1 The Gut Microbiota

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Abstract
The most densely populated microbial ecosystem that colonizes the human body is located in the gut and is commonly referred to as gut microbiota. This microbial community encompasses trillions of bacteria with an estimated biomass of 1.5 kg, a size that is similar to the liver, the largest organ in the body. It is tempting to consider the gut microbiota as an organ itself, composed of 1,000–1,200 cell types (species) that encode 150-fold more genes (microbiome) than we have in our own genome. The gut microbiota is highly dynamic and exhibits temporal (age) and spatial (along and across the length of the gut) variations. Furthermore, the intestinal microbial composition is responsive to host genetics, diet, ingested drugs, and a wide number of other environmental factors. The gut microbiota plays a fundamental role in human health, as it evolved specific functions that complement human metabolism and physiology. As an example, intestinal bacteria exhibit specific functions involved in fermentation of polysaccharides to bioavailable nutrients that may also act as signaling component. Moreover, intestinal bacteria take part in vitamin production, regulation of hormone synthesis, and maturation of the immune system. Hence, dysbiosis of the gut microbiota has been implicated in many human diseases such as inflammatory bowel disease, obesity, diabetes, and celiac disease.

Introduction

Microbes are the most abundant life form on Earth, and we are accordingly adapted to life in a microbial environment (Whitman et al. 1998). Evidence for the presence of microorganisms associated with the human body was provided at the end of the seventeenth century when Antonie van Leeuwenhoek observed that microorganisms, which he called "animalcules" (tiny animals), were associated with the mouth and feces of individuals in health and disease. While it is now well recognized that the human body provides many niches for a vast number of microbes, the importance of Leeuwenhoek's work remained unappreciated for a long time. The microorganisms, regularly found to colonize the different surface of the host, including skin, oral cavity, respiratory tract, urogenital, and gastrointestinal tract, and that peacefully coexist with their host, represent the so-called normal microbiota or microflora and their collective genomes – the gut microbiome, which is the major part of the metagenome (microbiome plus the human genome) (Dethlefsen et al. 2006). The largest collection of microorganisms is located in the gastrointestinal (GI) tract, which provides several functions for the host, including developmental, immunological, physiological, and nutritional functions, which may affect our life in health and disease (Drasar and Hill 1974; Guarner and Malagelada 2003; Nicholson et al. 2005).

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Microbial Diversity in the GI Tract

The human gut comprises members of the three domains of life on Earth – Bacteria, Archaea, and Eukarya (Finegold et al. 1983). Bacteria dominate this ecosystem where more than 90 % of the phylotypes are members of two of so far ten identified bacteria phyla (*Table 1.1*): the Bacteroidetes and the Firmicutes (Backhed et al. 2005; Turroni et al. 2008; Zoetendal et al. 2006). The Gram-positive Firmicutes include numerous different phylogenetic clusters of clostridia, with clusters IV (also known as *Clostridium leptum* group), IX, and XIVa (also referred to as *Clostridium coccoides* group) being the most abundant clusters (Collins et al. 1994). The predominant genera in these clusters are Clostridium, Eubacterium, Roseburia, and Ruminococcus. Two important groups of butyrate-producing bacteria are *Eubacterium rectale* and *Roseburia* species (members of Clostridium cluster XIVa), comprising 5–10 % of the total microbiota (Aminov et al. 2006; Scott et al. 2008), and *Faecalibacterium prausnitzii* (Clostridium cluster IV), comprising 5–15 % of the total microbiota (Eckburg et al. 2005; Hold et al. 2002; Scott et al. 2008). The Gram-negative genera Bacteroides and Prevotella represent the most well studied from the Bacteroidetes division. Furthermore, Actinobacteria, including the genera Bifidobacterium, Collinsella, and Atopobium, detected at high GC-content (guanine-cytosine content) Gram-positive bacteria, represent important members of the gut microbial community (Franks et al. 1998; Harmsen et al. 2002; Turroni et al. 2008; van der Waaij et al. 2005). Other members of the human gut microbiota, not recognized as dominant, are distributed between seven phyla – Cyanobacteria, Fusobacteria, Lentisphaerae, Proteobacteria, Spirochaetes, TM7, and Verrucomicrobia. The Verrucomicrobia were recently discovered and consist of a single species *Akkermansia muciniphila*, specialized in mucus degradation (Derrien et al. 2004). TM7 is also a newly identified phylum that is widely distributed in the environment and contained so far only uncultured bacteria (Hugenholtz et al. 2001). *Methanobrevibacter smithii* and *Methanobrevibacter stadtmanae* are the two methanogens that represent the Archaea domain in the gut microbial community and are highly prevalent in the GI tract of healthy individuals (Gill et al. 2006; Salonen et al. 2010).

Recent studies of the gut microbial ecosystem identified more than 1,000 species and possibly over 7,000 strains, of which the largest part (~80 %) remains uncultured (Backhed et al. 2005; Blaut and Clavel 2007; Rajilic-Stojanovic et al. 2007; Zoetendal et al. 2008). However, new approaches for culturing previously uncultured colonic microbes are being developed (Duncan et al. 2007; Ingham et al. 2007; Zoetendal et al. 2008). In addition, powerful tools for high-throughput sequencing of genomic DNA from minute quantities of sample and pyrosequencing of amplified microbial genes are providing new insights in the composition of the gut microbiota at high spatiotemporal resolution (Andersson et al. 2008; Marcy et al. 2007).

Host Factors That Affect the Distribution of the Gut Microbiota in the GI Tract

The mammalian GI tract is a compartmentalized system that consists of several distinct anatomical regions, ranging from the stomach to the rectum. Each of these anatomical sections is characterized by varying physicochemical features, such as transit rates of the luminal content, local pH, redox potential, availability of diet-derived compounds, and host secretions (e.g., hydrochloric acid, digestive enzymes, bile, and mucus). Hence, the composition and the abundance of the intestinal microbiota also vary throughout the different regions of the gut (*Fig. 1.1*). The upper GI tract consists of stomach, duodenum, and jejunum and contains a sparse microbiota, which concentration is less than 10^4 organisms per ml of digesta. The relatively low abundant endogenous microbial populations residing in this part of GI tract are affected firstly by the acid stress in the stomach and subsequently by bile acids and pancreatic enzymes released in the duodenum. In addition, the microbial colonization in this part of the small intestine is also impeded by the fast flow of food that causes a rapid wash out of the microbes. The microbial concentration increases toward the end of the small intestine and reaches densities of 10^7–10^8 bacterial cells per gram. The largest microbial concentration is located at the distal part of the GI tract where it reaches concentrations of 10^{12} bacteria per gram of stool, which likely is caused by reduced transit times and increased nutrient availability (*Fig. 1.1*). Thus, the gut microbiota of a single individual outnumbers the total human population of the world by a factor of 1,000 (Moore and Holdeman 1974).

Different microbial populations have been associated not only with the different anatomical regions of the GI tract but also with the latitudinal anatomical sites of the gut. The intestinal lumen forms a continuum with the external environment and is separated from the internal body environment by a single layer of intestinal cells, termed as epithelial surface, which is covered by a mucus layer. The composition of the microbiota associated with the mucus and epithelial crypts significantly differs from that present in the luminal content and the feces (Eckburg et al. 2005; Frank et al. 2007) (*Fig. 1.1*).

Establishment of Gut Microbiota: Succession and Colonization of the Infant GI Tract

The colonization of the intestinal lumen begins at birth when the sterile environment of the infant gut first is colonized by a simple microbial community which develops into a climax community at 2 years of life. The development of the newborn gut microbiota is a gradual and dynamic process that is determined by several factors such as mode of delivery, prematurity, maternal microbiota, type of feeding, illness and antibiotic therapy, and environmental hygiene (Wall et al. 2009). The initial colonization is a consequence of the contact and interaction with both the maternal vaginal and fecal microbes and the surrounding environment (Dominguez-Bello et al. 2010). Eventually, the

Table 1.1

Phylogenetic distribution of the human gastrointestinal microbial phylotypes

Phylum	Class	Order	Family/cluster
			Actinomycetaceae
		Actinomycetales	Corynebacteriaceae
Actinobacteria	Actinobacteria		Micrococcaceae
			Propionibacteriaceae
		Bifidobacteriales	Bifidobacterium
		Coriobacteriales	Corynebacteriaceae
			Rekenellaceae
			Bacteroidaceae
Bacteroidetes	Bacteroidetes	Bacteroidales	Prevotellaceae
			Porphyromonadaceae
			Unclassified
Cyanobacteria	Cyanobacteria	Chroococcales	Unclassified
	Asteroleplasma	Asteroleplasmatales	Asteroleplasmataceae
			Bacillaceae
	Bacilli	Bacillales	Staphylococcaceae
			Aerococcaceae
		Lactobacillales	Carnobacteriaceae
			Lactobacillaceae
			Leuconostocaceae
Firmicutes			Lactococcaceae
			Streptococcaceae
			Clostridium cluster I
			Clostridium cluster III
			Clostridium cluster IV
	Clostridia	Clostridiales	Clostridium cluster IX
			Clostridium cluster XI
			Clostridium cluster XIII
			Clostridium cluster XIVa
			Clostridium cluster XV
			Unclassified
			Clostridium cluster XVI
			Clostridium cluster XVII
	Mollicutes	Unclassified	Clostridium cluster XVIII
Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae
Lentisphaerae	Lentisphaerae	Lentisphaerae	Lentisphaeraceae
		Rhizobiales	Unclassified
	α-Proteobacteria	Sphingomonadales	Unclassified
		Unclassified	Unclassified
			Alcaligenaceae
	β-Proteobacteria	Burkholderiales	Oxalobacteraceae
Proteobacteria			Burkholderiaceae
		Aeromonadales	Aeromonadaceae
			Succinivibrionaceae
		Enterobacteriales	Enterobacteriaceae
	γ-Proteobacteria	Pasteurellales	Pasteurellaceae

Table 1.1 (continued)

Phylum	Class	Order	Family/cluster
		Pseudomonadales	Moraxellaceae
			Pseudomonadaceae
		Vibrionales	Vibrionaceae
		Xanthomonadales	Xanthomonadaceae
	δ-Proteobacteria	Desulfovibrionales	Desulfovironaceae
			Campylobacteraceae
	ε-Proteobacteria	Campylobacterales	Helicobacteraceae
Spirochaetes	Spirochaetes	Spirochaetales	Serpulinaceae
TM7	TM7	Unclassified	Unclassified
Verrucomicrobia	Verrucomicrobia	Vericomicrobiales	Vericomicrobiaceae
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae

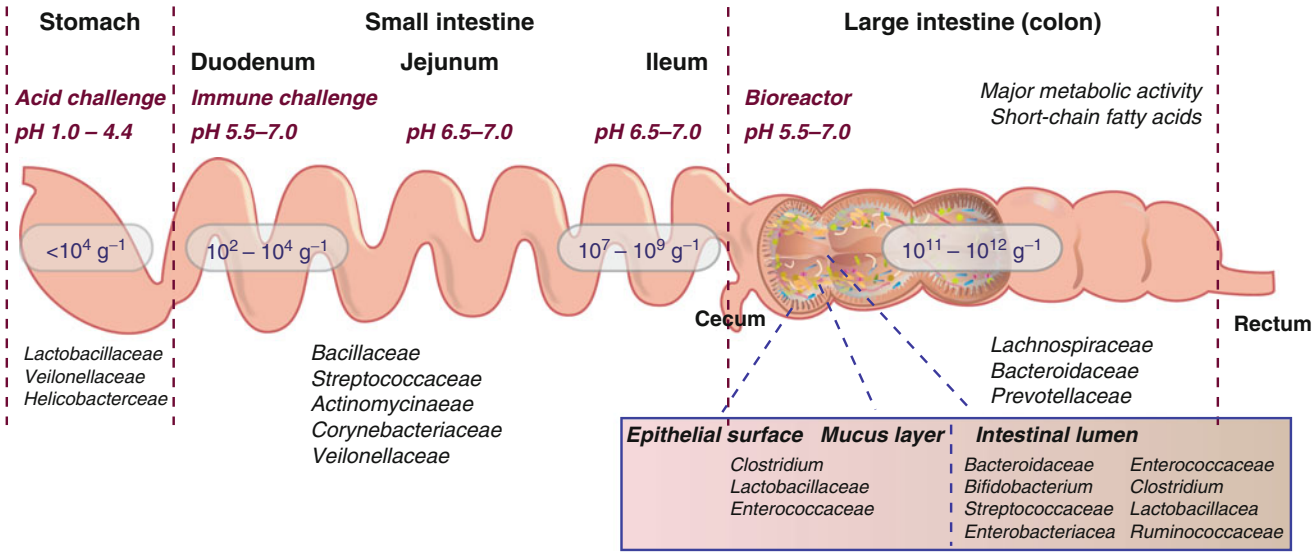

Fig. 1.1

Variations of the gut microbiota composition and numbers along the length of the GI tract. Major features that shape the gut microbiota into the different anatomical regions of the gut are indicated

newborn will be colonized only by those microbes to which it is exposed and that are capable of forming a permanent community in the neonatal GI tract. The succession of microbial populations in the infant gut starts with colonization by facultative anaerobes (*Escherichia coli* and *Streptococcus* spp.) due to the positive redox potential in the gut at birth. Microbial concentrations in the feces quickly reach levels of 10^8–10^{10} cells/g of feces within 1–2 days from birth. Gradually, the initial indigenous microbiota creates reducing conditions that are favorable for the proliferation of anaerobic bacteria. The anaerobic microbial community does not become established in the neonate's gut until the second month of life. Furthermore, the colonization process is strongly affected by the diet (i.e., breast milk and/or formula milk), which will be discussed further in this chapter. During the first month of life, bifidobacteria and *E. coli* are the predominant populations, followed by *Lactobacillus* spp., Bacteroidetes, and Gram-positive bacteria, all in similar quantities. During the first year of life, the microbial composition of the mammalian intestine is relatively simple and differs widely between individuals (Wall et al. 2009). Changes in the proportions of the dominant members of the neonates' gut microbiota appear after about one year of life, mainly as a result of the introduction of new food into the diet of the infant. *Lactobacillus* spp., *Bacteroides* spp., and clostridia numbers increase in this period of life, while bifidobacteria and *E. coli* decrease. Finally at the age of 2 years, the microbial community of the infant gut reaches a climax with a composition of microbes similar to that found in the adult intestine (Koenig et al. 2011). The colonization process of the infant gut, both in timing and composition, may play an important role in health and disease later in life. However, a number of factors, such as diet, host genotype (including the immune system), colonization history, aging,

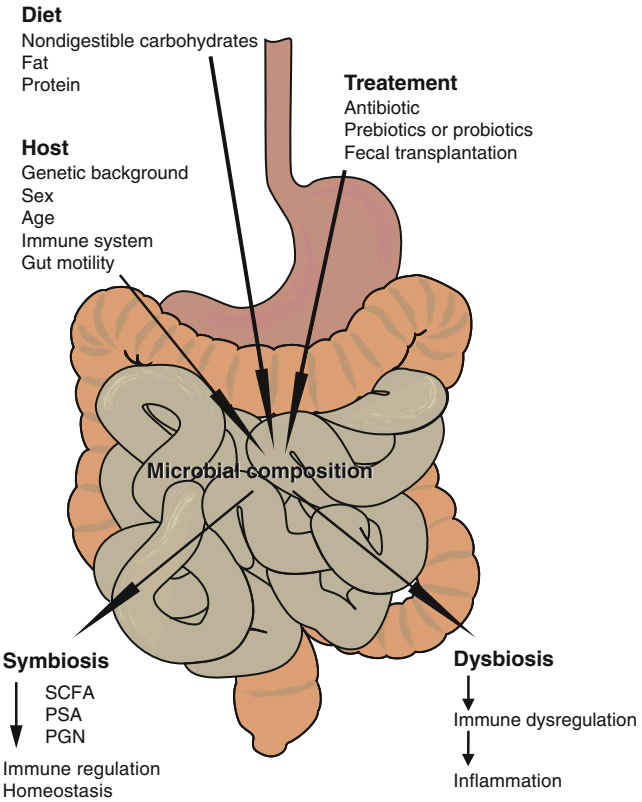

Fig. 1.2

Factors shaping gut microbiota composition. Abbreviations: *SCFA* short-chain fatty acids, *PSA* polysaccharide A, *PGN* peptidoglycan

disease, antibiotic treatment or medication, and stress, shape the final composition of the gut microbiota (Zoetendal et al. 2001) (*Fig. 1.2*).

Factors That Affect the Gut Microbiota Composition

Host Genetics

The genetic makeup of the host has an effect on the intestinal microbiota, as it has been shown in studies where related subjects, identical (monozygotic) and/or fraternal (dizygotic) twins, have been involved. In those culture-independent-based cohorts, high degree of similarity of the gut microbiota composition in monozygotic twins, higher than the similarity between random unrelated individuals, has been reported (Turnbaugh et al. 2009; Zoetendal et al. 2001). However, the study of Turnbaugh et al. reported that the microbiota of monozygotic twins overall was not significantly more similar than that of dizygotic twins suggesting that environment early in life may play an important role for the developing ecosystem. But among the different individuals, an extensive "core microbiome" at a functional (and metabolic) level has been stated, despite their different phylogenetic profiles (Turnbaugh et al. 2009). Furthermore, studies with germ-free (GF) hosts that receive interspecies gut microbiota transplants show that the mammalian hosts might be able to modulate their microbial lineages toward a composition that resembles the original one that is normally found in the conventional status (Rawls et al. 2006). Thus, the genetic makeup is likely to have a profound influence on the host microbiota composition and functionality, but more comprehensive studies are needed to elucidate the exact degree of dependence.

Diet

Diet, microbiota, and GI tract interactions in mammals are extremely complex and are the result of millions of years of coevolution between the higher vertebrates and their specific microbiota. As a consequence, any major change in lifestyle and diet is likely to place stress on the stability of these interactions and affect the entire GI tract ecophysiology. In the first stages of life, the effect of diet (breast vs. formula milk) dramatically influences the colonization pattern. Gut microbiota composition of breast-fed infant is dominated by bifidobacteria and lactic acid bacteria, while formula-feeding in most of the cases results in a more diverse community including bifidobacteria, Bacteroides, clostridia, and a number of facultative anaerobes such as staphylococci, streptococci, and Enterobacteriaceae (Palmer et al. 2007; Wall et al. 2009). Additionally, the number of pathogenic species, such as *E. coli*, *Clostridium difficile*, and some species of the *Bacteroides fragilis* group, is much lower in breast-fed infants, and incidence of *C. difficile* is higher in formula-fed babies. This selection of beneficial microbes in the gut microbiota of breast-fed infants is attributed to the composition of the human milk. The main compounds of the mother milk are oligosaccharides, which are known to act as substrates for fermentation in the distal gut and promote the growth of bifidobacteria (bifidogenic effect) (Wall et al. 2009). Bifidobacteria may also play an important role in the establishment of the microbial community that modulates the immune system (Bode 2009). Human milk is also a rich source of microbes, with numbers that reach up to 10^9 microbes per liter breast milk in a healthy mother, which also may affect microbial ecology in the infant gut (Moughan et al. 1992). Furthermore, introduction of solid food into the infant diet leads to a large compositional shift into the gut microbiota community (Koenig et al. 2011; Palmer et al. 2007).

Differences in human populations and geographic factors might also contribute to the variation of gut microbiota composition. In a recent study, the fecal microbiota of rural children from Burkina Faso (BF) and urban children in Italy was compared by means of 16S rDNA sequencing. During breast-feeding, no significant difference in the microbiota composition between the two geographically different cohorts was found. However, once solid food was introduced, significant enrichment of Bacteroidetes, of several microorganisms involved in polysaccharide degradation and depletion of Firmicutes, was observed in the BF children. These features were completely absent in the Italian children, and the selection of the microbes in the gut of BF children was attributed to the high level of plant polysaccharides

(fibers) present in the BF diet (De Filippo et al. 2010). Fiber fermentation by the gut microbiota leads to increase production of short-chain fatty acids (SCFA), such as acetate, propionate, and butyrate (see p8 in this chapter), and the amounts of the produced SCFA will change quickly when there is a switch in the diet. In this respect, significantly less SCFA were measured in the feces of the European children in comparison with those in the African cohort (De Filippo et al. 2010).

Close relation between dietary habits and the human genome has been shown by Perry et al. when higher copy of salivary amylase gene (AMY) was associated with increased uptake of starch (Perry et al. 2007). Similar evolution has been observed in the gut microbiome, where porphyranase genes were only found in the microbiota of Japanese individuals. Those genes encode for the enzyme that digests porphyrans, a carbohydrate found only in seaweed and that is present in the Japanese diet. Hehemann et al. hypothesize that gut bacteria use horizontal gene transfer to acquire porphyranase genes from the ingested microbes present in the seaweed (Hehemann et al. 2010).

