric surgery may present small intestine bacterial overgrowth (SIBO), a condition defined as greater than 105 bacteria (colony forming units) mL^{-1} of proximal jejunal aspiration (DiBaise 2008). SIBO is a common manifestation of obesity and a recent prospective study, including 378 patients with morbid obesity, reported that 15% of patients before undergoing RYGB had SIBO, and that this figure increased up to 40% after the operation (Paganelli et al. 2019).

In clinical practice, SIBO diagnosis is made from small bowel aspirate test, but this test is invasive and costly so the most practical detection method is the "therapeutic trial," by empirically administering treatment with antibiotics upon the presence of the clinical manifestations associated with SIBO (Adike and DiBaise 2018).

SIBO interferes to the weight loss process and increases the micronutrient deficiencies risk. It manifests with several gastrointestinal symptoms, including bloating, diarrhea, and nutrients malabsorption, all depending from the specific type of bacteria that overgrow into in the small intestine (Sachdev and Pimentel 2013). Mechanical stasis is frequently associated with RYGB and creation of blind loops. SIBO bacteria bear a resemblance to those normally found in the colon, either gram-negative aerobes and/or anaerobes species, such as *E. coli*, *Enterococcus* spp., *Klebsiella pneumonia*, or *Proteus mirabilis*, capable to metabolize undigested carbohydrates into SFCA and gas. The disproportionate growth of atypical bacteria in the proximal small intestine permits their competition with the human host for nutrients harvesting. Additionally, the inflammatory response following SIBO provokes alterations of the epithelial cells and provokes villous atrophy and/or stimulates the synthesis of inflammatory cytokines resulting to mucosal injury (Sabate et al. 2017).

It has been shown that SIBO also impairs the absorption of vitamins B_1 and B_{12}. In a retrospective analysis of 80 RYGB patients, 39 of them had lower B_1 levels than the reference range (Dukowicz et al. 2007). Twenty-eight of these patients had elevated folate levels in plasma, a marker suggesting the SIBO presence, and another 15 were also diagnosed with SIBO by undergoing glucose-hydrogen breath testing (Sachdev and Pimentel 2013). The persistent B_1 deficiency rapidly resolved after treating SIBO with antibiotic therapy (Dukowicz et al. 2007). Secondary megaloblastic anemia may be present following RYGB due to impaired B_{12} absorption. In a case report of two patients submitted to RYGB which were positive for SIBO postoperatively, although antibiotic treatment improved hemoglobin levels, mean cell volume was still increased while B_{12} level was below the normal range (Sachdev and Pimentel 2013).

The malabsorption of fat-soluble vitamins, like A, E, and D, arises due to the bacterial deconjugation of bile acids by small intestine bacteria leading to the formation of toxic lithocholic acid, which further aggravates the intestinal epithelial cell disfunction and subsidizes carbohydrate and protein malabsorption as well (Sabate et al. 2017). In contrast, in patients with SIBO, the vitamin K levels are within normal limits or even increased since bacteria are capable to synthesize menaquinone (Grace et al. 2013).

The reduced brush border enzyme activity as well as the substrate readiness generate impaired carbohydrate uptake, which small bowel bacteria can metabolize prematurely. Also, increased numbers of small bowel bacteria compete with the host for intraluminal protein, thus disturbing the amino acids and peptides absorption. Furthermore, patients with SIBO demonstrate diminished enterokinases levels which result to impaired proteolytic reactions and subsequently to disturbed activation of pancreatic zymogens (Grace et al. 2013).

6.6.3 Bile Acids and Gut Microbiota Interactions

The bacteria involved in the deconjugation of bile acids are mostly *Bacteroides* species, which were reported to be decreased in BS patients, and this alteration is correlated with decreased fat mass and improved glucose control (Damms-Machado et al. 2015). The gut bacteria contribution in deconjugation and fermentation of primary bile acids to secondary ones has different impacts on human metabolism; The primary bile acids foster metabolism improvement, while secondary bile acids do not but rather seem to initiate carcinogenic processes (Swann et al. 2011). In addition, GM benefit from the deconjugation of bile acids as it can consume glycine or taurine for its own metabolism (Dawson and Karpen 2015). Also, bile acids shape the GM population through regulation of their growth and colonization and impacting the structure of their cell membrane. It has been reported that bile acids exhibit antimicrobial effects on certain bacteria while they promote the growth of others (Wahlström et al. 2016).

It seems that FXR plays multiple roles in metabolism regulation. FXR is a major regulator of bile acid signaling in both the liver and intestine, controlling the enterohepatic cycle of them by inhibiting hepatic bile acid synthesis and intestinal absorption. Additionally, bile acids serve as a ligand for FXR and appear to control glucose metabolism via FXR-related pathways. In this way, bile acids expand their molecular repertoire as modulators for both glucose and lipids metabolism (Bozadjieva et al. 2018). Finally, genetic and pharmacological mouse models have demonstrated differential roles of liver and intestinal FXR signaling in glucose metabolism and weight management (Bozadjieva et al. 2018).

Bile acid levels are increased in response to BS, and it is suggested that they mediate weight loss and metabolic improvements after BS (Patti et al. 2009; Pournaras et al. 2012). Regarding RYGB, the plasma bile acids are increased due to the fast supply of undiluted bile to the distal L cells and activation of the TGR5 receptors (Peterli et al. 2013). Additionally, a significant increase in the 12a--hydroxylated/non-12a-hydroxylated bile acid ratio has been described following RYGB (Furet et al. 2010). In RYGB, bile acids do not mix with food until the latter part of the jejunum. Therefore, in obese rodents which underwent RYGB, the procedure produced significant weight loss and improvement in glucose tolerance independently from the weight (Kohli et al. 2013). This is also reported in a study where increased bile acid levels were found in T2DM patients who underwent RYGB, but they were decreased after a hypocaloric diet that resulted in similar weight loss in T2DM patients, suggesting that the increase in bile acids after BS is weight independent (Jahansouz et al. 2016).

It has been suggested that FXR is crucial for the positive outcomes of VSG on both weight loss and glycemic control, as FXR-deficient mice despite been submitted to VSG showed reduced ability to decrease body weight and improve glucose tolerance (Ryan et al. 2014). It is worth to note that increased bile acids levels are also found after VSG (Stefater et al. 2011; Nakatani et al. 2009). This implies that this is not simply due to rerouting of bile acid as in the case of RYGB, but rather a physiological change of bile acids regulation than simply an operation-related

displacement of the bile acids (Bozadjieva et al. 2018). Moreover, FXR is essential for the positive effects of VSG on weight loss and glycemic control (Bozadjieva et al. 2018; Ryan et al. 2014).

The hypothesis that bile acids exhibit a contributory role in mediating the effects of BS is not always granted. For instance, in a study comprising T2DM and normoglycemic patients who underwent RYGB, glucose metabolism improved shortly after surgery, but the total bile levels did not increase until 3 months postsurgery (Jørgensen et al. 2015). Another study reported decreased bile acid levels shortly after surgery and an increase at 2 years after it (Dutia et al. 2015). These data reveal the possibility that the relationship between the clinically relevant effects of BS procedures and the alterations of bile acid levels may be more complicated.

The gut-derived peptide FGF15/19 is a potential molecular and therapeutic marker to elucidate the positive metabolic effects of BS (Bozadjieva et al. 2018). FGF15/19 is expressed in ileal enterocytes of the small bowel and is released postprandially in response to bile acid absorption. Once released from the ileum, FGF15/19 enters the portal venous circulation and travels to the liver where it binds to its receptor FGFR4 and suppresses the de novo bile acid synthesis via reduction of cholesterol 7a-hydroxylase (*CYP7A1*) and gallbladder filling.

It has been reported that circulating FGF19 levels increase following BS, indicating FGF15/19 as a potential target to mediate the positive effects of BS. However, how the increased levels of FGF19 in patients following BS directly mediate the beneficial effects of the surgical procedure is still unclear. Future studies that apply BS in combination with animal models with tissue-specific deletion of FGF15 or FGFR1/4 may provide further insight into understanding the direct role of FGF15/19 signaling in mediating the effects of BS. The literature data indicate the need of more studies to fully understand the plethora of FGF15/19-mediated actions. Understanding these complex actions may help researchers to directly link the FGF15/19 increase with specific metabolic benefits of BS (Bozadjieva et al. 2018).

6.6.4 Micronutrient Deficiencies After Bariatric Surgery

Following BS, 30–70% of patients develop nutritional deficiencies which, if severe, can result to edema, hypoalbuminemia, anemia, and hair loss as well as peripheral neuropathy, Wernicke encephalopathy and beriberi, metabolic bone disease, and anemia (Bal et al. 2012). Micronutrient deficiencies are common after RYGB and VSG (Krzizek et al. 2018), and a prevalence up to 50% in mid- and long-term follow-up has been reported (Adike and DiBaise 2018). The underlying causes can be due to either surgery- or patient-related reasons (Alexandrou et al. 2014).

BS may lead to severe postoperative micronutrient deficiencies which persist despite vitamin and mineral supplementation. A variety of factors can contribute to micronutrient deficiency observed after BS including eating behavior, decreased absorption, SIBO, poor compliance to the suggested optimization of diet and to prescribed nutritional supplementation (Sweeney and Morton 2013).

It is well documented that after both RYGB and VSG, the restriction of food intake, the reduced appetite, as well as the changes of gastrointestinal hormones are common mechanisms for the observed weight loss (Patel et al. 2017). Furthermore, the complications observed after BS, such as nausea, vomiting, food intolerance, or SIBO, may result to vitamin and mineral deficiencies (van Rutte et al. 2014).

It is of interest to state that micronutrient deficiencies are manifested in a similar degree after VSG and RYGB, although fewer micronutrient deficiencies are to be expected after VSG, since the small bowel remains intact after this operation (Patel et al. 2017; Aarts et al. 2011). This observation leads to the assumption that BS-related micronutrient deficiencies must be explained by different mechanisms: Namely, VSG accelerates gastric emptying and gastroduodenal transit time and, furthermore, reduces the secretion of hydrochloric acid and of the intrinsic factor. All these changes, due to the gastric fundus resection, affect the gastrointestinal motility, and, therefore, the release and dissolution of several vitamins and minerals is diminished (Aarts et al. 2011).

On the other hand, after RYGB, the bypass of the remnant stomach and of the upper part of the small intestine exclude the exposure of the food bolus to the biliopancreatic secretions and therefore affect the vitamins and minerals absorption. It is worth to note that the degree of malabsorption is related to the length of the common channel (distal jejunum, ileum, and colon) rather than the length of the Roux limb (Ferraz et al. 2018). Additionally, diminished absorption may also occur in the common portion of the small intestine as an asynergia consequence between food bolus, bile acids, and pancreatic enzymes. Finally, following RYGB, the absorption of some micronutrients (especially vitamin B_{12}) can also be reduced due to a lower location of gastric juice output as a result of bypassing the distal stomach (Stefanidis et al. 2011).

Except the abovementioned BS-related variables of micronutrient deficiency, some patient-related causes can alter their postoperative micronutrient status. Thus, it has been reported that patients who underwent BS may exhibit substance and alcohol abuse as well as poor compliance to the nutritional supplementation protocol. Thus, a long-term (up to 7 years) follow-up study of more than 2000 BS patients reported that 20% of patients submitted to RYGB developed alcohol use disorder (King et al. 2017b). In a 2019 questionnaire-based survey on 533 BS patients slightly over half of the respondents reported nonadherence to micronutrient supplementation (Mahawar et al. 2019).

The main micronutrient deficiencies reported after both BS include vitamin B_{12}, folic acid, iron, thiamine (vitamin B_1), vitamin D, and calcium (Antoniewicz et al. 2019; Engebretsen et al. 2018). Other reports on nutritional deficiencies after weight loss surgery, particularly following mixed bariatric procedures, are for fat-soluble vitamins (liposoluble), namely, vitamin A (Eckert et al. 2010), vitamin E (Boylan et al. 1988), and vitamin K (Lupoli and Milone 2015), as well as for copper (Boylan et al. 1988), zinc, and selenium (Sallé et al. 2010; Hassan zadeh et al. 2019). Therefore, lifelong nutritional supplementation, especially regarding protein, iron, folate, calcium, vitamins B_1, and B_{12}, and D, is a critical part of the postsurgical management of BS-operated patients as those substances are the most affected (Bal et al. 2012).

6.7 Conclusion

Bariatric surgery, being the most effective treatment of severe obesity, has continuously expanding use in our modern era. From the other hand, the role of gut microbiota on the host's ability to maintain a healthy metabolism and digestion is widely recognized. However, our understanding of the linking mechanisms between obesity and concurrent changes in gut microbiota is not clear as it seems that bariatric surgery cannot fully restore the disrupted microbial balance provoked by obesity. Therefore, there is a growing interest regarding the effects of bariatric surgery on gut microbiota as the weight loss and improvement or remission of obesity related comorbidities after bariatric surgery are associated with significant alterations in gut microbiota composition.

The exact contributing mechanisms which induce the GM alterations after bariatric surgery are not clear as different factors have been suggested namely diet, weight loss, or surgery itself. Moreover, there are some side effects that are triggered from the onset of small intestine bacterial overgrowth, which affect the weight loss process of the patients who underwent bariatric surgery.

Still the impact of bariatric surgery is not well defined, as the microbiota alterations which are detected following surgery are not consistent, and they should be considered in the context of restricted energy intake and altered dietary quality. Moreover, no differences regarding GM modulation were observed among the two most currently performed weight loss surgery techniques, i.e., RYGB and VSG. In general, an increase in members of the phylum Bacteroidetes and Proteobacteria, as well as a decrease in members of the phylum Firmicutes is reported.

In summary, bariatric surgery seems to attempt to restore a healthier gut microbiome with a leaner metabolic profile, and this microbiome realignment potentially contributes to the observed reduced fat mass reduction, the increase of lean mass, as well as resolving the obesity related comorbidities. However, the mechanism by which microbes and microbial by-products restore the gut microbiota remains poorly understood, and microbiome manipulations that exploit the host–bacteria interaction after bariatric surgery still need to be developed.

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7 Gut Microbiome and Mental Stress-Related Disorders: The Interplay of Classic and Microbial Endocrinology

Charikleia Stefanaki, George Mastorakos, and George P. Chrousos

Abstract

The gastrointestinal (GI) tract contains its own autonomic "enteric nervous system", which is in dynamic homeostasis with the central nervous system of the organism, forming with it the so-called gut-brain axis. The GI tract, however, contains the gut microbiome, a remarkable "organ", irrevocably connected to its function and hence also to that of the gut-brain axis. The stress system of the organism, through its end-hormones, influences the gut–brain-gut microbiome axis, in various ways. Microbial endocrinology suggests that microorganisms carry receptors with high affinity for stress hormones, which may serve as organismal cues for the sustenance, reproduction, symbiotic functions and/or the virulence of the gut microorganisms. The gut microbiome may, thus, have a role in the onset, course and symptomatology of various stress-related mental health disorders. In this chapter, we review the latest findings on the interconnection of the gut microbiome and some stress-related mental disorders, under the light of Microbial Endocrinology.

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7.1 Introduction

The gastrointestinal (GI) tract, derived from the embryonic endoderm, has an immensely complex physiology. It is constituted of multiple cell types, which are dispersed into two planes: a vertical plane that allows one to recognize different layers of the bowel wall and a horizontal plane that develops into the esophagus, stomach, small intestine, colon, and anus. These diverse cell types permit specific physiologic functions to be carried out in each anatomic region through successive patterns of gene expression and organ development (Lu et al. 2019). Muscular sphincters compartmentalize the bowel, dividing it into regions with distinct functionality and radically different luminal environments. Neuronal monitoring of luminal contents permits ingested material to be transported at a rate that allows each compartment to accomplish its task. The enteric nervous system (ENS) is the largest component of the autonomic nervous system and is uniquely equipped with intrinsic microcircuits that enable it to orchestrate gastrointestinal function independently of the central nervous system (CNS) (Rao and Gershon 2016).

While digesting everything inside it by breaking it down into smaller, absorbable chemical substances, the gut withstands these processes and avoids autodigestion. Complex neuromuscular interactions allow the GI tract to move food and liquids from one section of the gut to the next, while at the same time controlling the passage of food in such a way that maximum digestion and absorption occurs in each of the appropriate sections (Schubert 2016). Even in a single organ, such as the small intestine, a differentiation gradient exists so that different substances are preferentially absorbed at different sites and through different cells, or cell compartments. Therefore, the GI tract serves as a major interface between the outside world and the rest of the body and serves as a major immune organ, with immune defense processes largely taking place primarily in the small bowel (Shariati et al. 2019; Sommer et al. 2017). The gut is continuously exposed to toxins and infectious organisms, yet it is capable of eliminating these agents without sustaining any harm. Failure in its defense processes may result in disease and this generally occurs when the integrity of the bowel wall is compromised.

The GI tract is, also, a major endocrine organ, as it regulates food and nutrient metabolism. There are multidirectional interactions between the CNS, the enteric nervous system, and the GI tract. Many studies have suggested a prominent role of the gut microbiome in these gut–brain interactions. The gut microbiome may influence emotions, behavior, the stress, and fatigue and pain systems, and/or the brain neurotransmitter systems. In addition, human gut microbiome perturbations by prebiotics, probiotics, symbiotics, and antibiotics exert modulatory effects on some of the above systems in humans and animals (Mayer et al. 2015). In this chapter, we

review the human gut–brain axis in relation to the gut microbiome and suggest interactions between classic (traditional) and microbial endocrinology (Mayer et al. 2015).

7.2 Stress, Stress-Related Mental Disorders, and the Concept of Microbial Endocrinology

Living organisms survive by maintaining an immensely complex dynamic and harmonious equilibrium, or homeostasis that is constantly challenged, or outright threatened by intrinsic or extrinsic disturbing forces, or stressors. The steady state required for successful adaptation is maintained by counteracting/reestablishing forces, the adaptive response, consisting of an extraordinary repertoire of physical, or mental reactions that counteract the effects of the stressors to reestablish homeostasis. Thus, stress is a state of disharmony, or threatened homeostasis. The adaptive response may be specific to stressors or generalized, and relatively "nonspecific."

Through its normally adaptive hormonal mediators, when excessive or prolongued, stress can lead to acute or chronic pathology, especially in individuals with increased genetic, constitutional, and/or epigenetic vulnerability. Many studies have shown that stress-related mental disorders usually have an upregulated hypothalamic–pituitary–adrenal (HPA) axis, however, the opposite may also be true (Table 7.1) (Kamradt et al. 2018; Anesiadou et al. 2020; Angeli et al. 2018).

Acute stress may trigger allergic manifestations, such as asthma, eczema, or urticaria, angiokinetic phenomena, such as migraines, hypertensive, or hypotensive attacks, different types of pain (such as headaches, abdominal, pelvic, and lowback pain), gastrointestinal symptoms (pain, indigestion, diarrhea, constipation), as well as panic attacks and psychotic episodes. Chronic stress may cause behavioral and/or neuropsychiatric and physical manifestations such as anxiety, depression, executive and/or cognitive dysfunction; cardiovascular phenomena, such as hypertension and atherosclerotic cardiovascular disease; metabolic disorders, such as obesity, the metabolic syndrome, and type 2 diabetes mellitus; neurovascular degenerative

Table 7.1 Some stress-related mental disorders (Chrousos 2009)

Melancholic depression
Anxiety disorders
Post-traumatic stress disorder (PTSD)
Obsessive-compulsive disorder
Chronic fatigue syndrome
Premenstrual syndrome[a]
Attention deficit/hyperactivity disorder (ADHD)

[a]No gut microbiome studies have been performed about the entity of premenstrual syndrome

disease; osteopenia and osteoporosis; and sleep disorders, such as insomnia or excessive daytime sleepiness. The pathogenesis of chronic stress-related disorders can also be explained by sustained, excessive secretion and effects of the major mediators of the stress and sickness syndromes, which influence the activities of multiple homeostatic systems. These disorders, thus, represent chronic, maladaptive effects of two physiological processes, whose mediators are meant to be secreted in a quantity- and time-limited fashion, but have gone awry.

Studies on the physiology of the stress response have revealed the irrevocable interrelations between the nervous and immune systems (Schauenstein et al. 2001; Ader et al. 1990; Abramov 1986; Elenkov and Chrousos 2002, 2006). Several studies have linked decreased 5-hydroxytryptamine (serotonin/5-HT), norepinephrine, and dopamine concentrations to depressive symptomatology (O'Mahony et al. 2015; Collins et al. 2012) and elevated norepinephrine, along with decreased GABA concentrations with symptoms of anxiety (Liang et al. 2015; Desbonnet et al. 2015). It seems like the aforementioned neurotransmitters play a role in immunologic functions. Serotonin may have an effect on a subtype of T cells, follicular B-helper T cells that are found in Peyer's patches and one of their roles being to help establish metabolic homeostasis in the gut (Wu et al. 2019). Norepinephrine regulates immunomodulation *via* the NF-κB signaling cascade of the β2-adrenergic receptors (Kolmus and Tavernier 2015), and epinephrine plays a major role in changing absorption rates as per the needs of the human body (Mittal et al. 2017). Dopamine induces many direct and omnipotent effects on many dopamine receptor (DR)-expressing immune cells, primarily T cells and dendritic cells (Levite 2016). GABA seems to cause an increase in IgA secretion in the presence and absence of lipopolysaccharide (LPS), having a significant protective effect against oxidative injury and attenuating the effects on intestinal immunity (Kubota et al. 2018). Thus, these neurotransmitters are able to regulate and control not only blood flow, but also affect gut motility, nutrient absorption, gastrointestinal innate immune system, and the microbiome. Evidence, also, indicates that glucocorticoids (GCs) and catecholamines, the major stress hormones, inhibit the production of proinflammatory cytokines, such as interleukin (IL)-12, tumor necrosis factor-alpha (TNF-α), and interferon gamma (IFN-γ), whereas they stimulate the production of anti-inflammatory cytokines, such as IL-10, IL-4, and transforming growth factor-beta (TGF-β) (Kubota et al. 2018; Green and Brown 2016; Lim et al. 2020; Kiank et al. 2010; Tache et al. 2018).

