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„Influences Of Gastrointestinal Microbiota And Short Chain Fatty Acids On Metabolic Syndrome; And Consequences Of Caloric Restriction On The Composition Of The GI Microbiota“

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Introduction

The human gut microbiota, our lifestyle, our diet and our health are clearly associated. Several studies provided detailed insight into host–microbiota interactions, for example with the metabolic system, immune system, or central nervous system. Culture-independent studies have revealed that a typical gut ecosystem harbors thousands of phylotypes from less than ten bacterial phyla dominated by *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (Salonen *et al.*, 2012). The collective genome of the intestinal microbes has 100 times more genes than the human genome (Qin *et al.*, 2010). All humans have an individual microbiome, so the discovery of the high-level clusters, such as enterotypes (Arumugam *et al.*), or microbial markers, such as the butyryl-CoA:acetate CoA-transferase gene (Louis & Flint, 2007) are of huge interest for routine and research analysis. A highly diverse microbiota seems to be the key to adult human health. Long-term dietary patterns have been found to be associated with specific microbiota compositions. Fecal transplantations indicate that homeostasis can be restored and indicate that diet–microbiota-induced disorders can be improved by therapeutic modification of the microbiota (Zoetendal & de Vos, 2014). The gut microbiota provides numerous fundamental functions to the host, including vitamin and metabolite supply and colonization resistance against pathogens (Neish, 2009). Insight into the specificity of complex carbohydrate conversion into SCFA is essential to understand the impact of diet on health (Zoetendal & de Vos, 2014).

The microbial production of short-chain fatty acids, acetaldehyde and inflammatory mediators have been shown to significantly impact upon the metabolic health of the host through pathways that influence satiety, gut permeability and immune function. In the small intestine, microbial metabolism alters the host bile acid profile affecting the interactions with dedicated bile acid receptors to influence both local and systemic cellular responses. Recent findings have, therefore, identified specific microbiota profiles and metabolites as predictors of disease risk as well as determining the microbial species (such as *Akkermansia muciniphila*) which correlate with health and disease. (Joyce & Gahan, 2014)

Microbiota over Life Time

The development of the gut microbiota primarily occurs during infancy and is influenced by multiple factors, including the neonate’s mother, prenatal exposure, gestational age, mode of delivery, feeding type, pre-, pro-, and antibiotic use and host genetics. It develops into a complex ecosystem through dramatic changes primarily in the first years of life (Favier *et al.*, 2002, Palmer *et al.*, 2007). In genetically susceptible individuals, changes in the gut microbiota induced by environmental factors may contribute to the development of immune-related disorders in childhood, including atopic diseases, inflammatory bowel disease, irritable bowel syndrome, and necrotizing enterocolitis. (Li *et al.*, 2014) The adult-like profile of the human intestinal microbiota is stably maintained over time in healthy adults (Faith *et al.*, 2011, Lozupone *et al.*, 2012). Although the mechanisms for the selection of the intestinal microbes are not fully understood, the resulting ecosystem is unique for each subject and is influenced by both genetics and environment (Zoetendal *et al.*, 2001, Turnbaugh & Gordon,

2009). This subject-specific microbiota consists of several hundred microbial species and remains remarkably stable over short-term intervals (Jalanka-Tuovinen *et al.*, 2011, Rajilic-Stojanovic *et al.*, 2012).

The microbiota in children is predominantly composed of *Firmicutes* and *Bacteroides*. *Roseburia/E. rectale*, a member of *Clostridia cluster XIVa*, represents a larger *Firmicutes* subgroup in comparison with cluster IV of which *F. prausnitzii* is a member. A higher proportion of *Roseburia/E. rectale/F. prausnitzii* is detected in children (Payne *et al.*, 2011). In obese children higher levels of butyrate could be observed (Payne *et al.*, 2011). In normal weight children higher levels of lactate could be observed compared to higher concentrations of butyrate in obese children, suggesting the presence of lactate-utilizing butyrate-producing species in obese children. Acetat/butyrate levels are comparable between lean and obese children. (Payne *et al.*, 2011)

Jost *et al.* 2014 demonstrate that the maternal gut microbiota remains stable over the perinatal period despite altered metabolic activity and low-grade inflammation. The total fecal SCFA concentrations, particularly the branched-chain fatty acids isobutyrate and isovalerate, were higher than for non-pregnant subjects throughout the perinatal period. This suggests metabolic changes and increased energy extraction via proteolytic in addition to saccharolytic fermentation, accompanied by low-grade inflammation based on fecal calprotectin levels.

Despite its individual gastro intestinal stability, the microbiota seems to change with age as significant differences between the composition of the microbiota of healthy middle-aged adults, elderly and centenarian subjects have been reported (Claesson *et al.*, 2012, Rajilic-Stojanovic *et al.*, 2012) In a pilot study by Rampelli *et al.* changes in microbiota composition in older people have been connected to immunosenescence and inflammaging (Franceschi *et al.*, 2000, Biagi *et al.*, 2013, Cevenini *et al.*, 2013, Cheng *et al.*, 2013). An important factor which affects the gut microbiota in ageing is diet (Yatsunenko *et al.*, 2012). Rampelli *et al.* found an age-related reduction in the abundance of genes in pathways involved in SCFA production. Based on differential abundance, the aged-type microbiota is structurally and functionally compromised, moving from a saccharolytic to a putrefactive metabolism. It is noteworthy that these microbiome alterations concord with the enrichment of genes belonging to pathobionts. Pathobionts are minor microbiota opportunists able to thrive in inflamed conditions, sustaining and nurturing physiological inflammation (Biagi *et al.*, 2013). According to these findings, 116 genes usually present in different metagenome project databases (Kurokawa *et al.*, 2007, Turnbaugh *et al.*, 2007, Qin *et al.*, 2010), reflecting the rearrangement of the core metabolic potential of the centenarian intestinal microbial ecosystem. This altered functional profile was associated with a relevant enrichment in “pathobionts”, i.e. opportunistic pro-inflammatory bacteria generally present in the adult gut ecosystem in low numbers. Finally, as a signature for long life we identified 116 microbial genes that significantly correlated with ageing. Collectively, our data emphasize the relationship between intestinal bacteria and human metabolism, by detailing the modifications in the gut microbiota as a consequence of and/or promoter of the physiological changes occurring in the human host upon ageing. (Rampelli *et al.*, 2013)

Gastrointestinal Microbiota and Low Grad Inflammation in Obesity and Related Metabolic Disorders

There is accumulating evidence that changes in gut microbiota can affect body physiology. While the exact mechanism remains unsolved, animal experimental and human observational studies provide tantalizing evidence that these changes in the intestine can be triggered by exposures to antimicrobial agents, especially in the context of certain dietary nutrients introduced into the gut, and that these changes can contribute to weight gain (Riley *et al.*, 2013). The gut microbiota bridge the indeterminate gap between food and weight by affecting the host metabolism through regulation of intestinal glucose absorption, lipogenesis and fat deposition due to microbiota-triggered epigenetic regulation. Housekeeping functions such as the central carbon metabolism or the amino acid synthesis of important protein complexes are present in every bacterium. Profiles of human gut microbiota reflect the metabolic cooperation between different phylotypes since no genus can degrade all substrates; for example to produce all short chain fatty acids (SCFAs). The ratio of *Bacteroidetes* to *Firmicutes* is significantly and positively correlated with reduced glucose tolerance, but negatively correlated with higher BMI. It is estimated that subjects with type 2 diabetes are relatively enriched with Gram-negative bacteria, belonging to the phyla *Bacteroidetes* and *Proteobacteria*, which can elicit endotoxemia (Larsen *et al.*, 2010) through increased plasma LPS (Cani *et al.*, 2008, Cani & Delzenne, 2009, Tsukumo *et al.*, 2009, Larsen *et al.*, 2010, Luoto *et al.*, 2010, Diamant *et al.*, 2011). In a high fat diet elevated LPS increase gut permeability, by affecting GLP-1, GLP-2 and PYY (Cani *et al.*, 2008, Diamant *et al.*, 2011). An absence of LPS receptor CD14 reduces the high-fat diet-induced effects on adipose tissue inflammation, blood glucose profiles, insulin resistance index, glucose-induced insulin secretion (Cani *et al.*, 2008, DiBaise *et al.*, 2008, Cani & Delzenne, 2009), hepatic steatosis, liver inflammation and adipose tissue macrophages infiltration (Cani *et al.*, 2008). Therefore, in addition to their involvement in inflammatory and metabolic reactions of the gut, gastrointestinal microbiota influence gut permeability and may play a role in intolerances. The groups *Faecalibacterium prausnitzii*, *Akkermansia* and *Lactobacilli* are associated with inflammatory immune regulation and the gut barrier (Louis & Flint, 2007, Furet *et al.*, 2010, Everard *et al.*, 2013, Kim *et al.*, 2013, Le Chatelier *et al.*, 2013). Obese children show significantly higher IgG antibody levels against food antigens compared with children of normal weight. In overweight people IgG antibodies may be associated with increased atherosclerosis risk and concomitantly increased CRP (C-reactive protein) levels (Wilders-Truschnig *et al.*, 2008). Other publications indicate that an elimination diet based on elevated IgG₄ values improves symptoms in patients with irritable bowel symptoms. The work of Zar *et al.* (2005) also shows a positive influence on psychological symptoms (Atkinson *et al.*, 2004, Zar *et al.*, 2005).

Short Chain Fatty Acids in Health and Disease

Microbial fermentation of hydrolyzed polysaccharides in the large intestine results in production of the SCFAs acetate, propionate and butyrate, branched chain fatty acids, lactate, formate, ethanol and mixed gases (e.g., CH₄, CO₂ and H₂) (Robert & Bernalier-Donadille, 2003). SCFAs resulting from colonic fermentation may provide an estimated additional 10% daily dietary energy to the host, which may be used for de novo hepatic triglyceride and glucose synthesis (Flint *et al.*, 2008). Hence, a mere

daily energy increase of 1%, providing an additional 20 kcal per day, based on a 2000 kcal per day diet, could result in nearly 1 kg of weight gain annually (Payne *et al.*, 2011).

SCFAs play an important role in the maintenance of health and the development of disease. The highest levels of SCFAs are found in the proximal colon, where they are used locally by enterocytes or transported across the gut epithelium into the bloodstream. Two major SCFA signalling mechanisms have been identified, inhibition of histone deacetylases (HDACs) and activation of G-protein-coupled receptors (GPCRs). Since HDACs regulate gene expression, inhibition of HDACs has a vast array of downstream consequences (Tan *et al.*, 2014). GPCRs, particularly GPR43, GPR41, and GPR109A, have been identified as receptors for SCFAs. Studies have implicated a major role for these GPCRs in the regulation of metabolism, inflammation, and disease. SCFAs have been shown to alter chemotaxis and phagocytosis, induce reactive oxygen species (ROS), change cell proliferation and function, have anti-inflammatory, antitumorigenic, and antimicrobial effects, and alter gut integrity. SCFAs are a major player in the maintenance of gut and immune homeostasis. Given the variety of effects of SCFAs, and that their levels are regulated by diet, they provide a new basis to explain the increased prevalence of inflammatory disease in westernized countries (Tan *et al.*, 2014).

***Faecalibacterium prausnitzii* Phylotypes in Obesity and its Consequences in Metabolic Disorders**

F. prausnitzii is one of the most common species in the gastro intestinal tract (GIT) of adults consuming a western diet (Scott *et al.*, 2013). *F. prausnitzii* is more common in subjects with high intestinal gut microbiota gene content and (Le Chatelier *et al.*, 2013) lower concentrations have consistently been observed in patients with Crohn's disease or colorectal cancer. *F. prausnitzii* has been discussed for its anti-inflammatory activity, regulating IL-12, IL-10, and IFN- γ (interferon- γ) in the gut (Furet *et al.*, 2010), and as a marker for a high bacterial gene content and gut well-being (Remely *et al.*, 2013).

F. prausnitzii has the ability to switch between diet- and host-derived substrates (Lopez-Siles *et al.*, 2012) and can convert sugars, dietary fibers, proteins and peptides to butyrate (Remely *et al.*, 2013) using mainly the butyryl-CoA:acetate CoA-transferase route. (Louis *et al.*, 2004).

Butyrate is thought to affect diet-induced obesity (Lin *et al.*, 2012) and chronic diseases. It provides additional energy (5-15 % of the total caloric intake) for gut epithelial cells, where the majority of butyrate is metabolized by the colonic epithelium and the remaining is most likely extracted by the liver. It has anti-carcinogenic and anti-inflammatory potential (Hamer *et al.*, 2008) and plays a role in adipose tissue expansion (Cani & Delzenne, 2011). Concentrations in the venous systemic serum range from 0.5 to 3.3 μmol (Hamer *et al.*, 2008), where butyrate acts as a signalling molecule (Brown *et al.*, 2003) via G-protein coupled receptors (GPCRs). *F. prausnitzii* belongs to the *Clostridium leptum* cluster, which has often been associated with type II diabetes (Gerritsen *et al.*, 2011) and Karlsson *et al.* (2013) observed a negative correlation between *Clostridium leptum* and HbA1c values (Karlsson *et al.*, 2013).

Lopez-Siles *et al.* report that 97% of *F. prausnitzii* cluster in two phylogroups (Lopez-Siles *et al.* 2012). The observed strains differed in substrate utilization, pH tolerance, and/or bile sensitivity. Phylogroup I and II strains revealed significant systemic differences in obese subjects compared to diabetic type 2

subjects. These results add evidence to the hypothesis that phylotypes of *F. prausnitzii* differ in the regulation of inflammation. *F. prausnitzii* may furthermore protect obese subjects from developing type II diabetes.

Effects of Short Chain Fatty Acid-Producing Bacteria on Epigenetic Regulation of Inflammatory Mediators such as TNF α , IL 6, FFAR3, TLRs and NF- κ B in Obesity and Related Metabolic Disorders

Adipose tissue produces and secretes several molecules such as leptin, resistin, adiponectin, and visfatin, as well as cytokines such as TNF- α , IL-6, MCP-1, and IL-1 (Kang, 2013). Various studies have shown that interleukin-6 (IL-6) is an upstream inflammatory cytokine that plays a central role in propagating the downstream inflammatory response. IL-6 release is stimulated by acute infections, chronic inflammatory conditions, obesity, and physiologic stress (Hartman & Frishman, 2014).

Intestinal epithelial cells (IECs) contribute especially to the symbiotic system by partially tolerating commensals and regulating mucosal inflammation (Takahashi *et al.*, 2009, Takahashi *et al.*, 2011). The higher expression of pro-inflammatory cytokines is coordinated mainly through distribution patterns of toll-like receptor (TLR) and NF (nuclear factor)- κ B, a key transcription factor in the inflammatory cascade. Toll-like receptors are common transmembrane proteins in the immune system, recognizing structural molecules derived from microbes (Creely *et al.*, 2007). Each TLR recognizes specific ligands, activating NF- κ B (Himes & Smith, 2010). LPS (lipopolysaccharide) promotes the transcription of pro-inflammatory molecules and thus induces an inflammatory signalling cascade dependent on the stimulated TLR and TIR (Toll/IL-1R homology) domain. Activation of intracellular kinases mediates the activation of the transcription factor NF- κ B, which induces expression of pro-inflammatory genes (TNF- α (tumor necrosis factor α), IL-1 (interleukin), IL-12) in the cell nucleus. These inflammatory markers initialize Th17 (CD4+ T) and Th1 cells and promote an increased generation of chemokines and cytokines (Lee *et al.*, 2010, Sommer & Backhed, 2012).

While optimal TLR-signalling is required for protection against microorganisms, inappropriate TLR expression and signalling can lead to hyper-responsiveness to bacterial ligands and thus enhance susceptibility to chronic inflammation (Furuta *et al.*, 2008). It can also lead to host-destructive pathology, for example inflammatory bowel disease, diabetes, and multiple sclerosis (Lee *et al.*, 2010, Sommer & Backhed, 2012). Metabolic endotoxemia correlates significantly with oxidative stress, macrophage infiltration markers, and all inflammatory markers triggering insulin resistance (PAI-1, IL-1, IL-6 and TNF- α) (Cani *et al.*, 2008, Cani & Delzenne, 2009, Caesar *et al.*, 2010, Musso *et al.*, 2010).

TLR4 senses bacterial LPS and saturated fatty acids (SFA), and also endogenous lipids that may contribute to the pathogenesis of lipid-induced insulin resistance (Shi *et al.*, 2006). In vivo, a high-fat diet induced the expression of TNF- α , IL-6, SOCS3, and MCP-1. Lauric acid is thought to initiate TLR4 signalling. It is hypothesized that increased fatty acid levels in obese individuals promote TLR4 signalling in adipocytes and macrophages to induce inflammatory signalling and suppress insulin-mediated regulation of glucose metabolism and insulin resistance (Shi *et al.*, 2006). Mice lacking TLR 4 or under antibiotic treatment, show a reduction of LPS, and are protected from systemic lipid infusion and relieved of metabolic syndrome symptoms (Diamant *et al.*, 2011). A repression of TLR4 gene

transcription is mediated by epigenetic mechanisms including DNA methylation and histone deacetylation (Takahashi *et al.*, 2011).

TLR2 senses lipoteichoic acid and FFAs (free fatty acids) (Bayarsaihan). Cell line studies demonstrated that a high CpG methylation of the TLR2 promoter correlates with a lower TLR2 expression (Haehnel *et al.*, 2002). Mice lacking the receptor are associated with lower body weight, lower serum glucose, and improved insulin sensitivity (Caricilli *et al.*, 2008, Kellermayer *et al.*, 2011). Furthermore, an involvement of TLR2 in the regulation of serum lipids is indicated (Himes & Smith, 2010).

Aim of the Thesis

The aim of this PhD thesis was to analyze microbiota shifts, especially in short chain fatty acid (SCFA) producers and in particular *F. prausnitzii*. Connections with nutrition, age, food intolerances, obesity and other metabolic disorders, and gut permeability, and interventions such as chemotherapy, antibiotic treatment, and probiotic intervention or weight reduction were investigated. Short chain fatty acids, in particular the butyrate pathways, were assessed.

A number of molecular methods were used to assess fecal samples, for example qPCR, qPCR-melt curve, PCR-DGGE, 454 sequencing, Pyrosequencing, Qiaxcel- fragment length polymorphism, targeting butyrate with the butyryl-CoA:acetate CoA-transferase gene, and cloning. A high resolution melt curve test for different *F. prausnitzii* phylotypes was generated. Blood samples were used to develop a test comparing interactions of inflammation-associated epigenetic markers with microbiota shift.

Hypothesis

The main hypothesis of my PhD thesis focused on the assumption that SCFA-producing bacteria, in particular butyrate-producing bacteria, influence the inflammatory responses underlying various complex diseases, including epigenetic regulatory pathways.

Main Objectives

- Does nutrition, age, antibiotic or chemotherapeutic treatment affect SCFA producers in the human GI tract?
- Are there differences in short chain fatty acid-producing bacteria with effects on inflammation? Are there different *F. prausnitzii* subgroups in the human GI tract?
- Are there microbiota shifts, especially in SCFA producers, in obesity, Type 2 diabetics, nutrition intervention, or food intolerance?
- Are there markers of GI microbiota interactions in obesity and Type 2 diabetes?

Secondary Objectives

- Development of analysis methods for short chain fatty acid-producing bacteria and SCFA production
 - Fragment Length Polymorphism (Qiaxcel) for SCFA producers
 - butyryl-CoA:acetate CoA-transferase gene for butyrate levels
 - HRM for *F. prausnitzii* phylotypes

Results Reported in Manuscripts

Short Chain Fatty Acid Producing-Bacteria

The microbiota composition is influenced by diet (Larsen *et al.*, 2010) and age (Mariat *et al.*, 2009), as well as genetic factors (Khachatryan *et al.*, 2008). The gastrointestinal microbiota produces short-chain fatty acids (SCFAs). Butyrate is of particular interest due to its anticarcinogenic and anti-inflammatory potential (Maslowski *et al.*, 2009), its effects on the intestinal barrier (Peng *et al.*, 2007), satiety (Cani *et al.*, 2009) and epigenetic regulation (Rada-Iglesias *et al.*, 2007). Two of the most important groups of butyrate producers are *Faecalibacterium prausnitzii* from the *Clostridium* cluster IV, and the *Eubacterium rectale*/*Roseburia* spp. from the *Clostridium* cluster XIVa (Walker *et al.*, 2010). Both clusters (now also known as *Ruminococcaceae* and *Lachnospiraceae*) consist of producers and nonproducers of butyrate (Pryde *et al.*, 2002). Isolated dietary compounds have been shown to promote growth of butyrate producers (Hernot *et al.*, 2009). For example, the consumption of inulin significantly stimulated growth of *F. prausnitzii* (Louis & Flint, 2009). In colonic *in vitro* model systems, resistant starch stimulated the growth of *E. rectale* (Leitch *et al.*, 2007).

Butyrate is easily taken up by the gut mucosa and fecal butyric acid levels give little information about the butyrate-producing capacity of the gut microbiota. Therefore, a function-based approach was suggested for the enumeration of butyrate-producing bacteria (Louis & Flint, 2007) targeting the butyryl-CoA:acetate CoA-transferase gene. Furthermore, the butyryl-CoA:acetate CoA-transferase route, using acetate as a cosubstrate, is suggested to be the most important route for butyrate production in the gut ecosystem (Duncan *et al.*, 2004). Alternative routes are via butyrate kinase and phosphotransbutyrylase, which are found in a minority of bacteria (Louis *et al.*, 2004) in the human gastrointestinal tract.

To evaluate the relevance of the butyryl-CoA:acetate CoA-transferase gene in butyrate-producing communities, we compared the amount of butyryl-CoA:acetate CoA-transferase gene in young omnivores, vegetarians and the elderly by quantitative PCR (qPCR) with a 16S rRNA gene quantification of *Clostridium* clusters IV and XIVa, considering the effects of ageing and nutrition. Published in our paper ***“Quantification of butyryl CoA:acetate CoA-transferase genes reveals different butyrate production capacity in individuals according to diet and age” (2011, FEMS Microbiology Letters).***

***Faecalibacterium prausnitzii* – One of the Main Butyrate Producers in the Human GI Tract**

Faecalibacterium prausnitzii is the most abundant bacterium in the human intestinal microbiota of healthy adults, representing up to 8% of the total bacterial population. *F. prausnitzii* is one of the main butyrate producers in the healthy human gut. Over the past five years, an increasing number of studies have clearly described the importance of this highly metabolically active commensal

bacterium as a component of the healthy human microbiota. Changes in the abundance of *F. prausnitzii* have been linked to dysbiosis in several human disorders (Miquel *et al.*, 2013). *F. prausnitzii* is more common in subjects with high intestinal gut microbiota gene content and (Le Chatelier *et al.*, 2013) lower concentrations have consistently been observed in patients with Crohn's disease or colorectal cancer. *F. prausnitzii* has been discussed for its anti-inflammatory activity, (reducing/increasing?) IL-12, IL-10, and IFN- γ (interferon- γ) in the gut (Furet *et al.*, 2010), and as a marker for a high bacterial gene content and gut well-being (Remely *et al.*, 2013). Information on its genetic diversity and its role in the regulation of inflammation are unclear.

F. prausnitzii has the ability to switch between diet- and host-derived substrates (Lopez-Siles *et al.*, 2012) and can convert sugars, dietary fibers, proteins and peptides to butyrate (Remely *et al.*, 2013) using mainly the butyryl-CoA:acetate CoA-transferase route (Louis *et al.*, 2004). Butyrate is thought to affect diet-induced obesity (Lin *et al.*, 2012) and chronic diseases. It provides additional energy (5-15% of the total caloric intake) for gut epithelial cells, where the majority of butyrate is metabolized by the colonic epithelium and the remaining is most likely extracted by the liver. It has anti-carcinogenic and anti-inflammatory potential (Hamer *et al.*, 2008) and plays a role in adipose tissue expansion (Cani & Delzenne, 2011). Concentrations in the venous systemic serum range from 0.5 to 3.3 μmol (Hamer *et al.*, 2008), where butyrate acts as a signalling molecule (Brown *et al.*, 2003) via G-protein coupled receptors (GPRs). *F. prausnitzii* belongs to the *Clostridium leptum* cluster, which has often been associated with type II diabetes (Gerritsen *et al.*, 2011) and Karlsson *et al.* (2013) observed a negative correlation between *Clostridium leptum* and HbA1c values (Karlsson *et al.*, 2013). Lopez-Siles *et al.* report that 97% of *F. prausnitzii* cluster in two phylogroups (Lopez-Siles *et al.*). The observed strains differed in substrate utilization, pH tolerance, and/or bile sensitivity, but phylogroup I and II strains did not reveal a systemic difference in healthy subjects (Lopez-Siles *et al.* 2012). Individual abundance of *F. prausnitzii* correlates with environmental factors, suggesting that different nutrients shape different *F. prausnitzii* strains. Acetate is known to stimulate the growth of *F. prausnitzii* (Duncan *et al.*, 2002) and different strains even distinguish between apple and citron pectin substrates for their growth (Lopez-Siles *et al.*, 2012).

Recently it has been reported that *F. prausnitzii* is able to colonize the GIT of germ-free rats after the colonization of the gut with *B. thetaiotaomicron* (Wrzosek *et al.*, 2013). *B. thetaiotaomicron* and *F. prausnitzii* are metabolically complementary. *B. thetaiotaomicron* may preshape the conditions in the GIT for the growth of *F. prausnitzii*. This could explain why *F. prausnitzii* only accounts for 4% of total gut bacteria in children from rural Burkina Faso compared to 25% in children with a western diet (De Filippo *et al.*, 2010). While *F. prausnitzii* has been suggested as a marker for gut health, this may only be valid for a western diet where a *Bacteroides* overgrowth, the predominant proteolytic bacteria, is combined with an efficient butyrate producer to buffer the disadvantages of a high protein and low fiber diet (Wrzosek *et al.*, 2013).

In our paper **“Faecalibacterium prausnitzii phylotypes in a diabetic, obese and lean control group”** (under review FEMS letters, 2014) *F. prausnitzii* phylotypes were analyzed in feces of obese patients without established insulin resistance, insulin-dependent type two diabetics and a lean control group. The objectives of the study were the analysis of the two phylotypes and the investigation of a possible

protective GIT effect. Several studies describe the involvement of the gut microbiota in obesity-associated metabolic disorders, type two diabetes, metabolic endotoxemia and low-grade inflammation (Louis & Flint, 2007, Remely et al., 2013). In our study the lean control group shows the highest abundance of *F. prausnitzii* copies, but the lowest butyryl-CoA:acetate CoA-transferase gene content. These results correspond with previous research where higher butyrate amounts were observed in overweight and obese participants compared to lean controls (Schwartz et al., 2010) as well as in the feces of obese mice (Turnbaugh et al., 2006). The butyryl-CoA:acetate CoA-transferase gene melt variant amplicons were divided into three areas as described by Louis & Flint (2009). These peaks represent bacteria related to *E. hallii* and *Anaerostipes coli*, *Roseburia/E. rectale spp.* and *F. prausnitzii*. The abundance of the butyryl-CoA:acetate CoA-transferase gene melt variant for *F. prausnitzii* is least in the lean control group (Figure 3). The distribution of the melt variant of the main butyrate producers in the obese and diabetic group is equal. *F. prausnitzii* contributes the least butyrate amount in lean controls (15%), compared to up to 42% in the obese and diabetic groups. Butyrate, in particular, has an important impact on promoting body energy expenditure via modulation of mitochondrial activity (Remely et al., 2013) and is an additional energy source promoting adiposity (Layden et al., Scott et al., 2013). In contrast, high-fat diets supplemented with butyrate prevent and even reverse insulin resistance in obese mice (Pegorier et al., 2004, Samuel et al., 2008). High butyrate levels in humans stimulate GLP-1 secretion and therefore increases of insulin, inhibit gastric emptying and increase feeling of satiety (Wichmann et al., 2013). Due to the inhibition of gastric emptying, butyrate is maybe excreted more slowly and accumulates, influencing the anti-inflammatory potential, pH and oxidative stress. The observed high *F. prausnitzii* levels compared to low butyrate levels in lean controls could also origin from different *F. prausnitzii* strains. Possibly the different strains have different anti-inflammatory potentials and with that a protective effect in obese subjects, preventing the development of type two diabetes. Remely et al. (2013) reported a lower proportion of inflammatory markers and *F. prausnitzii* in type two diabetic subjects indicating a higher incidence of low-grade inflammation (Furet et al., 2010). Strains belonging to pylogroup II were detected only in the diabetic group (Fig. 4 and 5). Although Lopez-Siles et al. 2012 reported a tendency for sequences to group individually, we found strains from Phylogroup I in all cloned samples (Lopez-Siles et al.).

Conditions Altering the Microbiome - Nutrition, Age, Antibiotic or Chemotherapeutic Treatment

Nutrition has been described as a rapid and consistent modulator for gut microbiota. (David et al. 2013) Several studies found similar microbiota-diet associations at three different stages of life, showing that diet modulates the microbiota regardless of gender or other confounding factors. (De Filippo et al., 2010, Wu et al., 2011, Claesson et al., 2012)

An animal protein-based diet has been associated with increases in species (e.c. *Bilophila wadsworthia*) which are connected with atherosclerosis and stroke. It has been published that a long

term diet with high intake of animal protein and fat increases *Bacteroides* (Wu *et al.*, 2011). High bile acid concentrations are associated with enteral disease.

In our paper **“Characterization of bacteria, clostridia and *Bacteroides* in feces of vegetarians using qPCR and PCR-DGGE”** we show that subjects with a vegetarian diet have a 12% higher abundance of total bacteria and higher amounts of gastro intestinal butyrate concentrations, and a tendency for less *Clostridium cluster* IV. PCR-DGGE PCA suggests a grouping of bacteria and members of *Clostridium cluster* IV. Two bands are significantly more frequent in omnivores than in vegetarians ($p < 0.005$ and $p < 0.022$). One is identified as *Faecalibacterium prausnitzii* sp..

Marked changes in the gut microbiota occur early on in life (Yatsunenکو *et al.*, 2012). After the initial colonization, communities undergo consecutive changes in composition and function until a relatively stable climax community is established (Lozupone *et al.*, 2012). A recent study by the ELDERMET consortium focused on elderly individuals living in communities and those in long-term care; they describe an association between high intestinal microbiota diversity and a healthy food diet (HFD). Diets with a low HFD score were associated with microbiota populations that were less diverse. Differences in dominant genera in *Prevotella* have been associated with diets rich in plant-based foods, while the individuals with a lower HFD harbored more *Bacteroides*. (Claesson *et al.*, 2012)

The diet in elderly individuals can change for a number of reasons, including loss of sensation of taste and smell, tooth loss and chewing difficulties. These changes may result in an increase in the consumption of high sugar/high fat foods and a reduction in the proportion of plant-based foods consumed (Jeffery & O'Toole, 2013).

In our paper **“Quantification of butyryl CoA:acetate CoA-transferase genes reveals different butyrate production capacity in individuals according to diet and age”** (FEMS letters, 2011) the elderly had significantly fewer copies of the butyryl-CoA:acetate CoA-transferase gene than young omnivores ($P=0.014$), while vegetarians showed the highest number of copies ($P=0.048$). The thermal denaturation of the butyryl-CoA:acetate CoA-transferase gene variant melting curve related to *Roseburia/Eubacterium rectale* spp. was significantly more variable in the vegetarians than in the elderly. The *Clostridium cluster* XIVa was more abundant in vegetarians ($P=0.049$) and in omnivores ($P<0.01$) than in the elderly group. The gastrointestinal microbiota of the elderly is characterized by decreased butyrate producing capacity, reflecting increased risk of degenerative diseases.

Understanding how the microbiota forms a stable, resilient state would enable development of strategies increasing the resilience of healthy microbiota or decreasing the resilience of unhealthy microbiota (Lozupone *et al.*, 2012). Antibiotic use in infants is associated with lower bacterial diversity and higher relative abundance of *Enterobacter* (Greenwood, C., 2014). Antibiotic treatment in adults has been associated with high inter-individual variance in disturbance and recovery (Dethlefsen & Relman, 2011). Long-term studies of the microbiota after antibiotic treatment indicate that post-antibiotic equilibrium states are themselves resilient. For example, clindamycin treatment affected *Bacteroides* in the gut for up to 2 years after treatment had finished (Jernberg *et al.*, 2007).

In our study **“Effects of antibiotic therapy on the gastrointestinal microbiota and the influence of *Lactobacillus casei*”** (Food and Agricultural Immunology, 2013) a combination of antibiotics and

probiotics could diminish the antibiotic-associated diarrhea (AAD) effect by 73%. Following antibiotic therapy, a decrease in the abundance of total bacteria, *Clostridium cluster IV* and XI, *Bifidobacterium* spp. and butyryl-CoA CoA transferase genes was observed, whereas *Enterobacteriaceae* increased. Probiotic (LcS) intervention reduced the antibiotic-associated decrease in the diversity of microbiota, increased the abundance of *Lactobacillus* spp. and reduced the antibiotic-induced decrease of *Bifidobacterium* spp. Antibiotic treatment affects the diversity and the composition of the microbiota, impairing butyrate production. Intervention with certain *Lactobacillus* strains may antagonise some of these changes, and more potent short-chain fatty acid-stimulating probiotics are needed for intervention in AAD.

The gut microbiota influences both local and systemic inflammation. Inflammation contributes to development, progression, and treatment of cancer. Iida *et al.* showed that an optimal response to cancer therapy requires an intact commensal microbiota that mediates its effects by modulating myeloid-derived cell functions in the tumor microenvironment. These findings underscore the importance of the microbiota in the outcome of disease treatment of cancer (Iida *et al.*, 2013). Viaud *et al.* also suggest that the gut microbiota help shape the anticancer immune response (Viaud *et al.*, 2013). A recent study shows that subcutaneous tumors fail to respond to immunotherapy and platinum chemotherapy after antibiotic treatment (Iida *et al.*, 2013).

In our paper **“Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting.”** (PLOS one, 2011) we investigated whether chemotherapy, with the presence or absence of antibiotics, against different kinds of cancer changed the gastrointestinal microbiota. Feces of 17 ambulant patients receiving chemotherapy with or without concomitant antibiotics were analyzed before and after the chemotherapy cycle at four time points in comparison to 17 gender-, age- and lifestyle-matched healthy controls. We targeted 16S rRNA genes of all *bacteria*, *Bacteroides*, *bifidobacteria*, *Clostridium cluster IV* and XIVa as well as *C. difficile* with TaqMan qPCR, denaturing gradient gel electrophoresis (DGGE) fingerprinting and high-throughput sequencing. After a significant drop in the abundance of microbiota ($p=0.037$) following a single treatment, the microbiota recovered within a few days. The chemotherapeutical treatment marginally affected the *Bacteroides* while the *Clostridium cluster IV* and XIVa were significantly more sensitive to chemotherapy and antibiotic treatment. DGGE fingerprinting showed decreased diversity of *Clostridium cluster IV* and XIVa in response to chemotherapy, with *cluster IV* diversity being particularly affected by antibiotics. The occurrence of *C. difficile* in three out of seventeen subjects was accompanied by a decrease in the genera *Bifidobacterium*, *Lactobacillus*, *Veillonella* and *Faecalibacterium prausnitzii*. *Enterococcus faecium* increased following chemotherapy. Despite high individual variations, these results suggest that the observed changes in the human gut microbiota may favor colonization with *C. difficile* and *Enterococcus faecium*. Perturbed microbiota may be a target for specific mitigation with safe pre- and probiotics.