Antibiotics

Antibiotic therapy is another factor that perturbs the ecology of the gut microbiota. Commonly used to remove or prevent a bacterial colonization in the human body, antibiotic treatments cause changes into the gut microbiota, persisting for a long time after discontinuation of the treatment (Dethlefsen et al. 2008; Dethlefsen and Relman 2011; Jernberg et al. 2007; Palmer et al. 2007). A major impact of the use of antibiotics on the indigenous microbiota is a long-term decrease of overall community diversity.

Age

The human gut microbiota is influenced by aging. Medications, most noticeably antibiotic treatments, naturally decline physiological functions. Additionally, changes in the quality and quantity of foods also alter gut microbial ecology in elderly people. Almost similar to what happens at the early stage of our life, the elderly gut is characterized by lower microbial biodiversity, increase in opportunistic facultative aerobes, such as Staphylococci, Streptococci, and Enterobacteriaceae, and decrease in the anaerobic microbiota, particularly in members of Bacteroidetes and Clostridium cluster IV and XIVa. Differently from the infant gut microbiota, the elderly type has lower levels of bifidobacteria (Biagi et al. 2010, 2011; Claesson et al. 2009).

Gut Microbiota Functionality: Metabolic Roles of Gut Microbiota

In terms of functional diversity, recent metagenomic-based studies have indicated that the gut metagenome has a coding capacity that exceeds that of the human genome by at least 150-fold and encodes biochemical pathways that humans have not evolved (Backhed et al. 2005; Gill et al. 2006; Kurokawa et al. 2007; Ley et al. 2006; Qin et al. 2010; Turnbaugh et al. 2007). A plethora of important functions that define the physiology of the host have been assigned to the gut microbiota, including defense against pathogens, immune system maturation, development of the intestinal microvilli, and nutrition (*Table 1.2*). The nutritional function of the gut microbiota is linked to the fermentation of nondigestible dietary fiber and the anaerobic conversions of peptides and proteins, which result in recovery of metabolic energy for the host (Acheson and Luccioli 2004; Hooper et al. 1998; Xu and Gordon 2003). Particularly important is the ability of the gut microbiota to process otherwise indigestible components of our diet because this activity not only provides energy sources but also promotes the maintenance of gut health (Guarner and Malagelada 2003; Savage 1986; Xu and Gordon 2003).

Table 1.2
Beneficial contributions of intestinal microbiota to human health

Maintenance of gut homeostasis
Renewal of intestinal epithelial layer
Regulation of intestinal barrier integrity
Recovery of intestinal epithelial injury
Intestinal angiogenesis
Improved bowel motility
Maturation and education of the immune system
Protection against pathogens (colonization resistance)
Improved energy harvest through digestion of complex fibers in food (i.e., resistant starch and dietary fibers)
Production of nutrients (SCFA, amino acids)
Production of vitamins (vitamin K, vitamin B12, and folic acid)
Metabolism of xenobiotics and procarcinogens
Development of the nervous system
Regulation of appetite and behavior

Until recently, the colon was only considered as a storage place for undigested food components. Based on its biochemical (metabolic) potential, the gut microbiota has been suggested as a "metabolic" organ by itself with metabolic potential which is comparable to that of the liver (O'Hara and Shanahan 2006). These functions include utilization of nondigestible carbohydrates, host-derived glycoconjugates (e.g., mucin), deconjugation, and dehydroxylation of bile acids, cholesterol reduction, biosynthesis of vitamins (K and B group) and isoprenoids, and metabolism of amino acids and xenobiotics.

Microbial Fermentation

The host lacks enzymatic capacity to degrade complex carbohydrates, such as polysaccharides or nondigestible carbohydrates

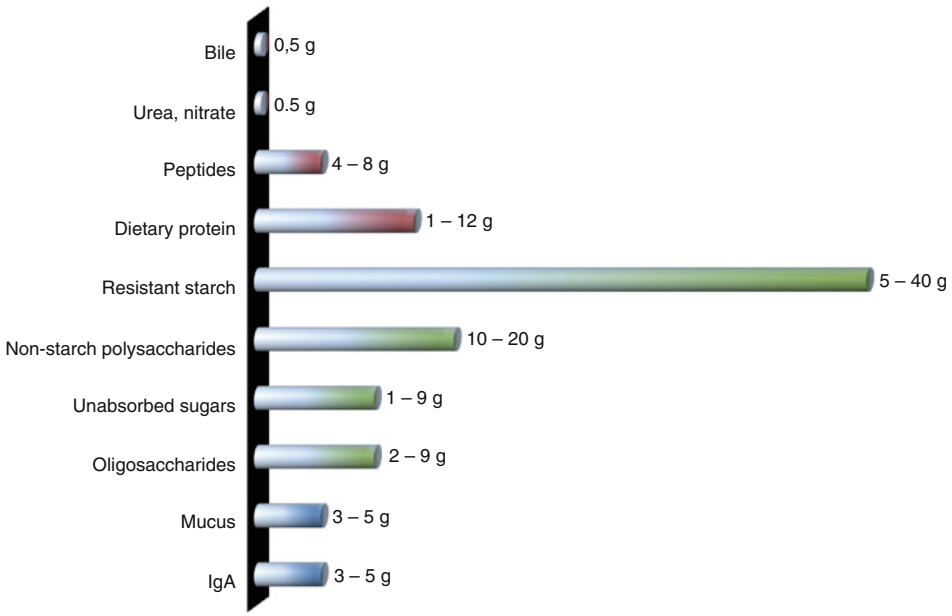

Fig. 1.3
Substrates available for fermentation in the human colon

(resistant starch, non-starch polysaccharides, and fibers of plant origin and nondigestible oligosaccharides), host-derived glycans (mucins, glycosphingolipids), and some proteins from the diet (Cummings and Englyst 1987). As a consequence, these food residues escape digestion in the small intestine and enter the colon where they are fermented by microorganisms (Ouwehand et al. 2005). The amount of dietary carbohydrates entering the colon varies in the range of 10–60 g/day, among which the most abundant carbohydrate is thought to be resistant starch (RS), which is starch recalcitrant to the activity of human amylases, followed by non-starch polysaccharide; unabsorbed sugars, such as lactose, raffinose, and stachyose; and oligosaccharides (*Fig. 1.3*) (Hughes et al. 2000; Macfarlane and Cummings 1991; Scott et al. 2008). These substrates consist of a complex assortment of macromolecules with diverse structures, and degradation requires an array of microbial hydrolytic enzymes produced by various members of the colonic community. Furthermore, during the multiphase conversions of dietary complex carbohydrates, cooperation via metabolic cross-feeding is an important process. Several classes of microbial activities can be observed to occur during such fermentations, and based on substrate utilization and metabolite formation, different functional groups in the colonic microbiota can be defined (*Table 1.3*). Nevertheless, there is still a lack of knowledge with respect to the key microbes involved in colonic fermentation of different relevant dietary carbohydrates. Firstly, enzymatic activities of the primary degraders of complex carbohydrates result in the release of large amounts of polysaccharides, which serve as substrates for the luminal microbiota. Furthermore, the colonic microbiota is involved in the breakdown of polysaccharides to oligosaccharides. Oligosaccharides are one of the most studied carbohydrates, and it has been shown that bacteria that primarily utilize oligosaccharides do not grow on polysaccharides (Rossi et al. 2005). During cross-feeding, the fermentation products released by one microorganism are utilized or serve as a growth factor for another population, often with an impact on the energy metabolism of one or both partners (Samuel and Gordon 2006; Wolin et al. 1997) (*Fig. 1.4*).

Few colonic bacteria, e.g., *Bacteroides* spp. and *Ruminococcus* spp., initiate the breakdown of insoluble substrates (Flint et al. 2008; Jindou et al. 2008; Rincon et al. 2005). In contrast, oligosaccharides, such as fructooligosaccharides (FOS) and galactooligosaccharides (GOS), and the polysaccharide inulin are preferentially fermented by *Bifidobacterium* spp. and *Lactobacillus* spp. A number of bacteria belonging to Clostridium cluster XIVa form the group of butyrate producers, as they produce butyrate as major fermentation product (Barcenilla et al. 2000; Pryde et al. 2002). Different fermentation pathways could be involved in butyrate synthesis in the human colon. The most widespread pathway among butyrate producers of the Clostridium cluster XIVa is the CoA-transferase route (*Fig. 1.4*), whereas another pathway involving butyrate-kinase activity is less common (Louis et al. 2004). A third group of butyrate producers are found to utilize D- and L-lactate for butyrate production: this group includes species belonging to *Eubacterium hallii* and *Anaerostipes caccae* and *Clostridium indolis* (Duncan et al. 2004). There are three distinct pathways for propionate synthesis, namely, the succinate decarboxylase pathway, preferred by *Bacteroides* spp., the acrylate pathway utilized by members of Clostridium cluster IX group (Louis et al. 2007), and the recently described propanediol pathway reported for *Roseburia inulinivorans* (Scott et al. 2006) (*Fig. 1.4*).