Thus, a systemic, excessive immune response, through activation of the stress system, stimulates an important negative feedback mechanism, which protects the organism from an "overshoot" of proinflammatory cytokines and other products of activated macrophages with tissue-damaging potential. Conditions that are associated with significant changes in stress system activity, such as acute or chronic stress, cessation of chronic stress, severe exercise, and pregnancy and the postpartum period, through modulation of the systemic or local pro/anti-inflammatory cytokine balance, may suppress or potentiate autoimmune disease activity and/or progression (Elenkov and Chrousos 2002). Thus, activation of the stress system, through direct and indirect effects of corticotropin-releasing hormone (CRH), may

influence the susceptibility of an individual to certain autoimmune, allergic, infectious, or neoplastic diseases. Antalarmin, a nonpeptide CRH antagonist, prevented several proinflammatory effects of CRH, thus revealing its therapeutic potential in some forms of inflammation (Elenkov et al. 1999).

Apart from the enteric nervous system, the GI tract contains one of the most important systems in the human body, the gut microbiome. The latter consists of microorganisms comprising the microbiome, i.e., bacteria, viruses, and fungi, and their collective genomes, also known as bacteriome, virome, and mycobiome (Stefanaki 2019). This symbiotic relation has been of utmost importance for the health and well-being of the host (Ghaisas et al. 2016; Ihekweazu and Versalovic 2018). It is of note that stress hormones exert great influence on the pathogenicity and virulence of bacteria, primarily because of downregulation of the immune system of the host (Dhabhar 2000). Moreover, catecholamines may increase the growth of bacteria, virulence-associated factors, adhesions and biofilm formation, and, consequently, influence the outcome of infections by these bacteria in many hosts. The siderophores and the ferric iron transport system play a vital role in the mechanism through which catecholamines stimulate bacterial growth, while exposure to stress hormones may enhance the expression of genes involved in bacterial virulence (Sarkodie et al. 2019).

A holistic approach to understanding the mechanisms by which stress influences the pathogenesis of infectious diseases has resulted in the development of the field of microbial endocrinology, as suggested by Mark Lyte (2016), who was the first showed that bacteria carry receptors for stress hormones that may stimulate them to enter, either the spore phase, or the reproduction phase, depending on the environment's concentrations of these hormones (Villageliu et al. 2018). This transdisciplinary field represents the intersection of microbiology with mammalian endocrinology and neurophysiology, and is based on the principle that microorganisms have evolved systems for utilizing neurohormones, which are widely distributed throughout nature, as environmental cues to initiate growth, or even pathogenic processes (Freestone et al. 2008).

It is only natural to assume that the fully independent enteric autonomic nervous system in combination with the gut microbiome could influence the onset, progression, and/or symptomatology of stress-related mental disorders.

7.3 Gut Microbiome and Melancholic Depression

The current literature supports bidirectional interactions between the gut microbiome and the brain. Gut microbiome composition correlated with neural activity and brain structure in humans, as assessed by functional and structural MRI (Tillisch et al. 2017; Cheung et al. 2019). A recent systematic review study showed that patients with depression presented with five phyla (*Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Fusobacteria*, and *Protobacteria*) in abundance; however, divergent results were observed across studies of all phyla. The largest number of differentiating taxa was within phylum *Firmicutes*, in which, nine families and 12 genera

differentiated the diagnostic groups. The majority of these families and genera were statistically different between the two groups in two studies. Family *Lachnospiraceae* differentiated the diagnostic groups in four studies. Across all five phyla, nine genera were higher in patients with depression (*Anaerostipes*, *Blautia*, *Clostridium*, *Klebsiella*, *Lachnospiraceae incertae sedis*, *Parabacteroides*, *Parasutterella*, *Phascolarctobacterium*, and *Streptococcus*) than controls, six were lower (*Bifidobacterium*, *Dialister,* *Escherichia/Shigella*, *Faecalibacterium*, and *Ruminococcus*), and six were divergent (*Alistipes*, *Bacteroides*, *Megamonas,* *Oscillibacter*, *Prevotella*, and *Roseburia*).

The authors concluded that, in general, the genera that have extensive capacity to metabolize carbohydrates, particularly mono- and disaccharides and their derivatives were found in reduced abundance in patients with depression. On the contrary, genera with the ability to metabolize proteins were found in increased abundance. Of note, protein metabolism or fermentation (bacterial putrefaction), a process that may divert essential amino acids from the host to the microbes, may result in production of toxic substances, such as ammonia, putrescine, and phenols. Another mechanism proposed was the decrease in certain bacteria, like *Bifidobacteria* that produce vitamins, such as ascorbate (vitamin C), biotin (B7), folate (B9), niacin (B3), pantothenic acid (B5), pyridoxine (B6), riboflavin (B2), and thiamine (B1), or precursors of neurotransmitters, such as tryptamine and neurotransmitters, such as GABA, serotonin, norepinephrine, and dopamine (Williams et al. 2014; Barrett et al. 2012; Valles-Colomer et al. 2019).

A major role of gut microbiome in depression has been confirmed by a number of randomized controlled trials employing probiotics in patients with depression. A recent meta-analysis concluded that probiotics were associated with a significant reduction in symptoms of depression, underscoring the need for additional research on this potential preventive strategy (Huang et al. 2016).

To our knowledge no comparative study exists about the mycobiome or virome of patients with depression.

7.4 Gut Microbiome and Anxiety Disorders

Anxiety disorders are often comorbid with gut functional disorders, suggesting a bidirectional relation between mental health and gut function. As in the case of depression and the gut microbiome, there are many theories on the phenomenon, seemingly valid, as many interventional studies that employed administration of probiotics, prebiotics, or symbiotics demonstrated auspicious results (Cheung et al. 2019; Noonan et al. 2020).

Interestingly, there are not many case control studies about the composition of the gut microbiome in patients with anxiety disorders (Aslam et al. 2018). In fact, there is only one study that has evaluated the gut microbiome in generalized anxiety disorder (GAD) patients *vs.* patients with depression and healthy controls. In this study, the researchers found that GAD was associated with decreased diversity and variation in bacterial populations. However, these changes were not reversed after

the patients achieved remission (Jiang et al. 2018). Microbial dysbiosis of these patients was characterized by prevalence of *Bacteroides*, lower prevalence of SCFA-producing genera, such as *Faecalibacterium*, *Eubacterium rectale*, *Lachnospira*, *Butyricicoccus,* and *Sutterella*. Also, the researchers found that the proportions of *Ruminococcus gnavus* and *Fusobacterium* were significantly increased, along with significant enrichment of *Escherichia–Shigella* in the patients with anxiety (Jiang et al. 2018). The specific bacterial signature mentioned above may have provoked signs of leaky gut and low grade, subclinical inflammation, granted that the presence of or exposure to pathogenic bacteria in the gut can increase anxiety-like behavior (Jiang et al. 2018).

There are no studies on potential differences in the gut mycobiome or virome between patients with anxiety and healthy controls.

7.5 Post-Traumatic Stress Disorder and Gut Microbiome

Inadequate immunoregulation and elevated systemic inflammation may be risk factors for post-traumatic stress disorder (PTSD), and microbial inputs are important determinants of immunoregulation (Leclercq et al. 2016; Hemmings et al. 2017). Many studies have been performed in humans and rats (Matharu et al. 2019; Pearson-Leary et al. 2020) and have all reported gut dysbiosis in the form of decreased total abundance of *Actinobacteria*, *Lentisphaerae*, and *Verrucomicrobia* (Hemmings et al. 2017), higher abundance of pathobionts (*Enterococcus* and *Escherichia/Shigella*), and lower autochthonous genera belonging to *Lachnospiraceaeae* and *Ruminococcaceae* (Bajaj et al. 2019).

It seems that the aforementioned data might suggest a potential link between the gut bacteriome and symptoms of PTSD; however, as far as the mycobiome and virome are concerned, there are no data reported. Also, a recent systematic review about the use of symbiotics in patients with past traumatic stress disorders was promising; however, to date, existent findings do not support a relation, in spite of extensive coverage of probiotics in the press (Pearson-Leary et al. 2020; Brenner et al. 2017).

7.6 Obsessive-Compulsive Disorder and Gut Microbiome

In 2014, JC Rees proposed a mechanism about the onset of obsessive compulsive disorder: the root cause of obsessive-compulsive disorder (OCD) was proposed to be a dysfunction in the constituency of the gut microbiome, resulting in increased susceptibility to obsessive thinking. Both stress and antibiotics were proposed as potential mechanisms by which gut microbiome was altered, preceding the onset of OCD symptomatology. Stressful life events known to trigger OCD, such as pregnancy, were remodelled to show the possibility of alterations of gut microbiota prior to onset of OCD symptoms (Rees 2014; Turna et al. 2016).

Studies in rats showcased obsessive-compulsive behavior accompanied by changes in several communities of bacteria, belonging to the order *Clostridiales* (class *Clostridia*, phylum *Firmicutes*) and, predominantly, in the *Lachnospiraceae* and *Ruminococcaceae* families. It was, then, suggested that changes in these microbes may serve to support the energy requirements of compulsive checking and obsessive-compulsive disorder (Jung et al. 2018).

In humans, OCD patients presented with lower species richness and evenness (α-diversity, Inverse Simpson) and lower relative abundance of three butyrate producing genera (*Oscillospira*, *Odoribacter,* and *Anaerostipes*) (Turna et al. 2020). No studies exist about the relationship between gut virome and mycobiome with OCD.

Also, no interventional study employing probiotics, prebiotics, or synbiotics exists in patients with obsessive compulsive disorder. Only a case report of a boy with autism spectrum disorder, OCD, and self-injurious behavior exists, in which treatment with *Saccharomyces boulardii* successfully reduced both types of symptoms (Kobliner et al. 2018).

7.7 Chronic Fatigue and Gut Microbiome

The breakdown of immune homeostasis following the development of gut inflammation, caused by gut dysbiosis, or stress, and the consequent increased intestinal permeability, is increasingly considered to be the ultimate source of the systemic immune activation, T helper 17/T regulatory cell imbalances, and maybe neurological disturbances, seen in autoimmune diseases, such as type 1 diabetes (Stefanaki et al. 2017), insulin resistance (Stefanaki et al. 2018), and inflammatory bowel disease.

Increased intestinal permeability, as confirmed by other studies (Giloteaux et al. 2016), and translocation of commensal antigens into the systemic circulation is, also, a likely cause of the severe fatigue and an almost bewildering range of neurocognitive, neuroimaging, and overall symptom presentations observed in patients with chronic fatigue syndrome (CFS) (Giloteaux et al. 2016; Morris et al. 2016; Proal and Marshall 2018). Preliminary evidence suggests that the enteric microbiota may play a role in the expression of neurological symptoms in chronic fatigue syndrome. Overlapping symptoms with the acute presentation of d-lactic acidosis has prompted the use of antibiotic treatment to target the overgrowth of the *Streptococcus* genus found in commensal enteric microbiome, as a possible treatment for neurological symptoms in chronic fatigue syndrome.

It has been reported that bacterial diversity was decreased in the CFS specimens compared to controls, in particular, a reduction in the relative abundance, and diversity of members, belonging to the *Firmicutes* phylum (Giloteaux et al. 2016). These results have also been reproduced again in other studies that have employed exercise that ultimately ameliorated the gut microbiome composition in these patients (Shukla et al. 2015).Other interventional studies employed antibiotics, such as

erythromycin, along with probiotics that gave propitious results, confirming the aforementioned hypothesis (Wallis et al. 2018). To our knowledge, no study about gut virome and mycobiome exists in patients with chronic fatigue.

7.8 Attention Deficit/Hyperactivity Disorder (ADHD) and Gut Microbiome

ADHD is a disorder with genetic and environmental cues, contributing to its onset. Disturbances in neurogloia have been implicated in this entity, along with immune dysfunction (Donev and Thome 2010). It has been shown that probiotic supplementation early in life may reduce the risk of neuropsychiatric disorder development later in childhood probably by mechanisms not limited to gut microbiome composition (Partty et al. 2015).

ADHD patients presented with slight increase in *Bifidobacterium* genus, a finding, later connected to diminished neural reward anticipation circuit and, thus, dysregulation of the dopaminergic system (Aarts et al. 2017). Another study found significantly higher scores in questionnaires about functional gastrointestinal symptoms in ADHD patients, a finding attributed to gut dysbiosis, in the form of overabundance of *Bifidobacteria* (Ming et al. 2018). Another recent study revealed higher abundances in the family *Bacteroidaceae*, at the genus level, *Prevotella*, *Neisseria*, and a negative correlation between scores of hyperactivity symptoms and α-diversity. Assuming a causal relationship, the reduced α-diversity that was found in ADHD patients might reflect a bacterial community involved in deviant neural transmission (Prehn-Kristensen et al. 2018).

Randomized controlled trials with promising results, employing micronutrient supplementation in ADHD patients, pointed to a decrease in the abundance of *Bifidobacteria* in the gut environment. It seems that micronutrient treatment did not drive large-scale changes in composition, or structure of the intestinal microbiome. The differential abundance and relative quantity of *Actinobacteria* was significantly decreased post-micronutrient treatment, and this was largely attributed to species from the genus *Bifidobacteria.* This was compensated by an increase in the relative quantity of species from the genus *Collinsella*. The researchers in that pilot study suggested micronutrient administration as a safe, therapeutic method to modulate *Bifidobacterium* populations, which could have potential implications for regulating ADHD behaviour (Stevens et al. 2019). The microbiome signature of ADHD was definitely the overabundance of *Bifidobacteria*, along with a decrease in diversity and *Lactobacillus* spp. abundance, a possibly neuroprotective species, in another study (Bull-Larsen and Mohajeri 2019).

Virome and mycobiome have not been evaluated in ADHD patients, just yet.

7.9 Conclusions

Data from contemporary studies indicate that the gut microbiome influences CNS development, function, and metabolism. Gut dysbiosis was associated with notable mental disorders, with significant neurological components. However, most of the data were collected in experimental animals, and extrapolation to humans should be done with great caution. Conlusions should be drawn only after a significant number of randomized controlled human trials have been performed. Moreover, it is not definitively established whether neurologic diseases depend on a generic modification of the gut microbiome or whether a single bacterial phylum or species plays a specific role for any single condition, except perhaps for ADHD. Interestingly, in most of the published studies, there is no evaluation of gut virome or mycobiome. Future studies of stress-related mental disorders should, thus, evaluate not only the gut bacteriome but also the virome and mycobiome, along with serum stress and inflammatory biomarkers. The field of human microbial endocrinology is still nascent, but promising. In the near future, it will definitely enlighten the path to the current conundrum of gut microbiome in health and disease.

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The Gut Microbiome in Serious Mental Illnesses

8

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Abstract

In the past few years, significant progress has been made in characterizing the function of the gut–brain axis, i.e., the interactions between the central nervous system, the enteric nervous system, and the gastrointestinal tract. Preclinical studies described the important role of the gut microbiota in gut brain interactions Furthermore, gut microbiome has been linked to various serious mental illnesses, such as schizophrenia, bipolar disorder, anxiety disorders, depression. This chapter will describe the possible mechanisms that enhance the connection between them and the gut microbiome.

Keywords

Mental illnesses · Schizophrenia · Bipolar disorder · Intestinal inflammation · Prebiotics · Probiotics

8.1 Introduction

In the past few years, significant progress has been made in characterizing the function of the gut–brain axis, i.e., the interactions between the central nervous system, the enteric nervous system, and the gastrointestinal tract. Preclinical studies

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described the important role of the gut microbiota in gut brain interactions (Mayer et al. 2015).

The gut microbiome, a dynamic ecological community of microorganisms (including mainly bacteria, but also protozoa, fungi, archaea, microbial eukaryotes, and viruses) inhabit the human body (Nguyen et al. 2019).

For decades, the importance of the human microbiome remained elusive, due to technical challenges in studying unculturable microorganisms (Eme and Doolittle 2015).

The advent of high-throughput sequencing techniques has made apparent that the microbiome is a rich and diverse ecosystem with implications for human health and disease (NIH Human Microbiome Portfolio Analysis Team 2019; Vrbanac et al. 2017).

Gut microbiota may influence brain function through neural, endocrine, and immune pathways (Rieder et al. 2017) related to the vagus nerve signaling of gut hormones, metabolism of tryptophan, the immune system, as well as microbial metabolic products, such as short chain fatty acids (SFCA) (Dinan and Cryan 2017).

For example, the gut microbiota may impair the integrity of the intestinal barrier. The resulting release of cytokines may signal to the brain through vagal activation or signaling across the blood–brain barrier. In addition, substances produced by the gut microbiota may be absorbed reaching the brain by the blood stream. The brain, in turn, may influence the gut microbiota through neuronal and endocrine pathways as well as through adopting health behaviors.

Thus, imbalance of gut microbiota may affect the brain and subsequently lead to dysfunctions related to psychiatric disorders such as emotional and cognitive abnormalities.

The human gut microbiota is divided into many groups called phyla. Gut microbiota is composed mostly of four main phyla that include among others *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* (Belizario and Napolitano 2015). While bacteria colonize the human body, including oral cavity, placenta, vagina, skin, and GIT, the majority of bacteria reside within the GIT, with the majority of predominantly anaerobic bacteria housed in the colon. In order to gain perspective of the magnitude of bacterial presence and potential effects on the host, the human body expresses 20,000 eukaryotic genes while the gut microbiome expresses 3.3 million prokaryotic genes (NIH Human Microbiome Portfolio Analysis Team 2019; Carbonetto et al. 2016).

The human gut microbiome is potentially more easily modifiable than human genome. Compared with the human genome, which is fixed from birth, the microbiome is a dynamic and highly variable environment (Caporaso et al. 2011) that is continuously formed by development (from birth through old age) (Contreras et al. 2010; Yatsunenko et al. 2012) and in response to intrinsic (e.g., immune system) and extrinsic (e.g., diet, exposure to drugs/medications, or physical geography) environmental factors.

The microbiome has emerged as the "new" biomarker of human health, by maintaining host physiology and homeostasis and particularly in developing and shaping the immune system (Duerkop et al. 2009; Forsythe and Bienenstock 2010).

8.2 Schizophrenia and Bipolar Disorder

Actually, the microbiome has a pivotal role across a range of medical conditions such as inflammatory bowel disease (Kostic et al. 2014; Koenig et al. 2011), obesity and metabolic diseases (Bouter et al. 2017; Hartstra et al. 2015), cancer (Schwabe and Jobin 2013), and chronic pulmonary diseases (O'Dwyer et al. 2016; Budden et al. 2017). Parallels can be drawn between these medical disorders and severe mental illnesses (SMI) which show a high prevalence of gut (Severance et al. 2015) and metabolic (De Hert et al. 2009; Hennekens et al. 2005) dysfunction.

Schizophrenia and bipolar disorder (BD) are not just severe "*mental"* illnesses but also severe "*physical"* illnesses (Jeste et al. 2011). Cardiovascular, cerebrovascular, and digestive diseases are the top three leading causes of natural death in schizophrenia (Saha et al. 2007). Microbial colonization of the gut is crucial for the normal development of immunity (Round and Mazmanian 2009). Thus, imbalance of the intestinal ecosystem may alter immune responses (Kamada et al. 2013) and contribute to systemic physiological dysfunctions, including elevated inflammation and oxidative stress, often observed in schizophrenia and bipolar disorder (Berk et al. 2011; Flatow et al. 2013; Kirkpatrick and Miller 2013). Therefore, microbiome research may contribute to a greater understanding of the pathogenesis and treatment of chronic mental illnesses (Nguyen et al. 2018a).

8.2.1 Microbiome and Inflammation in Psychotic Disorders

Gut microbiome has an important role in health and disease. One of the main roles of gut microbiome in mammals is that it guides the maturation and functioning of a host immune system, tuning it toward effect or regulatory directions. Furthermore, intestinal microbiota gut and CNS interact, forming the microbiome–gut–brain axis. This occurs via afferent and efferent neural, endocrine, nutrient, and immunological signals (Nguyen et al. 2018a; Tomova et al. 2015). For example, some intestinal microbes are associated with anxiety- and depression-like behavior as well as modulation of GABA-ergic, glutaminergic NMDA, and serotonergic 5HT1A receptors in the brain, whereas germ-free mice exhibit reduced anxiety-like behavior (Walker et al. 2015; Walters et al. 2014).

