Dirk Haller *Editor*

The Gut Microbiome in Health and Disease

Springer

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ISBN 978-3-319-90544-0 ISBN 978-3-319-90545-7 (eBook)
https://doi.org/10.1007/978-3-319-90545-7

Library of Congress Control Number: 2018949648

Printed on acid-free paper

This Springer imprint is published by the registered company Springer Nature Switzerland AG.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

The German Research Foundation (DFG) started in 2013 to fund the Priority Program SPP 1656 entitled "Intestinal Microbiota" (www.intestinal-microbiota.de) and thereby consolidated a concerted action of the German Society of Hygiene and Medical Microbiology (DGHM) to support microbiome research in Germany. An interdisciplinary network of scientists, including microbiologists, gastroenterologists, immunologists, nutrition scientists, and physicians, worked together over the past few years to achieve novel insights into the role of the gut microbiome in health and diseases. In addition to numerous scientific accomplishments, the consortium made an effort to use their complementary expertise in educating the next generation of young researchers. In 2018, the members of the Priority Program SPP 1656 organized the 1st Summer School on "Microbiome in Health and Disease" within the frame of the annual Seeon Conference (www.seeon-conference.de), aiming to establish a continuous platform for education in this rapidly developing area of science. In addition, and complementary to the Summer School, this book provides a comprehensive review on the gut microbiome and its functions in health and a variety of intestinal as well as extraintestinal diseases, covering basic principles of the gut microbial ecosystem (composition, metabolic activities, and evolution over time and life stages), its reciprocal interaction with the immune system, and the clinical implementation related to diagnosis and therapy. We focus on bacteria as the dominant type of microorganism in the intestine, despite the fact that viruses, archaea, phages, and fungi emerge as relevant players in the regulation of the bacterial ecosystem and host functions. Considering the need for a continuous education process of students and health professionals, this book provides a structured overview about the methodologies applied as well as the scientific and clinical aspects of microbiome–host interactions, highlighting perspectives on historic developments and controversies in the field.

Munich, Germany — Dirk Haller
June 2018

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Intestinal Microbiome in Health and Disease: Introduction

1

Dirk Haller

Abstract

At the end of the nineteenth century, Robert Koch and Louis Pasteur developed the concept that transmissible human diseases are caused by microbial infections and, thereby, revolutionized the view of physicians on how to prevent and treat epidemics. More than 100 years later, the next conceptual revolution implies that naturally occurring communities of "commensal" microbes, collectively called microbiome, in and on human body sites affect health and the development of numerous diseases. The intestine provides an explicitly large interface to the environment and is critically involved in immune and metabolic homeostasis, providing the conceptual basis that this spatially adapted communities of microorganisms affects human health. Immune, metabolic, and xenobiotic receptors sense and process microbial signals and thereby contribute to a mutualistic relationship between the microbiome and the host. It seems a plausible hypothesis that the microbiome, considered as the *forgotten organ*, coevolved with the mammalian host, leading to a symbiotic interdependence of this metaorganism. Increasing evidence suggests that "unfavorable or so-called dysbiotic" changes in the gut microbiome lead to a distortion of microbe–host homeostasis and potentially affect disease susceptibility. In this book, we discuss breakthroughs, challenges, and applications of microbiome research at a cutting-edge level.

At the end of the nineteenth century, Robert Koch and Louis Pasteur developed the concept that transmissible human diseases are caused by microbial infections and, thereby, revolutionized the view of physicians on how to prevent and treat epidemics. More than 100 years later, the next conceptual revolution implies that naturally occurring communities of "commensal" microbes in and on human body sites affect health and the development of numerous diseases. Over the past decade, large science consortia in Europe (MetaHIT; Metagenomics of the Human Intestinal Tract) and the USA (Human Microbiome Project) have started to acquire data on the genomic potentials, phylogenetic relationships, and functional properties of microbial communities, collectively called microbiome, in healthy and diseased human populations. The technical breakthroughs and affordability of next-generation sequencing (NGS) stimulated an enormous boost of scientific activities leading to almost 40,000 publications indexed under the search term "microbiome" in the database of the US National Library of Medicine (PubMed) (Fig. 1.1). A broad variety of disorders, including

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D. Haller (ed.), *The Gut Microbiome in Health and Disease*,
https://doi.org/10.1007/978-3-319-90545-7_1

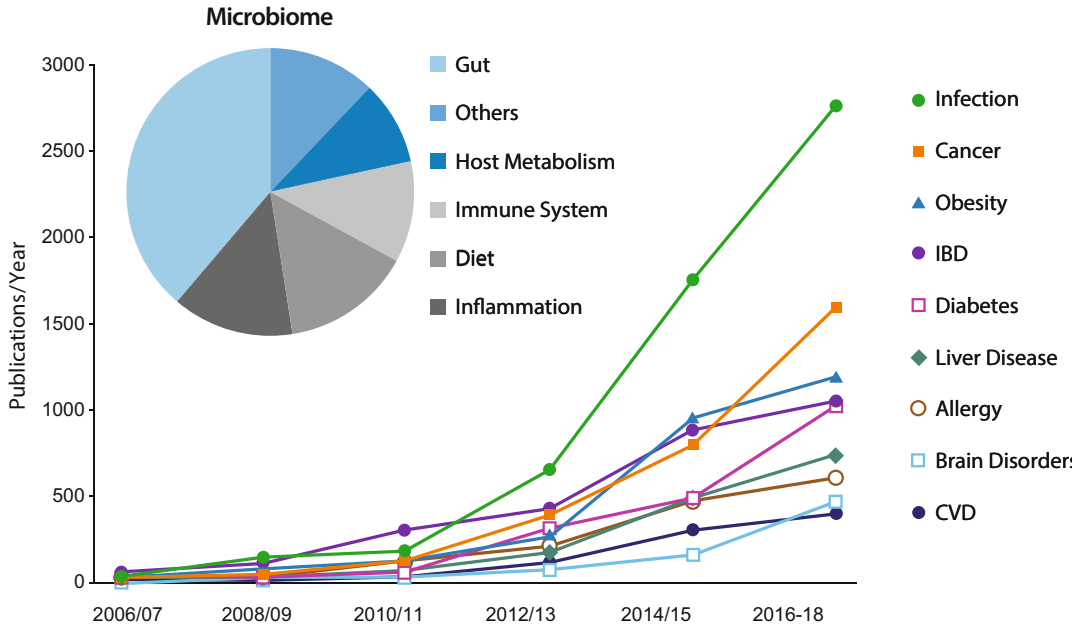

Fig. 1.1 Number of publications related to the search term "microbiome." Data were obtained by searching the database of the US National Library of Medicine (www.ncbi.nlm.nih.gov/pmc). The term "microbiome" retrieved a total of 39,592 publications (February 20, 2018). The pie chart illustrates the relative contribution of different aspects in microbiome research related to host organs (gut, total of 15,335 publications), host processes (inflammation, metabolism, immune system, total of 13,805 publications), or diet (total of 5709 publications). The annual contribution of publications related to disease categories is displayed between 2006 and 2018. Abbreviations: *IBD* Inflammatory Bowel Diseases, *CVD* Cardiovascular Disease

infectious as well as immune- and metabolically driven diseases, are associated with microbiome changes in the most densely colonized body site—the gut. Our digestive organ provides an explicitly large interface to the environment and is critically involved in immune and metabolic homeostasis, providing the conceptual basis that this spatially adapted community of microorganisms affects human health. Immune, metabolic, and xenobiotic receptors sense and process microbial signals and thereby contribute to a mutualistic relationship between the microbiome and the host. It seems a plausible hypothesis that the microbiome, considered as the *forgotten organ*, coevolved with the mammalian host, leading to a symbiotic interdependence of this metaorganism. Increasing evidence suggests that "unfavorable or the so-called dysbiotic" changes in the gut microbiome lead to a distortion of microbe-host homeostasis and potentially affect disease susceptibility. Nevertheless, the clinical relevance of microbiome changes remains speculative. Given the substantial interindividual variations in the microbiome of human populations and the pleiotropy of confounding factors, NGS-based analyses in cross-sectional studies are correlative and require validation in well-controlled replication studies using a careful selection of participants based on extensive phenotyping. The implementation of prospective (longitudinal) and treatment-naïve early-onset or birth cohorts may help to identify disease-relevant microbiome signatures in a progressive fashion and at very early stages. Disorders with low incidence require however prospective cohorts with probably unrealistic size in order to reach relevant numbers of cases. In addition to a better stratification of human phenotypes, the implementation of standardized protocols for sampling and analysis is needed to improve the reproducibility and comparability of microbiome signatures at a meaningful taxonomic resolution. An essential question arising from many human studies is whether microbiome alterations are the cause or simply the consequence of pathologies, exemplifying the need to better

understand the functional relationships of microbial communities with their host at the mechanistic level. One has to accept the fact that knowledge in this area of research is still not consolidated, and the major challenge is to establish a causal understanding of microbiome-host interactions and to address the obvious knowledge gaps. First, sample preparation and NGS technologies are subject of constant refinement complicated by methodological limitations for data interpretation. Bioinformatic algorithms need to cope with the inherent complexity, and the implementation of machine-learning algorithms is a growing need. Second, sequencing-based knowledge gain requires biological backup leading to the obvious need for an expansion on the isolation of yet uncultured taxa and the development of large-scale bacterial strain repositories. Third, the generation of disease-relevant gnotobiotic animal models, being colonized by either simplified or complex microbial consortia, is a prerequisite to unravel the mechanistic basis of microbe-host interactions. Finally, and based on the total sum of microbiome research, the aim must be to develop therapeutic and prognostic tools for targeted clinical implementation.

Composition and Function of the Gut Microbiome

2

Michael Blaut

Abstract

The human gastrointestinal tract harbors a plethora of microorganisms, most of which belong to the domain Bacteria. Owing to manifold effects on host physiology and host health, there is a growing interest in better understanding the role and function of gut microbial communities. Microbiota composition changes along the gastrointestinal tract in response to changes in the physicochemical conditions and substrate availability. Moreover, large interindividual differences are observed. One major function of the gut microbiota lies in the conversion of indigestible dietary carbohydrates and host-derived glycans to short-chain fatty acids, which provide energy to the host and have regulatory functions. Microbiome analysis has led to the notion of a "core microbiome" which encodes functions shared by human individuals. Gut microbial community members interact with each other and with the host constituting a functional microbial ecosystem. However, there are still major gaps in our understanding of the molecular mechanisms underlying such interactions.

