Analyzing the Functionality of the Human Intestinal Microbiota by Stable Isotope Probing

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....to my father - my motivation, and my husband Walter -my love and great support

..на баща ми - моят стимул за успех, и съпругът ми Walter - моята любов и подкрепа

ABSTRACT

The human gastro-intestinal (GI) tract comprises a series of complex and dynamic organs ranging from the stomach to the distal colon, which harbor immense microbial assemblages, with considerable diversity and significant metabolic activity that are vital for human health. For understanding the functionality of microbial communities, it is necessary to elucidate the role of individual species within a community. The aim of the current study was to explore the functionality of the human GI tract microbiota, particularly in the conversions of dietary carbohydrates, in the human large intestine using *in vitro* as well as *in vivo* experiments.

The functional capacity of the human intestinal microbiota in the fermentation of relevant [^{13}-C]-labeled dietary carbohydrates was analyzed using 16S ribosomal RNA (rRNA)-based Stable Isotope Probing (SIP). Integrated application of RNA-based SIP with high-throughput diagnostic microarray-based phylogenetic profiling and metabolic flux analysis resulted in identification of the primary degraders of potato starch, inulin and lactose under human colon-like conditions. Furthermore, metabolic cross-feeding networks were proposed, involving secondary fermentation processes. *Ruminococcus bromii* was identified as the key player in the degradation of potato starch based on molecular analysis of samples taken during the fermentation under human colon like conditions, but also through fermentation studies in mono-culture or co-culture with *Eubacterium rectale*. The latter experiments revealed a cross-feeding relationship between *R. bromii* and *E. rectale* that involved the H_2 produced by *R. bromii* from the starch degradation to increase production of propionate and acetate by *E. rectale*. Furthermore, species related to *Dorea longicatena* and *Bifidobacterium adolescentis* were identified as key members in inulin degradation, and *Actinobacteria*, particularly *Bifidobacterium* spp. and *Collinsella* spp., were found to be the main taxa involved in lactose fermentation under human colon like conditions. Moving beyond *in vitro* studies we explored the role of the gut microbiota in lactose fermentation with a pioneering study, where [U-^{13}C]-lactose was delivered directly to the terminal ileum of healthy human volunteers using a multi-lumen catheter that was also used for sampling of luminal content, which was further examined by RNA-SIP and phylogenetic microarray analysis. Individual patterns of the microbiota involved in lactose fermentation were observed, showing that in both volunteers different microbial populations were involved, which could be attributed to differences in the sampling location. A population related to *Lactobacillus plantarum* was found to be most actively involved in fermentation in one of the

volunteers during the trial, while in the other volunteer most probably members of the genera *Bifidobacterium* and *Colinsella* were the primary lactose degraders, in agreement with the *in vitro* model study. Further analysis on the metabolites that accumulated during the lactose degradation will help to reconstruct the metabolic pathways involved in intestinal metabolism.

Based on our exploratory research using RNA-based stable isotope probing together with high throughput phylogenetic analysis and metabolite profiling we could expand our knowledge on key microbial functions related to a healthy gut status.

Key words: gut bacteria, dietary carbohydrates, digestion, RNA-SIP, TIM-2, HITChip, human trial

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Outline

OUTLINE OF THIS THESIS

The human gut microbiota play a central role in health and well-being. Hippocrates was the first who in about 400 BC postulated the importance of the gut in human physiology. “Death sits in the bowels: a bad digestion is the root of all evil”, which, translated in the current research, underlines the importance of gut microbiota functionality to human health. With the work presented in this thesis we address specific metabolic activities of microbial groups active in the human gut and further describe microbe interactions, central for defining microbial food webs.

Chapter 1 provides a state of the art overview of the recently developed molecular approaches, used in different ecosystems, and their contribution to describe the functionality of the human gastrointestinal tract, with special attention for the residing microbiota. Furthermore, available *in vitro* models of the human gastrointestinal tract, including the TNO model of the human large intestine (TIM-2), are described and compared.

A substantial part of the human diet is represented by the daily intake of dietary carbohydrates, such as resistant starch and other carbohydrates which are not digested in the small intestine. **Chapter 2** details the application of RNA-based Stable Isotope Probing (SIP) in linking metabolic activity to microbial identity. Here, RNA-based SIP in combination with T-RFLP fingerprinting analysis of samples obtained from the TIM-2 *in vitro* model of the human colon revealed *Ruminococcus bromii* as the primary degrader of [^{13}-C]-labeled potato starch. Additionally, molecular and metabolic analyses suggested metabolic cross-feeding in the studied system, where populations related to *Ruminococcus bromii* are the primary starch degraders, while those related to *Prevotella* spp., *Bifidobacterium adolescentis* and *Eubacterium rectale* might be further involved in the trophic chain.

In **Chapter 3** we further explore the potential of RNA-based SIP to identify bacterial populations responsible for the degradation of [^{13}-C]-labeled lactose and inulin. In this study, the T-RFLP fingerprinting was complemented by a detailed phylogenetic analysis using the Human Intestinal Tract Chip (HITChip), a comprehensive phylogenetic microarray for the human intestinal tract microbiota. The results indicated the involvement of members of *Clostridium* cluster XIVa and *Actinobacteria* in the fermentation of inulin and lactose, either as a direct degrader of the studied substrates or involved in the trophic chain.

Defined pure and mixed cultures are excellent model systems to study the metabolism of individual bacterial populations but also their interactions with

other species. In **Chapter 4** we examine the ability of two bacterial species that are abundant in the human colon, *Eubacterium rectale* and *Ruminococcus bromii*, to convert potato starch in mono- and co-cultures. A cross-feeding relationship between the two species was observed that was described in detail.

To explore the potential of RNA-based SIP for the application in *in vivo* studies, as described in **Chapter 5**, [^{13}-C]-labeled lactose was delivered to the human intestine through a catheter. Further analysis of intestinal fractions containing RNA with low and higher density revealed the microbiota involved in the lactose fermentations *in vivo*.

Finally, **Chapter 6**, provides a general discussion of experiments described in this thesis. Furthermore, taking into account the impact of the gut microbiota on human well-being, we further discuss potential future research directions that will contribute to filling the gaps in the knowledge on the functionality of the gut microbiota.

1 CHAPTER
GENERAL INTRODUCTION

Tools for the Tract: Understanding the Functionality of the Gastro-Intestinal Tract

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Abstract

The human gastro-intestinal tract comprises a series of complex and dynamic organs ranging from the stomach to the distal colon, which harbor immense microbial assemblages that are known to be vital for human health. Until recently, most of the details concerning our gut microbiota remained obscure. Over the past several years, however, a number of crucial technological and conceptual innovations have been introduced to bring more light into composition and functionality of human gut microbiota. Recently developed high-throughput approaches, including next generation sequencing technologies and phylogenetic microarrays targeting ribosomal RNA gene sequences, allow for comprehensive analysis of diversity and dynamics of gut microbiota composition. Nevertheless, most of the microbes especially in the human large intestine still remain uncultured, and *in situ* functions of distinct groups of the gut microbiota are therefore largely unknown, but pivotal to understand their role in human physiology. Apart from functional and metagenomics approaches, stable isotope probing is a promising tool to link the metabolic activity and diversity of microbial communities, including yet uncultured microbes, in a complex environment. Advancements in current stable isotope probing approaches and integrated application with high-throughput diagnostic microarray-based phylogenetic profiling and metabolic flux analysis may facilitate application in human microbial ecology and will enable the development of innovative strategies to treat or prevent intestinal diseases of yet unknown etiology.

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Introduction

The human body typically harbors ten times more microbial cells than human cells, which is mainly due to the extremely high density of microorganisms found to be present in the human gastro-intestinal (GI) tract (Savage, 1977; Berg, 1996; Backhed et al., 2005). The vast majority of this microbiota is located particularly at the distal region of the human GI tract, the colon (Suau et al., 1999; Eckburg et al., 2005). From medical perspective, the importance of this part of the human GI tract as central to host health was acknowledged even by the earliest observers like Hippocrates 400 BC, who stated that "death sits in the bowel" (Kolida et al., 2000). Most of the clinical knowledge is focusing on the pathogenesis of a given disease and respectively on the proper therapies for medical treatments, which does not give a clear definition of health (Tannock, 2006; Arebi et al., 2008; Neish, 2009). However, there is growing evidence of the imperative impact of the colonic microbiota on human gut physiology and health, strongly affected by a number of microbial activities. These activities include, but are not restricted to, fermentation of dietary compounds that escape digestion in the upper GI tract, processing of mucosal cells shed in the small intestine, and degradation of intestinally secreted mucus (Xu and Gordon, 2003; Noverr and Huffnagle, 2004; Fava et al., 2006; Srikanth and McCormick, 2008). For understanding the functionality of microbial communities, it is necessary to elucidate the role of individual species within a community. However, it is estimated that approximately 80% of species comprising the human gut microbiota are yet to be cultured (Egert et al., 2006; Rajilic-Stojanovic et al., 2007). Hence, insights into the function and metabolic potential of these uncultured microbes are lacking. This indicates that culture-independent approaches are crucial to comprehensively study the ecology of the GI tract microbiota. In addition, our knowledge on the gut microbiota is in general restricted to the luminal part at the end of the colon, as this is excreted as feces. The other parts of the GI tract can so far only be accessed using invasive procedures. However, minimally invasive experimental techniques that can be used *in vivo,* can now be applied in combination with stable isotope probing (SIP) (Radajewski et al., 2000; Whiteley et al., 2006; Kreuzer-Martin, 2007) to link *in situ* microbial activity and diversity of GI tract microbiota. SIP is a powerful tool that can be used in human studies to delineate bacterial food webs that may ultimately influence human welfare (Pouteau et al., 2003; Kelleher, 2004; Dolnikowski et al., 2005).

This review presents promising strategies to delve into the functionality of the GI tract microbiota, including fermentation processes in the human colon. Recent

scientific advances discussed here could assist in expanding the knowledge of microbial determinants for a healthy gut defined based on key functional properties of gut microbiota. This will also enable the development of direct nutritional strategies for intestinal disease prevention and health promotion.

Overview of the human gut microbiota – microbial diversity

The human gut is one of the most densely populated ecosystems, comprising members of the three domains of life on Earth – Bacteria, Archaea and Eucarya (Finegold et al., 1983). Bacteria dominate this complex ecosystem, where > 90% of the phylotypes are member of two bacteria divisions: the *Bacteroidetes* and the *Firmicutes* (Backhed et al., 2005; Zoetendal et al., 2006; Turroni et al., 2008). The Gram-positive *Firmicutes* include numerous different phylogenetic clusters of *Clostridia,* with clusters IV, IX and XIVa being the most abundant clusters. The predominant genera are *Clostridium*, *Eubacterium*, *Roseburia* and *Ruminococcus*. Furthermore, the *Actinobacteria*, including the genera *Bifidobacterium* and *Atopobium,* represent important members of the gut microbial community (Franks et al., 1998; Harmsen et al., 2002; van der Waaij et al., 2005; Turroni et al., 2008). In terms of functional diversity recent metagenomics-based studies indicated that the gut microbiome has a coding capacity that vastly exceeds that of the human genome and encodes biochemical pathways that humans have not evolved (Backhed et al., 2005; Gill et al., 2006; Ley et al., 2006; Kurokawa et al., 2007; Turnbaugh et al., 2007).

Recent studies of the gut microbial ecosystem identified more than 1000 species and possibly over 7000 strains, of which the largest part (~80%) remains uncultured (Backhed et al., 2005; Blaut and Clavel, 2007; Rajilic-Stojanovic et al., 2007; Zoetendal et al., 2008). However, new approaches for culturing previously uncultured colonic microbes are being developed (Duncan et al., 2007a; Ingham et al., 2007; Zoetendal et al., 2008). In addition to this, new powerful tools for amplification and sequencing of genomic DNA from minute quantities of sample and barcoded pyrosequencing can be expected to give new insight in the composition of the gut microbiota at high spatio-temporal resolution (Marcy et al., 2007; Andersson et al., 2008).

Almost sterile at birth (DiGiulio et al., 2008), the development of the infant gut succeeds to extremely dense colonization, reaching at the age of two years a climax mixture of microbes similar to the microbiota found in the adult intestine (Wall et al., 2009). The composition of infant gut microbiota is determined by several factors that

include the mode of delivery, maternal microbiota, diet and environmental hygiene (Fanaro et al., 2003; Favier et al., 2003; Hallstrom et al., 2004; Palmer et al., 2007). In contrast to the developing infant gut microbiota, each healthy adult's gut appears to have a unique and stable microbiota, as evidenced by molecular fingerprinting, on the time scale of months (Zoetendal et al., 1998; Turnbaugh et al., 2007; Frank and Pace, 2008). Recent studies have also indicated aberrations in the composition of the human microbiome in obese individuals (Ley et al., 2006), as well as in individuals with a variety of other diseases (Zoetendal et al., 2008; Turnbaugh et al., 2009). Furthermore, Ley et al. (2006) reported that the composition of the human gut microbiota is responsive to dietary modulation for weight reduction.

Metabolic roles of gut microbiota

An important role of the human gut microbiota is that of a metabolic "organ", which delicately affects our physiology with functions that we have not had to evolve on our own (Backhed et al., 2005; Gill et al., 2006; Turnbaugh et al., 2007). The ability to process otherwise indigestible components of our diet is one of these vital microbial activities that significantly influences the gut environment and the host, such as energy source and in the maintenance of gut health (Savage, 1986; Guarner and Malagelada, 2003; Xu and Gordon, 2003).

Microbial performance, growth and metabolism, in the human colon depends to a large extent on the supply of substrates that resist digestion in the upper GI tract, and endogenous substrates, such as mucin, secreted by the host (Blaut and Clavel, 2007). The main dietary products, which serve as food for the colonic microbiota, are complex carbohydrates (starches, non-starch polysaccharides) and proteins (Cummings and Englyst, 1987). The majority of microorganisms in the human colon ferment carbohydrates and then switch to protein fermentation when these are not available anymore (Ouwehand et al., 2005). Carbohydrate metabolism is of great importance in the large intestine, as in terms of absolute numbers, the vast majority of culturable microorganisms are saccharolytic (Macfarlane and Macfarlane, 1997). Numerous different types of carbohydrates reach the colon, where their rates of fermentation are affected by the transition time and vary according to substrate availability, chemical structure and composition (Englyst et al., 1992). Several studies suggest that dietary carbohydrates are protective agents against several GI-disorders such as colorectal cancer (Topping and Clifton, 2001; Guarner, 2005). However, these studies were performed with animal models (Cassidy et al., 1994; Pool-Zobel, 2005; Young et al., 2005; Le Leu et al., 2007; Le Leu and Young, 2007).

The underlying mechanism of protection could be associated with the end products of these anaerobic bacterial fermentations, but other than metabolic interactions can not be excluded (Roediger, 1988). In the human colon, the end products of fermentation are short-chain fatty acids (SCFAs) such as butyrate, acetate, propionate, as well as other terminal products such as lactate. SCFAs lead to lowering of the luminal pH, increase in bacterial biomass and fecal bulk, and modification of the microbial composition, especially by stimulating the growth of beneficial bacteria including bifidobacteria and lactobacilli (Le Leu et al., 2005).

Butyrate, one of the major SCFAs, is the subject of studies aiming to understand its role in nourishing the colonic epithelium and in prevention of colon cancer (Cummings and Bingham, 1987; Bauer-Marinovic et al., 2006; Sengupta et al., 2006; Hamer et al., 2008). Recently, it was observed in healthy individuals that colonic butyrate application resulted in reduced visceral pain perception (Vanhoutvin et al., 2009). In contrast, colonic protein fermentation is often associated to an increased colon cancer risk as this fermentation results in the production of branched chain fatty acids and potentially toxic metabolites, such as amines, ammonia, phenolic compounds and thiols (Cummings et al., 1979; Bingham et al., 1996). This is also indicated by the fact that colon cancer mostly occurs at the distal end of the colon (Bufill, 1990; Muir et al., 2004). Therefore, an intake of more slowly fermentable carbohydrates could result in prolongation of the potentially beneficial saccharolytic activity, which would lead to an increased production and delivery of SCFA, particularly butyrate, to the distal colon (Jacobasch et al., 1999; Topping and Clifton, 2001; Wong et al., 2005).

Obviously, the diet affects colonic nutrition mainly through its effects on the gut microbiota. Growing evidence defined the roots of many colonic diseases and particularly colonic cancer risk to be determined by interactions between the diet and the gut microbiota. However, further studies should focus on unraveling the *in situ* functionality of the gut microbiota and improved understanding of the impact of the microbiota on host health and well-being. These must cope with the individuality and complexity of a microbial community in a largely inaccessible environment.

In vitro models of the human colon

The human colon is a largely inaccessible part of the GI tract, and a difficult area to study the gut microbiota and microbial activities *in vivo*. To this end, *in vitro* modeling represents an elegant way to study the microbial processes, such as carbohydrate and protein fermentation (Macfarlane and Macfarlane, 2007). *In vitro*

studies are less expensive in comparison to *in vivo* studies with human studies, and usually pure cultures, defined mixed cultures and stool material are used as inoculum. Furthermore, *in vitro* models allow for fast and reproducible experiments under standardized conditions. The strength of *in vitro* models, however, has also been questioned with respect to several issues. The degree to which the inoculum represents the human colonic microbiota (Drasar, 1988) and the precise mimicking of the colonic conditions (Edwards and Rowland, 1992) are recurring points of discussion. Another limitation of *in vitro* modeling is that it does not represent the colon as an open system with respect to the absence of fecal content removal, inevitably resulting in changes in bacterial composition and subsequently metabolic activity (Christian et al., 2003). Similarly, *in vitro* models lack host cells, their activity and interaction with the colonic microbiota. Nevertheless, besides these constraints, *in vitro* model systems can serve as tools (Table 1) to study microbe-mediated processes going on in the human colon and estimate the consequences of these activities on gut health.

Table 1. *In vitro* systems used to study the human gut microbiota.

***In vitro* model**	**Targeted part of the human GI-tract**	**Reference**
SHIME–model (Simulator of the Human Intestinal Microbial Ecosystem)	small intestine and colon	(Molly et al., 1993)
Continuous three-stage system	proximal and distal colon	(Macfarlane et al., 1998)
TIM-model (TNO intestinal model)	stomach, small intestine and colon	(Minekus et al., 1995; Minekus et al., 1999)
Continuous two-stage system	proximal and distal colon	(Brück et al., 2003)
Three-stage culture system	infant proximal, transverse and distal colon	(Cinquin et al., 2006)
Human proximal colon system	proximal colon	(Jiménez-Vera et al., 2008)

Many studies on fermentation characteristics of dietary relevant carbohydrates have been performed with the use of *in vitro* models of the gut (van Nuenen et al., 2003; Probert et al., 2004; van de Wiele et al., 2007; Jiménez-Vera et al., 2008). Additionally, *in vitro* modeling systems are also used to study human intestinal microbes able to colonize mucus and to establish biofilm communities (Macfarlane et al., 2005). Recently the TNO *in vitro* model of the large intestine – termed TIM-2 (Minekus et al., 1999) -was used in combination with isotopically labeled substrates to identify colonic populations actively involved in the fermentation of glucose (Egert et al., 2007) and potato starch (Kovatcheva-Datchary et al., 2009). An important advantage of this *in vitro* system is the fact that metabolites and water can be constantly removed from the module. In this computer-controlled model, parameters such as transit time and pH are regulated and, for example, age-dependent colon simulations can be realized. Moreover, peristaltic mixing is simulated and microorganisms reach physiological densities (~10^9–10^{10} ml^{-1}).

We have recently used high-throughput phylogenetic microarray analysis to compare the microbial community that colonized the TIM-2 model with the fecal community of randomly selected adult volunteers. The data indicated that TIM-2 microbiota is not significantly different from the fecal microbial community of the human volunteers with respect to composition and diversity of the major microbial groups (Fig. 1). This further reinforces the assumption that TIM-2 system well represents the human large intestinal microbiota (Kovatcheva-Datchary et al., 2009).

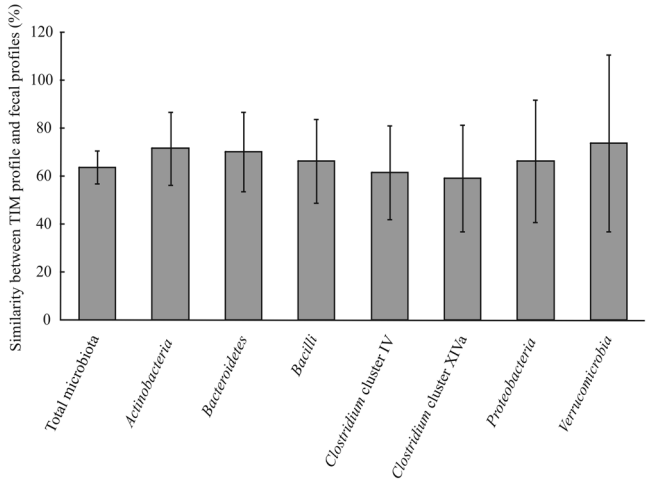

Figure 1. Similarity of the total microbiota and major phylogenetic groups between TIM-2 profile and human faecal profiles. Samples were analyzed using the Human Intestinal Tract Chip (HITChip) (Rajilic-Stojanovic et al., 2009)

Diagnostic tools to assess microbial diversity of the human gut

Our current knowledge about the microbial composition of the colonic ecosystem in health and disease is still limited. In order to be able to diagnose the presence and abundance of key players of the gut microbiota, a number of culture-independent approaches has been applied.

The gut microbiota composition is likely to be influenced by complex interactions between host, microbes and the environment. Diet is an important factor, which undoubtedly shape the gut microbiota, and has been explored in detail using the power of the molecular fingerprinting techniques and 16S ribosomal RNA gene sequencing (Hayashi et al., 2002; Bartosch et al., 2005; Abell et al., 2008). Recent work has reviewed some important aspects of the mammalian host-gut microbial relationship that could link gut microbiome to the human obesity (Ley, 2010; Musso et al., 2010; Vrieze et al., 2010). The influence of dietary factors was studied by Ley et al. (2006), where a high throughput sequencing approach was applied to characterize the fecal microbiota of 12 obese individuals who received either fat-restricted or carbohydrate-restricted low-caloric diets. It was shown that the stool samples of obese subjects were significantly enriched in *Firmicutes* and depleted in *Bacteroidetes,* in comparison to samples obtained from lean individuals. Recently, the differences in the human intestinal microbiota and fecal SCFA concentration of 98 lean and obese subjects were studied. The authors report higher proportion of *Bacteroidetes* in overweight and obese subjects versus lean individuals, and higher concentration of *Methanobrevibacter* in lean participants (Schwiertz et al., 2010). However, it has been concluded that not ratio of *Firmicutes* and *Bacteroidetes* is decisive to the development of obesity, but rather the metabolism of SCFA might play a considerable role (Schwiertz et al., 2010). In a separate study fluorescent *in situ* hybridization (FISH) analysis was applied to investigate the effect of reduced carbohydrate intake on fecal microbiota composition of twenty obese individuals (Duncan et al., 2007b). Progressive decrease as a fraction of total bacterial cells was observed in populations related to *Roseburia* spp., *E. rectale* and *Bifidobacterium* spp., after decreasing the carbohydrates intake. These data showed that dietary carbohydrate supply is an important factor for these microbial groups in order to maintain their populations in the human colon (Duncan et al., 2007b).

The two primary human inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are usually associated with unstable and disturbed composition of the gut microbiota in comparison with healthy individuals. In the last few years, a number of research groups have focused their activities on determining

the gut microbial composition in patients and to define how it is impacted by disease. The composition of the gut microbiota in concordant and discordant identical twins with CD, and healthy twins, was studied to identify members of the microbiota, which could be linked to CD incidence or development (Dicksved et al., 2008). Molecular fingerprinting analyses based on terminal-restriction length polymorphism (T-RFLP) of the 16S rRNA sequences revealed higher microbial diversity in the healthy twins compare to the CD twins. Moreover, the fecal microbiota of the healthy individuals was found to be less variable than those of CD twins. The microbial community profiles of individuals with ileal CD were significantly different from healthy individuals and those with colonic CD. Furthermore, a lower relative abundance of *B. uniformis* and higher abundance of *B. ovatus* was observed in all patients with ileal involvement in comparison to both healthy twins and twins with colonic disease (Dicksved et al., 2008). In another study, temperature gradient gel electrophoresis (TGGE) of PCR-amplified 16S rRNA gene fragments was applied to investigate the effect of enteral nutrition therapy on fecal microbiota in children with CD (Lionetti et al., 2005). This revealed differences in the microbial composition of healthy subjects and CD patients, but also within the latter, between patients in remission and relapse. Recently, 16S rRNA sequence data were collected from fecal and biopsy samples from CD and UC patients, and compared to those from healthy individuals (Frank et al., 2007). Significant differences between the gut microbiota of the two patient groups were detected, including a depletion in the level of *Firmicutes* and *Bacteroidetes* in comparison with the health control. Determining the differences in microbial composition in patients and healthy controls may thus provide novel therapeutic targets. For this purpose, high-throughput, cost-effective methods for microbiota characterization are needed. Recently the application of 454-pyrosequencing of hyper-variable regions of the 16S rRNA gene revealed taxonomic richness of the gut microbiota that exceed any previously reported estimates (Andersson et al., 2008; Dethlefsen et al., 2008). Pyrosequencing analyses were applied recently to study the role of the gut microbiota in the development of obesity. High bacterial diversity and particular enrichment of H_2-producing *Prevotellaceae* accompanied with high abundance of H_2-utilizing methanogenic *Archaea* was reported (Zhang et al., 2009).

Phylogenetic microarrays are high-throughput analytical tools, which can be used to measure diversity and abundance of the human gut microbiota. Recently, such a DNA microarray, the Human Intestinal Tract Chip (HITChip), was developed, combining the power of fingerprinting, phylogenetic and quantitative community analysis (Rajilic-Stojanovic et al., 2009). The HITChip targets over 1,000 phylotypes

of intestinal microbiota, and its application for the analysis of intestinal samples of patients and healthy individuals can provide novel insights into the relation between the gut microbiota and health or disease (Zoetendal et al., 2008). In a recent study the potential of HITChip approach was further explored, after comparative analysis of the phylogenetic array and pyrosequencing technologies. Additionally, it confirmed their high capacity for in-depth profiling of complex microbial communities (Claesson et al., 2009).

Diagnostic tools to assess microbial functionality of the human gut

To understand the complex changes in the gut microbiota composition that may predispose towards intestinal disorders or promote human health, techniques that can assay and link metabolic activity to the diversity of intestinal bacteria are needed. Recently explored metagenomics approaches allow the comprehensive study of phylogenetic, physical and functional properties of complex microbial communities, providing a full picture of microbiota dynamics (Handelsman, 2004). Because metagenomic analyses allow to study the phylogenetic diversity, as well as can provide an inventory of potential functions of gut microbiota, it can be used as a tool to link diversity to functionality (Booijink et al., 2007). Metagenomics screening approaches can be divided into functional and sequence-based driven analyses of collective microbial genomes in complex environments (Gabor et al., 2007). Sequence-based metagenomic investigations have started to reveal core metabolic functions of the gut microbiota. An early metagenomic study on two healthy adults showed that their fecal microbial metagenomes were enriched in genes involved in energy metabolism, which include also the production of short-chain fatty acids as pivotal energy supply for the intestine (Gill et al., 2006). Additionally, a recent study where metaproteomics analyses were applied to study the distal gut microbiota of a healthy twin pair, indicated more than 50% of the detected proteins to be involved in translation, energy production and carbohydrate metabolism (Verberkmoes et al., 2009). Comparison of metagenomics (Gill et al., 2006) and metaproteomics data (Verberkmoes et al., 2009) indicated matches in the fucose and butyrate colonic fermentation pathways. Recent large-scale comparative metagenomic analyses demonstrated a clear effect of diet and age on the gut microbiome (Kurokawa et al., 2007). Very recently a gene catalogue of all prevalent genes of the gut microbiome was generated by illumina-based metagenomic sequencing from the fecal material of 124 European individuals (Qin et al., 2010). This analysis provided a broad

overview of functions crucial for the bacteria in the human gut, but also pointed out the existence of a bacterial core for the different individuals (Qin et al., 2010).
However, an ongoing challenge for microbiologists is to be able to identify, which microbes in the human gut carry out a specific metabolic conversion, the products of which may promote intestinal disorders and/or gut health. Recently, isotope probing approaches have been developed, offering great potential to identify microbes that are involved in the metabolism of specific substrates. These molecular tools involve the usage of commercially prepared substrates highly enriched in a stable isotope (e.g. ^{13}C) or radioisotope (e.g ^{14}C), which is added to an environmental sample. Endogenous microbes that metabolize the labeled substrate will incorporate the isotope into components of the microbial cells that provide phylogenetic information (Radajewski et al., 2000; Manefield et al., 2002; Lueders et al., 2004b; Dumont et al., 2006a; Manefield et al., 2006). Such components are often referred to as biomarkers, and most commonly nucleic acids and fatty acids are used. FISH-microautoradiography (FISH-MAR) and isotope array technology both use radioactive tracers to study the incorporation of substrate. With FISH-MAR a direct monitoring of the incorporation of the substrate labeled with a radioactive isotope into single microbial cells is performed (Lee et al., 1999). In a different way the analyses with the isotope arrays are performed, requiring the isolation of the labeled biomarker. Ribosomal RNA is hybridized to oligonucleotide arrays to target the 16S rRNA of the bacteria of interest (Adamczyk et al., 2003). However, since both methods use radioisotopes, their application is limited, especially in animal and human subjects, and therefore stable isotopes can offer a safe and convenient alternative for *in vivo* analysis. SIP methodologies vary in the use of biomarker, but also the means by which biomarkers are analyzed for isotopic and phylogenetic content (Fig. 2). The first application of SIP was in the analysis of phospholipid fatty acids (PLFA) that can be extracted from environmental samples and analyzed by isotope-ratio mass spectrometry (IRMS) (Boschker et al., 1998). Microbial populations often have signature PLFA molecules, which allow identification of microbes that have incorporated the ^{13}C-substrate. However, the interpretation of the PLFA patterns of microbes for which there are no cultivated representatives remain still limited, which is the main restriction of the PLFA-SIP (Dumont and Murrell, 2005).

Nucleic acids (NA) have a higher phylogenetic resolution than PLFA-SIP and enable identification of active but, as yet uncultured, populations at the species level. NA-based SIP works on the principle of separation of isotopically labeled DNA or RNA from unlabeled NA. The isolated labeled DNA/RNA represents the microbial

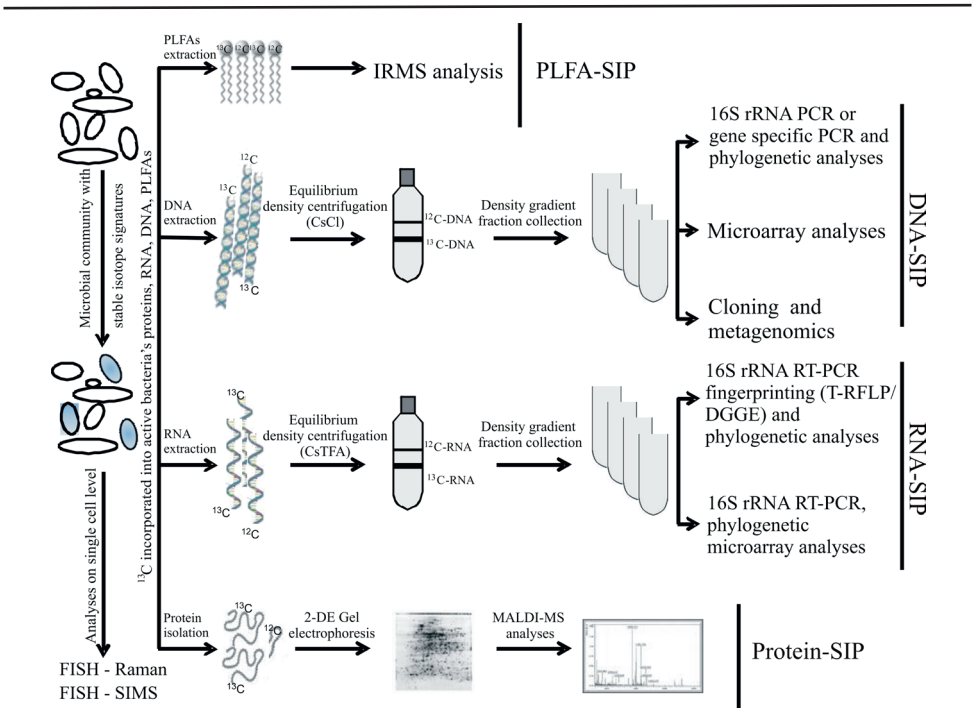

Figure 2. Summary of stable isotope probing (SIP) approaches, suitable diagnostic tools, to assess gut microbiota functionality and link it to phylogeny.

populations that incorporated the isotope into the biomarker through metabolic sequestration. NA-based SIP experiments have been applied in a large number of environmental studies focused on identification of bacteria that carry out specific degradative functions. Different culture independent techniques were used to monitor the ^{13}C-DNA/RNA. DNA-based SIP studies reported the application of polymerase chain reaction (PCR) analyses, targeting functional or taxonomic marker genes (Radajewski et al., 2000; Morris et al., 2002; Radajewski et al., 2002). As culture-independent microbial taxonomic marker the 16S rRNA and the encoding gene have been applied most frequently. Subsequent fingerprinting (e.g. denaturing gradient gel electrophoresis (DGGE) or T-RFLP), 16S rRNA clone library construction and/or microarray analyses, are further used to reveal the microbial populations involved in the degradation of the particular substrate (Dumont et al., 2006b; Cupples et al., 2007; Neufeld et al., 2007). DNA-SIP enables analysis of isotopically labeled functional genes (Dumont and Murrell, 2005), which provides a further functional view of

the active microbiota. Moreover, DNA-SIP in combination with metagenomics can provide broad insight into the genetic potential of microorganisms that were attributed to the *in situ* use of specific substrates (Egert et al., 2006). An important limitation of DNA-SIP is the requirement for DNA synthesis and cell division in order to obtain sufficient incorporation of the label into the DNA for gradient separation. Conversely, RNA occurs in greater cellular copy numbers, has a higher turnover rate than DNA and is produced independent of cellular replication. For this reason RNA will be labeled more rapidly than DNA, making it a highly responsive biomarker in SIP analyses (Manefield et al., 2002). Due to its greater buoyant density, isopycnic centrifugation of RNA is performed in cesium trifluoroacetate (CsTFA) rather than CsCl. Additionally, based on the reduced loading capacity of CsTFA, lower RNA loading amounts (250 - 500 ng ml^{-1}) (Lueders et al., 2004a; Whiteley et al., 2006; Egert et al., 2007) are essential for a successful separation in comparison with the DNA-SIP, where 5 µg ml^{-1} of DNA is an optimal concentration (Lueders et al., 2004a; Jensen et al., 2008). To analyze the fractionated RNA qualitative analyses as reverse transcriptase PCR-based fingerprinting methods (DGGE T-RFLP) and subsequent cloning and sequencing, or phylogenetic microarray analysis, are applied, which enable phylogenetic identification of the active microbial population (Lueders et al., 2004b; Egert et al., 2007; Kovatcheva-Datchary et al., 2009). Additionally, quantitative evaluation of the isopycnic RNA gradients can be performed using reverse transcriptase quantitative PCR (RT-qPCR), which leads to high precision and better resolution for recovery of the labeled nucleic acids (Lueders et al., 2004a). The success of NA-SIP depends mostly on the sufficient degree of labeling required for the separation of labeled and unlabeled nucleic acids by buoyant density centrifugation. To this end, extended incubation times are often required. However, increasing the time of nucleic acids enrichment has to be balanced in order to avoid changes that can occur in the bacterial community after addition of the substrate of interest. An example could be the effect of secondary degradation of the substrates (cross-feeding), which can affect bacterial diversity and metabolic activity (Belenguer et al., 2006). However, such cross-feeding effects can be instrumental in identifying food chains in the human intestinal systems. Recent studies with 16S rRNA-based SIP performed at *in vitro* conditions of the human intestine showed that a high concentration of labeled tracer is necessary to have good separation between the labeled and unlabeled fractions of the nucleic acids (Egert et al., 2007).

Protein-based stable isotope probing (Protein-SIP) is a novel approach, which analyzes specific metabolic activity of a single bacterial species within a community

which incorporate the labeled substrate using proteins as biomarker (Jehmlich et al., 2008; Jehmlich et al., 2010). The most important advantages of the protein analysis are its direct connection to physiological function, as proteins are known to catalyze the biochemical reaction. Thus, proteins are source of phylogenetic and functional information, making them ideal biomarkers for monitoring community structure and function.