Evidence of gastrointestinal (GI) inflammation has been reported in patients with schizophrenia. Elevated antibodies to anti-*Saccharomyces cerevisiae*, a marker of GI inflammation, have been found in people with first-episode psychosis, schizophrenia, and bipolar disorder.

Furthermore, serological surrogate markers of bacterial translocation correlated with serum CRP levels (Whiteford et al. 2013), suggesting that GI inflammation may contribute to systemic low-level inflammation. A contributing factor may be increased GI permeability, which is supported by studies finding elevated antibodies against food in people with schizophrenia (Woese and Fox 1977). These results show that patients with psychotic disorders may also suffer from both GI

inflammation and "leaky gut" syndrome. These could play a major role in immunological reactions that affect patients with psychotic diseases.

Schizophrenia and bipolar disorder are a leading global cause of disability (Whiteford et al. 2013) and rank among the most substantial causes of death worldwide (Walker et al. 2015). Compared with the general population, people with these psychiatric disorders have higher rates of chronic medical conditions and die younger (Brown 1997; Roshanaei-Moghaddam and Katon 2009). Excess deaths in these groups are not primarily from mental disorders themselves or suicide, but due to metabolic and cardiovascular diseases, cancers, and other chronic diseases (Hennekens et al. 2005; Nguyen et al. 2018a; Casey et al. 2009; Kupfer 2005). Here is to say, the gap in longevity between people with schizophrenia and general population has increased 37% since the 1970s, a fact that is very concerning regarding the development of the disease (Lee et al. 2018). Despite the enormous burden of serious mental illness (SMI), the underlying mechanisms associated with disease pathogenesis and progression are still not fully understood. The potential role of intestinal microbiota in the etiology of various human diseases has attracted considerable attention during the last decade. However, no article to our knowledge has systematically reviewed all the available studies of the microbiome in human, clinical populations of schizophrenia and BD. There are many gaps in scientific knowledge, not only for the potential implications in diagnosis and therapeutic interventions but also in the outlining of the directions in microbiome research in the future (Nguyen et al. 2018a).

8.3 Bipolar Disorder

8.3.1 Intestinal Inflammation

Bipolar disorder caused a high global disease burden with a lifetime prevalence of 1.0% for bipolar-I disorder, 1.1% for bipolar-II disorder, and 2.4% for sub threshold bipolar disorder (Sajatovic 2005). The exact pathogenesis of bipolar disorder is unclear. Diagnosis is based on clinical symptoms. Hence, it is frequently misdiagnosed with the consequence of poor therapeutic outcomes. So, it is of great importance to study the mechanism of bipolar disorder, searching for biomarkers which may facilitate the development of novel therapies (Ghaemi et al. 1999; Phillips and Kupfer 2013).

Pathologies throughout the gastrointestinal tract represent a frequent comorbidity in psychological diseases, including BD, that favors the idea of connection between GI pathology and psychological illness. According to the use of diagnostic criteria, irritable bowel syndrome (IBS) is estimated to affect about 11% of the general population (Lovell and Ford 2012).

In contrast, rates of comorbidity with psychiatric disorders range from 54% to 94% in those seeking treatment for IBS (Whitehead et al. 2002; Roy-Byrne et al. 2008). The results of a meta-analysis between two groups, one including 177,117 IBS patients and one with 192,092 healthy subjects, revealed a significant raise in

the prevalence of BD in the IBS group in contrast to healthy controls group (OR = 2.48, $p < 0.001$) (Tseng et al. 2016; Flowers et al. 2020).

Low-grade peripheral inflammation with further increases in proinflammatory cytokine levels have often been found in patients with BD during mood episodes (Bai et al. 2014). Patients with schizophrenia and BD exhibit antibody levels to fungal organisms such as *Saccharomyces cerevisiae* and *Candida albicans* (Severance et al. 2016; Debnath and Berk 2014).

In addition to gut translocation of microbes, BD patients also show increased exposure to other gut-related markers such as food-derived proteins from the GI system (Flowers et al. 2020; Severance et al. 2014).

8.3.2 Studies of Gut Microbiome and Bipolar Disorder

Sudo et al. (2004a) were the first to demonstrate that the presence of gut microbiota modulated the long-range hypothalamus–pituitary–adrenal reaction to stress. These experiments showed that germ-free (GF) mice (mice raised in a sterile environment and devoid of gut bacteria) exhibited an elevated stress response as measured by an increased adrenocorticotropic hormone and corticosterone release, compared to control mice with gut microbiota. Evans et al. analyzed the stool microbiome of clinical bipolar and control participants from the Prechter Longitudinal Study of Bipolar Disorder housed at the University of Michigan. The authors found significant differences in gut microbial communities between the bipolar and healthy control participants. Additionally, individuals with BD showed a decreased relative abundance of the gut microbe known as *Faecalibacterium* when compared to control participants (Evans et al. 2017). Specific gut microbes have also been linked to symptoms of mood in a clinical cohort of major depressive disorder. In this investigation, measures of species richness, or the total number of detected gut bacteria, were predictive of insomnia and depression, while abundance of *Enterobacteriaceae* was predictive of anxiety. In the same investigation, *Lactobacillus* abundance and *Enterococcus* abundance were also positively related to psychomotor agitation (Mason et al. 2017). Painold et al. found decreased measures of species richness and diversity detected in fecal microbial samples of individuals, with a BD diagnosis compared to healthy controls. Furthermore, there was a difference between BD patients and healthy control subjects. *Actinobacteria phylum* and Coriobacteriaceae were in higher abundance in BD patients, while in healthy controls were *Ruminococcaceae* and *Faecalibacterium* (Flowers et al. 2020; Severance et al. 2016; Painold et al. 2019).

8.3.3 Lithium, Antipsychotics, SSRIs, and Gut Microbiome

Lithium has been used for the treatment of bipolar disorder (BD) for the last decades, and recent studies with more reliable designs and updated guidelines have recommended lithium to be the treatment of choice for acute manic, mixed and depressive

episodes of BD, along with long-term prophylaxis (Won and Kim 2017). Although little is known about the interaction between lithium and gut microbiome, there are some studies which have investigated this interaction. Lithium did not exhibit antimicrobial activity against the gram-negative organism *Escherichia coli* or the gram-positive organism *Lactobacillus rhamnosus* in vitro. The authors did, however, observe an increase in species richness and diversity in the gut microbiota in rats fed with a lithium-supplemented chow, corresponding to approximately 150 mg/kg/day. Moreover, *Clostridium* spp. *Peptoclostridium*, *Intestinibacter,* and *Christenellaceae* genera were elevated after lithium administrations for treatment purposes (Lähteenvuo et al. 2018; Cussotto et al. 2019).

Atypical antipsychotics (AAP) are used for treatment of mental illnesses such as schizophrenia and bipolar disorder and considered to have fewer extrapyramidal effects than older antipsychotics. A plethora of studies about mental health have highlighted the role of AAP in heart and metabolic disease in patients with mental conditions (McEvoy et al. 2005). There is a link between gut microbiota, obesity, and metabolic disease, and, therefore, the contribution of the microbiome to the AAP-associated metabolic risk is currently being investigated. A recent in vitro study revealed a direct activity of antipsychotics against commensal microbes, specifically *Akkermansia muciniphila*, an organism associated with metabolic syndrome (Schneeberger et al. 2015)**.** A very interesting point is that in rat studies, many of these AAP-induced changes were more pronounced in female rats compared with males and were attenuated with coadministration of antibiotics (Davey et al. 2013).

In a BD human cohort, AAP treatment was associated with a decreased relative abundance of *A. muciniphila* and a decreased biodiversity in AAP-treated patients compared to non-AAP-treated BD patients (Flowers et al. 2017, 2019).

Specific SSRI, such as fluoxetine, has even been associated with an increased risk of developing a *Clostridium difficile* infection (Rogers et al. 2013). While the mechanism of action of SSRI for depression is not related with any antimicrobial effect of these drugs, potential changes in microbial communities may have an effect on other inflammatory or physiological parameters linked to mood. The common SSRI sertraline, fluoxetine, and paroxetine show activity against gram-positive bacteria such as *Staphylococcus* and *Enterococcus* species (Ayaz et al. 2015; Coban et al. 2009) and gram-negative bacteria such as *Pseudomonas aeruginosa* and *Klebsiella pneumonia (*Flowers et al. 2020*;* Kruszewska et al. 2012*)*.

8.4 Schizophrenia

Schizophrenia represents a major psychiatric disorder that includes positive symptoms (delusions, hallucinations, aberrant flow of thoughts) and negative symptoms (apathy, withdrawal, slowness). It is estimated that ~21 million people globally are affected and thus making it a significant physical and social morbidity (Hjorth et al. 2017; Marwaha and Johnson 2004; Szeligowski et al. 2020).

Until now, at least six studies have been published researching microbiome differences between healthy individuals and schizophrenia patients. At phylum level, *Proteobacteria* and *Firmicutes* were found at reduced levels in schizophrenia patients when compared to non-affected individuals. This is also the case for taxa within the class *Clostridia*, even though a single study identified this class to be enriched in schizophrenia. It is possible that the only truly consistent finding is the elevation of *Lactobacilli* in schizophrenia and people at increased risk of schizophrenia, which even correlated with symptom severity (Nguyen et al. 2018b; Schwarz et al. 2018).

Two key studies investigated the possibility that microbiome differences could serve as schizophrenia biomarkers. One investigation showed that the disorder is associated with changes in *Gammaproteobacteria* at class level, *Enterobacteriales* at order level, and *Bacteroides fragilis* at species level, while a second investigation determined that a panel consisting of *Aerococcaceae*, *Bifidobacteriaceae*, *Brucellaceae*, *Pasteurellaceae*, and *Rikenellaceae* is capable of distinguishing patients from healthy individuals (Zheng et al. 2019).

8.4.1 BDNF in Schizophrenia and Gut Microbiome

BDNF is a key neurotrophin involved in neurodevelopment, particularly in learning and memory processes. Neurodevelopmental models of schizophrenia often include BDNF alterations, focusing on their role in the cognitive dysfunction in the illness (Nieto et al. 2013). Reduced BDNF levels have been observed both in postmortem hippocampal samples and in the plasma of drug-naïve patients with schizophrenia, while low baseline BDNF levels are associated with worse response to antipsychotic treatment (Buckley et al. 2007; Rizos et al. 2008). In some studies, broad-spectrum antimicrobials have been found to significantly lower BDNF in mouse hippocampus, though another study with similar design found significantly increased BDNF levels in the hippocampus, paralleled by increased abundance of *Lactobacilli* and *Actinobacteria* (Bistoletti et al. 2019).

The recent studies have conflicting results, and it remains unclear whether the BDNF changes were mediated by the microbiome and/or the antibiotics themselves.

8.4.2 Prebiotics and Probiotics in Schizophrenia

Prebiotics are substrates utilized by host microorganisms, providing favorable conditions for "beneficial" bacteria (Gibson et al. 2017). They commonly include non-digestible fructan oligosaccharides (FOS) and galactan oligosaccharides (GOS), selectively degraded by *Bifidobacteria*. Recently, a study has shown the potential of using prebiotics as an adjunctive treatment in schizophrenia and was based on animal studies that explored two aspects of schizophrenia: cognitive dysfunction and antipsychotic-mediated weight gain (Szeligowski et al. 2020).

Although the mechanisms underlying the pro-cognitive effect of GOS in schizophrenia are not clear, prebiotic supplementation in rats was also found to increase responses of PFC pyramidal neurons to the application of NMDA and elevate cortical expression of GluN2B and GluN2A NMDA receptor subunits (Gronier et al. 2018; Savignac et al. 2013). Furthermore, elevated hippocampal levels of BNDF have been reported (Savignac et al. 2013). These changes are highly pertinent to schizophrenia, as NDMA hypofunction and decreased BDNF levels are thought to be involved in its pathogenesis and its associated cognitive impairment (Islam et al. 2017).

Probiotics contain living beneficial bacteria, typically from genera *Lactobacilli* and *Bifidobacteria* (Lara-Villoslada et al. 2007). A randomized, placebo-controlled trial of a combination of *Lactobacillus rhamnosus* and *Bifidobacterium lactis* Bb12 in schizophrenia did not change PANSS scores over the course of the 14-week trial (Dickerson et al. 2014), though a trend increase in plasma BDNF was observed (Tomasik et al. 2015). Recently, a probiotic supplement containing *Lactobacilli* and *Bifidobacterium bifidum* was given with vitamin D to schizophrenia subjects, which resulted in a significant improvement in the general and total PANSS scores, decreased circulating CRP levels, and enhanced total antioxidant capacity of plasma, indicating symptomatic improvement and reduced inflammation (Ghaderi et al. 2019). However, it is not clear which component of the intervention was responsible for those changes.

In conclusion, the results of probiotic trials are highly discrepant, which could reflect differences in the treatments used. There is, however, preliminary evidence that probiotic supplementation could benefit people with schizophrenia both in terms of symptoms and comorbid conditions, despite the apparent lack of effect on core aspects of the disorder (Szeligowski et al. 2020).

Since the microbiome is a complex and dynamic ecosystem, it is required more research in order to understand its role in host illness and its potential for the treatment of BD and schizophrenia.

8.5 Anxiety Disorders and Depression

Among other mental illnesses, the gut microbiome has been linked to various stress-related disorders, such as anxiety disorders or depression. The association of these specific disorders with the gut microbiome is mainly based on findings from preclinical and animal studies as well as association analysis of patient populations indicating relation between gut microbiota composition and modulation of stress physiology and behavioral patterns (Kim and Shin 2018; Foster and McVey Neufeld 2013; Foster et al. 2017).

Anxiety disorders and depression have a high prevalence, resulting in a reduced quality of life of the patients and in a high economic burden for the society (Mirzaei et al. 2019). Despite the availability of the evidence-based treatments in anxiety disorders and depression, a big proportion of patients do not follow any treatment (Eisenberg et al. 2011), or appear to be nonresponsive (Griffiths and Griffiths 2015),

or even experience new episodes throughout time (Curry et al. 2011). Therefore, studies on the gut microbiota of patients with these disorders have been increased in the last year, since they constitute an alternative approach that takes into account the study of other nonhuman genetic factors in the onset of these diseases and may offer direction on where to look for possible mechanistic pathways behind their etiopathogenesis and more effective treatments that will target gut–brain axis through the gut microbiome. A better understanding of the mechanisms behind the gut microbiome alterations associated to depression and anxiety disorders may open the way for new suggested therapeutic schemes. To date, there has been some early evidence of the microbial diversity involvement in anxiety-related disorders. Epidemiologic studies have shown association between specific antibiotics (e.g., fluoroquinolones) and occurrence of depression and anxiety (Kaur et al. 2016; Ahmed et al. 2011; Grassi et al. 2001). More specifically, there are studies in the field of psychiatry that provide evidence for link of some classes of antibiotics with depressive and anxiety disorders, through mechanisms that involve gut dysbiosis indicated by discharge of epithelial integrity molecules from the intestine into the blood of patients that do not present gastrointestinal distress. Many studies have also shown that treatment with antidepressants has antimicrobial effects, affecting anxiety and depression pathophysiology through modulations in brain biochemistry as well as in the gut microbial composition (Lieb 2004; Munoz-Bellido et al. 2000). An innovative study by Lach et al. first demonstrated enhanced gut permeability in patients suffering from depression and anxiety disorders as compared to healthy controls. This correlation was indicated by plasma biomarkers for gut permeability, namely zonulin, FABP2, and LPS. These specific findings were encouraging for further studies targeting gut microbiota for depression and anxiety disorder management (Lach et al. 2018).

8.5.1 Involvement of the Gut Microbiome in Anxiety Disorders

Experiencing anxiety is inextricably linked to dysregulation of the gut functionality. Gastrointestinal disturbances including upset stomach or nausea, or abdominal cramps and pain are among the most common symptoms related to the expression of anxiety (Walter et al. 2013). Furthermore, comorbidity of gastrointestinal disorders related to disturbances of the gut microbiota (e.g., irritable bowel syndrome, Crohn's disease, ulcerative colitis) with anxiety symptoms has been reported (Vos and Vos 2012; Neuendorf et al. 2016; Fond et al. 2014). Moreover, there is evidence that antibiotic administration in early life increases the risk of developing anxiety disorders as an adult (Lurie et al. 2015)**.** The increase of the risk for developing anxiety disorders is also associated to intestinal infections by pathogens (Bruch 2016).

Sudo et al. first demonstrated that germ-free (GF) mice exhibited increased stress reactivity. Subsequent studies have also shown that GF mice present decreased anxiety-like phenotype in comparison to pathogen-free mice, according to customized behavioral tests in mouse models, known as elevated plus maze. The reduction

in the anxiety-like behavioral pattern remained even after colonization with house-specific pathogen-free gut microbiota, indicating that alterations of the early life microbial composition affect the occurrence of later anxiety-like behavior in GF mice (Sudo et al. 2004b). Another study by Nishino et al. exploited open-field test showing that offsprings of colonized GF mice present decreased anxiety-like behavioral patterns in comparison to GF mice. Additionally, the specific paper also resulted in association between the predominant bacterial species of GF mice with their behavior (Nishino et al. 2013). In mice treated with antibiotics, the observed reduction of bacterial load was enhanced after triggering stress response in the animals through water avoidance stress test (Aguilera et al. 2013).

Regarding studies on anxiety-related disorders including humans, Jiang et al. reported that specific bacteria genera, namely *Faecalibacterium*, *Eubacterium rectale*, *Lachnospira*, *Butyricicoccus*, and *Sutterella*, are present in lower abundance in fecal samples from patients diagnosed with generalized anxiety disorder compared to healthy controls (Jiang et al. 2018).

8.5.2 Altered Gut Microbiota Diversity and Richness in Depression

Depending on the applied method for estimating microbial α- and β-diversity, several results have been extracted concerning the microbial diversity and richness modulations in patients suffering from depression in comparison to healthy controls (Barandouzi et al. 2020). A number of studies presented no significant differences in α and in phylogenetic microbial diversity between the two groups (Chen et al. 2018a; Naseribafrouei et al. 2014; Zheng et al. 2016). Other studies have indicated increased α-diversity and microbial richness (Kelly et al. 2016), whereas Kelly et al. reported a reduction of the total bacterial species, and Liu et al. resulted in a decreased α-diversity in patients with depression compared to healthy controls (Kelly et al. 2016; Liu et al. 2016). Concerning β-diversity of gut microbial communities from three related studies only two identified significant differences (Chen et al. 2018a; Zheng et al. 2016; Kelly et al. 2016). As indicated by these studies, no consistent directional findings were observed concerning the alterations of microbial diversity in patients suffering from depression.

To date, few studies have been published investigating the differences in the abundance of the microbiota between patients suffering from depression and healthy controls, not presenting through consistent findings. The findings of these studies are shown in Table 8.1.

