Ecophysiology of Novel Intestinal Butyrate-Producing Bacteria

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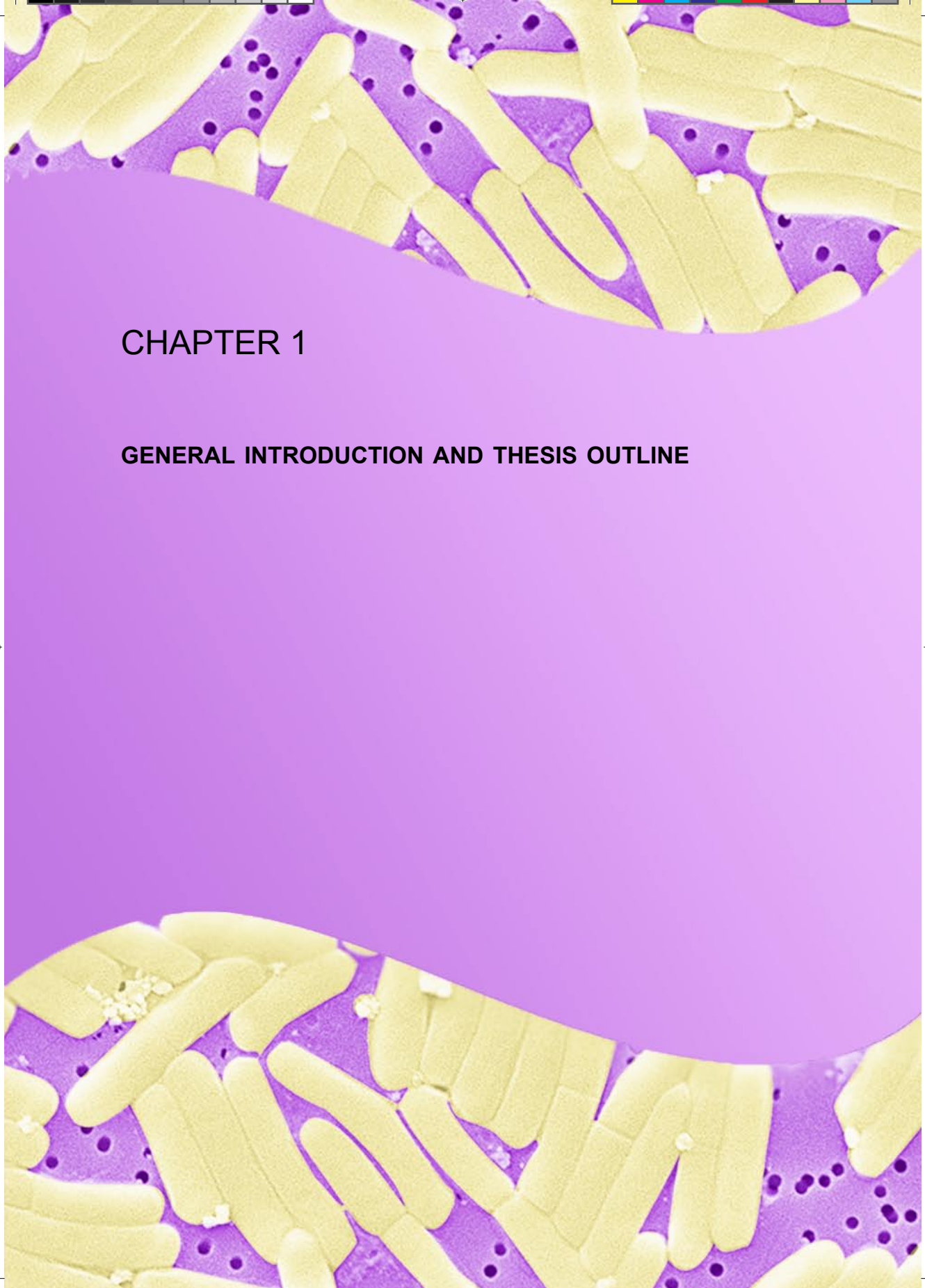

CHAPTER 1

GENERAL INTRODUCTION AND THESIS OUTLINE

The human body harbours a trillion of cells that are predominantly microbes, collectively known as microbiota. These microbes thrive in complex consortia in particular niches of the body and among individuals; their niche-specific assemblages are relatively conserved (Turnbaugh et al., 2007; Qin et al., 2010). The intestinal tract harbours the largest set of the human microbes that constitute a climax community in the colon. The colonic microbiota is part of the digestive system and contributes significantly to human health and well-being (Zoetendal et al., 2006). The human intestinal microbiota affect the host, depending on the diversity, species composition and metabolic output (Flint et al., 2012). Many culture-independent studies have been reported that focus on the diversity and composition of the human microbiota (Zoetendal et al., 2008; Yatsunenko et al., 2012). Yet, the function of these microbes is not fully understood and this is complicated by the fact that many are strict anaerobes. Moreover, it is highly challenging to predict the behaviour of an entire microbial community without knowing its composition or the function of the individual microbes. Hence, isolating microbes and growing these as pure cultures is a prerequisite for facilitating further research on their metabolic properties that might be instrumental in unravelling the mechanism of their actions. Studies on microbial diversity of colonic samples based on 16S rRNA analysis have shown that 75% of directly amplified sequences do not closely relate to known cultured bacteria (Flint et al., 2006). This shows that the majority of the intestinal microbes have never been cultured and some might have not been identified yet. A recent study reported on the first 1000 cultured species from the human intestinal tract and indicated that rapid progress is made with novel culturing approaches (Rajilić-Stojanović and de Vos, 2014). In the first comprehensive intestinal metagenomics study, it was estimated that at least 1150 bacterial species were present in 124 individuals (Qin et al., 2010). Other estimates referred to more than 2000 species belonging to 12 different phyla that have been listed as human-related isolates (Hugon et al., 2015). Moreover/however, the analysis of curated 16S rRNA libraries suggested the presence of 3500 species (Ritari et al., 2015), indicating that a multitude of uncultured bacteria are awaiting to be isolated in pure culture.

Short chain fatty acids (SCFAs) such as acetate, butyrate and propionate, are products of fermentation of dietary components in the gut. The SCFAs have different functions in the human body; butyrate is mainly fuelling the colonocytes, propionate is mainly metabolized in the liver, and acetate reaches the peripheral tissues (Guarner and Malagelada, 2003). In addition, SCFAs have an effect on colon morphology; lower the pH; increase in cell proliferation and faecal bulk and modification of the microbial composition. SCFAs also control body weight and insulin sensitivity (Canfora et al., 2015). Acetate stimulates cholesterol synthesis and propionate is a substrate for hepatic gluconeogenesis. Propionate has inhibitory effects on lipid and cholesterol synthesis and protective effects against inflammation and carcinogenesis (Hosseini et al., 2011). Moreover, SCFAs function as a signal to the host via two G-protein-coupled receptors (GPR41 and GPR43) that have been shown to affect the immune system in mice (Brown et al., 2003; Le Poul et al., 2003). Specifically, butyrate and butyrate-producing

bacteria have been identified as crucial components mediating colonic health (Sengupta et al., 2006; Hamer et al., 2008; Macfarlane and Macfarlane, 2012). Butyrate has various beneficial effects including the inhibition of colonic carcinogenesis, oxidative stress and inflammation as well as the enhancement of the colonic defence barrier function (Hamer et al., 2008). To get a better understanding of butyrate synthesis (butyrogenesis) in the human gut, it is necessary to know and characterize the assemblage of butyrate-producing bacteria. The ideal way to do this is by cultivating novel intestinal butyrate-producing bacteria and identifying their metabolic features. This thesis presents the outcome of isolating and characterising butyrate-producing bacteria from the human gut, investigating their butyrogenic metabolisms, and characterizing their interactions with other intestinal microbes.

The human gut ecosystem

The human gastrointestinal tract (GI tract) is a complex and dynamic ecosystem colonised by myriads of microbes (Flint et al., 2007; Zoetendal et al., 2008). It is anatomically composed of 5 main compartments: mouth, pharynx, esophagus, stomach, the small intestine and the large intestine, each with particular functions. Each distinct compartment along the GI tract is characterised by its own physicochemical conditions which include acidity, redox potential, nutrient availability, intestinal mobility and host secretions. After ingesting food through the mouth, it passes through the esophagus and enters the stomach for further mechanical and chemical digestion by acids and enzymes. The number of microbes in the stomach is typically 10^3 cells/g biomass due to the highly acidic condition (pH ≤ 2) and short transition time (2-6 h). The small intestine is mainly responsible for uptake and conversion of (simple) carbohydrates from the un-digested food components (Zoetendal et al., 2012). The microbial population in the small intestine is larger than in the stomach because of its less hostile conditions (pH 5-6). However, it still remains low (10^5-10^7 cells/g biomass) due to the presence of bile and the fast flow of food movement that results in rapid passage of bacteria. Fast-growing facultative anaerobic streptococci are dominant in this region and are mainly responsible for sugar conversion resulting in the formation of lactate and acetate, that can be used by either butyrate-producing bacteria or *Veillonella* spp. (Zoetendal et al., 2012). While most of the easily degradable food components are taken up in the small intestine, the large intestine is the site where most of the fermentation takes place. The more complex food components, such as plant cell walls and resistant starch, have such complex structures that they cannot be degraded by the digestive enzymes and reach the large intestine in intact forms. This also may apply to other sugar or protein polymers that are in a form that they escape degradation by the digestive enzymes. Altogether, these compounds are termed non-digested compounds throughout this thesis (Figure 1). The hydrolysis of such complex substrates (Figure 1) requires the action of specialized microbial consortia which possess enzymes able to degrade these compounds (Flint et al., 2006). The large intestine is metabolically the most active region in the GI tract because of the slow passage of nutrients and the high density of microorganisms that are mostly obligate anaerobes. It has been estimated that the large

intestine consists of around 10^{12} microbial cells/g biomass of which over 1000 species have been identified (Zoetendal et al., 2008; Rajilić-Stojanović and de Vos, 2014).

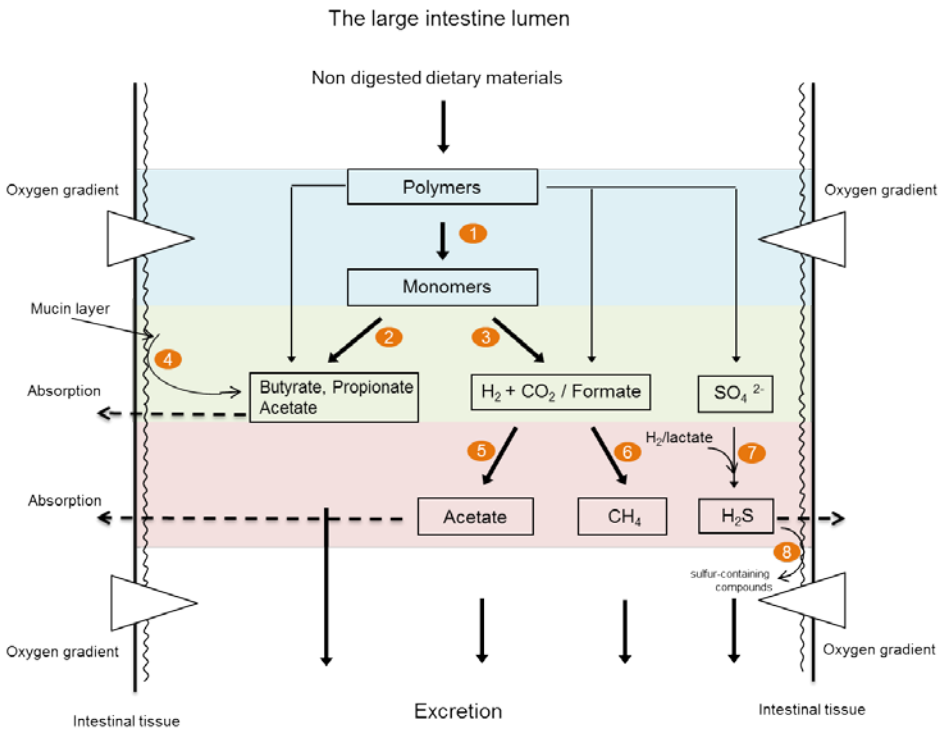

Figure 1. Anaerobic microbial degradation of food components in the large intestine. Step 1: Polymer hydrolysis; Step 2, 3: Fermentation leading to SCFAs and gases/formate. Step 4: Mucin degradation; Step 5, 6: Conversion of H_2/CO_2 or formate into acetate or CH_4; Step 7: SO_4^{2-} to H_2S reduction in presence of H_2 or lactate; Step 8: H_2S oxidation to thiosulfate and other sulfur-containing compounds by colonic tissue. The blue, green and pink boxes indicate hydrolysis of polymers, acidogenesis and acetogenesis/methanogenesis/ sulfidogenesis, respectively. The triangle indicates the oxygen gradient towards the lumen.

The microbial fermentation of non-digestible diets and host-derived components in the colon is indicated in Figure 1. These polymers derive from non-digestible dietary components and host mucins that are hydrolysed into monomers (step 1), which are further fermented into short chain fatty acids (butyrate, propionate, acetate – step 2) and gases (H_2, CO_2 or formate – step 3). Mucin degradation also contributes to SCFA production (step 4). These processes are performed by either single microbes or microbial consortia. While these SCFAs are absorbed by the colon and further metabolised by the host, H_2/CO_2 or formate are further converted

into acetate (step 5) or CH_4 (step 6). In addition, H_2S can be formed via respiration with sulfate (SO_4^{2-}) as terminal electron acceptor (step 7). Sulfate can be derived from many food products but also from the degradation of cysteine or methionine-containing proteins. Sulfur-containing components that are abundant in mammalian tissues and mucus can also be a source for colonic sulfate. When diffusing to colonic mucosa, the formed H_2S can be readily detoxified by colonic tissue to thiosulfate (step 8) (Furne et al., 2001). This thiosulfate can be either utilized by colonic bacteria (Stoffels et al., 2012) or disposed in the stool. Moreover, during inflammation it can be converted to other sulfur-containing compounds, such as tetrathionate that provides a competitive advantage for pathogens (Winter et al., 2010). Other major functions of intestinal microbiota are summarised below in Table 1.

Table 1: Major functions of the human gut microbiota

Functions of intestinal microbiota	References
Energy harvest and nutrient supply	Sonnenburg et al., 2005; Allen and Stabler, 2008; Turnbaugh and Stintzi, 2011
Inhibition of pathogens	Guarner and Malagelada, 2003
Cancer prevention	Segain et al., 2000; Boleij and Tjalsma, 2012
Normal gastrointestinal immune function	Hooper and Macpherson, 2010 Gassull, 2006; Hamer et al., 2008
Normal gut motility	Lewis and Heaton, 1997 Scheppach, 1994
Cardiovascular health	Wikoff et al., 2009
Mucin degradation	Derrien et al., 2004

Human microbiota

Human health is a result of the interplay between genes, microbiota and environment. While new-borns are virtually sterile, colonization is believed to start directly after birth. Firstly, rather simple communities of mainly facultative bacteria proliferate that subsequently develop into a more complex and diverse microbial community (Scholtens et al., 2012; Yatsunenko et al., 2012). Delivery mode and breast-feeding have a strong impact on the development of adult microbiota during the first year of life (Bäckhed et al., 2015). Moreover, antibiotic use in early life has shown to have an impact on the microbiota and health at later stages of life (Korpela et al., 2016). In adult life, the intestinal microbiota is highly diverse and resilient to diet, antibiotics and lifestyle. Diet is an important driver of the microbiota composition and changing dietary patterns may have considerable impact on the microbiota and health biomarkers (Zoetendal & de Vos 2014; O'Keefe et al 2014).

High-throughput techniques have been used to investigate the structure and function of our intestinal microbes by analysing thousands of samples from all over the world (Ley et al., 2006; Turnbaugh et al., 2006; Qin et al., 2012). Metagenomic analysis has revealed remarkable diversity within the colonic microbiota of healthy adults and was mainly composed of Firmicutes, Bacteroidetes and Actinobacteria with Proteobacteria and Verrucomicrobia as less abundant groups (Qin et al., 2010). Three enterotypes have been proposed in the gut microbiome of individuals across several nations and continents and these were found to be robust in many subsequent studies (Arumugam et al., 2011). However, as already demonstrated in the early phases of gut microbiota research, each individual carries a unique microbial community (microbiome) (Zoetendal et al., 1998). This personalized microbiome has been observed in all large-scale studies and relates to the unique acquisition and maintenance of the intestinal microbiota in early life. This also explains the observation that identical twins have a more similar microbiome than unrelated members in the same household (Tims et al., 2013). Once established, the human microbial composition is relatively stable over time and significant similarity was found in healthy adult subjects monitored over a time span of 10 years (Franks et al., 1998; Rajilić-Stojanović et al., 2013; Zoetendal and de Vos, 2014). While specific groups of microbes are affected and often depending on the disease, a general feature of a healthy microbiome is high diversity (Zoetendal and de Vos, 2014). In contrast, the microbiota of patients suffering from various diseases differ from healthy subjects and this is specifically observed in patients suffering from recurrent *Clostridium difficile* infection (CDI). These patients have a low microbial diversity, which is as low as in a few-months old baby. This is mainly due to the recurrent use of antibiotics in CDI patients and the infection by the antibiotic-resistant pathogen (van Nood et al., 2013). Remarkably, it has been convincingly shown that resetting this low diversity by faecal transplantation of a healthy donor, and not the use of more antibiotics, leads to the complete cure and is associated with a rapidly increasing microbial diversity in the GI tract of the patient (van Nood et al., 2013; Fuentes et al., 2014). This primary example demonstrates that in some cases “bugs are better than drugs” and this testifies for specific host-microbe interactions. A great variety of other diseases also have been associated with a reduced diversity, notably in inflammatory bowel disease patients. Patients also showed a loss in richness of *Faecalibacterium prausnitzii* and a decreased level of butyrate formation (Lopez-Siles et al., 2015).

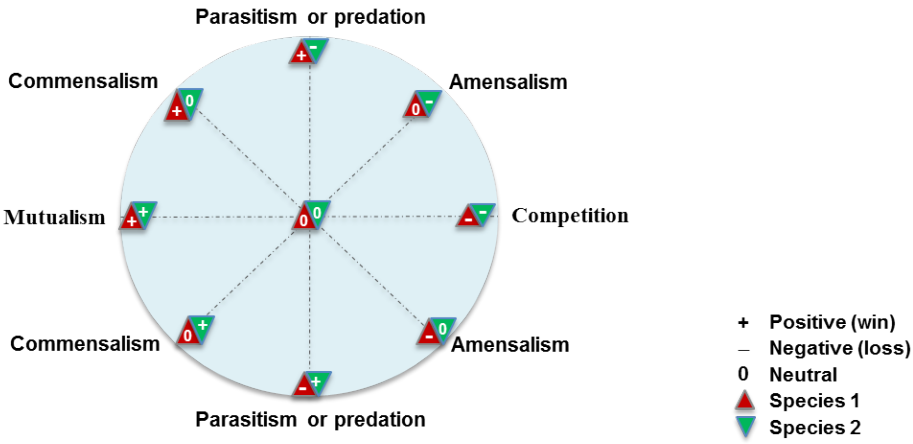

Figure 2: Overview of possible interactions between microorganisms. The wheel display summarizes possible pairwise interactions. For each interacting partner, there are three possible outcomes: positive (+), negative (−) and neutral (0). For instance, in parasitism, the parasite benefits from the relationship (+), whereas the host is harmed (−); this relationship is thus represented by the symbol pair +/−. This figure was modified after Faust and Raes, 2012.

The presence of more than 1000 species in the colon, of which the cell density is extremely high, creates opportunities for interactions not just between host and microbes, but also among microbes. There is always the mutualistic relationship of the human host and its colonic bacteria (Bäckhed et al., 2005). The host-microbe interaction is not just described as commensalism but also as mutualism and even syntrophy and must be in balance (Faust and Raes, 2012). In mutualism, commensal microbes usually provide beneficial molecules to the host while the host in turn supplies substrates from dietary intake or mucosal secretions to the microbes, meaning that the human host and microbiota both gain benefit from each other (Figure 2). In commensalism, however, some intestinal microbes stay in the gut and live on the products or intermediates of the microbial metabolism but do not affect the host. In some circumstances, pathogens might thrive in the gut and have a detrimental effect on the host. For instance the toxin produced by the pathogenic *Clostridium difficile* disrupts the intestinal epithelium, leading to an inflammatory response with clinical symptoms varying from mild diarrhoea to severe colitis (Kelly and LaMont, 2010). Within the intestinal microbiota, all possible interactions (Figure 2) have been described (Faust & Raes 2012). Metabolic syntrophic interactions between two or more microbial species have been reported to enhance the efficiency of glycan metabolism in the gut (Koropatkin et al., 2012). In another example of commensalism, *Bifidobacterium aldolescentis* was found to degrade resistant starch that released intermediates such as

lactate and acetate that could be used by butyrate-producing bacteria (*Eubacterium hallii* and *Anaerostipes caccae*) for growth (Belenguer et al., 2006). Microbes that grow on the same compound may compete for substrate use, such as acetogens while methanogens or sulphate-reducing bacteria (in the presence of sulphate) compete for instance for hydrogen (Figure 1). The human intestinal microbiota have now been associated with a total of 25 diseases. Potential health-promoting bacteria have been proposed such as *Bifidobacterium* spp., *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* (de Vos and de Vos, 2012). These microbes may assist in the recovery from diseases and they can be influenced by a diet in a predictable way, depending on the microbiota composition and diversity (Salonen et al., 2014). In return, dietary substrates that are available for the microbiota largely determine the metabolic output from the community (Louis et al., 2007a). Those metabolic outputs are mainly SCFAs that have a profound impact on gut health (Fernandes et al., 2014) and have been associated with a reduced risk of several diseases (Macfarlane and Macfarlane, 2012). In the next section, SCFA production in the human gut is discussed.

Short chain fatty acids production in the human gut

The different SCFAs are key end-products of colonic fermentation of not-digested dietary materials, such as resistant starch, plant cell wall material and oligosaccharides (Figure 1) (Macfarlane and Macfarlane, 2012). Mucus and protein degradation also contribute to SCFA production (Macfarlane et al., 1992; Smith and Macfarlane, 1998; Derrien et al., 2008). The degradation of proteins or amino acids also leads to a wide range of end products that include branched chain fatty acids, amines, phenols, indoles, thiols, NH_3 and H_2S, some of which have detrimental effects on intestinal health (Macfarlane and Macfarlane, 2012). Ammonium formed during protein degradation may be used to buffer the acids or used as nitrogen source for intestinal microbes. Propionate can be formed via three pathways which include succinate, acrylate and propanediol as distinct intermediates (Figure 3), and two pathways have been proposed for butyrate formation by intestinal bacteria (Figure 4) (Louis and Flint, 2009). The rate and amount of SCFA production in the colon depends on species composition, substrate availability and gut transit time (Wong et al., 2006). SCFAs contribute to about 5 to 15 % of the total caloric requirements for humans (Bergman, 1990). The ratio of acetate, propionate and butyrate is approximately 3:1:1 and the SCFA production decreases along the colon (Cummings et al., 1987; Schwiertz et al., 2010; Fernandes et al., 2014). It has been estimated that 5-10 % of the SCFAs is excreted in the faeces (Wong et al., 2006). The SCFA concentration is estimated to vary from 70 to 140 mM and from 20 to 70 mM at proximal and distal colon, respectively (Topping and Clifton, 2001). This decline in SCFA may be a result of reduced substrate availability (Noakes et al., 1996) and water depletion (Bowling et al., 1993) in the distal colon. Moreover, the absorption of SCFAs by the colonocytes has been reported to be greater in the distal as compared to the proximal part of the colon (Bowling et al., 1993).

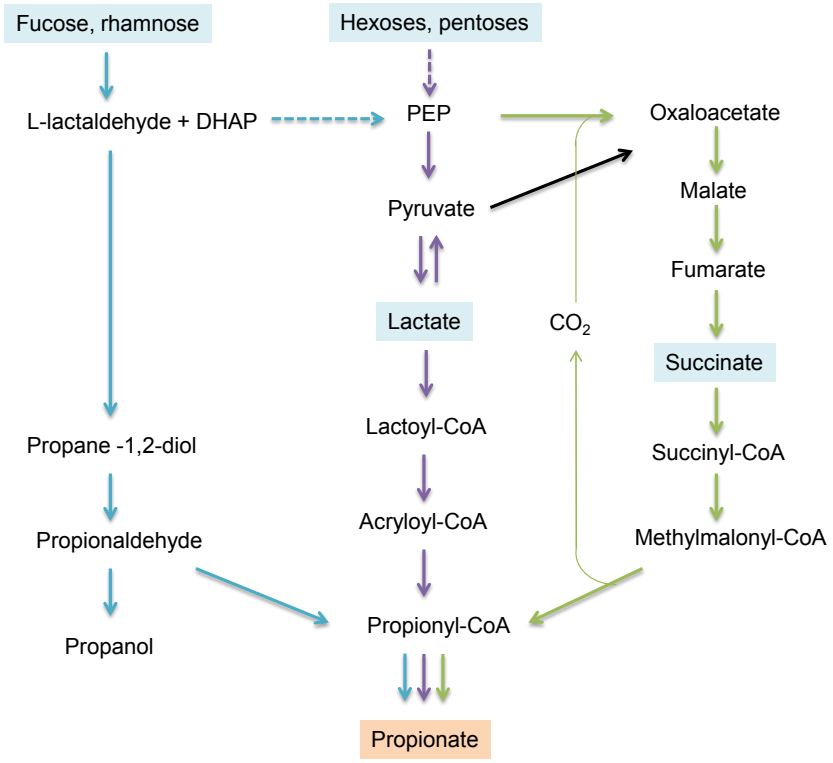

Figure 3: Pathways for propionate production in the gut. Blue, purple and green arrows indicate propanediol, acrylate and succinate pathways, respectively. Blue boxes indicate substrate and the orange box indicates products. This figure is modified from Reichardt et al., 2014.

Butyrate and its effects on the host

Butyrate is believed to be mainly derived from with carbohydrate fermentation in the large intestine. Butyrate levels can be increased by a diet high in resistant starch (McOrist et al., 2011). While consumption of soluble fibres results in a high production of SCFAs in the ileum due to their swift and easy degradation, insoluble fibres enhance faecal mass and colonic transit as well as SCFA production in the colon (Rose et al., 2007). Butyrate can also be supplied via oral substrates or rectal enemas (Scheppach et al., 1992). Supplementation of probiotic strains such as *Butyrivibrio fibrisolvens* (Ohkawara et al., 2005) and *Clostridium butyricum* (Araki et al., 2004) have been reported in animal models. Ingestion of substrates, which metabolize to butyrate in the colon, has been proven beneficial, for instance, the oligosaccharide acarbose (Weaver et al., 1997; Wolever and Chiasson, 2000), tributyrin (Conley et al., 1998). In addition, the use of butyrate tablets has been reported but clear health benefits have not been shown (Vernia et al., 2000; Roda et al., 2007).

Butyrate has a wide range of positive effects on the intestinal health. Butyrate transporters (monocarboxylate transporter-MCT1 and Na(+)/monocarboxylate transporter - SMCT1) were downregulated in human colon cancer tissue (Lambert et al., 2002; Paroder et al., 2006), leading to reduced uptake and metabolism of butyrate in the colonocytes. Experiments using in vitro cell systems have shown that butyrate shows i) anticarcinogenic effects to tumour cell lines, including the induction of apoptosis (Hinnebusch et al., 2002), ii) inhibition of cell proliferation (Comalada et al., 2006), or iii) promotion of a more differentiated phenotype (Hodin et al., 1996). Butyrate is believed to exert anti-inflammatory effects by inhibiting the nuclear factor kappaB (NF-kB) that is one of pro-inflammatory factors in the pathogenesis of inflammatory bowel disease (IBD) and other diseases (Segain et al., 2000; Klampfer et al., 2003). In addition, butyrate interferes with interferon-gamma signalling (IFN-ɣ) which plays an important role in the pathogenesis of IBD (Segain et al., 2000; Klampfer et al., 2003). Next to these direct effects of butyrate, it has been found that butyrate is capable of affecting gene expression in different cell types via DNA methylation (de Haan et al., 1986; Hamer et al. 2008). Moreover, other functions such as oxidative stress, are inhibited by butyrate in rats (Toden et al., 2007) and human colonocytes (Rosignoli et al., 2001). Remarkably, colonic administration of physiologically relevant concentrations of butyrate via an enema in human volunteers decreased visceral sensitivity (Vanhoutvin et al., 2009). Hence, butyrate may improve the function of the colonic defence barrier. Promotion of satiety has also been proposed, but the mechanisms by which this effect is mediated remains to be elucidated (Hamer et al., 2008).

Butyrate synthesis pathways in human gut

Intestinal bacteria are known to use two different pathways for butyrate production that differentiate in the enzymes involved in the final step of the pathway: one uses butyryl-CoA:acetate CoA transferase (directly converting butyryl-CoA to butyrate; BCT pathway) while the other exhibits butyrate kinase combined with phosphotransbutyrylase (Phosphotransbutyrylase (PTB) and butyrate kinase (BK) convert butyryl-CoA to butyryl-P and from butyryl-P to butyrate; PTB-BK pathway) (Louis and Flint, 2009). Those bacteria ferment sugars or intermediates (lactate/acetate) available in the GI tract (Louis and Flint, 2009) (see Figure 4). Both pathways yield ATP via substrate-level phosphorylation (SLP) as well as electron transport phosphorylation (ETP). It has been suggested that SLP and ETP could yield a maximum of 4.5 ATP per butyrate formed from glucose (Hackmann and Firkins, 2015). ATP is conserved through SLP during glycolysis (Fig. 4 – step 1) for both pathways and the conversion from acetyl-P to acetate (Fig. 4 – step 12) for the BCT pathway and the conversion from butyryl-P to butyrate (Fig. 4 – step 10) for the PTB-BK pathway. The conversion from crotonyl-CoA to butyryl-CoA is postulated as a major step (Fig. 4 – step 7) to conserve energy via the butyryl-CoA dehydrogenase (Bcd) electron-transferring flavoprotein (Etf) complex that generates a proton gradient via a membrane-associated NADH : ferredoxin

oxidoreductase (Li et al., 2008). It has also been reported that the Bcd/etf complex is able to produce superoxide in aerobic condition, that is certainly toxic to the cell (Chowdhury et al., 2015). This could explain why most butyrogens are oxygen sensitive. The Embden-Meyerhof-Parnas pathway (EMP) is the main route for glucose metabolism in intestinal bacteria and only minor contributions of Entner Doudoroff and transketolase-transaldolase pathways have been demonstrated (Miller and Wolin, 1996). Using the EMP glycolytic pathway, the conversion of glucose to 2 pyruvate generates 2 NADH and 2 ATP. Subsequently, the formed pyruvate is converted to acetyl-CoA that releases CO_2, H_2 or formate or electrons and protons. NADH can be either re-oxidised via lactate production (Fig. 4 – step 2) or the reduction of crotonyl-CoA to butyryl-CoA (Fig. 4 – step 7) or the conversion from acetoacetyl-CoA to 3-hydroxybutanoyl-CoA (Fig. 4 – step 5).

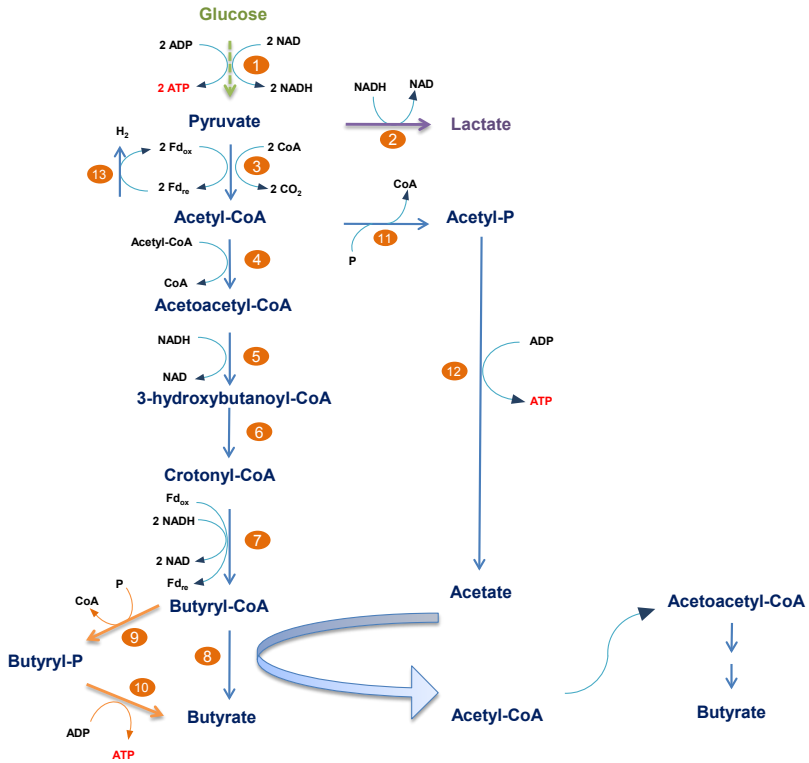

Figure 4: Butyrogenesis from carbohydrates. Schematic representation of BCT pathway (blue arrows) and PTB-BK pathway (orange arrows). Numbers refer to major steps or enzymes: 1: glycolysis (EMP pathway); 2: Lactate dehydrogenase; 3: Pyruvate:ferredoxin oxidoreductase; 4: Thiolase; 5: β-hydroxybutyryl-CoA dehydrogenase; 6: crotonase; 7: butyryl-CoA dehydrogenase; 8: Butyryl-CoA:acetate CoA transferase; 9: Phosphotransbutyrylase; 10: Butyrate kinase; 11: Phosphate transacetylase; 12: Acetate kinase. 13: hydrogenase. This scheme is modified from Duncan et al., 2002a.

The BCT pathway is found to be the most dominant butyrogenic metabolic route in the human gut (Louis and Flint, 2009; Vital et al., 2014). This pathway is present in bacteria belonging to the *Clostridium* cluster IV, XIVa and XVI (Louis et al., 2004). A PCR approach was developed to target the two key genes for the final steps in the butyrate pathway (phosphotransbutyrylase and butyrate kinase for the PTB-BK pathway; butyryl-CoA:acetate CoA transferase for the BCT pathway). When applied to 38 butyrate-producing human isolates, it was shown that the PTB-BK pathway had a 10-fold lower abundance than the BCT pathway (Louis et al., 2004). Apart from butyrate, butyrogenic bacteria also produce other compounds, such as lactate, acetate, formate and gases such as carbon dioxide and hydrogen. The proportion of these compounds depends on the environmental conditions and the activity of biosynthetic pathways (Louis and Flint, 2009).

The high cell density in the colon enables the intestinal microbes to exchange metabolites as well as genetic material. Horizontal gene transfer (HGT) is a special event in which bacteria transfer a gene from one cell to another. It has been suggested that the β-hydroxybutyryl-CoA dehydrogenase (Fig. 4 – step 5) was transferred among intestinal butyrate producers through HGT (Louis et al., 2007b). A metagenomic study has identified a signature of the transfer for the entire acetyl-CoA pathway (acetyl-CoA to butyrate including BCT and PTB-BK pathways) between Firmicutes families (Vital et al., 2014). This HGT event might be created by the substantially high amount of cells in the large intestine (10^{11} – 10^{12} cells/g biomass) and selective pressure in the gut environment (Smillie et al., 2011).

Several approaches have been used to identify the butyrate-producing community in the human gut. The qPCR approach targeting the terminal genes has been adopted to predict the presence of butyrate synthesis pathways (Louis et al., 2004; Louis and Flint, 2007). While such an approach seems to be relatively simple, it might lead to misinterpretation due to high similarity of targeted genes (Vital et al., 2013) or the presence of other CoA transferase genes (Eeckhaut et al., 2011). Therefore, a metagenomic approach has been used recently targeting all DNA and hence the genes of all pathways (Vital et al., 2014). With complex microbial communities, this metagenomic approach seems to be more robust as all genes of the pathway can be targeted and in some cases it may point at a potential growth substrate for a bacterial group of interest based on the pathway analysis. This metagenomic approach was used to screen over 3000 bacterial genomes in data of the human microbiome project (HMP) (based on 16S rRNA and metagenome information) for the presence of butyrate synthesis pathways (Vital et al., 2014). Interestingly, four butyrate synthesis pathways were detected in the human microbiome datasets, including the acetyl-CoA, lysine, hydroxyglutarate and 4-aminobutyrate/succinate pathways that showed different prevalence (Vital et al., 2014). These four pathways merge at a central step where crotonyl-CoA is converted to butyryl-CoA via the butyryl-CoA dehydrogenase complex that is coupled to an electron-transferring flavoprotein (Etf) complex that generates a proton gradient via a membrane-associated NADH : ferredoxin oxidoreductase (Buckel and Thauer, 2013). The lysine, 4-aminobutyrate/succinate

and hydroxyglutarate pathways, all convert amino acids (lysine, L-arginine and glutamate respectively) into butyrate and are commonly neglected as potential butyrogenic routes in the human intestine. Analysis of the abundance of the butyrogenic pathways showed that the acetyl-CoA pathway was most abundant, ranging from 46 % to 97.5 %. The lysine pathway emerged as the second dominant route among the four pathways, varying from 0.5 % to 49.7 %, and was detected in many phyla, indicating the significance of this pathway for intestinal butyrogenesis. However, the metagenomic analysis was done on only 15 individuals, which is a rather small cohort. An analysis on a large scale with higher numbers of volunteers at different ages, genders and continents might provide a more concise overview of the presence of the butyrogenesis pathway in the human gut. Moreover, metagenome data only reveal the presence of the pathways; expression or activity data are still needed to validate the predictions.

Lysine is an essential amino acid for the host; yet, lysine is often detected in human stool, suggesting that dietary lysine intake is either exceeding the demands by the host and microbes or is not available to these activities (Kirsner et al., 1949; Sukemori et al., 2003). Moreover, lysine synthesis from urea by microbes has also been reported in the human gut using ^{15}N tracer studies (Metges et al., 1999; Millward et al., 2000). When further analysing the distribution of microbial lysine, it was found that this contributed to approximately 5-9 % of the plasma lysine (Metges et al., 1999). This synthesized lysine not only increased the lysine supply to the host but could also serve as substrate for intestinal microbes.

The hydroxyglutarate and 4-aminobutyrate pathways where glutamate and L-arginine/ putrescine originally served as substrates, were the least abundant and only found in a few phyla of the human microbiome (Vital et al., 2014). This indicates that these two routes contribute a minor part to the overall butyrogenesis in the gut. Remarkably, no single contig has been detected that contained the entire pathway of either amino acid converting pathway, including the lysine pathway. In spite of this observation, the lysine pathway was found as the second most abundant conversion in the gut for butyrate formation (Vital et al., 2014). Hence, further research on butyrate production from amino acid or protein-derived substrates by intestinal microbes needs to be performed.

Phylogeny and substrate utilization of intestinal butyrate producing bacteria

Based on the analysis of butyryl-CoA:acetate CoA-transferase genes, it was shown that the majority (approximately 88 %) of butyrate-producing bacteria in the human gut have been cultivated (Louis et al., 2010). However, this study did not take into account amino acid-fermenting butyrogenic bacteria that are also present in high numbers in some humans (Vital et al., 2014). The butyrogenic bacteria are phylogenetically highly diverse and consist of members of the families Lachnospiraceae and Ruminococcaceae, which comprise 10 to 20 % of the total healthy intestinal microbiota (Figure 5). The most abundant groups involved in butyrate production appeared to be *Faecalibacterium* spp, belonging to *Clostridium* cluster IV

(within Ruminococcaceae) and the group of *Eubacterium* spp./*Roseburia* spp./*Anaerostipes* spp./*Coprococcus* spp., belonging to *Clostridium* cluster XIVa (within Lachnospiraceae) (Flint et al., 2014). Prior to the work described in this thesis, 3 species of the genus *Anaerostipes* were characterised: *Anaerostipes caccae* (Schwiertz et al., 2002), *Anaerostipes hadrus* (Allen-Vercoe et al., 2012) and *Anaerostipes butyraticus* (Eeckhaut et al., 2010). Metabolically, *Anaerostipes* spp. and *Eubacterium hallii* are similar as they are able to produce butyrate from glucose and lactate/acetate. This metabolic feature is highly important as it prevents colonic lactate accumulation, which is known to cause gastrointestinal disorder (Hove et al., 1994). *Eubacterium limosum* was isolated from human stool for more than half century ago (Moore and Cato, 1965) and this strain was present in some humans at low abundance (Schwiertz et al., 2000). Remarkably, *E. limosum* was able to convert lactate into butyrate and acetate while growing in PY broth (peptone-yeast extract) (Moore and Cato, 1965; Sato et al., 2008). *Faecalibacterium prausnitzii*, *Eubacterium rectale* and *Roseburia* spp. are net consumers of acetate in pure culture but no lactate consumption has been observed in these species (Duncan et al., 2002c; Duncan et al., 2006; Louis and Flint, 2009; Heinken et al., 2014). Members of the two families Lachnospiraceae and Ruminococcaceae used the acetyl-CoA pathway for butyrate production. Butyrate producing bacteria have been detected in early life microbiota (de Weerth et al., 2013) and slowly increased in abundance during the first years of life. Those include *Faecalibacterium* spp., *Eubacterium* spp., *Anaerostipes* spp., *Roseburia* spp. and *Coprococcus* spp. (Bäckhed et al., 2015). In addition, butyrate-producing bacteria were found to be in the top 15 prevalent genera of the human microbiota, including *Faecalibacterium* spp., *Roseburia* spp., *Eubacterium* spp. and *Anaerostipes* spp. (Arumugam et al., 2011). However, the prevalence and activity of the colonic butyrogenic community are greatly influenced by the dietary intake or eating habit (Vital et al., 2015). It was recently shown that a dietary swap from a western to a fibre-rich diet also resulted in a remarkable change in the microbial composition, as well as in the metabolome. Those individuals fed with a high-fibre diet showed an increase in saccharolytic fermentation and butyrogenesis (O'Keefe et al., 2015).

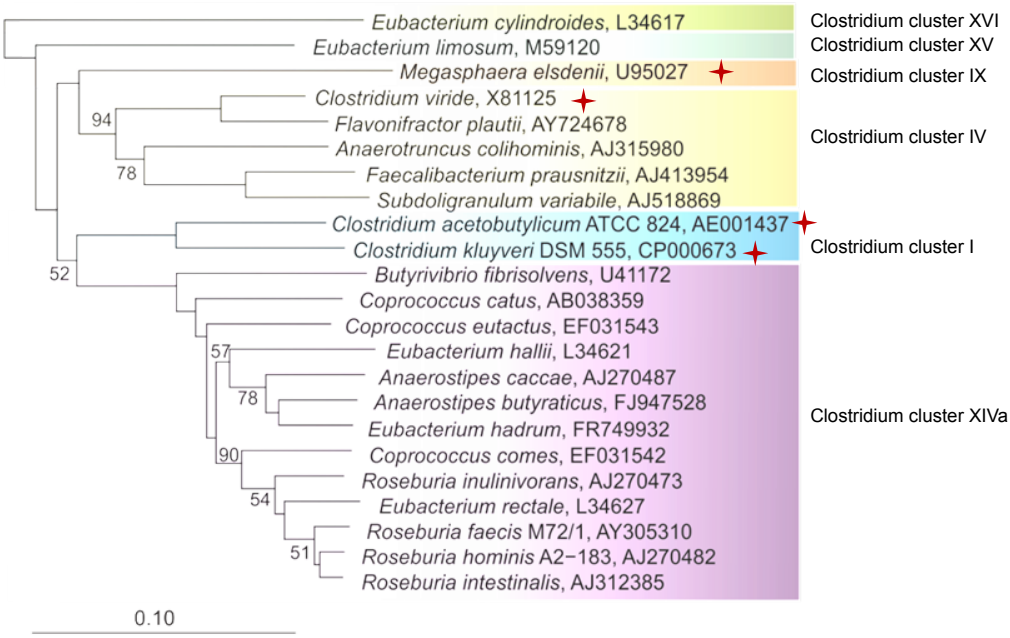

Figure 5: Phylogeny of intestinal butyrate producing bacteria. The neighbour-joining tree of 16S rRNA gene sequence was constructed using termini filter, Juke-cantor correction with 1000 replicates. Bootstraps values > 50 % are shown at branching points. The scale bar represents 10 % sequence divergence. Red stars indicated non-human associated isolates. Colour codes indicate different taxonomic groups. The figure shows the type species described prior to the start of the research in this PhD thesis.

Colonic butyrate formation is assumed to derive from either sugars that are released from the breakdown of complex carbohydrates (Louis and Flint, 2009) or from intermediates of cross-feeding of primary degraders and lactate-producing bacteria, for instance, lactate and acetate (Belenguer et al., 2006; Chassard and Bernalier-Donadille, 2006; Marquet et al., 2009; De Vuyst and Leroy, 2011). Some butyrogens are able to grow on monosaccharides while others can grow on disaccharides or polysaccharides. Many simple sugars are known to be substrates for butyrogens, such as glucose, fructose, mannose, galactose, arabinose, xylose but not rhamnose. Rhamnose is a hexose and is naturally present in many plants as a component of cell wall pectin polysaccharides (Ridley et al., 2001). Groups of *Anaerostipes* spp. and *Eubacterium* spp. are known to utilize a wide range of monosaccharides (Schwiertz et al., 2002; Allen-Vercoe et al., 2012) and hardly grow on polysaccharides. In contrast, several species of the Ruminococcaceae are capable of utilizing polymers, such as xylan that is well-degraded by *Roseburia intestinalis* (Duncan et al., 2002c), while other *Roseburia* spp. have found to form butyrate from inulin, starch (Duncan et al., 2006).This indicates that *Roseburia* spp. may play an important role in degrading starch from dietary fibres to produce butyrate in

the gut. The capability of using oligosaccharides, inulin and pectin has been reported for *F. prausnitzii* (Duncan et al., 2002b; Lopez-Siles et al., 2012). Moreover, a human trial indicated an increase of this bacterium when volunteers consumed inulin and oligofructose (Ramirez-Farias et al., 2009). *F. prausnitzii* has been shown to employ an extracellular electron shuttle to transfer electrons to oxygen, allowing it to grow at low levels of oxygen (Khan et al., 2012). This feature is likely to be involved in the detoxification of oxygen in the lumen. In addition, *F. prausnitzii* may reside near mucosa as this bacterium showed a capability of growing on N-acetylglucosamine, a mucosa-derived substrate (Lopez-Siles et al., 2012). Bacteria belonging to the group of *F. prausnitzii* are phylogenetically diverse and consist of two phylogroups based on 16S rRNA sequences. The two groups of *F. prausnitzii* showed no discrepancy in substrate utilization (Lopez-Siles et al., 2012).

Changing dietary fibre in the diet can alter the human intestinal microbiota composition and can affect the abundance and diversity of butyrate-producing bacteria (Duncan et al., 2007; Graf et al., 2015; O'Keefe et al., 2015). Diets have shown to be a major factor affecting the faecal butyrate-producing community in mammals, birds and reptiles (Vital et al., 2015). Changes in the abundance of these dominant butyrate-producing bacteria could be due to the indirect stimulation of the trophic chain involved in butyrate production or direct use of dietary components for butyrogenesis. The number of *Eubacterium* spp. and *Roseburia* spp. decreased significantly in human volunteers on a high-protein, low carbohydrate diet and increased in human volunteers on high carbohydrate diet, culminating in the observation that the abundance of these bacteria strongly correlated with the faecal butyrate level (Duncan et al., 2007; Russell et al., 2011; Walker et al., 2011). The same trial done on different diets showed that the cell number of *E. rectale* and *Ruminoccocus bromii* increased in resistant-starch fed volunteers (Salonen et al., 2014). These studies indicated that the *Roseburia*/*E. rectale* group is particularly dependent on residual dietary carbohydrates to maintain its competitiveness in the colon and provides in vivo support for the earlier demonstrated trophic chain that *R. bromii* and *E. rectale* might form on resistant starch (Kovatcheva-Datchary et al., 2009). This trophic chain can be explained by the capability of hydrolysing long chain carbohydrates including resistant starch with amylase activities of *R. bromii* (Ze et al, 2012). Inulin, oligofructose, raffinose and galactose-oligosaccharides have shown to increase the number of *F. prausnitzii* (Ramirez-Farias et al., 2009; Fernando et al., 2010; Davis et al., 2011). This result is supported by the capability of *F. prausnitzii* growing on an inulin and starch solution in pure culture (Duncan et al., 2002b).

Although amino acids are partially absorbed in the small intestine and liver, these are also catabolized by intestinal microbes (Metges, 2000). It has been shown that bacterial metabolism of nitrogen-containing compounds leads to a significant decrease of nitrogen in the large intestine (Hendriks et al., 2012). Also relatively large quantities of soluble protein and peptides can be present in the luminal content throughout the large intestine (Smith and Macfarlane, 1998). It has been estimated that about 13 grams of proteins and peptides will

reach the human large intestine every day (Smith and Macfarlane, 1998). Trypsin, a serine protease produced in the pancreas and found in the digestive system (Leiros et al., 2004), as well as other microbial proteolytic enzymes (Macfarlane and Allison, 1986), also contribute to releasing amino acids and peptides that are used by intestinal bacteria. In addition, a recent metagenomic study indicated that butyrate could be produced from amino acids (Vital et al 2014). About three decades ago, the proteolytic activity of human faecal samples was studied (Macfarlane and Allison, 1986). Short chain fatty acids including butyrate were produced from casein and bovine serum albumin in faecal slurries (Macfarlane and Allison, 1986). Another study on single amino acid fermentation by human microbiota showed that butyrate was also an end-product of amino acid fermentation, especially from glutamate and lysine (Smith and Macfarlane, 1997). Serine, lysine and glutamate were quickly degraded by faecal suspensions from omnivorous and vegetarian human donors (Richardson et al., 2013). Among these three amino acid fermentations, lysine was rapidly metabolised into butyrate and acetate. Unfortunately, the butyrogenic communities were not characterized.

Many foods need to pass cooking or other heating processes before they are ready to eat, including meat, fish, egg or bread. This cooking process creates a high number of advanced glycation end products (AGEs), which contribute to the taste and provide colour to the foods. These AGEs, formed via non-enzymatic Maiilard reaction of amino acids and sugars, are widely present in heated foods or beverages and could serve as substrates for intestinal bacteria (Takeuchi et al., 2015). AGEs occur at low concentration in natural foods but increase significantly after food processing. The amount of AGEs in daily uptake has been estimated about 75 mg for humans (Henle, 2003). The accumulation of the AGEs has been associated with pro-inflammatory processes in diabetes, insulin resistance and atherosclerosis (Kellow and Coughlan, 2015). The degradation of major AGEs, fructoselysine (FL) and Nε-(carboxymethyl)lysine (CML), has been studied to some extent. The Amadori product FL is formed from glucose and lysine at high temperature and has been identified as a key compound for CML formation in glycated protein (Li et al., 2012b). Hence, FL metabolism in the gut is of great interest. While FL was found to be rapidly degraded in 4 hours, CML was degraded slowly in 80 hours by faecal suspensions (Hellwig et al., 2015). In early studies it was found that *E. coli* could grow on FL in the presence of oxygen but this degradation did not result in the butyrate production (Wiame et al., 2002; Wiame and Van Schaftingen, 2004).

Lysine fermentation to butyrate and acetate is thermodynamically feasible as it is an exergonic reaction ($\Delta G^{o'}$ = -132.9 kJ mol^{-1}). Hence, there could be intestinal microbes that are able to use lysine as carbon and energy source. A recent metagenomic study showed that the lysine pathway often co-occurred with the acetyl-CoA pathway in genomes (Vital et al., 2014), indicative that colonic microbes are able to both utilize carbohydrate- and protein-derived substrates for butyrogenesis. Several candidates have been suggested that could carry out a lysine-degrading butyric fermentation such as Fusobacteria, Porphyromonas, Actinobacteria and Thermotogae. Fusobacteria have been characterised as lysine fermenters and are

abundantly present in the oral cavity and might be associated with periodontal disease as well as colorectal carcinoma (Loesche and Gibbons, 1968; Barker et al., 1982; Kapatral et al., 2002; Castellarin et al., 2012). *Porphyromonas* is mostly considered as asaccharolytic (Shah and Collins, 1988) and butyrate was detected as one end-product in peptone-yeast extract-glucose medium (Shah and Collins, 1988) but lysine fermentation by this group has not been reported. None of the cultivated Actinobacteria and Thermotogae have been identified to date as capable of producing butyrate. In conclusion, prior to the work described in this PhD thesis, there have been no intestinal species being cultured that are able to grow on lysine as sole carbon and energy source. Hence, there is the need of cultivating butyrogenic bacteria that utilize protein-derived substrates.

Microbial interactions between intestinal butyrogens and other microbes

The majority of butyrate-producing bacteria are not able to use complex carbohydrates. Therefore, it is crucial that they can use breakdown products released by primary degraders or intermediates released for their growth. The release of simple compounds creates an opportunity for metabolic interaction between different species. Metagenomic and other molecular studies have shown co-occurrence of networks involving different butyrate-producing bacteria together with other species (Arumugam et al., 2011). These in silico studies suggest that there may also be functional interactions between these microbial groups. However, experiments are required to validate these network interactions. Butyrate-producing bacteria have shown the capability of interacting with microbes belonging to several functional groups in vitro such as *Bifidobacterium* on starch (De Vuyst and Leroy, 2011), hydrogenotrophic microbes on xylan and cellulose (Robert et al., 2001; Chassard and Bernalier-Donadille, 2006), and *R. bromii* on resistant starch (Kovatcheva et al 2009). Metabolic cross-feeding was suggested between *E. hallii* or *A. caccae* and *Bifidobacterium* spp. on starch or fructo-oligosaccharides (FOS) that resulted in butyrate formation and lactate was suggested as an intermediate (Belenguer et al., 2006; Falony et al., 2009; De Vuyst and Leroy, 2011). *E. hallii* and *A. caccae* can be outcompeted by *Desulfovibrio piger* (Marquet et al., 2009) in the presence of exogenous sulphate for lactate consumption. Many butyrate producers generate hydrogen gas that facilitates the interaction with hydrogenotrophic species and these butyrogens include *E. hallii*, *Anaerostipes* spp. and *Roseburia* spp. The butyrogenic bacterium *R. intestinalis* is able to interact with hydrogenotrophic species on xylan (Chassard and Bernalier-Donadille, 2006). Hydrogen and acetate are transferred between xylanolytic *R. intestinalis* and *Blautia hydrogenotrophica* or *Methanobrevibacter smithii*, leading to butyrate production from oat xylan without hydrogen production (Chassard and Bernalier-Donadille, 2006). This cross utilization of metabolites might play a role in preventing hydrogen accumulation and might affect microbial fermentation and energy harvest in the gut. In another study, *Bacteroides thetaiotaomicron*, *E. rectale* and *M. smithii* were used to study the metabolic interaction using a gnotobiotic mouse model (Samuel and Gordon, 2006). This study showed that the presence of *M. smithii*, a dominant intestinal archaeon (Eckburg et al., 2005; Abell et al., 2006; Scanlan

et al., 2008), shifted the dietary fructan fermentation towards increased formation of formate. This enhanced the potential energy harvest from these fructans when co-colonised with *B. thetaiotaomicron* in mice (Samuel and Gordon, 2006).

The human microbiome is of interest for not only its diversity but also its functionality. Over the last decades, many studies have addressed the members of different taxonomic groups and described their correlation in human during health and disease. A variety of high throughput approaches have been used, including metagenomics, metaproteomics or metatranscriptomics (Verberkmoes et al., 2008; Gosalbes et al., 2011; Wang et al., 2015b). These approaches have been highly successful in describing the components of the intestinal microbiome (Zoetendal et al., 2008). However, cultured representatives are needed to bring these studies to the next level, as they are essential for addressing the impact of microbes on human health and disease and to study host-microbe interactions. Several recent studies have addressed the high throughput culturing of intestinal bacteria. One study embarked on a so-called culturomics approach where a high throughput plating system was applied in conjunction with rapid identification using mass spectometry (Lagier et al., 2012;

Pfleiderer et al., 2013). However, the success of this study cannot be assessed since the dozens of apparently identified strains have not been deposited in public culture collections. In another high throughput approach, bacterial strains were identified that revealed microbe-host phenotypes using bacterial communities in gnotobiotic mice (Faith et al., 2014). A total of 17 strains including one uncultured species were identified to express different phenotypes such as host adiposity modulation and immune modulation, but these were also not deposited in known culture collections. A high-throughput approach using microfluidic devices has been developed a few years ago for single-cell sorting and cultivation (Leung et al., 2012). When microfluidics-based and genetically targeted approaches were applied to isolate intestinal tract bacteria, a few new species could be cultured at the microcolony level but these rapidly lost viability (Ma et al., 2014). A multiplex phenotypic profiling approach employed 16 antibiotics in various combinations to enrich for antibiotic-tolerant phenotypes. This approach led to the identification of four bacterial species, two of which appeared as previously uncultured (Rettedal et al., 2014). These approaches all were unsupervised and can therefore lead to repeated isolation of the same bacteria. Targeted isolation of bacterial groups that are known to exist based on phylogenetic or other datasets is another approach. This has been described previously for uncultured *Ruminococcus obeum* (Zoetendal et al., 2002) but so far there has only been limited success of this approach to isolate target bacteria from the gut (Ma et al., 2014). In conclusion, there is a need to cultivate intestinal microbes and isolate representatives of new species.