Recently, new elegant tools have been developed that combine single-cell technologies with stable isotope analysis of microbial communities to monitor stable-isotope uptake at the single-cell level. These include technologies such as Raman microspectroscopy (Huang et al., 2004) and nano-secondary ion mass spectrometry (nano-SIMS) (Kuypers and Jørgensen, 2007). Raman spectroscopy analyses enable the detection of clear shifts in key regions (phenylalanine, proteins and nucleic acids) of bacterial Raman spectral profiles, which allow detection of the incorporation of the stable isotope into individual cell. Further, the Raman approach can be combined with FISH (Raman-FISH), which enables to establish the link between individual bacterial cells and their metabolic functions (Huang et al., 2007). Raman microspectroscopy is a nondestructive technique and allows the identification and the further physical separation of a single isotope-label cell for further subsequent cultivation or single cell genomic approaches (Wagner, 2009). The nano-SIMS technology analyses both stable- and radioactive-isotope content at single cell resolution (Kuypers and Jørgensen, 2007). Additionally, the nano-SIMS instruments are more sensitive as allow the quantification of several elements or their isotopes on a single cell level simultaneously, but also subcellular analysis can be also performed as their spatial resolution is much higher, which exceeds the capacity of a Raman microspectroscopy (Wagner, 2009). Combination of FISH-nanoSIMS allows the phylogenetic and isotopic analysis of a sample in a single scan. Nevertheless, this technology is far from becoming commonplace and affordable, mostly because of the high cost of the infrastructure required for nanoSIMS analysis. Furthermore, SIP techniques are suitable for obtaining qualitative and quantitative information about metabolic fluxes in the colon. Isotopically labeled compounds enable the selective study of that part of the microbial or host metabolism that involves the isotopic tracer. NMR and gas- or liquid-chromatography can be use to measure the labeled compounds and further identify active metabolic pathways (Bacher et al., 1998; Egert et al., 2006). RNA based-SIP was used to identify primary carbohydrate degrader at *in vitro* and *in vivo* condition and further provide the global idea off all different chapters where SIP approach was involved.

SIP *in vivo*

Further moving towards stable isotope probing *in vivo*, in which the labelled substrates of interest will be delivered to the human colon, will allow the exploration of the real power of this molecular approach. Moreover, SIP *in vivo* will lead to the development of a dynamic picture of the metabolic activity and diversity of the gut microbiota in health and disease. Optimizations on several limiting issues of the SIP application *in vivo* are required in order to be able effectively to explore the *in situ* function of the gut microbiota. The possibility to deliver labelled substrates effectively to the desired site of the human colon and the substrate homogenous distribution is an important once. Another key point is the origin of the sample, in most of the case biopsy, or luminal and /or faecal contents will be used to identify metabolically active phylogenetic groups through their degree of nucleic acid enrichment. Additionally the concentration of the labelled substrate is a further important factor, however recent *in vitro* studies (Egert et al., 2007; Kovatcheva-Datchary et al., 2009) could serve as a starting point and further if required the concentration can be adjusted in order to prevent dilution within the colon and ensure sufficient labeling of the microbial biomarkers.

The complete understanding of the metabolic activity of the gut microbiota will enable the development of direct strategies to treat or prevent intestinal diseases caused by microorganisms.

Future perspectives

Identification of prime functions of the human gut microbiota in maintaining human health inquire better understanding of its diversity and functionality, which can facilitate its manipulation. Most intestinal microbes have not been cultured and the *in situ* functions of distinct groups of the gut microbiota are largely unknown but pivotal to understand their role in health and disease. Technological advances in culture-independent microbiology have revolutionized gastroenterological microbiology. Recently introduced metagenomics approaches become extremely useful in addressing knowledge gaps on gut microbiota composition and started to reveal core metabolic functions of the gut microbiota. However, it is not known which members of the gut microbiota are involved in specific metabolic activities *in situ*. An important function of the gut microbiota is related to fermentation of non-digestible dietary residue, metabolites of which are considered to be essential for the intestinal health. New developments in stable isotope-based approaches can be used

in the identification of key players of the gut microbiota, which functions may have direct impact on human well being. Furthermore, extending the *in vitro* models to human feeding trials, in which relevant dietary oligo- and polymeric carbohydrates will be delivered to the human colon, will allow the exploration of the real power of these molecular approaches.

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CHAPTER 2

Linking Phylogenetic Identities of Bacteria to Starch Fermentation in an *In Vitro* Model of the Large Intestine by RNA-Based Stable Isotope Probing

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Abstract

Carbohydrates, including starches, are an important energy source for humans, and are known for their interactions with the microbiota in the digestive tract. Largely, those interactions are thought to promote human health. Using 16S ribosomal RNA (rRNA)-based stable isotope probing (SIP) we identified starch-fermenting bacteria under human colon like conditions. To the microbiota of the TIM-2 *in vitro* model of the human colon 7.4 g/l of [U-^{13}C]-starch was added. RNA extracted from lumen samples after 0 h (control), 2 h, 4 h and 8 h was subjected to density-gradient ultracentrifugation. Terminal-restriction fragment length polymorphism (T-RFLP) fingerprinting and phylogenetic analyses of the labeled and unlabeled 16S rRNA suggested populations related to *Ruminococcus bromii, Prevotella* spp. and *Eubacterium rectale* to be involved in starch metabolism. Additionally, 16S rRNA related to that of *Bifidobacterium adolescentis* was abundant in all analyzed fractions. While this might be due to the enrichment of high-GC RNA in high-density fractions, it could also indicate an active role in starch fermentation. Comparison of the T-RFLP fingerprints of experiments performed with labeled and unlabeled starch revealed *Ruminococcus bromii* as the primary degrader in starch fermentation in the studied model, as it was found to solely predominate in the labeled fractions. LC-MS analyses of the lumen and dialysate samples showed that, for both experiments, starch fermentation primarily yielded acetate, butyrate and propionate. Integration of molecular and metabolite data suggests metabolic cross-feeding in the system, where populations related to *Ruminococcus bromii* are the primary starch degrader, while those related to *Prevotella* spp., *Bifidobacterium adolescentis* and *Eubacterium rectale* might be further involved in the trophic chain.

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Introduction

The human colonic microbial community comprises a vast number of microbes representing a complex ecosystem, which has beneficial effects on the host (Finegold et al., 1983). Fermentation of dietary carbohydrates such as dietary fibers and resistant starch (RS) is an essential function of this community (Guarner and Malagelada, 2003). The end products from these microbial fermentations are short-chain fatty acids (SCFA) such as butyrate, propionate and acetate, which have an important function in host physiology (Scheppach, 1994). It has been suggested that specific SCFA reduce the risk of gastrointestinal disorders, cancer, and cardiovascular disease (Jenkins et al., 2000; Kendall et al., 2004). Particularly butyrate is subject of studies aiming to understand its role in nourishing the colonic epithelium and in prevention of colon cancer (Cummings and Bingham, 1987; Bauer-Marinovic et al., 2006; Sengupta et al., 2006). An intake of more slowly fermentable carbohydrates could result in prolongation of these beneficial fermentations and an increased production and greater delivery of SCFA, particularly butyrate, to the distal colon (Jacobasch et al., 1999; Topping and Clifton, 2001; Wong et al., 2005).

Over the last decade the introduction of molecular techniques in microbial ecology, especially the 16S rRNA-based approaches, has emphasized the extreme diversity of the human colonic microbiota and has indicated the limitations of culture-based approaches (Zoetendal et al., 2004). The most abundant bacterial groups shown by molecular screening to colonize the large intestine belong to *Bacteroidetes*, *Clostridium* clusters XIVa and IV, and *Actinobacteria* (Franks et al., 1998; Hold et al., 2002). However, one limitation of the rRNA-based techniques is that they do not provide a direct link to physiology and metabolic capacities. A recently introduced molecular approach, which allows linking the phylogenetic identity of a microorganism with its ecological function in a complex microbial community, is nucleic acid-based stable isotope probing (SIP) (Radajewski et al., 2000; Dumont and Murrell, 2005; Egert et al., 2006; de Graaf et al., 2007; Neufeld et al., 2007). This approach involves the incorporation of stable-isotope label into cellular biomarkers (including DNA and RNA), which are used to identify the microorganisms assimilating a labeled substrate and are involved in particular processes of interest. Especially the introduction of RNA-based SIP represents a significant advancement, as it does not require microbial growth, and has been used to link microbial function with phylogeny in a broad spectrum of different environments. Studies addressed phenol remediation in bioreactor systems (Manefield et al., 2002), identification of methylotrophs (Lueders et al., 2004b) or syntrophs (Lueders et al., 2004a) in

paddy soil, analysis of active methanogens in rice field soil (Lu and Conrad, 2005), and pentachlorphenol degraders in pristine grassland soil (Mahmood et al., 2005) or rhizosphere microbes (Rangel-Castro et al., 2005). Recently, RNA-based SIP was also used to investigate glucose-fermenting bacteria in an *in vitro* model of the human colon with microbial community representing the human small intestinal microbiota (Egert et al., 2007).

In the present study, 16S rRNA-based SIP was applied to identify the microbes involved in starch fermentation under conditions simulating the *in situ* situation in the human colon. The microbial community of an *in vitro* model of the large bowel was fed ^{13}C-labeled potato starch and the active populations were identified. We detected incorporation of the ^{13}C-label into the rRNA after 4 and 8 h of incubation. Terminal-restriction fragment length polymorphism (T-RFLP) fingerprinting and phylogenetic analyses of the labeled and unlabeled 16S rRNA revealed pronounced differences in the community structure of the analyzed microbiota. LC-MS analyses of the metabolites produced during starch fermentation showed strong increase of the measured SCFAs particularly after 4 h of incubation. Our study showed that RNA-based SIP combined with metabolic analyses is a useful tool for identifying metabolically active colonic bacteria and linking these to metabolites in the course of *in vitro* experiments. The current study also provides the basis for experimental approaches to determine microbial functionality in human subjects.

Experimental Procedures

In vitro model of the large intestine

The validated TNO *in vitro* model of the human colon (TIM-2), representing the conditions in the proximal colon and tested with a variety of fermentable substrates (Minekus et al., 1999; Rajilic-Stojanovic, 2007) was used in the current study. The inoculation of the *in vitro* colon model with a fresh standardized microbiota and the further preparation of the microbiota was performed as described (van Nuenen et al., 2003; Egert et al., 2007). Briefly, approximately 20 gram of faecal material from each of 7 individuals (healthy adults on a regular diet, who had not received antibiotic treatment for at least 2 months prior to faecal collection) was mixed in an anaerobic glove box and used to inoculate the TIM-2 system. After overnight stabilization and a 2 h starvation (Egert et al., 2007), 7.4 g/l of 98% [U-^{13}C]-labeled starch, isolated from potatoes grown in the presence of $^{13}CO_2$ (IsoLife, Wageningen, The Netherlands), was administered to the *in vitro* model, corresponding to a concentration of 40 mM

glucose-units. Luminal and dialysate samples (each approximately 2 ml) were taken at the time points 0 (control) h, 2 h, 4 h and 8 h after starch addition and stored at -80°C. A control experiment was performed with non-labeled potato starch (Sigma) as well.

RNA-extraction, purification and fractionation by density

Total RNA was extracted and purified from 250 µl luminal sample collected at the different time points as described previously (Egert et al., 2007). After purification and quantification approximately 1 µg of total RNA were loaded in cesium trifluoroacetate (CsTFA) centrifugation medium and resolved by density gradient centrifugation (Egert et al., 2007).
Gradients were subsequently fractionated as described by (Egert et al., 2007), albeit using a KDS200 syringe pump (KD Scientific Inc., Holliston, MA, USA). Afterwards, RNA from each fractions was precipitated for subsequent community analyses.

Amplification and fingerprinting of 16S rRNA

Amplification of the bacterial 16S rRNA for terminal restriction fragment length polymorphism (T-RFLP) analyses was done using a one-step reverse transcription-PCR (RT-PCR) system (Access Quick, Promega, Leiden, The Netherlands) as described previously (Egert *et al*., 2007). To detect all bacteria, 5'6-carboxyfluorescein (FAM) labeled forward primer 35f (CCT GGC TCA GGA TGA ACG) (Hayashi et al., 2004) and a modified reverse primer 907r (CCG TCA ATT YCT TTR AGT TT) (Muyzer et al., 1995) were used, where in the reverse primer a C was replaced by a Y. Both primers were used in order to increase *Bifidobacteria* detection at the analyzed samples. Subsequently, the fluorescently labeled RT-PCR amplicons were purified and quantified as described previously (Egert et al., 2007).
For T-RFLP analysis, aliquots (75 ng) of purified PCR products were digested for 3 h at 37ºC with 2.5 U of AluI or MspI (Promega) in a 10 µl reaction mixture following instructions of the supplier. To check for pseudo-T-RFs, which can lead to overestimation of the microbial diversity, mung bean nuclease treatment prior to the restriction digest was performed as described previously (Egert and Friedrich, 2003), except that the digestion was terminated with the PCR-purification kit. Size separation of terminal restriction fragments (T-RFs) was performed using an ABI 377 DNA sequencer (Applied Biosystems) as described earlier (Egert *et al.*, 2007).

Fragments between 50 and 1000 base pairs were analysed using Genescan 3.1 analytical software (Applied Biosystems), T-RFs ≤ 50 bp were excluded from the analysis to exclude artifacts caused by primer-dimers. The relative abundance of the T-RFs within a given T-RFLP profile was calculated as the peak area of the respective T-RF divided by the total peak area of all T-RFs detected within a fragment length range between 50 and 900 bp.

Cloning, sequencing and phylogenetic analysis of bacterial 16S rRNA fragments

Amplicons for sequence analyses were generated from rRNA of fractions 4 and 8 (4 h gradient) with the above mentioned primer set as described earlier (Egert *et al.*, 2007). Subsequently, the PCR-products were purified using the NucleoSpin Extract II kit (Macherey-Nagel). Fragment cloning and the selection of the clones for sequencing were performed according to (Egert *et al.*, 2007). Bi-directional sequencing of SP6-T7 PCR products was performed by BaseClear (Leiden, The Netherlands) using SP6 and T7 primers. Phylogenetic analyses were conducted using the ARB software package (Ludwig et al., 2004) as previously described (Egert et al., 2007). For tree construction with distance matrix-based neighbor joining, 773 sequence positions (*E. coli* positions 28-906) and a base-frequency filter for Bacteria, provided with the ARB package, were used.

To test for the significance of differences observed between both libraries, a Chi Square test of independence was performed (Mathbeans Project, http://math.hws.edu), using abundance of sequences in 4 phylogenetic groups (*Clostridium* clusters IV, XIVa, *Bacteroidetes*, *Actinobacteria*). In addition, the LibraryCompare software available at the RDP II website was used to assess the significance of differences observed for the individual groups (Cole *et al.*, 2007). A confidence threshold of 80% was applied. 16S rRNA gene sequences obtained in this study have been deposited at the GenBank database under accession numbers EU202927 – EU202948.

HITChip analysis

For phylogenetic analysis of TIM-2 microbiota from [U-^{13}C]-starch and [U-^{13}C]-inulin studies, the HITChip was used. This phylogenetic microarray consists of more than 4,800 oligonucleotides based on 16S rRNA gene sequences targeting over 1,100 intestinal microbial phylotypes. The procedure for hybridization and analysis was performed as described before (Rajilic-Stojanovic, 2007) with the only difference that a one-step reverse transcription-PCR (RT-PCR) system (Access Quick, Promega,

Leiden, The Netherlands) was used to amplify 16S rRNA fragments from RNA from selected gradient fractions, extracted from TIM-2 luminal samples collected at 0 h and 4 h after substrate addition, with the primers *T7prom*-Bact-27-for (5'-TGA ATT GTA ATA CGA CTC ACT ATA GGG GTT TGA TCC TGG CTC AG-3') and Uni-1492-rev (5'-CGG CTA CCT TGT TAC GAC-3'). After purification of the PCR products (NucleoSpin Extract II kit, Macherey-Nagel, Düren, Germany) the DNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop® Technologies, Wilmington, USA).

The 16S rRNA genes were transcribed *in vitro* from the T7 promoter using the Riboprobe System (Promega, La Jolla, USA) with a mix containing aminoallyl-rUTP (Ambion Inc., Austin, Texas, USA) at room temperature. The template DNA was digested with RNAse-free DNAse (Qiagen, Hilden, Germany), and RNA was purified (RNeasy Mini-Elute Kit, Qiagen, Hilden, Germany). Subsequently, the amino-allyl-modified nucleotides were labeled with CyDye (Post-Labeling Reactive Dye, Amersham Biosciences, Little Chalfont, UK). After stopping the reaction, labeled RNA was purified and quantified as described before.

Microarrays synthesized by Agilent Technologies (Agilent Technologies, Palo Alto, CA) in 8×15K format were used for hybridization with two samples, labeled with Cy3 and Cy5, respectively. The Cy3/Cy5 labeled target mixes were fragmented with 10× fragmentation reagent (Ambion Inc., Austin, Texas, USA). Hybridization on the arrays was performed at 62.5°C for 16 h in a rotation oven (Agilent). Slides were washed at room temperature in 2× SSC with 0.3% SDS for 10 min, followed by 0.1× SSC with 0.3% SDS at 38°C for 10 min and 0.06× SSPE for 5 min (Sambrook et al., 1989).

Data were extracted from microarray images using the Agilent Feature Extraction software, version 9.1 (www.agilent.com). Data normalisation and the further microarray analysis were performed using a set of R based scripts (http://r-project.org) in combination with a custom designed relational database which runs under the MySQL database management system (http://www.mysql.com). Similarity of the total microbiota composition based on the HITChip profiles was assessed by calculating Pearson's product moment correlation (Pearson's correlation) between hybridisation signals obtained for two samples for unique HITChip probes, and for different phylogenetic groups between signals obtained for two samples for phylogenetic group - specific HITChip probes. Pearson's correlation reflects the degree of linear relationship between analysed data sets. Ward's minimum variance method was used for the generation of hierarchical clustering of probe profiles by

calculating a distance matrix between the samples based on the squared difference between each pair of profiles (squared Euclidian distance – E^2) (Carter, 1989).

Quantitative Real-Time PCR

Real-time PCR was performed on an iCycler IQ real-time detection system associated with the iCycler optical system interface software version 2.3 (Bio-Rad, Veenendaal, the Netherlands). A reaction mixture (25 µL) consisted of 12.5 µL of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer set and 5 µL of the template cDNA diluted in water (10-fold) to avoid PCR inhibition. The cDNA was synthesized from the RNA collected in different fraction with different density. Reaction mixtures consisting of SuperScript™ III Reverse Transcriptase (Invitrogen), 8 µl RNA solution, 1 µl 50 µM random hexamer primer and 1 µl 10 mM dNTPs were incubated at 65°C for 5 min and then placed on ice for at least 1 min. The cDNA synthesis mix containing 4 µl 5 × First-Strand Buffer, 1 µl rRNasin® (40 U/µl), 1 µl SuperScript™ III RT (200 U/µl) and 1 µl 0.1 M DTT was then added to each RNA/primer sample which were then incubated first at 25°C for 5 min and afterwards at 55°C for 60 min. Reactions were terminated by exposure to 70°C for 15 min. Subsequently the cDNA was purified (High Pure PCR Cleanup Micro Kit, Roche Diagnostics GmbH, Mannheim, Germany) and used for qPCR analysis. A standard curve for quantification of total bacteria and *Ruminococcus bromii* was generated by using serially diluted 16S rRNA gene amplicons obtained from *Ruminococcus bromii* 51896 ATCC. For the quantification of methanogenic *Archaea*, *Methanobrevibacter smithii* 861^T was used as reference strain. In this assay, *mcrA* forward and reverse primers (Luton et al., 2002) were used to amplify the *mcrA* gene, known to be alternative to 16S rRNA in phylogenetic analysis of methanogen populations. All qPCR assays were performed in triplicate. Primers Eub341F and Eub534R (Smits et al., 2004) were used to estimate the total number of copies of the bacterial 16S rRNA in each sample. PCR was performed with an initial denaturation step of 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s, 60ºC for 30 s and 72ºC for 30 s. Primers RBR-5 and RBR-6 (Wang et al., 1997) were used to investigate the number of *Ruminococcus bromii* 16S rRNA copies in the sample with the following PCR program, initial step at 50 ºC for 2 min and an initial denaturation step of 95ºC for 3 min, followed by 35 cycles of 95ºC for 15 s, 62ºC for 45 s and 74ºC for 40 s. To estimate the number of methanogenic *Archaea* the primers *qmcrA*-F and *qmcrA*-R and the PCR program reported by (Denman et al., 2007) was used.

Metabolite analysis using LC-MS

To follow the metabolite production during fermentation of [U-^{13}C]-labeled starch in the same incubations as used for the RNA-SIP study a method minimizing the sample preparation and utilizing liquid chromatography-mass spectrometry (LC-MS) was applied (Meesters et al., 2007). This approach allowed to determine the concentrations of the individual isotopomers of each SCFA. Total concentration of SCFA were determined by GC analysis (van Nuenen et al., 2003), and were used to determine the cumulative production of the labeled metabolites in the TIM-2 system.

Results

RNA recovery and RT-PCR amplification of the 16S rRNA

To identify colonic bacteria that ferment starch, a standardized human fecal microbiota was supplemented with 7.4 g/l of uniformly ^{13}C-labeled starch in the TIM-2 *in vitro* model of the human large intestine, and fermentation was followed for 8 h. A control study, where 7.4 g/l of non-labeled starch was tested, was performed as well. RNA was extracted from luminal samples taken at different time points, and yielded 48 to 55 µg RNA per 250 µl of sample.

The fractionated centrifugation gradients from the [U-^{13}C]-starch experiment covered an average density range of 1.724 g ml^{-1} (fraction 14) to 1.829 g ml^{-1} (fraction 1) (Fig. 1 A). In the gradient fractions of the 0 h (control) sample all nucleic acids were detected at densities between 1.747 g ml^{-1} (fraction 11) and 1.793 g ml^{-1} (fraction 5). Four hours after the addition of the [U-^{13}C]-labeled starch, RNA was detected even in fraction 4 (1.802 g ml^{-1}), indicating the incorporation of the label in the RNA of starch-utilizing populations. Notably, the highest amount of RNA was detected in fraction 7 (1.777 g ml^{-1}, Fig. 1 B), whereas the maximal RNA concentration in the non-labeled starch experiment, for all the gradients from 0 h, 4 h and 8 h, was detected in fraction 8 (1.759 g ml^{-1}). Additionally, identical distribution of the RNA in all gradients from the different time points for the non-labeled starch fermentation was detected (data not shown). Furthermore, 8 h after [U-^{13}C]-labeled starch addition the maximal RNA concentration was detected in fraction 8 (Fig. 1 B). This most probably indicates that in this particular system all of the ^{13}C-label has been incorporated after 8 h and is already replaced by unlabeled carbon as a result of the RNA turn over. This is in line with the observed reduction in the amount of the detected RNA 8 h after starch addition in fractions 5 (1.793 g ml^{-1}) and 4 (1.802 g ml^{-1}) (Fig. 1 B).

Comparable results were obtained by RT-PCR amplification of bacterial 16S rRNA fragments present in the different fractions. The amplification of the 16S rRNA in the fractions of the control gradient (0 h) as well as the 2 h gradient yielded PCR-products for the fractions 5 (1.793 g ml^{-1}) to fraction 12 (1.739 g ml^{-1}) (Fig. 1 C). However, 4 h after starch addition also fraction 4 displayed a detectable PCR product.

These data corroborate the incorporation of the ^{13}C-label into the bacterial 16S rRNA. The amplification of the 16S rRNA from samples obtained during the course of the non-labeled starch fermentation yielded products for the fractions with a density between 1.742 g ml^{-1} to 1.791 g ml^{-1}.

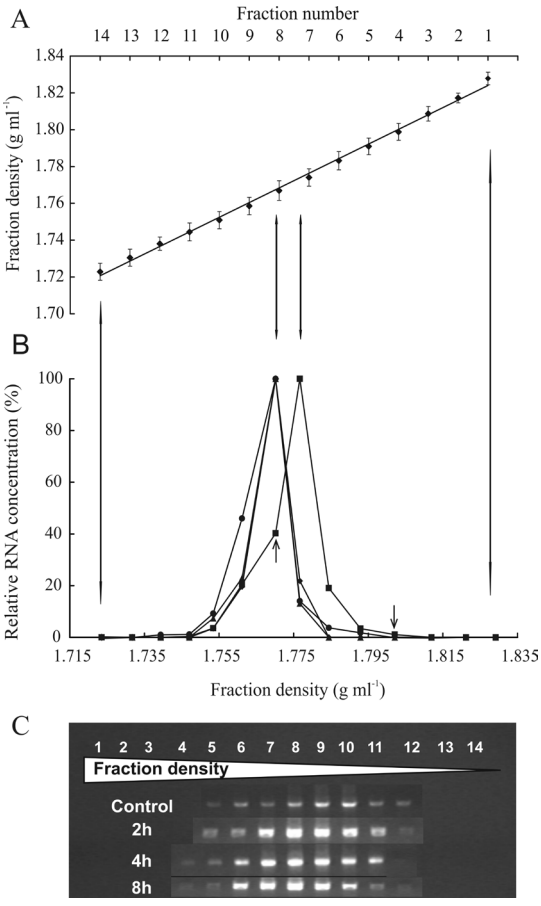

Figure 1. A. Densities of gradient fractions averaged over 4 gradients. For each fraction, the SEM was ≤ 0.001 g ml^{-1}. **B.** RNA concentration in gradient fractions. RNA was isolated from lumen samples of the colon model after incubation for 0 (control) h, 2 h, 4 h and 8 h with 7.4 g/l of [U-^{13}C]-starch. Values are given in relative units (%) to facilitate comparison between the gradients; RNA concentration in the fractions containing most RNA (100%) were 2.7 ng μl^{-1} (control), 2.5 ng μl^{-1} (2 h), 3.5 ng μl^{-1} (4 h) and 2.2 ng μl^{-1} (8 h). ♦, control; ▲, 2 h; ■, 4 h; ●, 8 h. The fractions used for the creation of "light" and "heavy" clone libraries are indicated by a small arrow, respectively. Large vertical arrows indicate corresponding fractions in figures A and B. **C.** RT-PCR-analysis results representing an ~900 bp fragment of bacterial 16S rRNA from the gradient fractions. The picture is assembled from 4 different stained agarose gels after electrophoresis of the amplicons. Fractions were obtained from ultracentrifugation gradients performed to density-resolve RNA isolated from lumen samples of the colon model after incubation for 0 (control) h, 2 h, 4 h and 8 h with 7.4 g/l of [U-^{13}C]-starch. Fraction numbers are indicated on top and correspond to those in A and B.

Fingerprinting of the bacterial 16S rRNA in gradient fractions

To compare the microbial community in the gradient fractions at different time points, T-RFLP fingerprint analysis was applied. The profile obtained after AluI digestion of the PCR products derived from fraction 8 from the 0 h (control; unlabeled) gradient, where the bulk of unlabeled RNA accumulated, displayed 12 major T-RFs sized 52, 62, 69, 131, 185, 237, 243, 246, 432, 624, 723, and 823 bp, respectively (Fig. 2 A). Identical T-RFs were detected in fraction 8 from the 4 h (Fig. 2 B) and the 8 h (Fig. 2 C) gradients. Minor variations in the relative height of peaks were observed, as a fragment of 62-bp was most abundant after 8 h, whereas the 185-bp peak was highest after 0 h and 4 h of incubation (compare Fig. 2 A - C). No changes in the number of the major T-RF peaks were detected for fractions 6 - 7 in any of the analyzed gradients. A strong decrease was, however, observed in the number of the

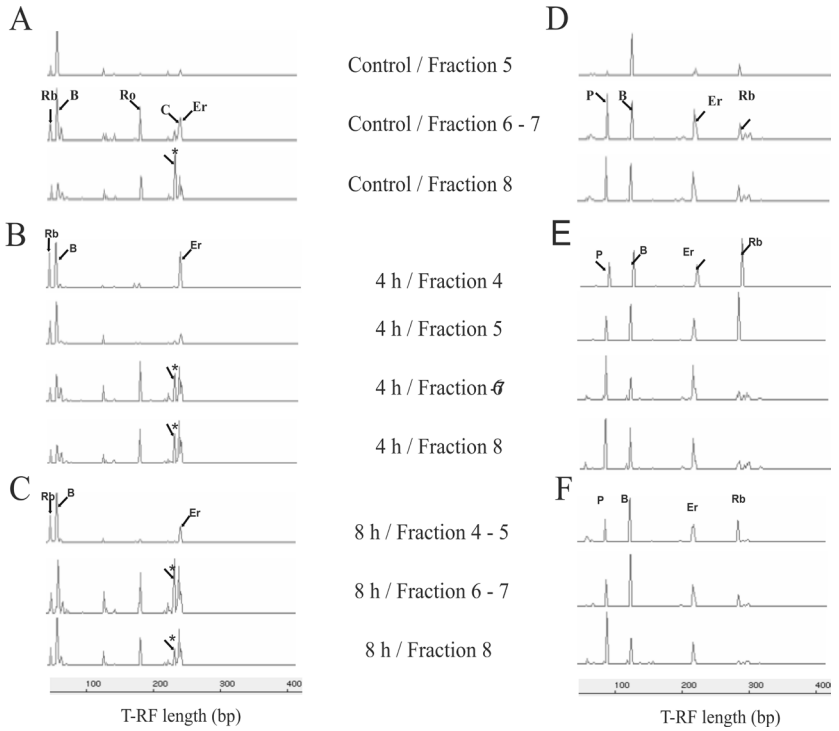

Figure 2. T-RFLP profiles of bacterial 16S rRNA obtained from fractions of the control (A), 4 h gradient (B) and 8 h gradient (C), after applying AluI as restriction endonuclease; control (D), 4 h gradient (E) and 8 h gradient (F), after applying MspI as restriction endonuclease. Assignment of T-RF peaks to bacterial phylotypes (Rb, *Ruminococcus bromii*; B, *Bifidobacterium adolescentis*; Ro, *Ruminococcus obeum*; C, uncultured *Clostridium* spp.; Er, *Eubacterium rectale*; P, *Prevotella* spp.). T-RFs marked with an asterisk were assigned as pseudo T-RFs as indicated in the text.

T-RF peaks for fractions of higher density (i.e. fractions 4 to 5; Fig. 2 A - C). A clear dominance of the 62-bp T-RF was detected in these fractions, surprisingly even in fraction 5 after 0 h incubation. Significant changes in the T-RFLP profiles of these high density fractions were found 4 h after ^{13}C-labeled starch addition, where an increase in the relative abundance of the T-RFs with a size of 52 bp and 246 bp was detected (Fig. 2 B, fraction 4).

The profile resulting after digestion of the PCR products with MspI did not differ in the number of peaks in fractions 6 to 9 from any of the analyzed gradients. However, a strong decrease in peak number was observed for fraction 5 of the control gradient, where the community was represented mainly by a T-RF with a size of 123 bp (Fig. 2 D). The same peak is present in all profiles, and probably is related to the 62-bp T-RF detected in all the T-RFLP profiles after using AluI endonuclease (see below). Similarity in the profiles resulting after digestion of the PCR products with MspI and AluI was also observed between the fractions from the 8 h gradient (Fig. 2 C and 2 F). Four hours after addition of the ^{13}C-labeled starch, a 7-fold increase in relative abundance of the T-RF with a size of 276 bp was detected only in the fractions containing labeled RNA (Fig. 2 E), in line with results found for AluI fingerprints. By contrast, the fingerprints of the community from the non-labeled starch experiment showed no increase in the T-RF sized 276 bp, which remained low in all analyzed fractions (Fig 3 A).

Individual fractions retrieved from the gradients differed significantly in RNA concentration, and thus different amounts of RNA were available as template for initial RT-PCR reactions. It was therefore assessed whether such differences in concentration, i.e. the dilution of the initial template, resulted in decline in both the number and the height of the peaks present in a T-RFLP profile as reported by Egert *et al.*, 2007. In the present study, template concentration did not significantly affect T-RFLP profiles, and the 237-bp T-RF observed after digestion with AluI was identified as the only major pseudo-T-RF using mung bean nuclease treatment (data not shown).

The relative abundance of the T-RFs within the T-RFLP profiles of the fractions 4 h after addition of labeled and non-labeled starch was calculated. Only the T-RFs that had a relative abundance of $\geq 2\%$ in all the analysed fractions were included for further analyses. The relative abundances of T-RFs sized 52 bp after digestion with AluI and the corresponding 276 bp after digestion with MspI, were the only ones that differed significantly for the labeled starch and non-labeled starch experiments (Fig. 3 A and B).

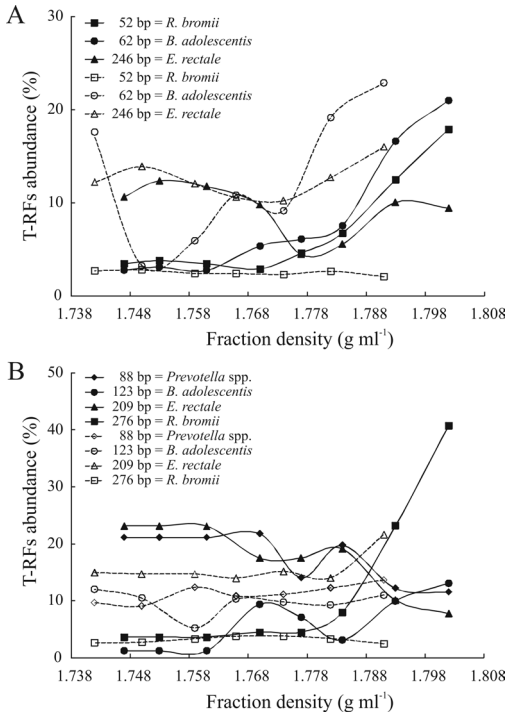

Figure 3. Relative abundance of different T-RFs, across the fraction density, after 4 h incubation with 7.4 g/l of [U-^{13}C]-starch (closed symbols) and 7.4 g/l of non-labeled starch (open symbols). The reported data represent the T-RFs generated after applying AluI as restriction endonuclease (A) and MspI (B) as restriction endonuclease.

Phylogenetic analyses

In order to identify the bacteria based on the density-resolved rRNA, and to assign phylogenetic groups to distinct T-RFs, bacterial clone libraries were generated from 16S rRNA template from the "heavy" (fraction 4, n = 72 clones) and "light" (fraction 8, n = 24 clones) fractions from the 4 h gradient. After validation of insert size and RFLP analyses of SP6/T7 PCR products, 23 RFLP groups were defined and representative clones were selected for sequencing.