Zheng et al showed though experiments on GF mice that depression-like behavior can be induced by the gut microbiome. The specific study performed colonization of GF mice with the gut microbiota of patients with depression, resulting in enhanced depression-like behavior compared to mice colonized with microbiota from healthy controls. Interestingly, the same paper highlighted that mice harboring depression microbiota exhibit modulations in host genes and metabolites involved in amino acid and carbohydrate metabolism. The enhanced carbohydrate

Table 8.1 Summary of studies related to gut microbial composition alterations in depression

Microbiota composition	Higher abundance in patients with depression compared to healthy controls	Lower abundance in patients with depression compared to healthy controls
Phylum level	Lin et al. (2017), Chen et al. (2018b)	Liu et al. (2016), Jiang et al. (2015)
Firmicutes		
Bacteroidetes	Liu et al. (2016), Jiang et al. (2015)	Chen et al. (2018a, b), Naseribafrouei et al. (2014), Zheng et al. (2016), Lin et al. (2017)
Actinobacteria	Chen et al. (2018a, b), Zheng et al. (2016), Jiang et al. (2015)	
Proteobacteria	Jiang et al. (2015)	Chen et al. (2018b)
Fusobacteria	Jiang et al. (2015)	
Family level	Zheng et al. (2016), Chen et al. (2018b)	
Actinomycineae		
Coriobacteriaceae	Chen et al. (2018a), Zheng et al. (2016)	
Bifidobacteriaceae	Chen et al. (2018b)	
Porphyromonadaceae	Chen et al. (2018b), Jiang et al. (2015)	
Clostridiaceae, *Streptomycetaceae*, *Nocardiaceae*	Chen et al. (2018b)	
Lactobacillaceae, *Streptococcaceae*, *Eubacteriaceae*, *Clostridiales incertae sedis XI*	Zheng et al. (2016)	
Thermoanaerobacteriaceae	Kelly et al. (2016)	
Fusobacteriaceae	Jiang et al. (2015)	
Veillonellaceae *Bacteroidaceae*		Zheng et al. (2016), Jiang et al. (2015)
Prevotellaceae		Kelly et al. (2016), Chen et al. (2018b), Jiang et al. (2015)
Sutterellaceae		Zheng et al. (2016), Chen et al. (2018b)
Oscillospiraceae, *Marniabilaceae*, *Chitinophagaceae*		Chen et al. (2018b)
Lachnospiraceae		Naseribafrouei et al. (2014), Zheng et al. (2016), Jiang et al. (2015)
Ruminococcaceae	Chen et al. (2018a, b)	Zheng et al. (2016), Jiang et al. (2015)
Acidaminococcaceae	Jiang et al. (2015)	Zheng et al. (2016)
Enterobacteriaceae	Jiang et al. (2015)	Chen et al. (2018b)
Erysipelotrichaceae	Zheng et al. (2016)	

(continued)

Table 8.1 (continued)

Microbiota composition	Higher abundance in patients with depression compared to healthy controls	Lower abundance in patients with depression compared to healthy controls
Rikenellaceae	Jiang et al. (2015)	Zheng et al. (2016), Chen et al. (2018b)
Genus level	Naseribafrouei et al. (2014), Jiang et al. (2015)	
Oscillibacter		
Blautia	Chen et al. (2018a), Zheng et al. (2016), Jiang et al. (2015)	
Holdemania	Kelly et al. (2016)	
Clostridium XIX	Lin et al. (2017)	Zheng et al. (2016)
Anaerostipes	Chen et al. (2018a), Zheng et al. (2016)	
Lachnospiraceae incertae sedis, *Parabacteroides*, *Parasutterella*	Jiang et al. (2015)	Zheng et al. (2016)
Anaerofilum, *Gelria*, *Turicibacter*	Kelly et al. (2016)	
Streptococcus	Zheng et al. (2016)	
Eggerthella	Chen et al. (2018a), Kelly et al. (2016)	
Klebsiella, *Escherichia/Shigella*	Lin et al. (2017)	
Paraprevotella	Zheng et al. (2016), Kelly et al. (2016)	
Coprococcus, *Clostridium XlVa*		Chen et al. (2018a), Zheng et al. (2016)
Lactobacillus		Aizawa et al. (2016)
Dialister		Kelly et al. (2016, Lin et al. (2017)
Bifidobacterium	Chen et al. (2018a)	Aizawa et al. (2016)
Roseburia	Chen et al. (2018a), Lin et al. (2017)	Chen et al. (2018a)
Lachnospiraceae incertae sedis	Lin et al. (2017)	Zheng et al. (2016)
Megamonas	Lin et al. (2017)	Chen et al. (2018a)
Clostridium XIX	Lin et al. (2017)	
Bacteroides	Chen et al. (2018a), Zheng et al. (2016)	Lin et al. (2017)
Prevotella	Zheng et al. (2016), Chen et al. (2018b)	Kelly et al. (2016), Lin et al. (2017)
Alistipes	Naseribafrouei et al. (2014), Lin et al. (2017)	Chen et al. (2018a)
Faecalibacterium	Chen et al. (2018a), Jiang et al. (2015)	Chen et al. (2018a), Lin et al. (2017)
Ruminococcus	Chen et al. (2018a)	Lin et al. (2017)

metabolism of mice with 'inputted' human 'depression microbiota' implies higher energy demands, which is in agreement with studies showing decreased glucose of patients with major depression disorder. Additionally, disturbed amino acid metabolism can be related to disturbed central and peripheral amino acid metabolism in patients with major depression. Despite the limited evidence, recent findings in this field provide promising results for elucidating the underlying pathological mechanisms in depression and for revealing future application of gut-mediated therapies in depression (Zheng et al. 2016).

8.5.3 Altered Gut Microbiota Composition in Relation to Antidepressant Medications

Several studies have investigated the effects of antidepressant treatment on the gut microbial communities. At the level of phyla, ***Bacteroidetes*** and ***Proteobacteria*** have been found to be increased, whereas ***Firmicutes***, ***Actinobacteria***, and ***Fusobacteria*** presented low abundance after treatment with selective serotonin reuptake inhibitors (SSRIs) or serotonin–norepinephrine reuptake inhibitors (SNRIs) (Jiang et al. 2015). At family level, increased composition of ***Bacteroidaceae***, ***Acidaminococcaceae***, ***Porphyromonadaceae***, ***Enterobacteriaceae***, and ***Rikenellaceae*** were observed in patients treated with antidepressants as compared to healthy controls. In addition, in the specific study it was observed that microbial genera ***Alistipes***, ***Bacteroides***, ***Parabacteroides***, ***Phascolarctobacterium***, and ***Roseburia***, were in higher abundance in patients with depression after being treated with antidepressants (Jiang et al. 2015). Lin et al. (2017) reported no differentiation in the microbial composition on phylum level in patients with depression between three different visits in 1 month after receiving escitalopram as treatment (SSRIs). Zheng et al. (2016) also reported no significant correlation between microbial community at the family level and the antidepressant treatment, even though most of the participating patients were drug naïve. Aizawa et al. (2016) also resulted in no significant differences of bacterial composition of patients treated with different antidepressant medication (Imipramine) dosage.

8.5.4 Possible Underlying Mechanisms Connecting Depression, Anxiety Disorders, and the Gut Microbiota

The underlying mechanisms of the findings supporting the connection between the gut microbiome and anxiety disorders, depression, and generally mental illnesses are yet not clear, but it has been hypothesized that reductions in the production of SCFA reported by the gut bacteria as documented in a study by van de Wouw et al. may result in disturbances in intestinal barrier, and further trigger brain abnormalities through mechanisms related to immune responses (van de Wouw et al. 2018). More specifically, the symbiotic gut microbiome and its products SCFA contribute in the intestinal mucosal barrier integrity and in the secretion of mucin, an essential

protein that protects gut from pathogens. The metabolite indolepropionic acid (IPA) produced by the gut microbiota is also essential for the intestinal barrier integrity and for the maintenance of the macrophages and T cells. Dysbiosis of beneficial bacteria leads to decreased production of beneficial substances, rendering the gastrointestinal barrier more susceptible to microenvironment modifications (Zhang et al. 2019). The gut microorganisms have been also accused for the inflammation caused in relation to psychological stress. The stress-induced intestinal permeability due to stress signals leads to increased translocation of microbiome toxic substances such as lipopolysaccharides (endotoxins) from gut into the circulatory system (Vanuytsel et al. 2014). Increase of harmful bacteria substances initiate immune system response in the blood, which finally results in neuroinflammation through microglia immune response, increase of inflammatory mediators and neurotoxins in the brain, as well as through impedance of neurotransmitters (Miller and Raison 2016). The hypothesis of elevated inflammatory response due to bacterial translocation derived from increased gut permeability has been also indicated in depression through studies that showed increased levels of *IgM and IgA* against the lipopolysaccharide (LPS) of the gut (Luna and Foster 2015).

8.5.5 Anxiety Disorders, Depression, and Probiotic Administration

Studies including animal models and healthy participants have provided evidence that probiotics can be proved helpful in mitigating symptoms such as stress, anxiety, and depression. Only a few studies though have investigated the effect of probiotics on alleviating the symptoms of patients diagnosed with clinical depression. More specifically, administration of probiotics as an adjunctive therapy to antidepressant treatment of patients with major depression disorder resulted in improvement of depression scores in comparison to patients receiving placebo. However, administration exclusively of probiotics on medication-free patients suffering from depression without any complementary antidepressant treatment led to no significant improvement of their symptoms compared to healthy controls.

Non-consistent conclusions concerning the impact of probiotics on anxiolytic symptoms in various studies have been extracted from the various performed studies, although the administration of the probiotic *Lactobacillus rhamnosus* has shown promising results. In a study that performed a meta-analysis of 22 preclinical and 14 clinical studies that investigated the anxiolytic potential of probiotics, it was shown that administration of probiotics cannot yet be considered an efficacious therapy in patients suffering from anxiety disorders, although anxiety-like behavior was improved in rodent models. Notably, the authors stated that further research on this field should be conducted, including higher dosages and longer duration of probiotic treatment (Reis et al. 2018).

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The Controversial Interplay of Gut Microbiome and Reproductive Function in Humans

9

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Abstract

In the human body, bacterial cells outreach human cells by approximately ten times and provide additional genes other than those present in human genome. Specifically the human gut is densely populated by a great variety of bacterial species that create an ecosystem, where bacterial populations and the human host have a mutualistic relationship. As the potential of the gut microbiome to affect many different functions of the human body has become obvious among scientists, many studies have tried to characterize how the gut microbiome changes in case of disease and which are the mechanisms potentially involved. The human reproductive function has been also studied in relation to the gut microbiome with the aim to understand what changes occur in the gut microbiome both under normal reproductive phases and reproductive pathology and whether these changes reflect pathogenetic mechanisms or results of the underlying condition.

Keywords

Microbiome · Reproduction · Gynecology · Female · Gut

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9.1 Potential Links Between Gut Microbiome and Reproductive Function

9.1.1 The Role of Steroid Sex Hormones

Steroid sex hormones play a key role in the endocrine regulation of the human reproductive function. Endogenous estrogens get conjugated and thus become inactive in the liver mainly through methylation, glucuronidation, and sulfonation. Part of these conjugated estrogens is then discharged with the bile into the small intestine. In the gut, several microbes have the ability to deconjugate these estrogen metabolites through secretion of enzymes—mainly b-glucuronidase, but also sulfatases (Pellock and Redinbo 2017). The process of deconjugation produces energy that can be utilized by the gut bacteria and is, therefore, beneficial for them. The deconjugated estrogens can be then reabsorbed into the systemic circulation and act as active estrogens, a process which is described by the term enterohepatic recycling (Adlercreutz et al. 1979). In addition to the aforementioned, gut microbiome can also convert estrogens, such as estrone to the more active estradiol, and glucocorticoids to androgens, which can in turn be converted to estrogens (Järvenpää et al. 1980; Ridlon et al. 2013). Consequently, gut microbiome has the ability to regulate the levels of the steroid sex hormones and potentially contribute to hypoestrogenic pathologies, such as obesity and metabolic syndrome that are known to affect pregnancy outcomes, hyperestrogenic pathologies, such as endometriosis and gynecological cancers, and pathologies involving hyperandrogenism, such as polycystic ovary syndrome (PCOS). Furthermore, the onset of puberty might also be affected by the gut microbiome, since steroid sex hormones are an important contributing factor. Apart from the aforementioned pathologies, gut microbiome could also affect the risk for perinatal psychiatric conditions since perinatal mental health can be influenced by the changes in the levels of steroid sex hormones that occur during pregnancy, labor, and postnatal. In addition, spore-forming bacteria that inhabit the gut can also enhance the biosynthesis of serotonin, and, consequently, the gut microbiome can further affect the risk of mental health illness (Yano et al. 2015).

While the gut microbiome could potentially affect the systemic levels of steroid sex hormones, it can conversely be affected by their levels. Both estrogens and androgens are thought to affect gut microbiome composition and mediate the observed sex differences (Org et al. 2016; Yurkovetskiy et al. 2013). Steroid receptors have been found in most immune cells; thus, steroid sex hormones can modulate immunity and affect inflammation, which may in turn influence the gut microbiome (Laffont et al. 2017; Fransen et al. 2017). Therefore, it is expected that the gut microbiome characteristics will change under pathologies accompanied by decreased or increased levels of estrogens or hyperandrogenism. It is, consequently, difficult to predict if any changes of the gut microbiome under such pathologies reflect underlying pathogenetic mechanisms, or the result of the disease, or both.

9.1.2 The Role of Short-Chain Fatty Acids (SCFAs)

Short-chain fatty acids (SCFAs) have a central role within the hypotheses regarding the potential links between gut microbiome and reproductive function as they have the potential to affect metabolic pathways, hormone secretion, and to induce epigenetic changes. SCFAs are the products of anaerobic fermentation of undigested polysaccharides by the gut microbiome (Natarajan and Pluznick 2014). The major SCFAs are the acetate, the propionate, and the butyrate (Natarajan and Pluznick 2014).

With regard to metabolic pathways, SCFAs can enhance the de novo lipogenesis in the liver and also serve as substrates for gluconeogenesis (Samuel et al. 2008). Glucose metabolism is further affected by systemic inflammation, which can be mediated by the SCFAs. SCFAs enhance the production of mucin by the intestinal goblet cells (Willemsen et al. 2003; Burger-van Paassen et al. 2009), and butyrate is an important source of energy for the colonic epithelial cells (Samuel et al. 2008). Both mucin and the colonic epithelial cells participate in the gut barrier function which, once impaired, predisposes the individual to increased permeability of the gut. As a result, lipopolysaccharides (LPS) can leak from the gut and activate the Toll-like receptors (TLR), which leads to increased inflammatory cytokines due to activation of the NF-κB (Kim et al. 2012). Their release in systemic circulation and the associated systemic inflammation lead to impaired glucose metabolism. Moreover, SCFAs bind to G-protein-coupled receptors (GPR), among which is the free fatty acid receptor-2 (FFA2/GPR43) that is also involved in impaired glucose tolerance (Fuller et al. 2015). Since impaired glucose tolerance is observed as a normal pregnancy is progressing, in gestational diabetes mellitus, and participates also in the pathogenesis of PCOS, gut microbiome characteristics may also correlate with the aforementioned conditions.

SCFAs can also affect the secretion of several hormones, including the glucagon-like peptide-1 (GLP-1), the peptide YY (PYY) from the intestinal L cells, leptin from the adipocytes, and ghrelin (Canfora et al. 2015). The binding of SCFAs with G-protein-coupled receptors, such as GPR41 and GPR43, is thought to trigger the secretion of several gut hormones mentioned above (Canfora et al. 2015). The increased secretion of the satiety hormones GLP-1, PYY, and leptin contributes to improved insulin sensitivity and decreased appetite (Canfora et al. 2015). Therefore, reduced SCFAs-producing bacteria could affect glucose metabolism also through endocrine regulation. With regard to leptin, this is a hormone that is also known to correlate with onset of puberty; this association has been proposed to be mediated by regulation of kisspeptin neurons and consequently gonadotropin-releasing hormone (GnRH) pulsatility (Matkovic et al. 1997). Therefore, the onset of puberty might be affected by the gut microbiome also through leptin regulation. In addition, changes in ghrelin and PYY levels can potentially induce changes in the levels of sex hormones that are secreted by the pituitary gland and the hypothalamus (Pinilla et al. 2006; Kluge et al. 2012). This further strengthens the hypothesis that the gut microbiome can cause changes in the levels of steroid sex hormones.

Finally, SCFAs can regulate the expression of different genes and induce epigenetic changes, which can also affect the offspring in case of pregnancy. SCFAs,

especially butyrate and acetate, can act as histone deacetylase inhibitors and lead to activation of gene expression by increasing histone acetylation and decreasing DNA methylation (Bhatia et al. 2009; Fathallah et al. 2007). For example, acetate can lead to increased acetylation of the forkhead box protein 3 (FoxP3) by inhibiting histone deacetylase (Thorburn et al. 2015). This is regarded as a potential mechanism that can explain the results of animal studies showing that acetate exposure during pregnancy leads to decreased risk of allergic airway disease in the offspring (Thorburn et al. 2015). Since gut microbiome participates in the production of SCFAs within the gut, scientists have formed the hypothesis that maternal gut microbiome may affect the child's risk for atopic and allergic diseases as a result of epigenetic changes induced by the SCFAs.

9.1.3 The Role of Amino Acids Homeostasis

Amino acids are the building blocks of proteins, hormones, and peptides that participate in signaling and metabolic pathways. Amino acid homeostasis in the body is regarded as important for the mammalian reproduction since certain amino acids are more abundant in body fluids, such as seminal fluid, uterine secretions, allantoic, and amniotic fluid (Dai et al. 2015). Furthermore, dietary supplementation with specific amino acids has been shown to improve reproductive function in animals and humans (Dai et al. 2015). The gut microbiome participates in the amino acid homeostasis by utilizing and metabolizing dietary amino acids and by de novo synthesizing essential amino acids. As a result of the amino acids' catabolism by the gut microbiome, several metabolites are produced, including nitrogenous products (e.g., ammonia and nitric oxide), phenolic, and indolic compounds. In case of high protein diet, the dietary intake of amino acids exceeds host's absorptive capacity and microbial needs, and the excessive amino acids are catabolized by the gut microbiome (Dai et al. 2015). Therefore, excessive amounts of amino acid metabolites are generated within the gut and can reach any organ via enteric absorption and systemic circulation. Among these metabolites, ammonia, phenolic, and indolic metabolites can be toxic for the reproductive organs (Dai et al. 2015). On the other hand, in case of malnutrition and protein restriction, the gut microbiome competes with the host for amino acids, which could result in reduced availability of important dietary amino acids for the reproductive organs (Dai et al. 2015). In both cases, gut microbiome can potentially affect reproductive function and contribute to reproductive tract diseases as a result of impaired homeostasis of amino acids in the human body.

9.1.4 The Role of Vitamin Synthesis, Iron Absorption, and Bacterial Translocation

Several other hypotheses have been proposed regarding other potential links between the gut microbiome and the reproductive function, which remain however less well-studied. These include de novo synthesis of vitamins (K and B group, such

as folic acid), which could mediate an association of gut microbiome characteristics with excessive bleeding during labor and birth defects, respectively, especially of the brain and the spinal cord (LeBlanc et al. 2013). Furthermore, gut microbiome has been shown to influence the dietary absorption of iron in the gut (Yilmaz and Li 2018); thus, the gut microbiome might contribute to the risk of adverse birth outcomes that are associated with iron deficiency, such as preterm birth and low birth weight. Finally, several scientific groups are working on the controversial hypothesis that bacterial translocation from the gut to remote tissues can occur; inflammation to remote tissues, such as placenta and cervix, due to bacterial translocation from the gut can affect their function and induce adverse pregnancy outcomes, as is preterm birth. This translocation is hypothesized to be facilitated by dendritic and CD18+ cells, but translocation of IgG bound bacteria has been also proposed (Rescigno et al. 2001; Vazquez-Terres et al. 1999; De Agüero et al. 2016). Even though some studies have isolated bacteria from previously thought sterile niches (e.g., amniotic fluid, umbilical cord blood, and placenta), it remains unclear whether bacterial translocation indeed takes place or these results arise from bacterial contamination of samples with very low bacterial biomass (Kuperman et al. 2020; O'Callaghan et al. 2020).

9.2 Associations of Gut Microbiome with Reproductive Function, Obstetrical, and Gynecological Outcomes

The proposed hypotheses for an interplay between the gut microbiome and human reproductive function warrant further research regarding potential associations between the gut microbiome characteristics and outcomes related to the reproductive tract and function. With regard to pregnancy, several studies have tried to unravel if and how the gut microbiome changes during normal pregnancy, what are the consequences of such changes, and if they are affected by adiposity before pregnancy or gestational weight gain. In addition, pregnancy complications, such as gestational diabetes mellitus and preeclampsia, and adverse pregnancy outcomes, such as preterm birth, have been studied in relation to the gut microbiome. Perinatal maternal mental health and offspring health outcomes have been examined, as well as central precocious puberty. Moreover, several studies have explored whether the gut microbiome could be involved in infertility and related diseases, especially PCOS and endometriosis. Finally, the gut microbiome of women with gynecological cancers has been studied and compared to that of otherwise healthy women. However, in contrast to obstetrical and gynecological outcomes that have been examined in various studies, the reproductive function of men has not been yet studied in relation to the gut microbiome in humans.

Scientific studies that examine the role of the gut microbiome in reproductive health and disease are based on the proposed hypotheses that could link the two. However, methodologies that could facilitate exploring these hypotheses in depth are still lacking. For example, it remains unclear which would be the ideal measure to capture the microbiome's ability to affect steroid sex hormones levels or to

produce SCFAs. Therefore, all association studies that link the gut microbiome with obstetrical and gynecological outcomes rely mainly on general gut microbiome characteristics, such as a- and b-diversity and the relative abundance of bacterial taxa. A-diversity is defined as the biodiversity within a given sample, and it is dependent upon the number of different bacterial taxa that are present in this sample and their relative abundance (Lozupone and Knight 2008). Therefore, a gut microbiome sample has low a-diversity, in case that few bacterial taxa are present and/or few bacterial taxa are numerically dominant because their relative abundance is much higher compared with the nondominant bacterial taxa. B-diversity describes how much of the biodiversity is shared between two samples (Lozupone and Knight 2008). Similar b-diversity is, thus, expected when two gut microbiome samples share many bacterial taxa, when the shared bacterial taxa are numerically dominant, and/or when the nonshared bacterial taxa are phylogenetically close. Lastly, the relative abundance of specific bacterial taxa is examined, with the aim to explore if specific bacterial taxa get enriched or depleted in case of disease. Even though the aforementioned are well defined and widely accepted measures to describe the gut microbiome, they are still not the perfect measures to test the hypotheses regarding the role of the gut microbiome in reproductive health and disease, but they rather provide an aggregate way to examine if any association of the gut microbiome with obstetrical and gynecological outcomes holds. In addition, some studies have performed metagenomic and metatranscriptomic analyses with the aim to provide a better insight into the functional characteristics of the gut microbiome. Finally, in order to assess whether any changes in gut microbiome are contributing to the examined outcomes or are their consequence, some research groups have transferred gut microbiome from humans to germ-free animal models and assessed the consequences on the animal model.

