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STUDIES ON THE HUMAN GUT MICROBIOTA

Relevance for maturation, inflammation and nutritional processes

| Professorial dissertation

Faculty 09 - Agricultural Sciences, Nutritional Sciences and Environmental Management

For Benjamin and Robin

OBSTACLES ARE THOSE FRIGHTFUL THINGS YOU SEE WHEN YOU TAKE YOUR EYES OFF YOUR GOAL

Henry Ford (1863 – 1974)

CONTENTS

For centuries, bacteria have been perceived as pathogenic organisms. Only in the last decades it has been recognized and accepted that all external human body surfaces have a natural resident microbiota (**Figure 1**)(1). Because of cell turnover, all human surfaces are coated with dead and desquamating cells, which provide an excellent nutrient source. In addition, body fluids and foods, which pass the human body via the mouth and intestine, supply the microorganisms with nutrients. With few exceptions (e.g. the stomach), the communities consist of large numbers of microbes and have a rather complex composition. This complexity of microbial communities found at many body sites is truly astounding; it has been estimated that the human body carries around 1000 different microbial taxa with 10^{13} to 10^{14} microorganisms in total, of which the collective genome ("microbiome") contains more than 150 times as many genes as the human genome (2).

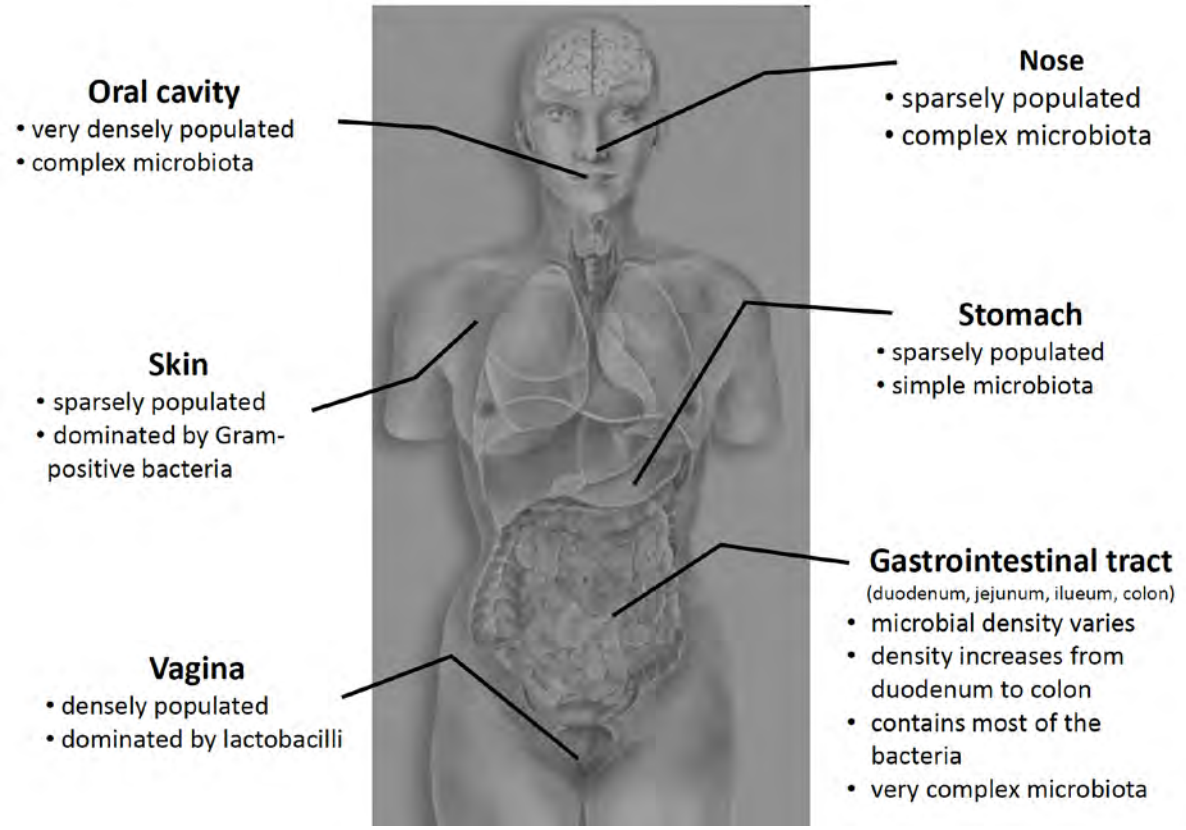


Figure 1 **Microbial communities inhabiting various sites of the human body**

The majority of microbes resides in the gut, especially the colon. In that respect it is not surprising that the human gut microbiota, which represents the major part of the human microbiome, is often referred to as the "forgotten organ" (3). However, most of these microorganisms have not been cultivated yet. Nevertheless, modern molecular approaches may enable us to detect their presence, their role in health and disease and to gain insight into their physiological traits. This human-microbe interaction is now often referred to as symbiotic.

The indigenous microbiota of the gastrointestinal tract

The importance of the gastrointestinal tract (GIT) and its microbial inhabitants for health has now been widely recognized. The intestinal mucosa is the largest interface between the human internal milieu and the outside world. It has been estimated that it covers an area of about 1000 m^2 (1).

Under normal circumstances, the GIT of a foetus is sterile. During the birth process and rapidly thereafter, microbes from the mother and the surrounding environment colonize the GIT of the infant, until a dense, complex bacterial community is established (4, 5). The first colonizers in humans are members of the taxa *Enterobacteriaceae* and *Lactobacillales.* The *Enterobacteriaceae* consume the oxygen present and thus decrease the redox potential in the intestine. It has been estimated that in newborns the redox potential is reduced within two days from 178 mV to -113 mV. In later life the redox potential ranges from – 200 mV to -300 mV (6). Due to the decrease of oxygen tension strictly anaerobic microbes increase in numbers. Breast-fed full term infants show a faecal bacterial composition, in which bifidobacteria predominate over potentially harmful bacteria, whereas in formula fed infants coliforms, enterococci, and bacteroidetes predominate (7). The process of colonisation is

greatly influenced by the successive shifts from exclusive breast-feeding to partial and full substitution of breast milk by formula feeding and finally to the introduction of solid food (8). Bacterial successions in infants have been widely documented, but there are relatively few studies on the changes in the microbiota during the aging process (7, 9-12).

A key problem in determining the composition of the human microbiota of the GIT is obtaining samples for analysis. Ethical and technical limitations do not allow sampling at all sites of the GIT. Hence, faecal samples are widely used for the determination of the colonic microbiota, as the composition of the microbiota of faecal samples and the distal colon resemble each other. Analysis of the microbial communities colonising the human GIT by culture and molecular techniques revealed a rather complex microbiota, with an estimated 500 different phylotypes present (13). Of the used methods, those sequencing the full-lenght of the 16S rDNA gene through PCR and cloning provide the most powerful taxonomic resolution (14). However, studies which used the conventional Sanger sequencing method have been forced to use rather small sample sizes due to its cost and labor input. Thus, only dominant members of the microbial communities have been described, leaving major parts of the diverse microbiota undetected (15).

Recent advances in sequencing technology, such as the 454 pyrosequencing approach, are changing our way to study microbial communities. First results of the MetaHit Consortium (http://www.metahit.eu), a project supported by the European Commission within the 7th FP program, indicate that the entire cohort of 124 European individuals harboured between 1,000 and 1,150 bacterial species and each individual at least 160 different species (**Table 1**)(2).

While large studies using deep sequencing methods have revealed a greater diversity of the human microbiota than previously estimated, even these studies have proved insufficient to fully understand these communities (16-19).

Though, even if the molecular approach became very popular, it is still important to try to cultivate the hitherto unculturable microorganisms. Culturing and characterisation alone allow for a better understanding of the physiological capabilities of the bacteria and their role in the human–microbe interactions. Over the last decade, several new genera and species from the human gut were newly isolated (20-22, 22, 23).

The human gut microbiota represents an enormous metabolic potential, which is by far greater than that of the human body. The metabolism of the gut microbiota is essential to the biochemical activity of the human body, resulting in the generation of absorbable products, the detoxification of compounds and production of vitamins and other beneficial nutrients (24). Hence, humans can be seen as superorganisms, whose metabolism represents the combination of both microbial and human features (25).

The gut microbiota also regulates many aspects of innate and acquired immunity, protecting the host from pathogen invasion and chronic inflammation. In contrast, imbalances in the composition of the gut microbiota have been associated with susceptibility to infections, immune disorders, and recently with insulin resistance and body weight gain (26, 27).

Table 1 Major groups of microorganisms isolated from human faecal samples

Domain	Phylum	Order	Genus
Eukarya	*Ascomycota*	*Saccharomycetales*	- *Candida*
Archaea	*Euryarchaeota*	*Methanobacteriales*	- *Methanobrevibacter* - *Methanosphaera*
Bacteria	*Firmicutes*	*Clostridiales*	- *Anaerostipes* - *Clostridium* - *Eubacterium* - *Ruminococcus* - *Roseburia* - *Dorea* - *Blautia* - *Faecalibacterium*
		Bacillales *Lactobacillales*	- *Staphylococcus* - *Streptococcus* - *Lactococcus* - *Lactobacillus*
	Bacteroidetes	*Bacteroidales*	- *Bacteroides* - *Prevotella* - *Porphyromonas* - *Alistipes*
	Proteobacteria	*Enterobacteriales*	- *Escherichia* - *Klebsiella* - *Proteus*
	Fusobacteria	*Fusobacteriales*	*Fusobacterium*
	Verrucomicrobia	*Verrucomicrobiales*	- *Akkermannsia*
	Actinobacteria	*Bifidobacteriales* *Coriobacteriales*	- *Bifidobacterium* - *Coriobacterium* - *Atopobium* - *Collinsella*

Microbiota and maturation

The presence of an autochthonous intestinal microbiota in infancy is critical for numerous physiologic processes including growth, angiogenesis, optimisation of nutrition, and stimulation of various arms of the innate and adaptive immune system (28-30). Thus, a dynamic balance exists between the gastrointestinal bacterial community, host physiology, and diet, all of which influence the initial acquisition, developmental succession, and eventual stability of the gut ecosystem. The microbial colonization process of human body habitats begins at birth, when the baby leaves the uterus. The initial microbial communities are assembled from organisms in the immediate surroundings: for vaginally delivered infants, the mother's vagina is a major source of the initial colonising bacteria, and for those born by caesarean section, the hands that touch the baby are a dominant source (31). Since the pioneering study of Tissier in 1900 (32), several studies have described the bacterial succession in this system based on analysis of the microbiota in infants' stools (**Figure 2**)(7, 8, 12, 33-38). Gut communities start with low phylogenetic and species richness, which increases over time (5). Time in this context reflects the rate of encounters with new bacteria, the increasing size of the gut or the proliferation of ecological niches that can promote diversity. The introduction of solid foods alters the relative proportion of bacterial phyla in the gut and is followed by the establishment of an adult-like microbiota with greater stability and characterized by a full suite of functions. Thus, infancy is a period of rapid colonisation by microbial consortia that can shift in response to events such as illness or changes in diet.

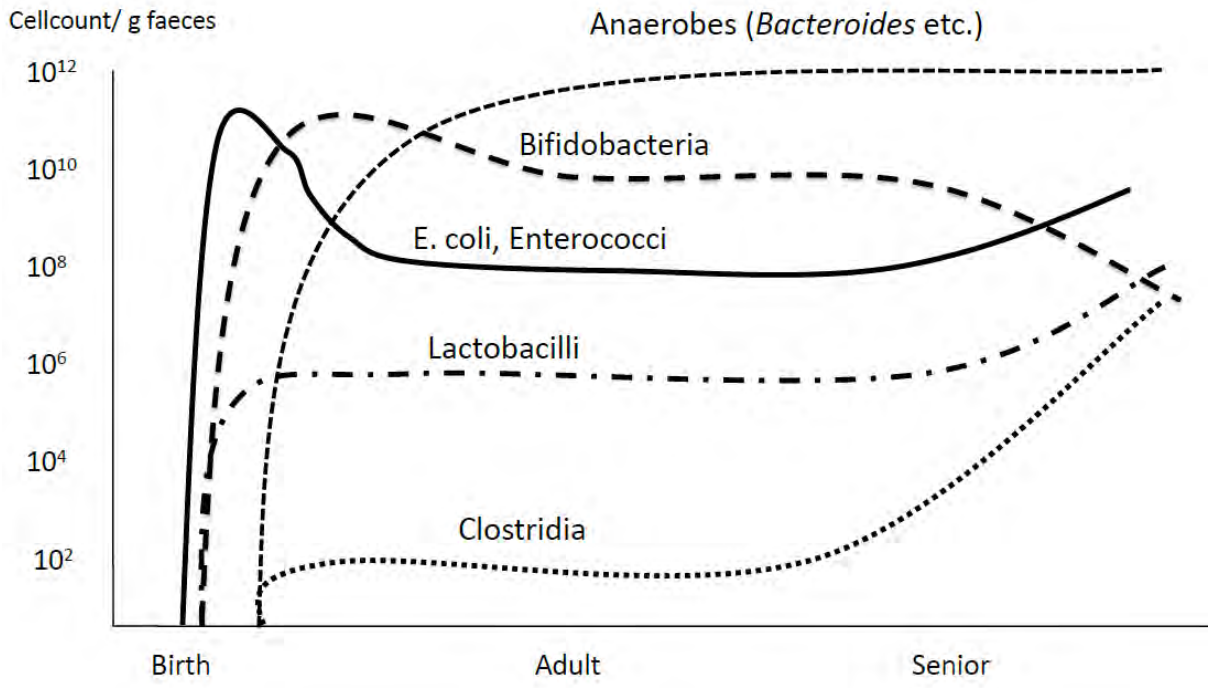


Figure 2 Culture dependent perspective of the bacterial succession in the gut (12)

In full-term vaginally delivered infants, colonization starts immediately after delivery, and enterobacteria and enterococci appear in faeces. The composition of the gut microbiota is profoundly influenced by the diet of the infant. Thus, in breast fed children prior to weaning, the faecal microbiota is dominated by species of the genus *Bifidobacterium,* which belongs to the phylum *Actinobacteria.* With age the number of bifidobacteria declines in the human body (4). They constitute about 90% of the infant's intestinal bacteria. When breast-fed infants' diets are changed to formulas based on cow's milk and solid food, bifidobacteria are joined by rising numbers of other bacteria found in the human body, such as *Bacteroides* spp., *Enterococcus* spp. and *Lactobacillus* spp. (39, 40). Lower numbers of bifidobacteria are found in formula-fed babies, which might account for a higher risk of diarrhoea, atopy and asthma. The prevalence of these diseases may be higher in babies, who are not breast-fed (41, 42). However, this assumption remains controversial, as several longitudal studies show

inconsistent results (43-45). But as bifidobacteria produce lactic acid instead of gas as a metabolic endproduct, people in general and particularly infants with a higher proportion of bifidobacteria tend to have less gas and digestive problems. In the very young, differences in the composition of the microbiota are a consequence of many factors, including an immature immune system, hospitalisation and nutrition.

Contributions to our knowledge on the composition of the paediatric microbiota have been gained by studies on the development of the intestinal microbiota in hospitalised pre-term infants in comparison to breast-fed full-term infants and during infancy. It is evident that the gut microbiota shows both a general and a host-specific pattern of maturation, which is most profound within the first year of life (8) and which depends on the environment of the host (46).

In contrast to children, whose intestinal microbiota is dominated by the phylum *Actinobacteria*, the adult microbiota is dominated by the phyla *Firmicutes* and *Bacteroidetes* (9). Major changes of the gut microbiota due to age are mainly seen within the last decades (more than 60 yrs) of life (46). It remains to be determined, whether these shifts reflect changes in the body itself, such as an altered mucosal innate immunity, or if it is an indirect consequence of different nutritional habits. However, adequate knowledge on the types of microorganisms as well as the events that influence the timing and process of colonisation, may provide opportunities to modulate the microbiota when necessary and thus, to enhance its function and benefit to the human host.

Microbiota and the immune system

The intestinal immune system is separated from the vast luminal microbiota by as little as a single epithelial layer. Gut lymphocytes furthermore undergo critical developmental transitions after encountering intestinal antigens in local lymphoid structures such as the Peyer's patches (47, 48). Gut microorganisms are known to interact with the host immune system, to prime it and maintain homeostasis (49, 50). A key immunoregulatory property of gut bacteria is their ability to vary their surface properties; one example is the synthesis of certain polysaccharides that induce an immune response (51, 52).

Studies in germ-free mice have indicated that gut bacteria influence the maturation and function of several components of the mucosal immune system. Furthermore, comparisons of germ-free and colonised mice have revealed that microbes drive the production of mucosal immunoglobulin A (53) through the involvement of dendritic cells (54). The majority of IgA is thought to be relatively unselective, because it binds with epitopes that are widely shared among gut bacteria (55). IgA is generally thought to limit microbial penetration into the mucosa, and it can trigger bacterial agglutination in the mucus, enhancing clearance via peristalsis (56, 57). In addition to IgA, the host produces antimicrobial proteins (e.g. defensins, cathelicidins, and C-type lectins) in all epithelial cell lineages (56, 58). Many are expressed constitutively (59). Others (e.g. C-type lectins) are triggered by the presence of bacteria (60). These compounds vary in their killing selectivity (29, 61). Naturally occurring levels of antimicrobial peptides are far lower in the lumen than in the mucosa (56), so their impact on microbial populations in the lumen is uncertain.

Microbiota and disease – a dysbiotic problem?

The history of the bowel toxaemia theory reaches as far back as Hippocrates, who stated in 400 BC: “death sits in the bowels” and “bad digestion is the root of all evil”. At the beginning of the last century, the Nobel laureate Elie Metchnikoff proposed that the gut microbiota produces small amounts of toxic substances, which damage the nervous and vascular system and lead to aging (62). The bowel toxaemia theory eventually evolved into the intestinal dysbiosis theory. A still popular, however unfavourable term for an imbalanced microbiota. It describes a condition of microbial imbalance in a given habitat on or within the body.

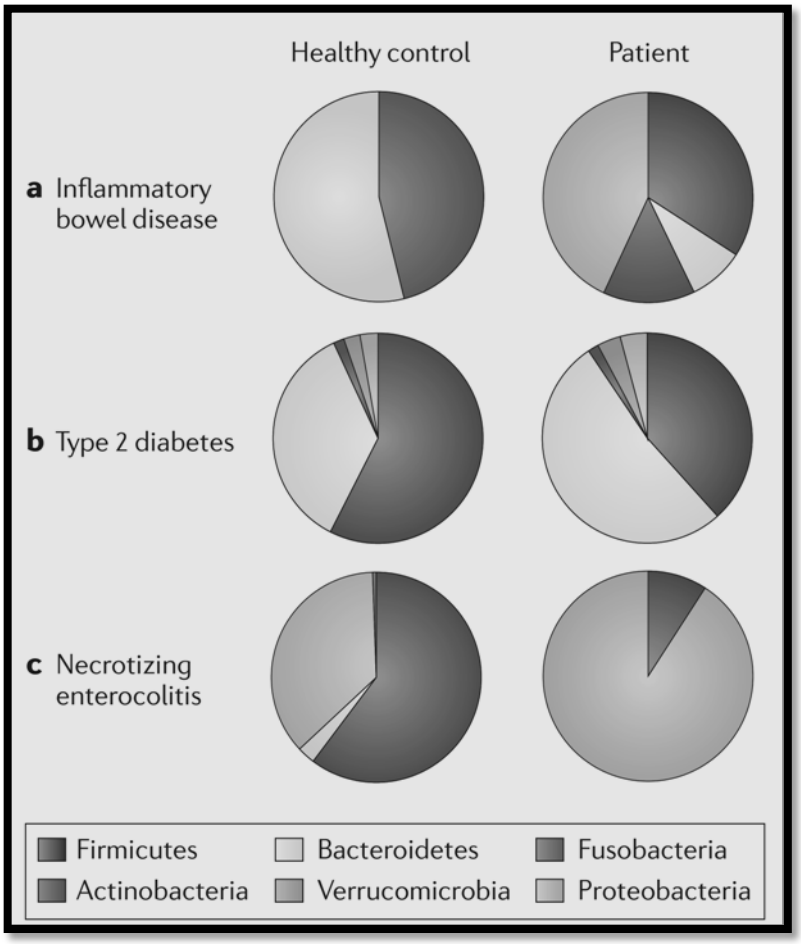


Figure 2 **Gut microbial dysbiosis associated with diseases**

Dysbiosis is most prominent in the digestive tract or on the skin, but can also occur on any exposed surface or mucous membrane such as the vagina, lungs, nose, sinuses, ears, nails, or eyes. Dysbiosis as a medical condition is not listed in the International Classification of Diseases. However, gut bacteria have long been known to influence a variety of diseases. It becomes more and more evident that the human microbiota is linked to diseases such as inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), allergy, and maybe diabetes and obesity (63-67)(**Figure 3**).

Dysbiosis and inflammatory bowel diseases

An increasing number of clinical and experimental findings has provided evidence for changes in the composition of the microbiota in the pathogenesis of IBD. Data from human studies corroborate the hypothesis that there is an association between the gastrointestinal flora and intestinal inflammation. The idea that a changed or dysbiotic microbiota may play a role in IBD was introduced by Shorter et al. in the 1970ies (68). Since then, paramount information has been gathered from animal and human studies in favour of the concept that a dysregulated response to the normal microbiota also plays a critical role in the development of IBD (69). The sites that are typically affected by inflammation in IBD patients are those, which are colonised by the highest numbers of bacteria, i.e. the distal ileum and the colon. Furthermore, the use of antibiotics has shown positive effects on the inflammation in IBD patients, thus supporting the notion that bacteria play a role in the development of the disease (70). However, it remains unclear whether the stimulus in IBD patients is a regular response to an as-yet unrecognized and persistent specific bacterial species or whether the inflammation represents a dysregulated response to antigens readily present in the intestine under normal conditions. In recent years, a decrease in the abundance and biodiversity of intestinal bacteria within the dominant phylum *Firmicutes* has

been observed repeatedly in Crohn's disease (CD) patients (71-74). Sokol et al. reported that the proportion of *Firmicutes*, and in particular of *Faecalibacterium prausnitzii*, which is a numerical important coloniser, was low in patients that exhibited endoscopic recurrence six months after surgery. Sokol et al. could show that by administering *F. prausnitzii* into a murine model of severe intestinal inflammation the inflammation was reduced (75).

Dysbiosis and obesity

Obesity results from an increased energy intake and subsequent alterations in the body's regulation of expenditure and storage. Recent evidence, primarily from investigations in animal models, suggests that the gut microbiota affects nutrient acquisition and energy regulation. Comparisons of the genomes of gut microorganisms with the genomes of microorganisms living elsewhere have shown that distantly related members of the Bacteria and even the Archaea use a remarkably similar gene portfolio for life in the gut. These genes include a diverse range of carbohydrate-active genes, adhesins and bile salt hydrolases to facilitate processing of the diet and bacterial retention in the gut (76, 77). The initial link between gut microbial ecology and obesity was made in mice by Ley et al. (**Figure 4**)(78). Results from a 16S rRNA gene sequence survey revealed that the bacterial communities in the ceca of obese mice had higher numbers of *Firmicutes* and fewer counts of *Bacteroidetes,* when compared to those of lean wild-type or heterozygous mice. Members of the phyla *Firmicutes* and *Bacteroidetes* represent up to 90% of the total human gut microbiota.

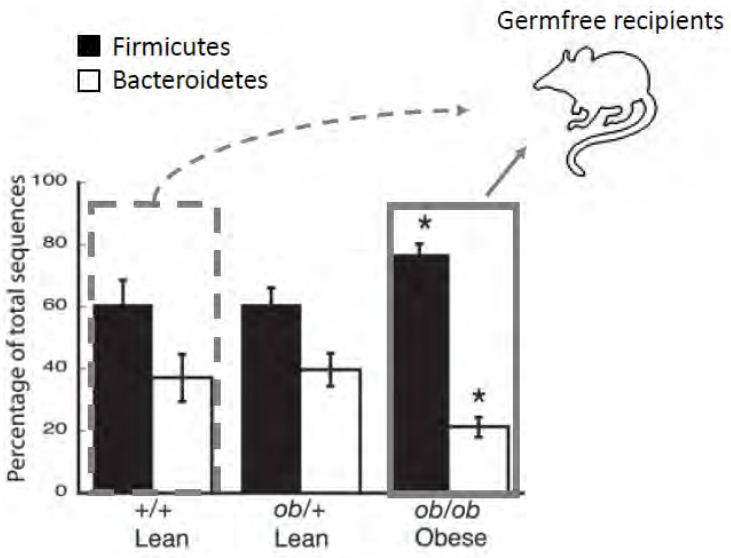


Figure 4 The adipose phenotype is transmissible by microbiota transfer (78)

A subsequent metagenomic analysis of these same microbial communities, which was based on shotgun sequencing of the microbial community DNA, showed an enrichment in genes involved in energy extraction from food in the obese host's microbiome relative to that of the lean host's microbiome (79). A microbiota with greater energy extraction efficiency resulted in less energy remaining in faeces and greater levels of short-chain fatty acids (SCFAs) in the cecum. Furthermore, when the luminal contents from the ceca of obese or lean mice were transferred to lean germ-free recipients, the mice receiving the microbes from the obese donors gained more weight over a 2-week period than recipients of the lean microbiota, despite equivalent food intake (79). Since then, several studies have been conducted on the effect of weight loss, pregnancy or physical activity on the composition of the gut microbiota in humans (17, 80-84).

Interactions among microorganisms in the gut appear to play an important role in host energy homeostasis, with hydrogen-oxidizing methanogens enhancing the metabolism of fermentative bacteria. Gill and co-workers found that the human gut microbiome of two

healthy lean subjects is enriched with many clusters of orthologous groups (COGs) representing key genes of the metabolic pathways of methanogens (25). Since *Methanobrevibacter smithii* is the major representative of the human gut methanogens (13), this finding led to the hypothesis that *M. smithii* may be a therapeutic target for the reduction of energy harvest in obese humans (85, 86).

Microbiota and nutritional processes

In a world of rapidly changing food habits and stressful life styles it is more and more acknowledged that a healthy digestive system is essential for an overall quality of life. Thus, the metabolic activities of the various microbial gut inhabitants came increasingly into focus. It is now widely accepted that the indigenous microbiota plays a crucial role in the maintenance of health by its metabolic capacity to ferment and transform food constituents like fibre and secondary plant metabolites.

Dietary fibres

The physiological effects of dietary fibres are significantly influenced by the degree, to which the fibres are fermented in the colon. Colonic fibre fermentation leads to the production of SCFA, gases (CO_2, CH_4, H_2 and H_2S) and microbial cell mass. The various SCFAs produced are metabolized by different pathways in the body. Propionate is utilized primarily in the liver and has been suggested to be a potential modulator of cholesterol synthesis and a precursor in liponeogenesis, which may influence body weight. Acetate is largely metabolized by peripheral tissues (i.e. muscle) or bacteria (87, 88). Whereas butyrate is the preferred energy source for colonocytes and thus is extensively metabolized by the colon (89). Butyrate and other SCFA have also been implicated in providing protection against

cancer (**Figure 5**)(90, 91). Hence, in recent years considerable efforts have been put into the detection and isolation of butyric acid producing bacteria (23, 92-95).

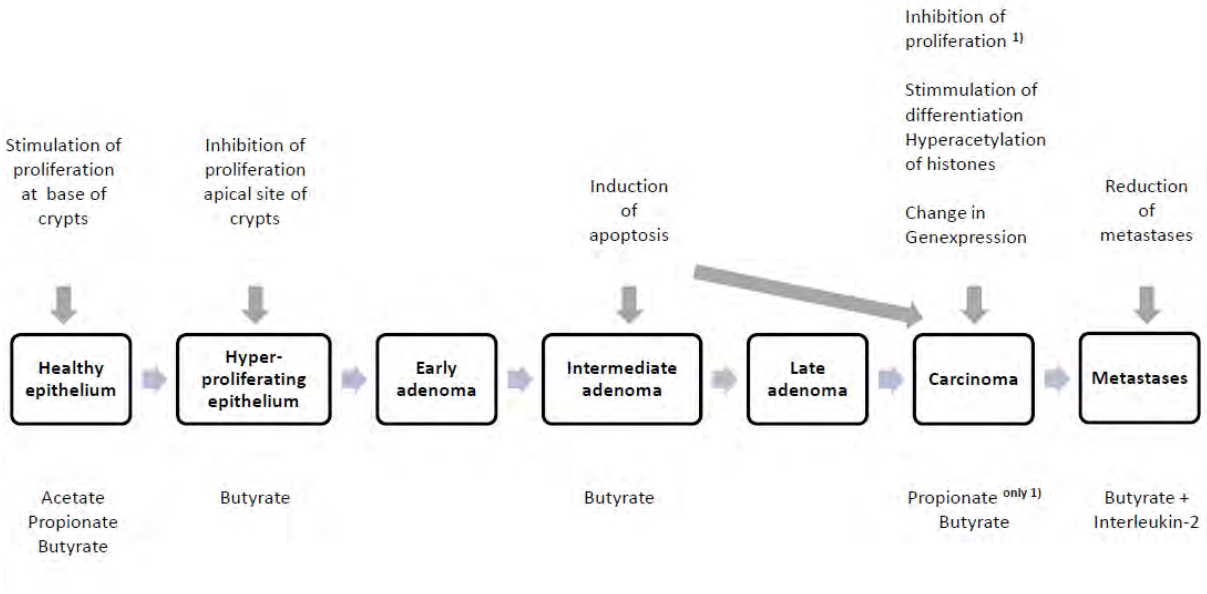


Figure 5 Proposed roles of SCFA in colon cancer prevention

Numerically, two of the most important groups appear to be *Faecalibacterium prausnitzii*, which belongs to the *Clostridium leptum* cluster (Clostridial cluster IV), and *Eubacterium rectale/Roseburia spp.*, which belong to the *Clostridium coccoides* group (Clostridial cluster XIVa) of firmicute bacteria. Estimates based on fluorescent *in-situ* hybridisation (FISH) and real-time PCR detection indicate that each of these groups typically accounts for around 5–10% of the total bacteria detectable in faecal samples from healthy human adult subjects.

A wide range of polysaccharides can be utilized by various butyrate-producing firmicutes, suggesting that these bacteria make an important contribution to the colonic fermentation of dietary components. Bacteria related to *Eubacterium hallii* and *Anaerostipes caccae* within the *C. coccoides* cluster were shown to be able to convert

acetate and lactate into butyrate, in addition to producing butyrate from carbohydrates (88, 94, 96).

Additionally, butyrate producers have been implicated to ameliorate IBD (71, 75). In particular, populations of *F. prausnitzii* appear to be reduced in faecal samples and on the gut mucosa of Crohn's disease patients (71). This may have a negative impact on the supply of butyrate for the gut epithelial cells. Furthermore, recent evidence strongly suggests that *F. prausnitzii* produces a separate anti-inflammatory factor (71).

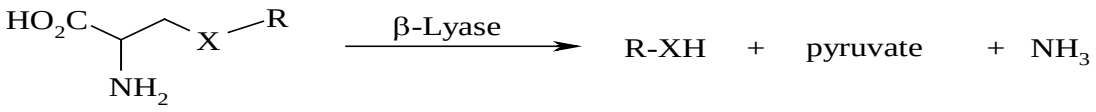
Aside from the studies on the positive effects of butyric acid, there has been considerable interest in the discovery of natural occurring compounds with a high potential of beneficial effects such as anti-inflammatory, antioxidant, vasodilatory, anticancerogenic, and antibacterial properties. Among these so-called protective agents there is a considerable number of sulphur- and especially selenium-containing compounds and flavonoids, which are widely distributed in plants and are ingested in high amounts with food.

Flavonoides

More than 5,000 different naturally occurring flavonoids have been described so far. Although it is known that human intestinal bacteria play a significant role in the degradation of flavonoids (97), there is a paucity of information on the species involved, their distribution in humans, and the mechanisms of degradation. *Clostridium orbiscindens* (98, 99)[meanwhile unified with *Eubacterium plautii* in a new genus as *Flavonifractor plautii* gen. nov., comb. nov.,(100)], *Eubacterium desmolans* (98), and *Eubacterium ramulus* (101), all isolated from human faecal samples, are known to convert flavonoids. However, only *E. ramulus* was further characterized with respect to its potential to degrade flavonoids, the pathways of conversion of flavonoids, and the organism's distribution in humans (101).

Selenium compounds

A further topic of interest for human health is the detection of compounds with potential anticancer activity and their transformation by gut microorganisms. Examples are selenium (Se) compounds, which have been shown to be more active than the corresponding sulfur compounds with respect to their anticancerogenic activities (102, 103). Furthermore, studies using cell culture, animal models and humans have demonstrated chemoprotective effects of certain selenium compounds on tumor development (104-106).



X = S; R = Me: *S*-methylcysteine
X = Se; R = Me : *Se*-methylselenocysteine
X = Se; R = *p*-$(CH_3O)C_6H_4CH_2$- : *Se*-*p*-methoxybenzylselenocysteine

Figure 6 β-Elimination of *S*- and *Se*-cysteine conjugates catalysed by β-lyases

Although it is suitable to describe effects of selenium in terms of the element, it must always be kept in mind that chemical form and dose are determinants of its biological activity as cancer preventive or toxic agent. The activation of such compounds is mainly realized by *β*-lyases (**Figure 6**), which are present in the kidney, liver and the gastrointestinal tract and various bacteria of the intestinal microbiota (107, 108). The *β*-lyase of bacteria in the gastrointestinal tract is a key enzyme, because it bio-activates *S*- and *Se*-cysteine conjugates.

Concluding remarks

Environmental factors and host genetics clearly interact in the acquisition and maintainance of the stability of a healthy gut microbiota. In turn, these three components — environment, host genetics and microbiome — interact to maintain homeostasis in the gut. The disruption of this stability by modifying one or more of the three interacting components may be a trigger for the development of diseases.

Although several studies show an effect of the host genotype on the microbiome using faecal and cecal samples, bacteria inhabiting specific locations of the gut, such as mucosal surface-associated communities, might be even more influenced by the host genetics, as they are more 'tightly' associated with the host. The rapidly decreasing cost of sequencing will allow future studies to include whole-genome sequencing of the host, so that the effects of rare human genes are also taken into account and epigenetic effects can be incorporated into disease models. A deeper characterisation of the microbiome through metagenomic, metatranscriptomic and metabolomic approaches, as well as strain resequencing to characterise populations of specific taxa within individuals, will also add powerfully to our understanding of the relevance of the microbiota for maturation, inflammation and nutritional processes.

Outline of this thesis

In this postdoctoral qualification thesis the effects and significance of the human microbiota and its members on the maturation, inflammation and nutritional processes in humans were investigated. In order to understand and exploit the impact of the gut microbes on human health and well-being, it is necessary to gain insight into the succession of the gut microbiota during aging.

Chapter 1 describes, how the bacterial composition is dependent on the environment of the host and is not necessarily influenced by birth weight, diet or antibiotic treatment. However, there are conflicting reports in the literature regarding the composition of the neonatal gastrointestinal tract (GIT) microbiota and the factors that shape it. Several studies have reported that Bifidobacteria almost always dominate the GIT microbiota of breast-fed infants by several weeks of age (8, 109-111), while others find that they occur in only a small fraction of infants, or are not numerically dominant (112). Because of the increased incidence of GIT problems in premature infants, the effect of gestational age has also been extensively studied. These studies have consistently shown that the microbiota of hospitalized, preterm infants differs from that of healthy, full-term babies (38, 113, 114). Attempts to associate specific microbes with the occurrence of necrotizing enterocolitis, a condition with suspected bacterial aetiology that is an important cause of morbidity and mortality in premature babies, have yielded mixed results (114, 115). Clearly, there is still much to be learned about the origins and development of the infant GIT microbiota and its influence on health and disease.

In the first year of life, the infant intestinal tract progresses from sterility to extremely dense colonization, ending with a mixture of microbes that is broadly very similar to that found in the adult intestine (109). **Chapter 2** and **Chapter 3** describe how the colonic

microbiota exhibits both a bacteria-specific and general pattern of maturation that is most profound within the first year of life. It remains to be shown, whether these changes reflect direct changes of the gut microbiota, the mucosal innate immunity, or indirect consequences of a changing nutrition.

In recent years, the gut microbiota has been linked to various beneficial or detrimental activities, one being obesity (78, 82, 116, 117). In **Chapter 4,** one of the first studies including a larger group (> 100 subjects) of lean, overweight, and obese healthy individuals on a western diet showed significant differences between the three groups and their respective microbiota composition. The most abundant bacterial groups in faeces of lean and obese subjects belonged to the phyla *Firmicutes* and *Bacteroidetes*. The ratio of *Firmicutes* to *Bacteroidetes* changed in favour of the *Bacteroidetes* in obese subjects, which is in contradiction to previously published data from a smaller group of individuals (118). Additionally, obese subjects had higher SCFA concentrations than lean subjects, especially propionate. Taking into account that propionate is mainly used in gluconeogenesis and liponeogenesis, this finding is rather intriguing as propionate may thus provide additional energy to the host metabolism. However, the diet-associated effect in the rate of microbial production, microbial cross feeding and mucosal absorption of SCFA on the overall faecal SCFA net concentration in intestinal contents is yet to be determined.

In a recent work published by Gill and co-workers (25), the human gut microbiome of two healthy subjects has been shown to be enriched with many clusters of orthologous groups (COGs) representing key genes of the metabolic pathways of methanogens. Since *Methanobrevibacter smithii* is the major representative of the human gut methanogens (13), this finding led to the hypothesis that *M. smithii* may be a therapeutic target for the reduction of energy harvest in obese humans (85, 86). However, this hypothesis could not be

confirmed as described in **Chapter 4**. The question, whether bacteria are responsible for obesity remains controversial, as several recent studies could show that diet is one of the most important factors shaping the microbial diversity in the gut (119-121). Furthermore, most findings in this field of research are based on mouse studies and the relevance to human biology requires further investigation.