The influence of commensal intestinal bacteria in the outcome of cancer treatment and the function of anticancer immunity pose new questions from a preclinical and clinical standpoint in the cancer field about how to handle antibiotic treatment in cancer therapy (Karin, 2014).

Microbiota Shift in Obesity, Type 2 Diabetes or Food Inconvenience

The healthy gut microbiota is highly variable. Understanding the variety can help to develop individualized therapies or tailored clinical trials. For example, the extent to which members of the same human family harbor similar microbes will determine whether the family history of microbiota-driven disease is informative (Lozupone et al., 2012). Understanding the compositional and functional differences in the gut microbiota can help to relate these differences to human health. Differences in the microbiota and the microbiome could help to explain the variation in the gut metabolic processes of individuals, including the metabolism of drugs and food (Clayton et al., 2009, Gonzalez et al., 2011, Lozupone et al., 2012). There is accumulating evidence that changes in the gut microbiota can affect body physiology. While the exact mechanism remains unsolved, animal experimental and human observational studies provide tantalizing evidence that these changes in the intestine can be triggered by exposures to antimicrobial agents, especially in the context of certain dietary nutrients introduced into the gut, and that these changes can contribute to weight gain (Riley et al., 2013). Gut microbiota bridge the indeterminate gap between food and weight by affecting the host metabolism through regulation of intestinal glucose absorption, lipogenesis and fat deposition. Housekeeping functions such as the central carbon metabolism or the amino acid synthesis of important protein complexes are present in every bacterium. Secondary metabolisms are related to a minimal metagenome, for example the biodegradation of complex sugars and glycans (Gerritsen et al., 2011). Profiles of human gut microbiota reflect the metabolic cooperation between different phylotypes since no genus can degrade all substrates; for example to produce all short chain fatty acids (SCFAs). The gut microbiota of all human beings fulfill core functions, but differ in specialist functions; as a consequence some communities are linked to human diseases and obesity more than others (Gerritsen et al., 2011). Observations of experiments with germ-free mice compared to conventionally- raised mice or after conventionalization showed a weight gain dependent on the gut microbiota and independent of diet (Musso et al., 2010). Studies on the relationship between gut microbiota composition and obesity show an increased number of *Firmicutes* and a reduction of *Bacteroidetes* in obese mice and humans compared to lean controls (Ley et al., 2006, Turnbaugh et al., 2006, DiBaise et al., 2008, Musso et al., 2010). A controlled diet and weight-loss reversed the observed ratio of *Firmicutes* to *Bacteroidetes* (Cani et al., 2008, Duncan et al., 2008, Musso et al., 2010, Schwartz et al., 2010). The number of *Bacteroidetes* depends on the weight loss rather than on caloric intake (DiBaise et al., 2008, Diamant et al., 2011) whereas the *Firmicutes* group remains unchanged and no correlation with total caloric intake can be detected (Cani et al., 2008). The ratio reaches a lean type profile after a one-year period of diet-induced weight loss (Wu et al., 2010). The abundance of *Firmicutes* in diabetic persons is significantly lower compared to non-diabetics, while *Bacteroidetes* and *Proteobacteria* are more abundant in diabetics. The ratio of *Bacteroidetes* to *Firmicutes* is significantly and positively correlated with reduced glucose tolerance, but negatively correlated with higher BMI. Especially the relative abundance of *Bacilli* is increased in diabetic mice models and humans. Relative abundances of *Actinobacteria* and *Verrucomicrobia* do not differ between diabetic and non-diabetic individuals. *Bacteroides-Prevotella* versus *C. coccoides* - *E. rectale* groups are positively associated with plasma glucose, but negatively correlated with BMI (Larsen et al., 2010). Wu et al. (2009) found a higher abundance of *Bacteroides* (frequency of 53,6 %), and a lower

abundance of the genus *Prevotella* (10,7%), the genus *Proteobacteria* (3,6%) and the phylum *Firmicutes* (10,8%) compared to healthy volunteers in sequence analysis of selected DGGE amplicons, whereas qPCR results indicated a lower copy number of *Bacteroides vulgatus* and *Bifidobacterium* in the fecal microbiota. The *Clostridium leptum* cluster was more abundant in the diabetic than in the healthy group (Backhed *et al.*, 2004, Musso *et al.*, 2010, Diamant *et al.*, 2011). It is estimated that subjects with type 2 diabetes are relatively enriched with Gram-negative bacteria, belonging to the phyla *Bacteroidetes* and *Proteobacteria*, which can elicit endotoxemia (Larsen *et al.*, 2010) through increased plasma LPS (Cani *et al.*, 2008, Cani & Delzenne, 2009, Tsukumo *et al.*, 2009, Larsen *et al.*, 2010, Luoto *et al.*, 2010, Diamant *et al.*, 2011). Obese individuals may have a microbiota more efficient in extracting energy, and may be enriched in enzymes for the breakdown of otherwise indigestible alimentary polysaccharides and in other transport proteins and fermentation enzymes which further induce insulin resistance and diabetes (Musso *et al.*, 2010). In a high-fat diet elevated LPS increase gut permeability by affecting GLP-1, GLP-2 and PYY (Cani *et al.*, 2008, Diamant *et al.*, 2011). An absence of LPS receptor CD14 reduces the high-fat diet-induced effects on adipose tissue inflammation, blood glucose profiles, insulin resistance index, glucose-induced insulin secretion (Cani *et al.*, 2008, DiBaise *et al.*, 2008, Cani & Delzenne, 2009), hepatic steatosis, liver inflammation and adipose tissue macrophages infiltration (Cani *et al.*, 2008). Metabolic endotoxemia is indicated by a change in the intestinal microbiota as antibiotic treatment reduces plasma LPS levels (Cani *et al.*, 2008, DiBaise *et al.*, 2008, Cani & Delzenne, 2009, Diamant *et al.*, 2011).

The objective of our study ***“Abundance and diversity of microbiota in type 2 diabetes and obesity” (Diabetes & Metabolism, 2014)*** was to investigate differences in the gut microbiota in obese subjects and type 2 diabetics and the response to intervention in order to assess its potential role in controlling metabolic syndrome. We characterized fecal microbiota at three time points in fourteen obese participants, nineteen lean controls and twenty-four type 2 diabetes patients. Obese subjects and type 2 diabetics received an intervention of nutritional counseling, type 2 diabetics an additional therapy with a GLP-1-agonist. The microbiota composition was analyzed for abundance and diversity by quantitative real-time polymerase chain reaction, denaturing gradient gel electrophoresis and high throughput sequencing. In type 2 diabetics an increase in diversity was observed with intervention whereas the values of lean controls remained unaffected. In the lean and obese groups, a lower *Firmicutes: Bacteroidetes* ratio correlated with lower BMI. In type 2 diabetics the ratio of *Firmicutes* to *Bacteroidetes* increased throughout the intervention period. Type 2 diabetics showed a significantly enhanced proportion of lactic acid bacteria before and after intervention; *Akkermansia* and *Enterobacteria* also showed a higher abundance in type 2 diabetics, increasing throughout the study period. *Archaea* were significantly more frequent in type 2 diabetics.

Not only the microbiota composition but also their metabolites, in particular butyrate, are considered to affect diet-induced obesity (Lin *et al.*, 2012)- and chronic diseases. Butyrate provides additional energy (5-15% of the total caloric intake) for gut epithelial cells, where the majority of butyrate is metabolized by the colonic epithelium and the remaining is most likely extracted by the liver (Remely *et al.*, 2013) and plays a role in adipose tissue expansion (Cani & Delzenne, 2011). Concentrations in the venous systemic serum range from 0.5 to 3.3µmol (Hamer *et al.*, 2008), where butyrate acts as a

signalling molecule (Brown *et al.*, 2003) via G-protein coupled receptors (GPRs). *F. prausnitzii* belongs to the *Clostridium leptum* cluster, which has often been associated with type 2 diabetes (Gerritsen *et al.*, 2011) and Karlsson *et al.* (2013) observed a negative correlation between *Clostridium leptum* and HbA1c values (Karlsson *et al.*, 2013).

In our paper **“*Faecalibacterium prausnitzii* phylotypes in a diabetic, obese and lean control group” (under review, FEMS letters, 2014)** *F. prausnitzii* phylotypes were examined in feces of obese patients without established insulin resistance, insulin-dependent type 2 diabetics and a lean control group. The fecal samples were analyzed with methods distinguishing between GC- content and fragment length. *F. prausnitzii* genes of each group were cloned and sequenced. The lean control group differed significantly from the diabetic group in results of qPCR ($p=0.02$), butyryl-CoA:acetate CoA-transferase gene melt curve variant for *F. prausnitzii* ($p=0.03$), high resolution melt curve analysis (HRMA)($p=0.006$), but not in automated fragment length polymorphism (ARISA). 22% of all sequenced clones matched with phylogroup II and 78% with phylogroup I. Strains assigned to phylogroup I belong to the diabetic group. These results add evidence to the hypothesis that phylotypes of *F. prausnitzii* differ in the regulation of inflammation. *F. prausnitzii* may furthermore protect obese subjects from developing type II diabetes.

The short chain fatty acids are believed to affect the epigenetic regulation of inflammatory reactions via FFARs (free fatty acid receptor) and other short chain fatty acid receptors.

In our paper **“Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity” (Gene, 2014)** we studied a potential interaction of the microbiota with epigenetic regulation in obese and type 2 diabetes patients compared to a lean control group over a four month intervention period. Intervention comprised a GLP-1 agonist (glucagon-like peptide 1) for type 2 diabetics and nutritional counseling for both intervention groups. The microbiota was analyzed for abundance, butyryl-CoA:acetate CoA-transferase gene and for diversity by polymerase chain reaction and 454 high-throughput sequencing. Epigenetic methylation of the promoter region of FFAR3 and LINE1 (long interspersed nuclear element 1) was analyzed using bisulfite conversion and pyrosequencing. The diversity of the microbiota as well as the abundance of *Faecalibacterium prausnitzii* were significantly lower in obese and type 2 diabetic patients compared to lean individuals. Results from *Clostridium cluster* IV and *Clostridium cluster* XIVa showed a decreasing trend in type 2 diabetics in comparison to the butyryl-CoA:acetate CoA-transferase gene according to melt curve analysis. During intervention no significant changes were observed in either intervention group. The analysis of five CpGs in the promoter region of FFAR3 showed a significantly lower methylation in obese subjects and type 2 diabetics with an increase in obese patients over the intervention period. These results disclosed a significant correlation between a higher body mass index and lower methylation of FFAR3. LINE-1, a marker of global methylation, showed no significant differences between the three groups or the time points, although methylation of type 2 diabetics tended to increase over time. Our results provide evidence that a different composition of gut microbiota in obesity and type 2 diabetes affect the epigenetic regulation of genes.

Food intolerances are an increasing global health problem. Wilders and Truschnig (Wilders-Truschnig *et al.*, 2008) report that obese subjects have higher IgG levels compared to lean controls. Interactions of low grade inflammation, genetics and environmental changes such as microbial- and stress factors are related to food intolerances. Whereas the analysis of IgE-mediated allergic responses is based on solid concepts, the roles of microbiota, gut permeability, and IgG antibodies remain widely unclear and are under fierce discussion for scientific relevance.

In our paper **“Abundance and diversity of GI microbiota rather than IgG₄ levels correlate with abdominal inconvenience and gut permeability in consumers claiming food intolerances”** (Endocrine, Metabolic & Immune Disorders - Drug Targets, 2014) we suggest an association of low gut permeability and reduced inflammation with an established microbial equilibrium. Self-reported abdominal inconvenience of participants related mainly to characteristics of microbiota and gut permeability. Anti-inflammatory effects of *Faecalibacterium prausnitzii* or *Lactobacilli* and gut barrier functions of *Akkermansia* may have a key role in food intolerances. The role of IgG₄ in linking food immune responses with intolerances remains unclear.

In this study we analyzed forty participants, under consultation of nutritional health professionals, for gastrointestinal discomfort and claimed food intolerances. Food frequency questionnaires addressed nutrition, lifestyle and present discomfort. Feces samples were analyzed for dominant microbiota using 16S rDNA-based methods and the fecal marker Calprotectin. Blood samples were analyzed for IgG₄ levels. The total microbial abundance significantly correlated with claimed discomfort ($R=-0.37$; $p=0.02$). The abundance and diversity of microbiota significantly correlated with low Calprotectin values ($R=-0.35$; $p=0.01$) and with higher abundance of *Faecalibacterium prausnitzii* ($R=0.78$; $p<0.01$) and *Akkermansia* ($R=0.82$; $p<0.01$). Participants with low discomfort showed enhanced *Clostridium Cluster XIVa* ($p=0.008$). An increased diversity also correlated with reduced antibodies against IgG₄ of egg white ($R=0.68$; $p<0.01$).

An association between IgG₄ levels and single aspects of gastrointestinal microbiota and gut permeability can be suggested by this study. *F. prausnitzii* plays an important role in convenience of study participants.

Impact and Marker of GI Microbiota Interactions

Changes in gut microbiota and thus cell wall components are involved in the epigenetic regulation of inflammatory reactions. An improved diet targeted to induce gut microbial balance and consequently even epigenetic changes of pro-inflammatory genes may be effective in the prevention of metabolic syndrome. Metabolic syndrome is associated with alterations in the structure of the gut microbiota leading to low-grade inflammatory responses. An increased penetration of the impaired gut membrane by bacterial components is believed to induce this inflammation, possibly involving epigenetic alteration of inflammatory molecules such as Toll-like receptors (TLRs).

In our paper **“Microbiota and epigenetic regulation of inflammatory mediators in type 2 diabetes and obesity.”** (Beneficial microbes, 2014) we evaluated changes in the gut microbiota and epigenetic DNA methylation of TLR2 and TLR4 in three groups of subjects: type 2 diabetics under glucagon-like

peptide-1 agonist therapy, obese individuals without established insulin resistance, and a lean control group. *Clostridium cluster IV*, *Clostridium cluster XIVa*, lactic acid bacteria, *Faecalibacterium prausnitzii* and *Bacteroidetes* abundances were analyzed by PCR and 454 high-throughput sequencing. The epigenetic methylation in the regulatory region of TLR4 and TLR2 was analyzed using bisulfite conversion and pyrosequencing. We observed a significantly higher ratio of *Firmicutes/Bacteroidetes* in type 2 diabetics compared to lean controls and obese subjects. Major differences were shown in lactic acid bacteria, with the highest abundance in type 2 diabetics, followed by obese and lean participants. In comparison, *F. prausnitzii* was least abundant in type 2 diabetics, and most abundant in lean controls. Methylation analysis of four CpGs in the first exon of TLR4 showed significantly lower methylation in obese individuals, but no significant difference between type 2 diabetics and lean controls. Methylation of seven CpGs in the promoter region of TLR2 was significantly lower in type 2 diabetics compared to obese subjects and lean controls. The methylation levels of both TLRs significantly correlated with body mass index. *Faecalibacterium prausnitzii*, a member of *Clostridium Cluster IV*, is known for its anti-inflammatory activity, decreasing the rates of NF- κ B activation and IL-8 secretion, increasing IL-12, IL-10, and IFN- γ (interferon- γ) (Furet *et al.*, 2010), and is also associated with improved insulin sensitivity (Karlsson *et al.*, 2013). A negative relationship between *Faecalibacterium prausnitzii* and inflammatory markers was shown in obese and diabetic patients after adjustment for BMI (Furet *et al.*, 2010). It is also associated with improved insulin sensitivity and diversity (Karlsson *et al.*, 2013). The proportions of *Faecalibacterium prausnitzii* were lower in type 2 diabetic patients, suggesting a higher incidence of low-grade inflammation, but also reduced microbial diversity (Furet *et al.*, 2010). Our data suggest that changes in gut microbiota and thus cell wall components are involved in the epigenetic regulation of inflammatory reactions.

Different compositions of gut microbiota in obese individuals and type 2 diabetics affect the epigenetic regulation of FFARs. These interactions between microbiota and epigenetic regulation may influence the satiety and hunger cycle. Especially butyrate may have an impact in strategies for regenerative medicine by promoting epigenetic remodeling and the expression of associated genes.

Our results from the publication **“Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity”** (2014, Gene) highlight the necessity to concentrate on gene-specific methylation to better understand the involvement of epigenetic regulation in the development of obesity and type 2 diabetes. The analysis of five CpGs in the promoter region of FFAR3 showed a significantly lower methylation in obese subjects and type 2 diabetics with an increase in obese patients over the intervention period. These results disclosed a significant correlation between a higher body mass index and lower methylation of FFAR3. Significant correlations were observed between butyrate and dietary factors, especially resistant starch. For example, the consumption of inulin stimulates the growth of *F. prausnitzii* and *E. rectale* (Louis & Flint, 2009). Duncan *et al.* (2008) reported an effect of butyrate-producing bacteria on weight loss, dependent on diet. A reduction in these species was observed during low-carb diets, but no changes were seen during moderate-carbohydrate intake (Duncan *et al.*, 2008). The signalling actions of SCFAs are mediated by the G-protein coupled receptors (FFARs). We observed a higher methylation in the promoter region of the FFAR3 gene in blood from the lean control group. Both intervention groups

had a lower methylation, with the lowest in type 2 diabetics. These differences of methylation between the groups result in a negative correlation between methylation and BMI. Type 2 diabetics showed no significant differences over the intervention period, whereas obese patients showed an increase in methylation after nutritional counseling, although they did not reach the methylation status of the lean control group. Since there are no other available investigations on the methylation of FFAR3 promoter region and expression, we expected a lower expression of FFAR3 receptor in the lean control group similar to Canani *et al.* (2011). In mice, an increase in leptin production caused by an overexpression of FFAR3 was observed after oral doses of propionate (Ichimura *et al.*, 2009). A knockdown of FFAR3 by siRNA inhibits the ability of propionate to induce leptin expression, mainly in adipocytes (Wu *et al.*, 2012). Leptin, exclusively produced in adipose tissue, influences the hunger-satiety cycle by suppressing the appetite and contributing to the fat metabolism. Although obese patients harbor many fat cells with correspondingly higher leptin levels, leptin is no longer able to contribute to satiety. Studies have demonstrated a positive correlation between leptin plasma levels and body fat mass. It was discussed that high leptin levels over time make the satiety cells insensitive to leptin, whereas a supplementation with SCFAs induces sensitivity or the production of leptin. Only few obese patients are affected by leptin deficiency, so therapies using recombinant leptin have not been successful (Schloegl *et al.*, 2010) and might affect therapies with SCFAs. These results are consistent with our results demonstrating a significant negative correlation between BMI and methylation levels found in the FFAR3 promoter region. On the other hand, FFARs are suggested to stimulate the secretion of satiety hormone PYY (Peptide YY), which inhibits gut motility, slows intestinal transit time, and enhances nutrient absorption. These results are underlined by a study with Gpr41-deficient mice and conventionally-raised mice. A deficiency of the receptor is associated with a lower expression of PYY and, as a consequence, with a higher energy harvest from the diet. Inhibition of Gpr41 activation may result in decreased extraction of energy from the diet and leaner individuals (Samuel *et al.*, 2008). However, current research indicates a FFAR3-independent influence of butyrate and propionate on body weight and food intake. In addition, FFAR3 is mentioned as playing a minor role in butyrate-stimulation of GLP-1 secretion. SCFAs increase the reduced GLP-1 secretion in obese subjects and type 2 diabetics and thus promote insulin secretion (Lin *et al.*, 2012). Therefore, a different microbial composition may contribute to reduced GLP-1 release. The main production of GLP-1 is located in the mucosal L cells, in the ileum and colon but also in pancreatic α -cells and in neurons (Vrieze *et al.*, 2010). Prebiotic treatment induced GLP-1 secretion in ob/ob mice, whereas a deletion of GLP-1 prevented the beneficial effects of prebiotics on weight gain, glucose metabolism and inflammatory pathway activation (Musso *et al.*, 2010). However, there is no evidence that an intervention with GLP-1 agonists for adjustment of blood glucose levels influences epigenetic methylation. Together, these findings suggest a role for FFAR3 in the microbiota-host interaction via circulating levels of enteroendocrine hormones that reduce gut motility and thus increase absorption of SCFAs, which may contribute to lipogenesis.

Methods

Analyzing the abundance of *F. prausnitzii* and fecal butyrate levels shows that there is no direct correlation. To detect butyrate levels it is necessary to measure these differently. Fecal butyrate levels depend on shifts in microbial cross feeding patterns, mucosal absorption and different transit times. Therefore the butyryl-CoA:acetate CoA-transferase gene can be used as a marker for butyrate production as the direct analysis in feces is hampered by rapid binding and degradation of butyrate in the gut (Schwiertz *et al.*, 2010, Duncan *et al.*, 2007). Assessing the synthesis of butyrate following the butyryl-CoA:acetate CoA-transferase gene pathway, discriminates bacteria using other described pathways such as butyrate kinase or phosphotransbutyrylase (Louis *et al.*, 2004). The butyryl-CoA:acetate CoA-transferase gene was amplified and thermally denatured using SYBR Green qPCR analysis. The melt curves from amplified PCR products were divided into three areas, as described by Louis & Flint (2009). These peaks represent bacteria related to *Eubacterium hallii* and *Anaerostipes coli* (82.5–85.0°C), *Roseburia/E. rectale spp.* (85.5–89.0°C) and *F. prausnitzii* (89.5–92.5°C) (Louis & Flint, 2009). Analyzing this gene gives additional information about further butyrate-producing bacteria. Qiaxcel – fragment length polymorphism discriminates between different fragment lengths of the amplified PCR products. The relative migration time can be assigned to a particular fragment length and this can be assigned to particular bacterial strains.

In our paper “*Faecalibacterium prausnitzii* phylotypes in diabetic, obese and lean control subjects” we could distinguish between *F. prausnitzii* phylotyp I with 160-161bp and phylotyp II with 153-155bp. High resolution melt curve analysis may be a promising tool for routine and research use to further analyze *F. prausnitzii* and phylotypes in low grade inflammation and obesity-associated metabolic disorders. 4 variants of *F. prausnitzii* can be distinguished with HRM, depending on the fragment lengths of the different phylotypes and mixtures of those in the different individuals. Diabetic group results differed significantly from the lean control group in HRM temperature ($p=0.0058$) and variance ($p=0.0068$), and from the obese group in HRM temperature ($p=0.047$) and variance ($p=0.049$).

Molecular microbiological analysis has increased understanding of the diversity and phylogeny of human gut microbiota and their functions. These modern techniques, including genotyping methods, are becoming increasingly important for species identification and for the differentiation of strains. The precise classification and identification of probiotic strains give a strong indication of their typical habitat and origin, safety and technical applicability, and provide possibilities for monitoring and product quality. The book chapter “**Detection and Identification Of Probiotic Microorganisms And Other Beneficial Organisms From The Human GI Tract.**” (Springer Verlag, 2011) provides an overview of probiotic strain characterization, gut metagenomics and the analytical methods (FISH, PCR, RAPD, DGGE, repPCR, PFGE, RFLP, microarray, high throughput sequencing) required for their study.

Discussion

Several studies describe the involvement of the gut microbiota in obesity-associated metabolic disorders, type two diabetes, metabolic endotoxemia and low-grade inflammation (Louis & Flint, 2007, Remely *et al.*, 2013). In this study we did not observe any significant differences in total bacterial abundance between type 2 diabetics, obese subjects and lean controls, as a possible result of the intervention. Larsen *et al.* also detected similar counts of total bacterial abundance in type 2 diabetics and the control group (Larsen *et al.*, 2010). Rather than the abundance of total bacteria or certain bacterial groups, the diversity of microbiota indicated by the richness of species or gene counts is now seen as an important indicator of microbiota resilience and gut health (Turnbaugh *et al.*, 2009). Shannon's diversity index showed no significant differences between the groups, whereas band pattern analysis showed significant differences between the lean control subjects and type 2 diabetics. In type 2 diabetics an increase of bands was seen with intervention whereas the values of lean controls remained unaffected. The analysis of one of the major butyrate producers (Louis & Flint, 2009) showed a lower abundance of *Faecalibacterium prausnitzii* in type 2 diabetics, followed by obese participants.

The diffusion of SCFAs or SCFA-transporters (Hartung *et al.*, 2008) together with their conversion to fat in the liver and the regulation of host genes that promote deposition of fat in lipocytes can cause an increase of adipose tissue (Tsukumo *et al.*, 2009). Butyrate, in particular, has an important impact on promoting body energy expenditure via modulation of mitochondrial activity. These results are consistent with the role of SCFAs as an additional energy source promoting adiposity. Research of human SCFA content in feces indicated a higher proportion of SCFAs in overweight and obese participants compared to lean controls, although the ratio of *Firmicutes*/*Bacteroidetes* was lower in overweight and obese individuals (Schwiertz *et al.*, 2010). A higher concentration of butyrate and acetate was measured in the caeca of obese mice, while propionate was not significantly different between the lean or obese group (Turnbaugh *et al.*, 2006). A higher ratio of *Firmicutes*/*Bacteroidetes* increases the energy harvest and the content of SCFAs and as such lipogenesis and accumulation in adipocytes (Diamant *et al.*, 2011). In contrast, high-fat diets supplemented with butyrate prevent and even reverse insulin resistance in obese mice (Gao *et al.*, 2009, Kootte *et al.*, 2012). Besides being an energy source for the epithelial cells, butyrate also influences a wide array of cellular functions. It may have an anti-carcinogenic and anti-inflammatory potential and play a role in satiety and oxidative stress (Hartung *et al.*, 2008). These observations suggest an effect of SCFAs on host inflammatory signalling processes via epigenetic control of gene expression (Qin *et al.*, 2010), although the precise role of SCFAs in signalling pathways is still unclear (Kootte *et al.*, 2012), and there is currently insufficient data to allow an estimation of optimal levels of fecal SCFAs for the promotion of colonic health.

Faecalibacterium prausnitzii has been identified as a dominant and conserved species in the healthy human gut microbiota and is described as playing a role in the pathology of low-grade inflammation (Tremaroli & Backhed, 2012). A negative relationship between *Faecalibacterium prausnitzii* and

inflammatory markers was observed in obese and diabetic patients, and remained after adjustment for BMI. The proportions of *Faecalibacterium prausnitzii* were lower in type 2 diabetic subjects indicating a higher incidence of low-grade inflammation (Furet et al., 2010). Butyryl-CoA:acetate CoA transferase gene analysis showed neither significant differences between the groups nor a correlation with qPCR analysis of *Faecalibacterium prausnitzii*. In addition, analysis of *Clostridium cluster IV* and *Clostridium cluster XIVa* was in line with analysis of butyryl-CoA:acetate CoA-transferase gene. These results correspond to previous research where higher butyrate amounts were observed in overweight and obese participants compared to lean controls (Schwiertz et al., 2010) as well as in the feces of obese mice (Turnbaugh et al., 2006). The butyryl-CoA:acetate CoA-transferase gene melt variant amplicons were divided into three areas as described by Louis & Flint (2009). These peaks represent bacteria related to *E. hallii* and *Anaerostipes coli*, *Roseburia/E. rectale spp.* and *F. prausnitzii*. The abundance of the butyryl-CoA:acetate CoA-transferase gene melt variant for *F. prausnitzii* is the least in the lean control group (Figure 3). The distribution of the melt variant of the main butyrate producers in the obese and diabetic group is equal. *F. prausnitzii* contributes the least butyrate amount in lean controls (15%), compared to up to 42% in the obese and diabetic groups. Butyrate, in particular, has an important effect in promoting body energy expenditure via modulation of mitochondrial activity (Remely et al., 2013) and is an additional energy source promoting adiposity (Layden et al., Scott et al., 2013). In contrast, high-fat diets supplemented with butyrate prevent and even reverse insulin resistance in obese mice (Pegorier et al., 2004, Samuel et al., 2008). High butyrate levels in humans stimulate GLP-1 secretion and therefore increases in insulin, inhibit gastric emptying and increase feeling of satiety (Wichmann et al., 2013). Due to the inhibition of gastric emptying, butyrate is maybe excreted more slowly and accumulates, influencing the anti-inflammatory potential, pH and oxidative stress. The observed high *F. prausnitzii* levels compared to low butyrate levels in lean controls could also originate from different *F. prausnitzii* strains. Possibly the different strains have different anti-inflammatory potentials and with that a protective effect in obese subjects, preventing the development of type two diabetes. Remely et al., (2013) reported a lower proportion of inflammatory markers and *F. prausnitzii* in type two diabetic subjects indicating a higher incidence of low-grade inflammation (Furet et al., 2010).

Strains belonging to phylogroup II were only detected in the diabetic group. Although Lopez-Siles et al. reported a tendency for sequences to group individually, we found strains from Phylogroup I in all cloned samples (Lopez-Siles et al. 2012).

Additionally, the relationship between dietary composition, intestinal microbiota, epithelial integrity, and obesity has been investigated (de Kort et al., 2010). Type 2 diabetes is associated with increased gut permeability, and this alteration includes reduced gastrointestinal motility (a consequence of diabetic autonomic neuropathy), intestinal enteropathy, and increased mucosal surface. These disturbances can lead to bacterial overgrowth thus possibly affecting gut permeability (de Kort et al., 2010).

Surprisingly, all investigated groups show a comparable nutritional behavior, consuming the same amounts of grains, vegetables, fruits, and meat-, dietary- and fish products. It is possible that the FFQ could not identify minor differences. The questionnaire used in the present study is carefully adapted

from the EPIC study; multiple related questions are used to validate responses (Kroke *et al.*, 1999). Health experts interviewed every participant personally in a face-to-face meeting. However, the limitation of FFQ for palliating answers is discussed widely. The comparable nutritional behaviors of the analyzed groups may not be responsible for differences in *F. prausnitzii* abundance. Walker *et al.* (2012) also demonstrated a limited response of *F. prausnitzii* to a change of diet.

Gender balance may play a role in studying *F. prausnitzii* and disease (Feng *et al.*, 2013) but Mueller *et al.* (2006) did not detect gender difference in the amount of fecal *F. prausnitzii* in healthy adults (Mueller *et al.*, 2006). We detected no gender difference, even if only the diabetic group was gender balanced.

Fecal butyrate levels depend on shifts in microbial cross feeding patterns, mucosal diffusion and different transit times. Therefore the butyryl-CoA:acetate CoA-transferase gene is used as a marker for butyrate production as the direct analysis in feces is hampered by rapid binding and degradation of butyrate in the gut (Schwartz *et al.*, 2010, Duncan *et al.*, 2007). Assessing the synthesis of butyrate following the butyryl-CoA:acetate CoA-transferase gene pathway discriminates bacteria using other described pathways (butyrate kinase, phosphotransbutyrylase) (Louis *et al.*, 2004). The expression of the butyryl-CoA:acetate CoA-transferase genes was not analyzed and will be a focus for further studies. The results of the different methods comparing GC content or fragment length correlated significantly.

In our pilot study we analyzed interactions of gastrointestinal microbiota, gut permeability, nutrition, and IgG₄ levels, and their impact on or correlation with food intolerances and well-being. High total bacterial abundance correlated with high amounts of *F. prausnitzii*, *Akkermansia*, low Calprotectin values, decreased inconvenience, and less abdominal pain. These results suggest that food antigens affect the quality of the pathological immune response in the gut (Stapel *et al.*, 2008). In subjects with a high abundance of butyrate-producing bacteria (*Faecalibacterium prausnitzii*, *Clostridium Cluster XIVa*) the absence of discomfort (convenience/ wellbeing) was significantly higher, despite high IgG₄ levels or nutritional aspects. The groups of *Lactobacilli*, *Faecalibacterium prausnitzii*, and *Akkermansia* have been widely discussed for their role in inflammatory immune regulation as well as in mucus- and gut barrier-associated aspects of microbiota (Everard *et al.*, 2013). *Akkermansia*, a mucin consuming and degrading species (Louis & Flint, 2007), discussed as a marker for gut wall permeability, also shows a tendency to correlate with convenience/ wellbeing. Calprotectin, a marker for inflammation and implicated in gut permeability (Le Chatelier *et al.*, 2013), correlates with the general abundance of all bacteria. Participants presenting with pain (46%) showed significantly higher Calprotectin values. However, those patients with a high diversity and abundance of bacteria seen as a balanced microbial equilibrium, showed typically low Calprotectin values.

An interaction between IgG₄ levels and single aspects of gastrointestinal microbiota and gut permeability can be suggested by the present results.

Our results encourage the further analysis of *F. prausnitzii* and phylotypes in studying low grade inflammation and obesity-associated metabolic disorders. High resolution melt curve analysis may even be a promising tool for routine and research use. *F. prausnitzii* phylotypes differ in obese

subjects with and without developed diabetes type II and may protect obese subjects from developing type 2 diabetes.

Conclusion

Short chain fatty acid producing-bacteria differ significantly in different life style conditions. Shifts in the main butyrate producers reflect clearly different stages of inflammation.

Intervention in the distribution of the short chain fatty acid producing-bacteria could turn out to be of central importance in complex diseases such as metabolic syndrome, food intolerance and diseases of central nervous system.

Summary

The human gut microbiota, our lifestyle, our diet and our health are clearly associated. The microbial genome has 100 times more genes than the human genome with more than 3 million genes. Several studies provided detailed insight into host–microbiota interactions, for example with the metabolic system, immune system and central nervous system. Recent findings have identified specific microbiota profiles and metabolites as predictors of disease risk as well as determining the microbial species which correlate with health and disease. The bacterial composition and its metabolites have an effect on the human host. And the host with its lifestyle has an impact on the microbiota shift. The analysis of short chain fatty acid producers, in particular *F. prausnitzii*, and their association with nutrition, age, food intolerances, obesity and metabolic disorders in humans, were the focus of my dissertation. *F. prausnitzii* is more common in subjects with high intestinal gut microbiota gene content and lower concentrations have consistently been observed in patients with inflammatory bowel disease or colorectal cancer. The microbial production of short-chain fatty acids has been shown to significantly impact upon the metabolic health of the host through pathways that influence satiety, gut permeability and immune function. Butyrate is of particular interest due to its anticarcinogenic and anti-inflammatory potential, its effects on the intestinal barrier, satiety and epigenetic regulation.