Outline of the thesis

The research described in this thesis aims to provide a better understanding of the metabolic flexibility of butyrate-producing bacteria in the human gut and to provide detailed insights in their metabolic potential. To do this, stool samples, that are easy to collect, were used to isolate novel butyrogenic bacteria using defined strict anaerobic mineral salt media as a selective factor. Subsequently, the butyrogenic pathways of these isolates were investigated employing genome, proteome and metabolome analysis in combination with ^{13}C NMR of ^{13}C-labeled isotopomer and activity assays. Moreover, the microbial interactions of some of the new butyrogens and other intestinal microbes were also addressed by performing the co-culture experiments.

Chapter 2 describes a novel butyrate-producing bacterium isolated from infant stool. The isolate was obtained using a classical cultivation technique and belongs to one of the top 15 abundant genera in the gut; *Anaerostipes* that belongs to *Clostridium* cluster XIVa of the Firmicutes. The biochemical and physiological properties of this novel strain were subsequently characterised. The strain was designated as *Anaerostipes rhamnosivorans* 1y-2^T because of its unique capability of converting rhamnose into butyrate. As other *Anaerostipes* isolates, *A. rhamnosivorans* not only utilized a wide range of sugars for butyrogenesis but also converted lactate plus acetate into butyrate. This is important to avoid lactate accumulation in the gut.

The microbial interactions between *A. rhamnosivorans* and other microbes when growing on several substrates are illuminated in **Chapter 3**. This butyrogenic bacterium is able to benefit in the co-culture with *Bacteroides thetaiotaomicron* on two pectin dietary fractions for butyrate production. Moreover, *A. rhamnosivorans* is capable of performing cross-feeding with *Blautia hydrogenotrophica* on lactate and a trace amount of acetate where hydrogen and acetate are transferred between the two species. As a result, lactate is completely depleted while the butyrate production level is maximized. The influence on the butyrogenic metabolism becomes more significant when pairing *A. rhamnosivorans* with *Methanobrevibacter smithii*, an intestinal methanogenic archaeon. In this co-culture, the growth of the methanogen pulls the fermentation towards its substrate hydrogen. Consequently, more butyrate is produced, less lactate is formed, and hydrogen and acetate are transferred between these two species.

Chapter 4 describes a new butyrogenic isolate from a mouse cecum; representing the novel genus *Intestinimonas* within *Clostridium* cluster IV. The isolate was named *Intestinimonas butyriciproducens* SRB521^T as it produces butyrate as a major product. A highly similar strain, *Intestinimonas* AF211, was isolated from a stool sample of a healthy individual. The strain *Intestinimonas* AF211 was classified as another strain of *I. butyriciproducens* as its 16S rRNA gene and metabolic properties were highly identical to that of the type strain, *I. butyriciproducens* SRB521^T, isolated from mouse. Interestingly, strain AF211 is able to perform butyrogenesis not only from sugars but also the amino acid lysine. Moreover, the strain AF211 is able to convert fructoselysine efficiently into butyrate, and acetate was found to affect the

fructoselysine fermentation, representing the impact of the environmental conditions where acetate is abundant in the gut. The pathway was fully elucidated using proteogenomic, ^{13}C-NMR and enzyme measurements. This led to the identification of butyryl-CoA : acetoacetate CoA transferase as the key enzyme in the lysine pathway in **Chapter 5**. It is shown that the strain AF211 is abundantly present in the gut and its genomic analysis revealed the presence of all genes for complete lysine degradation. As no other lysine-degrading butyrogens have yet been isolated from the gut, *I. butyriciproducens* AF211 can be considered as a missing link that couples protein metabolism and butyrate formation. Adding to that, the finding that *I. butyriciproducens* strain AF211 is capable of the butyrogenic conversion of fructoselysine, which is formed in heated foods via the Maillard reaction, is the first example of a dedicated degradation pathway for an Amadori product that is abundant and specific for cooked foods.

To investigate the metabolic potential and host specific features, the complete genomes and physiological properties of the *I. butyriciproducens* strain AF211 (human isolate) and SRB521^{T} (mouse isolate) were analysed and fully compared (**Chapter 6**). The results indicate that the butyrogenesis from lysine is a shared characteristic between the two *I. butyriciproducens* strains. Nevertheless, the human isolate seems to be more efficient than the mouse strain in converting sugars into butyrate, especially galactose and arabinose. Detailed comparative analysis revealed significant genomic rearrangements and variations in bacteriophages in the human strain. Altogether, this genomic and physiological characterisation provided support for different ecological adaptations of *I. butyriciproducens* to the mouse and human gut environment, suggesting the possible impact from different diets of the mouse and human. **Chapter 7** provides a summary of this thesis and the contribution of these findings to the current knowledge on butyrogenesis in the human gut. Additionally, it discusses future perspectives for research as well as a proposal for potential therapeutic application of the obtained isolates to promote butyrate production in the human gut.

CHAPTER 2

ANAEROSTIPES RHAMNOSIVORANS SP. NOV., A HUMAN INTESTINAL BUTYRATE FORMING BACTERIUM.

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Abstract

A novel butyrate-producing bacterium, strain 1y-2^T, was isolated from a stool sample of a 1-year old, healthy Dutch infant. The isolate was obtained by using lactate and acetate as sources of carbon and energy. The strain was Gram-variable, strictly anaerobic, spore-forming and formed curly rod-shaped cells that fermented glucose into butyrate, lactate, formate and acetate as main products. The G + C composition of the strain was 44.5 % and its major cellular fatty acids were $C_{12:00}$, $C_{19:1}$ iso I and $C_{16:00}$. Strain 1y-2^T was related to *Anaerostipes caccae* DSM14662^T based on 16S rRNA gene analysis with 3 % divergence but hybridization studies of their genomic DNA revealed only 33 % similarity. Moreover, strain 1y-2^T showed marked physiological and biochemical differences with known *Anaerostipes* species. Based on the phylogenetic, chemotypic and phenotypic criteria, we propose that strain 1y-2^T should be classified in the genus *Anaerostipes* as a novel species, *Anaerostipes rhamnosivorans* sp. nov. The type strain is 1y-2^T (= DSM 26241^T = KCTC 15316^T).

Introduction

The large intestinal tract is the most heavily colonized portion of the human body with cell numbers that can exceed 10^{11} per gram of content. A major metabolic function of the intestinal microbiota is the anaerobic conversion of polymeric sugars into short chain fatty acids (SCFAs), such as acetate, propionate and butyrate. These SCFAs fuel the enterocytes, while some have a signalling function. Hence, bacteria that produce these SCFAs are of special interest because of their vital roles in human colonic health. Butyrate, in particular, has been identified as a major energy source for colonocytes (Pryde et al., 2002). Butyrate may protect against cancer and ulcerative colitis (Hague et al., 1997), and has been associated with preventing the development of insulin resistance and obesity in mice (Flint et al., 2012). Therefore, studies on butyrate-producing bacteria are of specific importance for further understanding of their role in intestinal health and disease. All bacteria known to date that produce butyrate belong to the phylum Firmicutes, low G+C content Gram-positive bacteria. There are basically two metabolic avenues to produce butyrate. One is directly from sugars with or without the use of external acetate by intestinal bacteria such as *Faecalibacterium, Roseburia* and some *Clostridium* spp. The other one is via the conversion of acetic acid and lactic acid that are produced from mixed acid fermentation from sugars. The latter conversion is of specific interest as it removes the generated lactic acid where the accumulation of this compound may lead to intestinal damage. Bacteria such as those related to *Anaerostipes caccae* and *Eubacterium hallii* are the main intestinal representatives that are capable of producing butyrate from acetic and lactic acid rather than from sugars (Harry J. Flint, 2012).

Any microbial study of butyrate producers starts with their isolation from the originating ecosystem. Over the last decade, efforts to isolate butyrate producers from human stool have revealed that many different species are chiefly present within two clusters of Firmicutes: *Clostridium* cluster IV and cluster XIVa (Barcenilla et al., 2000; Louis et al., 2004). Recent analysis of the butyryl-CoA:acetate CoA-transferase gene, a key gene for butyrate production, has shown that there is still a fraction of not yet cultured butyrate-producing strains (Louis et al., 2010). In an effort to isolate these butyrate-producers, we performed an intensive screening procedure using a medium containing lactate and acetate as carbon and energy sources especially from *Clostridium* cluster XIVa, one of the most predominant and diverse clusters in the human intestine. A decade ago, a new genus in the cluster XIVa, *Anaerostipes*, was isolated (Schwiertz et al., 2002). This genus represents more than 2% of total colonic microbiota in the healthy colon (Harry J. Flint, 2012). Furthermore, this organism is believed to play an important functional role in gut ecosystem (Allen-Vercoe et al. 2012) due to its capability to produce butyrate from lactate (Muñoz-Tamayo et al., 2011). So far, only three isolates have been discovered: *Anaerostipes caccae*, and *A. hadrus* (formerly classified as *Eubacterium hadrum*) from the human intestine (Schwiertz et al., 2002) and *A. butyraticus,* from broiler chicken caecal content (Eeckhaut et al., 2010). Here, we report the isolation, phenotypic characterization and phylogenetic analysis of a novel isolate from an infant stool.

Based on our findings, we propose that this bacterium is classified as a new species within the *Anaerostipes* genus as *Anaerostipes rhamnosivorans* strain 1y-2^T.

Materials and methods

Strain 1y-2^T was obtained from a faecal sample of a 1-year healthy Dutch infant. Approximately 1-gram of fresh faecal slurry was collected by using a needle and syringe and transferred into an anaerobic 35-ml serum vial containing 10ml of 20mM phosphate buffer, 25 % glycerol (v/v), 0.5 g resazurine l^{-1} and 0.5 g cysteine l^{-1}. This suspension was thoroughly mixed and subsequently diluted serially up to 10^{10} times into a bicarbonate-buffered anaerobic medium consisting of (per litre): 0.53 g Na_2HPO_4 $2H_2O$, 0.41 g KH_2PO_4, 0.3 g NH_4Cl, 0.11 g $CaCl_2$-$2H_2O$, 0.10 g $MgCl_2$ $6H_20$, 0.3 g NaCl, 4.0 g $NaHCO_3$, 0.48 g Na_2S. $9H_2O$ as well as alkaline and acid trace elements (each 1 ml/litre) and vitamins (0.2 ml/litre) (Stams et al., 1993). The alkaline trace element solution contained the following (mM): 0.1 Na_2SeO_3, 0.1 Na_2WO_4, 0.1 Na_2MoO_4, and 10 NaOH. The acid trace element solution was composed of the following (mM): 7.5 $FeCl_2$, 1 H_3BO_4, 0.5 $ZnCl_2$, 0.1 $CuCl_2$, 0.5 $MnCl_2$, 0.5 $CoCl_2$, 0.1 $NiCl_2$, and 50 HCl. The vitamin solution had the following composition (gram per litre): 0.02 biotin, 0.2 niacin, 0.5 pyridoxine, 0.1 riboflavin, 0.2 thiamine, 0.1 cyanocobalamin, 0.1 p-aminobenzoic acid and 0.1 and pantothenic acid. This basal medium was supplemented with 40mM sodium lactate and 40mM sodium acetate added from 1M sterile anaerobic stock solutions. Incubations were done in 35-ml serum bottles sealed with butyl-rubber stoppers at 37 ^oC under a gas phase of 1.7 atm of N_2/CO_2 (80 : 20, v/v). The pH of the medium was 6.8. The inoculum amount was 1% (v/v). After 2-weeks of incubation, the highest dilution where the growth was observed was transferred into the second dilution row up to 10^{10}. For a primary identification of the microbial composition, genomic DNA was isolated from the highest grown dilution of the second row after 5 days that was amplified by the 16S rRNA gene primer sets 27F and 1492R (Weisburg et al., 1991) and used to generate a clone library of full 16S rRNA sequences using pGEM-T Easy Vector System (Promega). A total of 24 clones were selected for 16S rRNA gene sequencing using the same primer set as used for the cloning. Based on almost full 16S rRNA sequences, all clones were identified to have 97 % similarity with *Anaerostipes caccae* DSM 14662^T. To further purify the apparently new *Anaerostipes* spp. the culture was repeatedly transferred into new dilution series and eventually plated in 1.5 % (w/v) agar-containing bicarbonate buffered medium (agar noble; Difco) containing 40mM lactate and 40mM acetate as carbon and energy sources. A single colony was picked and transferred to a new plate , which was incubated in an anaerobic jar at 37 ^oC. These plating steps were repeated until a pure culture was obtained, that was designated strain 1y-2^T.

Cell morphology and purity of strain 1y-2^T was examined continuously with a phase-contrast microscope. Additional purity confirmation was performed using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene amplicons of strain 1y-2^T grown in different media including RCM, bicarbonate buffered medium with glucose and bicarbonate

buffered medium with lactate and acetate as carbon and energy sources (data not shown). The PCR primers were F-968-GC: 5' – CGCCCGGGGCGCGCCCCGGGCGGGGCGGG

GGCACGGGGGGAACGCGAAGAACCTTAC – 3' and R-1401: 5' - CGGTGTGTACAAG

ACCC – 3' (Nübel et al, 1996).

Genomic DNA of strain 1y-2^T was extracted and purified with a FastDNA Spin kit for soil (MP Biomedicals) following the manufacturer's instructions. A 16S rRNA gene was amplified from chromosomal DNA of strain 1y-2^T as described above. The PCR programme was started at 94 ^{o}C for 5 min and continued with 35 cycles consisting of 94 ^{o}C for 1 min 30 s, 52 ^{o}C for 30 s, and 72 ^{o}C for 1 min 30 s; and finally 72 ^{o}C for 10 min. The PCR products were subsequently purified using the Qiaquick PCR purification kit (Qiagen) and sequenced using a universal primer set: 27F, 1492R together with 533F: 5' - GTGCCAGCMGCCGCG - 3' (Lane, 1991) and 650R: 5' - TCCACGCCGTAAACGATGAGT - 3' (unpublished) to read from the middle of 16S rRNA gene. Sequencing of the amplified 16S rRNA gene was performed at GATC Biotech (http://www.gatc-biotech.com). Single fragments were checked for reading errors using Chromas and subsequently aligned using program DNASTAR. The 16S rRNA gene sequence was blasted using BLASTN search program (http://blast.ncbi.nlm.nih.gov/Blast) and Eztaxon 2.1 (Chun et al., 2007). The 16S rRNA sequences of the isolate and known related type strains were aligned with the CLUSTAL_X program (Thompson et al., 1994) and Kimura's two-parameter model (Kimura, 1983). The phylogenetic tree was constructed using the neighbour-joining and maximum parsimony algorithms (Saitou and Nei, 1987) with calculated evolutionary distances by the MEGA 5 program (Tamura et al., 2011). Bootstrap analysis was replicated 1000 times to obtain confidence levels for the branches (Felsenstein, 1985). DNA-DNA relatedness was determined reciprocally between strain 1y-2^T and *Anaerostipes caccae* DSM 14662^T obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) as described previously (Ley et al., 1970) with some changes (Huss et al., 1983). All strains were grown under the same conditions using RCM medium. The values were measured by using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with in-situ temperature probe.

Cell morphology, motility and spore formation of the isolate was observed using a LEICA DM 2000 Microscope, a JEOL-6480LV Scanning Electron Microscope (SEM) and a JEOL, JEM-1011 Transmission Electron Microscope (TEM) (Supplementary Fig. S2). For SEM observation, fresh wet samples were fixed in a solution of 2% (w/v) glutaraldehyde in phosphate buffer. After fixation the samples were filtered over a 0.2 µm polycarbonate membrane, rinsed with 100% ethanol and dried overnight at room temperature. The membranes with bacteria on top were attached to 15 mm diameter mounts with a double-sided carbon adhesive tab. Before observation under High Vacuum (HV), samples were coated in a JEOL JFC-1200 fine coater with a thin (10 nm) Au layer. The SEM was operated under HV conditions at 6 kV at 10 mm working distance (WD) and spot Size (SS) 20. For TEM imaging, 2-days grown cells

were fixed for 65 hours in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.2) at 4°C. After rising with 0.1 M sodium cacodylate buffer, a post fixation was done in 1% (w/v) OsO_4 and 1% (w/v) potassium ferricyanide in the same buffer for 1 hour at room temperature. The cells were then rinsed three times in demineralised water before embedding in 5% (w/v) gelatine. The gelatine solidified at 0 °C and subsequently dehydrated in a graded ethanol series (10, 30, 50, 70, 90, 100%) before being infiltrated with modified Spurr resin mixture (Serva). The samples were eventually sectioned to 70 – 80 nm on an ultra-microtome (Reichert, Ultracut S). Micrographs were taken with a JEOL JEM 1011 transmission electron microscope and a digital camera (Olympus, Veleta). The Gram reaction was done using standard methods (Plugge et al., 2000). Spore visualization was implemented by staining with malachite green following the manufacturers instruction (SIGMA).

To determine the optimum growth temperature and pH range, strain 1y-2^T was inoculated in 10 ml of Reinforced Clostridial Medium (RCM; Difco) and incubated at temperature range of 4 – 60 °C at intervals of 5 °C up to 8 weeks. The optimum pH was tested in duplicate in mineral medium using 20mM TRIS to buffer at pH higher than 7; 20mM PIPES to buffer from 6 to 7.5 and 20mM citrate to buffer at pH lower than 6 up to 4 weeks. Growth was determined in a range of pH 4-9, at intervals of 0.5 pH units (adjusted with 2N HCl and 2N NaOH). To define salt tolerance, the strain was grown in the bicarbonate-buffered medium with 1 to 10 % w/v sodium chloride and incubated up to 4 weeks. Media for pH and salt tolerance contained glucose as a sole carbon and energy source.

Strain 1y-2^T and other *Anaerostipes* species were pre-grown in RCM (Difco) before performing biochemical characterization using several commercial API test systems (bioMerieux). Carbohydrate assimilation was defined by using API 20A and API 50CHL kits. Enzyme profiling was performed using API rapid ID 32A and API ZYM kits. All tests were performed in duplicate. The results were interpreted following the manufacturer's instructions. Substrate utilization was determined by adding a final concentration of 40 mM into anaerobic bicarbonate-buffered medium, with 10% inoculum and 72 hours incubation at 37 °C. Tested substrates were D-lactate, L-lactate, D- glucose, D-galactose, D-fructose, D-mannose, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, D-arabinose, D-maltose, D-saccharose, D-trehalose, xylitol, D-tagatose, D-arabinose, inulin, laminarin, xylan. End products were determined using HPLC and GC methods as described previously (van Gelder et al., 2012).

The cellular fatty acid composition was analysed from cells grown in Wilkens Chalgren Broth (Oxoid) at 37°C for 24 hours. Cells were centrifuged at 4°C and 10000 rpm and subjected for cellular fatty acid extractions. The products were analysed by Agilent model 6890N gas chromatography (MIDI Sherlock, Newark, N.J.) as described previously (Miller, 1982; Kämpfer and Kroppenstedt, 1996). The G+C content of genomic DNA of strain 1y-2^T was determined based on draft genome data performed at Baseclear B.V. (the Netherlands).

Results and discussion

The isolate, strain 1y-2^T is a non-motile and strictly anaerobic, spore-forming, curved rod-shaped organism (Supplementary Fig. S2). The cells are 0.7 - 0.83 µm x 3 – 6 µm and sometimes occur in short chains of 2 to 5 cells. Cell morphology is slightly different in length dependent on the growth medium. Young cells stained Gram-positive while old cell stained Gram-negative (Supplementary Fig S2). After 1-day incubation on RCM Agar Medium (1.5 % agar, Difco) at 37 °C, strain 1y-2^T produced beige, circular and convex colonies with rough surfaces, 1–4 mm in diameter. Growth was observed between 15 - 45 °C and pH 5.5 – 9 with an optimum at 37°C or pH 6.5 - 7. Strain 1y-2^T can tolerate up to 3 % NaCl. No growth was observed when traces of oxygen were present in the medium without the reducing agent, as indicated by the pink colour of the bicarbonate buffered medium in which resazurin was present. No haemolytic activity is found in Columbia blood agar medium (Oxoid). Catalase and oxidase are negative. Indole is not produced. The strain can neither reduce nitrate to nitrite nor hydrolyse urea, gelatin and esculin.

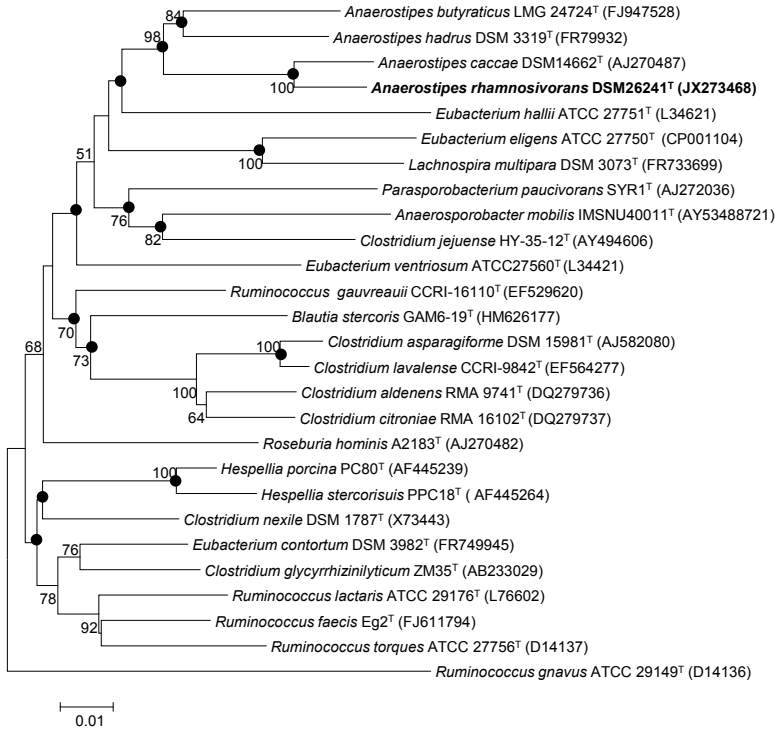

Figure 1: Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relation of strain 1y-2^T and closely related members of clostridium cluster XIVa. Bootstrap values > 50 % based on 1,000 replications are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in a tree generated with the maximum-parsimony algorithm. Bar, 1 % sequence divergence.

An almost full-length 16S rRNA gene sequence (1435bp) was obtained from strain 1y-2^T. Phylogenetic analysis based on 16S rRNA gene sequences showed that its closest relatives were members of cluster XIVa of the *Clostridium* subphylum of Gram-positive bacteria (Fig. 1), and it groups in the genus *Anaerostipes* in the lineage Firmicutes, Clostridia, Clostridiales, Lachnospiraceae. The pairwise comparison of the almost complete 16S rRNA sequence (> 1400 nt) of most related species revealed approximately 3 %, 6 % and 7 % sequence divergences between the isolate and *Anaerostipes caccae* DSM14662^T, *A. hadrus* DSM 3319^T, *A. butyraticus* LMG 24724^T, respectively. Moreover, the DNA-DNA relatedness between the isolate and A. *caccae* DSM14662^T is only 33% that is below the 70 % cut-off point for species classification (Wayne et al., 1987).

The biochemical properties of the isolate and 3 *Anaerostipes* type strains (obtained from DSMZ Braunschweig, Germany) using API ZYM, API rapid ID32A, API 20A system clearly showed different capabilities of fermenting the carbohydrates as well as possessing various enzyme profiles (Table 1). Strain 1y-2^T can be specifically distinguished from the other *Anaerostipes* species by its capacity to ferment rhamnose. The observed glucose fermentation stoichiometry was as follows (CO_2 content was calculated based on redox balance): 1 glucose → 0.57 lactate + 0.11 acetate + 0.46 butyrate + 0.45 formate + 0.31 H_2 + 0.58 CO_2 + 0.24 $C_5H_8O_2N$. Biomass was determined using a method as previously described (Van Gelder et al., 2012). Electron recovery was 94 %. Butyrate was found as the product of all sugar fermentations (data not shown). All *Anaerostipes* strains have been shown to utilize lactate and acetate to form butyrate in the molar ratio of 2:1:1.5 as described previously (Duncan et al., 2004) and so did strain 1y-2^T (Supplementary Fig. S1). Furthermore, strain 1y-2^T showed distinct glucose fermentation pattern compared to the other related species (Supplementary Table S1). All characteristics that differentiate strain 1y-2^T from the 3 other *Anaerostipes* strains are listed in Table 1. Overall, the isolate has distinct biochemical properties that discriminate it from other members of the *Anaerostipes* group.

Table 1: Selected physiological and biochemical tests that differentiate strain 1y-2^T from *Anaerostipes caccae* DSM 14662^T, *Anaerostipes hadrus* DSM 3319^T and *Anaerostipes butyraticus* LMG 24724^T.

Characteristic	*Anaerostipes* 1y-2^T	*Anaerostipes caccae* DSM 14662^T	*Anaerostipes hadrus* DSM 3319^T	*Anaerostipes butyraticus* DSM 22094^T
Gram stain	Variable	Variable [a]	+[b]	+[c]
Spore-forming activity	+	- [a]	- [b]	+ [c]
Cell size (µm)	0.46-0.53 x 3.0-7.0	0.5-0.6 x 2.0-4.0[a]	0.7-1.0 x 3.0-10.0[b]	5.0-15.0[c]
Metabolic end products (glucose fermentation)	L, B, F, H, A #	L, B, H, A #	L, B, F, H, A #	B, F #
D/L-lactate used	D, L	D, L [a]	D [b]	ND *, [c]
Esculin hydrolysis	-	+	w	w
Gelatin hydrolysis	-	+	-	-
Assimilation of				
Mannitol	+	+	w	w
Salicin	-	+	+	w
Rhamnose	+	-	-	-
D-Ribose	w	+	-	-
L-Xylose	-	+	-	-
Trehalose	+	w	w	w
Enzyme activity				
α-galactosidase	-	+	-	-
β-galactosidase	-	-	+	-
β-galactosidase 6 phosphate	-	-	-	w
Tyrosine arylamidase	w	-	-	-
Histidine arylamidase	w	w	+	+
Serine arylamidase	w	w	-	w
Alkaline phosphatase	-	+	-	w
Esterase (C4)	+	w	-	+
Esterase lipase (C8)	+	-	-	-
Naphthol-AS-BI-phosphohydrolase	+	w	w	w
α-glucosidase	-	-	w	+
Growth temperature (optimum, °C)	18-45 (37)	ND *, [a] (37)	35-45 [b] (37)	35-47 [c] (41)
GC content (%)	44.5	45.5-46 [a]	37-42 [b]	44 [c]

All data were obtained in this study.

Data taken from: a, (Schwiertz et al., 2002); b, (Allen-Vercoe et al.); c,(Eeckhaut et al., 2010).

*) ND: not determined; +, positive; -, negative; w, weak positive.

#) L: lactate; B: butyrate; F: formate; H: hydrogen; A: acetate

G+C composition of strain 1y-2^T is 44.5 %. The cellular fatty acids were mainly composed of $C_{12:00}$, $C_{19:1}$ iso I and $C_{16:00}$ and differed from the other members of *Anaerostipes* genus (Supplementary Table S2).

Based on a polyphasic approach, our data clearly show that strain 1y-2^T is significantly different from the other *Anaerostipes* species. Therefore, it is appropriate to designate strain 1y-2^T to a novel species within this genus, for which the name *Anaerostipes rhamnosivorans* 1y-2^T sp. nov. is proposed.

Description of *Anaerostipes rhamnosivorans* sp. nov.

Anaerostipes rhamnosivorans (rham.no.si.vo'rans. N.L. neut. n. rhamnosum rhamnose; L. v. vorare to devour; N.L. part. adj. *rhamnosivorans* devouring rhamnose).

Cells are non-motile, spore-forming anaerobic rods and often observed to elongate and form curly cells in old cultures. Cell size is 0.7 - 0.83 µm by 3 – 6 µm. They occur as single cells and sometimes in short chains of 2 to 5 cells. They stain Gram-positive (young cells) or Gram-negative (old cells). On RCM agar colonies are beige, circular and raised with rough surfaces, about 1–4 mm in diameter. The strain can grow at 15 - 45 ^oC (optimum at 37 ^oC) and pH 5.5 – 8 (optimum pH 6.5 - 7) and 3 % NaCl. Lactate, formate, butyrate and acetate are major metabolites from glucose fermentation and 1,2 propanediol, butyrate, acetate, formate are major products of rhamnose fermentation. The strain produces about 13mM butyrate from 16mM lactate (both D- and L-lactate can be use) plus acetate. Butyrate is detected in all sugar fermentations.

Activity for esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase and weak responses for arginine phosphatase, prolinearylamidase, tyrosine arylamidase, histidine arylamidase, serine arylamidase. Acid is produced from D-glucose, D-mannitol, D-saccharose, D-mannose, D-sorbitol, L-rhamnose, trehalose, D-galactose, D-fructose, L-sorbose, D-maltose, dulcitol, inositol, N-acetyl-Glucosamine, D-turanose, D-lyxose, D-tagatose, and weakly ferments D-arabinose, D-ribose, D-adonitol, methyl-α D-glucopyranoside, Xylitol, D/L-arabitol and Xylose. The major fermentation products are lactate and butyrate .

The G + C % is 44.5%. The major cellular fatty acids are $C_{12:00}$, $C_{19:1}$ iso I and $C_{16:00}$.

The type strain is 1y-2^T(= DSM 26241^T = KCTC 15316^T) and was isolated from infant stool in Wageningen, Netherlands, in 2012.

Acknowledgements

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Supplementary data

Supplementary Table S1: Glucose and rhamnose fermentation products of strain 1y-2^T and related *Anaerostipes* strains.

Data were obtained in duplicate after 48 hours growth in bicarbonate-buffered medium with 40mM of glucose and 40mM of rhamnose at 37 °C. Values are mean ± SD (mole/mole) from duplicates.

	Products (molar ratio)					
Strain	Lactate	Formate	Acetate	Butyrate	1,2 propanediol	Hydrogen
*A.*1y-2 DSM 26241^T	0.57±0.01	0.45±0.005	0.11±0.002	0.46±0.005	0	0.31±0.01
	0.029±0.024#	0.13±0.04#	0.35±0.007#	0.16±0.006#	0.77±0.035#	0.009±0.0004#
A. caccae DSM 14662^T	1.35±0.01	0.08±0.005	0	0.19±0.002	0	0.13±0.01
A. hadrus DSM 3319^T	0.03±0.001	0.96±0.01	0.31±0.01	0.60±0.01	0	0.59±0.02
A. butyraticus DSM22094^T	0.02±0.001	0.24±0.001	0.08±0.001	0.69±0.005	0	0.50±0.01

products of rhamnose fermentation of strain 1y-2^T

Supplementary Table S2: Cellular fatty acid composition of strain 1y-2^T and *Anaerostipes caccae* DSM 14662^T, *A. hadrus* DSM 3319^T and *A. butyraticus* LMG 24724^T.

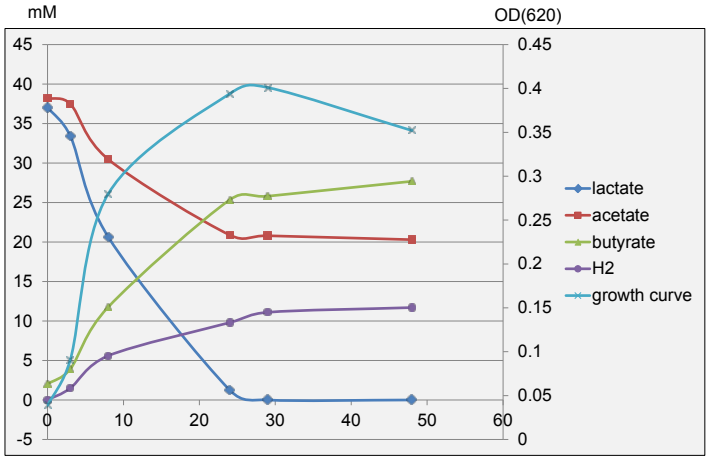
Data were obtained in this study. All strains were grown in Wilkens Chalgren Broth for 24 hours at 37 °C. Values are percentages of total cellular fatty acids.

Cellular fatty acids	*A.*1y-2^T	*A. caccae* DSM14662^T	*A. hadrus* DSM 3319^T	*A. butyraticus* DSM 22094^T
Saturated				
10 : 00	3.3	2.35	1.37	0.29
12 : 00	**38.79**	**32.23**	**49.05**	**44.9**
14 : 00	6.87	**9.43**	5.38	**12.19**
16 : 00	**12.1**	**8.32**	3.52	**8.63**
18 : 00	5.55	**9.98**	**13.3**	**12.58**
Unsaturated				
17 : 1 *w8c*	0.6	0.81	-	-
18 : 1 ω7c	0.59	0.76	-	-
18 : 1 ω9c	2.36	2.13	-	-
19 : 1 iso I	**20.55**	**28.94**	**12.41**	**8.91**
Summed feature 1 (13:0 3OH/15:1 i I/H)	0.31	0.46	1	1.92
Summed feature 2 (16:1 ISO I/14:0 3OH)	0.36	0.27	**8.76**	2.26
Summed feature 4 (17:1 ISO I/ANTEI B)	6.6	3.17	3.03	4.85
Unknown (ECL 14.959)	1.79	0.78	1.58	2.5

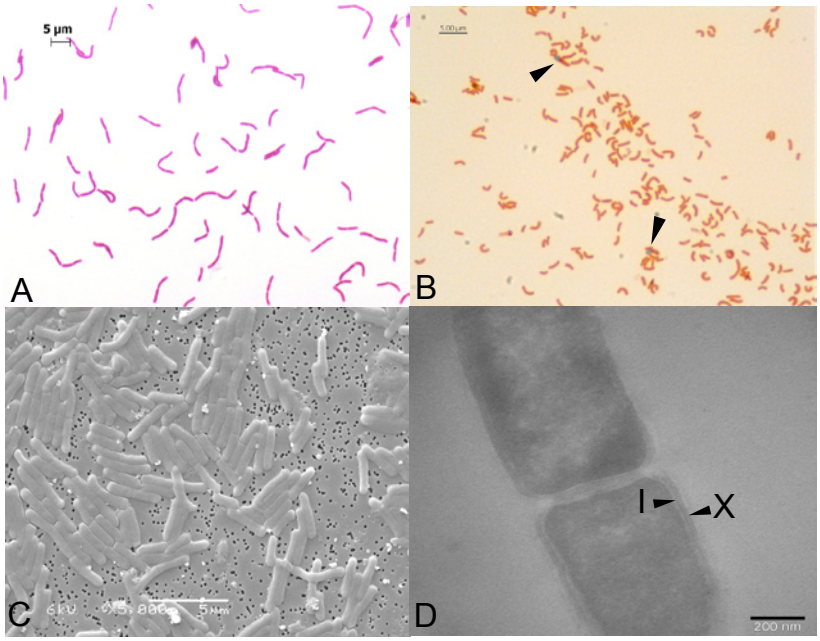
Products shown in bold are the dominant fatty acids.

-, not detected.

Supplementary Figure S1: Stoichiometry of strain 1y-2^T in bicarbonate-buffered medium containing 40mM lactate and 40mM acetate as carbon and energy sources.



Supplementary Figure S2: Images of strain 1y-2^T. A: Gram-staining cells taken by bright field microscopy; **B:** Light microscopic image of cells of strain 1y-2^T after staining with safranine and malachite green (SIGMA) for visualization of spores indicated by arrowheads. **C:** Scanning Electron Micrograph and **D:** Transmission Electron Micrograph, arrowheads indicate the presence of an inner membrane (I) and an outer layer (X) which resembles a S-layer, outer membrane or just exopolysaccharide.



CHAPTER 3

MICROBIAL INTERACTIONS THAT PROMOTE BUTYRATE PRODUCTION IN THE HUMAN INTESTINE

Manuscript in preparation.

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Abstract

The human intestinal tract harbors a microbial community that is highly diverse and complex. It has a vast metabolic capacity that includes the breakdown of complex carbohydrates into short chain fatty acids, such as acetate, propionate and butyrate. Because of the impact of butyrate on health, there is a considerable interest in butyrogenic bacteria and their role in the colonic anaerobic food chain. Here, we used *Anaerostipes rhamnosivorans* as a model for an intestinal butyrate producer, and studied its interactions *Blautia hydrogenotrophica*, a hydrogen-utilizing acetogen, *Methanobrevibacter smithii*, an intestinal methanogen, and with *Bacteroides thetaiotaomicron*, a degrader of plant cell wall pectins. We detected cross-feeding, a special type of mutualism, between *A. rhamnosivorans* and *Blautia hydrogenotrophica* on lactate and a small amount of acetate, resulting in a significant increase of butyrate formation. Interestingly, *A. rhamnosivorans* supported the growth of *M. smithii*, which in turn stimulated butyrate formation. A trophic chain, where one organism uses the product of the other organism for its growth, was observed when *A. rhamnosivorans* and *Bacteroides thetaiotaomicron* were grown on two different pectins. Our findings demonstrate three microbe-microbe interactions between *A. rhamnosivorans* at the one hand and an acetogen, a methanogen or a pectin-degrader at the other hand. In all cases butyrate production was increased, indicating a potential target for promoting colonic health.

Introduction

The human intestine is a mainly anaerobic ecosystem with microbial communities that are highly dynamic and regulated by a variety of host and environmental factors, including nutrition (Zoetendal and de Vos, 2014). Nevertheless, each individual carries a personalized gut microbiome and multiple studies revealed high-level structures, termed enterotypes, with similarities in composition (Arumugam et al., 2011). Three enterotypes have been described with *Bacteroides*, *Prevotella* and *Ruminococcus* as their main drivers. Either positive or negative co-occurrence of different taxonomic groups with these three main drivers have been proposed, that could be used for the prediction of species interactions at functional level. However, such microbial network predictions should be tested and hence it is essential to study the microbial interspecies interactions in vitro under physiologically relevant conditions that mimic the human intestinal tract.

Butyrate, a major metabolite of microbial fermentation in the colon is crucial to maintain a healthy gut as it fuels enterocytes, signals to the host and is a critical mediator of the colonic inflammatory response (Zimmerman et al., 2012). Butyrate-producing bacteria, one of the core functional groups in human intestine, are reduced in cell number in a variety of diseases, including inflammatory bowel disease (IBD) and diabetes (Clemente et al., 2012). Therefore, there is a great interest in defining microbial interactions that involve butyrogenic bacteria and influence butyrate production.

Lactate and hydrogen are important fermentation products of degradation of polysaccharides (Flint et al., 2012). The accumulation of lactate or hydrogen is known to result in gastrointestinal disorders (Hove et al., 1994). Lactate can be used by butyrate-producing bacteria in presence of acetate (Duncan et al., 2004). Bacteria that convert lactate and acetate into butyrate mostly belong to *Clostridium* cluster XIVa, including *Anaerostipes* spp. and *Eubacterium hallii*. Other intestinal butyrate producers convert glucose and acetate, such as *Faecalibacterium prausnitzii*, which belongs to *Clostridium* cluster IV and *Roseburia* spp. (Louis and Flint, 2009). Isotope labelling studies have identified lactate and acetate as important precursors of butyrate production in mixed communities from the gut (Bourriaud et al., 2005; Muñoz-Tamayo et al., 2011). *Methanobrevibacter smithii* is a major intestinal methanogenic archaeon, which is able to grow on H_2/CO_2 or formate (Eckburg et al., 2005; Abell et al., 2006; Scanlan et al., 2008). *Blautia hydrogenotrophica* is a predominant intestinal acetogen which is able to grow both on H_2/CO_2 and carbohydrates (Bernalier et al., 1996). This archaeon and acetogen have shown to efficiently remove hydrogen in coculture experiments on cellulose and xylan (Robert et al., 2001; Chassard and Bernalier-Donadille, 2006) and *M. smithii* consumed formate in a germ-free mouse model (Samuel and Gordon, 2006).

The composition of human microbiota and its activities are significantly impacted by the carbohydrate content of the dietary intake (Walker et al., 2011; O'Keefe et al., 2015). Therefore, understanding trophic chains utilising food-related carbohydrates and resulting in

butyrate formation would contribute to mechanistic insight. Pectin is one of the polymeric carbohydrates that is abundant in fruits and vegetables and frequently applied as gelling agent in food (Voragen et al, 2009). Pectin is a complex polysaccharide with mainly galacturonic acid (GalA) in the backbone, either present in long GalA sequences in homogalacturonan structural elements or the highly substituted rhamnogalacturonan I segments. The rhamnogalacturonan I backbone consists of dimers of rhamnose-galacturonic acid which galactose and arabinose rich side chains are connected to O-4 position of rhamnose (Voragen et al., 2009). Several species of *Bacteroides* are able to break down pectin (Jensen and Canale-Parola, 1986), including *Bacteroides thetaiotaomicron* (Dongowski et al., 2000). This abundant intestinal anaerobe also degrades malto-oligosaccharides and starch (Reeves et al., 1996). However, it is not known how *Bacteroides thetaiotaomicron* interacts with butyrate-producing bacteria on pectins, though a substantial amount of these polymers are present in daily food.

Anaerostipes rhamnosivorans is a butyrate-producing bacterium, newly isolated from infant stool (Bui et al., 2014). This strain belongs to genus *Anaerostipes*, which is among the top fifteen abundant genera in the human intestinal microbiome (Arumugam et al., 2011). The strain is able to perform mixed acid fermentations with many sugars, including rhamnose for its butyrogenesis. So far, butyrate production from rhamnose is a unique feature for intestinal microbes. *A. rhamnosivorans* produces butyrate from either sugars or lactate and acetate.

Here we present how *A. rhamnosivorans* metabolically interacts with a number of gut microorganisms, including *Blautia hydrogenotrophica* (former *Ruminococcus hydrogenotrophicus*), an acetogenic bacterium isolated from human stool (Liu et al., 2008); *Bacteroides thetaiotaomicron*, a degrader of various pectin substrates (Dongowski et al., 2000) and *Methanobrevibacter smithii*, the most abundant intestinal methanogen (Gaci et al., 2014). The results show the existence of syntrophic interactions, including trophic chains and cross-feeding, which leads to an increased production of butyrate by *A. rhamnosivorans* in all the cocultures.

Materials and methods

Growth of the microorganisms

Anaerostipes rhamnosivorans (DSM 26241^T) was isolated from infant stool (Bui et al., 2014), *Bacteroides thetaiotaomicron* (DSM 2079^T), *Blautia hydrogenotrophica* (DSM 10507^T), and *Methanobrevibacter smithii* (DSM 861^T) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The metabolisms of these microbes were indicated in Table 1.

All microorganisms were routinely maintained under strict anaerobic conditions in 120-ml serum bottles. Reinforced Clostridial Medium (RCM, DIFCO) was used to pregrow *A. rhamnosivorans*, *Bacteroides thetaiotaomicron* and *Blautia hydrogenotrophica*. *M. smithii* was grown in bicarbonate-buffered anaerobic media (Stams et al., 1993; van Gelder et al., 2012)

supplemented with 0.2 g/l yeast extract; 0.05 g/l cystein; 2 mM acetate as carbon source and H_2/CO_2 (80:20, v/v) at a pressure of 1.7 bar as energy source. All mixed culture experiments were performed in serum bottles sealed with butyl-rubber stoppers containing the bicarbonate buffered anaerobic media with certain substrates as carbon and energy sources at 37 °C under a gas phase of 1.7 atm of N_2/CO_2 (80:20, v/v). In addition, vitamins and sodium sulfide (1 mM) were added to the medium from sterile stock solutions as growth factor and reducing agent, respectively (Stams et al., 1993). All stock solutions were prepared, sterilized and kept in anaerobic serum bottles with a headspace of N_2.

Table 1: Microorganisms used in this study and their metabolisms.

Strains	Substrates	Major metabolites	References
Anaerostipes rhamnosivorans DSM 26241^T	Glucose	Butyrate, lactate, formate, acetate, hydrogen	Bui et al., 2014
	Rhamnose	1,2 - propanediol, butyrate, lactate, formate, acetate, hydrogen	
	Lactate/acetate	Butyrate, hydrogen	
Blautia hydrogenotrophica DSM 10507^T	H_2/CO_2	Acetate	Bernalier et al., 1996
	Glucose	Lactate, acetate	Liu et al., 2008
Bacteroides thetaiotaomicron DSM 2079^T	Glucose	Succinate, propionate, acetate	Pan and Imlay, 2001
	Rhamnose	1,2-propanediol	Patel et al., 2008
	Pectins	Acetate, propionate	Dongowski et al., 2000
Methanobrevibacter smithii DSM 861^T	H_2/CO_2 or formate	CH_4	Miller et al., 1982

Co-culture of *A. rhamnosivorans* and *Blautia hydrogenotrophica* with lactate

The coculture experiments were performed in 50 ml bicarbonate buffer medium supplemented with 2 g/l yeast extract, 2 g/l tryptone to facilitate the growth of *Blautia hydrogenotrophica*. Lactate was added from 1M sterile anaerobic stock solutions to a final concentration of 20 mM in the coculture as the carbon source. Equal amounts of the overnight cultures of each microorganism (2 %, v/v) were inoculated simultaneously into the medium to complete the cocultures. Monocultures of *A. rhamnosivorans* and *Blautia hydrogenotrophica* supplemented with 2 g/l yeast extract, 2 g/l tryptone in the bicarbonate buffered media containing lactate were used as controls.

Co-culture of *Anaerostipes rhamnosivorans* and *Methanobrevibacter smithii*

M. smithii and *A. rhamnosivorans* were pre-cultured in the media as described above. *M. smithii* was pregrown for a week with H_2/CO_2 (80:20, v/v) before the headspace was exchanged to N_2/CO_2 (80:20, v/v). The coculture was grown in 120-ml serum bottles with 50 ml bicarbonate buffered medium by adding 1ml of the overnight culture of *A. rhamnosivorans* into 1-week grown culture of *M. smithii*. Glucose from 1 M sterile stock solution was subsequently added to the coculture to make a final concentration of 20 mM. Monocultures of *A. rhamnosivorans* in the bicarbonate buffered medium containing 20 mM glucose as substrate were used as control. In addition, 1-week pregrown culture of *M. smithii* was exchanged the headspace into N_2/CO_2 (80:20, v/v) and supplemented with 20 mM glucose before the incubation as negative control.

Co-cultures of *A. rhamnosivorans* and *Bacteroides thetaiotaomicron*

All mixed culture experiments were performed using an anaerobic, 10 ml bicarbonate-buffered mineral medium (Stams et al., 1993). Due to various solubilities, pectin fractions were prepared in stock solutions with different concentrations (1 g/20 ml SBPOS; 0.2 g/20 ml RG-I apple; 1 g/20 ml SBP6230; 2 g/20 ml SBP; 0.4 g/20 ml RG-I potato; 1 g/20 ml SSPS) in anaerobic serum bottles and sterilized before adding into the medium. The overnight cultures of *A. rhamnosivorans* and *Bacteroides thetaiotaomicron* were used as inoculums. Two different coculture experiments were carried out.

In the first co-culture experiments, substrates were added into 9 ml basal medium to make the indicated quantities (Table 2). Complete medium was inoculated with 2 % (v/v) of pregrown cultures of each microorganism to prepare the cocultures. In parallel, *A. rhamnosivorans* and *Bacteroides thetaiotaomicron* were growing in monocultures in the bicarbonate buffered medium containing with SBP, SBP6230, SBPOS, RG-I potato and RG-I apple and SSPS (refer to Table 2 for full names) as substrates for the comparisons. Gas and liquid samples were collected after 8 days of incubation for H_2 and organic acid production.

Table 2: Dietary pectin fractions and their quantities in the initial cocultures of *A. rhamnosivorans* and *Bacteroides thetaiotaomicron*.

Substrates	Abbreviation	Quantity per bottle	References
Sugar beet pectin	SBP	2 g/l	Remoroza et al., 2012
Sugar beet pectin 6230	SBP6230	1 g/l	Buchholt et al., 2004
Sugar beet pectic oligosaccharides batch C7-1	SBPOS	1 g/l	Leijdekkers et al., 2015
Rhamnogalacturonan I from potato pectic fibre	RG-I potato (or MHR potato)	0.4 g/l	Schols and Voragen, 1994
Rhamnogalacturonan I Apple (Modified hairy regions Saponified)	RG-I apple (or MHR-S apple)	0.2 g/l	Coenen et al., 2007
Soy pectin	SSPS	1 g/l	Coenen, 2007

The second co-culture experiment was performed in the bicarbonate buffered medium with an increased amount of SBP and SSPS (4 g/l). The inoculum was 8% of *A. rhamnosivorans* and 2 % of *Bacteroides thetaiotaomicron* (v/v). Monocultures of *A. rhamnosivorans* and *Bacteroides thetaiotaomicron* in the same conditions were used for comparison. Gas and liquid samples were collected after 6 days of incubation for H_2 and organic acid production. All experiments were performed in duplicates.

Analytical methods

Glucose, organic acids and alcohols were measured on a Thermo Scientific Spectra HPLC system equipped with a Agilent Metacarb 67H 300 x 6.5 mm column kept at 45 °C and running with 10 mM arabinose as eluent. The detector was a refractive index detector. The eluent flow was 0.8 ml/min. All analyses were performed in duplicates. All cultures were sampled at different time intervals for HPLC.

H_2 and CH_4 measurement were performed by a Shimadzu GC-14B gas chromatography (Shimadzu, Kyoto, Japan) equipped with a Molsieve 13X column (Varian, Middelburg, The Netherlands) and a thermal conductivity detector. Injection volume was 0.2 ml and detector temperature was 150 °C. The carrier gas was argon at the flow rate of 30 ml/min. All analyses were performed in duplicates. All cultures were sampled at different time intervals for HPLC.

Enumeration of bacteria in cocultures by microscopic counting

Due to their distinct morphologies, the relative abundance of each microbe in the cocultures could be determined by microscopic counting using a Leica DM 2000 phase contrast microscope at 1000x magnification. Bacterial cultures were sampled at the exponential phase. Each microscopic slide was covered by a thin layer of soft agar suspension (1 % w/v Noble agar, DIFCO). Subsequently, cells were mixed and 2 µl cultures were put on the prepared slides and air-dried for 5 min before being observed. Images were captured by

using a Leica DMC2900 camera (Leica Microsystem, Germany). At least 50 microscopic fields were counted for each coculture. The values represented an average of all counted fields.

Results

Coculture of *A. rhamnosivorans* and *Blautia hydrogenotrophica* on lactate

A. rhamnosivorans is known to convert lactate and acetate to butyrate and hydrogen (Bui et al., 2014) while *Blautia hydrogenotrophica* has reported to convert H_2/CO_2 to acetate (Bernalier et al., 1996). In pure culture, *A. rhamnosivorans* metabolized lactate and acetate to butyrate, but butyrate formation stopped when acetate was depleted (Fig. 1A). In pure culture, *Blautia hydrogenotrophica* produced a small amount of acetate during the first 24 h, likely due to the utilization of the presence of yeast extract and peptone, but this production of acetate and lactate leveled off upon further incubation (Fig. 1B). Thus, none of the microbes can grow on lactate in monoculture. However, following inoculation at an OD_{600} of 0.07, the cocultures reached an OD_{600} of 0.4 after 60 hours, indicative of significant growth. Moreover, the added 20 mM lactate was completely utilized by the coculture and approximately 12 mM of butyrate was formed as a major product after 72 hours (Fig. 1C). Hydrogen remained at a very low concentration (below 0.05 mM) during the coculture experiment whereas acetate increased from 2 mM to 3.8 mM after 24 hours and slowly decreased afterwards. So the coculture of the butyrogenic bacterium *A. rhamnosivorans* and the hydrogenotrophic partner *Blautia hydrogenotrophica* converted lactate to butyrate rapidly and completely and the C-recovery was 99. 24 % (taken CO_2 production into account). Hydrogen and acetate levels were low, reflecting the balance between the production and utilization of these two compounds by *A. rhamnosivorans* and *Blautia hydrogenotrophica*. An equal ratio of *A. rhamnosivorans* and *Blautia hydrogenotrophica* was observed by microscopic counting, confirming the comparable activities of these two organisms in this coculture (Supplementary table 1 and Fig. 1).

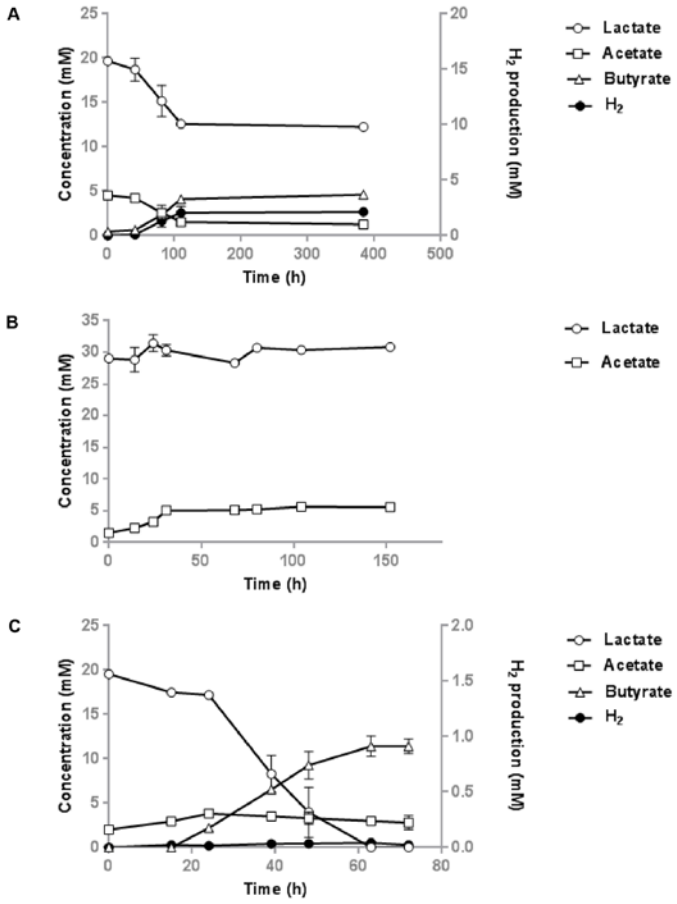

Figure 1: Lactate degradation and product formation in monocultures of *A. rhamnosivorans* (A), *Blautia hydrogenotrophica* (B) and cocultures of *A.rhamnosivorans* and *Blautia hydrogenotrophica* (C). Lactate, acetate and butyrate concentrations are shown on the left Y axis (mM), H_2 is plotted on the right Y axis (mM). All experiments were performed in duplicates and mean values were shown here.

Coculture of *A. rhamnosivorans* and *M. smithii* in glucose

A monoculture of *A. rhamnosivorans* converted 20 mM glucose to butyrate, lactate, formate, acetate and hydrogen (Fig. 2A), while there was no growth of *M. smithii* in glucose medium. After the rapid depletion of glucose within 24h, *A. rhamnosivorans* slowly converted the lactate and acetate formed to butyrate. This conversion was ongoing during 5-day incubation (Fig. 2A). However, in coculture with *M. smithii*, glucose fermentation and methane production occurred simultaneously (Fig. 2B). The maximum OD_{600} of the cocultures, on average 2.3, was similar to those of the monoculture. Glucose was consumed slightly faster during the

first 12 hours as compared to the monoculture (Fig. 2) but was completely depleted after 21 hours in both the monoculture and coculture. In the coculture, only a small amount of lactate was formed after 21 hours (about 2 mM) and this was completely degraded after 48 hours leading to a higher butyrate level. In contrast, lactate production was much higher in monoculture (about 15 mM). Consequently, after 54h incubation about 20 mM butyrate was formed in the coculture, which was higher than that of the monoculture of *A. rhamnosivorans* (about 15 mM). The net acetate production was always slightly higher in the coculture than in the monoculture of *A. rhamnosivorans*. In the co-culture, formate and H_2 increased within 12 hours and thereafter were rapidly converted to CH_4 indicating growth of the methanogen. About 5 mM CH_4 was formed at the end of growth in the coculture. Cell numbers of *A. rhamnosivorans* were 2.6 times higher than those of *M. smithii* (Supplementary table 2). The growth of methanogenic partner was obviously dependent on the butyrogenic partner, and in turn influenced the metabolism of its partner.