Although it is clear that an important symbiotic relationship between the autochthonous microbiota and its host has evolved over millions of years, most of the microorganisms and their functions have not been described yet. To isolate and study these symbiotic microorganisms still remains one of the achievable goals in science. In **Chapter 5** and **Chapter 6** two such organisms are described. Both organisms have been isolated during the quest for new butyrogenic bacteria. Butyric acid, produced within the intestinal lumen by bacterial fermentation of dietary carbohydrates, exerts a wide variety of effects on intestinal function. First of all, butyric acid is the preferred source of energy for colonocytes. It affects cellular proliferation, differentiation and apoptosis. Secondly, butyric acid has well documented anti-inflammatory effects. Inhibition of histone deacetylase activity, resulting in hyperacetylation of histones, and as a consequence suppression of nuclear factor kappa B activation, is a likely explanation. Thirdly, it has been proposed that butyric acid reinforces the colonic defence barrier by increasing production of mucins and antimicrobial peptides. Finally, it has been shown that butyric acid decreases intestinal epithelial permeability by increasing the expression of tight junction proteins. Anti-inflammatory activities, combined with a strengthening of the mucosal barrier integrity, are ideal properties for therapeutic compounds against IBD-like syndromes (91). Several studies have shown that, numerically, the majority of butyrate producing bacteria found in human faeces are highly oxygen-sensitive anaerobes belonging to the clostridial clusters IV and XIVa (94). Decreases in

members of the butyrate-producing clostridial clusters IV and XIVa have been reproducibly reported in the gut of IBD patients (75). As outlined in **Chapter 7** a dysbiotic microbiota is present in paediatric IBD patients, already. Although no major changes could be observed in patients with ulcerative colitis, except for a decrease of bifidobacteria in the active state of the disease, numbers of *Faecalibacterium prausnitzii* and bifidobacteria were reduced in children with active and inactive Crohn's disease. Additionally, numbers of *Escherichia coli* were increased in patients with active Crohn's disease only. Hence, it can be stated that the microbiota in children with Crohn's disease is characterized by a decrease of *F. praunsitzii* and an increase in *E. coli* cell numbers.

An approach to augment the amount of colonic butyrate is to increase the numbers of these butyrogenic species by dietary intervention. While certain dietary substrates such as resistant starch appear to be butyrogenic, it is not known to what extent these stimulate butyrate production directly, e.g. by promoting amylolytic species, or indirectly, e.g. through cross-feeding of fermentation products. A better understanding of the microbial ecology of colonic butyrate-producing bacteria will help to explain the influence of diet upon butyrate supply, and to suggest new approaches for optimising microbial activity in the large intestine.. Such an approach for the detection of quantitatively significant groups of butyrate-producing bacteria is presented in **Chapter 8** and their subsequent elevation by dietary intervention in **Chapter 9**. It could be shown that a diet rich in resistant starch elevates the butyrate levels, however, the selected *Eubacterium spp.* did not have a major impact on this process. Recent studies show that species of the cluster *Roseburia* are major butyrate producers in the human gut (94).

Next to the elucidation of the effects of butyrate and the manipulation of the accountable microbiota, there has been considerable interest in the discovery of natural

occurring compounds with a high potential for anti-inflammatory or antioxidative effects and cancer prevention. Two such compounds are flavonoids and selenium compounds. Although it is known that human intestinal bacteria play a significant role in the degradation of flavonoids and the activation of selenium compounds (97, 108), there is a paucity of information on the species involved, their distribution in humans, and the mechanisms of degradation. In **Chapter 10** and **Chapter 11** newly isolated organisms or numerically important bacteria from the human intestinal tract were shown to exhibit degradation or activation capabilities for flavonoids and selenium compounds, respectively. However, it remains to be elucidated, whether shifts in the numbers and pattern of intestinal bacteria affects the bio-conversion of these compounds.

Although this work provides new insights into the role of the human intestinal microbiota regarding health, our understanding of its complexity and function still remains in its infancy. A thorough understanding of how the microbiota interacts with the host will not only facilitate the development of diagnostic strategies and nutritional intervention. It will also enable us to fully appreciate the complexity of the unique universe that is our microbiota and that determines so much of who we are.

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Chapter 1

Development of the intestinal bacterial composition in hospitalized pre-term infants in comparison to breast-fed full-term infants

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Development of the Intestinal Bacterial Composition in Hospitalized Preterm Infants in Comparison with Breast-Fed, Full-Term Infants

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ABSTRACT

The establishment and succession of bacterial communities in hospitalized preterm infants has not been extensively studied. Because earlier studies depended on classical cultural techniques, their results were limited. This study monitored the establishment and succession of the neonatal microbiota in the first weeks of life by analyzing the 16S rDNA variety in fecal samples applying PCR-denaturing gradient gel electrophoresis (PCR-DGGE). Fecal samples from 29 preterm infants hospitalized in a neonatal intensive care unit, including samples from antibiotic-treated infants and one with neonatal necrotizing enterocolitis, were subjected to PCR-DGGE analysis. Daily DGGE profiles from all preterm infants during the first 4 wk were obtained and analyzed. In addition, feces of 15 breast-fed, full-term infants and a variety of clinical bacterial isolates were examined and compared with the PCR-DGGE profiles of the preterm infants. During the first days of life, the DGGE profiles were rather simple but increased in their complexity over time. It became obvious that not only the intraindividual band-pattern similarity increased over time, but also the interindividual. During the observation period, similarity values (C_s) increased in each preterm infant from 0 to 80%, whereas interindividual C_s increased from 18.1 to 57.4%, revealing the acquisition of a highly similar bacterial community in these infants. In contrast, C_s-values obtained for breast-fed, full-term infants were rather low (11.2%). *Escherichia coli*, *Enterococcus* sp., and *Klebsiella pneumoniae* were the bacteria most commonly found in all preterm infants. The interindividual bacterial composition in hospitalized preterm infants is more similar in comparison with breast-fed, full-term infants and is not necessarily influenced by birth weight, diet, or antibiotic treatment. (*Pediatr Res* **54: 393–399, 2003**)

Abbreviations
C_s, Sorenson's pair-wise similarity coefficient
DGGE, denaturing gradient gel electrophoresis
GI, gastrointestinal
NEC, necrotizing enterocolitis

The GI tract of a normal fetus is sterile. During the birth process and rapidly thereafter, microbes from the mother and the surrounding environment colonize the gastrointestinal tract of the infant until a dense, complex bacterial community is established. This enteric flora contributes not only to health by facilitating carbohydrate assimilation (1) and interaction with the developing immune system (2, 3), it also plays a significant role in disease (4–6). A dynamic balance exists between the GI bacterial community, host physiology, and diet, all of which influence the initial acquisition, developmental succession, and eventual stability of the gut ecosystem (7). In the birth process and soon thereafter, bacterial colonization starts and facultative anaerobic bacteria such as enterobacteria appear in feces. Because at this stage the composition of the gut bacterial community is strongly influenced by the diet, a shift in the bacterial composition can be observed (3, 8). A breast-fed, full-term infant shows a fecal bacterial composition in which bifidobacteria predominate over potentially harmful bacteria, whereas, in formula-fed infants, coliforms, enterococci, and bacteroides predominate (8–11). The process of colonization is greatly influenced by the successive shifts from formula feeding to weaning and finally to the introduction of solid food.

Although several studies monitored the bacterial communities in infants (8, 12), our picture of the neonatal microbiota is still limited, because the classical cultivation techniques only allow the identification of a limited number of bacteria. In recent years, the use of 16S rRNA gene sequences has greatly facilitated the study of GI tract ecology (13) because it allows the culture-independent analysis of the fecal microbiota. The rRNA gene sequences comprise highly conserved sequence

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domains interspersed with more variable regions. Consequently, comparative analyses of rRNA sequences can identify so-called signature sequence motifs. The simplest and currently the most widely used method to obtain 16S rRNA genes from fecal samples is through the use of PCR. rRNA genes can be amplified directly from total community DNA using rRNA-specific primers. By taking advantage of the highly conserved nature of rRNA, universal primers capable of annealing to rRNA genes from all three domains (*Archea, Bacteria, Eukarya*) or primers designed to amplify rRNA genes from a particular group of organisms can be used (14–18).

One of the 16S rRNA-gene based techniques is PCR-DGGE, a technique in which DNA is isolated from fecal samples and amplified by PCR using conserved 16S rDNA bacteria-domain primers (16, 17). In practice, total bacterial DNA from fecal samples is extracted and a region with a hypervariable nucleotide base sequence of the 16S rDNA gene is amplified by specific PCR primers. One of these bacteria-domain primers has a so-called "G + C clamp" of approximately 39 nucleotides attached to the 5' end. This "G + C clamp" prevents the two DNA strands from dissociating completely, even under highly denaturing conditions. The resulting mixture of 16S rDNA fragments is subjected to a denaturing gradient, established in a polyacrylamide gel with urea and formamide, to separate the fragments and generate a "genetic fingerprint" of the microbiota present. Although all PCR products are of approximately equal size, individual amplicons stop to migrate as the double-stranded products denature according to their G + C content. This approach thus allows separation of individual sequences based on G + C content, corresponding to the different microbial species within the sample. The vertical orientation of the denaturing gradient facilitates the simultaneous screening of many samples.

In a recent study, Favier *et al.* (19) used PCR-DGGE to study the development of the infant flora for up to 12 mo. They were able to show that band patterns during the first days of life were very simple, but they became more complex over time.

In contrast, not much information concerning the composition of the bacterial community of premature infants is available at the moment. Previous studies of the gut microbial community in premature infants relied on the application of classical cultural methods, and showed that these infants harbored a rather simple bacterial community (20–22). So far, only one study inspected the microbial composition of premature infants by molecular methods (23).

The purpose of this study was to obtain information on the fecal bacterial community of premature infants in comparison with breast-fed, full-term infants. A further goal of this study was to determine whether bacterial strains, which have been routinely isolated by classical cultural methods in the baby care unit, are major representatives of the premature infants' fecal composition.

MATERIAL AND METHODS

Subjects. The protocol of this study was submitted to the ethics commission of the Brandenburg state medical association, which approved the study without further consultation because the study solely involved stool analyses but not the examination of patients just for the study. Informed consent was obtained from the parents of the children who took part in this study. In the period April 2001 to January 2002, 29 preterm infants were delivered between wk 24 and 37, either vaginally (11 infants) or by cesarean section (18 infants), with a birth weight ranging from 830 to 2635 g. All infants were born in the Ernst von Bergman Klinikum, Potsdam, stayed for at least 4 wk in a baby incubator in the neonatal intensive care unit, and were subjected to a feeding regime reported earlier (22). All full-term infants (15 infants) were fully breast-fed, delivered vaginally on time, and were not hospitalized.

Preparation of samples. After birth, fecal samples of 29 premature infants were taken daily for 2 wk and on d 17, 21, 24, and 28. During the study period, seven infants showed high IL-6 levels (preterm infants no. 4, 9, 18, 19, 22, 23, and 28) and hence received an antibiotic treatment. All of these infants were subjected to a standard antibiotic therapy, receiving in the first 3 d cefotaxime (200 mg/kg/d) and piperazine (150 mg/kg/d). After d 3, vancomycin (15 mg/kg/d) and amikacin (15 mg/kg/d) were given until the inflammation was reduced. Fecal samples were collected during the whole observation period. One of these infants, treated from d 2 on, died from NEC on the d 4. Therefore, fecal samples from this infant could only be obtained on d 1 and 3 after birth. In addition, fecal samples were taken from 15 full-term infants 6 d after delivery. All samples were stored at 4°C until further processing.

Organisms and culture conditions. All strains used in this study are isolates obtained and identified during routine testing in the neonatal intensive care unit of the Department of Pediatrics of the Ernst von Bergmann Klinikum. Species identification was achieved by using the automated identification system Vitek (Bio Mérieux, Nürtingen, Germany), which is commonly used in hospitals for bacteria identification. The system allows the identification of aerobic and anaerobic bacteria down to the species level. However, the system is not able to distinguish between strains. In this study, we used the most frequently isolated bacterial species. For further analysis, all isolates were cultured at 37°C under oxic conditions on blood agar plates (Bio Mérieux).

DNA extraction. For DNA extraction, 100 mg of fresh feces (wet weight) were homogenized in 1 mL TN 150 [10 mM Tris-HCL (pH 8), 150 mM NaCl]. DNA was isolated as described earlier (19, 24).

For DNA extraction, bacterial cultures were grown aerobically overnight on blood agar plates. The cells were harvested and subjected to nucleic-acid extraction following protocol number 5 of the InViTek DNA-Isolation Kit III (InViTek GmbH, Berlin, Germany).

PCR amplification for DGGE. Primers *U968-GCf* (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and *L1401-r* (5' CGG TGT GTA CAA GAC CC) (25) were used to amplify the V6–V8 regions of the bacterial 16S rRNA from all infants. In addition, bifidobacteria-specific primers *Bif164-f* (5' GGG TGG TAA TGC CGG ATG) and *Bif662-GC-r* (5' CGC CCG CCG CGC GGC GGG CGG GGC GGG GCC ACG GGG GG CCA CCG TTA CAC CGG GAA) (15, 26, 27) were used to

perform PCR. Nucleotides comprising the "G + C clamps" are underlined.

PCR were performed with a *Taq* polymerase kit from InViTek (Berlin, Germany). Each 50-μL mixture contained deoxynucleoside triphosphate at a concentration of 10 mM, 2.5 U of *Taq* polymerase, 10 μmol of each primer, and 1 μL of a DNA solution obtained from the fecal and pure culture DNA extractions, respectively. PCR amplification was performed with a PCR thermal cycler (Hybaid, Heidelberg, Germany) under the following conditions: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 20 s (62°C for 20 s for *Bif164-f* and *Bif662-r* primers), and extension at 68°C for 40 s. After completion, an additional extension step was performed at 68°C for 7 min, and the samples were then chilled to 4°C.

DGGE of PCR amplicons. PCR fragments were separated by DGGE as described earlier (16, 24) using the Decode system (Bio-Rad, Hercules, CA, U.S.A.), with the following modifications. Polyacrylamide gels (dimensions, 200 × 200 × 1 mm) consisted of 8% (vol/vol) polyacrylamide (ratio of acrylamide-bisacrylamide, 37.5:1) and 0.5 × Tris-acetate-EDTA (TAE, pH 8.0) buffer; 100% denaturing acrylamide was defined as 7 M urea and 40% formamide. The gels were poured from the top by using a gradient maker and a pump (Econopump, Bio-Rad) set at a speed of 4 mL/min. We used 40–60% gradients to separate products amplified with universal primers. For the separation of products generated with the bifidobacteria-specific primers, we used a 45–55% gradient (15). Before polymerization of the gel, a stacking gel without denaturing chemicals was added, and an appropriate comb was subsequently inserted. Electrophoresis was performed first for 5 min at 200 V and subsequently overnight at 85 V in 0.5 × TAE buffer at a constant temperature of 60°C. The gels were stained with $AgNO_3$ as previously described (28). To allow a comparison between PCR-DGGE gels, internal standards were used. These standards consisted of *Enterococcus* sp., *Escherichia coli*, and *Klebsiella pneumoniae*, all of which were isolated in the neonatal intensive care unit. Each internal standard resulted in one prominent band, which allowed their specific distinction and identification in the fecal samples. In addition, the following species commonly isolated in the neonatal intensive care unit were used for band identification: *Citrobacter freundii*, *Enterobacter cloacae*, *Enterobacter aerogenes* (*Klebsiella mobilis*), *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*.

Estimation of microbial richness and diversity. The richness and diversity of the preterm and full-term infant fecal microbiota was estimated from the number of PCR-DGGE bands present (29). Each lane in a DGGE gel represents one given time point during the observation period. The band numbers and frequencies within a given infant were compared for various time points and between different infants. "Species" used in the indices refer to individual bands on the PCR-DGGE gels. These indices measure ecological diversity using various parameters, including species richness (the number of different species) and evenness (the distribution of individual species in the ecosystem) (30). Band number corresponds to the number of individual bands in a single lane. Band frequency was calculated by measuring the percentage of all samples at a given time point containing a specific band. C_s was used to quantify similarity (31–33). C_s values were determined by the following equation:

$$C_s = \left[\frac{2j}{(a+b)}\right] \times 100$$

Where a is the number of PCR-DGGE bands in lane 1, b is the number of PCR-DGGE bands in lane 2, and j is the number of common PCR-DGGE bands (30). Thus, two identical profiles will give a C_s value of 100%, whereas completely different profiles result in 0% similarity. Each sample of a given time point was compared with every other sample; therefore, mean percentage similarities (C_s values) can be compared for each time point in relation to other time points. A high C_s value indicates a highly similar PCR-DGGE pattern, which indicates a high similarity in microbiota composition.

Statistics. The data for each time point of the observation period are given as mean ± SD of 27 preterm infants and 15 full-term infants. Differences between the means were checked for significance by the paired t test, as described by Lorenz (34), and are indicated as p values.

RESULTS

Molecular analysis by DGGE of the fecal bacterial community of preterm and full-term infants. To characterize and compare bacterial succession in the large intestine of infants, fecal samples from 29 preterm infants and 15 full-term infants were analyzed using PCR-DGGE (Figs. 1–4). The investigation period extended over several months, as most of the infants were hospitalized at different times. Several bacteria commonly isolated from infants during routine microbial testing were included in the analysis (Table 1).

The strategy involved the extraction of DNA from fecal samples or selected bacterial strains followed by amplification with PCR of a fragment corresponding to region V6–V8 of the 16S rRNA gene using universal bacterial primers. In addition, bifidobacteria-specific primers were used for the fecal analysis of breast-fed, full-term infants. Analysis of PCR products by DGGE results in a fingerprint that represents the diversity of the rDNA nucleotide sequences originating from the various bacterial population groups present in the ecosystem. The bands in the profile represent most of the dominant microbial populations in the community, and their appearance and disappearance reflect changes in the microbial community composition. The intensity of the band may provide an estimate of the proportion of the target sequence in the respective sample.

Bacterial succession in preterm and full-term infants. The DGGE profiles of all preterm infants showed a low diversity in the 16S rDNA sequences during the first 3 d. Only a few bands were present and varied from one to several major bands. Following d 4 after birth, the band pattern became more constant in most infants. Ten days after birth, the band pattern became stable, with only minor changes (Fig. 1). The number of major bands in individual babies ranged from five to 20. Interestingly, the band patterns became increasingly similar in

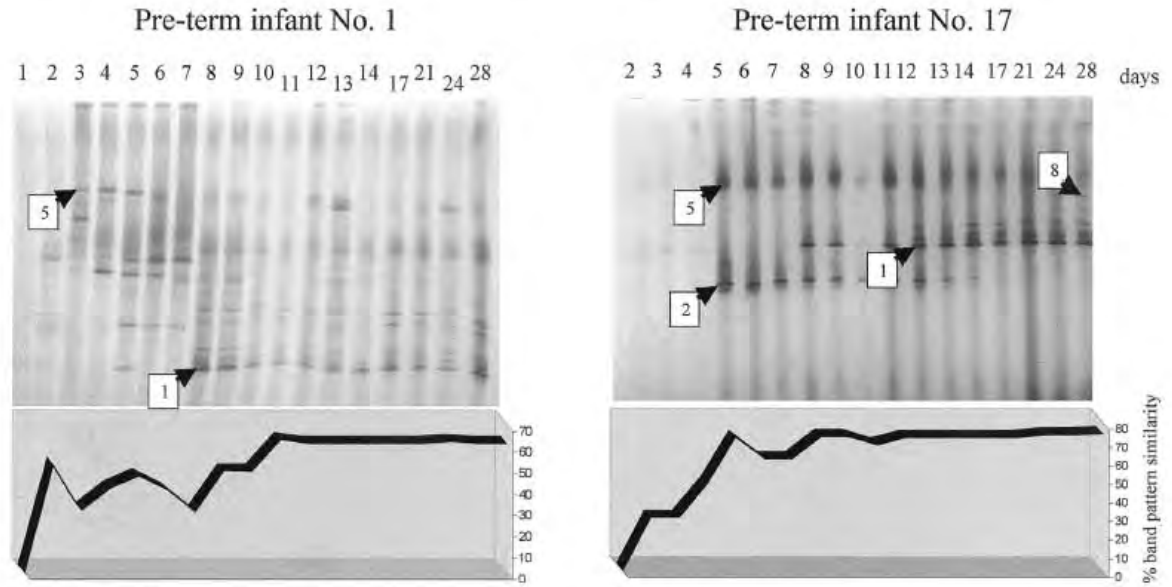

Figure 1. PCR-DGGE profiles representing the bacterial diversity in preterm infants 1 and 17 generated from samples taken on the indicated days. Band patterns do not represent the same positions in the PCR-DGGE gels. The bands identified from the PCR-DGGE profiles of known species are numbered and indicated by *arrowheads*. Numbers correspond to the numbers given in Table 1. Below the PCR-DGGE profiles, the band pattern similarity is illustrated for each infant over time.

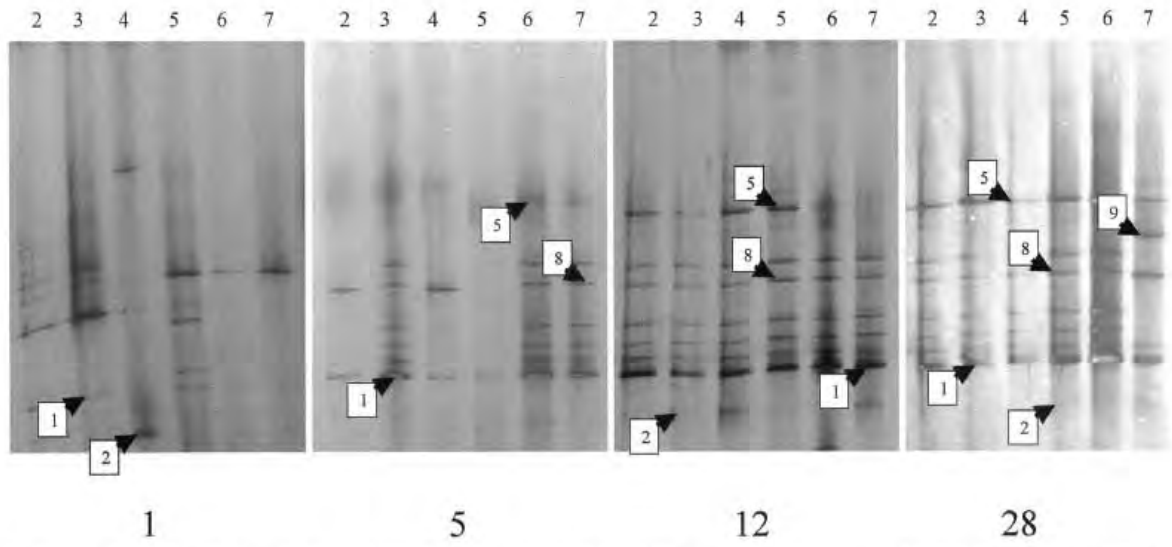

Figure 2. PCR-DGGE profiles representing the bacterial diversity in six of the tested 29 preterm infants are illustrated. Numbers on top indicate the specific preterm infant. Numbers below the gels refer to the sampling days after birth. Bands identified from PCR-DGGE profiles of known species are numbered and indicated by *arrowheads*. Numbers correspond to the numbers given in Table 1.

most infants after the wk 2 (Fig. 2). Several of the hospital isolates could be identified in the fecal samples (Figs. 1–3).

DGGE-profiles were obtained from full-term infants at one time point after the d 5 of life. In contrast to the preterm infants, the emerging band patterns were more diverse in the full-term infants. The number of the major bands varied from one to 12 (data not shown). There were few similarities in the band patterns between the individual infants. Using bifidobacteria-specific PCR-DGGE primers, several bands were obtained indicating that bifidobacteria made up the majority of species present (data not shown).

Comparison of fecal bacterial diversity. The quantity of samples containing specific PCR-DGGE bands was calculated to characterize the distribution frequency of these bands among

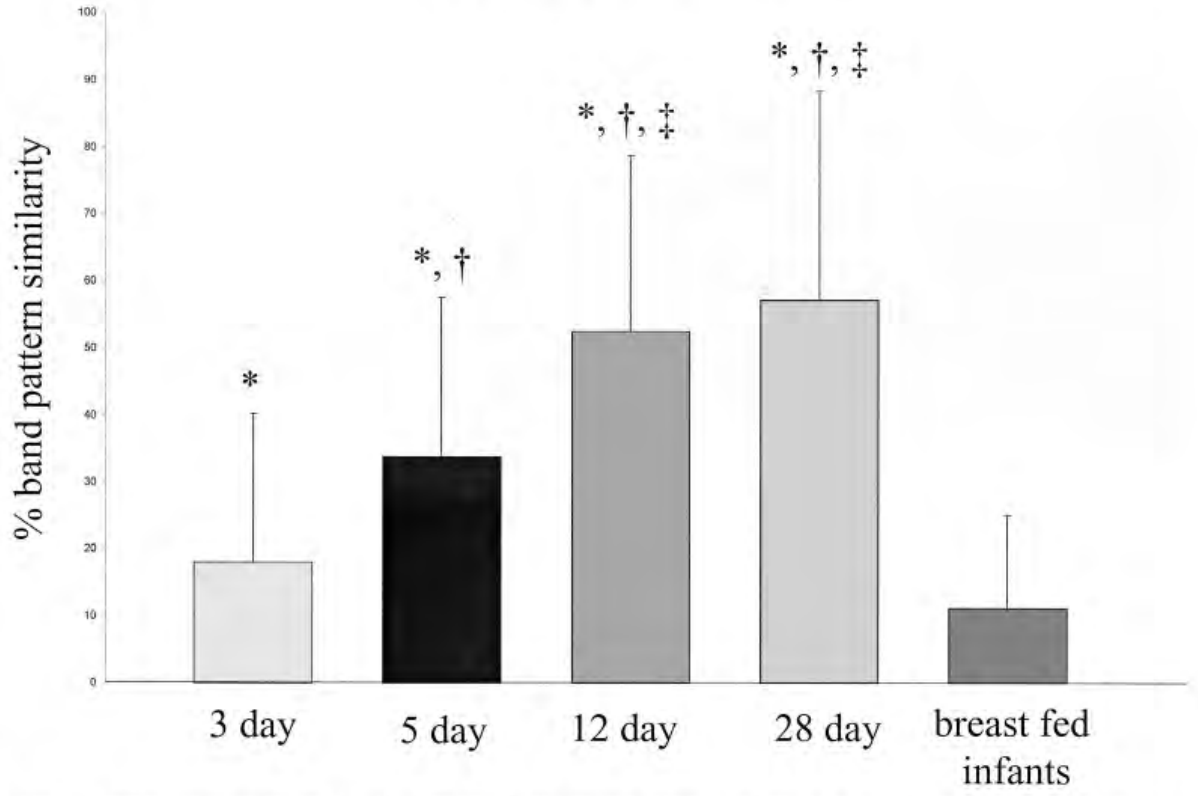

Figure 3. Percentage of similarities for PCR-DGGE banding patterns from fecal samples of preterm infants from the given time period. In addition, the similarity value for full-term, breast fed infants is given. *Significantly more similar than breast-fed infants ($p < 0.05$). †Significantly more similar than on d 3 ($p < 0.05$). ‡Significantly more similar than on d 5 ($p < 0.05$). For further information, see "Material and Methods."

the different sample days. It was apparent that the similarity of the PCR-DGGE band patterns increased over time (Figs. 1 and 3). Similarity values (C_s) for the various time points in each infant increased from 0 to over 70 or 80% (Figs. 1 and 3), whereas similarity between the individual preterm infants increased from 18.1 to 57.4% over time (Fig. 3). Several of the bacterial species routinely isolated from feces in the neonatal intensive care unit resulted in DGGE bands that were also observed in the profiles of the fecal samples analyzed. Bands corresponding to *E. coli*, *Enterococcus* sp., and *K. pneumoniae* were the most common in the preterm infants.

Profiles of full-term infants were only obtained at d 6 after birth. The C_s value of 11.2% in these infants was considerably lower than in the preterm infants, indicating that the composition of the bacterial community was highly variable between individual full-term infants.

Bacterial succession in antibiotic-treated preterm infants. The total antibiotic-treatment period in the prophylactically treated infants varied from three to 21 d. It is obvious that the bacterial community was not affected in any of these infants. For instance, in preterm infant no. 9, who was treated from his/her first to fourth day of life with antibiotics, a complex DGGE band pattern was even observed during the antibiotic regime (Fig. 4). The C_s value for this infant increased from 50 to more than 80% over the study period. Interestingly, this infant showed a rather complex band pattern, comparable to the infants not treated with antibiotics. However, for an antibiotic-treated infant, one might expect lower band pattern diversity.

In contrast, preterm infant no. 22 was treated with antibiotics from day 5–21. After antibiotic treatment, the numbers of bands was reduced, but remained stable until d 10. Beginning with d 10, a new band pattern established, but the complexity of the band pattern appeared to be low in comparison to that of infant no. 9 (Fig. 4). C_s values of the PCR-DGGE bands increased from 28 to 80% in this and the other antibiotic-treated preterm infants (nos. 4, 18, 19, and 23), who showed a comparable band pattern evolution (data not shown).

Preterm infant no. 28 received an antibiotic treatment from his/her second day on. This infant was diagnosed with NEC on the third day and subsequently died thereof. Two fecal samples from this infant were collected. Both samples showed a highly diverse band pattern, indicating that several bacterial species were present in the intestine at birth already (Fig. 4).

DISCUSSION

In the present study, we used samples obtained from preterm infants in their first 4 wk of life to follow the succession of the dominant bacterial species. We could show that the PCR-

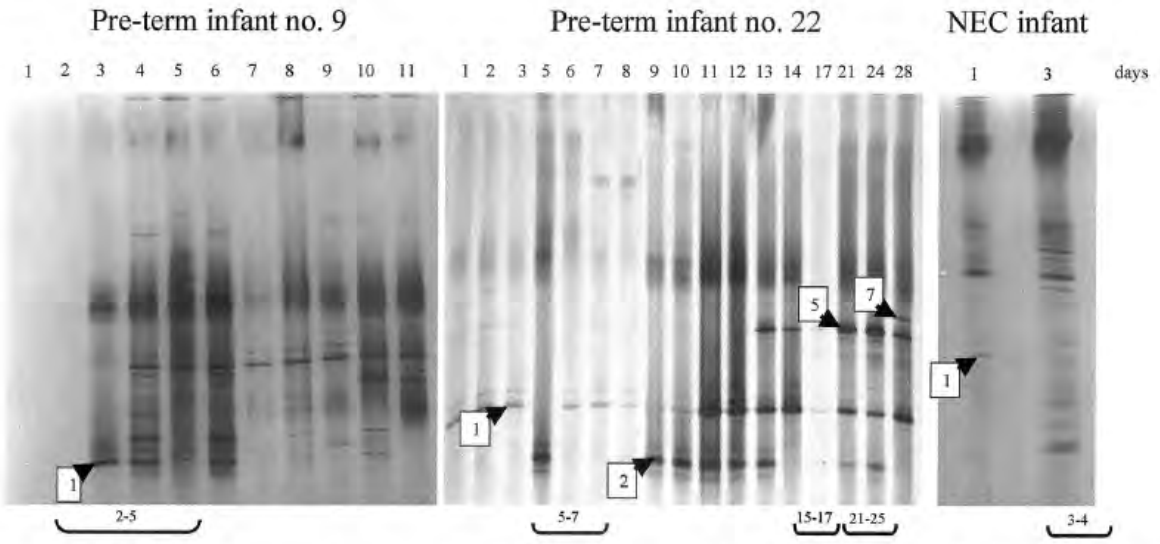

Figure 4. PCR-DGGE profiles representing the successive bacterial diversity in antibiotic-treated preterm infants and an infant diagnosed with NEC. Band patterns do not represent the same positions in the PCR-DGGE gels. Brackets indicate the time period of antibiotic treatment. Bands identified from PCR-DGGE profiles of known species are numbered and indicated by *arrowheads*. Numbers correspond to the numbers given in Table 1.

Table 1. *Bacteria commonly found during routine microbial testing in the neonatal intensive care unit*

No.	Bacteria Cultured and Identified	Band Pattern Identified in PCR-DGGE
1.	*Escherichia coli*	+
2.	*Enterococcus* sp.	+
3.	*Enterobacter aerogenes (Klebsiella mobilis)*	
4.	*Enterobacter cloacae*	
5.	*Klebsiella pneumoniae*	+
6.	*Staphylococcus aureus*	
7.	*Staphylococcus epidermidis*	+
8.	*Pseudomonas aeruginosa*	+
9.	*Citrobacter freundii*	+

DGGE patterns for all preterm infants were very simple at birth and that the diversity increased over time. These results are comparable to an earlier study (19). We could also show that the band profiles of our preterm infants became more similar to each other over time, indicating that all preterm infants harbored a similar bacterial composition. These results confirm previous studies, in which it was shown that cross-transmission of bacteria is a serious problem in hospitals (35, 36). In contrast to the 29 preterm infants, who showed a highly similar band pattern, the band patterns of breast-fed, full-term infants showed little similarity, indicating that the breast-fed, full-term infants acquired bacterial communities whose composition displayed much less similarity between individual infants in this group and also in comparison to the preterm hospitalized infants. This is also reflected by the rather low similarity index (C_s) of band patterns in the preterm infants. In contrast, the preterm hospitalized infants had an increasing C_s value over time. Several of the bands in the DGGE gels of the breast-fed, full-term infants, but not in the preterm infants, could be attributed to bifidobacteria. So far, only a few studies followed the bacterial succession in preterm infants (23, 37–39).

Hospitalization is known to have serious effects on bacterial composition (40). In the present study, we were able to show that preterm infants acquire a rather diverse bacterial community after birth, but, as a result of hospitalization, tend to develop a similar strain composition over time. This may indicate subject-specific factors responsible for the acquisition of the bacterial community in the GI tract, as put forward by Zoetendal *et al.* (41). The authors were able to show a positive relationship between the similarity indices for PCR-DGGE band patterns, which represent the bacterial community, and the genetic relatedness of the hosts. With regard to our results, the acquisition of a specific bacterial community is dependent on host factors and environmental factors, which, because of hospitalization, are altered. The composition of the neonatal fecal bacterial community is of great interest, as it is hypothesized that an inappropriate colonization of the premature intestine may play a role in the development of NEC (42). In our study, one hospitalized infant died of NEC during the course of the study. It is noteworthy that this incidence does not represent the average prevalence for NEC in the Ernst von Bergmann Klinikum, which is three out of 200 preterm infants. In the two fecal samples taken before death, a highly diverse band pattern became obvious. Because only one infant was diagnosed with NEC, we were not able to draw any realistic conclusion from this event on the role of the of bowel microbiota in premature infants in NEC. Nevertheless, it is worthwhile to note that the microbiota of this premature infant was already highly diverse at birth.

The PCR-DGGE method has the advantage of being independent of culturing, which may favor the growth of certain bacterial groups and prevent the growth of others, resulting in

a biased view of the bacterial community. However, the PCR-DGGE approach can also lead to some distortions, as some sequences may amplify better than others, and heteroduplexes may be formed during PCR (43). Because PCR-DGGE bands correspond to the relative G + C content of the amplified V6–V8 region of the 16S rDNA, bacterial species with similar G + C content in this amplified region may form assemblages and appear as a single band, resulting in fewer bands (17). Thus, PCR-DGGE experiments may not reflect the total composition of the bacterial community present. In addition, the presence of intragenomic 16S rDNA heterogeneity in bacteria strains may hamper the results (15).

In summary, we were able to show an increase in similarity of the bacterial communities in hospitalized preterm infants in contrast to breast-fed, full-term infants. A strikingly high similarity was observed between bacterial communities from different preterm infants regardless of birth weight, feeding regime, and antibiotic therapy, respectively, in contrast to full-term, breast fed infants. This result supports the notion that the initial colonization of the newborn's GI tract is highly dependent on the environment.

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Chapter 2

The effects of maturation on the colonic microflora in infancy and childhood

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Research Article

The Effects of Maturation on the Colonic Microflora in Infancy and Childhood

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The composition of colonic mircoflora and its changes with maturation have rarely been investigated in large samples. *Methods.* We used conventional microbiological testing to analyse the colonic flora (Kyberstatus, Institut forMicroecology, Herborn, Germany) of stool samples from 12 484 children with different intestinal and nonintestinal diagnoses. Stool samples were analysed for total colony forming units (CFU) (per g stool) and the abundance of *Bifidobacteria, Bacteroides sp., Escherichia coli, Enterococcus sp.,* and *Lactobacillus sp.* with respect to age, gender. A subset of 1089 infants was analysed for monthly changes within the first year of life. *Results.* Total CFU and individual microbial species were highest during the first year of life, decreased within the first 2 years, and then stabilized for the remaining childhood. In infants, the total CFU rose until month 5, declined with weaning, and peaked at 9–10 months. Significant effects of age, but not of gender, were found in *Bacteroides sp.* and *Lactobacilli.* However *Bacterioids sp.* and *Lactobacilli* increased with age, while *Enterococci* and *E. coli* decreased, and Bifidobacteria remained stable. *Conclusion.* Colonic microflora show both a bacteria-specific and general pattern of maturation which is most profound within the first year.