The phylogenetic analyses showed that both bacterial 16S rRNA clone libraries consisted of phylotypes belonging to three phyla, namely *Bacteroidetes*, *Firmicutes* (*Clostridium* clusters IV and XIVa) and *Actinobacteria* (*Bifidobacterium*) (Fig. 4). The "light" community comprised populations belonging to *Bacteroidetes* and *Firmicutes,* particularly to the *Clostridium* cluster XIVa. Here a clear predominance of sequences related to *Prevotella* spp. and *Ruminococcus obeum* was observed. In stark contrast to the clone library from the "light" fraction, bacterial clones retrieved from the "heavy' fraction had prevalence of sequences related to *Ruminococcus*

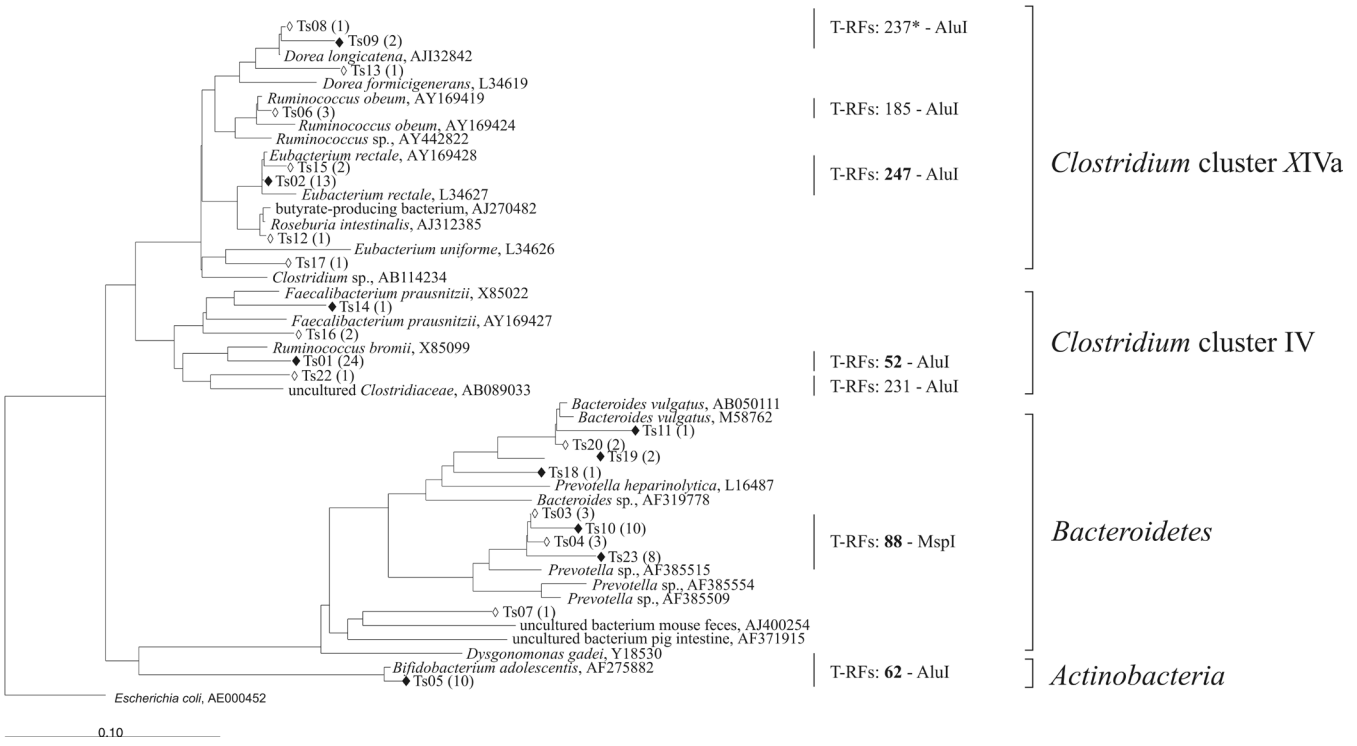

Figure 4. Phylogenetic tree presenting the positions of bacterial 16S rRNA sequences recovered from labeled and unlabeled RNA extracted from TIM-2 luminal content (respectively fraction 4 and fraction 8 of the 4 h gradient; see Fig. 1A). The scale bar represents 10% sequence difference. Different symbols were used to indicate clones from the light (◊) and heavy (♦) libraries, respectively, and their frequency in the library is indicated in brackets. Assignment of terminal restriction fragments for *Clostridium* cluster XIVa and IV, and *Actinobacteria* (AluI digestion), and *Bacteroidetes* (MspI digestion) from the corresponding T-RFLP profile (see Fig. 2 B and E) is based on sequence data; T-RFs marked with asterisk were assigned as pseudo T-RFs. T-RFs that were specifically observed in profiles of heavy fractions (Fig. 2) are depicted in bold. Accession numbers of sequences determined in this study, as well as those of references sequences are given.

bromii (*Clostridium* cluster IV), which represented 33% of the clones, *Eubacterium rectale* (*Clostridium* cluster XIVa - 18% of clones) and *Bifidobacterium adolescentis* (*Actinobacteria* - 14% of clones). The number of clones affiliated to *Prevotella* spp. was relatively high in the clone libraries obtained from both fractions (Fig. 4).
Overall, both libraries could be shown to significantly differ in the relative abundance of clones within the 4 major groups (*Clostridium* clusters IV, XIVa, *Bacteroidetes*, *Actinobacteria*), using a Chi Square test of independence ($p = 0.03$). In addition, trends for individual groups could be confirmed, albeit not in all cases at significance level due to the limited size of the libraries, using the Library Compare software available at the Ribosomal Database Project website (http://rdp.cme.msu.edu/); Cole *et al.*, 2007), with p-values ranging between 0.02 (*Ruminococcus obeum*), 0.12 (*Bifidobacterium* spp.) and 0.74 (*Prevotellaceae*) (data not shown).
In the T-RFLP profile of bacterial 16S rRNA of fraction 8 after 4 h incubation digested with AluI, it was estimated that more than 80% of all major T-RFs could be allocated to phylogenetic groups from the constructed clone library, mainly affiliated to *Clostridium* clusters IV and XIVa. The predominant T-RF with a size of 62 bp was assigned to *Bifidobacterium adolescentis*. The obvious strong dominance of this T-RF in all the fractions was probably caused by the high GC-content specific for *Bifidobacterium* spp., which leads to an increase in the density and appearance of the corresponding RNA in the RNA fractions with higher density (Neufeld et al., 2007). The T-RFs with a size of 52 bp and 246 bp, which increased strongly in fractions with labeled RNA (Fig. 2 B), could be assigned to *Ruminococcus bromii* and *Eubacterium rectale*, respectively.
In addition to these two populations, analyses of the profiles resulting after MspI restriction indicated that a T-RF with a size of 88-bp was strongly enriched in fraction 4 after 4 h incubation, compared to fraction 5 after 0 h incubation (Fig. 2 D and E). Based on phylogenetic analyses this peak corresponded to a population most closely related to a *Prevotella* species.

Community profiling of the TIM-2 microbiota using the HITChip

The microbiota composition of the initial community of TIM-2 for the [U-^{13}C]-starch experiment and that of two fractions from the 4 h gradient of the [U-^{13}C]-starch study (fraction 4 and fraction 8) was compared using the HITChip, comprehensive phylogenetic microarray for the human intestinal tract microbiota (Rajilic-Stojanovic, 2007). Furthermore, to assess the reproducibility of TIM-2 experiments using different [U-^{13}C]-substrates, a sample representing the initial community

of an additional experiment with [U-^{13}C]-inulin (Kovatcheva-Datchary et al., in preparation) was included as well. These analyses revealed that the initial microbiota in both experiments is highly similar - 98%, calculated as Pearson's product moment correlation between the respective HITChip fingerprints. Despite this high similarity of the initial microbiota, different microbial populations were identified by T-RFLP analysis as primary [U-^{13}C]-inulin degraders as compared to the [U-^{13}C]-starch study (Kovatcheva-Datchary et al., in preparation). As it could be anticipated, the profile of fraction 4 from the 4 h [U-^{13}C]-starch study, which represents only the part of the microbiota that is actively involved in starch degradation, showed lower diversity than the profiles of the initial samples and fraction 8 (Fig. 5).

In addition to the analysis of the total and fractions' microbiota from TIM-2 *in vitro* model, the total rRNA-based microbiota profiles of fecal samples of four randomly selected adult volunteers were generated using the HITChip (Fig. 5). Similarity between the initial total microbiota from the TIM-2 experiment and the total microbiota from faecal samples of the volunteers was found to be in average 65.6% (Standard deviation (SD) 4.6%). This was within the 2SD confidence range of the inter-individual similarity of the total faecal microbiota of the volunteers (average 74.2%, SD 4.3%). Furthermore, none of the subpopulations of the microbiota seemed to be majorly affected in TIM-2 *in vitro* experiments. More specifically, it was found that the similarity of the *Actinobacteria*-specific profiles of TIM-2 model samples and fecal samples was 71.3% (SD 6.8%), *Bacteroidetes*-specific profiles 68.5% (SD 6.6%), and *Proteobacteria*-specific profiles 67.8% (SD 8.2%). Members of the *Firmicutes* phylum appeared to be somewhat more affected in the *in vitro* model as similarity values were for *Bacilli* 62.7% (SD 5.9%), for *Clostridium* cluster IV 58.5% (SD 3.9%) and for *Clostridium* cluster XIVa 56.4% (SD 3.0%).

The diversity of the total initial microbiota profiles of TIM-2 model was found to be 1.7 (expressed as a number of the responding HITChip probes), which was, again, found to be in the range of diversity detected in faecal samples of volunteers (1.94 ± 0.18), further reinforcing the fact that the TIM-2 model used in this study well represents the human large intestinal microbiota.

Quantitative-PCR (qPCR) of R. bromii

The 16S rRNA-based ratios of *Ruminococcus bromii* to total bacteria from the experiment with [U-^{13}C]-starch and non-labeled starch indicated a strong increase in the *Ruminococcus bromii* population in the fraction with high density from the [U-^{13}C]-starch experiment in comparison with the non-labeled starch study (Fig. 6).

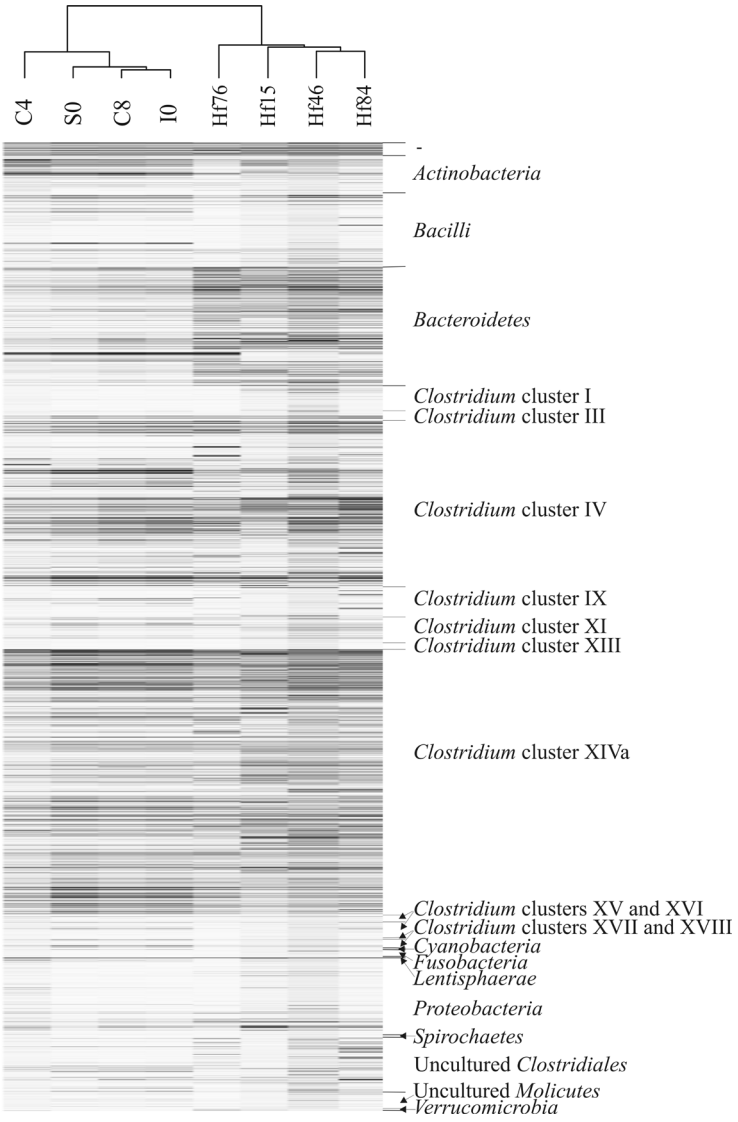

Figure 5. Phylogenetic HITChip fingerprints of the microbiota composition of the initial community of TIM-2 for the [U-^{13}C]-starch (S0) and [U-^{13}C]-inulin (I0) experiment, that of two fractions from the 4 h gradient of the [U-^{13}C]-starch study - fraction 4 (C4) and fraction 8 (C8), and the faecal microbiota of four adult volunteers (Hf76, Hf15, Hf46 and Hf 84). The highest phylogenetic ranks of probe specificity are given at the right side of the figure.

These data confirm the T-RFLP data, where after comparison of the T-RF abundance of the major T-RFs from the experiments with labeled and non-labeled starch a significant increase in the T-RF assigned to *Ruminococcus bromii* in the heavy fractions after incubation in the presence of labeled starch for 4 hours was detected. In none of the samples analyzed, methanogenic *Archaea* could be detected (data not shown).

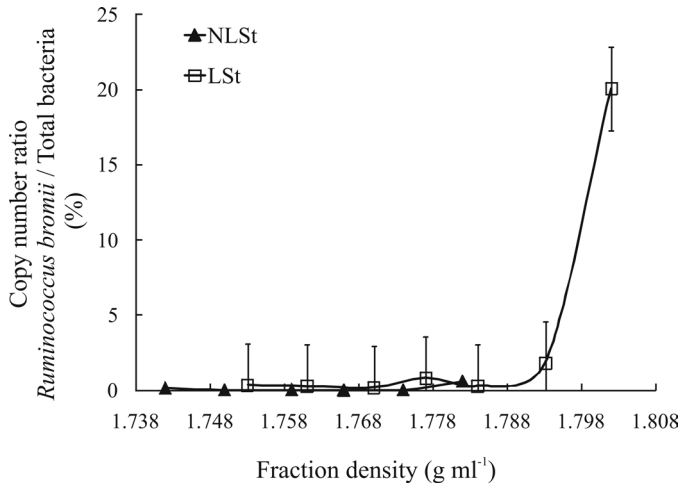

Figure 6. Ratio of 16S rRNA copy numbers across the fraction density after 4 h incubation with 7.4 g/l of [U-^{13}C]-starch (open symbols - LSt) and 7.4 g/l of non-labeled starch (closed symbols - NLSt). The reported data represent the copy number quantified after Real-Time PCR specific for *Ruminococcus bromii* and total bacteria, respectively. A standard curve was generated by using serially diluted 16S rRNA gene amplicons obtained from *Ruminococcus bromii* 51896 ATCC.

^{13}C-labeled metabolite production

LC-MS analyses were used to follow the production of metabolites from the ^{13}C-labeled starch in the same incubations as used for the RNA isolation. Acetate (~ 5 mmol), butyrate (~ 2 mmol) and propionate (~ 1 mmol) were identified as main fermentation products (Fig. 7 A). A strong increase of acetate, butyrate and propionate concentrations was detected particularly 4 h after starch addition.

LC-MS analysis allowed for the detection of the different isotopomers of acetate (M+0 [all ^{12}C-isotopomer]; M+1 [one ^{13}C atom, one ^{12}C]; and M+2 [both ^{13}C]), propionate (M+0, M+1, M+2 and M+3) and butyrate (M+0 through M+4).

The major acetate-isotopomer produced from the labeled starch was the M+2 isotopomer (Fig. 7 B), whereas there was only limited production of the M+1-isotopomer. The LC-MS method cannot discriminate between $^{13}C_1$-acetate or $^{13}C_2$-acetate. However, both are formed through the Wood-Ljungdahl reaction of coupling two CO_2 molecules, one of which is ^{13}C-labeled.

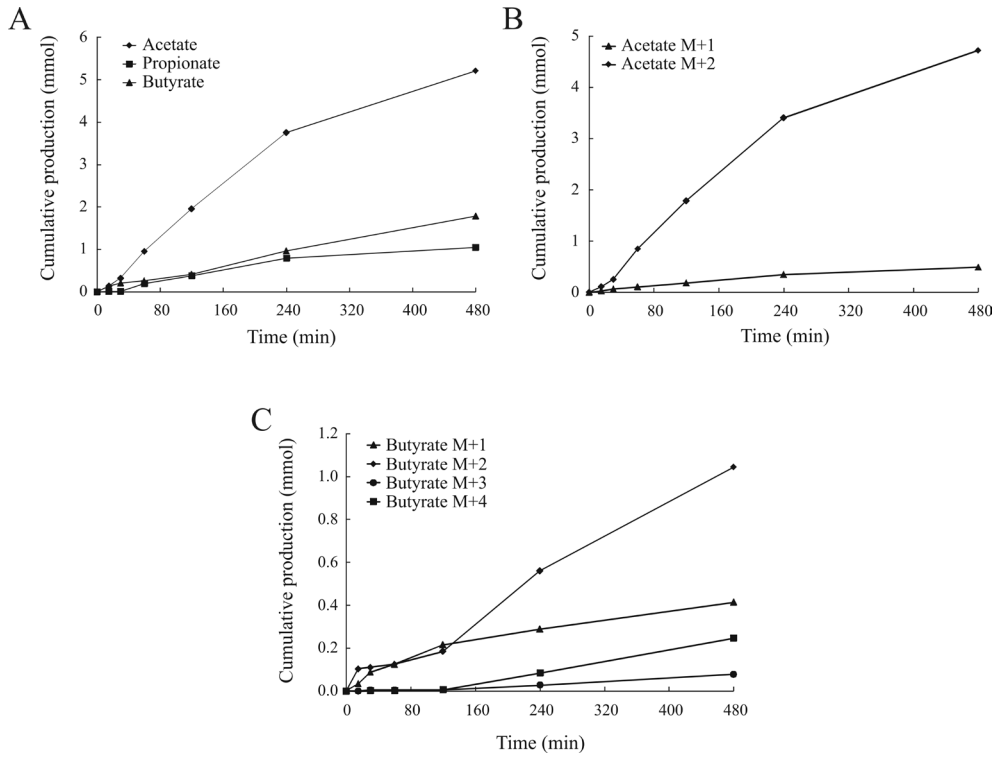

Figure 7. Cumulative production of the metabolites produced during fermentation of 7.4 g/l [U-^{13}C]-starch, analyzed by LC-MS. **A.** Sum of all isotopomers produced (M+1 and M+2 for acetate; M+1 – M+3 for propionate; M+1 – M+4 for butyrate). **B.** Acetate-isotopomers produced from labeled starch (acetate M+1 and acetate M+2). **C.** Butyrate-isotopomers produced from labeled starch (butyrate M+1, butyrate M+2, butyrate M+3 and butyrate M+4).

The major butyrate-isotopomer after 8 h was the M+2-isotopomer, the production of which was bi-phasic with an intermediate lag-phase during the first 2 h of incubation (Fig. 7 C). The M+3- and M+4-isotopomers were first detected after 2 h. Strikingly, the production of the M+1 isotopomer of butyrate showed a different profile, with a relatively rapid production during the first 2 h, and then a slower phase for the remaining 6 h. In both phases equivalent amounts were produced.

It should be noted that during the full 8 h of incubation unlabeled isotopomers of acetate and butyrate (M+0) were present as well (not shown in Fig. 7), due to either i) their production from other (unlabeled, non-carbohydrate) substrates, or ii) their

presence in the system at the start of addition of the labeled starch. Compared to acetate and butyrate, propionate production was relatively low, with approximately equal amounts of the M+1, M+2 and M+3 isotopomer, and similar kinetics of production (data not shown).

Discussion

Dietary carbohydrates, such as resistant starch, represent a substantial part of the human diet (Annison and Topping, 1994), and are known to affect the functioning of the human colon. Data from several animal studies showed increased large bowel butyrate levels and counts of *Bifidobacterium, Lactobacillus*, *Eubacterium* and *Bacteroides* spp. after consumption of resistant starch (Kleessen et al., 1997; Jacobasch et al., 1999; Bird et al., 2000; Le Blay et al., 2003; Wong et al., 2006). However, there is still lack of knowledge with respect to the key microbes involved in colonic breakdown of these carbohydrates. The objective of our study was to use [U-^{13}C]-potato starch as a substrate for the microbial community of an *in vitro* model system of the human colon, and to apply RNA-based stable isotope probing (RNA-SIP) to phylogenetically identify bacteria involved in the colonic fermentation of starch.

Clone libraries constructed from both unlabeled and labeled 16S rRNA constituted the most abundant bacterial groups normally found in human fecal samples. Additional analysis by the HITChip phylogenetic microarray confirmed these data and showed that our experimental model is in fact well-representing the fecal microbiota present *in vivo*, in human volunteers. Furthermore, HITChip analysis also revealed high similarity between experiments performed with different [U-^{13}C]-substrates with regard to initial composition, further supporting the good reproducibility of the model (Fig. 5). The *Clostridium* cluster XIVa was found to be predominant in the "light" library. According to previous reports on the human colonic microbiota composition, this cluster comprises 24 to 59% of the total bacterial phylotypes isolated from human fecal samples (Suau et al., 1999; Hold et al., 2002; Hayashi et al., 2003). A strong numerical increase in the *Clostridium* cluster IV was detected in the "heavy" library, especially concerning clones related to the *Ruminococcus bromii*. Significant changes in the "heavy" clone library were also detected in the *Bacteroidetes* phylum, mostly due to an increase in phylotypes related to *Prevotella* species.

The heavy fraction (fraction 4) appears to represent only a tiny percentage of the total RNA at the 4 h time point. This is based on the fact that only a very small amount

of rRNA becomes strongly labeled due to the high numbers of bacteria present, the fact that TIM is an open system, as well as the presence of other substrates. It is the fundamental principle of SIP that the sequences found here represent a snapshot of the most active substrate consumers in the system (Egert et al., 2006). Thus the amount of strongly labeled rRNA does not provide information with respect to the relative abundance of these groups in the system. This can be estimated from the fingerprints generated from the unlabeled RNA.

The *Bifidobacterium* group is also a vital member of the human fecal microbiota (Matsuki et al., 2004). *Bifidobacterium adolescentis* represented 7% of all sequences retrieved from both libraries. Most of the studies investigating 16S rRNA gene libraries of human adult colonic microbiota detected lower levels of *Bifidobacterium* species (Suau et al., 1999; Hayashi et al., 2002; Hold et al., 2002; Schwiertz et al., 2002). This has in general been explained by the high GC-content in *Bifidobacterium* species, resulting in incomplete DNA denaturation during the PCR reaction (Suau et al., 1999). However, a possible explanation of the relative high presence of *Bifidobacterium* spp. in our clone libraries in comparison with the studies mentioned above could be the selection of a suitable forward primer (35f), which increases the sensitivity in detection of bifidobacteria in human fecal samples. Similar results were reported by (Hayashi et al., 2004) after using the same forward primer for the construction of a 16S rRNA gene clone library from fecal samples of five healthy individuals.

An important function of the human colonic microbial community is the degradation and fermentation of dietary carbohydrates, which escape digestion in the upper intestinal tract (Guarner and Malagelada, 2003). To this end, the contribution of different groups of microorganisms linked in a food chain is necessary for performing of this fermentative process. Several studies reported *Bacteroides* and *Bifidobacterium* species as the major saccharolytic bacteria in experiments targeting human fecal microbiota (Macfarlane et al., 1997; Wang et al., 1999; Cummings et al., 2001). In a recent study, it was observed that *Ruminococcus bromii, Eubacterium rectale, Bifidobacterium adolescentis* and *Bifidobacterium breve* are able to attach to and colonize starch particles (Leitch et al., 2007). Our results based on 16S rRNA-SIP corroborate the involvement of *Ruminococcus bromii* in the starch fermentation under the *in vitro* conditions (Leitch et al., 2007). This is also in line with recent observations made by Abell et al. 2008 in an *in vivo* study, where the effect of non-starch polysaccharides (NSP) and resistant starch (RS) on microbiota composition was tested, revealing a significant increase of *Ruminococcus bromii* and closely

related phylotypes particularly with the RS diet. Comparison of the T-RF abundance of the major T-RFs from the experiments with labeled and non-labeled starch showed a significant increase in the T-RF assigned to *Ruminococcus bromii* in the heavy fractions after incubation in the presence of labeled starch for four hours. This was furthermore confirmed by quantitative PCR targeting total bacterial and *Ruminococcus bromii*-specific 16S rRNA fragments (Fig. 6). The most plausible explanation for this observation is that this species represents the population most actively assimilating starch in the studied system. Notably, these results are in agreement with the high frequency of clones related to *Ruminococcus bromii* detected in the "heavy" library. Whereas analysis of fingerprints generated from heavy and light fractions of RNA from the [U-^{13}C]-starch experiment suggested populations related to *Eubacterium rectale, Prevotella* spp. and *Bifidobacterium adolescentis* to be directly involved in the starch degradation, a comparison of the relative abundance of the T-RFs representing these species generated from the experiments performed with labeled and non-labeled starch did not reveal significant changes. Possibly these populations are not the primary [U-^{13}C]-starch degraders in the studied model, but rather consume metabolites of primary degradation by *Ruminococcus bromii* related species. The presence of species related to *Bifidobacterium adolescentis* to be relatively abundant not only in the fractions with heavier density but also in those with lower density could be related to a GC-bias that is known to affect SIP analyses (Rangel-Castro et al., 2005).

Amylolytic bifidobacterial strains are known to produce mostly acetate and lactate as end products from carbohydrate fermentation (Duncan et al., 2004b). Lactate is rarely detected as major fermentation end product of complex microbial communities (Le Blay et al., 2003), and some reports suggest that a considerable part of the lactate is converted to butyrate in the human colon (Duncan et al., 2004a; Bourriaud et al., 2005; Belenguer et al., 2006). The complete absence of lactate after starch fermentation in our experiment further supports this, but does not allow us to conclude with certainty whether amylolytic bifidobacteria are involved in the starch degradation in these experiments. In a separate experiment the TIM-2 microbial community was probed to ferment glucose, which is a common, natural substrate for microbial fermentations especially for the human small intestine microbiota. Here, [U-^{13}C]-glucose was fermented within 2 h, yielding mostly lactate, but also acetate, formate and butyrate as main metabolic products (Egert et al., 2007). This obvious difference in metabolites indicates that distinct microbial populations are responsible for microbial fermentations of the different substrates tested, which was

also confirmed with RNA-base stable isotope probing.
Starch is a substrate, which is known to stimulate the abundance of butyrate-forming bacteria, mostly belonging to the *Eubacterium rectale* group (Barcenilla et al., 2000), but the mechanisms are not yet clearly defined. Several studies have suggested that the major butyrogenic bacteria depend on other species, mostly acetate producers, but also initial degraders of complex carbohydrates (Duncan et al., 2002; Belenguer et al., 2006). The increase in the production of acetate and butyrate concomitant with enrichment of labeled 16S rRNA particularly for *Ruminococcus bromii,* suggests that the latter might interact with *Eubacterium rectale* by cross-feeding. It is tempting to hypothesize that in our system the butyrogenic effect is stimulated through a cooperative food-web, comprising *Ruminococcus bromii* and *Eubacterium rectale*, where *Ruminococcus bromii.* is degrading the starch and producing acetate, which the known butyrate-producer *Eubacterium rectale* uses to convert to butyrate. The main butyrate isotopomer observed was the M+2-isotopomer (56% of the ^{13}C-label, Fig. 7 C), indicating coupling of a fully ^{13}C-labeled acetate to a fully unlabeled acetate. This is most likely to occur when the labeled and unlabeled metabolite pool of acetate mix extracellularly, and corroborates our hypothesis of cross-feeding between *Ruminococcus bromii* and *Eubacterium rectale*. This also explains the lack of strong labeling of *Eubacterium rectale* RNA. This is further supported by the observation of an intermediate lag-phase during the 2 h in the production of the M+2 butyrate isotopomer, as well as the fact that the M+3 and M+4 isotopomers were detected only after 2 h. It is unclear whether the M+4 metabolite is produced by coupling of two labeled M+2-acetate molecules by cross-feeding, or that the M+4 butyrate is produced by a microorganism that feeds on the labeled starch directly. However, due to the time-lag of production of this M+4 isotopomer, the appearance of which coincides with the second phase of production of the M+2 butyrate isotopomer, it is likely to be formed due to cross-feeding. We hypothesize that the time-lag in production of these isotopomers is due to the fact that it takes time for the M+2 acetate isotopomer (required for production of M+2 and M+4 butyrate) to reach those microorganisms that cross-feed on this metabolite.

In conclusion, we could show that RNA-SIP can be used to identify bacteria involved in carbohydrate fermentation under conditions simulating those in the human colon. Additional fermentation studies are currently performed to reveal whether these allow to confirm the primary substrate consumers and to define possible cross-feeding processes. Furthermore, by extending the *in vitro* models to human feeding trials, in which relevant dietary oligo- and polymeric carbohydrates

will be delivered to the human colon, will allow the exploration of the real power of this molecular approach.

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CHAPTER 3

RNA-Based Stable Isotope Probing in Combination with Phylogenetic Microarray Analysis Implicates Members of *Clostridium* Cluster XIVa and *Actinobacteria* in Inulin and Lactose Metabolism in an *In Vitro* Model of the Human Colon

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Abstract

Using 16S ribosomal RNA (rRNA)-based Stable Isotope Probing (SIP) we identified bacteria fermenting inulin and lactose under human colon-like conditions. Two separate studies were performed to test the activity of the microbiota of the TIM-2 *in vitro* model of the human colon. Either 7.4 g/l of [U-^{13}C]-inulin or 7.4 g/l of [U-^{13}C]-lactose were added to the system. Lumen and dialysate samples were taken at frequent intervals and processed for metabolic and molecular analyses. RNA was extracted from luminal samples and was further subjected to density-gradient ultracentrifugation and subsequently separated into fractions of increasing density. Terminal-restriction fragment length polymorphism (T-RFLP) fingerprinting of 16S rRNA in these fractions suggested populations related to *Dorea longicatena*, a member of *Clostridium* cluster XIVa, and *Bifidobacterium adolescentis*, belonging to the *Actinobacteria*, to be most actively involved in inulin and lactose metabolism, respectively. These findings were confirmed and extended by analyzing the ^{13}C–enriched 16S RNA amplicons by a phylogenetic microarray, the Human Intestinal Tract Chip (HITChip). Moreover, the contribution of *Actinobacteria* to the inulin fermentation was detected as well. The HITChip analysis of selected fractions from the lactose experiment indicated that *Actinobacteria*, particularly *Bifidobacterium* spp. and *Collinsella* spp., were the main taxa involved in lactose fermentation. LC-MS analyses of the lumen and dialysate samples showed that both inulin and lactose fermentation primarily yielded acetate, butyrate and propionate. Additionally, NMR data indicated the presence of lactate 15 min after the start of both fermentations. Integration of molecular and metabolite data suggests members of *Clostridium* cluster XIVa and *Actinobacteria* to be involved in the fermentation of inulin and lactose, either as a direct degrader of the studied substrates or as being associated in the trophic chain.

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Introduction

The human body is inhabited by a complex microbial consortium, which represents only 1% to 2% of the body mass of a healthy human, but has immense impact on its health. The number of microbial cells associated with us is estimated to outnumber the human cells by 10-fold and the number of microbial genes is suggested to exceed those of the host by a factor of more than 100 (Savage, 1977; Berg, 1996; Backhed et al., 2005; Qin et al., 2010). Particularly in the human colon up to 100 trillion microbes, representing more than 1000 species, structure a microbiome that possesses many metabolic capabilities, which are lacking in the human host and make it essential for human life (Suau et al., 1999; Eckburg et al., 2005; Xu et al., 2007). Fermentation of dietary carbohydrates, such as plant polysaccharides like inulin and a variety of oligosaccharides, which escape digestion in the upper gastro-intestinal (GI) tract and reach the colon, is one of these vital microbial activities. This fermentation allows the microbiota to use these otherwise indigestible carbohydrates as an energy source, leading to the production of metabolites that are believed to contribute to maintaining gut health (Savage, 1986; Hooper et al., 2002; Guarner and Malagelada, 2003; Xu and Gordon, 2003). The end products from these microbial fermentations are short-chain fatty acids (SCFA) such as butyrate, propionate and acetate, as well as other intermediate products such as lactate. Increase in the production of SCFAs was reported to lower the luminal pH. This modifies the microbial composition in the colon as it suppresses the activity of pathogenic bacteria and stimulates the growth of bacteria such as bifidobacteria and lactobacilli, leading to an increase in the biomass and fecal bulk respectively (Cummings and Macfarlane, 1997; Topping and Clifton, 2001; Le Leu et al., 2005).

Dietary fructans such as inulin and fructo-ologosaccharides are well-studied prebiotics, and their health-promoting effect has been reported in a number of studies, using animals and humans (Djouzi and Andrieux, 1997; Kleessen et al., 1997; Reddy et al., 1997; Gallaher and Khil, 1999; Kleessen et al., 2001; Perrin et al., 2001; Oberreuther-Moschner et al., 2004). As a direct outcome of the inulin ingestion, the stimulation of bifidobacteria, known as bifidogenic effect, and an increase in butyrate concentration (butyrogenic effect) have been reported in *in vivo* studies (Gibson et al., 1995; Kleessen et al., 1997; Bouhnik et al., 1999; Kruse et al., 1999; Tuohy et al., 2001; Bouhnik et al., 2004; Kleessen et al., 2007; Ramirez-Farias et al., 2009). Different carbohydrates are digested to various degrees in the GI tract, and some enter the colon or pass through the entire intestine comparatively intact (Scheppach et al., 2001). Most disaccharides are completely digested in the small

intestine, however, the host genetic background can influence digestive efficacy significantly. Lactose is one of these disaccharides, and lactose intolerance results from insufficient or even absent lactase activity in the small intestine (Henslee and Jones, 1982; de Vrese et al., 2001; Vonk et al., 2003). In lactase-deficient individuals, consumption of lactose can cause variable abdominal symptoms, but also can have a positive effect on microbial fermentation, as lactose was proposed to be prebiotic when host lactase activity is limited (Gudmand-Hoyer, 1994; Szilagyi, 2002, 2004). Obviously, the diet affects colonic nutrition mainly through its effect on the gut microbiota. Hence, studies should unravel the *in situ* functionality of this microbiota and aim to contribute to our understanding of the impact of the intestinal microbiota on human health.

Numerous microbes inhabit the human intestine, many of which still remain uncharacterized or uncultured, which keeps their *in situ* functions largely unknown. A recently introduced molecular approach, which allows linking the metabolic activity and diversity of microbial communities in a complex environment, is nucleic acid-based Stable Isotope Probing (SIP) (Radajewski et al., 2000; Dumont and Murrell, 2005; Egert et al., 2006; de Graaf et al., 2007; Neufeld et al., 2007). This approach involves the incorporation of stable-isotope label into cellular biomarkers (including DNA and RNA), which are used to identify the microorganisms assimilating a labeled substrate and are involved in particular processes of interest. Especially the introduction of RNA-based SIP represented a significant advancement, as it does not require microbial growth, and has been used to link microbial function with phylogeny in a broad spectrum of different environments. Recently, RNA-based SIP was applied to identify acetate-metabolizing bacteria in the presence of iron oxides as electron acceptors (Hori et al., 2010), phenol-assimilating bacteria (Sueoka et al., 2009), or butyrate-producing bacteria under methanogenic conditions (Hatamoto et al., 2008). Furthermore, RNA-based SIP was also applied to study human intestinal bacteria in an *in vitro* model of the human colon in presence of [U-^{13}C]-glucose (Egert et al., 2007) and in the presence of [U-^{13}C]- potato starch (Kovatcheva-Datchary et al., 2009b).

The aim of the present study was to identify the microbes involved in inulin and lactose fermentation under conditions simulating the *in situ* situation in the human colon, using 16S rRNA based-SIP together with high-throughput diagnostic microarray-based phylogenetic profiling and metabolic flux analysis. Our study showed that RNA based-SIP combined with metabolic analyses is a useful tool for identifying colonic bacteria that metabolize inulin and lactose and linking these to

metabolites in the course of *in vitro* experiments. The current study also provides the basis for experiments to determine microbial functionality *in vivo* in human subjects.

Experimental Procedures

In vitro model of the large intestine

The validated TNO *in vitro* model of the human colon (TIM-2), representing the conditions in the proximal colon and tested with a variety of fermentable substrates (Minekus et al., 1999; Rajilic-Stojanovic, 2007; Kovatcheva-Datchary et al., 2009a) was used in the current study. The inoculation of the *in vitro* colon model with a fresh standardized microbiota and the further preparation of the microbiota was performed as described (van Nuenen et al., 2003; Egert et al., 2007; Kovatcheva-Datchary et al., 2009b). After overnight stabilization and a 2 h starvation (Egert et al., 2007), 7.4 g/l of 98% [U-^{13}C]-labeled inulin (Degree of polymerization (DP) 3 - >40 measured by Dionex HPLC), isolated from the taproots of chicory grown in the presence of $^{13}CO_2$ (IsoLife, Wageningen, The Netherlands) or 7.4 g/l of 98% [U-^{13}C]-labeled lactose (Omicron Biochemicals, Inc., South Bend, USA), was administered to the *in vitro* model, corresponding to a concentration of 40 mM hexose units. Luminal and dialysate samples (each approximately 2 ml) were taken at time points 0 h, 15 min, 30 min, 1 h, 2 h, 4 h and 8 h after substrate addition and stored at -80°C. Additional control experiments were performed with non-labeled inulin (Sensus, The Netherlands) and lactose (Sigma Aldrich, L-1768) as well.

RNA-extraction, purification and fractionation by density gradient centrifugation

Total RNA was extracted and purified from 250 µl of luminal samples collected at the different time points as described previously (Egert et al., 2007). After purification and quantification approximately 1 µg of total RNA was loaded in cesium trifluoroacetate (CsTFA) centrifugation medium and resolved by density gradient centrifugation. Gradients were subsequently fractionated as described earlier (Egert et al., 2007), albeit using a KDS200 syringe pump (KD Scientific Inc., Holliston, MA, USA) for fractionation. Afterwards, RNA from each fraction was precipitated for subsequent community analyses.

Amplification and fingerprinting of 16S rRNA

Amplification of the bacterial 16S rRNA for terminal restriction fragment length polymorphism (T-RFLP) analysis was done using a one-step reverse transcription-PCR (RT-PCR) system (Access Quick, Promega, Leiden, The Netherlands) as described previously (Egert et al., 2007). Primers and conditions for RT-PCR used to amplify the bacteria 16S rRNA have also been described previously (Kovatcheva-Datchary et al., 2009b).

For T-RFLP analysis, aliquots (50 ng) of purified PCR products were restricted for 3 h at 37ºC with 2.5 U of AluI (Promega) in a 10 µl reaction mixture following instructions of the supplier. Size separation of terminal restriction fragments (T-RFs) was performed at VHL (Wageningen, The Netherlands). T-RFs $\leq$ 50 bp were excluded from the analysis to exclude artifacts caused by primer-dimers. The relative abundance of the T-RFs within a given T-RFLP profile was calculated as the peak area of the respective T-RF divided by the total peak area of all T-RFs detected within a fragment length range between 50 and 900 bp.