Short Chain Fatty Acid Producing-Bacteria: Two of the most important groups of butyrate producers are *Faecalibacterium prausnitzii* from the *Clostridium* cluster IV, and the *Eubacterium rectale*/*Roseburia* spp. from the *Clostridium* cluster XIVa. Both clusters consist of producers and nonproducers of butyrate. Butyrate is easily taken up by the gut mucosa and fecal butyric acid levels give little information about the butyrate-producing capacity of the gut microbiota. Therefore, a function-based approach was suggested for the enumeration of butyrate-producing bacteria by Louis & Flint (2007), targeting the butyryl-CoA:acetate CoA-transferase gene. Furthermore, the butyryl-CoA:acetate CoA-transferase route, using acetate as a cosubstrate, is suggested to be the most important route for butyrate production in the gut ecosystem. To evaluate the relevance of the butyryl-CoA:acetate CoA-transferase gene in butyrate-producing communities, the amount of butyryl-CoA:acetate CoA-transferase gene was compared in all studies. This gene was amplified and thermally denatured using SYBR Green qPCR analysis. The amplicons were divided into three areas. These peaks represent bacteria related to *Eubacterium hallii* and *Anaerostipes coli*, *Roseburia*/*E. rectale* spp. and *F. prausnitzii* (Louis & Flint, 2009). With only one method of analysis the butyrate-producing capacity of the main butyrate producers could be measured.

***Faecalibacterium prausnitzii* – One of the Main Butyrate Producers in the Human GI Tract:**

Faecalibacterium prausnitzii is the most abundant bacterium in the human intestinal microbiota of western, healthy adults, representing up to 8% of the total bacterial population. *F. prausnitzii* is one of the main butyrate producers in the healthy human gut. *F. prausnitzii* has been discussed for its anti-inflammatory activity, and as a marker for a high bacterial gene content and gut well-being. 97% of *F. prausnitzii* cluster in two phylogroups. Analysis of the two phylotypes of *F. prausnitzii* with 5 different methods showed a significant difference when comparing obese and diabetic type 2

subjects to lean control subjects. That may mean that there is a protecting effect of *F. prausnitzii* preventing obese subjects from developing type 2 diabetes.

Conditions Altering the Microbiome - Nutrition, Age, Antibiotic or Chemotherapeutic Treatment: In our “age and nutrition” -related study we analyzed fecal samples of young healthy omnivores (24±2.5 years), vegetarians (26±5 years), and elderly (86±8 years) omnivores. Diet and lifestyle were assessed in questionnaire-based interviews. The elderly had significantly fewer copies of the butyryl-CoA:acetate CoA-transferase gene than young omnivores, while vegetarians showed the highest number of copies. The thermal denaturation of the butyryl-CoA:acetate CoA-transferase gene variant melting curve related to *Roseburia/Eubacterium rectale* spp. was significantly more variable in the vegetarians than in the elderly. The *Clostridium cluster XIVa* was more abundant in vegetarians and in omnivores than in the elderly group. Vegetarians had a 12% higher abundance of bacterial DNA than omnivores, a tendency for less *Clostridium cluster IV* and higher abundance of *Bacteroides* which, however, were not significant due to high inter-individual variations. PCA suggested a grouping of bacteria and members of *Clostridium cluster IV*. Two bands appeared significantly more frequently in omnivores than in vegetarians. One was identified as *Faecalibacterium* sp.. Gastrointestinal microbiota of the elderly is characterized by decreased butyrate production capacity, reflecting increased risk of degenerative diseases. These results suggest that the butyryl-CoA:acetate CoA-transferase gene is a valuable marker for gastrointestinal microbiota function.

In the probiotic intervention study analyzing the effects of intervention with *Lactobacillus casei shirota* on the incidence of antibiotic-associated diarrhea (AAD), *Clostridium difficile* infection (CDI) and changes in fecal microbiota, 56 patients were analyzed. As much as 18.5% of the antibiotic-treated group developed AAD, but only 5% of patients treated with antibiotics and LcS. Following antibiotic therapy, a decrease in the abundance of total bacteria, *Clostridium cluster IV* and XI, *Bifidobacterium* spp. and butyryl-CoA CoA transferase genes was observed, whereas *Enterobacteriaceae* increased. LcS intervention reduced the antibiotic-associated decrease in the diversity of microbiota, increased the abundance of *Lactobacillus* spp. and reduced the antibiotic-induced decrease of *Bifidobacterium* spp..

Antibiotic treatment affects the diversity and the composition of the microbiota, impairing butyrate production. Intervention with certain *Lactobacillus* strains may antagonize some of these changes, and more potent short-chain fatty acid-stimulating probiotics are needed for intervention in AAD.

In our “chemotherapy study” we analyzed feces of 17 ambulant patients receiving chemotherapy with or without concomitant antibiotics; patients were analyzed before and after the chemotherapy cycle at four time points in comparison to 17 gender-, age- and lifestyle-matched healthy controls. After a significant drop in the abundance of microbiota following a single chemocerapeutic treatment, the microbiota recovered within a few days. The chemotherapeutical treatment marginally affected the *Bacteroides* while the *Clostridium cluster IV* and *XIVa* were significantly more sensitive to chemotherapy and antibiotic treatment. DGGE fingerprinting showed decreased diversity of *Clostridium cluster IV* and *XIVa* in response to chemotherapy, with *cluster IV* diversity being particularly affected by antibiotics. The occurrence of *C. difficile* in three out of seventeen subjects was accompanied by a decrease in the genera *Bifidobacterium*, *Lactobacillus*, *Veillonella* and *Faecalibacterium prausnitzii*. *Enterococcus faecium* increased following chemotherapy.

Microbiota Shift in Obesity, Type 2 Diabetics or Food Inconvenience. In our “obesity study” we characterized fecal microbiota at three time points in obese and type 2 diabetes participants and lean controls. Obese participants and type 2 diabetics received an intervention of nutritional counseling, type 2 diabetics an additional therapy with a GLP-1-agonist. In type 2 diabetics an increase in diversity was observed with intervention whereas the values of lean controls remained unaffected. In the lean and obesity groups, a lower *Firmicutes: Bacteroidetes* ratio correlated with lower BMI. Type 2 diabetics showed a significantly enhanced proportion of lactic acid bacteria before and after intervention, also *Akkermansia* and *Enterobacteria* showed a higher abundance in type 2 diabetics, increasing throughout the study period. *Faecalibacterium prausnitzii* was least abundant in type 2 diabetics, and most abundant in lean controls. Results from *Clostridium cluster* IV and *Clostridium cluster* XIVa showed a decreasing trend in type 2 diabetics in comparison to the butyryl-CoA:acetate CoA-transferase gene and according to melt curve analysis. The diabetic group was found to differ significantly from the lean control group in the results of butyryl-CoA:acetate CoA-transferase gene melt curve variant analysis for *F. prausnitzii* and high resolution melt curve analysis. 22% of all sequenced clones matched with *F. prausnitzii* phylogroup II and 78% with phylogroup I. The strains assigned to phylogroup I were from the diabetic group. *F. prausnitzii* phylotypes differed in obese subjects with and without developed diabetes type 2 and may protect obese subjects from developing type 2 diabetes.

In the “non IgE-mediated food intolerance pilot study” we analyzed forty participants, under consultation of nutritional health professionals, for gastrointestinal discomfort and claimed food intolerances. Food frequency questionnaires addressed nutrition, lifestyle and present discomfort. The total microbial abundance significantly correlated with claimed discomfort. The abundance and diversity of microbiota significantly correlated with low Calprotectin values and with higher abundance of *Faecalibacterium prausnitzii* and *Akkermansia*. Participants with low discomfort showed enhanced *Clostridium* Cluster XIVa. An increased diversity also correlated with reduced antibodies against IgG₄ of egg white. The data suggest an association of low gut permeability and reduced inflammation with an established microbial equilibrium. Self-reported abdominal inconvenience of participants related mainly to characteristics of microbiota and gut permeability. Anti-inflammatory effects of *Faecalibacterium prausnitzii* or *Lactobacilli* and gut barrier functions of *Akkermansia* may have a key role in food intolerances. The role of IgG₄ linking food immune responses with intolerances remains unclear.

Impact and Marker of GI Microbiota Interactions: Changes in gut microbiota and thus cell wall components are involved in the epigenetic regulation of inflammatory reactions. An improved diet targeted to induce gut microbial balance and as a consequence epigenetic changes of pro-inflammatory genes may be effective in the prevention of metabolic syndrome. Methylation analysis of four CpGs in the first exon of TLR4 showed significantly lower methylation in obese individuals, but no significant difference between type 2 diabetics and lean controls. Methylation of seven CpGs in the promoter region of TLR2 was significantly lower in type 2 diabetics compared to obese subjects and lean controls. The methylation levels of both TLRs significantly correlated with body mass index. The analysis of five CpGs in the promoter region of FFAR3 showed a significantly lower methylation in obese subjects and type 2 diabetics with an increase in obese patients over the intervention period.

These results disclosed a significant correlation between a higher body mass index and lower methylation of FFAR3. Different compositions of gut microbiota in obese individuals and type 2 diabetics affect the epigenetic regulation of FFARs. These interactions between microbiota and epigenetic regulation may influence the satiety and hunger cycle. Especially butyrate may have an impact in strategies for regenerative medicine by promoting epigenetic remodeling and the expression of associated genes. Our data suggest that changes in gut microbiota and thus cell wall components are involved in the epigenetic regulation of inflammatory reactions.

Analyzing Methods: To assess the synthesis of butyrate following the butyryl-CoA:acetate CoA-transferase gene pathway we amplified and thermally denatured the product using SYBR Green qPCR analysis. The melt curves from amplified PCR products were divided into three areas. These peaks represent bacteria related to *Eubacterium hallii* and *Anaerostipes coli* (82.5–85.0°C), *Roseburia/E. rectale spp.* (85.5–89.0°C) and *F. prausnitzii* (89.5–92.5°C)(Louis & Flint, 2009). Analyzing this gene gives additional information about further butyrate-producing bacteria. Qiaxcel – fragment length polymorphism discriminates between different fragment lengths of the amplified PCR products. The relative migration time can be assigned to a particular fragment length and this can be assigned to particular bacterial strains. The two *F. prausnitzii* phylotypes could be distinguished by fragment length, phylotype I with 160-161bp and phylotype II with 153-155bp. Four variants of *F. prausnitzii* could be distinguished with HRM, depending on the fragment lengths of the different phylotypes and mixtures of those in the different individuals. Diabetic group results differed significantly from the lean control group in HRM temperature and variance, and from the obese group in HRM temperature and variance. High resolution melt curve analysis may be a promising tool for routine and research use for further analyzing *F. prausnitzii* phylotypes in low grade inflammation and obesity-associated metabolic disorders.

Zusammenfassung

Die menschliche Darmmikrobiota (Darmflora), unsere Ernährung und unsere Gesundheit sind eng miteinander verbunden. Das gastro intestinale Mikrobiome umfasst 3 Millionen mal mehr Gene als der Mensch. Wir sind sozusagen zusammengesetzte Wesen, gesteuert von unseren eigenen Genen und den Genen der Bakterien und Pilze die wir unser Leben lang erwerben. Zahlreiche Studien beschreiben den Einfluss der Bakterien und ihrer Metaboliten auf die Steuerung des menschlichen metabolischen Systems, Immunsystems, Zentral Nervösen Systems und Hormonkreislaufs. Mittlerweile wurden spezielle Bakterienzusammensetzungen beschrieben die in verschiedenen Krankheiten stärker vorkommen und Bakterienprofile die mit Gesundheit assoziiert sind. Nicht nur die Stoffwechselprodukte der Bakterien beeinflussen den Menschen, auch der Mensch und sein Lebensstil beeinflussen die Zusammensetzung der Mikrobiota.

In meiner Dissertation beschreibe ich das Wechselspiel von Buttersäure produzierenden Bakterien, im Speziellen *Faecalibacterium prausnitzii* und Ernährung, Alter, Nahrungsmittelunverträglichkeiten, Übergewicht und damit verbundene metabolische Krankheiten, sowie geringgradige Entzündungen im Mensch. Personen mit einer hohen, als positiv eingeschätzten, Gesamtbakterienzahl haben auch ein großes *F. prausnitzii* Vorkommen, wobei niedrige *F. prausnitzii* Mengen in Personen mit entzündlichen Darmkrankheiten oder Darmkrebs beobachtet werden. Die bakterielle Produktion von kurzkettigen Fettsäuren (SCFA) hat einen bedeutenden Einfluss auf die metabolische Gesundheit des Menschen. SCFAs regulieren Mechanismen der Sättigung, Darmdurchlässigkeit und Immunfunktionen. Buttersäure ist besonders interessant da es als antikanzerogen, entzündungshemmend und sättigungsregulierend gilt, und eine epigenetische Wirkung beschrieben wird.

Kurzkettige Fettsäuren produzierende Bakterien:

Zwei der wichtigsten bakteriellen Gruppen von Buttersäure (Butyrat) Erzeugern sind *Faecalibacterium prausnitzii* vom *Clostridium* Cluster IV und *Eubacterium rectale* / *Roseburia spp.* vom *Clostridium* - Cluster XIVa . Beide Cluster bestehen aus Butyrate Produzenten und Nichtproduzenten. Butyrat wird leicht von der Darmschleimhaut aufgenommen weshalb die Bestimmung der Buttersäure im Stuhl nur Vermutungen auf die tatsächliche bakterielle Butyrat- Produktion zu lässt.

Die Bestimmung der Kopien Anzahl des Butyryl -CoA: Acetat- CoA - Transferase-Gen, entwickelt von Louis & Flint 2007, bietet ergänzend einen funktionellen Ansatz. Butyryl -CoA : Acetat -CoA - Transferase- ist im letzten Schritt einer Umbaukaskade beteiligt, bei der Acetat mit Hilfe dieses Enzyms in Buttersäure umgewandelt wird. Dieser Aufbau ist die Hauptroute für die Bildung von Buttersäure im menschlichen Darm mit Hilfe von Bakterien. Das für dieses Enzym codierende Gen wurde in allen von uns durchgeführten Studien gemessen. Das Gen wurde amplifiziert und thermisch denaturiert mit SYBR Green qPCR Analyse. Die Amplikons (DNA Fragmente) wurden in drei Bereiche unterteilt. Die Peaks der Schmelzkurve können 3 Bakteriengruppen zugeordnet werden: *Eubacterium hallii* und *Anaerostipes coli* , *Roseburia* / *E. rectale* spp. und *F. prausnitzii*. Dadurch können mit nur einem Analyseverfahren die Butyrat- Produktion der Haupt Butyrat Produzenten gemessen werden.

***Faecalibacterium prausnitzii* - Einer der Haupt Butyrat Produzenten des menschlichen GI-Traktes:**

Faecalibacterium prausnitzii ist mit bis zu 8% im menschlichen Darm von gesunden Erwachsenen das am Häufigsten vorkommende Bakterium. *F. prausnitzii* ist für seine entzündungshemmende Aktivität bekannt, einschließlich Regulation von IL-12, IL-10 und IFN- γ , wodurch *F. prausnitzii* als Marker für eine hohe Bakteriendichte und damit verbunden als Marker für Darmwohlbefinden gilt. 97% der *F. prausnitzii* kann man 2 phylogenetische Gruppen zuordnen. Beim Vergleich dieser beiden Phylogruppen, mit 5 verschiedenen Methoden, unterschieden sich Typ 2 DiabetikerInnen von adipösen PatientInnen und der normalgewichtigen Kontrollgruppe signifikant. Dies könnte bedeuten dass *F. prausnitzii* eine schützende Wirkung für Adipöse hat, und vielleicht das Risiko senkt an Typ-2-Diabetes zu erkranken.

Ernährung , Alter , Antibiotika oder Chemotherapeutische Behandlung ändern das Mikrobiom:

In unserer Studie, die Einflüsse von Altern und Ernährung auf das Mikrobiom untersucht, analysierten wir Stuhlproben von jungen gesunden Mischköstlern ($24 \pm 2,5$ Jahre), Vegetariern (26 ± 5 Jahre) und älteren (86 ± 8 Jahre) Mischköstlern. Ernährung und Lebensweise wurden in Fragebogen basierten Interviews bewertet. Die älteren Menschen hatten signifikant weniger Kopien des Butyryl -CoA: Acetat- CoA - Transferase-Gens als junge Mischköstler, während Vegetarier die höchste Anzahl dieses Gens zeigten. Die thermische Denaturierung der Butyryl -CoA: Acetat- CoA -Transferase- Gen-Variante für *Roseburia/Eubacterium rectale* spp. war signifikant höher in den Vegetariern verglichen mit den älteren Menschen. Der *Clostridium* -Cluster XIVa war bei Vegetariern und Mischköstlern reichlicher vorhanden als in der älteren Gruppe. Vegetarier hatten verglichen zu den den Mischköstlern eine um 12% höhere Gesamtbakterien-DNA Menge, weniger *Clostridium* cluster IV und mehr *Bacteroides*. *Clostridium* cluster IV und eine weitere Bakteriengruppe clusterten in der PCA. Zwei Banden erschienen signifikant häufiger in Mischköstlern als in Vegetariern. Eine dieser Banden war *Faecalibacterium* sp..

Die gastrointestinale Mikrobiota von älteren Menschen ist gekennzeichnet durch eine verringerte Buttersäureproduktion, was ein erhöhtes Risiko von degenerativen Krankheiten widerspiegelt. Diese Ergebnisse legen nahe, dass das Butyryl -CoA: Acetat- CoA - Transferase-Gen ein wichtiger Marker für gastrointestinale Mikrobiota –Funktionen ist.

In unserer „Probiotika-Interventionsstudie“ untersuchten wir die Auswirkungen von *Lactobacillus casei* Shirota auf das Vorkommen von Antibiotika-assoziiierter Diarrhoe (AAD), *Clostridium difficile* – Infektion (CDI) und Änderungen der Mikrobiota bei 56 PatientInnen. 18,5% der mit Antibiotikum behandelten Gruppe entwickelten AAD, aber nur 5 % der mit Antibiotika und LCS behandelten PatientInnen. Nach Antibiotika-Therapie wurde ein Rückgang der Gesamtbakterien, *Clostridium* cluster IV und XI, von *Bifidobacterium* spp. und des Butyryl -CoA: CoA-Transferase -Gens beobachtet, während *Enterobacteriaceae* erhöht waren. LCS Intervention reduzierte die Antibiotika-assoziierte Abnahme der Mikrobiota Vielfalt. Antibiotika- Behandlung wirkt sich auf die Vielfalt, die Zusammensetzung und Buttersäurekapazität der Mikrobiota aus. Intervention mit speziellen *Lactobacillus*-Stämmen kann Antibiotika induzierten Veränderungen entgegenwirken. Spezielle

Probiotika mit Focus auf der Produktion von kurzkettigen Fettsäuren sind besonders bei der Therapie von AAD wünschenswert.

In unserer „Chemotherapie -Studie“ analysierten wir Stuhl von 17 ambulanten PatientInnen unter Chemotherapie, mit oder ohne begleitende Antibiotika, zu 4 Zeitpunkten: vor, zwei während und nach der Behandlung. Die Vergleichsgruppe bestand aus 17 gender-, alters- und Lifestyle – angepassten gesunden Kontrollen. Nach der ersten Behandlung zeigte sich ein deutlicher Rückgang der Gesamtbakterienzahl, welche aber schon nach wenigen Tagen wieder zu nahm. *Bacteroides* waren dabei nicht so stark betroffen, während die *Clostridium cluster* IV und XIVa deutlich empfindlicher auf Chemotherapie und Antibiotika-Behandlung reagierten. DGGE -Fingerprinting zeigte eine verringerte Vielfalt von *Clostridium cluster* IV und XIVa durch Chemotherapie, wobei der *Cluster* IV besonders stark auf Antibiotikabehandlung reagierte. Das Auftreten von *C. difficile* in drei der siebzehn Patienten wurde durch eine Abnahme der Gattungen *Bifidobacterium*, *Lactobacillus*, *Veillonella* und *Faecalibacterium prausnitzii* begleitet. Das Vorkommen von *Enterococcus faecium* erhöhte sich nach Chemotherapie.

Mikrobiota Verschiebung bei Übergewicht, Diabetes Typ 2 oder Nahrungsmittelunverträglichkeit:

In unserer „Adipositas Studie“ untersuchten wir die fäkale Mikrobiota zu drei Zeitpunkten bei Adipösen, Typ-2- DiabetikerInnen und normalgewichtigen Kontrollen. Adipöse und Typ -2- DiabetikerInnen erhielten eine Intervention und Ernährungsberatung, Typ -2-Diabetiker eine zusätzliche Therapie mit einem GLP-1 - Agonist. Bei Typ- 2-DiabetikerInnen wurde unter Intervention eine Erhöhung der Vielfalt beobachtet, während die bakterielle Vielfalt der schlanken Kontrollen gleich blieb. Das Verhältnis von *Firmicutes* zu *Bacteroidetes* korrelierte mit niedrigen BMI. Die Gruppe mit Diabetes Typ 2 hatte zu allen Zeitpunkten viele Milchsäurebakterien, sowie auch viele *Akkermansia* und *Enterobakterien*, welche sich durch die Intervention noch vermehrten. Dafür hatten sie am wenigsten *Faecalibacterium prausnitzii* im Vergleich zur normal gewichtigen Kontrollgruppe. *Clostridium cluster* IV und *Clostridium Cluster* XIVa nahmen während des Studienverlaufs ab. Die Gruppe mit Diabetes Typ 2 unterschied sich von der schlanken Kontrolle in den Schmelzkurvenvarianten des Butyryl -CoA: Acetat -CoA - Transferase-Gens für *F. prausnitzii*. 22% aller sequenzierten Klone konnten der *F. prausnitzii* Phylogruppe 2 und 78 % der Phylogruppe 1 zu geordnet werden. Stämme der Phylogruppe 1 wurden nur bei der Gruppe mit Diabetes detektiert. Stämme der unterschiedlichen *F. prausnitzii* Phylotypen unterschieden sich bei Übergewichtigen mit und ohne Typ-2- Diabetes signifikant, und geben damit einen Hinweis auf ein eventuelles Diabetesrisiko.

In der „Nicht -IgE- vermittelten Nahrungsmittelintoleranzpilotstudie“ wurden 40 TeilnehmerInnen untersucht die bei ErnährungsberaterInnen in Behandlung waren wegen Magen Darm Beschwerden und/oder vermuteten Nahrungsmittelunverträglichkeiten. Fragebögen betreffend der Nahrungsmittel-Aufnahme Häufigkeit, Lebensstil und Wohlbefinden wurden abgefragt. Es zeigte sich das ein beschriebenes Unwohlsein mit der Anzahl an Gesamtbakterien korrelierte. Die Gesamtbakterienanzahl sowie die bakterielle Vielfalt korrelierten mit den Calprotectinwerten und der Anzahl an *F. prausnitzii* und *Akkermansia*. TeilnehmerInnen die Wohlbefinden angaben, hatten viele Bakterien des Clostridien *Clusters* XIVa. Eine niedrige bakterielle Vielfalt korrelierte mit niedrigen Werten für IgG₄ Antikörper gegen Eiweiß.

Die Daten lassen Rückschlüsse zu, dass eine ausgewogene Mikrobiota mit einer niedrigen Darmdurchlässigkeit und geringen Entzündungswerten verbunden werden kann. Das beschriebene Unwohlsein konnte oft in Zusammenhang mit einer veränderten Mikrobiota und Darmdurchlässigkeit beobachtet werden. Eine Schlüsselrolle in der Erforschung von Nahrungsmittelunverträglichkeiten können hier die entzündungshemmenden *F. prausnitzii* und Laktobazillen haben, sowie die mit Darmdurchlässigkeit assoziierten *Akkermansia*. Die genaue Verknüpfung von Lebensmittel-unverträglichkeiten und IgG₄ Immunantworten bleibt unklar.

Marker und Wechselwirkungen der GI Mikrobiota:

Eine Änderung der mikrobiellen Zusammensetzung und eine daraus folgende Änderung der Zellwandbestandteile und Stoffwechselprodukte ändert die epigenetische Regulation von Entzündungsreaktionen. Eine verbesserte Ernährung die gezielt das mikrobielle Gleichgewichts des Darms aufrechterhalten kann, und somit die epigenetische Regulierung von entzündungsrelevanten Genen beeinflussen kann, könnte gut sein um Folgeerkrankungen des Metabolischen Syndroms vorzubeugen.

Die Methylierungsanalyse von vier CpGs im ersten Exon des TLR4 zeigte signifikant niedrigere Methylierungen bei übergewichtigen Personen aber keine signifikanten Unterschiede zwischen Typ-2- Diabetikern und schlanken Kontrollen. Methylierung von sieben CpGs in der Promotorregion des TLR2 war signifikant niedriger bei Typ 2 Diabetikern im Vergleich zu übergewichtigen Probanden und schlanken Kontrollen. Die Methylierung Niveaus beider TLRs korrelierten signifikant mit Body-Mass-Index. Die Analysen von fünf CpGs in der Promotorregion des FFAR3 zeigten eine signifikant geringere Methylierung bei Übergewichtigen und Typ -2-Diabetikern. Diese Ergebnisse zeigen einen signifikanten Zusammenhang zwischen einem höheren Body- Mass-Index und niedrigerer Methylierung von FFAR3. Eine geänderte Zusammensetzung der Darmmikrobiota beeinflusst Mechanismen der epigenetischen Regulierung von FFAR3. Diese Wechselwirkungen zwischen Mikrobiota und epigenetischer Regulation können Einfluss auf den Sättigungs und Hunger Zyklus haben. Dabei kann Butyrate für einen therapeutischen Ansatz besonders interessant sein. Die Expression von regenerativen Genen könnte mit Butyrate aktiviert werden. Unsere Daten legen nahe, dass die Mikrobiota an der epigenetischen Regulation von Entzündungsreaktionen beteiligt ist.

Analyse -Methoden: Die Verwendung der Analyse des Butyryl -CoA: Acetat- CoA - Transferase-Gen Wegs zur Messung der Buttersäureproduktion im Darm erfolgte mittels SYBR Green und qPCR. Die Schmelzkurven des amplifizierten PCR-Produktes wurden in drei Bereiche unterteilt. Diese Peaks lassen sich verschiedenen Bakteriengruppen zuordnen: *Eubacterium hallii* und *Anaerostipes coli* (82,5-85,0°C), *Roseburia/ E. rectale spp.* (85,5-89,0°C) und *F. prausnitzii* (89,5-92,5°C) (Louis & Flint, 2009). Die Analyse dieses Gens gibt zusätzliche Informationen über Butyrat -produzierende Bakterien. Qiaxcel - Fragmentlängen-Polymorphismus unterscheidet die amplifizierten PCR Produkte nach ihren Längen. Die relative Migrationszeit kann einer spezielle Fragmentlänge zugewiesen werden, und diese Länge kann speziellen Bakterienstämmen zugeordnet werden. Die beiden *F. prausnitzii* Phylotypen unterscheiden sich in ihren Fragmentlängen: für Phylotyp I mit 160 - 161bp (Basenpaaren) und für Phylotyp II mit 153- 155bp. *F. prausnitzii* strains unterscheiden sich in der hochauflösenden Schmelzkurve (HRM) in 4 Varianten, je nachdem, welche Fragmentlängen und in welcher Mischung die unterschiedlichen Phylotypen in den Individuen vorkommen. Die Gruppe mit Diabetes Typ 2

unterscheidet sich von der normalgewichtigen Kontrolle signifikant in HRM- Temperatur und Varianz. Die Hochauflösende Schmelzkurvenanalyse kann ein vielversprechendes Werkzeug für Routine-und Forschungszwecke zur weiteren Analyse von *F. prausnitzii* Phylotypen in geringgradigen Entzündungen und Adipositas -assoziierten Stoffwechselstörungen sein .

Appendix

Quantification of butyryl CoA:acetate CoA-transferase genes reveals different butyrate production capacity in individuals according to diet and age

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Introduction

Recent evidence suggests that 1000–1150 different species are capable of living in the gut ecosystem. An individual harbours at least 160 species (Qin et al., 2010), with high interindividual variations in species diversity and evenness. It has been reported that the microbiota composition is influenced by diet (Larsen et al., 2010) and age (Mariat et al., 2009), as well as genetic factors (Khachatryan et al., 2008). The gastrointestinal microbiota produces short-chain fatty acids (SCFAs). Butyrate is of particular interest due to its anticarcinogenic and anti-inflammatory potential (Maslowski et al., 2009), its effects on the intestinal barrier (Peng et al., 2007), satiety (Cani et al., 2009) and epigenetic regulation (Rada-Iglesias et al., 2007). Two of the most important groups of butyrate producers are *Faecalibacterium prausnitzii* from the *Clostridium* cluster IV, and the *Eubacterium rectale*/*Roseburia* spp. from the *Clostridium* cluster XIVa (Walker et al., 2010). Both clusters (now also known as *Ruminococcaceae* and *Lachnospiraceae*) consist of producers and nonproducers of butyrate (Pryde et al., 2002). Isolated dietary compounds have been shown to promote growth of butyrate producers (Hernot et al., 2009). For example, the consumption of inulin significantly stimulated growth of *F. prausnitzii* (Louis & Flint, 2009). In colonic in vitro model systems, resistant starch stimulated the growth of *E. rectale* (Leitch et al., 2007).

Butyrate is easily taken up by the gut mucosa and faecal butyric acid levels give little information about the butyrate-producing capacity of the gut microbiota. Therefore, a function-based approach was suggested for the enumeration of butyrate-producing bacteria (Louis & Flint, 2007) targeting the butyryl-CoA:acetate CoA-transferase gene. Furthermore, the butyryl-CoA:acetate CoA-transferase route, using acetate as a cosubstrate, is suggested to be the most important route for butyrate production in the gut ecosystem (Duncan et al., 2004). Alternative routes are via butyrate kinase and phosphotransbutyrylase, which are found in a minority of bacteria (Louis et al., 2004) in the human gastrointestinal tract.

To evaluate the relevance of the butyryl-CoA:acetate CoA-transferase gene in butyrate-producing communities, we compared the amount of butyryl-CoA:acetate CoA-transferase gene in young omnivores, vegetarians and the elderly by quantitative PCR (qPCR) with a 16S rRNA gene quantification of *Clostridium clusters* IV and XIVa considering the effects of aging and nutrition.

Materials and methods

Study subjects

Study groups consisted of 15 institutionalized, mobile elderly persons [age: 86 ± 8 years, body mass index (BMI) 21.75 ± 5.08], 15 young healthy vegetarians (age: 26 ± 5 years, BMI 21.02 ± 2.71) and 17 young healthy omnivores (age: 24 ± 2.5 years, BMI 22.68 ± 3.4) consuming a central European diet. All subjects were interviewed using a questionnaire assessing age, gender, body height, weight, individual health status, lifestyle and dietary habits. Approval was obtained from the Viennese Human Ethics committee (EK 07-153VK).

Sample material

Faeces was collected from each participant individually and stored at -18°C until processed. DNA was extracted using the DNA stool Mini Kit (Qiagen) following the manufacturer's protocol with minor modifications and immediately stored at -20°C . Efficiency and quality of extraction was controlled by photometry (Nanodrop) and gel electrophoresis.

Quantification of specific metabolic genes and 16S rRNA genes by qPCR

The butyryl-CoA:acetate CoA-transferase genes were amplified with degenerated primers BCoATscrF/R as listed in Table 1 (Louis & Flint, 2007) on a Rotor-Gene 3000A (Qiagen) using the SensiMix SYBR Kit (Quantace). Amplification of one of the faecal samples with BCoATscrF/R leads to a highly concentrated butyryl-CoA:acetate CoA-transferase gene mix. This purified PCR product was used for quantification of samples. Amplification with primer pair BcoATscr resulted in clearly distinguishable and assignable melt peaks. Total bacteria (Yu & Morrison, 2004) and *Clostridium clusters* IV and XIVa were quantified (Meier et al., 1999; Matsuki et al., 2004) on a Rotor-Gene 3000A (Qiagen) using the SensiMix Probe Kit (Quantace). The primers and probes used in this study are listed in Table 1. Samples were quantified using standards derived from one clone [clone library CleptF/R; Promega Vector System, specificity in library confirmed (Liszt et al., 2009) with known concentration in the case of *Clostridium cluster* IV and from a *Blautia coccooides* T pure culture for cluster XIVa].

Table 1. Primers and probes used for quantification of 16S rRNA genes and butyryl CoA:acetate CoA-transferase genes with qPCR Target organism

Table 1

Target organism	Primer/probe	Sequence (5'–3')	Size (bp)	References
BCCT gene	BCoATscrF	GCIGAICATTTACITGGAAY	530	Louis & Flint (2007)
		WSITGGCAYATG		
C. cluster IV	BCoATscrR	CCTGCCTTTGCAATRTCIACRAANGC	239	Matsuki <i>et al.</i> (2004)
	sg-Clept-F	GCA CAA GCA GTG GAG T		
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		
	Clept-P	(FAM)-AGG GTT GCG CTC GTT-(BHQ)		
C. cluster XIVa	195-F	GCA GTG GGG AAT ATT GCA	468	Meier <i>et al.</i> (1999)
	Ccocc-R	CTT TGA GTT TCA TTC TTG CGA A		
	Ccocc-P	(FAM)-AAATGACGGTAC		
		CTGACTAA-(BHQ)		
All bacteria	BAC-338-F	ACT CCT ACG GGA GGC AG	468	Yu <i>et al.</i> (2005)
	BAC-805-R	GAC TAC CAG GGT ATC TAA TCC		
	BAC-516-P	(FAM)-TGC CAG CAG CCG CGG		
		TAA TAC-(BHQ)		

Thermal denaturation of butyryl-CoA:acetate CoA-transferase amplicons

Melt curves from amplified PCR products were divided into three areas (Fig. 1b), as described by Louis & Flint (2009). These peaks were assigned to represent bacteria related to *Eubacterium hallii* and *Anaerostipes coli* (82.5–85.0 °C), *Roseburia/E. rectale* spp. (85.5–89.0 °C) and *F. prausnitzii* (89.5–92.5 °C) as illustrated in Fig. 1a and b.