The end products of microbial fermentation are gases such as hydrogen, carbon dioxide, and methane as well as short-chain fatty acids, which are the main products of the anaerobic

Table 1.3

Relevant metabolic features of members of the human gut microbiota

Microbial group	Species	Metabolic function	Produced metabolites
Bacteroidetes	*Bacteroidetes* spp. *Prevotella* spp. *B. thetaiotaomicron*	Polysaccharides breakdown; L-, S- Polysaccharides breakdown; L-, S- Polysaccharides breakdown, mucin degradation	Acetate, propionate, succinate Acetate, succinate, formate Acetate, propionate
Actinobacteria	*Bifidobacterium adolescentis* *B. longum* *B. bifidum* *Collinsella*	Carbohydrate metabolism Carbohydrate metabolism Carbohydrate metabolism Carbohydrate metabolism	Acetate, lactate Acetate, lactate, formate, ethanol Lactate, acetate, formate Lactate, acetate, formate
Clostridium cluster IV	*Ruminococcus bromii* *R. flavefaciens* *F. prausnitzii*	Carbohydrate metabolism Plant fibre breakdown Carbohydrate metabolism	Acetate, formate, H_2, ethanol Acetate Butyrate, formate, D-lactate
Clostridium cluster	*R. intestinalis* *R. inulinovarans* *E. hallii* *A. caccae* *E. rectale* *Coprococcus eutactus* *Dorea longicatena*	Carbohydrate metabolism Carbohydrate metabolism Carbohydrate metabolism; A-, L- Carbohydrate metabolism; A-, L- Carbohydrate metabolism; A- Carbohydrate metabolism; A- Carbohydrate metabolism	Butyrate, CO_2 Butyrate, propionate Butyrate Butyrate, CO_2 Butyrate, lactate, formate, H_2 Butyrate, formate, lactate Formate, acetate
Clostridium cluster IX	*Mitsoukella multiacida* *Megasphaera elsdenii*	Gluconic acid metabolism; A- Gluconic acid metabolism; A-, L-	Butyrate Butyrate, propionate
Proteobacteria	*Desulfovibrio* spp.: e.g., *Desulfovibrio piger* *Desulfovibrio desulfuricans*	SRB, L- SRB, L-	Acetate, H_2S
Verrucomicrobia/	*Akkermansia muciniphila* *Victivallis vadenis*	Mucin degradation Cellobiose degradation	Acetate, propionate Acetate
Archaea	*Methanobrevibacter* spp.	H_2-utilizer	CH_4

L lactate utilizer, *S* succinate utilizer, *A* acetate utilizer, *SRB* sulfate-reducing bacteria

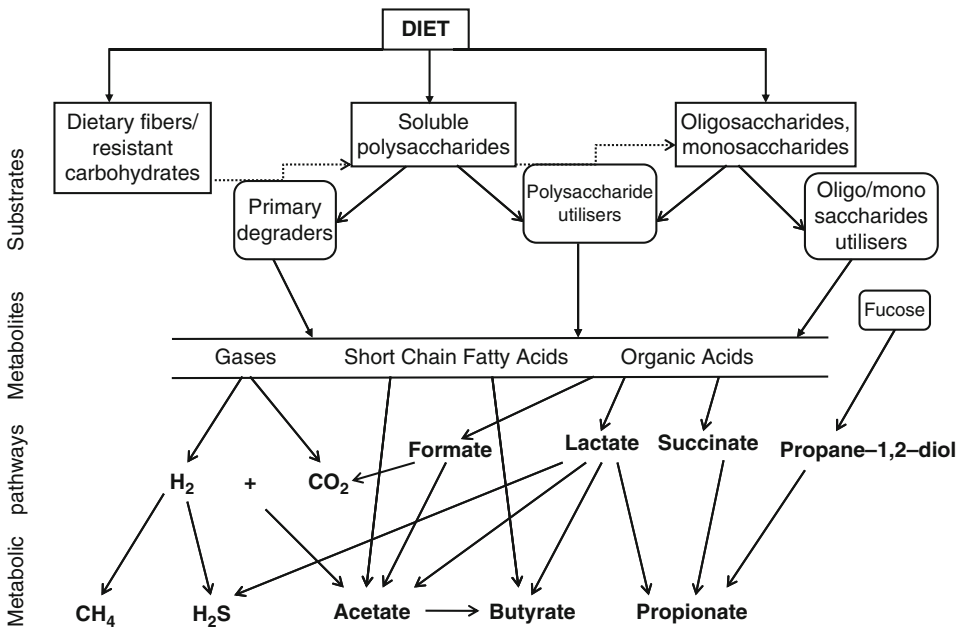

Fig. 1.4

Schematic representation of the conversions of dietary carbohydrates by the gut microbiota. Substrate utilization by different populations in the colon and metabolites produced as a result of the microbial anaerobic fermentations and cross-feeding interactions between primary carbohydrate degraders and other members of the gut microbiota

microbial fermentations occurring in the human colon. Particularly important are butyrate, propionate, and acetate, which have a fundamental role in host physiology (Mortensen and Clausen 1996; Scheppach 1994) (Fig. 1.4).

Physiologic Effects of Microbial Fermentation

SCFA with different carbon chain lengths (acetate (C2), propionate (C3), butyrate (C4), valerate (C5), and caproate (C6)) are produced in varying amounts depending on the nutrient uptake and the composition of the host gut microbiota. The concentrations of SCFA, measured in autopsy samples from sudden death victims, varied from 137 to 197 mmol/kg chime in the proximal colon and 86–97 mmol/kg chime in the distal colon. The SCFA molar ratio for C2:C3:C4:C5:C6 was found to be approximately 54:20:21:4:1 (Cummings et al. 1987; Macfarlane et al. 1992). SCFA are rapidly absorbed, and only 5–10 % of the produced SCFA are being excreted in the feces (Wong et al. 2006). SCFA lead to lowering of the luminal pH, increase in bacterial biomass and fecal bulk, and modification of the microbial composition, especially by stimulating the growth of beneficial bacteria including bifidobacteria and lactobacilli (Le Leu et al. 2005).

SCFA stimulate mucosal cell proliferation, mucus production, and mucosal blood flow and also affect peripheral metabolism. Butyrate, one of the major SCFA, is also an important energy source for the colonic epithelium and for the prevention of colon cancer (Bauer-Marinovic et al. 2006; Cummings and Bingham 1987; Hamer et al. 2008; Sengupta et al. 2006). Recently, it has been also observed in healthy individuals that colonic butyrate application resulted in reduced visceral pain perception (Vanhoutvin et al. 2009). Acetate is used as a substrate for liver cholesterol and fatty acid synthesis; it also increases colonic blood flow and oxygen uptake and enhances ileal motility by affecting ileal contractions (Scheppach 1994). Propionate is better absorbed than acetate from the human colon and can act as a substrate for gluconeogenesis in the liver. Both acetate and propionate may protect against hepatic cancer, as well as other cancers known to metastasize in the liver, such as breast and colon cancer (Chambers et al. 2002). Formate, succinate, and lactate are also released into the lumen as a result of the carbohydrate conversions by the colonic microbiota. These metabolites also have been reported to affect the host physiology and gut microbiota functionality and composition (Bergman 1990; Cummings and Macfarlane 1991).

Gut Microbiota in the Protein Degradation in the Distal Colon

After anaerobic conversion in the proximal colon, the digesta moves through the colon transversum to the distal colon, and during this passage, carbohydrate availability decreases while proteins and amino acids become the main energy source for the gut microbiota, particularly in the distal colon (Macfarlane et al. 1992). The large intestine has been described as a site of intense protein turnover (Macfarlane and Macfarlane 1995). Important proteolytic species associated with the human colon include species belonging to *Bacteroides, Propionibacterium, Clostridium, Fusobacterium, Streptococcus*, and *Lactobacillus* (Macfarlane and Cummings 1991). Approximately 13 g of dietary proteins enter the colon daily (Fig. 1.3). Additionally, other sources of protein in the colon are provided by endogenous material, e.g., pancreatic enzymes, mucus, and exfoliated epithelial cells. In the colon, nitrogenous residues are initially depolymerized by a mixture of residual pancreatic endopeptidases and bacterial proteases and peptidases (Macfarlane and Cummings 1991), which form short peptides and release amino acids for fermentation. In addition to SCFA, hydrogen, and CO_2, which are formed during protein fermentation, branched-chain fatty acids such as isobutyrate, isovalerate, and 2-methylbutyrate and other organic acids are also produced. Other minor components resulting from protein fermentation are ammonia, amines, phenols, and indoles.

Colonic protein fermentation is associated with increased risk for developing colon cancer, probably due to the production of branched-chain fatty acids and potentially toxic metabolites (i.e., amines, ammonia, phenolic compounds, and thiols) (Bingham et al. 1996; Cummings et al. 1979). A link between protein fermentation and colon cancer is also provided by the fact that colon cancer mostly affects the distal end of the colon (Bufill 1990; Muir et al. 2004), where protein fermentation also occurs. Therefore, it is suggested that an intake of more slowly fermentable carbohydrates could result in prolongation of the potentially beneficial saccharolytic activity and reduce protein fermentation (Jacobasch et al. 1999; Topping and Clifton 2001; Wong et al. 2005).

The Activity of Methanogens and Sulfate-Reducing Bacteria in the Human Colon

During the anaerobic colonic fermentation of carbohydrates and proteins, molecular hydrogen (H_2) is generated, which is further removed from the system through the metabolism of the colonic microbiota (Fig. 1.4). Based on their activity, three functional groups can be defined (Table 1.3). H_2 can serve as growth regulator for colonic methanogens such as *M. smithii* and *M. stadtmanae*, which generate methane (CH_4) as end product of H_2 oxidation. In presence of accessible sulfate (Christl et al. 1992) and mucins (Gibson et al. 1988) in the colonic environment, the sulfate-reducing bacteria (SRB) will outcompete the methanogenic microbes and oxidize H_2 to produce H_2S (hydrogen sulfide). If the colonic sulfate pool is sufficient, the sulfate-reducing bacteria (SRB) will dominate in this niche, while during sulfate-limiting conditions, methanogenic and acetogenic populations will dominate. Acetogens dispose H_2 by reduction of CO_2 to acetate. H_2 that is not utilized by the colonic microbiota can be removed from the colon by passing through the gut wall into the blood stream, where it is

transported to the lungs and then excreted in breath. Measurement of breath H_2 is thus a good indicator for colonic microbial fermentations.

Strategies to Assess Microbial Diversity of the Human Gut

The vast majority of the human intestinal microbiota is strictly anaerobic (99 %) and hard to enumerate using culture-dependent methods. Accordingly, our current knowledge of the microbial composition of the intestinal ecosystem in health and disease is still limited. Development of anaerobic culturing methods such as introduction of the Hungate roll-tube technique and the development of the anaerobic glove box was a major advance in the characterization of gut microbes (Aranki et al. 1969). These strategies are still used as standard tools for the isolation and cultivation of intestinal microorganisms. However, it is generally accepted that only a minor fraction of this important community has been isolated in pure culture as of yet.

An alternative to characterize and enumerate the gut microbiota by culturing is to analyze different biomarkers by the application of molecular or culture-independent techniques. Several biological compounds can serve as biomarkers including metabolites, proteins, RNA, DNA, and single cells. However, the prime biomarker used for phylogenetic analysis of the complex gut microbiota, similarly to other complex environment, is the gene encoding 16S ribosomal RNA (16S rRNA) (Woese 1987). One consequence of the 16S rRNA-based techniques is that only a minor fraction of the gut microbiota has been isolated in pure culture.