Cloning, sequencing and phylogenetic analysis of bacterial 16S rRNA fragments

A clone library generated in a previous study (Kovatcheva-Datchary et al., 2009b), where the same microbial community was examined to ferment [U-^{13}C]-starch was used in the current experiments to assign phylogenetic groups to distinct T-RFs.

HITChip analysis

For phylogenetic analysis of the microbiota in the TIM-2 samples from the [U-^{13}C]-inulin and [U-^{13}C]-lactose studies, the HITChip was used. This phylogenetic microarray consists of more than 4,800 oligonucleotide probes based on 16S rRNA gene sequences targeting over 1,100 human intestinal microbial phylotypes (Rajilic-Stojanovic et al., 2009). Target preparation, hybridization and washing, and data analysis were essentially performed as described before (Kovatcheva-Datchary et al., 2009b; Rajilic-Stojanovic et al., 2009). Gradient fractions stemming from the samples taken 15 min, 30 min and 1 h after addition of inulin and 30 min and 1 h after lactose supplementation were analysed with the HITChip. The selection of the fractions was based on the data from the metabolite profiling.

Metabolite analysis using LC-MS and NMR

To follow the metabolite production during fermentation of [U-^{13}C]-labeled inulin and [U-^{13}C]-labeled lactose in the same samples as used for the RNA-SIP study a method minimizing the sample preparation and utilizing liquid chromatography-mass spectrometry (LC-MS) was applied (Meesters et al., 2007). LC-MS analysis allowed to determine the concentrations of the individual mass isotopomers including acetate (M+0 [all ^{12}C-isotopomer], M+1 [one ^{13}C atom, one ^{12}C]; and M+2 [both ^{13}C]), propionate (M+0, M+1, M+2 and M+3) and butyrate (M+0 through M+4). For determination of positional isotopomers as well as for profiling of non-SCFA metabolites, one- and two-dimensional NMR was used as described previously (de Graaf et al., 2010). Total concentrations of SCFA were determined by GC analysis (van Nuenen et al., 2003), and were used to determine the cumulative production of the labeled metabolites in the TIM-2 system as described previously (de Graaf et al., 2010).

Results

RNA recovery and RT-PCR amplification of the 16S rRNA

In two independent studies a fresh standardized human fecal microbiota, populating the TIM-2 *in vitro* model of the human large intestine, was supplemented with 7.4 g/l of uniformly ^{13}C-labeled inulin or uniformly ^{13}C-labeled lactose. In parallel, control studies with the same amounts of non-labeled inulin and lactose were done. All fermentations were followed for 8 h, and luminal and dialysate samples were taken at 0 h, 15 min, 30 min, 1 h, 2 h, 4 h and 8 h after substrate addition. The amount of RNA extracted from luminal samples taken at different time points amounted to 50 to 86 µg and 20 to 43 µg RNA per 250 µl of sample for the ^{13}C-inulin and ^{13}C-lactose *in vitro* fermentations, respectively.

The fractionated isopycnic centrifugation gradients from the [U-^{13}C]-inulin experiment covered an average density range of 1.721 g ml^{-1} (fraction 15) to 1.832 g ml^{-1} (fraction 1) (Fig. 1 A), while from the [U-^{13}C]-lactose fermentation the average density ranged from 1.725 g ml^{-1} (fraction 14) to 1.833 g ml^{-1} (fraction 1) (Fig. 1 B). In the gradient fractions of the 0 h sample from the [U-^{13}C]-inulin fermentation, all nucleic acids were detected at densities between 1.750 g ml^{-1} (fraction 11) and 1.787 g ml^{-1} (fraction 6), whereas for the [U-^{13}C]-lactose fermentation RNA was found in

the range between 1.753 g ml^{-1} (fraction 10) and 1.784 g ml^{-1} (fraction 6). Fifteen minutes after the addition of [U-^{13}C]-labeled inulin, presence of RNA was detected in high density fraction 5 (1.796 g ml^{-1}), indicating the incorporation of the label into the RNA of inulin-utilizing populations. The highest amount of RNA at the same time point was detected in fraction 8 (1.772 g ml^{-1}, Fig. 1 A), as was the case for the 0 h time point. Furthermore, the RNA distribution in the gradients from the 30 min, 1 h, 2 h, 4 h and 8 h time points was similar to that reported for the 15 min sample. The only difference was observed for the 2 h gradient, where the maximal RNA concentration was detected in one of the high density fractions, i.e. fraction 7 (1.779 g ml^{-1}, Fig. 1 A).

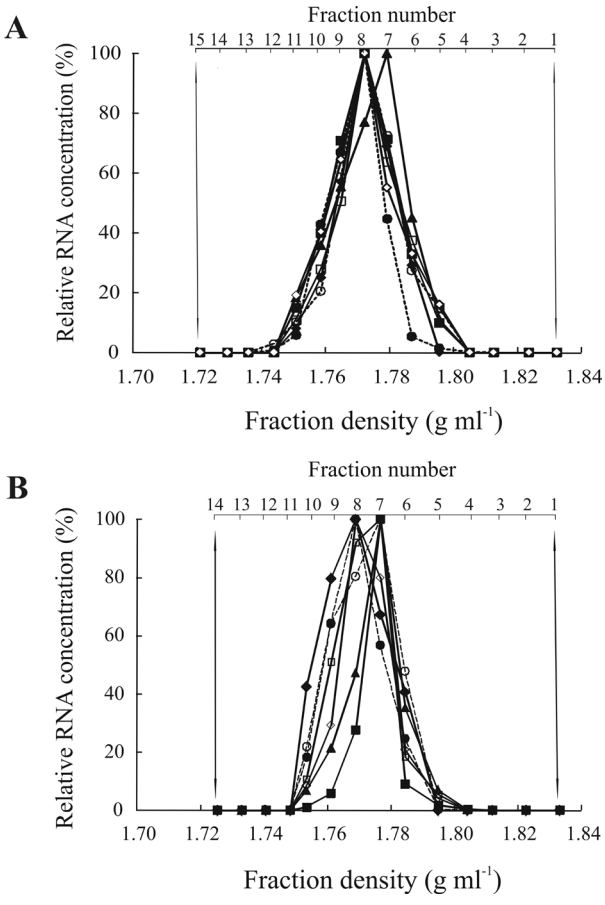

Figure 1. RNA concentration in gradient fractions. RNA was isolated from lumen samples of the colon model after incubation for 0 h, 15 min, 30 min, 1 h, 2 h, 4 h and 8 h with 7.4 g/l of [U-^{13}C]-inulin (**A**) or 7.4 g/l of [U-^{13}C]-lactose (**B**). Values are given in relative units (%) to facilitate comparison between the gradients; RNA concentration in the fractions containing most RNA (100%) for inulin were 3.1 ng μl^{-1} (0 h), 3.0 ng μl^{-1} (15 min), 2.4 ng μl^{-1} (30 min), 2.6 ng μl^{-1} (1 h), 2.8 ng μl^{-1} (2 h), 3.4 ng μl^{-1} (4 h) , 3.6 ng μl^{-1} (8 h) and for lactose were 3.0 ng μl^{-1} (0 h), 3.5 ng μl^{-1} (15 min), 3.4 ng μl^{-1} (30 min), 2.9 ng μl^{-1} (1 h), 2.8 ng μl^{-1} (2 h), 3.1 ng μl^{-1} (4 h), 2.9 ng μl^{-1} (8 h). ♦, 0 h; ● 15 min, ○ 30 min, ■ 1 h, ▲ 2 h, □ 4 h, ◊ 8 h. Vertical arrows indicate corresponding fractions in the figure. Densities of gradient fractions from the [U-^{13}C]-inulin (A) and [U-^{13}C]-lactose (B) studies are averaged over 7 gradients, the standard deviation of the densities of the different gradients were in the range of 0.002 and 0.003 g ml^{-1} for the inulin study and between 0.001 and 0.003 g ml^{-1} for the lactose (data not shown).

In contrast to the inulin fermentation, in the [U-^{13}C]-labeled lactose experiment, presence of RNA in fraction with higher density as compared to the 0 h sample was initially detected for the first time 30 min after the beginning of the study, namely in fraction 5 (1.795 g ml^{-1}, Fig. 1 B). The highest amount of RNA at the 0 h and 15 min time points was detected in fraction 8 (1.769 g ml^{-1}, Fig. 1 B), whereas for all other time points the maximal RNA concentration was detected in fraction 7 (1.777 g ml^{-1}, Fig. 1 B).

Identical distribution of the RNA in all gradients from the different time points was observed for both non-labeled inulin and lactose experiments. For the non-labeled inulin fermentation the average density range was 1.718 g ml^{-1} (fraction 15) to 1.825 g ml^{-1} (fraction 1) , while for the non-labeled lactose fermentation the values were in the range of 1.719 g ml^{-1} (fraction 14) to 1.821 g ml^{-1} (fraction 1) (data not shown). For all analyzed gradients from the non-labeled inulin and lactose experiments, the maximal RNA concentration was detected in fraction 9 (1.759 and 1.755 g ml^{-1}, respectively; data not shown).

Subsequently, the RNA from each of the fractions was recovered and the 16S rRNA was amplified by RT-PCR. The amplification of the 16S rRNA in the fractions of the 0 h gradient from the [U-^{13}C]-inulin fermentation yielded PCR-products from fraction 6 (1.787 g ml^{-1}) to fraction 11 (1.751 g ml^{-1}) (Fig. 2 A). However, 15 min after

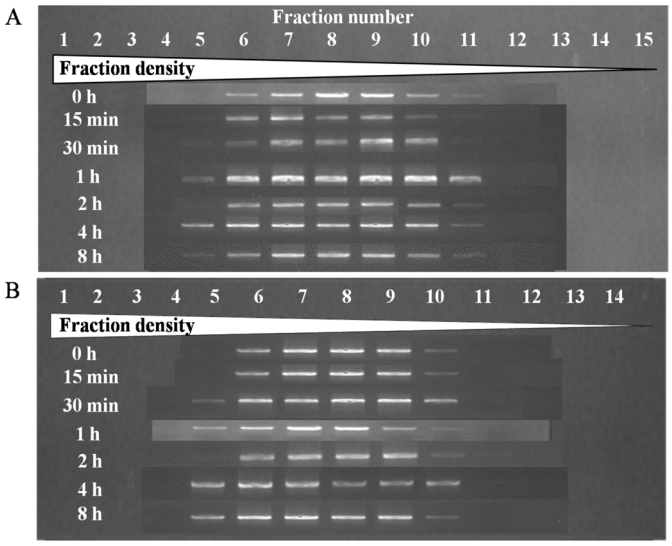

Figure 2. RT-PCR-analysis results representing an ~900 bp fragment of bacterial 16S rRNA from the gradient fractions from the [U-^{13}C]-labeled inulin study (**A**) and from the [U-^{13}C]-labeled lactose study (**B**). The picture is assembled from 7 different ethidium bromide-stained agarose gels after electrophoresis of the amplicons. Fractions were obtained from ultracentrifugation gradients performed to density-resolve RNA isolated from lumen samples of the colon model after incubation for 0 h, 15 min, 30 min, 1 h, 2 h, 4 h and 8 h with 7.4 g/l of [U-^{13}C]-inulin and [U-^{13}C]-lactose. Fraction numbers are indicated on top and correspond to those in Fig. 1A and 1B.

inulin addition a detectable product was also obtained for the high density fraction 5 (1.796 g ml^{-1}). As to the [U-^{13}C]-lactose fermentation, 16SrRNA RT-PCR performed with the fractions from the 0 h and 15 min gradients yielded PCR-products from the high density fraction 6 (1.784 g ml^{-1}) to fraction 10 (1.753 g ml^{-1}) (Fig. 2 B). Thirty minutes after [U-^{13}C]-lactose addition, an RT-PCR-product was detected also from the high density fraction 5 (1.795 g ml^{-1}). In summary, these data corroborate the incorporation of the ^{13}C-label into the bacterial 16S rRNA.

Fingerprinting of the bacterial 16S rRNA in graient fractions

T-RFLP fingerprints were generated for each gradient fraction from the different time points. The profile obtained after AluI restriction of the 16SrRNA RT-PCR products derived from fraction 8 from the 0 h gradient from the [U-^{13}C]-inulin fermentation, where the bulk of unlabeled RNA accumulated, displayed 12 major T-RFs sized 51, 60, 68, 128, 185, 242, 244, 428, 476, 623, 708, and 820 bp, respectively (Fig. 3 A). Identical T-RFs were detected in fraction 7 and 8 from all analyzed time points (Fig. 3 A). Only for the sample taken at 1 h, the fraction 7 fingerprint was not identical to the one from fraction 8 (Fig. 3 A). Minor variations in the relative height of peaks were observed, as fragments of 185 bp, 242 bp and 244 bp were most abundant after 8 h (Fig. 3 A). A clear dominance of the T-RF sized 60 bp was detected particularly in each of the fractions 15 min after ^{13}C-labeled inulin addition (Fig 3 A). However, for the 30 min sample, this peak was dominant in fractions 7 and 8, but not in fraction 5 and 6 (Fig 3 A). Changes in the T-RFLP profiles in fractions containing RNA with high density were found 30 min after ^{13}C-labeled inulin addition, where an increase in the T-RF sized 623 bp was detected (Fig. 3 A).

Additionally, the relative abundance of the T-RFs of the profiles of the fractions 15 min and 30 min after addition of labeled inulin and non-labeled inulin experiments was calculated. Only the T-RFs that had a relative abundance of $\geq$ 2% in all the analysed fractions were included for further analyses. A significant increase in the relative abundance of the T-RF sized 60 bp, was observed 15 min after addition of labeled and non-labeled inulin (Fig. 4 A). Furthermore, the relative abundance of the 623 bp T-RF was the only one that differed notably for the labeled inulin and non-labeled inulin experiments at 30 min (Fig. 4 A).

The fingerprint generated after AluI restriction of the 16S rRNA RT-PCR products derived from fraction 8 of the 0 h gradient from the [U-^{13}C]-lactose fermentation, where the bulk of unlabeled RNA accumulated, displayed 9 major T-RFs sized 51,

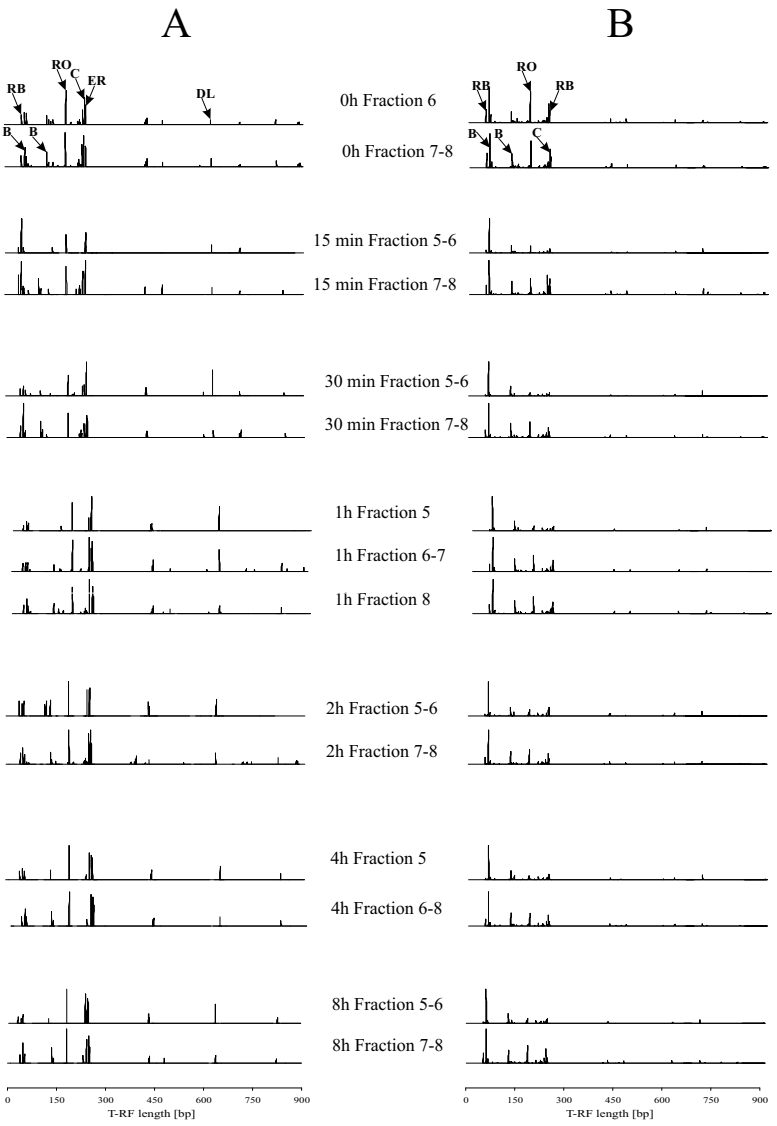

Figure 3. T-RFLP profiles of bacterial 16S rRNA obtained from [U-^{13}C]-inulin fractions (**A**) and from [U-^{13}C]-lactose fractions (**B**) of the 0 h, 15 min, 30 min, 1 h, 2 h, 4 h and 8 h gradients, after applying AluI as restriction endonuclease; Assignment of T-RF peaks to bacterial phylotypes (RB, *Ruminococcus bromii*, 51 bp; B, *Bifidobacterium adolescentis*, 61 bp; RO, *Ruminococcus obeum*, 185 bp; C, uncultured *Clostridium* spp., 242 bp; ER, *Eubacterium rectale*, 244 bp; DL, *Dorea longicatena*, 623 bp.

60, 68, 129, 185, 243, 246, 428, 476 and 623 bp, respectively (Fig. 3 B). Similar to the [U-^{13}C]-inulin study, the T-RFs detected in fraction 7 and 8 from all analyzed time points were identical (Fig. 3 B). A T-RF with a size of 60 bp was abundant in all profiles generated for the [U-^{13}C]-lactose experiment (Fig. 3 B). However, the relative abundance of the T-RF sized 60 bp was significantly different for the labeled lactose and non-labeled lactose experiments after 30 min and 1 h incubation (Fig. 4 B).

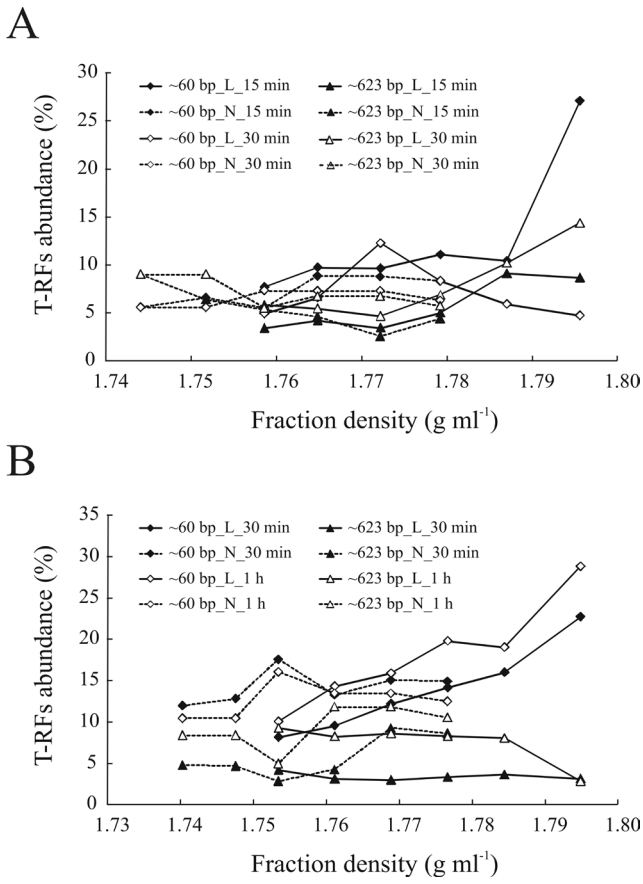

Figure 4. Relative abundance of T-RFs sized 60 bp and 623 bp, across the fraction density, (A) after 15 min (closed symbols) and 30 min (open symbols) incubation with 7.4 g/l of [U-^{13}C]-inulin (full line) and 7.4 g/l of non-labeled inulin (dashed line). (B) after 30 min (open symbols) and 1 h (closed symbols) incubation with 7.4 g/l of [U-^{13}C]-lactose (full line) and 7.4 g/l of non-labeled lactose (dashed line)

Phylogenetic analyses

The observed T-RFs were mapped to a reference set of 16S rRNA amplicons and the predominant T-RF with a size of 623 bp, which was detected to increase in fractions with high density (fraction 5) from the [U-^{13}C]-inulin fermentation, was assigned to *Dorea longicatena* (Fig. 3 A). The 60 bp T-RF, found to be strongly abundant in each profile generated for the [U-^{13}C]-lactose fermentation, but also in few fingerprints from the [U-^{13}C]-inulin experiment, was assigned to *Bifidobacterium adolescentis* (Fig. 3). Additionally, few T-RFs which were found to be abundant during the 8 h *in vitro* fermentations and which showed minor variations in relative height during the experiment, in particular the T-RFs sized 185 bp, 242 bp and 244 bp, were assigned to *Ruminococcus obeum*, *Clostridium* spp. and *Eubacterium rectale*, respectively (Fig. 3).

^{13}C-labeled metabolite production

To complement the microbial community analyses, LC-MS and NMR analyses of the metabolites produced during the [U-^{13}C]-inulin and [U-^{13}C]-lactose fermentations were performed using the same samples as used for the RNA isolation. During the [U-^{13}C]-inulin experiment, we identified as main fermentation products acetate (~ 4 mmol cumulative production), propionate (~ 2 mmol) and butyrate (~ 0.4 mmol), whereas during the [U-^{13}C]-lactose study mainly acetate (~ 6 mmol), propionate (~ 2 mmol) and butyrate (~ 0.4 mmol) were produced (Fig. 5). The LC-MS data showed an increase of acetate, butyrate as well as propionate concentrations particularly 15 min after inulin addition. In contrast to the [U-^{13}C]-inulin study, during the [U-^{13}C]-lactose fermentation the peak production of acetate was detected 1 h after the addition of the substrate, and for butyrate and propionate after approximately 2 h (Fig. 5 B). During both experiments, a continuous increase in butyrate concentrations was observed until the end of the incubation. In contrast, acetate and propionate increased during the [U-^{13}C]-inulin study for the first 2 h but then remained stable during the rest of the incubation. Similarly, during the [U-^{13}C]-lactose study, acetate reached rapidly the maximal concentrations after 1 h, while propionate continued to increase until 4th h.

LC-MS analysis allowed determination of the concentrations of the individual mass isotopomers of acetate, propionate and butyrate. The major acetate isotopomer produced from the labeled inulin and lactose was the M+2 isotopomer (Fig. 5 A), whereas there was no production of the M+1-isotopomer detected with either substrate. With both substrates, the major butyrate-isotopomer after 8 h was the

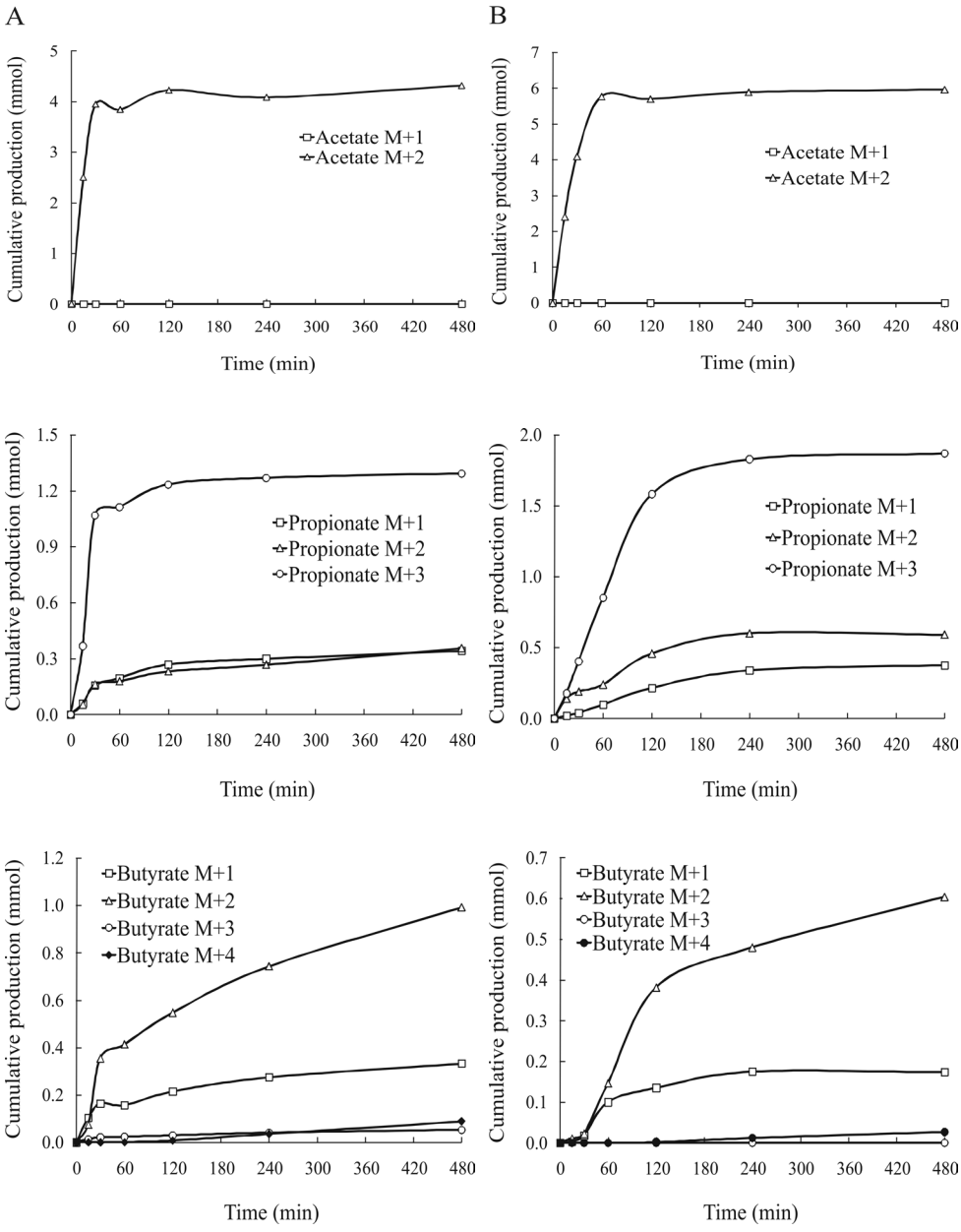

Figure 5. Cumulative production of different isotopomers of the main produced SCFA during the fermentation of 7.4 g/l [U-^{13}C]-inulin **(A)**, or 7.4 g/l [U-^{13}C]-lactose (**B**), analyzed by LC-MS.

M+2 isotopomer, which for the inulin experiment started to accumulate 15 min after addition of the ^{13}C-labeled substrate, whereas for lactose it appeared only after 1 h, and also in lower amounts in comparison with the inulin experiment (Fig. 5).
The M+4 butyrate isotopomer was first detected after 30 min in the inulin experiment and after 2 h with lactose, albeit in typically more than 10-fold smaller amounts than the M+2 isotopomer (Fig. 5). The production of the M+1 isotopomer of butyrate showed a profile similar to that of the M+2-isotopomer in both studies. In the inulin experiment the M+1 isotopomer concentration started to increase 15 min after substrate addition, whereas with lactose an increase was only observed after 1 h. Finally, in both experiments a relatively rapid production of the M+1 isotopomer was observed during the first 2 h, and then a slower phase for the remaining 6 h (Fig. 5).
During the full 8 h of incubation, unlabeled isotopomers of acetate and butyrate (M+0) were present in addition to the labeled ones in both fermentations. These isotopomers most probably were produced from other than the ^{13}C-labeled substrates, e.g. from proteins present in the system already at the start of the experiment.

In addition to the LC-MS analysis, NMR analysis was used to quantify non-SCFA metabolites derived from the ^{13}C-labeled substrates. Specifically lactate and succinate were observed and their production increased 15 min after the addition of either ^{13}C-labeled substrate. The concentrations of the ^{13}C-lactate in the [U-^{13}C]-inulin and [U-^{13}C]-lactose fermentations at time point 15 min were 1.2 mM and 4.6 mM, respectively. Increase in the ^{13}C-lactate at the 30 min was measured only in the [U-^{13}C]-lactose fermentation, where it reached a value of 7.3 mM. The measured ^{13}C-lactate at 30 min in the [U-^{13}C]-inulin fermentation was 0.4 mM. The time profiles of the ^{13}C succinate qualitatively mimicked those of lactate.

Community profiling of the TIM-2 microbiota during [U-^{13}C]-inulin and [U-^{13}C]-lactose fermentation using the HITChip

To provide information on the composition of the TIM-2 microbial community of the [U-^{13}C]-inulin and [U-^{13}C]-lactose studies at higher resolution, selected fractions were compared using the HITChip, a comprehensive phylogenetic microarray for the human intestinal tract microbiota (Rajilic-Stojanovic et al., 2009). For the [U-^{13}C]-inulin experiment fractions containing labeled (fraction 5) and unlabeled (fraction 8) RNA from time point 15 min, 30 min and 1 h were included in the analysis. Cluster analysis of the HITChip profiles indicated clear separation of the profiles of the fractions containing labeled RNA from those with unlabeled RNA (data not

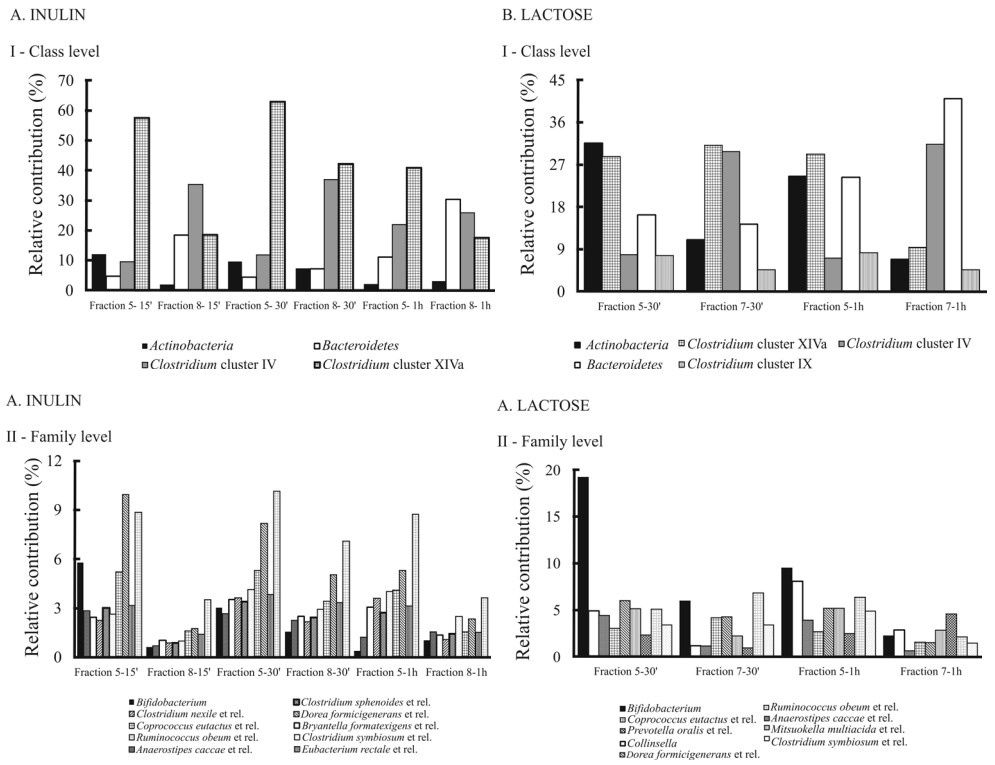

Figure 6. Relative contribution of phylotypes detected with the HITChip in fractions containing labeled (fraction 5) or unlabeled (fraction 7 for the [U-^{13}C]-lactose experiment and fraction 8 for the [U-^{13}C]-inulin experiment) RNA from three selected time points 15 min, 30 min and 1 h from the [U-^{13}C]-inulin study (**A**) and two selected time points 30 min and 1 h from the [U-^{13}C]-lactose study (**B**). I- higher taxonomic level – Class; II- lower taxonomic level – family.

shown). HITChip analysis indicated a strong increase of *Clostridium* cluster XIVa in fraction 5 of the 15 min sample of the [U-^{13}C]-inulin experiment (Fig. 6 A-I). The same dominance of this group was also observed for labeled RNA-containing fractions from the 30 min and 1 h samples, albeit with a slight decrease in relative abundance after 1 h. Furthermore, the most abundant subpopulation of *Clostridium* cluster XIVa in the heavy fractions belonged to the group of *Dorea formicigenerans* et rel., which also includes *Dorea longicatena* (Fig 6 A-II). This is in agreement with results from the T-RFLP fingerprinting analysis. Moreover, an increase in the *Bifidobacterium* group was detected as well, particularly in fraction 5 from the 15 min time point, again confirming the T-RFLP data (Fig. 6 A-II). An increase in the

relative abundance in fraction 5 was detected also for other members of *Clostridium* cluster XIVa, particularly in the groups of *Ruminococcus obeum*, *Coprococcus eutactus* and *Eubacterium rectale,* 15 min after addition of [U-^{13}C]-inulin (Fig. 6 A-II).

For the [U-^{13}C]-lactose study, fractions containing labeled (fraction 5) and unlabeled (fraction 7) RNA from 30 min and 1 h time points were included in the HITChip analysis, as an increase in the SCFA concentrations was detected only 30 min after ^{13}C-lactose addition. Cluster analysis of the HITChip profiles from the selected fractions indicated clear separation of the profiles of the fractions containing labeled RNA from those with unlabeled RNA (data not shown). In the fractions with heavy RNA, a strong increase in the group of *Actinobacteria* (Fig. 6 B-I), particularly *Bifidobacterium* and to a lesser extend *Collinsella,* was detected (Fig. 6 B-II), confirming the T-RFLP analysis. Additionally, an increase in *Prevotella melaninogenica, Prevotella oralis* and *Mitsuokella multiacida* was observed 30 min after [U-^{13}C]-lactose addition, however, after 1 h these groups increased also in the fractions containing unlabeled RNA (Fig. 6 B-II).

One hour after addition of [U-^{13}C]-lactose, an increase in *Clostridium* cluster XIVa was detected in the fractions with labeled RNA (Fig. 6 B-I), particularly populations belonging to *Ruminococcus obeum*, *Dorea formicigenerans, Clostridium symbiosium* and *Anaerostipes caccae* (Fig. 6 B-II).

Discussion

The human gut microbiota plays an important role in maintaining human health, among other mechanisms also through its ability to digest food compounds and to produce specific metabolites. Particularly in the human colon, a number of food components that escape digestion in the upper GI tract, serve as substrate for the colonic microbiota. Inulin is one of these complex compounds, and the vast majority (around 95% of the consumed polymer) reaches the colon in an unmodified form (Nilsson and Bjorck, 1988; Bach Knudsen and Hessov, 1995). A number of *in vitro* and *in vivo* animal and human studies have demonstrated a stimulatory effect of inulin not only on the colonic bifidobacterial population (Roberfroid et al., 1998; Kolida et al., 2002; Gibson et al., 2004), but also on butyrate production (Campbell et al., 1997; Le Blay et al., 1999; Tsukahara et al., 2003). Besides the above-mentioned complex molecules, also less complex carbohydrates can escape digestion in the stomach and the small intestine, and be further processed in the colon.

One example is the disaccharide lactose, which is normally digested in the small intestine by the enzyme lactase (Arola and Tamm, 1994), however, in individuals with insufficient or lack of enzyme activity, lactose is mostly fermented in the large intestine. Furthermore, recent studies have indicated a role of the colonic microbiota in lactose intolerance (He et al., 2006). Nevertheless, there is still lack of knowledge with respect to the key microbes involved in colonic breakdown of different relevant dietary carbohydrates. In the present study RNA-based SIP was used to identify bacteria involved in the fermentation of [U-^{13}C]-inulin and [U-^{13}C]-lactose under human colon like conditions as implemented in an *in vitro* model of the large intestine. Previously, using the same fecal inoculum, i.e. the same microbial community, we identified microbial populations involved in the fermentation of [U-^{13}C]-starch (Kovatcheva-Datchary et al., 2009b). Furthermore, based on phylogenetic microarray (HITChip) and sequence analysis of 16S rRNA gene fragments, we were able to show that the model we used in our experiments well-represents the human fecal microbiota present in human volunteers. In the present study, primary [U-^{13}C]-inulin and [U-^{13}C]-lactose degraders were identified by T-RFLP fingerprinting, and further characterized by HITChip analysis. These active populations were different from those previously shown to be involved in degradation of [U-^{13}C]-starch.