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2.1 Introduction

Prokaryotic microorganisms (Bacteria and Archaea) have conquered essentially every habitat on earth and may therefore be considered ubiquitous. They occupy environments that differ profoundly in their physicochemical conditions and the substrates available for growth. Microbial habitats range from marine and sweet water environments, deep-sea hydrothermal vents, soil, and air to plants and animals. The microbes thriving in a given habitat are optimally adapted to the conditions prevailing therein. Some microbial communities withstand even harsh conditions such as high temperature, high salinity, and low or high pH. The ability of prokaryotes to colonize essentially all habitats on earth reflects 4 billion years of evolution. Depending on the environment, prokaryotic organisms may be phototrophic, chemotrophic, lithotrophic, autotrophic, heterotrophic, and combinations thereof, indicating a high metabolic variability. Besides playing essential roles in the global cycles of carbon, nitrogen, and sulfur, prokaryotes also occur in and on animals and humans. They occupy various body sites including the skin, nose, throat, as well as the urogenital and gastrointestinal tracts. These habitats differ with respect to the availability of substrates and oxygen, but, at least in mammals, they all provide a constant temperature favoring microbial growth. The intestinal tracts of herbivores differ from those of carnivores or omnivores not only in their anatomies

D. Haller (ed.), *The Gut Microbiome in Health and Disease*,
https://doi.org/10.1007/978-3-319-90545-7_2

but also in the microbial communities they harbor reflecting adaptations to the respective preferred food source. There is evidence that the intestinal microbial communities coevolved with their respective host (Ley et al. 2008).

2.2 Distribution of Microbial Communities in the Human Gastrointestinal Tract

Environmental conditions in the human gastrointestinal tract are not uniform but differ considerably between the stomach and colon. It's therefore not surprising that the microbial communities resident in the various sections of the digestive tract differ in several aspects including cell density, composition, and metabolic activity.

2.2.1 Stomach

Between meals, the pH in the stomach of healthy adults is typically 1–2 but increases following food ingestion. Transit time through the stomach determined in eight healthy subjects with a magnet tracking system ranged between 5 and 133 min, with a median of 56 min (Worsoe et al. 2011). Transit time is influenced by food consistency (shorter for fluids than for solid and un-chewed food), osmolarity (longer for monosaccharides compared to polysaccharides), nutrient composition (longer for fats and carbohydrates), and energy density (longer for high-energy diets). The low pH of gastric juice largely prevents the growth of ingested microbes explaining the low density of $<10^3$ microbial cells per ml of gastric content. However, a culture-independent survey of microbial 16S rRNA gene sequences in 23 gastric mucosa biopsy samples revealed a diverse community of 128 phylotypes belonging to the phyla *Firmicutes* (36 phylotypes), *Bacteroidetes* (35 phylotypes), *Proteobacteria* (32 phylotypes), *Actinobacteria* (12 phylotypes), *Fusobacteria* (10 phylotypes) (Fig. 2.1), and minor components of other phyla (Bik et al. 2006). A high proportion of the detected sequences were assigned to oral bacteria, such as *Streptococcus salivarius*, *Streptococcus mitis*, *Streptococcus parasanguinis*, various *Prevotella* and *Porphyromonas* spp., *Rothia dentocariosa*, *Atopobium parvulum*, and *Fusobacterium nucleatum*. It may be surmised that the main habitat of many of these species is the oral cavity, from where they get into to stomach by swallowing. Nineteen of the 23 subjects harbored *Helicobacter pylori*. This organism resides in the mucus layer of the stomach and is known to secure its survival in the gastric environment by the production of urease, which catalyzes the release of ammonia (and carbon dioxide) from urea resulting in an increase of the pH in the immediate environment of the cell. Bacteria isolated from gastric contents include *Lactobacillus* spp. and *Streptococcus* spp., which are capable of surviving at relatively low pH.

2.2.2 Small Intestine

The small intestine represents the longest part of the digestive tract with changing conditions and increasing bacterial cell densities along its course. The relatively short residence time of intestinal contents, namely, 209–391 min with a median of 255 min (Worsoe et al. 2011), limits the growth of microorganisms to high density, in particular in the duodenum. Bacterial cell counts increase from the duodenum to the terminal ileum from approximately 10^4 to 10^8 per ml of intestinal content (Booijink et al. 2010; Finegold et al. 1983), and also the number of taxa detectable with culture-independent methods increases (Hayashi et al. 2005). The ileal effluents of ileostomy patients were reported to contain species of the *Lactobacillales*, *Clostridiales*, and the *Veillonella* group as well as *Streptococcus bovis*-related species at relatively high abundance, while species related to *Ruminococcus gnavus*, *Ruminococcus obeum*, and *Bacteroides plebeius* were present at lower relative proportions (Fig. 2.1) (Booijink et al. 2010). The microbial taxa detected in ileostomy effluent were in part the same as those retrieved from the small intestine of four healthy subjects and in part similar to those detected in their feces (Zoetendal et al. 2012). In general, the small intestinal microbiota

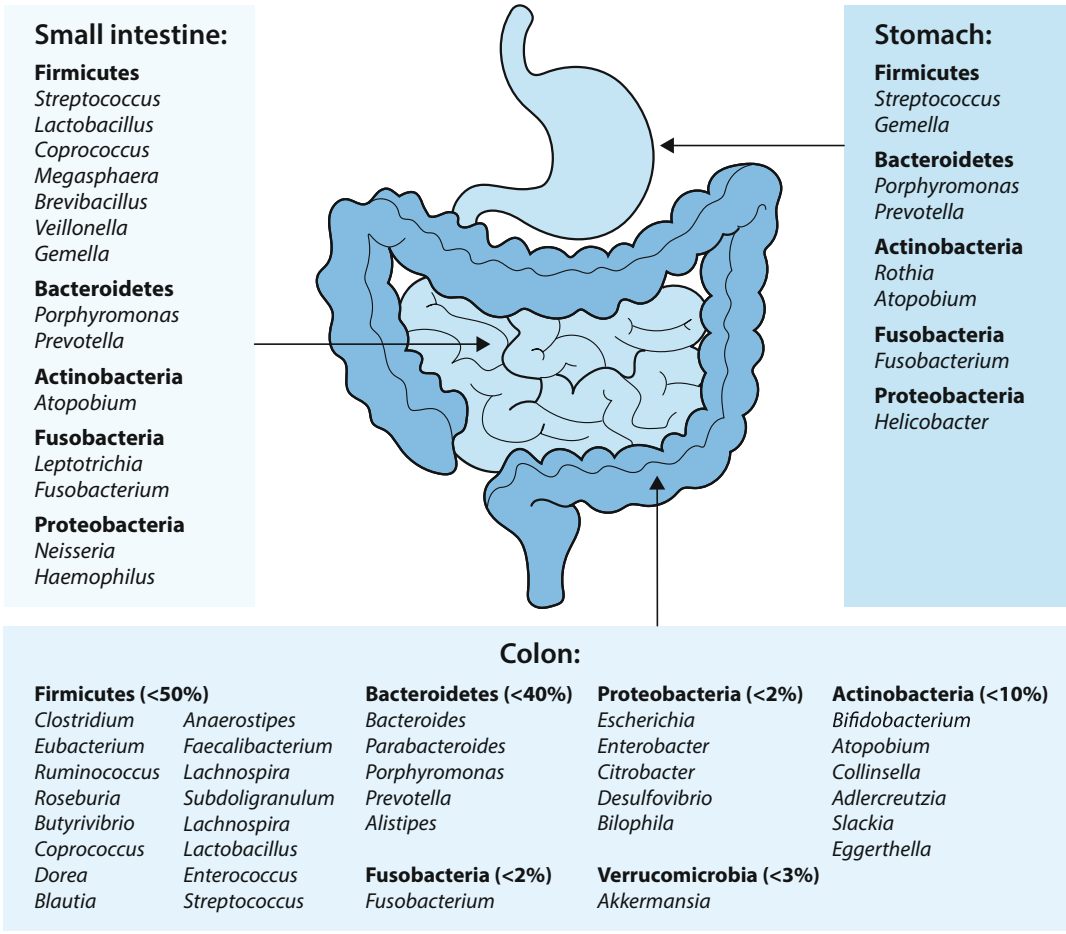

Fig. 2.1 Major bacterial genera encountered in the various sections of the gastrointestinal tract

composition was more variable among individuals and over time, when compared to the fecal microbiota. A more recent study, which compared the duodenal microbiota of 30 liver cirrhosis patients and 28 healthy subjects, reported the presence of the genera *Brevibacillus*, *Veillonella*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Leptotrichia*, *Atopobium*, *Megasphaera*, *Gemella*, *Haemophilus*, and *Neisseria* (Chen et al. 2016). A large fraction of microorganisms in the small intestine are facultative anaerobes, but their proportion decreases from the duodenum to the terminal ileum because oxygen becomes more and more limited and the redox potential decreases. It is important to note that the number of studies investigating the small intestinal microbiota is considerably smaller than the number dealing with the colonic or fecal microbiota.

2.2.3 Colon and Feces

Owing to a relatively long mean colonic transit time of 35 h (Metcalf et al. 1987), colonic microorganisms have more time to proliferate. The absorption of water and ions during passage of colonic contents also contributes to an increase in bacterial density from cecum (10^8 ml^{-1}) to distal colon (10^{11} ml^{-1}) (Sender et al. 2016b). Clearly, the colon is the most densely populated body site. The total number of microbial cells inhabiting the human body has until previously been estimated to exceed the number of host cells by a factor of 10 (Savage 1977). A more recent publication is in conflict with this estimate. Based on thorough considerations, the total number of microbial cells harbored by a reference male person (20–30 years of age with a weight

of 70 kg and a height of 170 cm) was estimated to be in the range of 4×10^{13} with more than 99% of these cells residing in the colon, while the number of human body cells is approximately 3×10^{13}, with red blood cells contributing 84% to this number (Sender et al. 2016a). Hence, the number of microbial cells in the human body is 1.3-fold higher than the number of body cells. However, if only nucleated cells are considered (0.3×10^{13}), this ratio increases to a factor of 10.