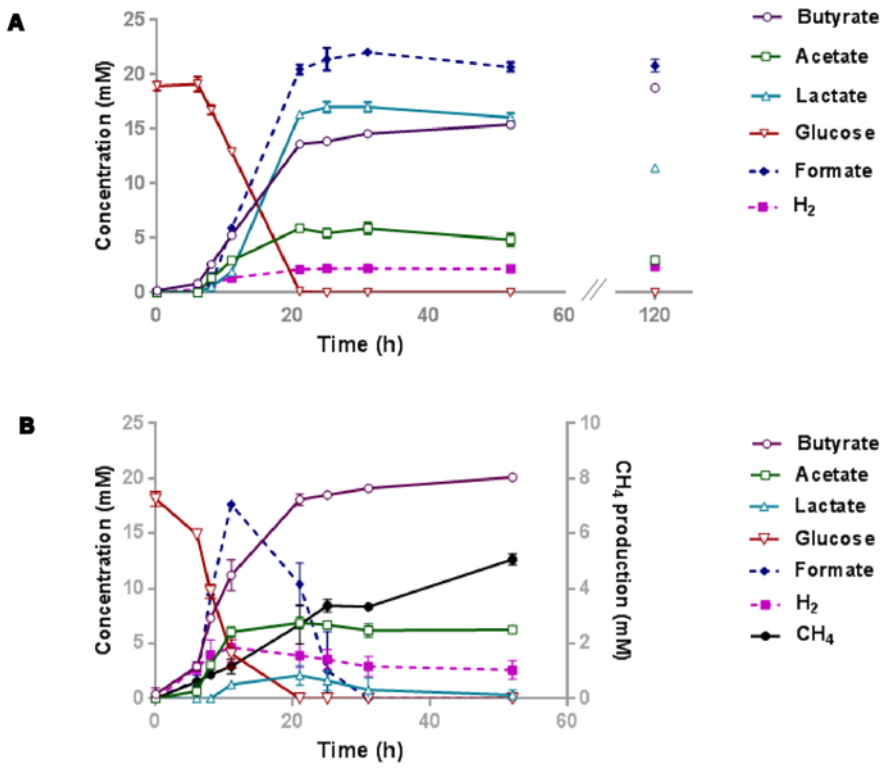

Figure 2: Glucose conversion and product formation in monocultures of *A. rhamnosivorans* (A) and cocultures of *A. rhamnosivorans* plus *M. smithii* (B). Secondary Y axis indicates the production of CH_4 and H_2 in mM. All experiments were done in duplicate and the means of the obtained values are shown.

Coculture of *A. rhamnosivorans* and *Bacteroides thetaiotaomicron* in pectins

Cocultures of *A. rhamnosivorans* and *Bacteroides thetaiotaomicron* were set up and initial experiments showed that different pectin fractions were degraded and that some butyrate was formed on SBP and SSPS but not on SBP6230, SBPOS, RG-I potato and RG-I apple (Table S1). This indicated that there was an effect of *Bacteroides thetaiotaomicron* on the growth of *A. rhamnosivorans* with two of these substrates. Although all pectins contain similar sugar building blocks linked through similar linkages, the amount and sequence of the different sugar building blocks vary amongst the pectins. SBP and SSPS were rich in galacturonic acid, rhamnose, arabinose and galactose. *A. rhamnosivorans* was not able to convert both substrates in monoculture while *Bacteroides thetaiotaomicron* showed different abilities to ferment the two distinctive pectin fractions SBP and SSPS, and acetate was a major product formed from these substrates (Table 3). The coculture experiments were repeated using higher amounts of these two substrates (4 g/l). The inoculum ratio was 4:1 (v/v) of pregrown cells of *A. rhamnosivorans* and *Bacteroides thetaiotaomicron*. The two bacteria were pregrown in RCM medium overnight, reaching an OD_{600} of 2 before being used as 2 % and 8% inoculum for *A. rhamnosivorans* and *Bacteroides thetaiotaomicron*, respectively. After 6 days of incubation, the cocultures reached an OD_{600} of 1.2 and 1.6 with SBP and SSPS, respectively. Acetate production was always higher on SSPS than on SBP, both under monoculture and coculture conditions. Altogether, *Bacteroides thetaiotaomicron* appeared to grow better with SSPS as compared to SBP. Interestingly, about 5 mM and 2 mM butyrate was formed in the cocultures with SBP and SSPS, respectively, suggesting growth of the butyrogenic partner and better growth on SBP as compared to SSPS. Trace amounts of hydrogen were detected in the cocultures, but not in the monocultures (1.2 mM with SBP and 0.5 mM with SSPS; Table 3). The fact that no lactate remained implied the conversion of the lactate formed into butyrate by *A. rhamnosivorans*. Ethanol and propionate production by *Bacteroides thetaiotaomicron* was slightly reduced while the acetate concentration stayed almost the same in the cocultures, suggesting that the presence of *A. rhamnosivorans* might influence the metabolic activity of its partner. In addition, no 1,2-propanediol was detected in any of the cocultures, indicating that rhamnose was not fermented or rhmanose fermentation was not resulted in 1, 2 propanediol formation. Microscopic cell counts indicated that *A. rhamnosivorans* was approximately 10 times less abundant than *Bacteroides thetaiotaomicron* after growth with both substrates while inoculum ratio was only 4, confirming the significant role of *Bacteroides thetaiotaomicron* (Supplementary table 3-A,B).

Table 3: Product formation from monocultures of *Bacteroides thetaiotaomicron* and cocultures of *A. rhamnosivorans* and *Bacteroides thetaiotaomicron* with SBP and SSPS.

Substrate	Strain	Time (d)	Lactate (mM)	Acetate (mM)	Succinate (mM)	Propionate (mM)	Ethanol (mM)	Butyrate (mM)	Hydrogen (mM)
SBP	Monoculture	T = 0	ND	3.25 ± 1.39	ND	ND	ND	ND	ND
		T = 6d	3.56 ± 0.36	25.5 ± 2.4	0.19 ± 0.05	2.09 ± 0.13	1.54 ± 0.27	ND	ND
	Coculture	T = 0	ND	5.63 ± 0.14	0.24 ± 0.17	ND	ND	1.85 ± 0.1	ND
		T = 6d	ND	27.96 ± 0.67	0.4 ± 0.1	ND	1.04 ± 0.1	6.83 ± 0.27	1.19
SSPS	Monoculture	T = 0	0.37 ± 0.04	1.8 ± 0.2	ND	ND	ND	ND	ND
		T = 6d	1.06 ± 0.16	38.25 ± 1.28	0.12 ± 0.04	0.96 ± 0.02	4.55 ±0.33	ND	ND
	Coculture	T = 0	0.37 ± 0.02	3.25 ± 0.3	0.04 ± 0.05	ND	ND	1.7 ± 0.15	ND
		T = 6d	0.05 ± 0.06	38.45 ± 0.31	0.19 ± 0.02	0.69 ± 0.35	3.33 ± 0.002	3.54 ± 0.2	0.53 ± 0.054

Product formation was determined after 6 days of growth. No production was observed in monoculture of *A. rhamnosivorans* in SBP or SSPS. Mean and data range are shown. ND: not detected. All experiments were done in duplicate (mM); 4 g/l of SSPS and 4 g/l SBP was added as substrate.

Discussion

Several intestinal bacteria belonging to *Clostridium* cluster XIVa contribute to intestinal butyrate formation (Louis et al., 2007a). This group is metabolically very versatile and many of its butyrate-producing bacteria perform a mixed acid fermentation with monomeric sugars while only a few can utilize dietary complex carbohydrates (Chassard and Bernalier-Donadille, 2006; Lopez-Siles et al., 2012; Scott et al., 2014). Several butyrate-producing bacteria have secondary interactions with hydrogen scavenging microorganisms (Duncan et al., 2004; Chassard and Bernalier-Donadille, 2006). Most of them rely on primary degraders that are able to utilize indigestible dietary fractions in the large intestine (Belenguer et al., 2006). Here, we investigate the interspecies metabolic interactions that may take place in the human intestine with special focus on the butyrate-producing bacterium *A. rhamnosivorans* alone and in combination with an acetogen, a pectin-utilizer and a methanogen.

Lactate is mostly converted into butyrate by intestinal microbial mixtures (Bourriaud et al., 2005), though detailed microbial community analysis to identify the main lactate degraders in the mixed community is missing. Lactate is produced by many intestinal microbes in vitro, however, it is not detected or at very low level in the human intestine due to its rapid conversion (Cummings and Marfarlane, 1991). Lactate accumulation is undesired, as it results in gastrointestinal disorders (Hove et al., 1994). One of the *Clostridium* cluster XIVa butyrate producers is *Eubacterium hallii*, which is able to convert lactate and acetate, produced by *Bifidobacterium adolescentis* from potato starch or fructo-oligosaccharides, into butyrate (Belenguer et al., 2006). In our coculture study, it was clearly shown that lactate was metabolized via efficient cross-feeding between *A. rhamnosivorans* and homoacetogen *Blautia hydrogenotrophica* by means of interspecies transfer of hydrogen and acetate. Previously, hydrogen transfer between xylanolytic or cellulolytic bacteria and hydrogenotrophic microbes was shown (Robert et al., 2001; Chassard and Bernalier-Donadille, 2006). The cross-feeding between *A. rhamnosivorans* and *Blautia hydrogenotrophica* resulted in simultaneous growth of both microorganisms. H_2/CO_2 conversion by the acetogenic species resulted in the formation of acetate, which was used with lactate by the butyrate-producing bacterium. The lactate conversion required a small amount of acetate to start up. These two reactions are depicted in Fig. 3. This cross-feeding between two dominant species in the human intestine may contribute to i) maintain a lactate balance in the gut, ii) prevent lactate and hydrogen accumulation, and iii) convert non-beneficial compounds (H_2, CO_2) to a beneficial compound (butyrate) in the gut.

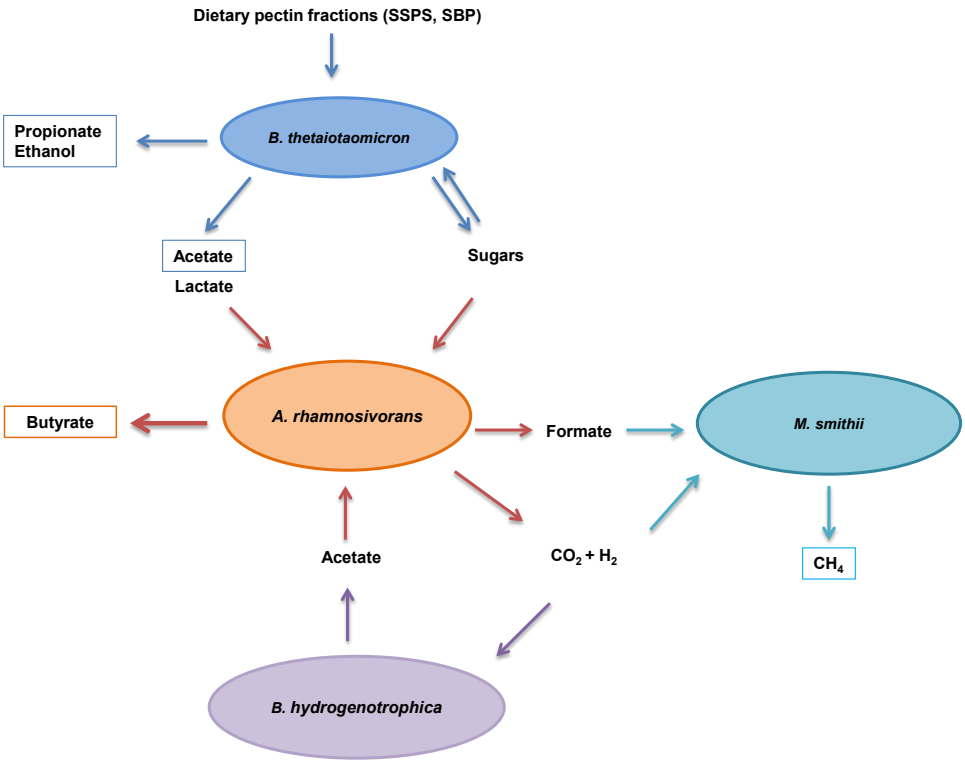

Figure 3: Schematic illustration of the interactions between *A. rhamnosivorans* and intestinal microbes in different substrates. Orange, blue, purple and indigo arrows indicate the conversion by *A. rhamnosivorans*, *Bacteroides thetaiotaomicron*, *Blautia hydrogenotrophica* and *M. smithii*, respectively. End products of the cocultures and monocultures are in rectangle panels while substrates and intermediates are not.

Some microbes are known to be highly specialized, such as *Ruminoccocus bromii*, a major starch degrader in the human colon. This bacterium is able to rapidly degrade starch but fails to grow on glucose (Ze et al., 2012), which becomes available for other intestinal bacteria. More than half of healthy adults harbor methanogenic archaea in their gut where these are likely to form syntrophic relationships with anaerobic bacteria (Chaudhary et al., 2015). In our study we observed a significant impact of the methanogen *M. smithii* on the metabolism of the butyrogenic bacterium *A. rhamnosivorans*. The presence of the methanogen led to a decrease in lactate formation, which was accompanied by an increased butyrate production. Clearly, the methanogenic partner triggered the production of hydrogen from glucose by the butyrogenic partner, resulting in a reduction of lactate formation. Consequently, increased levels of butyrate were obtained from glucose by *A. rhamnosivorans*. Evidently, H_2 and formate were transferred between the butyrogenic partner and the methanogen, and growth of the methanogen was dependent on the butyrogenic bacterium. As a result, no formate and

less H_2 were formed in the coculture. This coculture study demonstrates that *M. smithii* is able to consume formate efficiently in the presence of H_2. It has been previously shown that growth of this methanogenic archaeon enhanced the efficiency of dietary glycan-fermentation in the animal gut by removal of hydrogen and formate (Samuel and Gordon, 2006). Another study on co-occurrence of *M. smithii* and bacteria showed that several species of *Clostridium* cluster IV and XIVa were positively associated with *M. smithii* indicating a syntrophic relationship (Hansen et al., 2011). Hence, our coculture study between *A. rhamnosivorans*, a species belonging to *Clostridium* cluster XIVa, and *M. smithii* on glucose points to a metabolic interaction in humans that carry this methanogenic archaeon. By removing efficiently both hydrogen and formate, the methanogenic archaeon improves the substrate consumption rates and pulls the glucose fermentation towards butyrate production rather than towards lactate formation. As a result, butyrate formation is significantly enhanced. This facilitates the butyrate-producing bacteria in a highly competitive ecosystem as the gut to successfully compete for a substrate as well as harvest extra energy from the same substrate.

We also characterized the interaction between *A. rhamnosivorans* and the pectin-degrader *Bacteroides thetaiotaomicron* on a series of dietary pectins. Our results showed that the *A. rhamnosivorans* clearly benefited from the growth and activity of *B. thetaiotaomicron* degrading the SSPS and SBP pectin fractions. The OD values and acetate production of *Bacteroides thetaiotaomicron* indicated that this strain performed better growth in SSPS as compared to SBP. *Bacteroides thetaiotaomicron* facilitated growth of *A. rhamnosivorans* in a coculture experiment, as evidenced by the butyrate formation (Table 3). On the other hand both bacteria may compete for sugars generated from the pectin fractions. Acetate production levels in both monocultures and cocultures together with cell count results implied a dominating role of *Bacteroides thetaiotaomicron* in the interaction (Supplementary data 1 and Table 3). The higher butyrate production with SBP could be a result of the increased lactate release from this substrate by *Bacteroides thetaiotaomicron* that subsequently was utilized by *A. rhamnosivorans* to form butyrate (Table S1 and Table 3). The coculture experiments illuminated the syntrophic interaction between the two species when grown with SBP and SSPS. Other studies have also pointed to synthrophic butyrate production with *Bacteroides thetaiotaomicron* as primary degrader. In germ-free mice fed with glucans, it was found that *Bacteroides thetaiotaomicron* stimulated butyrate production when co-colonized with *E. rectale* (Shoaie et al., 2013). Moreover, pectin fermentation by *Bacteroides thetaiotaomicron* and other human gut microbes also resulted in butyrate formation (Dongowski et al., 2000). The interactions occurring on dietary pectins resulted in increased butyrate levels in vitro, which may be important for gut health (Fig. 3). Dietary pectins have a number of health-promoting benefits. A study in a rat model showed that apple pectin reduced the number of colon tumors (Ohkami et al., 1995). Orange peel pectin was shown to have a prebiotic effect that lead to an increased butyrate production which was correlated by an increase in the population of *Eubacterium rectale* in vitro (Manderson et al., 2005). Crude pectin from peels of apple,

carrot, lemon and orange has shown to stimulate the growth of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in vitro (Sen A, 2014). Pectin has shown to be potentially effective for cholesterol reduction and has anti-inflammatory and anti-carcinogenic effects (Tazawa et al., 1997; Bladergroen et al., 1999; Sherry et al., 2010). In our study, we observed that sugar beet pectin fractions promoted butyrate production and growth of the butyrate producing bacterium in vitro contributing to better understanding of another beneficial effect of SBP and SSPS on the gut bacteria.

This study revealed the occurrence of cross-feeding between anaerobes that naturally exist in the human intestinal tract and emphasized on those that are associated with butyrate formation. The butyrate-producing bacterium, *A. rhamnosivorans* benefits from the presence of some intestinal microorganisms or substrates for its growth and butyrate production. This may be due to its high metabolic flexibility using a wide variety of sugars as substrates for butyrogenesis, as well as its capacity to efficiently use lactate plus acetate for butyrate production. In addition, pectin is one of the most important sources of dietary fiber and has a number of health-promoting benefits. This coculture study evidently demonstrated that these pectin fractions promote the growth of the butyrate-producing bacterium and hence the butyrate level in the gut via syntrophic interactions. One of the implications is that these two pectins could be potential butyrogenic compounds to be used as prebiotic.

Acknowledgement

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Supplementary Table S1: Product formation of *Bacteroides thetaiotaomicron* and cocultures of *A. rhamnosivorans* and *Bacteroides thetaiotaomicron* in pectin fractions. The experiments were performed in bicarbonate buffer standard medium containing 2 g/l SBP; 1 g/l SBP6230; 1 g/l SBPOS; 0.4 g/l RG-I potato; 0.2 g/l RG-I apple; 1 g/l SSPS as substrates. The ratio of inocula was 1:1 (v/v) (2 % inoculum each). Product formation was determined after 8 days of incubation. All values were corrected for time zero. ND: not detected. Hydrogen was not detected in any of the incubations. *Bacteroides thetaiotaomicron* was grown in pure culture and coculture with *A. rhamnosivorans*. No growth and product formation was observed in monoculture of *A. rhamnosivorans* in any pectin fractions.

Substrates	Strains	Glucose (mM)	Lactate (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	OD 600nm
SBP	Monoculture	ND	1.05	17.64	2.29	ND	0.43
	Coculture	0.01	0	14.96	2.63	0.74	0.51
SBP 6230	Monoculture	0.07	ND	8.29	1.12	ND	0.32
	Coculture	ND	ND	7.82	1.66	ND	0.414
RG-I potato	Monoculture	ND	ND	2.59	ND	ND	0.27
	Coculture	0.10	ND	2.66	ND	ND	0.25
RG-I apple	Monoculture	ND	ND	1.21	ND	ND	0.15
	Coculture	ND	ND	ND	ND	ND	0.15
SSPS	Monoculture	ND	ND	9.82	ND	ND	0.27
	Coculture	ND	ND	11.46	0.82	0.71	0.33
SBPOS	Monoculture	ND	ND	6.80	0.78	ND	0.37
	Coculture	ND	ND	5.75	0.60	ND	0.38

Supplementary data 1: Cell count in coculture of *A. rhamnosivorans* and *Blautia hydrogenotrophica* in lactate. The counting was done in 106 fields. The ratio between *A. rhamnosivorans* and *Blautia hydrogenotrophica* is calculated in each field. The bold value is mean of all counted fields.

microscope field	number of *A. rhamnosivorans* cells	number of B. *hydrogenotrophica* cells	Ratio of *A.rhamnosivorans/B. hydrogenotrophica* per field
1	2	3	0.7
2	1	5	0.2
3	3	5	0.6
4	1	2	0.5
5	2	3	0.7
6	5	4	1.3
7	4	4	1
8	3	1	3
9	3	5	0.6
10	15	31	0.5
11	57	69	0.8
12	3	5	0.6
13	2	4	0.5
14	4	4	1
15	2	4	0.5
16	3	5	0.6
17	4	2	2
18	3	1	3
19	3	2	1.5
20	5	3	1.7
21	2	5	0.4
22	2	7	0.3
23	4	2	2
24	1	4	0.3
25	17	29	0.6
26	3	3	1
27	2	11	0.2
28	3	3	1
29	6	7	0.9
30	6	4	1.5
31	6	4	1.5
32	6	6	1
33	5	4	1.3
34	3	5	0.6
35	2	10	0.2
36	4	10	0.4
37	4	3	1.3
38	6	3	2
39	6	3	2
40	7	4	1.8
41	2	6	0.3
42	3	3	1
43	10	8	1.3
44	2	4	0.5
45	7	6	1.2
46	2	7	0.3
47	2	9	0.2
48	6	5	1.2
49	1	4	0.3
50	1	5	0.2
51	3	2	1.5
52	3	2	1.5
53	2	2	1
54	2	5	0.4
55	3	5	0.6
56	2	1	2
57	4	3	1.3
58	6	1	6
59	5	7	0.7
60	8	3	2.7
61	3	4	0.8
62	3	3	1
63	4	2	2
64	6	2	3
65	5	6	0.8
66	5	5	1
67	2	3	0.7
68	6	4	1.5
69	7	7	1
70	2	4	0.5
71	4	4	1
72	1	11	0.1
73	6	7	0.9
74	7	10	0.7
75	6	25	0.2
76	7	20	0.4
77	4	20	0.2
78	12	35	0.3
79	7	6	1.2
80	4	7	0.6
81	3	15	0.2
82	5	3	1.7
83	7	7	1
84	12	8	1.5
85	5	19	0.3
86	6	13	0.5
87	4	16	0.3
88	6	42	0.1
89	21	58	0.4
90	13	25	0.5
91	7	6	1.2
92	7	23	0.3
93	19	40	0.5
94	12	20	0.6
95	9	15	0.6
96	9	6	1.5
97	6	6	1
98	12	12	1
99	4	8	0.5
100	7	5	1.4
101	3	4	0.8
102	10	11	0.9
103	4	9	0.4
104	5	8	0.6
105	9	16	0.6
106	11	16	0.7
		average	**1**

Supplementary data 2: Cell count in coculture of *A. rhamnosivorans* and *M. smithii* in glucose. The counting was done in 48 fields. The ratio between *A. rhamnosivorans* and *M. smithii* is calculated in each field. The bold value is mean of all counted fields.

microscope field	number of *A. rhamnosivorans* cells	number of *M. smithii* cells	Ratio of *A.rhamnosivorans/M.smithii* per field
1	16	7	2.3
2	20	5	4
3	12	7	1.7
4	13	4	3.3
5	9	2	4.5
6	10	7	1.4
7	10	4	2.5
8	9	4	2.3
9	8	3	2.7
10	10	4	2.5
11	12	15	0.8
12	8	4	2
13	18	8	2.3
14	17	12	1.4
15	14	7	2
16	9	7	1.3
17	3	7	0.4
18	12	6	2
19	10	9	1.1
20	14	10	1.4
21	10	1	10
22	17	8	2.1
23	7	3	2.3
24	11	2	5.5

microscope field	number of *A. rhamnosivorans* cells	number of *M. smithii* cells	Ratio of *A.rhamnosivorans/M.smithii* per field
25	11	4	2.8
26	10	5	2
27	11	4	2.8
28	11	2	5.5
29	7	4	1.8
30	4	4	1
31	16	3	5.3
32	11	6	1.8
33	17	3	5.7
34	10	5	2
35	5	2	2.5
36	9	5	1.8
37	31	14	2.2
38	21	22	1
39	16	4	4
40	2	5	0.4
41	8	4	2
42	10	6	1.7
43	18	8	2.3
44	20	5	4
45	11	7	1.6
46	12	4	3
47	9	2	4.5
48	12	9	1.3
		Average	**2.6**

Supplementary data 3-A: Cell count in coculture of *Bacteroides thetaiotaomicron* and *A. rhamnosivorans* in SSPS. The counting was done in 84 fields. The ratio between *Bacteroides thetaiotaomicron* and *A. rhamnosivorans* is calculated in each field. The bold value is mean of all counted fields.

microscope field	number of *B. thetaiotaomicrons* cells	number of *A. rhamnosivorans* cells	Ratio of *B. thetaiotaomicron /A.rhamnosivorans* per field
1	117	7	16.7
2	94	4	23.5
3	75	7	10.7
4	112	6	18.7
5	65	7	9.3
6	91	5	18.2
7	102	4	25.5
8	67	8	8.4
9	94	6	15.7
10	41	9	4.6
11	65	6	10.8
12	49	12	4.1
13	62	10	6.2
14	54	8	6.8
15	63	7	9
16	64	4	16
17	92	5	18.4
18	104	6	17.3
19	71	9	7.9
20	40	5	8
21	50	9	5.6
22	55	6	9.2
23	35	6	5.8
24	35	5	7
25	37	7	5.3
26	45	4	11.3
27	47	4	11.8
28	47	5	9.4
29	42	7	6
30	43	7	6.1
31	24	3	8
32	38	1	38
33	26	7	3.7
34	51	2	25.5
35	62	9	6.9
36	33	4	8.3
37	67	5	13.4
38	22	2	11
39	182	8	22.8
40	175	10	17.5
41	112	7	16
42	45	7	6.4
43	42	4	10.5
44	118	10	11.8
45	11	3	3.7
46	15	4	3.8
47	27	5	5.4
48	8	2	4
49	49	5	9.8
50	31	6	5.2
51	13	3	4.3
52	39	3	13
53	9	3	3
54	17	1	17
55	11	2	5.5
56	19	4	4.8
57	11	3	3.7
58	17	1	17
59	17	2	8.5
60	22	3	7.3
61	13	7	1.9
62	11	4	2.8
63	6	5	1.2
64	6	3	2
65	13	5	2.6
66	36	1	36
67	19	2	9.5
68	8	1	8
69	30	7	4.3
70	14	4	3.5
71	19	4	4.8
72	31	6	5.2
73	20	3	6.7
74	7	4	1.8
75	24	1	24
76	12	3	4
77	11	6	1.8
78	18	4	4.5
79	13	4	3.3
80	23	2	11.5
81	19	7	2.7
82	14	8	1.8
83	21	6	3.5
84	35	6	5.8
		Average	**9.5**

Supplementary data 3-B: Cell count in coculture of *Bacteroides thetaiotaomicron* and *A. rhamnosivorans* in SBP. The counting was done in 74 fields. The ratio between *Bacteroides thetaiotaomicron* and *A. rhamnosivorans* is calculated in each field. The bold value

field	*thetaiotaomicrons* cells	*rhamnosivorans* cells	/*A.rhamnosivorans* per field
1	82	12	6.8
2	90	20	4.5
3	75	17	4.4
4	97	9	10.8
5	133	25	5.3
6	163	12	13.6
7	124	12	10.3
8	150	23	6.5
9	120	5	24
10	110	18	6.1
11	114	15	7.6
12	140	14	10
13	300	22	13.6
14	215	22	9.8
15	250	19	13.2
16	150	16	9.4
17	226	7	32.3
18	282	4	70.5
19	312	9	34.7
20	356	15	23.7
21	397	23	17.3
22	152	21	7.2
23	178	35	5.1
24	413	61	6.8
25	227	25	9.1
26	205	28	7.3
27	267	22	12.1
28	178	9	19.8
29	324	24	13.5
30	227	14	16.2
31	128	10	12.8
32	125	9	13.9
33	112	8	14
34	212	23	9.2
35	322	11	29.3
36	75	10	7.5
37	35	5	7
38	64	4	16
39	40	8	5
40	21	4	5.3
41	27	3	9
42	7	2	3.5
43	46	3	15.3
44	27	6	4.5
45	13	2	6.5
46	40	4	10
47	27	5	5.4
48	32	3	10.7
49	12	2	6
50	18	5	3.6
51	12	3	4
52	19	2	9.5
53	36	6	6
54	23	4	5.8
55	10	2	5
57	22	3	7.3
58	11	5	2.2
59	32	1	32
60	18	2	9
61	8	1	8
62	28	6	4.7
63	13	4	3.3
64	13	4	3.3
65	14	5	2.8
66	30	5	6
67	22	2	11
68	24	2	12
69	7	9	0.8
70	11	7	1.6
71	12	1	12
72	22	2	11
73	14	5	2.8
74	12	5	2.4
		Average	**10.8**

CHAPTER 4

INTESTINIMONAS BUTYRICIPRODUCENS GEN. NOV., SP. NOV., A NOVEL BUTYRATE-PRODUCING BACTERIUM FROM THE MOUSE INTESTINE

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Abstract

Whilst creating a bacterial collection of strains from the mouse intestine, we isolated a Gram-negative, spore-forming, non-motile and strictly anaerobic rod-shaped bacterium from the caecal content of a $TNF^{deltaARE}$ mouse. The isolate, referred to as strain SRB-521-5-I^T, was originally cultured on a reduced agar medium containing yeast extract, rumen fluid and lactic acid as energy and carbon sources. Phylogenetic analysis of partial 16S rRNA genes revealed that the species most closely related to strain SRB-521-5-I^T (GenBank accession no. KC311367) were *Flavonifractor plautii* and *Pseudoflavonifractor capillosus* (< 95 % sequence identity; 1436 bp). In contrast to *F. plautii* and *P. capillosus*, strain SRB-521-5-I^T contained a substantial amount of $C_{18:0}$ dimethylacetal. Additional major fatty acids were $C_{14:0}$ methyl ester, $C_{16:0}$ dimethylacetal and $C_{18:0}$ aldehyde. Strain SRB-521-5-I^T differed in its enzymatic profile from *F. plautii* and *P. capillosus* by being positive for dextrin, maltotriose, turanose, D,L-lactic acid and D-lactic acid methyl ester but negative for D-fructose. In reduced Wilkins-Chalgren-Anaerobe broth, strain SRB-521-5-I^T produced approximately 8 mM butyrate and 4 mM acetate. In contrast to *F. plautii*, the strain did not metabolize flavonoids. It shows intermediate resistance towards the antibiotics ciprofloxacin, colistin and tetracycline. Based on genotypic and phenotypic characteristics, we propose to create the name *Intestinimonas butyriciproducens* gen. nov., sp. nov. to accommodate strain SRB-521-5-I^T (= DSM 26588^T = CCUG 63529^T).

Introduction

The mammalian gut harbours a remarkable density and variety of bacterial species (Qin et al., 2010). These bacteria contribute to functions of importance for the mammalian host, e.g., the maturation of immune responses (Hörmannsperger et al., 2012), the regulation of energy homeostasis (Bäckhed et al., 2004), and the conversion of host-derived substrates such as mucin, bile acids and steroids (Bokkenheuser et al., 1977; Derrien et al., 2004; Ridlon et al., 2006) as well as dietary compounds such as indigestible carbohydrates and polyphenols (Clavel et al., 2006a; Roberfroid, 2007). Among important food components converted by gut bacteria, polyphenols have drawn much attention because of their possible beneficial health effects (Manach et al., 2005). To date, several gut bacteria able to convert polyphenols such as lignans (e.g., secoisolariciresinol), isoflavones (e.g., daidzein), flavonols (e.g., quercetin) and prenylflavonoids (e.g., xanthohumol) have been studied (Schneider, 1999; Clavel et al., 2006b; Clavel et al., 2009; Hanske et al., 2010). One of these polyphenol-converting bacteria, *Flavonifractor plautii*, is well known for its ability to metabolize the flavonoids quercetin, apigenin and luteolin (Schoefer et al., 2003). *F. plautii* is a butyrate-producing, strictly anaerobic bacterium originally described as [*Clostridium orbiscindens*] (Winter, 1991). In 2010, the species was reclassified as *F. plautii* after Carlier et al. isolated several strains from clinical samples (blood, pus and infected tissue samples) and demonstrated that [*C. orbiscindens*] and [*Eubacterium plautii*] were very much similar based on genotypic and phenotypic characteristics (Carlier et al., 2010). The only differences observed by Carlier et al. were the inability of [*E. plautii*] DSM 4000^T to produce spores and the motility of [*C. orbiscindens*] DSM 6740^T. The same authors also proposed the name *Pseudoflavonifractor capillosus* to accommodate the misclassified *Bacteroides* species [*Bacteroides capillosus*], a non-motile, Gram-negative, non-spore-forming bacillus originally isolated from faeces of human infants and referred to as [*Bacillus capillosus*] (Tissier, 1908). The present work deals with the description of a novel mouse intestinal bacterium related to *F. plautii* and *P. capillosus*.

Materials and methods

A caecal sample was collected from one 17-week-old female heterozygous TNF^{deltaARE} C57BL/6 mouse (Kontoyiannis et al., 1999) fed a standard experimental diet (ssniff GmbH, cat. no. S5745-E7020). Animal use was approved by the local institution in charge (animal welfare authorization 32-568, Freising District Office). The mouse dissection, bacterial isolation and cultivation procedures were as described previously (Clavel et al., 2010; Pfeiffer et al., 2012). Strictly anaerobic techniques were used (unless otherwise stated, the gas atmosphere was N_2/H_2, 90:10 for isolation in a Whitley H85 workstation and 100 % N_2 for sub-culturing in Hungate tubes). The selective agar medium used for isolation, based on the formulation by Postgate for the isolation of sulphate-reducing bacteria (Postgate, 1963), contained (per litre): 11.3 g M9 minimal salts (Sigma-Aldrich M6030), 1.5 g Na_2SO_4, 500 mg $FeSO_4$. 7 H_2O, 100 mg yeast extract, 100 mg $CaCl_2$, 100 mg $MgSO_4$. 4 H_2O, 50 mg $PdCl_2$, 2.5 mg phenosafranin and 5 ml

autoclaved rumen fluid which had been stored at 4 °C for 6 months. After autoclaving (121 °C, 15 min), the medium was allowed to cool down in a water bath (55 °C). Filter-sterilized cysteine, dithiothreitol and D,L-lactic acid sodium salt solution (Applichem A1831) were added to a final concentration of 0.05 % (w/v), 0.02 % (w/v) and 1 % (v/v), respectively. Bacteria from 10-fold serial dilutions of the caecal content were allowed to grow for 18 days. The purity of isolates was ensured by streaking twice onto selective agar plates. Purity was examined by observing cell morphology after Gram-staining and colony morphology. Wilkins-Chalgren-Anaerobe (WCA) broth (Oxoid) supplemented with 0.05 % (w/v) cysteine and 0.02 % DTT was used for sub-culturing. The genomic DNA of isolates was extracted and 16S rRNA gene sequences were analysed using primer 27F and 1492R as described previously (Pfeiffer et al., 2012). Primer 338F 5'-ACT CCT ACG GGA GGC AGC, 609F 5'-GGA TTA GAT ACC CBD GTA, 907F 5'-AAA CTY AAA KGA ATT GAC GG and 907R were also used for sequencing isolates of interest. The search-based approach DECIPHER was used to test for the presence of chimeras (Wright et al., 2012).

For transmission electron microscopy, cells of strain SRB-521-5-I^T were grown for 48 h in Reinforced Clostridial Medium (Difco). For transmission electron microscopy observation bacterial cells were fixed for 65 hours in 2.5 % glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) at 0 °C. After rinsing the cells was post fixed in 1% (w/v) OsO_4 and 1% (w/v) potassium ferricyanide for 1 hour at room temperature and brought in to 5 % (w/v) gelatine that was then solidified at 0 °C. After dehydration in a graded ethanol series the cells was infiltrated with modified Spurr resin mixtrure (Serva) and sectioned on an ultra-microtome (reichert, Ultracut S). Micrographs were taken with a JEOL JEM 1011 transmission electron microscope.

Strain SRB-521-5-I^T was analysed further using chemotaxonomic methods. Therefore, cell biomass was produced by cultivation at 37 °C in reduced WCA broth. Incubation time was 24 h (in 300 ml) for cellular fatty acid (CFA) and 72 h (in 1.5 l) for polar lipid and quinone analysis. Cells were pelleted by centrifugation (5 500 x g, 10 min), washed once in filter-sterilised PBS, stored at -80 °C prior to freeze-drying overnight and shipment at room temperature. Samples were measured by the Identification Service of the DSMZ. Details on the experimental procedures are available online (www.dsmz.de/services/services-microorganisms/identification). Genotypic analysis of strain SRB-521-5-I^T also included determination of the G + C content of DNA by HPLC at the German Collection of Microorganisms and Cell Culture (DSMZ). Classical phenotypic characterisation, e.g., growth features, motility, enzymatic tests and spore formation, was performed as described previously. The production of short chain fatty acids was determined in batch culture supernatants by means of gas chromatography (Clavel et al., 2010; Pfeiffer et al., 2012). Cells were grown in reduced WCA broth with a gas phase of N_2/CO_2 (80:20). The presence of the sporulation gene *spo0A* was tested by PCR as described previously (Brill and Wiegel, 1997) in strain SRB-521-5-I^T, *F. plautii* and *P. capillosus*.

Biolog enzymatic profiles (done in duplicate for each strain) were obtained as by the manufacturer's instructions (Biolog, CA, USA). Fresh 24-h-cultures in reduced WCA broth were used as inoculum. Cells were collected by centrifugation (5000 x g, 5 min) under anaerobic condition. After addition of Biolog-AN inoculating fluid (OD_{600nm} = ca. 0.3) and inoculation under aerobic condition, the plates were incubated at 37 °C for 24 h in a jar containing an Anaerogen sachet (Oxoid). Reactions were read visually and by measuring the OD at 590 nm. Substrates in wells characterised by a visible violet stain and an OD_{590nm} >0.05 after background subtraction (negative water control) were considered positive.

The susceptibility of the isolate towards eleven antimicrobial substances was tested using Etest strips (Bio-Stat Diagnostics, Stockport, UK), according to CLSI standard M11-A8. Briefly, a sterile swab was dipped into the inoculum suspension (fresh culture in reduced WCA broth) and the excess liquid was removed by turning the swab against the inside of the tube. The surface of reduced WCA agar plates was spread evenly with the swab. Plates were allowed to air-dry for 10-15 min and, using sterile forceps, Etest strips were applied to the inoculated agar surface. Two Etest strips were used per plate (with antimicrobial gradients in opposing directions) and bacteria were left to grow. The MIC values were read directly from the strips after 48 h incubation in a Don Whitley miniMACS anaerobic workstation ($N_2/H_2/CO_2$; 80:10:10). The range of concentrations tested was 0.016-256 µg/ml for all antibiotics but ciprofloxacin (0.002-32 µg/ml). Three independent repeats of duplicate experiments were carried out for each antibiotic.

Results and Discussion

Analysis using the BLAST program (Altschul et al., 1990) revealed that one of the isolated strains (SRB-521-5-I^T) was only distantly related to any known species with a standing name in nomenclature. Unambiguous alignment of partial 16S rRNA gene sequences (1475 bp) showed that the sequence of strain SRB-521-5-I^T (Genbank accession number KC311367) was: (i) 100 % similar to cloned sequences from the pig (AF371948) (Leser et al., 2002) and human intestine (HQ785725, HQ783112, HQ770450, FJ366757) (Turnbaugh et al., 2009; Li et al., 2012a); (ii) 100 % similar to two hitherto unclassified cultured strains isolated from the human gut (JX101685 and JX273469) as well as one mucin-degrading and butyrate-producing bacterium from the swine intestine (JX629258) (Levine et al., 2013); and (iii) ≤94.5 % similar to *Pseudoflavonifractor capillosus* (AY136666) and *Flavonifractor plautii* (AY724678). A phylogenetic tree based on the Neighbour-Joining method showed that strain SRB-521-5-I^T and the three yet unclassified strains mentioned above formed one cluster separated from *Pseudoflavonifractor* and *Flavonifractor* (Fig. 1). This grouping was confirmed using the Maximum-Likelihood method (Suppl. Fig. S1). A total of 261 full-length 16S rRNA gene sequences in the GenBank database matched the sequence of strain SRB-521-5-I^T with a similarity > 95 %. A majority of these sequences originated from intestinal samples in various species (human, mouse, chicken, zebra, cow, etc.), suggesting that this bacterial

group within *Clostridium* cluster IV has been repeatedly found in gut ecosystems. However, local BLAST analysis against 85 949 chimera checked and quality-filtered partial 16S rRNA gene sequences (V1–V3 region) from the caecum of conventional mice (Werner et al., 2011) revealed that only eight sequences matched that of strain SRB-521-5-I^T at a similarity > 95 %. The isolate had a G + C content of DNA of 58.4 mol%, which is in the range of those of most closely related species (*F. plautii*, 58-61.6 mol %; *P. capillosus*, 60 mol %).

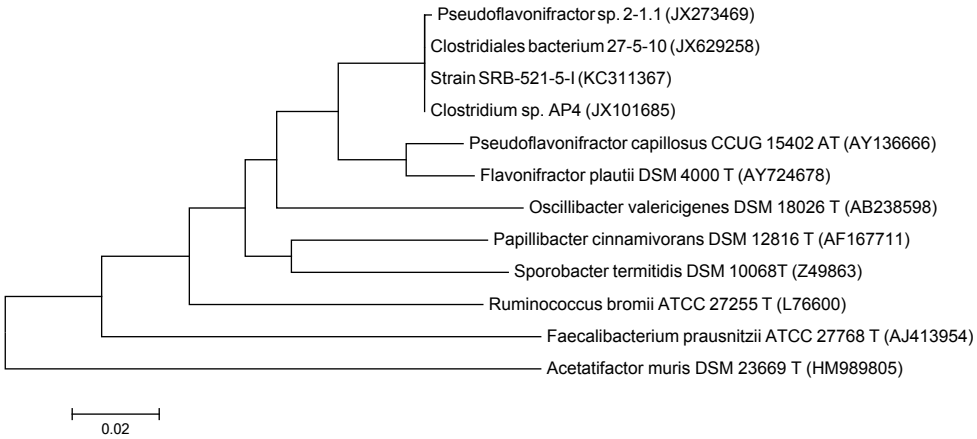


Figure 1. Phylogenetic tree of strain SRB-521-5-I^T and related species based on partial 16S rRNA gene sequencing. The accession numbers of the 16S rRNA gene sequences (1 436 bp) used to construct the tree are indicated in brackets. Sequences were aligned using Greengenes and the tree was constructed using the Neighbour-Joining method (Saitou and Nei, 1987) in MEGA5 (Tamura et al., 2011). The bar below the tree represents 2 nucleotide changes per 100 nucleotides.

The cellular fatty acid pattern of strain SRB-521-5-I^T is shown in Table 1. As in *F. plautii* and *P. capillosus*, major fatty acids were saturated (> 90 % of total fatty acids) and included $C_{14:0}$ methyl ester, $C_{14:0}$ dimethyl acetal and $C_{16:0}$ dimethyl acetal. However, the isolate showed a markedly lower proportion of $C_{16:0}$ aldehyde, $C_{16:0}$ methyl ester and $C_{16:0}$ dimethyl acetal, as well as a higher proportion of $C_{12:0}$ methyl ester, $C_{18:0}$ aldehyde and $C_{18:0}$ dimethyl acetal compared with the two other species (Table 1). Concerning polar lipids, strain SRB-521-5-I^T was characterised by the presence of diphosphatidylglycerol, eight glycolipids, six phosphoglycolipids, six phospholipids and three unidentified lipids (Suppl. Fig. S2). No respiratory quinones were detected in cells of strain SRB-521-5-I^T.

Table 1. Cellular fatty acid pattern of strain SRB-521-5-I^T and related species

Species: 1, *Intestinimonas butyriciproducens* (SRB-521-5-I^T); 2, *Flavonifractor plautii* (DSM 6740^T); 3, *Pseudoflavonifractor capillosus* (CCUG 15402A^T). Data were obtained in the present study. The three strains were grown and analysed under the very same conditions. Details for cell biomass production are given in the text. CFA were analysed at the DSMZ using the BHIBLA method (www.dsmz.de/services/services-microorganisms/identification). Values are percentages of total fatty acids.

Fatty acid	1	2	3
10:0 FAME	0.2	n.d	0.2
i-12:0 FAME	n.d	n.d	0.2
12:0 FAME	**4.8**	**0.2**	**2.8**
11:0 DMA	1.9	0.2	0.2
ai-13:0 FAME	n.d	n.d	0.1
13:0 FAME	0.2	n.d	0.3
i-14:0 FAME	0.2	0.1	2.5
14:1 CIS 9 FAME	0.9	0.2	n.d
14:0 FAME	33.1	28.3	33.3
14:0 DMA	**4.0**	**9.1**	**3.0**
i-15:0 FAME	0.2	0.8	n.d
ai-15:0 FAME	0.1	0.1	0.1
16:0 ALDE	**1.7**	**5.2**	**7.4**
15:0 FAME	n.d	0.5	0.4
i-15:0 DMA	n.d	n.d	0.1
i-16:0 FAME	n.d	n.d	0.3
16:1 CIS 9 FAME	0.9	0.2	0.2
16:1 CIS 11 FAME	n.d	0.2	n.d
16:0 FAME	**1.9**	**12.2**	**7.4**
16:1 CIS 9 DMA	0.2	n.d	n.d
16:0 DMA	**9.1**	**27.9**	**38.2**
18:0 ALDE	**7.8**	**2.7**	**0.3**
17:0 DMA	0.2	n.d	0.1
18:2 CIS 9,12 FAME	0.3	n.d	n.d
18:1 CIS 9 FAME	2.8	0.9	0.5
18:0 FAME	1.8	0.9	0.3
18:1 CIS 9 DMA	1.5	0.4	0.2
18:1 CIS 11 DMA	0.4	n.d	n.d
18:0 DMA	**23.0**	**8.1**	**0.6**
19:0 FAME	1.4	0.7	n.d
20:0 FAME	0.3	n.d	n.d
unidentified	1.4	2.2	1.4

Bold characters indicate CFA, for which substantial quantitative differences between strain SRB-521-5-I^T and the two related species were observed. Abbreviations: ai, anteiso; ALDE, aldehyde; DMA, dimethylacetal; FAME, fatty acid methyl ester; i, iso; n.d, not detected

Based on microscopic observation after Gram-staining, the isolate stained Gram-negative. The KOH test was positive (formation of mucoid string), which confirmed that cells of strain SRB-521-5-I^T are Gram-negative. A Gram-negative type cell wall structure was demonstrated as well by the presence of a periplasmic space in electron micrographs (Fig. 2).

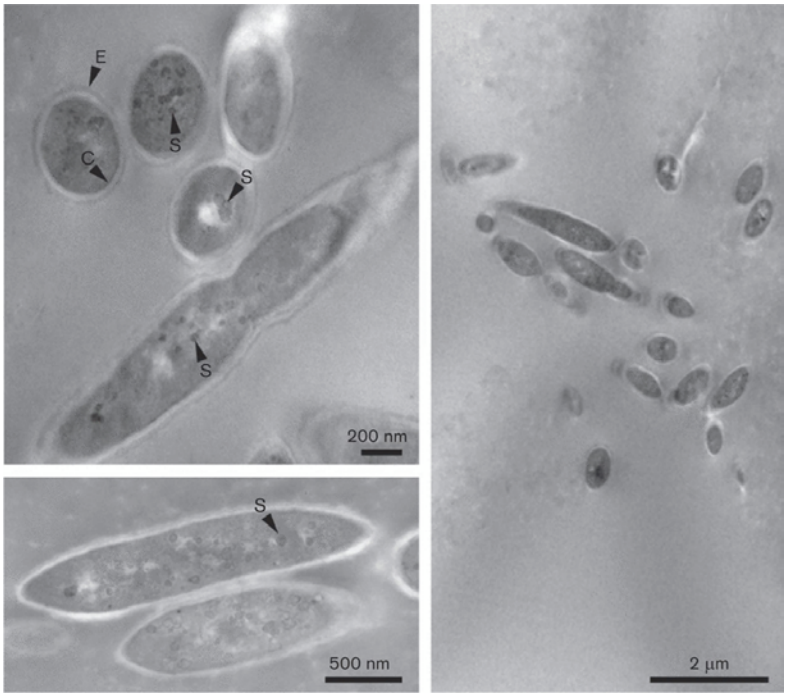


Figure 2. Cell morphology of strain SRB-521-5-I^T. Cells were analysed using transmission electron microscopy as explained in the text. Arrowheads indicate the presence of exocellular polymeric substances (E), a cytoplasmic membrane (C) and storage granules (S).

Small terminal endospores were detected by microscopic observation after 7 d of growth (Fig. S3). In contrast to *P. capillosus*, strain SRB-521-5-I^T and *F. plautii* were able to grow in reduced WCA broth after heat treatment (60°C, 20 min and 80 °C, 10 min under aerobic condition; tested in three independent experiments). In addition, the *spo0A* gene was amplified from genomic DNA of *F. plautii* and *P. capillosus* but that of strain SRB-521-5-I^T (Suppl. Fig. S4). After 48 h, strain SRB-521-5-I^T produced 3.6 mM acetate and 8.1 mM butyrate (n = 2 experiments) when grown in WCA medium. Minor amounts of lactate, isovalerate and valerate were also detected (< 0.5 mM). In contrast, *P. capillosus* produced acetate (3.0 mM) but no butyrate (< 0.1 mM). *F. plautii* produced 3.6 mM acetate and 7.1 mM butyrate. The results of enzymatic profiles for strain SRB-521-5-I^T are given in the species description. Comparative enzymatic patterns are shown in Suppl. Fig. S5. Discriminative features are listed in Table 2.

Table 2. Traits of strain SRB-521-5-I^T and most closely related species. Species: 1, *Intestinimonas butyriciproducens* (SRB-521-5-I^T); 2, *Flavonifractor plautii* (DSM 6740^T); 3, *Pseudoflavonifractor capillosus* (CCUG 15402A^T).

Features	1	2	3
Cell wall type	Gram-negative	v	Gram-negative
Motility	-	v	-
Spore formation*	+	v	-
spo0A (sporulation gene)	-	+	+
Heat treatment (80 °C, 10 min)	R	R	S
Butyrate production	+	+	-
Growth at 45 °C	+	slow	+
Flavonoids			
Apigenin	-	+	n.d
Eriodictyol	-	+	n.d
Luteolin	-	+	n.d
Naringenin	-	+	n.d
Quercetin	-	+	-
Taxifolin	-	+	n.d
Enzymatic test (Biolog)			
N-acetyl-D-glucosamine	+	-	+
Dextrin	+	-	-
Dulcitol	-	+	-
D-fructose	-	+	+
L-fucose	-	-	+
D-galactose	+	-	+
D-galacturonic acid	-	-	+
Gentiobiose	-	-	+
a-D-glucose	+	+	-
Glucose-1-phosphate	-	-	+
Lactulose	-	-	+
Maltose	-	-	+
Maltotriose	+	-	-
D-mannitol	-	+	-
Palatinose	-	-	+
D-sorbitol	+	+	-
Turanose	+	-	-
b-hydroxy-butyric acid	+	+	-
a-ketobutyric acid	-	+	-
a-ketovaleric acid	-	+	-
D,L-lactic acid	+	-	-
D-lactic acid methyl ester	+	-	-
Alaninamide	-	+	-
L-alanine	-	+	-
L-asparagine	-	+	-
Glycyl-L-methionine	-	-	+
Inosine	+	+	-
Thymidine	+	+	-
Thymidine-5'-monophosphate	+	+	-
Uridine-5'-monophosphate	+	+	-
Teicoplanin (MIC µg/ml)	0.029 ± 0.007	0.25 - 0.5	0.25 - 0.5
Vancomycin (MIC µg/ml)	0.482 ± 0.059	4 - 8	4 - 8
Major CFA beside 14:0 FAME	18:0 DMA	16:0 DMA	16:0 DMA

* Strain SRB-521-5-I^T was analysed by microscopic observation after Schaeffer-Fulton staining. Abbreviations: a, alpha; b, beta; CFA, cellular fatty acid; n.d, not determined; R, resistant; S, sensitive; v, variable.

The results of minimum inhibitory concentration (MIC) breakpoints (µg/ml) were expressed as means ± SEM (n = 6): cefotaxime, 0.029 ± 0.009; ciprofloxacin, 22.667 ± 4.341; clindamycin, 0.022 ± 0.005; colistin, 30.667 ± 4.341; erythromycin, 0.099 ± 0.015; metronidazole, <0.016; oxacillin, 0.750 ± 0.091; teicoplanin, 0.029 ± 0.007; tetracycline, 6.167 ± 0.910; tobramycin, 1.417 ± 0.369; vancomycin, 0.482 ± 0.059. Thus, the cationic cyclic polypeptide colistin, which interacts with the cytoplasmic membrane of Gram-negative bacteria, failed to inhibit the growth of strain SRB-521-5-I^T at concentrations <30 µg/ml. Ciprofloxacin, a broad-spectrum quinolone antibiotic that inhibits bacterial cell division, also showed a relatively high MIC value. The broad-spectrum antibiotic tetracycline, which inhibits protein synthesis by binding to the 30S subunit of ribosomes, showed an intermediate resistance profile according to the CLSI standard M100-S22 (<4 µg/ml, sensitive; 8 µg/ml, intermediate). Similarly to its closest phylogenetically related species (Carlier et al., 2010) , strain SRB-521-5-I^T was susceptible to the glycopeptide teicoplanin. However, the isolate seems to be more sensitive to the glycopeptide vancomycin than *F. plautii* and *P. capillosus* (0.5 vs. 4-8 µg/ml). Although vancomycin has usually no effect on Gram-negative bacteria due to low permeability of the outer membrane to such a large hydrophobic molecule (ca. 1.45 kDa), mutation-induced alteration of outer membrane composition and ensuing susceptibility to vancomycin has been reported in *Escherichia coli* (Shlaes et al., 1989). More recently, encapsulation of vancomycin in liposomes allowed passage through the outer membrane in a range of Gram-negative bacteria (Nicolosi et al., 2010) whilst combination of vancomycin and colistin had synergistic antimicrobial activity in multi-drug-resistant strains of *Acinetobacter baumannii* (Gordon et al., 2010). These findings suggest that vancomycin can be active against Gram-negative bacteria if allowed to penetrate the outer membrane. Additional analysis is required to elucidate the exact cell wall structure of strain SRB-521-5-I^T.

Finally, because *F. plautii* is known as a flavonoid-converting bacterium (Schoefer et al., 2003; Carlier et al., 2010), we tested the ability of strain SRB-521-5-I^T to metabolize 16 flavonoid compounds (apigenin, apigenin-7-glucoside, daidzein, eriodictyol, genistein, luteolin, luteolin-3-, 5- and 7-glucoside, naringenin, naringenin-7-neohesperiosid, phloretin, phloridzin, quercetin, rutin and taxifolin) by means of batch culture fermentation followed by liquid chromatography analysis, as described previously (Schoefer et al., 2003) . In contrast to *F. plautii*, the isolate was not able to convert any of the flavonoids tested. Template chromatograms showing the conversion of eriodictyol and luteolin by *F. plautii* compared with strain SRB-521-5-I^T are provided in Suppl. Fig. S6.

Based on the aforementioned genotypic and phenotypic traits, we propose that strain SRB-521-5-I^T represents a novel bacterial genus, for which the name *Intestinimonas butyriciproducens* is proposed. Features that help distinguishing strain SRB-521-5-I^T from related species are summarized in Table 2.

Description of *Intestinimonas* gen. nov.

Intestinimonas (In.tes.ti.ni.mo'nas. L. n. *intestinum*, gut, intestine; L. fem. n. *monas*, a monad, unit; N.L. fem. n. *Intestinimonas*, a unit (bacterium) isolated from the intestine)

Bacteria of the genus *Intestinimonas* are phylogenetically related to members of the genera *Flavonifractor* and *Pseudoflavonifractor* within the order Clostridiales, phylum Firmicutes. They are Gram-negative, strictly anaerobic, grow well in a temperature range of 25 to 45 °C and form terminal spores. Motility has not been observed. Major fermentation products in WCA broth are butyrate and acetate. Cellular fatty acids are mostly saturated (91.9 %), including $C_{14:0}$ methyl ester (33.1 %) and $C_{18:0}$ dimethyl acetal (23.0 %) as the two major fatty acids. Respiratory quinones have not been detected. The type species is *Intestinimonas butyriciproducens*.

Description of *Intestinimonas butyriciproducens* gen. nov., sp. nov.

Intestinimonas butyriciproducens (bu.ty.ri.ci.pro.du'cens. N.L. n. *acidum butyricum*, butyric acid; L. part. adj. *producens*, producing; N.L. part. adj. *butyriciproducens*, producing butyric acid).

The species has the aforementioned features of the genus. After 48 h of growth on reduced WCA agar, colonies are pinpoint to 1 mm in diameter, circular, entire, opaque, white, shiny and convex. Cells are straight fusiform rods (0.5 x 2-5 µm) that may be elongated and curved after 7 d of growth at 45 °C. They occur mostly single or sometimes in pairs. Positive for N-acetyl-D-glucosamine, dextrin, D-galactose, a-D-Glucose, glucose-6-phosphate, maltotriose, 3-methyl-D-glucose, D-sorbitol, turanose, b-hydroxy-butyric acid, D,L-lactic acid, D-lactic acid methyl ester, pyruvic acid, pyruvic acid methyl ester, L-serine, L-threonine, 2'-deoxy-adenosine, inosine, thymidine, uridine, thymidine-5'-mono-phosphate and uridine-5'-mono-phosphate. Negative for all other substrates in the Biolog AN plate. Does not metabolise flavonoids. Characterised by the presence of diphosphatidylglycerol, eight glycolipids, six phospho-glycolipids, six phospholipids and three unidentified lipids. The G + C content of DNA is 58.4 mol%. The type strain, SRB-521-5-I^T (=DSM 26588^T = CCUG 63529^T), was isolated from the caecal content of a 17-week-old female heterozygous TNF^{deltaARE} C57BL/6 mouse. It shows intermediate resistance towards the antibiotics ciprofloxacin, colistin and tetracycline.