9.2.1 Gut Microbiome Changes During Pregnancy

In a proof of concept study from Finland (Koren et al. 2012), 91 pregnant women were followed up during pregnancy and their gut microbiome was analyzed in early and late pregnancy with the aim to characterize if and how the gut microbiome changes during pregnancy. The authors reported that a-diversity was decreasing as pregnancy was progressing. Furthermore, stool samples obtained during the third trimester of pregnancy clustered separately than the ones from the first trimester, and women during the first trimester had a more homogeneous community structure among them than during the third trimester. Regarding relative abundance of bacterial taxa, Proteobacteria and Actinobacteria phyla showed increasing abundance as the pregnancy progressed, as did members of *Enterobacteriaceae* family and *Streptococcus* genus. On the contrary, the relative abundance of Clostridiales order was higher in the first trimester of pregnancy. Apart from the observational analysis of the gut microbiome during pregnancy, the researchers transferred stool from both early and late pregnancy to female germ-free mice. Interestingly, recipients of third trimester stool had reduced oral glucose tolerance, greater inflammation markers,

and increased adiposity compared with recipients of first trimester stool. Since it is well known that glucose resistance, inflammation, and adiposity increase during normal pregnancy in humans, this experiment provides evidence that the gut microbiome can actively affect host's metabolism.

A lot of research groups since the publication of the aforementioned study have also explored how the gut microbiome changes during pregnancy by comparing stool samples obtained at different time points during pregnancy, stool samples obtained during and after pregnancy, and stool samples from pregnant and nonpregnant women (Avershina et al. 2014; Dunlop et al. 2019; Ferrocino et al. 2018; Liu et al. 2017a; Kumar et al. 2015; Nuriel-Ohayon et al. 2019; Collado et al. 2008; DiGiulio et al. 2015; Bisanz et al. 2015; Sakurai et al. 2020; Goltsman et al. 2018; Rothenberg et al. 2019; Khan et al. 2019; Crusell et al. 2018; Smid et al. 2018). Their results have been heterogeneous as several studies reported no significant changes in gut microbiome during pregnancy and supported that the gut microbiome is relatively stable as pregnancy progresses (Avershina et al. 2014; Dunlop et al. 2019; DiGiulio et al. 2015; Bisanz et al. 2015; Sakurai et al. 2020). Even among the studies that support the hypothesis of an evolving gut microbiome during pregnancy, discrepancies in their results do exist. Regarding a-diversity, even though several studies support that it indeed decreases during pregnancy (Koren et al. 2012; Goltsman et al. 2018; Rothenberg et al. 2019; Khan et al. 2019; Crusell et al. 2018), others report an increase (Ferrocino et al. 2018; Smid et al. 2018) or no change at all (Liu et al. 2017a). With regard to the relative abundance of bacterial taxa, many changes have been reported to occur during pregnancy (Table 9.1). However, there has been little agreement between these results, so that none of the reported changes can be assumed to occur universally in pregnant women.

Results from metagenomic and metatranscriptomic analyses of the gut microbiome during pregnancy have been also published and provide some insight into the functional capacity of the gut microbiome. Researchers from USA reported that enterobactin biosynthesis decreases during pregnancy, whereas pyruvate to acetate and lactate fermentation increases (Goltsman et al. 2018). Researchers from Spain concluded that during pregnancy gut microbiome relies more on glucose, which can be stored as glycogen more effectively during pregnancy, and less on other carbohydrates (Gosalbes et al. 2019). Lastly, based on predicted metagenomic profiles, researchers from Italy reported that with increasing gestational age, there is an enrichment of glycolysis/gluconeogenesis, starch, sucrose, galactose, fructose, and mannose metabolism, and biosynthesis of amino acids, as well as a depletion of fatty acid metabolism, folate biosynthesis, and biotin metabolism (Ferrocino et al. 2018).

In conclusion, although experimental data support that the gut microbiome changes during pregnancy and actively affects maternal metabolism, the results of observational studies are largely heterogeneous. Even though the agreement between reported changes in relative abundance of specific bacterial taxa has been poor, gut microbiome most likely changes during pregnancy since the majority of the conducted studies report separate clustering of samples from early and late pregnancy and of samples from pregnant and nonpregnant women.

Table 9.1 Reported changes in relative abundance of bacteria taxa during pregnancy

Study	Geographical region	Reported changes in relative abundance of bacterial taxa during pregnancy
Collado et al. (2008)	Finland	↑ *Bifidobacterium* genus, *Clostridium histolyticum* group, *Bacteroides-Prevotella* group, *Staphylococcus aureus*, *Akkermansia muciniphila* species ↓ *Bacteroides fragilis*
Koren et al. (2012)	Finland	↑ Proteobacteria and Actinobacteria phyla, *Enterobacteriaceae* family, *Streptococcus* genus ↓ Clostridiales order
Avershina et al. (2014)	Norway	–
DiGiulio et al. (2015)	USA	–
Bisanz et al. (2015)	Tanzania	–
Kumar et al. (2015)	Finland	↑ Proteobacteria and Actinobacteria phyla ↓ *Bifidobacterium* genus, *Clostridium coccoides* group, *Clostridium leptum* subgroup, *Bacteroides fragilis* group, *Bacteroides-Prevotella* group, *Clostridium histolyticum* group, *Lactobacillus-Enterococcus* group, and *Akkermansia* muciniphila
Liu et al. (2017a)	South China	↑ Tenericutes phylum ↓ Firmicutes and Verrucomicrobia phyla
Goltsman et al. (2018)	USA	–
Smid et al. (2018)	USA	↑ *Actinomyces*, *Finegoldia*, *Anaerococcus*, *Eggerthella*, *Acidaminococcus*, *Pseudomonas*, and *Ralstonia* genera
Crusell et al. (2018)	Denmark	–
Ferrocino et al. (2018)	Italy	↑ Firmicutes phylum, *Lachnospiraceae* family, and genera *Blautia*, *Butyricicoccus*, *Clostridium*, *Coprococcus*, *Dorea*, *Faecalibacterium*, and *Ruminococcus* ↓ Actinobacteria and Bacteroidetes phyla, *Rikenellaceae* family, *Bacteroides* and *Collinsella* genera
Khan et al. (2019)	Saudi Arabia	↑ *Ruminococcaceae* family, *Faecalibacterium prausnitzii*, *Faecalibacterium* spp., and *Bacteroides vulgates* ↓ *Prevotella* and *Saturella* genera, and genera related to the phylum Firmicutes
Nuriel-Ohayon et al. (2019)	Israel	↑ *Bifidobacterium*, *Neisseria*, *Blautia*, and *Collinsella* genera ↓ Bacteroidales order, *Dehalobacterium,* and *Clostridium* genera
Dunlop et al. (2019)	USA (African American women)	–
Rothenberg et al. (2019)	USA	↑ Actinobacteria phylum
Sakurai et al. (2020)	Japan	↓TM7 phylum

9.2.2 The Role of Adiposity in Gut Microbiome Changes During Pregnancy

Several studies so far have explored potential associations between adiposity and gut microbiome characteristics during pregnancy. Since obesity and excessive weight gain during pregnancy are associated with unfavorable pregnancy and birth outcomes, researchers have compared stool samples from pregnant women based on their Body Mass Index (BMI) before pregnancy and their weight gain during pregnancy.

Thirteen studies (Koren et al. 2012; Collado et al. 2008; Sakurai et al. 2020; Crusell et al. 2018; Smid et al. 2018; Houttu et al. 2018; Aatsinki et al. 2018; Sugino et al. 2019; Santacruz et al. 2010; Gomez-Arango et al. 2016a; Zacarias et al. 2018; Faucher et al. 2020; Stanislawski et al. 2017) up to date have examined the potential relationship between BMI before pregnancy and gut microbiome during pregnancy. Higher a-diversity was associated with lower BMI in two studies (Sakurai et al. 2020; Stanislawski et al. 2017) and with higher BMI in one study (Faucher et al. 2020), whereas the remaining studies did not report any association. No study reported any difference in b-diversity related to woman's BMI before pregnancy. With regard to relative abundance of specific bacterial taxa, significant differences have been reported in some but not all of the studies, and these results exhibit great heterogeneity (Table 9.2).

To conclude, current evidence from human studies do not support differential a- and b-diversity during pregnancy across levels of BMI. With regard to differential relative abundance of specific bacterial taxa, most differences are reported either by a single study and are not confirmed by the remaining studies, or individual studies report conflicting results. A positive correlation of BMI with *Lachnospiraceae* family and *Staphylococcus* and *Acidaminococcus* genera is supported by more than one study, as is a negative correlation with *Bifidobacterium* genus. However, none of these associations should be regarded as definitive, before they are further confirmed by large prospective clinical studies.

With respect to weight gain during pregnancy, nine studies (Collado et al. 2008; Sakurai et al. 2020; Crusell et al. 2018; Smid et al. 2018; Aatsinki et al. 2018; Santacruz et al. 2010; Faucher et al. 2020; Stanislawski et al. 2017; Urwin et al. 2014) have reported results regarding its potential association with gut microbiome characteristics. Two studies (Smid et al. 2018; Faucher et al. 2020) have reported that a-diversity measures correlate positively with gestational weight gain, though the remaining studies did not support this finding. B-diversity measures were not reported to differ across different levels of gestational weight gain in any of the conducted studies. Regarding relative abundances of specific bacterial taxa, results have been diverse (Table 9.3).

In summary, gestational weight gain does not appear to correlate with dramatic changes in gut microbiome, which would be captured in a- and b-diversity indexes. However, it is possible that abundance of specific bacteria taxa differs, even though results of the studies have not been consistent in between them so far.

Table 9.2 Reported changes in relative abundance of bacterial taxa during pregnancy associated with increased Body Mass Index (BMI) before pregnancy

Study	Geographical region	Reported changes in relative abundance of bacterial taxa associated with increased BMI before pregnancy
Collado et al. (2008)	Finland	First trimester: ↑ *Bacteroides-Prevotella* group, *Staphylococcus aureus* ↓ *Clostridium* group Third trimester: ↑ *Bacteroides* genus
Santacruz et al. (2010)	Spain	↑ *Enterobacteriaceae* family, *Staphylococcus* genus, *Escherichia coli* ↓ *Bifidobacterium* and *Bacteroides* genera
Koren et al. (2012)	Finland	–
Gomez-Arango et al. (2016a)	Australia	↑ Actinobacteria phylum, *Lachnospiraceae* and *Rikenellaceae* families ↓ Tenericutes phylum
Stanislawski et al. (2017)	Norway	↑ *Lachnospiraceae* family and Clostridiales order ↓ *Ruminococcaceae*, *Clostridiaceae*, and *Christensenellaceae* families, *Finegoldia* and *Lachnospira* genera, and *Ruminococcus*, *Finegoldia*, *Parabacteroides*, and *Bifidobacterium* species
Houttu et al. (2018)	Finland	↑ *Prevotellaceae* family
Zacarias et al. (2018)	Finland	↑ Firmicutes phylum, families *Lachnospiraceae* and *Actinomycetaceae*, and genera *Coprococcus*, *Actinomyces*, *Blautia*, and *Holdemania* ↓ *Bacteroidaceae*, *Coriobacteriaceae*, and *Desulfovibrionaceae* families, and *Bacteroides* and *Methanobrevibacter* genera
Smid et al. (2018)	USA	–
Aatsinki et al. (2018)	Finland	–
Crusell et al. (2018)	Denmark	↑ *Porphyromonas*, *Acidaminococcus*, and *Ruminococcus* genera ↓ *Eggerthella*, *Ethanoligenens*, and *Sporobacter* genera, and an unclassified genus from *Erysipelotrichaceae* family
Faucher et al. (2020)	USA	27–29 weeks of gestation: ↑ genus *Bacteroides* 36–39 weeks of gestation: –
Sakurai et al. (2020)	Japan	–
Sugino et al. (2019)	USA	↑ *Acidaminococcus* and *Dialister* genera ↓ *Phascolarctobacterium* genus

Table 9.3 Reported changes in relative abundance of bacterial taxa during pregnancy associated with increased gestational weight gain

Study	Geographical region	Reported changes in relative abundance of bacterial taxa associated with increased gestational weight gain
Collado et al. (2008)	Finland	Early pregnancy: – Late pregnancy: ↑ *Bacteroides* genus
Santacruz et al. (2010)	Spain	↑ *Enterobacteriaceae* family and *Escherichia coli* ↓ *Bifidobacterium* and *Bacteroides* genera, and *Akkermansia muciniphila* species
Urwin et al. (2014)	UK	↓ Total bacteria, *Faecalibacterium prausnitzii* group
Stanislawski et al. (2017)	Norway	↑ *Blautia* genus Associations with unspecified bacterial taxa were also reported
Smid et al. (2018)	USA	↑ Bacteroidetes phylum
Aatsinki et al. (2018)	Finland	–
Crusell et al. (2018)	Denmark	↑ *Eisenbergiella* and *Lactobacillus* genera ↓ *Christensenella* and *Alistipes* genera
Faucher et al. (2020)	USA	27–29 weeks of gestation: ↑ *Bacteroides* genus Before labor: –
Sakurai et al. (2020)	Japan	–

9.2.3 Gut Microbiome and Gestational Diabetes Mellitus

The gut microbiome has the potential to affect glucose metabolism and insulin sensitivity in different ways. It has been, therefore, proposed that the gut microbiome may either participate in the pathogenetic mechanisms involved in gestational diabetes mellitus (GDM) or conversely it might adjust in case of GDM in order to ameliorate glucose metabolism and insulin sensitivity. In both scenarios, the gut microbiome would differ between pregnant women with gestational diabetes mellitus and pregnant women without. To explore this possibility, several studies have been conducted that compared GDM cases and healthy controls. Samples were obtained not only during pregnancy but also postpartum with the aim to explore if any changes in the gut microbiome persist after giving birth. Finally, pregnant women without GDM have been also examined, and gut microbiome characteristics have been associated with biomarkers related to glucose metabolism, insulin secretion, and sensitivity.

The gut microbiome during pregnancy in relation to GDM has been studied in nine case control studies (Koren et al. 2012; Ferrocino et al. 2018; Crusell et al. 2018; Gomez-Arango et al. 2016a; Cortez et al. 2019; Wang et al. 2018; Ye et al. 2019; Kuang et al. 2017; Mokkala et al. 2017). A-diversity did not differ between cases and controls in the majority of the conducted studies, though increased (Cortez et al. 2019) and decreased (Kuang et al. 2017) a-diversity in GDM cases has been

also reported. Similarly, b-diversity was reported to differ between cases and controls only in the minority of the conducted studies (Ye et al. 2019; Kuang et al. 2017). Nonetheless, significant differences in relative abundance of specific bacterial taxa and associations with biomarkers related to glucose metabolism have been reported in all but one study (Koren et al. 2012) and are shown in Table 9.4. However, the majority of the reported differences in relative abundance of bacterial taxa have been reported in one study but not confirmed by the remaining studies. Based on current evidence, higher relative abundance of *Lachnospiraceae* family, *Collinsella* and *Ruminococcus* genera, as well as lower relative abundance of *Faecalibacterium* and *Roseburia* genera in pregnant women with GDM compared with healthy controls have been reported in more than one study (Table 9.4).

It has been proposed that since glucose metabolism and insulin resistance are a continuum, any characteristics of the gut microbiome that have been linked to GDM might also associate with biomarkers related to glucose metabolism in pregnant women, regardless of GDM diagnosis. Two studies (Sakurai et al. 2020; Robinson et al. 2019) have been conducted up to date on this domain and have examined associations of gut microbiome characteristics with blood or urine biomarkers that reflect aspects of glucose metabolism, insulin secretion, and sensitivity. Few significant associations were reported and these were not in line with the results from the case control studies for GDM during pregnancy (Table 9.5).

Scientists have, also, explored whether any differences in gut microbiome of GDM patients persist after giving birth. Three studies (Crusell et al. 2018; Hasan et al. 2018; Fugmann et al. 2015) have been conducted so far on the topic and only one of them (Hasan et al. 2018) did not report any difference between women who had suffered from GDM during pregnancy and those who had not (Table 9.6). Of the reported differences in relative abundance of bacterial taxa that persisted after giving birth, only the association of *Collinsella* genus with previous GDM is in line with the data arising from more than one study that has examined GDM during pregnancy.

In summary, up to date several studies have examined the gut microbiome in patients with GDM during pregnancy and postpartum, as well as potential associations of gut microbiome characteristics with glucose metabolism associated biomarkers during pregnancy. Based on the research data that are currently available, a positive correlation of GDM diagnosis and insulin resistance with *Lachnospiraceae* family, *Collinsella* and *Ruminococcus* genus, as well as a negative correlation with *Faecalibacterium* and *Roseburia* genera have been reported by two or more observational studies. Notably, causality should not be inferred yet since experimental data that support a causal connection of any of the aforementioned bacterial taxa with GDM are lacking.

Table 9.4 Reported changes in relative abundance of bacterial taxa during pregnancy associated with gestational diabetes mellitus

Study	Geographical region	Reported changes in relative abundance of bacterial taxa during pregnancy associated with gestational diabetes mellitus (GDM)
Koren et al. (2012)	Finland	–
Gomez-Arango et al. (2016a)	Australia	• Positive correlation of Actinobacteria phylum, *Coriobacteriaceae* family, and *Collinsella* genus with insulin resistance, insulin levels, and c-peptide levels • Negative correlation of Tenericutes phylum with insulin resistance, insulin levels, and c-peptide levels • Positive correlation of *Ruminococcaceae* family with insulin and c-peptide levels • Positive correlation of *Coprococcus* genus with gastric inhibitory peptide • Negative correlation of *Ruminococcaceae* family with gastric inhibitory peptide • Negative correlation of *Ruminococcaceae* family with resistin
Kuang et al. (2017)	China	↑ *Parabacteroides*, *Megamonas*, and *Phascolarctobacterium* genera, species annotated to *Klebsiella*, *Catenibacterium*, *Coprococcus*, and *Citrobacter* ↓ Clostridiales order, *Coriobacteriaceae* family, *Ruminiclostridium*, *Roseburia*, *Eggerthella*, *Fusobacterium*, *Haemophilus*, *Mitsuokella*, and *Aggregatibacter* genera, species annotated to *Methanobrevibacter*, *Alistipes*, *Bifidobacterium*, and *Eubacterium* • Positive correlation of species annotated to *Eggerthella*, *Megamonas*, *Allofustis*, *Lachnospiraceae* family, and *Parabacteroides* genus with glucose levels at oral glucose tolerance test • Negative correlation of *Alistipes* species with glucose levels at oral glucose tolerance test
Mokkala et al. (2017)	Finland	↑ Clostridia class, *Ruminococcaceae* family, and an unidentified genus and species of family *Ruminococcaceae* • Positive correlation of *Ruminococcaceae* family with glucose levels
Wang et al. (2018)	China	↑ *Fusobacterium*, *Porphyromonas*, *Lactobacillus*, *Sneathia*, and *Campylobacter* genera ↓ *Faecalibacterium*, *Roseburia*, *Bacteroides*, and *Prevotella* genera
Crusell et al. (2018)	Denmark	↑ Actinobacteria phylum, *Collinsella*, *Rothia*, *Actinomyces*, *Desulfovibrio*, *Leuconostoc*, *Granulicatella*, and *Mogibacterium* genera, and species annotated to *Faecalibacterium* and *Anaerotruncus* ↓ *Marvinbryantia*, *Acetivibrio*, and *Anaerosporobacter* genera, and species annotated to *Clostridium* and *Veillonella* • Negative correlation of *Butyricicoccus* with insulin sensitivity • Positive correlation of *Prevotella* and *Faecalitalea* with stimulated two-hour plasma glucose level • Negative correlation of Verrucomicrobiales order and all parent taxa within *Verrucomicrobia* with insulin sensitivity • Positive correlation of *Christensenella* with fasting plasma glucose

(continued)

Table 9.4 (continued)

Study	Geographical region	Reported changes in relative abundance of bacterial taxa during pregnancy associated with gestational diabetes mellitus (GDM)
Ferrocino et al. (2018)	Italy	• Positive correlation of Bacteroidales order and *Prevotella* genus with glycated hemoglobin (HbA1c) • Negative correlation of *Faecalibacterium* genus with fasting glucose • Negative correlation of *Collinsella* and *Blautia* genera with insulin levels and insulin resistance indexes • Negative correlation of *Blautia* genus with HbA1c levels
Cortez et al. (2019)	Brazil	↑ *Lachnospiraceae* and *Christensenellaceae* families, and *Collinsella*, *Dorea*, *Subdoligranulum*, and *Ruminococcus* genera ↓ *Eubacterium* genus
Ye et al. (2019)	China	GDM cases under medication: ↑ *Blautia* and *Eubacterium* genera ↓ *Faecalibacterium* and *Subdoligranulum* genera GDM cases following dietary recommendations: –

Table 9.5 Reported associations of a-diversity and relative abundance of bacterial taxa with biomarkers related to glucose metabolism during pregnancy

Study	Geographical region	Reported associations of a-diversity and relative abundance of bacterial taxa with biomarkers related to glucose metabolism during pregnancy
Sakurai et al. (2020)	Japan	• Positive correlation of a-diversity with glycated hemoglobin (HbA1c)
Robinson et al. (2019)	Australia	• Positive correlation of *Roseburia* genus with ketonuria

Table 9.6 Reported postpartum changes in relative abundance of bacterial taxa associated with gestational diabetes mellitus

Study	Geographical region	Reported postpartum changes in relative abundance of bacterial taxa associated with gestational diabetes mellitus (GDM)
Fugmann et al. (2015)	Germany	↓ Firmicutes phylum
Hasan et al. (2018)	Finland	–
Crusell et al. (2018)	Denmark	↑ *Collinsella* and *Olsenella* genera, all taxa within phylum Actinobacteria, genus *Clostridium*, and the parent family *Clostridiaceae*, genera *Hafnia*, *Howardella*, and *Dehalobacter* ↓ *Fusobacterium* and the parent family *Fusobacteriaceae*, *Ruminococcus* genus

9.2.4 Gut Microbiome and Preeclampsia

Few studies have examined the role of gut microbiome in preeclampsia (Liu et al. 2017a; Lv et al. 2019; Wang et al. 2019a) or explored potential associations between gut microbiome characteristics and blood pressure (Gomez-Arango et al. 2016b), and there are discrepancies between their results.