The culture-independent techniques that have been successfully applied to study GI tract samples include cloning and subsequent sequencing of the 16S rRNA genes and fingerprinting techniques, such as temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), and terminal restriction length polymorphisms (T-RFLP) analysis. Conventional clone library analyses by Sanger sequencing are costly and time-consuming, preventing their application for the analysis of a larger number of samples in order to provide information of microbial composition at sufficient spatiotemporal resolution. TGGE/DGGE and T-RFLP are semiquantitative approaches that allow rapid profiling of the total microbiota. These fingerprinting techniques are mostly used to compare microbial communities and monitor their dynamics. Quantitative 16S rRNA gene-based techniques are fluorescence in situ hybridization (FISH) combined with flow cytometry and quantitative PCR (qPCR).

Phylogenetic microarrays are high-throughput analytical tools, which can be used to measure diversity and abundance of the human gut microbiota. Recently, such a DNA microarray, the Human Intestinal Tract Chip (HITChip), was developed, combining the power of fingerprinting and phylogenetic and quantitative community analysis (Rajilic-Stojanovic et al. 2009). In a recent study, the potential of the HITChip approach was further explored by performing a comparative analysis of the phylogenetic array and pyrosequencing technologies. This study confirmed the high capacity of the HITChip for in-depth profiling of complex microbial communities (Claesson et al. 2009). One limitation of the microarray technology is that these chips target only known sequences. The most recent approaches to enumerate the gut microbiota are based on next-generation sequencing technologies that generate larger amounts of sequences to a reduced price compared with Sanger sequencing. The Roche GS FLX systems developed by 454 life sciences (454 pyrosequencing) represent a sequencing platform that generates around one million DNA sequence reads of up to 450 base pairs each. Application of 454 pyrosequencing of hypervariable regions of the 16S rRNA gene has revealed that the taxonomic richness of the gut microbiota exceeds any previously reported estimates (Andersson et al. 2008; Dethlefsen et al. 2008). Additionally, 454 pyrosequencing analyses have opened new frontiers for the understanding of the role of the gut microbiota in health and disease. Recently, much effort has been put into using shotgun sequencing of the entire metagenome, which in addition to provide phylogenetic information also provide information about the functional capacity.

All together, the introduction of molecular techniques, especially 16S rRNA-based approaches, in microbial ecology over the last 10 years has emphasized the extreme diversity of the human colonic microbiota and has indicated the limitations of culture-based approaches. However, rRNA-based techniques are limited by the fact that they do not provide a direct link to the physiology and metabolic capacities of the intestinal microbiota. In this respect, functional studies are required.

Strategies to Assess Microbial Functionality of the Human Gut

To understand the complex changes in the gut microbiota composition that may predispose toward intestinal disorders or promote human health, techniques that can assay and link metabolic activity to the diversity of intestinal bacteria are needed.

Recently explored metagenomic approaches allow the comprehensive study of phylogenetic, physical, and functional properties of complex microbial communities, providing a full picture of microbiota dynamics (Handelsman 2004). Because metagenomic analyses allow the study of phylogenetic diversity and provide inventories of potential functions of the gut microbiota, they can be used as a tool to link diversity to functionality. Additionally, metagenomic strategies can be divided into functional and sequence-based analyses of collective microbial genomes in complex environments (Gabor et al. 2007). Sequence-based metagenomic investigations have started to reveal core metabolic functions of the gut microbiota. An early metagenomic study on two healthy adults showed that their fecal microbial metagenomes were enriched in genes involved in energy metabolism, which include also the production of SCFA as pivotal energy supply for the intestine (Gill et al. 2006). A recent study, where metaproteomic analyses were

applied to study the distal gut microbiota of a healthy twin pair, indicated more than 50 % of the detected proteins to be involved in translation, energy production, and carbohydrate metabolism (Verberkmoes et al. 2009). Comparison of metagenomic (Gill et al. 2006) and metaproteomic data (Verberkmoes et al. 2009) indicated matches in the fucose and butyrate colonic fermentation pathways. Large-scale comparative metagenomic analyses demonstrated a clear effect of diet and age on the gut microbiome (Kurokawa et al. 2007). A gene catalogue of all prevalent genes of the gut microbiome was generated by illumina-based metagenomic sequencing from the fecal material of 124 European individuals (Qin et al. 2010). This analysis provided a broad overview of functions crucial for the bacteria in the human gut but also pointed out the existence of a bacterial core for the different individuals (Qin et al. 2010). Metabonomics, quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification, is another omics strategy used to assay metabolic functions of the host as well as the gut microbiota (Nicholson 2006). It is estimated that >90 % of the metabolites found human urine are microbial derived. While metagenomics and metatranscriptomics aim to describe the metabolic potential of the gut microbiome, the metabonomics has "the capacity to measure the metabolic kinetic or flux of metabolites through an ecosystem at a particular point in time or over a time course" (Tuohy et al. 2009).

However, an ongoing challenge for microbiologists is to be able to identify which microbes in the human gut carry out a specific metabolic conversion, the products of which may promote intestinal disorders and/or gut health. A strategy, that offers a great potential to identify microbes that are involved in the metabolism of specific substrates, is the so-called stable-isotope probing (SIP). SIP-based approaches involve the usage of commercially prepared substrates highly enriched in a stable isotope (e.g., ^{13}C) or radioisotope (e.g., ^{14}C), which is added to an environmental sample. Endogenous microbes that metabolize the labeled substrate will incorporate the isotope into components of the microbial cells, thus providing phylogenetic information (Radajewski et al. 2000). SIP methodologies vary in the use of biomarkers but also in the means by which biomarkers are analyzed for isotopic and phylogenetic content. To explore the capacity of the gut microbiota in the fermentation of relevant dietary carbohydrates, we applied RNA-based SIP, which in combination with molecular identification tools, provides a direct link between the structure of a microbial community and the function of its members (Kovatcheva-Datchary et al. 2009). This approach is an effective strategy to clarify the functionality of the gut microbiota and to elucidate the role of individual species within the community in their natural environment. Moreover, RNA-based SIP allowed us to identify the primary degraders of tested substrates, and a phylotype related to *R. bromii* was reported to be primary degrader of potato starch, corroborating results that were reported in other in vivo and in vitro experiments (Abell et al. 2008; Leitch et al. 2007). Furthermore, more information on the functionality of the different microbes, found to be active in the colon ecosystem, could be generated after combining the data from the SIP technique with NMR and liquid-chromatography-mass spectrometry. Based on the detection of (partially) labeled metabolites, this allowed the identification of active metabolic pathways and delineation of food webs that may influence human health (de Graaf et al. 2007; Egert et al. 2007; Kovatcheva-Datchary et al. 2009). Integration of the molecular and metabolite data suggested metabolic cross-feeding where populations related to *R. bromii* were the primary starch degraders, while other members of the community related to *Prevotella* spp., *Bifidobacterium adolescentis*, and *E. rectale* were likely involved in this trophic web.

Protein-based stable-isotope probing (Protein-SIP) is a novel approach, which analyzes specific metabolic activity of a single bacterial species within a community that incorporates the labeled substrate in the cellular protein fraction (Jehmlich et al. 2008, 2010). The most important advantage of protein-based analysis is the direct connection to physiological function, as proteins are known to catalyze the biochemical reactions. Thus, proteins are source of phylogenetic and functional information, making them ideal biomarkers for monitoring community structure and function. Furthermore, SIP techniques are suitable for obtaining qualitative and quantitative information about metabolic fluxes in the colon. Isotopically labeled compounds enable the selective study of that part of the microbial or host metabolism that involves the isotopic tracer. NMR and gas- or liquid-chromatography can be used to measure the labeled compounds and further identify active metabolic pathways (Bacher et al. 1998; Egert et al. 2007).

The comprehensive understanding of the metabolic activity of the gut microbiota will enable the development of direct strategies to treat or prevent intestinal disorders caused by microorganisms in humans. Identification of the prime functions of the human gut microbiota in maintaining human health requires better understanding of its diversity and functionality, which can facilitate its manipulation. Most intestinal microbes have not been cultured, and the in situ functions of distinct groups of the gut microbiota are largely unknown but pivotal to understand their role in health and disease.

Relevance of Gut Microbiota for Human Disease

Many of the bacteria that stably inhabit the human gut establish a mutualistic relationship with the host and provide beneficial functions. Besides beneficial bacteria, the human GI tract is associated with several commensals that do not confer specific advantage or detriment. Finally, the gut microbiota also contains potentially harmful species, which are likely to be in closer contact with the intestinal epithelium and have been defined as pathobionts (Round and Mazmanian 2009; Sansonetti 2011). The sum of all symbiotic interactions between the host and its microbiota, and between different microbial species in the community, results in eubiosis, a balance condition that is essential for the maintenance of intestinal, immune, and metabolic

homeostasis (Table 1.2). The condition opposed to eubiosis is defined as dysbiosis and is characterized by qualitative and quantitative changes in composition, spatial distribution, and function of the symbiotic microbial community.

The composition of the gut microbiota in healthy individuals is stable at the phylum level, with Bacteroidetes and Firmicutes being the two dominant phyla. The prevalence of only two indicates that the gut microbiota is a highly adapted system that has coevolved with its host (Dethlefsen et al. 2007). However, enumeration of bacterial species revealed considerable variation among individuals and the abundance of shared species varies up to thousand folds (Qin et al. 2010). The variability at low taxonomical level indicates that the gut microbiota is endowed with functional redundancy and resilience to environmental stress, meaning that the composition of the microbial community may vary without alterations to its overall structure and function (Turnbaugh et al. 2009). However, if the perturbation is deep and/or repeated, the changes introduced eventually compromise the functionality of the community (Dethlefsen et al. 2008; Dethlefsen and Relman 2011). Given the broad contribution of the gut microbiota to human health, dysbiosis is bound to affect not only the intestine but also other, and far, organ systems (Fig. 1.5). In this section, the contribution of the gut microbiota to human disease will be discussed.