1. Introduction

Age-related changes in the abundance of nonpathogenic bacteria found in the human colon have rarely been investigated in larger samples, neither in patients with intestinal and nonintestinal disorders nor in healthy subjects. In contrast, the effects of maturation on colonic flora in infants and children have occasionally been studied, but mostly in small cohorts of children.

Deviations in colonic flora have been shown to be responsible for respiratory diseases [1], intestinal diseases [2], and, especially, dermatologic disorders [3–6] in the pediatric population. It has, for example, been argued that the amount of exposure to commensal and pathogenic bacteria in the very early days of life, such as following a Cesarian section, [7] may contribute to the occurrence of atopic dermatitis later in life [8]. A similar argument is the basis for the "hygiene hypothesis" [9], which argues that greater exposure to environmental pathogens during childhood, for example, while growing up on a farm or having contact with animals, would protect for immunologically mediated diseases later in life [10, 11], but this is still a matter of debate [12]. However, the importance of commensal microbiota for the development of normal innate immunity is well established [13].

One reason for the relative absence of large-scale investigations is the suspicion that bacterial colonies found in stool samples would not remain stable during transport and storage; therefore. providing an inaccurate estimate of bacterial counts when conventional microbiological tools are used. Hence, commercial assays for fecal microbiota have not been a widely accepted diagnostic tool in routine clinical assessment. One exception is the use of fecal microbiota in the diagnosis of the inflammatory bowel diseases, especially Crohn's disease and ulcerative colitis, since these are strongly associated with *Clostridium diff.* colonization in both children [14] and adults [15].

Both the recent interest in prebiotic and probiotic treatment for functional intestinal [16, 17], other intestinal

[18–21], and nonintestinal disorders [12–26], along with the availability of new molecular biological tools that are able to count different species, identify genetically different subspecies within each strain, and characterize normal human colonic microbiota and its variability, have created a new surge in research. Investigations regarding the effect of maturation on microbiota during childhood [27] and its change with aging [28] have recently been performed. However, as with most studies based on conventional microbiology, sample sizes have remained rather small thus far, as PCR technology is not yet easily available, and microarray chip technology that allows for the assessment of all bacterial species known to inhabit the human colon (in the range of >1000) is still very costly. This may, however, change in the near future.

The few studies that have assessed the effects of maturation on human fecal microbiota have shown that some bacterial species decline in their abundance with age, while other do not. This paper presents data from a large (>12,000 samples) conventional microbiological database of children with various intestinal and nonintestinal symptoms and diagnoses. Respective data regarding adult populations have recently been published [29]. The underlying hypothesis of this analysis is that the average bacterial abundance found in patients with various diseases and medical conditions may represent an approximation of what may be called the "normal" human fecal microbiota. Our assessment is based on conventional microbiological analyses that were performed in a commercial laboratory with GLP certification during the course of one year.

2. Material and Methods

2.1. Collection of Stool Samples for Microbiological Analysis. During the course of one year (2006), all fecal samples that were submitted by general practitioners for routine industrial microbiological analysis of nonpathogen colonic bacterial flora (Kyberstatus, Institute for Microecology, Herborn, Germany) were included in the study. In general, samples reached the laboratory within one day and were processed immediately.

To ensure that the transport did not have any effect on the cultured species, a storage study was performed with 20 fresh samples. In short, 0.2 g of faeces was serially diluted in 1 mL of phosphate-buffered saline (PBS, pH 7.2). The solution was vortexed for 5 seconds and serially diluted (to 10^{-9}) in PBS, pH 7.2. One mL of each dilution was plated onto enrichment or selective agar media.

The remaining feces were stored for three days at a temperature of 25°C, which represents the average temperature during shipment. Following the incubation period, the samples were processed as described and the results were compared. No significant discrepancy in the cell counts of the investigated microbiota could be detected within two days. Thus, it was concluded that a shipment of less than two days will have no effect on the composition of the cultivable microbiota. Only samples which arrived within one or two days of shipment were included in the study.

2.2. Identification and Enumeration of Microorganisms. Viable bacterial cell counts in feces were enumerated on the following selective media: Columbia blood agar (total cell count; BioMerieux, Nürtingen, Germany), U3G agar (enterobacteriacae, enterococci; Heipha, Heidelberg, Germany), Rogosa agar, (lactobacilli; Heipha), DIC agar (bifidobacteria; Heipha), Schaedler agar (bacteroides; Heipha), and SPM agar (clostridia; Heipha). Fecal samples were serially diluted in 1 mL of phosphate-buffered saline (PBS, pH 7.2) and subsequently plated on selective agar plates by a fully automated spiral plater capable of plating 12 agar plates simultaneously. Subsequently, the plates were incubated under either aerobic or anoxic conditions at 37°C for at least two days. Bacteria were first identified by Gram staining and colony morphologies. Additionally, identifications were performed by the API and VITEK systems (bioMérieux). All counts were recorded as the numbers of $\log_{10}$ CFU per mL of sample.

The following bacteria were routinely analyzed: *Clostridium sp.*, *Bifidobacteria*, *Bacteroides sp.*, subdominant (*E. coli*, *Enterococcus sp.*, *Lactobacillus sp.*), and other bacteria (*Pseudomonas sp.*, *Klebsiella sp.*, *Proteus sp.*, *Citrobacter sp.*, aerobic bacteria). Only bacteria that were identified in at least 50% of the respective subsamples (mentioned hereafter) were included into further analysis.

2.3. Additional Data. Since samples were from patients with various clinical diseases (Table 1), we collected additional data that was reported by the referring physician. This included age, gender, the presumed clinical diagnosis, and stool consistency and frequency. Stool pH was determined in the laboratory.

2.4. Data Analysis. After the data had been made anonymous, it was provided for further statistical analysis. Prior to the analysis, the sample was screened for identical patient IDs, and any second or subsequent analysis was excluded. Incomplete datasets were also excluded, except those where only gender information was missing.

The total age distribution allowed for the identification of a childhood sample ($n = 12.484$) (Figure 1) and an adult sample ($n = 35.292$). The adult study has been published recently [29]. A subset of 1089 infants (<1 year) was identified for the analysis of monthly changes within the first year of life.

Total CFU was analysed as well as the bacterial abundance for the following microbiota; *Bifidobacteria*, *Bacteroides sp.*, *lactobacilli*, *E. coli*, and *Enterococcus sp.* The percentage of subjects in which *Clostridium sp.* was identified was evaluated as well.

Prior to statistical analysis, data were normalized. The CFU of individual cell populations were compared to the total CFU identified by calculating the relative "abundance" of each bacterial species as ((specific CFU/total CFU) $*$ 100).

The subsets (infants, children) were analyzed separately by ANOVAs for each bacterial count (abundance) with the between factors "age" (in monthly intervals for the infants, in yearly intervals for children) and "gender." Post-hoc *t*

Table 1: Major diagnoses in children that initiated the stool analysis.

Organ classes	Main diagnoses	Children
Gastrointestinal	Unspecified	1862
	Irritable Bowel Syndrome	1188
	Crohn's disease	27
	Ulcerative colitis	64
	Diarrhea	771
	Constipation	296
	Candida	1061
	Food Intolerance	238
Respiratory	Unspecified	663
	Bronchitis	294
	Sinusitis	—
	Asthma	103
Urogenital	Unspecified	27
	Cystitis	—
	Genital mycosis	—
Dermatologic	Unspecified	40
	Dermatitis	256
	Psoriasis	28
	Acne	—
Allergic	Allergy, unspecified	229
	Neurodermititis	2184
	Recurrent urticaria	—
	Pollinosis	—
	Allergic asthma	230
	Food allergies	209
	Respiratory allergies	106
Rheumatologic	Arthritis	—
Others	Infection defence weakness	402
	Autoimmune diseases	—
	Malignom	—
Missing		421
Total		10699 (85%)*

* The remaining to the total of 12.484 are cases with unknown diagnoses.

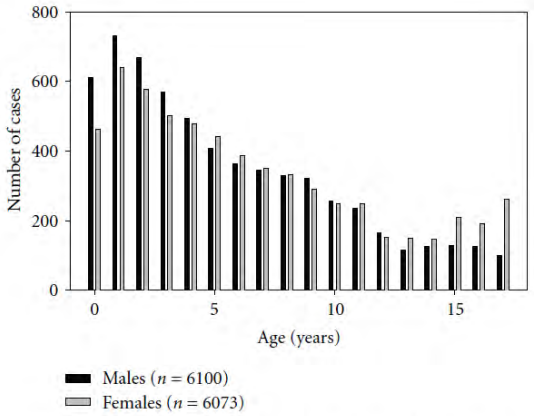

Figure 1: Age distribution (in years) in male and female subjects between 1 and 18 years.

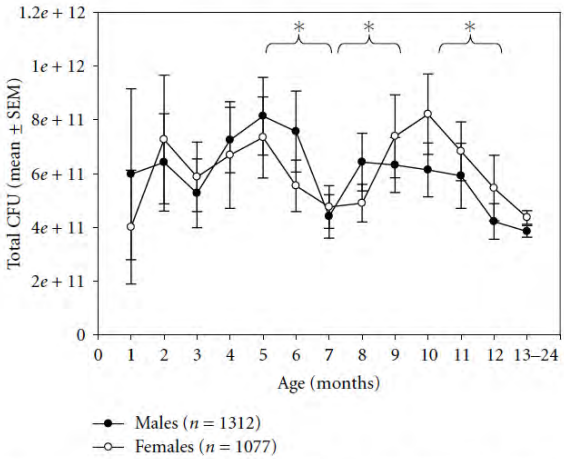

Figure 2: Total CFU (mean ± SEM) by age (months) and by gender. "∗" indicate significant difference in post-hoc T-test (Bonferroni corrected). A number of boys and girls are infants (604 : 459 m : f) plus children up to 2 years.

tests with Bonferroni correction to account for multiple comparisons were used to test for single differences (between months and years, resp.) within each dataset. Pearson's r was computed to test for intercorrelation between single measures. All data are given as mean ± SEM. A threshold of 0.05 was set to indicate statistical significance in all tests. All data were analyzed using the SPSS Version 13 Statistical Package.

3. Results

From the sample of 47,775, $n = 12483$ were children and adolescents less than 18 years, and among them, $n = 1089$ were infants and babies less than one year of age. Their stool samples were analyzed for a variety of clinical conditions (see Table 1), but without any documentation of the diagnostic accuracy.

3.1. Infants. The infant group (≤1 year) included 604 males and 459 females, 6.47 ± 0.92 months of age. ANOVA revealed a significant effect of age ($F = 3.94$, $P < 001$), but not of gender on total CFU (Figure 2). As can be seen, CFU increased within the first few months, declined to a first low at the time of weaning, and exhibited a second peak at months 10 and 11. It steadily declined thereafter to reach stable levels at age 5 or 6 (described in what follows).

ANOVA showed significant effects of age, but not of gender on individual bacterial abundance. All bacteria except *Lactobacilli* were present within the first month of life. However, *Enterococci* and *E. coli* decreased ($F = 3.098$, $P <$

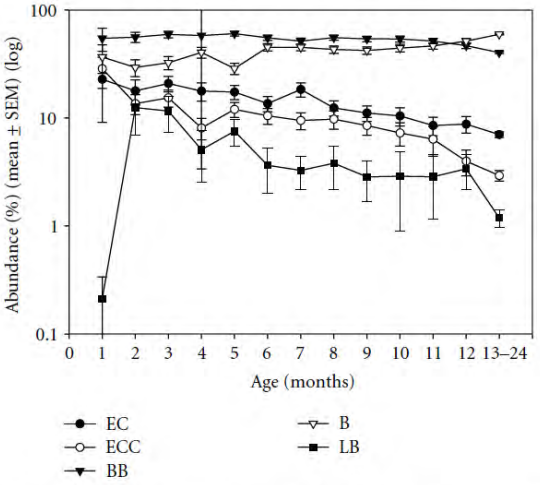

FIGURE 3: Abundance (%) (mean ± SEM) by age (months) of *E. coli*, *Enterococcus sp.*, as well as *Lactobacillus sp.*, *Bifidobacteria*, and *Bacteroides sp.*

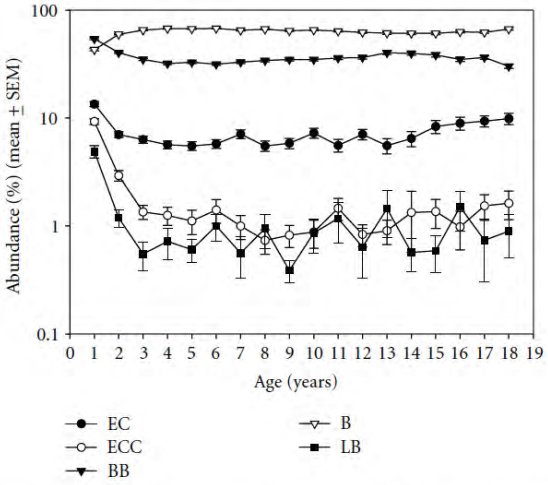

FIGURE 5: Abundance (%) (mean ± SEM) by age (years) of *E. coli*, *Enterococcus sp.*, as well as *Lactobacillus sp.*, *Bifidobacteria*, and *Bacteroides sp.*

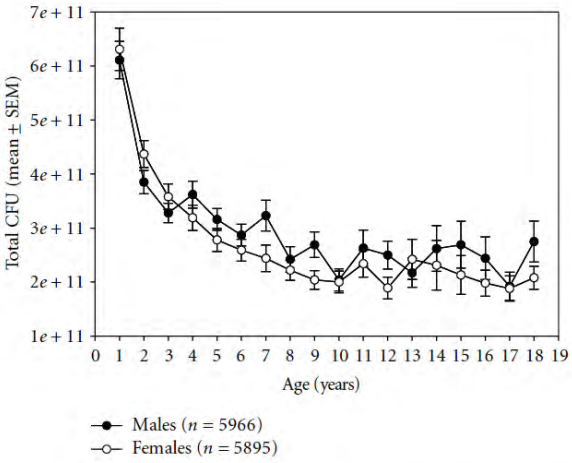

FIGURE 4: Total CFU (mean ± SEM) by age (years) and by gender.

.001 and $F = 2.884$, $P = .001$, resp.) while *Bacteroides sp.* increased ($F = 3.14$, $P < .001$), and *Bifidobacteria* remained stable ($F = 1.226$, $P = .265$) within the first year. *Lactobacilli* existed at high concentrations at month 2, but steadily decreased thereafter ($F = 2.143$, $P = .016$) (Figure 3). The percentage of *Clostridium sp.* declined from 3 to 2%.

3.2. Children. For all the 12,483 children (6.02 ± 0.04 years, 6100 : 6074 male: female, remaining = missing), total CFU significantly declined ($F = 126.74$, $P < .001$) from year 1 to year 10 and remained stable thereafter. Overall, females had a significantly lower bacterial count than males ($F = 19.24$, $P < .001$) (Figure 4).

Individual bacteria abundance showed the same age-related decline within the first few years for *Enterococci* ($F = 37.188$, $P < .001$) and *Lactobacilli* ($F = 9.888$, $P < .001$), while *E. coli* moderately decreased ($F = 8.679$, $P < .001$). Given the high proportion of their specific CFU, the time profile of *Bifidobacteria* and *Bacteroides sp.* was almost complementary. *Bifidobacteria* decreased overall ($F = 34.051$, $P < .001$), while *Bacteroides sp.* increased their proportion ($F = 43.465$, $P < .001$) (Figure 5). An independent effect of gender was noted for *Bifidobacteria* ($F = 4.982$, $P = .026$) and *E. coli* ($F = 12.203$, $P = .001$) with higher values for *Bifidobacteria*, but lower values for *E. coli* in males (data not shown). The percentage of *Clostridium sp.* remained low at around 2% (data not shown).

Stool consistency (rated between 1 = solid and 5 = liquid) decreased ($F = 16.108$, $P < .001$) and was significantly lower in females ($F = 51.735$, $P < .001$). Stool pH increased within the first years ($F = 74.425$, $P < .001$) and was overall higher in females ($F = 23.998$, $P < .001$) (Figures 6(a) and 6(b)).

Moderate negative correlations were found between pH and *Bifidobacteria* ($r = -.33$, $P < .001$), and positive correlations between pH and *E. coli* ($r = .24$, $P < .001$).

4. Discussion

Newborns are germfree at the time birth, and acquire bacteria from their immediate environment within the first hours of life: from their mothers' vaginal and fecal flora, and from the hospital or home environment, depending on the mode and location of delivery. Other factors that can influence the composition of the intestinal microflora in newborns are the environment during birth, maturity, hygiene measures, and the type of infant feeding [30].

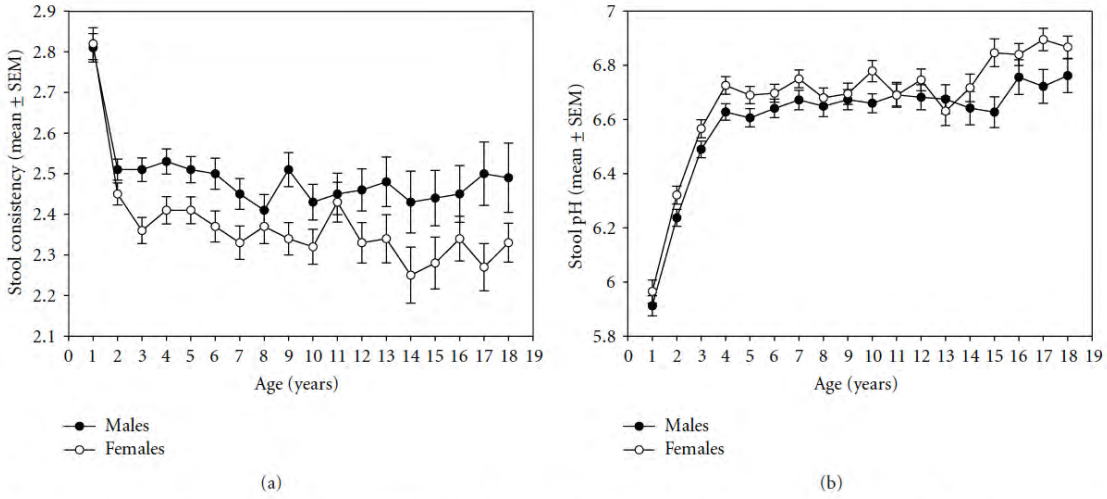

FIGURE 6: Stool consistency (mean ± SEM) (a) (1 = solid, 5 = liquid) and stool pH (mean ± SEM) (b) by gender and age (years).

Specifically, *E. coli*, *Enterobacter sp.*, and *Enterococci* colonize the gastrointestinal tract rapidly, and are often found in higher concentrations in newborns than in adults due to the fact that aerobes and facultative anaerobes are favored by the intestinal milieu at birth. When these species expand, they consume oxygen and allow anaerobic strains to follow, including *Bacteroides*, *Bifidobacteria*, and *Clostridia*. *Lactobacilli* are oxygen tolerant and are most likely derived from the mothers' fecal flora [31]. Infants delivered by Cesarian section do not come into contact with the maternal flora during delivery. Their colonization of *Bacteroides sp.* is delayed. This has been shown to be responsible for the development of atopic dermatitis later in life [32].

However, among infants, the variability of the composition and profile of intestinal microbiota remains high [33]. It is also subject to change according to differences in nutrition [34] and other cultural influences [35], as well as host genetics, which exert a strong influence over the composition of the fecal microflora [36].

Therefore, the exploration of the "normal" range of the gut microbiota composition during the development and maturation remains a methodological challenge. In a previous paper [29], we reported the data from a large (>35.000 samples) adult cohort under the assumption that the average bacterial abundance across all patients may represent an approximation of what may be called the "normal" human fecal microbiota. Using the current data from the same source, we applied this argument to infants and children.

In agreement with previous reports (summarized by Adlerberths et al. [31]), we report a high total bacterial load in the newborn intestine within the first months after birth that declines within the first years of life to levels that are maintained during childhood and thereafter [29]. The total CFU may also reflect changes associated with weaning, since a local trough can be found (see Figure 2) at around age 7 months, which may reflect this change in nutrition. This seems somewhat counter-intuitive, as one would expect to find an increase in the CFU once more types of foods are introduced in the diet. However, weaning may induce an initial decline of predominantly lactate acid metabolizing bacteria while other species may follow colonizing the colon at a slower rate.

During the first year, the composition of the microbiota changes. *E. coli* and *Enterococci* decline, while *Bacteroides sp.* increases 3-folds. *Bifidobacteria* B. in the infant stool samples remained stable during the first years. Different from many previous reports [31], *Lactobacilli* were not present during the first month, but represented as much as 10% of the identified CFU one month later (Figure 3). Controversy in literature on whether or not *Lactobacilli* are part of the early gut colonizers has been noted [31], and it has been stated that the *Lactobacilli* colonizing the infant's gut are likely not from their mothers' vaginal flora [37], but rather from maternal gut flora. Colonization with *Lactobacilli* is delayed after Cesarian section, but reported to be normal one month later [38]. Since we do not know the route of birth in our sample, we cannot exclude that this may be the cause of the late occurrence of LB in our study. Other reasons postulated [31] for this discrepancy are differences in the methods used to identify *Lactobacilli*, or differences in the colonization pattern during the last decades, as many studies derive from work earlier than 1990.

During the remaining childhood up to age 10, the total CFU constantly declines to levels at about 2×10 E11 (see Figure 4), while individual strains reach stable levels at age 3 (see Figure 5). These findings also support previous reports [4, 31]. Similar to our adult sample [29], females had significantly lower stool consistency (towards more solid stools) and higher stool pH. Although both measures are not linked, they likely express the same function. We cannot exclude, however, that this may be due to differences in body

weight and/or body mass. Unfortunately, the database did not allow adjusting for this.

Several other limitations of our analysis need to be addressed. One is that methods based on bacterial culture might not detect all species of the human microbiota. Additionally, obligate anaerobic bacteria might be at a disadvantage, and, therefore, the proportion of anaerobic bacteria to aerobic bacteria might not reflect the ratios in vivo. However, direct comparison of conventional culture analyses and molecular techniques have produced similar results, at least for the most abundant bacteria [39]. Other limitations refer to missing clinical data on medication intake, such as antibiotics. Nutritional habits and regular consumption of pre- and probiotic products may also have corroborated the results. For example, breastfeeding markedly influences the microbiotic flora in infants as does type of formula consumed and the time that supplementary food is introduced. Thus, we can only give an approximation of what may be called the 'normal' human faecal microbiota for this time period. Furthermore, the use of antibiotics severely influences intestinal flora. Since bacterial infection of the upper and lower respiratory tract is quite common, many children receive antibiotics during the first year of life. As these data were missing in our dataset, we cannot generalize our assumption. However, as stated in a previous paper [29], we reported the data from a large infant and child cohort (>12,000 samples) under the assumption that the average bacterial abundance across all patients may represent an approximation of what may be called the "normal" human fecal microbiota during infancy and childhood.

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Chapter 3

The effects of ageing on the colonic bacterial microflora in adults

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The Effects of Ageing on the Colonic Bacterial Microflora in Adults

Alterungseffekte auf die humane Kolon-Darmflora

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Zusammenfassung

Hintergrund: Die Zusammensetzung der Darmflora und deren Änderungen im Alter sind selten Gegenstand der Forschung gewesen, weder bei gesunden Probanden noch bei Patienten.

Methoden: Wir analysierten die fäkale Flora mittels konventioneller mikrobiologischer Kulturtechnik (Kyberstatus®, Institut für Mikroökologie, Herborn) aus Stuhlproben von 35292 Erwachsenen (46,3 ± 0,08 [18 bis 96] Jahre, 9564 Männer; 24784 Frauen; verbleibende = fehlend) mit unterschiedlichen intestinalen und nicht intestinalen Diagnosen auf die Gesamtflora (total colony forming units – CFU) (pro g Stuhl) wie auch auf die relative Häufigkeit (abundance) von *Bifidobacteria, Bacteroides spp., Escherichia coli, Enterococcus spp.* und *Lactobacillus spp.* im Hinblick auf Alter, Geschlecht und verfügbaren klinischen Daten (z. B. die Stuhlkonsistenz und den pH).

Ergebnisse: Die Gesamt-CFU war stabil und zeigt keine alters- und geschlechtsabhängige Veränderung. Individuelle bakterielle Species stiegen entweder konstant und signifikant mit dem Alter an (*E. coli, Enterococci spp.*), sanken mit steigendem Alter ab (*Bacteroides spp.*) oder blieben über die Lebensspanne hinweg gleich (*Lactobacilli, Bifidobacteria*). Gastrointestinale Diagnosen (Morbus Crohn, n = 198; Colitis ulcerosa, n = 515; Reizdarmsyndrome, n = 7765; andere GI-Diagnosen, n = 10478) zeigten tendenziell eine Spezifität des bakteriellen Profils; wurden die gastroenterologischen Diagnosen ausgeschlossen, änderten sich die Altersprofile der Restgruppe (n = 15619, 4197:11422) nicht.

Schlussfolgerungen: Eine konventionelle mikrobiologische Untersuchung der fäkalen Mikroflora zeigt sowohl bakterienspezifische als auch allgemeine Muster der Alterung der Flora, wobei sich in den letzten Dekaden des Lebens (älter als 60 Jahre) die profundesten Veränderungen zeigen.

Abstract

Background: The composition of the fecal mircoflora and its changes on ageing have rarely been investigated in large samples of both patients and volunteers.

Methods: We analysed the fecal flora by conventional microbiological testing (Kyberstatus®, Institute of Microecology, Herborn, Germany) of stool samples from 35292 adults (age: 46.3 ± 0.08 [18 to 96] years, 9564 males, 24784 females; remaining = missing data) with different intestinal and non-intestinal diagnoses for total colony-forming units (CFU) (per g stool) as well as relative abundance of *Bifidobacteria, Bacteroides spp., Escherichia coli, Enterococcus spp.*, and *Lactobacillus spp.* with respect to age, gender, and clinical data available (e. g., stool consistency and pH).

Results: The total CFU was stable and showed no age- or gender-related changes. Individual bacterial species constantly and significantly increased with age (*E. coli, Enterococci spp.*), or decreased at higher age (*Bacteroides spp.*), or were stable throughout the life span (*Lactobacilli, Bifidobacteria*). Gastrointestinal diagnoses (Crohn's disease, n = 198; ulcerative colitis, n = 515; irritable bowel syndrome, n = 7765; other GI diagnoses, n = 10478) tended to exhibit some specificity of the bacterial profile, and when GI diagnoses were excluded, the age-related bacterial profile of the remaining group (n = 15619, m:f = 4197:11422) was not different.

Conclusion: Conventional microbiological investigations of the fecal microbiota showed both bacteria-specific as well as a general pattern of ageing of the colonic microbiota, with the last decades (more than 60 years) demonstrating the most profound changes. It remains to be shown whether these changes reflect direct changes of the gut microbiota, the mucosal innate immunity, or indirect consequences of age-related altered nutrition.

cus spp., Lactobacillus spp. and other bacteria (*Pseudomonas spp., Klebsiella spp., Proteus spp., Citrobacter spp., aerobic bacteria*). Only bacteria that were identified in at least 50% of the samples (see below) were included into the further analysis; however, the percentage of subjects with CD was also assessed.

Detection of H_2O_2 production

Following identification, the lactobacilli were tested for hydrogen peroxide production as described previously [31]. Colonies that produced H_2O_2 on the agar appeared dark blue. Non-producers were colouless.

Additional data

Since samples were from patients with various clinical diseases (○ **Table 1**), additional data that were reported by the referring physician, included age and gender, the presumed clinical diagnosis, and stool frequency. Stool pH and consistency were determined in the laboratory.

Data analysis

After the data were made anonymous, they were provided for further statistical analysis. Prior to the analysis, the sample was screened for identical patient IDs, and any second or subsequent analysis was excluded, as were incomplete data sets except when the age and gender information was missing.

The total age distribution (○ **Fig. 1**) allowed a subdivision of the total sample into a childhood sample (n = 12484) and an adult sample (n = 35292). We here report only the data from the adult population.

Prior to the statistical analysis, data were normalized: the CFU of individual cell populations were related to the total CFU identified by calculating the relative "abundance" of each bacterial species as [(specific CFU/total CFU) × 100].

The sample was analyzed by ANOVAs for each bacterial count (abundance) with the between factors "age" (in 10-year intervals) and "gender" post-hoc t-tests with Bonferroni correction to account for multiple comparisons were used to test for single differences (between decades). Pearson's r was computed to test for intercorrelations between single measures. Subgroups of patients with inflammatory bowel diseases (IBD: Crohn's disease, ulcerative colitis), with functional bowel disorders (irritable bowel syndrome, IBS) and with "other GI disorders" (○ **Table 1**) were compared to the remaining group by ANOVA for all individual bacterial strains; in case of significance, diagnostic groups were compared pairwise by t-test with Bonferroni correction.

All data are given as mean ± SEM. A threshold of 0.05 was set to indicate statistical significance in all tests. All data were analyzed using the SPSS Version 13 Statistical Package.

Table 1 Diagnoses and diagnostic groups across patients (n = 35292).

diagnostic category	n	diagnoses	number
gastrointestinal	19262	unspecified	4890
		irritable bowel syndrome	7784
		Crohn's disease	198
		ulcerative colitis	415
		diarrhea	1714
		constipation	518
		suspected Candida colonization	3022
		suspected nutrients intolerance	117
respiratory	1264	unspecified	559
		bronchitis	155
		sinusitis	297
		asthma bronchiale	98
urogenital	581	unspecified	61
		cystitis	269
		genital mycosis	246
dermatological	1243	unspecified	89
		dermatitis	560
		psoriasis	271
		akne	161
allergic	3820	allergy, unspecified	392
		neurodermitis	1367
		urticaria	326
		pollinose	445
		allergic asthma	459
		nutrients allergy	614
		respiratory allergy	217
rheumatologic	245	arthritis	232
others	1703	immune deficiency	843
		autoimmune disease	355
		malignoma	476
missing	7163		7163
total	35292		34313 (97%)

Results

The sample consisted of n = 9567 males and n = 24784 females (remaining to 35292 = missing) (age: 46.3 ± 0.08 [18 to 96] years) stool samples that were collected due to different diagnoses (○ **Fig. 1**). Analysis of the total CFU did not reveal changes with age and remained stable within the different age groups. Additionally, no gender-related differences were observable (○ **Fig. 2**).

Among the 11 bacterial species or genera, which were routinely assessed, the following were present in at least 50% of samples, and were therefore subjected to further analysis: *Bifidobacteria, Bacteroides spp., E. coli, Enterococcus spp.*, and *Lactobacillus spp. Abundance of E. coli* and *Enterococci* significantly increased with age ($F = 12.9$, $p < 0.001$ and $F = 11.266$, $p < 0.001$, respectively), while *Bifidobacteria* decreased ($F = 34.94$, $p < 0.001$) and *Bacteroides spp.* increased moderately until age 70 and decreased

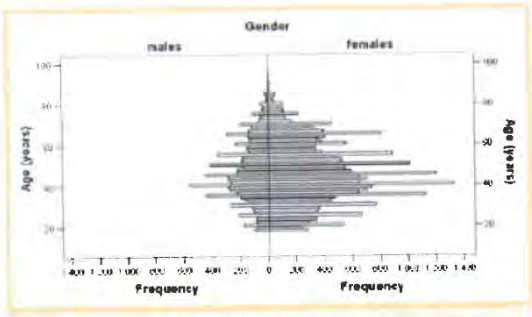

Fig. 1 Age distribution (in years) in male and female patients between 18 and 96 years.

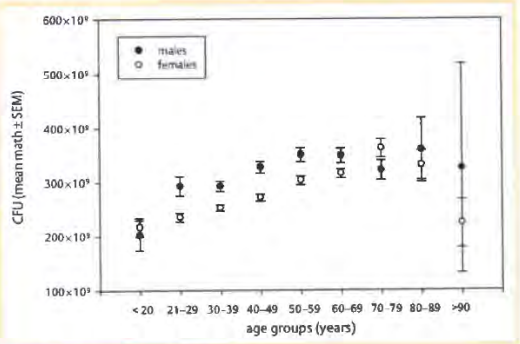

Fig. 2 Total CFU (mean ± SEM) and by gender.

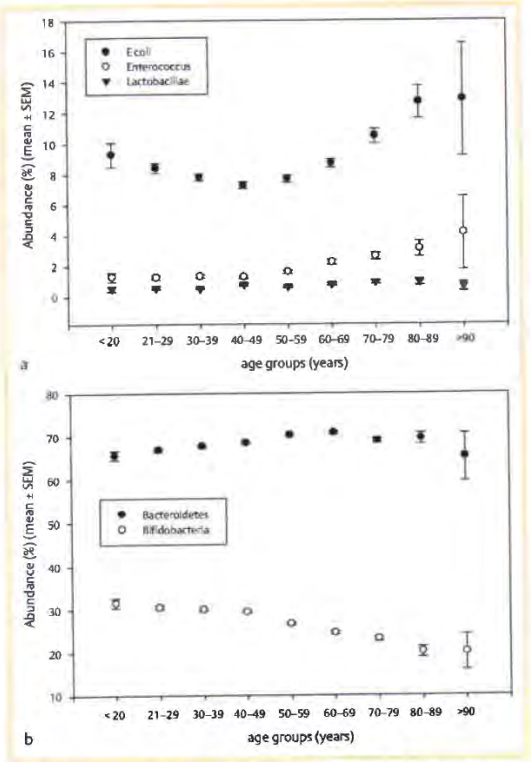

Fig. 3 Abundance (%) (mean ± SEM) by age (decades) of *E. coli*, *Enterococcus sp.*, *and Lactobacillus sp.* **a**, and of *Bifidobacteria* and *Bacteroides sp.* **b**.

thereafter, mainly in the very old ($F = 4.769$, $p < 0.001$), or remained unaltered like *Lactobacilli* (○ **Fig. 3a, b**).

The percentage of subjects with C. difficile was 17.3% across all ages and varied between 15 and 25%. Percent H_2O_2 producing bacteria remained stable between 50% and 60%.

At younger ages, *Bacteroides* and *Bifidobacteria* were significantly higher in men as compared to women, but for E. coli the abundance was higher in women; a significant interaction

Table 2 ANOVA results for age and gender effects on bacterial abundance of five species.

	age	gender	age × gender
E. coli	$F = 12.90$, $p < 0.001$	n.s.	$F = 3.61$, $p < 0.001$
Bifidobacteria	$F = 34.94$, $p < 0.001$	n.s.	$F = 2.28$, $p < 0.001$
Lactobacilli sp.	n.s.	n.s.	n.s.
Bacteroides sp.	$F = 4.769$, $p < 0.001$	n.s.	$F = 5.15$, $p < 0.001$
Enterococcus sp.	$F = 11.26$, $p < 0.001$	n.s.	n.s.

of gender with age for all three species (○ **Table 2**) indicates that the gender effect is reversed with age.

The adult sample included patients with Crohn's disease (CD) ($n = 198$) and ulcerative colitis (UC) ($n = 515$), 7765 patients with the diagnosis of irritable bowel syndrome (IBS), and 10,478 patients with "other gastrointestinal disorders" (○ **Table 1**, above). Comparison to the remaining group (no GI complaints, $n = 16,329$) revealed that the total CFU were significantly affected by the various diagnoses ($F = 6.648$, $p < 0.001$) and was elevated in CD and UC compared to the non-GI group ($p = 0.022$ and $p = 0.013$, respectively) (○ **Fig. 4a**). *Lactobacillus spp.* tended to be higher in UC ($p = 0.061$) but not in CD, and higher levels of *Enterococcus spp.* in UC and CD did not reach significance, but IBS patients showed a significantly ($p < 0.006$) lower abundance of *Bifidobacteria* (○ **Fig. 4b**).

The prevalence of C. difficile did not differ between patients with inflammatory bowel diseases and those with other diagnoses, and the percentage of H_2O_2 producers was similar in all groups.

When all diagnoses related to the gastrointestinal tract were excluded, the age-related bacterial profile of the remaining group ($n = 15619$, 4197:11422) showed the same pattern as described above (data not shown).

Stool consistency increased moderately but significantly over the years until age 60 and was lower in women, while stool pH remained stable until age 60 to increase thereafter; pH was significantly higher in women (○ **Fig. 5a, b**). Correlation between pH and individual bacteria was low but significant and positive for *E. coli* ($r = 0.32$, $p < 0.001$) and negative for *Bifidobacteria* ($r = -0.18$, $p < 0.001$) (data not shown).

Discussion

Age-related changes in the abundance of non-pathogenic bacterial strains of the human colon have rarely been investigated in larger samples, and gender effects have not been described, either in patients with intestinal and non-intestinal disorders or in healthy subjects.

We have here reported the data from a large (> 35000 samples) cohort under the assumption that the average bacterial abundance across all patients may represent an approximation of what may be called the "normal" human fecal microbiota.

The few studies that have assessed the ageing on the human colon microbiota have shown that some bacterial species decline in their abundance with age, while others increase [24–28]. Culture-based studies investigating the composition of fecal microbiota in aged individuals showed fewer total anae-

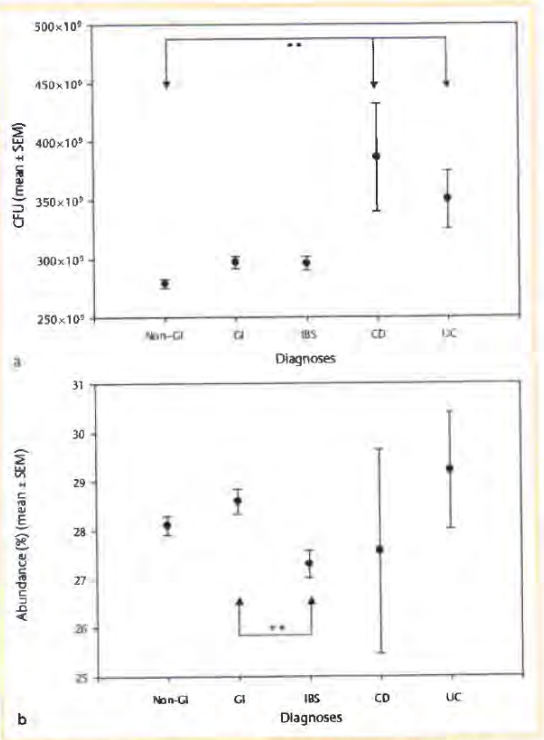

Fig. 4 Total CFU (mean ± SEM) **a** and abundance (%) (mean ± SEM) of *Bifidobacteria* **b** by diagnoses (non-GI = non-intestinal diagnoses, IBS = Irritable bowel syndrome; CD = Crohn's disease; UC = ulcerative colitis; GI = other gastrointestinal diagnoses); ** indicate significant differences in post-hoc t-testing.