Although there is no dispute about the fact that the number of microbial species or phylotypes in the colon is quite high when compared with other microbial habitats, estimations of the number of microbial species or phylotypes present in colonic contents or feces vary significantly. While Eckburg and coworkers detected 395 bacterial phylotypes in samples from multiple colonic mucosal sites and in feces of three healthy subjects (Eckburg et al. 2005), other researchers estimated the number of bacterial species found in the human intestinal tract to be approximately 800 (Backhed et al. 2005), while 16S rRNA gene sequence analysis of 190 resected tissue samples from patients with inflammatory bowel diseases and control subjects led to the estimation of 15,000 to 36,000 species (Frank et al. 2007). These differences may in part be explained by the error-prone 16S rRNA amplicon sequencing which can result in the detection of false positives, which can be circumvented by using low-error amplicon sequencing (Faith et al. 2013). The application of this method to fecal samples from 37 healthy adults, who were sampled several times over up to 296 weeks, revealed that these individuals harbored 101 ± 27 species. These few examples show the large range of numbers of bacterial species estimated to be present in the human intestine. In this context, it is important to specify whether the phylotype or species numbers given refer to all human fecal 16S rRNA gene sequences available in database, to those obtained from a group of human subjects, or to one individual only. For example, using shotgun sequencing, Qin et al. clearly stated that a cohort of 124 European individuals harbored 1000 and 1150 bacterial species and each individual at least 160 species, which are also largely shared (Qin et al. 2010). It may be deduced that the number of species present in the intestinal tract of a given individual is rather in the range of hundreds than of thousands. While species richness in the human gut is high, the 16S rRNA gene sequences affiliate with only a small proportion of the 92 presently known bacterial phyla with cultured representatives: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Cyanobacteria* (Hug et al. 2016). These phyla differ greatly in their relative contribution to bacterial cells in the microbiota. In one study involving 18 human subjects including monozygotic twins and their mothers, members of the *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* were reported to account for approximately $\geq$95% of bacterial cells in the gut microbiota (Turnbaugh et al. 2009). While the study participants differed considerably in the relative abundance of the phyla, they displayed high similarity in the relative abundance of gene categories among the collected samples, suggesting that different taxa can exert identical functions. A number of studies reported 16S rRNA gene sequences to be indicative of the presence of members of the *Cyanobacteria* in fecal samples. However, so far no representative of this phylum has been isolated from the intestinal habitat. Instead, phototrophic *Cyanobacteria* are typically found in oceans, lakes, rivers, and ponds. However, whole-genome reconstruction of human fecal metagenomic samples indicated the presence of a new candidate phylum closely related to *Cyanobacteria*, for which the authors proposed the designation *Melainabacteria*. Genome analysis suggested that both lineages, *Cyanobacteria* and *Melainabacteria*, had a common ancestor, which was a non-photosynthetic, anaerobic, and obligately fermentative bacterium (Di Rienzi et al. 2013).

Other numerically minor components of the human gut microbiota include methanogenic Archaea and eukaryotic yeasts, whose abundance, based on the proportion of these organisms' genes in the intestinal metagenome, is in the range of 0.8% and 0.1%, respectively (Qin et al. 2010). The Archaea are represented by *Methanobrevibacter smithii*, which converts H_2 and CO_2 or formate to methane, and by *Methanosphaera stadtmanae*, which in addition is capable of reducing methyl groups to methane. The amount of methane excreted by humans in breath is variable; approximately every other person harbors detectable populations of methanogens (Florin et al. 2000). Among

eukaryotic intestinal microorganisms, fungi are the most prominent members of the intestinal microbiota (Huffnagle and Noverr 2013). Intestinal fungi, referred to as gut mycobiome, have been much less studied than those of intestinal prokaryotes. Analysis of fecal samples from 98 healthy individuals led to the identification 66 fungal genera and an estimated number of 184 species (Hoffmann et al. 2013). *Saccharomyces*, *Candida*, and *Cladosporium* were the most prevalent genera, being found in 89%, 57%, and 42% of the samples, respectively. This investigation did not allow any conclusion on whether the detected fungi were resident or merely transient. Expert mycologists stated in a recent paper: "This diversity, while impressive, is illusory. If we examine gut fungi we will quickly observe a division between a small number of commonly detected species and a long tail of taxa that have been reported only once" (Suhr and Hallen-Adams 2015). A more recent study on the fecal mycobiota of healthy human vegetarians identified at least 46 distinct fungal OTUs affiliated with two phyla only: Ascomycota and Basidiomycota (Suhr et al. 2016). *Fusarium*, *Malassezia*, *Penicillium*, *Aspergillus*, and *Candida* (in decreasing order) were the most commonly detected genera. Even though fungi are considered important members of the microbial community in the human gut, knowledge about the role of these organisms in health and disease is still very limited in comparison to the bacterial members of the community. While viruses are not considered to be organisms, viruses can readily be detected in fecal samples. Sequencing of the metagenome and of DNA from virus-like particles in human fecal samples led to the identification of several thousand bacteriophage genomes referred to a virome (Minot et al. 2011). The role of the latter for the ecosystem is far from being understood.

2.3 Adaptation of Microbes to the Intestinal Environment

The term metagenome refers to the collective genome of all members of the microbiota in a given habitat, also referred to as microbiome. The proteins encoded in the human intestinal microbiome roughly reflect the functions required by intestinal bacteria to cope with the gut environment. A large proportion of the genes identified in the microbiome are related to fundamental functions required by every cell to grow and divide. Genes and proteins related to energy generation, synthesis of cellular components, and reproduction are found in every bacterial cell and are usually well conserved among bacteria. Examples include ribosomal proteins, RNA polymerase, and ATP synthase. Metabolic pathways including glycolysis and the tricarboxylic acid cycle are widely but not universally distributed among bacteria in general and intestinal bacteria in particular. For example, *Bifidobacterium* spp. degrade hexoses using the unique fructose-6-phosphate phosphoketolase pathway rather than by glycolysis. Moreover, in many strict anaerobes, the tricarbonic acid cycle is incomplete, and the remaining enzymes of the cycle preferentially fulfill anabolic functions. Physiology and the metabolic capacity of intestinal microorganisms are adapted to the conditions prevailing in the digestive tract.

2.3.1 Physicochemical Conditions in the Digestive Tract and Electron Transport

The intestine, especially the colon, is characterized by low oxygen partial pressure and highly reduced conditions with a redox potential (E_h) of approximately –215 mV, a value measured in the large intestine of pigs (Hornich and Chrastova 1981). Therefore, >99% of human fecal bacteria and also methanogenic Archaea are strict anaerobes. They cannot grow in the presence of oxygen because critical enzymes become inactivated under oxic conditions. Even though facultative aerobic or aerotolerant bacteria make up less than 1% of microbial cells in the human intestine, they play an important role. In particular facultative aerobes such as the *Enterobacteriaceae* are capable of utilizing oxygen as a terminal electron acceptor. For example, *Escherichia coli* is capable of expressing two ubiquinol-dependent oxidases, one of which has a low affinity for oxygen and a high turnover rate (cytochrome *o*-type oxidase),

while the other one (cytochrome *d*-type oxidase) has a high affinity for oxygen and a low turnover rate. If no oxygen is available, *E. coli* is capable of gaining energy anaerobically either by anaerobic electron transport using nitrate, trimethylamine-*N*-oxide, dimethyl sulfoxide, or fumarate as terminal electron acceptors. If none of these is available, *E. coli* gains energy by mixed acid fermentation. The metabolism of this facultative anaerobe is regulated in such a way that the most efficient mode of energy generation is turned on while less efficient, alternative modes are turned off. Bacteria that tolerate oxygen but cannot gain energy by respiration are called aerotolerant. Lactic acid bacteria, for example, exclusively gain energy by substrate-level phosphorylation involving the conversion of carbohydrates such as lactose to lactic acid and some minor fermentation products. In spite of the scarcity of oxygen in the human intestinal tract, small amounts of oxygen become available by swallowing air during meals and by diffusion of oxygen from blood circulation to the mucosal surface. In addition to facultative aerobes, which tolerate high oxygen partial pressures, some gut bacteria previously considered strict anaerobes are capable of utilizing oxygen as long as nanomolar concentrations are not exceeded (Baughn and Malamy 2004). *Bacteroides fragilis* and other *Bacteroides* spp. harbor a high-affinity cytochrome *bd*-type oxidase allowing ATP generation by aerobic respiration. The term nanaerobes has been coined for such bacteria (Baughn and Malamy 2004). *Faecalibacterium prausnitzii* is an oxygen-sensitive organism which nevertheless grows near the mucosal surface where the oxygen partial pressure is relatively high. This is possible because *F. prausnitzii* transfers electrons to extracellular flavins and thiols, present in the gut, to reduce oxygen (Khan et al. 2012). However, even though this mechanism enables the organism to reoxidize reduced electron carriers, ATP generation is less efficient than aerobic respiration.

2.3.2 Alternative Electron Acceptors

Anaerobes capable of transferring electrons derived from oxidation reactions onto external electron acceptors do not have to use valuable intermediates such as pyruvate as electron acceptors. For example, under anoxic conditions *E. coli* and other *Enterobacteriaceae* are capable of using nitrate or trimethylamine-*N*-oxide as electron acceptor. Nitrate may be formed in the inflamed gut in which NO levels are increased because of an upregulated nitric oxide synthase (iNOS). NO reacts with reactive oxygen species to peroxynitrate ($ONOO^-$) which isomerizes to nitrate. Accordingly, nitrate produced under inflammatory conditions not only stimulates the growth of *Salmonella* but also that of *E. coli* (Lopez et al. 2012; Winter et al. 2013). Various sulfur compounds including sulfate, thiosulfate, and tetrathionate may also serve as electron acceptors. Sulfate reaching the colon may be of dietary origin, but the majority is derived from sulfated mucins by the action of bacterial sulfatases (Christl et al. 1992). Sulfate-reducing bacteria in the human gut include species of the genera *Desulfovibrio*, *Desulfobacter*, and *Desulfobulbus* (Nava et al. 2012). Since sulfate (SO_4^{2-}) is a poor electron acceptor, it first needs to be activated to adenosine-5′-phosphosulfate (APS) ($SO_4^{2-} + ATP \rightarrow APS + PP_i$). APS is subsequently reduced to sulfite (SO_3^{2-}) in a two-electron transfer reaction and thereafter to sulfide (S^{2-}) in a six-electron transfer reaction. Some bacteria including *Bilophila wadsworthia* are capable of gaining sulfite from sulfonates such as taurine (Carbonero et al. 2012). Therefore, bile acids conjugated with taurine stimulate the growth of this organism. Trimethylamine-*N*-oxide, another electron acceptor used by *Enterobacteriaceae*, is formed by the oxidation of trimethylamine as catalyzed by host monooxygenases (Bennett et al. 2013). Trimethylamine in turn originates from the bacterial degradation of choline or carnitine in the human intestine.

2.4 Metabolic Activities of the Intestinal Microbiota

Besides conferring colonization resistance on the host and protecting against pathogens, the gut microbiota primes the immune system and provides enzymes that expand the metabolic capacity of the host. A major function of the

intestinal microbiota is the conversion of dietary and endogenous substrates that escape digestion including carbohydrates, proteins, secondary plant metabolites, and xenobiotics. The conversion of these substrates supports the growth of intestinal microbes by providing energy and metabolites for anabolic reactions. The gut microbiota's metabolic capacity has been proposed to rival that of the liver encompassing a wide range of reactions that reflect the low redox potential and the scarcity of oxygen available in most parts of the intestinal tract.