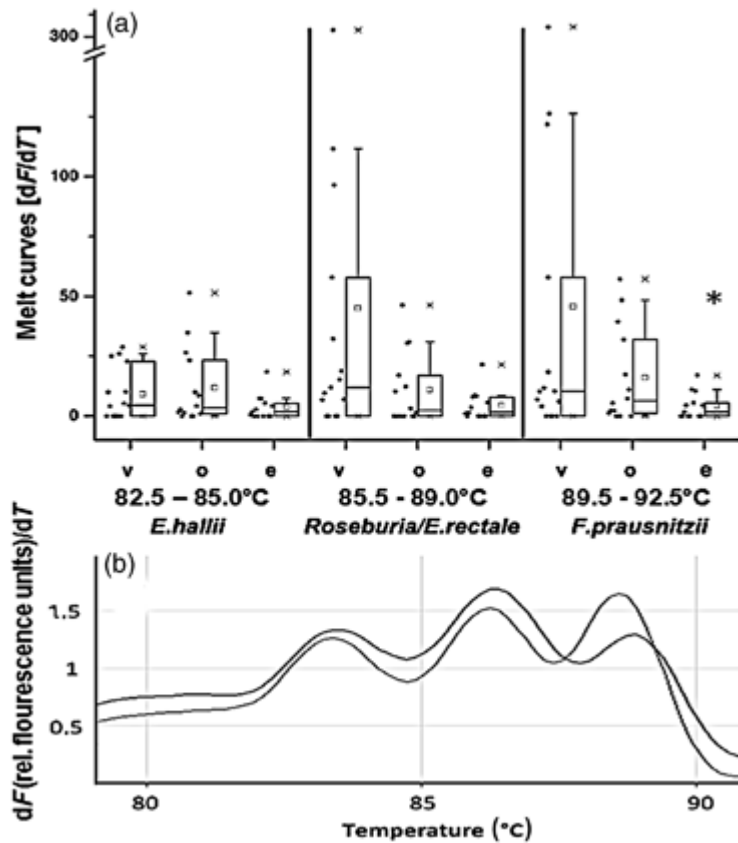


Figure 1

Figure 1. Thermal denaturation of butyryl CoA:acetate CoA-transferase gene of vegetarians (v), omnivores (o) and the elderly (e). (a) Box plots are grouped according to their melt curves representing bacteria related to *Eubacterium hallii* and *Anaerostipes coli* (82.5–85.0°C), *Roseburia/Eubacterium rectale* spp. (85.5–89.0°C) and *Faecalibacterium prausnitzii* (89.5–92.5°C). Rectangular marks represent mean levels, medians are indicated with a line, whisker ranges between 5th and 95th percentiles and box limits between the 25th and 75th percentile. The asterisk indicates a significant difference ($P < 0.05$). (b) Sample melt curves between 78 and 92°C of two elderly individuals representing the peaks related to *E. hallii* and *A. coli* (82.5–85.0°C), *Roseburia/E. rectale* spp. (85.5–89.0°C) and *F. prausnitzii* (89.5–92.5°C). dF/dT, rel. fluorescence units.

Data analysis

All statistical analysis (Spearman's rank, Kolmogorov–Smirnov, F- Kruskal–Wallis- and t-tests) was done using originpro 8G (<http://www.originlab.com>).

Results

Dietary analysis

Analysis of the dietary habits indicated similar consumptions of fruit and milk products in the individual groups. Vegetarians stated significantly more frequent consumption of vegetables (χ^2 ; $P < 0.03$) but similar patterns of consumption of carbohydrates, including whole-grain products. The elderly stated significantly more frequent consumption of meat and similar vegetable consumption (χ^2 test; $P < 0.04$) compared with omnivores. The exercise levels of vegetarians and omnivores were comparable.

Relative quantification

Vegetarians had $12 \pm 62\%$ more and the elderly had $31 \pm 21\%$ less 16S rRNA gene relative to absolute quantified genes compared with omnivores (Fig. 2a). Many SCFA-synthesizing bacteria belong to the *Clostridium* clusters IV and XIVa. The *Clostridium* cluster IV (Fig. 2b) was significantly more abundant in omnivores ($36.3 \pm 11.2\%$) than in the elderly ($27 \pm 11.7\%$, $P = 0.04$) quantified relative to total bacterial 16S rRNA genes. Vegetarians harboured $31.86 \pm 17.00\%$ of *Clostridium* cluster IV. The *Clostridium* cluster XIVa (Fig. 2c) was significantly more abundant in omnivores ($19.01 \pm 6.7\%$, $P > 0.01$) and vegetarians ($14.52 \pm 5.6\%$, $P = 0.049$) than in the elderly ($9.89 \pm 6.64\%$).

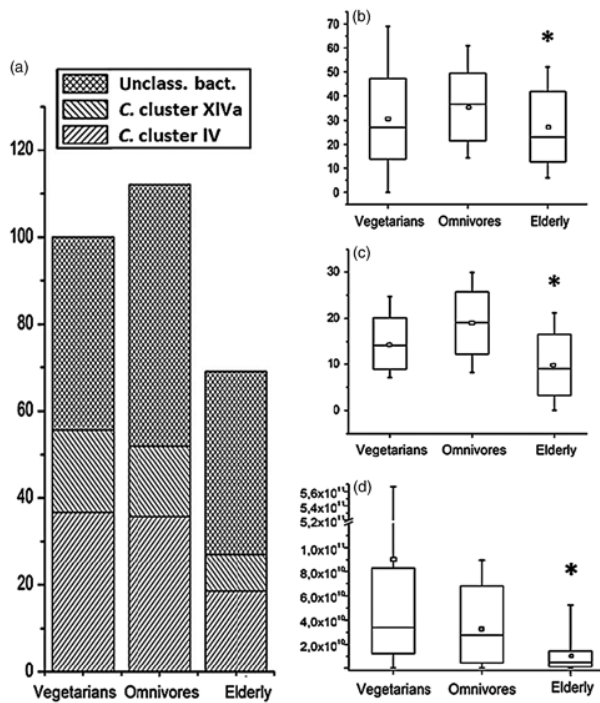


Figure 2

Figure 2. (a) Relative quantities (in %) of absolute amplified 16S rRNA genes in vegetarians and the elderly compared with omnivores. Absolute bacterial numbers of omnivores were set as 100%. (b, c) Relative quantities of total bacterial 16S rRNA genes (in %) of *Clostridium* cluster IV (b) and *Clostridium* cluster XIVa (c) based on group-specific qPCR of 16S rRNA genes in vegetarians and the elderly compared with omnivores. (d) qPCR quantification of the butyryl CoA:acetate CoA-transferase gene in faecal samples of vegetarians and the elderly compared with omnivores. Data are expressed in copy numbers of total DNA per 1 g faeces (d). Rectangular marks represent mean levels, medians are indicated with a line, whisker ranges between 5th and 95th percentiles, and box limits between 25th and 75th percentile. Asterisk indicates a significant difference (P<0.05) (b, c, d).

Butyryl-CoA:acetate CoA-transferase genes in faeces by qPCR

The elderly had significantly fewer copies ($1.52 \times 10^{11} \pm 1.36 \times 10^{10}$ copies g⁻¹ faeces) of the butyryl-CoA:acetate CoA-transferase gene compared with the omnivores ($4.96 \times 10^{11} \pm 3.22 \times 10^{10}$ copies g⁻¹ faeces, P=0.01) and the vegetarians ($1.37 \times 10^{12} \pm 1.47 \times 10^{11}$ copies g⁻¹ faeces, P=0.048) (Fig. 2d). The amount of the butyryl-CoA:acetate CoA-transferase gene did not correlate significantly with the amount of total bacteria.

Melt curve analysis

The *E. hallii*/*A. coli* melt peaks tend to be higher in vegetarians ($P=0.08$) and omnivores ($P=0.09$) than in the elderly. The abundance of *E. rectale*/*Roseburia* spp. melt peak differed significantly between vegetarians and the elderly ($P=0.04$). Melt peak attributed to *F. prausnitzii* was significantly lower in the elderly than in omnivores ($P=0.049$) (Fig. 1a).

Spearman's rank showed no significant correlation between the amount of the butyryl-CoA:acetate CoA-transferase gene and that of *Clostridium* clusters IV and XIVa at an individual level.

Discussion

Analysis of the overall abundance of bacterial 16S rRNA genes reveals that the vegetarians harboured more bacteria than the omnivores. The low numbers of bacteria in the elderly individuals (Fig. 2a) may reflect physiological alterations such as prolonged colonic transit time, reduced dietary energy requirement and food uptake (Morley, 2007). Figure 2b illustrates the significantly higher abundance of *Clostridium* cluster IV in omnivores. Mueller et al. (2006) detected the highest levels of the *Clostridium* cluster IV in a Swedish study population, whose dietary habits were characterized by a high consumption of fish and meat (Mueller et al., 2006). Despite high meat consumption in the elderly, the generally smaller capacity for energy harvest from food may decrease the abundance of *Clostridium* cluster IV (Li et al., 2008). The elderly gut microbiota is also characterized by a significantly lower relative contribution of *Clostridium* cluster XIVa compared with young study participants (Fig. 2c). A decrease of *Clostridium* cluster XIVa has already been described as an effect of the ageing process (Bartosch et al., 2004). Elderly study participants had lower physical activity levels and did not consume whole-grain products, whereas the other groups stated regular consumption of fibre-rich products.

Vegetarians and omnivores have significantly more copies of the butyryl-CoA:acetate CoA-transferase genes compared with the elderly (Fig. 2d). Although no clear correlation with *Clostridium* cluster IV and XIVa levels were found, the elderly tended to harbour fewer butyrate producers than did young individuals. Melt curve analysis showed that the butyryl-CoA:acetate CoA-transferase gene variant related to *E. rectale*/*Roseburia* spp. is significantly more variable in vegetarians than in the elderly (Fig. 1a). Correspondingly, *Clostridium* cluster XIVa seems to be more abundant in vegetarians. Biagi et al. (2010) found lower quantities of *Roseburia intestinalis*, *E. hallii* and *E. rectale* in the elderly (>75 years) than in young adults using the HITchip method. The abundance of the *Clostridium* cluster XIVa does not show significant correlations with the abundance of the butyrate gene variant as determined by melting curve analysis related to *Roseburia*/*E. rectale* spp., as this cluster also contains many nonbutyrogenic bacteria. As illustrated in Fig. 1a, the level of the melt peak attributed to *F. prausnitzii* was significantly lower in the elderly. This is of particular interest as this species has been reported to influence gut inflammation processes by exerting a butyrate-independent anti-inflammatory effect (Sokol et al., 2009).

The vegetarian diet may have favoured growth of the *Roseburia*/*E. rectale* spp. that carries the butyryl-CoA:acetate CoA-transferase gene, without causing an increase in the abundance of butyrate

producers in the entire *Clostridium cluster* XIVa. Omnivores and vegetarians had a similar potential to harbour butyryl-CoA:acetate CoA-transferase genes and members of *Clostridium clusters* IV and XIVa, possibly caused by a similar intake of fruit and carbohydrates.

These results suggest that the elderly group in this study harbours less total bacteria and an even lower abundance of *Clostridium clusters* IV and XIVa. Together with a lower abundance of the butyryl-CoA:acetate CoA-transferase gene, the results indicate that in the elderly, microbiota may be characterized by a low butyrate production capacity. In respect of the important nutritive, anti-inflammatory and anticarcinogenic potential of butyrate in the human colon, these findings demonstrate that these microbiota specificities may contribute to the development of degenerative diseases (Guigoz et al., 2008) and anorexia in advanced age (Donini et al., 2010).

Consideration of the results of the analysis must take into account methodological limitations. Despite the extraction controls discussed in Materials and methods, DNA extraction can be influenced by diet and the consistency of faeces. Previous studies reported a higher abundance of *Clostridium cluster* XIVa than cluster IV, which is not seen in our results (Fig. 2b and c). This points to the need to compare different standards for these clusters. Amplification of the 16S rRNA gene can be particularly biased due to possible multiple operons for this gene. The use of degenerated primers carries a certain risk for unspecific amplification of nontarget DNA. To estimate the accuracy of our amplification, we checked every PCR product in 2% agarose gels where all PCR products gave bands of the expected size. Our melt curve analysis assumes that the intensity of individual peaks represents the initial proportion of the different butyryl-CoA:acetate CoA-transferase gene variants.

In conclusion, the quantification of the butyryl-CoA:acetate CoA-transferase gene may be a suitable biomarker for butyrate production for an individualized assessment of gastrointestinal health and microbiota function in addition to analysis of gastrointestinal microbiota.

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Faecalibacterium prausnitzii phylotypes in diabetic, obese and lean subjects

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Abstract

Faecalibacterium prausnitzii is one of the most common species and one of the main butyrate producers in the healthy human gut. Information on its genetic diversity and its role in the regulation of inflammation is unclear. Two genetic phylotypes could be differentiated previously.

F. prausnitzii phylotypes were examined in feces of obese patients without established insulin resistance, insulin dependent type 2 diabetics and a lean control group. The fecal samples were analyzed with methods distinguishing in GC- content and fragment length. *F. prausnitzii* genes of each group were cloned and sequenced.

The lean control group differs significantly from the diabetic group in results of qPCR ($p=0.02$), Butyryl-CoA:acetate CoA-transferase gene melt curve variant for *F. prausnitzii* ($p=0.03$), high resolution melt curve analysis (HRMA) ($p=0.006$), but not in automated fragment length polymorphism (ARISA). 22% of all sequenced clones match with phylogroup II and 78% with phylogroup I. Strains assigned to phylogroup I belong to the diabetic group.

These results add evidence to the hypothesis that phylotypes of *F. prausnitzii* differ in the regulation of inflammation. *F. prausnitzii* may furthermore protect obese from developing type II diabetes.

Introduction

Faecalibacterium prausnitzii (*F. prausnitzii*), is one of the most common species in the gastro intestinal tract (GIT) of adults consuming a western diet (Scott *et al.*, 2013). *F. prausnitzii* are more common in subjects with high intestinal gut microbiota gene content and (Le Chatelier *et al.*) lower concentrations have consistently been observed in patients with Crohn's disease or colorectal cancer. *F. prausnitzii* has been discussed for its anti-inflammatory activity, including IL-12, IL-10, and IFN- γ (interferon- γ) in the gut (Furet *et al.*, 2010) and as a marker for a high bacterial gene content and gut well-being (Remely *et al.*, 2013).

F. prausnitzii have the ability to switch between diet- and host derived substrates (Lopez-Siles *et al.*, 2012) and can convert sugars, dietary fibers, proteins and peptides to butyrate (Remely *et al.*, 2013) using mainly the butyryl-CoA:acetate CoA-transferase route. (Louis *et al.*, 2004).

Butyrate is considered to affect diet-induced obesity (Lin *et al.*, 2012) and chronic diseases. It provides additional energy (5-15 % of the total caloric intake) for gut epithelial cells, where the majority of butyrate is metabolized by the colonic epithelium and the remaining is most likely extracted by the liver. It has an anti-carcinogenic and anti-inflammatory potential (Hamer *et al.*, 2008) and plays a role in adipose tissue expansion (Cani & Delzenne, 2011). Concentrations in the venous systemic serum range from 0.5 to 3.3 μmol (Hamer *et al.*, 2008), where butyrate acts as signaling molecule (Brown *et al.*, 2003) via G-protein coupled receptors (GPRs). *F. prausnitzii* belongs to the *Clostridium leptum* cluster, which has often been associated with type II diabetes (Gerritsen *et al.*, 2011) and Karlsson *et al.* (2013) observed a negative correlation between *Clostridium leptum* and HbA1c values (Karlsson *et al.*, 2013).

Lopez-Siles *et al.* report that 97% of *F. prausnitzii* cluster in two phylogroups (Lopez-Siles *et al.*) The observed strains differed in substrate utilization, pH tolerance, and/or bile sensitivity, but phylogroup I and II strains did not reveal a systemic difference in healthy subjects (Lopez-Siles *et al.*). Individual abundance of *F. prausnitzii* correlates with environmental factors, suggesting that different nutrients shape different *F. prausnitzii* strains. Acetate is known to stimulate the growth of *F. prausnitzii* (Duncan *et al.*, 2002) and growth of different strains even be distinguished between apple and citron pectin substrates (Lopez-Siles *et al.*).

Recently it has been reported that *F. prausnitzii* is able to colonize the GIT of germ free rats after the colonization of the gut with *B. thetaiotaomicron* (Wrzosek *et al.*, 2013). *B. thetaiotaomicron* and *F. prausnitzii* are metabolically complementary. *B. thetaiotaomicron* may preshape the conditions in the GIT for the growth of *F. prausnitzii*. This could explain why *F. prausnitzii* only counts for 4% of total gut bacteria in children from rural Burkina Faso compared to 25% additional high *Bacteroides* counts in children with a western diet (De Filippo *et al.*, 2010).

F. prausnitzii has been suggested as a marker for gut health, this might however only be valid for western diet, where a *Bacteroides* overgrowth, the predominant proteolytic bacteria, is combined with an efficient butyrate producer to buffer the disadvantages of a high protein and low fiber diet (Wrzosek *et al.*, 2013).

F. prausnitzii phylotypes are analyzed in feces of obese patients without established insulin resistance, insulin dependent type two diabetics and a lean control group. Objectives of the study were the analysis of the two phylotypes and the investigation of a possible protective GIT effect. Can this effect be attributed to the butyric acid production?

Materials and Methods

Sample collection and DNA extraction

Stool samples were collected, immediately frozen and kept at -20°C. Samples were extracted using the QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol. DNA concentration and quality was determined with a Pico100 (Picodrop, UK) and agarose gel-electrophoresis.

Study subjects

Described in Table 1.

Type 2 diabetic group received an additional therapy with GLP-1-Agonists (Liraglutid).

(Table 2) Table 1: Characterization of study participants

	number	gender	age $\pm$ SD (kg/m ²)	BMI $\pm$ SD (kg/m ²)
Lean	18	♀ 15 ♂ 3	26 $\pm$ 3	21.2 $\pm$ 1.9
Diabetic	24	♀ 10 ♂ 14	58 $\pm$ 9	38.4 $\pm$ 5.1
Obese	26	♀ 22 ♂ 4	42 $\pm$ 15	43.9 $\pm$ 12.1

Cloning and PCR-DGGE

One sample of each group was amplified using the FaePr primerpair (forward 5'-cccttcagtgccgcagt-3' and reverse 5'-gtcgcaggatgtcaagac-3')(Rinttilä *et al.*, 2004) and amplicon was transferred in the Promega pGEM®-T Easy Vector System, leading to 112 total clones.

45 clones were amplified with the FaePrGC primerpair and loaded to a 50-55% acrylamide gel, runtime was 4.5h at 170V in a BIO Rad DGGE. 18 clones were sequenced (LCG Genomics) and taxonomically identified using the online tools of the ribosomal database project (<http://rdp.cme.msu.edu/>) and BLAST (NCBI).

qPCR

Abundance of *F. prausnitzii* was analyzed using the FaePr primer pair by Rinttilä *et al.* [14] with 10 µl total reaction volume, 4pmol/µl primer concentration, 10ng DNA, and the SYBRgreen SensiMix (Bioline) in an AB-Stepone Plus Real-Time PCR System.

Butyryl-CoA:acetate CoA-transferase gene

Was amplified and thermally denatured with SYBR Green qPCR analysis. The amplicons were divided into three areas, as described by Louis & Flint (2009). These peaks are assigned to represent bacteria related to *Eubacterium hallii* and *Anaerostipes coli* (82.5–85.0 1C), *Roseburia/E. rectale* spp. (85.5–89.0 1C) and *F. prausnitzii* (89.5–92.5 1C)(Louis & Flint, 2009).

Qiaxcel – fragment length polymorphism

The amplified 16s rDNA was directly transferred to the QIAxcel high-resolution capillary electrophoresis using the DNA High Resolution Kit (1200) Cartridge and analyzed, compared to size marker 15 to 600bp length. Expected fragment length for phylotyp I is 160-161bp and for phylotyp II 153-155bp.

High resolution melt curve

The obtained clones are used as standards. All samples are analyzed with the FaePr primer pair and melt curve limit is set between $81.1 \pm 0.5^\circ\text{C}$. Reaction volume is 10 μl , 4pmol/ μl primer concentration, 10ng DNA, and the MeltDoctor™ HRM Master Mix (Life Technologies) analyzed in an AB-Stepone Plus Real-Time PCR System with the High ReSolution Melt (HRM) Software v2.0 (Applied Biosystems®).

Statistical analysis

Data is analyzed as duplicates and Kolmogorov Smirnov test, Spearman Correlation and t-test, are calculated using Origin Pro 8 (<http://www.originlab.de/>).

Results

Diabetic group results differ significantly from the lean control group in copies ($p=0.02$), butyryl-CoA:acetate CoA-transferase gene melt curve variant for *F. prausnitzii* ($p=0.03$), HRM temperature ($p=0.0058$) and variance ($p=0.0068$); and from the obese group in butyryl-CoA:acetate CoA-transferase gene melt curve variant for *F. prausnitzii* ($p=0.03$), HRM temperature ($p=0.047$) and variance ($p=0.049$)(Fig. 1).

Correlation analyzes with the control group agrees for all methods. The results correlate between base pair lengths from Qiaxcel compared to total *F. prausnitzii* copies, Melt temperature and Melt variance significant ($R=-0.47$, $R=0.43$, $R=0.43$).

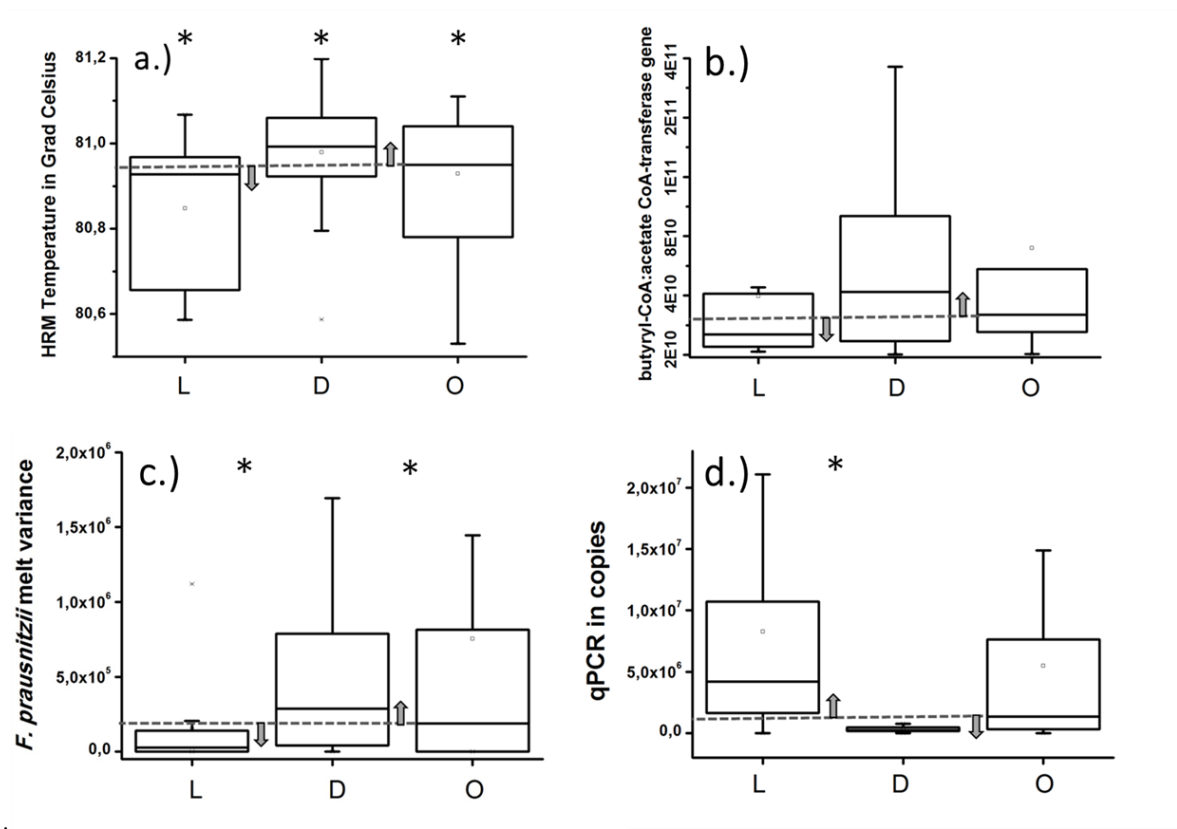


Figure 3

(Figure 3) Figure 1: Boxplot comparing Lean control-, Diabetic- and Obese groups for *F. prausnitzii* results with a.) HRM Temperatures in grad Celsius ($p=0.0058$, $p=0.047$); b.) butyryl-CoA:acetate CoA-transferase genes in copies; c.) butyryl-CoA:acetate CoA-transferase gene melt curve variant for *F. prausnitzii* in copies ($p=0.03$, $p=0.03$,) and d.) qPCR in copies ($p=0.02$).

Rectangular marks represent mean levels, medians are indicated with a line, whisker ranges between 5th and 95th percentiles and box limits between the 25th and 75th percentile.

HRM distinguishes in 4 different Variants

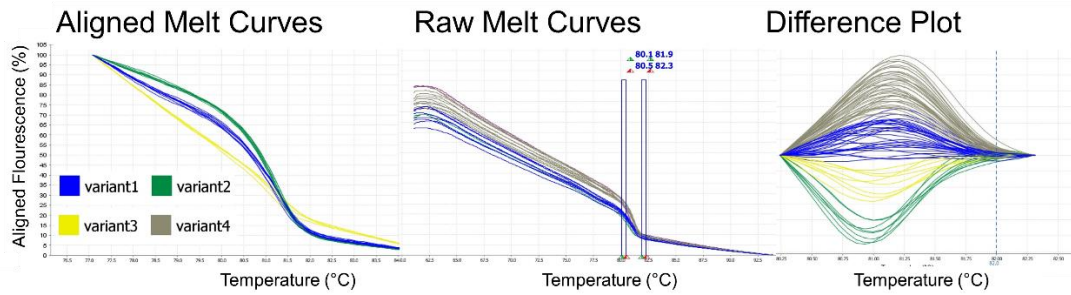


Figure 4

Figure 2: Difference Plot, Aligned- and Raw High Resolution Melt Curves of the 4 distinguishing variants of *F. prausnitzii* in lean control-, obese- and diabetic groups.

Butyryl-CoA:acetate CoA-transferase gene differs in *F. prausnitzii* melt variant depending to bodyweight

In all groups the melt curve quantity for *E.hallii*, *Roseburia*/ *E.rectale* and *F.prausnitzii* correlated positively with the total abundance of the butyryl-CoA:acetate CoA-transferase gene melt curve variants. In the diabetic group the butyryl-CoA:acetate CoA-transferase gene abundance correlated positively to HRM temperature ($R=0.56$, $p=0.004$).

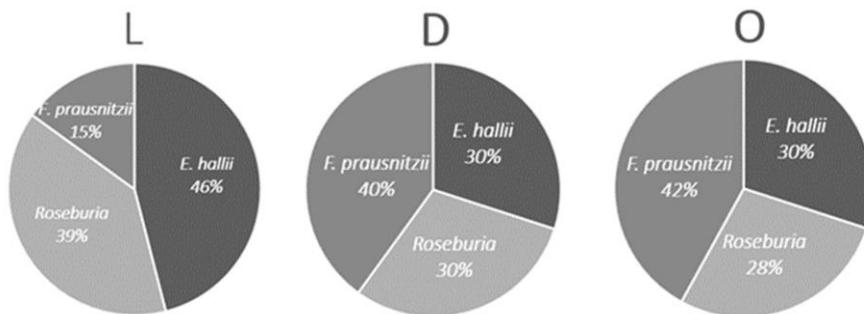


Figure 5

Figure 3: Relative distribution of butyryl-CoA:acetate CoA-transferase gene in lean control-, diabetic- and obese group according to their melt curves representing bacteria related to *F. prausnitzii*, *E. hallii* and *Roseburia*/ *E. rectale*.

Cloning and DGGE distinguishes in 2 Phylogroups:

Samples from the lean control group brought 43, from obese group 57 and from diabetic group 12 colonies. 14 of the sequenced clones align with 100% query cover and 100% identity to *F. prausnitzii* strain S4L/4 and ATCC 27768. Four of the clones cover with 100% identity with the strains L2-61, L2-15, HTF-75H, HTF-I, HTF-E, HTF-C, and HTF-A. These four different clones belong to the diabetic group.

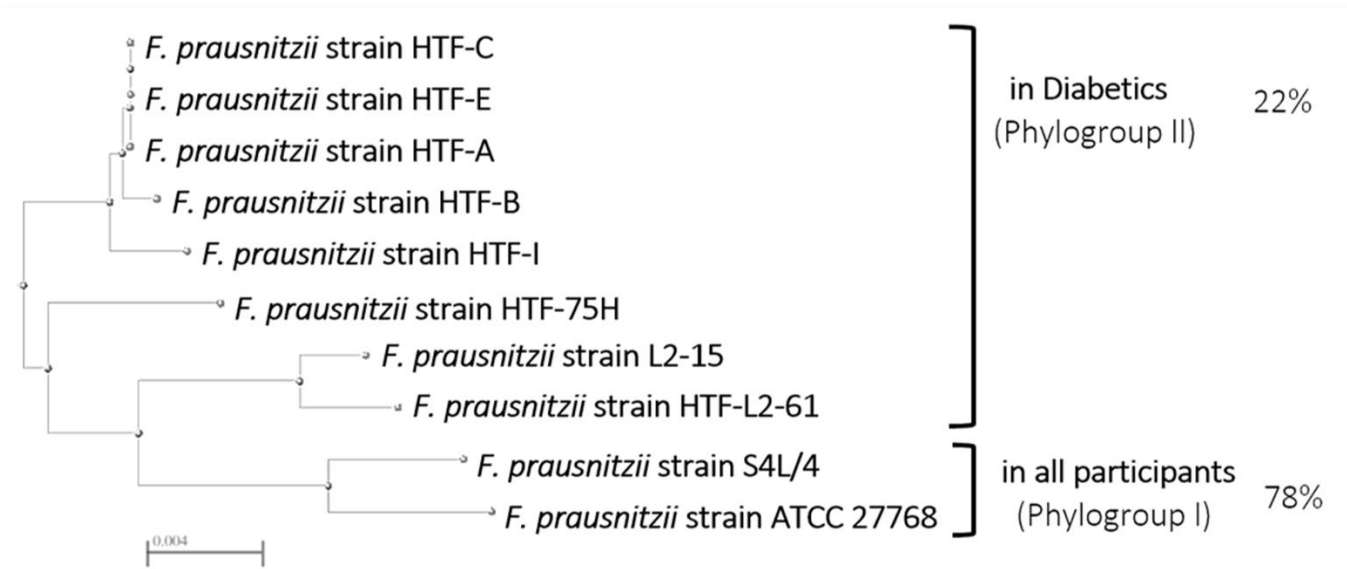


Figure 6

Figure 4: Neighbor-joining phylogenetic tree showing the 16S rRNA sequences matching with sequences from clones of obese, lean and diabetic individuals. 22% of sequenced clones match with phylogroup II, 78% match with phylogroup I.

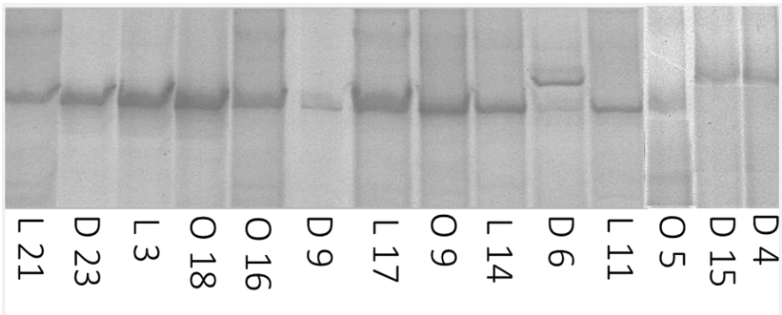


Figure 7

Figure 5: PCR-DGGE fingerprints from *F. prausnitzii* clones of obese, lean and diabetic individuals. Isolates are distributed in two separate bands that correlate with the Phylogroup designation as described by Lopez-Siles et al. 2012

Food Frequency Questionnaire (FFQ)

Analysis of the participant's FFQ did not reveal any significant differences in the consumption of grains, vegetables, fruits, and meat-, dietary- and fish products.

Differences were observed in the consumption of sweets, 40% of the diabetic group consumed less than once a week, 50% of the obese group consumed one to three times per week, and 28% of the lean individuals consumed sweets three to five times per week.

82% of the obese and 64% of the diabetic group never exercise, whereas 44% of the lean controls exercise 1-3 times per week.

HbA1C blood levels were determined at laboratory Health Center South in Vienna. The levels ranged between $7.5\% \pm 0.8\%$ in the type two diabetes group and $5.9\% \pm 0.9\%$ in the obese group.

Discussion

F. prausnitzii phylotypes are compared using different molecular methods, investigating differences between obese-, a diabetic-and a lean control group. The results of the diabetic group differ significantly from the lean group under most methods. Results for the obese group are in between as illustrated in Fig. 1.

Several studies describe the involvement of the gut microbiota in obesity-associated metabolic disorders, type two diabetes, metabolic endotoxemia and low-grade inflammation (Louis & Flint, 2007, Remely *et al.*, 2013). In our study the lean control group shows the highest abundance of *F. prausnitzii* copies, but the lowest butyryl-CoA:acetate CoA-transferase gene content. These results correspond to previous research where higher butyrate amounts are observed in overweight and obese participants compared to lean controls (Schwiertz *et al.*, 2010) as well as in the feces of obese mice (Turnbaugh *et al.*, 2006). The butyryl-CoA:acetate CoA-transferase gene melt variant amplicons were divided into three areas as described by Louis & Flint (2009). These peaks are assigned to represent bacteria related to *E. hallii* and *Anaerostipes coli*, *Roseburia/E. rectale* spp. and *F. prausnitzii*. The abundance of the butyryl-CoA:acetate CoA-transferase gene melt variant for *F. prausnitzii* is the least in the lean control group (Figure 3). The distribution of the melt variant of the main butyrate producers in obese and diabetic group is equal. *F. prausnitzii* contributes the minor butyrate amount in lean controls (15%), compared to the obese and diabetic groups with up to 42%. Butyrate, in particular, has an important impact on promoting body energy expenditure via modulation of mitochondrial activity (Remely *et al.*, 2013) and is an additional energy source promoting adiposity (Layden *et al.*, Scott *et al.*, 2013). In contrast, high-fat diets supplemented with butyrate prevent and even reverse insulin resistance in obese mice (Pegorier *et al.*, 2004, Samuel *et al.*, 2008). High butyrate levels in humans stimulate GLP-1 secretion and therefore increases of insulin, inhibits gastric emptying and increases feeling of satiety (Wichmann *et al.*, 2013). Due to the

inhibition of gastric emptying, butyrate is maybe excreted more slowly and accumulates, influencing the anti-inflammatory potential, pH and oxidative stress. The observed high *F. prausnitzii* levels compared to low butyrate levels in lean controls could also origin from different *F. prausnitzii* strains. Possibly the different strains have other anti-inflammatory potential and with that a protective effect in obese to develop diabetes type two. (Remely *et al.*, 2013) reported a lower proportion of inflammatory markers and *F. prausnitzii* in type two diabetic subjects indicating a higher incidence of low-grade inflammation (Furet *et al.*, 2010).

Strains belonging to pylogroup II were detected only in the diabetic group (Fig. 4 and 5). Although Lopez-Siles *et al.* report a tendency for sequences to group individually; we find strains from Phylogroup I in all cloned samples (Lopez-Siles *et al.*).