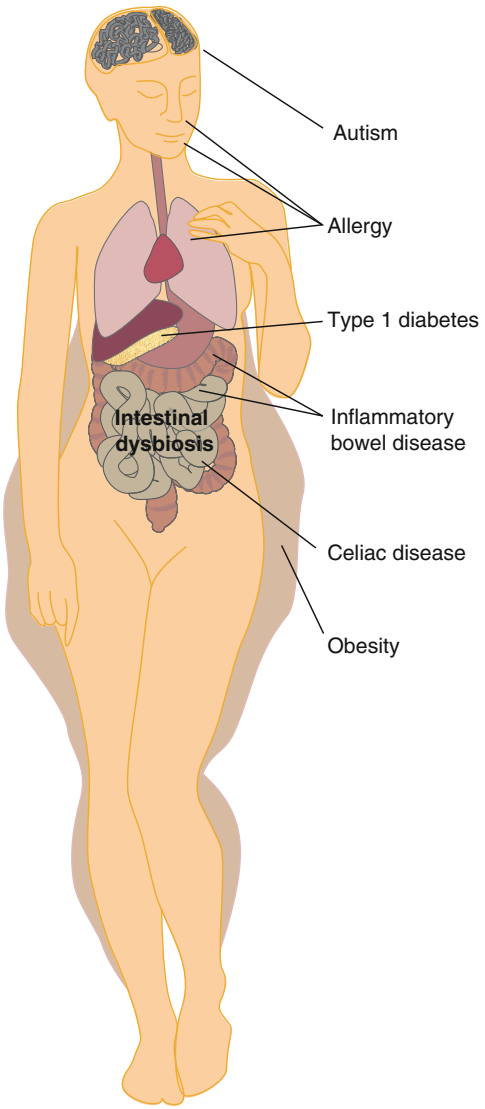

Fig. 1.5
Diseases that have been suggested to be affected by the gut microbiota

Inflammatory Bowel Disease (IBD)

The intestinal mucosa confronts the task of coexisting with symbiotic microbes while preventing microbial overgrowth and pathogen invasion. In health, the mucosal immune system recognizes microbial signals deriving from both symbionts and pathogens and mounts an adequate response that is not detrimental either locally or systemically. The induction of a proper response may be achieved by several mechanisms, such as (1) physical separation of the microbiota from the epithelial surface by the mucus layer or compartmentalization of microbial sensors (i.e., TLRs) to the basolateral membrane of intestinal epithelial cells, (2) evolution of less immunogenic antigens in commensal microbes, and (3) induction of tolerogenic immune responses by the gut microbiota. The microbiota-immune system bidirectional interaction results in the induction of innate immune responses, recruitment of lymphocytes, and activation of both pro-inflammatory (Th1 and Th17 lymphocytes) and anti-inflammatory (Treg cells) adaptive responses that, while preventing deleterious activation of the immune system against innocuous bacteria, also keep the mucosal immune system in a poised state against invading microbes. This condition has been defined as "physiological intestinal inflammation" and is fundamental for maintaining homeostasis at the mucosal interface (Fiocchi 2008; Sansonetti 2011).

Inflammatory bowel disease (IBD) refers to a group of disorders characterized by severe chronic and relapsing inflammation of the GI tract. The two most common forms of IBD in humans are Crohn's disease (CD), which targets the whole length of the GI tract inducing discontinuous inflammation, and ulcerative colitis (UC), which specifically affects the colon and is associated with continuous inflammation. The etiology of IBD is uncertain but strong evidence points to the involvement of an aberrant mucosal immune response to the intestinal microbiota in genetically predisposed subjects (Sartor 2008; Xavier and Podolsky 2007). In particular, IBD has been associated to abnormal activation of pro-inflammatory T cells that release pro-inflammatory cytokines (e.g., tumor necrosis factor (TNF) and interferon-γ (IFNγ)) and to the lack of immunoregulatory cytokines (e.g., interleukin 10 (IL10) and transforming growth factor-β (TGF-β)) that are produced by Treg cells. Susceptibility alleles are present in the population at high frequency but only confer a moderate increase in risk of IBD development. Furthermore, familial aggregation has been observed in IBD, the concordance of CD in monozygotic twins is 60 % while only 20 % for UC (Sun et al. 2011). Finally, increasing incidence of

the disease has been observed in countries with historically low rates of IBD. Taken together, these observations indicate that environmental factors are strongly involved in the pathogenesis of IBD and the gut microbiota could be considered as one.

Evidence for the involvement of gut microbiota in IBD has been found in the observations that antibiotic treatments ameliorate IBD symptoms and microbes or microbial molecules can be isolated from intestinal lesions. Moreover, genetically susceptible animals, if kept germ-free, do not develop the disease. Other environmental triggers of IBD could be the diet, the use of antibiotics, infections, smoking, and stress, which have also been shown to alter the gut microbiota. Recent studies aimed at the global profiling of the gut microbiota showed that both CD and UC are associated with intestinal dysbiosis of both the fecal and the mucosa-associated microbiota (Peterson et al. 2008). One consistent feature detected by several studies is the increased representation of facultative anaerobes (e.g., Proteobacteria, particularly Enterobacteriaceae) in IBD-associated gut microbiota. Increased numbers of these microorganisms have been proposed to result from the aberrant intestinal inflammation that characterizes IBD patients and that could provide a colonization advantage to facultative anaerobes. The gut microbiota of IBD patients is also found to be less diverse and depleted of specific members of the Firmicutes, of the *Clostridium leptum* group, and *F. prausnitzii* in particular (Manichanh et al. 2006; Sokol et al. 2008). The decrease of *F. prausnitzii* was observed in several studies and was associated with an increased risk of postoperative recurrence of ileal CD. This microbe was found to have anti-inflammatory properties due to the releases of a soluble factor that mitigated inflammation in a mouse model of colitis (Sokol et al. 2008). Besides the decrease in clostridia, a decrease in bifidobacteria was also reported both in the feces and in the mucosa-associated microbiota (Hansen et al. 2010; Joossens et al. 2011). As described in the previous sections, clostridia and bifidobacteria are important for the production of butyrate that, besides having a role for colonic cell nutrition, also induces intestinal anti-inflammatory responses (Maslowski et al. 2009). In agreement with these results, fecal extracts of IBD patients contain decreased amounts of butyrate and other SCFA (Marchesi et al. 2007) and increased levels of H_2S (Fava and Danese 2011; Sartor 2008). One recent metagenomic study has also shown that the fecal microbiota of IBD patients contains fewer genes than the microbiota of healthy individuals (Qin et al. 2010). Thus, dysbiosis characterized at the phylogenetic level also bears consequences for the functional diversity and the metabolic activity of the community.

The intestinal barrier also plays an important role in the pathogenesis of IBD, as defects in MUC2 (the major mucin in the human intestine) secretion from goblet cells are observed in UC patients. Several gut microbes secrete glycosidases that can remove the mucins' terminal sugars and expose the chain oligosaccharides and protein core to the action of other gut microbes, thus mediating further degradation of the mucin molecule. A recent study has shown that the mucosa of IBD patients, both in the presence and absence of inflammation, is more efficiently colonized by gut microbes in comparison with mucosa of healthy subjects (Png et al. 2010). The authors found that mucolytic bacteria are normally associated with the mucus of healthy individuals and argue that this mucolytic activity could be important for mucus turnover and stability of the community at the mucosal niche. However, *A. muciniphila*, the major described mucolytic microbe in healthy humans, was reduced in IBD patients, while other mucolytic bacteria, such as *Ruminococcus gnavus* and *R. torques*, were dominant in IBD non-inflamed patients, thus indicating a shift in the mucolytic community as a consequence of altered host factors but independent of intestinal inflammation. The general increase of mucosa-associated bacteria in IBD may be explained by aberrant mucolytic activity, which may increase substrate availability for other microbes, such as *B. vulgatus*, and promote their growth (Png et al. 2010). Additionally, less efficient killing of commensal microbes by the host immune response could also contribute to increase the number of mucosa-associated microbes, which in turn may contribute to increased mucosal inflammation and exacerbate the disease.

While IBD has been associated with alterations in the gut microbiota, the causal relationship between intestinal dysbiosis and disease development has not yet been proven, but recent data indicates that a colitogenic microbiota can be both a cause and a consequence of intestinal inflammation (Sekirov et al. 2010). T-bet deficient RAG2−/− mice lacking adaptive immunity and the ability to regulate inflammatory responses develop spontaneous inflammation that resembles UC. Treatment with antibiotics cures the diseases, indicating that the gut microbiota of these animals contains colitogenic microbes. In this model, colitogenic microbes seem to be selected by the host's predisposition to inflammation. However, transferring of gut microbiota from colitic T-bet deficient RAG2−/− mice to healthy RAG2−/− mice equipped with T-bet or to wild type (WT) animals was sufficient to induce colitis, thus showing that a dysbiotic microbiota can initiate disease (Garrett et al. 2007). The gut microbiota of T-bet deficient RAG2−/− mice was observed to contain higher levels of two Enterobacteriaceae, *Proteus mirabilis* and *Klebsiella pneumonia* (Garrett et al. 2010). Although these bacteria were not sufficient to induce the disease in susceptible mice, they were able to colonize the gut of WT mice and induce colonic inflammation if administered together with a specific pathogen-free microbiota (Garrett et al. 2010). So, selected members of the gut microbiota may be colitogenic, but their ability to induce the disease depends on host's genotype and intestinal inflammation. The nature of host-microbe interactions, which affect the eubiotic/dysbiotic composition of the gut flora, might be decisive for the development of IBD.

Autoimmunity and T1D

Autoimmune diseases are characterized by immune reactivity against self-antigens due to reduced self-tolerance. In health, self-tolerance is attained by elimination of autoreactive lymphocytes during their development in the bone marrow and in the thymus (central tolerance) as well as killing or inactivation of

mature self-reactive lymphocytes in peripheral organs (peripheral tolerance). In genetically predisposed individuals, exposure to environmental triggers may overcome the body's tolerogenic mechanisms and initiate autodestructive inflammatory responses. As mentioned below in this chapter, the gut microbiota has the potential to shape the development of systemic immune responses (Kranich et al. 2011), and in so doing, it may influence the development of autoimmune diseases, such as type 1 diabetes (T1D).

T1D is caused by the progressive T cell-mediated destruction of insulin-producing β-cells in the pancreas, which results in insulin deficiency and high blood glucose concentrations. This disease affects genetically predisposed children and young adults, and approximately 50 % of the genetic risk for T1D is linked to mutations in the human leukocyte antigen (HLA) genes, which encode for molecules that bind and present antigens to T cells. The incidence of T1D in affluent countries has increased during the past decades, and environmental factors have been claimed to have augmented the penetrance of risk genes (Harrison et al. 2008). Interestingly, an inverse association between infectious diseases and autoimmune diseases has also been noted (Bach 2002), similarly to the trend observed for allergic diseases. Clinical and experimental evidence indicates that the onset of T1D may be linked to the gut and the interaction between intestinal permeability, mucosal immune system, and microbiota. Indeed, humans at risk for T1D also display abnormal gut permeability, lack of intestinal Treg activation, aberrant responses to wheat and cow milk (Vaarala et al. 2008), and altered gut microbiota (Giongo et al. 2011). Profiling of the fecal microbiota in children at high risk for T1D revealed increased levels of Firmicutes and reduced levels of Bacteroidetes in children that progress toward diabetes while the opposite trend (i.e., decrease in Firmicutes and increase in Bacteroidetes) is observed for controls.