2.4.1 Substrates of the Intestinal Microbiota

Even though intestinal bacteria differ in how they generate energy, they share the same environment, i.e., the physicochemical conditions (pH, redox potential, temperature) at a given intestinal site, and they are dependent on the substrates coming from the diet (Table 2.1) or endogenous substrates provided by the host (Table 2.2). However, some bacterial population groups cross-feed other community members by converting these primary substrates into products that can be utilized further by bacteria that depend on these substrates; examples include lactate, formate, and hydrogen.

There is evidence that the intestinal microbiota coevolved with the respective animal host, suggesting that the bacteria resident in the digestive tract are optimally adapted to the specific environment and the nutritional habits of the host species. In humans, one of the main functions of the gut microbiota is the breakdown of dietary components that escape digestion by host enzymes. Nondigestible polysaccharides include resistant starch, plant cell wall components such as cellulose (β-[1→4] D-glucose), β-[1→3, 1→4] glucans, and pectins (α-[1→4]-linked D-galacturonic acid esterified by methanol to varying degree) as well as inulin (β-[2→1] fructose with a chain-terminating glucosyl moiety), which serves as a storage polysaccharide in various plants (Table 2.1). Some of these polymeric carbohydrates occur in conjunction with lignin and are referred to as dietary fibers, which represent the main substrate source for intestinal bacteria. However, the extent to which dietary fiber becomes utilized by intestinal bacteria depends on the physicochemical properties of the polymeric components, in particular on water solubility, water-binding capacity, and viscosity. These properties in turn depend on their chemical structure: type of carbohydrate units present, the way in which they are linked, and the degree of branching and polymerization. Dietary fiber may be categorized into structural polysaccharides originating from plant cell walls such as cellulose, pectin, xylan, mannan, and β-glucan and into storage carbohydrates such as inulin and starch.

Starch is the main carbohydrate source in a typical human diet. Humans are equipped with α-amylase, which is produced in salivary glands and pancreas and catalyzes the breakdown of starch to maltotriose and maltose. However, certain forms of starch, referred to as resistant starch, escape digestion because the glycosidic bonds cannot be accessed by human enzymes. Raw potatoes, green bananas, legumes, and unprocessed grains are typical sources of resistant starch. Moreover, heating and cooling of starch-containing foods such as potatoes and noodles may lead to the formation of retrograded starch, which represents one form of resistant starch. The intestinal microbiome has the capacity to depolymerize resistant starch and to utilize the cleavage products as sources of energy and carbon. Other indigestible dietary carbohydrates originate from whole-grain products, legumes, vegetables, fruits, and nuts. For the sake of completeness, it has to be mentioned that in addition to such complex carbohydrates, some mono- or oligosaccharides to a greater or lesser extent escape digestion in the small intestine and therefore become substrates of the intestinal microbiota. These include sugar alcohols such as sorbitol and xylitol, disaccharides such as lactulose (4-*O*-β-D-galactopyranosyl-D-fructofuranose), as well as oligosaccharides such as stachyose (α-D-galactopyranosyl-[1→6]-α-D-galactopyranosyl-[1→6]-α-D-glucopyranosyl-[1→2]-β-D-fructofuranoside), fructooligosaccharides, galactooligosaccharides,

Table 2.1 Substrates of dietary origin utilized by the intestinal microbiota

Dietary origin		
Category	Class	Composition or representative compounds
Polysaccharides	Resistant starch:	
	Amylose Amylopectin	α(1→4) Glucan α(1→4), α(1→6) Glucan (branched)
	Cellulose	β(1→4) Glycan
	Pectins	α(1→4), α(1→6) Galacturonan (methylesters) α(1→4) Galactan and mixed linked arabinans
	Pentosans	β(1→4) Xylan with some arabinose and uronic side chains
	Hexosans	β(1→4) Glucomannan, β(1→3), β(1→4) Glycan (single or mixed)
	Xyloglycans	β(1→4) Glucan with β(1→6)-linked xylose side chains
	Galactomannans (Guar gum)	β(1→4) Mannans with α(1→6)-linked galactose side chains
	Chitin	β(1→4) *N*-Acetylglucosamine
	Laminarin	β(1→3) Glucans
	Inulin	β(1→2) Fructan
Oligosaccharides	Stachyose	α(1→6) Galactosyl raffinose
	Raffinose	α(1→6) Galactosyl sucrose
	Lactose	β(1→4) Galactosyl glucose
	Lactulose	β(1→4) Galactosyl fructose
Sugar alcohols	Sorbitol	
	Xylitol	
Secondary plant metabolites	Flavonoids	– Quercetin, Luteolin, Cyanidin, Daidzein
	Tannins	– Polymers of ellagic acid, gallic acid, pyrogallic acid
	Glucosinolates	– Glucoraphanin, Sinigrin, Sinalbin, Glucobrassicin
	Lignin	– Cross-linked macromolecule formed from paracoumaryl, coniferyl, and sinapyl alcohol
Proteins	Sarcoplasmatic and myofibrillar proteins	

and xylooligosaccharides. In human and animal studies, the latter three have been demonstrated to stimulate the growth of bacterial population groups considered to be beneficial and to have health-promoting properties. They are referred to as prebiotics. However, the original concept has recently been challenged and been revised (Bindels et al. 2015).

2.4.2 Breakdown of Complex Carbohydrates

Humans and other mammals lack the enzymes required for the breakdown of the large variety of complex dietary carbohydrates. However, the human gut microbiome provides a wide range of depolymerizing enzymes enabling the host to take advantage of dietary fiber by utilizing the bacterial degradation products. Metagenomic studies revealed that the human colonic microbiome in comparison to all sequenced microbial genomes is enriched in genes involved in the breakdown of dietary polysaccharides, whereas genes encoding other functions such as energy production and lipid metabolism are underrepresented. Genes representing more than 80 different glycoside hydrolase families, also referred to as carbohydrate-active enzymes (CAZymes), were identified in the distal human gut microbiome (Gill et al. 2006). High-throughput functional screens enabled the isolation of 310 clones exhibiting β-glucanase, hemicellulase, galactanase, amylase, or pectinase activities with 26 clones

Table 2.2 Substrates of the intestinal microbiota provided by the host

Host		
Category	Class	Composition or representative compounds
Glycoproteins	Mucus	Protein backbone with characteristic carbohydrates (fucose, sialic acid, *N*-acetyl-galactosamine, *N*-acetyl-galactosamine) preferentially linked to serine and threonine residues
	Hyaluronate	Polymer of glucuronic acid β(1→3) *N*-acetyl-galactosamine
	Chondroitin sulfate	Polymer of glucuronic acid β(1→3) *N*-acetyl-galactosamine, the latter being sulfated in C4 and/or C6
	Mucosal surface glycoproteins	Fucosylated proteins
Proteins	Digestive enzymes	Trypsin
		Chymotrypsin
		Leucine aminopeptidase
		Elastase
		Lipase
		Nucleic acid hydrolase
	Desquamated epithelial cells	
Bile acids	Primary bile salts	Taurocholate, glycocholate Taurochenodeoxycholate, glycochenodeoxycholate
	Secondary bile salts	Deoxycholate and its taurine or glycine conjugates Lithocholate and its taurine or glycine conjugates

being particularly efficient in the degradation of raw plant polysaccharides (Tasse et al. 2010). Seventy-three CAZymes from 35 different enzyme families were discovered, 32 of which were highly homologous to prevalent genes found in the gut microbiome of 20 human individuals. The results obtained in this study are consistent with the occurrence of horizontal gene transfer among intestinal bacteria (Tasse et al. 2010).

The first step in the utilization of nondigestible polymeric carbohydrates by intestinal bacteria requires their breakdown, which results in the formation of oligomeric and monomeric carbohydrates (Fig. 2.2). This process involves various enzyme families such as glycoside hydrolases, polysaccharide lyases, glycosyltransferases, and carbohydrate esterases (Cantarel et al. 2009; Flint et al. 2012). These CAZymes may also be categorized according to the type of substrates they act on, namely, plant cell wall components such as cellulose, pectins, xylans, β-glucans, and mannans or storage carbohydrates such as inulin and fructooligosaccharides. Another type of CAZymes acts on glycans produced by the host in the form of mucins and other glycoproteins (see further below in Sect. 2.4.3). The availability of an increasing number of draft genomes of human intestinal bacteria and metagenomic analyses has helped to identify gene clusters encoding putative CAZymes (http://www.cazy.org/). However, the catalytic features and the regulation of the majority of these proteins have not yet been investigated. It is also important to note that in addition to the enzymes catalyzing the depolymerization of glycans, auxiliary proteins are required for substrate binding, transport, and regulation. They act hand in hand and efficiently provide bacterial substrates for energy generation.

2.4.3 Degradation of Glycans by Members of the *Bacteroidetes*

Early on, it was recognized that *Bacteroides* species play an important role in the degradation of nondigestible carbohydrates in the human colon (Salyers et al. 1977a). As a representative of the genus, *Bacteroides thetaiotaomicron* has been used as a model organism to study carbohydrate

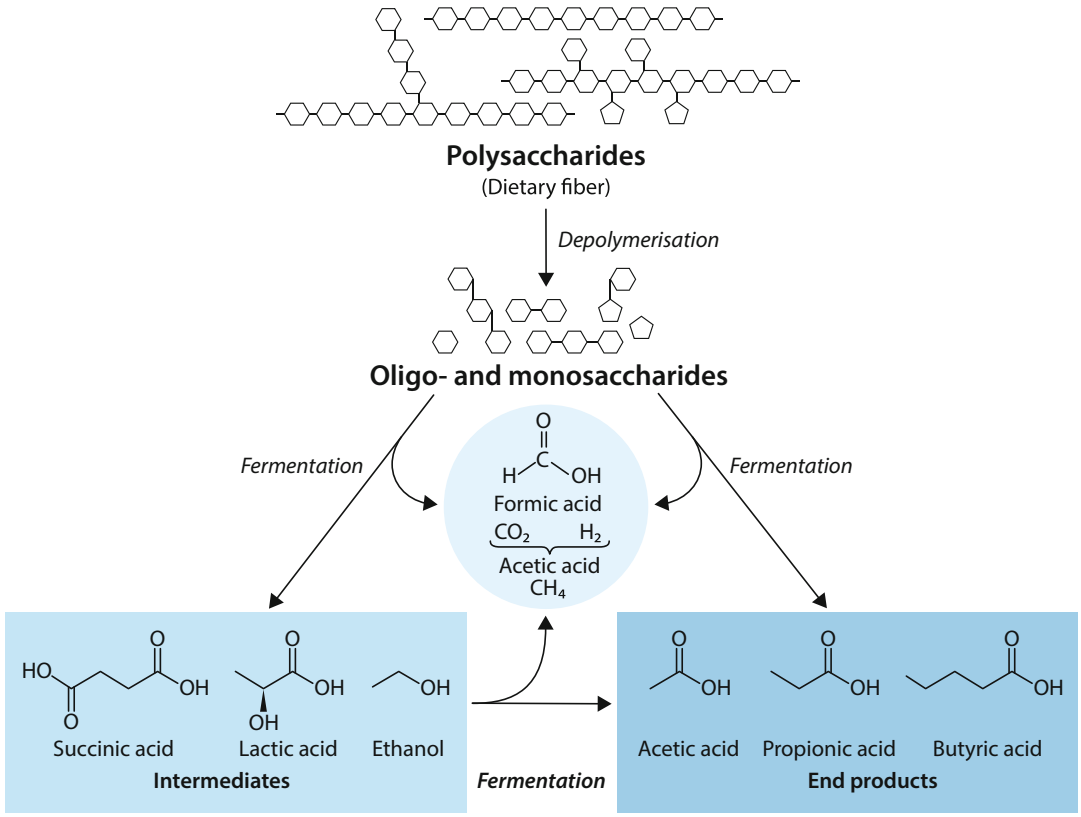

Fig. 2.2 Major steps in the breakdown of complex carbohydrates by the colonic microbiota