Surprisingly, all investigated groups show a comparable nutritional behavior, consuming the same amounts of grains, vegetables, fruits, and meat-, dietary- and fish products. Possibly the FFQ could not identify minor differences. The questionnaire used in the present study is carefully adapted from the EPIC study; multiple related questions are used to validate responses (Kroke *et al.*, 1999). Health experts interviewed every participant personally in a face-to-face meeting. However the limitation of FFQ for palliating answers is discussed widely. Maybe the comparable nutritional behaviors of the analyzed groups are not responsible for differences of *F. prausnitzii* abundance. Also Walker *et al.* 2012 (Walker *et al.*, 2012) demonstrated a limited response of *F. prausnitzii* to change of diet. Gender balance may play a role in studying *F. prausnitzii* and disease (Feng *et al.*, 2013) but Mueller *et al.* (2006) did not detect gender difference in the fecal *F. prausnitzii* amount of healthy adults (Mueller *et al.*, 2006). We detect no gender difference, even if only the diabetic group is gender balanced.

Fecal butyrate levels depend to shifts in microbial cross feeding patterns, mucosal absorption and different transit times. Therefore the butyryl-CoA:acetate CoA-transferase gene is used as a marker for butyrate production as the direct analysis in feces is hampered by rapid binding and degradation of butyrate in the gut (Schwartz *et al.*, 2010) (Duncan *et al.*, 2007). Assessing the syntheses of butyrate following the butyryl-CoA:acetate CoA-transferase gene pathway, discriminates bacteria using other described pathways (butyrate kinase, phosphotransbutyrylase) (Louis *et al.*, 2004). The expression of the butyryl-CoA:acetate CoA-transferase genes is not analyzed and will be a focus for further studies.

The results of the different methods comparing GC content or fragment length correlate significant.

Our results encourage the further analysis of *F. prausnitzii* and phylotypes in studying low grade inflammation and obesity-associated metabolic disorders. High resolution melt curve analysis may even be a promising tool for routine and research use.

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Characterization of Bacteria, *Clostridia* and *Bacteroides* in Faeces of Vegetarians

Using qPCR and PCR-DGGE Fingerprinting

Kathrin Liszt, Jutta Zwielehner, Michael Handschur, Berit Hippe, Roman Thaler, Alexander G.

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Abstract

Background/Aims: This study aimed to investigate the quantitative and qualitative changes of bacteria, *Bacteroides*, *Bifidobacterium* and *Clostridium* cluster IV in faecal microbiota associated with a vegetarian diet. **Methods:** Bacterial abundances were measured in faecal samples of 15 vegetarians and 14 omnivores using quantitative PCR. Diversity was assessed with PCR-DGGE fingerprinting, principal component analysis (PCA) and Shannon diversity index. **Results:** Vegetarians had a 12% higher abundance of bacterial DNA than omnivores, a tendency for less *Clostridium* cluster IV ($31.86 \pm 17.00\%$; $36.64 \pm 14.22\%$) and higher abundance of *Bacteroides* ($23.93 \pm 10.35\%$; $21.26 \pm 8.05\%$), which were not significant due to high interindividual variations. PCA suggested a grouping of bacteria and members of *Clostridium* cluster IV. Two bands appeared significantly more frequently in omnivores than in vegetarians ($p = 0.005$ and $p = 0.022$). One was identified as *Faecalibacterium* sp. and the other was 97.9% similar to the uncultured gut bacterium DQ793301. **Conclusions:** A vegetarian diet affects the intestinal microbiota, especially by decreasing the amount and changing the diversity of *Clostridium* cluster IV. It remains to be determined how these shifts might affect the host metabolism and disease risks.

Background/Aims: This study aimed to investigate the quantitative and qualitative changes of bacteria, *Bacteroides*, *Bifidobacterium* and *Clostridium* cluster IV in faecal microbiota associated with a vegetarian diet. **Methods:** Bacterial abundances were measured in faecal samples of 15 vegetarians and 14 omnivores using quantitative PCR. Diversity was assessed with PCR-DGGE fingerprinting, principal component analysis (PCA) and Shannon diversity index. **Results:** Vegetarians had a 12% higher abundance of bacterial DNA than omnivores, a tendency for less *Clostridium* cluster IV ($31.86 \pm 17.00\%$; $36.64 \pm 14.22\%$) and higher abundance of *Bacteroides* ($23.93 \pm 10.35\%$; $21.26 \pm 8.05\%$), which were not significant due to high interindividual variations. PCA suggested a grouping of bacteria and members of *Clostridium* cluster IV. Two bands appeared significantly more frequently in omnivores than in vegetarians ($p = 0.005$ and $p = 0.022$). One was identified as *Faecalibacterium* sp. and the other was 97.9% similar to the uncultured gut bacterium DQ793301. **Conclusions:** A vegetarian diet affects the intestinal microbiota, especially by decreasing the amount and changing the diversity of *Clostridium* cluster IV. It remains to be determined how these shifts might affect the host metabolism and disease risks.

Introduction

Vegetarianism has been associated with a decreased risk for diseases such as heart diseases and various cancers and has been linked to a lower BMI and an overall decline in mortality [1]. Different lifestyle factors and diets were shown to have a significant impact on the faecal microbiota [2, 3]. The highly diverse microbiota of the human gastrointestinal tract has been associated with colorectal cancer [4], inflammatory bowel diseases [5], obesity [6] and metabolic syndrome [7]. Two of the most predominant subpopulations in the human faecal microbiota are the *Clostridium* cluster IV (*Clostridium leptum* subgroup) and *Bacteroides* [8, 9]. Some members of these populations are fibrolytic and produce butyrate [10, 11]. The metabolic activities of these organisms have a significant influence on colonic health as butyrate is known as the major energy source for colonocytes [10] and as a possible epigenetic regulator of gene expression in colonic epithelium [12]. Epigenetic modifications have also been implicated in changes of gene expression associated with a vegetarian diet [13, 14]. Stimulation of Bifidobacterium has previously been shown after prebiotic intervention with inulin and fructo- oligosaccharides [15]. Bifidobacterium has been shown to be involved in the prevention of atopic disease [16] and the prevention of obesity and insulin resistance via enhanced barrier function of the gut epithelium [17]. The previous molecular analysis of vegetarian gut microbiota of a single individual revealed that Clostridium cluster XIVa, Clostridium cluster IV, and Clostridium cluster XVIII were major components [18]. In the present study, we applied quantitative PCR (qPCR) and PCR-DGGE fingerprinting to investigate the dominant microbiota in 14 young omnivores and 15 vegetarians assessing *Clostridium* cluster IV, *Bacteroides* and *Bifidobacterium*. Analyses of faecal samples have been shown to reflect adequate important changes [19] .

Material and Methods

Participants

Twenty-nine healthy young individuals, 15 vegetarians (aged 19-34 years, BMI 22.06 ± 3.82) and 14 omnivores (aged 21-31 years, BMI 21.02 ± 2.71), were compared. A questionnaire about dietary habits and health activities was given to all participants. Exclusion criteria were the use of antibiotics, chemotherapeutic treatment, and pre- and probiotics 3 months prior to sampling.

Sampling and DNA Extraction

Stool samples were immediately stored at -20°C after sampling. DNA was extracted using the DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol with minor modifications and immediately stored at -20°C .

TaqMan qPCR

Bacterial 16S rRNA was quantified by TaqMan qPCR using previously published primers and probes [20–23]. TaqMan Probe Clept-P (AGG GTT GCG CTC GTT) was designed for *Clostridium cluster IV* in this study. DNA of *Bacteroides thetaiotaomicron* and *Bifidobacterium longum*, clone CL16 and one faecal sample were used to generate standard curves for comparison of PCR reaction efficiencies among different experiments and enumeration of all bacterial groups. Relative abundances of bacterial subgroups were calculated in relation to total 16S rRNA gene copies for each individual.

PCR-DGGE Fingerprinting

16S rRNA coding regions were amplified using group-specific primers [17, 24–29]. Reference markers containing fragments of 16S rRNA coding regions were loaded to each gel in triplicate to allow gel-to-gel comparison. DGGE gels were prepared as described previously [30].

Statistical Analysis

We analyzed qPCR results by F test and Student's t test. Food frequency data and interesting bands of DGGE fingerprints were analyzed based on 2 approximation as implemented in SPSS15.

PCR-DGGE band comparison tables were created in GelComparII and analyzed with principal component analysis (PCA). The plots show the transformed data with the first two principal components as x- and y-axis

Results

Dietary Aspects

Analysis of the participants' dietary habits indicated similar consumption patterns of liquids, alcohol, fruits, grains and milk products in both groups. Exercise levels were comparable. Omnivores stated significantly less frequent (2 test; $p = 0.027$) consumption of vegetables than vegetarians. Three of the vegetarians assessed followed a vegan diet; all others followed a lacto-ovo vegetarian diet.

Five vegetarians said that they eat fish a few times a year.

Relative Quantitation

Bifidobacterium, members of *Clostridium cluster IV* and *Bacteroides* were quantified as percentage of the total bacterial DNA. No cross-reactivity of group-specific primers and probes with nontarget strains could be detected. Test-retest variations were between 2.7 and 5.2%; values after relative quantification varied for less than 4%. Vegetarians showed 12% higher counts of bacterial DNA than omnivores but these differences were not significant due to high interindividual deviations. The mean proportion of *Clostridium cluster IV* in stool samples of vegetarians was $31.86 \pm 17.00\%$ and in

omnivores $36.64 \pm 14.22\%$. The mean percentage of *Bacteroides* in vegetarians was $23.93 \pm 10.35\%$ and in omnivores $21.26 \pm 8.05\%$, while the mean proportion of *Bifidobacterium* in vegetarians ($1.52 \pm 1.29\%$) was unchanged compared to omnivores ($1.59 \pm 1.73\%$). PCR-DGGE Quantitative Analysis. The highly diverse datasets of all bacterial groups were subjected to PCA, which extracts underlying components within the dataset, separating samples according to their variance. This procedure resulted in a separation of omnivores and vegetarians according to their *Clostridium* cluster IV fingerprint. Some grouping was also visible in the dominant bacteria dataset (fig. 1). The mean numbers of bands observed with a primer pair (341–518) targeting most bacteria were 20.1 ± 3.3 for omnivores and 18.07 ± 3.7 for vegetarians. The fingerprints of bacterial subgroups were similar for all participants. Band patterns of *Bacteroides* were composed of 9.5 ± 2.9 bands, *Clostridium* cluster IV of 12.75 ± 3.37 bands and 13.2 ± 3.1 bands related to *Bifidobacterium* spp. Shannon diversity indices based on the DGGE fingerprinting were similar for all bacterial groups. However, two bands (fig. 2) from *Clostridium* cluster IV were more prevalent in omnivores than in vegetarians (2 test; $p = 0.005$; $p = 0.022$).

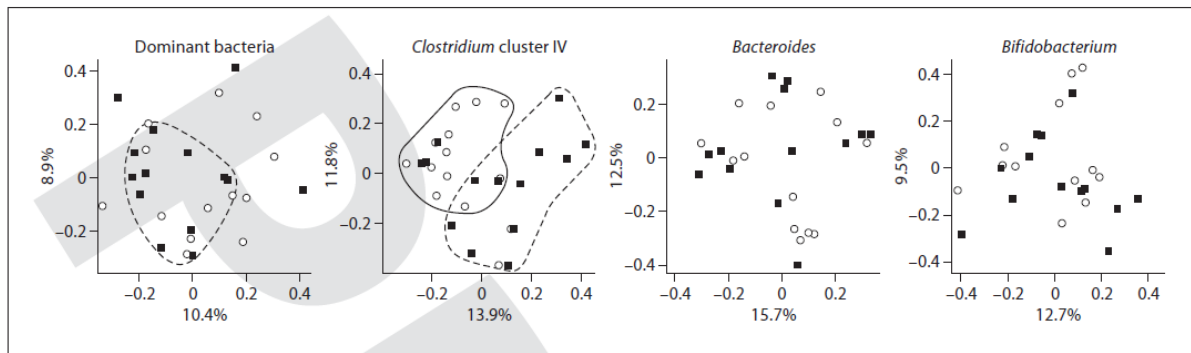


Figure 8

Fig. 1. PCA of DGGE fingerprints of 16S rRNA coding regions of dominant bacteria, *Clostridium* cluster IV,

Bacteroides and *Bifidobacterium* in faecal samples. y = Omnivores; + = vegetarians.

Discussion

We found 12% higher counts of bacterial DNA in intestinal microbiota of vegetarians than in omnivores. A decreased transit time of stool correlating with higher bacterial cell mass due to higher dietary fibre intake [31, 32] as well as prebiotic consequences might contribute to this observation.

We could also see a tendency for higher abundance of *Bacteroides* and lower abundance of *Clostridium* cluster IV in faecal microbiota of vegetarians compared to omnivores. *Bacteroides* can utilize a wide variety of carbon sources, and they account for the majority of polysaccharide digestion in the large intestine [33, 34]. Nevertheless, members of *Clostridium* cluster IV have the same ability.

We assume that dietary fibre encourages the growth of *Bacteroides*. *Clostridium cluster IV* and *Bacteroides* also play an important role in the hydrolysis and fermentation of endogenous mucins and probably dietary protein, as well as in the conversion of bile acids and the production of toxins [34, 35]. In a cross-sectional study of different European populations, Mueller et al. [36] detected the highest levels of the *Clostridium cluster IV* in the Swedish study population, whose dietary habits were characterized by a high consumption of fish and meat. Therefore, we suppose that higher meat consumptions may increase the abundance of the *Clostridium cluster IV* in the gut microbiota. A higher prevalence of this subgroup was discussed to be associated with obesity and cancer [4, 6].

A previous study [18] involving only 1 patient discussed whether *Clostridium cluster XIVa* was affected by a vegetarian diet. We could not find noticeable differences for this subgroup in our samples (data not shown). PCR-DGGE fingerprinting of dominant bacteria and *Clostridium cluster IV* indicated a grouping of vegetarians and omnivores in our study. Clustering of *Clostridium cluster IV* fingerprints might be due to the observation that two sequences identified as *Faecalibacterium sp.* and *Ruminococcaceae sp.* were more prevalent in omnivores than in vegetarians, probably due to the effects of higher meat consumption as discussed for *Clostridium cluster IV* in general. Despite enormous interindividual variations, we observed a tendency for smaller abundances of *Clostridium cluster IV* in vegetarians. This might suggest a smaller capacity for energy gain from food in vegetarians. Higher - abundances of fibrolytic *Bacteroides* were found and might compensate for reductions in *Clostridium cluster IV*. This shift in these nutritionally important bacterial subgroups might account for the distinct grouping of omnivores and vegetarians in PCA of dominant bacterial fingerprints. In summary, it remains to be determined whether these shifts result in differential metabolite profiles that might in turn affect host metabolism and disease risks.

Acknowledgments

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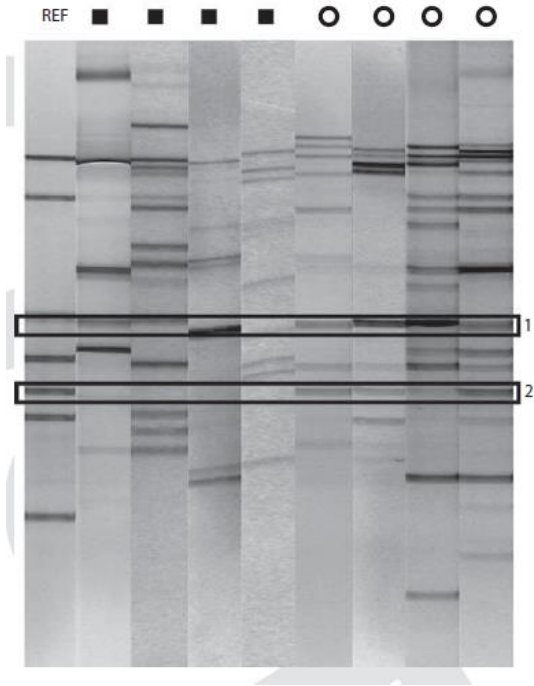


Figure 9

Fig. 2. PCR-DGGE band patterns of 16S rRNA coding regions of *Clostridium cluster IV* amplified with primer pair sg-Clept-F-GC/ sg-Clept-R. Bands that were observed more frequently in omnivores than in vegetarians are indicated with a black box. 1 = *Clostridium* sp. BI-114 (similarity 94.7%) and uncultured bacterium DQ793301 (similarity 97.9%); 2 = 96.7% similar to *Faecalibacterium prausnitzii* and 99.5% similar to *F. prausnitzii* AJ270469. γ = Omnivores; + = vegetarians; REF = reference lane.

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Effects of antibiotic therapy on the gastrointestinal microbiota and the influence of *Lactobacillus casei*

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Abstract

Effects of intervention with *Lactobacillus casei* Shirota (LcS) on the incidence of antibiotic-associated diarrhoea (AAD), *Clostridium difficile* infection (CDI) and changes in faecal microbiota were analysed using *C. difficile* ELISA (678 patients), qPCR using 16S rRNA group-specific primers, *C. difficile*-toxin kit and polymerase chain reaction/denaturing gradient gel electrophoresis (56 patients).

As much as 18.5% of antibiotic treated group developed AAD, but only 5% of patients treated with antibiotics and LcS. Following antibiotic therapy, a decrease in the abundance of total Bacteria, *Clostridium* cluster IV and XI, *Bifidobacterium* spp. and butyryl-CoA CoA transferase genes was observed, whereas Enterobacteriaceae increased. LcS intervention reduced the antibiotic-associated decrease in the diversity of microbiota, increased the abundance of *Lactobacillus* spp. and reduced the antibiotic-induced decrease of *Bifidobacterium* spp.

Antibiotic treatment affects the diversity and the composition of the microbiota impairing butyrate production. Intervention with certain *Lactobacillus* strains may antagonise some of these changes, and more potent short-chain fatty acid-stimulating probiotics are desirable for intervention in AAD.

Introduction

The human gut contains approximately 10–100 trillion organisms (Zhu, Wang, & Li, 2010), including all three domains of life: Bacteria, Archaea and Eukarya (Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005); recently three functional subtypes ('enterotypes') have been described (Arumugam et al., 2011). The gastrointestinal microbiota is dominated by two divisions of Bacteria: Bacteroidetes and Firmicutes (Backhed et al., 2005; Wang, Heazlewood, Krause, & Florin, 2003). Ninety-five per cent of the Firmicutes belong to the *Clostridium*, while large variations can be observed among the *Bacteroidetes* phylotypes (Eckburg et al., 2005).

A high microbial diversity supports a stable gut ecosystem, providing the host with protection against pathogens (Backhed et al., 2005). This stability can be influenced by many factors including intestinal pH, microbial interactions, environmental temperature, physiologic factors, peristalsis, bile acids, host secretions, immune responses, drug therapy and bacterial mucosal receptors (Thompson-Chagoyan, Maldonado, & Gil, 2007). If this ecosystem is disturbed and there is a reduction in the abundance or group specific shifts, pathogens are more able to colonise and cause harm.

Antibiotic treatment is one factor that allows pathogens to proliferate, and antibiotics can result in both short- and long-term effects on the human gut microbiota. Bacterial diversity may decrease, the relative proportions of different species can change, new species can be introduced and/or existing species completely eradicated (Dethlefsen & Relman 2011; Sommer & Dantas, 2011). Proteobacteria, for example, which are normally low in abundance, are increased at the expense of the normally dominant *Firmicutes* and *Bacteroidetes*. There is also continuing concern about the increasing rise in antibiotic resistance (Sommer & Dantas, 2011), all of which makes it important that antibiotic-associated changes to the intestinal microbiota are characterised and to find ways to resist these changes and achieve a rapid return to the original composition (De La Cochetiere et al., 2005).

Antibiotic-associated diarrhoea (AAD) is a common complication of antibiotic use. Nearly all types of antibiotics are reported to induce AAD, but those with a spectrum of activity including anaerobic bacteria have been particularly associated with higher rates of AAD (Breves et al., 2000). Clinically mild AAD is mostly caused by the toxic effects of the antibiotics themselves on the intestinal mucosa, or their pharmacological effects on gut motility, functional disturbances of intestinal bile acid or carbohydrate metabolism (Hogenauer, Hammer, Krejs, & Reisinger, 1998), accompanied by a decrease in the microbial metabolism of complex carbohydrates to short-chain fatty acids (SCFAs). SCFAs are essential for sodium and water uptake (Breves et al., 2000), as well as having a role as important energy sources for intestinal epithelial cells (Hamer et al., 2008), and anti-inflammatory, epigenetic and genetic activities (Canani et al., 2011). More severe AAD-causing complications include electrolyte imbalances, dehydration, pseudomembranous colitis, toxic megacolon or death. These complications can be caused by infection with *Clostridium difficile* (a member of *Clostridium* cluster XI), or other infectious agents including *C. perfringens*, *Staphylococcus aureus*, *Klebsiella oxytoca*, *Candida* species and *Salmonella* species (Breves et al., 2000; Hogenauer et al., 1998).

Intestinal overgrowth with *C. difficile* is a particularly dangerous complication of antibiotic intake. The pathogen causes severe diarrhoea and pseudomembranous colitis, entities associated with outbreaks having a high rate of mortality for hospitalised patients (Thompson-Chagoyan et al., 2007). This anaerobic spore-forming bacterium is believed to be responsible for 5–20% of all cases of AAD (Hogenauer et al., 1998), for virtually all cases of pseudomembranous colitis (Bartlett, 1987), and a frequent cause of outbreaks (Arumugam et al., 2011; Hensgens et al., 2011; McFarland, Beneda, Clarridge, & Raugi, 2007). *Clostridium difficile* releases at least two potent toxins that cause mucosal damage and inflammation of the colon (Hogenauer et al., 1998).

The almost complete inhibition of butyrate fermentation may be an important pathophysiological factor for the development of AAD, because butyrate has an essential role in ensuring the morphological and functional integrity of colonocytes (Breves et al., 2000). The effect of decreasing SCFA production could be compensated by probiotic intervention. This has been shown with *Saccharomyces boulardii* although the yeast restored acetate and propionate fermentation but not butyrate (Breves et al., 2000).

Saccharomyces boulardii has the strongest evidence with regard to reducing incidence of AAD and/or *C. difficile*-associated diarrhoea (McFarland, 2010) but evidence is also available for probiotic bacteria

including *Lactobacillus rhamnosus* GG (McFarland, 2006), *L. acidophilus* CL1285 and *L. casei* LBC80R (Gao, Mubasher, Fang, Reifer, & Miller, 2010), *L. rhamnosus* GG, *L. acidophilus* La-5 and *Bifidobacterium* Bb-12 (Wenus et al., 2008), *L. casei* DN-114 001 with the yogurt cultures *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Hickson et al., 2007) and *L. casei* Shirota (Lewis, Thomas, & Weir, 2009; Martinez, Paz, & O'Donnell, 2003). The safety of *Lactobacillus casei* Shirota (LcS) has been confirmed in several studies (Snydman, 2008; Whelan & Myers, 2010).

The rationale for combining a probiotic with antibiotic therapy is that the antibiotic kills any vegetative *C. difficile* cells in the intestine and eliminates their toxigenic effect, the probiotic helps to re-establish the protective intestinal microbiota so that when any residual spores germinate, their outgrowth and colonisation is prevented by the newly restored microbiota barrier (McFarland, 2006).

In the present study, the effects of using a probiotic with antibiotic therapy on the incidence of AAD and *C. difficile* infection (CDI) were investigated in a group of 678 hospitalised patients receiving antibiotics. The probiotic was a fermented milk drink containing LcS administered during and for three days after a course of antibiotics. Changes in faecal microbiota were analysed in a subgroup of 56 subjects. The results suggest an important role of LcS in antagonising antibiotic-induced changes in the intestinal microbiota with regard to its abundance, diversity and SCFA-producing capacity.

Material and methods

Subjects

The study was performed at the department of Internal Medicine of the General Hospital Oberpullendorf (Austria). Ethical clearance was given by the local ethical committee (Ethikkommission Burgenland). For the clinical study, patients given antibiotics were divided into two cohorts. Group AP (antibiotic + probiotic) comprised 340 patients (182 females and 158 males with a mean age of 71 years) fulfilling the following criteria: (1) they received a daily probiotic drink containing the probiotic strain LcS (one 65 ml bottle containing a minimum of 6.5×10^9 live cells of LcS) whilst taking antibiotics and for 3 days after the antibiotic treatment ceased; (2) they were treated with appropriate antibiotics administered either by oral or parenteral route according to their clinical indication (penicillins, cephalosporins, quinolones, clindamycin and vancomycin); (3) they were on a ward where all patients and the staff received probiotic (regardless of antibiotic therapy). Evaluation of compliance showed that at least 80% of the patients and 95% of the staff ingested the probiotic drink on a daily basis. Group A (on antibiotics but not given probiotic) comprised 338 patients (172 females and 166 males with a mean age of 69 years). The following exclusion criteria were defined: (1) diarrhoea on admission or within the previous week of the admission; (2) intake of high-risk antibiotics within one month before admission to the hospital (cephalosporins, clindamycin, aminopenicillin and quinolones were predefined as high-risk antibiotics); (3) recurrent diarrhoea or chronic intestinal diseases associated with diarrhoea; (4) known re-current CDI. The patient groups

did not differ significantly in terms of demographic characteristics or severity of disease. Of note, both groups received similar antibiotic regimens and there was no change in the infection prevention policy over the trial period. All subjects agreed to participate in the study and gave their informed consent.

Faecal samples were taken for further laboratory study from the sub-set of 56 subjects (aged 60±22 years), which were divided into four groups (Table 1). Patients of groups A and AP had been treated with antibiotics; patients of groups AP and P had received a daily fermented milk drink (Yakult®) containing the probiotic strain *L. casei* Shirota. The control group © consisted of healthy adults receiving neither antibiotics nor probiotics.

(Table 2) Table 1. Characterisation of participant groups.

Table 3

Group Treatment and/or intervention

AP	Antibiotic treatment and intake of a probiotic drink containing <i>Lactobacillus casei</i> Shirota
A	Antibiotic treatment only
P	Intake of a probiotic drink containing <i>L. casei</i> Shirota
C	Control group

Clinical endpoints

Primary endpoint: The occurrence of diarrhoea, defined as more than two abnormally loose bowel movements (i.e. unformed or liquid stools that were strictly defined as stool taking up the shape of the container and confirmed as such by a nurse) per day for the duration of three or more days.

Secondary endpoint: The incidence of CDI, defined as the onset of diarrhoea (as described above) plus detection of toxin A or toxin B in stool samples by immunoassay (TOX A/B QUICK CHEK®, Wampole®).

Sampling and DNA extraction from stool

Faecal samples were taken at three time points within the course of a week: before antibiotic treatment and/or intake of probiotic (day 0) and two times during antibiotic treatment and/or intake of probiotic (after 3 and 5 days). Samples of the control group were taken at similar time points. All samples were immediately stored at -70°C.

For DNA extraction, 200 mg of frozen stool sample was treated twice for 45 s in a bead-beater (Mini-Beadbeater-8) with one intervention minute on ice. The DNA was then extracted using QIAamp DNA Stool Mini Kit (QUIAGEN) following the manufacturers' protocol and immediately stored at -20°C.

Quantification of total bacteria, bacterial subgroups and detection of butyryl-CoA CoA transferase genes by q-PCR

For TaqMan q-PCR of total bacteria, *Clostridium* cluster IV and XIVa, all *Bacteroides* and *Bifidobacterium* spp., a reaction mix with the total volume of 10 µL, 5 µL SensiMix™ Probe Kit (TaqMan®) (3 mM MgCl₂), 1 µL of each primer, 1 µL of TaqMan®-probe (Table 2) and 10 ng template were used. For SYBR Green Real Time PCR of *Lactobacillus* spp., Enterobacteriaceae (4 mM MgCl₂) and *Clostridium* cluster XI, a reaction mix with the total volume of 10 µL, 5µL SensiMix™ SYBR No-ROX Kit (SYBR® Green) (3 mM MgCl₂), 1 µL of each primer (Table 3) and 10 ng template were used. Assays were carried out in a StepOnePlus™ Instrument (96 wells) using Real-Time PCR System Version 2.1 (Applied Biosystems).

Table 2. Primers and TaqMan®-probes targeting 16rRNA coding regions of bacteria

Table 4

Target organism	Primer/probe	Sequence (5'–3')	Size (bp)	Conc. [pmol/µL]	References
All bacteria	BAC-338-F	ACT CCT ACG GGA GGC AG	468	10	Yu, Lee, Kim, and Hwang (2005)
	BAC-805-R	GAC TAC CAG GGT ATC TAA TCC		10	
	BAC-516-P	(6-FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		2	
<i>Clostridium</i> cluster IV (<i>Clostridium leptum</i>)	sg-Clept-F	GCA CAA GCA GTG GAG T	239	4	Matsuki, Watanabe, Fujimoto, Takada, and Tanaka (2004)
	sg-Clept-R	CTT CCT CCG TTT TGT CAA		4	
	Clept-Pa	(FAM)-AGG GTT GCG CTC GTT- (BHQ-1)		2	
All bacteroides	AllBac296f	GAG AGG AAG GTC CCC CAC	106	3	Layton et al. (2006)
	AllBac412r	CGC TAC TTG GCT GGT TCA G		3	
	AllBac375Bhqr	(6-FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)		1	
<i>Bifidobacterium</i> spp.	Fwd primer	GCG TGC TTA ACA CAT GCA AGT C	125	3	Penders et al. (2005)
	Rev primer	CAC CCG TTT CCA GGA GCT ATT		3	
	Probe	(6-FAM)-TCA CGC ATT ACT CAC CCG TTC GCC-(BHQ-1)		1.5	
<i>Clostridium</i> cluster XIVa (Lachnos-piraceae)	195-F	GCA GTG GGG AAT ATT GCA	538	7	Meier, Amann, Ludwig, and Schleifer (1999)
	Ccocc-R	CTT TGA GTT TCA TTC TTG CGA A		7	

Target organism	Primer/probe	Sequence (5'–3')	Size (bp)	Conc. [pmol/μL]	References
	Ccocc-P	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)		1.5	

Table 3. Primers targeting 16rRNA coding regions of bacteria (SYBR® Green) and butyryl-CoA CoA transferase genes.

Table 5

Target organism	Primer	Sequence (5'–3')	Size (bp)	Conc. [pmol/μL]	References
<i>Clostridium</i> cluster XI	C Cluster XI F	ACG CTA CTT GAG GAG GA	180	3	Song, Liu, and Finegold (2004)
	C Cluster XI R	GAG CCG TAG CCT TTC ACT			
Enterobacteriaceae	LPW69	AGC ACC GGC TAA CTC CGT	492–509	3	Woo, Leung, Leung, & Yuen (2000)
	pB-00608 r	GAA GCC ACG CCT CAA GGG CAC AA	834–856	3	Ootsubo et al. (2002)
<i>Lactobacillus</i> spp.	Lac1	AGC AGT SGG GAA TCT TCC A	352–700	4	Walter et al. (2001)
	Lac2	ATT YCA CCG CTA CAC ATG		4	

For comparison of PCR reaction efficiencies among different experiments, one faecal sample and tenfold series of DNA dilutions of following type strains were used: *Clostridium leptum* DSM 753, *Clostridium blautia*, *Bifidobacterium longum* DSM 20211, *L. casei*, *Escherichia coli* 1029 and a bacterial mix containing *C. leptum* DSM 735, *C. blautia*, *C. difficile* 301968DNA, *S. aureus*, *Enterococcus faecium* DSM 20477, *Listeria monocytogenes*, *L. casei*, *Bacteroides thetaiotaomicron* DSM 2079, *E. coli* 1029, *Citrobacter caseri*, *Enterobacter cloacae* 1252, *Salmonella typhimurium* SL7207, *B. longum* subsp. *suis* DSM 20211.

Standard curves were created using serial dilutions of known concentrations of organisms containing the respective amplicons for each set of primers, and quantification was done. All templates were determined in duplicate, and the average used for calculation.

Euroclone® *C. difficile* A/B kit was used for specific *C. difficile* toxin determination, following the user manual.

Butyryl-CoA CoA transferase genes were detected as described previously (Hippe et al., 2011; Louis & Flint, 2007).

PCR/DGGE

Polymerase chain reaction (PCR) was used to amplify 16S rRNA gene sequences from total bacteria in faecal samples and type strains for further use in denaturing gradient gel electrophoresis (DGGE) analysis.

Assays were carried out in a 96-well Gradient Thermal Cycler (Labnet MultiGene™), using a ready-to-use GoTaq® Green Master Mix (Promega) with 1.5 mM MgCl₂ and the specific primer sets 341f-GC 5'-CCT ACG GGA GGC AGC AG-3' (Muyzer, de Waal, & Uitterlinden, 1993) and 518r 5'-ATT ACC GCG GCT GCT GG-3' (Neefs, Van De Peer, De Rijk, Goris, & De Wachter, 1991). The DGGE gel was prepared as described previously (Muyzer & Smalla, 1998) with a linear gradient of 25–65%, using a gradient mixer (Hoefer SG 30) and a peristaltic pump. A reference marker was generated, containing fragments of 16S rRNA genes from cultured bacteria and clones generated from faecal material: *Bac. thetaiotaomicon*, *Ent. faecium*, *C. leptum* 16, *E. coli*, *C. coccoides* 43, *L. reuteri* and *B. longum*.

Statistical analysis

Fisher's exact test was performed to compare the rates of AAD and CDI. Faecal analysis data were statistically analysed using the program OriginPro 8 (OriginLab®).

To compare two unpaired groups of interval values the parametric two-sample *t*-test, and of interval values the non-parametric Mann–Whitney *U*-test was used. For three unpaired group comparison of interval values the parametric one-way ANOVA and of ordinal values the non-parametric Kruskal–Wallis ANOVA were used. P-values less than 0.05 were defined as statistically significant.

Results

AAD and *C. difficile* infection

In the control group, 63 of 338 patients developed AAD, equivalent to an incidence of 18.6%. Of the patients suffering from AAD, an immunoassay proved that 21 had CDI, demonstrating a CDI incidence of 6.2% in this control group. On the other hand, 17 of 340 patients in the intervention group developed AAD, equivalent to an AAD incidence of 5%. One of the patients suffering from AAD had a positive immunoassay, confirming CDI. As a result, the incidence of CDI in the intervention group was 0.3%. Compared to the control group, the probiotic intervention group demonstrated a relative risk reduction of 73.2% for development of AAD ($p < 0.001$) and 95.3% for CDI ($p < 0.001$) (based on the immunoassay).

Among the faecal samples for microbiota analysis, one patient in group A tested positive using the qPCR Euroclone® *C. difficile* A/B kit but no one in group AP (Table 4). This test detected all three *C. difficile* strains provided from the Division for Human Medicine of the Austrian Agency for Health and Food Safety (AGES). Two patients in group A and one patient in group AP developed AAD.