Evidence for the role of the gut microbiota in T1D can also be found in experimental studies. The incidence of the disease depends on housing conditions and microbial status of animal facilities, with specific pathogen-free (SPF) facilities displaying higher rates of disease (Bach 2002). Besides, the lack of a gut microbiota promotes development of T1D in non-obese diabetic (NOD) mice (Rossini et al. 1979). These results suggest that the gut microbiota exerts a protective role against the development of T1D. A recent study showed that NOD mice that lacked the toll-like receptor (TLR) adapter molecule MyD88, a mutation that altered the intestinal microbiota, were protected from T1D. However, the protection was lost when the mice were rederived as GF or if the gut microbiota was altered through antibiotic treatment (Wen et al. 2008), thus indicating that alterations in an individual's microbiota may play a role in the induction of T1D.

Celiac Disease

In healthy individuals, the presentation of food antigens to the gut immune system results in systemic unresponsiveness to the same antigens, a phenomenon known as oral tolerance. Perturbation of the homeostatic mechanisms that govern oral tolerance can lead to abnormal activation of the gut immune system and damage to the intestinal mucosa, with negative consequences for body's digestive functions and nutrition. Celiac disease is a chronic inflammatory disorder that primarily affects the upper small intestine and is caused by an abnormal immune response to gluten in genetically predisposed subjects who have specific HLA-DQ alleles (i.e., HLA-DQ2/8 alleles, which occur in 30–40 % of the general population but their presence is not sufficient for developing the disease). This enteropathy is prevalent in children, estimated to affect as many as 1 % of the European and North American population, and the therapy for the disease is adherence to a gluten-free diet. Gluten is the principal storage protein in wheat, barley, and rye, but it is poorly digested in the human upper GI tract. The partial digestion of gluten produces toxic polypeptides rich in proline and glutamine that, after deamidation by the enzyme tissue transglutaminase, can bind to the molecules HLA-DQ2 (present in 95 % of the patients) or HLA-DQ8 (present in most of the remaining patients) on the surface of antigen-presenting cells (APCs). The interaction of loaded APCs with reactive T cells leads to the release of pro-inflammatory cytokines and tissue damage.

It has been noted in recent years that environmental factors associated to a decreased or increased risk of celiac disease (i.e., milk-feeding type, breast-feeding, and viral infections) also influence the composition of the intestinal microbiota, and a number of studies have shown that dysbiosis of the gut microbiota is linked to celiac disease (Sanz et al. 2011). The composition of the fecal microbiota of children with active celiac disease was characterized by a decrease in Bifidobacterium, *Clostridium histolyticum*, *C. lituseburense*, and *F. prausnitzii*, while there was an increase in the levels of bacteria belonging to the Bacteroides-Prevotella group (De Palma et al. 2010). The composition of both fecal and mucosa-associated duodenal communities showed a decrease in Bifidobacterium in children with active and non-active disease, while the fecal community of children in the active phase was enriched in Staphylococcus and *E. coli*. However, the count of these two microorganisms was normalized after treatment with a gluten-free diet (Collado et al. 2008a, 2009). The comparison of the mucosa-associated microbiota of children with active disease and in remission, after a 9-month treatment with a gluten-free diet, also revealed that the total diversity of the microbial community was higher in patients in comparison to healthy controls. However, the communities of celiac disease patients were less stable than the healthy ones (Schippa et al. 2010). Rod-shaped bacteria, likely *Clostridium* sp., *Prevotella* sp., and *Actinomyces graevenitzii*, were found to be associated with the epithelium of celiac disease pediatric patients, both active and on a gluten-free diet, during the so-called Swedish epidemics (Forsberg et al. 2004; Ou et al. 2009). In that period, a fourfold increase in celiac disease was observed in 2-year-old children that followed a twofold increase in gluten consumption. Besides the alterations in the gut microbiota, the composition of the fecal SCFA pools of pediatric patients and first-degree relatives was shown to be significantly altered when

compared to healthy controls (Tjellstrom et al. 2005, 2007). The pool of SCFA also differed in asymptomatic children in comparison to healthy controls and was found to resemble the fecal SCFA profile of symptomatic patients (Tjellstrom et al. 2010). These results indicate that the gut microbiota of celiac disease patients also has specific metabolic properties that are different from the microbiota of healthy subjects.

Allergy

Allergic diseases result from the induction of abnormal antibody-mediated immune responses to innocuous environmental antigens. Although genetics play a major role in a person's predisposition to allergy, the increased incidence of allergies in affluent countries since the 1950s is hard to explain solely in terms of genetic factors. As an example, the prevalence of asthma was found to be higher in West Germany in comparison to former East Germany, despite the common genetic background of the population (Bach 2002). Many aspects of everyday life have changed as a result of improved socioeconomic conditions in several countries, and theories have been proposed to explain the increase of allergic as well as other diseases. A couple of decades ago, an inverse correlation between the prevalence of allergic rhinitis and family size was observed by David Strachan (Strachan 1989), which resulted in the formulation of the "hygiene hypothesis." This theory suggests that infections early in childhood could have a protective role against allergic diseases by driving the neonate's immune response, which is Th2-skewed, toward a Th1 response. Indeed, healthy infants exhibit a decrease in Th2 responses after birth while infants that develop allergic diseases show an opposite pattern (Isolauri et al. 2009).

More recently, several authors have proposed that the "hygiene hypothesis" should be modified in order to include a role for the gut microbiota (Bjorksten 2009; Shreiner et al. 2008). The newer hypotheses not only explain the increase in allergy but also the increase of other diseases, such as IBD, T1D (a disease with a Th1-biased immune response), and obesity (Isolauri et al. 2009). The immune system of newborn infants is immature, and the cross talk between the gut microbiota and the immune system is critical for the development toward tolerance or allergy, as shown by the observation that GF mice cannot generate oral tolerance (Lewis et al. 2006; Maeda et al. 2001; Shreiner et al. 2008). Ultimately, the sequential and regulated colonization of the human gut during the first years of life shapes the adult physiology and may have lifelong influences on the organism's well-being. The "microbiota hypothesis" (Shreiner et al. 2008) and the "microbial deprivation hypothesis" (Bjorksten 2009) suggest that continuous exposure to a eubiotic gut microbiota along with its postnatal ecological succession in the intestine, more than sporadic infections, are important factors for the prevention of allergy and other diseases linked to the gut microbiota.

The composition of the gut microbiota is altered between allergic and nonallergic children, as revealed by several studies applying both culture-dependent and culture-independent techniques. Reduced diversity in the fecal microbiota of infants that develop allergy is observed early in life (i.e., 18 months) (Wang et al. 2008), and studies in children up to 5 years of age have shown that differences in gut microbiota composition could be detected before the onset of the disease (Kalliomaki et al. 2001; Sjogren et al. 2009). In particular, decreased Lactobacillus and Bifidobacterium levels and early colonization with *Clostridium* spp. were often positively associated with the development of allergy. In a study comparing the fecal microbiota of Estonian and Swedish children, Björksten and colleagues observed increased incidence of allergy in Sweden in comparison to Estonia. However, the fecal microbiota of allergic children from either country had a more similar composition that differed from the nonallergic population. These findings suggested that intestinal dysbiosis may be a common feature in allergy, independent of geographical and other differences possibly existing between the two countries (Bjorksten et al. 1999; Voor et al. 2005). Although a causative relationship between intestinal dysbiosis and allergy has not been established thus far, experimental evidence shows that alteration of the intestinal microbiota by antibiotic treatment and oral exposure to *Candida albicans* can mediate a break in airway tolerance to fungal allergens (Noverr et al. 2005). In summary, the lack of early exposure of infants to specific microorganisms and/or the elimination of beneficial microbes from the intestinal environment due to modern lifestyle in affluent countries may explain the increased incidence of immunologically mediated diseases in those countries.

Autism Spectrum Disorders (ASD)

The gut microbiota has been suggested to affect organs other than the intestine and as far away as the brain (Heijtz et al. 2011). Autism spectrum disorders (ASD) include a number of related neurodevelopmental conditions that can be detected early in childhood and are characterized by communicative, social, and behavioral problems. The prevalence of ASD in the general population is close to 1 %, with an increasing trend in the last few decades, which is partly explained by the greater attention reserved to autism in the media and the improved detection and/or diagnosis of the disease. Little is known about the etiology of ASD, but genetics and environmental triggers have been equally implied (Bailey et al. 1996; Herbert et al. 2006). Autistic children experience a range of dietary and gastrointestinal problems, and the associated symptoms have been connected to intestinal dysbiosis and overgrowth of potentially pathogenic and neurotoxin-producing microbes, such as clostridia (Bolte 1998; Parracho et al. 2005). Oral administration of vancomycin, an antibiotic active against Gram-positive bacteria, has been shown to improve ASD, but the benefits were limited to the period of treatment, and the condition regressed within 2 weeks from the discontinuation of the antibiotic (Sandler et al. 2000). As vancomycin is only minimally absorbed in the intestine, its effects are likely to be mediated by its activity on the gut microbiota. A circumstantial explanation of these results was given by

hypothesizing a reduction in neurotoxin-producing clostridia following vancomycin treatment while recolonization of the intestinal environment by clostridial spores would cause the relapse of the disease. It is also interesting that ASD patients often have a history of multiple exposures to antibiotics, which are known to alter the structure of the gut microbiota. Treatment with gluten-free and casein-free diets has been associated with improved gastrointestinal symptoms and behavior in some children (Knivsberg et al. 2002; Whiteley et al. 2010), thus providing support for the hypothesis that the gut microbiota may play a role in ASD (Finegold 2008). By using culture-based techniques, the fecal microbiota of ASD patients was found to contain ten times more clostridia than healthy controls, with selected species only present in autistic individuals (Finegold et al. 2002). In another study, increased levels of *C. histolyticum* were detected (Parracho et al. 2005). Finally, a recent profiling of the fecal microbiota by pyrosequencing of the 16S rRNA gene revealed a variation in the diversity of the gut microbiota associated with ASD: in particular, an increase in Bacteroidetes and a decrease in Bifidobacterium were observed in severely autistic children compared with healthy controls (Finegold et al. 2010).