utilization in detail. Transcriptome analysis of *B. thetaiotaomicron* recovered from mono-associated mice fed either a polysaccharide-rich or a simple sugar diet revealed that the organism not only induces glycoside hydrolases in a diet-dependent manner but also expresses outer membrane proteins engaged in polysaccharide binding (Sonnenburg et al. 2005). *B. thetaiotaomicron* preferably utilizes simple carbohydrates or host glycans when dietary polysaccharides are not available, indicating a high degree of metabolic flexibility. Detailed studies of the starch utilization system of *B. thetaiotaomicron* led to a model widely used as a paradigm for the degradation of polysaccharides by *Bacteroides* species (Cho et al. 2001; Shipman et al. 2000). Two sets of proteins are involved in starch utilization by *B. thetaiotaomicron*. One set, which is referred to as starch utilization system (Sus), encompasses seven proteins contributing to starch degradation (SusABCDEFG) and a regulatory protein (SusR). The *mal* (maltose) regulon with the regulatory protein MalR also plays a role in starch utilization besides controlling the expression of *malA*, an α-glucosidase gene (Cho et al. 2001). Deletion of *malR* attenuates *sus* gene expression, indicating that SusR in conjunction with MalR control their expression. SusR activated by maltose, maltotriose, or longer glucose oligomers binds to the promoter region located upstream of *susB* and thereby activates the transcription of *susBCDEFG*. As *susA* is located upstream of *susB*, it has its own promoter (Reeves et al. 1997) (Fig. 2.3). SusDEF are lipoproteins anchored in the outer membrane, where they form a complex capable of binding starch (Shipman et al. 2000). SusG, which is also anchored in the outer membrane, is an α-amylase cleaving starch molecule bound to SusDEF (Shipman et al. 1999). The products formed by SusG are

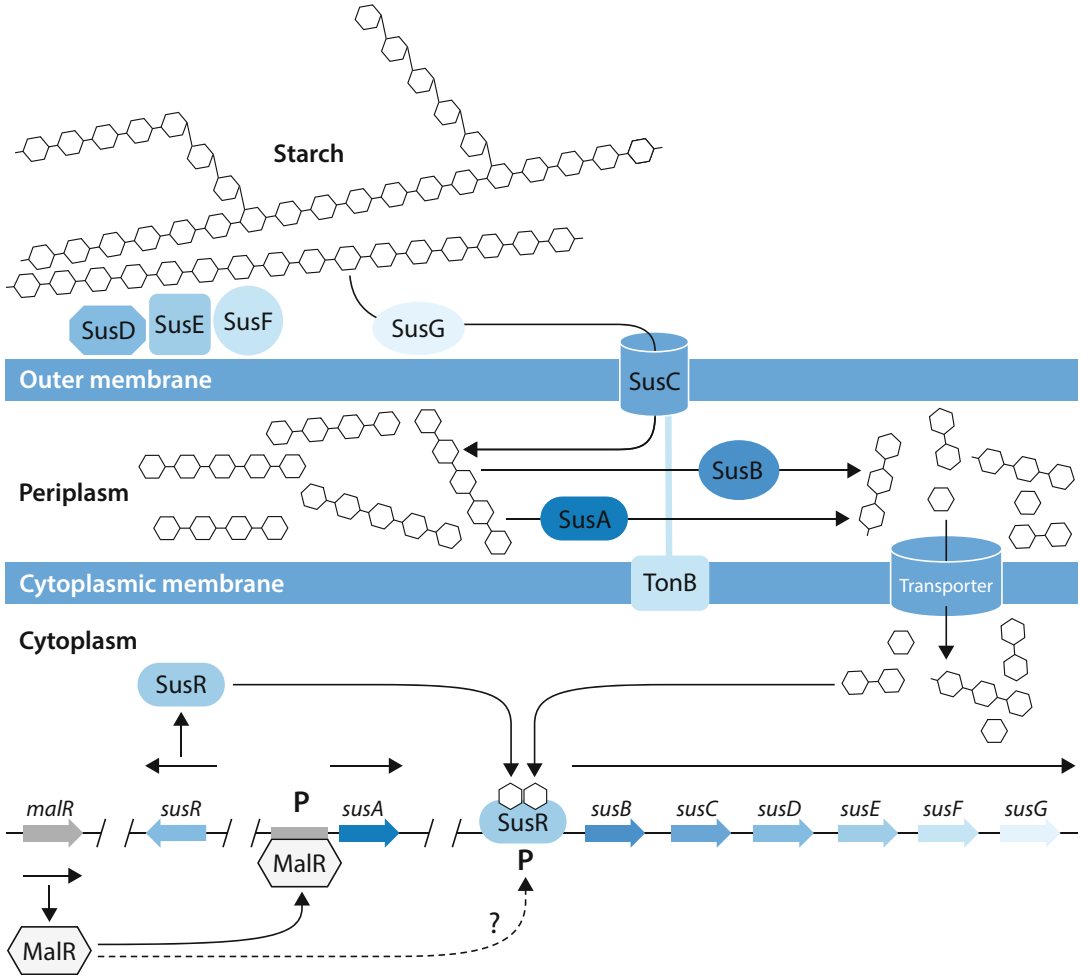

Fig. 2.3 Starch utilization by *Bacteroides thetaiotaomicron* as catalyzed by the starch utilization system (Sus). Mal refers to the maltose regulon

sufficiently small to reach the periplasm through the TonB-dependent transporter SusC located in the outer membrane. TonB is a complex in the cytoplasmic membrane of Gram-negative bacteria; it promotes the transport of various nutrients including complexed iron and cobalamin across the outer membrane (Schauer et al. 2008). Interestingly SusD is required for the binding and the subsequent uptake of starch through the TonB-dependent SusC complex. SusA and SusB, which are located in the periplasm, exhibit α-amylase and α-glucosidase activity, respectively, resulting in the release of mainly maltotriose and maltose, which are produced from the starch chunks released by SusG and transported into the periplasm. In turn, maltose and maltotriose are subsequently transported into the cytoplasm for further degradation and fermentation.

Inspection of the genome of *B. thetaiotaomicron* revealed the presence of approximately 90 *sus*-like gene loci, referred to as polysaccharide utilization loci (PULs) accounting for 18 % of this organism's genome (Martens et al. 2008; Sonnenburg et al. 2005). Approximately two thirds of these Sus-like PULs probably serve the degradation of plant-derived dietary polysaccharides, while one third plays a role in the degradation of host-derived glycans such as present in mucins and other glycoproteins. The gene clusters for proteoglycan degradation also

contain genes encoding sulfatases and esterases that catalyze the removal of the corresponding functional groups from glycans. It is important to note that *sus*-like gene loci have not only been identified in the *B. thetaiotaomicron* genome but also in genomes of other *Bacteroidetes*. *Bacteroides* genomes are enriched in genes involved in glycan utilization compared with genomes from other bacterial groups of the gut microbiota. A comparison of the genomes of *B. thetaiotaomicron, Bacteroides fragilis, Bacteroides vulgatus*, and *Parabacteroides distasonis* (previously *Bacteroides distasonis*) with those of two non-gut *Bacteroidetes* species predicted that *B. thetaiotaomicron* has the largest number of glycoside hydrolases and polysaccharide lyases for the degradation of both plant and host glycans indicating that *B. thetaiotaomicron* is capable of utilizing a wide range of glycans. Based on these findings *B. thetaiotaomicron* was designated a generalist, whereas *P. distasonis*, which has the smallest genome among these species and the smallest repertoire of genes involved in carbohydrate degradation, environmental sensing, and gene regulation, has been considered a specialist (Xu et al. 2007). Only two classes of enzymes involved in glycan degradation are more abundant in *P. distasonis* than in the three *Bacteroides* species, namely, α-amylase-like proteins, *N*-acetylhexosaminidases, and polysaccharide deacetylases. The latter two are required for the degradation of epithelial glycans, which contain *O*-acetylated carbohydrates such as sialic acid. By deacetylating such glycans, *P. distasonis* not only provides substrates for itself but also for other members of the microbiota devoid of the corresponding enzymes. This is one example of cross-feeding, which is a characteristic of cooperative links among members of microbial communities in anoxic environments. The genome of *B. vulgatus* indicates that the repertoire of glycan-degrading enzymes is intermediate between that of *P. distasonis* and *B. thetaiotaomicron*, respectively, and that it possesses the most complete set of pectin-degrading enzymes including methyl esterase, acetyl esterase, and polygalacturonase (Xu et al. 2007). These analyses show that the degradation abilities of the four representative *Bacteroidetes* species overlap to some extent but they also reveal a certain degree of specialization enabling each species to occupy an ecological niche.

One well-studied example of host glycan utilization is *B. thetaiotaomicron*'s ability to cleave off fucose residues from the ileal epithelium decorated with this carbohydrate and to utilize it as a substrate (Bry et al. 1996). Fucosylation of ileal epithelium in germ-free mice starts 17 days after birth but comes to an end at approximately 28 days of age. This fucosylation program continues or restarts only when the mice are associated with *B. thetaiotaomicron* or with a complete mouse intestinal microbiota. Colonization induces fucosyltransferases in the host, which catalyze the decoration of the epithelial surface with fucose. *B. thetaiotaomicron* mutants, in which the fucose-utilization genes have been deleted, fail to induce the fucosylation program, and are also less efficient in colonizing the mouse intestine compared with wild-type mice (Bry et al. 1996). The transcriptional regulator FucR acts as a fucose sensor. FucR binds to the promoter of the fucose-utilization genes and thereby represses their transcription (Hooper et al. 1999). If fucose is present, it binds to FucR, which leads to the release of FucR from the promoter unblocking the transcription of the fucose-utilization genes. It has been proposed that the *B. thetaiotaomicron* chromosome harbors a second locus, called *csp* (control of signal production), encoding a protein that induces fucosylation in the host. In the proposed model, expression of Csp is also regulated by FucR, which in conjunction with fucose blocks csp transcription. If fucose is absent, csp transcription is no longer blocked (Hooper et al. 1999).