Acknowledgements

The heterozygous TNF ^{deltaARE} mouse model of ileitis was a generous gift from Dr George Kollias (Biomedical Sciences Research Centre Alexander Fleming, Vari, Greece). We are grateful to: Professor J.P. Euzeby (Ecole Nationale Vétérinaire, Toulouse, France) for his help with etymology; Sabine Schmidt and Bärbel Gruhl (DIfE), Caroline Ziegler and Simone Daxauer (TUM) for technical assistance; co-workers of the DSMZ for contributing to the phenotypic characterization of strain SRB-521-5-I ^{T}; and Tiny Franssen-Verheijen (Laboratory of Virology, Wageningen University, The Netherlands) for transmission electron microscopy. This research has been financially supported by ERC grant 250172 – Microbes Inside. We thank Willem M. de Vos for valuable advice.

Supplementary information

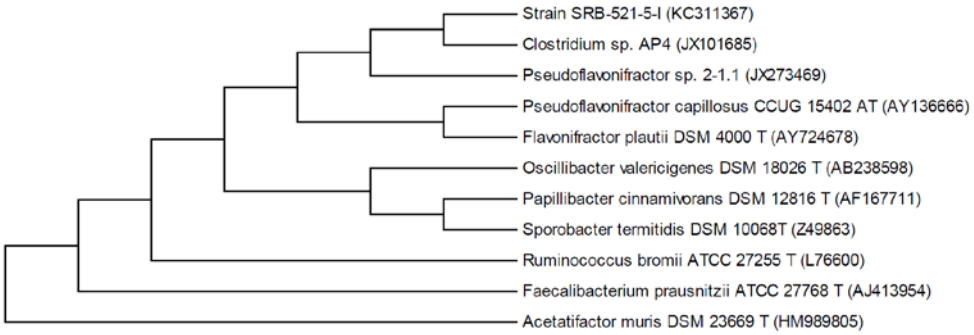


Figure S1: Phylogenetic tree of strain SRB-521-5-I^T and related species based on partial 16S rRNA gene sequencing. The accession numbers of the 16S rRNA gene sequences (1 436 bp) used to construct the tree are indicated in brackets. Sequences were aligned using Greengenes and the tree was constructed using the Maximum Parsimony method in MEGA5 (Tamura et al., 2011). Tree #1 out of 2 most parsimonious trees (length = 761) is shown. The consistency index is 0.725361 (0.607143), the retention index is 0.527149 (0.527149), and the composite index is 0.382374 (0.320055) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Subtree-Pruning- Regrafting (SPR) algorithm (Nei and Kumar, 2000) with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1356 positions in the final dataset.

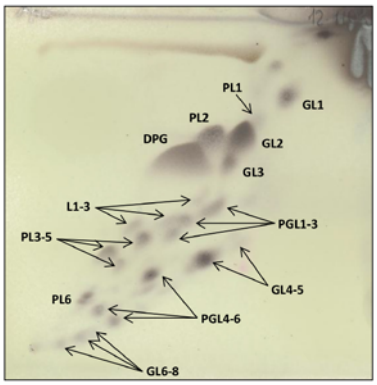


Figure S2: Polar lipid patterns of strain SRB-521-5-I^T. The analysis was carried out by the Identification Service of the DSMZ (Braunschweig, Germany) and Dr. B.J. Tindall. Abbreviations: DPG, diphosphatidylglycerol; GL, glycolipids; L, unidentified lipid; PGL, phosphoglycolipid; PL, phospholipids.

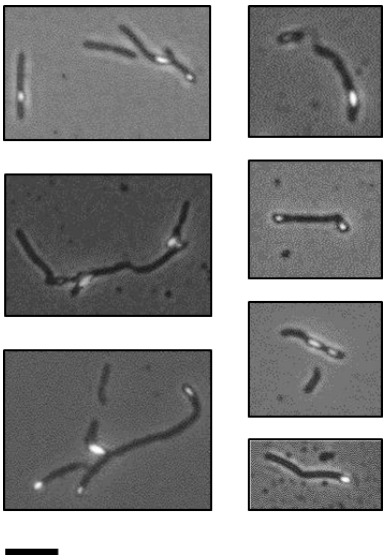


Figure S3. Microscopic pictures (100X) of spores. Strain SRB-521-5-I^T was grown at 45 °C for 7 d in reduced WCA broth. Cells were stained according to the Schaeffer-Fulton method. The bar represents 5 µm.

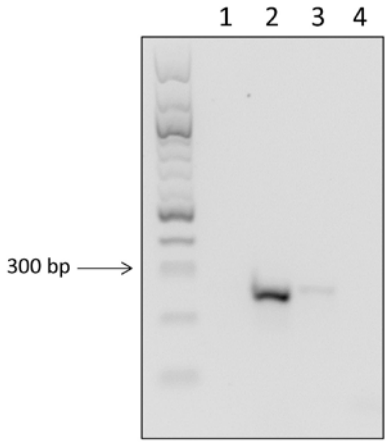


Figure S4. Occurrence of the sporulation gene *spo0A*. The procedure for gene amplification was as described by Brill & Wiegel (1997). 1, *Intestinimonas butyriciproducens* SRB-521-5-I^T; 2, *Pseudoflavonifractor capillosus* CCUG 15402A^T; 3, *Flavonifractor plautii* DSM 6740^T; 4, negative water control

Figure S5. Biolog enzymatic profile of strain SRB-521-5-I^T and related species. 1, *Intestinimonas butyriciproducens* SRB-521-5-I^T; 2, *Flavonifractor plautii* DSM 6740^T; 3, *Pseudoflavonifractor capillosus* CCUG 15402A^T. Enzymatic profiles were obtained as by the manufacturer's instructions (Biolog, CA, USA) (see body text). Positive wells, i.e., characterized by a visible violet stain and an OD_{590nm} > 0.05 after background substraction (negative water control), are marked with „+". Red dots indicate reactions that differ between the isolate and each reference species.

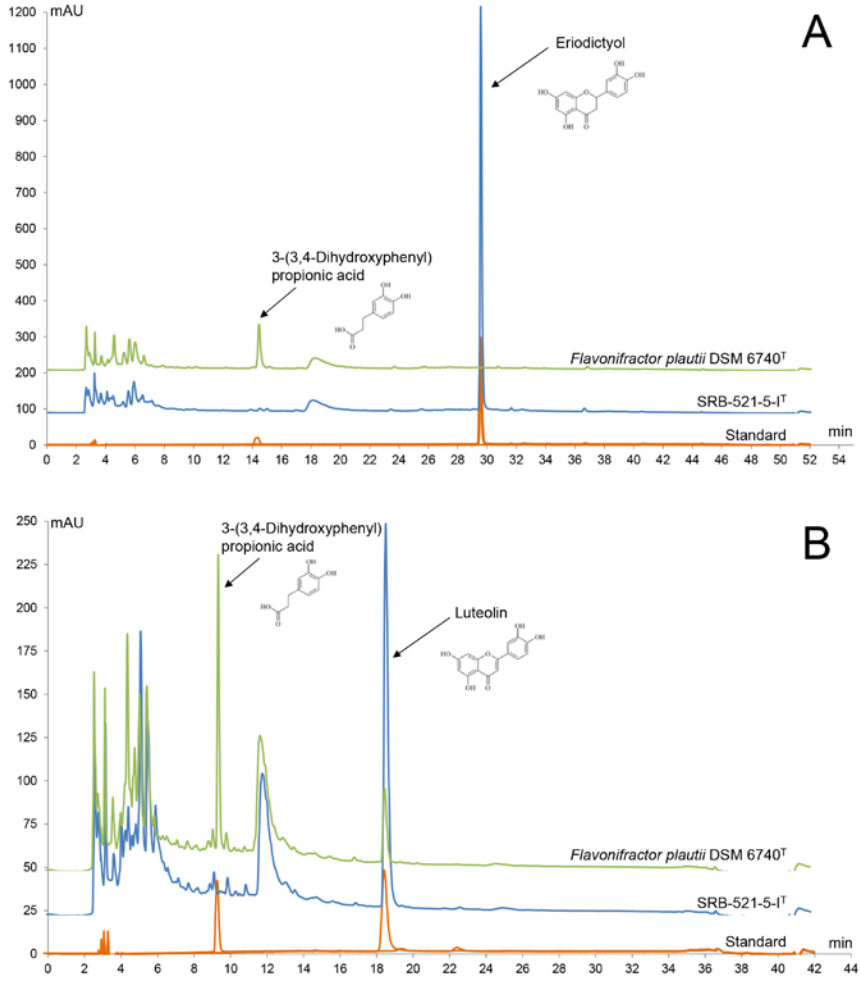


Figure S6. Rp-HPLC-UV elution profiles of growing cultures of strain SRB-521-5-I^T and *Flavonifractor plautii* DSM 6740^T. Two strains were incubated in Wilkins-Chalgren-Anaerobe broth (with 0.05 % (w/v) cysteine) under anoxic conditions (N_2/CO_2, 80:20) for 48 h with 0.5 mM eriodictyol (A) or luetolin (B). *F. plautii* converted eriodictyol and luetolin to 3-(3,4-dihydroxyphenyl) propionic acid. Fermentation of flavonoids and analysis by liquid chromatography were carried out as described previously (Schoefer et al. 2003). mAU, milli-absorbance units.

CHAPTER 5

PRODUCTION OF BUTYRATE FROM LYSINE AND THE AMADORI PRODUCT FRUCTOSELYSINE BY A HUMAN GUT COMMENSAL

This chapter has been published as:

Bui, T.P.N., Ritari, J., Boeren, S., de Waard, P., Plugge, C.M., and de Vos, W.M. (2015) Production of butyrate from lysine and the Amadori product fructoselysine by a human gut commensal. *Nature Communications* 6.

Abstract

Human intestinal bacteria produce butyrate, which has signalling properties and can be used as energy source by enterocytes thus influencing colonic health. However, the pathways and the identity of bacteria involved in this process remain unclear. Here we describe the isolation from the human intestine of *Intestinimonas* strain AF211, a bacterium that can convert lysine stoichiometrically into butyrate and acetate when grown in a synthetic medium. *Intestinimonas* AF211 also converts the Amadori product fructoselysine, which is abundantly formed in heated foods via the Maillard reaction, into butyrate. The butyrogenic pathway includes a specific CoA transferase that is overproduced during growth on lysine. Bacteria related to *Intestinimonas* AF211 as well as the genetic coding capacity for fructoselysine conversion are abundantly present in colonic samples from some healthy human subjects. Our results indicate that protein can serve as a source of butyrate in the human colon, and its conversion by *Intestinimonas* AF211 and related butyrogens may protect the host from the undesired side effects of Amadori reaction products.

Introduction

Our intestinal tract contains trillions of microbes that impact our health (Sommer and Backhed, 2013). The metabolic capacity of these microbes is enormous (Qin et al., 2010) and includes the production of short chain fatty acids (SCFAs), such as butyrate that serves as a major source of energy for the intestinal enterocytes and signals to the host (Hamer et al., 2008). Butyrogenic gut bacteria belong to the Lachnospiraceae and Ruminococcaceae (Rajilić-Stojanović and de Vos, 2014) and recent studies showed an inverse correlation between these butyrogenic Firmicutes and inflammatory bowel disease, type 2 diabetes and various other disorders (Harry J. Flint, 2012; Qin et al., 2012). Moreover, there is considerable interest in these butyrogens, since butyrate reduces proinflammatory signals (Hamer et al., 2008) and has a protective role in maintaining a healthy colon (Scheppach et al., 2001; Duncan et al., 2002a). Thus, it is of paramount importance to identify the microbes that are capable of producing butyrate and understand the mechanisms underlying its production.

Most butyrate produced in the human intestine is assumed to derive from carbohydrates, while some specialized bacteria have been found that convert lactate plus acetate into butyrate (Barcenilla et al., 2000; Belenguer et al., 2006). All of these produce butyrate via the acetyl-CoA pathway (Louis et al., 2014) (converting acetyl-CoA to butyrate) that involves a complex cascade of reactions, in which butyrate CoA-transferase (But) or butyrate kinase (Buk) are key enzymes. In a recent (meta)genome-based study, it was predicted that the But mediated route was ten-fold more abundant than that mediated by Buk, while these two enzymes showed high diversity among butyrogens (Louis et al., 2004). Three other routes for butyrate synthesis, the glutamate, succinate and lysine pathway, have been described. Based on the distribution of the genes in the intestinal metagenome libraries, the acetyl-CoA pathway was the most prevalent followed by the lysine pathway. Yet, no intestinal bacterium has been reported that contained all genes coding for butyrogenesis from this amino acid (Vital et al. 2014).

In addition to carbohydrates, proteins are crucial dietary compounds that are receiving increased attention to their use in special diets. Proteolytic enzymes in the upper intestinal tract generate amino acids that are rapidly taken up by the host, but also may reach the colon, partly depending on the intake. Moreover, protein recycling in the colon has been described, adding to the interest in colonic amino acid fermentations. Amadori products (fructosamines), formed via non-enzymatic reactions between reducing sugars and free amino groups of proteins during the food heating process, are of significant interest as they have shown to be associated with the aging process and several chronic diseases (Deppe et al., 2011). Fructoselysine is a key intermediate in the formation of advanced glycation endproducts (AGEs) (Deppe et al., 2011) and not yet any organism has been known to convert fructoselysine into butyrate. Only a few microbes have been reported to metabolise lysine into butyrate, and they derive from ecosystems other than the gut. A lysine degradation pathway was described

over 50 years ago in *Clostridium sticklandii* isolated from black mud (Stadtman and White, 1954). More recently, this pathway was detected in *Clostridium subterminale* SB4 isolated from sewage and *Fusobacterium nucleatum* isolated from the oral cavity, and eight genes encoding a lysine fermentation pathway were identified from the genome of *Fusobacterium nucleatum* (Kreimeyer et al., 2007). Several enzymes involved in butyrogenesis from lysine have been biochemically characterized (Chirpich et al., 1970; Baker et al., 1972; Yorifuji et al., 1977; Barker et al., 1978), but so far no intestinal microorganism has been found to contain the entire pathway in spite of the predicted presence of the lysine fermentation pathway in the human intestine (Baker et al., 1972; Yorifuji et al., 1977; Kreimeyer et al., 2007; Vital et al., 2014). Here we describe the butyrogenic conversion of lysine and the Amadori product fructoselysine by a novel *Intestinimonas* strain from a healthy volunteer and characterize its butyrate synthesis pathway by using a combined physiological, biochemical and proteogenomic approach.

Materials and Methods

Enrichment, isolation and growth

Strain *Intestinimonas* AF211 was isolated from a stool of a healthy adult. The fecal sample was enriched in an anaerobic bicarbonate buffered mineral salt medium (Stams et al., 1993) containing 40mM lactate and 40mM acetate as energy and carbon source. The headspace was filled with CO_2/N_2 (1:4) at 1.5 atm and incubation was at 37°C. Subsequently, the enrichment culture was transferred to Reinforced Clostridium Medium (RCM, Difco) in serial dilutions and plated at least 3 times on RCM agar, which resulted in an axenic culture. The purity of the strain, designated as strain AF211, was confirmed by 16S rRNA gene sequencing and microscopy. The strain was routinely maintained in RCM medium at 37°C. A phylogenetic tree of the 16S rRNA of *Intestinimonas* AF211 (GenBank accession JX273469) and closely related strains was constructed as described previously (Bui et al., 2014) (Supplementary Fig. 1).

For physiological characterization, different amino acids and amino acid derivatives were tested including 20mM of L-lysine, fructoselysine (USBiological, USA), glutamate, glutamine, glycine, proline, arginine, aspartate and methionine which were added into the bicarbonate buffered medium from 1M sterile anoxic stock solutions. For sugar utilization 20mM glucose plus 20mM acetate were added into the bicarbonate buffered medium from 1M stock solutions. The growth was determined via product formation by High Performance Liquid Chromatography (Bui et al., 2014) (HPLC) and OD measurement by a spectrophotometer at a wavelength of 600nm. Generation times were calculated employing Gompertz modelling (Zwietering et al., 1990)

Analytical methods

Lysine was quantified on a HPLC using a Polaris C18-A column (Agilent) running at 45°C and a UV-visible detector at wavelength of 436 nm. Flow rate was 0.5ml/min. A eluent mobile

phase consisted of 24 mM acetic acid: 8% acetonitrile (pH 6.6) as solvent A and acetonitrile:2-propanol (60:40) as solvent B. The gradient elution was used from 95% eluent A and 5% eluent B to 25% eluent A and 75% eluent B for first 15 min. The column was subsequently washed with 100% eluent B for 7 min before the next sample injection. Norleucine (4mM) was used as internal standard. The volatile fatty acid formation was measured on a Thermo Scientific Spectra HPLC system equipped with a Agilent Metacarb 67H 300 x 6.5 mm column kept at 45°C and running with 10mM arabinose as an eluent. The detector was a refractive index detector. The eluent flow was 0.8 ml/min. Gas production was performed as previously described (van Gelder et al., 2012). All analyses were performed in duplicate. Ammonium was determined using Spectroquant@ test kit according to manufacture instruction. Fructoselysine was separated by ion exchange chromatography and quantified by post column reaction with ninhydrin using photometric detection at 570nm (International Organization for Standardization (ISO) 13903).

Nuclear magnetic resonance

Strain AF211 was cultivated in a bicarbonate buffered medium containing 20 mM of of [2-^{13}C]L- lysine or [6-^{13}C]L- lysine or lysine plus [2-^{13}C]acetate. The growth conditions were as described above. 13C-labelled lysine was purchased from Campro Scientific (Veenendaal, The Netherlands). Samples were taken from an overnight culture and centrifuged at 10000 g. D_2O (50 µL; 99.9 atom %, Sigma Aldrich) was added to the supernatants (0.5 ml) and subsequently transferred in NMR tubes (Campro scientific). ^{1}H-decoupled ^{13}C NMR spectra were recorded at a probe temperature of 300K on a Bruker Avance-III-500 spectrometer located at the Wageningen NMR Centre (WNMRC), Wageningen, the Netherlands. Chemical shifts are expressed in ppm relative to the C-6 of added [6-^{13}C] lysine at 41.75 ppm, added [2-^{13}C] lysine at 57.19 ppm, formed [2-^{13}C] butyrate at 42.33 ppm, formed [4-^{13}C] butyrate at 15.95 ppm and formed [2-^{13}C] acetate at 25.99 ppm (Biological Magnetic Resonance Data Bank, http://www.bmrb.wisc.edu/metabolomics/metabolomics_standards). For the Heteronuclear Multiple Bond Correlation (HBMC) spectra 400 experiments of 8 scans were recorded resulting in a measuring time of 50 minutes, using a standard Bruker pulse sequence. The products were identified based on chemical shifts as compared to database as mentioned above. In the HMBC experiment no decoupling is used (Fig 3b). Therefore, the single bond couplings will result in double cross-peaks split by the large single-bond coupling. The active coupling between 5 and 10 Hz of 2- and 3-bond couplings remains within the width of a cross-peak in a HMBC spectrum, resulting in single cross-peaks. When the carbon attached to the proton involved in a three-bond coupling is also enriched, this cross-peak will be split by the large single bond coupling with this carbon. These splittings are visible as extra peaks next to the single cross-peaks between H-2 and C-4 and between H-4 and C-2 (arrows in the figures), indicating double enrichment of both C-2 and C-4. The calculation was shown in Fig 3c. Both the split and non-split HMBC cross peaks with C-4 at H-2 and with C-2 at H-4 were integrated. The integrals of the split and non-split cross peaks at a single proton are

comparable, since they are based on the same three-bond couplings and experience similar relaxation behaviour. The split cross peaks of H-2 with C-4 and of H-4 with C-2 show a small difference due to sensitivity of the HMBC experiment for difference in relaxation and difference in coupling values. Since these split cross peaks refer to the same population of [2,4-^{13}C] butyrate, the factor needed to equalize them can also be used to correct the value of the non-split values, thus revealing the percentages of all three possible fractions of labelled butyrate.

Proteomics

The protein abundances in cultures growing with different substrates were investigated with liquid chromatography – mass spectrometry/mass spectrometry. Strain AF211 was grown in triplicate in 500 ml bicarbonate buffer medium containing with 20mM lysine and 40mM glucose plus 40mM sodium acetate as carbon and energy sources. Yeast extract was supplemented in the culture of glucose plus acetate (2g/l). Cells were collected in the exponential phase by centrifugation at 10000xg at 4^{o}C for 20 min. Cell pellets were washed twice in 100mM Tris-HCl, pH 7.5, 1mM dithioerythreitol (DTE) and suspended in 1ml of SDT-lysis buffer which contained 100mM Tris/HCl pH7.5, 4 % SDS and 0.1M dithiotreitol. Protein extractions, separation, tryptic digestion and analysis were performed as described previously (Oosterkamp et al., 2013). An *Intestinimonas* AF211 database downloaded from Uniprot (http://www.uniprot.org) was used together with a contaminant database that contains sequences of common contaminants for instance, trypsin, keratin, bovine serum albumin. The proteomics result contained peptides and proteins with a false discovery rate (FDR) of less than 1% and proteins with at least 2 identified peptides of which should be unique and one should be unmodified without any reversed hits. The normal logarithm was taken from protein label free quantitation (LFQ normalized with respect to the total amount of protein and all of its identified peptides) intensities. Zero “Log LFQ” values were replaced by a value of 5.4 (just below the lowest value) to make sensible ratio calculations possible. Relative protein quantitation of sample to control was done with Perseus 1.3.0.4 by applying a two sample T-test using the “LFQ intensity” columns obtained with FDR set to 0.05 and S0 set to 1.

Preparation of *Intestinimonas* AF211 cell extract

Strain AF211 was grown in 150 ml anaerobic bicarbonate buffered medium containing 20mM lysine or 40mM glucose plus 40mM acetate as carbon and energy source. Cells of strain *Anaerostipes rhamnosivorans* DSM26241^{T} (Bui et al., 2014) grown in 150 ml anaerobic bicarbonate buffered medium containing 20mM glucose were used as a negative control of the enzyme assay. Cells were harvested in the exponential phase by centrifugation at 10000xg at 4 ^{o}C for 20 min. Cell pellets were washed twice in an anaerobic buffer containing 100mM Tris-HCl pH7.5, 1mM dithioerythreitol (DTE) and suspended in 1ml of the same buffer. Cells were disrupted by sonication 5 times x 30s and the cell suspension was cooled on ice for 30s in between. Finally the suspension was centrifuged for 10 min at 8000xg. The cell free extract was transferred to serum bottles, flushed with N_2, and either stored at -20 ^{o}C or used directly

for enzyme activity assays. All steps were performed in an anaerobic chamber with a N_2/H_2 (96:4; v/v) atmosphere, circulated over a palladium catalyst to eliminate traces of oxygen.

Detection of acetoacetyl-CoA transferase activity.

CoA transferase activity was determined using a spectrophotometric assay (Barker et al., 1978) at 310 nm, 25°C with 100mM Tris-Cl pH 8.1, 20mM $MgCl_2$, and 50µM butyryl-CoA plus 10mM lithium acetoacetate as substrates. For enzyme activity assay the formation of acetoacetyl-CoA (E_{310nm} = 15.1 $mM^{-1}cm^{-1}$) from butyryl-CoA and acetoacetate was followed by measuring the increase in A_{310nm} by means of an absorbance recording spectrophotometer. Total volume of the mixture was 1.0 ml. The activities were expressed as U/mg of total proteins (U = µmol/min). All assays were done with biological duplicates and 4 to 6 replicate measurements were performed. Total protein was quantified using Qubit®2.0 Fluorometer (Invitrogen) according to manufacturer's instructions.

Phylogenetic analysis of CoA transferase

For the construction of a CoA transferase phylogenetic tree, all butyryl-CoA:acetate CoA transferases (Ato) and butyryl-CoA:4-hydroxybutyrate CoA transferases (4Hbt) from known intestinal butyrate producing bacteria according to references (Charrier et al., 2006; Louis and Flint, 2009) were retrieved from the NCBI database (Fig. 4). Selected 4-hydroxybutyrate CoA transferases from *Clostridium kluyveri*, *C. acetobutyricum* and *C. tetani* were also included (Charrier et al., 2006). Butyryl-CoA-acetoacetate CoA tranferases (Ato) from *C. sticklandii* DSM519, *Fusobacterium nucleatum* ATCC25586 and *C. acetobutylicum* ATCC824 were collected from their genomes (Gerischer and Dürre, 1990; Kapatral et al., 2002; Fonknechten et al., 2010). These CoA transferases are catalysing either butyrate or butanol formation. All amino acid sequences of 4Hbt and AtoA/C/D from strain *Intestinimonas* AF211 were aligned with retrieved sequences using the CLUSTAL_X program. A phylogenetic tree was constructed using the neighbour-joining algorithm by the MEGA 5 with 1000 bootstraps to obtain confidence levels for the branches.

Quantification of *Intestinimonas* in the human colon.

Stool samples of 10 Dutch healthy volunteers at different age and gender were collected (Supplementary Table 3). Genomic DNA was isolated via the bead-beating protocol described previously (Salonen et al., 2010). 16S rRNA sequences of phylogenetically related species were retrieved from GenBank (www.ncbi.nlm.nih.gov) and used to perform multiple alignments by using CLUSTALW. Primers for qPCR were designed using DNASTAR program according to Walter et al.(Walter et al., 2001). Primers were PFF590f: 5'-AAAACTATGGGCTCAACCCA-3' and PFF702r: 5'- GTCAGTTAATGTCCAGCAGG-3' to quantify *Intestinimonas* AF211 which resulted in a 100bp amplicon. Total bacteria were quantified using BAC1396F and PROK1492R primer pairs (Suzuki et al., 2000). 16S rRNA gene of strain AF211 was used for optimizing temperature and primer concentration and for making standard curves. The programme that

was used to amplify the partial 16S rRNA gene of strain AF211 was as following: 95°C for 5 min and 35 cycles consisting of 95°C for 30s, 56.7°C for 10s, and 72°C for 30 s; 95°C for 1 min and 60°C for 1 min and total bacteria as followed: 95°C for 10 min of 95°C for 20s, 56.3°C for 30s, and 72°C for 30s; 95°C for 1 min and 60°C for 1min. Both were followed by a melting curve analysis. DNA copies were calculated from standard curves and subsequently used for the strain quantities.

Metagenome analysis

The metagenomic protein sequence data from fecal samples of 65 Human Microbiome Project subjects were obtained from MG-RAST (http://metagenomics.anl.gov/). The following samples were included: SRR063550, SRR063552, SRR063553, SRR063555, SRR063556, SRR063558, SRR063561, SRR063562, SRR063564, SRR063565, SRR061730, SRR063567, SRR063568, SRR063570, SRR063571, SRR063573, SRR063574, SRR063576, SRR063577, SRR063579, SRR063580, SRR063538, SRR063582, SRR063585, SRR063586, SRR063588, SRR063589, SRR063802, SRR063899, SRR063900, SRR063902, SRR063903, SRR063540, SRR063905, SRR063906, SRR063908, SRR063909, SRR063539, SRR063542, SRR063545, SRR063548, SRR063551, SRR063554, SRR063541, SRR063557, SRR063560, SRR063563, SRR063566, SRR063569, SRR063572, SRR063575, SRR063578, SRR063581, SRR063584, SRR063543, SRR063587, SRR063801, SRR063898, SRR063901, SRR063904, SRR063907, SRR063544, SRR063546, SRR063547, SRR063549.

AF211 butyrogenic pathway protein homologs were searched from the metagenomic data by using Usearch v. 8.0.1517 with settings usearch_global and id=0.6. The results were analysed in R v. 3.1.1 software environment (http://www.R-project.org) (Edgar, 2010).

Results

Lysine degradation by *Intestinimonas* AF211

In a search for new butyrogens, strain AF211 was isolated from the stool of a healthy subject using a mineral bicarbonate-buffered medium with lactate and acetate under strict anoxic conditions (butyrate was found as the main end product). Strain AF211 contained 2 copies of the 16S rRNA gene that differed in a single nucleotide, and subsequent phylogenetic analysis showed that this strain belonged to *Clostridium* cluster IV (Lachnospiraceae) of the Firmicutes phylum. Further analysis showed that this 16S rRNA sequence was highly similar to that of the mouse intestinal isolate *Intestinimonas butyriciproducens* DSM26588^T (Kläring et al., 2013), indicating that the newly isolated strain AF211 belongs to the genus *Intestinimonas* (Supplementary Fig. 1). However, strain *Intestinimonas* AF211 was a human isolate and showed physiological differences with *I. butyriciproducens* from mouse (to be reported elsewhere). Butyrate was found to be a major metabolite from all substrates where growth was observed (Supplementary Table 1). *Intestinimonas* AF211 hardly grew on glucose in mineral medium (generation time of 88 days) but its growth rate tripled in the presence of acetate and

yeast extract. In media containing 20 mM glucose with 20 mM acetate and 2 % yeast extract, *Intestinimonas* AF211 produced up to 4.4 mM butyrate and trace amounts of lactate and ethanol (2 weeks incubation). Remarkably, the strain grew much better (generation time of 7.5 h) in L-lysine but not in any other natural amino acids or D-lysine (Supplementary Table 1).

To study the capacity to convert lysine into butyrate, *Intestinimonas* AF211 was grown in bicarbonate buffered medium containing L-lysine as the sole carbon and energy source. A total of 16.8 ± 0.4 mM lysine was converted into 14.2±0.6 mM butyrate, 15.6 ± 0.7 mM acetate and 22.1 ± 0.5 mM NH_3 when the cells reached the stationary phase after 2 days at 37 °C (Fig. 1a). This suggests that part of the released ammonia is sequestered into proteins during anabolism of *Intestinimonas* AF211. Hence, we propose the fermentation reaction as: $C_6H_{14}O_2N_2 + 2H_2O \rightarrow C_4H_8O_2 + C_2H_4O_2 + 2NH_3$. Clearly, *Intestinimonas* AF211 is capable of growing in defined media with L-lysine as sole carbon and energy source with a maximum growth rate of 0.1 h^{-1}.

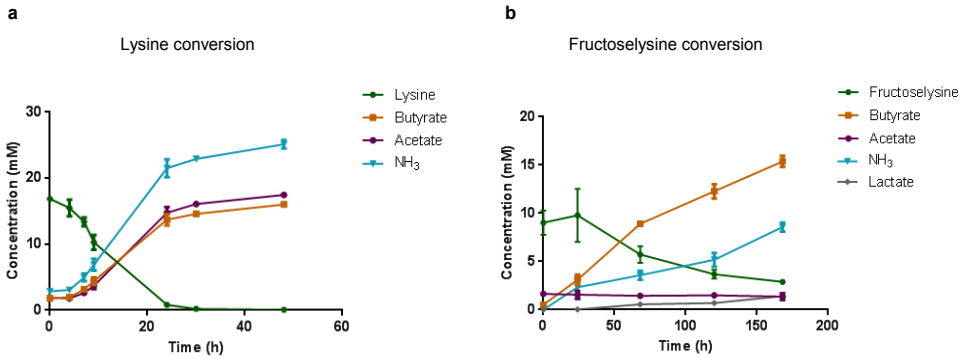


Figure 1: L-lysine and fructoselysine conversion by strain *Intestinimonas* AF211 throughout time. Carbon recovery was 87% and 101% for the lysine (**a**) and fructoselysine (**b**) utilization, respectively. Exact concentrations of substrates and products and OD values are included in Supplementary Table 1. Values are mean of biological duplicates. Error bars indicate the standard deviations.

Growth of *Intestinimonas* AF211 on fructoselysine

As fructoselysine is a key intermediate in the formation of AGEs, we addressed its degradation by *Intestinimonas* AF211 and found it to be readily metabolised (Fig. 1b; generation time 24 hours). Butyrate, NH_3 and lactate were formed as the main products. The conversion equation is proposed as: $C_{12}H_{24}O_7N_2 + 2\ H_2O \rightarrow 2\ C_4H_8O_2 + 2\ NH_3 + 1\ CO_2 + 1\ C_3H_6O_3$. We anticipate that the fructoselysine pathway includes the simultaneous degradation of both lysine and the sugar moiety in two branches (termed lysine pathway and acetyl-CoA pathway; Fig. 2). Some deviations in the butyrate/fructoselysine ratio were observed (Supplementary Tables 1 and 2). These may be attributed to the different environmental conditions but also be caused by

the intracellular balance between the two branches of the fructoselysine pathway (see Fig. 2). However, the initial concentration of acetate was predicted to be a major factor affecting the butyrate/fructoselysine ratio. We experimentally verified this and observed that in the presence of extra acetate, fructoselysine was completely converted into butyrate and NH_3 (Supplementary Table 2) according to the equation: $C_{12}H_{24}O_7N_2 + C_2H_4O_2 + H_2O \rightarrow 3\ C_4H_8O_2 + 2\ NH_3 + 2\ CO_2$. The growth rate in fructoselysine plus acetate was significantly higher than that in fructoselysine alone (see Supplementary Table 2), which could be due to stimulation of butyrate production via external acetate, leading to an increased energy gain by generating a proton motive force via the membrane-associated Rnf complex and consequently, no extra NADH needed for lactate formation (Fig. 2). The influence of acetate on the growth of fructoselysine resembles the human gut environment where acetate is abundantly present (Louis et al., 2014). Previously, the conversion of fructoselysine has been reported for a few bacteria, including *E.coli*, which also converts psicoselysine (Wiame et al., 2002; Wiame and Van Schaftingen, 2004). However, none of these bacteria are capable of butyrogenesis. To provide further support for the butyrogenic pathway we focused on the lysine and glucose degradation by using a combined approach of NMR, proteogenome and enzyme activity assays.

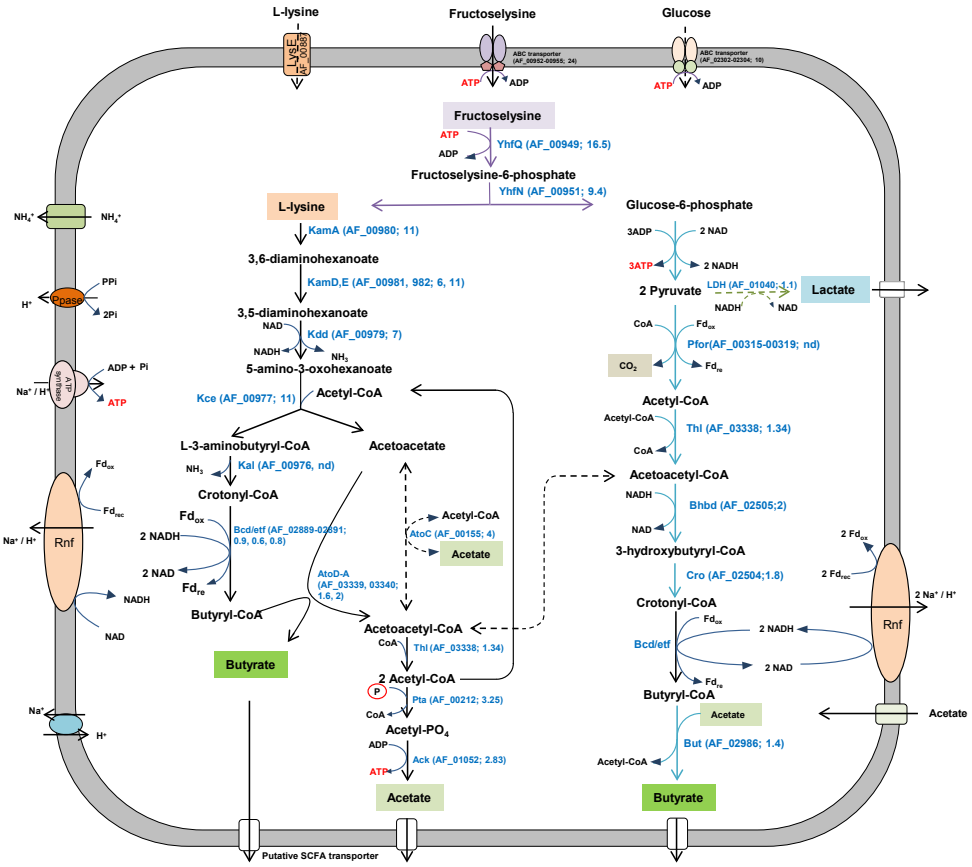
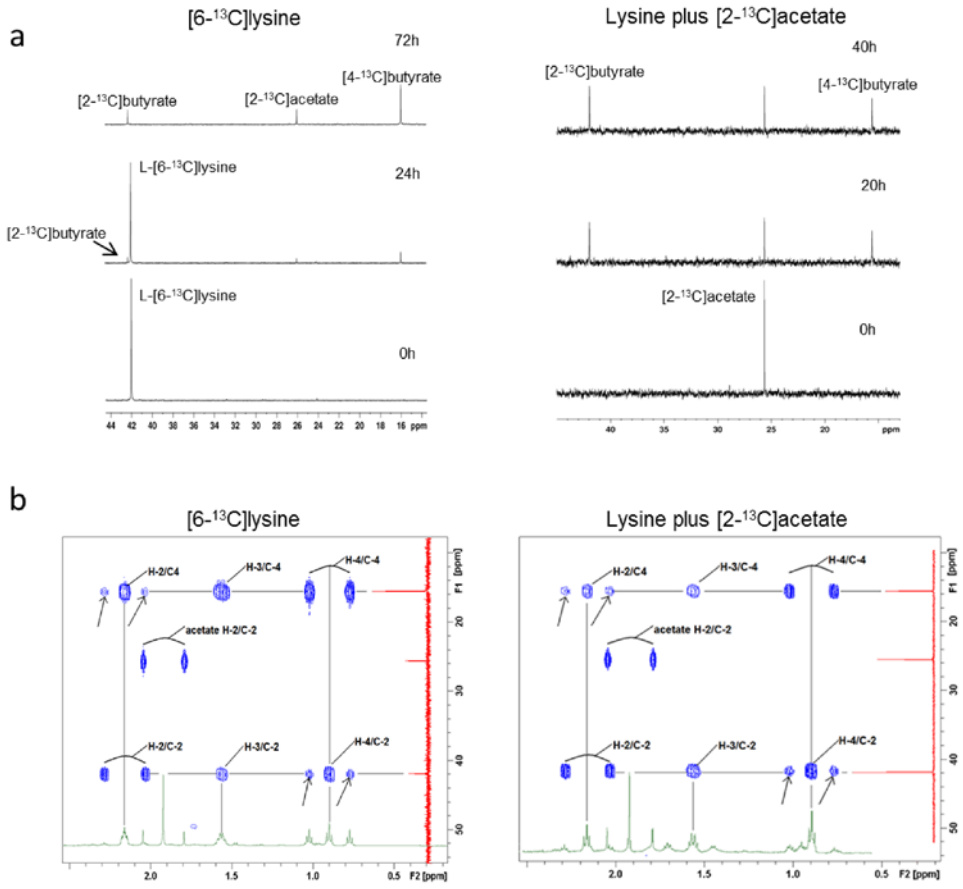


Figure 2: Model for fructoselysine metabolism in *Intestinimonas* AF211. The locus tag and fold induction of the proteins based on the proteomic data are indicated in the brackets. Rnf, Proton pumping Rnf cluster; Ppase, Pyrophosphatase; Fd, ferredoxin; nd, not detected. According to our model, fructoselysine is taken up by ABC transporter (AF_00952-00955), which is phosphorylated by fructoselysine kinase (AF_00949) to form fructoselysine-6-phosphate that is subsequently cleaved by fructoseamine deglycase (AF_00951) into lysine and glucose-6-phosphate (indicated by the purple arrows). Lysine is then degraded via the lysine pathway (black arrows) while glucose-6-phosphate is metabolised via glycolysis and the acetyl-CoA pathway (blue arrows) as described in the text (see also Supplementary Fig. 4). There are various links between the lysine and acetyl-CoA pathways and the one at the level of acetoacetyl-CoA, involving acetate to generate acetoacetate and acetyl-CoA, is indicated (dashed black arrows). For simplicity, only the key reactions are shown and other links or the conversion of acetyl-CoA formed via acetyl-CoA pathway to acetate have been omitted. Fructoselysine, lysine, butyrate, acetate, lactate and CO_2 are indicated in different colours highlighting their distinctive positions in the pathways. ATP is in red to

indicate reactions that either require or generate energy via substrate-level phosphorylation and electron transport chain. Lactate formation is dependent on the amount of exogenous acetate and redox state (hence both lactate formation and acetate uptake are indicated by dashed green arrows). Therefore, the overall fructoselysine stoichiometry depends on the presence or absence of exogenous acetate as well as environmental conditions and activity of biosynthetic pathways. In the presence of acetate (such as in the human colon), fructoselysine is converted in approximately 3 butyrate (2 butyrate are formed via acetyl-CoA pathway and 1 butyrate through the lysine pathway), while no lactate is produced; however, when no exogenous acetate is present, fructoselysine is converted into approximately 2 butyrate and 1 lactate (see Supplementary Table 2 for details).

Elucidation of the lysine degradation pathway using ^{13}C-NMR

To elucidate the butyrogenic pathway of *Intestinimonas* AF211, we applied *in vivo* ^{1}H-decoupled ^{13}C-NMR analysis of the culture supernatants of cells grown with L-[2-^{13}C]lysine, L-[6-^{13}C] lysine and lysine plus [2-^{13}C]acetate as described previously (Plugge et al., 2001). Growth of *Intestinimonas* AF211 on L-[6-^{13}C]lysine resulted in its complete conversion into [4-^{13}C]butyrate, [2-^{13}C]acetate, [2-^{13}C]butyrate and [2,4-^{13}C]butyrate (Fig. 3a, left). Proton detected multiple-bond spectroscopy was also performed with the supernatant and based on the Heteronuclear Multiple-Bond Correlations (HMBC) we could estimate the percentages of [4-^{13}C]butyrate, [2-^{13}C]butyrate and [2,4-^{13}C]butyrate out of all labelled butyrate (Fig. 3b, left). Combination of this quantitative and kinetic analysis showed that at all time points the main product formed from L-[6-^{13}C]lysine was [4-^{13}C]butyrate (71 %; Fig. 3c), indicating that cleavage of lysine occurred between the C2 and C3 residues. Both [4-^{13}C]butyrate, [2-^{13}C]butyrate and [2,4-^{13}C]butyrate were detected in the cells grown in lysine plus [2-^{13}C]acetate (Fig. 3a-b, right). This is indicative of simultaneous operation of the acetyl-CoA and lysine degradation pathways (Fig. 2) and explained the formation of minor amounts of [2-^{13}C]acetate, [2-^{13}C]butyrate and [2,4-^{13}C] butyrate in L-[6-^{13}C]lysine. Altogether, these data provide molecular evidence that the lysine pathway was substantially active and generated the intermediates for the acetyl-CoA pathway in *Intestinimonas* AF211. Similar results supporting the simultaneous operation of the two pathways were obtained by using D,L-[2-^{13}C]lysine in growing cells of *Intestinimonas* AF211, and the NMR analysis also confirmed the exclusive selectivity for L-lysine (Supplementary Fig. 2). HPLC analysis confirmed the nearly complete conversion of lysine to equimolar amounts of butyrate and acetate as indicated above (Fig. 1a). The molecular events that explain the observed isotopomers derived from [^{13}C]lysine were reconstructed (Supplementary Fig. 3) and the deduced metabolic pathway revealed 10 enzymatic reactions that were further characterized by genomic, proteomic and enzyme studies (Fig. 2).



c

Substrates	Products (%)		
	[4-^{13}C]butyrate	[2-^{13}C]butyrate	[2,4-^{13}C]butyrate
[6-^{13}C]lysine	71	22.5	6.4
Lysine + [2-^{13}C]acetate	39	55	6

Figure 3: Elucidation of lysine pathway via ^{1}H-decoupled ^{13}C-NMR spectrum and 2D HMBC spectrum. a; High-resolution ^{1}H-decoupled ^{13}C-NMR spectra showing L-[6-^{13}C]lysine ^{13}C-labelled fermentation products. [2-^{13}C]butyrate, [2-^{13}C]acetate and [4-^{13}C]butyrate had a chemical shift of 42.33ppm, 25.99ppm and 15.95 ppm, respectively. **b;** 2D HMBC spectrum for [6-^{13}C]lysine is shown. **c;** Percentages of labelled butyrate fractions (see Supplementary Figs. 2 and 3 for more details).

Proteogenomic analysis of the metabolic pathways

The complete genome of *Intestinimonas* AF211 was determined using single molecule next generation sequencing revealing a circular chromosome of 3,376,476 bp, predicted to encode 3359 genes (NCBI accession number CP011307; to be reported elsewhere). Gene candidates for the fructoselysine and lysine metabolic pathways and linked energy-generating conversions were identified in the annotated genome of *Intestinimonas* AF211 (Fig. 2; Supplementary Fig. 4). These included an operon-like cluster (AF_00949-00955) with genes for fructoselysine and psicoselysine uptake and degradation (Deppe et al., 2011) and a lysine utilization operon (AF_00976-00982) coding for 6 key enzymes involved in converting lysine into butyrate (Supplementary Fig. 4).

To confirm the presence and gene expression of the lysine degradation pathway in *Intestinimonas* AF211, proteins extracted from cells grown on either lysine or glucose and acetate (GA) were analysed by semi-quantitative proteomics (Supplementary Fig. 4; Supplementary Data 1). *Intestinimonas* AF211 was found to produce all proteins involved in the conversion of glucose and acetate into butyrate and employed the acetyl-CoA pathway, similar to other members of Ruminococcaceae and Lachnospiraceae (Duncan et al., 2002a). Moreover, the proteins involved in the lysine pathway (encoded by the cluster AF_00976-00982) were not only highly abundant but also differentially and approximately 10-fold induced during growth on lysine compared to GA, except for 3-aminobutyryl-CoA ammonia-lyase (encoded by AF_00976), which was not detected. The latter protein is rather small (14 kDa) and may have been lost during the protein extraction. Another annotated Kce candidate (AF_02981) was found to be reduced 10-fold during the growth in lysine and is present at very low level, pointing to another function rather than being involved in the lysine pathway. Two predicted 3-ketoacyl-CoA thiolases (AF_03338, AF_00601) were 1.3-fold and 1.8-fold more abundant in lysine grown cells than GA grown cells. AF_03338 is located in the same gene cluster operon with AtoD-A (AF_03339-03340; see below), which is a lysine-inducible key enzyme in the lysine pathway (Barker et al., 1978). Thus, AF_03338 is likely to be co-expressed under lysine growth conditions, while the product of AF601 might participate in the simultaneous conversion of acetate into butyrate. Several predicted butyryl-CoA dehydrogenase (bcd) genes were detected in the genome but only one of these (AF_02889) formed an operon-like cluster with the Etf complex genes (Herrmann et al., 2008) (AF_02890-02891). The reactions catalysed by ferredoxin oxidoreductase plus the exergonic NADH-dependent reduction of crotonyl-CoA to butyryl-CoA show a possible chemi-osmotic energy conservation via the Rnf complex (Li et al., 2008). This is essential under all growth conditions and hence the proteins encoded by this gene cluster were always detected (Fig. 2). The phosphate transacetylase (AF_00212) and acetate kinase (AF_01052) were found to be induced approximately 3-fold in lysine-grown cells compared to GA-grown cells, indicative of the involvement of these two enzymes in the lysine pathway. Remarkably, proteome analysis revealed the fructoselysine operon-like genes to be induced up to 25-fold during growth on lysine (Supplementary Fig.

4; Supplementary Data 1), most likely due to a high amount of lysine which was also an intermediate in the fructoselysine pathway (Fig. 2).

Phylogeny of CoA transferases and enzyme activity

A crucial reaction involved in butyrate formation from lysine is the transfer of a CoA moiety from one molecule to another, catalysed by members of the CoA transferase family. Remarkably, analysis of the genome of *Intestinimonas* AF211 predicted the presence of 14 such enzymes, and a phylogenetic tree was generated based on these and CoA transferases from intestinal bacteria and environmental isolates (Fig. 4a). These include the CoA transferases AtoD-A, 4Hbt and But, for which experimental evidence for their involvement in butyrate synthesis has been described in several anaerobes, including *Clostridium* SB4, *Roseburia* spp., *Faecalibacterium prausnitzii*, *Clostridum acetobutylicum* (Barker et al., 1978; Boynton et al., 1996; Duncan et al., 2002a). Five well-separated clades of enzymes could be distinguished. One clade included the But enzymes from the main butyrate producers in the human intestine, belonging to the Lachnospiraceae, and Ruminococcaceae (Rajilić-Stojanović and de Vos, 2014) that are all capable of butyrogenesis from glucose or lactate plus acetate (Duncan et al., 2002a; Charrier et al., 2006). The *Intestinimonas* AF211 enzyme encoded by AF_02986 belonged to this But clade and was detected in the proteome under both growth conditions, indicating that it participated in the acetyl-CoA pathway rather than 4-aminobutyrate or glutamate pathways. The second clade harbored the 4Hbt enzymes and included several predicted CoA transferases of *Intestinimonas* AF211 but none was detected at the protein level. The third and fourth clade held the α and β subunits of the AtoD-A enzymes, respectively, that are encoded by 2 juxtaposed genes in the well-studied anaerobes: *F. nucleatum*, *C. sticklandii* and *C. acetobutylicum* (Barker et al., 1982; Wiesenborn et al., 1989; Bennett and Rudolph, 1995; Kapatral et al., 2002; Fonknechten et al., 2010). Within these two AtoD-A subunit clades, 3 gene pairs of *Intestinimonas* AF211 were clustered. However, only one of these, encoded by AF_03339-03340, was highly abundant and induced two-fold under lysine growth conditions, indicative of its involvement in butyrate formation via the lysine pathway (Fig. 2). A fifth cluster of acetyl-CoA:acetoacetate CoA transferases (AtoC) contained enzymes not only from *Intestinimonas* AF211 (indicated by AF_00155, AF_02540 and AF_01396) but also from *A. rhamnosivorans*, which is capable of butyrogenesis from glucose as well as from acetate plus lactate (Bui et al., 2014). The 4-fold protein induction of AtoC (AF_00155) during growth on lysine indicated the involvement of this CoA transferase in lysine degradation. We propose its role to be the balancing of acetyl-CoA, released from the acetyl-CoA pathway and providing either additional acetoacetate or acetoacetyl-CoA, in line with the simultaneous activity of the lysine and acetyl-CoA pathway during butyrogenesis from lysine (see above).

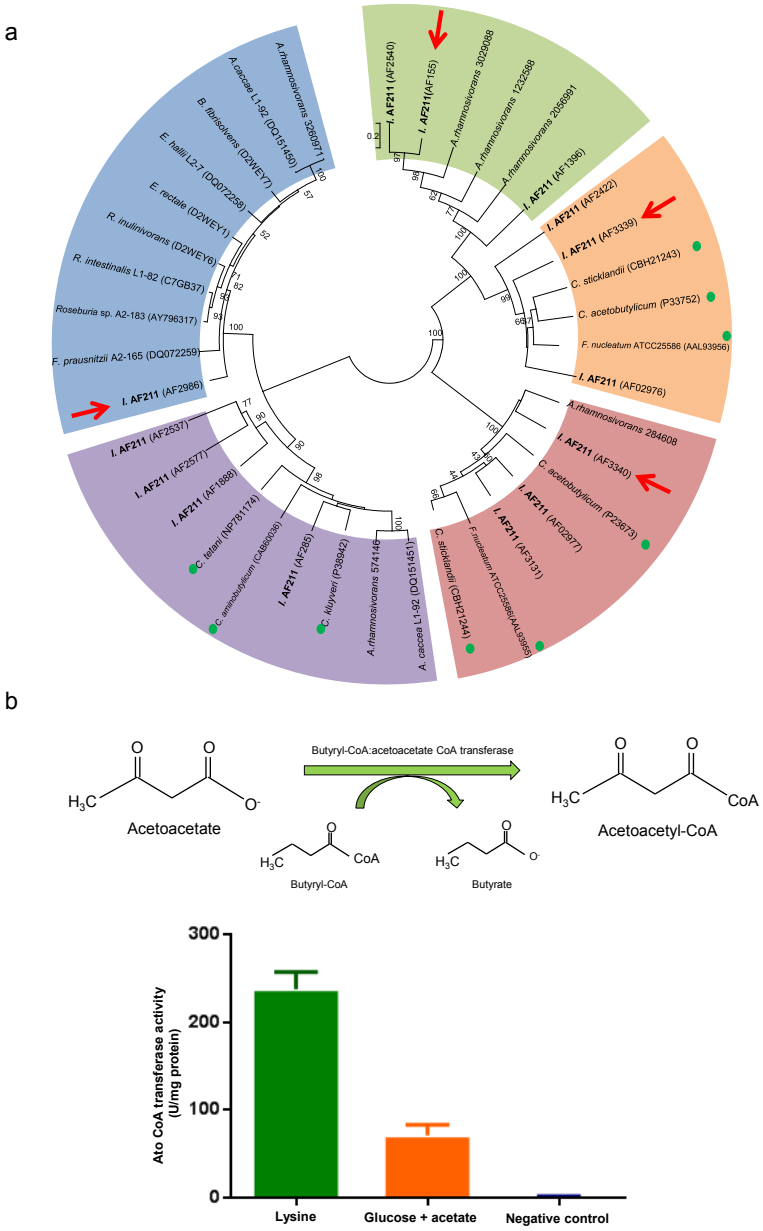


Figure 4: Phylogeny of CoA transferase and enzyme activity. **a**: Phylogenetic tree of predicted CoA transferases from *Intestinimonas* AF211 (bold) and other representative anaerobes. The tree was based on sequences from butyryl-CoA:acetate CoA transferase (But, in blue), butyryl-CoA:4-hydroxybutyrate CoA transferase (4Hbt, in purple), butyryl-CoA:acetoacetate CoA transferase (Ato) alpha subunit (AtoD, in orange), beta subunit (AtoA, in brown) and acetyl-CoA:acetoacetate CoA transferase (AtoC, in green), respectively. Green

dots indicate non-intestinal isolates. *Intestinimonas* AF211 proteins induced during growth on lysine are indicated by the red arrows. **b**: Butyryl-CoA:acetoacetate CoA transferase activity in crude cell extracts. Each measurement was performed with biological duplicates and 4 to 6 replicate measurements. Values represent mean of replicates. Error bars indicate standard deviations. Green and red bars represent the enzyme activity of *Intestinimonas* AF211 grown in lysine and glucose plus acetate, respectively; blue bar is the negative control with *Anaerostipes rhamnosivorans* DSM26241^T grown on glucose which is not capable of lysine fermentation.

To further provide support for the pivotal conversion catalysed by AtoD-A, its activity in *Intestinimonas* AF211 was studied by incubating cell-free extracts under anaerobic conditions with acetoacetate and butyryl-CoA and monitoring the production of acetoacetyl-CoA (Barker et al., 1978; Wiesenborn et al., 1989). We found reproducible and high activity of this enzyme activity in cells grown on lysine (237 units/mg protein), which was 3.5-fold reduced in cells grown with GA (71 units/mg protein) (Fig. 4b). As the observed activity induction coincided with the protein quantitation from the proteome analysis, we deduce that AtoD-A (encoded by AF_03339-03340) is probably the butyryl-CoA:acetoacetate CoA transferase involved in the butyrogenic lysine pathway.

***Intestinimonas* and fructoselysine genes in the human gut**

As metagenome analysis indicated that the lysine pathway has high abundance in the human intestine (Vital et al., 2014), we studied the presence of *Intestinimonas* related to AF211 in a series of healthy subjects by using a specific 16S rRNA-based qPCR (Supplementary Table 3). In 5 out of 10 subjects, 0.2-10 % of the 16S rRNA sequences were derived from *Intestinimonas* spp. since all qPCR products showed the expected nucleotide sequence. From fecal DNA of the remaining 5 subjects, amplicons were generated but sequence analysis showed four of them to derive from *Ruminococcus* spp. as their 16S rRNAs fortuitously amplified with the used primers, indicating it is not possible to correctly estimate the level of *Intestinimonas* spp. in these subjects (Supplementary Table 3). When analysing the deep metagenome information obtained in 65 subjects characterised in the Human Microbiome Project, we could identify many of the genes for lysine degradation in over half of the subjects but the genes involved in fructoselysine degradation were only observed in half a dozen individuals as indicated by the presence of the key gene fructoselysine kinase (Supplementary Fig. 5).

The observed abundance and prevalence of *Intestinimonas* spp. level is in good agreement with the metagenome-predicted presence of the lysine degradation pathway (Vital et al., 2014), suggesting that *Intestinimonas* is the key species converting lysine and fructoselysine into butyrate in the human gut. This is supported by the recent isolation of a similar but antibiotic-resistant strain from a healthy subject (Rettedal et al., 2014). However, it is evident that not all humans have intestinal metagenomes that are equipped with genes for the conversion of fructoselysine, the major Amadori product.