(Table 5) Table 4. Group distribution concerning antibiotic-associated diarrhoea (AAD) and *Clostridium difficile* infection (CDI).

Table 6

	A (no intervention)	AP (intervention)
Euroclone® <i>C. difficile</i> A/B kit 1	(n=15)	0 (n=10)
Clinical study	AAD: 63 (n=338)(18.5%)CDI: 21 (6.2%) AAD: 17 (n=340) (5%)CDI: 1 (0.3%) AAD: RRR 73.2% ($p<0.001$) CDI: RRR 95.3% ($p<0.001$)	

A: antibiotic treatment; AP: antibiotic treatment and intake of a probiotic drink containing *Lactobacillus casei* Shirota; n: sample size; RRR: relative risk reduction.

Total bacteria

In the faecal analysis of the subset of subjects, both antibiotic-treated groups had a significant lower number of copies per gram than the control groups ($p=8.4 * 10^{-4}$), particularly at time points two (AP: $p=0.02$; A: $p=0.03$) and three (AP: $p=0.003$; A: $p=0.02$) compared to group C. Within the antibiotic groups, a mean decrease of copies per gram was observed (Figure 1A).

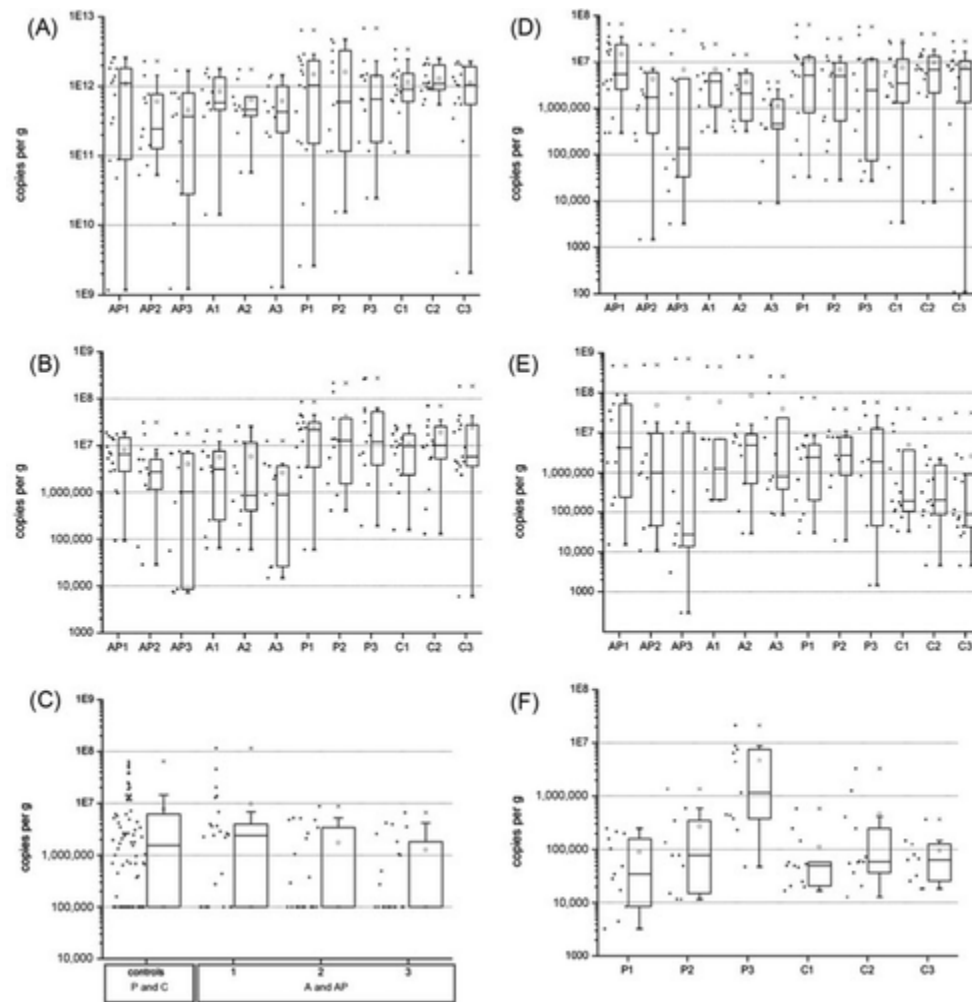


Figure 10

Figure 1. 16S rRNA qPCR of (A) total bacteria, (B) *Clostridium* Cluster IV, (C) *Clostridium* cluster XI, (D) *Bifidobacterium* spp., (E) Enterobacteriaceae, (F) *Lactobacillus* spp.

(A: antibiotic treatment; AP: antibiotic treatment and intake of a probiotic drink containing *Lactobacillus casei* Shirota; P: intake of a probiotic drink containing *L. casei* Shirota; C: control group; 1: day 0, 2: day 3, 3: day 5).

Denaturing gradient gel electrophoresis band pattern analysis (Figure 2) of 16S rRNA coding regions of total bacteria amplified with primer pair 341GC-518 revealed that on average two more bands could be detected in group AP, increasing from time point 1 to 3, in comparison to group A (A1:A3 10:8 bands, AP1:AP3 8:9 bands), indicating a higher diversity was maintained in the AP group.

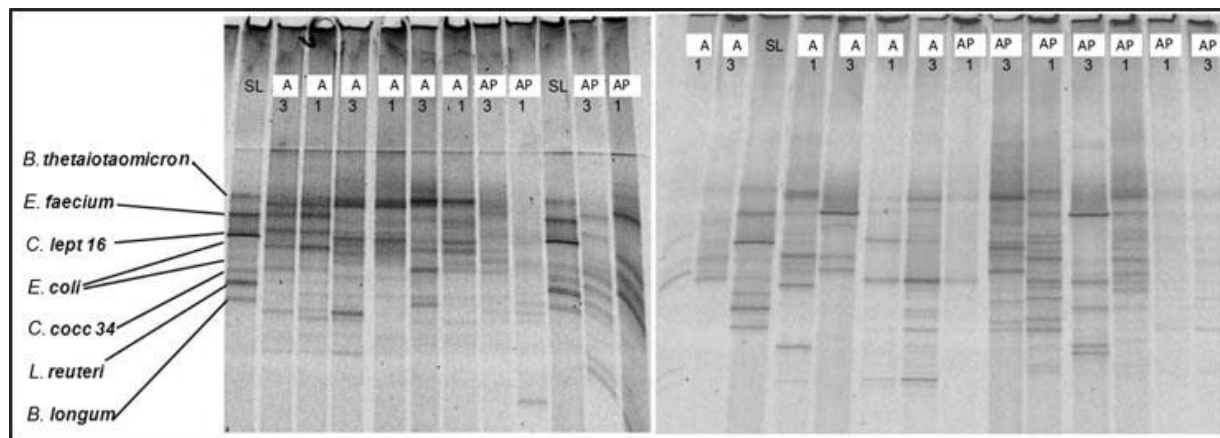


Figure 11

Figure 2. PCR-DGGE fingerprinting of 16S rRNA coding regions amplified with primer pair 341GC-518.

(A: antibiotic treatment; AP: antibiotic treatment and intake of a probiotic drink containing *Lactobacillus casei* Shirota; P: intake of a probiotic drink containing *L. casei* Shirota; C: control group; 1: day 0, 3: day 5, SL: standard line).

Relative amount of bacteria

Most striking is that in patients receiving antibiotics, the *Clostridium* Clusters IV and XIV are clearly reduced compared to the healthy controls and at the first time point the Enterobacteriaceae are clearly increased (Figure 1). Furthermore, in patients under antibiotic treatment, there were more bacteria which cannot be identified by the primers used in this study (Figure 3).

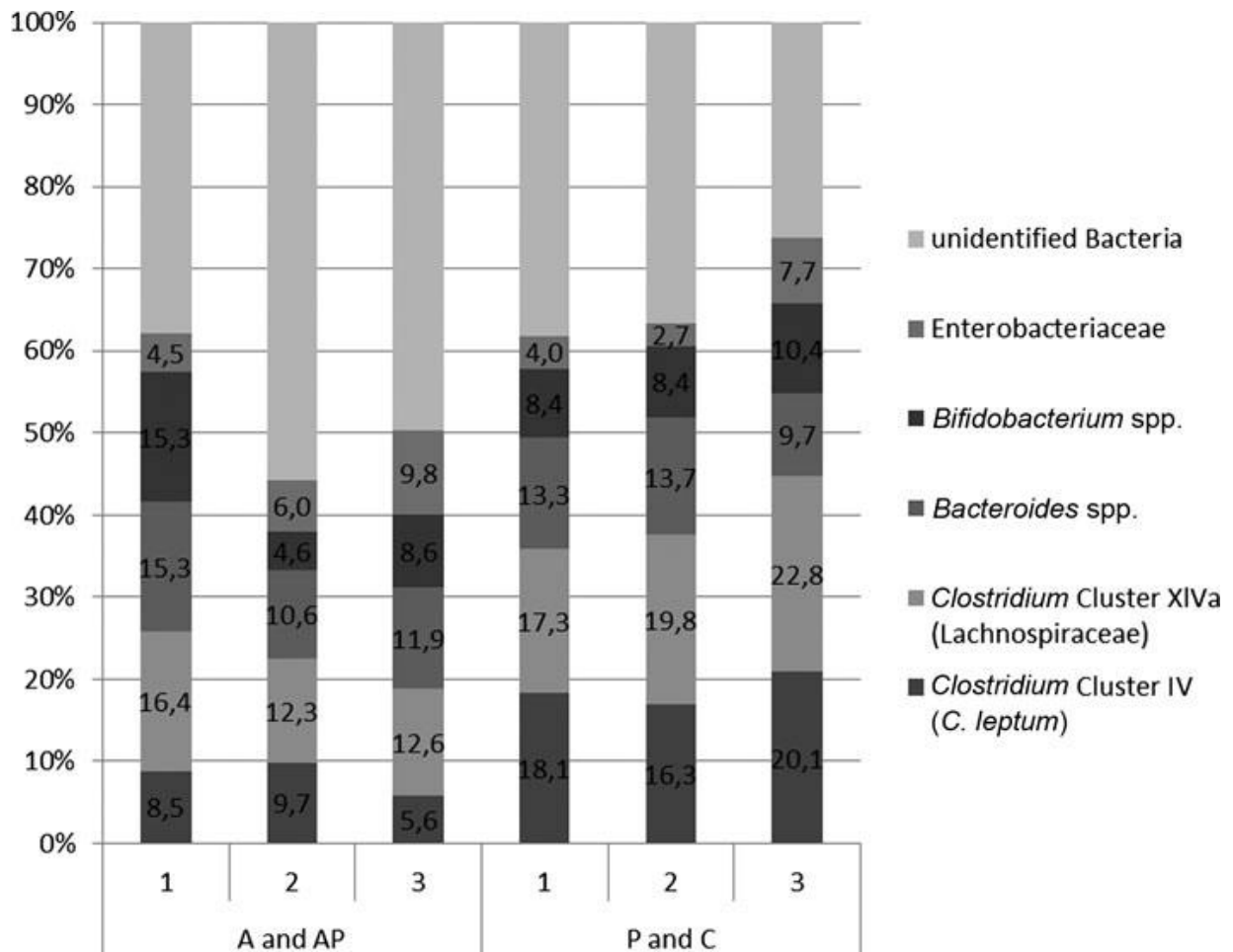


Figure 12

Figure 3. Percentage of bacterial subgroups in relation to the analysed bacteria. In patients under antibiotic treatment, there were more bacteria which cannot be identified by the primers used in this study.

(A: antibiotic treatment; AP: antibiotic treatment and intake of a probiotic drink containing *Lactobacillus casei* Shirota; P: intake of a probiotic drink containing *L. casei* Shirota; C: control group; 1: day 0, 2: day 3, 3: day 5).

Bacterial subgroups

Clostridium cluster IV (*C. leptum* subgroup): The values of the groups receiving antibiotics were on average lower by a half-power than the two control groups not on antibiotics. This difference was statistically significant ($p=6.31 \cdot 10^{-7}$). Group AP and A and group P and C did not differ from each other (Figure 1B).

Clostridium cluster XI: After commencement of the antibiotic treatment, the abundance of *Clostridium* cluster XI decreased, whereas in the control groups values remained constant (Figure 1C).

All *Bacteroides*: Evaluation of the results revealed no remarkable differences.

Bifidobacterium spp.: The mean number of copies per gram of both antibiotic groups was lower than those of the control groups ($p=0.051$). The average numbers of copies fell in group AP and A, but in group AP this decrease stopped, after the LcS intake was begun and levels started to rise again slightly (Figure 1D).

Enterobacteriaceae: In the antibiotic groups AP and A, the number of copies per gram was higher by a power than those of both groups without antibiotic treatment (P and C) ($p=0.06$). Furthermore, it was apparent that individual values were widely spread. There was a statistically significant difference between all four groups ($p=6.9 * 10^{-5}$) but not within the three time points of each group (Figure 1E).

Lactobacillus spp.: In group P, the values of each time point differed significantly ($p=0.01$) and the number of copies per gram increased significantly from time point 1 to 3 ($p=0.02$) as well as from time point 2 to 3 ($p=0.045$). At time point 3, the number of copies per gram was significantly higher in group P than in group C ($p=0.04$) (Figure 1F).

Butyryl-CoA CoA transferase gene

In patients receiving antibiotics (group AP and A), the amount of butyryl-CoA CoA transferase genes was lower than that in the controls ($p=1.08 * 10^{-5}$). The graph also showed a difference in values between the two groups on antibiotic treatment, group AP and A; however, this was not statistically significant (Figure 4).

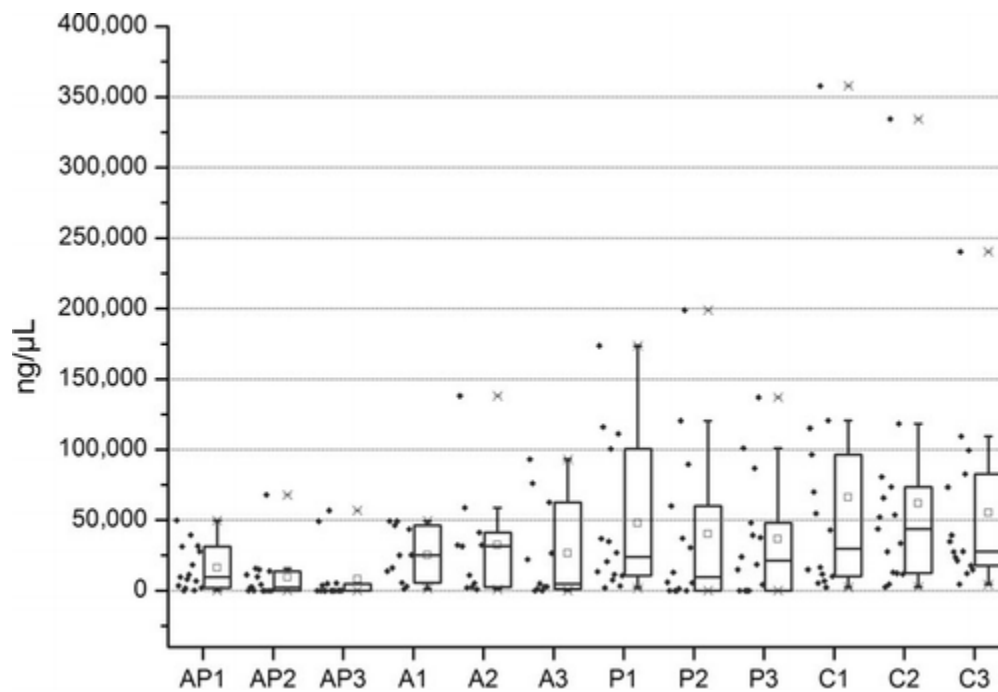


Figure 13

Figure 4. Abundance of butyryl-CoA CoA transferase genes in group A (antibiotic treatment), AP (antibiotic treatment and intake of a probiotic drink containing *Lactobacillus casei* Shirota), P (intake of a probiotic drink containing *L. casei* Shirota) and C (control group) (1: day 0, 2: day 5, 3: day 5). Amplification was done by 16S rRNA qPCR with primer pair BCoATscrF/R.

Discussion

Results of a clinical trial including 678 patients suggest that AAD is significantly decreased by giving the probiotic *L. casei* Shirota in combination with antibiotic therapy. We used 16S rRNA based qPCR and PCR/DGGE to analyse possible differences in faecal microbiota of subjects receiving antibiotics and/or a combined antibiotic/*L. casei* Shirota intervention.

Antibiotics clearly changed the composition of the gastrointestinal microbiota as indicated by a change in the abundance of subgroups and in their diversity. As described in recent studies (De La Cochetiere et al., 2005; Dethlefsen & Relman 2011; Jakobsson et al., 2010; Sommer & Dantas, 2011), our results showed that antibiotic intake caused group-specific shifts, and in general a reduction of bacterial abundance.

The quantitative analysis by qPCR revealed that the total amount of bacteria decreased as a result of antibiotic treatment, either with or without probiotic intake. However, further qualitative analysis using DGGE indicated that diversity remains higher when the probiotic was consumed during and after antibiotic therapy. This result certainly merits further investigation with a bigger sample size.

It was also observed that the abundance of the bacterial subgroups *Bifidobacterium* spp., *Clostridium* clusters IV and XI was reduced during antibiotics, whereas the abundance of Enterobacteriaceae increased. A dramatic decline in the Actinobacteria phylum, which includes Bifidobacteria, following antibiotic intake has been described previously (Jakobsson et al., 2010). Bifidobacteria are inulin and starch degraders; lactate is the primary fermentation product and is converted to butyrate by secondary fermenters (Dethlefsen, Eckburg, Bik, & Relman, 2006) thus a decline in Bifidobacteria represents a decrease in an important SCFA-producing group.

Short-chain fatty acids result from anaerobic microbial fermentation of indigestible carbohydrate or dietary fibre (Fredstrom, Lampe, Jung, & Slavin, 1994). The main SCFAs are acetate, propionate and butyrate, which stimulate the colonic blood flow and fluid and electrolyte uptake (Topping & Clifton, 2001). Furthermore, indirect actions of butyrate are believed to involve the hormone-neuro-immuno system (Guilloteau et al., 2010). As described previously, SCFAs are also essential for sodium and water uptake. Reduced production of SCFAs is linked to pathogenic mechanisms causing diarrhoea. Therefore, the observation that combined use of *L. casei* Shirota antagonises the drop of Bifidobacteria caused by antibiotics could be linked to the clinical results showing reduced AAD incidence with the probiotic. This is in line with the previous reports of increasing abundance of Bifidobacteria during LcS intake (Bian et al., 2011; Nagata et al., 2011).

The decline of *Clostridium* cluster IV would also have a strong impact on SCFA-production. This subgroup represents 22% of the total faecal bacteria, with *Faecalibacterium prausnitzii* strains being the most abundant (Lay et al., 2005). Together with *Clostridium* cluster XIVa, cluster IV contains the

main butyrate-producing bacteria in human gut, and in particular strains related to *F. prausnitzii* (Pryde, Duncan, Hold, Stewart, & Flint, 2002). These results are in line with the observed lower values of butyryl-CoA CoA transferase gene in antibiotic-treated patients. Butyrate has also been implicated in the down-regulation of bacterial virulence (Guilloteau et al., 2010); a decrease in butyrate allows pathogen growth in the intestinal ecosystem. In the present study, the butyryl-CoA CoA transferase gene was used as a marker for butyrate production because direct analysis of SCFAs in faeces is hampered by the rapid binding and degradation of SCFAs in the gut. Clearly, results from the analysis of the butyryl-CoA CoA transferase gene focus on the prediction of butyrate production and not on the actual production which is difficult to assess.

The abundance of *Clostridium* cluster XI was decreased in patients who were prescribed antibiotics and declined during their treatment. *Clostridium* cluster XI is a heterogeneous phylogenetic cluster including opportunistic pathogens, such as *C. difficile* (Nadal et al., 2009). *Clostridium difficile* is a Gram-positive, spore-forming, anaerobe that can be carried asymptotically in the human intestinal tract. Antibiotics suppressing the intestinal microbiota can allow *C. difficile* to proliferate and subsequently cause intestinal damage, inflammation and clinical disease (Lawley et al., 2009). Results of the present clinical study suggest that consumption of *L. casei* Shirota efficiently prevents the development of CDI and reduces development of AAD. In this study, CDI was evaluated by TOX A/B QUICK CHEK®, Wampole® immune assay for all the patients on antibiotics in the study. For the molecular analysis of the subset of 56 samples, Euroclone® *C. difficile* A/B kit was used. Problems and different outcomes in the detection of *C. difficile* using different test systems have been widely discussed (Alcala et al., 2008; Eastwood, Else, Charlett, & Wilcox, 2009; Luna et al., 2011).

Administration of probiotics upon administration of antibiotics has been discussed for developing antibiotic resistance and the use of probiotics is critically discussed in critically ill patients (Singhi & Baranwal, 2008). Although *L. casei* is vancomycin resistant, *L. casei* Shirota does not have vanA or vanB genes which confer transferable vancomycin resistance (Kiwaki and Kushiro, 2010).

Conclusions

This study also confirmed that antibiotic treatment, as well as the underlying disease requiring this therapy, was mainly associated with changes in the diversity and composition of the gastrointestinal microbiota, accompanied by a lower occurrence of butyryl-CoA CoA transferase genes. Pathogens are more able to colonise a disturbed intestinal ecosystem; reduction in both bacterial diversity and production of SCFAs might significantly contribute to problems following antibiotic treatment. It is apparent that intervention with *L. casei* Shirota antagonises some of these changes. Successful probiotic intervention in AAD might therefore be directed at the problem of reduced production of SCFAs that occurs after antibiotic treatment.

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Changes in Human Fecal Microbiota Due to Chemotherapy Analyzed by TaqMan-PCR, 454 Sequencing and PCR-DGGE Fingerprinting

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Background

We investigated whether chemotherapy with the presence or absence of antibiotics against different kinds of cancer changed the gastrointestinal microbiota.

Methodology/Principal Findings

Feces of 17 ambulant patients receiving chemotherapy with or without concomitant antibiotics were analyzed before and after the chemotherapy cycle at four time points in comparison to 17 gender-, age- and lifestyle-matched healthy controls. We targeted 16S rRNA genes of all bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster IV and XIVa as well as *C. difficile* with TaqMan qPCR, denaturing gradient gel electrophoresis (DGGE) fingerprinting and high-throughput sequencing. After a significant drop in the abundance of microbiota ($p = 0.037$) following a single treatment the microbiota recovered within a few days. The chemotherapeutical treatment marginally affected the *Bacteroides* while the *Clostridium* cluster IV and XIVa were significantly more sensitive to chemotherapy and antibiotic treatment. DGGE fingerprinting showed decreased diversity of *Clostridium* cluster IV and XIVa in response to chemotherapy with cluster IV diversity being particularly affected by antibiotics. The occurrence of *C. difficile* in three out of seventeen subjects was accompanied by a decrease in the genera *Bifidobacterium*, *Lactobacillus*, *Veillonella* and *Faecalibacterium prausnitzii*. *Enterococcus faecium* increased following chemotherapy.

Conclusions/Significance

Despite high individual variations, these results suggest that the observed changes in the human gut microbiota may favor colonization with *C. difficile* and *Enterococcus faecium*. Perturbed microbiota may be a target for specific mitigation with safe pre- and probiotics.

Introduction

The human intestinal ecosystem can be pictured as a microbial organ within a host organism involving a dynamic interplay between food, host cells and microbes [1]. The microbiota plays several significant roles in the digestion of food, energy regulation, generation of short-chain fatty acids, vitamin synthesis, prevention of colonization by pathogens and protection against cell injury [2], [3], [4]. Moreover, the gut microbiota influences the host by directing intestinal epithelial cell proliferation and differentiation, pH, and the development of the immune system [1]. Recent culture-independent molecular studies on healthy individuals have shown that the intestinal microbiota is specific to the host [5] and resilient to modifications over time, as it is able to form an alternative stable state after disruption [6], [7]. A healthy microbiota contains a balanced composition of many

classes of bacteria [8]. The fecal microbiota is dominated by three groups of anaerobic bacteria: the *Clostridium coccoides* group -clostridial cluster *XIVa* (reclassified as *Blautia coccoides* [9]), the *Clostridium leptum* group - *Clostridium* cluster *IV*, and the *Bacteroides* [10], [11]. All three groups are known to positively affect the gut health through nutrient absorption, production of short chain fatty acids (SCFAs) and epithelial cell maturation [12], [13]. Moreover, the subgroup bifidobacteria seems to be an important part of the gastrointestinal tract (GI) microbiota, being involved in the prevention of atopic disease, obesity and insulin resistance via enhanced barrier function of the gut epithelium [14].

To prevent the invasion of endogenous bacteria from oral cavity and the GI tract into the blood stream, three defense mechanisms are considered to be relevant: innate immunity, mechanical mucosal barrier, and colonization resistance [15]. However, chemotherapy damages the rapidly generated mucosal cells of the GI and the use of antibiotics disrupts the ecological balance, allowing pathogens such as *Clostridium difficile* to grow [16], [17]. This bacterium is thought to be the causative agent in up to 20% of antibiotic-associated diarrhea (AAD) cases [18]. It is evident that the intestinal microbial ecosystem has an important but incompletely defined role in mucosal protection [19].

Mucositis is a major oncological problem, caused by the cytotoxic effects of cancer chemotherapy and radiotherapy [20]. Approximately 40% of patients receiving standard dose chemotherapy and up to 100% of patients receiving high dose chemotherapy and stem cell or bone marrow transplantation suffer from abdominal pain, ulceration, bloating and vomiting [21], [22]. Although gastrointestinal disturbances (mucositis, diarrhea and constipation) and immunosuppression are well recognized side-effects of cancer treatment, very little research has been conducted into the underlying mechanisms and the changes in the composition of the microbiota. Because of these changes, nutrient absorption and other intestinal functions involving the microbiota may also be altered [23].

For this reason, we investigated shifts in fecal microbiota of patients receiving cancer chemotherapy with or without antibiotics in comparison to healthy control individuals. Prescription of antibiotics may become necessary in some individuals due to bacterial infection [24]. Samples were taken at four time points before and after chemotherapy to study changes in fecal microbiota over the course of time. In this study, we aimed to clarify how chemotherapy agents influence total fecal bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster *IV*, *Clostridium* cluster *XIVa* and *C. difficile* using culture-independent methods assessing abundance and diversity. Four samples were also analyzed with 454 high-throughput sequencing.

Results

PCR-DGGE fingerprinting analysis shows decreased diversity of Clostridium clusters IV and XIVa in response to medical treatment compared to healthy individuals

DGGE fingerprinting analyses of all bacteria, *Clostridium* cluster *IV* and *Clostridium* cluster *XIVa* indicate a highly diverse dataset between individuals and uniqueness of fecal microbiota. Table 1

shows the average number of bands in cancer patients at the three time points and for controls over all time points. It becomes apparent that the average number of bands within *Clostridium* cluster IV declined immediately after chemotherapy (T1), followed by a recovery at T2. The average number of *Clostridium* cluster XIVa bands decreased after onset of chemotherapy and remained low also at T2. The datasets were subjected to principal component analysis (PCA). PCA extracts underlying components of samples according to their variance. Figure 1A illustrates the bacterial fingerprints of sample P01 over time. Figure 1B displays the PCA analysis of all bacteria. Most samples taken after chemotherapy are grouped together with all other samples. Patients who receive antibiotics are indicated as black symbols. They cluster together with the samples taken after chemotherapy and also with the majority of samples before chemotherapy and healthy controls. There are two exceptions though: Two samples from P07 after chemotherapy under antibiotic treatment are outliers in the lower right part of the PCA plot. P07 received blood stem cell transplantation resulting in a sharp decline in bacterial abundances as measured with quantitative PCR. The first two principal components explain 17.4% of variance.

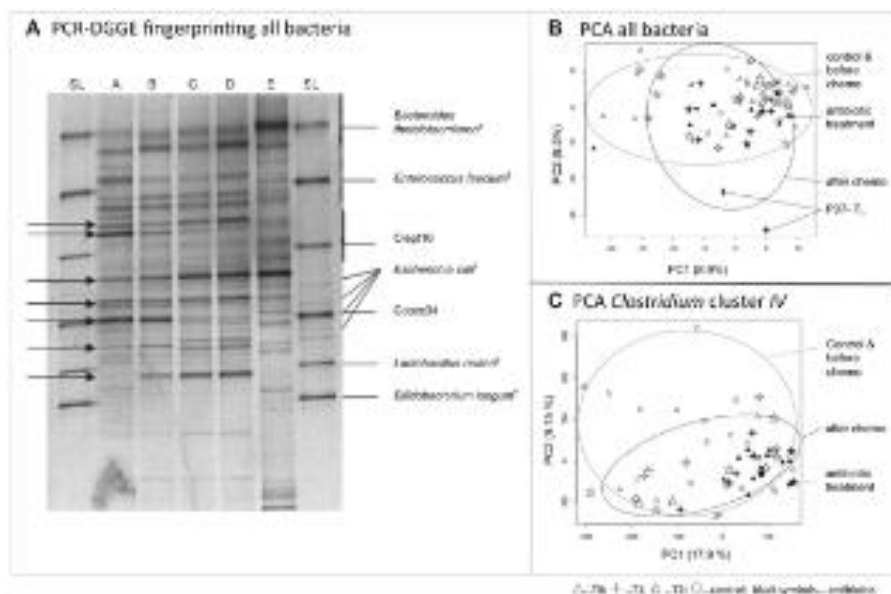


Figure 14

Figure 1. A PCR-DGGE fingerprinting of 16S rRNA coding regions of dominant bacteria over time. Bands that become stronger or nearly disappear following a single chemotherapeutic treatment are indicated with arrows. B Principal Component analysis (PCA) based on dominant bacteria PCR-DGGE fingerprinting. The two outliers in the lower right corner of the plot are two samples of P07 following blood stem cell transplantation. C PCA illustrating the development of *Clostridium* cluster IV diversity in the course of chemotherapy and antibiotic treatment. Cluster IV diversity drops right after chemotherapy, causing a grouping of samples. Samples under antibiotic treatment (indicated as grey dots) group even closer, indicating a strong influence of antibiotics on *Clostridium* cluster IV diversity. A, sample of P01 before chemotherapy B, C and D, samples of P01 after chemotherapy; E, healthy control; SL, unrelated standard lane; black symbols... patients under chemotherapy and antibiotic treatment.

Figure 1C shows the principal components analysis of Clostridium cluster IV. DGGE fingerprints of individuals after chemotherapy are found to be less variable than healthy controls and patients before onset of treatment. Although overlapping, PCA resulted in grouping of band patterns before and after chemotherapy. Additional effects by antibiotic treatment became evident: Antibiotic treatment significantly reduced the diversity within the Clostridium cluster IV ($p = 0.00003$) with Shannon diversity index being 1.4 ± 0.7 compared to patients under chemotherapy alone 2.1 ± 0.6 . In the PCA plot, samples affected by antibiotics are found in the lower right corner of the plot. This means that they are grouped according to their variance along principal component (PC) 1 and 2. These two PCs explain 17.9% and 9.15% of the variance in the dataset, underlining the validity of this interpretation. Principal components analysis of Clostridium cluster XIVa is not shown.

Table 1. Number of bands observed in PCR-DGGE fingerprinting in oncology patients before chemotherapy (T0), immediately after chemotherapy (T1) and 5–9 days after chemotherapy (T2) and healthy controls averaged over all time points.

[Table 7](#)

Time point	All bacteria	<i>Clostridium</i> cluster IV	<i>Clostridium</i> cluster XIVa
T ₀	18.9±4.6	14±7.0	8±3.2
T ₁	19.7±4.9	10±6.0	4.9±3.6
T ₂	19.6±3.6	15±6.0	5.2±2.6
control	19.2±3.5	12.0±5.0	8.9±3.0

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Chemotherapeutic treatment with or without antibiotics decreases absolute bacterial numbers in comparison to healthy controls

To study whether chemotherapy with or without antibiotics changes the human GI microbiota composition in contrast to healthy individuals and over time, we investigated absolute numbers and relative percentages of bacterial subgroups. Absolute numbers give an indication about the direct antimicrobial effects of the treatments. Relative quantification is able to identify which bacterial subgroups are particularly affected and helps to describe the community disruption induced by chemotherapy with or without antibiotics. In absolute numbers, oncology patients harbored significantly less bacteria ($p < 0.05$) than healthy control (figure 2). From already low bacterial counts before chemotherapy, bacterial abundance significantly declined further ($p = 0.037$) immediately after chemotherapy (T1) and recovered 5–9 days later (T2) in comparison to time points before treatment (T0). Absolute numbers of bacteria in different time points of healthy controls are following a lognormal distribution in contrast to microbiota abundances in oncology patients. The decrease in total bacteria following chemotherapy ($p = 0.037$) was significantly greater than any variation in copy numbers observed in healthy controls ($p = 0.027$). The observed decrease after chemotherapy affected the *Bacteroides* ($p = 0.044$), the bifidobacteria ($p = 0.034$) and *Clostridium*

cluster *IV* ($p = 0.049$) as shown in figure 3. There were also fewer absolute numbers of *Clostridium* cluster *XIVa*, but this difference was not significant. All patients with fever showed an increase in total fecal microbiota (see figure 3). In sample P07 a sharp decline affecting all bacteria and bacterial subgroups was observed at T1 (figure 3), following blood stem cell transplantation and medical intervention.

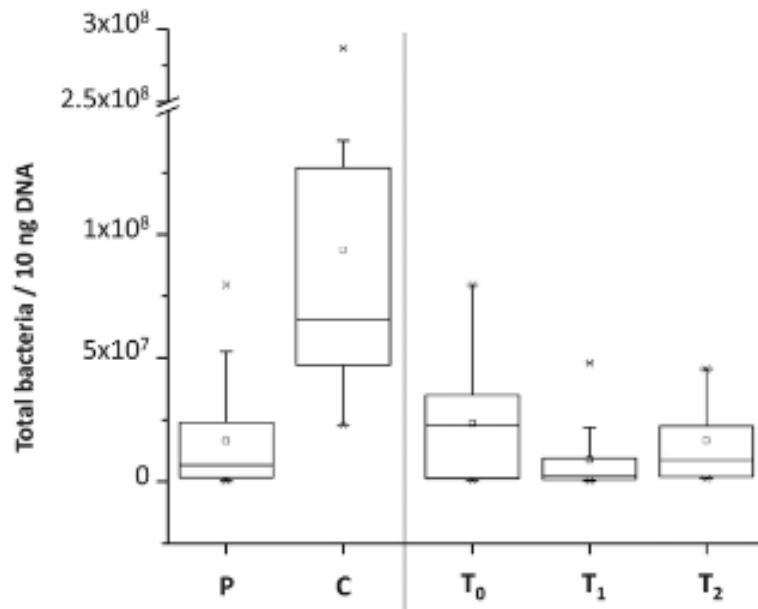


Figure 15

Figure 2. TaqMan qPCR quantification of bacterial 16S rRNA coding regions showing lower abundance in patients undergoing chemotherapy and antibiotic treatment (P) than healthy controls ©.