Studies investigating the regulation of glycan utilization by intestinal bacteria and the consequences for their growth are scarce. However, an investigation on the utilization of fructans shed some light on the principal mechanisms (Sonnenburg et al. 2010). Genetic and functional differences among *Bacteroides* species in PULs targeting various fructans were found to predict the competitiveness of these bacteria in the intestinal tract. In *B. thetaiotaomicron*

regulation of fructan utilization involves a hybrid two-component signaling sensor that controls the expression of the corresponding gene cluster (Sonnenburg et al. 2010). The gene content of this fructan utilization locus differs among *Bacteroides* species and thereby determines the specificity and the type of fructans that can be utilized. For example, only *B. thetaiotaomicron*, which possesses an extracellular β-[2→6] endo-fructanase, is able to grow on levan.

The amount and type of carbohydrates accessible by community members have a major impact on the abundance of microbial population groups (McNulty et al. 2013). Therefore, the differences in fecal microbiota composition, in particular in the abundance of *Prevotella* and *Xylanibacter* species, observed between children from Burkina Faso and Italy, can be attributed to differences in the intake of fiber and starch (De Filippo et al. 2010). A recent mouse study suggests that certain community members may even get lost completely, if the substrates they require for growth are not available for a longer period of time, i.e., over generations (Sonnenburg et al. 2016).

2.4.4 Horizontal Gene Transfer of PULs

Several lines of evidence indicate that PULs genes can be horizontally transferred among members of the intestinal microbiota (Tasse et al. 2010) and between bacteria resident in the digestive tract and bacteria ingested with food (Hehemann et al. 2010). *Zobellia galactanivorans*, a marine member of the *Bacteroidetes*, is capable of degrading the sulfated polysaccharide porphyrin, which is present in marine red algae, and utilizing it as a growth substrate. There is evidence that the genes encoding the porphyranases, agarases, and accessory proteins required for porphyrin degradation have been transferred to the gut bacterium *Bacteroides plebeius*. Interestingly, this species was isolated exclusively from Japanese individuals (Kitahara et al. 2005) who consumed porphyrin-containing seaweed and thereby probably also ingested *B. plebeius* present on it. Intestinal microbiome analyses revealed that the genes encoding porphyranase and agarase are frequently found in Japanese subjects but not in North American individuals.

2.4.5 Degradation of Complex Carbohydrates by *Firmicutes*

Investigations into the breakdown of complex carbohydrates by intestinal bacteria have largely concentrated on *Bacteroidetes* even though it is clear that members of the *Firmicutes*, in particular *Ruminococcaceae* and *Lachnospiraceae*, also play an important role in polysaccharide degradation (Flint et al. 2012). For example, in human subjects the consumption of a diet rich in resistant starch led to an increase in the abundance of intestinal bacteria related to *Ruminococcus bromii* (Abell et al. 2008). *R. bromii* not only outperformed *B. thetaiotaomicron* in the degradation of resistant starch but also promoted its utilization by other starch-degrading bacteria including *Eubacterium rectale*, *Bifidobacterium adolescentis*, and *B. thetaiotaomicron* (Ze et al. 2012). Therefore, bacteria related to *R. bromii* have been proposed to play a critical role in the initial steps of resistant starch degradation. However, amylases have also been identified in *Roseburia inulinivorans* and other *Roseburia* species (Flint et al. 2012). While *Bacteroides* species employ several proteins anchored in the outer membrane to capture and cleave starch, amylolytic Gram-positive gut bacteria such as *R. inulinivorans* take advantage of amylases that are bound to the bacterial cell wall. In conjunction with a variable number of carbohydrate-binding modules on the cell surface, they effectively bind and cleave starch (Ramsay et al. 2006). Interestingly, starch also induces the formation of flagella in *R. inulinivorans*, which possibly help the organism to reach the substrates (Scott et al. 2011). Nine to 13 putative glycohydrolase genes were identified in the genomes of *Roseburia* and *E. rectale*, but their exact roles are not yet clear (Ze et al. 2012). A considerable number of intestinal *Firmicutes* play a role in the degradation of complex carbohydrates of plant origin. For

example, human strains of *Ruminococcus albus*, *Roseburia intestinalis*, and *Faecalibacterium prausnitzii* utilize galactomannan, xylan, and pectin, respectively (Chassard et al. 2007; Lopez-Siles et al. 2012; Salyers et al. 1977b). A β-fructofuranosidase in *R. inulinivorans* catalyzes the depolymerization of fructans of different chain lengths. This activity is linked to an ATP-dependent sugar carrier, and expression of the corresponding genes was increased in the presence of inulin. Various mucin degraders including *Ruminococcus torques* have also been identified among the *Firmicutes* (Hoskins 1993). It may be concluded that *Firmicutes* play an important role in the degradation of both plant and host glycans, but in comparison with glycan-degrading *Bacteroidetes*, knowledge on mechanistic details is relatively limited.

2.4.6 Formation of Short-Chain Fatty Acids by Bacterial Fermentation in the Colon

The depolymerization may be considered the first step in the utilization of glycans (Fig. 2.2). Further steps include the fermentation of the cleavage products, i.e., monomeric and oligomeric saccharides. To some extent they become available to bacteria lacking enzymes for the breakdown of complex polysaccharides (cross-feeding). Bacterial population groups in the gut differ in the pathways they employ for the fermentation of these saccharides and in the respective spectrum of fermentation products. In the overall fermentation process in the colonic ecosystem, lactate, succinate, and ethanol merely represent intermediates (Fig. 2.2), which are converted further by other bacterial taxa. These activities give rise to the major end products of bacterial fermentation in the colon, namely, the short-chain fatty acids (SCFA) acetate, propionate, and butyrate, which are formed at an approximate molar ratio of 60:23:17. Total SCFA concentrations in the colon are in the range of 90–120 mM (Cummings et al. 1987). However, both the molar ratios and the concentrations of colonic SCFA are highly variable and depend on the type and amount of dietary fiber ingested. The majority (95%) of the SCFA formed by the colonic microbiota becomes absorbed (Topping and Clifton 2001). Following their absorption, acetate and butyrate may become oxidized in body tissues providing energy to the host. SCFA, preferentially butyrate, provide up to 70% of the energy required by colonic epithelial cells (Roediger 1980). Propionate may serve as a gluconeogenic substrate in the liver and acetate as a substrate for lipogenesis (Cummings 1995). However, SCFA also play various regulatory roles. For example, by inhibiting histone deacetylase, butyrate influences gene expression, which in colon cancer cells results in cell cycle arrest and activates apoptosis (Lazarova et al. 2013). Thus, SCFA play an important role in maintaining homeostasis of the colonic mucosa. SCFA have also been recognized as ligands of the G-protein-coupled receptors FFAR2 (free fatty acid receptor 2) and FFAR3, earlier referred to as GPR43 and GPR41 (Brown et al. 2003), which are expressed in ileal and colonic enteroendocrine L cells, adipocytes, and immune cells. FFAR2 activation triggers the release of leptin from adipocytes (Xiong et al. 2004) and the excretion of peptide YY (Tazoe et al. 2008) and glucagon-like peptide from enteroendocrine cells (Tolhurst et al. 2012). Since these molecules reduce appetite (Wren and Bloom 2007), they have been proposed to play a role in the control of appetite regulation. However, recent animal studies cast some doubts on such a role of intestinal FFAR (Lin et al. 2012; Tang et al. 2015).

2.4.7 Bacteria Involved in SCFA Formation

The majority of intestinal bacteria produce acetate, some in larger and others in smaller quantities. Homofermentative and heterofermentative lactic acid bacteria produce no or only small amounts of acetate. The third major group of lactate-producing intestinal bacteria, bifidobacteria, produces considerable amounts of acetate in addition to lactate (2 glucose → 2

lactate + 3 acetate). Major propionate producers include various *Bacteroides* species, *Veillonella parvula*, *Dialister succinatiphilus*, *Phascolarctobacterium succinatutens*, *Selenomonas ruminantium*, and *Megasphaera elsdenii*, many of which also produce succinate as a by-product or an intermediate that can be taken up again to be converted to propionate. Three different propionate formation pathways have been identified: the methylmalonyl-CoA pathway, the acrylate pathway, and the propanediol pathway. Primers targeting genes characteristic of either pathway were used to test the presence of the corresponding genes in representative human gut species (Reichardt et al. 2014). The majority of bacterial species were found to use the methylmalonyl-CoA pathway.

Butyrate-producing human fecal bacteria capable of utilizing lactate include strains related to *Eubacterium hallii*, *Anaerostipes caccae*, and distant relatives of *Clostridium indolis* (Duncan et al. 2004). Butyrate-forming human colonic bacteria include *R. intestinalis*, *E. rectale*, and *F. prausnitzii*, all of which convert glucose but not lactate to butyrate (Duncan et al. 2002).

Lactate is a major source for both propionate and butyrate. Some organisms are capable of producing both butyrate and propionate depending on the substrate. In the presence of acetate, *Coprococcus catus* converts lactate mainly to propionate; in contrast, mainly butyrate is formed from fructose with net consumption of acetate (Reichardt et al. 2014). Experiments in fecal slurries moreover suggest that the pH is a major factor influencing the conversion of lactate in the ecosystem and the propionate/butyrate ratio (Belenguer et al. 2007).

2.4.8 Utilization of Hydrogen and Formate

In addition to SCFA, the gut microbiota produces formic acid and the gases H_2 and CO_2 (Fig. 2.2), which are partly excreted and partly utilized. H_2 is produced by various bacterial population groups in the colon. For example, *Enterobacteriaceae* such as *E. coli* produce H_2 and CO_2 from formate catalyzed by formate-hydrogen lyase, while strict anaerobes such as *Clostridium* species and other *Firmicutes* may release H_2 in the course of pyruvate oxidation as catalyzed by pyruvate: ferredoxin oxidoreductase. The reduced ferredoxin produced in this reaction is used by hydrogenase for the reduction of two protons to produce H_2. The latter and formate play a role in methanogenesis and acetogenesis. Approximately 50% of humans excrete methane. The intestinal archaeon *Methanobrevibacter smithii* produces CH_4 from H_2 and CO_2 ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$) or formate ($4HCOOH \rightarrow 3CO_2 + CH_4 + 2H_2O$). Homoacetogenic bacteria such as *Blautia hydrogenotrophica* or *Blautia producta* may also take advantage of H_2 and CO_2 and/or formate (Bernalier et al. 1996; Liu et al. 2008). Using the Wood-Ljungdahl pathway, these bacteria catalyze the formation of acetate ($2CO_2 + 4H_2 \rightarrow CH_3–COOH + 2H_2O$ or $4HCOOH \rightarrow CH_3–COOH + 2CO_2 + 2H_2O$) (Ragsdale 2006). H_2 is an important product of anaerobic fermentation as it enables anaerobes to reoxidize electron carriers without the need to use intermediates such as pyruvate as electron acceptors, enabling a higher ATP gain per hexose metabolized compared to other fermentations.