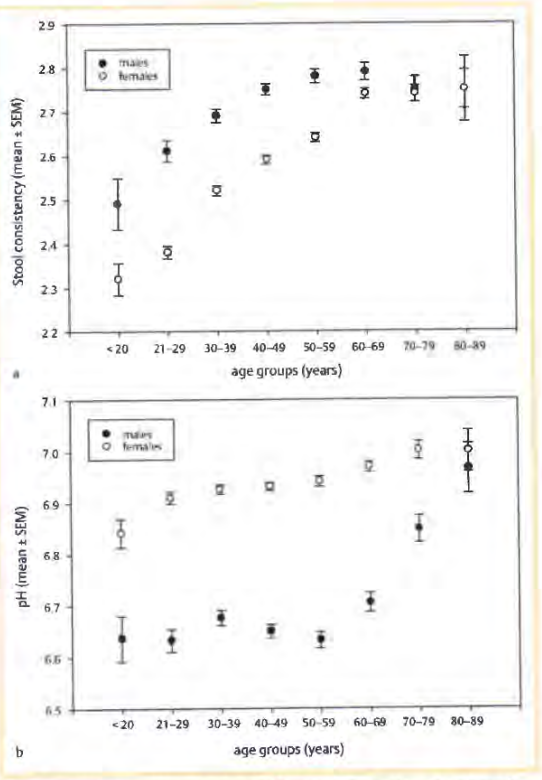

Fig. 5 Stool consistency (mean ± SEM) **a** (1 = solid, 5 = liquid) and stool pH (mean ± SEM) **b** by age.

robes and *Bifidobacteria* [24, 25, 32]. In contrast Enterobacteriaceae and overall counts of endotoxin-producing Gram-negative bacteria were significantly increased in the elderly [24, 25, 32]. The diminished *Bifidobacteria* counts [26, 27] with concomitant increases in *Enterobacteriaceae* [24, 27, 28] and increased dominant bacterial diversity at the species level [27, 33] were confirmed using molecular approaches.

It is unknown so far whether this is the consequence of ageing per se or whether it may reflect age-related changes in nutritional habits [7, 32] of other factors. The influence of the diet on composition of the intestinal microbiota is well documented. *Bifidobacteria* are described to be present in higher levels in individuals who consume a western diet as compared to individuals who subsisted on a native Japanese diet [3]. Additionally, the gut microbiota of vegetarians differs from that of individuals on a Western diet in that the former is dominated by clones belonging to the Clostridium rRNA subcluster XIVa (also called Clostridium coccoides cluster) and the Clostridium rRNA cluster XVIII (also called Clostridium ramosum cluster) [5, 33]. In a strict vegetarian, the detection of Faecalibacterium prausnitzii failed [5]. In line with this, Mueller et al. detected the highest levels of Faecalibacterium prausnitzii in a Swedish study group, a population whose dietary habits are characterized by a high consumption of fish and meat [7]. Furthermore, higher numbers of Bifidobacteria but significantly lower levels of anaerobic bacteria and lecithinase-negative clostridia were detected in rural elderly compared to urban elderly in Japan, who have a lower intake of dietary fiber [4]. Additionally it has been demonstrated that the consumption of fructooligosaccharides leads to an increase in fecal *Bifidobacteria* [4, 34–36].

In our study group no effect of age on the proportion of Lactobacilli was detectable. In contrast, Mueller et al. report on age-dependent differences in elderly living in France and Germany showing age-dependent increased levels of *Lactobacilli* and *Enterococci*. In line with our findings, elderly living in Sweden or Italy showed no age-related changes in the proportion of *Lactobacilli*. However, the Swedish group had the highest levels of *Lactobacilli* [7].

The limitations of our analysis need to be addressed: one is that methods based on bacterial culture might not detect all species of the human microbiota, additionally obligate anaerobic bacteria might be disadvantaged and therefore the proportion of anaerobic bacteria to aerobic bacteria might not reflect the ratios in vivo. However, direct comparison of conventional culture analyses and molecular techniques have produced similar results, at least for the most abundant bacteria [39]. Other limitations refer to missing clinical data on medication intake, e.g., antibiotics, in these patients, and nutritional habits, e.g., the regular consumption of pre- and probiotic products that may have corroborated the results. This refers also to the diagnostic accuracy of the specific GI diagnoses (UC, CD, RDS). Finally, the fecal flora that was analysed here may not reflect the mucosal flora.

Es müsste jedoch geklärt werden, ob dies direkte Veränderungen der Flora sind, Folge der altersbedingte Änderungen des mukosalen Immunsystems oder ob sie infolge von Änderungen der Ernährung im Alter auftreten.

Introduction

Age-related changes in the abundance on non-pathogenic bacteria of the human colon have rarely been investigated in larger samples, neither in patients with intestinal and non-intestinal disorders nor in healthy subjects. Additional gender differences in the human fecal microbiota have rarely been evaluated.

When the "normal" intestinal microbiota were the subject of investigation, the colonization was usually related to specific diagnoses such as inflammatory bowel diseases [1, 2], to cancer pathogenesis [1, 3], or to other diseases with immunological consequences [1]. In healthy subjects, the bacterial microbiota of non-pathogenic species has been related to restrictive nutritional habits such as vegetarian or vegan diets [4, 5], or to cultural differences based to nutritional habits, e.g., in Japan, the United States and the Mediterranean area [6, 7]. Eventually, dietary habits have been interpreted – with caution – as being related to the prevention of cancer or the treatment of inflammatory diseases [8, 9].

One reason for the relative absence of large-scale investigations was and still is the suspicion that bacterial colonies in respective stool samples would not remain stable during transport and storage, and that this would corroborate the correct estimate of bacterial counts with conventional microbiological tools. Hence, commercial assays for fecal microbiota have not gained wider acceptance as diagnostic tools in routine clinical assessment, e.g., in patients with gastrointestinal disorders. One exception is the strong association between inflammatory bowel diseases, especially Crohn's disease and ulcerative colitis, with Clostridium difficile colonization [2, 10, 11].

With the recent interest in prebiotic and probiotic treatment in functional [12, 13], but also in other intestinal [14–19] and non-intestinal disorders [20–22] on the one hand, and the availability of molecular biological tools for counting the different species, but also to identify the genetically different subspecies within each strain, characterization of the normal human colonic microbiota and its variability has prompted a new surge for studies. Both investigations in maturation of the microbiota during childhood [23] and its change with ageing [7] have recently been performed. However, as with most studies based on conventional microbiology, samples sizes have been rather small so far, as PCR technology is not yet frequently available, and microarray chip technology that does allow assessment of all bacterial species known to inhabit the human colon (in the range of > 1000) is still very costly. This may, however, change in the very near future.

The few studies that have assessed the ageing of the human fecal microbiota, have shown that some bacterial species decline in their abundance with age, while others increase [24–28]. It is unknown so far, whether this is the consequence of ageing per se or whether it may reflect age-related changes of nutritional habits [7] or other factors. One of the most striking findings was, however, a decline of the diversity of subspecies of individual bacterial strains, e.g., of the Lactobacilli spp., with ageing, that may indicate rather a direct effect of age on the colonic microbiota [29, 30].

We here report data from a large (> 35000 samples) conventional microbiological database in an adult population with various intestinal and non-intestinal symptoms and diagnoses. The underlying hypothesis of this analysis is that across different diseases and medical conditions, the average bacterial abundance across all patients may represent an approximation of what may be called the "normal" human fecal microbiota. Our assessment is based on conventional microbiological analyses that were performed in a commercial laboratory with GLP certification during one year.

Material and Methods

Collection of stool samples for microbiological analysis

During the course of one year (2006), all fecal samples that were sent in by general practitioners for routine microbiological analysis of non-pathogen fecal bacterial flora (KyberStatus, Institute of Microecology, Herborn, Germany) were included. In general, samples reached the laboratory within one to two days and were processed immediately.

To ensure that the transport did not have any effect on the cultured species, a storage study was performed with 20 fresh samples. In short, a 0.2 g aliquot of feces was serially diluted in 1 mL of phosphate-buffered saline (PBS, pH 7.2). The solution was vortexed for 5 s and serially diluted (to 10^{-9}) in PBS, pH 7.2. One mL of each dilution was plated onto enrichment or selective agar media.

The remaining feces were stored for three days at a temperature of 25 °C, which represents the average temperature during shipment. Following the incubation period the samples were processed as described and the results were compared. No significant discrepancy in the cell counts of the investigated microbiota could be detected within two days. Thus, it was concluded that a shipment time of up to two days will have no effect on the composition of the cultivable microbiota. Only samples which took one or two days of shipment were included in the study.

Identification and enumeration of microorganisms

Viable bacterial cell counts in feces were enumerated on the following selective media: Columbia blood agar (total cell count; BioMerieux, Nürtingen, Germany), U3G agar (*Enterobacteriacae, Enterococci;* Heipha, Heidelberg, Germany), Rogosa agar, (*Lactobacilli;* Heipha), DIC agar (*Bifidobacteria*; Heipha), Schaedler agar (*Bacteroides sp.*; Heipha) and SPM agar (*Clostridia*; Heipha). Fecal samples were serially diluted in 1 mL of phosphate-buffered saline (PBS, pH 7.2) and subsequently plated on selective agar plates by a fully automated spiral plater capable of plating 12 agar plates simultaneously. Subsequently, the plates were incubated under aerobic (24 h) or anoxic (48 h) conditions at 37 °C for at least two days. Bacteria were first identified by Gram staining and colony morphologies. Additionally, identifications were performed by the API® and VITEK® systems (bioMérieux). All counts were recorded as the numbers of $\log_{10}$ CFU per mL of sample.

The following bacteria were routinely analyzed: *Clostridium difficile, Bifidobacteria, Bacteroides spp., Escherichia coli, Enterococ-*

In summary, we found, no impact of ageing or gender on total CFU of intestinal microbiota. The analysis of CFU of *E. coli* and Enterococci revealed an increase in abundance of *E. coli* and a intendency of Enterococci which was related to ageing. In contrast, the abundance of *Bacteroides spp* showed a negative correlation with ageing. However, studies based on molecular detection techniques have to be performed to exclude effects on p. ex. obligate anaerobic bacteria by suboptimal environmental conditions.

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Chapter 4

Microbiota and SCFA in lean and overweight healthy subjects

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Microbiota and SCFA in Lean and Overweight Healthy Subjects

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Obesity has recently been linked to the composition of human microbiota and the production of short chain fatty acids (SCFAs). However, these findings rely on experimental studies carried out using rather small and defined groups of volunteers or model animals. Our aim was to evaluate differences within the human intestinal microbiota and fecal SCFA concentration of lean and obese subjects. A total of 98 subjects volunteered to take part in this study. The BMI in kg/m^2 of 30 volunteers was within the lean range, 35 were overweight and 33 were obese. The fecal microbiota was characterized by real-time PCR analyses. With the primers used herein we were able to cover 82.3% (interquartile range of 68.3–91.4%) of the total microbiota detectable with a universal primer. In addition, the concentration of SCFA was evaluated. The total amount of SCFA was higher in the obese subject group (P = 0.024) than in the lean subject group. The proportion of individual SCFA changed in favor of propionate in overweight (P = 0.019) and obese subjects (P = 0.028). The most abundant bacterial groups in faeces of lean and obese subjects belonged to the phyla *Firmicutes* and *Bacteroidetes*. The ratio of *Firmicutes* to *Bacteroidetes* changed in favor of the *Bacteroidetes* in overweight (P = 0.001) and obese subjects (P = 0.005). Our results are in line with previous reports suggesting that SCFA metabolism might play a considerable role in obesity. However, our results contradict previous reports with regard to the contribution of various bacterial groups to the development of obesity and this issue remains controversial.

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INTRODUCTION

Obesity is a complex disease which is not completely understood and which is considered by the World Health Organization to be a global epidemic with more than 1 billion adults overweight—one third of these being clinically obese (1). Reasons for obesity can be given in the increased consumption of more energy-dense, nutrient-poor foods containing high levels of sugar and saturated fats in combination with reduced physical activity. However, it has been suggested that several other factors contribute to the development of obesity, such as genetic factors (e.g., Prader–Willi syndrome) (2), underlying illnesses (e.g., hypothyroidism) (3), medications (e.g., atypical antipsychotic) (4,5), high glycaemic diets (6,7), stress (8,9), smoking cessation (10,11), virus infections (12–14) and recently also bacteria (15,16).

The microbiota of the human gastrointestinal tract has been studied extensively due to its role both in disease causation and in maintaining gut health. One of the important activities of the large intestinal microbiota is to break down substrates such as resistant starch and dietary fibre, which are not completely hydrolysed by host enzymes in the small intestine (17–19). The main fermentation products ensuing from this fibre breakdown are the short chain fatty acids (SCFAs) acetate, propionate, and butyrate (20), which can be utilised for lipid or glucose *de novo* synthesis (21). The bacterial SCFAs thus provide an additional source of energy for the body.

Recently, it has been hypothesised that an increased ratio of *Firmicutes* to *Bacteroidetes* may make a significant contribution to the pathophysiology of obesity. By comparison with wild-type animals Ley and co-workers demonstrated that genetically obese (ob/ob) mice show an increased proportion of the *Firmicutes* and a decrease in *Bacteroidetes*, (22). A further report indicated that the proportion of *Bacteroidetes* 16S rRNA sequences was diminished in faeces from 12 obese human subjects (23). Furthermore, Gill and co-workers found that the human gut microbiome of two healthy subjects is enriched with many clusters of orthologous groups representing key genes in the pathway of methanogens (24). This finding

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led to the hypothesis that *Methanobrevibacter smithii* may be a therapeutic target for the reduction of energy harvest in obese humans (25,26), as *M. smithii* is the major representative of the human gut methanogens (27).

The purpose of this study was to investigate whether the proposed role of SCFA and microbiota composition in obesity, which was based on proof of principle experiments, can be confirmed in a larger study which did not exclude all confounding factors.

METHODS AND PROCEDURES

Volunteer recruitment

Lean and obese volunteers of both sexes were recruited from the Institute of Microecology and from the obesity consultation hours at the University of Giessen and Marburg. In total 98 volunteers (34 males and 64 females) took part in the study. All of the samples collected were analysed. The volunteers were aged 47 ± 13 year (mean ± s.e.m.: range 14–74 year). The BMI in kg/m^2 of 30 volunteers was within the normal range (18.5–24.9), while 35 were overweight (25.0–29.9) and 33 were obese (≥30.0). Of the latter, 17 were classified as obesity class 1 (30–35), 11 as class 2 (35–40), and 5 as class 3 (>40). No antibiotics had been taken in the 6 months prior to the study. All participants subsisted primarily on a western diet and all volunteers provided informed, signed consent.

Collection and preparation of stool samples for analysis

From the fresh stool sample provided from each volunteer DNA (200 mg) was extracted using the Easy Mag DNA Isolation system (BioMerieux, Nuertingen, Germany) according to the manufacturer's instructions. The remaining sample was stored at −20 °C for metabolic analysis.

Determination of SCFA and fecal gross energy content

Human stool samples to be analysed for SCFA were freeze-dried and subsequently analysed using gas chromatograph as previously described (28). Briefly, the sample was weighed (~80 mg dry matter) and an extraction solution (1 ml) containing oxalic acid (0.1 mol/l), sodium azide (40 mmol/l), and an internal standard (caproic acid 0.1 mmol/l) was added. The solution was extracted for 60 min on a horizontal shaker and then centrifuged (10 min at 16000 × g). Concentrations of the SCFA were determined in the supernatant using an Agilent 6890N gas chromatograph with flame ionization detection equipped with a capillary column Innowax 30 m × 530 µm ×.0.1 µm (Agilent). ChemStation software was used for data processing. Bomb calorimetry was performed on fecal samples of all lean, overweight and obese volunteers. Aliquots of fecal samples were freeze-dried for 48 hours and the gross energy content measured using an adiabatic bomb calorimeter, calorimetric cooling system and oxygen station (models C7000, C7002, and C7048, respectively, from IKA-Analysentechnik, Heitersheim, Germany). The calorimeter energy equivalent factor was determined using benzoic acid standards.

Primer selection for quantitative PCR (qPCR)

Primers were selected to recognise similar bacterial groups as defined by previously released 16S rRNA targeting probes used for fluorescence in-situ hybridisation analysis. A particular fluorescence in-situ hybridisation-defined group containing the appropriate target sequence in the ARB program (31) was selected and subsequently qPCR primers were designed with the ARB program to amplify the same group of commensal bacteria (29). Discriminating nucleotides were chosen to be at the 3'end of the primer and a specific primer was combined with a universal primer that does not exclude any members belonging to that particular group. qPCR primers for bacterial groups were stringently selected using the Primer Designer programme to avoid primer-dimer formation and yield products of 100–300 base-pairs (Table 1). The standard line was based on actual counting of cultured bacteria and correlated directly to the C_t values of the qPCR. We validated the qPCR data to actual bacterial counts as described by Barman and colleagues (30). Specificity of the different qPCR primer sets was tested elsewhere (31–38). Several of the primers mentioned have already been used in at least one other published study (39). Enumeration of predominant bacteria in the fecal samples by qPCR

Quantitative PCR amplification and detection were carried out using the primers described in Table 1. PCR amplification and detection was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Darmstadt, Germany) in optical-grade 96-well plates sealed with optical sealing tape. Each reaction mixture (25 µl) was composed of 12.5 µl of QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 2 µl primer mix (10 pmol/µl each), 9 µl sterile distilled H_2O, and 1.5 µl stool DNA (10 ng/µl). For the negative control, 2 µl of sterile distilled H_2O was added to the reaction solution instead of the template DNA solution. A standard curve was produced using the appropriate reference organism to quantify the qPCR values into number of bacteria/g. The standard curves were prepared in the same PCR assay as for the samples. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was carried out following amplification to distinguish the targeted PCR product from the nontargeted PCR product. The melting curves were obtained by slow heating at temperatures from 55 to 95 °C at a rate of 0.2 °C/s, with continuous fluorescence collection. The data was analysed using the ABI Prism software.

The real-time PCRs were performed in triplicate, and average values were used for enumeration. PCR conditions were optimized based on those described in the literature (31–38). The amplification programme consisted of one cycle of 95 °C for 15 min and then 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. This programme was used for all qPCR-primers with the exception of the qPCR-primers for the detection of the genus *Methanobrevibacter*. Here, the annealing temperature was 58 °C for 30 s (38).

In all assays the amplification efficiency was higher than 90%, and the standard curve showed a linear range across at least 5 logs of DNA concentrations with a correlation coefficient >0.99. The lowest detection limits of all assays were as low as 10–100 copies of specific bacterial 16S rDNA per reaction, corresponding to 10^4—10^5 copies per gram of wet-weight faeces. The data was analysed using the ABI Prism software. Table 1 shows the primer sets for the targeted bacterial groups.

Statistical analyses

All statistical analyses were performed using SPSS (SPSS, Chicago, IL). The normality of the data was checked using the nonparametric Kolmogorov–Smirnov test with Lilliefors correction. Depending on the normality of the underlying data, the ANOVA test or the Mann-Whitney-U-test was used to perform statistical analyses. The χ^2-test statistic was used to analyze the proportion of volunteers harbouring methanogens. Correlations between the parameters measured were appraised by calculation of nonparametric Spearman correlation coefficients and subsequent visual inspection of scatter plots. Additionally, only the significant Spearman correlations that were also significant in a partial correlation analysis with "age" and "gender" as control variables were considered. Test results of all analyses were considered significant at an alpha of $P < 0.05$.

RESULTS

Changes in SCFA concentrations and fecal energy content

The major SCFA found in the stool samples were acetate, propionate, butyrate, and valerate as well as iso-valerate and iso-butyrate (Table 2). The differences in the stool SCFA concentrations between the lean, overweight and obese subjects were considerable. The mean total SCFA concentration in fecal samples of obese volunteers was by more than 20% higher in total than of lean volunteers ($P = 0.024$). The highest increase

Table 1 16S rRNA gene-targeted group-specific primers used in this study

Target	Primer name	Primer sequence (5′-3′)	Reference probe/primers	Reference
Total bacteria	UniF340 UniR514	ACTCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGGC		(40)
Firmicutes				
C. leptum group (clostridial cluster IV)	C-lept-F1123 C-lept-R1367	GTTGACAAAACGGAGGAAGG GACGGGCGGTGTGTACAA	Clept1240 GTTTTRTCAACGGCAGTC	(32)
C. coccoides group (clostridial cluster XIVa)	Univ-F338 C.coc-R491	ACTCCTACGGGAGGCAGC GCTTCTTAGTCAGGTACCGTCAT	Erec482 GCTTCTTAGTCARGTACCG	(33)
E. cylindroides group	Univ-F338 E.cyl-R399	ACTCCTACGGGAGGCAGC CATTGCTCGTTCAGGGTTC	Ecyl387 CGCGGCATTGCTCGTTCA	(35)
Lactobacilli/enterococci	Lab-F362 Lab-R677	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	Lab158 GGTATTAGCAYCTGTTTCCA	(34)
Ruminococcus flavefaciens subgroup	Rum-F476 RumR794	CAGCAGCCGCGGTAATA CCCACACCTAGTAATCATCGTT	Rfla729 AAAGCCCAGTAAGCCGCC	(36)
Veillonella	Veil-F71 VeilR413	AYCAACCTGCCCTTCAGA CGTCCCGATTAACAGAGCTT	Veil223 AGACGCAATCCCTCCTT	(35)
Bacteriodetes				
Bacteroides	Bact-F285 Univ-R338	GGTTCTGAGAGGAGGTCCC GCTGCCTCCCGTAGGAGT	Bac303 CAATGTGGGGGACCTT	(31)
Prevotella	Prevo-F449 PrevoR757	CAGCAGCCGCGGTAATA GGCATCCATCGTTTACCGT	Prev803 CARGTTAAACGATGGATGCC Prev1335 GGTCGGGTTGCAGACC	(32)
Actinobacteria				
Bifidobacterium	Bifido-F143 Univ-R338	CTCCTGGAAACGGGTGGT GCTGCCTCCCGTAGGAGT	Bif153 ACCACCCGTTTCCAGGAG	(37)
Archea				
Methanobrevibacter genus	Mbb-F1 Arch-R1386	CTCCGCAATGTGAGAAATCG GCGGTGTGTGCAAGGAGC	—	(38)

Table 2 Mean SCFA (mmol/l) concentration and gross energy content (kJ/g) in dry feces of volunteers with different BMI

BMI	Category	*n*	Acetate	Propionate	Iso-butyrate	Butyrate	Iso-valerate	Valerate	Total SCFA	Energy
18.5–24.9	Normal	30	50.5 ± 12.6	13.6 ± 5.2	1.8 ± 0.9	14.1 ± 7.6	2.7 ± 2.1	1.9 ± 0.7	84.6 ± 22.9	21.7 ± 1.3
25–30	Overweight	35	56.0 ± 18.2	18.3 ± 7.9[a]	1.6 ± 0.9	18.5 ± 10.1	2.3 ± 1.7	2.0 ± 1.1	98.7 ± 33.9[a]	21.6 ± 1.6
>30	Obesity	33	59.8 ± 18.3	19.3 ± 8.7[a]	1.7 ± 1.2	18.1 ± 10.0	2.8 ± 2.0	2.3 ± 1.1	103.9 ± 34.3[a]	21.6 ± 1.6

[a]Significant increase in comparison to lean volunteers ($P < 0.05$).

was seen for propionate with 41% ($P = 0.002$), followed by butyrate (28%, $P = 0.095$). A numerical increase of valerate (21%), and acetate (18%) concentrations was observed. In addition, this resulted in changes in the proportions of individual to total SCFA. The propionate proportion was thus higher in overweight (18.7%, $P = 0.019$) and obese (18.3%, $P = 0.028$) volunteers than in lean volunteers (15.9%). No considerable increase or change in the proportions of the iso-SCFA was observed.

To estimate the efficiency of nutrient utilisation, gross energy content in fecal samples was determined. The mean content in fecal samples of lean, overweight and obese volunteers was noted as 21.7, 21.6, and 21.6 kJ/g dry matter, respectively, and was just numerically different between the BMI classes studied.

Quantification of the predominant bacterial groups in the stool

qPCR analyses were performed to quantify individual bacterial groups in stool samples collected from the study group. The most abundant bacterial groups in lean and obese subjects (Table 3) were the gram-positive bacteria belonging to the *Clostridium leptum* group and the *Clostridium coccoides* group as well as the gram-negative *Bacteroides* spp., which altogether made up a median proportion of 96.3% (interquartile range of 89–99.2%) of all detected bacteria. Species of the genera *Veillonella*, *Bifidobacterium*, *Prevotella*, *Lactobacillus*, *Enterococcus*, *Methanobrevi-bacter*, and the *Eubacterium cylindroides* group accounted for the remaining minor proportion of all bacteria. Some of these microbial groups were present in slightly different concentrations in faeces of lean,

Table 3 Fecal microbiota composition of lean (n = 30), overweight (n = 35), and obese subjects (n = 33)

	BMI		
Population	<25	25–30	>30
Firmicutes			
Clostridium leptum group	10.4 ± 0.35	10.3 ± 0.64	10.2[a] ± 0.71
Ruminococcus flavefaciens subgroup	10.3 ± 0.41	9.9[a] ± 1.02	9.9[a] ± 1.15
Clostridium coccoides group	10.3 ± 0.34	10.3 ± 0.63	10.0 ± 0.50
Lactobacillus/ Enterococcus	7.0 ± 0.53	6.8 ± 0.64	6.9 ± 0.45
E. cylindroides group	7.7 ± 1.24	6.9 ± 1.65	7.7 ± 1.85
Veillonella	6.5 ± 0.59	6.4 ± 0.83	6.3 ± 1.44
Bacteroidetes			
Bacteroides	10.2 ± 0.47	10.4 ± 0.49[b]	10.2 ± 0.74
Prevotella	6.6 ± 1.42	5.9 ± 2.00	5.9 ± 2.01
Actinobacteria			
Bifidobacterium	8.7 ± 0.69	8.5 ± 0.81	8.3[a] ± 0.76
Archaea			
Methanobrevibacter[c]	8.0 ± 3.92	7.3 ± 3.73	6.2[a] ± 3.24
Total cell count	10.9 ± 0.20	11.1 ± 0.34	10.9 ± 0.42
Sum of all detected species	10.8 ± 0.21	11.1 ± 0.35	10.9 ± 0.42
Proportion of Firmicutes (%)	73.1	47.7[a]	51.0[a]
Proportion of Bacteroidetes (%)	22.9	46.8[b]	45.0[b]
Ratio Firmicutes/ Bacteroidetes	3.3	1.1[a]	1.2[a]

Median $\log_{10}$cells/g of feces (dry weight) ± s.d.
[a]Significant decrease in comparison to lean volunteers ($P < 0.05$). [b]Significant increase in comparison to lean volunteers ($P < 0.05$). [c]Median numbers calculated solely on the basis of methanogen carriers (63.3, 45.7, and 33.3% of lean, overweight, and obese individuals, respectively).

overweight and obese volunteers. Overweight volunteers harboured significantly higher fecal concentrations of the genus *Bacteroides* than lean ($P = 0.002$) but not than obese volunteers ($P = 0.145$). On the other hand, both overweight ($P = 0.006$) and obese volunteers ($P = 0.011$) exhibited lower cell numbers of the *Ruminococcus flavefaciens* subgroup. In addition, in obese volunteers numerically or even significantly lower fecal concentrations of the *C. leptum* group ($P = 0.07$) and of the genus *Bifidobacterium* ($P = 0.02$), respectively, were noted. The *C. leptum* group, which is made up to an extensive part by the *Ruminococcus flavefaciens* subgroup, belongs to the bacterial division of *Firmicutes*, which therefore accounted for a significantly smaller proportion of the total sum of all microorganisms studied in overweight ($P = 0.001$) and obese volunteers ($P = 0.002$) compared with lean volunteers. In turn, the median proportion of the bacterial division *Bacteroidetes* of the total sum of species studied was higher in overweight (46.8%, $P = 0.001$) and obese (45.0%, $P = 0.006$), respectively, than in lean volunteers (22.9%), since the *Bacteroidetes* count remained largely unaffected by BMI. On the whole, this led to a lower ratio of *Firmicutes* to *Bacteroidetes* in overweight ($P = 0.001$) and obese volunteers ($P = 0.005$). Furthermore, species of the genus *Methanobrevibacter* were detected in a smaller proportion of overweight (45.7%, $P = 0.155$) and obese volunteers (33.3%, $P = 0.017$) compared to lean volunteers (63.3%). Simultaneous, obese carriers of methanogens exhibited a lower cell count of *Methanobrevibacter* spp. than lean carriers ($P = 0.018$).

An analysis of correlations between the parameters measured and the BMI supports the results obtained from the comparison of the different BMI groups as significant correlations between the BMI and propionate ($P = 0.003$), the propionate proportion of SCFA ($P = 0.005$), and the concentration of *Bifidobacterium* ($P = 0.003$) and *Methanobrevibacter* ($P < 0.005$) were detectable which even remain significant following correction to take into consideration the influence of age and gender using partial correlation analysis. Within the subgroup of obese participants no additional dependencies between the BMI and other parameters could be detected.

DISCUSSION

In recent years the importance of the gut microbiota to health has been widely acknowledged. Recent reports of a possible correlation between the human gut microbiota and obesity has placed the focus on new aspects of the significance of the human microbiota to wellbeing (16,22,26,40). It has been assumed that the proportional representation of the *Firmicutes* and *Bacteroidetes* may play a role in the development of obesity (22,40). Members of both groups produce SCFA from dietary compounds which escape digestion in the small intestine, thus supplying the host with an additional amount of energy. Normal colonic epithelia derive 60–70% of their energy supply from SCFA, particularly butyrate (41). Propionate is largely taken up by the liver and is a good precursor for gluconeogenesis, liponeogenesis and protein synthesis (42,43). Acetate enters the peripheral circulation to be metabolised by peripheral tissues and is a substrate for cholesterol synthesis (21). Within our study group total SCFA content increased significantly from 84.60 mmol/l in lean to 103.87 mmol/l in obese subjects. Additionally, the propionate concentration increased significantly from lean to obese subjects as well as its proportion of total SCFA.

High fecal concentrations of total or individual SCFA might also be the result of increased microbial production, shifts in microbial cross feeding patterns, low mucosal absorption or even the rate of transit alone. Nevertheless, it is known that changes in concentration and proportion of individual SCFA are concurrent with changes in bacterial groups (44–46). In order to examine the importance of the microbial composition on these effects, a panel of quantitative PCR (qPCR) primers for major groups of the microbiota, which account for up to 90% of the total cells, was employed (32,44). Known propionate producers belong to the genera *Bacteroides* and *Prevotella*, whose joint cell numbers were more numerous in overweight

($P = 0.001$) volunteers. It is worthy of mention that the proportion of *Bacteroidetes* significantly increased in overweight and obese subjects.

Our results are in accordance with two reports by Duncan and co-workers, who showed that despite weight loss there was no change in the relative counts of the *Bacteroides* spp. or the percentage of *Firmicutes* (44,47), thus assuming that not the ratio of *Firmicutes* and *Bacteroidetes* is important but rather the amount of SCFA produced. When carbohydrate intake was lowered in their study, the acetate proportion increased, butyrate decreased and propionate remained unaffected (order unchanged) (44). Subsequently, leaner people had a higher ratio of acetate to butyrate and propionate. The respective ratio was also higher—though only slightly—in lean volunteers of our study. The increase of the proportion of *Bacteroides* spp. which are the major representatives of the *Bacteroidetes,* from lean to obese subjects is in concordance with reports by Collado and co-workers (48) but in contrast to earlier reports which linked an increased ratio of *Firmicutes to Bacteroidetes* and high amounts of SCFA to obesity (16,22,23). As previously reported by others, we also measured higher SCFA concentrations. On the other hand, we were unable to identify any correlation of obesity with higher proportions of *Firmicutes* or higher concentrations of methanogenic *Archaea* in obese volunteers. Both observations have been made in proof of principle experiments and are proposed to be one driving factor of obesity (16,22,23,40). It seems that under field conditions with unrestricted human volunteers as in our study, other lifestyle-related factors, such as intensity and regularity of exercise as well as total daily energy intake, might be much more important in causing and maintaining obesity. These factors, as possible confounding variables, were excluded in previously reported well-defined experiments using in part even genetically-modified animals or strictly-controlled human studies. In our opinion, this could be the most probable explanation for the results obtained in our study which are principally different and in contradiction to those obtained in the afore-mentioned studies, although methodological differences in DNA extraction protocols as well as primer design may have caused additional variation. Therefore, owing to fundamental differences in "proof of principle experiments" and field studies and the contrasting results reported here, it seems premature to suggest a certain type of gut microbiome to be a biomarker for obesity in humans or to identify methanogens as a potential therapeutic target as done in the past (16). Many more clinical studies are needed here to establish a consistent theory on the extent of the influence of intestinal microbiota on obesity. Interestingly, the reduction of *Bacteroidetes* and bifidobacteria has an influence on obesity (49).

We see it rather difficult to draw definite conclusions on the importance of various bacterial groups in obesity, as not all influencing parameters such as diet, genetic background, habitation, and overall fitness were taken into account.

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DISCLOSURE

The authors declared no conflict of interest.

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Chapter 5

Anaerostipes caccae gen. nov., sp. nov., a new saccharolytic, acetate-utilising, butyrate-producing bacterium from human faeces

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SYSTEMATIC AND APPLIED MICROBIOLOGY

Anaerostipes caccae gen. nov., sp. nov., a New Saccharolytic, Acetate-utilising, Butyrate-producing Bacterium from Human Faeces

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Summary

Two strains of a previously undescribed *Eubacterium*-like bacterium were isolated from human faeces. The strains are Gram-variable, obligately anaerobic, catalase negative, asporogenous rod-shaped cells which produced acetate, butyrate and lactate as the end products of glucose metabolism. The two isolates displayed 99.9% 16S rRNA gene sequence similarity to each other and treeing analysis demonstrated the faecal isolates are far removed from *Eubacterium* sensu stricto and that they represent a new subline within the *Clostridium coccoides* group of organisms. Based on phenotypic and phylogenetic criteria, it is proposed that the two strains from faeces be classified as a new genus and species, *Anaerostipes caccae*. The type strain of *Anaerostipes caccae* is NCIMB 13811^T (= DSM 14662^T).

Key words: *Anaerostipes caccae* – 16S rRNA – taxonomy – phylogeny – human faeces

Introduction

The human intestinal tract harbours an immense diversity of bacteria and the total number of resident bacteria has been estimated to reach 10^{14} cells (SAVAGE, 1977; SUAU et al. 2001). Although the human gut flora has been studied intensively over several decades using traditional culture-based approaches and phenotypic methods of identification, it is now universally recognised that knowledge of the diversity of species present is far from complete. Culture-independent rRNA-based studies (such as PCR rRNA cloning/sequencing and rDNA TGGE) have revealed that the majority of the dominant species within the gut have so far eluded taxonomic description (WILSON and BLITCHINGTON 1996; ZOETENDAL et al. 1998; SUAU et al. 1999). In a recent investigation of butyrate-producing bacteria isolated from human faeces, using 16S rRNA as a tool to aid identification, numerous novel isolates were recovered within the *Clostridium coccoides* rRNA lineage which did not correspond to recognised species (BARCENILLA et al. 2000). One of the organisms isolated was a strictly anaerobic, non spore-forming, rod-shaped bacterium designated L1-92 (BARCENILLA et al. 2000). In a subsequent and independent investigation of the human faecal flora, a second *Eubacterium*-like strain designated P2 was isolated which displayed a high degree of relatedness (16S rRNA) to the butyrate-producing strain L1-92 described by BARCENILLA et al. (2000). In this article we report the phenotypic characteristics of these two isolates and the results of a phylogenetic analysis. Based on the findings presented, we propose the novel bacterium from human faeces be classified as a new genus and species, *Anaerostipes caccae*.

Materials and Methods

Cultures and cultivation

Strains L1-92^T and P2 were isolated from faecal samples from two different healthy volunteers that had not undergone antibiotic therapy in the previous six months. Strains L1-92^T was isolated as described by BARCENILLA et al. (2000) and deposited in the National Collection of Industrial and Marine Bacteria (UK) under accession number NCIMB 13811^T. For the isolation of strain P2, fresh faecal samples were transferred into an anaerobic workstation (MK3; DW Scientific, Shipley, UK) and diluted serially ten-fold up to 10^{10} in Sorensen buffer (25 mM KH_2PO_4, 33 mM $Na_2HPO_4 \times 12\ H_2O$, 0.04% (v/v), thioglycolic acid, 0.06% (w/v), cysteine, pH 6.8). Aliquots (1 ml) of the dilutions were plated onto starch plates containing (l^{-1}): 4 g $NaHCO_3$, 0.348 g K_2HPO_4, 0.227 g KH_2PO_4, 0.5 g NH_4Cl, 2.25 g NaCl,

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0.5 g $MgCO_3 \times 7\ H_2O$, 0.07 g $CaCl_2 \times 2H_2O$, 5 mg $FeSO_4 \times 7H_2O$, 0.15 mg $NaSeO_3$, 2 g tryptically digested peptone from casein, 2 g yeast extract, 2 g soluble starch, 15 g agar, 1 mg resazurin, 3 ml trace element solution SL 10 (WIDDEL et al., 1983) and 0.5 mg cysteine × HCl, at pH 7.0. Following autoclaving, 1 l medium was supplemented with 20 ml of a filter-sterilised vitamin solution as described by WOLIN et al. (1964). Single colonies were isolated and re-streaked until pure cultures were obtained.