Discussion

Here we describe the isolation of a butyrate-producing bacterium, *Intestinimonas* AF211, abundantly present in the intestine of some humans. The bacterium can use fructoselysine, a key intermediate in the formation of AGEs, as sole carbon and energy source by converting this into mainly butyrate and NH_3. The predicted fructoselysine pathway includes the simultaneous degradation of both lysine and sugar moiety. By determining the metabolic route of the ^{13}C-labelled lysine conversion by NMR, in combination with enzyme measurements and proteogenomic analysis, the butyrogenic pathway from lysine was fully elucidated and also indicated the presence of the fructoselysine pathway in *Intestinimonas* AF211. The use of fructoselysine as carbon and energy source for butyrogenesis is unique and *Intestinimonas* AF211 is the first intestinal bacterium to harbour the complete pathway for the conversion of lysine into butyrate (Fig. 1), previously predicted based on metagenomic data (Vital et al., 2014). Lysine is an essential amino acid that is cleaved from dietary proteins by pancreatic trypsin, producing peptide chains with a C-terminal arginine or lysine residue (Leiros et al., 2004), The generated lysine-containing peptides can be utilized via the proteolytic activity of *Intestinimonas* AF211 as evidenced by its growth on different protein-derived substrates (Supplementary Table 4) and the several-fold induction of various aminopeptidases under lysine growth condition (Supplementary Table 5). Collectively, one can consider the butyrogenic conversion of lysine by *Intestinimonas* AF211 as a specific example of host-microbe interactions.

Degradation of L-lysine was previously observed in complex media by *Fusobacterium nucleatum*, suggesting that this gram-negative bacterium and potential pathogen can use L-lysine as energy source but its use as carbon source is not clear (Barker et al., 1982; Rogers et al., 1998; Kreimeyer et al., 2007). *Clostridium sticklandii* uses L-lysine as an electron donor in the Stickland reaction and lysine was only degraded in the stationary phase when other amino acids were depleted (Fonknechten et al., 2010). Despite the fact that a few bacteria have been reported to degrade lysine to butyrate, recent (meta) genomic analysis which included genomes from these isolates indicated that none of the genomes analysed had genes for the entire pathway (Vital et al., 2014). Additionally, *Escherichia coli* and *Bacillus subtilis* were found to be capable of degrading fructoselysine but none of them produces butyrate (Deppe et al., 2011). However, *Intestinimonas* AF211 contained all genes involved in degradation of fructoselysine to butyrate (Fig. 2) while proteomic analysis revealed that these genes were induced under the lysine growth conditions.

Remarkably and unlike other butyrogenic bacteria, *Intestinimonas* AF211 was found to contain over a dozen genes coding for CoA transferases (Fig. 4a). We hypothesize that this may help the bacterium to be more flexible to act on a broad range of substrates. The integrated analysis of the *Intestinimonas* AF211 genome, proteome and activity measurements revealed that a specific acetoacetyl-CoA transferase AtoD-A (AF_03339-03340) was abundantly

expressed under lysine degradation conditions and hence predicted to be the key enzyme involved in butyrate synthesis. As this is the first described lysine pathway in the intestinal ecosystem, the lysine utilization operon and the identified AtoD-A genes may have application as markers for butyrogenesis from lysine.

An important corollary of the abundant presence of lysine-degrading butyrogenic *Intestinimonas* AF211 and relatives in the intestine of some humans is the fact that amino acids can serve as source of butyrate formation. Since butyrate has presumed health benefits as an energy source for colonocytes and is a vital molecule to maintain intestinal integrity, this may nuance suggestions that protein fermentation, notably in the distal colon, has a negative health impact (Windey et al., 2012). Remarkably, we observed that *Intestinimonas* AF211 is also capable of growth on fructoselysine as sole carbon and energy source resulting in the conversion of fructoselysine into butyrate (Supplementary Table 1). The Amadori product fructoselysine is abundant in cooked foods and is formed via the non-enzymatic Maillard reaction of reducing sugars and amino acids during the heating process. As humans are unique in the consumption of cooked products at a large scale, it would be of interest to determine whether the fructoselysine gene cluster detected in *Intestinimonas* AF211 has been recently acquired and is not present in the gut microbes of other primates. The production of fructoselysine from cooked foods has various impacts, including the loss of essential amino acids and a reduced protein digestibility. Fructoselysine is key product leading to the formation of AGEs in the human body that have been associated with chronic diseases and development of diabetes complication (Brownlee, 1994; Baynes and Thorpe, 2000; West et al., 2014). Moreover, recent food interventions in mice showed the deleterious effect of AGEs on these and other diseases (Cai et al., 2014). The butyrogenic conversion of fructoselysine by *Intestinimonas* AF211 illustrates the important role of this anaerobe in the human intestinal tract whereas the observation that some but not all human carry genes for fructoselysine degradation indicates the potential for specific interventions with the newly discovered strain. In conclusion, our study underlines the need for cultivating novel microbes to get a comprehensive understanding of the intestinal metabolic processes and the beneficial effect on the human host. In addition, *Intestinimonas* AF211 and related bacteria may play an important role in the intestinal tract by maintaining protein balance and gut homeostasis.

Acknowledgments

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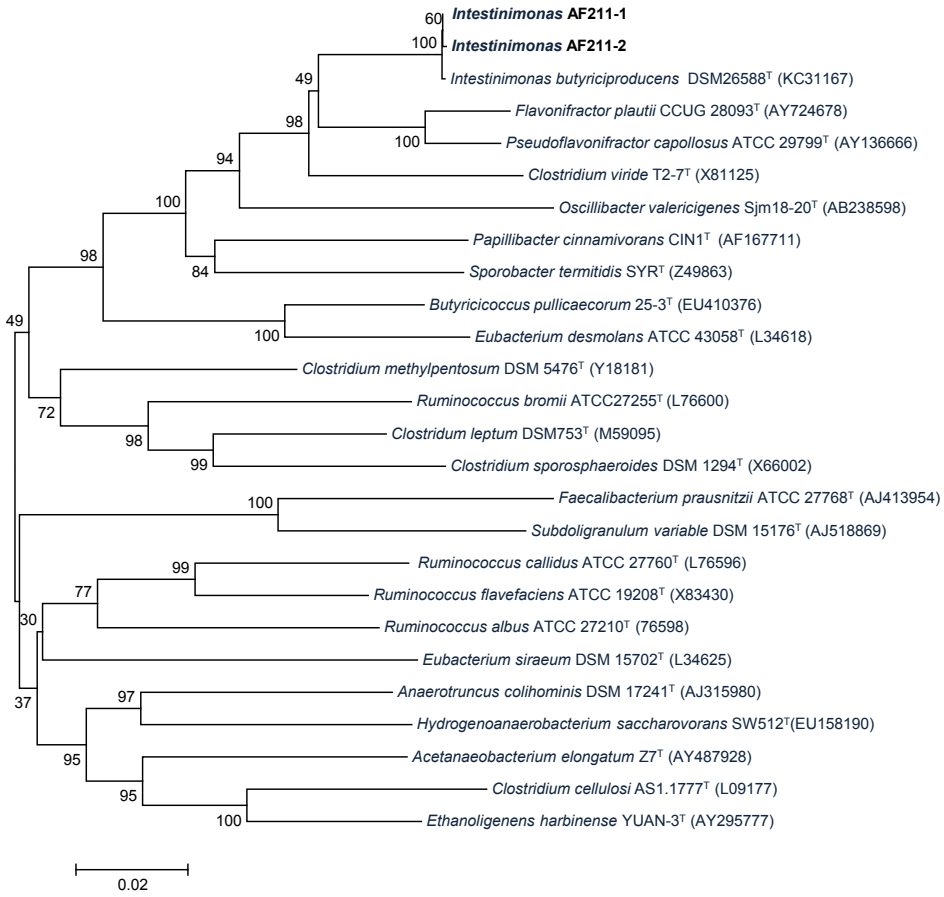
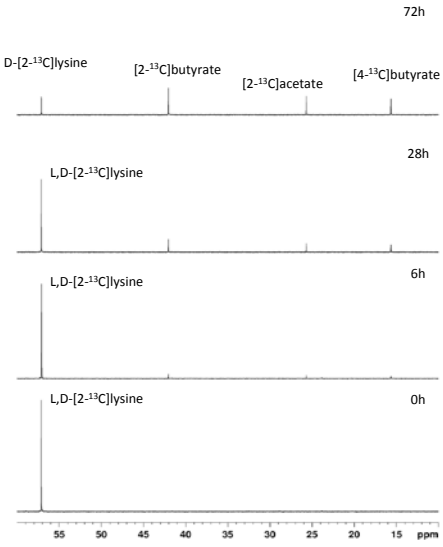
Supplementary information

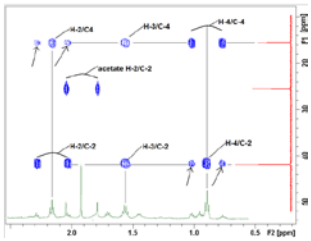


Supplementary Figure S1: Phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relation of strain AF211 and closely related members of *Clostridium* cluster IV. Bootstrap values > 50 % based on 1,000 replications are shown at branching points. Bar, 1 % sequence divergence.

a



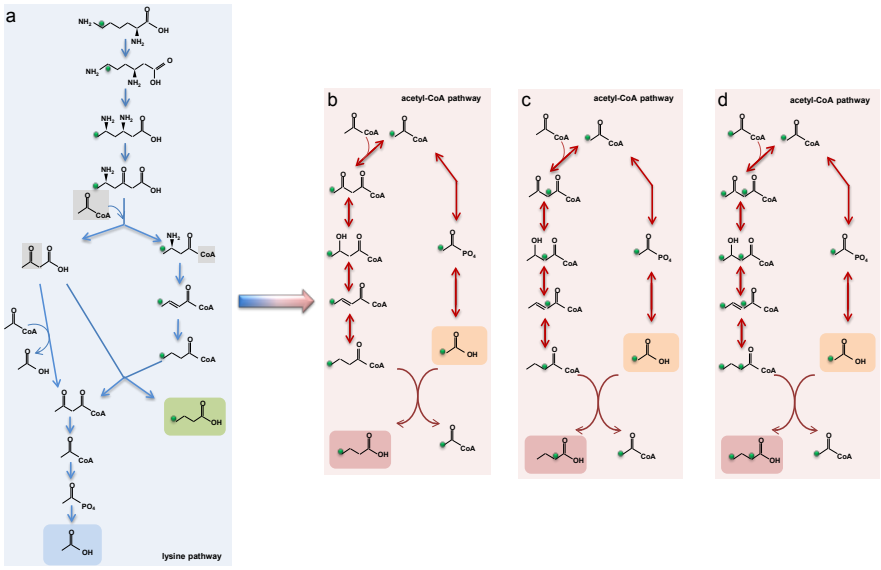
b



c

Product	Percentage (%)
[4-^{13}C]butyrate	23
[2-^{13}C]butyrate	59
[2,4-^{13}C]butyrate	17

Supplementary Figure S2. Elucidation of lysine pathway via ^{1}H-decoupled ^{13}C-NMR spectrum and 2D HMBC spectrum when grown in L,D-[2-^{13}C]lysine. **a**. High-resolution ^{1}H-decoupled ^{13}C-NMR spectra showing L,D-[2-^{13}C]lysine fermentation products. [2-^{13}C] butyrate, [2-^{13}C]acetate and [4-^{13}C]butyrate had a chemical shift of 42.33ppm, 25.99ppm and 15.95 ppm, respectively. **b**. 2D HMBC spectrum for L,D-[2-^{13}C]lysine is shown. **c**; Percentages of labelled butyrate fractions.

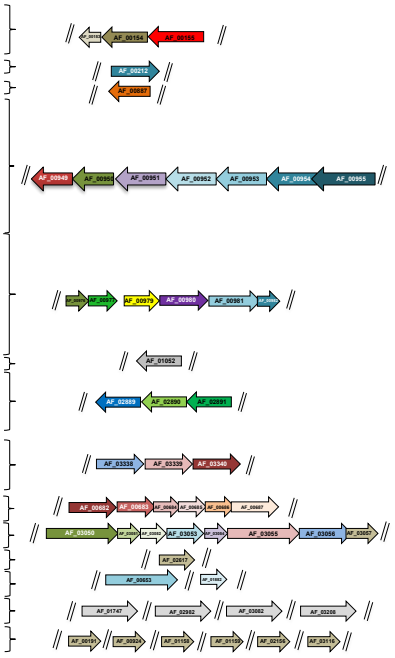


Supplementary Figure S3: The fate of ^{13}C-labelled carbon in *Intestinimonas* AF211. a: Proposed lysine pathway where L-[6-^{13}C]lysine resulted in [4-^{13}C]butyrate (in green). **b, c, d:** Acetyl-CoA pathway fed by intermediates of the lysine pathway. This pathway resulted in [2-^{13}C]butyrate, [4-^{13}C]butyrate, [2,4-^{13}C]butyrate and [2-^{13}C]acetate formation upon different combinations of either acetyl-CoA and [2-^{13}C]acetyl-CoA or 2 molecules of [2-^{13}C]acetyl-CoA. When labelled acetyl-CoA was plenty in the medium it also incorporated in the lysine pathway which ended up in [2-^{13}C]acetate production.

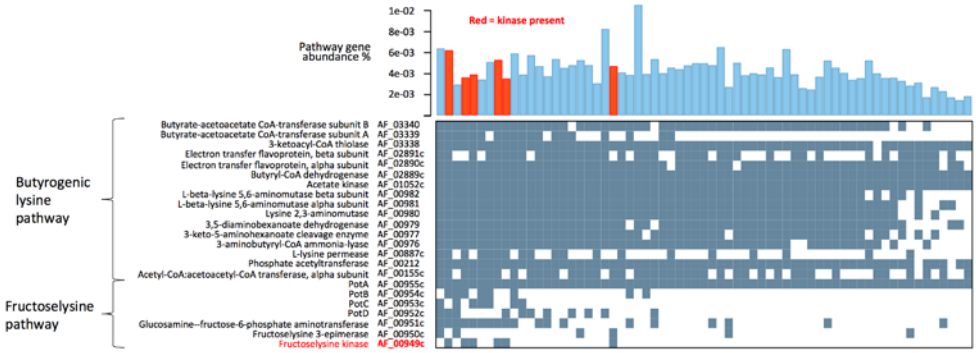
(a) Locus tag

Functions	Gene code	Locus tag	Fold induction
Uncharacterised protein	-	AF_00153	1.8
D-β-hydroxybutyrate permease	-	AF_00154	9.28
Acetyl-CoA:acetoacetate CoA transferase	AtoC	AF_00155	4.08
Phosphate acetyltransferase	Pta	AF_00212	3.25
L-Lysine permease	LysE	AF_00887	ND
Fructoselysine kinase	YhfQ	AF_00949	16.51
Fructoselysine 3-epimerase	YhfOP	AF_00950	ND
Fructosamine deglycase	YhfN	AF_00951	9.42
Spermidine putrescine ABC transporter permease component PotD	PotD	AF_00952	2.78
Spermidine putrescine ABC transporter permease component PotC	PotC	AF_00953	24.8
Spermidine putrescine ABC transporter permease component PotB	PotB	AF_00954	ND
Spermidine putrescine ABC transporter permease component PotA	PotA	AF_00955	20.21
3-aminobutyryl-CoA ammonia lyase	Kal	AF_00976	ND
3-keto-5-aminohexanoate cleavage enzyme	Kce	AF_00977	10.87
3,5-diaminobexanoate dehydrogenase	Kdd	AF_00979	7.07
Lysine 2,3-aminomutase	KamA	AF_00980	11.11
L-beta-lysine 5,6-aminomutase alpha subunit	KamD	AF_00981	6.25
L-beta-lysine 5,6-aminomutase beta subunit	KamE	AF_00982	11.26
Acetate kinase	Ack	AF_01052	2.83
Butyryl-CoA dehydrogenase	Bcd	AF_02889	0.93
Electron transfer flavoprotein α subunit	Etfα	AF_02890	0.60
Electron transfer flavoprotein β subunit	Etfβ	AF_02891	0.74
3-ketoacyl-CoA thiolase/Acetyl-CoA acetyltransferase	Thl	AF_03338	1.34
Butyrate-acetoacetate CoA-transferase α subunit	AtoD	AF_03339	1.57
Butyrate-acetoacetate CoA-transferase β subunit	AtoB	AF_03340	2.03
Proton pumping Rnf cluster (C, D, G, E, A, B subunit)	Rnf	AF_00682-00687	0.83; 0.83; ND (G,E,A); 0.92
V-type ATP synthase cluster (I, K, E, C, F, A, B, D subunit)	ATP synthase	AF_03050-03057	5.31; 308; 2.07; 5.01; -; 2.28; 2.53; 3.78
Inorganic Pyrophosphatase	Ppase	AF_02617	0.83
Ammonium transporter	NH_3 transporter	AF_00653, AF_01882	ND
Putative short chain fatty acids transporter (AF_01747, AF_02982, AF_03082, AF_03208)	SCFA trasporter	4 copies	0.15 (AF_01747)
Na+/H+ antiporter (AF_00191, AF_00924, AF_01158, AF_01159, AF_02156, AF_03116)	Antiporter	6 copies	0.56 (AF_01159); 0.3 (AF_03119)

(b) Gene arrangement



Supplementary Figure S4: Proteins involved in the fructoselysine and lysine conversion pathway (a) and their gene organization (b). The fold induction was deduced from the proteome analysis of *Intestinimonas* AF211 cells grown on lysine and GA (glucose plus acetate). ND: not detected.



Supplementary Figure S5: Overview of butyrate pathway gene presence in Human Microbiome Project data. The matrix shows the presence (blue) and absence (white) of each gene in the two butyrogenic pathways in 65 HMP samples. The barplot shows the pathway gene numbers normalised by the read numbers in each sample. The red bars indicate the samples where sequences related to the fructoselysine kinase gene were found.

Supplementary Table S1: Metabolites from glucose; lysine and fructoselysine fermentation. Strain AF211 was grown in 17 mM L-lysine or 9 mM fructoselysine or 20 mM glucose plus 20 mM acetate. Values are means of duplicates ± standard deviation. The incubation time was 2 weeks for sugar fermentation, 2 days for lysine and 7 days for fructoselysine. ND: not detected. NA: not applicable. Carbon recovery data took into account the formation of CO_2. There was not any growth observed in D-lysine, glutamate, glutamine, glycine, proline, arginine, methionine and aspartate.

Substrate	Consumption (mM)		Production (mM)					Carbon recovery %	OD
	substrate	acetate	lactate	ethanol	acetate	butyrate	NH_4^+		
Fructoselysine	6.1 ± 1.0	0.29 ± 0.5	1.4 ± 0.2	ND	NA	14.9 ± 0.8	8.6 ± 0.5	101 ± 0.7	0.34
L-Lysine	16.8 ± 0.4	NA	ND	ND	15.6 ± 0.7	14.2 ± 0.6	22.1 ± 0.5	87 ± 2.5	0.36
Glucose	4.0 ± 0.2	2.8 ± 0.6	1.2 ± 0.5	1.7 ± 0.2	ND	4.4 ± 0.1	ND	83 ± 11.0	0.2

Supplementary Table S2: Acetate effect on fructoselysine growth. Strain *Intestinimonas* AF211 was grown in 4.3 mM fructoselysine without or with 10 mM acetate in duplicate. The incubation time was 6 days. ND: not detected. NA: not applicable. Carbon recovery data took into account the formation of CO_2. The OD values increased three-fold under all conditions but the growth rate were approximately 1.5 times higher on fructoselysine with acetate than on fructoselysine alone. The product balances also changed and without acetate, 1 fructoselysine was converted to 2 butyrate and 1 lactate while with acetate 1 fructoselysine was converted to 3 butyrate.

Substrate	Substrate consumption (mM)		Product (mM)				Carbon recovery %	Growth rate (h^{-1})
	Fructoselysine (added)	Acetate	Butyrate	Lactate	NH_4^+	Acetate		
Fructoselysine 1	4.3	NA	8.9	2.4	7.3	0.22	98 %	0.04
Fructoselysine 2	4.3	NA	8.5	2.9	9.4	0.5	96 %	
Fructoselysine-acetate 1	4.2	1.9	10.8	ND	6	NA	105 %	0.06
Fructoselysine-acetate 2	4.2	3.7	11.6	ND	7.9	NA	102 %	

Supplementary Table S3: Quantification of *Intestinimonas* AF211 in human fecal samples. A summary of the qPCR results and Sanger sequencing data is provided. Total DNA of *Intestinimonas* AF211 was amplified with 95% efficiency, compared to the 16S rRNA amplicon while total DNA of *Pseudoflavonifractor capilosus* or *Flavonifractor plautii* did not amplify with the *Intestinimonas* primers.

Volunteers	Total 16S copy number	*Intestinimonas* AF211 copy number	Percentage (%)	Sequencing check
1	7.92E+04	2.67E+03	1.7	***Intestinimonas butyriciproducens***
2	1.80E+05	1.76E+04	4.9	*Ruminococcus bromi*
3	1.45E+05	1.07E+04	3.7	*Ruminococcus bromi*
4	1.98E+05	1.68E+04	4.2	No data
5	8.27E+04	2.50E+02	0.15	***Intestinimonas butyriciproducens***
6	2.54E+05	1.27E+03	0.15	***Intestinimonas butyriciproducens***
7	1.50E+05	2.95E+04	9.8	***Intestinimonas butyriciproducens***
8	6.15E+04	1.31E+03	1.1	***Intestinimonas butyriciproducens***
9	9.79E+04	1.63E+02	0.09	*Ruminococcus bromi*
10	6.34E+04	7.44E+03	2.4	*Ruminococcus bromi*
gDNA AF211	7.77E+05	7.43E+05	95.58	

Supplementary Table S4: Product formation in different protein sources. Short chain fatty acid production was analysed after a week growth in the bicarbonate buffered media plus 10g/l of tryptic soy broth without dextrose (BD), tryptone (BD), casitone (BD), vegetable peptone (BD), yeast extract (BD), bacterial peptone (BD), casein hydrolysate (BD), methyllysine (SIGMA), dimethyllysine (SIGMA) or hydroxylysine (SIGMA).

Substrates (10g/l)	Acetate (mM)	Propionate (mM)	Butyrate (mM)
Tryptic Soy Broth w/o dextrox	1.85	1.76	2.23
Tryptone	3.74	3.13	3.95
Casitone	4.52	0.81	3.46
Vegetable Peptone	1.09	0	0.55
Yeast Extract	3.20	3.04	5.04
Bacterial Peptone	0.81	0	0.70
Casein Hydrolysate	6.77	0.65	3.52
Methyllysine	0	0	0
Dimethyllysine	0	0	0
Hydroxylysine	0	0	0

Supplementary Table S5: Aminopeptidases found and detected from the whole proteome. The fold induction was deduced from the proteome analysis of *Intestinimonas* AF211 cells grown on lysine and GA (glucose plus acetate).

Functions	locus tags	Fold induction
Methionine aminopeptidase (EC 3.4.11.18)	AF_03023c	4.15
Tripeptide aminopeptidase (EC 3.4.11.4)	AF_00964	2.17
Aminopeptidase YpdF (MP-, MA-, MS-, AP-, NP- specific)	AF_01181	2.75
peptidase M18, aminopeptidase I	AF_01776c	3.09
Tripeptide aminopeptidase (EC 3.4.11.4)	AF_02694	7.03
Deblocking aminopeptidase (EC 3.4.11.-)	AF_01656c	-1.46
Deblocking aminopeptidase (EC 3.4.11.-)	AF_01657c	1.19
Deblocking aminopeptidase (EC 3.4.11.-)	AF_01658c	-1.07

Supplementary Data 1: Can be found in Nature Communications site with the link below http://www.nature.com/ncomms/2015/151201/ncomms10062/full/ncomms10062.html

CHAPTER 6

COMPARATIVE GENOMICS AND PHYSIOLOGY OF BUTYRATE-PRODUCING BACTERIUM *INTESTINIMONAS BUTYRICIPRODUCENS* STRAINS REVEAL HOST-SPECIFIC FEATURES

Manuscript in preparation

Thi Phuong Nam Bui, Sudarshan Shetty, Ilias Lagkouvardos, Jarmo Ritari, Francois Douillard, Lars Paulin, Thomas Clavel , Caroline M. Plugge and Willem M. de Vos

Abstract

Intestinimonas is a newly described bacterial genus with representative strains present in the intestinal tract of human and other animals. Although members of this genus carry out important metabolic functions, including the production of butyrate from both sugars and amino acids, there is to date no data on their diversity, ecology and physiology. Using a comprehensive phylogenetic approach, *Intestinimomas* was found to include at least 3 species that colonize primarily the human and mouse intestine. We focused on the most common and cultivable species of the genus, *Intestinimonas butyriciproducens*, and performed detailed genomic and physiological comparisons of strains SRB521^{T} and AF211, isolated from the mouse and human gut, respectively. The complete 3.3-Mb genomic sequences of both strains were highly similar with 98.8 % average nucleotide identity, testifying for their assignment to one single species. However, thorough analysis revealed significant genomic rearrangements, variations in phage-derived sequences, and the presence of new CRISPR sequences and metabolic genes present only in the human strain. While both strains were capable of producing butyrate from sugars and lysine, the human strain AF211 was more efficient in these conversions than the mouse isolate. Notably, this was observed with arabinose and galactose, two sugars found abundantly in the human but not the mouse diet. In conclusion, the present genomic and physiological characterisation of *I. butyriciproducens* provides evidence for different host-specific features of strains originating from the mouse and human gut.

Introduction

The gut microbiome is a living organ within the human body that contains highly complex and diverse microbial communities contributing significantly to human health (Zoetendal et al., 2006). While the majority of these communities are strictly anaerobic bacteria that are hard to culture (Flint et al., 2006), an increasing number has successfully been isolated, and presently over 1000 cultured species have been reported (Rajilić-Stojanović and de Vos, 2014). In spite of this, only a small number of these intestinal anaerobes have been characterized at the genomic level and often draft genomes have been assembled. Complete genomes have the advantage that the full genetic repertoire can be studied, and repetitive sequences can be revealed.

The chemical compound butyrate and butyrate-producing bacteria have gained increasing attention as they both play an important role in maintaining gut homeostasis and intestinal epithelial integrity (Hamer et al., 2008). Butyrate is a major substrate for colonocytes and is reported to have anti-carcinogenic, anti-inflammatory and anti-oxidative effects (Hinnebusch et al., 2002; Klampfer et al., 2003; Hamer et al., 2008). Interestingly, a decreased amounts of butyrogenic bacteria have been observed in inflammatory bowel diseases, colon cancer and type II diabetes (Flint et al., 2012; Qin et al., 2012; de Vos and Nieuwdorp, 2013). Till date several butyrate-producing bacteria have been isolated that mainly belong to *Clostridium* clusters XIVa and IV (Lachnospiraceae and Ruminococcaceae, respectively). These bacteria mainly produce butyrate via the acetyl-CoA pathway (Louis et al., 2010). A recent study based on comparative analysis of metagenomes reported three other routes of butyrate production where amino acids serve as substrates, including lysine and glutamate (Vital et al., 2014). All these butyrogenic pathways merged at an energy generating step where crotonyl-CoA is converted to butyryl-CoA by means of a butyryl-CoA dehydrogenase complex (Bcd/etf). The oxidation of the reduced ferrodoxin and reduction of NADH from this conversion by the membrane Rnf complex create a proton motive force (Li et al., 2008). The lysine pathway that includes a specific acetoacetyl-CoA transferase involved in the butyrate formation step was estimated to be the second dominant butyrogenic pathway in the gut. However, no cultured isolates capable of performing this relevant reaction had been described (Vital et al., 2014). Using a classical cultivation approach with human stool as inoculant, we isolated strain *Intestinimonas* AF211, which is capable of converting lysine completely and efficiently into butyrate and acetate (Bui et al., 2015). This feature is unique for the gut ecosystem and links two important metabolic features, butyrogenesis and amino acid fermentation in the intestinal tract. The complete 16S rRNA gene sequence of strain AF211 revealed more than 99 % similarity to *Intestinimonas butyriciproducens* SRB521^T isolated from mouse caecum (Kläring et al., 2013), indicating that these two strains belong to the same species. This species belongs to a newly described genus *Intestinimonas,* which is one of the genera within *Clostridium* cluster IV. It is of interest to note that *Faecalibacterium prausnitzii.* another butyrogenic species within *Clostridium* cluster IV has found to be highly abundant in human

(Arumugam et al., 2011) but not in mice (Xiao et al., 2015).

Predictions based on complete genome sequences combined with physiological and other functional studies are powerful tools for improving our understanding of the lifestyle and role of bacteria. Genomic approaches can also be used to gain insight into the genetic potential of an organism and assist in predicting its ecological role. Presently, only a few genomes of intestinal butyrate-producing bacteria have been characterised including that of *Faecalibacterium prausnitzii*.(Heinken et al., 2014) and *Roseburia inulinivorans* (Scott et al., 2006). More recently, a partial genome sequence of *Anaerostipes hadrus* PEL 85 was reported (Kant et al., 2015) and a number of partial genomes of other butyrate-producing bacteria are available but not yet characterised. In the present study, we focus on the recently describe genus *Intestinimonas* and described its phylogenetic and host distribution. Moreover, we compare the complete genome sequences of the human isolate *I. butyriciproducens* AF211 and the mouse isolate *I. butyriciproducens* SRB521^T. Finally we describe the physiological characteristics of both strains to confirm their predicted metabolic features and provide explanations of their adaptation to different hosts.

Materials and methods

Strains and growth condition

I. butyriciproducens strain AF211 was obtained from a stool sample of a healthy individual (Bui et al., 2015) and *I. butyriciproducens* strain SRB521^T was isolated from the caecum of a mouse (Kläring et al., 2013). Bacteria were routinely grown in anaerobic Reinforced *Clostridium* Medium (RCM, Difco) in 120-ml serum bottles sealed with butyl-rubber stoppers at 37 ^oC under a gas phase of 1.7 atm of N_2/CO_2 (80 : 20, v/v).

Metadata search of *Intestinimonas* spp. using 16S rRNA gene amplicons

The 16S rRNA gene of the type strain of *I. butyriciproducens* strain SRB521^T (NR_118554) was used for a similarity query (95 %) against all 16S rRNA samples available from the Sequence Read Archive (SRA) using the Integrated Microbial Next Generation Sequencing platform (www.imngs.org). IMNGS is an innovative web tool, which routinely checks and retrieves raw data from SRA and uniformly processes them to searchable operational taxonomic units (OTU) tables using a modified version of the UPARSE pipeline (Edgar, 2013). Of note, IMNGS processes SRA samples individually, generating a list of OTUs for each sample, leading to redundancy of OTUs between samples. This feature is essential for flexibility of the system and easy comparison of OTU profiles between samples. Hence, the term sample-specific OTUs is used to refer to OTUs generated by IMNGS, and these OTUs that can be used to address targeted microbial diversity.

Based on RDP classification (Cole et al., 2014), all 16S rRNA reads that matched the sequence of *I. butyriciproducens* but were not categorized as unknown Ruminococcaceae

(the current lineage of *Intestinimonas*) were removed. A local BLAST (Altschul et al., 1990) database was built with the remaining reads, and those with >95 % similarity to representative sequences of the two neighbouring genera *Flavonifractor* and *Pseudoflavonifractor* were removed. Remaining reads (referred to as true SRA-derived *Intestinimonas* sequences) were aligned using SINA (Pruesse et al., 2012) in SILVA (Quast et al., 2013). A segment of highest coverage spanning the V4 hypervariable region was identified and the alignment was trimmed around this region (approx. 300 nt). Pairwise similarity across all remaining sequences covering this region was calculated in ESPRIT (Sun et al., 2009). Based on clusters calculated at 5 % dissimilarity (considered as genus level cut-off in the present analysis) using average linkage, reads that fell into the cluster containing the reference *Intestinimonas* sequence were selected and their pairwise distance matrix was used to produce a multidimensional scaling (MDS) plot in the R programming environment. For the sake of clarity, points in the MDS plot were clustered in hexagonal bins with the number of sequences in each bin visualized as a color gradient. A representative amplicon sequence together with the longest 100 % identical clone-derived sequences from GenBank were selected for each of the most abundant bins, and their pairwise similarity to *I. butyriciproducens* was calculated. Additional clones classified as *Intestinimonas* were collected from SILVA and those that formed a monophyletic group with the reference *Intestinimonas* sequence were kept. These clones, the clones aforementioned matching most abundant bins in the MDS plot and 16S rRNA sequences from the two neighbouring genera and the outgroup *Faecalibacterium prausnittzii* were used for calculation of a phylogenetic tree using Maximum Likelihood based on the General Time Reversible model with invariant sites and 500 bootstraps in MEGA6 (Tamura et al., 2013). All positions containing gaps and missing data were eliminated, leaving a total of 1,316 positions in the final dataset. For ecological reconstruction, each SRA-derived read from the filtered *Intestinimonas* dataset was assigned to the closest sequence out of the 5 selected clones mentioned above and the reference *I. butyriciproducens* sequence using BLAST. Sample-specific environmental information corresponding to the reads was represented as histograms next to the tree. Finally, all short reads (5,732) that were closest to the sequences within the monophyletic *Intestinimonas* branch were clustered to species-like groups (approx. 3% similarity) using CROP (Hao et al., 2011).

Genome sequencing and assembly

Cells of strain AF211 and SRB521^T grown in RCM (overnight cultures) were used for genomic DNA extraction performed using ZR Fungal/Bacteria DNA MiniPrep kit (ZYMO, USA) according to manufacturer's instructions. Sequencing effort included the use of two complementary platforms based on the Illumina and PacBio technologies.

Genome sequencing of two 15kb libraries using with PacBio RS II instrument using P4/C2 chemistry (Pacific Biosciences, Menlo Park CA, USA) at the DNA sequencing and genomics laboratory, Institute of Biotechnology, University of Helsinki, Finland. Data processing and

filtering was done with PacBio SMRT analysis pipeline v2.2 and the Hierarchical Genome Assembly Process (HGAP) protocol (http://www.pacb.com/devnet/). Filtering was run with minimum subread length 500 and polymerase read length quality 0.80. For pre-assembly, settings were: minimum seed read length 7000, split target into chunks 1 and alignment candidate per chunk 24. Assembly was performed by the Celera V1 assembler with parameters genome size 3,000,000, target coverage 30, overlapper error rate 0.06, overlapper mini length 40 and overlapper K-mer 14. Assembly polishing was done with Quiver.

In addition, genome sequences were also collected using a HiSeq2000. This resulted in 3,202,992 and 2,759,530 paired reads for AF211 and SRB521^{T}, respectively, which were used to correct for possible PacBio sequencing errors. For the HiSeq paired-end reads, all rRNA reads were removed with SortMeRNA v1.9 using default settings except for an increased error value of 20% for the adaptors, and using the reverse complement of the adaptors as well (Kopylova et al., 2012). Quality trimming was performed with PRINSEQ Lite v0.20.0 with a minimum sequence length of 40 and a minimum quality of 30 on both ends and as mean quality of read (Schmieder and Edwards, 2011). For assembly, Ray v2.3 was used (Boisvert et al., 2012). Duplicate contigs in both the genome assemblies of AF211 and SRB521T were discarded if they had a hit with at least 99% sequence identity within a bigger contig, which spanned at least 98% of the contig query length. Furthermore, contigs with length <500bp were discarded. Next, Illumina contigs were aligned to the PacBio assembly using promer algorithm from the MUMmer 3.0 software package (Kurtz et al., 2004) with the parameters show-tiling -c -R -i 50. The alignment was processed in R (http://www.r-project.org/) using in-house scripts to extract the Illumina sequence and combined with PacBio assembly at gap positions to produce the final closed genome sequence. A circular element was detected within this assembly, based on the BLASTP results of the predicted proteins (e-value 0.0001), and this added to the final assembly result. Scaffolding of the contigs was done with SSPACE-LongRead and the PacBio CCS reads using default settings (Boetzer and Pirovano, 2014). The annotation was done by RAST server (Aziz et al., 2008). Functional prediction of proteins was verified manually by BLASTing the amino acid sequences in Pfam (Finn et al., 2014), Brenda, Interpro (Hunter et al., 2012) and Uniprot databases. For a global overview of the genome features, the circular genome map was created using the virtual machine CGView Comparison Tool (CCT) (Grant et al., 2012).

Specialized annotations

The core genome of *I. butyriciproducens* was calculated using Spine and the pan-genome was identified using AGEnt (Ozer et al., 2014). For genomes of two strains, specific annotations for CRISPR genes was done using CRISPRfinder (Grissa et al., 2007). Target phages for CRISPR were identifies using CRISPRtarget using Genbank Phage, and ACLAME genes databases with an E-value cut-off of 0.1 (Biswas et al., 2013). The CRISPRTarget tool identifies target phages using different databases and the search algorithm has its own scoring system with

a score of 20 which was kept as default minimum as suggested by the authors (Biswas et al., 2013). All the spacers targeting phages in both SRB521^T and AF211 had an acceptable score of 21. Prophage genes in the genomes were annotated using PHAST (PHAge Search Tool) (Zhou et al., 2011). The PHAST uses a scoring system based on three methods (i) Presence of known phage genes/proteins; (ii) >50% genes/proteins in the region are related to a known phage; and (iii) <50% genes/proteins in the region are related to a known phage (Zhou et al., 2011). In general, if a given phage region's total score is < 70, it is marked as incomplete; between 70 to 90, it is marked as questionable; if >90, it is marked as intact. The carbohydrate active enzymes (CAZymes) were annotated using the amino acid sequences with default search parameters (Park et al., 2010). Genomic islands were identified using online IslandViewer and a standalone program i.e. SeqWord Gene Island Sniffer that identifies genomic islands based on the analysis of oligonucleotide usage variations in DNA sequences (Bezuidt et al., 2009; Langille and Brinkman, 2009; Dhillon et al., 2013).

Phenotypic characterization

To investigate carbohydrate assimilation and detection of enzyme activities, API 20NE, API rapid 32, API ZYM were used with overnight-grown cells of both strains. The Gram reaction was determined using standard methods (Plugge et al., 2000). In-vivo DNA-DNA hybridization was done at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), as described previously (Ley et al., 1970) with some modifications (Huss et al., 1983).

For cellular fatty acid analysis, cells of both strains were grown in RCM medium for 5 days before being centrifuged (10,000 rpm) for 10 min at 4 °C. Pellets were stored at -80 °C and later used to extract cellular fatty acids as described previously (Kämpfer and Kroppenstedt, 1996). Products were analysed by Agilent model 6890N gas 199 chromatography (MIDI Sherlock, Newark, N.J.) as described previously (Miller, 1982; Kämpfer and Kroppenstedt, 1996).

Bile tolerance was tested in RCM supplemented with OX-Bile (SIGMA ALDRICH) (0.1; 0.2; 0.3; 0.4; 0.5% (w/v)). Growth was determined via OD measurement and product formation using HPLC as described previously (van Gelder et al., 2012).

Growth on carbohydrates

I. butyriciproducens strains were grown in 50 ml anaerobic bicarbonate buffered medium (Stams et al., 1993) with 2g/l yeast extract. Ten mM of carbohydrates were added from 1M sterile stock solutions. Tested carbohydrates were glucose, fructose, maltose, mannitol, cellobiose, lactose, raffinose, xylose, D-mannose, saccharose, galactose, D,L- arabinose and sorbitol Samples were collected at different time points and subjected to GC and HPLC analysis as described previously (van Gelder et al., 2012).

Antibiotic susceptibility tests

The E-test was done to identify minimal inhibitory concentrations (MICs) according to the manufacturer's protocol (bioMérieux, France). Both strains were pre-grown in 50 ml RCM broth (overnight cultures) and 50µl were spread on RCM agar plates (1.5 % w/v agar) until the agar surface was dry and the liquid was absorbed by the agar. Two E-test strips were placed in one plate to test one antibiotic. Results were noted according to manufacturer's protocol (bioMérieux, France). Antibiotics included ciprofloxacin, cefotaxime, erythromycin, oxacillin, teicoplanin, tetracycline, tobramycin, vancomycin and sulfamethoxazole. The concentration range was 0.016 – 256 µg/ml for oxacillin, tetracycline, tobramycin and vancomycin, and 0.016 – 32 µg/ml for ciprofloxacin, cefotaxime, erythromycin, teicoplanin and sulfamethoxazole. MIC values were recorded directly from the strips after 48 hours and rechecked after 4 days. Duplicate experiments were performed for each antibiotic.

Results and discussion

Diversity and ecology of *Intestinimonas*

Intestinimonas is a newly described genus with peculiar metabolic functions, but its phylogeny and ecological distribution remain unknown. Thus, we made an inventory of all its potential members by analysing 16S rRNA amplicon sequences available in SRA, including approximately 55,000 samples from various host habitats and environmental sources. This similarity search delivered 21,313 *Intestinimonas*-like sample-specific OTUs from a total of over 703,000 amplicon reads. After filtering (including *I. butyriciproducens* lineage match, exclusion of reads >95 % similar to neighbouring genera or ESPRIT-based clusters), 9,887 OTU sequences were kept and their pairwise distances were projected in two dimensions using MDS (Fig. 1A). Based on the number of sequences in hexagonal bins, four main islands emerged. The short SRA-derived reads with the highest abundance in these bins had similarity >95 % to *I. butyriciproducens* (NR_118554), suggesting the presence of possibly 4 additional species within the genus (>97 % cut-off for species classification). However, when larger clone sequences with 100 % identity to those reads were compared, they showed overall similarity <95 %, pointing at different genera (Fig. 1A), indicating that the V4 region of 16S rRNA genes does not offer strong resolving power for this genus. Therefore, to avoid inflating the number of predicted species, all SRA reads that matched those other genera-type clones were removed. Hence, only the short reads with best match to the reference sequence of *I. butyriciproducens* and the neighbouring (species) type clones (FJ676152 and FJ374227) were considered for final estimation of the sequence-supported diversity and ecology of *Intestinimonas*. Clustering using CROP resulted in the formation of 8 none-singleton clusters, where the vast majority of OTU sequences (99 %) fell into only 3 major species clusters (data not shown). These results mirrored the distribution observed in the clone-based tree (red dashed-line box containing *Intestinimonas* spp. in Fig. 1B). Interestingly, whereas clone FJ374227 seems not to represent an abundant *Intestinimonas* species, the SRA-based analysis supports the existence of

another species without clone representative. Ecological assessment revealed that 90 % of *I. butyriciproducens*-like sequences were predicted to originate from human samples (Fig. 1B). In contrast, the species represented by clone FJ676152 clustered with reads that originate from various animal samples. The third and smallest species represented by clone FJ374227 was characterized by the murine gut as primary sample origin. *I. butyriciproducens* strains have also been isolated from swine faeces, signifying its widespread presence in mammals (Levine et al., 2013; Rettedal et al., 2014). As *I. butyriciproducens*-like sequences represented a major clade within the genus and were most prevalent in humans, but also present in the murine host, we performed in-depth genomic and physiological comparison of strains AF211 and SRB521^{T}, isolated from human and mouse, respectively.

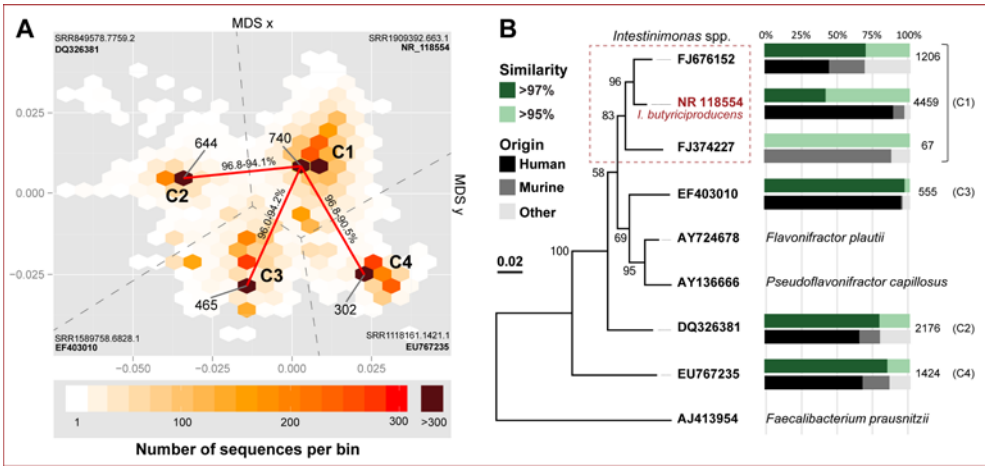


Figure 1: 16S rRNA-based diversity and host distribution of *Intestinimonas*. A. MDS plot of pairwise distances between all short-read OTUs (n = 9,887) within the genus *Intestinimonas* extracted from all samples (55,073) stored in SRA. Sequences were binned in hexagons color-coded according to the number of sequences they contained. The reads formed separated clusters (C1-C4) around highly abundant cluster cores, for which the exact number of supportive SRA-derived OTUs are shown in panel B. Similarity distances between representative short amplicon reads and clones from the three neighboring cores and the one containing the reference 16S rRNA sequence of *I. butyriciproducens* (cluster C1) are shown next to the red lines connecting them, respectively; the corresponding sequence accession for each cluster core are given in the corresponding corner. The grey dash lines indicate midpoints between cores after assignment of each point (OTU) to its closest center. B. Maximum likelihood tree of *Intestinimonas*-related isolates and clone sequences representative of most abundant amplicon reads (Fig 1A) together with selected neighboring genera and outgroup. The tree with the highest log likelihood (i.e. representing the most probable tree topology) is shown. The tree was calculated using 500 bootstraps and percentages of congruent trees are shown next to branches. The tree is drawn to scale, with branch lengths measured in the

number of substitutions per site (the bar represents the phylogenetic distance that corresponds to 2 % nucleotides substitution). The box with red dashed-line indicates members of the genus *Intestinimonas*. The encoded sequences FJ676152 and FJ374227 derive from cow (Durso et al., 2010) and beetle larvae (Andert et al., 2010), respectively.

Phenotypic characterization of *I. butyriciproducens* strains

A global physiological and biochemical characterization of *I. butyriciproducens* strains AF211 and SRB521^T was performed by using API Rapid 32A, API 20NE, API ZYM kits. The results showed slightly different capacities of carbohydrate fermentation in which strain SRB521^T showed N-acetyl-β-glucosaminidase activity but this was not found with strain AF211 (Table S1). Interestingly, analysis of cellular fatty acid composition, an important chemotaxonomic characteristic for microbial classification, revealed significant differences between the two strains (Table S2). More than 20 % content difference was observed in major fatty acids, such as $C_{14:00}$; iso-$C_{19:1}$ I and $C_{18:00}$. The cellular fatty acids have a major role in maintaining the integrity and viability of microbial cells, and may change in composition dependent on the environmental conditions. Recently, differences in intra-species cellular fatty acid composition in *Lactobacillus plantarum* strains were found to be correlated to the origin of isolation (Garmasheva et al., 2015). The strains AF211 and SRB521^T have been isolated from different hosts and hence are likely to be adapted to the intestinal tracts that differ considerably in pH, concentration and composition of bile, and exposure to food components.

In the gut environment, bacteria need to tolerate several stress conditions. One of these is bile, which can act as a surfactant and has the ability to affect the phospholipids and proteins of cell membranes (Begley et al., 2006). Bacteria are known to tolerate bile via two mechanisms: one is by using bile salt hydrolases (BSH) to hydrolyse bile and the other is by using efflux pumps to remove bile that passes through the outer membrane (Begley et al., 2006; Jones et al., 2008). The genomes of both AF211 and SRB521^T lacked the coding sequence for canonical BSHs but indicated the presence of genes for several MDR efflux pumps that could aid in tolerance to bile. In vitro investigation of bile tolerance showed growth of strains AF211 and SRB521^T after 3 days of incubation in 0.1% bile (Fig. 2) but no growth was observed at higher concentration of bile. However, while strain AF211 grew slightly faster and to a higher OD than strain SRB521^T without bile, these differences were much more pronounced in the presence of 0.1 % bile (Fig. 2). The better growth of strain AF211 with bile could be attributed to the exposure on different bile components from the human and the mouse gut (Sayin et al., 2013), signifying the impact of host environments on bacterial metabolism. Remarkably, strain AF211 showed an improved adaptation to bile acid although it is known that the bile flux in mouse is higher than in man (Hofmann, 2001).

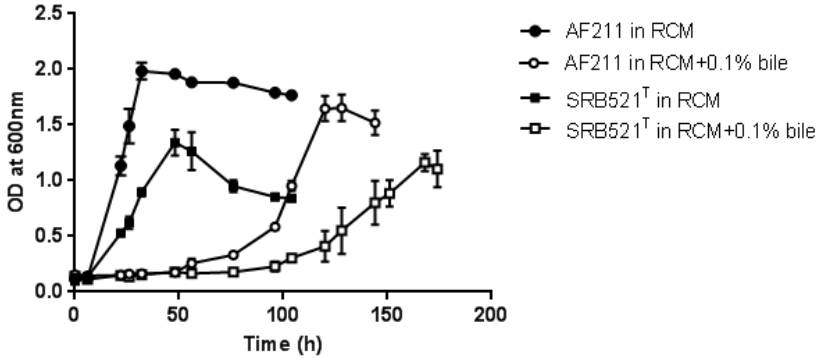


Figure 2: Growth of strains AF211 and SRB521^T in the presence and absence of 0.1 % bile. Strains were grown in RCM and RCM plus 0.1 % bile (Oxgall). Values are mean of duplicates. Error bars are standard deviations.

Comparative genomics - general features of the *I. butyriciproducens* genomes

I. butyriciproducens strains AF211 and SRB521^T both have a single circular chromosome with similar genome features (Fig. 3A). No plasmid sequences were detected in either of the genomes. The genome size of AF211 is 112,795 bp larger than that of SRB521^T, indicating larger genetic potential in the human isolate that is also reflected in the higher number of coding sequences i.e. 3,363 vs. 3,268 in the mouse isolate. Furthermore, the average nucleotide identity (ANI) between the two isolates confirmed that they belong to the same species. Additional genomic features such as the distribution of GC content, GC-skew and location of genes including tRNA, rRNA, prophage genes are indicated in Fig. 3B.

A

	AF211	SRB521^{T}
Host	Human	Mouse
Accession number	CP011307	CP011524
Genome Size (Base pairs)	3,376,476	3,263,681
GC content (%)	59.1	59.4
RNA genes	57	58
Number of Coding Sequences	3363	3268
Number of Subsystems	348	338
Average nucleotide Identity (ANI)	98.80 %	98.80 %

B

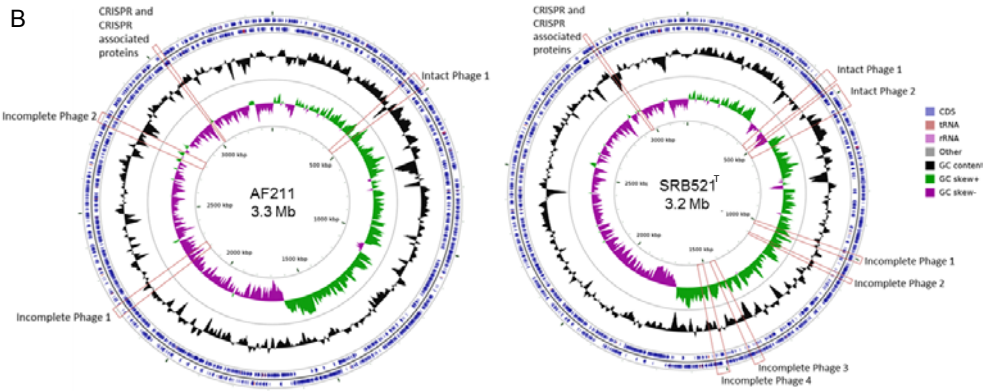


Figure 3: General genome features (A) and genome maps (B) of *Intestinimonas butyriciproducens* isolates. From the outer circle inward, coding regions are marked on the first two rings: genes encoded on the positive strand (outside) and if encoded on the negative strand (inside). The third ring (black) shows local GC content. The innermost graph shows the CG skew. Phage regions and CRISPR associated genes are marked within red lines.

Pan- and Core genome

The pan-genome of *I. butyriciproducens* was calculated to contain 4,762 coding DNA sequences (CDS), of which 2,612 CDS (2,603,042bp) were considered as core. The average number of CDS predicted per genome was approximately 3,427, indicating that an average of 76.21 % of the genome of each isolate was part of the core. The overall subsystem level core and accessory genome features were further analysed. This revealed that approximately 22.8% of the genome sequences were unique to one of the isolates signifying the strain level differences in the genomic content of AF211 and SRB521^{T}. The additional coding capacity in the AF211 was mostly assigned to genes coding for hypothetical proteins, mobile element proteins and restriction enzymes that were located on genomic islands (Fig. S2).

Host-associated flexible genomic regions in *I. butyriciproducens*

To identify whether genomic features of *I. butyriciproducens* AF211 and SRB521^T could be linked with the difference in the human gut and mouse gut environment, a detailed analysis was made of their genomes. As already evident from differences in GC skew (Fig. 3), the two genomes carry a variety of genomic rearrangements including large inversions and integration of foreign regions of DNA (Fig. 4).

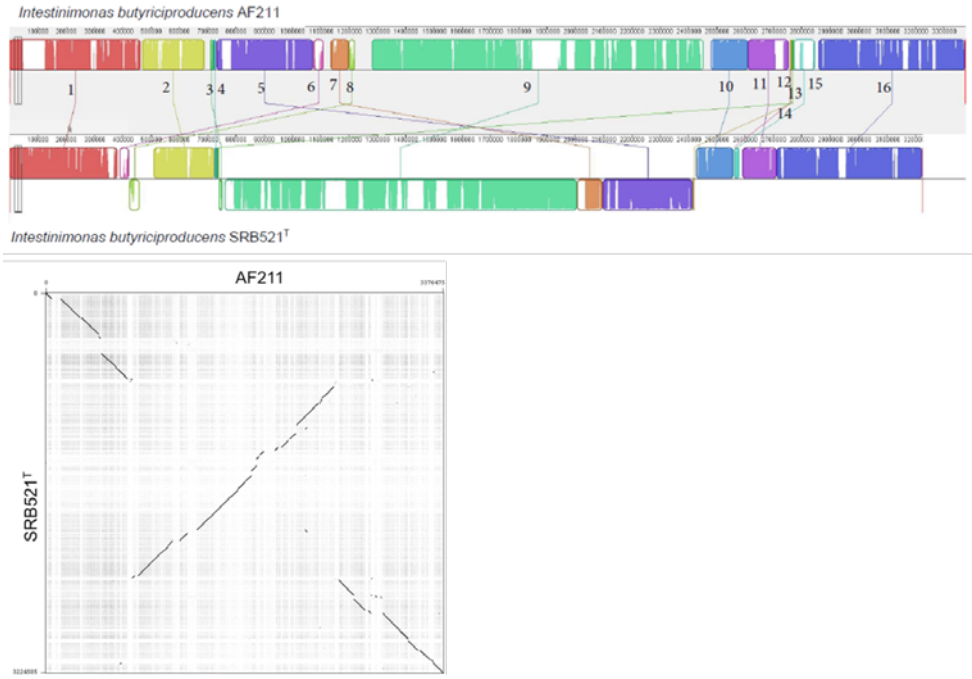


Figure 4: Alignment of the genome sequences of *I. butyriciproducens* strain AF211 and SRB521^T. A: The genome of strain AF211 was used as reference for global alignment using progressive MAUVE. The numbers indicate the locally collinear blocks (LCBs) that were identified in both the genomes. Conserved and highly related regions are coloured, and low-identity unique regions are in white (colourless). LCBs below the mid-line in strain SRB521^T are inverted relative to the strain AF211 sequence. **B**: Dot plot alignments of AF211 and SRB521^T using Gepard. SRB521^T is on the y-axis and AF211 is on the x-axis and the genomes are starting with the origin of replication at position 1. The black lines represent regions of similarity while the breaks in this line (syntenic line) represent regions of genomic variations at a given locus between the two genomes.

Alignment of the genomes of AF211 and SRB521^T revealed the presence of 16 locally collinear blocks (LCBs) with several regions of inversion and rearrangement (Fig. 4A). Moreover, an obvious X-pattern was observed when the genomes of AF211 and SRB521^T were compared in a dot blot, indicating a symmetric chromosomal inversion, involving approximately one third of the genome around the origin of replication (Fig. 4B). Not surprisingly, genes for a site-specific recombinase (SRB521_00768) and a probable integrase/recombinase (SRB521_002435c) flanking the inverted region in genome of SRB521^T were detected. One of the LCBs, which includes a site-specific recombinase gene (SRB521_00768), is located at 738168-739784 bp in mouse isolate and corresponding LCB in human isolate with the same gene (AF_02777) is located at around 2770592-2772208 bp, signifying the role of this gene in genomic rearrangement.

In addition, we searched the genomes for the presence of genomic islands to identify non-self-mobilising elements that code for proteins with diverse functions. The AF211 genome contained 24 genomic islands (GIs) while SRB521^T had 18 (Fig. S2A). These include the already noted (incomplete) prophages (see Fig. 1 and Fig. S3) and series of other predicted gene clusters that are part of the GIs (Table S2). Remarkably, the majority of the genes located on the GI's encode hypothetical proteins in both AF211 (56%) and SRB521^T (63%).