Microbial taxa involved in colonic inulin fermentation

Fingerprinting analysis of fractions containing heavy and light RNA indicated a strong increase in relative abundance of *Bifidobacterium adolescentis* 15 min after addition of inulin. However, this effect was observed in all fractions from this particular time point. This could be related to a G+C-bias that is known to affect SIP analyses, especially concerning populations with high G+C content in their nucleic acids, such as bifidobacteria (Rangel-Castro et al., 2005). However, 30 min after substrate addition no increase in the T-RF representing *B. adolescentis* was detected in fractions with higher density, arguing against a possible G+C bias. This observation could be confirmed by HITChip analysis, which also indicated an increase in the *Bifidobacterium* group in fraction 5 (i.e. heavy RNA) at 15 min, in comparison with the fraction with light RNA, and no raise after 30 min. Furthermore, quantitative real time PCR analysis was used to confirm the increase in *Bifidobacterium* in the heavy fraction at 15 min, but not after 30 min (data not shown). This temporal trend is in line with that observed for lactate production,

which peaked after 15 min, followed by a decrease after 30 min. Additionally, an increase in butyrate was observed at the same time when lactate decreased, which is an indication for a cross-feeding in the studied microbial community (Duncan et al., 2004a; Belenguer et al., 2006). Hence, our molecular data, in combination with metabolite analysis, indicate that *Bifidobacterium* spp. are mostly active in the first 15 min of the inulin fermentation, contributing to the lactate pool formed by inulin fermentation. This is in good agreement with the well-established bifidogenic effect of inulin (Kleessen et al., 1997; Falony et al., 2006; Kleessen et al., 2007; van de Wiele et al., 2007; Ramirez-Farias et al., 2009).

By analysing the temporal development of the microbiota in the heavy fractions following addition of the [U-^{13}C]-inulin, we found with HITChip analysis an increase of *Clostridium* cluster XIVa after 15 min. Particularly, the biggest difference between heavy and light fractions was observed for *Dorea formicigenerans* et rel., which includes *Dorea longicatena*. With T-RFLP analysis an increase in a T-RF assigned to *Dorea longicatena* in the labeled fractions was also detected. It thus seems that this member of *Clostridium* cluster XIVa is another key inulin degrader in the studied model. This extends previous studies that pointed to the involvement of members of this particular cluster in inulin degradation (Duncan et al., 2003; Langlands et al., 2004). *Dorea* species were recently designated in a novel genus (Taras et al., 2002), with *Dorea longicatena* and *Dorea formicigenerans* being the best described members. It has been described that *Dorea* spp. can form lactate as end product from glucose depending on the growth conditions, but not butyrate (Taras et al., 2002). However, *Dorea longicatena* might play an important function in the gut as in a recent study it has been reported to be one of the species that has a higher ratio of adult- as well as infant gut-enriched genes than other environmental bacteria (Hattori and Taylor, 2009).

Inulin is known to increase butyrate production in the colon, which could be due to several mechanisms. Firstly, butyrate could be produced directly in the colon by butyrate producing bacteria, mostly reported to be members of *Clostridium* cluster IV (e.g. *Faecalibacetrium prausnitzii*) and *Clostridium* cluster XIVa (e.g. *Roseburia intestinalis*, *Roseburia inulinivorans*, *Eubacterium rectale*) some of which have previously been reported to utilize inulin *in vitro* (Duncan et al., 2004b; Ramirez-Farias et al., 2009; Louis et al., 2010). Secondly, cross-feeding between different members of the colonic microbiota has been suggested as another possible mechanism for increased butyrate production in the colon (Kanauchi et al., 1999; Pryde et al., 2002; Duncan et al., 2004a; Belenguer et al., 2006). Nevertheless, fully

labeled butyrate was not detected at the beginning of the [U-^{13}C]-inulin fermentation. The mainly detected butyrate isotopomer was the M+2 isotopomer, which can be formed by coupling of one fully ^{13}C-labeled acetate to unlabeled acetate, or alternatively from lactate. This indicates that the butyrate produced in the studied system most probably is derived from cross-feeding. In contrast, the major acetate and propionate isotopomers from the ^{13}C-inulin fermentation were fully labeled M+2 acetate and M+3 propionate. These isotopomers accumulated from the beginning of the fermentation and most probably resulted from the direct degradation of inulin by *Dorea longicatena* and *Bifidobacterium adolescentis*.

Butyrate can not be produced by bifidobacteria (Makras et al., 2006), however, lactate, acetate, formate, ethanol and small amounts of succinate are known products of the fermentative metabolism of these bacteria (Wolin et al., 1998; van der Meulen et al., 2004; van der Meulen et al., 2006). It is tempting to speculate that in our system the butyrogenic effect is the result of a cooperative food-web, comprising *Dorea longicatena, Bifidobacterium adolescentis*, and species related to *Coprococcus eutactus* and *Eubacterium rectale*, found to be abundant in both labeled and unlabeled RNA fractions. Previous studies reported that *Coprococcus* related species are net producers of acetate, and harbor butyrate kinase and butyryl-CoA:acetate-CoA transferase activity, indicating that these species can produce butyrate directly, but also via acetate (Duncan et al., 2002; Duncan et al., 2004b). Most probably, *D. longicatena* and *B. adolenscentis* constitute initial inulin degraders, producing acetate, propionate and lactate. These metabolites could then be used by the known butyrate-producer *Eubacterium rectale* and possibly additional members of *Clostridium* cluster XIVa, also known as butyrate-producers. As the main detected butyrate isotopomer was the M+2 isotopomer, probably no direct conversion of inulin into butyrate took place. Several studies suggest that a considerable part of the lactate in the human colon is converted to butyrate (Duncan et al., 2004a; Bourriaud et al., 2005; Belenguer et al., 2006). This is experimentally supported in the present study, as in the first 15 min of the fermentation a considerable amount of lactate is accumulated, which decreased again after 30 min, concomitant with detection of the M+2 butyrate isotopomer, supporting our hypothesis for possible cross-feeding. However, further detailed isotopomer kinetic analysis is required to discriminate between M+2 acetate and M+3 lactate as precursors for M+2 butyrate. A decrease in the *Clostridium* cluster IV and *Bacteroidetes* group in the fractions with heavy RNA in comparison with the light fractions was observed for all the analyzed time points, indicating that these groups are not directly involved in inulin fermentation in

the studied system, which is in line with human *in vivo* trials (Kleessen et al., 1997; Kleessen et al., 2007).

Fermentation of lactose under human colon-like conditions

In a second set of incubations, we studied lactose fermentation. Lactose is a disaccharide, which becomes available to the colonic microbiota only in case of specific disaccharide-digestion deficiencies or as a result of defects in the corresponding transport system (Southgate, 1989). The importance of studying lactose in our study is mostly related to the lack of knowledge on the effect of this substrate on the colonic microbiota. This is surprising in view of the fact that lactose mal-digestion is very common around the world (Sahi, 1974; Scrimshaw and Murray, 1988; Sahi, 1994). T-RFLP analysis indicated a strong increase only in the population related to *Bifidobacterium adolescentis* during the whole *in vitro* fermentation in fractions containing labeled and unlabeled RNA. However, HITChip analysis, providing compositional data at higher resolution than T-RFLP fingerprinting, pointed towards enrichment of 16S rRNA of several other taxa in the fraction containing heavy RNA, 30 min after addition of the labeled substrate. The biggest changes were detected in the *Actinobacteria* group, where an approximately 3 fold increase in the relative contribution to the total signal was observed for *Bifidobacterium* and *Collinsella* populations. This increase reached 4-fold in the heavy fraction 1 h after [U-^{13}C]-lactose addition. The bifidogenic effect of lactose has been reported in several studies, addressing the direct effect of lactose (Makivuokko et al., 2006), oligosaccharides derived from lactose (Cardelle-Cobas et al., 2009), or fermented milk (Sairanen et al., 2007). The increase of *Bifidobacterium* and *Collinsella* spp. in the fractions with heavy RNA from the beginning of the experiment indicates that these species represent the key players in lactose degradation in the studied *in vitro* model. Moreover, during the first hour of [U-^{13}C]-lactose fermentation, a strong increase in the production of fully labeled acetate and lactate was detected, which most probably were derived from the direct conversion of [U-^{13}C]-lactose by the two members of the *Actinobacteria*, that are known to convert carbohydrates to acetate, lactate and formate (Kageyama and Benno, 2000; Jacobs et al., 2010). It has been suggested that members of the genus *Collinsella* are an indicator of a healthy intestinal microbiota as a higher incidence of *Collinsella aerofaciens* was found in subjects with a low risk of colon cancer (Moore and Moore, 1995), and a lower abundance of *Collinsella* sp. has been associated with samples from irritable bowel syndrome (IBS) subjects (Kassinen et al., 2007). *Prevotella melaninogenica* is another member

of the TIM-2 microbiota, which was observed to increase in the heavy fraction 30 min after addition of [U-^{13}C]-lactose as compared to the light fractions from the same time point. Contradictory to this, after 1 h, *P. melaninogenica* was detected to increase only in the fractions containing light RNA comparing with the 30 min time point. A similar trend was also observed for other members of the *Bacteroidetes,* including *Prevotella oralis* and *Mitsuokella multiacida.* The latter has recently been reclassified to *Clostridium* cluster IX (Jousimies-Somer and Summanen, 2002) but is a known acid-utilizing bacteria (Tsukahara et al., 2002). *M. multiacida*, probably together with the two *Prevotella* spp., is involved in propionate production either directly from lactose, or alternatively via the acrylate pathway, during which lactate is converted to propionate. Both metabolic routes lead to fully labeled propionate, which was indeed the major detected propionate isotopomer.

T-RFLP analysis indicated 16S rRNA of populations related to *Ruminococcus obeum*, *Eubacterium rectale* and a member of *Clostridium* cluster XIVa to be abundant in all fractions from the 0 h time point, but also in all fractions containing unlabeled RNA after the first 15 min of the experiment. The HITChip profiles did not indicate differences in *Clostridium* cluster XIVa between the fractions with heavy and light RNA after 30 min. However, after 1 h an approximately 3-fold increase of *Clostridium* cluster XIVa was detected in the labeled fraction compared to the 30 min time point fraction with labeled RNA. A recent study in which 11 lactose-intolerant subjects were fed a diet supplemented with yogurt and bifidobacteria, reported an increase in the *Eubacterium rectale*/*Clostridium coccoides* group as determined by fluorescent *in situ* hybridization (FISH) analysis of the fecal microbiota (He et al., 2008). The detected raise in *Clostridium* cluster XIVa in the current study was largely attributed to groups known as butyrate-producers, such as *Clostridium symbiosium* and *Anaerostipes caccae,* the latter of which is a well studied lactate-utilizer, known to convert lactate to butyrate (Belenguer et al., 2006). These two groups might be involved in a similar food web as the one detected in the inulin study, where the produced lactate was further converted to butyrate. In accordance, an increase in the M+2 isotopomer of butyrate was detected also 1 h after beginning of the lactose fermentation. Besides this, some other members of *Clostridium* cluster XIVa, including *Ruminococcus obeum* and *Dorea formicigenerans,* which also contributed to the increase in *Clostridium* cluster XIVa observed after 1 h in the fractions with heavy RNA, are likely to be involved in the propionate production. The increase detected in the propionate M+1, M+2 and M+3 isotopomers, particularly at 1 h after [U-^{13}C]-lactose addition, supports this conclusion.

In conclusion, to explore the *in situ* functions of the human colonic microbiota we applied RNA-based SIP in combination with high-throughput microarray-based phylogenetic profiling and metabolic flux analysis. This allowed us to identify bacteria directly involved in the fermentation of relevant dietary carbohydrates under conditions simulating those in the human colon. Human feeding trials, in which carbohydrates are delivered directly to the human colon, are going to reinforce the great potential of this methodology for the elucidation of metabolic interactions in complex food webs such as represented by the colonic microbiota.

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CHAPTER 4

Cross-Feeding Between *Ruminococcus bromii* and *Eubacterium rectale* During Anaerobic Degradation of Potato Starch

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Abstract

Resistant dietary carbohydrates, including potato starch, are not digested in the upper gastrointestinal (GI) tract, but are fermented to short-chain fatty acids (SCFAs) and other organic acids by the colonic microbiota. These substrates are comprised of a complex assortment of macromolecules with diverse structures, the degradation of which requires an array of hydrolytic enzymes produced by various bacteria. Here we examine the ability of two bacterial species belonging to the *Firmicutes* that are abundant in the human colon, *Eubacterium rectale* and *Ruminococcus bromii*, to convert potato starch in mono- and co-cultures. Previously we identified populations closely related to these organisms as key players in starch degradation in an *in vitro* model of the human colon. The mono-culture experiments indicated that *R. bromii* is able to grow better on potato starch than *E. rectale.* The fermentation primarily yielded glucose, acetate, formate, hydrogen and ethanol. In contrast, pure cultures of *E. rectale* ferment potato starch poorly and yield only small amounts of lactate and propionate in the initial phase of growth. However, during the co-cultivation of both species, lower amounts of glucose and ethanol accumulated than in the *R. bromii* mono-culture, and a strong increase in propionate and acetate production was detected. Quantitative PCR indicated an increase in *R. bromii* 16S rRNA gene copy number in both mono- and co-culture studies. Increase in the *E. rectale* copy number was detected only in the co-culture. In conclusion, potato starch fermentations with mono- and co-cultures of *R. bromii* and *E. rectale* confirmed that *R. bromii* is a key player in resistant starch conversions in the large bowel. A cross-feeding relation between both bacteria was found, where the conversion of carbohydrates by the co-culture resulted in the accumulation of acetate and propionate.

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Introduction

A vital function of the colonic microbiota is the utilization of dietary substrates, which have not been digested by the human digestive enzymes in the upper gastro-intestinal (GI) tract and reach the large intestine, where they are fermented by the colonic microbiota (Savage, 1986; Guarner and Malagelada, 2003; Xu and Gordon, 2003). The end products from these microbial fermentations are short-chain fatty acids (SCFA), such as butyrate, propionate and acetate, which have an important function in the host physiology (Scheppach, 1994; Mortensen and Clausen, 1996). Fermentation of non-digested carbohydrates causes a lowering of the luminal pH, increase in bacterial biomass and fecal bulk, and modification of the microbial composition, especially by stimulating the growth of potentially beneficial bacteria, including bifidobacteria and lactobacilli (Young and Le Leu, 2004; Le Leu et al., 2005). Resistant starch (RS) is such a dietary compound, which provides a source of fermentable substrate for the colonic microbiota (Cummings and Englyst, 1987; Asp, 1992; Topping et al., 2003). Moreover, RS has been shown to reduce the accumulation of harmful by-products from protein fermentation (Birkett et al., 1996). Several animal studies have reported that RS also can modify the colonic microbiota and may reduce the risks of developing colorectal cancer (Silvi et al., 1999; Bauer-Marinovic et al., 2006; Toden et al., 2006; Clarke et al., 2008). The effect of RS on human gut microbiota is however unclear, but essential to understand gut microbiota functionality in the colon. In a previous *in vitro* study using 16S ribosomal RNA (rRNA)-based stable isotope probing (SIP) we identified a population closely related to *Ruminococcus bromii* as the primary degrader of potato starch, a specific type of RS (Kovatcheva-Datchary et al., 2009). Furthermore, after integration of molecular and metabolite data we obtained evidence for a cross-feeding process during the fermentation of potato starch, where *R. bromii* is the primary starch degrader and *Eubacterium rectale* is further involved in the trophic chain. In the present study we addressed the role of *R. bromii* and *E. rectale* growing in pure cultures and in co-culture in the degradation of potato starch and explored the cross-feeding relationship between the two species.

Experimental Procedures

Microorganisms and media

R. bromii (ATCC 51896) and *E. rectale* (ATCC 33656) were purchased from the American Type strain Culture Collection (ATCC). Both bacterial cultures were maintained in a medium composed of the following (concentration per 1 L): 1 g cellobiose, 4 g glucose, 1 g maltose, 1 g starch, 50 ml rumen fluid, 12.5 g beef extract (Oxoid, Basingstoke, UK), 30 g peptone (Oxoid), 5 g yeast extract (Oxoid), 1 mg resazurin, 0.5 g L-cystein, 0.005 g Vitamin K_1 and 5 mg hemin, 150 ml mineral solution I (composition per 1 L solution – 6 g K_2HPO_4), 150 ml mineral solution II (composition per 1 L solution – 6 g KH_2PO_4, 12 g $(NH_4)SO_4$, 12 g NaCl, 1.2 g $MgSO_4$, 1.2 g $CaCl_2$) and 2 ml trace element solution (composition per 100 ml solution - 0.5 mg $ZnSO_4.7H_2O$, 0.15 mg $MnCl_2.4H_2O$, 1.5 mg H_3BO_3, 1 mg $CoCl.6H_2O$, 0.1 mg $NiCl_2.6H_2O$, 0.15 mg $Na_2MO_4.2H_2O$, 7.5 mg $FeCl_2.4H_2O$). The pH of the medium was adjusted to 6.7 with 1M NaOH, the medium was shortly boiled to remove O_2 and cooled down under oxygen-free N_2 flow to prevent oxygen diffusion into the medium prior to distribution into 120-ml serum bottles. Subsequently, the gas phase in the closed serum bottles was replaced by a gas mixture of H_2/CO_2 (80/20 % v/v). The bacterial cultures were stored at - 80˚C in serum tubes containing the media mentioned earlier, supplemented with 25% (vol/vol) glycerol as a cryoprotectant.

Fermentation experiments

Mono- and co-culture fermentations were carried out in a basal bicarbonate-buffered medium composed of the following (concentration per 1 L): 0.9 g K_2HPO_4, 0.9 g KH_2PO_4, 1.8 g $(NH_4)SO_4$, 1.8 g NaCl, 0.09 g $MgSO_4$, 0.09 g $CaCl_2$, 4 g $NaHCO_3$, 2 g yeast extract (Oxoid), 1 mg resazurin, 1 g L-cystein, 5 mg Vitamin K_1 and 5 mg hemin, 2 ml trace elements (composition as mentioned above). The pH of the medium was adjusted to 6.5. Before the medium were distributed to 120-ml serum bottles 2.5 g of potato starch (Sigma, Cat. No.S4251, Zwijndrecht, The Netherlands) was added. Subsequently, the gas phase in the closed serum bottles, which contained 40 ml of the fermentation media, was replaced by a gas mixture of H_2/CO_2 (80/20 % v/v). Before inoculation 2.5 ml of filter-sterilized calcium-vitamins solution and the same volume of bicarbonate solution were added to the batches. Additionally for the ^{13}C-glucose fermentation filter sterilized 1M [U-^{13}C]-glucose (Sigma, Cat. No.389374) was added to a final concentration of 20 mM to the serum bottles as a sole

C-source. The calcium-vitamins solution composition was as follow (concentration per 100 ml): 0.22 g $CaCl_2.2H_2O$, 0.20 g $MgCl_2.6H_2O$ and 2 ml vitamins solution. The vitamins solution had the following composition (per 100 ml solution): 20 mg biotin, 100 mg pyridoxamine, 50 mg thiamine, 50 mg nicotinamide, 50 mg cobalamin, 50 mg p-aminobenzoic acid, 50 mg lipoic acid, 20 mg folic acid and 50 mg riboflavine. The bicarbonate solution contained 0.88 g NH_4HCO_3, 7.46 g $NaHCO_3$ and 0.40 g Na_2SO_4 dissolved in 100 ml demineralized water. Inocula were prepared as follows: strains were transferred from -80˚C stock culture to maintenance medium and incubated anaerobically at 37˚C for 48 h. During the fermentation experiment the transferred volume was 10% (vol/vol). Before being transferred to the fermentation media *R. bromii* and *E. rectale* were subcultured twice on maintenance medium and also on fermentation medium to be adapted to the experimental condition. The purity of the culture was checked by molecular and microscopic analysis. The fermentation of potato starch was followed for 96 h at 37˚C. Triplicate bottles were inoculated, and samples were taken from the same bottles at 0 h, 2 h, 6 h, 10 h, 24 h, 48 h, 72 h and 96 h to measure metabolite production, protein content and for quantification of the two bacterial species in mono- and co-cultures. All samples were stored at -20°C for further analysis.

DNA extraction and quantitative PCR

One milliliter aliquots of well-homogenized microbial cultures were concentrated by centrifugation (15 700 RCF, 5 min). DNA was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals, Germany) with mechanical cell disruption by repeated bead beating, following the instruction of the manufacturer.

Quantitative PCR (qPCR) was performed on an iCycler IQ real-time detection system associated with the iCycler optical system interface software version 2.3 (Bio-Rad, Veenendaal, the Netherlands). A reaction mixture (25 µL) consisting of 12.5 µL of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer set and 5 µL of the template DNA diluted in water (10-fold to avoid PCR inhibition). The standard curves for quantification of *R. bromii* and *E. rectale* were generated by using serially diluted 16S rRNA gene amplicons obtained from *R. bromii* (ATCC 51896) and *E. rectale* (ATCC 33656), respectively. All qPCR assays were performed in triplicate. Primers Rflbr730F and Clep866mR (Ramirez-Farias et al., 2009) were used to estimate the number of copies of the 16S rRNA gene of *R. bromii* in the samples from the mono- and co-cultures. PCR was performed with an initial denaturation step of 95ºC for 3 min, followed by 40 cycles of 95ºC for 30 s,

60ºC for 30 s and 72ºC for 30 s. Primers E.rec-F and E.rec-R (Balamurugan et al., 2008b; Balamurugan et al., 2008a) were used to investigate the number of copies of the 16S rRNA gene of *E. rectale* in the samples collected during the potato starch fermentation in mono- and co-cultures with the following PCR program: an initial denaturation step of 95ºC for 10 min, followed by 44 cycles of 95ºC for 30 s, 60ºC for 30 s and 72ºC for 30 s.

Analytical procedures

pH and protein concentration

The high starch concentration interfered with the ability to assess bacterial growth in the mono- and co-cultures directly, by following the absorption at 600 nm (OD_{600}). Therefore, Lowry colorimetrical protein assay was applied to follow bacterial growth in mono- and co-cultures. For this purpose 1 ml bacterial suspension was immediately centrifuged after sampling for 15 min at 15 700 RCF in the presence of 1 ml 0.6 M NaCl. Subsequently, cell pellets were hydrolyzed by adding 1 ml of 1 M NaOH and incubating for 30 min at 50ºC. The hydrolysates were further analyzed for protein content following the Lowry protein assay (Lowry et al., 1951). Additionally, as an indication for bacterial growth and metabolite production, the pH of the mono- and co-cultures of *R. bromii* and *E. rectale* during the potato starch fermentation was measured in 1 ml bacterial culture.

Analysis of the produced metabolites during the fermentation of potato starch and ^{13}C-glucose

For the potato starch fermentation 1 ml aliquots of well-homogenized microbial cultures were harvested by centrifugation (15 700 RCF, 15 min). Subsequently 500 µl of the supernatant were transferred into a crimp cap vial and was mixed with 500 µl 0.2N H_2SO_4 solution containing 20 mM crotonate as internal reference standard. The metabolites produced during the fermentation of potato starch were determined by injection of 20 µl supernatant into a high pressure liquid chromatography (HPLC) equipped with refractive index detector (Spectra System, RI-150) and CromoQuest 4.2.34 software. We used an organic acid column MetaCarb 67H (6.5 by 300 mm, Varian Inc., Palo Alto, USA), maintained at 30ºC, and 0.01N H_2SO_4 as eluent at a flow rate of 0.8 ml/min. Quantification of organic acids, glucose and ethanol was done by using calibration curves of acetate, propionate, lactate, formate, glucose and ethanol in concentrations between 2.5 and 100 mM.

During the [U-^{13}C]-glucose fermentation 5 ml aliquots of well-homogenized microbial cultures were harvested by centrifugation (15 700 RCF, 15 min). To the total volume of the collected sample 0.5 ml D_2O was added in a 10 mm NMR tube. ^{13}C NMR spectra were recorded at room temperature on a Bruker AV 300 wb spectrometer located at the Wageningen NMR Centre. Chemical shifts were measured relative to β glucose C-1 calibrated at 96.8 ppm. Additionally, the metabolites production from the ^{13}C-glucose fermentation was determined by HPLC as described above.

Results

Bacterial growth during potato starch fermentation

The anaerobic degradation of potato starch by *R. bromii* and *E. rectale* in pure cultures was determined and compared with that in co-culture experiments (Fig. 1). The inoculum ratio used for the co-culture fermentation was 1:1. The growth was followed by protein determinations and no significant growth of *E. rectale* was observed during 96 h of incubation (Fig. 1 A). However, a two-fold increased protein yield was observed in the samples from the *R. bromii* mono-culture and the co-culture (Fig. 1 A). The pH of the mono-culture of *E. rectale* did not change during the whole fermentation, while for the mono-culture of *R. bromii* the pH dropped from 6.5 to 5.5 (Fig. 1 B) until the end of the experiment. A strong decrease in the pH value was observed for the co-culture, where it changed from 6.5 to 4.25 (Fig. 1 B).

Quantification of R. bromii and E. rectale populations in mono- and co-cultures during the fermentation of potato starch

Real-time PCR analysis indicated an increase in *R. bromii* 16S rRNA gene copy numbers 24 h after the beginning of the incubation in the mono-culture, and after 48 h in the co-culture fermentation. At the end of the experiment the quantified *R. bromii* copy number in the co-culture incubation were approximately 2-fold more than in the monoculture (Fig. 2 A). In corroboration with the data from the protein assay, *E. rectale* showed poor growth in the mono-culture on potato starch from the beginning of the study. A 2-fold increase in the *E. rectale* 16S rRNA gene copy number in the mono-culture was measured only 10 h after incubation with potato starch, followed by a fast decrease until the end of the experiment, probably as a result of cell lysis (Fig. 2 B). However, in the co-culture incubation a 3-fold increase was measured in

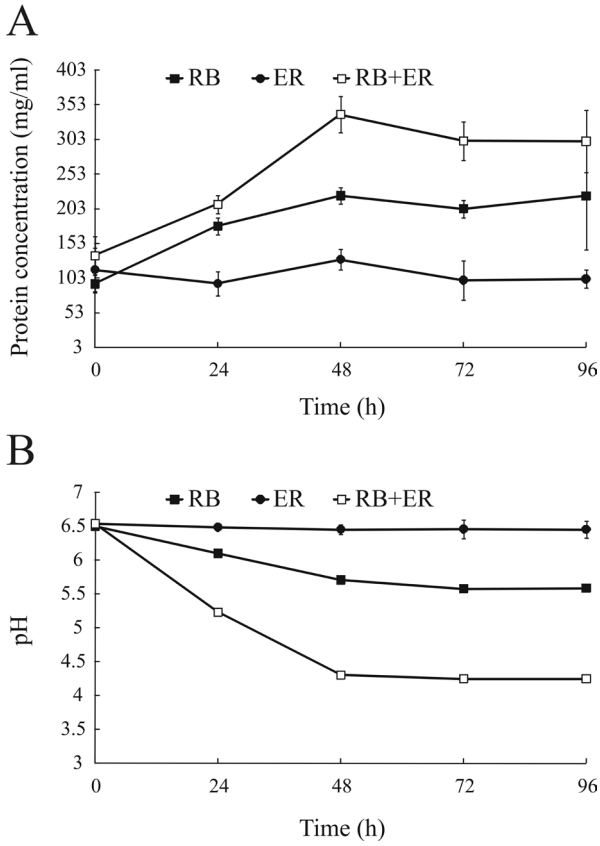


Figure 1. Protein concentration (**A**) and pH (**B**) changes during the anaerobic degradation of potato starch from *Ruminococcus bromii* (RB) and *Eubacterium rectale* (ER) in mono- and co-cultures. Error bars represent the standard deviation of triplicate measurements. When the standard deviation is smaller than the symbol, it is not presented.

the *E. rectale* 16S rRNA gene copy number at the end of the experiment (Fig 2 A). The increase in the number of 16S rRNA gene copies of both bacteria during the co-culture incubation corroborate with the data from the protein assay and observed acidification of the media (Fig 1).

Quantification of metabolites produced during the anaerobic conversion of potato starch in mono- and co-cultures of R. bromii and E. rectale

The utilization of potato starch by the mono-culture of *R. bromii* yielded glucose (~ 110 mM), ethanol (~ 16 mM), acetate (~ 10 mM) and formate (~ 7 mM) (Fig. 3). During the first 10 h of the *R. bromii* mono-culture incubation an approximately 3-fold increase of only ethanol was observed (Fig. 3 F). From 24 h, a strong accumulation of continuously produced glucose was detected until the end of the experiment (Fig.

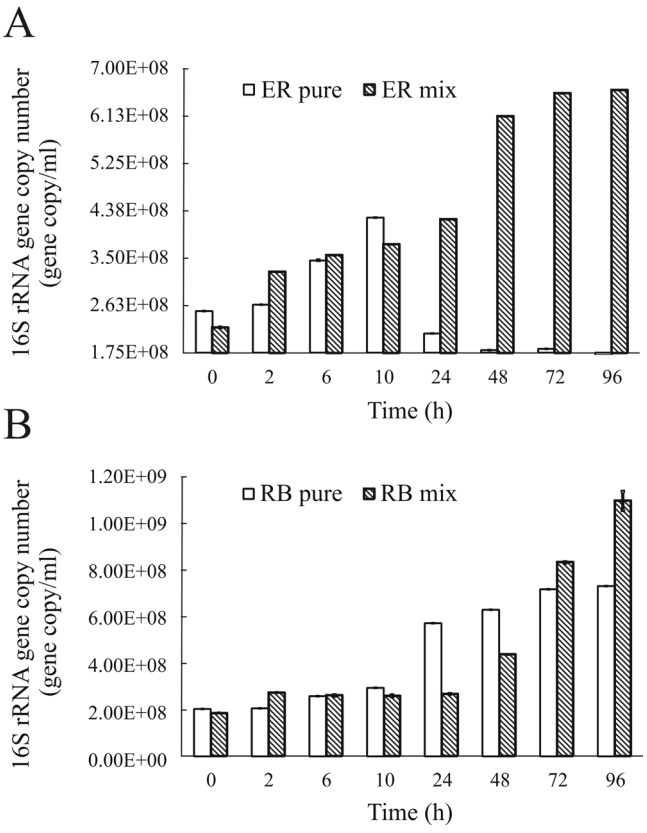


Figure 2. Changes in the 16S rRNA gene copies of *Eubacterium rectale* (ER) (**A**) and *Ruminococcus bromii* (RB) (**B**) during the anaerobic degradation of potato starch in mono- and co-cultures. Error bars represent the standard deviation of triplicate measurements. When the standard deviation is smaller than the symbol, it is not presented.

3 A). Additionally, an increase in acetate production and appearance of formate was observed after 24 h of incubation in the presence of potato starch. The acetate concentrations increased from about 2.5 mM after 10 h to 4.3 mM after 24 h, and at the end of the experiment approximately 10 mM acetate were measured in the *R. bromii* mono-culture fermentation (Fig. 3 B). About 6.5 mM formate was formed after 24 h, while at the end of the incubation only small changes in the formate concentration were measured (Fig. 3 D). During potato starch utilization by *E. rectale* mono-cultures, small amounts of lactate were detected, with higher concentration than in the co-culture after 10 h of incubation (3.5 mM, Fig. 3 E). Additionally, in the *E. rectale* mono-culture, about 2.6 mM lactate was detected from the beginning of the experiment, which most probably was residual from the inoculum (Fig. 3 E). The level of lactate detected in the *E. rectale* mono-culture increased within 2 h, but thereafter decreased again. These results confirm data obtained by the protein assays,

pH measurements and qPCR analyses, indicating that *E. rectale* grows poorly in high concentration of potato starch.

The metabolites pattern of the conversion of potato starch in the co-culture was different from that obtained with the different mono-cultures. The co-culture incubation yielded glucose (~ 22 mM), ethanol (~ 9 mM), acetate (~ 27 mM), formate (~ 6 mM), propionate (~ 55 mM) and lactate (~ 3 mM) (Fig 3). During the incubation of potato starch in the co-culture, the detected glucose concentration was 5-fold lower than the levels detected in the *R. bromii* mono-culture (Fig. 3 A). Similarly, ethanol production was observed to increase in the *R. bromii* mono-culture after 24 h and while initially about 4.9 mM ethanol was found, at the end of the experiment its concentration increased to 16 mM (Fig. 3 F). However, in the co-culture incubation after 24 h the concentration of ethanol was similar to that in the mono-culture (~ 4.9 mM), but at the end of the experiment approximately 8.9 mM of ethanol were measured (Fig. 3 F). No changes in the formate pattern in comparison with the *R. bromii* mono-culture were observed in the co-culture incubation (Fig. 3 D). However, a strong increase in the production of acetate and particularly propionate was observed in the co-culture. Accumulation of propionate in the *E. rectale* and *R. bromii* mono-culture incubations with potato starch was not observed. In contrast, in the co-culture a strong increase in the propionate concentration was measured after 48 h, which reached approximately 55 mM at the end of the study (Fig. 3 C). The level of acetate after 48 h in the co-culture with potato starch was approximately 3.5 fold higher in comparison with the mono-culture of *R. bromii* (Fig. 3 B). During the potato starch utilization by the co-culture, no lactate was detected at 0 h, the level of lactate after 6 h was about 2.3 mM and it reached approximately 3.2 mM at the end of the incubation (Fig. 3 E).

H_2 was calculated from the redox balance. The fermentation balance calculated for the *R. bromii* mono-culture fermentation was as follows:

7.75 glucose → 7.8 acetate + 7.1 formate + 14.8 ethanol + 15.5 CO_2 + 8.5 H_2

Consequently, when growth is neglected in the mono-culture experiment the *R. bromii* forms 8.5 mM hydrogen. The fermentation balance calculated for the co-culture fermentation was as follows:

43.45 glucose → 24.7 acetate + 6.4 formate + 3.2 lactate + 49.4 propionate + 7.7 ethanol + 26 CO_2

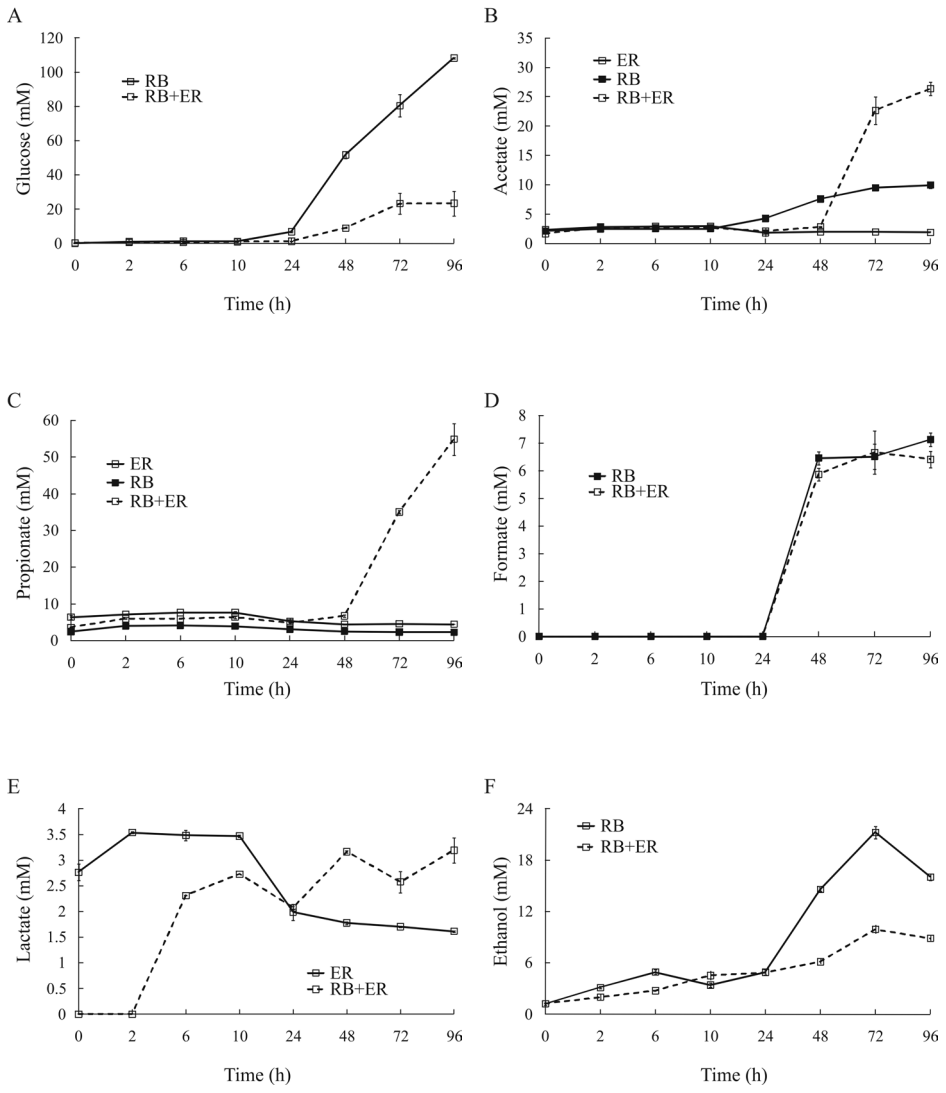


Figure 3. Metabolites produced from the coversion of potato starch by mono- and co-cultures of *Ruminococcus bromii* (RB) and *Eubacterium rectale* (ER). (**A**) glucose; (**B**) acetate; (**C**) propionate; (**D**) formate; (**E**) lactate; (**F**) ethanol. Error bars represent the standard deviation of triplicate measurements. When the standard deviation is smaller than the symbol, it is not presented. Cultures for which specific metabolites were not detetced at any time throughout the incubation were not included in the respective graph.