Phenotypic characterisation

All incubations were performed using strictly anaerobic conditions. For morphological and physiological studies both strains were grown on Columbia blood agar (bioMérieux), in ST medium (SCHWIERTZ et al. 2000) or in a medium used for culturing acetogenic bacteria (KAMLAGE et al. 1997) but modified by adding 0.5 g Proteose-Peptone No.2 (Difco) per litre (HA medium). The morphology of the isolates was examined by phase contrast microscopy. Growth was monitored by changes in pH and optical density, measured at 600 nm. Cells were tested for catalase and oxidase as described by SMIBERT and KRIEG (1994). Other biochemical features were determined with the API rapid ID32A, API ZYM and API50 CHL systems (BioMérieux) according to the manufacturer's instructions. Experiments with resting cells were performed as described by KAMLAGE et al. (1997). Acetate, butyrate, propionate, valerate, *iso*-valerate and hydrogen were determined by gas chromatography (HARTMANN et al. 2000; SCHNEIDER et al. 1999). Succinate, ethanol, formate, acetate, D-lactate, L-lactate and glucose were determined enzymatically (BERGMEYER and GRASSL, 1984). Acetate utilisation was tested on M2GSC medium (MIYAZAKI et al. 1997) and the acetate concentrations determined by capillary GC as described in (RICHARDSON et al. 1989).

DNA base composition

The mol%G + C content of DNA was determined by HPLC according to MESBAH et al. (1989) except that the methanol content of the chromatographic buffer was decreased to 8% and the temperature was increased to 37 °C.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA genes of the isolates were amplified by PCR and directly sequenced using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, USA) and an automatic DNA sequencer (model 377; Applied Biosystems). The closest known relatives of the new isolates were determined by performing database searches. These sequences and those of the known related strains were retrieved from the GenBank or the Ribosomal Database Project (RDP) databases and aligned with the newly determined sequences using the program DNATools (RASMUSSEN, 1995). The resulting multiple sequence alignment was corrected manually using the program GeneDoc (NICHOLAS et al. 1997) and a distance matrix was calculated using the program DNADIST (using Kimura's two-parameter correction) (FELSENSTEIN, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR and the stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE FELSENSTEIN, 1989).

Results and Discussion

The two faecal isolates (L1-92^T and P2) were non spore-forming, non-motile, strictly anaerobic, rod-shaped organisms. Cells were 0.5–0.6 µm wide × 2.0–4.3 µm long and occurred sometimes in short chains of approximately 2 to 4 cells. In the exponential growth phase cells stained Gram-positive, while stationary phase cells stained Gram-negative. Cultures in ST medium had ropy sediment with little or no turbidity. Both strains formed white-opaque, non-haemolytic colonies on Columbia blood agar and Wilkens Chalgren agar; the colonies were approximately 1 to 3 mm in diameter, circular, convex, smooth and shiny. Using traditional testing the two strains produced acid from D-fructose, fructooligasaccharides (FOS), D-glucose, D-galactose, inositol, maltose, D-mannose, ribose (weak), soluble starch, sucrose, L-sorbose and sorbitol. Strain L1-92^T produced acid (weak) from salicin whereas strain P2 did not. Aesculin was hydrolysed only by strain L1-92^T. Both organisms failed to produce acid from L-arabinose, cellobiose, glycerol, inulin, lactose, lactulose, melibiose, melezitose, L-rhamnose, D-trehalose or D-xylose. Using the commercially available API 50 system both strains displayed very similar carbohydrate reactions: acid was produced from adonitol, D-arabitol, L-arabitol, D-arabinose, dulcitol, erythritol, galactose, D-glucose, D-fructose, inositol, D-lyxose, maltose, D-mannose, mannitol, melibiose, α-methyl-D-glucoside, N-acetyl-glucosamine, D-raffinose (weak), ribose (weak), L-sorbose, sorbitol, sucrose, D-tagatose, D-turanose and xylitol but not from amygdalin, L-arabinose, cellobiose, D-fucose, β-gentiobiose, gluconate, glycerol, glycogen, inulin, lactose, 5-keto-gluconate, melezitose, α-methyl-D-mannoside, β-methyl-D-xyloside, rhamnose, trehalose, D-xylose or L-xylose. Different results were obtained for arbutin, salicin and 2-keto-gluconate: strain L1-92^T produced acid from arbutin and salicin but not from 2-keto-gluconate, whereas strain P2 failed to produce acid from the former two substrates but produced acid albeit weakly from 2-keto-gluconate. In addition strain L1-92^T hydrolysed aesculin whereas strain P2 showed a weak reaction.

The enzyme profiles of the two strains were identical using the API ZYM and API rapid ID32A systems. Using the API ZYM kit, both strains gave positive reactions only for acid phosphatase and phosphoamidase, with all other tests being negative. Using the API rapid ID32A system both isolates displayed activity for arginine dihydrolase and α-galactosidase and were weakly nitrate reductase positive. To ascertain further information about the catabolic potentials of the two iolates, resting cells in 50 mM anoxic potassium phosphate buffer (pH 7.0, 1 mg of resazurin per litre, 5 mM dithioerythritol) were incubated with glucose under N_2/CO_2 (80/20) and the degradation products analysed. The fermentation balance was as follows (CO_2 content was calculated from redox balance): 1 glucose → 0.2 acetate + 0.9 lactate + 0.4 butyrate + 1.2 H_2 + 1 CO_2. The carbon recovery in this experiment was 95%. Growth of L1-92^T and P2 on anaerobic M2GSC revealed that both strains were net acetate utilisers in this medium (utilising 6.29 and 6.41 mM respectively). The DNA base composition values for the two faecal isolates L1-92^T and P2 were 46.0 and 45.4 mol% G + C, respectively. The genotypic relatedness of the isolates was further investigated by comparative 16S rRNA gene sequence analysis. Almost complete sequences of L1-92^T

(1455 bp) and P2 (1506bp) were determined. Pair-wise analysis showed that the two strains had almost identical 16S rRNA gene sequences (99.9% sequence similarity) and searches of GenBank and RDP databases revealed that the isolates were closely related to members of the *Clostridium coccoides* group of organisms (*Clostridium* rRNA cluster XIVa, [COLLINS et al., 1994]). The novel bacterium (as exemplified by isolate L1-92^T) formed a distinct sub-line within this cluster, but did not display a particularly close nor statistically significant association (as shown by bootstrap re-sampling) with any recognised species of the *Clostridium coccoides* group (Fig. 1).

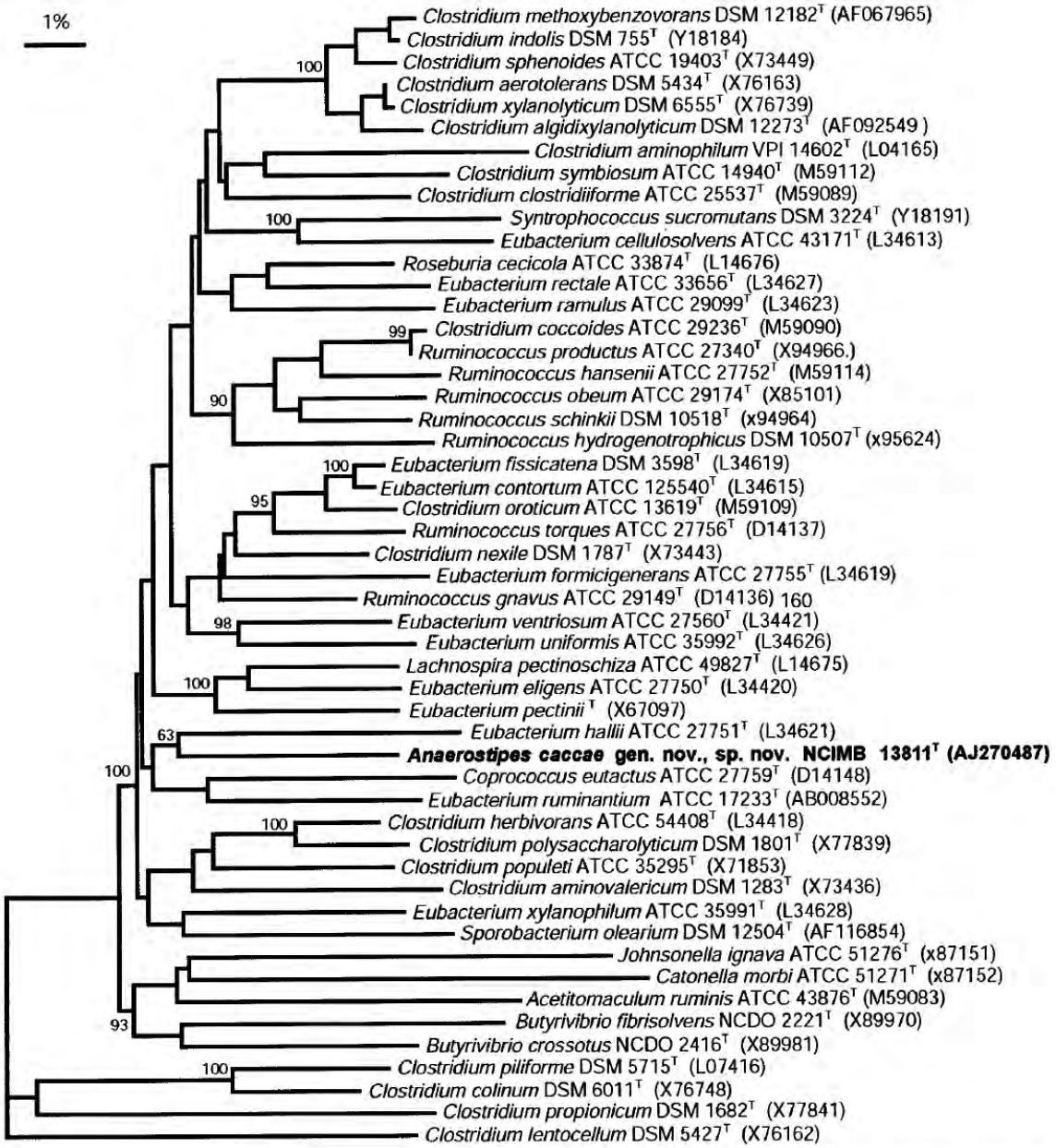

Fig. 1. Unrooted phylogenetic tree showing the phylogenetic relationships of *Anaerostipes caccae* gen. nov., sp. nov. within the *Clostridium coccoides* rRNA group or organisms. The tree constructed using the neighbor-joining method was based on a comparison of approximately 1300 nucleotides. Bootstrap values expressed as a percentage of 500 replications are given at the branching points.

Table 1. Characteristics which are useful in differentiating *Anaerostipes caccae* from some miss-classified *Eubacterium* species of the *Clostridium coccoides* rRNA group.

Test/Species	1[a]	2	3	4	5	6	7	8	9	10	11	12	13
Acid end products	A[b], B, L	A, B, L	A, E, F	A, E, F L	A, E, F	A, E, F L	A, B, But, F	A, B, F L	A, B, L	A, E, F L	A, B, F L	A, B, F	A, B, F L, S
Motility	–	v[c]	–	+	v	–	–	–	v	–	–	+	–
Acid from:	–	v	–	–	–	–	–	–	v	nd	–	nd	–
Amygdalin													
L-Arabinose	–	–	+	–	v	v	–	–	+	+	–	–	+
Cellobiose	–	v	v	+	–	–	–	+	+	nd	–	+	+
Dulcitol	+	–	–	–	–	–	nd	nd	–	nd	nd	nd	–
Fructose	+	–	+	+	+	+	+	+	+	v	+	–	v
Galactose	+	–	+	–	+	+	+	nd	+	+	+	–	–
Glucose	+	+	+	+	+	+	+	+	+	+	+	–	+
Inulin	–	–	–	–	–	–	nd	nd	+	nd	–	nd	–
Lactose	–	v	v	+	–	v	+	+	v	+	–	–	–
Maltose	+	+	+	–	+	+	+	v	+	+	+	–	+
Melezitose	–	–	–	–	–	–	–	–	+	nd	–	nd	–
Melibiose	+	–	v	–	–	–	–	+	+	v	–	–	–
Raffinose	+	–	+	–	–	–	–	+	+	nd	–	nd	–
Ribose	+	–	+	–	v	v	–	–	–	nd	–	nd	–
Starch	+	–	–	–	–	–	–	–	v	nd	v	nd	+
Sucrose	+	+	+	–	+	–	v	–	+	nd	v	nd	–
Trehalose	–	v	–	–	+	–	–	–	v	nd	–	nd	–
Xylose	–	–	v	–	v	v	–	–	+	+	–	–	v

[a] 1 – *Anaerostipes caccae;* 2 – *E. cellulosolvens;* 3 – *E. contortum;* 4 – *E. eligens;* 5 – *E. fissicatena;* 6 – *E. formicigenerans;* 7 – *E. hallii;* 8 – *E. ramulus;* 9 – *E. rectale;* 10 – *E. uniforme;* 11 – *E. ventriosum;* 12 – *E. xylanophilum;* 13 – *E. ruminantium.*
[b] A – Acetic acid; B – butyric acid; But – butanol; E – ethanol; F – formic acid; L – lactic acid.
[c] v – variable; nd – not determined.

It is evident from the results of the taxonomic investigation that the two faecal isolates belong to a hitherto undescribed species. As a strictly anaerobic non spore-forming rod, the unidentified bacterium somewhat resembles the genus *Eubacerium*. It is now recognised however that the genus *Eubacterium* is phenotypically and phylogenetically diverse and should be restricted to *Eubacterium limosum*, the type species of the genus, and its close relatives (viz: *E. barkeri, E. callanderi* and *E. aggregans;* WILLEMS and COLLINS, 1996). The human isolates described here are phylogenetically only remotely related to *E. limosum* and close relatives (i.e. *Eubacterium* sensu stricto, WILLEMS and COLLINS, 1996), with the latter species forming a different cluster (rRNA cluster XV, COLLINS et al. 1994) and displaying approximately 20% sequence divergence with the unidentified rod-shaped bacterium. Hence the new feacal bacterium cannot be assigned to the genus *Eubacterium* sensu stricto. Phylogenetically the novel rod-shaped bacterium belongs to the *C. coccoides* rRNA group of organisms. The *Clostridium coccoides* group is a phylogenetically diverse supra-generic grouping embracing organisms bearing a variety of generic names (e.g. *Butyrivibrio, Clostridium, Coprococcus, Lachnospira, Roseburia, Ruminococcus, Sporobacterium, Syntrophococcus*) and includes several miss-classified *Eubacterium* species. Although tree topology and 16S rRNA sequence divergence values clearly show the unidentified bacterium warrants classification as a new species, its generic placement is problematic. Phylogenetically the novel bacterium is only distantly related to recognised species and genera within the *Clostridium coccoides* group (displaying divergence values of greater than 10%). The unidentified bacterium is also phenotypically incompatible with all currently named genera within this rRNA cluster. For example the new rod-shaped bacterium can be distinguished from *Clostridium* spp. and *Sporobacterium* in not producing endospores, from *Coprococcus, Lachnospira* and *Ruminococcus* by cellular morphology and end-products of glucose metabolism, and from *Butyrivibrio* and *Roseburia* in being non-motile and end-products of glucose metabolism. The human faecal bacterium can also be biochemically readily distinguished from miss-classified *Eubacterium* species (such as *E. hallii*) within the *C. coccoides* cluster (see Table 1). These miss-classified eubacterial species invariably display 10% or more sequence divergence with the unidentified bacterium and therefore cannot be considered members of the same genus. Thus it is clear that the novel faecal bacterium reported here, is both phylogenetically and phenotypically incompatible with *Eubacterium* sensu stricto and all recognised genera within the *C. coccoides* rRNA cluster, and merits classification in a separate genus. Therefore based on the presented findings we propose that the unknown rod-shaped organism be classified as a new genus and species, *Anaerostipes caccae*.

Description of *Anaerostipes*

Anaerostipes (an. ae. ro. sti'pes) Gr. pref. *an* not; Gr. n. *aer* air; *anaero* not living in air; L. masc. n. *stipes* club of stick; *Anaerostipes*, a stick not living in air) consists of non-motile, non spore-forming rod-shaped cells. Cells are Gram-positive, but in older cultures may stain Gram-negative. Strictly anaerobic and catalase and oxidase negative. Non-haemolytic. Glucose and some other sugars may be fermented. Butyrate, acetate and lactate are the major products of glucose metabolism. Acetate is utilised. Arginine dihydrolase, phosphoamidase, and α-galactosidase are produced. Gelatin and urease are not hydrolysed. Indole is not produced. Nitrate is reduced. The DNA G + C content is 45.5–46.0 mol%. The type species is *Anaerostipes caccae*.

Description of *Anaerostipes caccae* sp. nov.

Anaerostipes caccae (cac'cae, pronounced kak'ka Gr. n. kakke faeces: N.L. gen. n. caccae of faeces) cells consist of non-motile, non spore-forming rods. Individual cells are 0.5–0.6 µm in width × 2.0–4.0 µm in length and occur in chains of up to 4 cells. Cells stain Gram-positive but older cultures (>16 h) may stain Gram-negative. On Columbia blood agar, white-opaque colonies are formed that are 1–3 mm in diameter, circular, convex, smooth, shiny and sticky, which are non haemolytic. The strains are strictly anaerobic and catalase and oxidase negative. Butyrate, acetate and lactate are the major products of glucose metabolism. Acetate is utilised. Using conventional tests acid is produced from D-fructose, FOS, D-glucose, D-galactose, inositol, maltose, D-mannose, ribose (weak), soluble starch, sucrose, L-sorbose and sorbitol. Acid may or may not be produced from salicin. Acid is not produced from L-arabinose, cellobiose, glycerol, inulin, lactose, lactulose, melibiose, melezitose, L-rhamnose, D-trehalose or D-xylose. Using the miniaturised API 50 system acid is produced from adonitol, D-arabitol, L-arabitol, D-arabinose, dulcitol, erythritol, galactose, D-glucose, gluconate, D-fructose, inositol, D-lyxose, maltose, D-mannose, mannitol, melibiose, α-methyl-D-glucoside, N-acetyl-glucosamine, D-raffinose (weak), ribose (weak), L-sorbose, sorbitol, sucrose, D-tagatose, D-turanose and xylitol but not from amygdalin, L-arabinose, cellobiose, D-fucose, β-gentiobiose, glycerol, glycogen, inulin, lactose, 5-keto-gluconate, melezitose, α-methyl-D-mannoside, β-methyl-D-xyloside, rhamnose, trehalose, D-xylose or L-xylose. Acid may or may not be produced from arbutin, salicin and 2-keto-gluconate. Aesculin hydrolysis is variable. Using API ZYM and API rapid ID32A systems activity is detected for acid phosphatase and phosphoamidase, and arginine dihydrolase, α-galactosidase and nitrate reductase (weak reaction) respectively. No activity is detected for alanine arylamidase, alkaline, phosphatase, arginine arylamidase, α-arabinosidase, chymotrypsin, cystine arylamidase, esterase C4, ester lipase C8, α-fucosidase, β-galactosidase, β-galactosidase-6-phosphate, α-glucosidase, β-glucosidase, β-glucuronidase, glycine arylamidase, glutamylglutamic acid arylamidase, histidine arylamidase, leucine arylamidase, leucylglycine arylamidase, lipase C14, α-mannosidase, N-acetyl-α-glucosaminidase, phenylalanine arylamidase, pyroglutamic acid arylamidase, proline arylamidase, serine arylamidase, trypsin, tyrosine arylamidase, valine arylamidase or urease. Gelatin is not hydrolysed and indole is not produced. The DNA G + C content is 45.5–46.0 mol%. Isolated from human faeces. The type strain is L1-92^T (= DSM 14662^T = NCIMB 13811^T). The GenBank accession number of the 16S rRNA sequence of the type strain is AJ270487.

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Chapter 6

Ruminococcus luti sp. nov., isolated from a human faecal sample

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SYSTEMATIC AND APPLIED MICROBIOLOGY

Ruminococcus luti sp. nov., Isolated from a Human Faecal Sample*

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Summary

A strain of an unidentified strictly anoxic, Gram-postive, non-motile *Ruminococcus*-like bacterium was isolated from a human faecal sample. The organism used carbohydrates as fermentable substrates, produced acetate, succinate, and hydrogen as the major products of glucose metabolism, and possessed a G + C content of 43.3 mol%. The morphological and biochemical characteristics of the organism were consistent with its assignment to the genus *Ruminococcus* but it did not correspond to any recognized species of this genus. Comparative 16S rRNA gene sequencing showed the unidentified bacterium represents a previously unrecognised sub-line within the *Clostridium coccoides* rRNA group of organisms. The nearest relative of the unknown bacterium corresponded to *Ruminococcus obeum* but a 16S rRNA sequence divergence value of >3% demonstrated it represents a different species. Based on the presented findings a new species, *Ruminococcus luti*, is described. The type strain of *Ruminococcus luti* is BInIX^T (DSM 14534^T, CCUG 45635^T).

Key words: *Ruminococcus luti* sp. nov. – faecal flora – 16S rRNA – phylogeny – taxonomy

Introduction

The composition of the human intestinal microflora has a profound influence on health and disease through its involvement in nutrition, pathogenesis, and immunology of the host (CUMMINGS and MACFARLANE, 1991; GIBSON and ROBERFROID, 1995). A proper comprehension of the diversity of species present in this ecosystem is therefore of considerable importance. The human gut harbours a great variety of organisms and is thought to comprise of several hundred species. The diversity of organisms within the human gut had until recently been studied almost exclusively by conventional culture-based methodologies. However, the application of culture-independent molecular-based approaches (e.g. whole cell in situ hybridization, PCR rDNA gene amplification/sequencing and rDNA TGGE) in the past few years has provided evidence that we currently have a vastly incomplete picture of the gut microflora (SIMMERING et al., 1999; ZOETENDAL et al., 1998). For example estimates of culturability of bacteria in the gut vary from 10–50% (LANGENDIJK et al., 1995; WILSON and BLITCHINGTON, 1996), and in a recent rDNA phylogenetic inventory of human faeces, it was shown that the great majority of species had so far eluded taxonomic description (SUAU et al., 1999). In the course of an investigation of taxonomically problematic organisms from human faeces we have characterised a hitherto unknown strictly anaerobic Gram-positive, non-motile, coccoid-shaped organism. Based on the reported findings, we propose a new species from human faeces, *Ruminococcus luti*.

Materials and Methods

Culture and cultivation

Strain BInIX^T (DSM 14534^T, CCUG 45635^T) was isolated from a faecal sample obtained from a healthy person who had not undergone antibiotic therapy for the previous 6 months. Fresh faecal samples were collected, stored immediately in an

*The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Ruminococcus luti* strain DSM 14534^T is AJ133124.

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anoxic workstation and diluted serially in Soerensen-buffer (0.1 mol l^{-1} KH_2PO_4, 0.1 mol l^{-1} $Na_2HPO_4 \times 2\ H_2O$, 2.0 g l^{-1} agar, 30 mmol l^{-1} thioglycolic acid). Aliquots were plated on Wilkins-Chalgren-Anaerobic agar (WCA) which was supplemented with inulin (10 g/l; Raftiline, Orafti, Belgium) instead of glucose and single colonies were repeatedly picked and streaked out until pure cultures were obtained. All incubations were done at 37 °C under strictly anoxic conditions. The anoxic techniques applied were essentially those of HUNGATE (1969) and BRYANT (1972). A gas phase of N_2 and CO_2 (80:20, by vol.) was used unless otherwise indicated. For morphological and physiological studies both strains were grown on Columbia blood agar (bioMérieux, Nürtingen, Germany), in ST medium (SCHWIERTZ et al., 2000) or in a medium (BIC) used by SCHNEIDER et al. (1999). Solid media were prepared by adding 1.5% agar to the media and pouring the media inside an anoxic workstation (MK3; DW Scientific, Shipley, UK) into petri dishes. The gas atmosphere inside the chamber consisted of $N_2/CO_2/H_2$ (80:10:10, by vol.).

Morphological and biochemical characteristics

The cell morphology of the isolate was examined by phase contrast microscopy (Axioplan 2, Zeiss, Germany). The biochemical features of the isolate were determined using the Vitek® system, the API ID32A system, API ZYM system and the API 50 CH system according to the manufacturer's instructions (bioMérieux), except that the latter preparation was incubated inside an anoxic workstation. Conventional biochemical and physiological tests were also performed with growing and with resting cells as described earlier (KAMLAGE et al., 1997). The production of H_2, acetate, butyrate, propionate, valerate and isovalerate was determined by gas chromatography as described elsewhere (HARTMANN et al., 2000; SCHNEIDER et al., 1999). Glucose, ethanol, formate, succinate, D- and L-lactate were determined enzymatically using commercially available detection kits (r-biopharm, Darmstadt, Germany) or as described by BERGMEYER (1974).

DNA isolation and G + C content determination

DNA isolation was performed by following protocol number five of the InViTek DNA Isolation Kit III (InViTek GmbH, Berlin, Germany). The DNA mol% G + C content was determined by HPLC after digestion of isolated DNA with P1 nuclease and alkaline phosphatase as reported previously (MESBAH and WHITMAN, 1989; KAMLAGE et al., 1997).

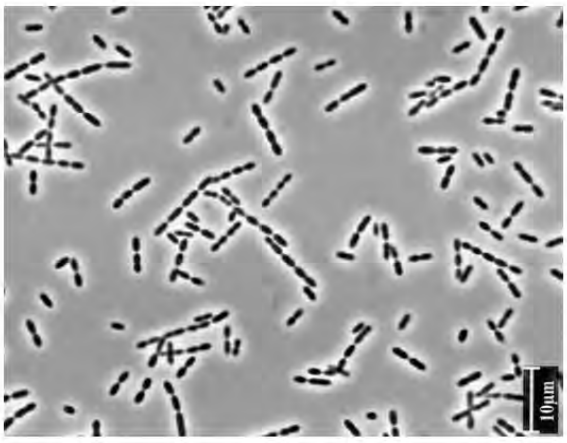

Fig. 1. Light micrograph of isolate BInIX^T grown in ST-medium for 12 h at 37 °C. Bar, 10 µm.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene of the isolate was amplified by PCR and directly sequenced using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, USA) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the new isolate were determined by performing database searches. These sequences and those of other known related strains were retrieved from the GenBank or the Ribosomal Database Project (RDP) databases and aligned with the newly determined sequence using the program DNA-Tools (RASMUSSEN, 1995). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated using the programs PRETTY and DNADIST (using Kimura's two-parameter correction) (FELSENSTEIN, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR and the stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (FELSENSTEIN, 1989).

Results and Discussion

The faecal isolate consisted of strictly anoxic, Gram-positive, non-sporing, non-acidfast, non-motile, coccoid-shaped cells which were about 0.7–0.9 µm in diameter and occurred in chains of up to 10 cells. Figure 1 depicts a light-microscopic picture of strain BInIX^T. On Columbia-blood-agar after 72 hours, the strain formed smooth, in the middle opaque and translucide at the border, non-haemolytic cream coloured colonies of about 1 mm in diameter. The isolate fermented glucose, mannose, L-arabinose, D-arabinose, cellobiose, melibiose, ribose, starch, sorbitol, fructose, D-xylose, lactulose, maltose, lactose, raffinose, N-acetyl-glucosamine, sucrose, L-fucose, fructo-oligosaccharides (FOS), inulin, galactose and trehalose but not L-sorbose, glycerol, erythritol, inositol, mannitol, melezitose, pyruvate, rhamnose, salicin or peptone. It did not reduce nitrate, failed to liquefy gelatin, and was indole, urease and catalase negative. Analysis with the Vitek system showed the strain hydrolysed the respective p-nitrophenyl derivatives of β-D-galactopyranoside, α-D-galactopyranoside, β-D-glucopyranoside, α-D-glucopyranoside, α-L-fucopyranoside, β-D-xylopyranoside, α-L-arabinofuranoside and N-acetylglucosamine. Hydrolysis of the p-nitroanilide derivatives of L-lysine, L-glutamate (γ-position) and β-D-lactoside was weak or variable. In contrast, the p-nitrophenyl derivatives of β-D-fucopyranoside, β-D-glucuronide, α-D-mannopyranoside and the p-nitroanilide derivates of L-leucine, L-alanine, N-benzoyl-DL-arginine and of L-proline were not degraded. Using commercial API systems activities for esterase C4, ester lipase C8, acid phosphatase, phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-N-acetylglucosaminidase, β-glucosidase, alkaline phosphatase, α-arabinosidase, α-mannosidase and α-fucosidase were detected but not cystine-arylamidase, arginine-arylamidase, glycine-arylamidase, histidine-arylamidase, lipase C14, leucine-arylamidase, valine-arylamidase, trypsin, prolin-arylamidase, phenylalanin-arylamidase, pyroglutamic acid-arylamidase, tyrosin-arylamidase, alanin-arylamidase, serin-arylamidase,

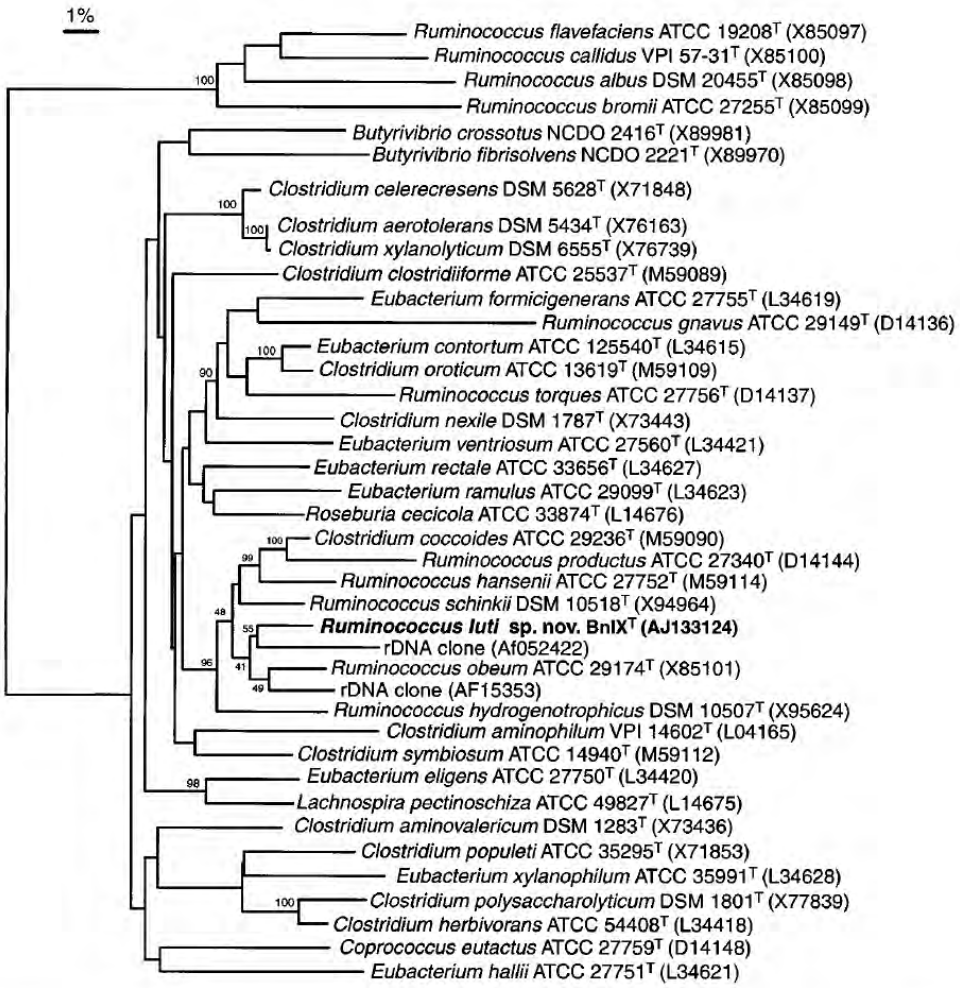

Fig. 2. Unrooted phylogenetic tree showing the phylogenetic relationships of strain BnIX^T within the *Clostridium coccoides* group of organisms. The tree constructed using the neighbour-joining method was based on a comparison of approximately 1300 nucleotides. Bootstrap values expressed as a percentage of 500 replications are given at the branching points.

glutamic acid-decarboxylase, chymotrypsin or β-glucuronidase. Resting cells of the isolate converted glucose mainly to acetate, succinate and hydrogen. In addition, small amounts of ethanol, CO_2 and L-lactate were produced, but no formate. The fermentation balance was as follows (CO_2 content was calculated from redox balance): 1 glucose → 1.74 acetate + 0.55 succinate + 0.64 H_2 + 0.02 ethanol + 0.07 CO_2 + 0.04 L-lactate. The carbon recovery in this experiment was 98.3%.

The cellular morphology and biochemical features of the strain were consistent with the genus *Ruminococcus* (BRYANT, 1986). Determination of the G + C content of the DNA of strain BInIX^T gave a value of 43.3 mol% which is within the range of 39 to 46 mol% reported for other *Ruminococcus* species. To ascertain the phylogenetic position of the unknown bacterium, comparative 16S rRNA gene sequencing was conducted. The almost complete sequence (>1400 nt) of the 16S rRNA gene of the strain BInIX^T was determined. Sequence database searches revealed that the strain was closely related to species of the *Clostridium coccoides* rRNA group of species (cluster XIVa, COLLINS et al. 1994). Highest sequence relatedness was found with *Ruminococcus obeum* (96.6%). A tree constructed by neighbour-joining depicting the phylogenetic affinities of the new isolate is shown in Fig. 2 and shows the organism represents a hitherto unknown species within the *Clostridium coccoides* rRNA group.

The results of the treeing analysis clearly show the unknown faecal isolate is a member of the *Clostridium coccoides* group of organisms. The *Clostridium coccoides*

rRNA cluster embraces a phenotypically diverse spectrum of organisms bearing a variety of generic names, including *Butyrivibrio*, *Clostridium*, *Coprococcus*, *Eubacterium*, *Lachnospira*, *Roseburia* and *Ruminococcus*. Within this major grouping, the unidentified coccus formed a distinct sub-line within a small subcluster of species which included several described ruminococcal species (*Ruminococcus productus*, *Ruminoccocus hansenii*, *Ruminococcus obeum*, *Ruminococcus hydrogenotrophicus*, *Ruminococcus schinkii*), *Clostridium coccoides*, and some clones derived from uncultured faecal organisms (Fig. 2). Although tree topology and sequence divergence values (>3%) show the unidentified coccus warrant classification as a novel species, its generic assignment is somewhat problematic. Phenotypically the unknown coccus closely resembles *Ruminococcus* but *Ruminococcus flavefaciens*, the type species of the *Ruminococcus* genus, is phylogenetically only distantly related and is found in a different rRNA group (designated rRNA cluster IV, COLLINS et al., 1994). Although it is now recognized that the genus *Ruminococcus* is heterogeneous and is in need of revision (RAINEY and JANSSEN, 1995; WILLEMS and COLLINS, 1995; RIEU-LESME et al. 1996) based on the currently recognized definition (BRYANT, 1986) this is the only appropriate home for the novel faecal coccus. We recognize, however, that as with many other ruminococcal species (e.g. *Ruminococcus hydrogenotrophicus*, *Ruminococcus obeum* and *Ruminococcus schinkii*) this placement may require emendation, if the genus *Ruminococcus* is itself revised, at some future date. Therefore based on the presented finding, we propose the unknown coccus be designated a new species, *Ruminococcus luti*.

Description of *Ruminococcus luti* sp. nov.

Ruminococcus luti (lu´ti. L. gen. neut. n. *luti* of faeces). Cells are Gram-positive, non-sporing, non-motile, and coccoid-shaped (0.7–0.9 µm in diameter) and are occurring in chains of up to 10 cells. Strictly anaerobic and catalase negative. On Columbia-blood agar, the cells form smooth, opaque, non-haemolytic colonies which are cream in colour, have an entire margin, a convex elevation and a butyraceous texture. Acetate, succinate and hydrogen are the major products of glucose fermentation, but also smaller amounts of ethanol, carbon dioxide and L-lactate are produced. Acid is produced from glucose, mannose, L-arabinose, D-arabinose, cellobiose, melibiose, ribose, starch, sorbitol, fructose, D-xylose, lactulose, maltose, lactose, raffinose, N-acetyl-glucosamine, sucrose, L-fucose, fructo-oligosaccharides (FOS), inulin, galactose and trehalose but not from L-sorbose, glycerol, erythritol, innositol, mannitol, melezitose, pyruvate, rhamnose, salicin or peptone. The respective p-nitrophenyl derivatives of β-D-galactopyranoside, α-D-galactopyranoside, β-D-glucopyranoside, α-D-glucopyranoside, α-L-fucopyranoside, β-D-xylopyranoside, α-L-arabinofuranoside and N-acetylglucosamine are hydrolysed. Hydrolysis of the p-nitroanilide derivatives of L-lysine, L-glutamate (γ-position) and β-D-lactoside were weak or variable. The p-nitrophenyl derivatives of β-D-glucuronide, α-D-mannopyranoside, β-D-fucopyranoside and β-D-lactoside, and the p-nitroanilide derivatives of L-leucine, L-alanine, N-benzoyl-DL-arginine and of L-proline are not hydrolysed. Esterase C4, ester lipase C8, acid phosphatase, phosphohydrolase, α-galactosidase, β-galactosidase, β-N-acetylglucosaminidase, β-glucosidase, alkaline phosphatase, α-arabinosidase, α-mannosidase and α-fucosidase are produced. Lipase C14, cystine-arylamidase, arginine-arylamidase, glycine-arylamidase, histidine-arylamidase, leucine-arylamidase, valine-arylamidase, trypsin, chymotrypsin, prolin-arylamidase, phenylalanin-arylamidase, pyroglutamic acid-arylamidase, tyrosin-arylamidase, alanin-arylamidase, serin-arylamidase, glutamic acid-decarboxylase or β-glucuronidase are not produced. Nitrate is not reduced and gelatin is not hydrolysed. Oxidase and urease are negative. The DNA G + C content is 43.3 mol%. Isolated from human faeces. The type strain of *Ruminococcus luti* type (BInIX^T) has been deposited in the DSMZ as DSM 14534^T and in the CCUG as 45635^T.