Three case control studies (Liu et al. 2017a; Lv et al. 2019; Wang et al. 2019a) have been conducted in China, which involved in total 122 preeclampsia patients and 173 controls. In two of these studies (Liu et al. 2017a; Lv et al. 2019), measures of a- and b-diversity did not differ between cases and controls. However, in one study (Wang et al. 2019a) a-diversity was lower in preeclampsia patients, although not statistically significantly, and b-diversity differed between cases and controls. Differences in relative abundance of bacterial taxa were reported in all three studies, but there was little agreement in their results (Table 9.7). In particular, only lower relative abundance of *Faecalibacterium* genus in pregnant women with preeclampsia was reported in more than one of the conducted studies (Lv et al. 2019; Wang et al. 2019a). In addition, the hypothesis that increased permeability of the gut due to dysbiosis contributes to preeclampsia pathogenesis was also explored in one of these studies (Wang et al. 2019a). Researchers observed that plasma and fecal levels of LPS were significantly higher in preeclampsia patients, as was the functional modules related to LPS biosynthesis (Wang et al. 2019a). Interestingly, in the other study (Lv et al. 2019), the women's diastolic and systolic blood pressure correlated positively with the genera that were enriched in preeclampsia patients, whereas the fetal features, such as birth weight and gestational length, correlated positively with the genera that were depleted in preeclampsia patients. However, these associations

Table 9.7 Reported changes in relative abundance of bacterial taxa associated with preeclampsia

Study	Geographical region	Reported changes in relative abundance of bacterial taxa associated with preeclampsia
Liu et al. (2017a)	South China	↑ *Cyanobacteria phylum*, *Clostridium perfringens*, and *Bulleidia moorei* ↓ *Coprococcus catus*
Lv et al. (2019)	China	Antenatally: ↑ *Blautia*, *Ruminococcus*, *Bilophila*, and *Fusobacterium* genera ↓ Fusobacteria, Tenericutes, and Verrucomicrobia phyla, *Faecalibacterium*, *Gemmiger*, *Akkermansia*, *Dialister*, and *Methanobrevibacter* genera Postnatally: –
Wang et al. (2019a)	China	↑ Bacteroidetes, Proteobacteria, and Actinobacteria phyla, Bacteroidia and Gammaproteobacteria classes, Enterobacteriales order, *Enterobacteriaceae* family, and species *Bacteroides coprocola* and *Bacteroides fragilis* ↓ Firmicutes phylum, Clostridia class, Clostridiales order, *Ruminococcaceae* and *Rikenellaceae* families, *Faecalibacterium* and *Alistipes* genera, and species *Bacteroides stercoris*

have not been examined in any other study and their replicability is questionable. Furthermore, in this study participants provided stool samples not only antenatally but also postnatally (Lv et al. 2019). Significant differences between preeclampsia cases and controls were observed only within the antenatal samples, indicating that any change in gut microbiome in preeclampsia patients is not expected to last after giving birth (Lv et al. 2019). Lastly, in the same study (Lv et al. 2019), the functional composition of the stool samples was also predicted with the aim to provide insight into the mechanisms potentially involved in the observed differences between preeclampsia patients and controls. Some functional modules were depleted in preeclampsia patients and participated in carbohydrate, amino acid, vitamin, and cofactor metabolism, ATP synthesis and photosynthesis, carbon fixation, two-component regulatory system, and the transport systems of various small molecules (Lv et al. 2019). On the contrary, several functional molecules that participate in saccharide transport systems were enriched in preeclampsia patients (Lv et al. 2019).

The potential associations of blood pressure with gut microbiome characteristics have been also explored in a study of 205 overweight or obese pregnant women from Australia (Gomez-Arango et al. 2016b). Both systolic and diastolic blood pressure negatively correlated with *Odoribacteraceae* and *Clostridiaceae* families, while systolic blood pressure negatively correlated also with *Christensenellaceae* family, members of the Bacteroidales order, and genera *Blautia* and *Odoribacter*. As many of the aforementioned bacteria are butyrate-producing bacteria, the researchers further hypothesized that reduced bacterial butyrate production may associate with increased blood pressure during pregnancy. To test this hypothesis, they quantified the expression of But and Buk genes, which are the main bacterial genes responsible for the butyrate production. Indeed, systolic and diastolic blood pressure were negatively correlated with Buk expression but not with But expression. However, it remains unclear whether the aforementioned results hold only within pregnant women without hypertensive disorders or can be extrapolated to patients with hypertensive disorders of pregnancy.

9.2.5 Gut Microbiome and Preterm Birth

Up to date, studies that examine the gut microbiome characteristics in relation to giving birth preterm have been scarce (Shiozaki et al. 2014; Dahl et al. 2017). They have important methodological differences and their results are heterogeneous. Thus, drawing firm conclusions from these studies is still premature.

The association of gut microbiome characteristics with gestational age at onset of labor and at birth has been examined in a study from Japan (Shiozaki et al. 2014). Forty-one pregnant women that provided stool samples were divided in three groups, namely women with preterm labor and birth, women with preterm labor but term birth, and lastly women with term labor and birth. Bacteria from the genus *Clostridium* had lower relative abundance in all women with preterm labor; even lower was their abundance if the woman actually gave birth preterm. In addition,

decreased relative abundance of genus *Bacteroides* and increased abundance of the order Lactobacillales were reported in the group of women with preterm labor and birth, but did not show an exposure–response relationship when the group of women with preterm labor but term birth was taken into consideration.

The association of preterm birth with gut microbiome characteristics has been also examined in a study from Norway (Dahl et al. 2017), in which 121 women with vaginal birth gave stool samples 4 days postpartum. In women with preterm birth, lower a-diversity was observed than in women with term birth, but there was no difference in b-diversity. The relative abundance of phylum Firmicutes was higher, whereas the relative abundance of phylum Actinobacteria was lower in women who had given birth preterm. In addition, in the preterm birth group, the relative abundance of Clostridiales order and *Bifidobacterium* and *Streptococcus* genera were decreased. However, in this study, all stool samples were obtained after birth. If the gut microbiome indeed changes as the pregnancy progresses, the results of this study could actually reflect reverse causality as the samples were not taken at the same gestational age for all women.

9.2.6 Association of Gut Microbiome with Blood Biomarkers During Pregnancy

Biomarkers reflect specific biological processes in the human body or the aggregate effect of more than one process. Blood biomarkers exist for several of the mechanisms which have been proposed to participate in the hypothesized links between gut microbiome and reproductive function. Glucose metabolism, which has been discussed conjointly with gestational diabetes mellitus, and lipid metabolism during pregnancy, intestinal permeability, inflammation, adiposity, appetite, and folic acid and iron homeostasis are among them. Therefore, blood biomarkers have been used to examine the association of gut microbiome characteristics with the mechanisms potentially involved in the controversial interplay of gut microbiome and reproductive function in humans.

With respect to lipid metabolism during pregnancy, three studies (Sakurai et al. 2020; Santacruz et al. 2010; Gomez-Arango et al. 2016a) so far have examined it in relation to the gut microbiome; however, their results have not been consistent. In an early study that used real-time qPCR rather than sequencing-based techniques to analyze the gut microbiome of pregnant women, total cholesterol was positively correlated with total bacteria count and *Staphylococcus* (Santacruz et al. 2010). In addition, increased high-density lipoprotein (HDL) and decreased triglycerides were associated with more abundant *Bacteroides* genus. Conflicting results arise from another study from Australia (Gomez-Arango et al. 2016a), in which data from 70 pregnant women were analyzed. Increased triglycerides, increased very low-density lipoprotein (VLDL), and decreased HDL were associated with increased relative abundance of *Collinsella* genus. In contrast to the aforementioned results, no significant association was observed in early or late pregnancy in a study from Japan (Sakurai et al. 2020), once adjusting the analyses for relevant confounding factors.

The hypothesis that dysbiosis of the gut microbiome can lead to distorted gut barrier function and increased gut permeability, which results in LPS leak from the gut and consequently increased inflammation, has been also examined. Zonulin blood levels were assessed in a study from Finland (Mokkala et al. 2016), in which 92 overweight or obese women were recruited. Zonulin is involved in the function of tight junctions between cells of the intestinal wall, and increased levels of zonulin associate with increased gut permeability. Pregnant women with lower zonulin had higher a-diversity, and statistically significant differences in relative abundance of bacterial taxa were also reported (Table 9.8). High sensitivity C-reactive protein and haptoglobin, which are both inflammation markers, were assessed in another study from Finland (Zacarias et al. 2018) that involved 54 pregnant women. There were indications that a-diversity might correlate negatively with inflammation markers, but these associations were not consistent when different indexes of a-diversity were used. In addition, several associations between relative abundance of bacterial taxa and inflammation markers were reported (Table 9.8). Lastly, researchers from USA have also performed a study on this domain (Hantsoo et al. 2019); they

Table 9.8 Reported changes in relative abundance of bacterial taxa associated with distorted gut barrier function and increased inflammation

Study	Geographical region	Reported changes in relative abundance of bacterial taxa associated with distorted gut barrier function and increased inflammation
Santacruz et al. (2010)	Spain	• Positive correlation of ferritin with *Enterobacteriaceae* family and *E.coli* • Negative correlation of ferritin with *Bifidobacterium* genus
Mokkala et al. (2016)	Finland	• Positive correlation of zonulin with *Bacteroidaceae* and *Veillonellaceae* families, *Bacteroides* and *Blautia* genera • Negative correlation of zonulin with *Faecalibacterium* genus and *Faecalibacterium prausnitzii*
Zacarias et al. (2018)	Finland	• Positive correlation of high sensitivity C-reactive protein and haptoglobin with *Holdemania*, *Coprococcus*, and *Blautia* genera • Negative correlation of high sensitivity C-reactive protein and haptoglobin with *Coriobacteriaceae* family, *Bacteroides* and *Methanobrevibacter* genera
Hantsoo et al. (2019)	USA	• Positive correlation of IL-6 AUC with *Bacteroides* genus • Negative correlation of IL-6 AUC with Clostridiales order, *Lachnospiraceae* and *Enterobacteriaceae* families, and *Dialister* genus • Positive correlation of TNF-α AUC with *Bacteroides*, *Prevotella*, and *Megasphaera* genera • Negative correlation of TNF-α AUC with *Ruminococcaceae* family • Positive correlation of CRP AUC with *Ruminococcaceae* family and *Megasphaera* genus • Positive correlation of cortisol response to stress with *Rikenellaceae* family and *Dialister* genus • Negative correlation of cortisol response to stress with *Bacteroides* genus

recruited 19 pregnant women and measured inflammation and stress markers at four time points, both before and after stress was induced by Trier social stress test, and calculated the area under the curve (AUC) for several inflammation-related biomarkers. All reported associations of interleukin 6 (IL-6) AUC, tumor necrosis factor alpha (TNF-α) AUC, C-reactive protein (CRP) AUC, and cortisol response to stress with relative abundance of bacterial taxa are shown in Table 9.8. Finally, ferritin, which is an acute phase protein that reflects inflammation but also iron storages, has been studied in relation to the gut microbiome in a study from Spain (Santacruz et al. 2010), and the results are also presented in Table 9.8. However, these results should be interpreted with caution, as real-time qPCR was used to analyze the gut microbiome and not sequencing-based techniques as in the other studies. In summary, the aforementioned studies exhibit considerable heterogeneity in the reported results. Nonetheless, there is relative agreement that more abundant *Blautia* and *Bacteroides* genera associate with higher gut permeability and increased inflammation markers during pregnancy, even though results in the opposite direction have been reported for *Bacteroides* genus.

With the aim to explore the association of gut microbiome with adiposity and appetite, researchers from Australia studied 70 overweight or obese pregnant women and measured the blood levels of leptin and ghrelin (Gomez-Arango et al. 2016a). Higher leptin blood levels were associated with more abundant *Lachnospiraceae* and *Ruminococcaceae* families. Higher ghrelin, which stimulates food intake, correlates positively with BMI, and is involved in regulation of glucose metabolism, was associated with higher relative abundance of *Bacteroidaceae* family and lower relative abundance of *Prevotellaceae* family.

Last but not least, the involvement of gut microbiome in iron and folic acid homeostasis has been examined in an early study of 50 pregnant women from Spain (Santacruz et al. 2010), in which real-time qPCR was used to analyze the gut microbiome. More abundant *Bacteroides* and *Bifidobacterium* genera were associated with increased folic acid. In addition, more abundant *Enterobacteriaceae* family and *E.Coli* and less abundant *Bifidobacterium* genus were associated with higher ferritin, reduced transferrin, and increased transferrin saturation index.

9.2.7 Gut Microbiome and Central Precocious Puberty

An association of gut microbiome characteristics with onset of puberty has been hypothesized based on the fact that the gut microbiome has the ability to affect the levels of steroid sex hormones and regulate leptin secretion. This hypothesis has been further strengthened by the results of an animal study, which showed that fecal transplant from male adult mice to female prepubertal mice resulted in elevated testosterone levels (Markle et al. 2013). Up to date, only one study has examined this hypothesis in humans (Dong et al. 2020). In particular, researchers from China examined the gut microbiome of 25 girls with idiopathic central precocious puberty (ICPP) and 23 controls and reported that a-diversity was higher among ICPP cases and b-diversity differed between cases and controls (Dong et al. 2020). They further

Table 9.9 Changes in gut microbiome characteristics associated with idiopathic central precocious puberty, as reported in Dong et al. study (Dong et al. 2020)

Outcome	Reported statistically significant results
Differences in relative abundance of bacterial taxa	↑ *Ruminococcus*, *Gemmiger*, *Roseburia*, *Coprococcus*, *Oscillibacter*, *Clostridium XIVb*, *Barnesiella*, *Coprobacter*, *Psychrobacter,* *Holdemania*, *Acinetobacter*, and *Pseudomonas* genera, *Clostridium* sensu stricto, *Bacteroides plebeius*, *Bacteroides coprocola*, *Gemmiger formicilis*, *Ruminococcus bromii*, *Coprobacter fastidious*, *Psychrobacter fulvigenes*, and *Roseburia inulinivorans* species ↓ *Ruminococcus gnavus* species
Differences in functional categories	↑ Signal transduction, cell motility, environmental adaptation ↓ Carbohydrate, glycan, and energy metabolism, cellular processes and signaling, folding, sorting and degradation, signaling molecules and interaction, and metabolic diseases
Associations of LH, FSH, and insulin resistance with relative abundance of bacterial taxa	• Positive correlation of FSH with *Fusobacterium* genus • Positive correlation of LH with *Gemmiger* genus • Negative correlation of LH with *Romboutsia* genus • Positive correlation of insulin resistance with *Gemmiger*, *Ruminococcus*, *Megamonas*, and *Bifidobacterium* genera
Associations of LH, FSH, and insulin resistance with functional categories	–

observed that many bacterial taxa were enriched in ICPP cases, while one bacterial species was depleted (Table 9.9) (Dong et al. 2020). In this study, researchers further examined the functional capacity of the gut microbiome based on predicted functional profiles and reported that several functional categories were enriched or depleted in ICPP cases (Table 9.9) (Dong et al. 2020). In addition, they observed that follicle stimulating hormone (FSH), luteinizing hormone (LH), and insulin resistance correlated with the relative abundance of the affected bacterial taxa, but not with the affected functional categories (Table 9.9) (Dong et al. 2020). Finally, they concluded that the differences in relative abundance of bacterial taxa, but not in functional categories, were similar to those observed in case of obesity (Dong et al. 2020). Given that obesity is one of the drivers of ICPP due to its effect on leptin levels, the authors of this study proposed that obesity might affect the onset of puberty also through altering the gut microbiome (Dong et al. 2020). However interesting these results are, there is a major limitation in this study design. Namely, 10 out of 23 girls in the control group were prepubertal; if gut microbiome changes during puberty in response to change of steroid sex hormones levels, the aforementioned findings might reflect the result of puberty in the ICPP group rather than a driving cause of puberty's onset (Org et al. 2016; Yurkovetskiy et al. 2013). It is, therefore, crucial for future studies to compare groups of individuals at the same pubertal stage and evaluate whether gut microbiome differences are present before the onset of puberty in case of ICPP.

9.2.8 Gut Microbiome and PCOS

Polycystic ovary syndrome (PCOS) is characterized by insulin resistance, hyperandrogenism, polycystic ovarian morphology, and chronic anovulation. In addition, central adiposity and metabolic syndrome are more often diagnosed in PCOS patients than in women without PCOS. As follows, PCOS is characterized by a great heterogeneity with regard to patients' phenotypes. It is, therefore, possible that gut microbiome characteristics may vary depending on the patient's phenotype.

In a proof of concept study from China (Qi et al. 2019) that involved 50 PCOS patients and 43 BMI matched controls, a-diversity did not differ between cases and controls, but b-diversity did. *Bacteroides vulgatus* was enriched in patients with PCOS, and the abundance of bile salt hydrolase genes within the *B. vulgates* species was significantly increased in individuals with PCOS compared with controls. In addition to the microbiome analyses, the researchers transplanted stool from PCOS patients and controls in female germ-free mice. Interestingly, mice transplanted with stool from PCOS patients displayed insulin resistance and negatively affected reproductive function compared with mice transplanted with stool from healthy controls. Furthermore, ovaries from mice transplanted with stool from PCOS patients had increased numbers of cyst-like follicles and fewer corpora lutea; these mice exhibited higher levels of testosterone and LH and decreased number of pups per litter. Furthermore, when the researchers administered *B. vulgatus* and heat-killed *B. vulgatus* to mice, they observed that insulin resistance, negatively affected reproductive function, ovarian morphology, and hormone profile were to an extent depending on *B. vulgates*. As follows from the aforementioned, this study is a proof of concept study as it demonstrated that the gut microbiome from PCOS patients can induce PCOS-like features in animal models and is, thus, regarded to participate in the pathogenetic mechanisms of PCOS.

Several other case control studies have been performed and have compared the gut microbiome characteristics between PCOS patients and healthy controls. Minimal differences were reported in a study from Austria (Lindheim et al. 2017) that compared 24 patients with PCOS with 19 controls. In particular, a-diversity was lower in PCOS patients, whereas b-diversity differed but only when the rare bacterial taxa were taken into consideration. Statistically significant differences were observed only in bacterial taxa with a relative abundance <1% and are presented in Table 9.10. Similarly, in another study from Spain (Insenser et al. 2018), the gut microbiome of 15 PCOS patients was compared to that of 16 control women and also 15 control men and few differences were observed. A- and b-diversity measures did not differ between the groups, but there were statistically significant results regarding the relative abundance of several bacterial taxa (Table 9.10).

The role of polycystic ovarian morphology (PCOM) in gut microbiome characteristics has been explored in a large study from Poland (Torres et al. 2018), in which 73 patients with PCOS, 42 women with PCOM, and 48 healthy controls were recruited. A-diversity was highest in healthy controls and lowest in PCOS patients, while in women with PCOM a-diversity was not significantly different from PCOS patients or controls. Notably, measures of a-diversity correlated negatively with

Table 9.10 Reported changes in relative abundance of bacterial taxa associated with polycystic ovary syndrome (PCOS)

Study	Geographical region	Reported changes in relative abundance of bacterial taxa associated with PCOS
Liu et al. (2017b)	China	↑ *Bacteroides* genus in obese PCOS patients compared with controls ↓ *Akkermansia*, *Bacteroides*, *Clostridium*, *Lactobacillus*, *Oscillibacter*, and unclassified genera from family *Ruminococcaceae* in obese PCOS patients and obese controls compared with nonobese controls
Lindheim et al. (2017)	Austria	↓ Tenericutes phylum and an unclassified genus from the Bacteroidetes phylum • Positive correlation of Tenericutes phylum and an unclassified genus from the Bacteroidetes phylum with a-diversity
Insenser et al. (2018)	Spain	↑ *Catenibacterium*, *Kandleria*, and *Oribacterium* genera compared with control women ↓ *Raoultella* genus compared with control men
Torres et al. (2018)	Poland	↑ *Porphyromonas* spp., *Bacteroides coprophilus*, *Blautia* spp., and *Faecalibacterium prausnitzii* ↓ *Anaerococcus* spp., *Odoribacter* spp., *Roseburia* spp., and *Ruminococcus bromii*
Zeng et al. (2019)	China	↑ *Bacteroidaceae* family, *Bacteroides* genus ↓ *Prevotellaceae* family, *Prevotella* genus ↓ *Lachnospiraceae* family and *Faecalibacterium* genus in PCOS patients with insulin resistance ↑ Ratio of *Firmicutes* to *Bacteroidetes* in PCOS patients without insulin resistance, but ↓ in case of insulin resistance ↑ *Lachnospiraceae* and *Ruminococcaceae* families in PCOS patients without insulin resistance
Zhang et al. (2019)	China	↑ *Parabacteroides*, *Bacteroides*, *Lactobacillus*, *Oscillibacter*, *Escherichia*, *Shigella*, and *Clostridium* genera ↓ *Faecalibacterium*, *Lachnospira*, *Bifidobacterium*, and *Blautia* genera • Positive correlation of *Bifidobacterium animalis* and *Faecalibacterium prausnitzii* with SCFAs • Negative correlation of *Bacteroides* genus with SCFAs
Qi et al. (2019)	China	↑ *Bacteroides vulgatus*

total testosterone level, hyperandrogenism, number of menses per year, and hirsutism, but did not correlate with age, BMI, or insulin sensitivity measures. With regard to b-diversity, this was not different between the study groups, but it did differ based on presence or absence of hyperandrogenism. Moreover, several bacterial taxa were reported enriched or depleted in PCOS patients compared with otherwise healthy controls (Table 9.10).