Quantification of the metabolites produced during the fermentation of [U-^{13}C]-glucose in mono-culture of R. bromii and co-culture of *R. bromii* and *E. rectale*

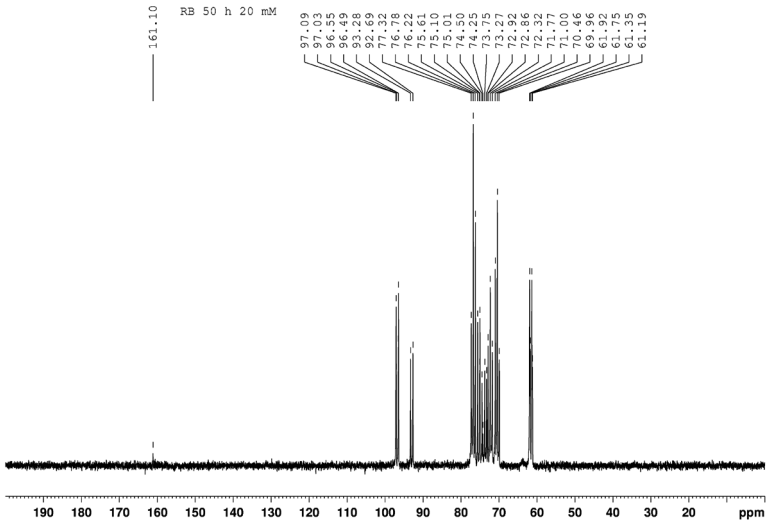
The HPLC data showed that *R. bromii* is able to degrade potato starch, however, strong accumulation of glucose was observed until the end of the experiment, which could be an indication that *R. bromii* can not grow on glucose or there is another factor, which limits the conversion of carbohydrates by *R. bromii.* Hence the conversion of ^{13}C-labeled glucose by *R. bromii* was tested and the metabolites formed were analyzed by NMR (Fig. 4). The NMR profiles of the mono-culture of *R. bromii* grown in presence of 20 mM of ^{13}C-glucose indicated poor conversion of glucose during 50 h of fermentation (Fig. 4 A-I). However, after 168 h of growth in the presence of ^{13}C-labeled glucose, we detected appearance of ^{13}C enriched acetate, formate and ethanol. But still the feature of the ^{13}C-labeled glucose was detected indicating that less than 60% of the added glucose is converted. Subsequently, a co-culture incubation of *R. bromii* and *E. rectale* (inoculated in ratio 1:1) in presence of ^{13}C-glucose was performed. The conversion of glucose by the co-culture was faster, and after 24 h the presence of enriched metabolites was detected, particularly acetate, propionate, succinate, lactate and ethanol (Fig. 4 B-I). In comparison with the *R. bromii* mono-culture no ^{13}C-glucose remained after 168 h of incubation (Fig. 3 B-II). Rather, after 50 h of incubation ^{13}C-glucose was already almost depleted (data not shown).

Discussion

The degradation of complex carbohydrates, including potato starch used here, requires a set of enzymatic activities, which are usually displayed by mixed microbial communities rather than by single species. In order to fully understand and explore the metabolic food-webs that are active within the human gut ecosystem, it is essential to identify bacteria that are key to the utilization of dietary carbohydrates. To this end, defined pure and mixed cultures are excellent tools to study the metabolism of individual bacterial populations as well as interspecies interactions (Belenguer et al., 2006; van der Meulen et al., 2006).

R. bromii is one of the predominant bacterial species in the human fecal microbiota and is known to ferment carbohydrates, but it was also reported to grow poorly on glucose (Moore et al., 1972; Herbeck and Bryant, 1974). A number of studies performed in the early 1980's pointed to the nutritional requirements of

A-I



A-II

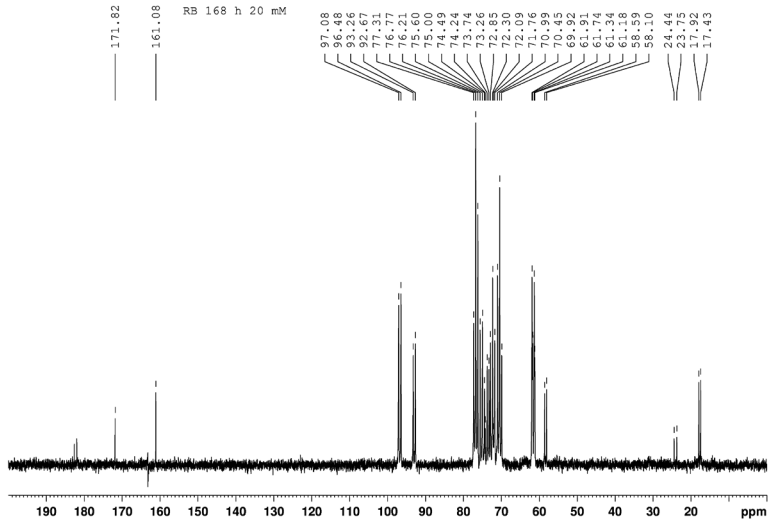
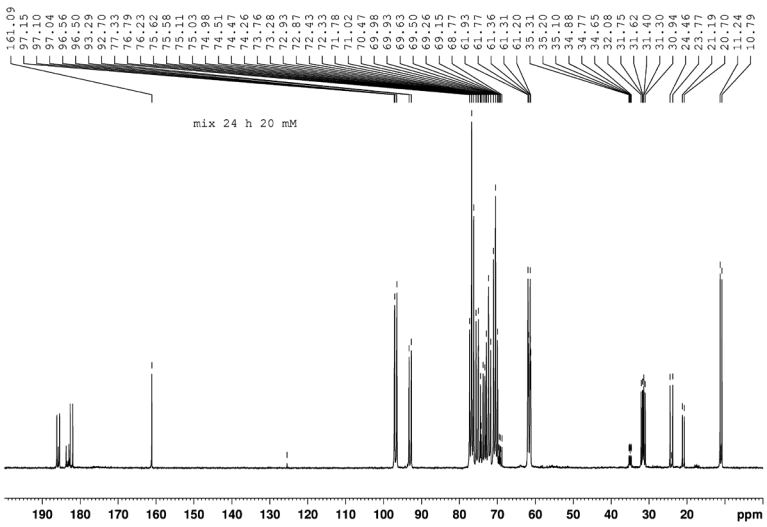


Figure 4 -A. Occurrence of ^{13}C-label as measured in NMR-spectra after 50 h (A-I) and 168 h (A-II) anaerobic conversion of ^{13}C-glucose by mono- culture of *Ruminococcus bromii* (RB). Chemical shifts (ppm): 61.19 – 97.09 glucose; 17.43, 17.92, 58.1 and 58.59 ethanol; 23.75, 24.44, and 181.9 acetate; 161.08 CO_2; 171.82 formate.

B-I



B-II

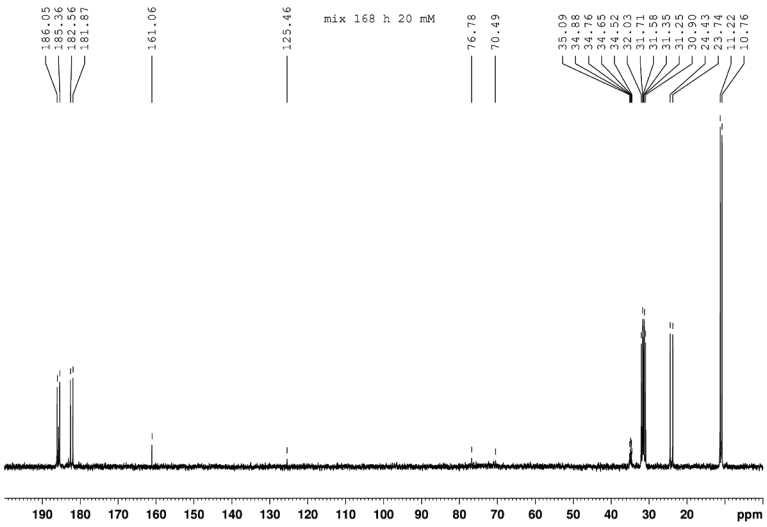


Figure 4 -B. Occurrence of ^{13}C-label as measured in NMR-spectra after 24 h (B-I) and 168 h (B-II) incubation of ^{13}C-glucose by co- culture of *Ruminococcus bromii* and *Eubacterium rectale* (mix). Chemical shifts (ppm): 61.19 – 97.09 glucose; 17.43, 17.92, 58.1 and 58.59 ethanol; 23.75, 24.44, and 181.9 acetate; 161.08 CO_2; 171.82 formate; 10.79, 11.24, 30.94-32.07, 185.4 – 186.1 propionate; 35.2 succinate; 68.7 and 69.3 lactate.

this bacterium and its strong amylolytic capacity (Moore et al., 1972; Herbeck and Bryant, 1974), but the potential of this species to degrade complex carbohydrates was not clarified (Moore et al., 1972; Leitch et al., 2007). Recent studies reported on the effect of RS on human colonic microbiota *in vivo* and *in vitro,* and further suggested *R. bromii* as key player in the conversion of RS (Leitch et al., 2007; Abell et al., 2008; Kovatcheva-Datchary et al., 2009).

In the current study we explored the potential of *R. bromii* to degrade potato starch. Furthermore, a co-culture experiment with *R. bromii* and *E. rectale* was performed to get insight into interspecies interactions of the two bacteria. Our data indicated that *R. bromii* degrades potato starch, whereas *E. rectale* hardly grew in mono-culture on this substrate. Furthermore, in the *R. bromii* mono-culture experiment considerable accumulation of glucose was detected until the end of the experiment, indicating that *R. bromii* has the enzymatic potential to break down potato starch to monosaccharides, whereas the further conversions of the generated glucose occurs only slowly. This was confirmed by the very inefficient utilization of glucose by a *R. bromii* in mono-culture (Fig. 4 A).

During utilization of potato starch by the co-culture, the level of accumulated glucose dropped significantly, indicating the further conversion of the glucose to other metabolites. The data from the qPCR analysis for the mono- and co-cultures pointed at a delay in the growth of *R. bromii*. Additionally, we also detected a delay in the accumulation of the produced metabolites (Fig 4 B). A possible explanation could be that growth of *R. bromii* is inhibited by the accumulation of products from the primary potato starch degradation. This could be caused by the increased production of H_2 during starch conversion. Similar effects were observed with *Ruminococcus albus* growing on cellulose (5 g) in mono- and co-culture (Miller and Wolin, 1995). In mono-culture *R. albus* ferments cellulose to acetate, ethanol, CO_2 and H_2. When a H_2-utilizing bacteria was grown together with *R. albus* an increased acetate formation and decreased ethanol formation was measured. We also observed a decrease in the ethanol production and an increase in the acetate formation of the co-culture during the degradation of potato starch, which is in relation to the effect mentioned above. *Ruminococcus* spp. are known to produce H_2 during the fermentation of carbohydrates (Miller and Wolin, 1973, 1979). In a separate experiment, mono- and co-cultures of *R. bromii* and *E. rectale* were grown in the presence of glucose, during which we observed the accumulation of H_2 in the mono-culture but its depletion in co-culture (data not shown). The inhibitory effect of H_2 is likely to prevent *R. bromii* from growing on glucose in mono-culture, and explains the further accumulation of glucose during the utilization of potato starch.

The strong increase in the level of accumulated glucose and the increase measured after qPCR analysis in the *E. rectale* population in the co-culture is strong evidence for an interaction between the two species. It is likely that *R. bromii* breaks down the potato starch to glucose, which is further used by *E. rectale* as carbon and energy-source. Another explanation for the decreased level of glucose in the co-culture could be that *E. rectale* utilizes the H_2 produced by *R. bromii* during the potato starch degradation and opening its capacity to convert the accumulated glucose.

Numerous species belonging to the *Clostridium* cluster XIVa (Collins et al., 1994) are able to utilize starch and produce butyrate (Barcenilla et al., 2000; Sharp and Macfarlane, 2000). *Clostridium* spp. are a very diverse group whitin the human gut bacteria, which based on 16S rRNA gene sequence analysis are distributed over ten distinct phylogenetic clusters, (Collins et al., 1994; Stackebrandt et al., 1999). *Ruminococcus bromii* is the first isolated human intestinal *Ruminococcus* species (Moore et al., 1972), which based on the 16S rRNA gene sequencing is grouping within one of these clusters known as *Clostridium* cluster IV (Collins et al., 1994). *Eubacterium rectale* belongs to the *Eubacterium* genus, similar to *Clostidium*, and further related to *Clostridium* cluster XIVa (Collins et al., 1994). It has been reported that butyrogenic species, mostly saccharolytic clostridia, better adapt to fast growth rate and high concentrations of the substrate, while for examples *Bacteroides* spp. compete better in complex communities at low growth rates under conditions of limiting substrate availability (Sharp and Macfarlane, 2000). However, *E. rectale* showed poor growth in the presence of 5 g of potato starch (Fig. 1). Similar results were obtained when even a higher concentration of RS (15 g) was used in a pilot human study (Schwiertz et al., 2002). Earlier studies with RS reported an increase in acetate, propionate and butyrate concentrations (Jenkins et al., 1998; Le Blay et al., 1999; Schwiertz et al., 2002). In the present study we detected high levels of acetate and propionate in the co-culture, but no accumulation of butyrate, neither in the mono-cultures nor in the co-culture. Unexpectedly, in the *E. rectale* mono-culture, the production of propionate from potato starch was detected. We could speculate that *E. rectale* does not produce butyrate under the tested conditions, because of the high potato starch concentration. This could be analogous to earlier observations showing that *Eubacterium* spp. do not play a major role in butyrate formation from high concentrations of RS *in vivo* (Schwiertz et al., 2002). Alternatively, it could be possible that the produced butyrate reported in previous studies is generated not from the carbohydrate sources but from other ingredients of the media (e.g. yeast extract) or available nutrients in the colon. Additionally, most of the *in vitro* studies where the

butyrogenic effect of RS was reported were performed with fecal inoculum and/or in complex media, which could also be linked to the butyrate increase. Interestingly, in a recent study, where the effect of potato starch on the human fecal microbiota and SCFAs was tested on minimal media, no significant changes after fluorescent *in situ* hybridization (FISH) analysis in the *E. rectale* population were detected during the fermentation. In contrast, a decrease in the *E. rectale* population was detected after 5 h when the starch was the sole carbohydrate, but after 10 h a slight increase was observed (Lesmes et al., 2008). Additionally, these authors reported the production of acetate throughout the entire experiment, the appearance of propionate after 10 h and the production of butyrate only after 24 h in 5-fold smaller amounts than propionate (Lesmes et al., 2008). Nevertheless, it is difficult to link the propionate production from RS to the activity of *E. rectale* in this system, as for the inoculum fecal material was used. The authors reported an increase in the populations of *Bacteroides* spp., *Bifidobacterium* spp. and *Atopobium* spp. in presence of RS after 5 h, where still no propionate is measured, which could indicate that the accumulated propionate is produced by the *E. rectale* population, or is an intermediate product from cross-feeding activity (Lesmes et al., 2008).

In summary, we conclude that most probably in the studied co-culture system of *R. bromii* and *E. rectale*, both species are equally involved in the conversion of the potato starch. *R. bromii* actively degrades the potato starch to glucose, ethanol, H_2, acetate and formate, while *E. rectale* further utilizes the H_2 to convert the ethanol and lactate to propionate. Additionally, the lower concentrations of H_2 due to the activity of *E. rectale* facilitates *R. bromii* to grow on glucose and further produce higher amounts of acetate.

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CHAPTER 5

Identification of Microbial Populations Involved in the Degradation of Lactose in the Human Intestinal Tract

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Abstract

The importance of the gut microbiota and its immense metabolic potential that affects health and disease, is receiving extensive attention. However, due to the difficulty to access the complex human colon ecosystem it is still an open question, which microbes are responsible for specific fermentation processes. Here we report a pilot study, where the ability of the human gut microbiota to ferment lactose was explored *in situ*. A multi-lumen feeding catheter was introduced naso-duodenally in two healthy individuals (male and female, Caucasian). After positioning into the duodenum, progression of the catheter through the small intestine by normal peristaltic movements was followed by fluoroscopic imaging. After positioning in the terminal ileum, a bolus of 1 g [U-^{13}C]-labeled lactose was infused into the intestinal lumen. Luminal samples were taken through the catheter at different time points over a period of 4 h following the infusion. RNA was extracted from the samples and density-resolved by ultra-centrifugation in caesium trifluoracetate. The extracted heavy and light RNA was subjected to phylogenetic analysis using the HITChip, a comprehensive diagnostic microarray for the human intestinal tract microbiota. Comparison of unlabeled RNA-derived profiles obtained from fractions with the lowest density, inter-individual variation and stability of the intestinal microbiota was observed for both volunteers. Principle component analysis of the HITChip profiles indicated that the luminal samples taken from volunteer A had an ileum-like microbial composition whereas those from volunteer B were more fecal-like, which could indicate positioning of the catheter in the proximal colon instead of in the terminal ileum. Most probably because of this difference in location of substrate infusion and sampling, different bacterial populations were found to be involved in the lactose conversion in the two volunteers. The fractions with RNA with higher density showed minor increases in populations

related to *Akkermansia*, *Atopobium*, *Megasphaera elsdenii* and *Faecalibacterium prausnitzii* for volunteer A. Additionally after 2 h, strong increase in signals indicative of *Lactobacillus plantarum*, *Bacteroides stercoris* and a *Desulfovibrio* sp. was observed. For volunteer B, in fractions with higher density, increase was observed of *Megasphaera elsdenii*, which most probably is a key player in the lactose metabolism. Confirming our previous result from lactose *in vitro* fermentation an increase in *Bifidobacterium* and *Collinsella* after 4 h was found in volunteer B. In conclusion, inter-individual differences were found with respect to populations involved in lactose degradation *in vivo*. However, in both volunteers, similar cross-feeding processes are probably involved in the further assimilation of metabolites from the lactose conversion, which remains to be confirmed by metabolite analyses.

Manuscript in preparation

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Introduction

The human colon comprises a complex and dynamic microbial consortium with large numbers, considerable diversity, and significant metabolic activity that are essential for human life (Backhed et al., 2005; Qin et al., 2010), including a range of metabolic activities which are lacking in the human host (Suau et al., 1999; Eckburg et al., 2005; Xu et al., 2007). Recently, a first comprehensive analysis of the coding capacity has been reported and found to exceed by far that of the human genome (Qin et al., 2010). However, a large fraction of the microbiota still remains unexplored thus making the relationship between colonic microbial activity, and health and disease a controversial issue. Dietary carbohydrates are important energy sources and are known for their interactions with the microbiota in the intestinal tract (Savage, 1986; Hooper et al., 2002; Guarner and Malagelada, 2003; Xu and Gordon, 2003). The main fermentation products that are produced from carbohydrates are the short-chain fatty acids (SCFA), such as butyrate, propionate and acetate, as well as other intermediate products such as lactate (Macfarlane and Gibson, 1997). The bacterially-produced SCFA constitute an important energy source for the body (Roy et al., 2006; Wong et al., 2006). Different carbohydrates are digested to various degrees in the intestinal tract, and while some are fermented in the colon, others pass through the entire intestine in a relatively intact form (Scheppach et al., 2001). Most disaccharides are completely digested in the small intestine, however, host genetic background can influence digestive efficacy significantly. Lactose is one of these disaccharides, and lactose intolerance results from insufficient or even lack of lactase activity in the small intestine (Henslee and Jones, 1982; de Vrese et al., 2001; Vonk et al., 2003). Colonic metabolism of lactose has been suggested to play a role in the degree of intestinal discomfort associated with lactose intolerance. Data from *in vitro* lactose fermentation studies indicate higher rates of SCFA and lactate production in lactose intolerant subjects compared to tolerant subjects, when translated into an *in vivo* situation may result in temporary accumulation of SCFA into the lumen, causing symptoms associated with lactose intolerance (Vonk et al., 2003; He et al., 2006; He et al., 2008). Therefore, it is important to unravel the lactose metabolism *in situ* and understand the contribution of the colonic microbiota.

Recently, a molecular approach, termed nucleic acid-based stable isotope probing (SIP), has been developed that allows linking the metabolic activity and microbial diversity of communities in complex ecosystems (Radajewski et al., 2000; Dumont and Murrell, 2005; Egert et al., 2006; de Graaf et al., 2007; Neufeld et al., 2007). This approach involves a process of exposing microorganisms to a substrate

of interest enriched with stable isotopes (usually ^{13}C) and analyzing the subsequent isotopic incorporation into cellular biomarkers including DNA and RNA. Subsequent characterization of the labeled biomarkers allows identifying the microorganisms assimilating the labeled substrate. Especially the introduction of RNA-based SIP represented a significant advancement, as it does not require microbial growth, and has been used to link microbial function with phylogeny in a broad spectrum of different environments. Examples include, but are not limited to, identification of acetate-metabolizing bacteria in the presence of iron oxides as electron acceptors (Hori et al., 2010), phenol-assimilating bacteria (Sueoka et al., 2009), or butyrate-producing bacteria under methanogenic conditions (Hatamoto et al., 2008). Furthermore, RNA-based SIP was also applied to study human intestinal bacteria in an *in vitro* model of the human colon in the presence of [U-^{13}C]-glucose (Egert et al., 2007) and in the presence of [U-^{13}C]-potato starch (Kovatcheva-Datchary et al., 2009). As a next step, application of SIP *in vivo*, in which the labeled substrates of interest is delivered directly to the human colon, will allow to test hypotheses based on observations made in *in vitro* model experiments, providing a more comprehensive understanding of nature and inter-individual variation of metabolic activities and interactions within the gut microbiota.

Here we report, to the best of our knowledge, the first human trial with a substrate that is suitable for RNA-SIP. We selected lactose as the substrate as we aimed to identify the microbes responsible for the *in situ* degradation of this disaccharide in the terminal ileum. The substrate was infused in the terminal ileum through a naso-gastric catheter. We show that RNA based-SIP together with high-throughput diagnostic microarray-based phylogenetic profiling is a useful tool for identifying metabolically active human gut bacteria in human studies. The current study also provides the basis for further experiments to determine the microbial functionality *in vivo* in human subjects.

Experimental Procedures

Subjects

Two healthy subjects, a 28-year old male and 24-year old female (using contraceptives), both lean individuals, with normal BMI (18-25) and no history of bowel disorders or surgery, took part in the study. None of them had received antibiotic treatment for at least 2 months prior to inclusion, both were non smoking,

had no known history of use of drugs, and had no known diabetes type 1 or 2. Both volunteers gave their written informed consent prior to participation. The study was approved by the Medical Ethics Committee of University Hospital Maastricht and conducted in accordance with the principles of the 'Declaration of Helsinki' (52nd WMA General Assembly, Edinburgh, Scotland, Oct 2000).

Design

The day prior to the study, the subjects consumed a standardized meal. After an overnight fast, a catheter was positioned as described below. Once the catheter was in position in the terminal ileum, a bolus of 5 ml containing 1 g [U-^{13}C]-lactose (Omicron Biochemicals, Inc., South Bend, USA) was infused through the catheter into the terminal ileum. Luminal samples were collected for further analysis.

Catheter design

The catheter was constructed of a multilumen pvc tubing (Mui Scientific, Ontario, Canada) with a length of 2.5 m. The outer diameter of the catheter was 4.6 mm. It consisted of a large central lumen with a diameter of 2.2 mm, surrounded by 12 small lumina with an inner diameter of 0.4 mm each. At the tip of the catheter an inflatable latex balloon was attached with a volume of maximally 10 ml. The infusion port was located 11 cm proximal to the balloon and was connected to two of the small lumina. The sampling port was connected to the large central lumen and was located 1 cm proximal to the balloon. The balloon itself was connected to a small lumen to enable inflation and deflation of the balloon. The other 9 lumina were closed.

Catheter positioning

The catheter was introduced naso-duodenally by a gastroenterologist and aided through the pylorus using a guide wire in the catheter. The position of the tip of the catheter was checked by intermittent fluoroscopy. After the position of the tip was well beyond the pylorus, the guide wire was withdrawn from the catheter and the balloon attached at the tip of the catheter was inflated with 10 ml of air. The progression of the catheter was then aided by the natural peristalsis of the intestine. To prevent fluid accumulation in the intestinal lumen due to the blockade caused by the balloon, which may result in abdominal complaints, the balloon was deflated for a few minutes every 20 minutes. Lunch (coffee and a sandwich with jam) and dinner (pasta bolognese) were offered. This stimulated the peristaltic movements of the

intestine. The position of the catheter was checked again after dinner by fluoroscopy. At 9 PM the volunteers received a last snack (sugar waffle) before going to sleep. Catheter position was checked again fluoroscopically in the morning. If the target position was not reached yet, the subjects were offered a breakfast (sandwich with marmalade) to stimulate peristalsis. Once the catheter reached the terminal ileum (Fig. 1), the infusion of the [U-^{13}C] lactose started. At the end of the test day, a gastroenterologist gently pulled out the catheter, after intravenous infusion of 1 ml of Buscopan® 20 mg/ml (butylscopalamine bromide for intramuscular or intravenous injection, Registration number for medication: RVG 03837, Boehringer Ingelheim, Alkmaar, The Netherlands).

Sampling of luminal contents

Luminal content was sampled through the ileal catheter each hour until 4 h after start of ^{13}C-lactose infusion. If fluid sampling was unsuccessful, 5 ml of deoxygenated water (prepared under O_2 free conditions) was injected to dilute the luminal content and facilitate sampling. The samples were immediately placed in sterile eppendorf tubes. To the luminal samples intended for RNA-SIP analysis an equal volume of RNA later was added, and samples were further stored on ice until the end of the experiment. After arrival in the laboratory, the samples were immediately stored at -80ºC until they were further processed for RNA extraction.

RNA-extraction, purification and fractionation by density gradient centrifugation

Total RNA was extracted and purified from 250 µl luminal samples collected at the different time points as described previously (Egert et al., 2007). After purification and quantification approximately 1 µg of total RNA was loaded in cesium trifluoroacetate (CsTFA) centrifugation medium and resolved by density gradient centrifugation. Gradients were subsequently fractionated as described by (Egert et al., 2007), but using a KDS200 syringe pump (KD Scientific Inc., Holliston, MA, USA). Afterwards, RNA from each fraction was precipitated for subsequent community analyses.

Amplification of 16S rRNA and HITChip analysis

To examine all the fractions for presence of rRNA, we amplified the 16S rRNA by RT-PCR using a one-step reverse transcription-PCR (RT-PCR) system (Access

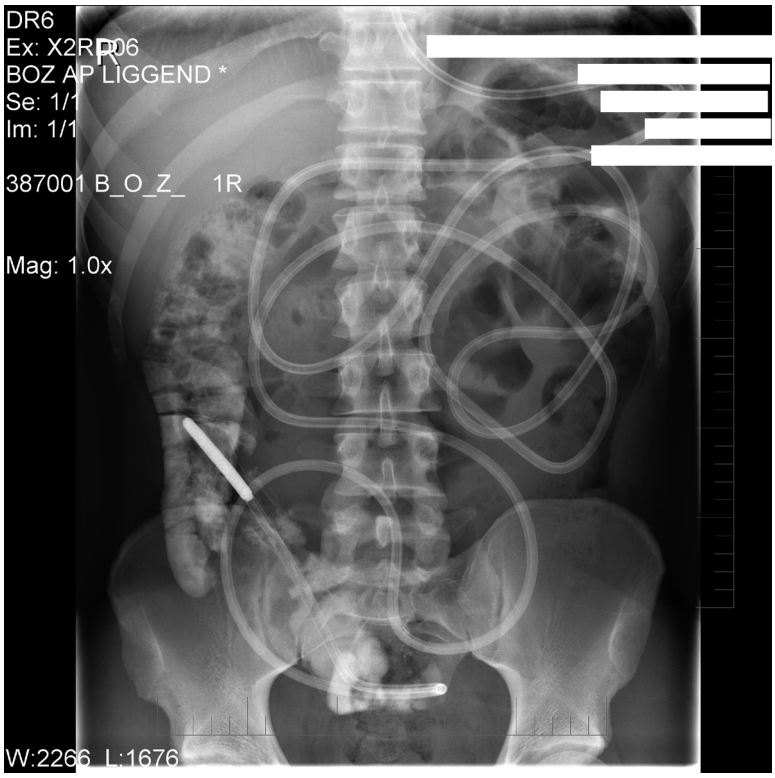

Figure 1. A fluoroscopic image of a volunteer indicating the positioning of the catheter through the gastro-intestinal tract. The picture shows the catheter tip entering the proximal colon. Colon structures were made visible for fluoroscopic imaging by administering of 10 ml of Lipiodol ® Ultra Fluide 480 mg Jood/ml (Guerbet Nederland BV, Gorinchem, The Netherlands). The Lipiodol® was diluted in Saline in ratio 1:3. In the experiments described here, the catheter was placed with the tip positioned in the terminal ileum.

Quick, Promega, Leiden, The Netherlands) with primers and conditions for RT-PCR as described previously (Egert et al., 2007; Kovatcheva-Datchary et al., 2009). Subsequently, phylogenetic analysis of the amplicons was performed using the HITChip (Rajilic-Stojanovic et al., 2009). This phylogenetic microarray consists of more than 4,800 oligonucleotide probes based on 16S rRNA gene sequences targeting over 1,100 human intestinal microbial phylotypes. Based on the data from the RNA recovery assay and the 16S rRNA RT-PCR analysis gradient fractions for further HITChip analysis were selected. Subsequently, cDNA was synthesized from the RNA from the selected fractions with different density. Reaction mixtures

consisting of SuperScript™ III Reverse Transcriptase (Invitrogen), 8 µl RNA solution, 1 µl 50 µM random hexamer primer and 1 µl 10 mM dNTPs were incubated at 65°C for 5 min and then placed on ice for at least 1 min. The cDNA synthesis mix containing 4 µl 5 × First-Strand Buffer, 1 µl rRNasin® (40 U/µl), 1 µl SuperScript™ III RT (200 U/µl) and 1 µl 0.1 M DTT was then added to each RNA/primer sample which were then incubated first at 25°C for 5 min and afterwards at 55°C for 60 min. Reactions were terminated by exposure to 70°C for 15 min. Afterwards, the cDNA was used as template for 16S rRNA PCR analysis for further preparation of the samples for the HITChip analysis as described before (Rajilic-Stojanovic et al., 2009). After purification of the PCR products (High Pure PCR Cleanup Micro kit, Roche Diagnostics GmbH, Mannheim, Germany) the DNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop® Technologies, Wilmington, USA). Target preparation, hybridization and washing, and data analysis was essentially performed as described before (Kovatcheva-Datchary et al., 2009; Rajilic-Stojanovic et al., 2009). Similarly, all bioinformatic analyses were performed as described previously (Kovatcheva-Datchary et al., 2009; Rajilic-Stojanovic et al., 2009).

Results

Volunteer A

RNA recovery and RT-PCR amplification of the 16S rRNA

In a pilot study a multi-lumen feeding catheter was introduced into the terminal ileum of two healthy individuals and a total amount of 1 g of [U-^{13}C]-lactose was infused. The conversion of the [U-^{13}C]-lactose by the intestinal microbiota was followed for 4 h. Luminal samples were taken for the first volunteer (A) at 0 h, 0.5 h, 1 h, 2 h, 3 h and 4 h. The amount of RNA extracted from luminal samples taken at different time points amounted from 2.4 µg/ml to 12 µg/ml. In previous *in vitro* studies with [U-^{13}C]-lactose the extracted RNA concentrations were 80 µg/ml to 172 µg/ml RNA (Chapter 3 of this thesis).

The extracted RNA was further isopycnically centrifuged to resolve the ^{13}C-labeled rRNA (heavy) from the background of ^{12}C-unlabeled rRNA (light). The fractionated gradients of rRNA extracted from the samples of volunteer A covered an average density range of 1.730 g ml^{-1} (fraction 14) to 1.835 g ml^{-1} (fraction 1)

(Fig. 2 A). In the gradient fractions of the 0 h sample all nucleic acids were detected at densities characteristic for unlabeled RNA between 1.746 g ml^{-1} (fraction 12) and 1.775 g ml^{-1} (fraction 8). The highest amount of RNA at this time point was detected in fraction 10 (1.759 g ml^{-1}). One hour after the addition of [U-^{13}C]-labeled lactose presence of heavy labeled RNA was detected in fraction 7 (1.783 g ml^{-1}), and the highest amount of RNA shifted to fraction 9 (1.767 g ml^{-1}, Fig. 2 A). Furthermore, the RNA distribution in the gradients from the 2 h, 3 h and 4 h time points, was similar to that for the 1 h sample. The only difference was observed for the maximal RNA concentration which was detected again in fraction 10 (1.759 g ml^{-1}) from the 2 h time point onwards as it was observed for 0 h (Fig. 2 A).

Subsequently, the bacterial 16S rRNA fragments present in the different fractions were amplified by RT-PCR and quantified (Fig. 2 B). It was observed that the 16S rRNA in the fractions of the 0 h gradient for both volunteers yielded detectable

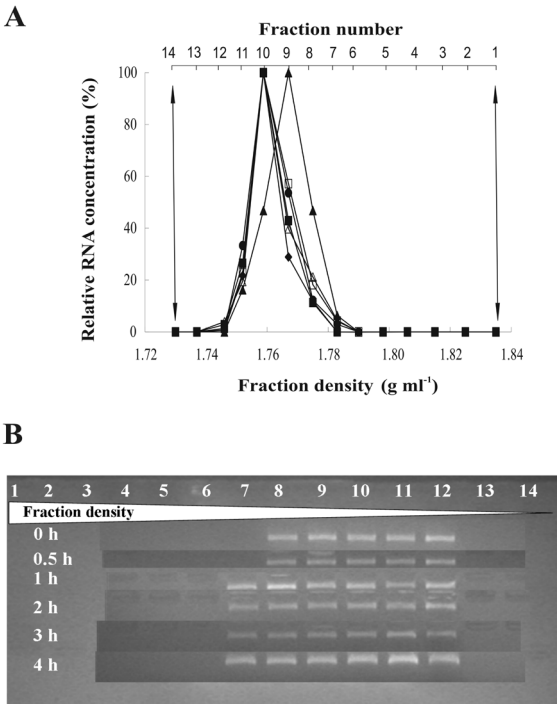

Figure 2. A. RNA concentration in gradient fractions for volunteer A. RNA was isolated from lumen samples of the human intestine at 0 h, 0.5 h, 1 h, 2 h, 3 h and 4 h after infusion with 1 g of [U-^{13}C]-lactose. Values are given in relative units (%) to facilitate comparison between the gradients; ♦, 0 h; ■ 0.5 h, ▲ 1 h, ● 2 h, □ 3 h, Δ 4 h. Vertical arrows indicate corresponding fractions in the figure. Densities of gradient fractions from the [U-^{13}C]-labeled lactose study are averaged over 6 gradients, and standard deviations of the densities of the different gradients were in the range of 0.003 and 0.004 g ml^{-1}(data not shown). **B**. RT-PCR-analysis results representing an ~900 bp fragment of bacterial 16S rRNA from the gradient fractions. The picture is assembled from 6 different ethidium bromide-stained agarose gels after electrophoresis of the amplicons. Fractions were obtained from ultracentrifugation gradients performed to density-resolve RNA isolated from lumen samples after incubation for 0 h, 0.5 h, 1 h, 2 h, 3 h and 4 h with 1 g of [U-^{13}C]-lactose. Fraction numbers are indicated on top and correspond to those in A.

products from fraction 8 (1.775 g ml^{-1}) to fraction 12 (1.746 g ml^{-1}) (Fig. 2 A). However, after 1 h a detectable product was also obtained with the high density fraction 7 (1.783 g ml^{-1}), confirming the results from the RNA recovery analysis.

Community profiling of the human intestinal microbiota

To specify the composition of the microbial communities in the fractions with low and high density, the extracted RNA was reverse transcribed into cDNA and analyzed on the HITChip, a phylogenetic microarray (Rajilic-Stojanovic et al., 2009). From each time point, two fractions were analyzed, *viz.* the one with the highest density and the one which contained the highest amount of RNA, according to RNA recovery analysis (Fig. 2).