Prophages and CRISPR systems in *I. butyriciproducens*

There has been considerable attention for the presence and function of bacteriophages in the human intestine. Specifically, the intestinal mucosa has been identified as a source for these phages, potentially indicating their role in maintaining microbiota dynamics (Barr et al., 2013; Wootton, 2013). As these phages may vary between mouse and man, it was of interest to analyse the natural defence systems of the two *I. butyriciproducens* strains, including the analysis of prophages known to provide superinfection immunity. Comparative analysis of the genomes of AF211 and SRB521^T demonstrated major differences in the prophage elements. The genome of AF211 revealed one intact and 2 incomplete prophages while that of SRB521^T was predicted to have two intact and 4 incomplete prophages (see Fig. S3). Prophage integration sites in the genomes of both strains were found to have significantly different G + C content (Fig. 3B). In the AF211, the prophages had varying G+C content, intact prophage 1 and incomplete prophage 1 had a higher G+C content of 61.77% and 62.42% respectively and incomplete prophage 2 had a lower G+C content of 54.95% when compared to the overall G+C content of 59.4% for AF211. In case of SRB521^T genome, the intact prophage 1 had G+C content of 59.29%, while that of intact prophage 2 was 64.15%. Additionally in the SRB521^T genome, two of the incomplete prophages had higher 63.84% and 64.10%, while for the other two it was lower, 55.66% and 54.90% respectively. The observed differences in the sequence suggest that the genes were acquired from both high and low GC content bacterial taxa. The comparison of prophage sequences between the two genomes revealed that these two strains do not share any prophage sequences, indicating exposure to different prophages

in human and mouse gut. This observation is also supported by the CRISPR/Cas system search in the two genomes. Both strains possessed clustered regularly interspaced short palindromic repeats (CRISPR/Cas system) in their genomes. The CRISPR array involves associated proteins to confer resistance to phages. The CRIPSR arrays contained the same 33 bp long direct repeat (5'- ATTTCAATCCACGCCCCCCGTGTGGGGGGCGAC- 3'). The number of spacers was 58 and 21 for strain AF211 and SRB521^T, respectively. Interestingly, only two spacers were shared between the two strains (Fig. S2B). Blast analysis of the spacer regions revealed no sequence with complete identity to phage database entries, which can be attributed to the lack of phage sequences in Genbank Phage, and ACLAME genes databases. However, spacer 11 of the CRISPR system in strain SRB521^T targeted the phage minor tail protein in prophage reported in Nocardia farcinica IFM 10152. Moreover, of significant interest is the finding that spacer 46 of the CRISPR system in strain AF211 appeared to target phages that made up 18% of total virus like particle (VLP) contig sequences in metagenomic DNA from human fecal samples (Minot et al., 2011). Harbouring an immune system against this highly abundant viral family could be beneficial for survival of AF211 in the human gut.

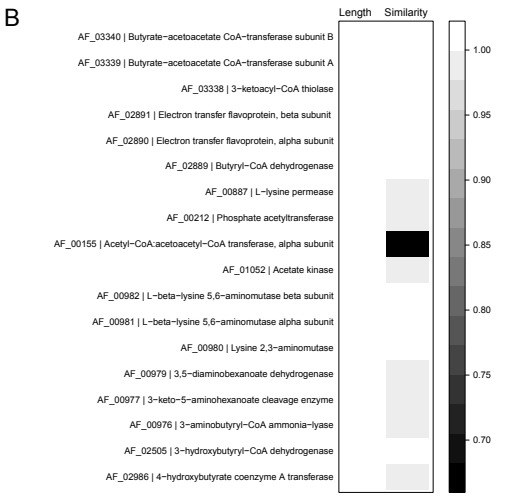
Butyrogenesis from sugars and lysine

As the intestine of men and mice differ considerably in the composition, load and kinetics of dietary components, it was of importance to address the metabolic capacity of the two *I. butyriciproducens* strains. Genomic analysis revealed the presence of a complete glycolytic pathway while the pentose phosphate and the tricarboxylic acid pathways were incomplete in both strains. In vitro tests confirmed a mixed acid fermentation using variety of carbohydrate substrates, which resulted in production of butyrate as a major product and ethanol, isobutyrate or lactate as minor metabolites. The two strains grew poorly in hexose sugars but growth was much improved by the addition of acetate (Fig. 5A). A similar feature was previously described in *Faecalibacterium prausnitzii.* the presence of acetate increased energy harvest from glucose by this species (Heinken et al., 2014) via stimulation of acetyl-CoA pathway involved butyryl-CoA dehydrogenase (Bcd) electron-transferring flavoprotein (Etf) complex that generates a proton motive force via a membrane-associated Rnf complex (Li et al., 2008). Comparison of the genes invovled in acetyl-CoA pathway between the two genomes revealed high similarity in butyryl-CoA transferases which is the key enzyme in butyrate formation (Fig. 5B). The CAZyme analysis did not detect any polysaccharide lyases and in vitro testing confirmed the inability of both strains to utilize polysaccharides. Strain AF211 was observed to be characterized by a higher number of genes related to auxiliary activites, carbohydrate esterases, glycoside hydrolases and glycoside transferases (Fig. S1). Genes coding for sugar phosphorylation such as hexokinase, galactosekinase, xylulosekinase were detected, demonstrating potential to grow on hexose. Indeed laboratory tests showed good growth on galactose, arabinose, glucose and some growth on fructose, maltose and lactose in presence of 10mM acetate and 2g/l yeast extract but mannitol, cellobiose, raffinose, xylose, D-mannose, saccharose or sorbitol did not support growth (Fig. 5A). Butyrate production

was higher in culture of strain AF211 compared to SRB521^T when grown on galactose and arabinose. These two sugars are abundantly present in human diet, for instance, cereal grain, fruits , but not present in standard mouse diets. Therefore, the improved growth of the human strain AF211 on these sugars, in particular galactose or arabinose, may be a consequence of the adaptation to the human diet of the human isolate while the mouse isolate has less contact with these sugars.

A

Substrate	Strain	Consumption (mM)		Production (mM)			
		Glucose	Acetate	Lactate	Isobutyrate	Ethanol	Butyrate
GALACTOSE ACETATE	AF 211	0.3	4.95	0	1.20	1.93	6.83
	SRB 521^T	0.6	1.19	0.4	0.89	0	3.05
FRUCTOSE ACETATE	AF 211	0	1.58	0.1	1.01	1.04	3.47
	SRB 521^T	0.7	1.07	0	0.91	0	3.42
MALTOSE ACETATE	AF 211	0	0.76	0.1	0.97	0	2.40
	SRB 521^T	1.3	0.54	0	0	0	1.70
LACTOSE ACETATE	AF 211	1.15	1.06	0	1.05	0	2.75
	SRB 521^T	1.08	0.50	0.46	0	0	2.00
ARABINOSE ACETATE	AF 211	0.61	5.16	0.35	0.84	0.99	7.30
	SRB 521^T	0.89	0.12	0.44	2.63	0	2.11



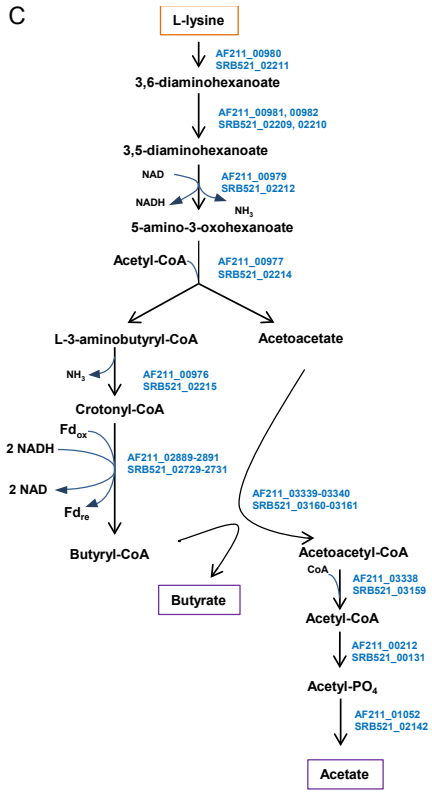


Figure 5: Butyrogenesis in strain AF211 and SRB521^T. A: Fermentation profiles of AF211 and SRB521^T in 10mM sugars and 10mM acetate for 2 weeks incubation. Product formation is calculated in mM. The highlighted values indicated significant discrepancy in butyrate formation and acetate consumption. Values were mean of duplicates and standard deviation were below 10 %. **B**: Genes involved in butyrate formation in strain AF211 and strain SRB521^T. The dark colour indicates low similarity of sequences of strain SRB521^T as compared to that of strain AF211. **C**: Lysine conversion pathway in both strains: locus tags are indicated in blue.

The amino acid lysine has been found as substrate for butyrogenesis in *I. butyriciproducens* AF211 (Bui et al., 2015). The strain SRB521^T showed similar capability of producing butyrate and acetate from lysine (Fig. S4) and the genome of this strain also coded for the entire lysine

pathway (Fig. 5C). Genes involved in the lysine pathway in strain SRB521^T and AF211 were compared (Fig. 5B) and found to have the same length and high nucleotide identities (above 95%), except for the acetyl-CoA: acetoacetate CoA transferase gene (about 70 %) (Fig. 5B-C). Thus, the lysine pathways could be a common feature of *I. butyriciproducens* species, irrespective of the host environment. This unique capacity of *I. butyriciproducens* to grow on lysine positions this strain at the cross-road between protein metabolism and butyrogenesis in the gut ecosystem.

Antibiotic resistance of *I. butyriciproducens*

The ability of antibiotics to modulate the gut ecosystem is well known. Strain AF211 and SRB521^T showed a similar sensitivity profile to the tested antibiotics (Fig. 6A). Several resistance mechanisms appear to be encoded by the genomes of the two strains (Fig. 6B). Multidrug resistance efflux pumps were abundantly present with >20 copies in both strains. These are non-specific transport systems used to export (or import) toxic compounds in many bacteria and involved in housekeeping functions (Poelarends et al., 2000). Antibiotic resistance analysis using the E-test showed highest minimum inhibitory concentration (MIC) on tetracycline (>256 µg/ml) for both strains and lowest MIC on teicoplanin (0.032 µg/ml for AF211 and 0.047 µg/ml for SRB521^T). These results were higher from that earlier reported for strain SRB521^T (Kläring et al., 2013) as the E-test was done in different media and incubation condition that might result in the different MIC values (Huys et al., 2002). Tetracycline is known as protein synthesis inhibitor that binds to the 30S subunit of ribosomes. High resistance might be due to the presence of tetracycline resistance of ribosome protection type in combination with multiple resistance efflux pumps, as demonstrated in *Pseudomonas aeruginosa* (Li et al., 1994) and *E. coli* (Okusu et al., 1996). The two strains were sensitive to teicoplanin, vancomycin, cefotaxime and oxacillin, which are known as inhibitors of cell wall synthesis in Gram-positive bacteria, but they showed relatively high MIC values with ciprofloxacin and sulfamethoxazole (Fig. 6B). Although (remnants of) some vancomycin resistance genes (vanB, vanW, vanS and vanR) were detected in the two genomes, these were not sufficient to provide vancomycin resistance, which requires a complex operon with at least 5 genes (Kruse et al., 2014). A set of genes predicted to code for resistance to inorganic compounds including arsenic resistance protein, cobalt-zinc-cadmium resistance protein, and copper resistance protein, were also found as part of the defence system of bacteria.

A

Antibiotics	*I. butyriciproducens* SRB521^T (ug/ml)	*I. butyriciproducens* AF211 (ug/ml)
Ciprofloxacin	>32	>32
Cefotaxime	0.064	0.064-0.05
Erythromycin	1	0.75-1
Oxacillin	0.38-0.5	0.38
Teicoplanin	0.032	0.047
Tetracycline	>256	>256
Tobramycin	1.5-2	2
Vancomycin	0.75	0.75
Chloramphenicol	0.75	0.75
Sulfamethoxazole	>32	> 32

B

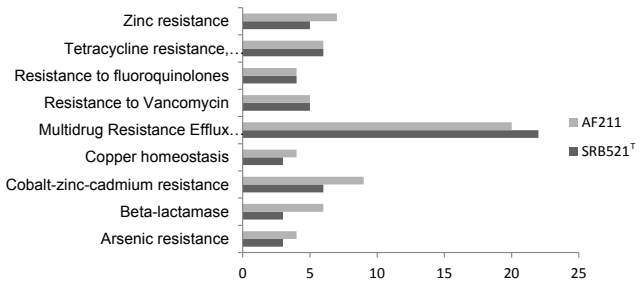


Figure 6: Resistance genes in strain AF211 and SRB521^T. A: Numbers of resistance genes in the genomes of *I. butyriciproducens* AF211 and SRB521^T. **B**: Minimum inhibitory concentration of the 2 strains (µg/ml).

Oxidative stress, transport systems and vitaimin production in *I. butyriciproducens*

The human intestine is considered as an anaerobic ecosystem, especially the large intestine, even though there is a gradient of oxygen from the epithelial layer outward (Albenberg et al., 2014). The reduction of oxygen results in production of superoxide radicals, hydrogen peroxide and hydroxyl radicals that are extremely toxic for cells (Fridovich, 1995). Hence, it is important for intestinal microbes that possess defence systems to prevent accumulation of these reactive oxygen species. Moreover, the oxidation of substrates and various xenobiotics might generate toxic oxidizing compounds in the environment, which intestinal anaerobes need to deal with for survival (Brioukhanov and Netrusov, 2004). Several genes predicted to have protective role under oxidative stress were detected in both strains (Table S3). Among them, CoA-disulfide reductase, rubrerythrin, superoxide reductase, and alkyl hydroperoxide reductase subunit C-like protein were detected in the proteome of strain AF211 (Bui et al 2015), indicative of active functions of these proteins. Superoxide reductase (EC 1.15.1.2) and manganese superoxide dismutase (EC 1.15.1.1) aid in lowering superoxide concentration and this process is carried out in the cytosol (Sheng et al., 2014) while catalase (EC 1.11.1.6) prevents accumulation of hydrogen peroxide. These enzymatic activities have been tested in strict anaerobes including Clostridia, *Bacteroides* spp., acetogens, sulfate

reducers and methanogens indicating a wide distribution among anaerobes (Brioukhanov and Netrusov, 2004). It has been reported that cell starvation also results in the synthesis of antioxidative defence systems apart from O_2 (Rocha and Smith, 1997). Genes encoding the protein rubrerythrin were most abundantly present in the genomes of either strain (6-8 copies). Rubrerythrin has a protective effect under oxidative stress, although its mechanism of action still remain unknown (Mukhopadhyay et al., 2007). In addition, a gene for the peroxide stress regulator PerR was found in both genomes. Interestingly, alkyl hydroperoxide reductase subunit C (AhpC) was found to be encoded by strain AF211 but not SRB521^T. AhpC is involved in organic peroxide detoxification (Rocha and Smith, 1999). The presence of different oxidative stress defence systems in two anaerobic strains enhances their survival in the gut environment where there is always a gradient of oxygen toward lumen.

Membrane transporters that facilitate the exchange of ions, nutrients and metabolites with the environment are essential for bacterial metabolism. A total of 102 genes were annotated as membrane transporters in the genome of strain AF211, and 86 in strain SRB521^T for the same mechanisms of transport system (Fig. S5). This suggests that strain AF211 has an improved overall capability of exchanging molecule compounds with its environment. For both strains, ECF class transporters and ABC transporters were the most prevalent groups (10 to 30 copies) while symporter and antiporter were the least abundant (<2 copies) (Fig. S5). The genomic analysis showed that strain AF211 had more copies of genes for ECF class transporters (30 versus 24), ABC transporters (47 versus 43), Nickel/ Cobalt transporter (9 versus 4) and TRAP transporter (8 versus 6) than strain SRB521^T, and this might result in better capability of transporting nutrients. The ECF transporters are present in various microbial lineages and responsible for transporting vitamins (Rodionov et al., 2009). ABC transporters for oligopeptides, dipeptides and branched-chain amino acids were found, indicating the capability of taking up these compounds, which agrees with our previous report of growth on different protein-derived sources (Bui et al., 2015). Galactose and multiple sugar ABC transporters were predicted from the genomes of both strains. Although sugar phosphotransferase systems (PTS) are often present in Gram-positive bacteria, no PTS system was found in the genomes.

Vitamin B12 is an important molecule that modulates the gut microbiota and host symbiosis (Degnan et al., 2014) and is produced by intestinal microbes (Allen and Stabler, 2008). This vitamin is an essential cofactor for bacterial metabolism; as a result there is microbial competition for uptake of this metabolite. In vitro experiments revealed that strain AF211 produced a pseudo-vitamin B12 (personal communication with Prof. Vieno Piironen and Bhawani Chamlagain in Helsinki University). Although we did not test for vitamin B12 synthesis in strain SRB521^T, genomic analysis of strain SRB521^T showed identical vitamin B12 synthesis pathway to that of strain AF211, indicating the capability of strain SRB521^T to produce a pseudo-vitamin B12 (data not shown). However, some genes of the corresponding

metabolic pathway appeared to be missing, but this can be attributed to the shortcomings of annotations currently available in present genome databases. Both isolates had vitamin B12 ABC transporter comprising of btuF (SRB521_01945, SRB521_01948 and AF_01368c, AF_01372c), btuC (AF_01370c, SRB521_01947) and iron B12 siderophore hemin periplasmic substrate-binding component of the ABC transporter. This suggests that the *I. butyriciproducens* strains have the ability to exchange B12 with the surrounding environment.

Conclusions

The 16S rRNA amplicon metadata search revealed that *I. butyriciproducens* is the most common known species within the *Intestinimonas* genus and is most prevalent in humans. Comparative genomic and physiological analysis of *I. butyriciproducens* strains AF211 and SRB521^T isolated from the human and mouse intestine, respectively, confirmed the unique capability of using lysine for growth as a major feature of the genus. Lysine is abundant in the gut as it is part of human diet and is released via microbial activity. Converting lysine not only prevents amino acid lost but also provides butyrate, an energy source for colonocytes, and releases ammonium, which can be used as nitrogen source by bacteria and neutralises acidity created by SCFA production. Interestingly, the two strains of *I. butyriciproducens* contained 2 butyrate synthesis pathways, where both lysine and simple sugars can serve as energy source. However, physiological data suggested that AF211 is more efficient in utilizing human-specific sugars such as arabinose and galactose in presence of acetate. Better growth of strain AF211 in comparison to strain SRB521^T was observed in media with or without bile. Both strain AF211 and SRB521^T are sensitive to most tested antibiotics except tetracycline and have similar mechanisms to prevent oxidative stress. In conclusion, the present study provides detailed genomic and physiological insight into newly discovered *I. butyriciproducens* strains with unique metabolic capabilities and supports for different ecological adaptations of *I. butyriciproducens* to the mouse and human gut environment.

Acknowledgement

This research was partly supported by ERC Advanced Grant 250172 - MicrobesInside from the European Research Council and the Netherlands Organization for Scientific Research (Spinoza Award and SIAM Gravity Grant 024.002.002) to WMdV.

Supplementary information

Supplementary Table S1: Physiological and biochemical comparison of *I. butyriciproducens* strain AF211 and SRB521^T. The experiments were performed by using API Rapid 32 (A), API 20NE (B), API ZYM (C). +: positive response; -: negative response; w: weak response; vw: very weak response.

A

API rapid 32	AF211	SRB521^T
Urease	-	-
arginine dihydrolase	-	-
α-galactosidase	-	-
β-galactosidase	-	-
β-galactosidase 6 phosphate	-	-
α-glucosidase	-	-
β-glucosidase	-	-
α-arabinosidase	-	-
β-glucuronidase	-	-
N-acetyl-β-glucosaminidase	-	+
mannose fermentation	-	-
rafinose fermentation	-	-
glutamic acid decarboxylase	-	-
α-fucosidase	-	-
reduction of nitrates	-	-
idole production	-	-
alkaline phosphatase	-	-
arginine arylamidase	-	-
proline arylamidase	-	-
leucyl glycine arylamidase	+	w
phenylalanine arylamidase	-	-
leucine arylamidase	-	-
pyroglutamic acid arylamidase	-	-
tyrosine arylamidase	-	-
alamine arylamidase	-	-
glycine arylamidase	-	-
histidine arylamidase	w	w
glutamyl glutamic acid arylamidase	-	-
serine arylamidase	w	w

B

API 20NE	AF211	SRB521^T
indole	-	-
urease	-	-
glucose	w	w
manitol	vw	-
lactose	vw	-
saccharose	vw	-
maltose	vw	-
salicin	vw	-
xylose	vw	-
arabinose	vw	-
gelatin	-	-
esculin	-	-
glycerol	-	-
cellobiose	-	-
mannose	-	-
melezitose	vw	-
raffinose	vw	-
sorbitol	vw	-
rhamnose	vw	-
trehalose	vw	-
catalase	vw	-

C

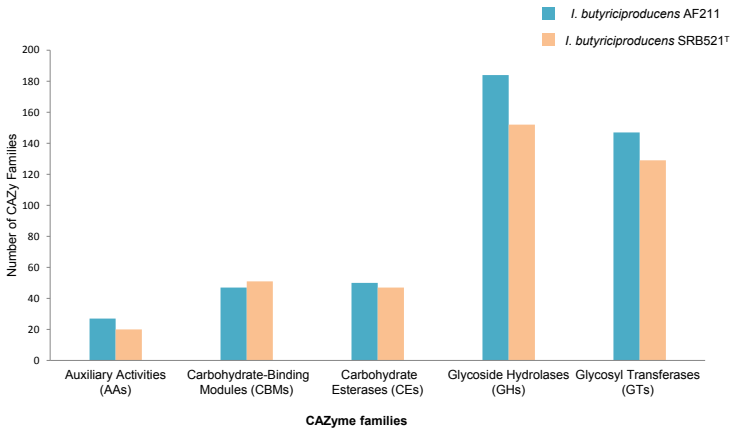
API ZYM	AF211	SRB521^T
Alkaline phosphatase	-	-
esterase E4	w	w
Esterase lipase C8	w	w
Lipase C14	-	-
Leucine arylamidase	-	-
Valine arylamidase	-	-
Cystine arylamidase	-	-
Trypsin	-	-
α-chymotrypsin	-	-
Acid phosphatase	w	+
Naphthol-AS-BI-phosphohydrolase	+	+
α-galactosidase	-	-
β-galactosidase	-	-
β-glucuronidase	-	-
α-glucosidase	-	-
β-glucosidase	-	-
N-acetyl-β-glucosaminidase	-	-
α-mannosidase	-	-
α-fucosidase	-	-

Supplementary Table S2: Cellular fatty acid composition of the strain AF211 and SRB521^T. Data were obtained in the present study. All strains were grown in RCM for 5 days at 37 °C. Values are percentages of total cellular fatty acids.

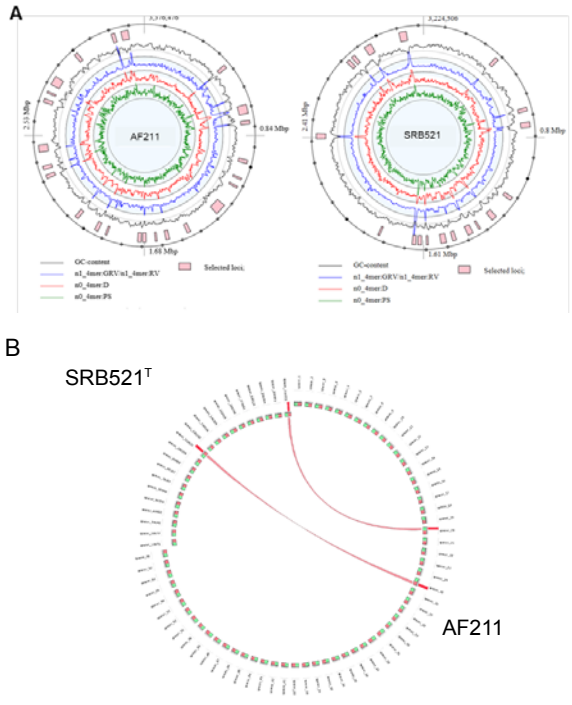
Cellular fatty acids	*I. butyriciproducens* AF211	*I. butyriciproducens* SRB521^T
12 : 00	4.33	3.15
14 : 00	32.08	39.83
16 : 00	3.12	2.3
18 : 00	12.79	4.06
19 : 00	ND	4.95
19 : 1 iso I	31.69	37.9
20 : 00	2.91	1.53
Summed feature 1 (15:1 iso H/13:0 3OH)	1.94	0.91
Summed feature 4 (17:1 iso I/antaiso B)	9.05	4.83

Supplementary Table S3: Number of genes associated to oxidative stress in AF211 and SRB521^T

Functions	*I. butyriciproducens* SRB521^T	*I. butyriciproducens* AF211
CoA-disulfide reductase	1	1
Peroxide stress regulator PerR, FUR family	1	1
Catalase	2	2
Manganese superoxide dismutase	2	2
Rubredoxin	2	3
Rubrerythrin	6	6
Superoxide reductase	2	2
Alkyl hydroperoxide reductase subunit C-like protein	0	1

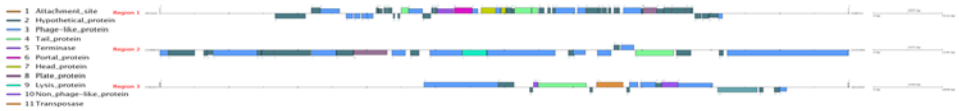


Supplementary Figure S1: CAZyme families detected in AF211 and SRB521^T

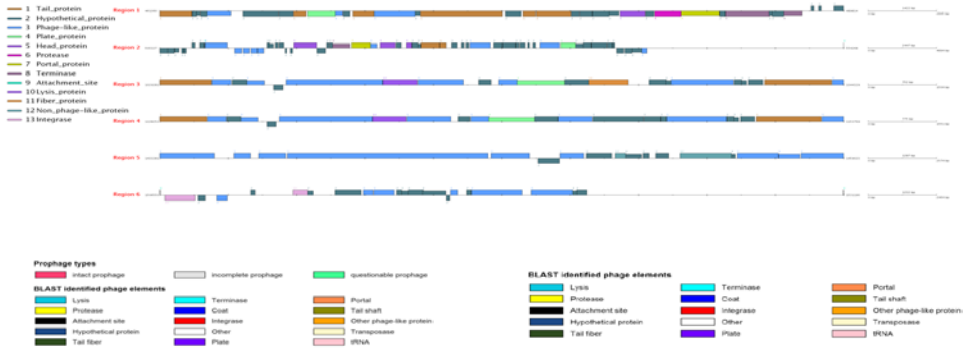


Supplementary Figure S2: Genomic islands and spacer comparison. A: Positions of Genomic islands in both AF211 and SRB521^T as predicted by SeqWord Sniffer. **B:** Spacer comparison.

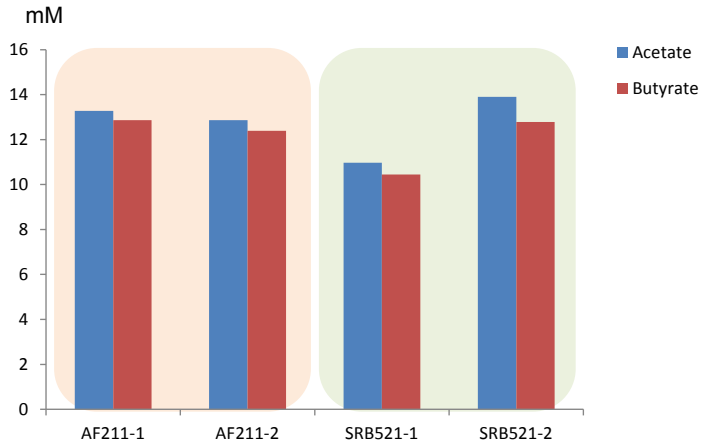
Prophage genes detected in *I. butyriciproducens* AF211



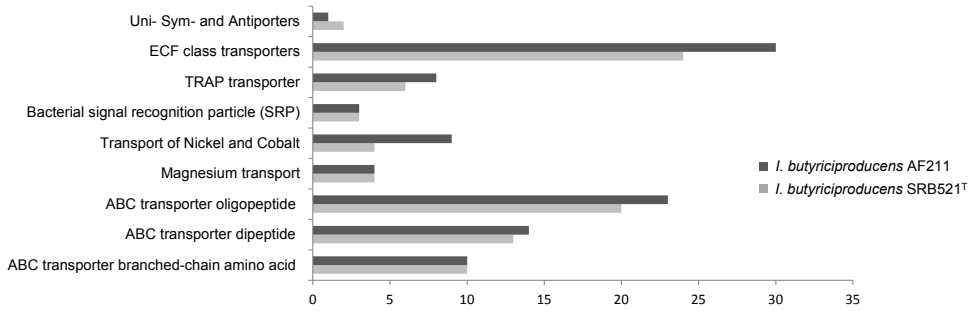
Prophage genes detected in *I. butyriciproducens* SRB521^{T}



Supplementary Figure S3: Prophage regions detected in *I. butyriciproducens* strain AF211 and SRB521^{T} genome sequences



Supplementary Figure S4: Lysine fermentation by AF211 and SRB521^{T}. Product formation was determined after 2 days of growth (mM). Orange and green boxes indicated the results from duplicate experiments of strain AF211 and SRB521^{T}, respectively. 15 mM L-lysine was added as substrate.



Supplementary Figure S5: Different transport systems in the genomes of AF211 and SRB521^T

CHAPTER 7

GENERAL DISCUSSION

Butyrogenic bacteria produce butyrate that is well known to be vital to intestinal health (Hamer et al., 2008). Several butyrogens are known to inhabit the human intestinal tract and estimations based on their 16S rRNA sequences indicate that this functional group is rather abundant, representing up to 20% of total microbiota (Louis et al., 2007a). Most of intestinal butyrate producers contain the so-called “butyryl-CoA transferase pathway” (BCT) except two *Coprococcus* species that utilize the butyrate kinase pathway (PTB-BK) (Flint et al., 2014). Many different culture-independent approaches have been applied to quantify butyrate-producing bacteria in the gut. Based on the presence of the key butyrogenesis genes that code for butyryl-CoA: acetate CoA-transferase and butyrate kinase, it has been suggested that most (more than 88%) of the butyrogens have been cultivated (Louis et al., 2010). Hence, only limited efforts to cultivate novel butyrogens have been reported in recent years. However, we should keep in mind that this approach was based on detection of the key genes of the two canonical butyrate production pathways and this analysis may not capture other butyrogenic pathways or genes. This may have led to an underestimation of the butyrogenic microbes. A recent metagenomics study showed the presence of butyrate synthesis pathways apart from acetyl-CoA pathway including lysine, hydroxyglutarate and 4-aminobutyrate/succinate routes in the human gut, although no intestinal microorganisms have been identified to possess these entire pathways (Vital et al., 2014). In the current work, new butyrate-producing isolates (Figure 1) are described with specific attention for their ecophysiology, their genomes, and their interactions with other intestinal microbes. The results of this study did not only expand the culture collection of intestinal butyrate producers with three new isolates (**Chapter 2, 4, 5**), but also provided the basis for the discovery of a new butyrogenic pathway that had not been considered as a potential route for butyrogenesis in the gut. This pathway represents another branch of the intestinal butyrogenesis in the gut where the amino acid lysine is converted into butyrate (**Chapter 5**). Consequently, this study significantly contributes to a further understanding of intestinal butyrogenesis. Butyrate is well known to be mainly associated with carbohydrate breakdown in the large intestine (Pryde et al., 2002; Louis et al., 2010). Lactate plus acetate are known substrates for butyric fermentation by some butyrogens such as *Anaerostipes* spp. and *Eubacterium hallii* (Duncan et al., 2004; Bourriaud et al., 2005) and *Eubacterium limosum* (lactate as only substrate for butyrate production) (Moore and Cato, 1965). In our study, we discovered that the amino acid lysine can serve as sole carbon source for some colonic butyrogens. Moreover, the Amadori product fructoselysine was also found to be readily metabolized into butyrate (**Chapter 5**). This is of interest in the context of human gut health and nutrition and suggests a potential approach to promote butyrate production in the colon.

New butyrogens in the gut

There is a great interest in obtaining functional insight in the intestinal microbiome. In recent years, several high throughput technologies have been developed to identify intestinal microbes by using targeted phenotypes, including antibiotic tolerance (Rettedal et al., 2014)

or specific host-microbe phenotypes (Faith et al., 2014). Personalized gut microbiota culture collections have been obtained when transplanting the fecal samples of healthy donors into gnotobiotic mice and culture collections could be manipulated via diets (Goodman et al., 2011). Another study suggested that strains involved in complex diseases could be identified via microbial inheritance study (Faith et al., 2015). Several high throughput devices have been developed to provide alternative cultivation platforms, such as the micro-Petri dish using a solid surface (Ingham, 2007), microfluidic devices that capture single cells (Leung et al., 2012) and laser tweezers Raman spectroscopy for monitoring spore germination (Wang et al., 2015a). However, when these targeted technologies or new platforms were used to culture strains from human intestinal ecosystem, only a few new and deposited isolates were obtained. Two novel bacteria have been isolated using antibiotic tolerance phenotypes including strain P1C11 (95 % similarity to *Eubacterium contortum*) and strain P2C1 (93 % similarity to *Oscillibacter ruminantium*) with sequenced genomes, but these two bacteria have not been deposited (Rettedal et al., 2014). A set of 11 new species has been identified from stool sample of a anorexia nervosa patient by MALDI-TOF, however, the bacteria were not properly taxonomically classified and these bacteria are not available for other researchers (Pfleiderer et al., 2013; Lagier et al., 2012). A novel *Oscillibacter* species (93 % similarity to *Oscillibacter valericigenes*) was cultivated using gene-targeted microfluidic cultivation approach without any deposition, while another new isolate could not be grown axenically (Ma et al., 2014). Hence, there remains a need to cultivate novel microbes to get a comprehensive understanding of the intestinal microbes, notably in relation with their beneficial impact on the human host such as butyrogens, and to have these novel microbes available for the scientific community. In the present study, we isolated the novel species *Anaerostipes rhamnosivorans* from the stool of a baby (**Chapter 2**) and *Intestinimonas butyriciproducens*, which belonged to the novel genus *Intestinimonas*, from either the stool of a healthy subject (**Chapter 5**) and or the mouse cecum (**Chapter 4**).

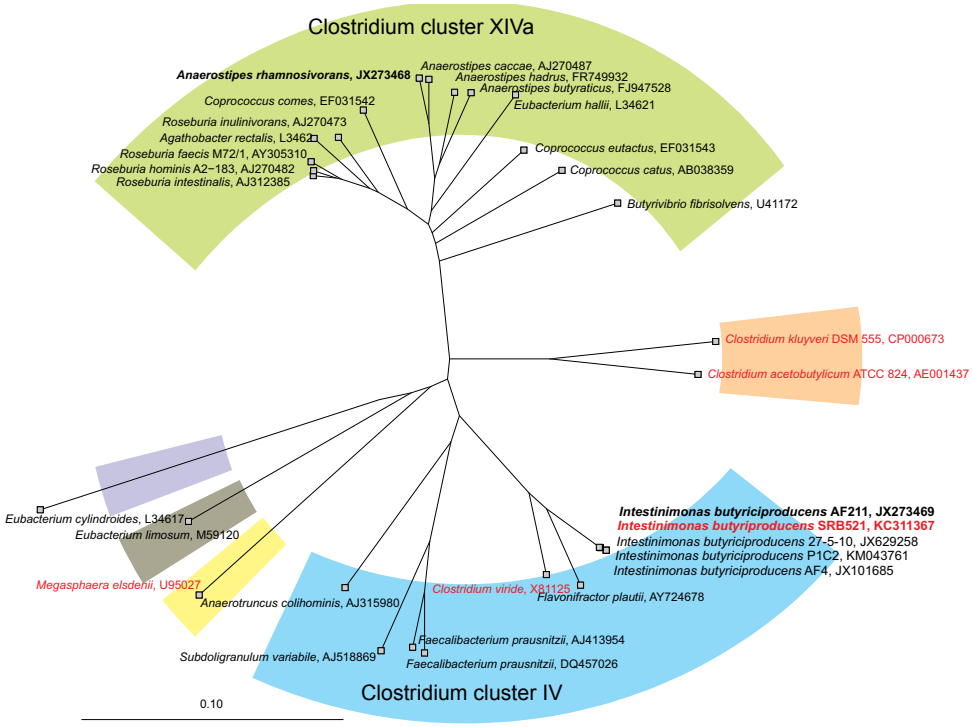


Figure 1: Phylogenetic tree of butyrate producing bacteria. The tree was constructed based on sequences of 16S rRNA genes retrieved from SILVA database. *Clostridium* cluster XIVa, IV, I, XVI, XV and IX are indicated in green, blue, pink, yellow, grey and purple, respectively. Species isolated from human source are in black while none-human isolates are in red. Strains reported in this thesis are in bold that are *Anaerostipes rhamnosivorans*, *Intestinimonas butyriciproducens* AF211 and *Intestinimonas butyriciproducens* SRB521^{T}. Bar represents 10% sequence divergence.

All of those are butyrate-producing bacteria belonging to the two dominant groups for butyrate synthesis in the gut:*Clostridium* cluster XIVa (*A. rhamnosivorans*) and *Clostridium* cluster IV (*I. butyriciproducens*) (Figure 1). So far, there have been more than a dozen of butyrate producers isolated that belong to *Clostridium* cluster XIVa, while only a few are classified to belong to *Clostridium* cluster IV (Figure 1). The phylogeny of these butyrate producers is rather diverse and they do not form a monophyletic group, although they share a similar metabolic phenotype. While they all form butyrate as an end product, there is a large divergence in the metabolic capability. While some butyrate producers are able to convert lactate plus acetate or glucose into butyrate (*Anaerostipes* spp. and *Eubacterium hallii*), others do not utilize lactate but consume acetate (*Faecalibacterium prausnitzii*, *Roseburia* spp and *Agathobacter rectalis*, recently reclassified from *Eubacterium rectale* (Rosero et al., 2015)). *Faecalibacterium prausnitzii* species have been reported to consist of two phylogenetic groups sharing the same metabolic features including substrate utilization, bile sensitivity and pH tolerance (Lopez-Siles et al., 2012).

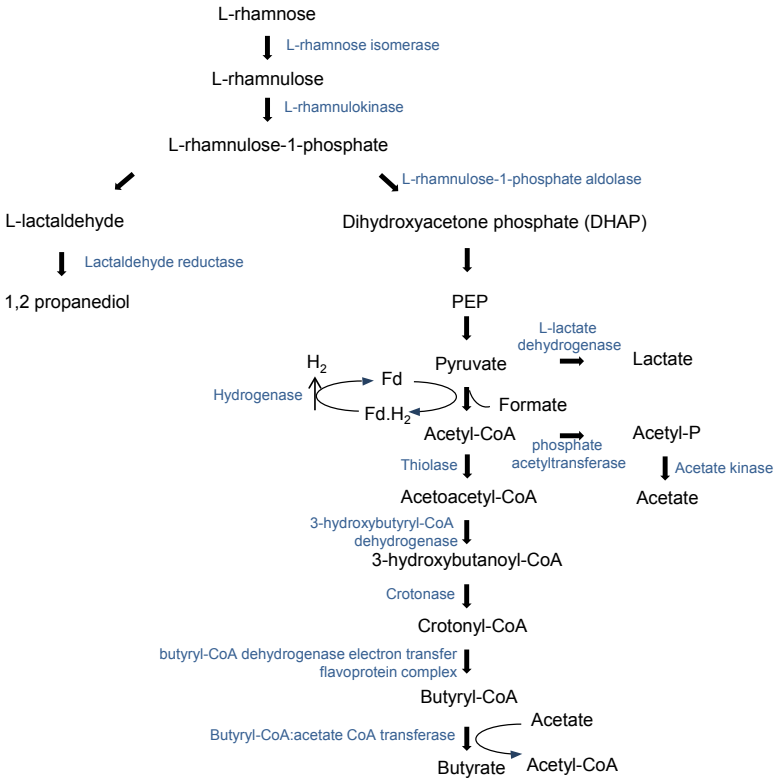


Figure 2: Predicted rhamnose fermentation pathway by *A. rhamnosivorans*. Genes involved are indicated in blue and these genes are found in the genome.

Unlike other *Anaerostipes* species, *A. rhamnosivorans* is not only able to produce butyrate from lactate and acetate or glucose, but does also convert rhammose into butyrate, extending the current known sugar substrates for butyrate production. The complete genome of *A. rhamnosivorans* was recently obtained using PacBio sequencing technology (manuscript in preparation). Its genome size was 3,588,860 bp and predicted to encode 3556 genes. The genomic analysis revealed the presence of the acetyl-CoA pathway as well as a complete rhamnose fermentation pathway (Figure 2). There is an increased attention for infant microbiome research, as early colonization may have long-term effects on the adult microbiota and consequently, on human health (Scholtens et al., 2012). Exposure to antibiotics in early life has been shown to be associated with the development of several diseases such as asthma or allergic diseases (Hoskin-Parr et al., 2013). The human milk oligosaccharides have been shown to stimulate the growth of beneficial bacteria, such as *Bifidobacteria* in infants (Thurl et al., 2010). *A. rhamnosivorans* could be one of first butyrate-producing bacteria colonising the infant intestine as *Anaerostipes* is among those genera that were detected in fecal samples from very young infants (de Weerth et al., 2013; Bäckhed et al., 2015).

Several bacteria have been reported to convert lysine into butyrate but they originated from other ecosystems rather than the gut. Those include the oral pathogenic *Fusobacterium nucleatum*, *Clostridium sticklandii* isolated from black mud, and *Clostridium subterminale* SB4 isolated from sewage. *A. rhamnosivorans* is likely to employ the acetyl-CoA pathway for butyrate production (**Chapter 2**) but *Intestinimonas butyriciproducens* has both the acetyl-CoA (BCT plus PTB-BK) and lysine pathway for butyrate synthesis (**Chapter 5**) that increases its metabolic flexibility upon substrate availability in the human gut. To our knowledge, this is the first example of an intestinal butyrogen that is known to have more than one butyrate synthesis pathway.

Table 1: Overview of most important cultivated butyrogens from the human GI tract.

Name	Acetyl-CoA pathway	Lysine pathway	Prevalence*	Genome	Isolation source	References
Intestinimonas butyriciproducens	+	+	0.15-9.8	complete	Human, mouse	Bui et al 2015
Faecalibacterium prausnitzii	+	-	3.8-15.4	complete	human	Flint et al 2006 Louis et al 2007
Subdoligranulum variabile	ND	ND	ND	partial	human	Holmstrøm et al 2004 Eckburg et al 2005
Anaerotruncus colihominis	ND	ND	ND	partial	human	Lawson et al 2004
Eubacterium cylindroides	+	ND		ND	human	Cato et al 1974
Eubacterium limosum	ND	ND	0.001	partial	human	Schwiertz et al 2000
Eubacterium hallii	+	-	0-3	complete	human	Louis et al 2007
Eubacterium rectale	+	-	2.3-8.8	complete	human	Duncan et al 2002 Aminov et al 2006 Duncan et al 2006
Roseburia intestinallis	+	-		partial	human	
Roseburia hominis	+	-		partial	human	
Roseburia faecis	+	-		partial	human	
Roseburia inulivorans	+	-		complete	human	
Anaerostipes caccae SSC/2, SS2/1	+	-	0-2	partial	human	Schwiertz et al 2002, Louis et al 2009
Anaerostipes hadrus	+	-	ND	partial	human	Allen-Vercoe et al 2012
Anaerostipes rhamnosivorans	+	-	ND	complete	human	Bui et al 2013
Coprococcus catus,	+	-	0.035	partial	human	Holdeman et al 1974, Louis et al 2007 Reichardt et al 2014
Coprococcus eutactus	+ (PTB-BK)	-	ND	partial	human	
Coprococcus comes	+ (PTB-BK)	-	ND	partial	human	

The prevalence indicates the percentages compared to the total number of bacteria. +, presence, -, absence, ND: not determined.

I. butyriciproducens was found to be a key player in butyrogenesis from lysine in the gut as its prevalence in Dutch healthy subjects was estimated to range from 0.15 to 10 % of the total intestinal microbiota based on a qPCR approach (**Chapter 5**). This was in agreement with the predicted abundance of the lysine pathway based on a metagenome survey in American subjects (Vital et al., 2014). However, *I. butyriciproducens* was not present at detectable levels in all adults studied. When analysing the deep metagenome information obtained from 65 subjects by the Human Microbiome Project, over half of subjects carried the genes for the lysine pathway, but the fructoselysine degradation pathway genes were only observed in half

a dozen subjects (approximately 10 %) (**Chapter 5**). This indicates that there might be other intestinal bacteria present that are capable of utilising lysine but not fructoselysine.

I. butyriciproducens represents a new cultured representative of a previously detected group of uncultured bacteria from several mammals, including human, mouse and pig (**Chapter 4**). The fact that we have isolated *I. butyriciproducens* using a conventional approach indicates that this approach other than high throughput technologies may be instrumental in obtaining new isolates. Some progress has been made for the isolation of several new bacteria. However, thorough physiological and proteogenomic analyses, that are the key to explore novel metabolisms, are lacking. Interestingly, the 16S rRNA sequence of *I. butyriciproducens* was found to be highly similar (>99 %) to that of a pig isolate (Levine et al., 2013) and two human isolates (Pfleiderer et al., 2013; Rettedal et al., 2014). Despite the different host origins, a detailed genomic and physiological comparison between *I. butyriciproducens* strains isolated from human and mouse indicated the capability of fermenting lysine to be the common feature of these species (**Chapter 5**). While the human strain AF211 was isolated using an anaerobic medium containing lactate plus acetate as substrates with N_2/CO_2 in the gas phase (**Chapter 5**), the mouse strain SBB521^T was obtained using a medium containing lactate and sulphate as electron donor and acceptor, respectively with N_2/H_2 in the gas phase (**Chapter 4**). Remarkably, another strain of *I. butyriciproducens* was isolated from the mucosa surface of swine but has not been taxonomcially identified yet (Levine et al., 2013). This swine strain was reported to have the capability to degrade mucin but we did not observe this feature in the human and mouse isolate (data not shown). This is supported by the genomic analysis of the human and mouse strains that did not reveal the presence of canonical genes involved in mucin degradation, such as sialidases, fucosidases, exo- and endo-β-N-acetylglucosaminidases, sulfatases (Derrien et al., 2010; Tailford et al., 2015). The metadata analysis on 16S rRNA amplicons of unknown Ruminococcaceae from the Sequence Read Archive (SRA) predicted that the *Intestinimonas* genus contained at least 3 species, with *I. butyriciproducens* being the most common (**Chapter 6**). The species *I. butyriciproducens* was estimated to be most prevalent in humans and mice. The physiological and genomic comparison between the human and mouse isolates indicated differences in efficiency in metabolising some sugars, growth rate, cellular fatty acid composition and genome architecture. The high throughput approaches including metagenomics have been implemented to define the microbial community in humans at high taxonomic orders, for instance genus level. However, there might be much functional divergence at species or even strain level. Novel sequencing technologies and bioinformatics approaches are needed to facilitate the identification at lower taxonomic levels. For instance, the whole genome focused array SNP typing (WG-FAST) method has been developed to discriminate metagenome data at the species level (Sahl et al., 2015). Another approach using metagenomic data, where single nucleotide polymorphisms (SNPs) are being targeted, could identify genomic variations within species (Schloissnig et al., 2013).

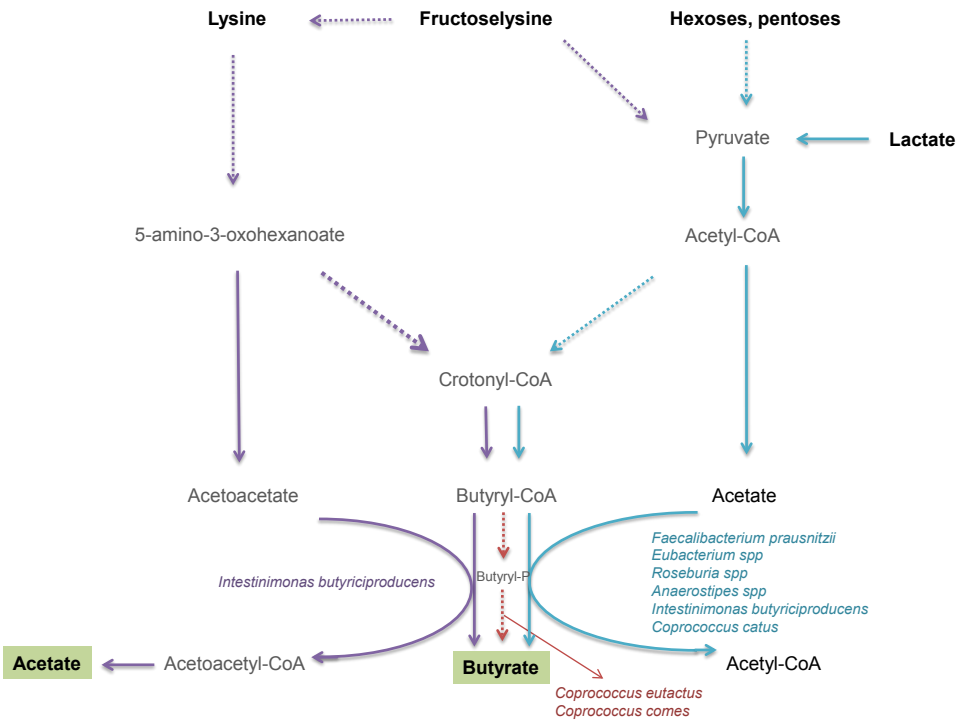


Figure 3: Butyrogenesis by intestinal microbes. Blue arrows indicate the routes for butyrogenesis from carbohydrate while purple arrows are for amino acid-derived substrates (lysine and fructoselysine). Dashed purple, blue arrows indicate multiple steps within corresponding pathways. Red dashed arrows indicate the butyrate production via butyrate kinase. The last step in BCT pathway is indicated in blue and catalysed by butyryl-CoA:acetate CoA-transferase while the last step in the lysine pathway is indicated in purple and catalysed by butyrate:acetoacetyl-CoA transferase AtoD-A. Bacteria that are able to perform these pathways are indicated in the corresponding colours.

We have not only discovered novel butyrate-producing bacteria but also a novel intestinal butyrate synthesis pathway, in which the amino acid lysine and the Amadori product fructoselysine serve as substrates (Figure 3). While there are many butyrate-producing bacteria including *Faecalibacterium prausnitzii*, *Anaerostipes* spp., *Eubacterium* spp., *Roseburia* spp., *Coprococcus* spp., known to have acetyl-CoA pathway, *I. butyriciproducens* AF211 is the only one so far possessing the lysine and fructoselysine pathway as well as acetyl-CoA pathway (Figure 3). Genes involved in entire fructoselysine degradation pathway have been identified in the genome of *I. butyriciproducens* AF211 **Chapter 5**).

Butyrate synthesis pathways

The acetyl-CoA (BCT plus PTB-BK) pathway is known as the only butyrate synthesis pathway when carbohydrates are fermented (Figure 3). In our study, we demonstrated that butyrate is also produced from lysine and fructoselysine (Figure 3). Butyrate production from hexoses or the intermediates lactate/acetate has been well characterised. Acetate is known to strongly stimulate the growth of *Faecalibacterium prausnitzii* on glucose (Duncan et al., 2002b; Heinken et al., 2014). This has been rationalized as acetate is predicted to generate additional ATP by means of a proton motive force (Louis and Flint, 2009). This energy conservation is generated via butyryl-CoA dehydrogenase (Bcd) electron-transferring flavoprotein (Etf) complex that creates a proton gradient via a membrane-associated NADH : ferredoxin oxidoreductase (Li et al., 2008). For *I. butyriciproducens* this additional ATP significantly increased the growth yield in presence of acetate (**Chapter 5**). The sugar fermentation was much improved when acetate was added (**Chapter 6**). It is often seen that other reduced metabolites (such as formate, lactate, acetate) are also produced when butyrate is produced from hexoses (**Chapter 2**), while the lysine fermentation leads to the production of only acetate and butyrate (**Chapter 5**). The presently described intestinal butyrate-producers are capable of utilizing the acetyl-CoA pathway only for butyrate formation. In contrast, *I. butyriciproducens* is able to employ both acetyl-CoA and lysine pathways simultaneously. Hence, we anticipated induction of proteins involved in the lysine pathway under lysine growth condition. Therefore, a detailed proteome analysis of grown cells in lysine and glucose/acetate was performed. The proteogenomic results indicated the presence and operation of the entire lysine and fructoselysine pathway. This included the lysine utilization operon (AF_00976-00982) coding for 6 enzymes involved in converting lysine into butyrate and acetate (Figure 4). An operon-like cluster (AF_00949-00955) with genes for fructoselysine and psicoselysine uptake and degradation was also found and the abundance of these proteins was significantly increased during the growth in lysine (**Chapter 5**). The high concentration of lysine in the medium induces the production of proteins involved in the uptake and degradation of fructoselysine. The conversion of fructoselysine into lysine and glucose-6-phosphate has been previously reported as a reverse reaction in *E. coli*, which is not able to produce butyrate from any substrate (Wiame et al., 2002). The operation of the acetyl-CoA pathway was also observed when lysine was used as growth substrate that is probably due to the shared step of central conversion of crotonyl-CoA to butyryl-CoA catalysed by butyryl-CoA dehydrogenase/Etf complex (Li et al., 2008; Buckel and Thauer, 2013). We identified the specific butyrate:acetoacetyl-CoA transferase AtoD-A (AF03339-03340) involved in butyrate formation in the lysine pathway, butyryl-CoA transferase But (AF_02986) involved in butyrate formation in acetyl-CoA pathway and acetocetyl-CoA AtoC (AF_00155) that is involved in balancing the intermediates acetoacetate and acetoacetyl-CoA of the two pathways (Figure 4 and **Chapter 5**).

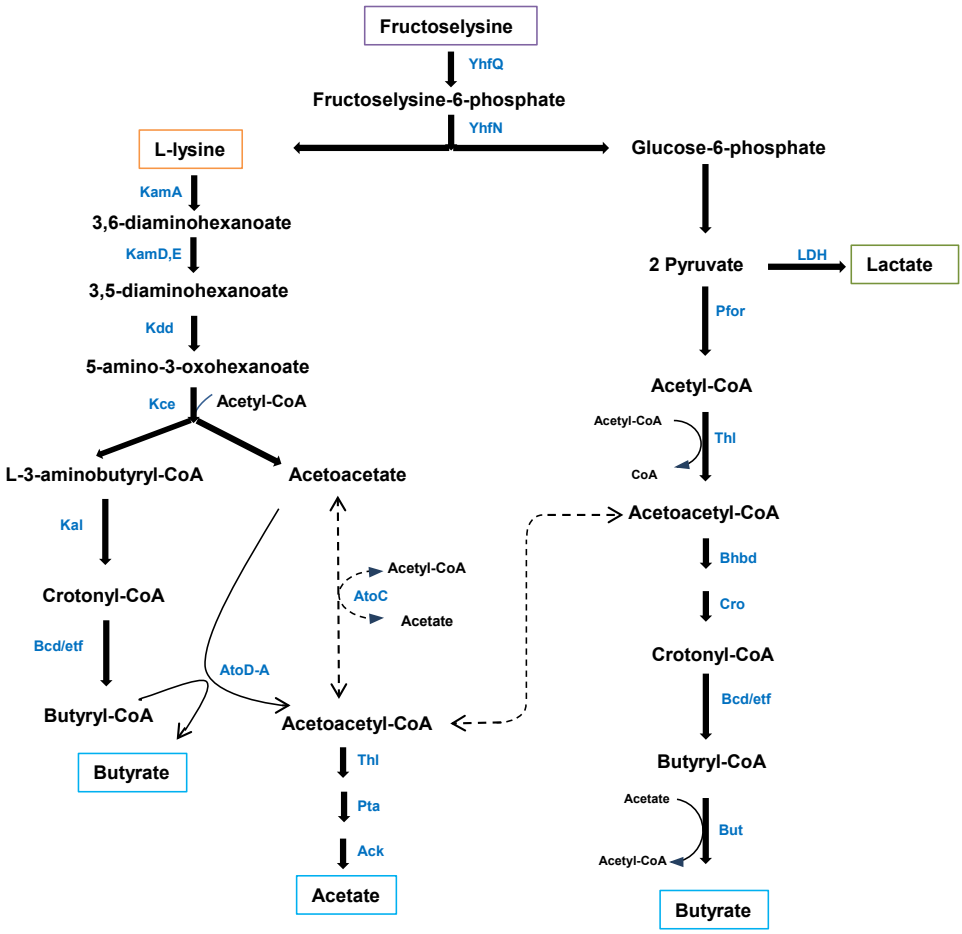


Figure 4: Fructoselysine degradation by *I. butyriciproducens* AF211. Substrates and products are indicated in boxes. Intracellular transfer of intermediates between the lysine and acetyl-CoA pathway is indicated in dashed arrows. YhfQ: fructoselysine kinase; YhfN: fructosesamine deglycase; KamA: lysine 2,3-aminomutase; KamD, E: L-beta-lysine 5,6-aminomutase alpha and beta subunit; Kdd: 3,5-diaminohexanoate dehydrogenase; Kce: 3-keto-5-aminohexanoate cleavage enzyme; Kal: 3-aminobutyryl-CoA ammonia lyase; Bcd/etf: butyryl-CoA dehydrogenase electron transfer flavoprotein complex; AtoD-A: butyrate-acetoacetate CoA-transferase alpha and beta subunit; AtoC: Acetyl-CoA: acetoacetate CoA transferase; Thl: thiolase; Pta: phosphate acetyltransferase; Ack: acetate kinase; Pfor: pyruvate:ferredoxin oxidoreductase; LDH: lactate dehydrogenase; Bhbd: 3-hydroxybutyryl-CoA dehydrogenase; Cro: crotonase; But: butyryl-CoA transferase.