It is pertinent to note that the closest described relative of the new species, *Ruminococcus obeum*, is also found in human faeces. *Ruminococcus luti* can, however, be distinguished from *Ruminococcus obeum* by producing succinate as one major fermentation product but only trace amounts of ethanol, and in fermenting trehalose but not rhamnose. In addition, *Ruminococcus luti* can be differentiated from *Ruminococcus obeum* by its enzyme profiles with the most prominent differences in β-galactosidase and α-fucosidase activities (Table 1).

Table 1. Semiquantitative enzymatic profiles[1] of *Ruminococcus luti* sp. nov. and *Ruminococcus obeum* ATCC 29174^T.

Enzyme	*R. luti* sp. nov.	*R. obeum*
alkaline phosphatase	0.8	2
acid phosphatase	3.3	4.3
phosphohydrolase	2	3
α-galactosidase	3.3	3
β-galactosidase	1	4.7
α-glucosidase	4	4
β-glucosidase	4.7	3.7
esterase (C4)	2	1
esterase lipase (C8)	1	0.3
γ-fucosidase	1.6	N. D.

[1]activity number: 1 = 5 nmol; 2 = 10 nmol; 3 = 20 nmol; 4 = 30 nmol; 5 = >40 nmol substrate hydrolysed
N. D. – not detected

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Chapter 7

Microbiota in pediatric inflammatory bowel disease

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Microbiota in Pediatric Inflammatory Bowel Disease

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Objective To test the hypothesis that compared with controls, children with inflammatory bowel disease (IBD) exhibit differences in the relationships between gut microbiota and disease activity.

Study design Children and adolescents (n = 69; median age, 14 years) with IBD and 25 healthy controls (median age, 14 years) were recruited for the study. The disease activity was determined according to the Pediatric Ulcerative Colitis Activity Index or the Pediatric Crohn Disease Activity Index. Cell counts of 9 bacterial groups and species in the fecal microbiota were monitored by real-time polymerase chain reaction analysis.

Results Although no major changes were observed in patients with ulcerative colitis, except for a decrease in bifidobacteria in the active state of IBD, children with active and inactive Crohn's disease (CD) had lower numbers of *Faecalibacterium prausnitzii* and bifidobacteria ($P < .05$), and patients with active CD had higher numbers of *Escherichia coli* ($P < .05$).

Conclusions The microbiota in children with CD is characterized by decreased numbers of *F praunsitzii* and increased numbers of *E coli*. *(J Pediatr 2010;157:240-4).*

The incidence of inflammatory bowel disease (IBD) in children living in North America was reported to be about 8 per 100 000 in 2003, with the incidence of Crohn's disease (CD) being more than twice that of ulcerative colitis (UC).[1] A study from France reported that in children and adults with IBD, the incidence of CD has increased significantly, but the incidence of UC has decreased, in recent years.[2] The pathogenesis of IBDs remains poorly understood. A specific question is why an increasing number of patients are acquiring the disease significantly earlier in life. Along with host genetic defects or defective host immunoregulation, an imbalance in the intestinal microbiota is considered crucial for the development of chronic intestinal inflammation.[3] Thus, dysbiosis is thought to increase the vulnerabilty of the gut mucosa and possibly to be a factor in the development of IBD.[4-7] Several studies have noted reductions in potentially beneficial microbes, such as *Bifidobacterium* species in CD and UC and the butyric acid–producing *Faecalibacterium prausnitzii* cluster in CD.[5,8,9]

The *F prausnitzii* cluster represents a subgroup of the *Clostridium leptum* group, which comprises fibrolytic- and butyrate-producing microorganisms contributing to processes important to colonic health.[10,11] *C leptum* and *C coccoides* groups comprise the majority of the phylum *Firmicutes*, one of the dominant bacteria phyla next to *Bacteroidetes* in the normal human fecal microbiota.[12]

To date, few studies have investigated the composition of the intestinal microbiota in children.[13,14] The aim of the present study was to examine the composition of the gut microbiota of pediatric patients with IBD to determine whether any imbalances of the commensal microbiota exist and, if so, whether they are correlated with disease activity.

Methods

Sixty-nine patients (age range, 1-20 years; median, 14 years) with documented IBD were recruited in the University of Erlangen's Pediatric Gastroenterology Unit. Disease activity was determined according to the Pediatric Ulcerative Colitis Activity Index (PUCAI)[15] or the Pediatric Crohn Disease Activity Index (PCDAI).[16] Active UC (AUC) was defined as a PUCAI >10, active CD (ACD) was defined as PCDAI >10, CD in remission (CDR) was defined as a PCDAI ≤10, and UC in remission (UCR) was defined as a PUCAI ≤10. In addition, 25 healthy children (age range, 5-19 years; median, 14 years) were recruited as controls. Each participant or a parent, when appropriate, provided informed consent. The study was approved by the university's Ethics Committee. No antibiotic treatment was provided during the 4 weeks before the analysis.

ACD	Active Crohn's disease	PCDAI	Pediatric Crohn Disease Activity Index
AUC	Active ulcerative colitis		
CD	Crohn's disease	PUCAI	Pediatric Ulcerative Colitis Activity Index
CDR	Crohn's disease in remission		
FISH	Fluorescence in situ hybridization	qPCR	Quantitative polymerase chain reaction
GI	Gastrointestinal		
IBD	Inflammatory bowel disease	UC	Ulcerative colitis
IL	Interleukin	UCR	Ulcerative colitis in remission

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From the fresh stool sample provided by each subject, DNA was extracted using the Easy Mag DNA Isolation system (BioMerieux, Nuertingen, Germany) according to the manufacturer's instructions. Primers were selected to recognize similar bacterial groups as previously published 16S rRNA–targeting probes used for fluorescence in situ hybridization (FISH) analysis. A particular FISH-defined group containing the appropriate target sequence in the ARB program (The ARB Project; http://www.arb-home.de/) was selected, and quantitative polymerase chain reaction (qPCR) primers were designed with the ARB program to amplify the same group of commensal bacteria.[17] Discriminating nucleotides were chosen to be at the 3′ end of the primer, and a specific primer was combined with a universal primer that does not exclude any members of that particular group. Primers for bacterial groups were stringently selected using the Primer Designer program (The ARB Project) to avoid primer-dimer formation and yield 100- to 300-bp products (Table I; available at www.jpeds.com). The standard line was based on actual counting of cultured bacteria and correlated directly to the Ct values of the qPCR. We validated the qPCR data through comparison with actual bacterial counts as reported by Barman et al.[18] The specificity of the various qPCR primer sets had been tested previously,[19-26] and several of the primers had been used in at least one other previous study.[27,28]

Quantitative PCR amplification and detection were carried out using the primers listed in Table I. PCR amplification and detection was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Darmstadt, Germany) in optical-grade 96-well plates sealed with optical sealing tape. Each reaction mixture (25 μL) comprised 12.5 μL of QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 2 μL of primer mixes (10 pmol/μL each), 9 μL of sterile distilled water, and 1.5 μL of stool DNA (10 ng/μL). For the negative control, 2 μL of sterile distilled water instead of the template DNA solution was added to the reaction solution. A standard curve was produced using the appropriate reference organism to quantify the qPCR values into numbers of bacteria per gram. The standard curves were prepared using the same PCR assay as used for the samples. The fluorescent products were detected in the final step of each cycle. A melting curve analysis was carried out after amplification to distinguish the targeted PCR products from the nontargeted PCR products. The melting curves were obtained by slow heating at temperatures of 55°C-95°C at a rate of 0.2°C/second, with continuous fluorescence collection.

Real-time qPCR was performed in triplicate, and average values were used for enumeration. PCR conditions were optimized based on those described in the literature.[19-26] The amplification program used for all primers consisted of one cycle of 95°C for 15 minutes and then 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds.

In all assays, the amplification efficiency was >90%, and the standard curve showed a linear range across at least 5 logs of DNA concentrations with a correlation coefficient >0.9. The lowest detection limit of all assays was as low as 10-100 copies of specific bacterial 16S rDNA per reaction, corresponding to 10^4-10^5 copies per gram of wet-weight feces. All data were analyzed using ABI Prism software (Applied Biosystems, Darmstadt, Germany).

Statistical Analyses

All statistical analyses were performed using SPSS (SPSS Inc., Chicago, Illinois). The normality of the data was checked using the nonparametric Kolmogorov-Smirnov test with Lilliefors correction. Before analysis for treatment differences, data were subjected to the Levene test for homogeneity of variances. Depending on the normality of the underlying data, analysis of variance or the Mann-Whitney *U* test was used for statistical analyses. Differences were considered significant at $P < .05$.

Results

Diagnoses included 21 ACD, 19 CDR, 13 AUC, and 16 UCR (Table II). Median activity indexes were 24 for ACD (range, 13-60), 0 for CDR (range, 0-10), 25 for AUD (range, 20-70), and 0 for UCR (range, 0-5). The IBD and healthy control groups did not differ in terms of age or sex.

Quantification of Predominant Bacterial Groups in Stool

Quantitative PCR analyses were performed to quantify individual bacterial groups in stool samples collected from the study group. Based on the universal bacterial primer, total bacterial cell numbers (log count per gram of feces) did not differ between patients with UC or CD and the healthy controls. No changes in cell numbers between the various disease states were detected. The most abundant bacterial groups detected were members of the *Bacteroides* and *Prevotella* genus, which form the majority of the phylum *Bacteroidetes*, and gram-positive bacteria belonging to the clostridial cluster XIVa (*C coccoides* group) or the clostridial cluster IV (*C leptum* group), which represent the majority of the phylum *Firmicutes* (Table II; Figures 1 and 2). Two other groups detected were *Escherichia coli*, the major representative of the *Proteobacteria* within the human microbiota, and the genus *Bifidobacterium*, the major representative of the *Actinobacteria*. Overall, with the primers used herein, we were able to cover a median of 90% of the total microbiota detectable with the universal primer.

Changes in bacterial groups were detected in the patients diagnosed with CD and UC (Table II; Figures 1 and 2). *Bifidobacterium* cell counts were lower in patients with AUC compared with healthy controls. Interestingly, patients with ACD and patients in remission had significantly lower fecal concentrations of *Bifidobacterium* and *F prausnitzii* compared with healthy controls ($P < .05$), and patients with ACD had significantly higher concentrations of *E coli* ($P < .05$) (Table II).

Table II. Median concentrations by range, $\log_{10}$ cells/g of feces, of microbiota in patients with IBD and controls

	PUCAI		PCDAI		Controls
	≤10 (n = 16)	>10 (n = 13)	≤10 (n = 19)	>10 (n = 21)	0 (n = 25)
Firmicutes	10.11 ± 0.40	10.19 ± 0.50	9.53 ± 0.92	10.02 ± 0.80	10.09 ± 0.34
C leptum group (clostridial cluster IV)	9.97 ± 0.41	9.96 ± 0.51	9.44 ± 0.99	9.84 ± 0.82	9.94 ± 0.29
F prausnitzii	9.52 ± 0.69	9.53 ± 0.53	8.93 ± 1.27*	8.89 ± 2.16*	9.59 ± 0.48
C coccoides group (clostridial cluster IVa)	9.24 ± 0.56	9.25 ± 0.65	8.87 ± 2.77	9.28 ± 0.90	9.56 ± 0.49
Lactobacillus/Enterococcus	5.12 ± 2.27	5.40 ± 2.33	5.53 ± 1.51	5.49 ± 2.45	5.24 ± 2.07
Eubacterium cylindroids	7.61 ± 1.35	7.49 ± 1.25	6.85 ± 1.90	7.41 ± 1.90	7.13 ± 1.10
Bacteroidetes	9.77 ± 2.39	9.93 ± 0.41	9.87 ± 1.28	9.92 ± 2.19	9.70 ± 0.65
Bacteroides spp	9.77 ± 2.39	9.93 ± 0.41	9.87 ± 1.28	9.92 ± 2.19	9.70 ± 0.65
Prevotella spp	6.49 ± 2.15	6.32 ± 2.50	6.14 ± 1.74	6.46 ± 1.91	7.41 ± 1.70
Actinobacteria					
Bifidobacterium spp	6.87 ± 1.15*	7.12 ± 1.31	6.98 ± 1.40*	7.01 ± 1.06*	7.51 ± 0.64
Proteobacteria					
E coli	7.01 ± 1.42	7.81 ± 1.46	7.68 ± 1.40	8.72 ± 1.62†	5.97 ± 2.07
Total cell count	10.31 ± 0.35	10.37 ± 0.23	10.06 ± 0.58	10.24 ± 0.55	10.30 ± 0.28

*Significant decrease compared with controls ($P < .05$).
†Significant increase compared with controls ($P < .05$).

Compared with healthy controls, in patients with ACD, the median *E coli* count increased from 9.5×10^5 ($\log_{10}$ 5.97) to 5.3×10^8 ($\log_{10}$ 8.72) cells per gram of feces, representing up to 3% of the total microbiota (Figure 2). Interestingly, the ratio of *Firmicutes* to *Bacteroidetes* was changed in favor of *Bacteroidetes* in the ACD group compared with healthy controls. In patients with CDR, the ratio increased further in favor of *Bacteroidetes* (Figure 1). However, because the individual numbers of the various bacterial groups representing the large phyla *Firmicutes* (clostridial clusters IV and XIVa) and *Bacteroidetes* (*Bacteroides* spp) were so widely scattered, no significant changes in proportions could be detected.

Discussion

CD and UC are thought to be the result of continous microbial antigenic stimulation of pathogenic immune responses developing as a consequence of host genetic defects in mucosal barrier function, innate bacterial killing, or immunoregulation. The intestinal microbiota appears to be the target of immune reactivity, as has been demonstrated in various genetic studies and animal models of mucosal inflammation. Numerous studies have used molecular techniques to detect changes in the composition of fecal microbiota in patients with IBD;[3] however, little data are available on the microbiota in children, which is a group distinct from adults in terms of disease onset and severity.[13,29]

In contrast to previous results in adults,[5,9] we detected no major changes in microbiota composition in patients with UC, except for decreased *Bifidobacterium* in patients with AUC (Table II and Figure 1). This might be because our study population was rather small. Because our study population was rather young, it also might be speculated that changes in microbiota composition in patients with UC may occur later in life. In contrast, our patients with CD exhibited changes in microbiota composition (Table II and Figure 1). Decreased numbers of *Firmicutes*, particularly

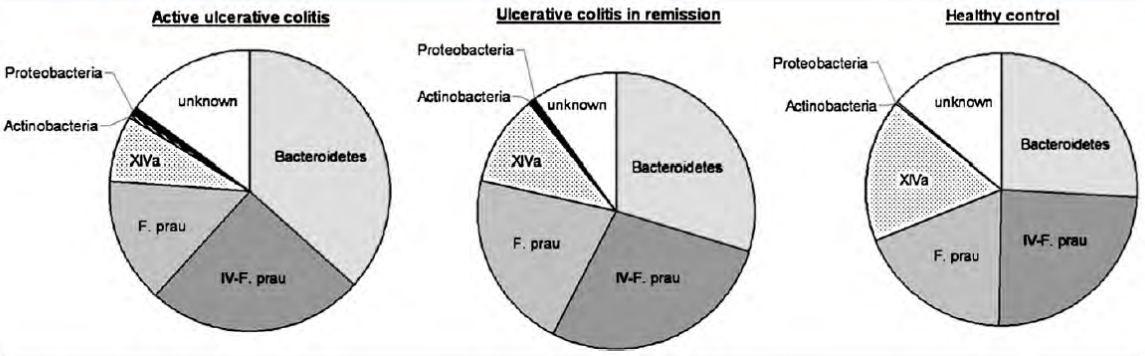

Figure 1. Mean proportions of different bacterial groups in feces of children diagnosed with ulcerative colitis compared with healthy controls (assessed by qPCR; Table II). IV, clostridial cluster IV; XIVa, clostridal cluster XIVa; F. prau, *F prausnitzii*. *F prausnitzii* is a member of clostridial cluster IV.

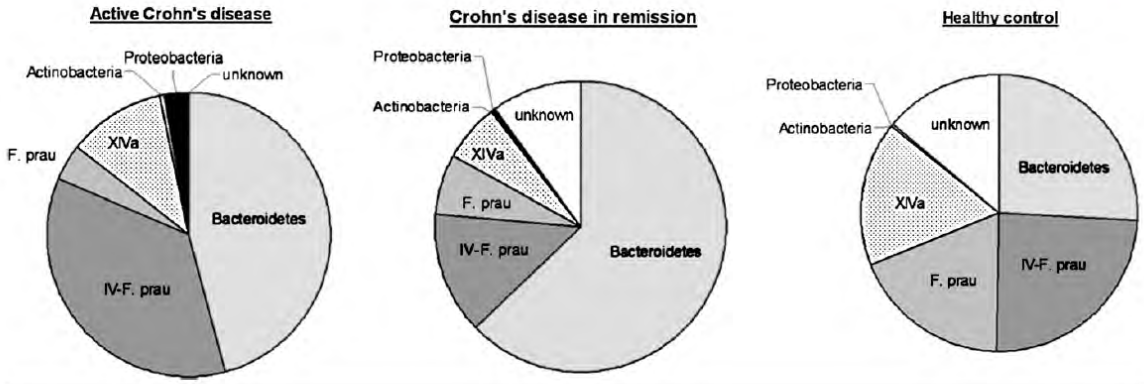

Figure 2. Mean proportions of different bacterial groups in feces of children diagnosed with Crohn's disease compared with healthy controls (assessed by qPCR; **Table II**). IV, clostridial luster IV; XIVa, clostridial cluster XIVa; F. prau, *F prausnitzii*. *F prausnitzii* is a member of clostridial cluster IV.

those of clostridial clusters XIVa and IV groups, have been reported in patients with CD.[9] Other analyses have detected decreased concentrations of *F prausnitzii*, a predominant species of the clostridial cluster IV, in patients with CD.[8,30]

In our study group of children and adolescents with CD, *F prausnitzii* likewise was decreased in the active and the remission phase of the disease (**Table II**). The median number of *F prausnitzii* cells in healthy controls was 3×10^9 (log 9.59), which represented 18.62% of the total detectable bacterial species. In patients with ACD and CDR, the total number of *F prausnitzii* cell was decreased to $<10^9$ cells per gram of feces and to proportions of <5% and <8% of the total microbiota, respectively (**Table II** and **Figure 2**). Interestingly, although the median total *F prausnitzii* cell count was decreased in both groups, counts $<1 \times 10^6$ were more common in patients with CDR (data not shown).

It can be speculated that a dramatic reduction in the quantities of microbes that provide metabolic services to the host gastrointestinal (GI) tract exacerbate certain forms of IBD. Butyrate, which is produced exclusively by bacterial metabolism, is an important source of energy for colonic epithelial cells and may enhance the integrity of the epithelial barrier and modulate the GI immune system. Butyrate also has been reported to modulate inflammation in IBD, possibly by down-regulating the production of proinflammatory cytokines. The *F prausnitzii* group of organisms is second to the *Roseburia* group as the most abundant group of butyrate-producing bacteria within the human gut.[11] *F prausnitzii* may be important not only for its provision of butyrate to the host, but also for its anti-inflammatory effects. *F prausnitzii* A2-165 has been shown to release high interleukin (IL)-10/IL-12 cytokine levels from peripheral blood mononuclear cells. It also can reduce IL-1β–induced IL-8 secretion by Caco-2 cells, and its supernatant can abolish tumor necrosis factor-α–induced NF-kB activity in HT-29 cells. Furthermore, both *F prausnitzii* A2-165 and its supernatant were found to reduce scores and blood measures of inflammation in TNBS-induced colitis in Balb/c mice, and, when administered intraperitoneally, its supernatant protected mice from death induced by TNBS.[8] In addition, *Bifidobacterium* counts were decreased, even though *E coli* counts were increased, in patients with ACD. These results are in accordance with other studies in which increased *E coli* counts and increased proportion of *E coli* in the GI microbiota were associated with CD and lower bifidobacteria counts.[9,31]

Although marked alterations in fecal and mucosal bacterial communities are seen in IBD, whether these alterations cause the disease or are due to changes in the gut environment resulting from inflammatory reactions and extensive tissue destruction is unclear. We have demonstrated that the microbiota changes in IBD are already present at a young age, at least in patients with CD. ■

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Table I. 16S rRNA gene-targeted group and species-specific primers used in this study

Target	Primer name	Primer sequence (5′-3′)	Reference
Total bacteria	UniF340	ACTCCTACGGGAGGCAGCAGT	32
	UniR514	ATTACCGCGGCTGCTGGC	
Firmicutes			
C leptum group (clostridial cluster IV)	C-lept-F1123	GTTGACAAAACGGAGGAAGG	20
	C-lept-R1367	GACGGGCGGTGTGTACAA	
C coccoides group (clostridial cluster XIVa)	Univ-F338	ACTCCTACGGGAGGCAGC	21
	C.coc-R491	GCTTCTTAGTCAGGTACCGTCAT	
E cylindroides group	Univ-F338	ACTCCTACGGGAGGCAGC	23
	E.cyl-R399	CATTGCTCGTTCAGGGTTC	
Lactobacilli/Enterococci	Lab-F362	AGCAGTAGGGAATCTTCCA	22
	Lab-R677	CACCGCTACACATGGAG	
F prausnitzii	PrausF480	CAGCAGCCGCGGTAAA	28
	PrausR631	CTACCTCTGCACTACTCAAGAAA	
Bacteroidetes			
Bacteroides spp.	Bact-F285	GGTTCTGAGAGGAGGTCCC	19
	Univ-R338	GCTGCCTCCCGTAGGAGT	
Prevotella spp.	Prevo-F449	CAGCAGCCGCGGTAATA	20
	PrevoR757	GGCATCCATCGTTTACCGT	
Proteobacteria			
E coli	EcoliF395	CATGCCGCGTGTATGAAGAA	28
	EcoliR470	CGGGTAACGTCAATGAGCAAA	
Actinobacteria			
Bifidobacterium spp.	Bifido-F143	CTCCTGGAAACGGGTGGT	25
	Univ-R338	GCTGCCTCCCGTAGGAGT	

Chapter 8

Oligonucleotide Probes That Detect Quantitatively Significant Groups of Butyrate-Producing Bacteria in Human Feces

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Oligonucleotide Probes That Detect Quantitatively Significant Groups of Butyrate-Producing Bacteria in Human Feces

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16S rRNA-targeted oligonucleotide probes were designed for butyrate-producing bacteria from human feces. Three new cluster-specific probes detected bacteria related to *Roseburia intestinalis*, *Faecalibacterium prausnitzii*, and *Eubacterium hallii* at mean populations of 2.3, 3.8, and 0.6%, respectively, in samples from 10 individuals. Additional species-level probes accounted for no more than 1%, with a mean of 7.7%, of the total human fecal microbiota identified as butyrate producers in this study. Bacteria related to *E. hallii* and the genera *Roseburia* and *Faecalibacterium* are therefore among the most abundant known butyrate-producing bacteria in human feces.

The microbiota of the gastrointestinal tract of humans has been studied extensively because of the role played by gut bacteria both in disease and in the maintenance of gut health (7, 17, 27). One important activity of the large intestinal microbiota is to break down substrates, such as resistant starch and plant cell wall polysaccharides. The main fermentation products are the short-chain fatty acids acetate, propionate, and butyrate. Of these, butyrate is known to play an important role in the metabolic welfare of colonocytes (19, 20) and is also implicated in providing protection against cancer and ulcerative colitis (3–5). Despite this prominent role, the taxonomy, population structure, and dynamics of predominant butyrate-producing bacteria in the human intestinal tract are poorly understood.

There is no simple way to selectively isolate butyrate-producing bacteria, and the majority of those recovered from nonselective isolation have proved to be highly oxygen sensitive (2). The purpose of the present study was, therefore, to design 16S rRNA-targeted oligonucleotide probes for butyrate-producing bacteria, including recent isolates from the human gut, many of which represent new species (8, 9, 21). The majority of these butyrate-producing isolates belong to the clusters XIVa and IV of clostridia (6) (Fig. 1 and 2), which account for a significant proportion of total bacterial diversity in the human large intestine on the basis of 16S rRNA sequence analyses (12, 25).

Four broad-specificity probes were designed to target the small subunit rRNA of bacteria related to *Eubacterium hallii*, the recently reclassified *Faecalibacterium prausnitzii* (formerly *Fusobacterium prausnitzii*) (9), *Coprococcus eutactus*, and *Roseburia intestinalis* clusters (Table 1 and Fig. 1 and 2). The latter probe is predicted to recognize *R. intestinalis*, *Eubacterium rectale*, and *Eubacterium ramulus* as well as the butyrate-producing *Eubacterium* isolates A2-194 and L1-83 and the *Roseburia* isolate A2-183 from the study of Barcenilla et al. (2). In addition, six more specific probes were designed to recognize *R. intestinalis* (8), *Anaerostipes caccae* (21), the *Eubacterium* isolates L1-83 and A2-194, *Coprococcus* isolate L2-50, and *E. rectale* isolate A1-86. The new probes were designed with the ARB (16) software package, checked against the Ribosomal Database Project (RDP) and EMBL databases, and named according to the nomenclature suggested by the Oligonucleotide Probe Database (OPD) (1). The probe sequences have also been deposited in the ProbeBase data bank (15). The specificity of the newly designed probes was tested by whole-cell in situ hybridization against a panel of 120 reference strains derived from the human and animal gastrointestinal tract as described by Schwiertz et al. (22) and also against the new target butyrate-producing strains. Hybridization temperatures (T_H) are given in Table 1. All newly designed probes hybridized only to the respective target organisms but not to any of the other organisms tested. The exception was the L1-83 probe, which showed weak cross-reactivity with *Eubacterium* sp. strain A2-194.

Fresh fecal samples from 10 healthy volunteers of both sexes, aged 28 to 56, who had consumed a Western diet and had not received any antibiotic treatment at least 3 months prior to the study, were collected and fixed as described elsewhere (24). Hybridization and enumeration were performed as described previously (22), with the lower limit of detection of 10^7 cells g^{-1} of dry feces. In addition to analysis with the newly designed probes described above, the fecal samples were analyzed with 11 *Eubacterium* species-specific probes described previously (22–24). Broad-specificity probes or probe mixes that targeted all eubacteria were applied (14), along with the *Ruminococcus-Eubacterium-Clostridium* cluster probe (Erec482) (10), the *Clostridium lituseburense* group probe (Clit135) (13), the *Clostridium histolyticum* group probe (Chis150) (10), and the *Eubacterium cylindroides* group probe (Ecyl387) (11).

Cell counts for the target organisms in the 10 subjects are summarized in Table 2. Each subject harbored at least three groups of butyrate producers, with a mean of 7.7% of the total fecal microbiota identified as butyrate producers in this study. The Fpra655 (*F. prausnitzii*) probe detected between 1.4 and 5.9% (mean, 3.8%) of the total fecal microbiota in all 10

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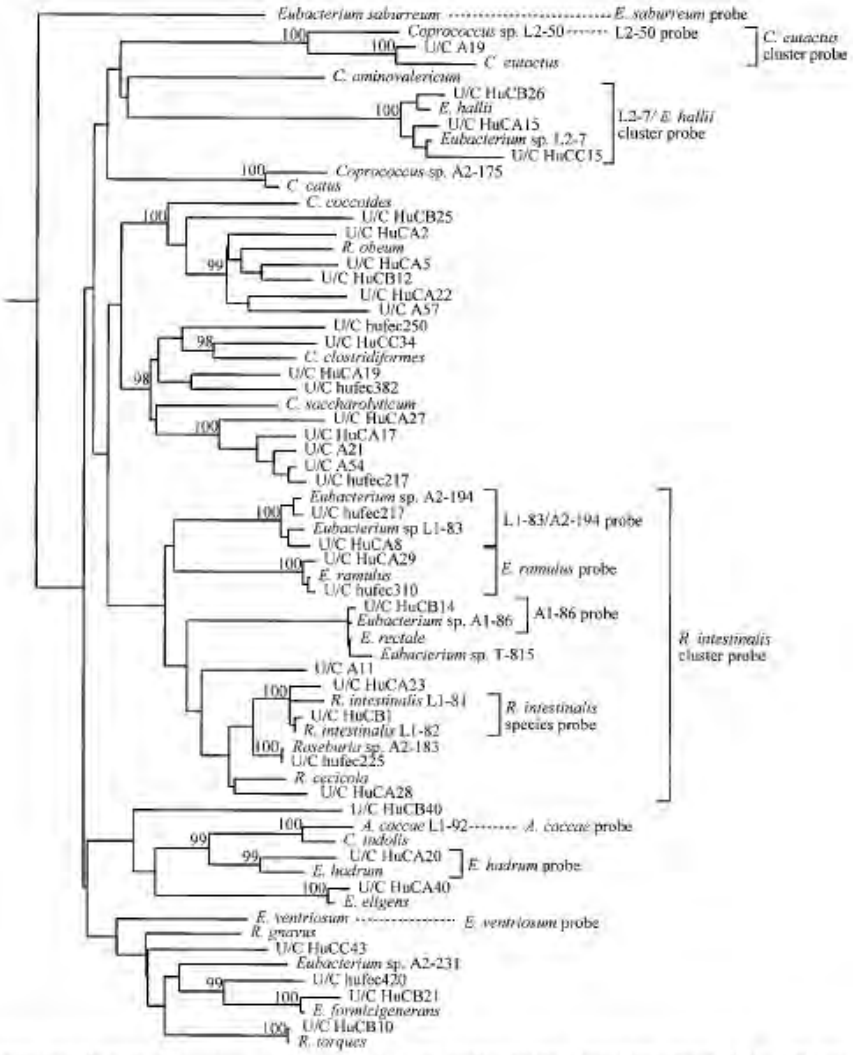

FIG. 1. Phylogenetic tree showing the coverage of the newly designed probes within *Clostridium* cluster XIVa. The tree was constructed by using neighbor-joining analysis of a distance matrix obtained from a multiple-sequence alignment. Bootstrap values (expressed as percentages of 1,000 replications) are shown at branch points; values of 97% or more were considered significant.

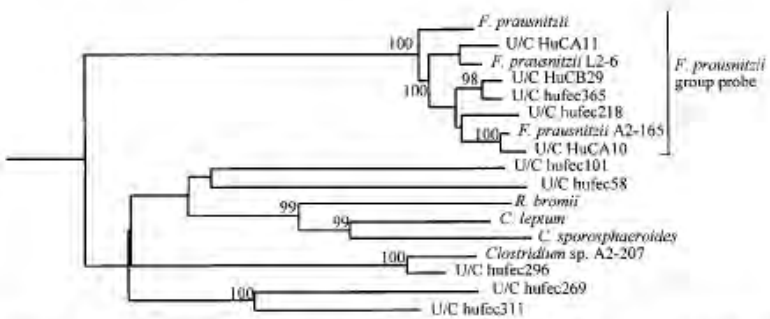

FIG. 2. Phylogenetic tree showing the coverage of the newly designed *F. prausnitzii* probe within *Clostridium* cluster IV. The tree was constructed by using neighbor-joining analysis of a distance matrix obtained from a multiple-sequence alignment. Bootstrap values (expressed as percentages of 1,000 replications) are shown at branch points; values of 97% or more were considered significant.

TABLE 1. Probes used during the study

Probe name	OPD name[a]	Target species with zero mismatches	Probe sequence 5′-3′	T_H (°C)	Reference or source
Species-specific probes					
A. caccae	S-S-Acac-0194-a-A-18	*A. caccae*	CTA TAC TGC CAG GGC TTT	46	This study
R. intestinalis	S-S-Rint-1102-a-A-18	*R. intestinalis*	GCT TAC CCG CTG GCT ACT	46	This study
Eubacterium sp. strain L1-83[b]	S-St-xxxx-0576-a-A-18	*Eubacterium* sp. strains L1-83 and A2-194	AGC CTT CCG CCT GCG CTC	58	This study
Coprococcus sp. strain L2-50[b]	S-St-xxxx-0060-a-A-18	*Coprococcus* sp. strain L2-50	CAC CGA TCT TCT CTC GTT	54	This study
E. rectale sp. strain A1-86[b]	S-S-Erec-0207-a-A-18	*E. rectale* strain A1-86	GGT GGT GTA CAA GAC CCG	52	This study
E. barkeri	S-S-Ebar-1237-a-A-18	*E. barkeri, E. aggregans*	CCT TTG TCC CAA CCC ATT	51	22
E. biforme	S-S-Ebif-0462-a-A-18	*E. biforme*	CAC TCA CTC ATC ATT CCC	51	22
E. cylindroides	S-St-Ecyl-0461-a-A-18	*E. cylindroides*	ACC CAC GGA TCA TTC CCT	51	22
E. cylindroides	S-St-Ecyl-0466-a-A-18	*E. cylindroides*	CCG TCA CCC ACA TAG CAT	51	22
E. hadrum	S-*-Ehad-0579-a-A-20	*E. hadrum*	GAC TTG CCA TAC CAC CTA CG	54	22
E. limosum	S-*-Elim-1433-a-A-18	*E. callanderi, E. limosum*	GCG GTT CTC TCA CAG GCT	51	22
E. moniliforme	S-S-Emon-0084-a-A-18	*E. moniliforme*	CCG CTA ATC CAT TTC CCG	51	23
E. multiforme	S-S-Emul-0183-a-A-18	*E. multiforme*	GTT CCT TCA TGC GAA GGT	51	23
E. ramulus	S-S-Eram-0997-a-A-18	*E. ramulus*	ACA TGT TCT GTC ACC GGG	46	24
E. saburreum	S-S-Esab-1467-a-A-18	*E. saburreum*	AGT TAT CCT CCC TGC CTT	48	23
E. ventriosum	S-S-Even-0066-a-A-18	*E. ventriosum*	TCT GTC CAA GGT GCT TCG	55	22
Group-and cluster-specific probes					
E. hallii L2-7/ *E. hallii*	S-*-Ehal-0578-a-A-18	*E. hallii* L2-7, *E. hallii*	TTG CAC TGC CAC CTA CGC	58	This study
E. cylindroides cluster	S-*-Ecyl-0387-a-A-18	*C. innocuum, E. biforme, E. cylindroides, E. dolichum, E. tortuosum, Streptococcus pleomorphus*	CGC GGC ATT GCT CGT TCA	46	11
Ruminococcus-Eubacterium-Clostridium cluster	S-*-Erec-0482-a-A-19	For details see Franks et al. (10)	GCT TCT TAG TCA RGT ACC G	50	10
C. eutactus	S-*-Ceut-0705-a-A-21	*C. eutactus, Coprococcus* sp. strain L2-50	GTC AGT AGC AGT CCA GTA AGT	54	This study
F. prausnitzii	S-*-Fpra-0655-a-A-18	*F. prausnitzii* A2-165 and L2-6	CGC CTA CCT CTG CAC TAC	58	This study
R. intestinalis subcluster	S-*-Rint-0623-a-A-18	*Eubacterium* sp. strains L1-83 and A2-194, *E. rectale* sp. strains A1-86, T1-815, and *Roseburia* sp. strain A2-183, and *R. cecicola, R. intestinalis, E. rectale, E. ramulus*	TTC CAA TGC AGT ACC GGG	54	This study
C. histolyticum	S-*-Chis150-a-A-23	For details see Franks et al. (10)	TTA TGC GGT ATT AAT CTY CCT TT	50	10
C. litusebarense	S-*-Clit135-a-A-19	For details see Franks et al. (10)	GTT ATC CGT GTG TAC AGG G	50	10

[a] Standardized probe name in accordance with the OPD (1).
[b] No valid systematic name was available.

subjects, which is in agreement with previous evidence, by using a different probe, indicating that this is one of the most abundant species in human feces (26). Also found in all 10 subjects was the *R. intestinalis* cluster (by using the Rint603 probe), which accounted for 0.9 to 5.0% (mean, 2.3%) of the total microbiota. Thus, the *R. intestinalis* and *F. prausnitzii* groups, which are likely to consist largely if not wholly of butyrate-producing strains, together accounted for not less than 3% and up to 10.9% (mean, 6.1%) of total eubacterial cells in the subjects studied. Organisms detected by the Ehal578 (*E. hallii*) probe were also widespread, being found in nine subjects and accounting for up to 2.4% (average of 0.6%) of the total microbiota. Recent work by Harmsen et al. (11) with a different probe showed that *E. hallii* and its close relatives can account for up to 3.6% of the total human fecal microbiota.