2.4.9 Utilization of Proteins by Intestinal Bacteria

Based on measurements of nitrogen digestibility in ileostomized patients, it has been estimated that 5–10% of the ingested dietary protein reaches the colon (Darragh and Hodgkinson 2000). Endogenous proteins, in particular digestive host enzymes, are an additional source of protein for bacterial fermentation in the colon. The proteolytic activity of host proteases decreases from proximal to distal colon indicating that they become inactivated due to bacterial proteolysis (Gibson et al. 1989). Depending on diet, the total daily amount of protein entering the colon has been estimated to vary between 6 and 18 g (Cummings and Macfarlane 1991; Yao et al. 2016). The majority of colonic bacteria prefer

carbohydrates over proteins for energy generation, but many species are also capable of utilizing proteins, peptides, and amino acids, alternatively or simultaneously. Protein can be used for energy generation and for biosynthetic purposes as they deliver both carbon and nitrogen for microbial growth. Protein degradation in the colon occurs in several steps and involves different bacteria. The first step in protein utilization is proteolysis, which results in the release of peptides and amino acids (Fig. 2.4). Strains of *B. fragilis* and *B. vulgatus* use cell-bound proteases for the cleavage of proteins, while strains of the genera *Clostridium*, *Propionibacterium*, and *Bacillus* take advantage of extracellular proteases. Fecal *Streptococcus* and *Staphylococcus* isolates possess both forms of proteases (Macfarlane et al. 1986). The majority of intestinal bacteria prefer ammonia over amino acids as a source of nitrogen. However, organisms such as bifidobacteria and clostridia, which utilize oligopeptides for anabolic purposes, retain only certain amino acids of absorbed peptides and excrete the remaining ones (Hespell and Smith 1983).

The pathways used by colonic bacteria for amino acid fermentation in the colon may differ among bacterial species. However, the initial steps of bacterial amino acid breakdown are restricted to only a few: oxidative deamination, reductive deamination, simple deamination, and decarboxylation resulting in α-oxo-acids, carboxylic acids, enoates, and (poly)amines, respectively (Fig. 2.4). Intestinal bacteria such as *Clostridium sticklandii* utilize pairs of different amino acids: one of the amino acids is subjected to oxidative deamination, while the other one undergoes reductive deamination, a process referred to as Stickland reaction. As a consequence of the release of ammonia, the luminal pH increases from proximal to distal colon indicating protein becoming more important as an energy source as carbohydrates become increasingly exhausted (Macfarlane et al. 1992). Amino acid fermentation results in the formation of SCFA, formate, H_2,

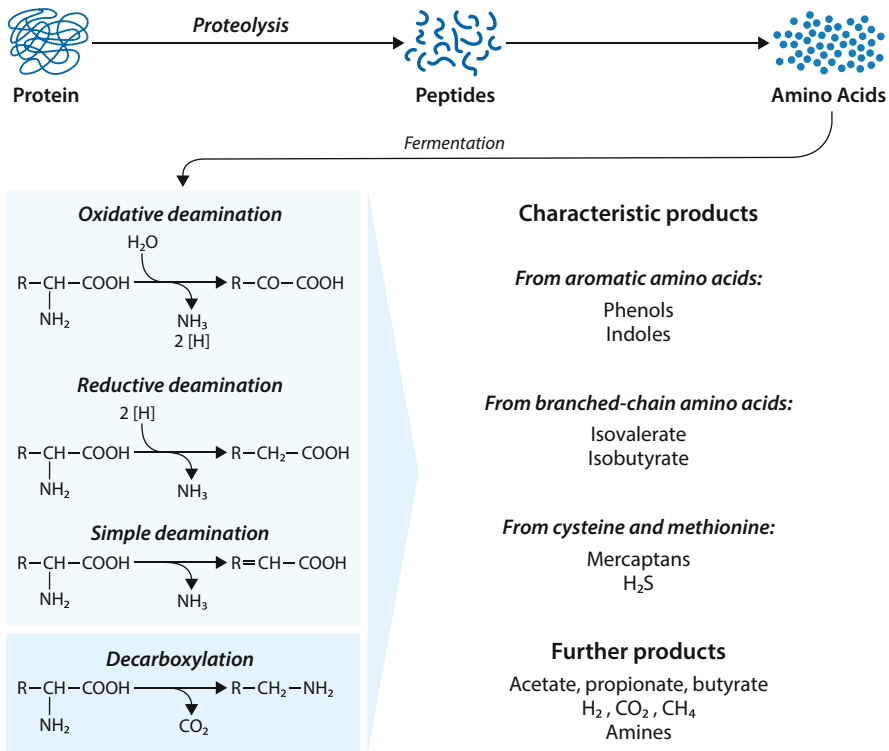

Fig. 2.4 Bacterial proteolysis in the large intestine

and CO_2 with the latter three serving as potential substrates for methanogenesis and homoacetogenesis, i.e., the same products also formed from carbohydrates. Products characteristic of amino acid fermentation include branched-chain fatty acids such as isovalerate and isobutyrate, which are formed from branched-chain amino acids; phenols and indoles, which stem from the fermentation of the aromatic amino acids tyrosine, phenylalanine, and tryptophan; as well as amines. Colonic fermentation of the sulfur-containing amino acids cysteine and methionine gives rise to hydrogen sulfide (H_2S) and mercaptans. A high protein intake is accompanied by increased bacterial sulfide generation in the human colon (Magee et al. 2000).

2.4.10 Conversion of Bile Acids

Even though bile acids are not a major source of energy for the gut microbiota, they may suppress the growth of bacteria sensitive to these detergents. They play a role in fat digestion and are ligands for the farnesoid X receptor (FXR), the liver X receptor (LXR), and the G-protein-coupled receptor TGR5; and they undergo conversion by the gut microbiota (Jones et al. 2008). They are synthesized in the liver from cholesterol, conjugated with glycine or taurine, and subsequently stored in the gall bladder. When required, they are excreted into the gut to solubilize dietary fat and support the formation of micelles. Many intestinal bacteria are able to deconjugate the primary bile acids to the corresponding unconjugated forms. Many intestinal bacteria including *Clostridium perfringens, Lactobacillus plantarum, Lactobacillus johnsonii, Bifidobacterium bifidum*, *B. longum*, and *B. adolescentis* harbor bile salt hydrolase genes (Ridlon et al. 2006). Metagenomic analysis revealed that bile salt hydrolases are enriched in the human gut microbiome and that they are present in all major bacterial divisions as well as in the archaeal methanogens *M. smithii* and *M. stadtmanae*, suggesting that this activity is relevant to survival in the mammalian gastrointestinal tract (Jones et al. 2008). The unconjugated bile acids may be further converted by bile acid dehydroxylases and hydroxysteroid dehydrogenases. Intestinal bacteria harboring enzymes involved in bile acid conversion include *E. lenta*, *C. perfringens*, *B. producta*, *B. fragilis*, *B. thetaiotaomicron*, *E. coli*, *Clostridium absonum*, *Clostridium sordellii*, *Clostridium innocuum*, *Clostridium scindens*, *Clostridium hylemonae*, *Clostridium bifermentans*, *Clostridium limosum*, *Clostridium leptum*, and *Clostridium paraputrificum* (Ridlon et al. 2006). A recent study revealed intestinal bacteria such as *Ruminococcus gnavus* favor the growth of *Bacteroides* spp. owing to their ability to detoxify deoxycholic acid by converting it to the corresponding 3-β-hydroxy bile acid epimer isodeoxycholic acid (Devlin and Fischbach 2015). Dehydroxylation of bile acids, which occurs in a position-specific and stereo-selective way, was studied in *Clostridium scindens* in detail. The eight genes required for bile acid dehydroxylation are organized in the *bai* (bile acid-inducible) operon (*baiBCDEAFGHI*) encoding the 27 kDa 3α-hydroxysteroid dehydrogenase (BaiA), the 58 kDa bile acid CoA ligase (BaiB), the 70 kDa 3-dehydro-4-chenodeoxycholic acid/cholic acid steroid oxidoreductase (BaiCD), the 72 kDa 3-dehydro-4-ursodeoxycholic acid/7-epi cholic acid steroid oxidoreductase (BaiH), the 19.5 kDa 7α-dehydratase (BaiE), a hypothetical 22 kDa 7-β-dehydratase (BaiI), the 47.5 kDa bile acid CoA hydrolase and a hypothetical bile acid CoA transferase (BaiF), and the 50 kDa transmembrane protein (BaiG), which catalyzes H^+-dependent bile acid transport (Ridlon et al. 2006). It is important to note that none of the bacterial enzymes acting on the bile acids cleaves the steroid ring structure.

Potential benefits for intestinal bacteria may arise from the utilization of the glycine or taurine moieties of bile acids as carbon or nitrogen source. Utilization of taurine by *B. longum* as a nitrogen source is in accordance with the finding that the bile salt hydrolase gene is co-transcribed with the glutamine synthetase adenylyltransferase gene (*glnE*), which is part of the nitrogen regulation cascade (Tanaka et al. 2000). There is evidence that taurine stimulates growth of the colitogenic *B. wadsworthia* by providing the electron acceptor for sulfite reduction (see Sect. 2.3.2).

2.4.11 Conversion of Secondary Plant Metabolites

In addition to carbohydrates and protein, diet may contain non-nutritive secondary plant metabolites such as polyphenols, which are found in grains, fruits, and vegetables. Polyphenols such as lignans and flavonoids have been reported to exert beneficial health effects. Therefore, their uptake, bioavailability, and biological activities in humans have been studied (Clavel et al. 2006b; Hollman and Katan 1999). The chemical structure of polyphenols and the composition of the intestinal microbiota affect the fate of these compounds in the digestive tract. Polyphenols are usually glycosylated, and, depending on the extent of absorption, they pass into the colon where they undergo conversion by intestinal bacteria. For example, intestinal bacteria convert the lignan secoisolariciresinol diglucoside (SDG) to enterolactone in several steps (Axelson et al. 1982). Various *Bacteroides* and *Clostridium* spp. are capable of deglycosylating SDG, but *Clostridium saccharogumia* turned out to be the most effective species of the strains tested (Clavel et al. 2006a, 2007). *Butyribacterium methylotrophicum*, *B. producta*, *Eubacterium callanderi*, and *E. limosum* are capable of catalyzing the second step, namely, the *O*-demethylation of matairesinol, whereas *C. scindens* and *E. lenta* dehydroxlyate the *O*-demethylated matairesinol to enterodiol (third step). The last step in this pathway, the conversion of enterodiol to enterolactone, is catalyzed by *Lactonifactor longoviformis* (Clavel et al. 2007). A defined consortium of four species, each catalyzing one of the four reactions, converts SDG to enterodiol and enterolactone. Gnotobiotic rats associated with this community excreted the two metabolites in urine and feces when fed a flaxseed diet, which is rich in SDG (Woting et al. 2010). The ability of humans to convert SDG to enterodiol and enterolactone is widely distributed among humans with women tending to harbor higher concentrations of enterolactone-producing intestinal bacteria (Clavel et al. 2005).