The intracellular transfer of intermediates between the two pathways was observed when growing the *I. butyriciproducens* on lysine and ^{13}C-labelled acetate. This intracellular transfer was likely to take place also during fructoselysine degradation, as lysine and glucose-6-phosphate are released after initial fructoselysine cleavage (Figure 4). This resulted in the complete depletion of lysine and sugar moiety, and butyrate was detected as the major product. The impact of exogenous acetate was also observed in the fructoselysine metabolism and its addition results in a maximum of 3 butyrate formed from 1 fructoselysine (**Chapter 5**). This feature is likely triggered by environmental conditions in the gut ecosystem where butyrate is highly preferable and acetate is used to be plentifully present. Acetate is formed by many groups of intestinal bacteria and can make up more than half of total fecal SCFAs (Table 2 - (Cummings et al., 1987)). The impact of acetate on the fructoselysine fermentation could be considered as an environmental factor. It could also be that the high concentration of intestinal acetate stimulates the growth of acetate-consuming microbes with an efficient acetate-utilizing metabolism to avoid excess colonic acetate. The overproduction of acetate might result in lowering pH in the gut environment considering that the pKa of acetate is 4.7. Moreover, the production of ammonia from lysine/fructoselysine may neutralize acidification of the gut from mixed acid fermentations. It would be interesting to investigate if other cultured isolates carry the lysine pathway by screening their genomes or continue cultivation of lysine degrading bacteria from stool samples using lysine as a sole carbon source (Figure 5).

Table 2: Short chain fatty acid (SCFA) production in different locations of the human intestine.

	Acetate	Propionate	Butyrate	Lactate	Total SCFA productions
Jejunum	0.6 ± 0.6	-	-	2.0 ± 1.2	<1
Ileum	7.9 ± 4.1	1.5 ± 1	2.3 ± 1.3	13.5 ± 5.5	13 ± 6
Caecum	69.1 ± 5	25.3 ± 3.7	26.1 ± 3.8	4.5 ± 1.4	131 ± 9
Colon-A	63.4 ± 6.8	26.7 ± 4	24.5 ± 4.2	3.1 ± 1.7	123 ± 12
Colon-T	57.9 ± 5.4	23.1 ± 2.8	24.4 ± 2.2	3.5 ± 2.2	117 ± 9
Colon-D	43.5 ± 11.1	14.2 ± 3.1	14.7 ± 2.9	3.1 ± 2.1	80 ± 11
Sigmoid/rectum	50.1 ± 16.2	19.5 ± 6.7	17.9 ± 5.6	1.5 ± 1.5	100 ± 30

Values represent mmol/kg contents. -: under detection limits or less than 0.1 mmol/kg. NH3 was not determined. Data collected from Cummings et al., 1987. The ascending, transverse and descending colon is indicated by colon-A, colon-T and colon-D, respectively.

A recent metagenomics survey indicated that there are two other butyrate synthesis pathways in the human gut (Vital et al., 2014). These are α- hydroxyglutarate and 4-aminobutyrate/succinate pathways where glutamate and 4-aminobutyrate serve as substrates (Vital et al., 2014). This finding suggests that there is another fraction of the glutamate metabolism contributing to the butyrate formation in the gut. The amino acid glutamate is a major constituent of dietary protein and consumed as a food additive in the form of monosodium glutamate. Moreover, glutamate is also formed via transamination of many amino acids including arginine, ornithine, proline, histidine and glutamine (Brosnan, 2000). Dietary glutamate is metabolised rapidly to carbon dioxide in the intestinal enterocyte and this is a major metabolic fate in the intestine (Burrin and Stoll, 2009). However, glutamate can also be efficiently fermented by the intestinal microbiota (Smith and Macfarlane, 1997). Based on the metagenome analysis of 15 stool samples of healthy individuals, it was estimated that the abundance of the hydroxyglutarate pathway was 2.5 % at average, ranging from 0.8 % to 9.6 % of all butyrate synthesis pathways (Vital et al., 2014). Thus, butyrogenic amino acid and specifically glutamate degradation is of interest for future research (Figure 5).

Impact of butyrate production from protein

Butyrate formation is mainly associated with carbohydrate breakdown in the large intestine while the protein fermentation is mostly regarded as detrimental to the gut (Macfarlane and Macfarlane, 2012). Some metabolites of microbial fermentation of aromatic amino acids, however, have been found to be beneficial for human health, for instance, phenylacetic and 4-hydroxylphenylacetic acid that are known as antioxidants in living cells (Russell et al., 2013). In our study we show that protein-derived sources also promote butyrate production via lysine and fructoselysine fermentation pathways (**Chapter 5**). This contributes considerably to the understanding of microbial metabolism in the context of gut health and nutrition. Moreover, it opens up a new possibility of targeted approaches to improve gut health. Lysine is one of the essential dietary amino acids that mammals cannot synthesize. Hence these higher organisms must ingest the amino acid as lysine or lysine-containing proteins via their diets. Eggs, meat, soy, beans peas, cheese and certain fish are known to be sources of lysine as they have a high lysine content varying from 5 % to 9 % of total protein. There is also a significant fraction of microbial lysine synthesis that contributes to host lysine homeostasis (Metges et al., 1999). It has been suggested that intestinal bacteria can salvage urea and incorporate this into amino acids which are later absorbed by the host (Jackson, 1995). Urease activity was detected in many intestinal anaerobes (Suzuki et al., 1979) and the transfer of nitrogen from urea to amino acid in the infant intestine has been suggested, although the bacteria responsible for the urea conversion are not well defined (George M et al., 1996; Millward et al., 2000). In plants, some fungi, algae and most bacteria, lysine is synthesized from aspartic acid and ammonia (Scapin and Blanchard, 2006; Liu et al., 2010).

Our study suggests that lysine degradation via microbial activity is also taking place in the

gut (**Chapter 5**). This confirms and extends previous descriptions of lysine conversion by a fecal suspension resulting in the butyrate formation (Smith and Macfarlane, 1997, 1998). The lysine pathway has been reported as the second most abundant in the gut (Vital et al., 2014). This suggests that lysine degradation by microbes might be a substantial factor depleting lysine and contributing to butyrate production in the gut. Therefore, lysine equilibrium must be settled to maintain lysine homeostasis amongst dietary supply, host absorption, biosynthesis and microbial degradation, lysine excretion. The amount of amino acid lysine supply varies depending on our dietary intake and may explain the high variety in the prevalence and presence of *Intestinimonas* spp. in the GI tract of healthy volunteers (**Chapter 5**). Given the fact that lysine is often detected in the fecal samples (Kirsner et al., 1949; Smith and Macfarlane, 1998; Sukemori et al., 2003) the lysine uptake and endogenous microbial lysine must be sufficient for both the human host and to sustain microbial activity. Hence, lysine-fermenting butyrogenic bacteria including *I. butyriciproducens* might play an important role in the intestinal tract by maintaining the lysine balance and the gut homeostasis.

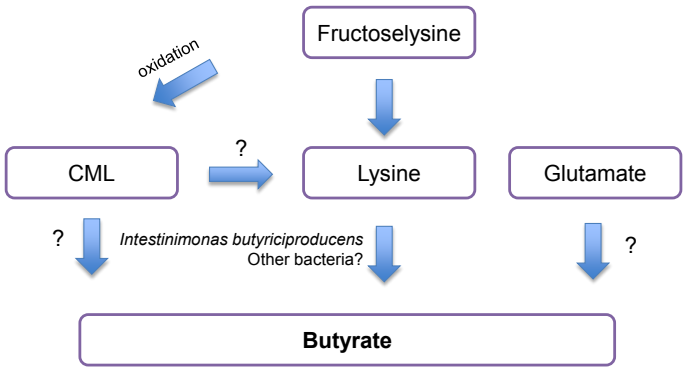


Figure 5: Butyrogenesis from protein metabolism – future perspectives.

The Amadori product fructoselysine could be utilised by *I. butyriciproducens* AF211 and was found to be fully metabolised to butyrate. *Escherichia coli* and *Bacillus subtilis* have been found to degrade fructoselysine under aerobic conditions but do not utilize lysine when provided as substrate and do not form butyrate (Wiame et al., 2002; Wiame and Van Schaftingen, 2004). Amadori products are implied as advanced glycation end products (AGEs) that are formed when reducing sugars react in a non-enzymatic way with amino acids in proteins and other macromolecules during heating process or storage of foods. In particular, frutoselysine is one of common Amadori products and is formed between lysine and glucose molecules during heating treatments of foods. Subsequently, the formed fructoselysine could be oxidised to N-carboxymethyllysine (CML), an oxidative degradation product of fructoselysine and often used as a biomarker for long-term protein damage (Luevano-Contreras and Chapman-Novakofski, 2010), and this oxidative conversion is dependent on pH, temperature and gases (Ahmed et al., 1986). In addition, AGEs have been implicated in the progression of aging and

age-related chronic diseases (Glenn and Stitt, 2009; Semba et al., 2009). AGEs are produced in foods during heating process (dietary AGEs or exogenous AGEs) but also in humans (endogenous AGEs) (Luevano-Contreras and Chapman-Novakofski, 2010). Increasing the time and exposure to heat can accelerate the formation of dietary AGEs in processed foods. Dietary intake of AGEs contributes significantly to the body AGE pool (He et al., 1999; Šebeková et al., 2001; Vlassara et al., 2002; Uribarri et al., 2005). AGEs are suggested to play an important role in healthy aging as well as chronic disease morbidity (Luevano-Contreras and Chapman-Novakofski, 2010). There are several aromatic compounds that can be potential inhibitors of the formation AGEs *in vivo*, such as vitamin C, E, aspirin, salicylic (Rahbar and Figarola, 2003). Natural phenols may inhibit the negative effects of the AGEs on vascular smooth muscle cells, for instance resveratrol (Mizutani et al., 2000) or eliminate the effect of AGEs on gene expression of receptor for AGEs in hepatic stellate cells, such as curcumin (Tang and Chen, 2014). This discovery of a bacterium capable of producing butyrate from lysine and the Amadori product fructoselysine indicates that, apart from sugars, protein fermentation also leads to butyrate production. Hence, further research should explore colonic butyrogenesis from protein fermentation, instead of the sole focus on sugar degradation. Our study indicates a potential approach employing microbial therapy to prevent the accumulation of intestinal fructoselysine as well as promote the butyrate production from protein. As high levels of food AGEs have been associated with age-related dementia (Cai et al., 2014), which is a chronic or persistent disorder of the mental processes caused by brain disease or injury, it could be interesting to study the presence and prevalence of *I. butyriciproducens* species as well as fructoselysine pathway in a healthy cohort and a dementia cohort, that might have an indication for intervention study. It might also be interesting to perform mouse experiments to study whether the colonisation of *I. butyriciproducens* in the mouse gut affects the host phenotype. Eating habits also influence on the amount of fructoselysine intake. It is clear that only some of humans do have the fructoselysine pathway (**Chapter 5**). It has been shown that the plasma level of AGEs (specifically CML) in vegetarians was higher than that of omnivores (Šebeková et al., 2001). This could be explained by the activity of intestinal microbes, which are able to degrade CML, in omnivores but not or less active in vegetarians. Recently, the degradation of CML by intestinal microbes has been observed (Hellwig et al., 2015). One of the major differentiating factors between human and other primates is that humans eat mainly processed foods while other primates consume raw products, such as fruit, leaves, insects etc. Genomic analysis revealed the presence of fructoselysine degradation cluster in the mouse isolate, suggesting a capability of the mouse *I. butyriciproducens* to utilize fructoselysine. However, in the present practice mouse feed is always heated to prevent contamination. Moreover, the human and mouse strains are genomically very similar, suggesting a recent evolutionary history (**Chapter 6**). It would be interesting to investigate the presence of the fructoselysine degradation pathway in vegetarians, vegans and raw food eaters but also in other primates or animals. It might explain the evolution of intestinal microbes that acquire the fructoselysine degradation after the cooking invention. It would also be interesting to

see if other amino acid derivatives can be used as substrate by intestinal microbes. In this context, it would be of interest to test the capability of *I. butyriciproducens* AF211 to degrade carboxymethyllysine (CML), which has been associated with increased oxidative damage to collagen in diabetes (Baynes, 1991) (Figure 5).

Coherent microbial interaction in GI tract

Living in a high-density ecosystem, like the human gut ,creates an opportunity for intestine microbes to interact with each other. Several studies addressed microbial interactions in the gut, including *Bifidobacterium* and butyrate-producing bacteria on starch (Duncan et al., 2004) as well as butyrate-producing bacteria and *Desulfovibrio piger* on lactate plus exogenous sulphate (Marquet et al., 2009). We have extended these interaction studies and tested a variety of gut-relevant microbes for their capacities to form trophic chains or be involved in cross feeding. We used *A. rhamnosivorans* as a model for a butyrate-producing bacterium and investigated the interaction between this butyrate producer with primary degrader *Bacteroides thetaiotaomicron* and acetogenic bacterium *Blautia hydrogenotrophica* and methanogenic archaeon *Methanobrevibacter smithii* (Figure 6 - **Chapter 3**). Cocultures were incubated in the presence of pectins as these polymers are abundantly present in our foods and contain rhamnose residues. However, we did not observe rhamnose fermentation via 1,2 propanediol production (**Chapter 3**). This is likely due to the limited access to rhamnose residues in the pectin fractions as rhamnose is a branched sugar within a complex backbone (Olano-Martin et al., 2002; Coenen, 2007; Voragen et al., 2009). Another possibility is that 1,2 propanediol (as reduced end product) was not produced, as redox was well balanced. It has been well established that substrate availability determines the microbial community (Louis et al., 2007a). Therefore, the cross-feeding between *A. rhamnosivorans* and *Bacteroides thetaiotaomicron* in two dietary pectin fractions could provide proof of principle that the butyrogen is able to benefit for butyrate formation from different dietary substrates via the partner. Another cross-feeding we observed was that with *A. rhamnosivorans* and *Blautia hydrogenotrophica* in a medium containing lactate and a small amount of acetate. Despite the fact that there are many groups of lactate-producing bacteria in the human intestine that contribute significantly to intestinal lactate production; lactate is hardly detected as end product in human stool. This shows that the lactate consumption is rapidly carried out by different groups of bacteria. This was demonstrated in coculture of *A. caccea* and *E. hallii* with *Bifidobacterium adolescentis* (Duncan et al., 2004) and also with *Desulfovibrio piger* (Marquet et al., 2009). In our experimental set up, lactate was also quickly consumed in the coculture of the butyrogenic lactate-utilizing bacterium *A. rhamnosivorans* and the hydrogenotrophic acetogenic bacterium *Blautia hydrogenotrophica* and these bacteria were able to continue this metabolic interaction till all substrate was depleted. This cross-feeding not only prevents the lactate and hydrogen accumulation but also contributes significantly to butyrate formation. The cross-feeding between butyrogenic xylanolytic *Roseburia intestinalis* or cellulolytic microbes and hydrogenotrophic species have been suggested on xylan and cellulose in vitro, respectively (Robert et al., 2001; Chassard

and Bernalier-Donadille, 2006). Interspecies hydrogen transfer has been reported to occur. We observed the same phenomenon in the coculture of the butyrate producer (*Anaerostipes rhamnosivorans*) and the acetogenic bacterium (*Blautia hydrogenotrophica*) on one hand and the butyrate producer and the methanogenic archaeon (*Methanobrevibacter smithii*) on the other hand. The latter is interesting as it changes the product profile of the butyrogenesis in the coculture. Instead of forming lactate and acetate as the butyrogenic species does in the pure culture, in coculture lactate levels were much lower in the coculture. Consequently, much more butyrate was produced. That is likely due to the growth of methanogenic partner that consumes formate and hydrogen and pulls the metabolism towards forming hydrogen and formate rather than lactate (**Chapter 3**). Although the role of this methanogenic archaeon to remove intestinal hydrogen and/or formate is well accepted (Samuel et al., 2007), the impact of this archaeon to elevate butyrate production was a novel finding. Our study showed the in vitro metabolic interactions between predominant intestinal microbes; hence, that may represent coherent interactions in the gut ecosystem.

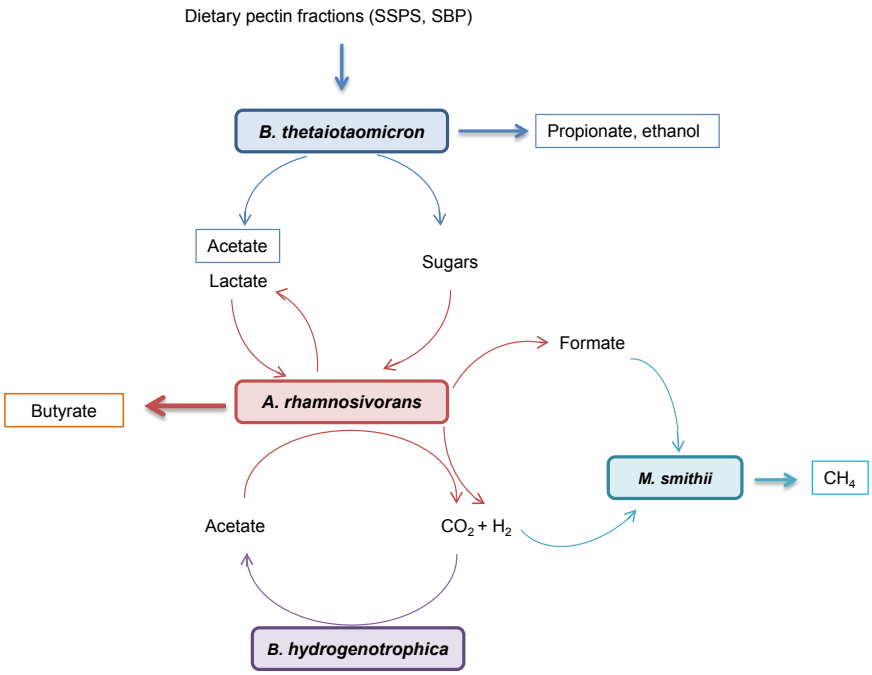


Figure 6: Schematic illustration of the interactions between *A. rhamnosivorans* and intestinal microbes in different substrates. Orange, blue, purple and indigo arrows indicate the conversion by *A. rhamnosivorans*, *B. thetaiotaomicron*, *B. hydrogenotrophica* and *M. smithii*, respectively. End products of the cocultures and monocultures are in rectangle panels while intermediates are not.

Concluding remarks and perspectives

Butyrate and butyrate-producing bacteria play a vital role to maintain a healthy intestine. This thesis gained insight in the process of intestinal butyrogenesis and expanded the sources for butyrate production to rhamnose and amino acid lysine and its derivative, fructoselysine (Figure 4, 5). Whether or not other amino acids can serve as source for butyrate production requires further investigation. The results not only contribute to better understanding of the colonic butyrogenesis but also indicate a potential approach for enhancing intestinal butyrate production as well as reducing fructoselysine – a precursor of AGEs that are associated with aging and chronic disease. Therefore, *Intestinimonas butyriciproducens* could be used as biomarker for a healthy gut. This strain also has potential as a functional microbe, which could improve gut barrier function via its butyrate formation. The cultivation of *A. rhamnosivorans* from an infant stool also indicates that this strain might play a role as one of the first butyrate-producing bacteria that colonises in the infant gut. The interactions between *A. rhamnosivorans* and *Bacteroides thetaiotaomicron*, *Blautia hydrogenotrophica* and *M. smithii* in different combinations clearly indicate the high metabolic flexibility that allows the bacterium to benefit from different types of microbes for optimal butyrate production.

APPENDICES

References

Abell, G., Conlon, M., and McOrist, A. (2006) Methanogenic archaea in adult human faecal samples are inversely related to butyrate concentration. *Microbial Ecology in Health and Disease* 18: 154 - 160.

Ahmed, M.U., Thorpe, S.R., and Baynes, J.W. (1986) Identification of N epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *Journal of Biological Chemistry* 261: 4889-4894.

Albenberg, L., Esipova, T.V., Judge, C.P., Bittinger, K., Chen, J., Laughlin, A. et al. (2014) Correlation between intraluminal oxygen gradient and radial partitioning of intestinal microbiota in humans and mice. *Gastroenterology* 147: 1055-1063.

Allen-Vercoe, E., Daigneault, M., White, A., Panaccione, R., Duncan, S.H., Flint, H.J. et al. (2012) *Anaerostipes hadrus* comb. nov., a dominant species within the human colonic microbiota; reclassification of *Eubacterium hadrum* Moore et al. 1976. *Anaerobe* 5: 523-529.

Allen, R.H., and Stabler, S.P. (2008) Identification and quantitation of cobalamin and cobalamin analogues in human feces. *American Journal of Clinical Nutrition* 87: 1324-1335.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410.

Aminov, R.I., Walker, A.W., Duncan, S.H., Harmsen, H.J.M., Welling, G.W., and Flint, H.J. (2006) Molecular diversity, cultivation, and improved detection by fluorescent in situ hybridization of a dominant group of human gut bacteria related to *Roseburia* spp. or *Eubacterium rectale*. *Applied and Environmental Microbiology* 72: 6371-6376.

Andert, J., Marten, A., Brandl, R., and Brune, A. (2010) Inter- and intraspecific comparison of the bacterial assemblages in the hindgut of humivorous scarab beetle larvae (Pachnoda spp.). *FEMS Microbiology Ecology* 74: 439-449.

Araki, Y., Andoh, A., Takizawa, J., Takizawa, W., and Fujiyama, Y. (2004) *Clostridium butyricum*, a probiotic derivative, suppresses dextran sulfate sodium-induced experimental colitis in rats. *International Journal of Molecular Medicine* 13: 577-580.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R. et al. (2011) Enterotypes of the human gut microbiome. *Nature* 473: 174-180.

Aziz, R., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R. et al. (2008) The RAST server: Rapid annotations using subsystems technology. *BMC Genomics* 9: 75.

Bäckhed, F., Ley, R., Sonnenburg, J., Peterson, D., and Gordon, J. (2005) Host-bacterial mutualism in the human intestine. *Science* 307: 1915 - 1920.

Bäckhed, F., Ding, H., Wang, T., Hooper, L.V., Koh, G.Y., Nagy, A. et al. (2004) The gut

microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences* 101: 15718-15723.

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P. et al. (2015) Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host & Microbe* 17: 690-703.

Baker, J.J., Jeng, I., and Barker, H.A. (1972) Purification and properties of l-erythro-3,5-diaminohexanoate dehydrogenase from a lysine-fermenting clostridium. *Journal of Biological Chemistry* 247: 7724-7734.

Barcenilla, A., Pryde, S.E., Martin, J.C., Duncan, S.H., Stewart, C.S., Henderson, C., and Flint, H.J. (2000) Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Applied and Environmental Microbiology* 66: 1654-1661.

Barker, H.A., Kahn, J.M., and Hedrick, L. (1982) Pathway of lysine degradation in *Fusobacterium nucleatum*. *Journal of Bacteriology* 152: 201-207.

Barker, H.A., Jeng, I.M., Neff, N., Robertson, J.M., Tam, F.K., and Hosaka, S. (1978) Butyryl-CoA:acetoacetate CoA-transferase from a lysine-fermenting *Clostridium*. *Journal of Biological Chemistry* 253: 1219-1225.

Barr, J.J., Auro, R., Furlan, M., Whiteson, K.L., Erb, M.L., Pogliano, J. et al. (2013) Bacteriophage adhering to mucus provide a non–host-derived immunity. *Proceedings of the National Academy of Sciences* 110: 10771-10776.

Baynes, J.W. (1991) Role of oxidative stress in development of complications in diabetes. *Diabetes* 40: 405-412.

Baynes, J.W., and Thorpe, S.R. (2000) Glycoxidation and lipoxidation in atherogenesis. *Free Radical Biology and Medicine* 28: 1708-1716.

Begley, M., Hill, C., and Gahan, C.G.M. (2006) Bile salt hydrolase activity in probiotics. *Applied and Environmental Microbiology* 72: 1729-1738.

Belenguer, A., Duncan, S.H., Calder, A.G., Holtrop, G., Louis, P., Lobley, G.E., and Flint, H.J. (2006) Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. *Applied and Environmental Microbiology* 72: 3593-3599.

Bennett, G.N., and Rudolph, F.B. (1995) The central metabolic pathway from acetyl-CoA to butyryl-CoA in *Clostridium acetobutylicum*. *FEMS Microbiology Reviews* 17: 241-249.

Bergman, E.N. (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species: *Physiological Reviews* 70: 567-590 .

Bernalier, A., Willems, A., Leclerc, M., Rochet, V., and Collins, M.D. (1996) *Ruminococcus*

hydrogenotrophicus sp. nov., a new H_2/CO_2-utilizing acetogenic bacterium isolated from human feces. *Archives of Microbiology* 166: 176-183.

Bezuidt, O., Lima-Mendez, G., and Reva, O. (2009) SeqWord Gene Island Sniffer: a program to study the lateral genetic exchange among bacteria. *World Academy of Science, Engineering and Technology* 58: 1169-11274.

Biswas, A., Gagnon, J.N., Brouns, S.J., Fineran, P.C., and Brown, C.M. (2013) CRISPRTarget: bioinformatic prediction and analysis of crRNA targets. *RNA biology* 10: 817-827.

Bladergroen, B.A., Beynen, A.C., and Geelen, M.J.H. (1999) Dietary pectin lowers sphingomyelin concentration in VLDL and raises hepatic sphingomyelinase activity in rats. *The Journal of Nutrition* 129: 628-633.

Boetzer, M., and Pirovano, W. (2014) SSPACE-LongRead: scaffolding bacterial draft genomes using long read sequence information. *BMC Bioinformatics* 15: 211.

Boisvert, S., Raymond, F., Godzaridis, É., Laviolette, F., and Corbeil, J. (2012) Ray Meta: scalable de novo metagenome assembly and profiling. *Genome Biology* 13: R122-R122.

Bokkenheuser, V.D., Winter, J., Dehazya, P., and Kelly, W.G. (1977) Isolation and characterization of human fecal bacteria capable of 21-dehydroxylating corticoids. *Applied and Environmental Microbiology* 34: 571-575.

Boleij, A., and Tjalsma, H. (2012) *Gut* bacteria in health and disease: a survey on the interface between intestinal microbiology and colorectal cancer. *Biological Reviews* 87: 701-730.

Bourriaud, C., Robins, R.J., Martin, L., Kozlowski, F., Tenailleau, E., Cherbut, C., and Michel, C. (2005) Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. *Journal of Applied Microbiology* 99: 201-212.

Bowling, T.E., Raimundo, A.H., Grimble, G.K., and Silk, D.B.A. (1993) Reversal by short-chain fatty acids of colonic fluid secretion induced by enteral feeding. *The Lancet* 342: 1266-1268.

Boynton, Z.L., Bennet, G.N., and Rudolph, F.B. (1996) Cloning, sequencing, and expression of clustered genes encoding beta-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase, and butyryl-CoA dehydrogenase from *Clostridium acetobutylicum* ATCC 824. *Journal of Bacteriology* 178: 3015-3024.

Brill, J.A., and Wiegel, J. (1997) Differentiation between spore-forming and asporogenic bacteria using a PCR and Southern hybridization based method. *Journal of Microbiological Methods* 31: 29-36.

Brioukhanov, A.L., and Netrusov, A.I. (2004) Catalase and superoxide dismutase: Distribution, properties, and physiological role in cells of strict anaerobes. *Biochemistry* 69: 949-962.

Brosnan, J.T. (2000) Glutamate, at the interface between amino acid and carbohydrate

metabolism. *The Journal of Nutrition* 130: 988.

Brown, A.J., Goldsworthy, S.M., Barnes, A.A., Eilert, M.M., Tcheang, L., Daniels, D. et al. (2003) The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *Journal of Biological Chemistry* 278: 11312-11319.

Brownlee, M. (1994) Glycation and diabetic complications. *Diabetes* 43: 836-841.

Buchholt, H.C., Christensen, T.M.I.E., Fallesen, B., Ralet, M.C., and Thibault, J.F. (2004) Preparation and properties of enzymatically and chemically modified sugar beet pectins. *Carbohydrate Polymers* 58: 149-161.

Buckel, W., and Thauer, R.K. (2013) Energy conservation via electron bifurcating ferredoxin reduction and proton/Na+ translocating ferredoxin oxidation. *Biochimica et Biophysica Acta - Bioenergetics* 1827: 94-113.

Bui, T.P.N., de Vos, W.M., and Plugge, C.M. (2014) *Anaerostipes rhamnosivorans* sp. nov., a human intestinal, butyrate-forming bacterium. *International Journal of Systematic and Evolutionary Microbiology* 64: 787-793.

Bui, T.P.N., Ritari, J., Boeren, S., de Waard, P., Plugge, C.M., and de Vos, W.M. (2015) Production of butyrate from lysine and the Amadori product fructoselysine by a human gut commensal. *Nature Communications* 6.

Burrin, D.G., and Stoll, B. (2009) Metabolic fate and function of dietary glutamate in the gut. *The American Journal of Clinical Nutrition* 90: 850-856.

Cai, W., Uribarri, J., Zhu, L., Chen, X., Swamy, S., Zhao, Z. et al. (2014) Oral glycotoxins are a modifiable cause of dementia and the metabolic syndrome in mice and humans. *Proceedings of the National Academy of Sciences* 111: 4940-4945.

Canfora, E.E., Jocken, J.W., and Blaak, E.E. (2015) Short-chain fatty acids in control of body weight and insulin sensitivity. *Nature Reviews Endocrinology* 11: 577-591.

Carlier, J.-P., Bedora-Faure, M., apos, ouas, G., Alauzet, C., and Mory, F. (2010) Proposal to unify *Clostridium orbiscindens* Winter et al. 1991 and *Eubacterium plautii* (Séguin 1928) Hofstad and Aasjord 1982, with description of *Flavonifractor plautii* gen. nov., comb. nov., and reassignment of *Bacteroides capillosus* to *Pseudoflavonifractor capillosus* gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 60: 585-590.

Castellarin, M., Warren, R.L., Freeman, J.D., Dreolini, L., Krzywinski, M., Strauss, J. et al. (2012) *Fusobacterium nucleatum* infection is prevalent in human colorectal carcinoma. *Genome Research* 22: 299-306.

Cato, E.P., Salmon, C.W., and Holdeman, L.V. (1974) *Eubacterium cylindroides* (Rocchi) Holdeman and Moore: Emended description and designation of neotype strain. *International Journal of Systematic and Evolutionary Microbiology* 24: 256-259.

Charrier, C., Duncan, G.J., Reid, M.D., Rucklidge, G.J., Henderson, D., Young, P. et al. (2006) A novel class of CoA-transferase involved in short-chain fatty acid metabolism in butyrate-producing human colonic bacteria. *Microbiology* 152: 179-185.

Chassard, C., and Bernalier-Donadille, A. (2006) H_2 and acetate transfers during xylan fermentation between a butyrate-producing xylanolytic species and hydrogenotrophic microorganisms from the human gut. *FEMS Microbiology Letters* 254: 116-122.

Chaudhary, P., Gaci, N., Borrel, G., O'Toole, P., and Brugère, J.F. (2015) Molecular methods for studying methanogens of the human gastrointestinal tract: Current status and future directions. *Applied Microbiology and Biotechnology* 99: 5801-5815.

Chirpich, T.P., Zappia, V., Costilow, R.N., and Barker, H.A. (1970) Lysine 2,3-aminomutase: Purification and properties of a pyridoxal phosphate and S-adenosylmethionine-activated enzyme. *Journal of Biological Chemistry* 245: 1778-1789.

Chowdhury, N.P., Kahnt, J., and Buckel, W. (2015) Reduction of ferredoxin or oxygen by flavin-based electron bifurcation in *Megasphaera elsdenii*. *FEBS Journal* 282: 3149-3160.

Chun, J., Lee, J.H., Jung, Y., Kim, M., Kim, S., Kim, B.K., and Lim, Y.W. (2007) EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *International Journal of Systematic and Evolutionary Microbiology* 57: 2259-2261.

Clavel, T., Doré, J., and Blaut, M. (2006a) Bioavailability of lignans in human subjects. *Nutrition Research Reviews* 19: 187-196.

Clavel, T., Saalfrank, A., Charrier, C., and Haller, D. (2010) Isolation of bacteria from mouse caecal samples and description of *Bacteroides sartorii* sp. nov. *Archives of Microbiology* 192: 427-435.

Clavel, T., Henderson, G., Engst, W., Dore, J., and Blaut, M. (2006b) Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. *FEMS Microbiology Ecology* 55: 471-478.

Clavel, T., Charrier, C., Braune, A., Wenning, M., Blaut, M., and Haller, D. (2009) Isolation of bacteria from the ileal mucosa of $TNF^{deltaARE}$ mice and description of *Enterorhabdus mucosicola* gen. nov., sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 59: 1805-1812.

Clemente, Jose C., Ursell, Luke K., Parfrey, Laura W., and Knight, R. (2012) The impact of the gut microbiota on human health: an integrative view. *Cell* 148: 1258-1270.

Coenen, G.J. (2007) Structural characterization of native pectins. In. Wageningen University: Wageningen University.

Coenen, G.J., Bakx, E.J., Verhoef, R.P., Schols, H.A., and Voragen, A.G.J. (2007) Identification of the connecting linkage between homo- or xylogalacturonan and rhamnogalacturonan type

I. *Carbohydrate Polymers* 70: 224-235.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y. et al. (2014) Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research* 42: D633-D642.

Comalada, M., Bailón, E., de Haro, O., Lara-Villoslada, F., Xaus, J., Zarzuelo, A., and Gálvez, J. (2006) The effects of short-chain fatty acids on colon epithelial proliferation and survival depend on the cellular phenotype. *Journal of Cancer Research and Clinical Oncology* 132: 487-497.

Conley, B.A., Egorin, M.J., Tait, N., Rosen, D.M., Sausville, E.A., Dover, G. et al. (1998) Phase I study of the orally administered butyrate prodrug, tributyrin, in patients with solid tumors. *Clinical Cancer Research* 4: 629-634.

Cummings, J.H., Pomare, E.W., Branch, W.J., Naylor, C.P., and Macfarlane, G.T. (1987) Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 28: 1221-1227.

Davis, L.M.G., Martínez, I., Walter, J., Goin, C., and Hutkins, R.W. (2011) Barcoded pyrosequencing reveals that consumption of galactooligosaccharides results in a highly specific bifidogenic response in humans. *PLoS One* 6: e25200.

de Haan, J.B., Gevers, W., and Parker, M.I. (1986) Effects of sodium butyrate on the synthesis and methylation of DNA in normal cells and their transformed counterparts. *Cancer Research* 46: 713-716.

de Vos, W.M., and de Vos, E.A.J. (2012) Role of the intestinal microbiome in health and disease: from correlation to causation. *Nutrition Reviews.*70: S45-S56.

de Vos, W.M., and Nieuwdorp, M. (2013) Genomics: A gut prediction. *Nature* 498: 48-49.

De Vuyst, L., and Leroy, F. (2011) Cross-feeding between bifidobacteria and butyrate-producing colon bacteria explains bifdobacterial competitiveness, butyrate production, and gas production. *International Journal of Food Microbiology* 149: 73-80.

de Weerth, C., Fuentes, S., and de Vos, W.M. (2013) Crying in infants. *Gut Microbes* 4: 416-421.

Degnan, Patrick H., Taga, Michiko E., and Goodman, Andrew L. (2014) Vitamin B12 as a modulator of gut microbial ecology. *Cell Metabolism* 20: 769-778.

Deppe, V., Bongaerts, J., O'Connell, T., Maurer, K.-H., and Meinhardt, F. (2011) Enzymatic deglycation of Amadori products in bacteria: mechanisms, occurrence and physiological functions. *Applied Microbiology and Biotechnology* 90: 399-406.

Derrien, M., Vaughan, E.E., Plugge, C.M., and de Vos, W.M. (2004) *Akkermansia muciniphila*

gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology* 54: 1469-1476.

Derrien, M., Collado, M.C., Ben-Amor, K., Salminen, S., and de Vos, W.M. (2008) The mucin degrader *Akkermansia muciniphila* is an abundant resident of the human intestinal tract. *Applied and Environmental Microbiology* 74: 1646-1648.

Derrien, M., van Passel, M.W.J., van de Bovenkamp, J.H.B., Schipper, R.G., de Vos, W.M., and Dekker, J. (2010) Mucin-bacterial interactions in the human oral cavity and digestive tract. *Gut Microbes* 1: 254-268.

Dhillon, B.K., Chiu, T.A., Laird, M.R., Langille, M.G., and Brinkman, F.S. (2013) IslandViewer update: improved genomic island discovery and visualization. *Nucleic Acids Research* 41: W129-W132.

Dongowski, G., Lorenz, A., and Anger, H. (2000) Degradation of pectins with different degrees of esterification by *Bacteroides thetaiotaomicron* isolated from human gut flora. *Applied and Environmental Microbiology* 66: 1321-1327.

Duncan, S.H., and Flint, H.J. (2008) Proposal of a neotype strain (A1-86) for *Eubacterium rectale*. Request for an opinion. *International Journal of Systematic and Evolutionary Microbiology* 58: 1735-1736.

Duncan, S.H., Louis, P., and Flint, H.J. (2004) Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Applied and Environmental Microbiology* 70: 5810-5817.

Duncan, S.H., Barcenilla, A., Stewart, C.S., Pryde, S.E., and Flint, H.J. (2002a) Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. *Applied and Environmental Microbiology* 68: 5186-5190.

Duncan, S.H., Hold, G.L., Harmsen, H.J.M., Stewart, C.S., and Flint, H.J. (2002b) Growth requirements and fermentation products of *Fusobacterium prausnitzii*, and a proposal to reclassify it as *Faecalibacterium prausnitzii*.gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 52: 2141-2146.

Duncan, S.H., Hold, G.L., Barcenilla, A., Stewart, C.S., and Flint, H.J. (2002c) *Roseburia intestinalis* sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. *International Journal of Systematic and Evolutionary Microbiology* 52: 1615-1620.

Duncan, S.H., Aminov, R.I., Scott, K.P., Louis, P., Stanton, T.B., and Flint, H.J. (2006) Proposal of *Roseburia faecis* sp. nov., *Roseburia hominis* sp. nov. and *Roseburia inulinivorans* sp. nov., based on isolates from human faeces. *International Journal of Systematic and Evolutionary Microbiology* 56: 2437-2441.

Duncan, S.H., Belenguer, A., Holtrop, G., Johnstone, A.M., Flint, H.J., and Lobley, G.E. (2007) Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Applied and Environmental Microbiology* 73: 1073-1078.

Durso, L.M., Harhay, G.P., Smith, T.P.L., Bono, J.L., DeSantis, T.Z., Harhay, D.M. et al. (2010) Animal-to-animal variation in fecal microbial diversity among beef cattle. *Applied and Environmental Microbiology* 76: 4858-4862.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M. et al. (2005) Diversity of the human intestinal microbial flora. *Science* 308: 1635-1638.

Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26: 2460-2461.

Edgar, R.C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods* 10: 996-998.

Eeckhaut, V., Van Immerseel, F., Pasmans, F., De Brandt, E., Haesebrouck, F., Ducatelle, R., and Vandamme, P. (2010) *Anaerostipes butyraticus* sp. nov., an anaerobic, butyrate-producing bacterium from *Clostridium* cluster XIVa isolated from broiler chicken caecal content, and emended description of the genus *Anaerostipes*. *International Journal of Systematic and Evolutionary Microbiology* 60: 1108-1112.

Eeckhaut, V., Van Immerseel, F., Croubels, S., De Baere, S., Haesebrouck, F., Ducatelle, R. et al. (2011) Butyrate production in phylogenetically diverse Firmicutes isolated from the chicken caecum. *Microbial Biotechnology* 4: 503-512.

Faith, J.J., Colombel, J.F., and Gordon, J.I. (2015) Identifying strains that contribute to complex diseases through the study of microbial inheritance. *Proceedings of the National Academy of Sciences* 112: 633-640.

Faith, J.J., Ahern, P.P., Ridaura, V.K., Cheng, J., and Gordon, J.I. (2014) Identifying gut microbe-host phenotype relationships using combinatorial communities in gnotobiotic mice. *Science Translational Medicine* 6: 220ra211-220ra211.

Falony, G., Verschaeren, A., De Bruycker, F., De Preter, V., Verbeke, K., Leroy, F., and De Vuyst, L. (2009) In vitro kinetics of prebiotic inulin-type fructan fermentation by butyrate-producing colon bacteria: Implementation of online gas chromatography for quantitative analysis of carbon dioxide and hydrogen gas production. *Applied and Environmental Microbiology* 75: 5884-5892.

Faust, K., and Raes, J. (2012) Microbial interactions: from networks to models. *Nature Reviews Microbiology* 10: 538-550.

Felsenstein, J. (1985) Confidence-limits on phylogenies - an approach using the bootstrap.

Evolution 39: 783 - 791.

Fernandes, J., Su, W., Rahat-Rozenbloom, S., Wolever, T.M.S., and Comelli, E.M. (2014) Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. *Nutrition & Diabetes* 4: e121.

Fernando, W., Hill, J., Zello, G., Tyler, R., Dahl, W., and Kessel, A.V. (2010) Diets supplemented with chickpea or its main oligosaccharide component raffinose modify faecal microbial composition in healthy adults. *Beneficial Microbes* 1: 197-207.

Finn, R.D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R.Y., Eddy, S.R. et al. (2014) Pfam: the protein families database. *Nucleic Acids Research* 42: D222-D230.

Flint, H.J., N. A. Logan, H.M.L.-S., and Oyston, P.C.F. (2006) The significance of prokaryote diversity in the human gastrointestinal tract. Prokaryotic Diversity: *Cambridge University Press*.

Flint, H.J., Duncan, S.H., Scott, K.P., and Louis, P. (2007) Interactions and competition within the microbial community of the human colon: links between diet and health. *Environmental Microbiology* 9: 1101-1111.

Flint, H.J., Scott, K.P., Louis, P., and Duncan, S.H. (2012) The role of the gut microbiota in nutrition and health. *Nature Reviews Gastroenterology and Hepatology* 9: 577-589.

Flint, H.J., Duncan, S.H., Scott, K.P., and Louis, P. (2014) Links between diet, gut microbiota composition and gut metabolism. *Proceedings of the Nutrition Society* 74: 1-10.

Fonknechten, N., Chaussonnerie, S., Tricot, S., Lajus, A., Andreesen, J., Perchat, N. et al. (2010) *Clostridium sticklandii*, a specialist in amino acid degradation:revisiting its metabolism through its genome sequence. *BMC Genomics* 11: 1-12.

Franks, A.H., Harmsen, H.J.M., Raangs, G.C., Jansen, G.J., Schut, F., and Welling, G.W. (1998) Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Applied and Environmental Microbiology* 64: 3336-3345.

Fridovich, I. (1995) Superoxide radical and superoxide dismutases. *Annual Review of Biochemistry* 64: 97-112.

Fuentes, S., van Nood, E., Tims, S., Heikamp-de Jong, I., ter Braak, C.J.F., Keller, J.J. et al. (2014) Reset of a critically disturbed microbial ecosystem: faecal transplant in recurrent *Clostridium difficile* infection. *ISME J* 8: 1621-1633.

Furne, J., Springfield, J., Koenig, T., DeMaster, E., and Levitt, M.D. (2001) Oxidation of hydrogen sulfide and methanethiol to thiosulfate by rat tissues: a specialized function of the colonic mucosa. *Biochemical Pharmacology* 62: 255-259.

Gaci, N., Borrel, G., Tottey, W., O'Toole, P.W., and Brugère, J.-F. (2014) Archaea and the human gut: New beginning of an old story. *World Journal of Gastroenterology* 20: 16062-16078.

Garmasheva, I., Vasyliuk, O., Kovalenko, N., Ostapchuk, A., and Oleschenko, L. (2015) Intraspecies cellular fatty acids heterogeneity of *Lactobacillus plantarum* strains isolated from fermented foods in Ukraine. *Letters in Applied Microbiology* 61: 283-292.

Gassull, M.A. (2006) Review article: the intestinal lumen as a therapeutic target in inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics* 24: 90-95.

George M, Nord CE, Ronquist G, Hedenstierna G, and L., W. (1996) Faecal microflora and urease activity during the first six months of infancy. *Upsala Journal of Medical Sciences* 101: 233 - 250.

Gerischer, U., and Dürre, P. (1990) Cloning, sequencing, and molecular analysis of the acetoacetate decarboxylase gene region from *Clostridium acetobutylicum*. *Journal of Bacteriology* 172: 6907-6918.

Glenn, J.V., and Stitt, A.W. (2009) The role of advanced glycation end products in retinal ageing and disease. *Biochimica et Biophysica Acta - General Subjects* 1790: 1109-1116.

Goodman, A.L., Kallstrom, G., Faith, J.J., Reyes, A., Moore, A., Dantas, G., and Gordon, J.I. (2011) Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. *Proceedings of the National Academy of Sciences* 108: 6252-6257.

Gordon, N.C., Png, K., and Wareham, D.W. (2010) Potent synergy and sustained bactericidal activity of a vancomycin-colistin combination versus multidrug-resistant strains of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy* 54: 5316-5322.

Gosalbes, M.J., Durbán, A., Pignatelli, M., Abellan, J.J., Jiménez-Hernández, N., Pérez-Cobas, A.E. et al. (2011) Metatranscriptomic approach to analyze the functional human gut microbiota. *PLoS One* 6: e17447.

Graf, D., Di Cagno, R., Fåk, F., Flint, H.J., Nyman, M., Saarela, M., and Watzl, B. (2015) Contribution of diet to the composition of the human gut microbiota. *Microbial Ecology in Health and Disease* 26: 26164-26175

Grant, J.R., Arantes, A.S., and Stothard, P. (2012) Comparing thousands of circular genomes using the CGView Comparison Tool. *BMC genomics* 13: 202.

Grissa, I., Vergnaud, G., and Pourcel, C. (2007) CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic Acids Research* 35: W52-W57.

Guarner, F., and Malagelada, J.R. (2003) Gut flora in health and disease. *The Lancet* 361: 512-519.

Hackmann, T.J., and Firkins, J.L. (2015) Electron transport phosphorylation in rumen butyrivibrios: unprecedented ATP yield for glucose fermentation to butyrate. *Frontiers in Microbiology* 6: 622.

Hague, A., Singh, B., and Paraskeva, C. (1997) Butyrate acts as a survival factor for colonic epithelial cells: Further fuel for the in vivo versus in vitro debate. *Gastroenterology* 112: 1036-1040.

Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F.J., and Brummer, R.J. (2008) Review article: the role of butyrate on colonic function. *Alimentary Pharmacology & Therapeutics* 27: 104-119.

Hansen, E.E., Lozupone, C.A., Rey, F.E., Wu, M., Guruge, J.L., Narra, A. et al. (2011) Pan-genome of the dominant human gut-associated archaeon, *Methanobrevibacter smithii*, studied in twins. *Proceedings of the National Academy of Sciences* 108: 4599-4606.

Hanske, L., Loh, G., Sczesny, S., Blaut, M., and Braune, A. (2010) Recovery and metabolism of xanthohumol in germ-free and human microbiota-associated rats. *Molecular Nutrition & Food Research* 54: 1405-1413.

Hao, X., Jiang, R., and Chen, T. (2011) Clustering 16S rRNA for OTU prediction: a method of unsupervised Bayesian clustering. *Bioinformatics* 27: 611-618.

Harry J. Flint, K.P.S., Sylvia H. Duncan, Petra Louis and Evelyne Forano (2012) Microbial degradation of complex carbohydrates in the gut. *Gut microbes* 3: 289 - 306.

He, C., Sabol, J., Mitsuhashi, T., and Vlassara, H. (1999) Dietary glycotoxins: inhibition of reactive products by aminoguanidine facilitates renal clearance and reduces tissue sequestration. *Diabetes* 48: 1308-1315.

Heinken, A., Khan, M.T., Paglia, G., Rodionov, D.A., Harmsen, H.J.M., and Thiele, I. (2014) Functional metabolic map of *Faecalibacterium prausnitzii.* a beneficial human gut microbe. *Journal of Bacteriology* 196: 3289-3302.

Hellwig, M., Bunzel, D., Huch, M., Franz, C.M.A.P., Kulling, S.E., and Henle, T. (2015) Stability of individual Maillard reaction products in the presence of the human colonic microbiota. *Journal of Agricultural and Food Chemistry* 63: 6723-6730.

Hendriks, W.H., van Baal, J., and Bosch, G. (2012) Ileal and faecal protein digestibility measurement in humans and other non-ruminants – a comparative species view. *British Journal of Nutrition* 108: S247-S257.

Henle, T. (2003) AGEs in foods: Do they play a role in uremia? *Kidney International Supplement* 63: S145-S147.

Herrmann, G., Jayamani, E., Mai, G., and Buckel, W. (2008) Energy conservation via electron-transferring flavoprotein in anaerobic bacteria. *Journal of Bacteriology* 190: 784-791.

Hinnebusch, B.F., Meng, S., Wu, J.T., Archer, S.Y., and Hodin, R.A. (2002) The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *The Journal of Nutrition* 132: 1012-1017.

Hodin, R., Meng, S., Archer, S., and Tang, R. (1996) Cellular growth state differentially regulates enterocyte gene expression in butyrate-treated HT-29 cells. *Cell Growth Differ* 7: 647-653.

Hofmann, A.F. (2001) Bile secretion in mice and men. *Hepatology* 34: 848–850

Holdeman, L.V., and Moore, W.E.C. (1974) New genus, *Coprococcus*, twelve new species, and emended descriptions of four previously described species of bacteria from human feces. *International Journal of Systematic Bacteriology* 24: 260-277.

Holmstrøm, K., Collins, M.D., Møller, T., Falsen, E., and Lawson, P.A. (2004) *Subdoligranulum variabile* gen. nov., sp. nov. from human feces. *Anaerobe* 10: 197-203.

Hooper, L.V., and Macpherson, A.J. (2010) Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nature Reviews Immunology* 10: 159-169.

Hörmannsperger, G., Clavel, T., and Haller, D. (2012) Gut matters: Microbe-host interactions in allergic diseases. *Journal of Allergy and Clinical Immunology* 129: 1452-1459.

Hoskin-Parr, L., Teyhan, A., Blocker, A., and Henderson, A.J.W. (2013) Antibiotic exposure in the first two years of life and development of asthma and other allergic diseases by 7.5 yr: A dose-dependent relationship. *Pediatric Allergy and Immunology* 24: 762-771.

Hosseini, E., Grootaert, C., Verstraete, W., and Van de Wiele, T. (2011) Propionate as a health-promoting microbial metabolite in the human gut. *Nutrition Reviews.*69: 245-258.

Hove, H., Nordgaard-Andersen, I., and Mortensen, P.B.b. (1994) Faecal DL-lactate concentration in 100 gastrointestinal patients. *Scandinavian Journal of Gastroenterology* 29: 255-259.

Human Microbiome Project Consortium (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486: 207-214.

Hugon, P., Dufour, J.C., Colson, P., Fournier, P.E., Sallah, K., and Raoult, D. (2015) A comprehensive repertoire of prokaryotic species identified in human beings. *The Lancet Infectious Diseases* 15: 1211-1219.

Hunter, S., Jones, P., Mitchell, A., Apweiler, R., Attwood, T.K., Bateman, A. et al. (2012) InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acids Research* 40: 4725.

Huss, V.A.R., Festl, H., and Schleifer, K.H. (1983) Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Systematic and Applied*

Microbiology 4: 184-192.

Huys, G., D'Haene, K., and Swings, J. (2002) Influence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method. *Letters in Applied Microbiology* 34: 402-406.

Ingham, C.J., Sprenkels, A., Bomer, J., Molenaar, D., van den Berg, A., van Hylckama Vlieg, J.E., de Vos, W.M. (2007) The micro-Petri dish, a million-well growth chipfor the culture and high-throughput screening of microorganisms. *Proceedings of the National Academy of Sciences* 104: 18217–18222

Jackson, A.A. (1995) Salvage of urea-nitrogen and protein requirements. *Proceedings of the Nutrition Society* 54: 535-547.

Jensen, N.S., and Canale-Parola, E. (1986) *Bacteroides pectinophilus* sp. nov. and *Bacteroides galacturonicus* sp. nov.: two pectinolytic bacteria from the human intestinal tract. *Applied and Environmental Microbiology* 52: 880-887.

Jones, B.V., Begley, M., Hill, C., Gahan, C.G.M., and Marchesi, J.R. (2008) Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proceedings of the National Academy of Sciences* 105: 13580-13585.

Kämpfer, P., and Kroppenstedt, R.M. (1996) Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Canadian Journal of Microbiology* 42: 989-1005.

Kant, R., Rasinkangas, P., Satokari, R., Pietilä, T.E., and Palva, A. (2015) Genome sequence of the butyrate-producing anaerobic bacterium *Anaerostipes hadrus* PEL 85. *Genome Announcements* 3.

Kapatral, V., Anderson, I., Ivanova, N., Reznik, G., Los, T., Lykidis, A. et al. (2002) Genome sequence and analysis of the oral bacterium *Fusobacterium nucleatum* strain ATCC 25586. *Journal of Bacteriology* 184: 2005-2018.

Kellow, N.J., and Coughlan, M.T. (2015) Effect of diet-derived advanced glycation end products on inflammation. *Nutrition Reviews.*73: 737-759.

Kelly, C.P., and LaMont, J.T. (2010) *Clostridium difficile* — More difficult than ever. *New England Journal of Medicine* 363: 1585-1585.

Khan, M.T., Duncan, S.H., Stams, A.J.M., van Dijl, J.M., Flint, H.J., and Harmsen, H.J.M. (2012) The gut anaerobe *Faecalibacterium prausnitzii*.uses an extracellular electron shuttle to grow at oxic-anoxic interphases. *ISME J* 6: 1578-1585.

Kimura, M. (1983) The Neutral Theory of Molecular Evolution: *Cambridge University Press*.

Kirsner, J.B., Sheffner, A.L., and Palmer, W.L. (1949) Studies on amino acid excretion in man. III. Amino acid levels in plasma and urine of normal men fed diets of varying protein content.

Journal of Clinical Investigation 28: 716-722.

Klampfer, L., Huang, J., Sasazuki, T., Shirasawa, S., and Augenlicht, L. (2003) Inhibition of interferon γ signaling by the short chain fatty acid butyrate. *Molecular Cancer Research* 1: 855-862.

Kläring, K., Hanske, L., Bui, N., Charrier, C., Blaut, M., Haller, D. et al. (2013) *Intestinimonas butyriciproducens* gen. nov., sp. nov., a butyrate-producing bacterium from the mouse intestine. *International Journal of Systematic and Evolutionary Microbiology* 63: 4606-4612.

Kontoyiannis, D., Pasparakis, M., Pizarro, T.T., Cominelli, F., and Kollias, G. (1999) Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* 10: 387-398.

Kopylova, E., Noé, L., and Touzet, H. (2012) SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 28: 3211-3217.

Koropatkin, N.M., Cameron, E.A., and Martens, E.C. (2012) How glycan metabolism shapes the human gut microbiota. *Nature* Reivews *Microbiology* 10: 323-335.

Korpela, K.S., A , Virta, L., Kekkonen, R., Forslund, K., Bork, P., de Vos, M. W (2015) Intestinal microbiome is related to lifetime antibiotic use in Finnish pre-school children. *Nature Communication*s 7.

Kovatcheva-Datchary, P., Egert, M., Maathuis, A., Rajilić-Stojanović, M., De Graaf, A.A., Smidt, H. et al. (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.

Kreimeyer, A., Perret, A., Lechaplais, C., Vallenet, D., Médigue, C., Salanoubat, M., and Weissenbach, J. (2007) Identification of the last unknown genes in the fermentation pathway of lysine. *Journal of Biological Chemistry* 282: 7191-7197.

Kruse, T., Levisson, M., de Vos, W.M., and Smidt, H. (2014) vanI: a novel d-Ala-d-Lac vancomycin resistance gene cluster found in *Desulfitobacterium hafniense*. *Microbial Biotechnology* 7: 456-466.

Kurtz, S., Phillippy, A., Delcher, A., Smoot, M., Shumway, M., Antonescu, C., and Salzberg, S. (2004) Versatile and open software for comparing large genomes. *Genome Biology* 5: R12.

Lagier, J.C., Armougom, F., Million, M., Hugon, P., Pagnier, I., Robert, C. et al. (2012) Microbial culturomics: paradigm shift in the human gut microbiome study. *Clinical Microbiology and Infection* 18: 1185-1193.

Lambert, D.W., Wood, I.S., Ellis, A., and Shirazi-Beechey, S.P. (2002) Molecular changes in the expression of human colonic nutrient transporters during the transition from normality to malignancy. *British Journal of Cancer* 86: 1262-1269.

Lane, D.J. (1991) 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics pp. 115-175. Edited by E. Stackebrandt; & M. Goodfellow. Chichester: Wiley.