T₀, samples taken before a single shot of chemotherapy; T₁, 1–2 days after chemotherapy; T₂, 5–9 days after chemotherapy; Asterisk indicates a significant difference at $p < 0.05$.

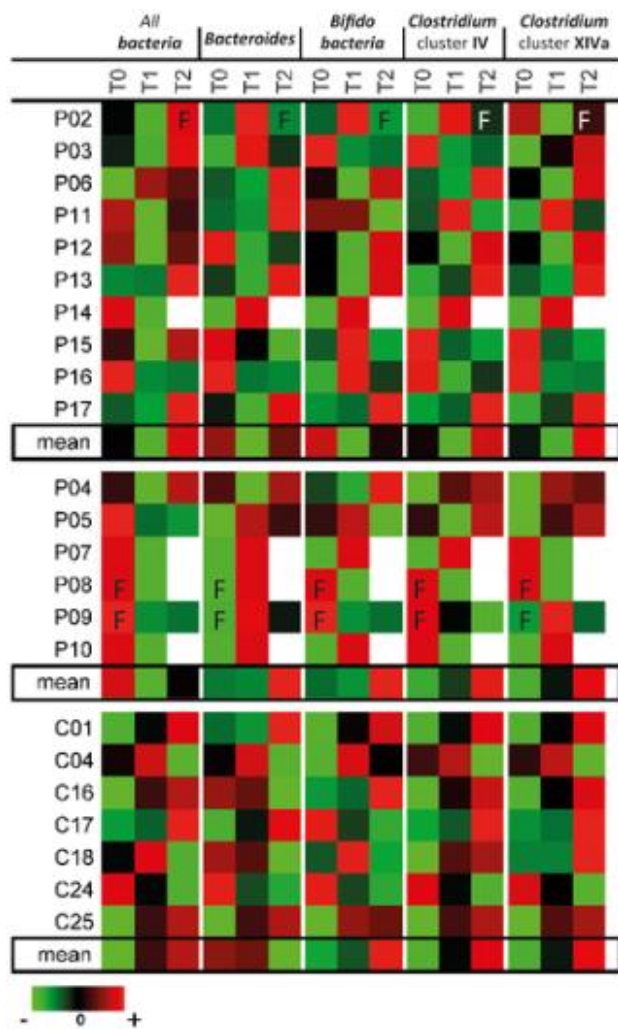


Figure 16

Figure 3. Abundances of bacterial 16S rRNA coding regions over time in oncology patients (P) and healthy controls ©.

The declined abundances of bacteria, *Bacteroides*, *Clostridium* cluster XIVa, *Clostridium* cluster IV and bifidobacteria immediately after chemotherapy (T₁) were observed to recover several days after treatment (T₂). Patients P04, P08 and P13 had never received chemotherapy before; P04, P05, P07, P08, P09 and P10 took antibiotics. Values were z-scored for presentation in this heatmap showing changes over time rather than absolute abundances. T₀, before chemotherapy; T₁, 1–2 days after chemotherapy; T₂, 5–9 days after chemotherapy; F, fever; S, blood stem cell transplantation.

Patients who received antibiotics had highest abundances of all bacteria ($p = 0.000003$) amongst all patients (data not shown). This bacterial overgrowth affected the *Bacteroides*, the bifidobacteria and *Clostridium* clusters IV and XIVa, since relative abundances of those subgroups did not stand out significantly. Thus, patients were grouped according to their chemotherapeutic cycle regardless whether or not they received antibiotics. The influence of antibiotics on the species composition as assessed with PCR-DGGE fingerprinting is discussed in the previous section.

***Clostridium* cluster XIVa shows great alterations due to chemotherapeutical interventions, while the *Bacteroides* and bifidobacteria seem to be marginally affected**

Relative quantification of *Clostridium* cluster XIVa as percentage of total bacterial DNA showed that oncology patients harbored significantly less *Clostridium* cluster XIVa ($p = 0.047$) than healthy controls. The mean proportion of *Bacteroides* in stool samples was $26 \pm 12\%$ in chemotherapy patients and $22 \pm 14\%$ in healthy individuals. The mean percentage of bifidobacteria in patients was $0.8 \pm 1.4\%$ and 0.3 ± 0.6 in controls. Patients harbored on average $16 \pm 9\%$ of *Clostridium* cluster IV and $18 \pm 12\%$ of *Clostridium* cluster XIVa, while controls harbored $20 \pm 12\%$ and $24 \pm 15\%$ of clostridial clusters IV and XIVa.

***Clostridium* cluster XIVa higher before chemotherapy than after**

The mean percentage of *Clostridium* cluster XIVa before chemotherapy was $22 \pm 13\%$ compared to after chemotherapeutic cycles with $19 \pm 12\%$. The average amount of *Bacteroides*, bifidobacteria and *Clostridium* cluster IV were $26 \pm 11\%$, $1.4 \pm 2\%$ and $16 \pm 9\%$ at time points before chemotherapy and $28 \pm 14\%$, $0.5 \pm 1.2\%$ and $18 \pm 12\%$ after chemotherapy. Figure 3 illustrates the development of the microbiota in the course of antibiotic treatment. Data were normalized for clarity, so that changes in abundances from time point T0 (before onset of treatment) to T1 (1–4 days after chemotherapy) and T2 (5–9 days after chemotherapy) rather than relative abundances are shown. It can be seen that chemotherapy causes a dramatic reduction of microbiota abundance immediately after chemotherapy, affecting all subgroups. As mentioned above, the significant decrease in all bacteria following chemotherapy was significantly greater than any variation in copy numbers observed in healthy controls ($p = 0.027$).

***C. difficile* colonization found in individuals receiving chemotherapeutic and antibiotic treatment**

To find out whether the chemotherapeutic and antibiotic disruption favors the growth of pathogens, we investigated the abundance of *C. difficile*. Three out of seventeen patients receiving chemotherapy harbored *C. difficile* (data not shown). Patient P09 harbored *C. difficile* at all time points investigated. Mean proportion over all four samples of P09 was recorded as $0.4 \pm 0.7\%$, yet the highest level (1.22% of total bacteria) occurred at sampling point T1 immediately after chemotherapeutic and antibiotic treatment. *C. difficile* was detected in P11 (3.90% of all analyzed bacteria) after chemotherapeutic intervention at time point T1. P14 carried *C. difficile* in low abundance directly after onset of chemotherapy (0.003% of all analyzed bacteria). Samples of patients P09 and P11 at T0 and T1 were further analyzed in 454 sequencing.

High throughput sequencing

High throughput sequencing showed a dramatic increase in sequences within the Peptostreptococcaceae towards sequences 98.9–100% similar to *C. difficile*T (figure 4): *Clostridium* bartlettii related sequences (98.1–100% similarity) were only detected before chemotherapy (T0).

After chemotherapy (T1), 63 sequences 98.9–100% similar to *C.difficile* appeared in samples P11 and P09. In accordance with the phylogenetic classification by the ribosomal database project, they are shown as ‘unclassified *Peptostreptococcaceae*’ in figure 5.

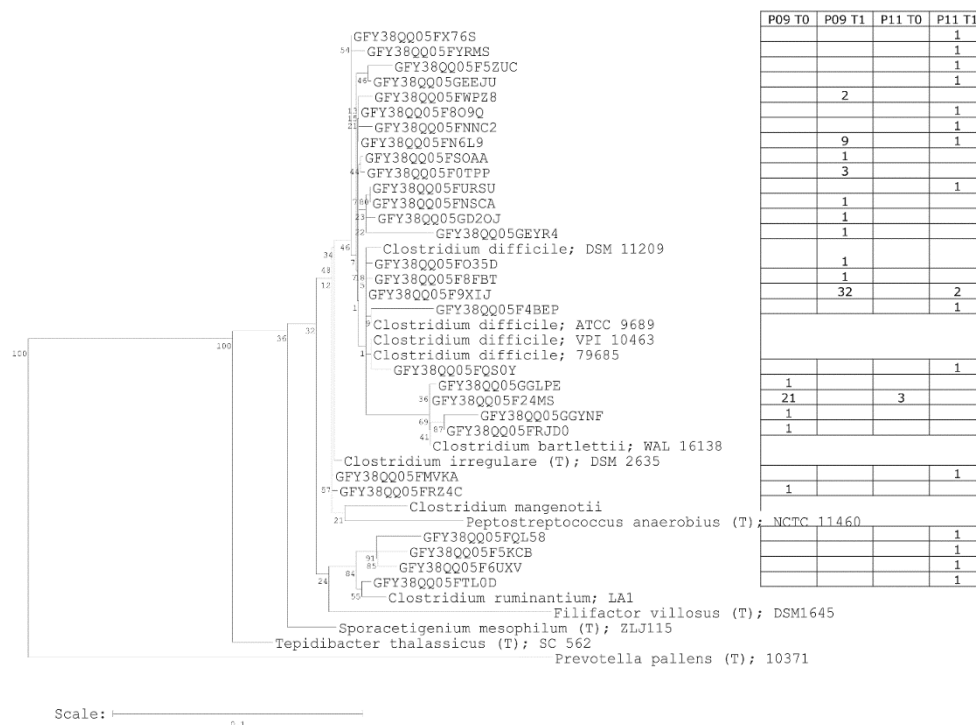


Figure 17

Figure 4. Phylogenetic tree showing the *Peptostreptococcaceae* found in samples from two oncology patients before and after chemotherapy.

Identical sequences were grouped; the table on the right hand side shows their abundances in the 454 sequencing dataset. Sequences with >98.9% similarity to *Clostridium difficile* appeared only in samples taken immediately after chemotherapeutic cycles. Numbers indicate bootstrap values after 100 resamplings.

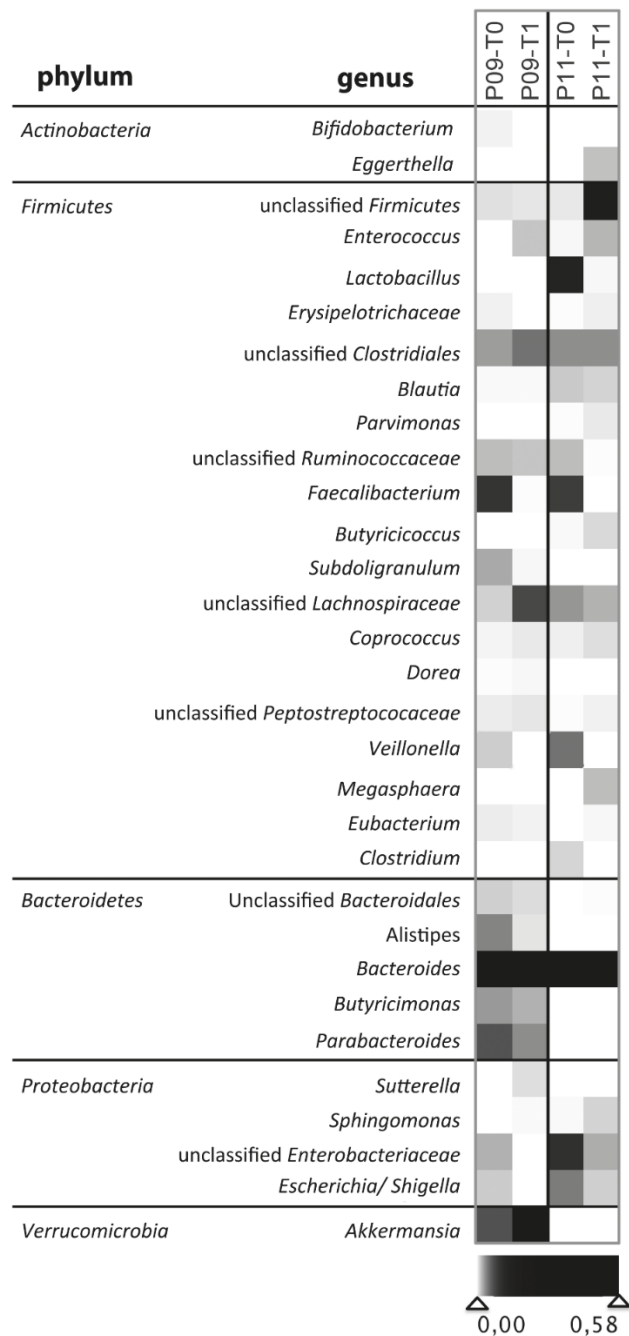


Figure 18

Figure 5. Heatmap showing abundances within the 454 sequencing dataset on the genus level. High throughput sequencing of samples P09 and P11 before (T₀) and after therapy (T₁) further helped to characterize the influence of a single chemotherapeutic cycle on the GI-microbiota. P11 was treated with chemotherapy alone and P09 also received antibiotic treatment.

Furthermore pronounced reductions of *Faecalibacterium* spp. as well as lactobacilli, *Veillonella* spp., bifidobacteria (in P09) and *E.coli/Shigella* became apparent in response to chemotherapy (figure 5).

The abundance of lactobacilli decreased in both patients after chemotherapy, in P09 from already low levels. Individual P11 did not receive concomitant antibiotics, whereas P09 did. In P09 and P11 *Faecalibacterium* spp. decreased dramatically from 9.5% and 8.3% to 0.07% and 0.00%, respectively. In both individuals *Enterococcus faecium* increased following chemotherapy. Furthermore, less abundant sequences appeared that were attributable to bacterial genera not detected before chemotherapy. These genera are: *Eggerthella*, *Megasphaera*, *Parvimonas* (only in P11), *Anaerostipes*, *Eubacterium*, *Anaerococcus*, *Methylobacterium*, *Holdemania*, *Turicibacter*, *Akkermansia*, *Sutterella* (only in P09), *Sphingomonas*, *Anaerotruncus*, *Coproccoccus*, *Streptococcus* and *Dorea*. Species with abundance <0.01% of all sequences are not shown in figure 5. The number of *Blautia* species from *Clostridium* cluster XIVa remained constant in the 454 sequencing datasets before and after chemotherapy.

Discussion

Chemotherapeutic and antibiotic use is associated with severe side effects such as mucositis, diarrhea and constipation. These side effects increase the cost of health services and are often life threatening [22]. Chemotherapeutic and antibiotic treatment has a detrimental impact on the host microbial ecosystem, which is important for host mucosal protection [19] and thereby increases the risk of infection [17]. Overgrowth of species with potential pathogenicity such as toxigenic *C. difficile* and inflammatory complications are among the most common serious complications of chemotherapy and antibiotic treatment among patients with cancer [15], [17].

We investigated how the use of cancer chemotherapy (in some individuals together with antibiotic treatment) perturbs the fecal microbial ecosystem during the course of therapy. We assessed if the microbiota is able to return to its original profile after chemotherapeutic and antibiotic intervention with special interest in the abundance of *C. difficile*. We used a combination of molecular methods including high-throughput sequencing to compare diversity (PCR-DGGE) and abundance (qPCR) of all bacteria, *Bacteroides*, bifidobacteria, *Clostridium* cluster IV, *Clostridium* cluster XIVa and *C. difficile* between groups and different time points of chemotherapy. The majority of previous studies on the effect of chemotherapy on human fecal microbiota used standard microbiological culture techniques [16], [22]. Other studies have focused on the colonization of pathogenic bacteria [17], [25] in patients with cancer and chemotherapy-induced diarrhea [22], [26]. As mentioned above, we used feces as source of information. Fecal microbial communities are composed of autochthonous gut members and transient bacteria. Even though the fecal microbiota might be different from the adherent microbiota, we chose fecal samples to investigate the microbial composition of the intestinal microbiota because they are easy to collect, are less invasive and reflect shifts in microbial population composition [11].

In this study, we assessed species richness using PCR-DGGE fingerprinting. Each lane of a PCR-DGGE gel represented a microbial fingerprint of a fecal sample; each band within a lane corresponded to one bacterial species, although different species may sometimes be represented by the same band [17]. It has also been observed that one bacterial strain may form several bands due to multiple 16S rRNA operons, e.g. *E.coli* (figure 4A). The limitations of DGGE in microbial analysis have been

previously described [27]. Nevertheless, substantial information about species composition can be obtained from very complex microbial communities such as the gut microbiota [27]. We found decreased species richness immediately after the chemotherapeutic shot, especially within *Clostridium* cluster IV where the number of different bands decreased from 14 ± 7 before chemotherapy (T_0) to 10 ± 6 bands shortly after (T_1). The microbiota recovered to a richness of 15 ± 6 *Clostridium* cluster IV bands per individual, but at a different composition, as evidenced by the grouping of samples in principal components analysis.

For quantification of fecal microbiota we used the strains *Bacteroides*

thetaiotaomicron^T, *Bifidobacterium longum* ssp. *longum*^T and *C. difficile* as well as the clones CL16 and CC34 as standards. However, a mixture of different strains for qPCR standards might result in a more accurate image of the human microbiota. Therefore absolute amounts should be considered as semi-quantitative.

Grouping oncology patients with and without antibiotic treatment poses a risk to falsely interpret the effects of antibiotic treatment as effects of chemotherapy. Patients who received antibiotics had significantly higher bacterial abundances than patients without antibiotics. This observation might be the reason for antibiotic treatment rather than its effect [24]. The abundance of bacterial subgroups, also *Clostridium* cluster IV, changed together with total bacteria both in patients with and without antibiotics. The sharp reduction of bacteria immediately after chemotherapy equally affected patients with and without antibiotics. In PCR-DGGE analysis we found that the all bacteria and *Clostridium* cluster XIVa fingerprints did not differ significantly in patients *with* or *without* antibiotics. This indicates that the use of antibiotics does not fully explain the observed changes. Previous work [17] has also found additional effects of chemotherapy in cases under prophylactic antibiotic treatment. Although the *Clostridium* cluster IV abundance did not differ significantly due to antibiotics, PCR-DGGE fingerprints showed grouping of patients under antibiotic treatment in principal components analysis.

Despite high individual variations, we show a significantly lower absolute bacterial load in feces of patients receiving chemotherapy in comparison to healthy controls. These findings are in line with data from van Vliet *et al.* (2009) who reported 100-fold lower total bacterial numbers during chemotherapy than in healthy controls.

The abundance of fecal microbiota decreased after a single cycle of chemotherapy. After the end of chemotherapeutic administration the bacterial abundance recovered within a few days, sometimes even showing a “rebound-effect” with numbers elevating above initial levels. Relative numbers of *Clostridium* cluster IV and XIVa showed great alterations due to chemotherapeutical interventions, while the bifidobacteria seemed to be less affected. In agreement with previous results [16] increased counts of *Bacteroides* spp. were found in patients undergoing chemotherapy. Nyhlén *et al.* (2007) also reported significant increases in yeast in patients, making it a focus for further research in immunocompromised patients. Samples taken immediately after chemotherapy had a lower diversity within *Clostridium* cluster IV. Antibiotics strongly contributed to the reduced diversity of cluster IV but

were not alone responsible for this effect. A few days later we observed a quantitative recovery, but not a recovery of the composition as evidenced by clustering of DGGE fingerprints.

The incidence of *C. difficile* in subjects P09 and P11 immediately after chemotherapy is accompanied by a decrease of the genera *Bifidobacterium*, *Lactobacillus* and *Clostridium* cluster IV. Sequences attributable to *Faecalibacterium prausnitzii* decreased dramatically from 9% to zero. The anti-inflammatory *F. prausnitzii* was associated with dietary fiber in colonic fermentation of healthy subjects [28] and found at low abundance in individuals suffering from inflammatory bowel diseases [29] [30], [31]. *Enterococcus faecium* increased following chemotherapy, possibly filling the ecological niches vacated by the lactobacilli and bifidobacteria. *Enterococcus faecium* is a facultative pathogenic bacterium causing life-threatening infections especially in nosocomial settings [32]. *Enterococcus faecium* has previously been found to increase in wastewater upon treatment [33]. The acquisition of multi-resistant *E. faecium* strains has been described in hospital environments under high selective antibiotic pressure. Under such conditions probiotic strains were demonstrated as unable to prevent nosocomial infection [34].

After chemotherapy less abundant sequences appeared that were not detected before treatment. These genera are: *Eggerthella*, *Megasphaera*, *Parvimonas*, *Anaerostipes*, *Eubacterium*, *Anaerococcus*, *Methylobacterium*, *Holdemania*, *Turicibacter*, *Akkermansia*, *Sutterella*, *Sphingomonas*, *Anaerotruncus*, *Coprococcus*, *Streptococcus* and *Dorea*. *Eggerthella lenta* was described to convert dietary lignans to the bioactive enterolactone [35]. *Megasphaera* spp. have been described as propionate-producers that utilize lactate [36] comparable to *Veillonella* spp. that were no longer detected by 454 sequencing after chemotherapy. The butyrate-producing *Anaerostipes caccae* and *Eubacterium hallii* utilize lactate as well. They were suggested to compete for lactate with sulfate-reducing bacteria such as *Desulfobacter piger* whose preferred co-substrate is lactate. High concentrations of sulfate are toxic for the gut epithelium and may contribute to bowel disease [37]. Microorganisms of the genus *Methylobacterium* are facultative methylotrophic, gram-negative rods that are ubiquitous in nature and rarely cause human disease, except in subjects with pre-existing immunosuppression. For instance, in 2010, a case of *M. fujisawaense* infection was described in a patient with relapsed acute leukemia undergoing unrelated allogeneic hematopoietic stem cell transplantation [38]. *Turicibacter* is a poorly known genus previously found in weaned piglets, known to be susceptible to chlortetracycline [39]. In humans, *Turicibacter* spp. have been found in the ileal pelvic pouch of a former ulcerative colitis patient [40]. *Akkermansia muciniphila* is a common mucin-degrading bacterium of the human GI. Its prevalence has been described to be 10^8 cells/g feces in adults, decreasing with age [41]. *Dorea* spp. are mucosa-associated bacteria of the human GI that are members of the *Clostridium coccoides* rRNA group of organisms [42], [43].

Further research is needed to elucidate if there is a causal relationship between growth of *C. difficile* and decreased abundance of lactobacilli, bifidobacteria and *Clostridium* cluster IV, especially the anti-inflammatory *Faecalibacterium prausnitzii*. The increase of mucus-degrading bacteria might be a result of *C. difficile* and probably also *E. faecium* associated inflammation of the gut epithelium. Mucus hypersecretion is a common symptom of irritable bowel syndrome, ulcerative colitis and bacterial infections of the gut epithelium [44], [45]. The lactate-utilizing microbiota shifted from *Veillonella*

spp. to *Anaerostipes*, *Eubacterium* and *Megasphaera* spp. This change may be interpreted as a beneficial adaptation, because lactate could otherwise be used as a co-substrate for sulfate-reduction. Sulfate-reducing bacteria, however, were not detected here.

The oncology patients assessed here suffered from a variety of malignancies and received different chemotherapy treatment regimes. Only two participants (P01 and P08) had never received any cancer therapy before, while all others had a history of chemotherapeutic treatment. Therefore, the observed changes are likely to be influenced by previous cycles of chemotherapy. For example, the significantly lower bacterial abundance in cancer patients before chemotherapy in comparison to control could be a consequence of previous treatments. The results presented here illustrate changes due to a single chemotherapeutic cycle, but cannot rule out, that these changes occurred as a consequence of several chemotherapeutic cycles over the course of several years. Six cancer patients also received antibiotics. These patients were characterized by significantly elevated abundances of bacteria. This finding confirms the diagnosis 'bacterial infection' for which antibiotic treatment was prescribed. *Clostridium* cluster IV PCR-DGGE profiles revealed a shift in species composition by chemotherapy, and even more so by antibiotics. Thus we conclude that antimicrobial treatment significantly reduces the species richness of the *Clostridium* cluster IV, with the anti-inflammatory *Faecalibacterium prausnitzii* being the most abundant representative. Van Vliet *et al.* (2009) tested the effect of chemotherapy *in vitro* and showed a direct bacteriostatic effect of chemotherapeutics on bacterial growth.

Further research is needed to show whether changes in bacterial colonization play a role in the development and maintenance of mucosal barrier function, infection and inflammation [17].

The use of prebiotics, probiotics and bacterial products, such as butyrate to prevent mucosal barrier injury and its complications could be a promising concept in restoring impaired functions or enhancing specific desirable functions of the microbiota. The use of pre- and probiotics to affect the composition and metabolic activity of the fecal microbiota in times of cancer chemotherapy and immunosuppression might be part of future research.

In conclusion, chemotherapy treatment causes changes in fecal microbiota, which coincide with the development of *C. difficile* infection in some patients. These changes in microbiota may have systemic effects and may contribute to the development of chemotherapy-induced mucositis, influencing important beneficial functions of the microbial ecosystem.

Materials and Methods

Ethics statement

The Viennese Human Ethics committee (3., Thomas-Klestil-Platz 8/2) under the chair of Dr. Karin Spacek, approved the proposal of the project "Analysis of microbiota in feces of patients with immunosuppression". Votum: EK 07-153-VK, 2008. From all participants involved in the study written consent was obtained.

Study participants and study design

Seventeen subjects receiving ambulant chemotherapy with or without antimicrobial therapy (aged 59±13 y, BMI 27±6) from the Sozialmedizinisches Zentrum Ost (SMZ Ost) in Vienna and seventeen healthy individuals (aged 65±18 y, BMI 24±5) joined this study. Four fecal samples within two weeks were collected of each ambulant oncology patient in order to collect samples before and after a single immune-suppressive chemotherapy cycle. The four samples obtained from every patient were grouped into three groups: samples taken before the day of chemotherapy (T0), samples taken 1–4 days after chemotherapy (T1) and samples taken 5–9 days after chemotherapy (T2). Healthy individuals also donated four samples during two weeks. Gender ratio among healthy controls was 56% female, 44% male. Oncology patients were 47% female and 53% male. Three out of seventeen patients (P04, P08, P13) had never received any chemotherapy before, while the others had a history of chemotherapy. Anonymous medical records reported types of malignancies as well as chemotherapeutic and antimicrobial treatment as shown in table 2.

Table 2. Relevant clinical data of study participants undergoing immunosuppressive chemotherapy.

Table 8

name	diagnosis	chemotherapeutic treatment	antimicrobial treatment	other condition
P01	urothel carcinoma	gemcitabine, cisplatinum		
P02	plasmocytoma, multiple myeloma	bortezomib, dexamethasone		rheumatism fever at 4 th sampling
P03	Non-Hodgkin lymphoma	bendamustine		diabetes II, adipositas, hypertension
P04	ovarian fibroma	taxol, carboplatin	levofloxacin	
P05	multiple myeloma	bortezomib, doxorubicin, dexamethasone	cotrimoxazole	osteoporosis
P06	mamma carcinoma	pegylated liposomal doxorubicin hydrochloride, gemcitabine		
P07	Non-Hodgkin lymphoma	high dose radiation therapy and PBSCT	cotrimoxazole, piperacillin, tazobactam	fever at 2 nd and 3 rd sampling
P08	monocytic leukemia	cytarabine, idarubicin	cotrimoxazole, piperacillin, tazobactam	fever at 2 nd sampling
P09	acute leukemia	high dose Ara-C, radiated erythrocyte concentrate	piperacillin, tazobactam	fever at 2 nd sampling
P10	Non-Hodgkin lymphoma	ifosamid, etoposid, methotrexat	levofloxacin	
P11	Acute lymphoblastic leukemia	cytarabine, methotrexat		adipositas, hypertension
P12	small intestinal tumor	cetuximab		
P13	rectal tumor	capecitabine, oxaliplatin		
P14	thymus tumor	taxol, carboplatin, bevacizumab, radiation		
P15	Acute lymphoblastic leukemia	cyclophosphamide, methotrexate, doxorubicin, cytarabine, vincristine		diabetes II
P16	Acute lymphoblastic leukemia	cytarabine, mitoxantrone		
P17	colon tumor	oxaliplatin, capecitabine, bevacizumab, irinotecan, monoclonal antibodies		

PBSCT... peripheral blood stem cell transplant.
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We interviewed all study participants assessing age, gender, body length, weight, health status (chronic and acute diseases), and life-style aspects such as alcohol consumption and physical activity. Dietary habits were assessed using a food frequency questionnaire. Exclusion criteria for healthy controls were (a) antimicrobial medication (b) chemotherapeutic treatment and (c) pre- and probiotics at least three months before sample collection. Approval for this study was obtained from the Viennese Human Ethics committee (3., Thomas-Klestil-Platz 8/2).

Stool sample processing

After collection, study participants immediately stored their samples at -18°C in their homes. Stool samples were still frozen when brought to the laboratory and then immediately stored at -70°C. A 200 mg aliquot of each sample was treated twice for 45 s in a bead-beater (Mini-Beadbeater-8). Thereafter DNA was extracted using the QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol. The DNA was stored at -20°C until analysis.

Type strains

We used type strains, known to be part of the human gastrointestinal microbiota and cloned sequences to design a DGGE standard lane marker. Type strains *Bacteroides thetaiotaomicron* DSM 2079T, *Enterococcus faecium* DSM 20477T, *Lactobacillus reuteri* ATCC 55730T, *Bifidobacterium longum* ssp. *longum* DSM 20097T, *Escherichia coli* IMBH 252/07 and clones CL16 and CC34 (see below) were used for creating a comparable standard lane marker for DGGE gels analyzing all bacteria. *E.coli* IMBH 242/07 gave 4 bands due to its multiple operons for the 16S rRNA gene.

Clone library

To create a standard lane marker for DGGE analysis and to identify members of the *Clostridium* cluster *XIVa* we constructed clone libraries from two stool samples of healthy volunteers. For this purpose PCR products amplified with primers 195-F [46] and Ccocc-R [47] were inserted into a p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Nucleotide sequences were corrected for primer and vector sequences in CodonCodeAligner (www.codoncode.com) and taxonomically identified using the online tools of the ribosomal database project (<http://rdp.cme.msu.edu/>). The clone library used for creating a standard lane marker for DGGE analysis of *Clostridium* cluster *IV* has previously been described [48]. Clones CL16 (*Clostridium leptum* 16) and CC34 (*Clostridium coccoides* 34) were also used as positive controls in Taqman qPCR.

Polymerase chain reaction (PCR)

PCR was carried out amplifying 16S rRNA gene sequences from bacteria in fecal samples, type strains and cloned sequences for DGGE analysis as well as for creation of the clone library using group- and kingdom- specific primers (table 3). The PCR reaction mixture consisted of ready-to-use mastermix (Promega) with 1.5 mM MgCl₂, 500 nM of primers and 2 µl of template DNA. When amplifying fecal samples, bovine serum albumin (Fermentas) was added to a final concentration of 400 µg/ml. We used a Robocycler (Stratagene) for all amplifications.

Table 9

Target organism	Primer	Sequence (5'-3')	Ann. temp (°C)	Reference
All bacteria	27f	GTGCTGCAGAGTTTGATCCTGGCTCAG	57	[52 T., Blocker 1989]
	985r	GTAAGTTCTTCGCGTT	57	[53]
	341f-GC	CCT ACG GGA GGC AGC AG	55	[49]
	518r	ATT ACC GCG GCT GCT GG	55	[54]
<i>Clostridium</i> cluster IV	sg-Clept-F-GC	GCA CAA GCA GTG GAG T	55	
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		[55]
<i>Clostridium</i> cluster XIVa	Ccocc-F-GC	AAATGACGGTACCTGACTAA	55	
	Ccocc-R	CTTTGAGTTTCATTCTTGCGAA		[55]
GC-clamp		CGCCCGGGCGCGCCCGGGCGGCCCGGGGCACCGGGGG		[49]

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Table 3. 16S rRNA gene primers used for PCR-DGGE fingerprinting.

PCR-DGGE-fingerprinting

DGGE was performed as previously described [49]. Primer pairs and annealing temperatures to analyze the diversity of (a) all bacteria, (b) *Clostridium* cluster IV and (c) *Clostridium* cluster XIVa are described in table 3. PCR products were separated by polyacrylamide gels with a denaturing gradient of 30–60% for predominant bacteria, 30–50% for *Clostridium* cluster IV and 35–50% for *Clostridium* cluster XIVa. Electrophoresis was performed for 9 h at 129 V at 60°C (predominant bacteria), 5 h at 200 V at 60°C (*Clostridium* cluster IV) and 7 h at 200 V at 60°C (*Clostridium* cluster XIVa). Standard lane markers were created for each DGGE analysis assay to ensure reliable gel-to-gel comparison. These standard lane markers (described above) were loaded in triplicate on each gel to adjust for gradient-variations between gels. We analyzed PCR-DGGE fingerprints using GelComparII (www.applied-maths.com). When generating the band comparison, a 1% tolerance was selected. Principal components analysis (PCA) was applied on quantitative band comparison datasets in 'R' (www.r-project.org) using the default settings. Shannon diversity index was calculated on quantitative band information as well with the default settings implemented in the 'vegan' package in 'R' (www.r-project.org). Shannon index is defined as $H = -\sum p_i \ln p_i$, where p_i is the proportional abundance of species i . In short, the higher the Shannon index is, the higher is the diversity. For interpretation of results, samples were grouped into three groups: samples taken before the day of chemotherapy (T0), samples taken 1–4 days after chemotherapy (T1) and samples taken 5–9 days after chemotherapy (T2).

The abundance of bacteria and bacterial subgroups was measured by 16S rRNA gene-targeting TaqMan qPCR in a Rotorgene 3000 (Corbett Life Science). Primers, annealing temperatures and expected product sizes are shown in table 4. Each sample was analyzed in duplicate. Amplifications were carried out in a total volume of 10 µl consisting of 5 µl Taq-Man SensiMix DNA Kit (Quantance), 1 µl of each primer and Taq-Man probe (concentrations see table 4) and 10 ng of bacterial DNA. Amplification programs included an initial denaturation at 95°C for 10 min followed by 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C (all bacteria, *Clostridium* cluster IV), 56°C (*Clostridium* cluster XIVa), 58°C (*C. difficile*) or 60°C (*Bacteroides*, bifidobacteria) for 30 s and extension at 72°C for 50 s.