The HITChip analyses of the selected fractions for volunteer A indicated that the *Proteobacteria* constitute the most abundant bacterial group in both fractions at the beginning of the experiment (Fig. 3 A). However, after 0.5 h, the profile completely changed particularly for the fraction with lower density, where members of *Clostridium* cluster XIVa and *Bacilli* were most dominant (Fig. 3 B). The relative abundance of *Proteobacteria* spp. was still high in the fraction with higher density, however, *Bacilli* were the dominant group in the low density fraction, particularly species related to *Streptococcus bovis* et rel., *Streptococcus intermedius* et rel. and *Streptococcus mitis* et rel.. The presence of members of *Clostridium* cluster XIVa was also detected (Fig. 3 A). After 1 h, a shift of the RNA from low density fraction to the higher density fraction (Fig. 2) was detected.

Members of *Bacilli* and *Clostridium* cluster XIVa were still the dominant groups in the fraction with lower density (Fig. 3 B). Increase in the abundance of bifidobacteria was observed in the fraction with higher density, followed by a decrease after 1 h from the [U-^{13}C]-lactose infusion (Fig. 3 A). A minor increase (approximately 2-fold) was detected in several specific genus-level phylogenetic groups in the fractions with higher density. These included in particularly, the taxa *Akkermansia*, *Atopobium*, *Megasphaera elsdenii* et rel. and *Feacalibacterium prausnitzii* et rel., which dropped immediately at 2 h (Fig. 4). Other changes in the microbial composition related to the increase at genus-level in fractions with higher density were observed also after 2 h. More specifically, the relative abundance increased in the fraction with higher density for *Lactobacillus plantarum* (>10 fold) and *Lactobacillus gasserii* (~5 fold), *Bacteroides stercoris* (> 10 fold), *Desulfovibrio* et rel. (>10 fold) and *Roseburia intestinalis* et rel. (> 2.7 fold) (Fig. 4). Furthermore, at 4 h a strong increase in *Bacteroidetes* was observed in both fractions (Fig. 3).

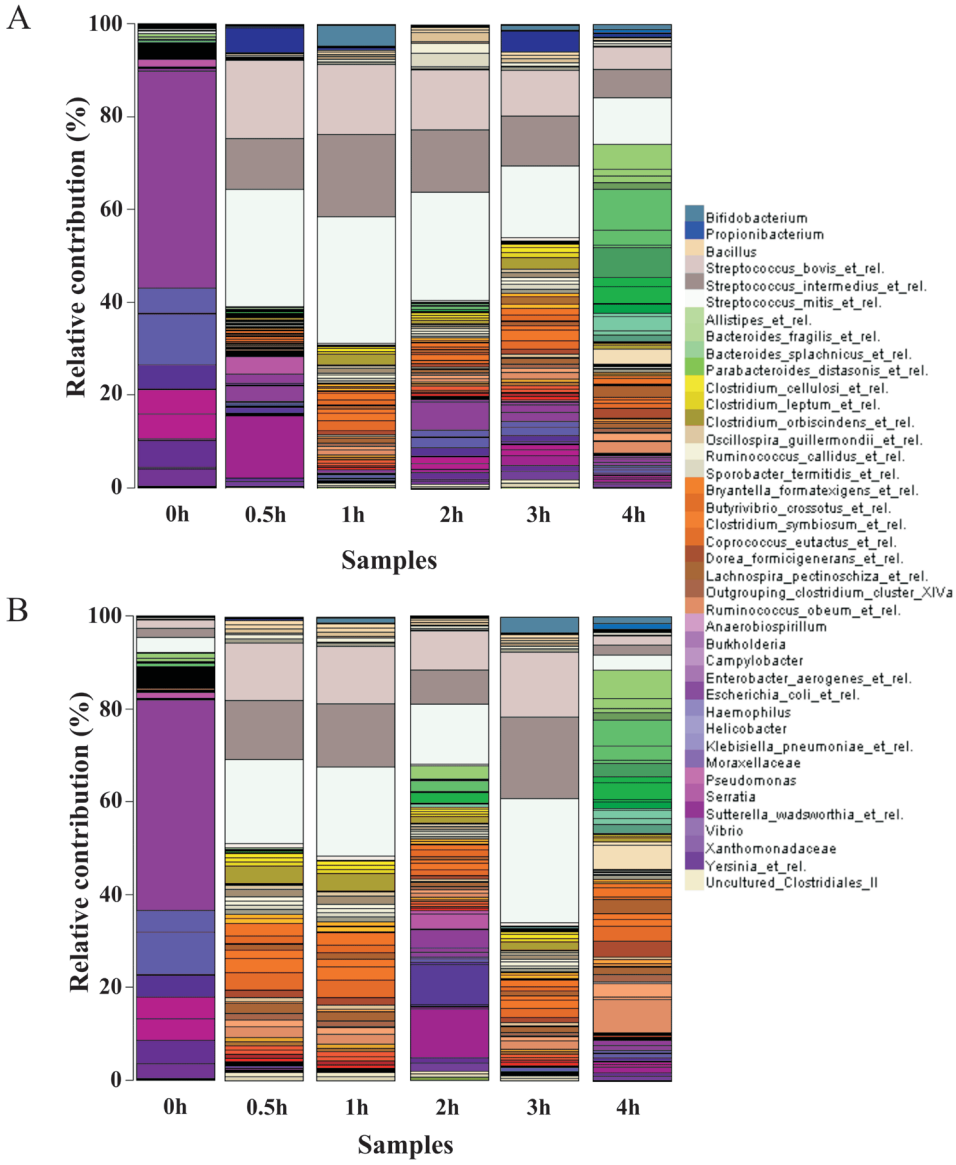

Figure 3. Relative contribution of phylotypes detected with the HITChip in fractions with higher density (**A**) and those with lower density containing the highest RNA concentration (**B**), for volunteer A from the [U-^{13}C]-lactose *in vivo* trial, collected at different time points. In the legend the 40 genus-level phylogenetic groups contributing most to the profiles are indicated.

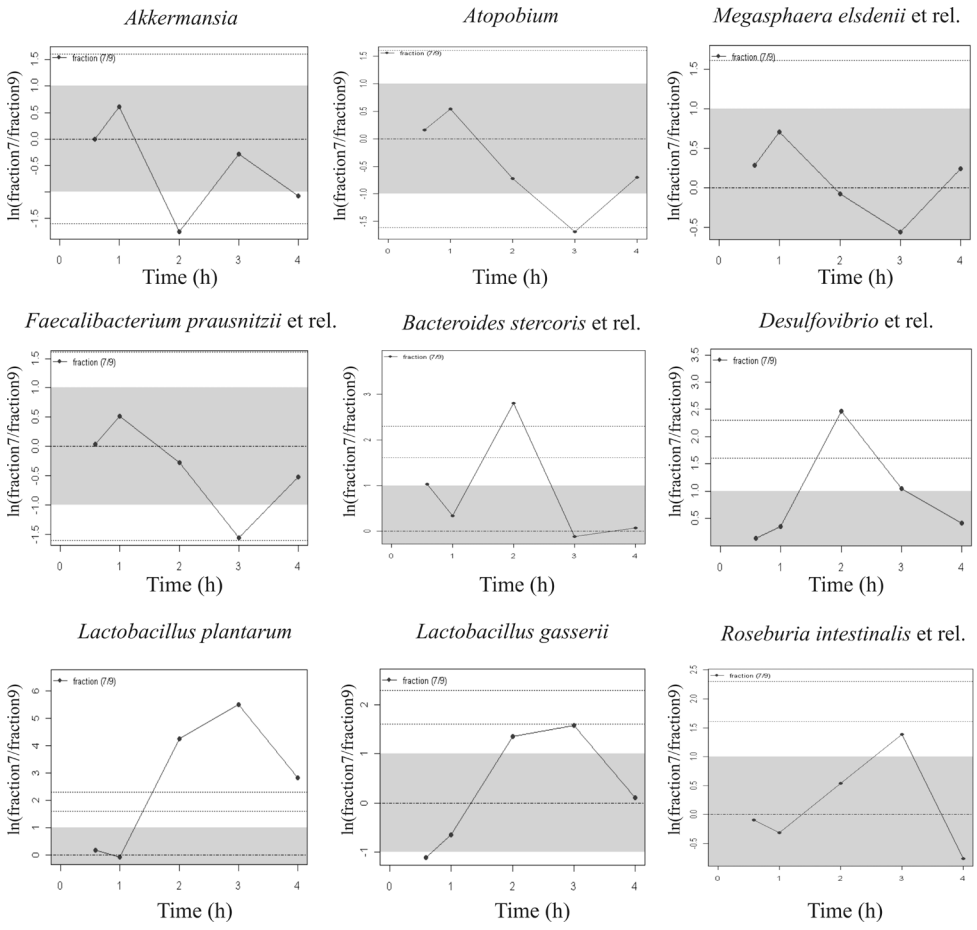

Figure 4. Development in time of the differences in relative abundances of the genus-like bacterial groups expressed as natural logarithm of the fraction 7 to fraction 9 ratio. The grey area visualizes minor ratios, which are between -2.72 and 2.72 (natural logarithm = 1). Dashed horizontal lines are drawn at no differences between the relative abundances of fraction 7 and 9 (natural logarithm = 0), and at differences of 5 and 10 fold between the relative abundances of the fractions.

In order to assess to what extent the microbiota profiles obtained from the volunteers resembled those normally observed for human intestinal samples, principal component analysis (PCA) of the fractions with higher and lower density was performed. For volunteer A, this indicated a clear grouping of the low and high density samples taken at 4 h with fecal samples and samples from a previous *in-vitro*

study with [U-^{13}C]-lactose (Fig. 5). In contrast, the samples with higher density from time points 0.5 h, 1 h, 2 h and 3 h were more closely clustered with ileal effluent samples from individuals with an ileostoma (Booijink, 2009) (Fig. 5 A). The samples with higher density and lower density from the 0 h time point did not cluster with any of the samples (Fig. 5). Additionally, only the samples from the 1 h and 3 h with lower density grouped together with the ileostoma samples (Fig. 5 B).

Species belonging to *Clostridium* cluster IV, particularly *Clostridium leptum* et rel., *Clostridium cellulosi* et rel. and *Ruminococcus callidus* et rel. showed to be positively correlated with the lower density fraction from 1 h and 3 h, while species belonging

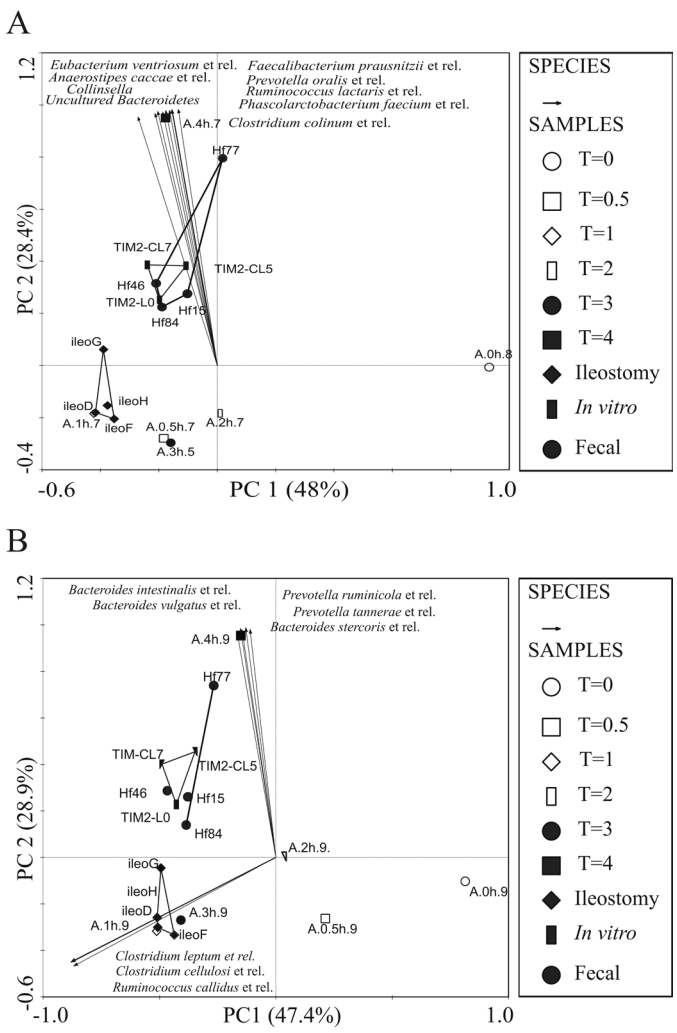

Figure 5. PCA plot depicting the microbiota of the fractions 8 from time point 0 h and fraction 7 from 0.5 h, 1 h, 2 h, 3 h and 4 h, from the [U-^{13}C]-lactose *in vivo* trial with volunteer A. (**A**), and fractions 9 from time point 0 h, 0.5 h, 1 h, 2 h, 3 h and 4 h (**B**). Additionally, ileal effluent microbiota of 4 individuals with an ileostoma (ileo), microbiota of the fecal material of four healthy human subjects (Hf) and the microbiota from three samples from a previously performed *in vitro* study with [U-^{13}C]-lactose (TIM-2) were included. Percentage values at the axes indicate contribution of the principle component to the explanation of the total variance in the dataset. The phylogenetic groups that contributed at least 98% (A.) and at least 97% (B.) to the explanatory axis used in the plot are presented as vectors.

to *Bacteroidetes* were positively correlated with the 4 h low density fraction (Fig. 5 B), which had a more fecal like composition.

Volunteer B

RNA recovery and RT-PCR amplification of the 16S rRNA

RNA was extracted from luminal samples taken at 0 h, 1 h, 2 h, 3 h, 4 h and 4.5 h, as sampling at 30 min for this volunteer was not possible. The amount of RNA extracted from the luminal samples taken at different time points amounted from 3.2 µg/ml to 16 µg/ml, which was higher than for volunteer A.

The fractionated isopycnic centrifugation gradients of RNA extracted from samples of volunteers B covered the same average density range as for volunteer A: 1.730 g ml^{-1} (fraction 14) to 1.835 g ml^{-1} (fraction 1) (Fig. 6 A). The presence of RNA after isopycnic centrifugation in the gradient fractions of the 0 h sample was detected (as for volunteer A), at densities between 1.746 g ml^{-1} (fraction 12) and 1.775 g ml^{-1} (fraction 8), with the highest amount of RNA being found in fraction 10 (1.759 g ml^{-1}). The presence of RNA in fractions with higher density in comparison to the 0 h sample was detected only 4 h after the beginning of the study, namely in fraction 7 (1.783 g ml^{-1}, Fig. 6 A). This contrasts with the kinetics observed with volunteer A where labeled RNA could already be detected after 1 h. The highest amount of RNA for all time points was measured in fraction 10 (1.759 g ml-1, Fig. 6 A), as for 0 h. The RT-PCR amplification of bacterial 16S rRNA fragments present in the different fractions was in agreement with the results from the RNA recovery measurements. RT-PCR amplification of the 16S rRNA in the fractions of the 0 h gradient yielded PCR-products from fraction 8 (1.775 g ml-1) to fraction 12 (1.746 g ml-1) (Fig. 6 B). A detectable product was also obtained for fraction 7 (1.783 g ml-1) after 4 h and 4.5 h (Fig. 6 B).

Community profiling of the human intestinal microbiota

For volunteer B, fractions 8 and 10 from 0 h, 1 h, 2 h and 3 h were included for HITChip analysis. For the 4 h and 4.5 h time point fraction 7 and fraction 10 were analysed. A relatively stable profile of the composition of the microbiota in the fractions with lower density was detected over the entire experimental period of 4.5 h with volunteer B (Fig. 7 B). Phylotypes belonging to *Bifidobacterium*, *Bacilli* and *Clostridium*

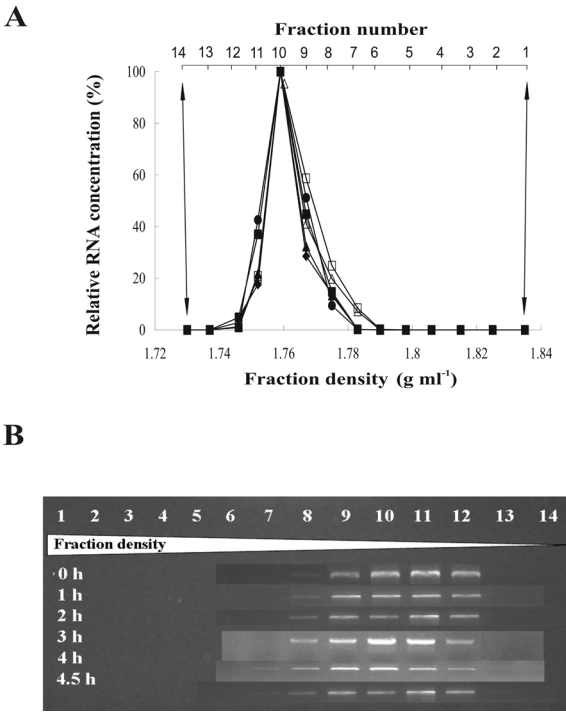

Figure 6. **A.** RNA concentration in gradient fractions for volunteer B. RNA was isolated from lumen samples of the human intestine at 0 h, 1 h, 2 h, 3 h, 4 h and 4.5 h after infusion with 1 g of [U-^{13}C]-lactose. Values are given in relative units (%) to facilitate comparison between the gradients; ♦, 0 h; ■ 1 h, ▲ 2 h, ● 3 h, □ 4 h, ∆ 4.5 h. Vertical arrows indicate corresponding fractions in the figure. Densities of gradient fractions from the [U-^{13}C]-labeled lactose study are averaged over 6 gradients, the standard deviation of the densities of the different gradients was 0.003 g ml^{-1} for all the fractions (data not shown). **B**. RT-PCR-analysis results representing an ~900 bp fragment of bacterial 16S rRNA from the gradient fractions. The picture is assembled from 6 different ethidium bromide-stained agarose gels after electrophoresis of the amplicons. Fractions were obtained from ultracentrifugation gradients performed to density-resolve RNA isolated from lumen samples after incubation for 0 h, 1 h, 2 h, 3 h, 4 h and 4.5 h with 1 g of [U-^{13}C]-lactose. Fraction numbers are indicated on top and correspond to those in A.

cluster XIVa dominated the community. The contribution of *Proteobacteria* spp. to the signal intensity in fractions with low density was more pronounced at the 0 h time point in comparison to the other time points (Fig. 7 B). However, differences between the lower and higher density fractions were observed at later time points. *Bacilli* were the dominant members in the higher density fractions until the end of the experiment. At the 0 h time point *Bacteroidetes* contributed approximately 10% to the signal intensity. However, after 1 h this group contributed to a lesser extend to the signal intensity in the fraction with higher density. Interestingly, after 2 h the presence of *Bacteroidetes* was detected again and an increase of several members of this group was observed in the fraction with higher density in comparison with the low density fraction. A particularly strong increase in *B. fragilis* et rel. (> 10-fold) and *B. ovatus* (> 10-fold) was observed (Fig. 8). Additionally, a more than 5-fold increase was observed for *Megasphaera elsdenii* in the fraction with higher density

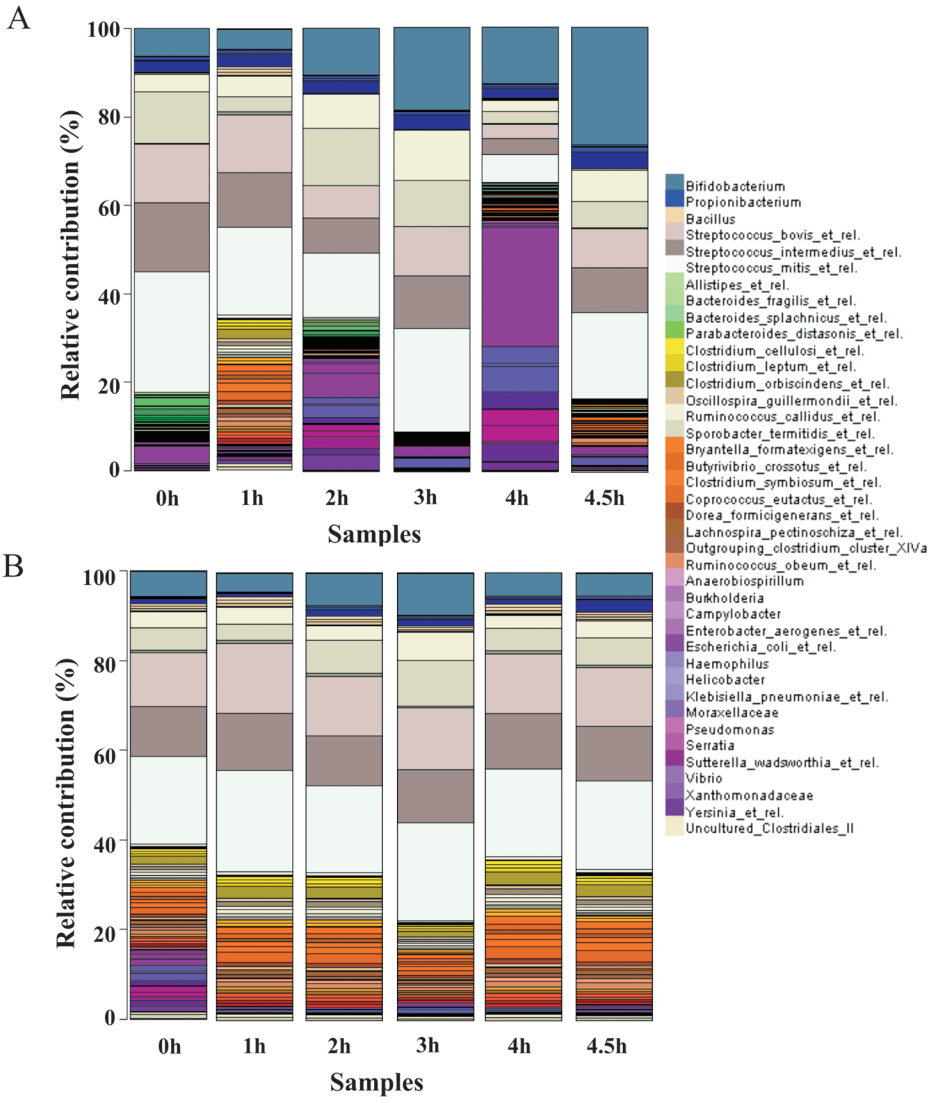

Figure 7. Relative contribution of phylogenetic groups detected with the HITChip in fractions with higher density (**A**) and lower density, which contain the highest RNA concentration (**B**) for volunteer B, collected at different time points. In the legend the 40 genus-level phylogenetic groups contributing most to the profiles are indicated.

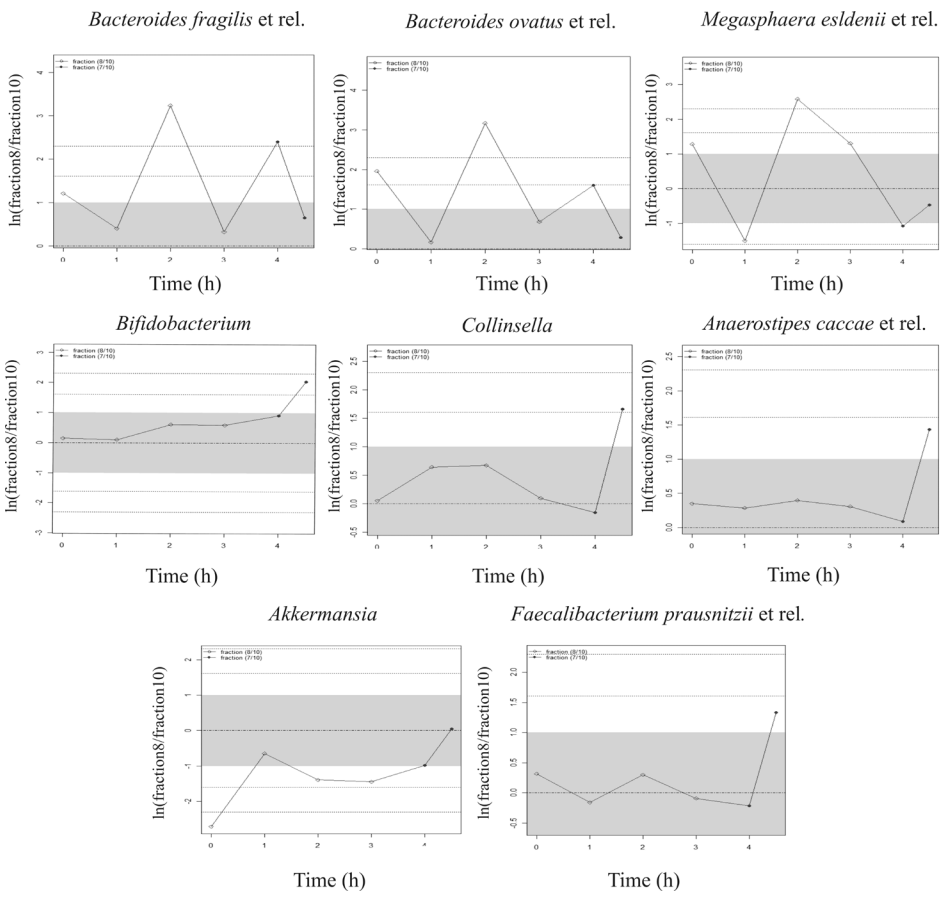

Figure 8. Development in time of the differences in relative abundances of the genus-like bacterial groups expressed as natural logarithm of the fraction 8 to fraction 10 ratio. The grey area visualizes minor ratios, which are between -2.72 and 2.72 (natural logarithm = 1). Dashed horizontal lines are drawn at no differences between the relative abundances of fraction 8 and 10 (natural logarithm = 0), and at differences of 5 and 10 fold between the relative abundances of the fractions.

in comparison to the lower density fraction (Fig. 8). Unexpectedly, at the 4 h time point in the fraction with higher density the presence of *Proteobacteria* was detected. After comparing the fraction with higher density at the 4.5 h time point with the fraction with lower density, a strong increase in *Bifidobacterium* (~8-fold), *Collinsella* (> 5-fold) and *Anaerostipes caccae* (~4-fold) (Fig. 8). Additionally, an

increase in *Akkermansia* spp. and *Faecalibacterium prausnitzii* was detected also with volunteer B after the 4 h time-point where label incorporation in 16S rRNA was first observed using RT-PCR (Fig. 6).

After visualizing the bacterial composition of the samples with higher density on a PCA plot, only the sample from the 1 h time with high density grouped with ileal effluent samples from subjects with an ileostoma and faecal samples. The higher density fraction from the 4 h sample did not group with any of the samples, and showed a positive correlation with a species belonging to *Proteobacteria*, particularly

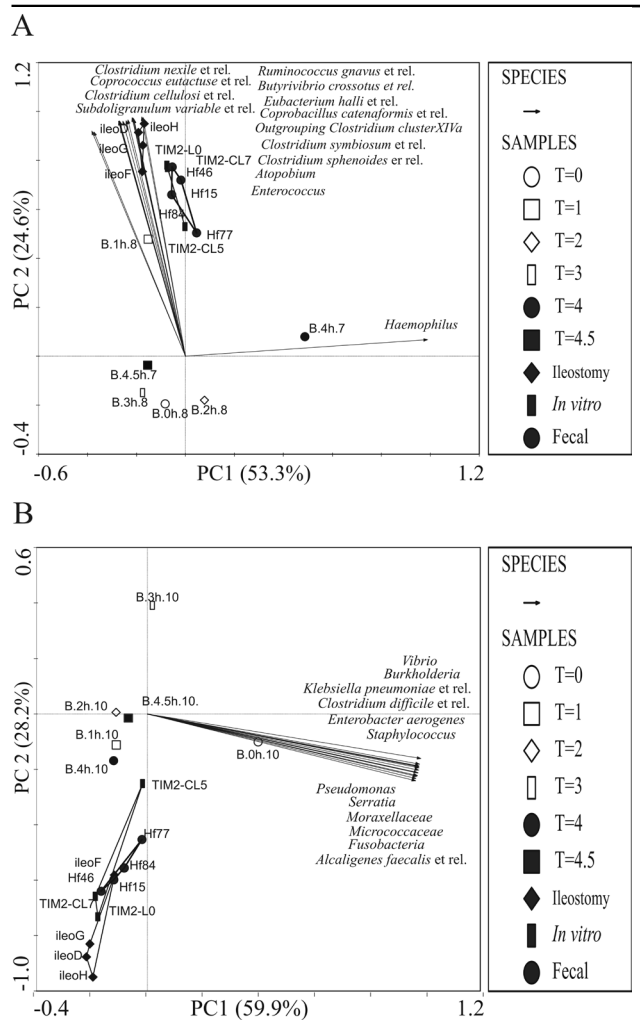

Figure 9. PCA plot depicting the microbiota of the fractions 8 from time points 0 h, 1 h, 2 h, 3 h, and fraction 7 for 4 h and 4.5 h, from the [U-^{13}C]-lactose *in vivo* trial with volunteer B (**A**), and fractions 10 from time points 0 h, 1 h, 2 h, 3 h, 4 h and 4.5 h (**B**). Additionally, the microbiota of 4 individuals with an ileostoma (ileo), microbiota of the faecal material of four healthy human subjects (Hf) and the microbiota from three samples from a previously performed *in vitro* study with [U-^{13}C]-lactose (TIM-2) were included. Percentage values at the axes indicate contribution of the principle component to the explanation of the total variance in the dataset. The phylogenetic groups that contributed at least 99% to the explanatory axis used in the plot are presented as vectors, valid for the both plots.

Heamophilus (Fig. 9 A). Additionally, the samples with lower density also did not group with the ileum and fecal samples. Moreover, the 0 h sample with lower density did not group with the other samples and a positive correlation with a number of groups within the *Proteobacteria* was observed (Fig. 9 B).

Discussion

The objective of this study, which describes pioneer experiments towards the application of RNA-based SIP under *in vivo* conditions in the human intestinal tract, was to explore the functionality of the microbiota in the conversions of relevant dietary carbohydrates in the terminal ileum of healthy individuals. The substrate tested in the current study was lactose. There is limited knowledge on the effect of lactose on the gut microbiota, even though it has been well established that lactose intolerance is endemic around the world (Sahi, 1974; Scrimshaw and Murray, 1988; Sahi, 1994). It has been reported that 2% of lactose is the theoretical daily maximum of lactose reaching the colon in lactase positive populations (Männistö et al., 2003), whereas most of the lactose reaches the colon in lactose maldigesters, the largest part of the world population.

Here we applied RNA-based stable isotope probing to identify members of the human intestinal microbiota involved in *in situ* lactose degradation in the human terminal ileum. After isopycnic separation of the RNA the range of the measured density was in agreement with previously reported data from *in vitro* studies, where ileal-like and fecal microbiota were studied (Egert et al., 2007; Kovatcheva-Datchary et al., 2009). Based on the data from the RNA quantification, for volunteer A, we observed presence of RNA with higher density in comparison with the 0 h sample from 1 h after infusion of [U-^{13}C]-lactose, whereas for volunteer B this occurred only after 4 h. From our previous experiments, where the fermentation of [U-^{13}C]-lactose was studied under *in vitro* conditions simulating the human colon using fecal material as inoculum, incorporation of the ^{13}C-label was detected 30 min after addition of the substrate (Kovatcheva-Datchary et al., manuscript in preparation). Because of the lack of knowledge on the optimal concentration of the ^{13}C-labeled substrate, in similar studies performed with humans, we used 1 g of [U-^{13}C]-lactose as was previously applied during the *in vitro* experiments.

Focusing on the changes that appear in the fractions with higher density from the 1 h time point, where heavy RNA was detected in volunteer A, with those containing RNA with lower density, minor changes could be detected. More specifically,

increase in *Akkermansia*, *Atopobium*, *Megasphera elsdenii* and *Faecalibacterium prausnitzii*, was observed. However, *Akkermansia* spp. are not reported to ferment lactose, and the preferable growth substrate for this bacteria is mucin (Derrien et al., 2004). It has been recently reported that *Akkermansia muciniphila* starts to colonize the human intestinal tract from early life (Collado et al., 2007). Additionally it has been described that *Akkermansia* and *Faecalibacterium prausnitzii* are present in the microbial community of humans with acute appendicitis, but their increase was not found to be related to the severity of the appendicitis (Swidsinski et al., 2009). Interestingly, an increase in both of these species was observed with volunteer B as well, albeit only after 4 h. Also higher concentrations of *Atopobium* have been reported in the faces of Asian infants after administration of probiotic product (Mah et al., 2007).

In the current study we followed the *in situ* conversion of lactose in the human intestine for a period of 4 - 4.5 h and we directly sampled from the location where the substrate was infused via a multi-lumen catheter. After comparison of the microbial community of both individuals in the fractions with lower density, the presence of species belonging to *Bacilli* and *Clostridium* cluster XIVa was detected in most of the fractions. In general, the microbial community in the fractions with lower density derived from volunteer B was more stable than that from volunteer A. However, further analysis on the composition of the microbiota in fecal samples from the two volunteers is needed to confirm this status. Unexpectedly, for volunteer A at the 0 h time point in both gradient fractions, *Proteobacteria* represented the major taxa in the community. However, 2 h after infusion of the labeled substrate, changes related to increase of phylotypes in the fraction with higher density were detected for two taxa of lactic acid bacteria, particularly *Lactobacillus plantarum* and *Lactobacillus gasserii*, and one *Bacteroides* sp. for volunteer A. Also an increase in *Desulfovibrio* spp. was observed at the same time point. *Desulfovibrio* spp., are well known sulfate reducing colonic bacteria, which in complex systems have been shown to outcompete for the lactate-producing members of *Clostridium* cluster XIVa, particularly *Eubacterium halii* and *Anaerostipes caccae*, converting lactate to acetate with concomitant production of H_2S (Marquet et al., 2009). Lactose is a compound, which can be converted by a number of bacteria, making it difficult to draw a final conclusion, but based on the data presented here it is most likely that in this study the lactobacilli are converting the lactose. In addition, we observed that the profile at 4 h for both fractions for volunteer A changed into a more fecal-like composition. The reason for this could be that during the *in vivo* experiment

the catheter continued to progress further in the intestinal tract and reached the colon. Additionally, the PCA analysis indicated that the microbial composition of the samples from the 1 h is similar to those of ileal effluent from individuals with an ileostoma, which could indicate that at this time point the catheter was positioned in the terminal ileum.

Interestingly, for volunteer B different members of the community were shown to be involved in the lactose degradation. Also here after 2 h changes were observed, indicating increases in phylotypes in the community in the fraction with higher density. Members of *Bacteroidetes* increased in the fractions with higher density (more than 10 fold) but also a species related to *Megasphaera elsdenii*. The latter microorganism is a well known lactate-utiliser, and it has been shown that it is able to counteract hyperlactate production in the large intestine (Hashizume et al., 2003). However, data from the metabolite profiling have to be analyzed, which will help to build the hypothesized cross-feeding relation between the identified species. Focusing on time point 4.5 h, where the presence of RNA was detected in the fraction with higher density, a strong increase in *Bifidobacterium* and *Collinisella* was detected in addition to the changes observed in *Akkermansia* and *Faecalibacterium prausnitzii*. Similar observations are described in **Chapter 3,** where we address the fate of ^{13}C labeled lactose in a laboratory *in vitro* colon model. Moreover, a member of *Clostridium* cluster XIVa related to *Anaerostipes caccae* et rel., also increased at the 4.5 h time point in the fraction with higher density. This latter species is also known to utilize lactate and convert it to butyrate (Marquet et al., 2009). PCA analysis grouped the samples with lower density from the different time points with fecal samples, which could indicate that for volunteer B the catheter progressed distally during the experiment, with the catheter tip located in the proximal colon, whereas the catheter remained in the terminal ileum throughout the test in volunteer A. This could also explain the observed differences on the microbial composition level.

In conclusion, using a multi-lumen catheter we were able to access and sample the human lower intestinal tract and applying RNA-based SIP together with high-throughput microarray-based phylogenetic profiling to explore the potential of the intestinal microbiota to convert lactose in the human intestine. Furthermore, the current human feeding trial will serve as starting point for further similar studies, which will allow to dig deep into the great microbial potential of the gut microbiota.

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CHAPTER 6
GENERAL DISCUSSION

GENERAL DISCUSSION AND CONCLUDING REMARKS

Introduction

An astonishing microbial diversity thrives on our planet and its activities are essential for the functioning of all ecosystems, including the human body. Consequently, a detailed understanding of the functions of microbes in their natural environment is required to understand our biology and the biology of the world around us. The aim of the work presented in this thesis was to explore the functionality of the human gastrointestinal (GI) tract microbiota, particularly in the fermentation of dietary carbohydrates. RNA-based stable isotope probing was applied together with high throughput phylogenetic analysis and metabolite profiling to expand our knowledge on key microbial functions related to healthy a gut status. The obtained results presented in this thesis contribute to a sound basis for the development of nutritional strategies for intestinal disease prevention and health promotion.