Langille, M.G., and Brinkman, F.S. (2009) IslandViewer: an integrated interface for computational identification and visualization of genomic islands. *Bioinformatics* 25: 664-665.

Lawson, P.A., Song, Y., Liu, C., Molitoris, D.R., Vaisanen, M.L., Collins, M.D., and Finegold, S.M. (2004) *Anaerotruncus colihominis* gen. nov., sp. nov., from human faeces. *International Journal of Systematic and Evolutionary Microbiology* 54: 413-417.

Le Poul, E., Loison, C., Struyf, S., Springael, J.Y., Lannoy, V., Decobecq, M.E. et al. (2003) Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell cctivation. *Journal of Biological Chemistry* 278: 25481-25489.

Leijdekkers, A.G.M., Huang, J.H., Bakx, E.J., Gruppen, H., and Schols, H.A. (2015) Identification of novel isomeric pectic oligosaccharides using hydrophilic interaction chromatography coupled to traveling-wave ion mobility mass spectrometry. *Carbohydrate Research* 404: 1-8.

Leiros, H.K.S., Brandsdal, B.O., Andersen, O.A., Os, V., Leiros, I., Helland, R. et al. (2004) Trypsin specificity as elucidated by LIE calculations, X-ray structures, and association constant measurements. *Protein Science* 13: 1056-1070.

Leser, T.D., Amenuvor, J.Z., Jensen, T.K., Lindecrona, R.H., Boye, M., and Møller, K. (2002) Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Applied and Environmental Microbiology* 68: 673-690.

Leung, K., Zahn, H., Leaver, T., Konwar, K.M., Hanson, N.W., Pagé, A.P. et al. (2012) A programmable droplet-based microfluidic device applied to multiparameter analysis of single microbes and microbial communities. *Proceedings of the National Academy of Sciences* 109: 7665-7670.

Levine, U.Y., Looft, T., Allen, H.K., and Stanton, T.B. (2013) Butyrate-producing bacteria, including mucin degraders, from the swine intestinal tract. *Applied and Environmental Microbiology* 79: 3879-3881.

Lewis, S.J., and Heaton, K.W. (1997) Increasing butyrate concentration in the distal colon by accelerating intestinal transit. *Gut* 41: 245-251.

Ley, J.D., Cattoir, H., and Reynaerts, A. (1970) The quantitative measurement of DNA hybridization from renaturation rates. *European Journal of Biochemistry* 12: 133-142.

Ley, R.E., Turnbaugh, P.J., Klein, S., and Gordon, J.I. (2006) Microbial ecology: Human gut microbes associated with obesity. *Nature* 444: 1022-1023.

Li, E., Hamm, C.M., Gulati, A.S., Sartor, R.B., Chen, H., Wu, X. et al. (2012a) Inflammatory bowel diseases phenotype, *C. difficile* and NOD2 genotype are associated with shifts in human ileum associated microbial composition. *PLoS One* 7: e26284.

Li, F., Hinderberger, J., Seedorf, H., Zhang, J., Buckel, W., and Thauer, R.K. (2008) Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*. *Journal of Bacteriology* 190: 843-850.

Li, L., Han, L., Fu, Q., Li, Y., Liang, Z., Su, J., and Li, B. (2012b) Formation and inhibition of Nε-(carboxymethyl)lysine in saccharide-lysine model systems during microwave heating. *Molecules* 17: 12758.

Li, X.Z., Livermore, D.M., and Nikaido, H. (1994) Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. *Antimicrobial Agents and Chemotherapy* 38: 1732-1741.

Liu, C., Finegold, S.M., Song, Y., and Lawson, P.A. (2008) Reclassification of *Clostridium coccoides*, *Ruminococcus hansenii*, *Ruminococcus hydrogenotrophicus*, *Ruminococcus luti*, *Ruminococcus productus* and *Ruminococcus schinkii* as *Blautia coccoides* gen. nov., comb. nov., *Blautia hansenii* comb. nov., *Blautia hydrogenotrophica* comb. nov., *Blautia luti* comb. nov., *Blautia producta* comb. nov., *Blautia schinkii* comb. nov. and description of *Blautia wexlerae* sp. nov., isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology* 58: 1896-1902.

Liu, Y., White, R.H., and Whitman, W.B. (2010) Methanococci use the diaminopimelate aminotransferase (DapL) pathway for lysine biosynthesis. *Journal of Bacteriology* 192: 3304-3310.

Loesche, W.J., and Gibbons, R.J. (1968) Amino acid fermentation by *Fusobacterium nucleatum*. *Archives of Oral Biology* 13: 191-202.

Lopez-Siles, M., Khan, T.M., Duncan, S.H., Harmsen, H.J.M., Garcia-Gil, L.J., and Flint, H.J. (2012) Cultured representatives of two major phylogroups of human colonic *Faecalibacterium prausnitzii* can utilize pectin, uronic acids, and host-derived substrates for growth. *Applied and Environmental Microbiology* 78: 420-428.

Lopez-Siles, M., Martinez-Medina, M., Abellà, C., Busquets, D., Sabat-Mir, M., Duncan, S.H. et al. (2015) Mucosa-associated *Faecalibacterium prausnitzii* phylotype richness is reduced in patients with inflammatory bowel disease. *Applied and Environmental Microbiology* 81: 7582-7592.

Louis, P., and Flint, H.J. (2007) Development of a semiquantitative degenerate real-time PCR-based assay for estimation of numbers of butyryl-Coenzyme A (CoA) CoA transferase genes in complex bacterial samples. *Applied and Environmental Microbiology* 73: 2009-2012.

Louis, P., and Flint, H.J. (2009) Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiology Letters* 294: 1-8.

Louis, P., Hold, G.L., and Flint, H.J. (2014) The gut microbiota, bacterial metabolites and

colorectal cancer. *Nature* Reviews *Microbiology* 12: 661–672.

Louis, P., Scott, K.P., Duncan, S.H., and Flint, H.J. (2007a) Understanding the effects of diet on bacterial metabolism in the large intestine. *Journal of Applied Microbiology* 102: 1197-1208.

Louis, P., McCrae, S.I., Charrier, C., and Flint, H.J. (2007b) Organization of butyrate synthetic genes in human colonic bacteria: phylogenetic conservation and horizontal gene transfer. *FEMS Microbiology Letters* 269: 240-247.

Louis, P., Young, P., Holtrop, G., and Flint, H.J. (2010) Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environmental Microbiology* 12: 304-314.

Louis, P., Duncan, S.H., McCrae, S.I., Millar, J., Jackson, M.S., and Flint, H.J. (2004) Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon. *Journal of Bacteriology* 186: 2099-2106.

Luevano-Contreras, C., and Chapman-Novakofski, K. (2010) Dietary advanced glycation end products and aging. *Nutrients* 2: 1247-1265.

Ma, L., Kim, J., Hatzenpichler, R., Karymov, M.A., Hubert, N., Hanan, I.M. et al. (2014) Gene-targeted microfluidic cultivation validated by isolation of a gut bacterium listed in Human Microbiome Project's most wanted taxa. *Proceedings of the National Academy of Sciences* 111: 9768-9773.

Macfarlane, G.T., and Allison, C. (1986) Utilisation of protein by human gut bacteria. *FEMS Microbiology Letters* 38: 19-24.

Macfarlane, G.T., and Macfarlane, S. (2012) Bacteria, colonic fermentation, and gastrointestinal health. *Journal of AOAC International* 95: 50-60.

Macfarlane, G.T., Gibson, G.R., Beatty, E., and Cummings, J.H. (1992) Estimation of short-chain fatty acid production from protein by human intestinal bacteria based on branched-chain fatty acid measurements. *FEMS Microbiology Letters* 101: 81-88.

Manach, C., Williamson, G., Morand, C., Scalbert, A., and Rémésy, C. (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. The *American Journal of Clinical Nutrition* 81: 230S-242S.

Manderson, K., Pinart, M., Tuohy, K.M., Grace, W.E., Hotchkiss, A.T., Widmer, W. et al. (2005) In vitro determination of prebiotic properties of oligosaccharides derived from an orange juice manufacturing by-product stream. *Applied and Environmental Microbiology* 71: 8383-8389.

Marquet, P., Duncan, S.H., Chassard, C., Bernalier-Donadille, A., and Flint, H.J. (2009) Lactate has the potential to promote hydrogen sulphide formation in the human colon. *FEMS Microbiology Letters* 299: 128-134.

McOrist, A.L., Miller, R.B., Bird, A.R., Keogh, J.B., Noakes, M., Topping, D.L., and Conlon, M.A. (2011) Fecal butyrate levels vary widely among individuals but are usually increased by a diet high in resistant starch. *The Journal of Nutrition* 141: 883-889.

Metges, C.C. (2000) Contribution of microbial amino acids to amino acid homeostasis of the host. *The Journal of Nutrition* 130: 1857S-1864S.

Metges, C.C., El-Khoury, A.E., Henneman, L., Petzke, K.J., Grant, I., Bedri, S. et al. (1999) Availability of intestinal microbial lysine for whole body lysine homeostasis in human subjects. *American Journal of Physiology - Endocrinology and Metabolism* 277: E597-E607.

Miller, L.T. (1982) Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *Journal of Clinical Microbiology* 16: 584-586.

Miller, T., Wolin, M., de Macario, E., and Macario, A. (1982) Isolation of *Methanobrevibacter smithii* from human feces. *Applied and Environmental Microbiology* 43: 227 - 232.

Miller, T.L., and Wolin, M.J. (1996) Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora. *Applied and Environmental Microbiology* 62: 1589-1592.

Millward, D.J., Forrester, T., Ah-Sing, E., Yeboah, N., Gibson, N., Badaloo, A. et al. (2000) The transfer of N from urea to lysine in the human infant. *British Journal of Nutrition* 83: 505-512.

Mizutani, K., Ikeda, K., and Yamori, Y. (2000) Resveratrol inhibits AGEs-induced proliferation and collagen synthesis activity in vascular smooth muscle cells from stroke-prone spontaneously hypertensive rats. *Biochemical and Biophysical Research Communications* 274: 61-67.

Moore, W.E.C., and Cato, E.P. (1965) Synonymy of *Eubacterium limosum* and *Butyribacterium rettgeri*: *Butyribacterium limosum* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 15: 69-80.

Mukhopadhyay, A., Redding, A.M., Joachimiak, M.P., Arkin, A.P., Borglin, S.E., Dehal, P.S. et al. (2007) Cell-wide responses to low-oxygen exposure in *Desulfovibrio vulgaris* Hildenborough. *Journal of Bacteriology* 189: 5996-6010.

Muñoz-Tamayo, R., Laroche, B., Walter, É., Doré, J., Duncan, S.H., Flint, H.J., and Leclerc, M. (2011) Kinetic modelling of lactate utilization and butyrate production by key human colonic bacterial species. *FEMS Microbiology Ecology* 76: 615-624.

Nicolosi, D., Scalia, M., Nicolosi, V.M., and Pignatello, R. (2010) Encapsulation in fusogenic liposomes broadens the spectrum of action of vancomycin against Gram-negative bacteria. *International Journal of Antimicrobial Agents* 35: 553-558.

Noakes, M., Clifton, P.M., Nestel, P.J., Le Leu, R., and McIntosh, G. (1996) Effect of high-amylose starch and oat bran on metabolic variables and bowel function in subjects with hypertriglyceridemia. *The American Journal of Clinical Nutrition* 64: 944-951.

O'Keefe, S.J.D., Li, J.V., Lahti, L., Ou, J., Carbonero, F., Mohammed, K. et al. (2015) Fat, fibre and cancer risk in African Americans and rural Africans. *Nature Communications* 6.

Ohkami, H., Tazawa, K., Yamashita, I., Shimizu, T., Murai, K., and Kobashi, K. (1995) Effects of apple pectin on fecal bacterial enzymes in azoxymethane-induced rat colon carcinogenesis. *Japanese Journal of Cancer Research* 86: 523 - 529.

Ohkawara, S., Furuya, H., Nagashima, K., Asanuma, N., and Hino, T. (2005) Oral administration of *Butyrivibrio fibrisolvens*, a butyrate-producing bacterium, decreases the formation of aberrant crypt foci in the colon and rectum of mice. *The Journal of Nutrition* 135: 2878-2883.

Okusu, H., Ma, D., and Nikaido, H. (1996) AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *Journal of Bacteriology* 178: 306-308.

Olano-Martin, E., Gibson, G., and Rastell, R. (2002) Comparison of the in vitro bifidogenic properties of pectins and pectic-oligosaccharides. *Journal of Applied Microbiology* 93: 505 - 511.

Oosterkamp, M.J., Boeren, S., Plugge, C.M., Schaap, P.J., and Stams, A.J.M. (2013) Metabolic response of *Alicycliphilus denitrificans* strain BC toward electron acceptor variation. *Proteomics* 13: 2886-2894.

Ozer, E.A., Allen, J.P., and Hauser, A.R. (2014) Characterization of the core and accessory genomes of *Pseudomonas aeruginosa* using bioinformatic tools Spine and AGEnt. *BMC Genomics* 15: 737.

Pan, N., and Imlay, J.A. (2001) How does oxygen inhibit central metabolism in the obligate anaerobe *Bacteroides thetaiotaomicron*. *Molecular Microbiology* 39: 1562-1571.

Park, B.H., Karpinets, T.V., Syed, M.H., Leuze, M.R., and Uberbacher, E.C. (2010) CAZymes analysis toolkit (CAT): web service for searching and analyzing carbohydrate-active enzymes in a newly sequenced organism using CAZy database. *Glycobiology* 20: 1574-1584.

Paroder, V., Spencer, S.R., Paroder, M., Arango, D., Schwartz, S., Mariadason, J.M. et al. (2006) Na(+)/monocarboxylate transport (SMCT) protein expression correlates with survival in colon cancer: Molecular characterization of SMCT. *Proceedings of the National Academy of Sciences* 103: 7270-7275.

Patel, E.H., Paul, L.V., Patrick, S., and Abratt, V.R. (2008) Rhamnose catabolism in *Bacteroides thetaiotaomicron* is controlled by the positive transcriptional regulator RhaR. *Research in Microbiology* 159: 678-684.

Pfeiffer, N., Desmarchelier, C., Blaut, M., Daniel, H., Haller, D., and Clavel, T. (2012) *Acetatifactor muris* gen. nov., sp. nov., a novel bacterium isolated from the intestine of an obese mouse. *Archives of Microbiology* 194: 901-907.

Pfleiderer, A., Lagier, J.C., Armougom, F., Robert, C., Vialettes, B., and Raoult, D. (2013) Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample. *European Journal of Clinical Microbiology & Infectious Diseases* 32: 1471-1481.

Plugge, C.M., Zoetendal, E.G., and Stams, A.J.M. (2000) *Caloramator coolhaasii* sp. nov., a glutamate-degrading, moderately thermophilic anaerobe. *International Journal of Systematic and Evolutionary Microbiology* 50: 1155-1162.

Plugge, C.M., van Leeuwen, J.M., Hummelen, T., Balk, M., and Stams, A.J.M. (2001) Elucidation of the pathways of catabolic glutamate conversion in three thermophilic anaerobic bacteria. *Archives of Microbiology* 176: 29-36.

Poelarends, G.J., Mazurkiewicz, P., Putman, M., Cool, R.H., Veen, H.W., and Konings, W.N. (2000) An ABC-type multidrug transporter of *Lactococcus lactis* possesses an exceptionally broad substrate specificity. *Drug Resistance Updates* 3: 330-334.

Postgate, J.R. (1963) Versatile medium for the enumeration of sulfate-reducing bacteria. *Applied Microbiology* 11: 265-267.

Pruesse, E., Peplies, J., and Glöckner, F.O. (2012) SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* 28: 1823-1829.

Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S., and Flint, H.J. (2002) The microbiology of butyrate formation in the human colon. *FEMS Microbiology Letters* 217: 133-139.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C. et al. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464: 59-65.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F. et al. (2012) A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490: 55-60.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P. et al. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 41: D590-D596.

Rahbar, S., and Figarola, J.L. (2003) Novel inhibitors of advanced glycation endproducts. *Archives of Biochemistry and Biophysics* 419: 63-79.

Rajilić-Stojanović, M., and de Vos, W.M. (2014) The first 1000 cultured species of the human gastrointestinal microbiota. *FEMS Microbiology Reviews* 38: 996-1047.

Rajilić-Stojanović, M., Heilig, H.G.H.J., Tims, S., Zoetendal, E.G., and de Vos, W.M. (2013) Long-term monitoring of the human intestinal microbiota composition. *Environmental Microbiology* 15: 1146-1159.

Ramirez-Farias, C., Slezak, K., Fuller, Z., Duncan, A., Holtrop, G., and Louis, P. (2009) Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and

Faecalibacterium prausnitzii. British Journal of Nutrition 101: 541-550.

Reeves, A.R., D'Elia, J.N., Frias, J., and Salyers, A.A. (1996) A *Bacteroides thetaiotaomicron* outer membrane protein that is essential for utilization of maltooligosaccharides and starch. *Journal of Bacteriology* 178: 823-830.

Remoroza, C., Cord-Landwehr, S., Leijdekkers, A.G.M., Moerschbacher, B.M., Schols, H.A., and Gruppen, H. (2012) Combined HILIC-ELSD/ESI-MSn enables the separation, identification and quantification of sugar beet pectin derived oligomers. *Carbohydrate Polymers* 90: 41-48.

Rettedal, E.A., Gumpert, H., and Sommer, M.O.A. (2014) Cultivation-based multiplex phenotyping of human gut microbiota allows targeted recovery of previously uncultured bacteria. *Nature Communications* 5.

Richardson, A.J., McKain, N., and Wallace, R.J. (2013) Ammonia production by human faecal bacteria, and the enumeration, isolation and characterization of bacteria capable of growth on peptides and amino acids. *BMC Microbiology* 13: 6-6.

Ridley, B.L., O'Neill, M.A., and Mohnen, D. (2001) Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* 57: 929-967.

Ridlon, J.M., Kang, D.-J., and Hylemon, P.B. (2006) Bile salt biotransformations by human intestinal bacteria. *Journal of Lipid Research* 47: 241-259.

Ritari, J., Salojarvi, J., Lahti, L., and de Vos, W. (2015) Improved taxonomic assignment of human intestinal 16S rRNA sequences by a dedicated reference database. *BMC Genomics* 16: 1056.

Roberfroid, M. (2007) Prebiotics: The concept revisited. *The Journal of Nutrition* 137: 830S-837S.

Robert, C., Del'Homme, C., and Bernalier-Donadille, A. (2001) Interspecies H_2 transfer in cellulose degradation between fibrolytic bacteria and H_2-utilizing microorganisms from the human colon. *FEMS Microbiology Letters* 205: 209-214.

Rocha, E.R., and Smith, C.J. (1997) Regulation of *Bacteriodes fragilis* katB mRNA by oxidative stress and carbon limitation. *Journal of Bacteriology* 179: 7033-7039.

Rocha, E.R., and Smith, C.J. (1999) Role of the alkyl hydroperoxide reductase (ahpCF) gene in oxidative stress defense of the obligate anaerobe *Bacteroides fragilis*. *Journal of Bacteriology* 181: 5701-5710.

Roda, A., Simoni, P., Magliulo, M., Nanni, P., Baraldini, M., Roda, G., and Roda, E. (2007) A new oral formulation for the release of sodium butyrate in the ileo-cecal region and colon. *World Journal of Gastroenterology* 13: 1079-1084.

Rodionov, D.A., Hebbeln, P., Eudes, A., ter Beek, J., Rodionova, I.A., Erkens, G.B. et al. (2009)

A novel class of modular transporters for vitamins in prokaryotes. *Journal of Bacteriology* 191: 42-51.

Rogers, A.H., Chen, J., Zilm, P.S., and Gully, N.J. (1998) The behaviour of *Fusobacterium nucleatum* chemostat-grown in glucose- and amino acid-based chemically defined media. *Anaerobe* 4: 111-116.

Rose, D.J., DeMeo, M.T., Keshavarzian, A., and Hamaker, B.R. (2007) Influence of dietary fiber on inflammatory bowel disease and colon cancer: Importance of fermentation pattern: *Nutrition Reviews.* 65: 51-62

Rosero, J.A., Killer, J., Sechovcová, H., Mrázek, J., Benada, O., Fliegerová, K. et al. (2015) Reclassification of *Eubacterium rectale* (Prévot et al., 1967) in a new genus *Agathobacter* gen. nov., as *Agathobacter rectalis* comb. nov., within the family Lachnospiraceae, and description of *Agathobacter ruminis* sp. nov., from the rumen. *International Journal of Systematic and Evolutionary Microbiology.* 66: 768-773

Rosignoli, P., Fabiani, R., De Bartolomeo, A., Spinozzi, F., Agea, E., Pelli, M.A., and Morozzi, G. (2001) Protective activity of butyrate on hydrogen peroxide-induced DNA damage in isolated human colonocytes and HT29 tumour cells. *Carcinogenesis* 22: 1675-1680.

Russell, W.R., Duncan, S.H., Scobbie, L., Duncan, G., Cantlay, L., Calder, A.G. et al. (2013) Major phenylpropanoid-derived metabolites in the human gut can arise from microbial fermentation of protein. *Molecular Nutrition & Food Research* 57: 523-535.

Russell, W.R., Gratz, S.W., Duncan, S.H., Holtrop, G., Ince, J., Scobbie, L. et al. (2011) High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. *The American Journal of Clinical Nutrition* 93: 1062-1072.

Sahl, J., Schupp, J., Rasko, D., Colman, R., Foster, J., and Keim, P. (2015) Phylogenetically typing bacterial strains from partial SNP genotypes observed from direct sequencing of clinical specimen metagenomic data. *Genome Medicine* 7: 52.

Saitou, N., and Nei, M. (1987) The neighbor-joining method - a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406 - 425.

Salonen, A., Nikkilä, J., Jalanka-Tuovinen, J., Immonen, O., Rajilić-Stojanović, M., Kekkonen, R.A. et al. (2010) Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: Effective recovery of bacterial and archaeal DNA using mechanical cell lysis. *Journal of Microbiological Methods* 81: 127-134.

Salonen, A., Lahti, L., Salojarvi, J., Holtrop, G., Korpela, K., Duncan, S.H. et al. (2014) Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. *ISME J* 8: 2218-2230.

Samuel, B.S., and Gordon, J.I. (2006) A humanized gnotobiotic mouse model of host–

archaeal–bacterial mutualism. *Proceedings of the National Academy of Sciences* 103: 10011-10016.

Samuel, B.S., Hansen, E.E., Manchester, J.K., Coutinho, P.M., Henrissat, B., Fulton, R. et al. (2007) Genomic and metabolic adaptations of *Methanobrevibacter smithii* to the human gut. *Proceedings of the National Academy of Sciences* 104: 10643-10648.

Sato, T., Matsumoto, K., Okumura, T., Yokoi, W., Naito, E., Yoshida, Y. et al. (2008) Isolation of lactate-utilizing butyrate-producing bacteria from human feces and in vivo administration of *Anaerostipes caccae* strain L2 and galacto-oligosaccharides in a rat model. *FEMS Microbiology Ecology* 66: 528-536.

Sayin, Sama I., Wahlström, A., Felin, J., Jäntti, S., Marschall, H.-U., Bamberg, K. et al. (2013) Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metabolism* 17: 225-235.

Scanlan, P., Shanahan, F., and Marchesi, J. (2008) Human methanogen diversity and incidence in healthy and diseased colonic groups using mcrA gene analysis. *BMC Microbiology* 8: 79.

Scapin, G., and Blanchard, J.S. (2006) Enzymology of bacterial lysine biosynthesis. In *Advances in Enzymology and Related Areas of Molecular Biology*: John Wiley & Sons, Inc., pp. 279-324.

Scheppach, W. (1994) Effects of short chain fatty acids on gut morphology and function. *Gut* 35: S35-S38.

Scheppach, W., Luehrs, H., and Menzel, T. (2001) Beneficial health effects of low-digestible carbohydrate consumption. *British Journal of Nutrition* 85: S23-S30.

Scheppach, W., Sommer, H., Kirchner, T., Paganelli, G.M., Bartram, P., Christl, S. et al. (1992) Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology* 103: 51-56.

Schloissnig, S., Arumugam, M., Sunagawa, S., Mitreva, M., Tap, J., Zhu, A. et al. (2013) Genomic variation landscape of the human gut microbiome. *Nature* 493: 45-50.

Schmieder, R., and Edwards, R. (2011) Quality control and preprocessing of metagenomic datasets. *Bioinformatics* 27: 863-864.

Schneider, H., Schwiertz, A., Collins, M. D. & Blaut, M. (1999) Anaerobic transformation of quercetin-3-glucoside by bacteria from the human intestinal tract. *Archive of Microbiology* 171: 81-91.

Schoefer, L., Mohan, R., Schwiertz, A., Braune, A., and Blaut, M. (2003) Anaerobic degradation of flavonoids by *Clostridium orbiscindens*. *Applied and Environmental Microbiology* 69: 5849-5854.

Schols, H.A., and Voragen, A.G.J. (1994) Occurrence of pectic hairy regions in various plant cell wall materials and their degradability by rhamnogalacturonase. *Carbohydrate Research* 256: 83-95.

Scholtens, P.A.M.J., Oozeer, R., Martin, R., Amor, K.B., and Knol, J. (2012) The early settlers: intestinal microbiology in early life. *Annual Review of Food Science and Technology* 3: 425-447.

Schwiertz, A., Le Blay, G., and Blaut, M. (2000) Quantification of different *Eubacterium* spp. in human fecal samples with species-specific 16S rRNA-targeted oligonucleotide probes. *Applied and Environmental Microbiology* 66: 375-382.

Schwiertz, A., Taras, D., Schäfer, K., Beijer, S., Bos, N.A., Donus, C., and Hardt, P.D. (2010) Microbiota and SCFA in lean and overweight healthy subjects. *Obesity* 18: 190-195.

Schwiertz, A., Hold, G.L., Duncan, S.H., Gruhl, B., Collins, M.D., Lawson, P.A. et al. (2002) *Anaerostipes caccae* gen. nov., sp. nov., a new saccharolytic, acetate-utilising, butyrate-producing bacterium from human faeces. *Systematic and Applied Microbiology* 25: 46-51.

Scott, K.P., Martin, J.C., Duncan, S.H., and Flint, H.J. (2014) Prebiotic stimulation of human colonic butyrate-producing bacteria and bifidobacteria, *in vitro*. *FEMS Microbiology Ecology* 87: 30-40.

Scott, K.P., Martin, J.C., Campbell, G., Mayer, C.-D., and Flint, H.J. (2006) Whole-genome transcription profiling reveals genes up-regulated by growth on fucose in the human gut bacterium "*Roseburia inulinivorans*". *Journal of Bacteriology* 188: 4340-4349.

Šebeková, K., Krajčovičová-Kudláčková, M., Schinzel, R., Faist, V., Klvanová, J., and Heidland, A. (2001) Plasma levels of advanced glycation end products in healthy, long-term vegetarians and subjects on a western mixed diet. *European Journal of Nutrition* 40: 275-281.

Segain, J.-P., de la Blétière, D.R., Bourreille, A., Leray, V., Gervois, N., Rosales, C. et al. (2000) Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease. *Gut* 47: 397-403.

Semba, R., Ferrucci, L., Sun, K., Beck, J., Dalal, M., Varadhan, R. et al. (2009) Advanced glycation end products and their circulating receptors predict cardiovascular disease mortality in older community-dwelling women. *Aging Clinical and Experimental Research* 21: 182-190.

Sen A, M.S., Kale RD (2014) Fruit waste pectin in enhancing the establishment of probiotic bacteria. J*ournal of Nutritional Health & Food Engineering* 1: 2373-4310.

Sengupta, S., Muir, J., and Gibson, P. (2006) Does butyrate protect from colorectal cancer? *Journal of Gastroenterology and Hepatology* 21: 209 - 218.

Shah, H.N., and Collins, M.D. (1988) Proposal for reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus,

Porphyromonas. *International Journal of Systematic and Evolutionary Microbiology* 38: 128-131.

Sheng, Y., Abreu, I.A., Cabelli, D.E., Maroney, M.J., Miller, A.-F., Teixeira, M., and Valentine, J.S. (2014) Superoxide dismutases and superoxide reductases. *Chemical Reviews* 114: 3854-3918.

Sherry, C.L., Kim, S.S., Dilger, R.N., Bauer, L.L., Moon, M.L., Tapping, R.I. et al. (2010) Sickness behavior induced by endotoxin can be mitigated by the dietary soluble fiber, pectin, through up-regulation of IL-4 and Th2 polarization. *Brain, Behavior, and Immunity* 24: 631-640.

Shlaes, D.M., Shlaes, J.H., Davies, J., and Williamson, R. (1989) *Escherichia coli* susceptible to glycopeptide antibiotics. *Antimicrobial Agents and Chemotherapy* 33: 192-197.

Shoaie, S., Karlsson, F., Mardinoglu, A., Nookaew, I., Bordel, S., and Nielsen, J. (2013) Understanding the interactions between bacteria in the human gut through metabolic modeling. *Scientific Reports* 3: 2532.

Smillie, C.S., Smith, M.B., Friedman, J., Cordero, O.X., David, L.A., and Alm, E.J. (2011) Ecology drives a global network of gene exchange connecting the human microbiome. *Nature* 480: 241-244.

Smith, E.A., and Macfarlane, G.T. (1997) Dissimilatory amino acid metabolism in human colonic bacteria. *Anaerobe* 3: 327-337.

Smith, E.A., and Macfarlane, G.T. (1998) Enumeration of amino acid fermenting bacteria in the human large intestine: effects of pH and starch on peptide metabolism and dissimilation of amino acids. *FEMS Microbiology Ecology* 25: 355-368.

Sommer, F., and Backhed, F. (2013) The gut microbiota - masters of host development and physiology. *Nature Reviews Microbiology* 11: 227-238.

Sonnenburg, J.L., Xu, J., Leip, D.D., Chen, C.-H., Westover, B.P., Weatherford, J. et al. (2005) Glycan foraging in vivo by an intestine-adapted bacterial symbiont. *Science* 307: 1955-1959.

Stadtman, T.C., and White, F.H. (1954) Tracer studies on ornithine, lysine and formate metabolism in an amino acid fermenting clostridium. *Journal of Bacteriology* 67: 651-657.

Stams, A.J.M., Van Dijk, J.B., Dijkema, C., and Plugge, C.M. (1993) Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. *Applied and Environmental Microbiology* 59: 1114-1119.

Stoffels, L., Krehenbrink, M., Berks, B.C., and Unden, G. (2012) Thiosulfate reduction in *Salmonella enterica* is driven by the proton motive force. *Journal of Bacteriology* 194: 475-485.

Sukemori, S., Ikeda, S., Kurihara, Y., and Ito, S. (2003) Amino acid, mineral and vitamin levels in hydrous faeces obtained from coprophagy-prevented rats. *Journal of Animal Physiology and Animal Nutrition* 87: 213-220.

Sun, Y., Cai, Y., Liu, L., Yu, F., Farrell, M.L., McKendree, W., and Farmerie, W. (2009) ESPRIT: estimating species richness using large collections of 16S rRNA pyrosequences. *Nucleic Acids Research* 37: e76.

Suzuki, K., Benno, Y., Mitsuoka, T., Takebe, S., Kobashi, K., and Hase, J. (1979) Urease-producing species of intestinal anaerobes and their activities. *Applied and Environmental Microbiology* 37: 379-382.

Suzuki, M.T., Taylor, L.T., and DeLong, E.F. (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. *Applied and Environmental Microbiology* 66: 4605-4614.

Tailford, L., Crost, E., Kavanaugh, D., and Juge, N. (2015) Mucin glycan foraging in the human gut microbiome. *Frontiers in Genetics* 6. 81

Takeuchi, M., Takino, J.-i., Furuno, S., Shirai, H., Kawakami, M., Muramatsu, M. et al. (2015) Assessment of the concentrations of various advanced glycation end-products in beverages and foods that are commonly consumed in Japan. *PLoS One* 10: e0118652.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731-2739.

Tang, Y., and Chen, A. (2014) Curcumin eliminates the effect of advanced glycation end-products (AGEs) on the divergent regulation of gene expression of receptors of AGEs by interrupting leptin signaling. *Lab Investigation* 94: 503-516.

Tazawa, K., Okami, H., Yamashita, I., Ohnishi, Y., Kobashi, K., and Fujimaki, M. (1997) Anticarcinogenic action of apple pectin on fecal enzyme activities and mucosal or portal prostaglandin E2 levels in experimental rat colon carcinogenesis. *Journal of Experimental & Clinical Cancer Research* 16: 33 - 38.

Thompson, J., Higgins, D., and Gibson, T. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673 - 4680.

Thurl, S., Munzert, M., Henker, J., Boehm, G., Müller-Werner, B., Jelinek, J., and Stahl, B. (2010) Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *British Journal of Nutrition* 104: 1261-1271.

Tims, S., Derom, C., Jonkers, D.M., Vlietinck, R., Saris, W.H., Kleerebezem, M. et al. (2013) Microbiota conservation and BMI signatures in adult monozygotic twins. *ISME J* 7: 707-717.

Tissier, H. (1908) Recherches sur la flore intestinale normale des enfants âgés d'un an à cinq ans. *Annales de l'Institut Pasteur* (Paris) 22: 189-208.

Toden, S., Bird, A.R., Topping, D.L., and Conlon, M.A. (2007) Dose-dependent reduction of dietary protein-induced colonocyte DNA damage by resistant starch in rats correlates more highly with caecal butyrate than with other short chain fatty acids. *Cancer Biology & Therapy* 6: 253-258.

Topping, D., and Clifton, P. (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological Reviews.*81: 1031 - 1064.

Turnbaugh, P., Ley, R., Mahowald, M., Magrini, V., Mardis, E., and Gordon, J. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444: 1027 - 1131.

Turnbaugh, P.J., and Stintzi, A. (2011) Human health and disease in a microbial world. *Frontiers in Microbiology* 2: 190.

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R., and Gordon, J.I. (2007) The Human Microbiome Project. 449: 804-810.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E. et al. (2009) A core gut microbiome in obese and lean twins. *Nature* 457: 480-484.

Uribarri, J., Cai, W., Sandu, O., Peppa, M., Goldberg, T., and Vlassara, H. (2005) Diet-derived advanced glycation end products are major contributors to the body's AGE pool and induce inflammation in healthy subjects. *Annals of the New York Academy of Sciences* 1043: 461-466.

van Gelder, A.H., Aydin, R., Alves, M.M., and Stams, A.J.M. (2012) 1,3-Propanediol production from glycerol by a newly isolated *Trichococcus* strain. *Microbial Biotechnology* 5: 573-578.

Vanhoutvin, S.A.L.W., Troost, F.J., Kilkens, T.O.C., Lindsey, P.J., Hamer, H.M., Jonkers, D.M.A.E. et al. (2009) The effects of butyrate enemas on visceral perception in healthy volunteers. *Neurogastroenterology & Motility* 21: 952-976.

van Nood, E., Vrieze, A., Nieuwdorp, M., Fuentes, S., Zoetendal, E.G., de Vos, W.M. et al. (2013) Duodenal infusion of donor feces for recurrent *Clostridium difficile*. *New England Journal of Medicine* 368: 407-415.

Verberkmoes, N.C., Russell, A.L., Shah, M., Godzik, A., Rosenquist, M., Halfvarson, J. et al. (2008) Shotgun metaproteomics of the human distal gut microbiota. *ISME J* 3: 179-189.

Vernia, P., Monteleone, G., Grandinetti, G., Villotti, G., Di Giulio, E., Frieri, G. et al. (2000)

Combined oral sodium butyrate and mesalazine treatment compared to oral mesalazine alone in ulcerative colitis. *Digestive Diseases and Sciences* 45: 976-981.

Vital, M., Howe, A.C., and Tiedje, J.M. (2014) Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. *mBio* 5. e00889-14

Vital, M., Gao, J., Rizzo, M., Harrison, T., and Tiedje, J.M. (2015) Diet is a major factor governing the fecal butyrate-producing community structure across mammalia, aves and reptilia. *ISME J* 9: 832-843.

Vital, M., Penton, C.R., Wang, Q., Young, V.B., Antonopoulos, D.A., Sogin, M.L. et al. (2013) A gene-targeted approach to investigate the intestinal butyrate-producing bacterial community. *Microbiome* 1: 1-14.

Vlassara, H., Cai, W., Crandall, J., Goldberg, T., Oberstein, R., Dardaine, V. et al. (2002) Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proceedings of the National Academy of Sciences* 99: 15596-15601.

Voragen, A.J., Coenen, G.-J., Verhoef, R., and Schols, H. (2009) Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry* 20: 263-275.

Walker, A.W., Ince, J., Duncan, S.H., Webster, L.M., Holtrop, G., Ze, X. et al. (2011) Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J* 5: 220-230.

Walter, J., Hertel, C., Tannock, G.W., Lis, C.M., Munro, K., and Hammes, W.P. (2001) Detection of *Lactobacillus, Pediococcus, Leuconostoc,* and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* 67: 2578-2585.

Wang, S., Yu, J., Suvira, M., Setlow, P., and Li, Y.-q. (2015a) Uptake of and resistance to the antibiotic berberine by individual dormant, germinating and outgrowing spores as monitored by laser tweezers Raman spectroscopy. *PLoS One* 10: e0144183.

Wang, W.L., Xu, S.Y., Ren, Z.G., Tao, L., Jiang, J.W., and Zheng, S.S. (2015b) Application of metagenomics in the human gut microbiome. *World Journal of Gastroenterology* : WJG 21: 803-814.

Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I. et al. (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic Bacteriology* 37: 463-464.

Weaver, G.A., Tangel, C.T., Krause, J.A., Parfitt, M.M., Jenkins, P.L., Rader, J.M. et al. (1997) Acarbose enhances human colonic butyrate production. *The Journal of Nutrition* 127: 717-723.

West, R.K., Moshier, E., Lubitz, I., Schmeidler, J., Godbold, J., Cai, W. et al. (2014) Dietary

advanced glycation end products are associated with decline in memory in young elderly. *Mechanisms of Ageing and Development* 140: 10-12.

Wiame, E., and Van Schaftingen, E. (2004) Fructoselysine 3-epimerase, an enzyme involved in the metabolism of the unusual Amadori compound psicoselysine in *Escherichia coli*. *Biochemical Journal* 378: 1047-1052.

Wiame, E., Delpierre, G., Collard, F., and Van Schaftingen, E. (2002) Identification of a pathway for the utilization of the Amadori product Fructoselysine in *Escherichia coli*. *Journal of Biological Chemistry* 277: 42523-42529.

Wiesenborn, D.P., Rudolph, F.B., and Papoutsakis, E.T. (1989) Coenzyme A transferase from *Clostridium acetobutylicum* ATCC 824 and its role in the uptake of acids. *Applied and Environmental Microbiology* 55: 323-329.

Wikoff, W.R., Anfora, A.T., Liu, J., Schultz, P.G., Lesley, S.A., Peters, E.C., and Siuzdak, G. (2009) Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proceedings of the National Academy of Sciences* 106: 3698-3703.

Windey, K., De Preter, V., Louat, T., Schuit, F., Herman, J., Vansant, G., and Verbeke, K. (2012) Modulation of protein fermentation does not affect fecal water toxicity: A randomized cross-over study in healthy subjects. *PLoS One* 7: e52387.

Winter, J., Popoff, M. R., Grimont, P. & Bokkenheuser, V. D. (1991) *Clostridium orbiscindens* sp. nov., a human intestinal bacterium capable of cleaving the flavonoid C-ring. *International Journal of Systematic and Evolutionary Microbiology* 41: 355-357.

Wolever, T.M.S., and Chiasson, J.L. (2000) Acarbose raises serum butyrate in human subjects withimpaired glucose tolerance. *British Journal of Nutrition* 84: 57-61.

Wong, J.M.W., de Souza, R., Kendall, C.W.C., Emam, A., and Jenkins, D.J.A. (2006) Colonic health: Fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology* 40: 235-243.

Wootton, L. (2013) Phage biology: A new barrier at mucosal surfaces. *Nature Reviews Microbiology* 11: 430-431.

Wright, E.S., Yilmaz, L.S., and Noguera, D.R. (2012) DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. *Applied and Environmental Microbiology* 78: 717-725.

Xiao, L., Feng, Q., Liang, S., Sonne, S.B., Xia, Z., Qiu, X. et al. (2015) A catalog of the mouse gut metagenome. *Nature Biotechnology* 33: 1103-1108.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M. et al. (2012) Human gut microbiome viewed across age and geography. *Nature* 486: 222-227.

Yorifuji, T., Jeng, I.M., and Barker, H.A. (1977) Purification and properties of 3-keto-5-aminohexanoate cleavage enzyme from a lysine-fermenting *Clostridium*. *Journal of Biological Chemistry* 252: 20-31.

Ze, X., Duncan, S.H., Louis, P., and Flint, H.J. (2012) *Ruminococcus bromii* is a keystone species for the degradation of resistant starch in the human colon. *ISME J* 6: 1535–1543.

Zhou, Y., Liang, Y., Lynch, K.H., Dennis, J.J., and Wishart, D.S. (2011) PHAST: a fast phage search tool. *Nucleic Acids Research* 39: 347-352

Zimmerman, M.A., Singh, N., Martin, P.M., Thangaraju, M., Ganapathy, V., Waller, J.L. et al. (2012) Butyrate suppresses colonic inflammation through HDAC1-dependent Fas upregulation and Fas-mediated apoptosis of T cells: *American Journal of Gastrointestinal and Liver Physiology* 302: G1405-G1415

Zoetendal, E.G., Ben-Amor, K., Harmsen, H.J.M., Schut, F., Akkermans, A.D.L., and de Vos, W.M. (2002) Quantification of uncultured *Ruminococcus obeum*-like bacteria in human fecal samples by fluorescent in situ hybridization and flow cytometry using 16S rRNA-targeted probes. *Applied and Environmental Microbiology* 68: 4225-4232.

Zoetendal, E.G., and de Vos, W.M. (2014) Effect of diet on the intestinal microbiota and its activity. *Current Opinion in Gastroenterology* 30: 189-195

Zoetendal, E.G., Akkermans, A.D.L., and De Vos, W.M. (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Applied and Environmental Microbiology* 64: 3854-3859.

Zoetendal, E.G., Vaughan, E.E., and De Vos, W.M. (2006) A microbial world within us. *Molecular Microbiology* 59: 1639-1650.

Zoetendal, E.G., Rajilić-Stojanović, M., and de Vos, W.M. (2008) High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut* 57: 1605-1615.

Zoetendal, E.G., Raes, J., van den Bogert, B., Arumugam, M., Booijink, C.C.G.M., Troost, F.J. et al. (2012) The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *ISME J* 6: 1415-1426.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., and van 't Riet, K. (1990) Modeling of the bacterial growth curve. *Applied and Environmental Microbiology* 56: 1875-1881.

Summary

The human intestinal tract harbours trillions on microbial cells, predominantly anaerobes. The activity and physiology of these anaerobes is strongly associated with health and disease. This association has been investigated for a long time. However, these has not been fully understood. One of the reasons is the limited availability of cultured representatives. It is estimated that there may be more than 3000 species colonised in the gut of healthy individuals, however, only a bit over 1000 species have been isolated and characterised. Among the intestinal microbes, butyrate-producing bacteria are of special interest as butyrate produced, is crucial to maintain a healthy gut. In addition, butyrate-producing bacteria have shown a reverse correlation with several intestinal diseases. In **Chapter 2** we described a novel species *Anaerostipes rhamnosivorans* 1y-2^T isolated from an infant stool. This strain belonged to genus *Anaerostipes* within *Clostridium* cluster XIVa. *A. rhamnosivorans* had a capability of converting rhamnose into butyrate that is unique within intestinal butyrate-producing bacteria. The genomic analysis also revealed the entire rhamnose fermentation pathway as well as the acetyl-CoA pathway for butyrate production. This bacterium is able to produce butyrate from a wide range of sugars as well as lactate plus acetate. In **Chapter 3**, we described the microbial interactions between *A. rhamnosivorans* and *Bacteriodes thetaiotaomicron* in dietary pectins; *Blautia hydrogenotrophica* in lactate and small amount of acetate; *Methanobrevibacter smithii* in glucose. We observed that *A. rhamnosivorans* was able to benefit from its partners in all cocultures for butyrate production. This is likely due to its high metabolic flexibility. While the interaction between *A. rhamnosivorans* and *B. thetaiotaomicron* appeared as syntrophy, the interaction between *A. rhamnosivorans* and hydrogenotrohic microbes were cross-feeding type where hydrogen was transferred between two species. The latter resulted in an increase in butyrate level. In **Chapter 4** we described a novel species *Intestinimonas butyriciproducens* SRB521^T representing a novel genus Intestinimonas from a mouse caecum within *Clostridium* cluster IV. This bacterium produced butyrate and acetate as end products from Wilkins-Chalgren-Anaerobe broth.

Butyrate production is assumed to derive from carbohydrate employing acetyl-CoA pathway. No gut bacterium is known to convert proteins or amino acids to butyrate although butyrogenic pathways from amino acid degradation have been detected in the human gut using metagenomic approach. In **Chapter 5** we discovered a novel butyrate synthesis pathway from the amino acid lysine and the Amadori product fructoselysine in *Intestinimonas butyriciproducens* AF211 that was isolated from human stool. This strain appeared to grow much better in lysine as compared to sugars although lysine and acetyl-CoA pathways were both detected in its complete genome. Moreover, the strain AF211 was able to metabolise efficiently fructoselysine into butyrate, and acetate was found to affect the fructoselysine fermentation, representing the impact of the environmental conditions where acetate is abundant in the gut. While the lysine pathway was found in the gut of many individuals, the fructoselysine pathway was present in only half of those samples. The finding that strain *I. butyriciproducens* AF211 is capable

of the butyrogenic conversion of amino acid lysine and fructoselysine, an Amadori product formed in heated foods via the Maillard reaction, indicated a missing link that coupling protein metabolism and butyrate formation. As this Amadori product has been implicated to play a role in aging process, the use of strain AF211 as fructoselysine clearance in the gut needs further investigation. In **Chapter 6** we performed genomic and physiological comparison between the *I. butyriciproducens* strain AF211 (human isolate) and SRB521^{T} (mouse isolate). *I. butyriciproducens* was the most abundant species within the Intestinimonas genus and highly prevalent in humans based on metadata analysis on 16S amplicons. We confirmed that the butyrogenesis from lysine was a shared characteristic between the two *I. butyriciproducens* strains. We also observed the host specific features including tolerance to bile, cellular fatty acid composition, more efficient capability of converting sugars into butyrate, especially galactose and arabinose, in the human strain AF211. In addition, genomic rearrangements as well as variations in bacteriophages differed among strains.

Samenvatting

Het menselijke spijsverteringskanaal bevat triljoenen microbiële cellen, voornamelijk bestaande uit anaerobe micro-organismen. De activiteit en fysiologie van deze anaeroben is sterk geassocieerd met gezondheid en ziekte. Deze associatie is lange tijd een onderwerp van studie geweest, maar nog niet alles is opgehelderd. Een van de redenen daarvoor is de gelimiteerde beschikbaarheid van representatieve pure cultures. Het is geschat dat de darm van gezonde individuen gekoloniseerd is door meer dan 3000 verschillende soorten micro-organismen terwijl er maar iets meer dan 1000 soorten geïsoleerd en gekarakteriseerd zijn. Van alle microben in het spijsverteringskanaal is men in het speciaal geïnteresseerd in butyraat-producerende bacteriën. Dit komt omdat butyraat cruciaal is voor een gezond spijsverteringsstelsel. Daarnaast is de aanwezigheid van butyraat-producerende bacteriën negatief gecorreleerd is met verschillende spijsverteringziekten. In **hoofdstuk 2** beschrijven we een nieuwe bacteriesoort genaamd *Anaerostipes rhamnosivorans* 1y-2^T, dat geïsoleerd is uit de ontlasting van een pasgeborene. De stam hoort bij het genus *Anaerostipes* binnen *Clostridium* cluster XIVa. *A. rhamnosivorans* is in staat om rhamnose om te zetten naar butyraat. Dit is tot nu toe een unieke eigenschap binnen de butyraat-producerende darmbacteriën. Analyse van het genoom heeft aangetoond dat *A. rhamnosivorans* de volledige metabole route ('pathway') voor rhamnose-fermentatie en de volledige acetyl-CoA pathway voor butyraatproductie in haar genen codeert. De bacterie kan ook butyraat produceren uit lactaat en acetaat en uit een verscheidenheid aan suikers. In **hoofdstuk 3** beschrijven we de microbiële interacties tussen *A. rhamnosivorans* en verscheidende andere bacteriën op verschillende groei-substraten: met *Bacteriodes thetaiotaomicron* op dieet-pectines als substraat; met *Blautia hydrogenotrophica* op lactaat en een kleine hoeveelheid acetaat; met *Methanobrevibacter smithii* op glucose. We observeerden dat *A. rhamnosivorans* voordeel haalde uit elk partnerschap voor de productie van butyraat. Dit komt waarschijnlijk door zijn hoge metabole flexibiliteit. Terwijl de interactie tussen *A. rhamnosivorans* en *B. thetaiotaomicron* syntroof leek te zijn, wees de interactie met hydrogenotrofe bacteriën op "cross-feeding", waar waterstof tussen de twee soorten doorgegeven wordt. Het laatste zorgde voor een toename in het butyraatniveau. In **hoofdstuk 4** beschrijven we een nieuwe soort geïsoleerd uit muizenfeces, genaamd *Intestinimonas butyriciproducens* SRB521^T, dat een nieuw genus vormt binnen *Clostridium* cluster IV. Deze bacterie produceert butyraat en acetaat als eindproducten wanneer het groeit op Wilkins-Chalgren-Anaerobe extract.

Het wordt in het algemeen aangenomen dat butyraat via de acetyl-CoA pathway geproduceerd wordt. Tot nu toe is er namelijk geen enkele bacterie ontdekt die butyraat kan produceren uit eiwit of aminozuren. Wel zijn in de menselijk darm, door middel van metagenoom-gebaseerde analyses, pathways ontdekt waarin butyraat geproduceerd zou kunnen worden tijdens de degradatie van aminozuren. In **hoofdstuk 5** beschrijven we een nieuwe butyraat-synthese pathway vanuit het aminozuur lysine en het Amadori product fructolysine in *Intestinimonas butyriciproducens* AF211. Deze stam is geïsoleerd uit menselijke feces en leek veel beter te

groeien op lysine dan op suikers alhoewel naast de lysine pathway ook de acetyl-CoA pathway gedetecteerd was in het complete genoom. Ook was stam AF211 capabel om fructolysine om te zetten naar butyraat. Acetaat beïnvloedde de fermentatie van fructolysine, wat de invloed laat zien van omgevingsfactoren in de darm waar acetaat in overvloed aanwezig is. Terwijl in de darm van veel individuen de lysine pathway gevonden is, was de fructolysine pathway alleen aanwezig in de helft van die individuen. De bevinding dat stam *I. butyriciproducens* AF211 butyraat kan produceren uit lysine en het Amadori product fructolysine (gevormd tijdens de Maillard reactie die optreedt tijdens verhitting van voedsel), is een 'missing link' dat het eiwit –en butyraatmetabolisme in de darm koppelt. Er zijn aanwijzingen dat fructolysine een rol speelt tijdens veroudering. Stam AF211 zou daardoor toegepast kunnen worden voor het verwijderen van fructolysine in de darm, maar daar is meer onderzoek voor nodig. In **hoofdstuk 6** beschrijven we de vergelijking tussen *I. butyriciproducens* stam AF211 (humaan isolaat) en SRB521^T (muis isolaat) op genomisch en fysiologisch niveau. Gebaseerd op metadata analyse van 16S amplicons is aangetoond dat *I. butyriciproducens* de meest voorkomende soort binnen het Intestinimonas genus is en sterk aanwezig is in mensen. We hebben bevestigd dat butyraat synthese van lysine een gedeelde eigenschap is tussen beide *I. butyriciproducens* stammen. We hebben ook gastheer-specifieke eigenschappen zoals tolerantie voor gal, cellulaire vetzuur compositie en efficiëntie in de capaciteit om suikers om te zetten in butyraat (voornamelijk galactose en arabinose) in de humane stam AF211 gedetecteerd. Daarbij zagen we verschillen in genomische samenstelling en variaties in bacteriofaag-sequenties tussen de stammen.

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Nam Bui was born on December 6th 1984 in Bac Giang province, Vietnam. In 2002, she obtained her BSc diploma on Food Technology at Hanoi University of Technology, Vietnam, after which she got a scholarship to do a master in Kyung Hee University in South Korea in 2008. There she screened for bacteria that were present in fermented food and ginseng soil that could tolerate a high concentration of ginsenosides and had activities to convert ginsenosides to pharmaceutical valuable products. She finished her master in 2010 after which, she worked for 6 months in the Laboratory for Molecular Infection Medicine Sweden (MIMS), Umea University, Sweden. In May 2011 she moved to Netherlands and joined Microbial Physiology group at Laboratory of Microbiology of Wageningen University. During her PhD, she studied intestinal butyrate-producing bacteria under the supervision of Prof. Dr Willem M. de Vos and Dr Caroline M. Plugge. Currently Nam is working as a post-doctoral researcher at the same lab.

List of publications

Bui, T.P.N., Ritari, J., Boeren, S., de Waard, P., Plugge, C.M., and de Vos, W.M. (2015) Production of butyrate from lysine and the Amadori product fructoselysine by a human gut commensal. *Nature Communications* 6.

Bui, T.P.N., de Vos, W.M., and Plugge, C.M. (2014) *Anaerostipes rhamnosivorans* sp. nov., a human intestinal, butyrate-forming bacterium. *International Journal of Systematic and Evolutionary Microbiology* 64: 787-793.

Kläring, K.*, Hanske, L.*, **Bui, T.P.N**.*, Charrier, C., Blaut, M., Haller, D. et al. (2013) *Intestinimonas butyriciproducens* gen. nov., sp. nov., a butyrate-producing bacterium from the mouse intestine. *International Journal of Systematic and Evolutionary Microbiology* 63: 4606-4612.

Bui, T.P.N., Schols, H. A., Stams, A.J.M., de Vos, W.M., and Plugge, C.M. Microbial interactions that promote butyrate production in the human intestine. Manuscript in preparation.

Bui, T.P.N., Shetty, S., Lagkouvardos, I., Ritari, J., Douillard, F., Paulin, L., Clavel, T., Plugge, C.M., and de Vos, W.M. Comparative genomics and physiology of butyrate-producing bacterium *Intestinimonas butyriciproducens* strains reveal host-specific features. Manuscript in preparation.

Bui, T.P.N., Kim, Y.-J., In, J.-G., and Yang, D.-C. (2011) *Lactobacillus koreensis* sp. nov., isolated from the traditional Korean food kimchi. *International Journal of Systematic and Evolutionary Microbiology* 61: 772-776.

Bui, T.P.N., Kim, Y.-J., Kim, H., and Yang, D.-C. (2010) Rhodanobacter soli sp. nov., isolated from soil of a ginseng field. *International Journal of Systematic and Evolutionary Microbiology* 60: 2935-2939.

Kim, H.-B., Srinivasan, S., Sathiyaraj, G., Quan, L.-H., Kim, S.-H., **Bui, T.P.N**. et al. (2010a) *Stenotrophomonas ginsengisoli* sp. nov., isolated from a ginseng field. *International Journal of Systematic and Evolutionary Microbiology* 60: 1522-1526.

Kim, Y.-J., Kim, M.K., **Bui, T.P.N**., Kim, H.-B., Srinivasan, S., and Yang, D.-C. (2010b) *Microbacterium ginsengiterrae* sp. nov., a β-glucosidase-producing bacterium isolated from soil of a ginseng field. *International Journal of Systematic and Evolutionary Microbiology* 60: 2808-2812.

*Contribute equally

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

D I P L O M A

For specialised PhD training

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE) declares that

Bui Thi Phuong Nam

born on 6 December 1984 in Bac Giang, Vietnam

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 22 April 2016

the Chairman of the SENSE board	the SENSE Director of Education
Prof. dr. Huub Rijnaarts	Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

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The SENSE Research School declares that **Ms Bui Thi Phuong Nam** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 38.5 EC, including the following activities:

SENSE PhD Courses

- Environmental Research in Context (2011)
- Research in Context Activity: Co-organising SENSE Symposium 'Novel Anaerobes', Wageningen (2014)

Other PhD and Advanced MSc Courses

- Microbial Physiology, Wageningen University (2011)
- Scientific Writing, Wageningen University (2012)
- Techniques for writing and presenting a scientific paper, Wageningen University (2014)
- ARB software course, Wageningen University (2014)

Management and Didactic Skills Training

- Teaching practicals for the BSc course 'Microbial Physiology' (2012, 2014)
- Teaching practicals for the BSc and MSc course 'Research Methods Microbiology' (2013)
- Supervising MSc student with thesis entitled 'Characterisation of a novel butyrate producing bacterium isolated from the mouse intestine' (2013)

Oral Presentations

- *Enrichment and Isolation of butyrate producing microorganisms from the GI tract*. SENSE PhD trip 'Microbiology in USA and Canada', 15 April-1 May 2013, Boston, United States
- *Butyrate production from lysine by a human gut commensal*. EMBO Meeting - The human Microbiome, 10-12 June 2015, Heidelberg, Germany

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