The role of adiposity in PCOS patients and its potential effect on gut microbiome was further explored in a study from China (Liu et al. 2017b), in which 33 PCOS patients and 15 healthy controls were recruited and studied based on their BMI. A-diversity was lower in PCOS patients, but also in case of obesity, resulting in the lowest a-diversity among obese PCOS patients and the highest in nonobese healthy controls. Regarding b-diversity, only nonobese controls clearly separated from the other groups, with obese controls having more similar b-diversity with PCOS patients rather than nonobese controls. Results regarding relative abundance of bacterial taxa are presented in Table 9.10.

In addition to the aforementioned, metabolic pathways potentially involved in the differences observed between PCOS patients and healthy controls have been identified in two studies from China (Zhang et al. 2019; Zeng et al. 2019) involving in total 55 PCOS patients and 34 healthy controls. In one study (Zhang et al. 2019), b-diversity was significantly different between PCOS cases and controls and relative abundance of bacterial taxa differed, as well (Table 9.10). Based on shotgun metagenomic sequencing, several metabolic pathways were increased in the PCOS group; these were involved in fructose, mannose, thiamine, and biotin metabolism, the citrate cycle, lipopolysaccharide and folate biosynthesis, bacterial chemotaxis, cationic antimicrobial peptide resistance, flagellar assembly, the phosphotransferase system, and one carbon pool by folate. On the contrary, metabolic pathways involved in propionate metabolism, valine, leucine, isoleucine, and fatty acid biosynthesis, ABC transporters, and bacterial secretion systems were depleted in the PCOS group. In the other study (Zeng et al. 2019) that had much smaller sample size, the researchers explored the role of insulin resistance in gut microbiome changes among PCOS patients and, therefore, grouped PCOS patients based on insulin resistance. Results regarding relative abundance of bacterial taxa revealed that insulin resistance was, to an extent, driving the observed differences (Table 9.10). Based on predicted functional profiles, genes related to lipid, amino acid, and carbohydrate metabolism were enriched in PCOS patients. Metabolic pathways involved in zeatin and N-glycan biosynthesis, arachidonic acid metabolism, and the digestive system were depleted in PCOS patients. In addition, several metabolism associated pathways, such as those involved in biosynthesis of steroid hormones and lipopolysaccharides, differed significantly within PCOS patients depending on insulin sensitivity.

In summary, several studies have been conducted with regard to the gut microbiome in PCOS patients. Even though their results regarding relative abundance of bacterial taxa are not in line, there is relative agreement that a-diversity is lower in PCOS patients and that PCOM, levels of androgens, adiposity, and insulin resistance are important factors that contribute to gut microbiome characteristics in PCOS patients. Most importantly, experimental data support the hypothesis that the gut microbiome does not solely change in response to PCOS, but can also induce PCOS symptoms and features.

9.2.9 Gut Microbiome and Endometriosis

The gut microbiome of women with endometriosis, as compared to women without, has been examined in a single study form Turkey so far (Ata et al. 2019). Twenty-eight women were recruited, of which 14 suffered from endometriosis stages 3 or 4 and 14 served as controls. Although a- and b-diversity did not differ between the two groups, genera *Sneathia*, *Barnesiella*, and *Gardnerella* were less abundant in the endometriosis patients. Furthermore, genera *Escherichia* and *Shigella* were more abundant in the stool samples from endometriosis patients with severe bowel involvement requiring colon resection.

9.2.10 Gut Microbiome and Female Fertility

Female fertility can be impaired as a result of several underlying conditions that have distinct pathogenetic mechanisms. It is therefore expected that any difference in the gut microbiome in case of infertility could vary depending on the underlying condition. Endometriosis and PCOS, which are both contributing to female infertility, have been studied in relation to the gut microbiome, although subgroups of patients with infertility have not been studied in particular. Regarding other causes of infertility, chronic anovulation has been examined in a study from Japan (Sasaki et al. 2019); eight women with chronic anovulation, who had normal gonadotropin levels and no apparent endocrinological disorder, were studied in comparison to 24 women with regular menstrual cycles. Although diversity measures and relative abundance of bacterial phyla did not differ between the two groups, a difference in relative abundance of several derivative bacterial taxa was observed. Women with chronic anovulation exhibited increased relative abundance of *Tissierellaceae* family and *Prevotella* and *Dialister* genera compared to women with regular menstrual cycles. They also exhibited decreased relative abundance of Betaproteobacteria class, Bacteroidales and Clostridiales orders, *Rikenellaceae*, *Lachnospiraceae*, and *Peptococcaceae* families, and *Oscillospira*, *Ruminococcus*, and *Allobaculum* genera.

9.2.11 Gut Microbiome and Gynecological Cancers

Up to date, three studies (Nam et al. 2013; Wang et al. 2019b; Sims et al. 2019) have examined whether the gut microbiome characteristics differ in case of gynecological cancer. In an initial small case control study from South Korea (Nam et al. 2013), nine patients with gynecological cancer and six healthy controls provided stool samples. Out of the nine cancer patients, seven suffered from cervical cancer and were already under chemotherapy, whereas the remaining two patients suffered from endometrial cancer and had not received treatment yet. In this study, a-diversity did not differ between cases and controls, but b-diversity and relative abundance of

Table 9.11 Reported changes in relative abundance of bacterial taxa associated with gynecological cancers

Study	Geographical region	Reported changes in relative abundance of bacterial taxa associated with gynecological cancers
Nam et al. (2013)	South Korea	↑ Actinobacteria phylum, *Clostridiaceae*, *Eubacteriaceae*, *Enterococcaceae*, and *Streptococcaceae* families ↓ Fusobacteria phylum, *Prevotellaceae*, *Oscillospiraceae*, and *Fusobacteriaceae* families
Wang et al. (2019b)	China	↑ Proteobacteria phylum, Gammaproteobacteria class, Enterobacteriales, Aeromonadales, Oceanospirillales, and Alteromonadales orders, *Enterobacteriaceae*, *Pseudomonadaceae*, *Succinivibrionaceae*, and *Halomonadaceae* families, *Parabacteroides*, *Escherichia*, *Shigella*, and *Roseburia* genera ↓ *Acidaminococcaceae* family, *Phascolarctobacterium* genus
Sims et al. (2019)	USA	↑ *Prevotella*, *Porphyromonas*, and *Dialister* genera ↓ *Lachnospiraceae* family, *Blautia,* and *Alistipes* genera

specific bacterial taxa did (Table 9.11). However, these differences could arise either from the neoplastic disease per se or from the chemotherapy that the majority of patients had received in this study.

With the aim to to disentangle the effect of cervical cancer from the effect of chemotherapy on the gut microbiome, researchers from China compared stool samples from eight cervical cancer patients before they received any treatment and five healthy controls (Wang et al. 2019b). They observed that a-diversity was higher in cancer patients, though that difference was not statistically significant, and that b-diversity was different between the two groups. They, additionally, reported many statistically significant differences in relative abundances, but these results should be interpreted with caution as the sample size of the study was very small (Table 9.11).

Apart from the aforementioned studies, whose small sample size was a major limitation in generalizing their results, another case control study was conducted in USA and involved 42 cervical cancer patients before any treatment and 46 controls (Sims et al. 2019). Unfortunately, sampling method differed between cases and controls as rectal swabs were used among cancer patients and stool samples were obtained from controls; therefore, differences between the two groups could also arise due to differential sampling method. Nonetheless, the authors of the study reported that a-diversity was higher in patients, but this difference was limited to patients above 50 years old. Furthermore, b-diversity was significantly different between the two groups, as was the relative abundance of various bacterial taxa (Table 9.11).

In summary, although all conducted studies that examine the gut microbiome in patients with gynecological cancers agree that a difference in b-diversity exists and indicate that a difference in a-diversity might also exist, their results regarding relative abundance of bacterial taxa are not in line with each other. Differences between the conducted studies regarding the study population, the sampling methods used, and their small sample size contribute to this heterogeneity of results.

9.2.12 Gut Microbiome and Perinatal Mental Health

Two studies, so far, have examined the potential association of gut microbiome characteristics with anxiety and depression symptoms during pregnancy. One study from the Netherlands (Hechler et al. 2019) involved 70 pregnant women that provided stool samples during the third trimester of pregnancy and revealed some differences in relative abundances of bacterial taxa, although a- and b-diversity were similar. In particular, higher general anxiety was associated with decreased relative abundance of the genera *Oscillospira*, *Eubacterium*, and *Megamonas*, as well as increased relative abundance of the genera *Oxalobacter*, *Rothia*, *Acetitomaculum*, *Acidaminococcus*, *Staphylococcus*, and unclassified genera within the families *Peptococcaceae* and *Peptostreptococcaceae*. General stress, pregnancy-related stress, fear of giving birth, and fear of giving birth to a child with disability did not correlate with any of the gut microbiome characteristics. Depression and anxiety symptoms have been also examined in another study from Finland (Aatsinki et al. 2018) and no significant differences were observed.

The research hypothesis that the amount of adverse childhood experiences (ACEs) is associated with gut microbiome characteristics during pregnancy has been also explored in a study from USA (Hantsoo et al. 2019). This hypothesis was based on results from an animal study regarding early prenatal stress (Jašarević et al. 2017); differential regulation of the hypothalamus–pituitary gland–adrenal glands axis was suspected as the key driver of any difference in gut microbiome, implicating the role of cortisol. In this study of 48 pregnant women, a- and b-diversity did not differ between pregnant women who had experienced multiple ACEs during their own childhood and those who had not. However, history of multiple ACEs was associated with higher relative abundance of *Prevotella* genus and a trend toward lower abundance of *Erysipelotrichaceae* family and *Phascolarctobacterium* genus was also observed.

9.2.13 Gut Microbiome and Offspring Health

The first study to explore in humans the hypothesis that the gut microbiome of the mother may be contributing to the child's risk for atopy was conducted in USA (Lange et al. 2012). In this study, 60 pregnant women were recruited and provided stool samples during the third trimester of pregnancy. Their children were followed up until they were 6 months old, when the presence of wheeze and eczema was

assessed. The researchers used quantitative cultivation, rather than sequencing-based techniques, and observed that counts of total aerobes and enterococci, which are facultative anaerobes, were higher in mothers whose children developed wheeze. Since then, two studies (West et al. 2015; Tanabe et al. 2019) have examined this hypothesis in humans and were both based on sequencing techniques to analyze the gut microbiome. In one study from Australia (West et al. 2015), 19 atopic pregnant women provided stool samples. Of their children, nine developed IgE-associated eczema within the first two and a half years of their life and were considered as cases, whereas ten children did not and served as controls. The gut microbiome was characterized by higher relative abundance of Bacilli class and *Streptococcus* genus, and lower a-diversity of the Bacteroidetes phylum in mothers of cases than in mothers of controls. The other study (Tanabe et al. 2019) has been conducted in Japan and 59 pregnant women were recruited. They provided stool samples both in early and late pregnancy and their children were examined for infancy dermatitis until they reached 4 months of age. In early pregnancy, mothers of children with infancy dermatitis showed decreased relative abundance of Actinobacteria phylum and *Bifidobacterium* genus in their gut microbiome compared with mothers of children without infancy dermatitis. However, in late pregnancy, mothers of children with infancy dermatitis showed only decreased a-diversity of Proteobacteria phylum compared to mothers of children without infancy dermatitis. To conclude, the studies that have examined so far whether maternal gut microbiome affects the child's risk for atopy have yield conflicting results.

Finally, there has also been one study from Japan (Tachibana et al. 2017) that explored whether the proportion of bacteria belonging to the Firmicutes phylum in maternal gut microbiome is associated with methylation patterns in regions of diabetes-associated genes in umbilical cord samples. Even though some associations were observed, these all lost statistical significance once maternal age and BMI were taken into consideration. Since no other studies have examined any similar outcomes and the sample size of this study was very small, drawing any conclusions is still immature.

9.3 Conclusions

Several hypothesis have been proposed regarding the potentially bidirectional association of gut microbiome with reproductive function, obstetrical, and gynecological outcomes in humans. Steroid sex hormones, short-chain fatty acids (SCFAs), amino acids, vitamin synthesis, iron absorption, and bacterial translocation from the gut have been proposed to facilitate this association. As a result, gut barrier function, systemic inflammation, hormone secretion, and lipid and glucose metabolism can be affected by the gut microbiome via different mechanisms and, in turn, affect the reproductive function.

Normal pregnancy and adverse pregnancy outcomes, such as preeclampsia, gestational diabetes mellitus, and preterm birth, have been studied in relation to the gut microbiome. Furthermore, central precocious puberty, polycystic ovary syndrome

(PCOS), endometriosis, and chronic anovulation have been examined, along with perinatal mental health, offspring health, and gynecological cancers. Even though the proposed links between gut microbiome and reproductive function are not restricted to females, male reproductive function and fertility have not been studied in humans up to date.

Studies that have transferred gut microbiome from human subjects, such as PCOS patients and pregnant women, to germ-free animal models have shown that the gut microbiome can induce in the animal models metabolic and reproductive features, which are similar with the ones observed in humans from which the gut microbiome samples were obtained. However, the results of studies that examine a- and b-diversity and relative abundance of bacterial taxa in relation to obstetrical and gynecological outcomes exhibit great heterogeneity. Several potential sources of this heterogeneity can be identified. First, the number of comparisons performed in these studies is very large; in addition, there are no solid hypotheses to support which specific bacterial taxa are expected to be more or less abundant. As a result of the aforementioned, the probability of type I error is inflated and false-positive results are expected more frequently. Even though different statistical corrections for multiple testing have been applied by the researchers, they might not be sufficient, a situation previously seen in the field of genetic epidemiology. Furthermore, the majority of the conducted studies have small sample size and neither a priori nor a posteriori power calculations are reported. Methodological differences also exist between the studies and may contribute to the observed heterogeneity of results. A minority of the conducted studies used nonsequencing-based techniques to analyze the gut microbiome, while whole genome sequencing and sequencing of different variable regions of the 16S rRNA gene have been applied in the majority of the conducted studies. In addition, some studies report differences only among the bacterial taxa with relative abundance >1%, whereas others report results also regarding the rarer taxa. Consequently, heterogeneity due to differential reporting of results is also expected. Last but not least, the gut microbiome in humans has been shown to differ based on race, lifestyle, dietary, and cultural habits, and therefore, samples drawn from different populations might vary considerably with regard to their gut microbiome (Senghor et al. 2018). Under this assumption, the differences in relative abundance of specific bacterial taxa that are observed in relation to an obstetrical or gynecological outcome in one population might not be present in another population. Nonetheless, the functional change of the gut microbiome in relation to this obstetrical or gynecological outcome and the effect of this change on the human host could still be one and the same, regardless of the population from which the sample of the study was drawn.

To sum up, even though solid hypotheses have been proposed regarding the potential links between gut microbiome and reproductive function in humans, great heterogeneity in results of observational studies in humans is present. Based on currently available data, gut microbiome analysis cannot be used for diagnosis or risk stratification in obstetrics and gynecology. Even so, probiotics, prebiotics, and symbiotics are currently tested regarding their ability to induce changes in the gut

microbiome and affect the risk of adverse obstetrical and gynecological outcomes. In conclusion, the interplay of the gut microbiome with reproductive function in humans remains controversial and research on this topic is still ongoing.

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Gut Microbiome on Allergies

10

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Abstract

Allergy refers to a hypersensitivity reaction initiated by specific immunologic mechanisms. Different forms of allergic diseases include anaphylaxis, urticaria, angioedema, allergic rhinitis, rhinoconjunctivitis, allergic asthma, serum sickness, allergic vasculitis, hypersensitivity pneumonitis, atopic dermatitis (eczema), contact dermatitis and granulomatous reactions, and food- or drug-induced hypersensitivity reactions. Usually, allergies initiate during the first 3 months of life, while genetic background is of utmost significance. Environmental factors that differentiated in the past few decades, such us climate changes, increased atmosphere pollution, nutrition, and the use of caesarean section that affects microbial colonization, are believed to strongly influence the growing allergy rates. Changes in environment and diet produce dysbiosis in gut, skin, and/or lung microbiome, inducing significant changes in the microbiota, directly affecting the immunological mechanisms implicated in the prevention of allergic diseases.

Keywords

Allergies · Barrier function · Gut–lung axis · Asthma · Atopic dermatitis

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10.1 Allergic Diseases

10.1.1 What Is Allergy?

Allergy was first described in 1996, as "specifically altered reactivity of the organisms." Today this definition has been modified and refers to a hypersensitivity reaction initiated by specific immunologic mechanisms (Johansson et al. 2004). Different forms of allergic diseases include anaphylaxis, urticaria, angioedema, allergic rhinitis, rhinoconjunctivitis, allergic asthma, serum sickness, allergic vasculitis, hypersensitivity pneumonitis, atopic dermatitis (eczema), contact dermatitis and granulomatous reactions, and food- or drug-induced hypersensitivity reactions. Usually, allergies initiate during the first 3 months of life, while genetic background is of utmost significance. Environmental factors that differentiated in the past few decades, such us climate changes, increased atmosphere pollution, nutrition, and the use of caesarean section that affects microbial colonization, are believed to strongly influence the growing allergy rates (De Martinis et al. 2017).

10.1.2 Introduction to Mechanisms of Allergic Diseases

The basic principles that govern the inflammatory process share common characteristics of various allergic conditions, including asthma, allergic rhinitis or rhinosinusitis, and atopic eczema. Inflammation due to allergy requires Immunoglobin E (IgE)-dependent activation of mucosal mast cells and eosinophil infiltration that is orchestrated by increased numbers of activated CD4+ T helper type 2 (Th2) lymphocytes (Galli and Tsai 2012). A protein capable of instructing the immune system to start producing IgE antibodies is called a primary sensitizer (allergen). Several structural and functional properties have been identified that contribute to allergenicity.

The innate immune system of the airways, gastrointestinal tract, and skin experience a constant exposure to many potential allergens. Similarly to microbial agents, allergens can engage innate pattern recognition receptors (PRRs), and thus leading to pathologic allergic/inflammatory immune responses. Although the circumstances leading to resulting in immunity in humans remained clouded, evidence suggests that allergic susceptibilities can originate in the innate immune system (Wills-Karp 2010).

The innate immune system responds to early infectious and inflammatory signals, by activating and instructing the adaptive immune system for antigen-specific T and B lymphocyte responses, and immunologic memory development. Important mediators of this process involve lipids, purines, cytokines, chemokines, and reactive oxygen species. Both innate and adaptive immune mechanisms are of major importance. Allergic inflammation is mediated by interplay between structural tissue cells and inflammatory cells (mast cells, basophils, lymphocytes, dendritic cells, eosinophils) and to a smaller degree, neutrophils (Murdoch and Lloyd 2010).

Adaptive immune responses require of naive CD4+ T cells activation and differentiation into effector cells. CD4+ Th2 cells are mediate allergic inflammation. IgE antibody production is controlled mostly by Th2 cells. Activated Th2 cells stimulate IgE production in B cells through a combination of different signals that include secreted cytokine (IL-4 or IL-13) and cell surface (CD40L). Distinct mechanisms of immune-mediated diseases are IgE-mediated hypersensitivity, antibody-mediated cytotoxicity, complex immune reaction, delayed hypersensitivity response, antibody-mediated activation inactivation of biologic function, cell-mediated cytotoxicity, and granulomatous reaction.