The role of gut microbiota in carbohydrate fermentation

The human GI tract microbiota has an important role in maintaining gut health. It has been suggested that the gut microbiota should be treated as a separate organ - "microbial organ", based on its collective metabolic potential (O'Hara and Shanahan, 2006; Cani and Delzenne, 2007). Furthermore, this metabolic repertoire delicately affects our physiology with functions that we have not had to evolve on our own (Backhed et al., 2005; Gill et al., 2006; Turnbaugh et al., 2007). The complex microbiota residing in the gut possess an array of activities associated with the fermentation of dietary compounds that escape digestion in the upper GI tract, processing of mucosal cells shed in the small intestine, and degradation of intestinally secreted mucus (Xu and Gordon, 2003; Noverr and Huffnagle, 2004; Ouwehand et al., 2005; Fava et al., 2006; Srikanth and McCormick, 2008) (Table 1). The ability to process nondigestible dietary fiber and related carbohydrates (resistant starch or oligosaccharides) is a crucial function of the colonic microbiota that significantly influences the gut environment and the host, by providing the means to use the carbohydrates as an energy source and in the maintenance of gut health (Savage, 1986; Guarner and Malagelada, 2003; Xu and Gordon, 2003; O'Hara and Shanahan, 2006).

The microbiota has access only to those dietary residuals that escape digestion by the host enzymes in the upper GI tract. A vast range of polysaccharide substrates from the diet reach the colon and is further converted by the colonic microbiota (Young et al., 1996). Plant cell-wall polysaccharides, storage polysaccharides (e.g.

Table 1. Relevant metabolic features of members of the human gut microbiota.

Microbial group	Species	Metabolic function	Produced metabolites	Reference
Bacteroidetes	*Bacteroidetes* spp.	Polysaccharides breakdown; L-, S-	Acetate, propionate, succinate	(Flint and Bayer, 2008) (Scott et al., 2008)
	Prevotella spp.	Polysaccharides breakdown; L-, S-	Acetate, succinate, formate	(Louis et al., 2007)
	B. thetaitaomicron	Polysaccharides breakdown, mucin degradation	Acetate, propionate	(Xu et al., 2007)
Actinobacteria	*Bifidobacterium adolescentis*	Carbohydrate metabolism	Acetate, lactate	(Marquet et al., 2009)
	B. longum	Carbohydrate metabolism	Acetate, lactate, formate,	(Falony et al., 2006)
	B. bifidum	Carbohydrate metabolism	ethanol, succinate	(Ramirez-Farias et al., 2009)
	Collinsella	Carbohydrate metabolism	Lactate, acetate, formate	(Nagai et al., 2009)
Clostridium cluster IV	*Ruminococcus bromii*	Carbohydrate metabolism	Acetate, formate, H_2, ethanol	(Abell et al., 2008; Kovatcheva-Datchary et al., 2009)
	R.. flavefaciens	Plant fibre breakdown	Acetate	(Robert and Bernalier-Donadille, 2003; Flint et al., 2008)
	F. prausnitzii	Carbohydrate metabolism	Butyrate, formate, D-lactate	(Duncan et al., 2002b)
Clostridium cluster XIVa	*R. intestinalis*	Carbohydrate metabolism	Butyrate, CO_2	(Falony et al., 2006)
	R. inulinovarans	Carbohydrate metabolism	Butyrate, propionate	(Duncan et al., 2006; Scott et al., 2006)
	E. halli	Carbohydrate metabolism; A-, L-	Butyrate	(Schwiertz et al., 2002; Duncan et al., 2004; Falony et al., 2006; Marquet et al., 2009)
	A. caccae	Carbohydrate metabolism; A-, L-	Butyrate, CO_2	
	E. recatle	Carbohydrate metabolism, A-	Butyrate, lactate, formtate, H_2	(Duncan and Flint, 2008)
	Coprococcus eutactus	Carbohydrate metabolism; A-	Butyrate, formate, lactate	(Holdeman and Moore, 1974)
	Dorea longicatena	Carbohydrate metabolism	Formate, acetate	(Taras et al., 2002)
Clostridium cluster IX	*Mitsoukella multiacida*	Gluconic acid metabolism; A-	Butyrate	(Tsukahara et al., 2002)
	Megasphaera elsdenii	Gluconic acid metabolism; A-, L-	Butyrate, propionate	
Proteobacteria	*Desulfovibrio* spp.: e.g.			(Willis et al., 1997; Marquet et al., 2009)
	Desulfovibrio piger	SRB, L-	Acetate, H_2S	
	Desulfovibrio desulfuricans	SRB, L-	Acetate, H_2S	
Verrucomicrobia / Lentisphaera	*Akkermansia muciniphila*	Mucin degradation	Acetate, propionate	(Derrien et al., 2004)
	Victivallis vadensis	Cellobiose degradation	Acetate	(Zoetendal et al., 2003)
Archaea	*Methanobrevibacter* spp.	H_2-utilizer	CH_4	(Conway de Macario and Macario, 2009)

L- lactate utiliser; S- succinate utiliser; A- acetate utiliser, SRB – sulfate reducing bacteria.

inulin), oligosaccharides and resistant starch are the main compounds that reach the colon (Macfarlane and Gibson, 1997) and further serve as food for the colonic microbiota. These substrates consist of a complex assortment of macromolecules with diverse structures, the degradation of which requires an array of bacterial hydrolytic enzymes produced by various bacterial members of the colonic community. There are only few colonic bacteria known to date that are able to initiate the breakdown of insoluble substrates and these predominantly belong to *Bacteroides* spp. and *Ruminococcus* spp. (Rincon et al., 2005; Flint et al., 2008; Jindou et al., 2008) (**Chapter 2**). Furthermore, during the sequential breakdown of complex carbohydrates, cooperation via metabolic cross-feeding is an important process (Fig. 1). Several classes of microbial activities can be observed to occur during such fermentations. As a first step, enzymatic activities of the primary degraders of complex carbohydrates result in the release of large amounts of polysaccharides (Fig. 1 I), which serve as substrates mostly for planktonic microbiota (Dehority,

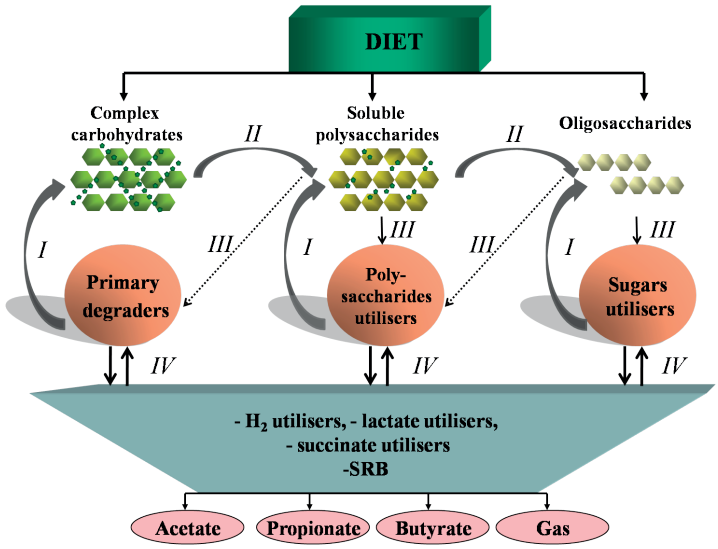

Figure 1. Schematic representation of the conversions of non-digestible carbohydrates by the anaerobic gut microbiota. I. Primary substrates degradation; II. Sequential degradation of dietary substrates; III. Substrate utilization by different populations in the colon; IV. Cross-feeding interactions between primary carbohydrate degraders and other members of the colonic microbiota. SRB - sulfate reducing bacteria.

1991). Furthermore, planktonic bacteria break down the polysaccharides and oligosaccharides that are released (Fig. 1 II). Oligosaccharides are one of the most studied carbohydrates, and it has been shown that bacteria that primarily utilize oligosaccharides do not grow on polysaccharides (Rossi et al., 2005). Additionally, cross-feeding during which the fermentation products released by one microorganism are utilized or serve as a growth factor for another population, often has impact on the energy metabolism of one or both partners (Wolin et al., 1997; Samuel and Gordon, 2006) (Fig. 1 IV) (**Chapters 2-4**). The main products from the anaerobic microbial fermentations occurring in the human colon are SCFAs, such as butyrate, propionate and acetate, which have an important function in host physiology (Scheppach, 1994; Mortensen and Clausen, 1996). It has been recognized that the composition of the gut microbiota is crucial for the relationship between the fermentable carbohydrate and the produced metabolites (Pryde et al., 2002).

A number of bacteria belonging to *Clostridium* cluster XIVa, form the group of butyrate-producers, based on their ability to produce butyrate as major product from carbohydrate fermentation (Barcenilla et al., 2000; Pryde et al., 2002) (Table 1). However, different fermentation pathways could be involved in butyrate synthesis in the human colon. The most widely spread pathway among different butyrate producing bacteria of the *Clostridium* cluster XIVa is the pathway known as the CoA-transferase route (**Chapter 2**), whereas another pathway involving butyrate-kinase activity is less common (Louis et al., 2004). Acetate is a co-substrate in the reaction where CoA-transferase activity is used for butyrate synthesis. There are a number of other fermentation pathways, where the synthesis of the other SCFA is involved, such as the succinate decarboxylase pathway and acrylate pathway (Louis et al., 2007), and the recently described propanediol pathway (Scott et al., 2006) for propionate synthesis. To investigate the use and importance of these pathways, it has been shown that the use of isotopically labeled substrates in fermentation studies is a suitable strategy in defining these different routes (**Chapter 3**). Additionally, molecular hydrogen (H_2) plays an important role in anaerobic ecosystems, such as the gut. Increase in H_2 production often decreases the metabolic activity of bacteria. However, such inhibition can be overcome by addition of a H_2-utiliser to the system, upon which for instance an increase in acetate is measured (Miller and Wolin, 1995). In **Chapter 4** such cross-feeding interaction is reported and further visualized in Figure 2. Based on experimental evidence we concluded that the H_2 produced by *R. bromii* during the starch degradation serves as electron donor for *E. rectale*, leading to an increase in SCFA amounts and the production of lactate. Additionally,

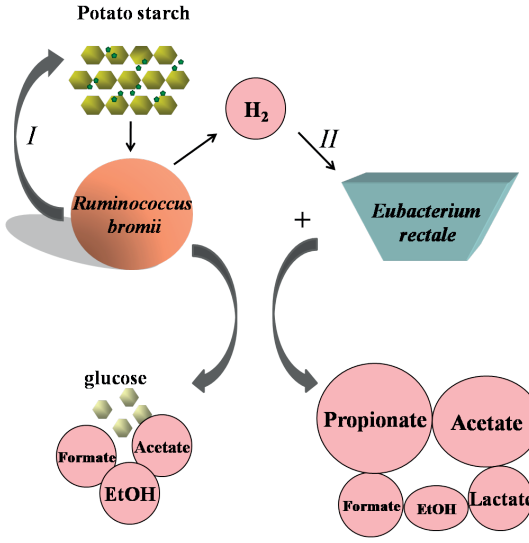

Figure 2. Interaction of a potato starch degrading *R. bromii* strain with hydrogen utilizing *E. rectale*-like bacterium (**Chapter 4**). I. Primary starch degradation; II. Cross-feeding interaction between the primary starch degrader and a hydrogen utilizing bacteria.

carbohydrate fermentation by bifidobacteria has been reported to involve another cross-feeding relation, as a result of which the lactate-utilizing *E. halii* produces butyrate (Belenguer et al., 2006). An earlier study described the mechanism of butyrate formation by colonic butyrate producers as *Roseburia* spp. and *Faecalibacterium prausnitzii*, which showed to be among the most active butyrate producers in the colon (Duncan et al., 2002).

Exploring the functionality of the human gut microbiota in the anaerobic fermentation of dietary carbohydrates

An effective strategy to delve into the functionality of the gut microbiota is to elucidate the role of individual species within the community in their natural environment. To explore the capacity of the gut microbiota in the fermentation of relevant dietary carbohydrates, we applied RNA-based Stable Isotope Probing (SIP), which in combination with molecular identification tools, provide a direct link between the structure of a microbial community and the function of its members (Kovatcheva-Datchary et al., 2009). In **Chapter 2** and **Chapter 3**, the capacity of the human fecal microbiota to ferment dietary carbohydrates such as starch, inulin and lactose was explored with RNA-based SIP analysis. Differences in the set of microbes involved in the conversion of carbohydrates in *in situ* conditions of the human colon were

described, by combining RNA-based SIP with high-throughput microarray-based phylogenetic profiling using the HITChip (**Chapter 3** and **Chapter 5**) (Rajilic-Stojanovic et al., 2009). With the presented research we additionally confirmed the power of the HITChip technology for high- throughput fingerprinting, phylogenetic and quantitative community analysis. To this end, the TIM-2 model of the human colon was used (Minekus et al., 1999) (**Chapter 1**). Moreover, RNA-based SIP allowed us to identify the primary degraders of the tested substrates, as it is reported in **Chapter 2,** where a phylotype related to *Ruminococcus bromii* was reported to be the primary degrader of potato starch, corroborating results that were reported in other *in vivo* and *in vitro* experiments (Leitch et al., 2007; Abell et al., 2008). Furthermore, more information on the functionality of the different microbes, found to be active in the colon ecosystem, could be generated after combining the data from the SIP technique with NMR and liquid-chromatography-mass spectrometry. The detection of (partially) labeled metabolites allowed the identification of active metabolic pathways and delineation of food webs that may influence human health (de Graaf et al., 2007; Egert et al., 2007; Kovatcheva-Datchary et al., 2009). In **Chapter 2**, the integration of the molecular and metabolite data suggested metabolic cross-feeding in the system during potato starch degradation, where populations related to *Ruminococcus bromii* are the primary starch degraders, while other members of the community, which were related to *Prevotella* spp., *Bifidobacterium adolescentis* and *Eubacterium rectale*, might be further involved in the trophic web.

Moving towards SIP *in vivo,* **Chapter 5** describes a pioneering and pilot *in vivo* trial, addressing the ability of the human intestinal microbiota to ferment carbohydrates *in situ* in the human colon. This study serves as a starting point for further *in vivo* experiments and further optimization on decisive issues for the SIP application *in vivo*, such as delivery of the labeled substrate, improvement of the sampling procedure, as well as the amount of the labeled substrate needed for unequivocal identification of microbial populations actively involved in the degradation of a given carbohydrate of interest, which is further discussed in **Chapter 5**.

Future perspectives

The comprehensive understanding of the metabolic activity of the gut microbiota will enable the development of direct strategies to treat or prevent intestinal disorders caused by microorganisms in humans but also in animals. Particularly in ruminants, the composition of the microbial community involved in degradation of polysaccharides

is quite similar to the human colonic microbiota. The knowledge derived from the SIP studies described here, may be a starting point in the optimization of the food intake of animals to improve their performance after acute acidosis, which is caused by increased lactate production after fermentation of readily fermentable carbohydrates. New animal nutritional supplements could also be developed based on the research described in the current thesis. Additionally, the data from the *in vivo* trial, described in **Chapter 5**, could help to clear the mechanism of acute acidosis based on the high lactate concentration.

Furthermore, new *in vivo* trials are expected to follow, which will benefit from the accumulated data from the *in vitro* but also the *in vivo* studies. The application of the SIP approach as a tool in medical research may bring new information for disease treatment, and prevention, and further contribute to describe the impact of gut microbiota on human health and well-being. Identification of the prime functions of the human gut microbiota in maintaining human health requires a better understanding of its diversity and functionality, which can facilitate its manipulation. Most intestinal microbes have not been cultured and the *in situ* functions of distinct groups of the gut microbiota are largely unknown but pivotal to understand their role in health and disease. Another part of the gut microbiota functionality, which needs to be further explored, is the conversion of protein that occurs usually in the distal colon, producing metabolites that are known to have toxic effects on the host (Macfarlane and Macfarlane 1995). Moreover, during carbohydrate fermentation, proteins are converted to microbial biomass (Birkett et al., 1996). In contrast, most carbohydrate fermentations occur in the proximal colon. Therefore it has been suggested that prolonged carbohydrate fermentation, mostly by the use of more slowly fermentable dietary carbohydrates, could reduce the incidence of proteolytic fermentations (Gibson, 1998; Gibson et al., 2004). This hypothesis needs to be further tested. It can be considered that with the current thesis, and particularly with the experiments described in **Chapter 2** and **Chapter 3**, we have generated the relevant knowledge, which can be used for optimizing carbohydrate fermentations. Additionally, further exploration of protein fermentations using the SIP approach, or even the new developments in stable isotope-based approaches described in **Chapter 1**, can be used in the identification of key players of the gut microbiota. Furthermore, the accumulated knowledge from both types of fermentations could serve in the development of new functional food components.

Finally, the use of metagenomics as a tool to analyse genomic DNA of the microbiota has expanded our knowledge on the microbial diversity, but also

functionality (Qin et al., 2010). The application of metagenomics with DNA-based SIP will facilitate the identification of functions from the targeted environment. Additionally, the combination of these approaches will allow to detect very rare and particular populations. Moreover the combination of SIP with metaproteomics, which is a very direct link for identification of proteins involved in specific metabolic processes (Jehmlich et al., 2008; Jehmlich et al., 2010), will further increase the sensitivity of this approach and allow to identify specific metabolic activities that can be linked to the phylogenetic identity of involved microorganisms.

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SUPPLEMENTS

*Summary * Samenvatting * Résumé * Резюме*

*Acknowledgements * About the Author*

*List of Publications * VLAG Activities*

SUMMARY

The human gastrointestinal (GI) tract comprises a series of complex and dynamic organs that are involved in food intake, food perception and food conversion. The colon embodies the most distal part of the GI tract, which is the main site of microbial colonization, and harbors immense microbial assemblages, known to be vital for human well-being. The human gut microbiota directly impacts on human physiology, in a beneficial or harmful manner through its collective metabolic activity.

The work presented in this thesis focused on deciphering the functional capacity of the human intestinal microbiota for the fermentation of relevant [^{13}-C]-labeled dietary carbohydrates using 16S ribosomal RNA (rRNA)-based Stable Isotope Probing (SIP). To this end, we combined RNA-based SIP with molecular identification tools and metabolite profiling. This resulted in identification of the primary degraders of potato starch, inulin and lactose under human colon-like conditions. Furthermore, metabolic cross-feeding networks were proposed, involving secondary fermentation processes. *Ruminococcus bromii* was identified as the key player in the degradation of potato starch based on molecular analysis of samples taken during the fermentation in the TIM-2 model of the human colon, but also through fermentation studies in mono-culture or co-culture with *Eubacterium rectale*. We further explored the metabolic conversion of potato starch in a simple co-culture fermentation system. This revealed a cross-feeding relationship between the two species that involved the consumption by *E. rectale* of H_2 produced by *R. bromii* from the starch degradation, leading to increased production of propionate and acetate. Furthermore, species related to *Dorea longicatena* and *Bifidobacterium adolescentis* were identified as key members of the tested community in inulin degradation. Moreover, species within the *Actinobacteria*, particularly *B. adolescentis* and *Collinsella,* were further described to be involved in lactose degradation at conditions simulating the human colon. Moving beyond *in vitro* studies we explored the role of the gut microbiota in lactose fermentation with a pioneering study, where [U-^{13}C]-lactose was delivered directly to the terminal ileum of healthy human volunteers using a multi-lumen catheter that was also used for sampling of luminal content to explore the community involved in lactose degradation directly in the human intestine. Individual patterns of the microbiota involved in the lactose fermentation were observed, showing that in both volunteers different microbial populations were involved, which

could be attributed to differences in the sampling location. A population related to *Lactobacillus plantarum* was found to be most pronounced in one of the volunteers during the trial, while with the other volunteer most probably members of the genera *Bifidobacterium* and *Colinsella* were the primary lactose degraders, in agreement with the *in vitro* model study. Furthermore, *Ananerostipes caccae* was found to be involved in the trophic chain, which could be related to its capacity to convert the lactate produced by the primary lactose degraders into butyrate. Further analysis of the accumulated metabolites during the lactose degradation will help to reconstruct the metabolic pathways involved in intestinal metabolism.

In conclusion, the results obtained in the framework of this thesis indicate that the integration of the RNA-based SIP approach and high-throughput diagnostic microarray-based phylogenetic profiling with metabolic flux analysis, appears a promising approach to reveal bacteria that are actually involved in the carbohydrate fermentations, not only in model systems, but more importantly also in the intestinal tract of man.

SAMENVATTING

Het menselijk maag-darmstelsel bestaat uit een serie van complexe en dynamische organen die betrokken zijn bij de opname, perceptie en vertering van voedingsstoffen. Het colon, ofwel de dikke darm, belichaamt het meest distale gedeelte van het maag-darmstelsel, is de voornaamste plaats van microbiële kolonisatie en huisvest immense verzamelingen van bacteriën, waarvan bekend is dat ze belangrijk zijn voor de gezondheid van de mens. Door hun gezamenlijke metabole activiteit heeft deze menselijke darm microbiota een directe invloed op de fysiologie van de mens, hetzij bevorderlijk of nadelig.

Het werk dat gepresenteerd is in dit proefschrift richtte zich op het ontrafelen van het functionele vermogen van de menselijke darm microbiota om relevante [^{13}-C]-gelabelde voedselkoolhydraten te fermenteren, gebruik makend van 16S ribosomaal RNA (rRNA)-gebaseerd Stabiele Isotoop Probing (SIP). Hiertoe hebben we RNA-gebaseerd SIP gecombineerd met moleculaire identificatie methodes en analyse van gevormde metabolieten. Dit resulteerde in de identificatie van de primaire verantwoordelijken voor de afbraak van aardappelzetmeel, inuline en lactose, onder omstandigheden die het menselijke colon het dichtst benaderen. Daarnaast zijn metabole cross-feeding netwerken voorgesteld, mede gebaseerd op secundaire fermentatie processen. *Ruminococcus bromii* werd aangetoond een sleutelrol te spelen bij de afbraak van aardappelzetmeel, gebaseerd op de moleculaire analyse van monsters die genomen zijn tijdens de fermentatie in het TIM-2 model van het menselijke colon, alsmede middels studies van mono-cultuur fermentaties of co-cultuur fermentaties met *Eubacterium rectale*. Uit laatstgenoemde co-cultuur experimenten kwam een cross-feeding relatie naar voren tussen de twee soorten, waarbij de waterstof die wordt geproduceerd door *R. bromii* bij de afbraak van zetmeel door *E. rectale* wordt gebruikt voor productie van propionaat en acetaat. Verder zijn soorten gerelateerd aan *Dorea longicatena* en *Bifidobacterium adolescentis* geïdentificeerd als belangrijkste micro-organismen, binnen de geteste microbiota, bij de afbraak van inuline. Bovendien is beschreven dat soorten binnen de *Actinobacteria*, met name *B. adolescentis* en *Collinsella,* betrokken zijn bij de afbraak van lactose onder condities die de menselijke colon simuleren. In een baanbrekende studie waarbij we verder zijn gegaan dan *in vitro* studies hebben we de rol van de microbiota onderzocht bij lactose fermentatie. Hierin werd [U-^{13}C]-

lactose direct in het laatste gedeelte van het ileum ingebracht bij gezonde vrijwilligers middels een multi-lumen katheter welke ook gebruikt werd voor monstername van de darminhoud om de microbiota, die betrokken is bij de afbraak van lactose, direct in de menselijke darm te bestuderen. In beide vrijwilligers werden verschillende patronen van de microbiota betrokken bij de fermentatie van lactose waargenomen, wat onder andere toegeschreven zou kunnen worden aan verschillen in locatie van monstername. Een populatie gerelateerd aan *Lactobacillus plantarum* werd gevonden als meest geprononceerd in een van de vrijwilligers tijdens de testperiode, terwijl bij de andere vrijwilliger meest waarschijnlijk leden van de genera *Bifidobacterium* en *Colinsella* primair verantwoordelijk zijn voor de afbraak van lactose, wat in overeenstemming is met de *in vitro* model studie. Daarnaast werd gevonden dat *Ananerostipes caccae* betrokken is bij lactose fermentatie in een latere fase. Dit zou gerelateerd kunnen worden aan zijn vermogen om lactaat, wat gevormd wordt door de voornaamste lactose afbrekende bacteriën, om te zetten in butyraat. Verdere analyse van de opgebouwde metabolieten tijdens de afbraak van lactose zal helpen om de metabole routes te reconstrueren die betrokken zijn bij het metabolisme in de darmen.

Concluderend kunnen we stellen dat de resultaten die verkregen zijn in het kader van dit proefschrift aangeven dat de integratie van de RNA–gebaseerde SIP benadering en de fylogenetische beschrijving, gebaseerd op high-throughput diagnostische mirco-arrays, samen met de metabole flux analyse een veelbelovende benadering blijkt te zijn om bacteriën te identificeren die werkelijk betrokken zijn bij de koolhydraat fermentaties, niet alleen in modelsystemen, maar, belangrijker nog, ook in het maag-darmstelsel van de mens.

RÉSUMÉ

Le tube digestif de l'homme comprend une série d'organes complexes qui sont impliqués dans la perception des aliments, la digestion du bol alimentaire, ainsi que son assimilation. Le colon représente la partie distale du tube digestif qui est le site principal de colonisation microbienne et abrite d'immenses assemblages microbiens, connus pour être vitaux pour le bien-être de l'Homme. Le microbiote du tube digestif humain influences directement la physiologie humaine de façon positive ou négative en fonction de l'activité métabolique collective.

Le travail présenté dans cette thèse se concentre sur le décodage de la capacité fonctionnelle du microbiote intestinal humain dans la fermentation d'hydrates de carbone diététiques [^{13}-C] marqués en utilisant un échantillonnage d'isotopes stables (Stable Isotope Probing, SIP) basé sur l'ARN 16S ribosomale. A cette fin, nous avons combiné la technique SIP basée sur l'ARN avec des outils d'identification moléculaire et de profilage de métabolites. Cette étude a permis une identification de dégradeurs primaires de l'amidon de pomme de terre, de l'inuline et du lactose dans des conditions semblables au colon humain. En outre, un croisement des réseaux métaboliques ont été effectué en incluant des procédés de fermentation secondaire. *Ruminococcus bromii* a été identifié comme le principal responsable de la dégradation de l'amidon de pomme de terre en se basant sur l'analyse moléculaire d'échantillons prélevé durant la fermentation du modèle du colon humain TIM-2, mais également par des études en mono-culture ou co-culture de fermentation d'*Eubacterium rectale*. Nous avons en outre exploré la conversion métabolique d'amidon de pomme de terre dans une fermentation en co-culture. Cela a révélé une relation d'alimentation croisée (cross feeding) entre les deux espèces. Le H_2 produit par *R. bromii* à partir de la dégradation de l'amidon accroît la production de propionate par *E. rectale*. De plus, les espèces liées à *Dorea longicatena* et *Bifidobacterium adolescentis* assimilent l'inuline et les bactéries *B. adolescentis* et *Collinsella* dégradent le lactose dans des conditions similaires au colon humain.

Une étude pionnière 'in-vivo' avec des volontaires ayant consommé du lactose a permis d'établir que *Lactobacillus plantarum*, *Bifidobacterium* et *Colinsella* sont les principales bactéries qui dégradent le lactose.

En conclusion, les résultats présentés dans cette thèse indiquent que l'intégration de différentes techniques novatrices ont permis d'identifier les bactéries

qui sont impliquées dans la fermentation des glucides et ce, non seulement, dans le cadre de modèles *in-vitro* mais également dans le tube digestif humain.

РЕЗЮМЕ

Човешката храносмилателна система се състои от сложни и функционални органи, които участвуват в поемането, възприемането и преработването на храната. Дебелото черво е най-отдалечената част на стомашно-чревния тракт, което е главното място на микробиално колонизиране и убежище на големи микробиални струпвания, доказали жизненоважната си функция върху здравето ни. Изследванията върху активността на човешката чревна микробиота в стомашно-чревния тракт са необходими за да опознаем по-добре ролята на микроорганизмите върху човешкото здраве и болести. Това ще позволи да бъдат разработени стратегии, които да повлияят на/ или предотвратят стомашно-чревни заболявания. Консумирането на пребиотични въглехидрати е една от стратегиите, чрез която се стимулират полезни чревни ферментации.

Целта на настоящата докторантура е дешифрирането на функционалния капацитет на човешката чревна микробиота по време на разграждането на сложни въглехидрати. За целта бе използвана методика, известна като 16S рибозомна РНК (рРНК) стабилно изотопно сондиране, където [^{13}-C]-белязани диетични въглехидрати бяха тествани. За да бъдат идентифицирани микроорганизмите, които директно разграждат пребиотични въглехидрати, като картофено нишесте, инулин и лактоза, в условията на дебелото черво, РНК-стабилно изотопно сондиране беше комбинирано с молекулярни методи и метаболитно профилиране. Установени бяха метаболитни системи на кръстосано хранене, включващи вторични ферментационни процеси. Беше установено, че *Ruminococcus bromii* е ключовият микроорганизъм в разграждането на картофеното нишесте, въз основа на молекулярен анализ на проби, взети по време на ферментация в модел, стимулиращ условията в човешкото дебело черво, а също и посредством ферментационни изследвания с монокултура или с ко-култура на *Eubacterium rectale*. Показано беше взаимодействие по време на кръстосано хранене между двата вида микроорганизми, където произведения H_2 от *R. bromii* при директното разграждане на нишестето бе инкорпориран и резултираше в повишено продуциране на пропионат и ацетат от *E. rectale*. В допълнение микроорганизми, сродни с *Dorea longicatena* и *Bifidobacteriun adolescentis* бяха идентифицирани като ключови бактерии при разграждане на инулина. А видове от *Actinobacteria*, в частност *B. adolescentis* и *Collinsella* бяха

показани, че разграждат лактозата при същите условия и в същата микробна популация.

За да проучим ролята на чревната микробиота в разграждането на лактоза, ние проведохме *in vivo* изследвания със здрави доброволци. Това е първото по рода си изследване, където изотоп на лактоза беше директно доставен в последната част на тънкото черво на доброволците чрез мулти-каналов катетър. Същият бе използван и за взимане на проби от съдържанието на лумена, за да се изследва чревната микробиота, разграждаща лактозата в условията на човешкия чревен тракт. Наблюдавани бяха индивидуални особености на микробиотата, която ферментира лактозата, показващи че и при двамата доброволци участват различни микробиални популации, което може би е свързано и с различната локализация на катетъра при взимане на пробата. Беше открита популация *Lactobacillus plantarum*, която беше сред най-срещаната микробна група, разграждаща лактозата при единия от доброволците по време на опита, докато при другия доброволец - най-вероятно видове от родовете *Bifidobacterium* и *Colinsella*, което беше доказано и с *in vitro* изследване. Освен това беше показано, че *Ananerostipes caccae* се включваше в трофичната верига най-вероятно чрез неговата способност да превръща продуцирания лактат от основните разграждачи на лактозата в бутират. Следващи анализи на акумулираните метаболитни продукти по време на лактозната деградация ще бъдат от помощ за пренасочване на метаболитните пътища, включени в чревния метаболизъм.

В заключение, получените резултати от тази дисертация показват, че интегрирането на РНК-стабилно изотопно сондиране с високо-диагностични анализи на база филогенетично профилиране и метаболитни анализи е надежден подход за идентифициране на бактерии, които в действителност участват във ферментацията на въглехидратите, не само в моделни системи, но и с по-голяма значимост в чревния тракт на човека.

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Petia

ABOUT THE AUTHOR

Petia Kovatcheva-Datchary (maiden name Petia Kovatcheva) was born on the 12th of February 1977 in Chirpan, Bulgaria. She obtained her M.Sc in Biotechnology (1999) at the University of Food Technologies, Bulgaria. She performed her practical thesis at the Laboratory of Food Microbiology, Wageningen University under the supervision of Dr. Ralf Hartemink and Dr. Rob Nout, on studying the survival of microorganisms, involved in the fermentation of a Bulgarian traditional fermented beverage, in conditions simulating the gastrointestinal tract. After her graduation she worked as research assistant at the Bulgarian Academy of Sciences. Her activities were mainly focused on biological active substances (alkaloids, natural colorants and antioxidants) of the secondary metabolism of plant tissue and cell cultures. In 2001 she participated in a training program on extraction, purification and separation of biological active substances from plants in the University of Reims Champagne-Ardenne, France. In 2002 she was involved in an international program in Germany on "The Biotechnology as a Contribution to Food Security". As a result from this course in 2003 she was awarded with a scholarship from the German Academic Exchange Service (DAAD) to take a part in a project on development of a plant virus's diagnosis system based on Microarrays. The last project was based at the Hamburg University of Technology. In March 2005 she began her PhD study at the Laboratory of Microbiology at Wageningen University under supervision of Prof. Dr. Willem M. de Vos, Dr. Hauke Smidt and Dr. Koen Venema. The aim of her project, which was a part of the activity of TI Food and Nutrition (formerly known as WCFS), was to find out the link between the structure and the *in situ* functional characterization of the active intestinal microbial community. The results of this research are presented in this thesis. This publication is the culmination of this PhD study.

LIST OF PUBLICATIONS

Kovatcheva-Datchary, P., Egert, M., Maathuis, A., Rajilic-Stojanovic, M., de Graaf, A.A., Smidt, H., de Vos, W.M., and Venema, K. (2009) Linking phylogenetic identities of bacteria to starch fermentation in an *in vitro* model of the large intestine by RNA-based stable isotope probing. *Environmental Microbiology* **11**: 914-926.
-Winner of TIFN Publication Prize 2008 (Bioingredients and Functionality);
-Winner of the NVvM/NVMM Kiemprize 2009 (Microbial Ecology).

Kovatcheva-Datchary, P., Zoetendal, E.G., Venema, K., de Vos, W.M., and Smidt, H. (2009) Review: Tools for the tract: understanding the functionality of the gastrointestinal tract. *Therapeutic Advances in Gastroenterology* **2**: s9-22.

Kovatcheva-Datchary, P., Egert, M., Maathuis, A., de Graaf, A.A., Smidt, H., de Vos, W.M., and Venema, K. RNA-based stable isotope probing in combination with phylogenetic microarray analysis implicates members of *Clostridium* cluster XIVa and *Actinobacteria* in inulin and lactose metabolism in an *in vitro* model of the human colon. Manuscript in preparation.

Kovatcheva-Datchary, P., Zhang L., de Waard P., van As H., Smidt, H., Stams A.J.M., de Vos, W.M., and Venema, K. Cross-feeding between *Ruminococcus bromii* and *Eubacterium rectale* during anaerobic degradation of potato starch. Manuscript in preparation.

Kovatcheva-Datchary, P., Vanhoutvin S., Troost F.J., Tims S., Heilig H.G., de Vos, W.M., Venema, K., and Smidt, H. Identification of microbial populations involved in the degradation of lactose in the human intestinal tract. Manuscript in preparation.

Overview of Completed Training Activities

Discipline specific courses	
Systems Biology: Principles of ~omics data analysis,VLAG & EPS, Nijmegen	2005
Micro-array technology, production and application, EMBO, Mexico, Mexico	2005
Ecophysiology of the GI-tract, VLAG & WIAS, Wageningen	2007
ARB – a software environment for sequence data, Ribocon / PRI, Wageningen	2007
HITChip course, MIB, Wageningen	2008
General courses	
Teaching and supervising thesis students, WUR, Wageningen	2005
Project & Time Management, PE&RC, Wageningen	2005
Techniques for writing and presenting a scientific paper, PE&RC, Wageningen	2005
French language, CENTA, Wageningen	2005
VLAG PhD week, VLAG, Bilthoven	2005
Meetings	
Darmendag, Maastricht (*poster presentation*)	2005
Symposium of "Biomedicine in the Post-genomic Era", EMBO – INER, Mexico, Mexico (*poster presentation*)	2005
Harvesting Microbial Diversity,Mining, Methods and Metagenomes, WCFS (TIFN), Osterbeek (*poster presentation*)	2006
158th meeting of the Society for General Microbiology, SGM, Warwick, UK (*oral presentation*)	2006
RRI-INRA symposium on Gut Microbiology, Aberdeen, UK, (*poster presentation*)	2006
ISME-11, Vienna, Austria (*poster presentation*)	2006
Darmendag, Groningen (*oral presentation*)	2006
Conference on Gastrointestinal Function, Chicago, USA (*oral presentation*)	2007
NVvM meeting, Papendal (*oral presentation*)	2007
INRA-RRI symposium on Gut Microbiology, France (*oral presentation*)	2008
ISME-12, Carins, Australia (*poster presentation*)	2008
Darmendag, Utrecht (*oral presentation*)	2008
TIFN Food Summit, Wageningen (*oral presentation*)	2009

Optional activities

Preparing PhD research proposal	2005
WCFS/TIFN project meetings C-012	2005-9
PhD/Postdoc meetings, Laboratory of Microbiology, Wageningen	2005-10

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