The species-specific probes for *R. intestinalis* and *Eubacterium hadrum* were positive for eight subjects. Bacteria related to *Eubacterium* sp. strains L1-83 and A2-194 and *E. ramulus* were each found in six subjects, while relatives of *E. rectale* sp. strain A1-86 were detected in four subjects. *Eubacterium biforme* and *Eubacterium ventriosum* were detected in only two subjects, and *Coprococcus* sp. strain L2-50 was detected in only one subject. In a PCR-based analysis the *Coprococcus* cluster was shown to account for up to 8% of total bacterial diversity in one human individual (25). Species probes for *Anaerostipes*

caccae, *Eubacterium barkeri*, *E. cylindroides*, *Eubacterium dolichum*, *Eubacterium saburreum*, *Eubacterium limosum*, *Eubacterium moniliforme*, and *Eubacterium multiforme* failed to detect cells above the limit of detection in samples from any of the 10 subjects. Negative results were also obtained with the cluster probes Chis150 and Clit135, but the Ecyl387 group probe detected significant numbers in two individuals.

The Erec482 probe used for the detection of the whole *Ruminococcus-Eubacterium-Clostridium* cluster (cluster XIVa) detected between 5.2 and 26.4% of total fecal bacteria in the 10 volunteers tested here. These numbers are essentially in agreement with previously published data (10, 22, 23). Nonoverlapping probes designed to recognize butyrate-producing species within the Erec482 cluster, namely Rint603, Ehal578, Ehad579, and Event66, together accounted for between 10.2 and 85% (mean, 43%) of the Erec482 signal. Thus, in some subjects (subjects 2, 4, and 7) almost all of the Erec482 representatives were closely related to known butyrate producers, while in others the remaining Erec482 diversity may correspond to species that do not produce butyrate (e.g., *Ruminococcus* sp.) but might also include groups of butyrate producers that have yet to be targeted.

We have now designed and validated probes that target most of the presently known butyrate-producing species or groups from the human gut within the *Clostridium* clusters IV and XIVa. The main conclusion is that butyrate producers accounted for, on average, 7.7% of the bacteria in the 10 subjects studied, with the most abundant groups by far being *R. intestinalis* and *F. prausnitzii*. Interestingly, the proportion of bacterial cells belonging to *Clostridium* cluster XIVa was lower in this set of volunteers than for those of previously published data sets (10, 18). This highlights interindividual differences possibly due to diet or geographic location. Also, the fact that the narrower strain or species-specific probes tested here did not detect bacteria in all fecal samples further emphasizes the diversity of the colonic microbiota at the strain level.

TABLE 2. Quantification of the various bacterial components known to produce butyric acid within the human fecal microbiota[a]

Target	Probe[b]	No. of cells/g (dry wt) and percentage of the total microbiota in volunteer no.[b]									
		1	2	3	4	5	6	7	8	9	10
All eubacteria	Universal	11.7	11.8	11.6	11.8	12.2	11.7	12	11.7	11.5	11.9
Ruminococcus-Eubacterium-Clostridium cluster	Erec482	10.7 (5.2)	10.7 (8.06)	11 (26.4)	10.5 (5.2)	10.9 (5.3)	10.9 (15)	10.8 (6.3)	10.6 (8.4)	10.8 (19.7)	10.9 (8.8)
E. hallii cluster	Ehal578	8.8 (0.13)	10.1 (2)		8.3 (0.03)	9.1 (0.1)	9 (0.17)	10.4 (2.4)	8.8 (0.12)	9.2 (0.48)	9 (0.12)
R. intestinalis cluster	Rint603	9.6 (0.87)	10.4 (3.88)	10 (2.6)	10 (1.66)	10.6 (2.2)	10 (2.07)	10.4 (2.74)	9.8 (1.22)	10.2 (4.98)	10 (1.16)
R. intestinalis	Rint1102	8.4 (0.06)	8.4 (0.03)		9.5 (0.44)	9.5 (0.22)	9.4 (0.47)		8.5 (0.06)	8.8 (0.2)	9.2 (0.2)
Eubacterium sp. strains L1-83 and A2-194			8.4 (0.03)			10.1 (1.28)	9.1 (0.24)		9.4 (0.51)	8.8 (0.2)	9.4 (0.26)
E. rectale sp. strain A1-86	Erec207	8.8 (0.12)					8.5 (0.06)	8.5 (0.04)			9.5 (0.34)
E. ramulus	Eram997		8.5 (0.05)	8.1 (0.03)			8.9 (0.01)		8 (0.02)		8.6 (0.05)
Coprococcus sp. strain L2-50							8.8 (0.035)				
E. hadrum	Ehad579	9.1 (0.4)	9.1 (0.2)	8.5 (0.1)	9 (0.14)	9.3 (0.01)	9.5 (0.56)	9.2 (0.17)			9.2 (0.2)
E. ventriosum	Event66				10.1 (1.7)						10.2 (1.7)
E. cylindroides group	Ecyl387		10.2 (2.5)						9.9 (1.7)		
E. biforme	Ebif462		10.2 (2.5)						9.9 (1.7)		
F. prausnitzii cluster	Fprau655	10.1 (2.7)	10.5 (4.6)	10.2 (4.1)	10.4 (3.75)	10.8 (3.6)	10.5 (5.53)	10.1 (1.35)	10.5 (5.89)	10.2 (4.9)	10.2 (1.8)

[a] All probes were negative for *E. barkeri*, *E. multiforme*, *E. moniliforme*, *E. saburreum*, *E. cylindroides*, *E. dolichum*, *E. limosum*, *A. caccae*, *C. litseburense*, and *C. hiranoiense*.

[b] Counts of bacteria are expressed as numbers of organisms $\log_{10}$ per gram of feces (dry weight). Coefficient of variation due to assay error of the fluorescent in situ hybridization method was 0.16 (13). The percentage of the total microbiota was calculated by using counts from the universal eubacterial probe (11B).

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Chapter 9

Influence of resistant starch on the SCFA production and cell counts of butyrate producing Eubacterium spp. in the human intestine

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Influence of resistant starch on the SCFA production and cell counts of butyrate-producing *Eubacterium* spp. in the human intestine

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Aims: The genus *Eubacterium*, which is the second most common genus in the human intestine, includes several known butyrate producers. We hypothesized that *Eubacterium* species play a role in the intestinal butyrate production and are inducible by resistant starch.

Methods and Results: In a human pilot study species-specific and group-specific 16S rRNA-targeted, Cy3 (indocarbocyanine)-labelled oligonucleotide probes were used to quantify butyrogenic species of the genera *Eubacterium*, *Clostridium* and *Ruminococcus*. Following the intake of RS type III a significant increase in faecal butyrate but not in total SCFA was observed. However, increase in butyrate was not accompanied by a proliferation in the targeted bacteria.

Conclusions: The tested *Eubacterium* species have the capacity to produce butyrate but do not appear to play a major role for butyric acid production in the human intestine.

Significance and Impact of the Study: In view of the fact that the bacteria responsible for butyrate production are largely unknown, it is still difficult to devise a dietary intervention to stimulate butyrogenic bacteria in a targeted way.

INTRODUCTION

Dietary fibre, which is not hydrolysed by host enzymes in the small intestine, undergoes bacterial degradation in the large intestine. The main fermentation products are the short-chain fatty acids (SCFA) acetate, propionate and butyrate. The latter has been shown to be the preferred energy substrate of the colonocytes (Roediger 1980). Butyrate has also been implicated in providing protection against cancer (Hill 1995). Since Englyst *et al.* (1987) reported that starch exhibits *in vitro* the strongest butyrogenic effect of all carbohydrates tested, several studies have demonstrated that resistant starches stimulate bacterial butyrate formation (Jenkins *et al.* 1998; Le Blay *et al.* 1999). Despite this, however, little is known about the predominant butyrate-producing bacteria in the human intestinal tract. The most obvious reason lies in the fact that there is no simple way to enrich and isolate butyrate producers. Each random isolate has to be tested for its ability to produce butyrate. So far, only a few reports have contributed to the description of the human butyrate-producing flora (Barcenilla *et al.* 2000; Sharp and Macfarlane 2000).

The choice of bacteria in this study was based on reports by Moore and Holdeman (1974), Finegold *et al.* (1983), Simmering *et al.* (1999) and Schwiertz *et al.* (2000), and included known butyrate producers (Moore and Holdeman Moore 1986): *Eubacterium barkeri*, *Eu. biforme*, *Eu. cylindroides*, *Eu. dolichum*, *Eu. hadrum*, *Eu. limosum*, *Eu. moniliforme*, *Eu. multiforme*, *Eu. rectale*, *Eu. ramulus*, *Eu. saburreum*, *Eu. tortuosum* and *Eu. ventriosum*. The species were chosen on the basis of their numerical importance and availability of probes for whole cell *in-situ* hybridization experiments. The objective of our study was to test the hypothesis that resistant starch (RS) type III stimulates the growth of the butyrogenic species of the genera *Eubacterium*, *Clostridium* and *Ruminococcus* and thereby induces higher faecal SCFA concentrations.

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MATERIALS AND METHODS

Oligonucleotide probes

16S rRNA-targeted probes were chosen to detect *Eubacterium* species known to produce butyrate, or bacterial groups known to include butyrogenic species (Moore and Holdeman Moore 1986; Franks *et al.* 1998). These probes included species-specific probes for the detection of *Eubacterium* species (Simmering *et al.* 1999; Schwiertz *et al.* 2000) and bacterial group probes for the detection of *Ruminococcus*, *Eubacterium* and *Clostridium* species (Erec482, Franks *et al.* 1998). In addition, species-specific probes for the detection of *Eu. multiforme*, *Eu. rectale*, *Eu. saburreum* and *Eu. tortuosum* were designed and validated for whole cell *in-situ* hybridization experiments as described earlier (Schwiertz *et al.* 2000). All used probes are summarized in Table 1.

Starch

The resistant starch (RS) product was made from native pea starch (Cosucra, Fontenoy, Belgium). The starch was enzymatically debranched and a 15% gel (w/w) was retrograded at room temperature for 24 h. The RS type III-content of the product was 50%, determined according to Englyst *et al.* (1992). The daily intake was 15 g RS type III product.

Subjects and pilot study design

Five healthy subjects (four females and one male) participated in a 28-d pilot study. The age ranged from 25 to 64 years. None of the subjects took antibiotics during the preceding three months and during the pilot study. The volunteers maintained their usual lifestyles and dietary intakes throughout the study period. During the study period of 28 d each subject consumed in addition to its unrestricted diet 15 g of RS type III product. The Ethical Committee of Brandenburg approved the protocol (no. eK 170–5/kmo, 8.10.1998) and all persons were asked to give their informed consent before the beginning of the study.

Sampling

Fresh faecal samples were collected from each subject at the beginning of the study, on the last day of RS-intake, and finally on the 14th day following the end of RS-intake.

Hybridization and analysis of faecal flora

For the analysis by whole cell in-situ hybridization a 0·2 g (wet weight) faeces specimen was added to 1·8 ml of sterile resuspension buffer containing 50% (v/v) ethanol-phosphate-buffered saline (139 mol l^{-1} NaCl, 10 mol l^{-1} NaH_2PO_4-Na_2HPO_4 at pH 7·2). The suspension was mixed by inverting and vortexing the tube for 5–10 min. The fixed samples were stored at –20 °C until used. Hybridization and enumeration were performed as described earlier (Schwiertz *et al.* 2000).

Data analysis and statistics

The data for each time point of the human pilot study are given as both individual data and the means and S.D. of five

Table 1 Probes used in the study and their corresponding sequences

Probe name	OPD designation*	Sequence (5′–3′)	Target	Reference
Ebar1237	S-S-Ebar-1237-a-A-18	CCTTTGTCCCAACCCATT	*Eu. barkeri*	Schwiertz *et al.* 2000
Ebif462	S-S-Ebif-0462-a-A-18	CACTCACTCATCATTCCC	*Eu. biforme*	Schwiertz *et al.* 2000
Ecyl	S-St-Ecyl-0461-a-A-18	ACCCACGGATCATTCCCT	*Eu. cylindroides*	Schwiertz *et al.* 2000
	S-St-Ecyl-0466-a-A-18	CCGTCACCCACATAGCAT		
Edol183	S-S-Edol-0183-a-A-18	TGTCTCCGAGATGCCTCG	*Eu. dolichum*	Schwiertz *et al.* 2000
Ehad579	S-*-Ehad-0579-a-A-20	GACTTGCCATACCACCTACG	*Eu. hadrum*	Schwiertz *et al.* 2000
Elim1433	S-*-Elim-1433-a-A-18	GCGGTTCTCTCACAGGCT	*Eu. limosum*	Schwiertz *et al.* 2000
Emon84	S-S-Emon-0084-a-A-18	CCGCTAATCCATTTCCCG	*Eu. moniliforme*	Schwiertz *et al.* 2000
Emul183	S-S-Emul-0183-a-A-18	GTTCCTTCATGCGAAGGT	*Eu. multiforme*	this study
Erec834	S-S-Erec-0834-a-A-18	CGAGAAGCAAUGCUUCCC	*Eu. rectale*	this study
Erec838	S-S-Erec-0838-b-A-20	CGGCACCGAGAAGCAATGCT	*Eu. rectale*	this study
Eram997	S-S-Eram-0997-a-A18	ACATGTTCTGTCACCGGG	*Eu. ramulus*	Simmering *et al.* 1999
Esab1467	S-S-Esab-1467-a-A-18	AGTTATCCTCCCTGCCTT	*Eu. saburreum*	this study
Etor727	S-S-Etor-0727-a-A-18	AGACCAGGCAACCGCCTT	*Eu. tortuosum*	this study
Etor129	S-S-Etor-0129-b-A-18	CCAGTTACATGGGTAGGT	*Eu. tortuosum*	this study
Even66	S-S-Even-0066-a-A-18	AACTATTGTCCCCCGACA	*Eu. ventriosum*	Schwiertz *et al.* 2000
Eub338	S-D-Bact-0338-a-A18	GCTGCCTCCCGTAGGAGT	Bacteria	Amann *et al.* 1990
Erec482	S-*-Erec-0482-a-A-19	GCTTCTTAGTCA(A/G)GTACCG	*Eu. rectale*-Cluster	Franks *et al.* 1998

*Standardized probe name according to proposal of Alm *et al.* (1996); OPD, oligonucleotide probe database.

subjects. Differences between the means were checked for significance by the paired *t*-test, as described by Lorenz (1992), and are indicated as *P*.

RESULTS

Probe design and specificity testing

Several 16S rRNA-targeted oligonucleotide probes for the detection of *Eu. multiforme*, *Eu. rectale*, *Eu. saburreum* and *Eu. tortuosum* were designed based on comparative analysis using the ARB software program (Strunk and Ludwig 1996) and checked with the RDP- and the EMBL-databases. The probes designed for the detection of *Eu. multiforme* (S-S-Emul-0183-a-A-18) and *Eu. saburreum* (S-S-Esab-1467-a-A-18) hybridized to the corresponding target organism but not to the approximately 100 intestinal organisms used in the specificity testing (Schwiertz *et al*. 2000). The optimal hybridization temperatures of the designed probes for the hybridization experiments were 48 °C for *Eu. saburreum* and 51 °C for *Eu. multiforme*, respectively. None of the probes designed for the detection of *Eu. rectale* and *Eu. tortuosum*, respectively, worked in whole cell *in-situ* hybridization experiments.

Enumeration of total and specific bacterial populations

To determine the impact of RS on the bacterial flora, five subjects were tested for their faecal bacterial composition in response to the consumption of RS. Cell counts and incidences of target organisms and groups in the five subjects are summarized in Table 2. The lower detection limit was 10^7 cells g^{-1} dry weight faeces. Subjects harboured at least one butyrate-producing *Eubacterium* species in each of their collected faecal samples. *Eubacterium hadrum* was detected in all five subjects. *Eubacterium ramulus* was found in four subjects while *Eu. biforme* was detected in only three subjects. No other *Eubacterium* species (*Eu. barkeri*, *Eu. cylindroides*, *Eu. dolichum*, *Eu. saburreum*, *Eu. limosum*, *Eu. moniliforme*, *Eu. multiforme* and *Eu. ventriosum*) could be detected throughout the study in the faeces of any of the subjects. Organisms detected with the REC-cluster probe were found in all subjects at concentrations of $1{\cdot}0 \times 10^{10}$ to $3{\cdot}7 \times 10^{11}$ cells g^{-1} dry weight of faeces. RS-intake did not lead to a significant change in any of the bacterial groups and species detected. Fourteen days after the intervention period the cell numbers of *Eu. hadrum* and *Eu. ramulus* increased significantly in comparison to the beginning of the study. Changes in the cell counts of *Eu. biforme*, the REC-cluster and total bacteria were not significant at this time.

SCFA analysis in faecal samples

The major SCFA found in the faecal samples were acetate, propionate and butyrate (Table 3); the concentrations of iso-butyrate and iso-valerate, were very low (< 16 μmol g^{-1} dry weight) and are therefore not shown. The differences in the faecal SCFA concentrations between the five subjects were considerable. During the period of RS intake the faecal concentration of total SCFA increased in

Table 2 Bacterial counts determined with whole cell *in-situ* hybridization for the different subjects during the study period

Subject	1			2			3		
Study period*	T_0	T_1	T_2	T_0	T_1	T_2	T_0	T_1	T_2
Total microflora	11·9†	11·3	12·4	12·1	11·6	11·6	11·9	11·8	11·6
Eu. rectale-cluster	10·6	10·7	11·6	10·4	10·7	10·1	10·8	10·6	11·1
Eu. biforme	10·2	9·1	9·7	–	–	–	9·5	9·9	10·1
Eu. hadrum	9·1	9·1	9·7	8·8	8·7	8·9	8·0	8·7	8·4
Eu. ramulus	8·8	8·4	9·3	8·5	8·8	9·3	8·5	8·9	8·6
Subject	4			5			Mean subjects 1.5 (S.D.)		
Study period	T_0	T_1	T_2	T_0	T_1	T_2	T_0	T_1	T_2
Total microflora	11·9	11·7	11·6	10·5	11·0	10·9	11·7 (0·7)	11·5 (0·3)	11·6 (0·5)
Eu. rectale-cluster	11·2	10·6	10·7	10·0	10·0	10·0	10·6 (0·5)	10·5 (0·3)	10·7 (0·7)
Eu. biforme	–	–	–	8·1	9·1	8·9	9·3 (1)	9·4 (0·5)	9·6 (0·6)
Eu. hadrum	8·5	8·9	9·3	8·2	8·9	8·5	8·5 (0·4)	8·9 (0·5)	9·0 (0·5) ‡
Eu. ramulus	8·0	9·3	8·8	–	–	–	8·5 (0·3)	8·9 (0·4)	9·0 (0·4) ‡

*T_0, beginning of study; T_1, last day of RS intake; T_2, at the 14th day following the study.
†Cell numbers are expressed as the number of organisms (log_{10}) per g^{-1} dry weight of faeces.
‡Significantly higher than T_0 ($P < 0{\cdot}05$).

Table 3 SCFA concentrations and relative proportions of the SCFA for the human pilot study

Subject	1						2					
	Total SCFA in μmol g^{-1} dry weight			Relative proportion in % of total SCFA			Total SCFA in μmol g^{-1} dry weight			Relative proportion in % of total SCFA		
Study period*	T_0	T_1	T_2	T_0	T_1	T_2	T_0	T_1	T_2	T_0	T_1	T_2
Total SCFA	274·2	469·2	435·9				197·1	295·3	207·2			
Acetate	188·1	298·2	271·7	68·6	63·5	62·3	134·8	161·8	130·2	68·4	54·8	62·8
Propionate	31·2	75·4	82·1	11·4	16·0	18·8	32·8	58·3	39·0	16·6	19·7	18·8
Butyrate	54·8	95·6	82·1	20·0	14·0	18·8	29·6	75·1	38·0	15·0	25·5	18·3
Subject	3						4					
	Total SCFA in μmol g^{-1} dry weight			Relative proportion in % of total SCFA			Total SCFA in μmol g^{-1} dry weight			Relative proportion in % of total SCFA		
Study period	T_0	T_1	T_2	T_0	T_1	T_2	T_0	T_1	T_2	T_0	T_1	T_2
Total SCFA	325·9	516·8	304·3				356·3	404·2	344·6			
Acetate	224·6	358·6	193·2	68·9	69·4	63·5	221·2	256·0	230·2	62·1	63·3	66·8
Propionate	45·6	61·5	27·6	14·0	11·9	9·1	81·9	77·5	62·9	22·9	19·2	18·2
Butyrate	55·5	96·5	83·5	17·0	18·7	27·4	62·2	70·6	51·5	17·4	17·5	14·9
Subject	5						Subjects 1–5 × S.D.					
	Total SCFA in μmol g^{-1} dry weight			Relative proportion in % of total SCFA			Total SCFA in μmol g^{-1} dry weight			Relative proportion in % of total SCFA		
Study period	T_0	T_1	T_2	T_0	T_1	T_2	T_0	T_1	T_2	T_0	T_1	T_2
Total SCFA	811·7	680·6	690·0				393·0 (241·7)	473·2 (142·6)	396·4 (183·5)			
Acetate	495·6	391·7	353·7	61·0	57·6	51·3	253·0 (140·4)	293·3(90·4)	235·8 (83·9)	65·8 (4)	61·7 (5·7)	61·3 (5·9)
Propionate	257·4	241·6	289·8	31·8	35·5	42·0	89·8 (95·9)	102·9 (78)	100·3 (108)	19·3 (8·2)	20·5 (9)	21·4 (12·2)
Butyrate	58·7	47·3	46·5	7·2	6·9	6·7	52·2 (12·9)	77·0 (20·3)†	60·3 (21·09)	15·3 (4·9)	16·5 (6·8)	17·2 (7·5)

*T_0, beginning of study; T_1, last day of RS intake; T_2, at the 14th day following the study.
†Significantly higher than T_0 ($P < 0·05$).

four of the five subjects. With the exception of one subject, the increase in SCFA was accompanied by a rise in the butyrate concentration. Following the intervention period, a significant increase in the faecal butyrate concentration was observed, while the relative proportions of the single SCFA remained unchanged. At the 14th day after the last RS-intake, the mean SCFA concentrations were lower than during the period of RS consumption (Table 3) and not different from those measured at the beginning of the study.

DISCUSSION

Short-chain fatty acids (SCFA) can be derived in large quantities from bacterial fermentation of dietary fibre in the large bowel (McIntyre *et al.* 1993). Although not proven, a high fibre intake has been associated with a reduced risk of colon cancer (McIntyre *et al.* 1993; Hill 1995). One of the SCFA, butyrate, is known to play a key role in the energy metabolism of the colonic epithelial cells and is thought to be important in the maintenance of colonic health in humans (Mortensen and Clausen 1996). In particular, its ability to modify nuclear architecture and induce death by apoptosis in colon cancer cells is of great interest (Heerdt *et al.* 1997). It is known that bacterial butyrate production is stimulated particularly by resistant starches (Wang *et al.* 1999; Sharp and Macfarlane 2000).

Since butyrate-producing strains of the genus *Eubacterium* are found in rather high cell counts (Finegold *et al.* 1983; Simmering *et al.* 1999; Schwiertz *et al.* 2000) we hypothesized that these species might play a role in the reported increase of butyrate following the intake of resistant starch. To test this assumption we performed a human pilot study, in which we investigated the influence of resistant starch on butyrate-producing *Eubacterium* species and on bacterial groups known to include butyrogenic species detectable with the Erec482-probe (Finegold *et al.* 1983; Franks *et al.* 1998). The enumeration of the species was done by whole cell *in-situ* hybridization. The cell counts obtained in our study are essentially in agreement with the published data, which were obtained with either classical methods (Finegold *et al.* 1983) or whole cell *in-situ* hybridization (Franks *et al.* 1998; Simmering *et al.* 1999; Schwiertz *et al.* 2000). No correlation between cell counts of the targeted organisms, RS intake and the increase in the butyrate concentration could be drawn. It may be speculated that other bacterial population groups were responsible for the observed butyrate increase after RS intake. A significant increase in cell numbers was observed only for *Eu. hadrum* and *Eu. ramulus* at day 14 after the last RS intake. This phenomenon may be due to dietary compounds not investigated in this study, as the increase was only significant 14 d after the last RS intake. RS intake cannot be held responsible for the detected increase in *Eu. hadrum* and *Eu. ramulus* cell counts.

The fact that not all *Eubacterium* species could be detected during this study can be explained by the detection limit of whole cell *in-situ* hybridization, which is approximately 10^7 cells g^{-1} faeces dry weight (Schwiertz *et al.* 2000). However, it cannot be excluded that the cell counts for *Eu. rectale* and *Eu. tortuosum* increased during RS intake. No specific probe could be developed for either of the two organisms. This may be due to inaccessibility of the target site (Fuchs *et al.* 1998).

Sharp and Macfarlane (2000) showed that butyrogenic species require a high supplementation with RS. Furthermore, their study indicates that saccharolytic clostridia are best adapted to fast growth rates and high substrate concentrations and that resistant starch granules are advantageous for their growth. As several of the *Eubacterium* species are close relatives of the clostridia, it could be speculated that such conditions might also be favourable for the eubacteria, while our chosen intake of 15 g RS III d^{-1} per person might have been insufficient to stimulate the growth of the bacterial species investigated.

Earlier studies with resistant starch reported an increase in the SCFA and butyrate concentrations (Jenkins *et al.* 1998; Le Blay *et al.* 1999). The results of this human pilot study are consistent with these reports as we found a rise in total SCFA after RS intake in four of the five subjects and a significant increase in the butyrate concentration. However, there was no increase in the proportions of butyrate relative to the other SCFA.

So far, no key butyric acid producing bacteria have been identified, although numerous species belonging to the cluster XIVa of the clostridia (Collins *et al.* 1994) are able to utilize starch and produce butyrate (Barcenilla *et al.* 2000; Sharp and Macfarlane 2000). Since species-specific probes for these bacteria are not available, the use of the REC probe, which detects most of the species from cluster XIVa, is presently the best way to describe the impact of resistant starch on butyrogenic bacteria from this cluster. However, the intake of resistant starch did not result in an increase in cell numbers detectable with this probe.

In summary, we have shown that the intake of RS type III leads to an increase in the faecal concentrations of SCFA and butyrate, but does not stimulate a number of selected butyrogenic *Eubacterium* species. This indicates that these organisms do not play a major role in butyrate formation from starch.

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Chapter 10

Anaerobic degradation of flavonoids by Clostridium orbiscindens

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Anaerobic Degradation of Flavonoids by *Clostridium orbiscindens*

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An anaerobic, quercetin-degrading bacterium was isolated from human feces and identified as *Clostridium orbiscindens* by comparative 16S rRNA gene sequence analysis. The organism was tested for its ability to transform several flavonoids. The isolated *C. orbiscindens* strain converted quercetin and taxifolin to 3,4-dihydroxyphenylacetic acid; luteolin and eriodictyol to 3-(3,4-dihydroxyphenyl)propionic acid; and apigenin, naringenin, and phloretin to 3-(4-hydroxyphenyl)propionic acid, respectively. Genistein and daidzein were not utilized. The glycosidic bonds of luteolin-3-glucoside, luteolin-5-glucoside, naringenin-7-neohesperidoside (naringin), quercetin-3-glucoside, quercetin-3-rutinoside (rutin), and phloretin-2′-glucoside were not cleaved. Based on the intermediates and products detected, pathways for the degradation of the flavonol quercetin and the flavones apigenin and luteolin are proposed. To investigate the numerical importance of *C. orbiscindens* in the human intestinal tract, a species-specific oligonucleotide probe was designed and tested for its specificity. Application of the probe to fecal samples from 10 human subjects proved the presence of *C. orbiscindens* in 8 out of the 10 samples tested. The numbers ranged from 1.87×10^8 to 2.50×10^9 cells g of fecal dry mass^{-1}, corresponding to a mean count of 4.40×10^8 cells g of dry feces^{-1}.

Flavonoids are widely distributed in plants and are ingested in considerable amounts with food. More than 5,000 different naturally occurring flavonoids have been described so far. They have been proposed to have beneficial effects on human health based on their anti-inflammatory, antioxidant, vasodilatory, anticancerigenic, and antibacterial properties (for reviews, see references 3, 12, and 21). Although it is known that human intestinal bacteria play a significant role in the degradation of flavonoids (22), there is a paucity of information on the species involved, their distribution in humans, and the mechanisms of degradation. So far, *Clostridium scindens* (30), *Clostridium orbiscindens* (30, 31), *Eubacterium desmolans* (30), and *Eubacterium ramulus* (24), all isolated from human fecal samples, are known to convert flavonoids. However, only *E. ramulus* (24) was further characterized with respect to its potential to degrade flavonoids, the pathways of conversion of flavonoids, and the organism's distribution in humans.

In this study, quercetin-degrading fecal isolates were identified as *C. orbiscindens*, whose ability to degrade flavonoids was first described by Winter et al. (30, 31) but not analyzed in detail. Therefore, the *C. orbiscindens* strains isolated were tested for their range of flavonoids converted and the degradation pathways that were employed. Population levels of *C. orbiscindens* in 10 human subjects were determined.

MATERIALS AND METHODS

Media and growth conditions. For cultivation of *C. orbiscindens* strains I1 to I6, which were isolated in our study, the anoxic techniques of Hungate (14) and Bryant (7) were applied. Cultures were grown under strictly anoxic conditions in 16-ml tubes and were fitted with butyl rubber stoppers and screw caps. The tubes contained 10-ml Wilkens-Chalgren anaerobe (WCA) broth (Oxoid, Basingstoke, United Kingdom). The cultures were incubated overnight at 37°C under a gas phase of N_2 and CO_2 (80:20, vol/vol). For cultivation on plates, WCA agar (Oxoid) was used. Plating of the cells was carried out in an anaerobic cabinet (model MK 3; DW Scientific, Shipley, United Kingdom), and the plates were incubated in anaerobic jars (Merck, Darmstadt, Germany) at 37°C for 48 h.

Chemicals. The flavonoids and their respective glycosides were purchased from Roth (Karlsruhe, Germany), except for genistein and daidzein, which were obtained from Acros Organics (Geel, Belgium). Phloroglucinol, 3,4-dihydroxyphenylacetic acid, 3-(3,4-dihydroxyphenyl)propionic acid, 3-(4-hydroxyphenyl) propionic acid, and 1,6-diphenyl-1,3,5-hexatriene were purchased from Fluka (Deisenhofen, Germany).

Isolation of quercetin-degrading bacteria. For the isolation of quercetin-degrading bacteria, the fluorescence-quenching test (26) was applied. A fecal sample from a healthy male adult was serially diluted in Soerensen buffer (25 mM KH_2PO_4, 33 mM Na_2HPO_4, 0.04% [vol/vol] thioglycolic acid, 0.06% [wt/vol] cysteine [pH 6.8]) under anoxic conditions. Circular nylon membranes (82-mm diameter; Roche Diagnostics GmbH, Mannheim, Germany) were soaked in a mixture of 350 μl of 1 mM 1,6-diphenyl-1,3,5-hexatriene (DPH) and 350 μl of 20 mM quercetin and transferred onto WCA agar plates. One hundred microliters each of the dilutions from 10^{-7} to 10^{-9} were streaked on the membranes, and the membranes were incubated under anoxic conditions at 37°C for 48 h. Five plates were inoculated for each dilution. For detection of fluorescence, the plates were inspected with a UV lamp (wavelength, 365 nm; BioMérieux, Nürtingen, Germany).

Degradation experiments. The degradation experiments were carried out in 16-ml tubes fitted with butyl rubber stoppers. The tubes contained 9.8 ml of WCA broth and a gas phase of N_2 and CO_2 (80:20, vol/vol). An aliquot of 100 μl from a stock solution of luteolin-3-glucoside, luteolin-5-glucoside, naringenin-7-neohesperidoside (naringin), quercetin-3-glucoside, quercetin-3-rutinoside (rutin), phloretin-2′-glucoside, luteolin, apigenin, eriodictyol, naringenin, genistein, daidzein (all at 50 mM concentrations), quercetin, taxifolin (both at concentrations of 50 and 100 mM), or phloretin (10 to 60 mM in 5 mM steps) in dimethyl sulfoxide was added to the medium under anoxic conditions. The media were inoculated with 100 μl of an exponentially growing culture of *C. orbiscindens* and incubated at 37°C. Samples of 400 μl were taken immediately after inoculation, hourly from 2 to 12 h and at 24 and 48 h. The samples were centrifuged at 12,000 × *g* for 5 min, and 100 μl of the supernatant was subjected to high-performance liquid chromatography (HPLC) analysis. The pellets were each dissolved in 400 μl of methanol to analyze flavonoids and their products that precipitated in the pellets. The resulting solutions were centrifuged at 12,000 × *g* for 5 min, and 100 μl of the supernatant was analyzed by HPLC.

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TABLE 1. Aligned sequences of the oligonucleotide probe C.orb0179 and the 16S rRNA sequences of *C. orbiscindens* and phylogenetically related organisms

Probe or organism	Sequence[a]
C.orb0179 3′	TACTACGTCAACCCAGCG 5′
Clostridium orbiscindens 5′	AUGAUGCAGUUGGGUCGC 3′
Eubacterium plautii 5′	....A.............. 3′
Acanthamoeba castellanii 5′	..C.G.....G.....U 3′
Clostridium viride 5′	........ACG..A.... 3′
Streptococcus sp. 5′	.A......A...CA.... 3′
Streptococcus anginosus 5′	.A......A...CA.N.. 3′

[a] Nucleotides different from those of the target sequence are shown. N, nucleotide not determined.

HPLC. The flavonoids and their aromatic degradation products were determined by HPLC in the reversed-phase mode according to the method of Braune et al. (5). Methanol and 2% aqueous acetic acid served as the mobile phase and were used to form gradients as follows: from 5 to 30% methanol in 20 min, from 30 to 50% methanol in 5 min, from 50 to 65% methanol in 5 min, 65% methanol maintained for 5 min, and from 65 to 100% methanol in 7 min. The flow rate was 0.8 ml min^{-1}. For analysis of the apigenin and luteolin degradation and the separation of naringenin and phloretin, the above-mentioned conditions and the following gradients were applied: from 5 to 50% methanol in 10 min and from 50 to 60% methanol in 20 min, followed by 2 min at 100% methanol.

The intermediates and products observed in the flavonoid degradation experiments were identified by their retention times and their UV spectra in comparison to those of reference substances by using HPLC with a UV diode array detector.

Identification of bacterial isolates. The six isolates I1 to I6 were identified by comparative 16S rRNA gene sequence analysis. The analyses were performed in the laboratory of M. D. Collins, University of Reading, as follows. The 16S rRNA genes of the isolates were amplified by PCR as described by Hutson et al. (15). The PCR products were purified using a Prep-A-Gene kit (Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions. They were directly sequenced using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and an automatic DNA sequencer (model 373 A; Applied Biosystems). The closest known relatives of the isolates were determined by searching the EMBL and GenBank databases with the FASTA program of the Genetics Computer Group package (9).

Design and validation of a species-specific oligonucleotide probe. An oligonucleotide probe (S-S-C.orb-0179-a-A-18 [hereinafter abbreviated to C.orb0179]) targeting a hypervariable region of the 16S rRNA from *C. orbiscindens* was designed by using the Arb software package (29), the Check-Probe function of the Ribosomal Database Project software package (18), and the EMBL database. Table 1 depicts an alignment of probe C.orb0179 and the 16S rRNA target sequences of *C. orbiscindens* and related organisms. The dissociation temperature of C.orb0179, determined according to the method of de los Reyes et al. (8), was 48°C.

C.orb0179 was checked for its specificity towards the target organism by whole-cell in situ hybridization with the target organism and 100 intestinal strains of human or animal origin (28). All bacterial species used for specificity testing were grown at 37°C under strictly anoxic conditions in a complex medium with N_2 and CO_2 (80:20, vol/vol) as the gas phase or on Columbia blood agar plates (BioMérieux) in anoxic jars. For whole-cell in situ hybridization, the bacteria were fixed as described by Roller et al. (20) and Amann et al. (1). The fixed bacteria were hybridized on silanized, Teflon-coated microscopic slides with probes whose 5′ ends were labeled with Cy3 according to the procedures of Roller et al. (20) and Schwiertz et al. (28). As a positive control, an equimolar mixture of five *Bacteria*-specific probes (Eub338, Eub785, Eub927, Eub1055, and Eub1088) (16) was used. The fluorescing cells were viewed with either an Optiphot-2 (Nikon, Düsseldorf, Germany) or an Axioplan-2 (Zeiss, Jena, Germany) microscope equipped with filters for epifluorescence microscopy.

Quantification of *C. orbiscindens* organisms in fecal samples. To determine the occurrence of *C. orbiscindens* in humans, C.orb0179 was applied to fecal samples. Fresh fecal samples were collected and fixed according to the method of Schwiertz et al. (28) from 10 healthy volunteers of both sexes aged 31 to 57 years who consumed a Western diet and had not received antibiotics for at least 6 months prior to the study. The cells detected with the C.orb0179 probe were enumerated and related to the bacteria detected with the *Bacteria*-specific-probe mixture. The cell counts of *C. orbiscindens* obtained by whole-cell in situ hybridization were compared to the plate counts calculated from the fluorescing colonies identified by the quenching test.

RESULTS AND DISCUSSION

Isolation of quercetin-degrading bacteria. For the isolation of quercetin-degrading bacteria, the fluorescence-quenching test was applied (26). Agar plates equipped with nylon membranes, quercetin (structure in Fig. 1), and DPH were incubated with dilutions of human feces. After 48 h of incubation at 37°C, the agar plates were observed under UV light. Plates inoculated with fecal dilutions of 10^{-8} and 10^{-9} showed several fluorescing spots with a diameter of approximately 1 cm (Fig. 2). Although no distinct colonies were observed within these fluorescing zones, bacteria could be isolated as follows. The centers (5 by 5 mm) of the fluorescing areas were cut out of the filter with a sterile scalpel under anoxic conditions and transferred to WCA broth. The steps comprising the spreading of cells on plates with nylon membranes, the excision of the centers of the fluorescing areas, and the transfer of the excised filter pieces to WCA broth were repeated until the isolates were pure cultures. Six isolates were obtained by this procedure.