10.1.3 Barrier Function and Microbiome

The microbiota is a promising modulator of allergic disease responses. Environmental and nutritional changes may lead to gut and skin dysbiosis and changes in lung microbiome such us quantitative changes and in turn this could affect the immunological mechanisms implicated in the prevention of allergic diseases (Myles 2019). The constant presence of microbial exposure requires adequate detection mechanisms and scaling of responses to avoid unnecessary inflammation and tissue damage. A plethora of antimicrobial mechanisms allow microorganism clearance that could otherwise harm the host. The system's vast redundancy halts microbial resistance, although many mechanisms are most effective against bacteria in their planktonic phase and less effective against bacteria in biofilms (Koo et al. 2017). The main primary epithelial effector mechanisms in host defence against infection involve mucus production, mechanism of mucociliary transport, production of antimicrobial peptides (AMPs), reactive oxygen (ROS) and nitrogen species (NOS), antiviral interferons, and autophagy. Mucus is an essential element of mucociliary clearance and is an extracellular gel that is composed of water, mucins, and several associated molecules. The coordination of cilia's beating with mucus provides an essential mechanism for the clearance of inhaled or aspirated particulates or microbes via mucociliary transport (MCT). Decreased clearance of pathogens and inflammatory mediators results in inflammation, infection, and tissue degeneration. Moreover, the AMPs are small peptides (~10–50 amino acids) with antimicrobial activity against bacteria, viruses, and fungi. Many of them to act as modulators of inflammation, repair, regeneration, and other important cellular processes. Epithelial cells of mammals produce AMPs of the defensin and cathelicidin families. ROS and NOS form a nonspecific antimicrobial mechanism that targets all bacteria and viruses, mainly through the NADPH oxidases DUOX1 and DUOX2. DUOX-derived ROS have been shown to contribute to the antimicrobial activity. A more specific mechanism is the production of interferons, shortly after virus infection. Detection of viral infection by the aforementioned membrane-bound and intracellular recognition mechanisms also triggers the production of type I interferons (IFN-α and IFN-β) and type III interferons (IFN-λ). Interferons induce the expression of a range of genes encoding proteins that interfere with viral replication and protein synthesis and trafficking. It should be noted that autophagy is a homeostatic

mechanism that delivers unwanted cellular components to lysosomes for degradation. It holds a role in cellular stress response, differentiation, and development and the clearance of toxic components and (intracellular) pathogens (Wesemann and Nagler 2016).

10.1.4 The Gut–Lung Axis

The decreasing number of infections in developing countries seems to be one of the leading factors of autoimmune and allergic diseases. The underlying mechanisms are complex and involve several subsets of regulatory T cell and innate immune receptors such as Toll-like receptors (TLRs). The modern lifestyle affects microbiota composition, and this can lead to epigenetic changes that influence the regulatory network of immune responses. The "hygiene hypothesis" suggested a link between microbes and allergy (Bloomfield et al. 2006). The hygiene hypothesis recently included the broad use of antibiotic use and vaccinations, as other lifestyle changes have reduced childhood infections and altered the microbiota. Moreover, other important perinatal and early postnatal factors include caesarean birth and milk formula feeding. Another recently reported issue is the adoption of high fat and low-fiber diet, which has profound consequences for the intestinal microbiome's composition.

Recent studies highlight the gut microbiome as a key player influencing remotely other organs, mucosal, and hematopoietic immune functions. The interaction of different mucosal barriers, including the gut–lung cross talk, is likely to be mediated by locally resident microbes and circulating immune cells, but further studies are needed to understand this issue (Frati et al. 2018) entirely. Until now, the available treatment options cannot completely cure the diseases. Instead, their use aims to reduce symptomatology. Recent studies in asthma physiology and mechanism have identified possible therapeutics that can target innate immunity and the microbiota. The maturation process of gut microbiota over the first year of life is crucial for asthma development, and it is modifiable by early life *Lactobacillus* supplementation. The early life gut microbial development seems rather distinct, but offers a novel strategy for early life preventive interventions (Durack et al. 2018). Taking into account all the experimental data collected on the gut–lung axis, it seems that the manipulation of the airway and gut microbiome, particularly in early life, might be a promising preventive strategy of asthma initiation and exacerbation. Further studies in pathophysiology and inflammation alteration due to microbiome composition, in combination with the interaction of significant risk factors for asthma development, such as host genetics and tobacco smoke, would allow an optimization of current treatments and in managing this chronic lung condition. Additionally, improving our understanding of the microbiome's role in these diseases, novel therapeutic strategies of modifying the microbiome through diet, probiotics, or fecal or selected bacterial transfers may be developed (Vieira et al. 2016).

10.2 Human Gut Microbiome and Atopy

10.2.1 Atopic Dermatitis

Chronic inflammatory disorders of the skin, such as atopic dermatitis (AD), have been recently associated to bacterial dysbiosis. The relevance of AD, often associated with other allergic diseases, has significantly increased in the last decades. Interestingly, studies of allergic diseases have found a correlation with gut microbiome dysbiosis, although the underlying mechanisms remain veiled unclear. An initial study of AD patients found enrichment for *Faecalibacterium prausnitzii* in the gut (Huang et al. 2017). In summary, changes in environment and diet result to dysbiosis in gut, skin, and/or lung microbiome, inducing significant quantitative and qualitative changes in the microbiota, directly affecting the immunological mechanisms implicated in the prevention of allergic diseases (Pascal et al. 2018).

10.2.2 Human Gut Microbiome and Implications in Food Allergy

10.2.2.1 Role of the Gut Microbiota in the Pathogenesis of Food Allergy

During the past two decades, studies suggest that the epidemiology of food allergy (FA) has shown a dramatic increase in the prevalence and severity of clinical manifestations, the risk of persistence into more advanced ages which lead in an increased number of medical visits. The latter results in increased costs due to hospital admissions and treatments that burden both healthcare systems and individual families (Loh and Tang 2018). A long list of aliments that includes more than 170 foods have been identified as triggers of FA. These, include among others, tree nuts, eggs, peanuts, fish, shellfish, milk, wheat, soy, and seeds, with national and geographical variations concerning the most common FA (Osborne et al. 2011).

The gastrointestinal tract (GIT) keeps the balance in the activities of Th1 and Th2 cells by regulating Th17 and T regulatory (Treg) cells in the lamina propria. This action results to a crucial organ that regulated the immune function in developing either effector or tolerant responses to different antigens. Immune dysfunction in allergic diseases such as asthma and atopy seem to be related to differences in the gut microbiome function and composition. The gut microbiome constitutes an overly complex ecosystem of fungi, viruses, and even archaea, although bacteria are the most prominent components. Its composition is generally formed during the first 3 years of life. Current knowledge suggests that its colonization may begin in utero, contrary to the fetus's widely held dogma as a sterile environment (Perez-Munoz et al. 2017). Although its early formation, the microbiome composition seems to be highly dynamic. The dynamic nature of the microbiomes depends on host-associated factors such as age, diet, and environmental conditions, with the major phyla being *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. The antigenic factors in the GIT arise commonly from dietary factors or gut microbiota, affecting immune tolerance by promoting Treg cells to these dietary factors

crucial to avoid an immune response to dietary antigens (Wu and Wu 2012). Changes in bacterial communities or diversity (dysbiosis) of GIT microbiome can disrupt mucosal immunological tolerance, leading to allergic diseases, including atopic dermatitis, food allergy, and even respiratory allergic diseases such as asthma (Plunkett and Nagler 2017).

Another factor that can also contribute to FA is the low IgA levels at the intestinal surface barrier. GIT microbiota can stimulate dendritic cells (DCs) in the Peyer's patches (the digestive type of mucosa lymphoid-associated tissue) to activate B cells, leading to specific IgA antibodies production through class switching (Tezuka and Ohteki 2019). This stimulation may occur through the production by members of the microbiome of metabolites, such as short-chain fatty acids (SCFAs). Thus, the intestinal lumen's immune tolerance network can be considered to include the gut microbiota, their metabolic products, dietary factors, epithelial cells, DCs, IgA antibodies, and regulatory T cells.

Several other factors such as caesarean versus vaginal delivery, low versus rich fiber diet, breastfeeding, and/or early life antibiotic exposure may be associated with microbiota dysbiosis resulting to FA (Wesemann and Nagler 2016).

10.2.2.2 Interventions in Food Allergy

The Role of Diet

Recent evidence by metagenomics and metabolomics analysis implicates the diet–gut microbiome axis as key modulators of the immune system's maturation. A systematic review by Garcia-Larsen et al. (2018) supports in detail the relationship between maternal diet during pregnancy and lactation and FA during childhood. It seems that maternal diet, up to the first 24 months of age (baby diet), may affect the risk of developing FA (Netting et al. 2014; Wopereis et al. 2014). A healthy diet characterized by high levels of fruits, vegetables, and homemade foods is associated with less FA at 24 months (Grimshaw et al. 2014). The Mediterranean diet (MD) is characterized as a balanced diet and has a protective role against allergic disease in children during pregnancy and early life (Castro-Rodriguez et al. 2016).

Short-chain fatty acids (SCFAs) are fatty acids derived from intestinal microbial fermentation of indigestible foods. SCFAs are the main energy source of colonocytes, making them crucial to gastrointestinal health due to their immunomodulatory effects. This mechanism is one of the strongest connections between diet, gut microbiome, and allergic diseases. Major SCFAs are acetate, propionate, butyrate, and valerate (Louis and Flint 2009). SCFAs influence epigenetically several nonimmune (tight junction proteins, mucus production) and immune functions (macrophages, neutrophils, dendritic cells (DCs), T and B cells) involved in the immune tolerance network (Schauber et al. 2003). Butyrate deficiency has been observed in allergic children (Sandin et al. 2009). Bacteria-produced SCFAs have been studied and have been attributed explicitly to butyrate production by spore-forming *Clostridiales*. A "post-biotic" potential approach has been suggested based on the use of SCFAs against FA (Berni Canani et al. 2019).

Probiotics

Food and Agriculture Organization of the United Nations and the World Health Organization define probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host". Probiotics offer the benefits by promoting the appropriate balance of gut microbiota. Different studies have shown that timing is of utmost importance. The results of effectiveness may rely on the time of intervention and aspects of the current microbiota composition.

In the case of food allergy, the coadministration of bacterial adjuvants with oral immunotherapy (OIT) has been suggested as a possible treatment option. Probiotic therapy with *Lactobacillus rhamnosus* increases efficacy when coadministered with peanut OIT—producing desensitization in 82% of treated patients or with hydrolyzed casein in milk allergic patients, in which an increase of fecal butyrate levels was found. However, other *Lactobacillus* sp. strains and/or *Bifidobacteria* spp. did not demonstrate any effect in preventing allergic diseases (Tang et al. 2015). Clinical studies have shown that the oral administration of probiotics may benefit allergic rhinitis patients. Local nasal administration of *Lactococcus lactis* NZ9000 can affect local and systemic immune responses against *S. pneumoniae* (Medina et al. 2008). Furthermore, it has been suggested that probiotics can prevent eczema and show favorable effects in other allergic diseases, including asthma. Another interesting approach, based on the intranasal application of bacterial products (endotoxin or flagellin), has demonstrated immunomodulatory ability, mimicking the effect of probiotics, for the lung in different animal models, reducing experimental asthma by either reestablishing the expression of the ubiquitin modifying enzyme A20 at the endothelial barrier or inducing Tregs. Therefore, it seems that the optimal periods to apply probiotic intervention are before, during, and just after birth represents (Rodriguez et al. 2015). Nevertheless, clinical studies based on clinical trial methodologies should be carried out to validate the above results and determine the optimal probiotics to use.

Prebiotics

Prebiotics represent nondigestible food components which selectively stimulate the growth of beneficial microorganisms. Studies suggest that fibers and oligosaccharides improve immunity and metabolism. The treatment of pregnant and lactating mice increases the proportions of *Lactobacillus* spp., and *Clostridium leptum* promotes a long-term protective effect against FA in the offspring. Contradicting results have been reported by evaluating the effect of prebiotics intake in modulating asthma. Recently, it has been proposed that although the addition of prebiotics to infant food may reduce the risk of eczema, it is not clear whether their use may affect other allergic diseases, including asthma (Rodriguez et al. 2015).

Symbiotic

When using a combination of prebiotics and probiotics produces synergistic health benefits, it is described as a symbiotic (Markowiak and Slizewska 2017). In mice models that were used for food allergy studies, both the microbiome and diet seem to be involved in the allergy processes. More specific, in FA mice models, both the

microbiome and diet can affect the development of food tolerance by the induction of Treg cells. A recent meta-analysis study concluded that there are positive effects for eczema treatment. However, further randomized, placebo-controlled longitudinal studies are still required in this field of clinical research (Pascal et al. 2018).

10.2.3 Gut Microbiome and Asthma

Certain physiological features of the respiratory tract may favor the immigration and, finally, the installation of a dysbiotic microbiota, influencing pulmonary diseases' susceptibility. The lungs' primary function is to transfer oxygen from the air into the bloodstream, exchanging for CO_2. There are temperature variations along the respiratory tract, from the mouth/nose to the alveoli. The respiratory system gradually warms the air to 37 °C. The different levels of pressure and temperature between the upper and lower respiratory tract may affect bacterial communities. The pulmonary epithelium is composed of ciliated and secretory cells but is not continuous from the upper respiratory tract to the alveoli (Evans et al. 2010). In bronchi, the mucous cells are located in a submucosal gland that produces mucus, and moving toward the bronchiole, mucus is produced by club and goblet cells. Type I and II pneumocytes are from the alveolar epithelium, which secretes a surfactant rather than mucus. Mucus is consisted mostly of water and complex polysaccharides, such as mucins. The most dominant mucins in human airways are MUC5AC (from goblet cells), MUC5B (from submucosal glands), and MUC2, which is produced in only small amounts creating the gel texture. Water and mucins form a thin mobile layer that is supported by a periciliary layer covering the cilia. In a healthy individual, the mucus layer provides an effective defense mechanism against epithelial injury or infections. On the other hand, the excessive mucus production contributes to obstruction in several respiratory diseases (pneumonia, asthma, chronic obstructive pulmonary diseases, cystic fibrosis) (Proud and Leigh 2011). This obstruction may lead to even more mucus production, making it increasingly difficult for the cilia to transport the accumulative mucus out of the lungs. As long as the mucus remains in the airways, it seems to favor the selection of certain bacteria, leading to pathogens' installation. Flynn et al. (2016) have shown that some bacteria present in sputum use mucins to produce metabolites, such as propionate, which can be used by *Pseudomonas aeruginosa*. The maintenance or selection of the microbiota is also determined by the nutrient sources available in the particular ecological niche. In the GIT, nutrient sources are present at high abundance (due to food breakdown) and are capable of supporting microbial growth. The microbes of the intestinal tract are commensal because they can share the food we eat. Commensal bacteria are the best characterized, particularly in the gastrointestinal tract, where their density increases from an estimated 104 to 108 per milliliter of luminal contents in the small intestine to ~1011 organisms per milliliter of luminal content in the colon, the highest bacterial density of any environment analyzed (Walter and Ley 2011). In addition to this large community of bacteria, the gastrointestinal tract contains more immune cells than any other organ. Maintenance of

homeostasis between microorganism and the immune system is critical for the organism's well-being. Exciting new research is beginning to identify the mechanisms by which the microbiota's beneficial functions regulate tolerance to dietary antigens (Tan et al. 2016). On the contrary, most of the lung nutrients derive from host compounds, such as Igs, cytokines, defensins, lactoferrins, and mucins. These differences in lung biotic (cell layers) and abiotic (temperature, pressure, mucus, surfactant) environments may have a major impact on bacterial communities' installation and location, particularly if they lead to certain bacteria being selected and becoming predominant in disease processes.

Asthma is a common chronic respiratory disease worldwide characterized by shortness of breath and cough, among other symptoms. It affects all ages but frequently begins in childhood (Carraro et al. 2014). The symptoms are associated with variable expiratory airflow impairment, i.e., breathing difficulty with prolonged expiration due to bronchoconstriction (airway narrowing), airway wall thickening, and increased mucous production. Epidemiological studies have estimated that 250,000 deaths can be linked to this disease each year, and more than 600 million people have asthma-related symptoms (D'Amato et al. 2016). Asthma is a complex disease that includes multiple phenotypes with diverging clinical and pathophysiological characteristics (Kuruvilla et al. 2019). Asthma initiation and exacerbation may depend on individual susceptibility, viral infections, allergen exposure, tobacco smoke exposure, and outdoor air pollution.

Gut microbiota provides antigenic stimulation to the immune system, educating its early life (Murk et al. 2011). Thus, the composition of the gut microbiota can play an essential role in shaping immune phenotype. Earlier studies of infant stool specimens have found that gut colonization patterns within the first 3 months of life differed between infants who developed allergic sensitization at 12 months of age. Specifically, more *Clostridia* and fewer *Bifidobacteria* species were identified from atopic children compared to nonatopic children. Different species within a specific bacterial family also may have different immune-stimulatory effects, as has been reported for *Bifidobacteria* and *Lactobacilli* (Ouwehand et al. 2001; Mileti et al. 2009).

Newer studies have implicated other bacterial species or bacterial diversity in the gut with the development of asthma. In a prospective study of 117 children classified by the Asthma Predictive Index (API) (Huffaker and Phipatanakul 2014), the prevalence of *Bacteroides fragilis* and other anaerobic bacteria cultured from fecal samples taken at 3 weeks of age was higher in API-positive vs. API-negative subjects (Vael et al. 2008). In a birth cohort study of 411 children at high-risk for asthma, stool samples collected at 1 and 12 months after birth were analyzed by 16S rRNA-based denaturing gradient gel electrophoresis (DGGE) and also by conventional cultures. As estimated from DGGE band analysis, reduced bacterial diversity was inversely associated with allergic sensitization in the first 6 years of life, though not with the development of asthma (Bisgaard et al. 2011). Together, evidence from studies of the gut and environmental microbiota indicates that decreased exposure to a diversity of microbes, including specific microbial consortia, has negative implications for immune health that affect allergy and asthma risks.

As with the esophagus and fetus, the lung has long been thought of as sterile; however, recent evidence has shown it to harbor various bacteria phyla, including *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, even in healthy subjects. Like the gut, the lung microbiome changes rapidly in the first years of life before stabilizing. Colonization occurs gradually in healthy children, starting with *Staphylococcus* or *Corynebacterium*, followed by *Moraxella* or *Alloiococcus*. Differences in levels and diversity of the lung microbiome have been found between healthy people and patients with asthma and allergic diseases, with an increase of *Proteobacteria* in the latter; moreover, their presence has been linked to increased severity of asthma, probably through the upregulation of Th17-related genes (Hilty et al. 2010; Huang et al. 2011).

Although it is not the main subject of this chapter, early colonization with *Haemophilus influenzae*, *Moraxella catarrhalis*, and Streptococcus pneumoniae has associated with recurrent wheezing and asthma. Importantly, as well as bacteria, viruses will also influence asthma development, as demonstrated with human rhinovirus infections of the nasopharynx in early life (Teo et al. 2015). Associations have been found between the composition of the lung and gut microbiome and the risk of respiratory allergic disease development, indicating that both gut and lung mucosa may function as a single organ, sharing immunological functions.

10.3 Factors Affecting Microbiome Diversity of Allergic Diseases

The delivery method in childbirth can produce profound differences in the infant gut microbiome, with decreased levels of *E. coli*, *Bifidobacterium*, and *Bacteroides* species in children born using caesarean section compared with children delivered vaginally. Cesarean-born infants typically have a microbiome enriched with *Staphylococcus* and *Streptococcus*, comparable with the maternal skin microbiome (Fujimura and Lynch 2015). These differences appear to be associated with a higher risk of allergic diseases and asthma. Transfer of maternal vaginal microbes at birth may mitigate these effects. Time of gestation may also be a factor: premature births are associated with the gut microbiome's alterations, but not atopic sensitization (Dunn et al. 2017).

There is growing evidence that early life exposure is critical for the microbiome and that gut microbial dysbiosis heavily influences immune system development. Potential factors include perinatal exposure to maternal or infant diet, antibiotic use, and contact with older siblings. Data from different populations show that the highest interindividual microbial variability occurs during the first 3 years. Noteworthy, contact with the microbiome can start before birth since a low-abundance microbiota in the placenta and meconium have been found. Microbial exposure during the first months of life induces the innate immune system's activation in different ways, with consequences for FA (Aagaard et al. 2014).

Another critical factor influencing gut microbiome diversity is infant feeding, and especially breastfeeding, which has been shown to increase colonization by

Lactobacilli and *Bifidobacteria (*Vangay et al. 2015*)*. Breast milk contains oligosaccharides and a wide range of fatty acids, which will affect the gut microbiome and its capacity to produce metabolites that protect against allergies and asthma through the development of Treg cells (Lluis et al. 2014). The intake of unprocessed milk also produces this effect during the first year of life, probably related to higher levels of peptides in the serum fraction and unsaturated omega-3 fatty acids. Other dietary components, such as polyphenols and fish oils, are crucial for microbiome diversity (Kaliannan et al. 2015).

Antibiotic introduction in the 1950s is associated with an increased incidence of allergy. This is thought to be caused by antibiotics inducing dysbiosis, which has been shown to impact AD and asthma development directly. Age of first exposure of allergy could be critical since the maternal intake of antibiotics during pregnancy increases the risk of allergy in children, and antibiotic use in the first month of life has been associated with cow's milk allergy (Metsala et al. 2013). Intrapartum antibiotics have been shown to lead to a modified microbiome in children at 3 and 12 months. Other studies revealed that antibiotics affect the microbiome in older subjects. Antibiotic administration is associated with severe allergic airway inflammation in neonates, but not in adults (Honda and Littman 2012). Even small doses of antibiotics can affect microbiome; however, the associations between antibiotic consumption and allergic diseases increase with the number of antibiotics prescribed, and variable effects have been found for different antibiotic families. Some studies have showed that beta-lactam antibiotics are the most common triggers when FA is diagnosed before 2 years of age, while macrolides are associated with FA when diagnosed later (Lapin et al. 2014). Additional studies are needed for asthma to clarify whether it is the infection rather than the antibiotics themselves that increase susceptibility (Hirsch et al. 2017).

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