Isoflavones represent a subgroup of flavonoids, which like the lignans have been implicated in preventive effects against hormone-related cancers and cardiovascular disease as well as in alleviating menopausal symptoms. These effects have mainly been attributed to one of its bacterial transformation products, namely, equol, which undergoes urinary excretion (Setchell and Clerici 2010). Isoflavones mainly occur in their glycosylated form. Interestingly, some intestinal bacteria, e.g., the *Lachnospiraceae* strain CG19-1 and *Eubacterium cellulosolvens*, are capable of cleaving the more stable *C*-glycosides in addition to the more common *O*-glycosides (Braune and Blaut 2012; Braune et al. 2016). Daidzein and genistein are major isoflavones present in soy. They may undergo metabolization by intestinal bacteria such as *Adlercreutzia equolifaciens* and *Slackia isoflavoniconvertens*, which have been identified as equol formers (Maruo et al. 2008; Matthies et al. 2009). In *S. isoflavoniconvertens,* daidzein induces the expression of eight genes involved in its conversion, three of which were found to encode daidzein reductase, dihydrodaidzein reductase, and tetrahydrodaidzein reductase, respectively (Schroder et al. 2013). Heterologous expression of the latter two resulted in the reduction of dihydrodaidzein to equol. In the meantime, quite a number of intestinal bacteria metabolizing flavonoids including isoflavones and enzymes involved have been identified (Braune and Blaut 2016).

Following the consumption of soy, 33–50% of healthy subjects excreted equol, while 80–90% excreted the biologically less active *O*-desmethylangolensin, in addition or alternatively (Atkinson et al. 2004). One organism shown to convert daidzein to *O*-desmethylangolensin is *Eubacterium ramulus* (Schoefer et al. 2002). Which pathway dominates depends on the composition of the gut microbiota. These examples highlight the fact that intestinal bacteria convert a wide range of non-nutritive metabolites and that there may be alternative conversion pathways resulting in several intermediates and end products.

2.4.12 Core and Variable Microbiome and/or Microbiota

The intestinal tract environment favors bacteria that have the capacity to grow therein. Since bacteria in the gut share the same environment, it can be surmised that they have certain gene functions in common in addition to the so-called housekeeping functions required by all living cells. Therefore, it is not surprising that the microbiomes from different individuals share a high proportion of gene functions, including genes that encode enzymes required to degrade dietary fiber and host glycoproteins. These have been referred to as core microbiome because they represent metabolic activities that are found in every subject, while others are only present in some individuals but absent from others (Turnbaugh et al. 2009). The latter category, which has been termed the variable microbiome, includes methanogenesis, oxalate degradation, conversion of isoflavones, and the utilization of porphyrin from marine red algae (see Sect. 2.4.5) (Atkinson et al. 2004; Hehemann et al. 2010; Kumar et al. 2002; Wolin and Miller 1983). The idea of a core microbiome, which encompasses key functions and is shared by each individual, is a useful concept as it reflects distinct environmental influences in a given habitat. However, the value of defining a core microbiota (not microbiome!), which encompasses key species shared among humans, is questionable because their relative abundance is highly variable (Turnbaugh et al. 2009).

2.5 Conclusions

The microbial communities inhabiting the human intestinal tract play a major role in the breakdown of dietary components that cannot be utilized by the host and in the conversion of host metabolites. By expanding the metabolic capacity of the host and interacting with the host immune system, the gut microbiota profoundly affects host physiology. Even though metagenomics, transcriptomics, and proteomics have increased our knowledge about important functions of the intestinal microbiota, there still is a gap in our understanding of the exact molecular mechanisms underlying microbe-microbe or host-microbe interactions. Therefore, identifying the exact role of members of the gut microbiota and their competitive or cooperative links is of major importance. Considerable differences in microbiota composition among human individuals and populations impede the elucidation of the mechanisms underlying the role of intestinal bacteria in various diseases. Moreover, it often is not possible to find out whether disease-related changes in the gut microbiota are cause or consequence of the disease. We should strive to identify microorganisms that play critical roles in physiological and pathophysiological processes, with the ultimate goal to identify the bacterial molecules involved and their targets in the host.

Controversy

Analysis of human fecal samples revealed differences in the relative abundance of key taxa. Three robust patterns or clusters, referred to as enterotypes, were identified (Arumugam et al. 2011). The three enterotypes are characterized by differences in the relative abundance of *Bacteroides*, *Prevotella*, or *Ruminococcus*. While the enterotypes did not correlate with gender, age, or body weight, long-term dietary habits were reported to influence the enterotype (Wu et al. 2011). The underlying concept was subsequently extended to other mammalian hosts including the mouse (Wang et al. 2014). Quite a number of scientists have found the enterotype concept appealing and conducted similar analyses. Several studies confirmed that the fecal microbiota of human subjects can be categorized into two of the three proposed enterotypes, namely, *Bacteroides* and *Prevotella*, while the *Ruminococcus* enterotype was usually not found. In a Korean study, 72% of the fecal samples collected from Korean monozygotic twin pairs belonged to the same enterotype and 2 years later the affiliation with either enterotype was still the same for 80% of the individuals (Lim et al. 2014). However, this also means that 20% of the subjects changed from one enterotype to the

other during this time. Interestingly, a more recent study in Taiwanese adults, which was based on the analysis of 181 fecal samples, identified *Escherichia* rather than *Ruminococcus* as a representative genus of a third enterotype (Liang et al. 2017).

A recent in vitro study revealed that the fermentation of various fermentable polysaccharides is determined by the enterotype of the fecal donor (Chen et al. 2017). The inoculum with a dominance of *Prevotella* versus *Bacteroides* was dominated by fiber-fermenting bacteria. In agreement with this observation in children in Burkina Faso, who consumed a diet rich in fermentable fiber, *Prevotella* accounted for 53% of intestinal bacteria but were absent in age-matched Italian children (De Filippo et al. 2010). In spite of these interesting observations, the value of the enterotype concept has been challenged for several reasons (Knights et al. 2014): Dominant genera including *Ruminococcus* and *Bacteroides* are highly variable among individuals belonging to the same enterotype. Available datasets propose continuous abundance in gradients rather than discrete clusters despite the fact that the absence of *Prevotella* as observed in Italian children (De Filippo et al. 2010) inevitably results in discrete clustering. Most importantly, the affiliation of human subjects with a given enterotype may vary over time arguing against the notion that enterotypes are discrete states (Knights et al. 2014). Indeed, a large cohort study revealed that the microbiota profile of the majority of the study subjects corresponded to one of the enterotypes, while others had intermediate profiles, impeding a clear assignment to an enterotype (Huse et al. 2012). Based on these findings, it may be concluded that the enterotype concept, however appealing it may appear, does not really promote a better understanding of the gut microbiome.

History

The ubiquitous existence of microorganisms only became evident with the invention of the light microscope by Antonie van Leeuwenhoek (1632–1723) and the studies of Louis Pasteur (1822–1895) and Robert Koch (1843–1910), which revealed that bacteria catalyze reactions and may cause infections. Theodor Escherich (1857–1911) was one of the first researchers, who became interested in the role of intestinal bacteria in the digestive tract, in particular of infants. He isolated a fecal bacterium that later on was named after him, namely, *Escherichia coli*. For a long period of time, bacteria have primarily been perceived as culprits even though most bacteria known to date are nonpathogenic. This might explain that the intestinal fermentation was considered a detrimental process. The British surgeon William Arbuthnot-Lane (1856–1943) removed the colon from some of his patients because he assumed that the colonic fermentation led to an "autointoxication." For a long time, the investigation of the intestinal microbiota was impeded, because adequate methods for handling strict anaerobes were not yet available. So the exploration of the ecosystem only started after pioneers such as Robert E. Hungate (1906–2004) and Sydney M. Finegold (born 1921) developed methods for the isolation and handling of strict anaerobes (Hungate 1969; Sugihara et al. 1974). These early researchers and others laid the foundation for the field. They isolated and described a considerable number of bacterial species and tried for the first time to link the gut microbiota to health and disease (Finegold et al. 1975). The development of cultivation-independent methods (Amann et al. 1995) facilitated and accelerated the characterization of various microbial habitats, including that of human fecal samples (Suau et al. 1999). Steady improvements in sequencing methods

and simultaneously decreasing costs have made metagenome sequencing a readily available tool, enabling researchers to assess all microbial gene sequences in an ecosystem and, in conjunction with transcriptomics and metabolomics, to characterize the metabolic potential of intestinal microbial communities (Dumas et al. 2006; Gill et al. 2006; Jiang et al. 2016). However, the prediction of gene functions depends on the correct annotation, which in turn is largely based on work of scientists who previously isolated bacteria and characterized their enzymes and genes. Isolation of new community members and identification of new gene functions can be tedious and usually receive little appreciation by the scientific community. This may be the reason that this important work is presently neglected even though a large proportion of gene functions have not yet been identified and gene functions predicted and annotated based on sequence similarity have not been experimentally verified. Therefore, it is still necessary to isolate as many bacteria as possible and to study their genes and enzymes.

Highlights

- The digestive tract of human and animals is colonized by microbial communities encompassing bacteria, archaea, and fungi, referred to as gastrointestinal microbiota.
- The gastrointestinal microbiota has coevolved with the host, and its members are well adapted to the different sections of the digestive tract, which differ in the physicochemical conditions and the availability of substrates. The composition of the intestinal microbiota at species level is highly variable among humans.
- The majority of intestinal microorganisms are strictly anaerobic bacteria, which gain energy by fermenting dietary fiber and endogenous substrates mainly to short-chain fatty acids, carbon dioxide, molecular hydrogen, and methane.
- The collective genome (metagenome) of all members of the intestinal microbiota represents the intestinal microbiome which encodes the functions of all community members. While a large proportion of functions are shared among the microbiomes of human individuals, some activities are only observed in certain human populations.
- Bacterial groups in the intestinal tract interact with each other and with the host. The majority of interactions are of mutualistic or commensal character. However, the mechanisms underlying such interactions are incompletely understood.

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