Identification of the isolates. All six isolates (I1 to I6) formed white colonies on WCA agar. The rod-shaped bacteria (1 to 2 μm in length) stained gram positive and formed short chains. They were strictly anaerobic and formed subterminal spores. The identification was accomplished by comparative 16S rRNA gene sequence analysis in which four of the unknown isolates displayed 100% similarity and two of the isolates showed 99% similarity to the 16S rRNA gene sequence of *C. orbiscindens* (DSM 6740). The phenotypic properties of the isolates mentioned above were in good agreement with the description of this species by Winter et al. (31).

Degradation of quercetin by *C. orbiscindens*. The degradation experiments were carried out with *C. orbiscindens* strain I2. Growing cells of *C. orbiscindens* I2 converted 0.5 mM quercetin in 6 h completely to a single product (Qu1) (data not shown). The retention time of Qu1 in HPLC analysis was 12.3 min, and the UV spectrum revealed maxima at 237.8 and 286.0 nm. By comparison with the commercially available standard, the compound was identified as 3,4-dihydroxyphenylacetic acid. At a concentration of 1 mM, taxifolin (structure in Fig. 1), an intermediate in quercetin degradation by *E. ramulus* (5), was degraded to 3,4-dihydroxyphenylacetic acid and an additional compound (Ta1) (Fig. 3a). Ta1 had a retention time of 18.6 min and a UV spectrum with maxima at 235.6 and 295.0 nm. It was identified as alphitonin (structure in Fig. 1) by comparison with the pure substance. Since alphitonin is not commercially available, the purified intermediate of taxifolin degradation by *E. ramulus*, identified previously by nuclear magnetic resonance analysis as alphitonin (5), was used as the reference. Alphitonin was not detected during the transformation of 0.5 or 1 mM quercetin. The time course of the degradation of 1 mM taxifolin is shown in Fig. 4a. At a concentration of 0.5 mM, taxifolin was completely transformed within 5 h to 3,4-dihydroxyphenylacetic acid but alphitonin was not detected.

C. orbiscindens was isolated by Winter et al. (30, 31) and was reported to be capable of flavonoid ring cleavage. However,

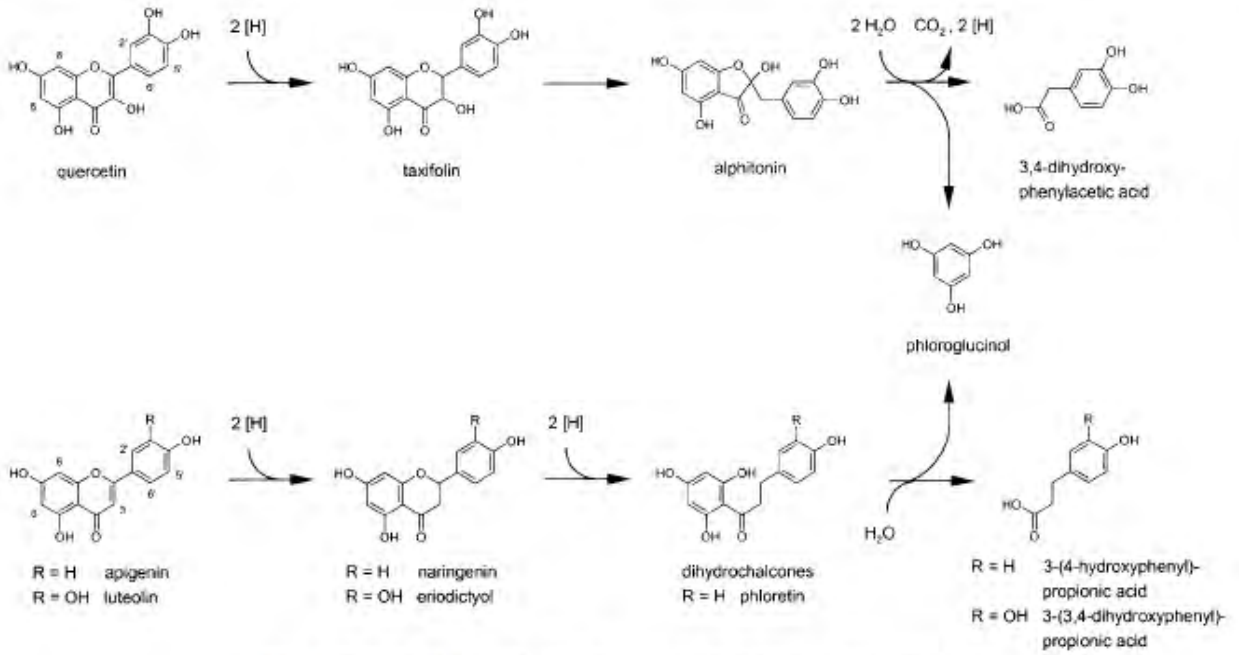


FIG. 1. Pathways of quercetin, apigenin, and luteolin degradation by *C. orbiscindens*.

Winter et al.'s investigations on flavonoid degradation were limited to identifying the end products of the degradation of quercetin, kaempferol, and naringenin as 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, and phenylacetic acid, respectively (30). Our investigations described here revealed that *C. orbiscindens* I2 apparently follows the same pathway for the degradation of quercetin as that described for *E. ramulus* (5). The initial reduction of the double bond in the 2,3 position of quercetin results in the formation of taxifolin (Fig. 1). The following ring contraction to the identified isomeric alphitonin probably occurs by a ring-opening recyclization mechanism via a chalcone or diketone structure. This reaction may either be catalyzed enzymatically or take place spontaneously. The ring contraction to alphitonin leads to a benzylic CH_2 group, which finally occurs in 3,4-dihydroxyphenylacetic acid. The hydrolytic opening of the five-membered ring of alphitonin leads to the formation of phoroglucinol and 3,4-dihydroxyphenylpyruvic acid (unpublished results). The ensuing steps, which yield 3,4-dihydroxyphenylacetic acid, are postulated to follow the well-known reactions of the bacterial phenylpyruvic acid breakdown in the course of phenylalanine degradation (11, 25).

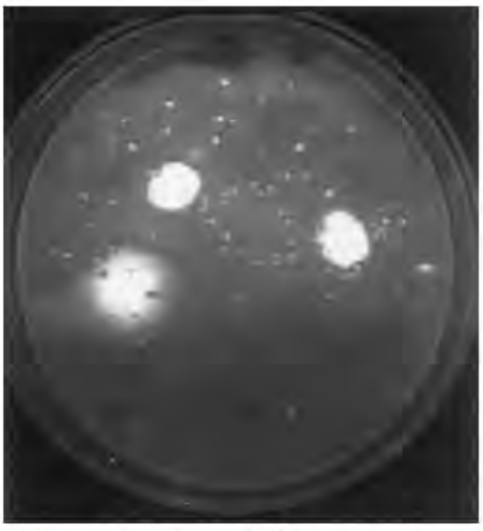


FIG. 2. Human fecal dilution (10^{-8}) spread out on an agar plate prepared to perform the fluorescence-quenching test. The fluorescing zones result from the bacterial degradation of quercetin, which quenches the fluorescence of a fluorescent additive. Some of the small colonies show weak autofluorescence.

Degradation of apigenin and luteolin by *C. orbiscindens*. Growing cells of *C. orbiscindens* I2 converted a 0.5 mM concentration of the flavone apigenin (structure in Fig. 1) to two intermediates, Ap1 and Ap2, with retention times of 21.3 and 20.8 min, respectively (Fig. 3b), and a final product with a retention time of 13.9 min (Ap3), detected by HPLC analysis after 5 h of incubation. The time course of apigenin degradation is depicted in Fig. 4b. Ap1 was identified as phloretin (structure in Fig. 1) by comparison of its retention time and UV spectrum (maxima at 234.6 and 294.7 nm) with those of the commercially available standard. Comparison of the retention time of Ap2 with that of the reference compound revealed that it is identical to naringenin (structure in Fig. 1). Using the same procedure, Ap3 was identified as 3-(4-hydroxyphenyl) propionic acid. Ap3 and the corresponding standard had identical UV spectra, with maxima at 234.5 and 281.6 nm.

Growing cells of *C. orbiscindens* converted 0.5 mM naringenin to 3-(4-hydroxyphenyl)propionic acid. Phloretin was de-

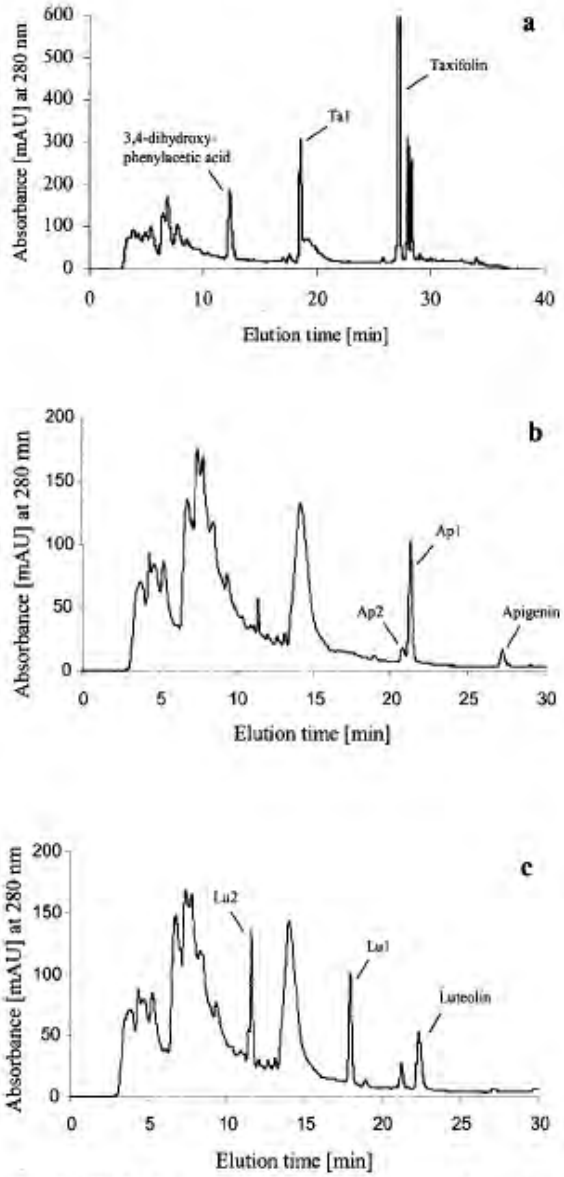


FIG. 3. HPLC elution profile of the supernatant of growing *C. orbiscindens* cultures incubated under anoxic conditions for 22 h with 1 mM taxifolin and Ta1 (alphitonin) (a); for 3 h with 0.5 mM apigenin, Ap1 (phloretin), and Ap2 (naringenin) (b); and for 6 h with 0.5 mM luteolin, Lu1 (eriodictyol), and Lu2 [3-(3,4-dihydroxyphenyl)propionic acid] (c). mAU, milli-absorbance units.

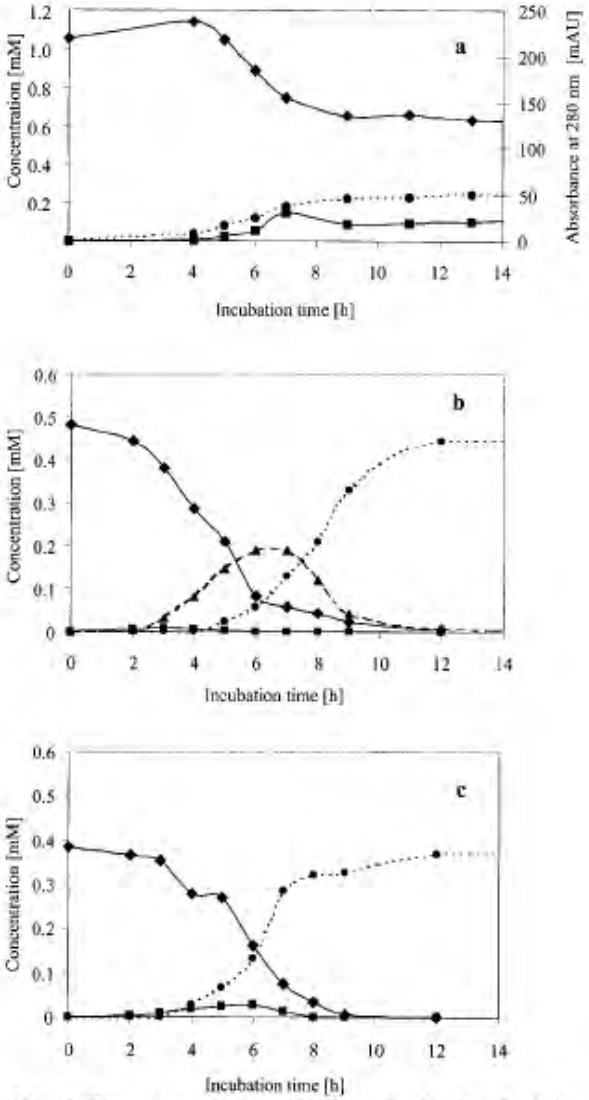


FIG. 4. Time course of flavonoid conversion by *C. orbiscindens* during growth in WCA broth. (a) Concentrations of taxifolin (♦), alphitonin (■), and 3,4-dihydroxyphenylacetic acid (●). Absorbances at 280 nm indicate concentrations of alphitonin. mAU, milli-absorbance units. (b) Concentrations of apigenin (♦), phloretin (▲), naringenin (■), and 3-(4-hydroxyphenyl)propionic acid (●). (c) Concentrations of luteolin (♦), eriodictyol (■), and 3-(3,4-dihydroxyphenyl)propionic acid (●).

graded to the same end product but only at substrate concentrations of <0.3 mM. Higher concentrations of phloretin inhibited the growth of *C. orbiscindens* I2.

The conversion of luteolin (0.5 mM) (structure in Fig. 1) yielded one intermediate with a retention time of 18.0 min (Lu1) and an end product with a retention time of 11.6 min (Lu2) (Fig. 3c). The time course of luteolin degradation is shown in Fig. 4c. Comparison of retention times and UV spectra of Lu1 with maxima at 234.9 and 293.9 nm and of Lu2 with maxima at 236.9 and 286.0 nm with those of the commercially available standards led to their identification as eriodictyol (structure in Fig. 1) and 3-(3,4-dihydroxyphenyl)propionic acid, respectively.

Growing *C. orbiscindens* cultures also converted 0.5 mM eriodictyol to 3-(3,4-dihydroxyphenyl)propionic acid. Phloroglucinol, a proposed intermediate in flavone and flavonol degradation by *E. ramulus* (24), was shown to be degraded by growing cells of *C. orbiscindens* within 7 h of incubation.

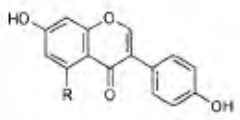
The resulting intermediates and products observed in flavone transformation by *C. orbiscindens* support the hypothetical degradation pathway of flavones described for *E. ramulus*

(5, 23). Presumably, the double bond in the 2,3 position of the aglycon is reduced in a first step to a flavanone as shown in Fig. 1. Subsequently, an isomerization to the corresponding chalcone structure takes place. The chalcone is reduced to a dihydrochalcone, and this compound is hydrolyzed to phloroglucinol and a phenylpropionic acid derivative. Phloroglucinol is further degraded to acetate and butyrate.

Although the degradation of flavones and flavonols seems to follow the same pathways in *C. orbiscindens* and *E. ramulus*, there are some important differences. While no intermediates could be detected during flavonoid transformation by growing cells of *E. ramulus* (23), several intermediates accumulated under similar conditions in experiments with *C. orbiscindens*. Another peculiarity of flavonoid degradation by *C. orbiscindens* could give some hints as to why bacteria degrade flavonoids at all. Apart from using flavonoids as electron acceptors or to gain additional energy from the degradation of phloroglucinol (6, 17), bacteria may have to detoxify these compounds. Phloretin, for example, inhibited the growth of *C. orbiscindens* at higher concentrations and may therefore be toxic to the organism. Apparently, the phloretin hydrolase, which catalyzes the cleavage of phloretin to 3-(4-hydroxyphenyl)propionic acid and phloroglucinol (unpublished results), is the bottleneck in the transformation of the flavone apigenin, as phloretin accumulated to comparatively high concentrations in the medium. This accumulation may be due to a very low phloretin hydrolase activity in *C. orbiscindens* and may also explain the growth inhibition by phloretin. *C. orbiscindens* may not be able to detoxify the compound in time, in contrast to *E. ramulus*, which was not inhibited at higher concentrations of phloretin (23). Antibacterial effects of flavonoids have been observed previously. Quercetin, myricetin, and morin inhibit the growth of *Bacillus cereus*, *Bacillus subtilis*, *Bacillus mycoides*, and *Staphylococcus aureus* (10). More recently, several bacterial strains were tested for their sensitivity to various flavonoids. The tested flavonoids showed antibacterial activity, but the intensity of the inhibitory effect was variable and dependent on the bacterial strain tested. Apigenin, for example, inhibited the growth of *Pseudomonas mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhi, *Escherichia coli*, *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Klebsiella pneumoniae* (2).

Degradation of flavonoid glycosides and isoflavones by *C. orbiscindens*. *C. orbiscindens* I2 did not convert flavonoid glycosides such as luteolin-3-glucoside, luteolin-5-glucoside, naringenin-7-neohesperidoside (naringin), quercetin-3-glucoside, quercetin-3-rutinoside (rutin), and phloretin-2′-glucoside. This fact is not surprising, as *C. orbiscindens* has already been described by Winter et al. as an asaccharolytic organism (30, 31). Therefore, *C. orbiscindens* is dependent on the deglycosylating activities of human tissues such as the small intestine and liver (19) and bacteria such as *E. ramulus*, *Enterococcus casseliflavus* (24), and *Bacteroides* sp. (4) for flavonoid degradation. In contrast to *E. ramulus*, *Enterococcus cassiflavus* and *Bacteroides* sp. deglycosylate the flavonoid only to take advantage of the sugar moieties. The aglycon is not used any further by these species and becomes available for organisms such as *C. orbiscindens*.

C. orbiscindens I2 also did not degrade the isoflavones daidzein and genistein (Fig. 5). In contrast, *E. ramulus* wK1 is able to convert the two isoflavones (27). It can therefore be deduced that the enzymes involved in isoflavone transformation are different from the ones involved in flavone or flavonol degradation.



R = H daidzein
R = OH genistein

FIG. 5. Structures of daidzein and genistein.

Prevalence of *C. orbiscindens*. The species-specific probe C.orb0179 was developed and applied to human fecal samples in order to estimate the prevalence of *C. orbiscindens* in the human intestinal tract. *C. orbiscindens* was detected in the feces of 8 out of the 10 subjects tested. The cell numbers ranged from 1.87×10^8 to 2.50×10^9 cells g of dry feces^{-1}, corresponding to a mean count of 4.40×10^8 cells g of dry feces^{-1}. Total numbers of bacterial cells detected by whole-cell in situ hybridization using the *Bacteria*-specific probe mixture were in the range of 3.80×10^{11} to 1.27×10^{12} cells g of dry feces^{-1}. The numbers determined for *C. orbiscindens* are equivalent to 0.12% of the total number of fecal bacteria.

For comparison, dilutions of a fecal sample were spread on agar plates with quercetin-containing membranes prepared for the quenching test. The fluorescent spots considered as resulting from quercetin degraders were enumerated. The mean count was 5.1×10^8 cells g of wet feces^{-1}. Taking a factor of 3 for the conversion of wet weight to dry weight, the calculated number of 1.5×10^9 cells g of dry feces^{-1} is within the range of cell counts determined by whole-cell in situ hybridization in fecal samples of the 10 subjects described above. Since all quercetin-degrading clones isolated from the plates were identified as *C. orbiscindens*, it might be concluded that all *C. orbiscindens* cells detected by whole-cell in situ hybridization are capable of quercetin degradation.

In parallel, the cell counts of *E. ramulus* were determined with whole-cell in situ hybridization of the same set of fecal samples (13). *E. ramulus* was detected in the feces of 6 out of 10 subjects at a mean concentration of 3.16×10^8 cells g of dry feces^{-1}. These numbers account for 0.04% of all fecal bacteria and are comparable to the cell counts of *C. orbiscindens* determined herein. Considering all these results together, *C. orbiscindens* may be as important as *E. ramulus* for flavonoid degradation in the human intestinal tract.

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Chapter 11

Bioactivation of selenocysteine derivatives by β-lyases present in common gastrointestinal bacterial species

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Bioactivation of Selenocysteine Derivatives by β-Lyases Present in Common Gastrointestinal Bacterial Species

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Abstract: Studies in cell cultures and animal models have demonstrated cancer chemopreventive effects of certain selenium compounds. Here we describe the screening of cysteine *S*-conjugate β-lyase activity in bacterial species that are implicated in the bio-activation of sulfur- and selenocysteine derivatives.

We screened a range of bacterial species commonly found in the human intestine for β-lyase activity on Se-*p*-methoxybenzylselenocysteine and the natural occurring S-methylcysteine and Se-methylselenocysteine conjugates. A high-performance liquid chromatography (HPLC)-assisted assay was established to determine specific activities of each strain. Of the 29 tested bacterial species, 22 showed specific activities towards the test compound reaching up to 10.1 U/mg protein, thereby accounting for 75% of total fecal activity (13.3 U/mg protein).

Lysates of four bacterial strains *(Bacteroides distasonis, Bacteroides vulgatus, Enterococcus faecalis, and Enterococcus faecium),* which exhibited high specific activities towards the test compound and which are known to be present at high numbers in the human intestine, were characterized further. Our results indicate that β-lyase activity is widely distributed in human intestinal bacteria and might play a key role in the bioactivation of selenocysteine derivatives.

Key words: Selenium, cancer prevention, gut flora, intestine, bioactivation, beta-lyase, Se-methylselenocysteine

Introduction

In the last two decades there has been a considerable interest in the discovery of naturally occurring compounds with a high potential in cancer prevention [1, 2]. Colon cancer in particular, with nearly a half-million cases per year worldwide [3], is a major target in dietary cancer prevention.

Intervention studies such as the Nutritional Prevention of Cancer (NPC) trial have provided strong evidence for the efficacy of selenium as an anticancer agent [4]. This placebo-controlled trial showed a 50% reduction in total

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cancer incidence in a large, selenium-adequate U.S. population treated with 200 µg selenium as selenium-enriched yeast. Further analysis of the NPC trial on colorectal carcinoma incidence revealed a significant decrease in risk associated with selenium supplementation of subjects in the lowest tertile of baseline selenium and current smokers [5]. These results gave rise to a huge phase III study, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), designed to test the efficacy of selenium (200 µg selenomethionine) and vitamin E (400 mg DL-α-tocopherol), both alone and in combination, in the prevention of prostate cancer [6]. Studies in cell cultures and animal models have demonstrated cancer chemopreventive effects of certain selenium compounds [7–9]. Several studies have indicated that selenium and sulfur compounds display anticancer activities, with the former being effective to a much higher degree [2, 10]. Among these compounds there is a substantial number of sulfur- and especially selenium-containing derivatives of methionine and cysteine, which have been found in *Allium* and *Brassica* species [11–13]. In rats, selenium-enriched broccoli proved very effective in the prevention of chemically induced colon cancer [14].

Recent advances in analytical techniques revealed a number of new selenium-containing natural products discovered in selenium-enriched plants and yeasts [15]. Thus, garlic *(Allium sativum)* and selenium-enriched yeast *(Saccharomyces cervisiae)* exhibit a large number of cysteine- and selenocysteine-conjugates, such as S-methylcysteine, Se-methylselenocysteine (both Figure 1), and γ-glutamyl-Se-methylselenocysteine [13, 16–18].

Although it is suitable to describe effects of Se in terms of the element, it must always be kept in mind that chemical form and dose are determinants of its biological activity as a cancer-preventive agent or toxicant. Certain selenium metabolites such as methylselenol (MeSeH) have potent anticancer activity [7, 19–21]. MeSeH can be formed by methylation of H_2Se, directly liberated from selenomethionine by cystathionine γ-lyase [22] or generated from Se-methylselenocysteine by β-lyases [23], which are present in the kidney, liver, or the gastrointestinal tract and various bacteria therein [24–27]. Other enzymes can also catalyze β-lyase reactions as has been shown for cystathionine γ-lyase [28] and β-lyases/glutamine transaminase K [29]. Thus, the bacterial β-lyases might represent key enzymes for the gastrointestinal conversion and bioactivation of dietary-derived S- and Se-conjugates. The first respective general overview on β-lyase activity present in gastrointestinal bacteria was published by Larsen [24]. Out of 43 tested intestinal bacteria, only 27 showed β-lyase activity and only members of the genus *Bacteroides* and the species *Clostridium ramosum, Peptostreptococcus productus,* and *Streptococcus faecalis*

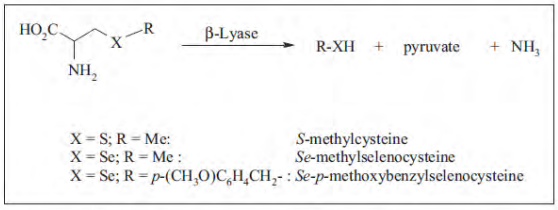

Figure 1: β-Elimination of S- and Se-cysteine conjugates catalyzed by β-lyases.

have been reported to be found frequently in high numbers in human fecal samples [30–32]. However, *Eubacterium limosum,* which has been reported to exhibit the highest β-lyase activities, is not a common member of the human gut microbial community [33]. Therefore, it is hard to delineate the importance of the gastrointestinal bacteria for MeSeH generation *in vivo*.

Using Se-*p*-methoxybenzylselenocysteine (Se-MBS) (Figure 1) as a substrate, we established a high-performance liquid chromatography (HPLC)-based screening assay for β-lyase activities of gastrointestinal bacteria known to be present in high numbers in human fecal samples, such as members of the genera *Bacteroides, Bifidobacterium, Clostridium, Enterococcus, Eubacterium,* and *Lactobacillus* [30–35]. Furthermore we investigated substrate specificity of four highly active bacterial strains with two physiologically relevant, naturally occurring S- and Se-cysteine conjugates. Our results point to the importance of bacterially-derived enzymes for the bio-conversion of dietary-derived precursors to chemopreventive agents such as MeSeH.

Materials and Methods

Chemicals and synthesis

Selenocysteine was purchased from Euburon Chemicals (Belgium). S-methylcysteine was purchased from Aldrich (Germany). Se-*p*-methoxybenzylselenocysteine (Se-MBS) and Se-methylselenocysteine were synthesized from selenocysteine according to procedures reported previously [16, 36]. ^1H NMR and ^{13}C NMR spectra of the synthesized compounds were recorded at 300 MHz on a Bruker AMX 300 with Me_4Si (δ 0) as the internal standard.

Organisms and culture conditions

All reference strains used in this study were obtained from the sources indicated (DSMZ, Deutsche Sammlung von

Mikroorganismen und Zellkulturen, Braunschweig, Germany; IFM, Institute for Microecology, Herborn, Germany; ATCC, American Type Culture Collection, Rockville, MD, USA). All IFM strains are human fecal isolates from our laboratory and were identified using the Vitek® System (bioMérieux, Nuertingen, Germany). All strains were cultured at 37°C in ST-medium (ST) under strictly anoxic conditions with N_2/CO_2 (80:20, v/v) as gas phase [37, 38], which contained per liter: 9 g tryptically digested peptone from meat, 1 g proteose peptone, 3 g meat extract, 4 g yeast extract, 6 g glucose, 3 g NaCl, 2 g Na_2HPO_4, 0.5 mL Tween 80, 0.25 g L-cystine, 0.25 g L-cysteine • HCl, 0.1 g $MgSO_4 • 7\ H_2O$, 5 mg $FeSO_4$ ($7\ H_2O$, 3.4 mg $MnSO_4 • 2\ H_2O$, pH 7.

Preparation of cell free extracts

The cell and fecal sample extracts were prepared in the presence of oxygen at 4°C from cultures grown overnight in ST medium or fresh feces. The samples were centrifuged (10000 × g, 15 minutes), washed twice with 100 mM potassium phosphate buffer (pH 7.0), re-suspended in the same buffer supplemented with DNase, and ruptured by twofold passage through a French pressure cell at 130 MPa (Aminco, Silver Springs, USA). Cell-free extracts were obtained by centrifugation at 14000 × g for 20 minutes at 4°C.

Enzyme activity

The specific β-lyase activity was determined by following pyruvate formation [39]. Se-*p*-methoxybenzylselenocysteine, S-methylcysteine, and Se-methyl-selenocysteine, respectively, were incubated at various concentrations (10 µM–0.9 mM) with bacterial lysates in 50 mM sodium borate buffer at 37°C and pH 8.6, which is the optimal pH for cytosolic β-elimination reactions [40]. The incubation volume was 100 µL. After 20 minutes, the reaction was stopped with 500 µL of 14% *o*-phenylenediamine in 3 N HCl. Following heating for 45 minutes at 60°C, the amount of derivatized pyruvate was analyzed by HPLC. Incubations without the cell-free extract and without substrate, respectively, were used to correct for spontaneous and non β-lyase-mediated degradation. Protein content was determined as described by Bradford [41].

Analysis of pyruvate formation from *Se*- and *S*-conjugates by HPLC

Fifty µL of derivatized incubation mixture were analyzed using an HPLC system which consisted of a L-6200A pump, a L-7480 fluorescent detector, an AS-2000A autosampler (all Merck-Hitachi, Darmstadt, Germany) with a 100-µL sample loop, a solvent degasser, and a 125 × 4 mm, 4 µm-particle-sized RP-18 column (Sepserv, Berlin). The mobile phase consisted of water and methanol (60:40, v:v) and the flow was set to 0.5 mL/minute. Pyruvate formation was calculated using an external calibration curve. Derivatized pyruvate eluted at 12.65 minutes and was detected with excitation at 336 nm and emission at 420 nm.

Results and Discussion

In the present study we analyzed β-lyase activities of several bacterial species present in the human intestinal tract. Se-MBS has been shown to be a good substrate for renal β-lyase, therefore it was chosen as test substrate [36]. Since β-lyase activity of *Eubacterium limosum* has been extensively studied [42], we obtained exemplary kinetic data with *Eubacterium limosum* as a criterion strain. Enzyme activity followed Michaelis-Menten kinetics. We obtained K_m (1.6 µM) and V_{max} (56.5 U/ mg protein) values; however we suppose that more than one enzyme can possibly exhibit β-lyase activity and thus Michaelis-Menten constants are not suitable to describe the β-lyase activity of the bacterial lysates used in this study. Since *Eubacterium limosum* is not present in high numbers in human fecal samples [33], it is unlikely that this organism plays a major role in the intestinal activation of selenocysteine compounds.

The same applies for most of the strains that were used by Larsen since they are not commonly found in high numbers in the gut [24]. Therefore, we extended the given list to numerically more important species. Our main focus was on members of the genera *Bacteroides, Bifidobacterium, Clostridium, Enterobacteriaceae, Eubacterium,* and *Lactobacillus* which have been reported to be present abundantly in human feces [30, 32, 33, 43, 44].

Considerable β-lyase activities for Se-MBS were found in 22 of 29 tested bacterial strains and in fresh feces, indicating a general distribution of β-lyases among gastrointestinal bacteria, as reported previously [24]. High levels of β-lyase activity (> 1 U/mg protein) were detected only in 10 of the 30 strains and in fresh feces (Table I). In general, the highest β-lyase activity was found among species of the genera *Bacteroides* and *Enterococcus* (Table I).

To decide whether these bacteria might be important for bio-conversion of dietary-derived chemopreventive substances, all four bacterial species showing the highest β-lyase activities *(Bacteroides distasonis, B. vulgatus, Enterococcus faecalis, E. faecium)* were further tested with S-methylcysteine and Se-methylselenocysteine. Both compounds are major natural ingredients of garlic *(Alli-*

Table 1: Specific β-lyase activities for Se-(4-methoxybenzyl)-L-selenocysteine in intestinal bacteria and their numerical occurrence in human fecal samples

Species	Strain	β-lyase activity U/mg protein (S.D.)	Range (mean)[a]	Reference to bacterial occurrence
Total bacteria		13.3 (± 3.6)	11.2–11.4 (11.3)	[33, 48]
Bacteroides distasonis	DSM 20701	9.2 (± 0.07)	9.3–12.5 (10.6)	[30, 48, 49]
Bacteroides sp.(B. fragilis)	IFM	1.7 (± 1)	7.5–11.8 (10.3)	[30]
Bacteroides sp. (B. vulgatus)	IFM	7.4 (± 4)	8.6–13.5 (10.5)	[32]
Bacteroides thetaiotaomicron	DSM 2079	2.8 (± 1)	6.3–12 (10.3)	[49]
Bifidobacterium adolescentis	ATCC 15703	0.65 (± 0.18)	5.7–13.4 (10.3)	[30]
Bifidobacterium catenulatum	ATCC 27539	0.43 (± 0.02)	[b]	[50]
Bifidobacterium infantis	ATCC 15697	0	6.1–12.4 (9.9)	[30]
Bifidobacterium longum	ATCC 15707	0.32 (± 0.03)	9.1–11.3 (10.4)	[30]
Clostridium acetobutylicum	ATCC 824	0	7.6	[30]
Clostridium barati	DSM 601	0.05 (± 0.05)	5.4–8.7 (7.1)	[30]
Clostridium butyricum	DSM 10702	0	10.4	[30]
Clostridium cellobioparum	DSM 1351	0.03 (± 0.03)	5.7–6.5 (6.1)	[30]
Clostridium clostridiforme	DSM 933	0.10 (± 0.05)	9.6–11.1 (10.3)	[30]
Clostridium difficile	DSM 1296	0.60 (± 0.08)	4.8	[30]
Clostridium innocuum	DSM 1286	0.09 (± 0.0)	n.d.[c]	[43]
Clostridium pasterianum	DSM 525	0.24 (± 0.03)	10.0	[30]
Clostridium perfringens	DSM 756	0.08 (± 0.07)	3.8–12.5 (6.9)	[30]
Clostridium sartagoforme	DSM 1292	1.01 (± 0.76)	8.3–10.9 (9.6)	[30]
Clostridium sordellii	DSM 2141	4.01 (± 1.67)	9.9	[30]
Clostridium sporosphaeroides	DSM 1294	0	5.5–10.9 (8.4)	[30]
Enterococcus sp. (E. faecalis)	IFM	10.1 (± 2.43)	5–6 (5.5)	[44]
Enterococcus sp. (E. faecium)	IFM	9.32 (± 2.27)	5–6 (5.5)	[44]
Eubacterium barkeri	ATCC 25849	0.10 (± 0.05)	n. d.	[33]
Eubacterium biforme	DSM 3989	0	7.59–9.1 (8.35)	[33]
Eubacterium limosum	DSM 20543	4.24 (± 0)	n.d.	[33]
Eubacterium moniliforme	DSM 3984	2.44 (± 0.06)	n.d.	[33]
Eubacterium multiforme	DSM 20694	0	n.d.	[33]
Eubacterium rectale	ATCC 33656	0	5.7–11.6 (9.4)	[33]
Lactobacillus fermentum	DSM 20052	0	3.6–11.5 (8.3)	[30]

[a] Range and mean count of bacteria expressed as number of organisms log10 per gram feces (dry weight in persons who consumed Western diet), [b] no direct counts given, [c] not detected. Data are expressed as mean ± SD from four individual experiments

Table II: Specific β-lyase activities for Se-methyl-L-cysteine and S-methyl-L-cysteine for most abundant bacterial species

Species	Specific β-lyase activity[a] (U/mg)	
	Se-methyl-L-cysteine (± SD)	S-methyl-L-cysteine (± SD)
Bacteroides distasonis	0.3 (0.05)	0.3 (0.04)
Bacteroides vulgatus	0.9 (0.09)	0.3 (0.05)
Enterococcus faecalis	1.3 (0.05)	1.1 (0.05)
Enterococcus faecium	1.2 (0.08)	1.9 (0.08)

[a] substrate concentrations used were 100 µM. Data are expressed as mean ± SD from four individual experiments

um sativum), selenium-enriched yeast and broccoli [45, 46]. Our results indicate that all four bacteria tested displayed a high β-lyase activity for these physiologically important compounds (Table II). Since we did not isolate a purified β-lyase, we did not determine K_m and V_{max} values.

Amino acids are mainly absorbed in the small intestine via sodium-dependent transporters. As both *Enterococcus* species are mainly found in the human small intestine [47], they may play a key role in the bio-activation of sulfur- and selenocysteine-derivatives from the diet *in vivo*.

In summary, we were able to show that numerically important bacteria from the human intestinal tract exhibit high β-lyase activity on S- and Se-containing compounds. The contribution of the gastrointestinal bacteria to the chemopreventive effects of selenium-enriched yeast ob-

served in the NPC trial might have been crucially important. It remains to be elucidated whether shifts in the amount and pattern of intestinal bacteria affects the bioconversion of dietary-derived substrates for β-lyase. Inter-individual variations in the composition of the intestinal flora may lead to a differentiated response to cancer protective food (i.e. broccoli and garlic). However, given the high capacity of the β-lyases expressed by the abundant intestinal bacteria, it appears safe to conclude that the enzymatic conversion of chemopreventive precursors of e.g. MeSeH is not limiting in a healthy human gastrointestinal tract.

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