Microbial metabolism has also been studied in the context of ASD. A recent ^{1}H-NMR metabolomic profiling of urine from ASD patients, unaffected siblings, and healthy controls has revealed decreased levels of the mammalian-microbial cometabolites hippurate and phenylacetylglutamine (Yap et al. 2010). These aromatic compounds are produced by the gut microbiota as a result of benzoate metabolism, and the decrease in their levels in individuals with ASD was suggested to derive from diminished production of benzoic acid by the gut microbiota. The authors of this study also notice that depletion of hippurate and phenylacetylglutamine was observed after oral treatment with vancomycin, thus providing a link for the functional role of the gut microbiota in ASD.

Obesity and Type 2 Diabetes

The prevalence of obesity has been dramatically increasing during the last 30 years, and the latest estimate says that today as many as 1.5 billion people are overweight and least 300 million of them obese. Obesity is recognized as a chronic disease associated with a low-grade systemic inflammation and is a major risk factor for type 2 diabetes mellitus (T2D), hypertension, and cardiovascular disease. Altogether, these diseases are known as the metabolic syndrome and are one of the major public health concerns. Particularly alarming is also the hypothesis that obesity could be transmissible, as maternal obesity seems to be a risk factor for adulthood obesity (Lawlor et al. 2007). Development of obesity depends on the interplay of genetic and nongenetic factors (i.e., age, diet, and lifestyle) governing the balance between energy intake and expenditure. The range of these factors goes beyond individual nutritional habits and amount of physical activity: complex regulatory mechanisms, link digestion of food, and absorption of nutrients in the intestine with hypothalamic regulation of energy balance and feeding behavior.

The intestinal microbiota acts at the interface between the food that we ingest and the nutrients that we absorb, and consequently, it plays a fundamental role in nutrition. During the coevolution of the human species and its gut microbiota, a metabolic partnership has evolved and the microbial symbionts that inhabit the gut have provided the genes for the degradation of dietary fibers and production of amino acids and vitamins that are not encoded by the human genome. Research conducted on GF mice has proven that the gut microbiota is an environmental factor that affects energy homeostasis. In these studies, the gut microbiota has been shown to (1) promote monosaccharide absorption in the intestine, (2) increase the amount of calories that can be extracted from the food through fermentation of nondigestible fiber, (3) increase the storage of these calorie in fat tissue, and (4) decrease fatty acid oxidation in muscle (Bäckhed et al. 2004). Additionally, microbial production of SCFA also influences the hormonal regulation of glucose homeostasis, intestinal motility, satiety, and feeding behavior by affecting the levels of ghrelin, peptide YY (PYY), and glucagon-like peptide-1 (GLP-1) (Cani and Delzenne 2009).

Recent studies in humans have shown that obesity is associated with intestinal dysbiosis. The first human studies on the impact of the gut microbial community in obesity showed that the fecal microbiota of obese individuals differed greatly from that of lean controls at the phylum level: the obese microbiota was enriched in Firmicutes and depleted in Bacteroidetes, so displaying a high Firmicutes/Bacteroidetes ratio. This ratio was observed to decrease upon weight loss after either low-carbohydrate or low-fat diets as a consequence of increased Bacteroidetes levels (Ley et al. 2006). In later studies, the obese microbiota was shown to be characterized by decreased phylogenetic diversity and enrichment in gene coding for enzymes involved in energy harvesting (Turnbaugh et al. 2009). Although an increased Firmicutes/Bacteroidetes ratio in obesity was shown also in other studies (Santacruz et al. 2010; Turnbaugh et al. 2009), several reports did not confirm this initial observation (Collado et al. 2008b, Schwiertz et al. 2010), and it was suggested that increased concentrations of fecal SCFA could be more relevant for obesity than the variation in the composition of the gut microbiota (Schwiertz et al. 2010). However, differences at lower taxonomical levels than phylum level are detected in a large number of studies. Early fecal samples of infants that developed obesity by the age of 7 had high Staphylococcus counts while corresponding samples of infants that remained lean were dominated by Bifidobacterium, *B. longum*, and *B. breve* in particular (Kalliomaki et al. 2008). Additionally, weight loss in obese adolescents subjected to a low-calorie diet was associated to differences in the gut microbiota (Santacruz et al. 2010): high weight loss was observed in subjects whose intestinal community before the beginning of the intervention was enriched in *B. fragilis*, *C. leptum*, and *B. catenulatum*, while containing lower numbers of *C. coccoides*, *B. breve*, and *B. bifidum*. Finally, intestinal colonization by methanogens, such as *M. smithii*, has been reported as an additional factor that may affect the development of obesity (Million et al. 2011; Schwiertz et al. 2010). Hence, although there is no consensus for

a clear compositional profile of the obese microbiota, dysbiosis and differences in SCFA are two features consistently associated with obesity.

Obesity and T2D are characterized by a low-grade systemic inflammation, which has been suggested to derive from the infiltration of immune cells (macrophages) in adipose, liver, and muscle tissue. Infiltrating macrophages are proposed to release pro-inflammatory factors that alter the host's homeostatic metabolic signaling and cause resistance to insulin. Nevertheless, the role of macrophages and the identity of the inflammatory triggers are not clarified. Recent data indicate that the gut microbiota might contribute to the development of the low-grade inflammation associated with insulin resistance in metabolic diseases. In this context, lipopolysaccharide (LPS) has been suggested as a microbial trigger of inflammation. Increased plasma levels of LPS were originally observed in mice fed a high-fat diet and then associated with increased gut permeability (Cani et al. 2007). Infusion of physiologic concentrations of LPS and high-fat feeding were observed to induce inflammation, insulin resistance, and increased fat mass deposition in mice. In these obese mice, high-fat feeding was also shown to reduce fecal levels of Bifidobacterium and Bacteroides. Similarly, the interplay between metabolic endotoxemia (i.e., high LPS level in plasma), consumption of high-fat diet, and development of obesity and T2D was confirmed in humans, and recent data indicate that visceral adiposity correlates with increased gut permeability in overweight women (Gummesson et al. 2011). A few studies have analyzed the correlation between gut microbiota, T2D, and inflammation and have shown that *F. prausnitzii* is depleted in T2D patients (Furet et al. 2010). Additionally, the abundance of Firmicutes seems to decrease while the abundance of β-Proteobacteria increases in T2D in comparison to healthy controls. In this context, the ratios Bacteroidetes/Firmicutes and Bacteroides-Prevotella/*C. coccoides-E. rectale* were observed to be correlated with plasma levels of glucose (Larsen et al. 2010).

Therapeutic Modulation of the Gut Microbiota

The observation that intestinal dysbiosis is associated with human diseases opens to the possibility of manipulating the intestinal microbiota in order to prevent or cure pathological conditions. To achieve this goal, it is necessary to provide a better understanding of the intrinsic features that characterize a "healthy gut microbiota" as opposed to a disease-associated gut microbiota. For example, it needs to be confirmed that a "core human microbiome" exists at the species level (Qin et al. 2010) and at the genetic level (Turnbaugh et al. 2009). At the same time, the mechanisms and microbial players involved in the etiology of specific diseases need to be characterized, and, besides the phylogenetic composition and the genetic ability of the community, the functionality of the microbiota needs to be assessed. The possibility of developing therapeutic measures based on the modulation of the gut microbiota is complicated by the observation that each individual is endowed with a unique microbiota. Hence, individuality in the gut microbiota might also require a personalized therapy based on the knowledge of subjective microbial requirements.

Ilya Mechnikov, who was awarded a Nobel Prize in 1908 together with Paul Ehrlich for their work on immunity, was the first to hypothesize that the use of live bacteria could be associated to health-promoting effects. At that time, he drove his conclusion by observing that a population of elderly healthy Bulgarians consumed fermented milk, to which he attribute beneficial effects. Today, several approaches are being developed for the manipulation of the gut microbiota, namely, the use of probiotics, prebiotics, synbiotics, and, recently, the transplantation of intestinal microbiota.

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit for the host" (FAO/WHO 2002). Prebiotics are "non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host" (Roberfroid 1998). It is implicit in the last definition that, in order for the prebiotic to produce health benefits, the gut microbiota needs to contain the microorganism(s) able to degrade the fibers that are supplemented. Therefore, synbiotics are developed and consist in mixtures of probiotic(s) and prebiotic(s). In the mixture, the prebiotic part is selected in order to promote the growth and/or function of the associated probiotic(s), which commonly is able to ferment only a selected range of fibers. As an example, amylase-resistant starch selectively increases the numbers of bifidobacteria and lactobacilli while decreases the levels of Enterobacteriaceae. Probiotics and prebiotics will be discussed further in dedicated chapters of The Prokaryotes.

Another possible therapeutic intervention that has recently received renewed attention is transplantation of fecal microbiota from healthy donors. The first report on fecal transplantation dates back to 1958 and describes the treatment of four patients affected by pseudomembranous colitis, a pathology caused by opportunistic infection of *Clostridium difficile* (CDI). Treatment of pseudomembranous colitis is clinically challenging as this pathology is characterized by fulminant disease and recurrent infections. Up to now, the treatment of 200 cases of refractory CDI has been described, with a success rate of 90 % and no ill effects reported (Khoruts and Sadowsky 2011). More recently, a clinical study was initiated that showed that the transplanted microbiota quickly colonizes the gut of the receivers and represents the dominant microbiota for at least 1 month (Khoruts and Sadowsky 2011). For CDI, this stable association results in the resolution of symptoms, thus indicating that the patients' dysbiotic microbiota is likely to cause the disease.

Recent findings have suggested that fecal transplantation may be a feasible approach for the treatment of metabolic diseases. In a preliminary report, Koote and colleagues have shown that transplantation of fecal microbiota from healthy donors to newly diagnosed metabolic syndrome patients (i.e., obese subjects with impaired fasting glucose), but not

autologous transplantation, significantly improved glucose metabolism after 6 weeks although no effect was observed on body weight (Kootte et al. 2011). Besides the relevance of this approach for the development of new therapeutic strategies, transplantation of human microbiota in animal models will add to the current knowledge of the mechanisms of human disease and might be useful for testing the applicability of microbial interventions for the treatment of human diseases associated with intestinal dysbiosis.

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