Table 10

Target organism	Primer and probe	Sequence (5' - 3')	Size (bp)	Conc. (nM)	Reference
<i>Bifidobacterium</i> spp.	Forward primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	
	Reverse primer	CAC CCG TTT CCA GGA GCT ATT		300	
	Probe	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)		150	[56]
<i>Bacteroides</i>	AlIBac296f	GAG AGG AAG GTC CCC CAC	106	300	
	AlIBac412r	CGC TAC TTG GCT GGT TCA G		300	
	AlIBac375Bhqr	(FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)		100	[53]
All bacteria	BAC-338-F	ACT CCT ACG GGA GGC AG	468	1000	
	BAC-805-R	GAC TAC CAG GGT ATC TAA TCC		1000	
	BAC-516-P	(FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		200	[50]
<i>Clostridium</i> cluster IV	sg-Clept-F	GCA CAA GCA GTG GAG T	239	400	
	sg-Clept-R3	CTT CCT CCG TTT TGT CAA		400	[47]
	Clept-P ⁺⁺	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		200	This study
<i>Clostridium</i> cluster XIVa	195F	GCA GTG GGG AAT ATT GCA		500	[46]
	CcoccR	CTT TGA GTT TCA TTC TTG CGA A		500	
	CcoccP	(6-FAM)-AAATGACGGTACCTGACTAA-(BHQ-1)		150	[47]
<i>Clostridium difficile</i>	CdiffF	TTG AGC GAT TTA CTT CGG TAA AGA		1000	[56]
	CdiffR	TGT ACT GGC TCA CCT TTG ATA TTC A	151	1000	
	CdiffP	(6-FAM)-CCA CGC GTT ACT CAC CCG TCC G-(BHQ-1)		200	

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Table 4. Primers and probes used for TaqMan qPCR quantification of 16S rRNA genes.

We used tenfold serial DNA dilutions of type strains *Bacteroides thetaiotaomicron*^T, *Bifidobacterium longum* ssp. *longum*^T and *C. difficile* as well as cloned sequences and one fecal sample to construct standard curves for comparison of PCR reaction efficiencies among different experiments.

We quantified DNA of *Bacteroides thetaiotaomicron*^T, *Bifidobacterium longum* ssp. *longum*^T and *C. difficile*, using the nanodrop method and calculated DNA copies/μl through mean G+C content of each strain. Clones CL16 and CC34 were amplified with the SP6 Promoter Primer (Promega, Cat.# Q5011) and the T7 Promoter Primer (Promega, Cat.# Q5021) and the PCR product quantified using a nanodrop machine. Knowing the sequences of these two PCR products and their flanking vector sequences we could quantify the copy numbers and use it as standards. Relative percentages of bacterial subgroups were calculated in relation to total rRNA gene copies amplified with primer pair BAC-338-F and BAC-805-R [50].

We reviewed sensitivity of PCR reactions with stepwise dilutions of standard curve DNA until we achieved sensitive detection levels of PCR. The specificity was confirmed using non-target DNA.

High throughput sequencing

In total, four samples (P09-T0, P09-T1, P11-T0, P11-T1) were amplified with primer 525F (5'-TCAGCAGCCGCGGTAATAC -3') and 926R (5'-TCCGTCAATTCCTTTGAGTTT -3') using a high-fidelity DNA polymerase (Phusion®, Finnzymes, Thermo Fisher Scientific) and submitted to 454 barcode sequencing (AGOWA, Berlin, Germany), resulting in a total of 113000 reads. The sequences were trimmed and aligned using the pyro pipeline of the ribosomal database project (<http://rdp.cme.msu.edu/>). Only sequences longer than 150 bp were retained, resulting in 3886 to 6811 sequences per sample with the average lengths of 366 to 368 bp. All analyses were performed using the online tools of the ribosomal database project pyro pipeline (<http://rdp.cme.msu.edu/>). Results of the phylogenetic classification are shown as a heatmap [51]. The *Peptostreptococcaceae*,

harbouring also *C.difficile*, from all four datasets were analyzed in more detail using the online tools of the ribosomal database project. 100% similar sequences were grouped and their abundances shown together with a phylogenetic tree.

Data analysis

Statistical evaluation of differences between groups (chemotherapy and control) and changes within the chemotherapy group (all time points before and after chemotherapy) was carried out using the OriginPro version 8 (OriginLab, Northampton, MA). For two group comparisons of independent ordinal and interval values we used the two-sample t-test and the nonparametric Mann-Whitney U-test. For the analysis of related data we used the paired sample t-test or the non-parametric Wilcoxon signed-rank test. P values <0.05 were considered statistically significant. To show the decline in abundance immediately after chemotherapy qPCR results were plotted in heatmaps [51]. Values were z-scored for presentation in this heatmap showing changes over time rather than absolute abundances.

Dietary aspects

We assessed the participants' dietary habits using a food frequency questionnaire. All study participants (patients and controls) were omnivores and showed similar consumption patterns of liquids, alcohol, fruits, vegetables, grains and milk products. Healthy controls stated more frequent consumption of fruits, whole grain products and alcohol several times a week compared to patients receiving chemotherapy.

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Author Contributions

Conceived and designed the experiments: JZ RR AGH. Performed the experiments: JZ AP CL BH OJS. Analyzed the data: JZ CL BH AP OJS RR AGH. Contributed reagents/materials/analysis tools: EK MR RR. Wrote the paper: JZ AGH.

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Abundance and diversity of microbiota in type 2 diabetes and obesity

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Abstract

Inflammatory reactions leading to the progression of metabolic syndrome contribute to changes in composition of the GIT microbiota.

We characterize fecal microbiota at three time points in fourteen obese participants, nineteen lean controls and twenty-four type 2 diabetes patients. Obese and type 2 diabetics received an intervention of nutritional counseling, type 2 diabetics an additional therapy with a GLP-1-Agonist. The microbiota composition was analyzed for abundance and diversity by quantitative real-time polymerase chain reaction, denaturing gradient gel electrophoresis and high throughput sequencing.

In type 2 diabetics an increase of diversity was observed with intervention whereas the values of lean controls remained unaffected. In the lean and obesity groups, a lower *Firmicutes: Bacteroidetes* ratio correlated with lower BMI. In type 2 diabetics the ratio of *Firmicutes* to *Bacteroidetes* increased throughout the intervention period. Type 2 diabetics showed a significantly enhanced proportion of lactic acid bacteria before and after intervention, also *Akkermansia* and *Enterobacteria* showed a higher abundance in type 2 diabetics, increasing throughout the study period. *Archaea* were significantly more frequent in type 2 diabetics.

Whether differences seen in abundance of certain groups and diversity of microbiota reflect different underlying inflammatory mechanisms of type 2 diabetes and obesity or rather the progression of the metabolic disease remains unclear and needs long-term investigation.

Keywords

GLP-1-Agonist; metabolic syndrome; ratio *Firmicutes: Bacteroidetes*; lactic acid bacteria; *Akkermansia*; *Prevotella*; Enterotypes

Abbreviations

ANOVA- analysis of variance
AMPK - AMP-activated protein kinase
BMI – body mass index
DGGE – denaturing gradient gel electrophoresis
DSM – Deutsche Stammsammlung von Mikroorganismen
FFG – Austrian Förderungsgesellschaft
FFQ – food frequency questionnaire
FIAF - fasting-induced adipose factor (or angiopoietin-like protein 4)
GI – gastro intestinal
GLP – glucagon-like peptide
Gpr – G-protein coupled receptor
HbA_{1c} – glycol hemoglobin
IL– interleukin
LAB – lactic acid bacteria
LPS - lipopolysaccharide
MCP-1 – monocyte chemo attractant protein-1
P – probability
PAI-1 - plasminogen activator inhibitor-1
PC – principal component
PCA – principal component analysis
PCR – polymerase chain reaction
PYY – Peptid YY
qPCR – quantitative polymerase chain reaction
rDNA – ribosomal DNA
SCFA – short chain fatty acid
T – time point
TLR – toll like receptor
TNF α – tumor necrosis factor alpha
UPGMA - unweighted pair group method with arithmetic mean
UK – United Kingdom

Introduction

The gut microbiota is an integral part of a complex network coordinating the physical and chemical elements of the intestinal barrier together with the immune, sensory, neuromotor- and enteroendocrine systems (Verdu, 2009). It bridges the indeterminate gap between food and weight by affecting the host metabolism through regulation of intestinal glucose absorption, lipogenesis and fat deposition. Housekeeping functions such as the central carbon metabolism or the amino acid synthesis of important protein complexes are present in every bacterium. Secondary metabolisms are related to a minimal metagenome, for example the biodegradation of complex sugars and glycans (Gerritsen *et al.*, 2011). Profiles of human gut microbiota reflect the metabolic cooperation between different phylotypes since no genus can degrade all substrates; for example establishing all short chain fatty acids (SCFAs). SCFAs have a role in the regulation of energy metabolism, immunity and adipose tissue expansion (Delzenne & Cani, 2011). Gut microbiota of all human beings fulfill core functions, but differ in specialist functions, as a consequence some communities are linked to human diseases and obesity more than others (Gerritsen *et al.*, 2011).

Observations of experiments with germ-free mice compared to conventional raised mice or after conventionalization showed independent from diet a weight gain according to gut microbiota (Musso *et al.*, 2010). Studies on the relationship between gut microbiota composition and obesity show an increased number of *Firmicutes* and a reduction of *Bacteroidetes* in obese mice and humans compared to lean controls (Ley *et al.*, 2006, Turnbaugh *et al.*, 2006, DiBaise *et al.*, 2008, Musso *et al.*, 2010). A controlled diet and weight-loss reversed the observed ratio of *Firmicutes* to *Bacteroidetes* (Cani *et al.*, 2008, Duncan *et al.*, 2008, Musso *et al.*, 2010, Schwiertz *et al.*, 2010). The number of *Bacteroidetes* depends on the weight loss rather than on caloric intake (DiBaise *et al.*, 2008, Diamant *et al.*, 2011) whereas the *Firmicutes* group remains unchanged and no correlation with total caloric intake can be detected (Cani *et al.*, 2008). The ratio reaches a lean type profile after a one-year period of diet-induced weight loss (Wu *et al.*, 2010). The abundance of *Firmicutes* in diabetic persons is significantly lower compared to non-diabetics, while *Bacteroidetes* and *Proteobacteria* are more abundant in diabetics. The ratio of *Bacteroidetes* to *Firmicutes* is significantly and positively correlated with reduced glucose tolerance, but negatively correlated with higher BMI. Especially the relative abundance of *Bacilli* is increased in diabetic mice models and humans. Relative abundances of *Actinobacteria* and *Verrucomicrobia* do not differ between diabetic and non-diabetic individuals. *Bacteroides-Prevotella* versus *C. coccoides* - *E. rectale* groups are positively associated with plasma glucose, but negatively correlated with BMI (Larsen *et al.*, 2010). Wu *et al.* (2009) found a higher abundance of *Bacteroides* (frequency of 53,6 %), and lower abundance of genus *Prevotella* (10,7 %), genus *Proteobacteria* (3,6 %) and phylum *Firmicutes* (10,8 %) compared to healthy volunteers in sequence analysis of selected DGGE amplicons, whereas qPCR results indicate a lower copy number of *Bacteroides vulgatus* and *Bifidobacterium* in the fecal microbiota. The *Clostridium leptum* cluster was more abundant in the diabetic than in the healthy group (Backhed *et al.*, 2004, Musso *et al.*, 2010, Diamant *et al.*, 2011). It is estimated that subjects with type 2 diabetes are relatively enriched with Gram-negative bacteria, belonging to the phyla *Bacteroidetes* and *Proteobacteria*, which can elicit endotoxemia (Larsen *et al.*, 2010) through increased plasma LPS (Cani *et al.*, 2008, Cani &

Delzenne, 2009, Tsukumo *et al.*, 2009, Larsen *et al.*, 2010, Luoto *et al.*, 2010, Diamant *et al.*, 2011). Obese individuals may have a microbiota more efficient in extracting energy and may be enriched in enzymes for the breakdown of otherwise indigestible alimentary polysaccharides and in other transport proteins and fermentation enzymes and further induce insulin resistance and diabetes (Musso *et al.*, 2010). Metabolic endotoxemia significantly correlates with oxidative stress, macrophage infiltration markers and all inflammatory markers triggering insulin resistance (PAI-1, IL-1, IL-6 and TNF- α) (Cani *et al.*, 2008, Cani & Delzenne, 2009, Caesar *et al.*, 2010, Musso *et al.*, 2010). In a high fat diet elevated LPS increase gut permeability, by affecting GLP-1, GLP-2 and PYY (Cani *et al.*, 2008, Diamant *et al.*, 2011). An absence of LPS receptor CD14 reduces the high-fat diet induced effects on adipose tissue inflammation, blood glucose profiles, insulin resistance index, glucose-induced insulin secretion (Cani *et al.*, 2008, DiBaise *et al.*, 2008, Cani & Delzenne, 2009), hepatic steatosis, liver inflammation and adipose tissue macrophages infiltration (Cani *et al.*, 2008). Metabolic endotoxemia is indicated by a change in the intestinal microbiota as antibiotic treatment reduces plasma LPS levels (Cani *et al.*, 2008, DiBaise *et al.*, 2008, Cani & Delzenne, 2009, Diamant *et al.*, 2011). An early modification of the microbiota with probiotics or prebiotics might be a beneficial influence (Diamant *et al.*, 2011).

The impact of the GLP-1-Agonist Liraglutide on blood glucose level, weight management and systolic blood pressure was conducted e.g. in the LEAD (Liraglutide Effect and Action in Diabetes) and in the DURATION study programs. GLP-1-Agonists lead to a glucose-dependent increase of insulin secretion in beta cells and suppress the glucagon secretion in pancreatic alpha cells. Additionally, it slows the gut passage and therefore decreases the postprandial spikes in blood glucose. Furthermore, GLP-1-Agonists decrease body weight, Liraglutid showed in one study that 25 % of patients lost significant weight, on average 7.7 kg (Nauck *et al.*, 2009).

The objective of this study was to investigate differences of gut microbiota in obese and type 2 diabetics, and the response to intervention for estimating its potential role in controlling metabolic syndrome.

Materials and methods

Ethics statement

The project “Abundance and diversity of microbiota in type 2 diabetes and obesity” was approved by the Viennese Human Ethics committee. We received a signed form of consent from all study participants for using stool samples and data obtained from food frequency questionnaires for the analyses presented here.

Study participants and study design

Twenty-four insulin-dependent type 2 diabetes patients (aged 56 +/- 9) were enrolled in cooperation with a Diabetes Outpatient Clinic. In addition we enrolled fourteen obese participants with no established insulin resistance (aged 38 +/- 14) and nineteen healthy lean controls (aged 30 +/- 8) (Table 1). Obese and type 2 diabetics received an intervention of nutritional counseling, type 2 diabetics an additional therapy with GLP-1-Agonists (Liraglutid). To ensure comparable data, patients

were interviewed for their history of gastrointestinal diseases, use of antibiotics, probiotics or prebiotics. BMI, age and lifestyle habits were assessed in combination with a retrospective food frequency questionnaire.

Fecal samples were collected at three occasions; before treatment, during and after treatment, with a distance of 1 month between the first two time points and 3 months between the second and third time point. Controls also donated three fecal samples over the course of four months.

Table 1: Characterization of participant groups.

Table 11

Group	characterization	rate	BMI ($\pm$ SD) [kg/m ²]
LC	Lean control group, no treatment	19	21.78 ($\pm$ 2.35)
OC	Obese patients, intervention with nutritional counseling	14	33.71 ($\pm$ 3.97)
D	Type 2 diabetic, treatment with nutritional counseling and GLP-1 Agonist	24	38.01 ($\pm$ 5.81)

Stool sample processing and extraction

Stool samples were stored immediately after collection at -18 °C in subject's home freezer and at -70 °C upon arrival in the laboratory.

Total bacterial DNA was extracted from fecal samples using the QIAamp® DNA Stool mini kit (QIAGEN, GmbH, Germany) according to the manufacturer's protocol. Additional samples were treated in FastPrep™ Lysing Matrix E tubes twice for 45 sec in a bead-beater (Mini-Beadbeater-8) with an intervening minute on ice. DNA concentration and quality was determined by picodrop (Picodrop, UK) and agarose gel-electrophoresis.

Clone library

For further analysis in qPCR we created clone libraries from dominant members of *Akkermansia muciniphila*, *Prevotella intermedia* and *Lactobacillus casei* in two stool samples of healthy volunteers. PCR-products amplified with group specific primers (table 2) were inserted into p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Clone libraries were screened according to Schabereiter-Gurtner et al., 2001 (Schabereiter-Gurtner *et al.*, 2001). The obtained nucleotide sequences from Sanger sequencing were corrected for vector sequences and taxonomically identified using the ribosomal database project 10 (<http://rdp.cme.msu.edu/>).

Real-time qPCR

Bacterial abundance was quantified by qPCR using TaqMan qPCR and SYBR Green qPCR in a Rotorgene 3000 (Corbett Life Science) using 16S rDNA group specific primers (Tables 2-3). Specificity

was checked with the ProbeMatch function of the ribosomal database project 10 (<http://rdp.cme.msu.edu/>). The PCR reaction mixture and serial DNA dilution of typically strains were prepared according to Pirker et al. 2012 (Pirker *et al.*, 2012).

Table 2: Primers and TaqMan®-probes targeting 16S rDNA coding regions of bacteria and archaea.

Table 12

Target organism	Primer/Probe	Sequence (5' - 3')	Size (bp)	Conc. [pmol/μL]	Reference
All <i>Bacteria</i>	Fwd primer	ACT CCT ACG GGA GGC AG	468	10	[1]
	Rev primer	GAC TAC CAG GGT ATC TAA TCC		10	
	Probe	(6-FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		2	
<i>Clostridium</i> cluster IV (<i>Ruminococcaceae</i>)	Fwd primer	GCA CAA GCA GTG GAG T	239	4	[2]
	Rev primer	CTT CCT CCG TTT TGT CAA		4	
	Probe	[3]-AGG GTT GCG CTC GTT-(BHQ-1)		2	
<i>Cluster XIVa</i> (<i>Lachnospiraceae</i>)	Fwd primer	GCA GTG GGG AAT ATT GCA	477	5	[4]
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A		5	
	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)		1,5	
<i>Bacteroidetes</i>	Fwd primer	GAG AGG AAG GTC CCC CAC	106	3	[5]
	Rev primer	CGC TAC TTG GCT GGT TCA G		3	
	Probe	(6-FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)		1	
<i>Bifidobacterium</i> spp.	Fwd primer	GCG TGC TTA ACA CAT GCA AGT C	125	3	[6]
	Rev primer	CAC CCG TTT CCA GGA GCT ATT		3	
	Probe	(6-FAM)-TCA CGC ATT ACT CAC CCG TTC GCC-(BHQ-1)		1,5	
Archaea	Fwd primer	ATT AGA TAC CCG GGT AGT CC	1044– 1059	4	[7]
	Rev primer	GCC ATG CAC CWC CTC T		4	
	Probe	(6-FAM)-AGG AAT TGG CGG GGG AGC AC-(BHQ-1)		4	

Table 3: Primers (SYBR® Green) targeting 16S rDNA coding regions of bacteria and butyryl-coenzyme A (CoA) CoA transferase genes.

Table 13

Target organism	Primer	Sequence (5' - 3')	Size (bp)	Conc. [pmol/μL]	Reference
Lactic Acid Bacteria (LAB)	Fwd primer	AGC AGT SGG GAA TCT TCC A	352-700	4	[8]
	Rev primer	ATT YCA CCG CTA CAC ATG		4	
BcoAT gene	Fwd primer	GCI GAI CAT TTC ACI TGG AAY WSI TGG CAY ATG	~540	27	[9]
	Rev primer	CCT GCC TTT GCA ATR TCI ACR AAN GC		27	
Enterobacteria	Fwd primer	AGC ACC GGC TAA CTC CGT	492-509	3	[10]
	Rev primer	GAA GCC ACG CCT CAA GGG CAC AA	834 - 856	3	[11]
Prevotella	Fwd primer	CACCAAGGCGACGATCA	1458	2,5	[12]
	Rev primer	GGATAACGCCYGGACCT		2,5	
Akkermansia	Fwd primer	CAGCACGTGAAGGTGGGGAC	1505	2,5	[13]
	Rev primer	CCTTGCGGTTGGCTTCAGAT		2,5	

PCR/DGGE

The diversity of total bacteria was measured by DGGE using the primer set 341f-GC 5'-CCT ACG GGA GGC AGC AG-3' (Muyzer *et al.*, 1993) and 518r 5'-ATT ACC GCG GCT GCT GG-3' (Neefs *et al.*, 1991). The endpoint PCR was carried out with a ready-to-use GoTaq® Green Master Mix (Promega) with 1.5 mM MgCl₂ in a 96-well Gradient Thermal Cycler (Labnet MultiGene™).

DGGE gels were prepared as described previously (Muyzer & Smalla, 1998) with a linear gradient of 25–65 % for total bacteria using a peristaltic pump. The reference marker contained 16S rDNA gene fragments of *Lactobacillus reuteri* DSM 20016, *Escherichia coli* 1029, *Enterococcus faecium* DSM 20477, *Clostridium blautia coccoides* DSM 935, *Clostridium leptum* DSM 753, *Bifidobacterium longum* sp. *longum* DSM 20219 and *Bacteriodes thetaiomicron* DSM 2079.

High throughput sequencing

Sixteen samples were analyzed with Roche GS FLX+ Titanium next generation sequencing (LGC sequencing GmbH, Berlin). For alignment and further analyses we used the pyro pipeline of the ribosomal database project 10 (<http://rdp.cme.mus.edu/>) and qiime. We then performed significance tests, UPGMA clustering, and principal coordinate analysis in qiime. We used jackknifing to determine results.

Statistical analysis

We used the non-parametric Mann-Whitney U test and for three unpaired groups the non-parametric Kruskal Wallis ANOVA for comparison of the non-parametric unpaired values in OriginPro version 8 (OriginLab, Northampton, MA). P values < 0,05 were determined as statistically significant. DGGE-gels were evaluated with Gel compare II (Applied Maths).

Results

All used methods indicate high individual variability. However the variation within samples of the same individual over time was consistently lower than variation between subjects. Our approach was to first compare the abundance of each bacterial group individually, then to calculate the *Firmicutes* to *Bacteroidetes* ratio and finally to compare our results with discussed enterotypes. We applied PCR-DGGE fingerprinting to estimate the species richness of all bacteria and selected sixteen samples for 454 sequencing to obtain in-depth data on microbial diversity.

Analyses of quantitative GI microbiota composition

We did not detect any differences between the three groups in total bacterial abundance. However, the microbial composition showed significant differences between type 2 diabetics, lean controls and obese participants. The most abundant bacterial groups in lean, obese and type 2 diabetics were the Gram-positive bacteria belonging to the *Clostridium* Cluster IV and the *Clostridium* Cluster XIVa as well as the Gram-negative *Bacteroidetes*, which altogether made up a median proportion of more than 95 % of all detected bacteria. The ratio of *Firmicutes*: *Bacteroidetes* increased in type 2 diabetics under dietary intervention and therapy with GLP-1-Agonist during the study period (T3 p = 0.04) from an already higher *Firmicutes*: *Bacteroidetes* ratio at the first time point (p = 0.02). In the obese and lean groups, a lower *Firmicutes*: *Bacteroidetes* ratio correlated with lower BMI. *Clostridium* Cluster IV and *Clostridium* Cluster XIVa abundances were not significantly different between the groups or time points, but we observed a decrease in intervention of type 2 diabetics. Lactobacilli were significantly more abundant in type 2 diabetics at baseline (p=0.01) and increased over the study period (T3 p = 0.004; figure 1), compared to the other groups. High throughput sequencing confirmed these

findings. Overall type 2 diabetics were enriched in *Firmicutes* especially *Clostridiales* and *Bacilli*, especially *Lactobacillales*. In accordance *Bacteroidetes* were less abundant in diabetics, resulting in a higher *Firmicutes: Bacteroidetes* ratio. Between the first and third time point *Bacteroidetes* decreased and *Firmicutes* increased. Sequencing and qPCR were congruent in finding a *Firmicutes: Bacteroidetes* ratio of 1 in lean controls (figures 2 and 3). No significant changes could be detected within the *butyryl-CoA: acetate CoA-transferase* gene abundance between the study groups, this butyrate production gene tended to be more abundant in type 2 diabetics.

Archaea as well as species of the genera bifidobacteria, enterobacteria, *Akkermansia* and *Prevotella* accounted for the remaining proportion of the microbiota. Enterobacteria were more abundant in type 2 diabetics at all time points compared to the control group. This observation was significant at the first two time points despite greater variability in diabetics (T1 $p = 0.04$; T2 $p = 0.01$). Greater abundance of enterobacteria was also detected in obese study participants. We observed no significant differences of *Prevotella* and bifidobacteria between the three groups or time points. *Akkermansia* increased throughout the study period in type 2 diabetics, with an already higher initial abundance compared to lean controls and obese subjects in data obtained from qPCR as well as in 454 sequencing results ($p = 0.16$).

At all three time points *Archaea* were significantly ($p = 0.05$) more frequent in the type 2 diabetics (18 ± 5) compared to control groups, followed by obese patients with $4 (\pm 0)$ and lean controls (5 ± 3).

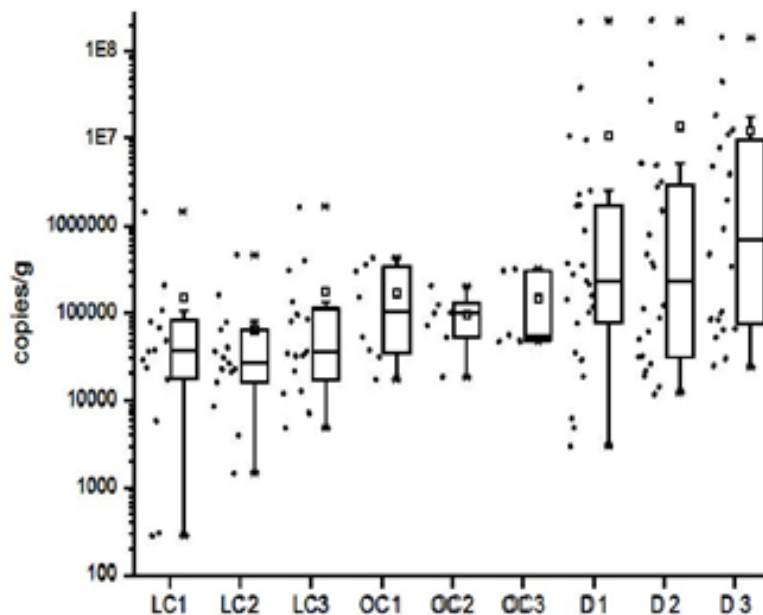


Figure 19

Figure 1: 16S rDNA qPCR quantification with primer pair for lactic acid bacteria showing significant higher abundance in type 2 diabetics (D) than obese patients (OC) and lean controls (LC).

(T1, samples taken before intervention; T2, samples taken after 1 month of intervention; T3, samples taken after 4 month of intervention)

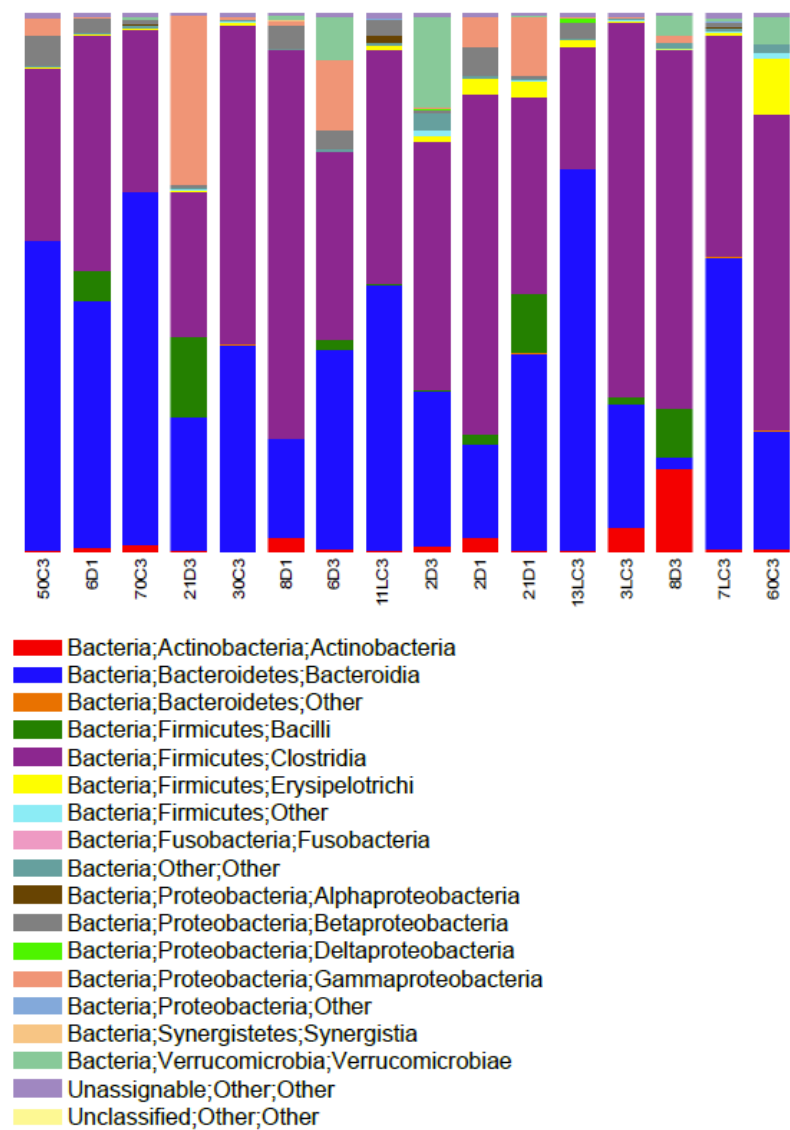


Figure 20

Figure 2: Bar Charts of 454 sequencing results reflect qPCR analysis with increasing abundance of Firmicutes especially Clostridiales and Bacilli in type 2 diabetics (D) resulting in an increasing ratio of Firmicutes:Bacteroidetes between the time points (T1, samples taken before intervention; T3, samples taken after 4 month of intervention). Verrucomicrobia and enterobacteria are also higher abundant in type 2 diabetics and enriched in obese patients (OC). Lean controls (LC) showed an according lower abundance of named bacterial 1E8 groups and higher abundance of Bacteroidetes.

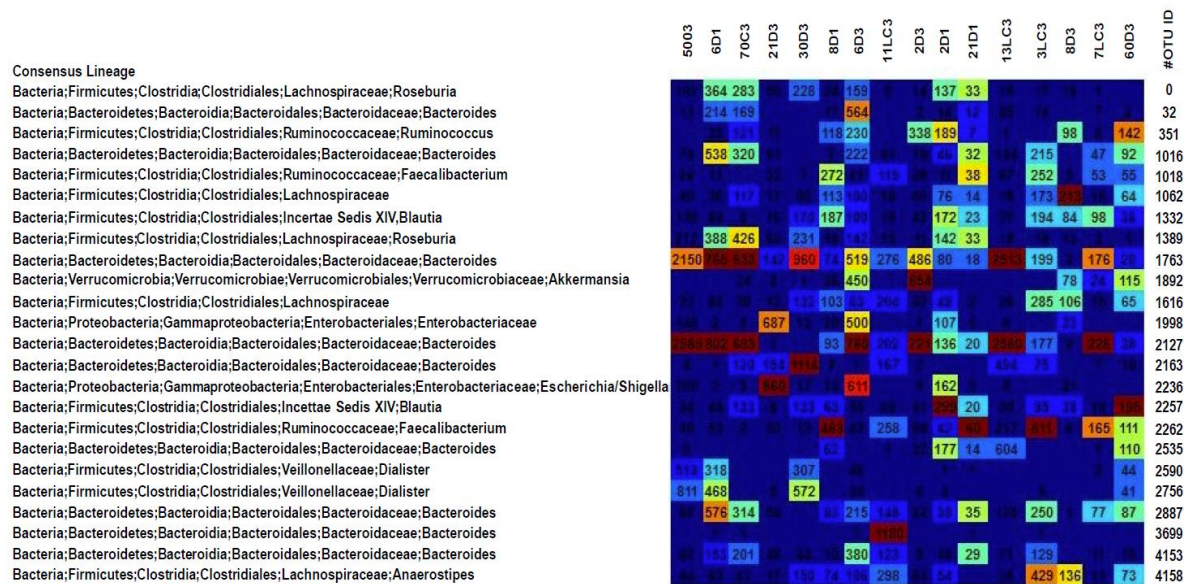


Figure 21

Figure 3: Heatmap showing abundances within the 454 sequencing dataset between the groups and time points (OC – obese patients, LC – lean control; D1 – type 2 diabetics time point one; D3-type 2 diabetics time point 3) The OUT heatmap displays raw OTU counts per sample, where the counts are colored based on the contribution of each OTU to the total OTU count present in that sample, blue indicates contributions with low percentage of OTUs to sample, whereas red contributes for a high percentage of OTUs.

Diversity analyses

DGGE fingerprinting indicated highly diverse individual differences of the microbiota. The dataset was subjected to principal component analysis (PCA). The first two principal components (PCs) explained 15.6 % and 12.0 % of variance (data not shown). Although overlapping, PCA showed grouping of band patterns according to the three groups of study participants. Between the groups a ranking of diversity was observed, as type 2 diabetes patients had the lowest diversity, followed by obese participants and the highest diversity in the lean control group. Differences between type 2 diabetics and lean controls were significant at time point 2 ($p = 0.03$) and time point 3 ($p = 0.01$) (Table 4).

Shannon's diversity index of 454 sequencing results showed no significant differences between the three groups (D 5.19 +/- 2.62; LC 6.48 +/- 0.47; OC 4.51 +/- 2.60). Lean controls had a higher Shannon index and a higher number of sequences (683.3 +/- 59.6) compared to obese controls (571.5 +/- 48.2) and type 2 diabetics (T1 645.5 +/- 22.6; T3 619.0 +/- 62.3).

PCA was also calculated on the 454 sequencing data. The first three PCs explained 44.6 %, 17.2 % and 8.3 % of the variance in the data. Samples of the diabetic group, obese group and the control group at phylum level were clustered along PC 1 and PC 2 (figure 4). In the PCA of sequencing results, samples of one participant at the two different time points and also the three different groups were

grouped according to their variance along principal components. The first PCA was attributed to *Firmicutes* and *Verrucomicrobia*, it contributed most to the separation along PC1 and *Proteobacteria*, *Actinobacteria* and *Bacteroides* contributed to the clustering along PC2. PCA of multiple genera showed grouping of the subjects with diabetes (figure 4). Additionally Jackknifing did not affect the UPGMA unifrac clustering results (figure 5).

Food Frequency Questionnaire (FFQ)

Analysis of the participant's FFQ did not reveal any differences in the consumption of grain, vegetables, fruits, meat, dietary products and fish. Mostly type 2 diabetics consumed sweets less than once a week (40.0 % T1, 28.0 % T2) in contrast 28.0 % of lean individuals consumed sweets three to five times per week and 50.0 % of obese controls consumed sweets one to three times per week. However, study participants stated an average consumption of sweets 1-15 times per week. The main differences are seen in life style patterns due to the lower physical activity in obese (37.5 % T1, 12.5% T3 never do sports) and type 2 diabetics (64.0 % T1, 52.0 % T3 never do sports), whereas 44.0 % of lean controls did sports 1-3 times per week. HbA_{1c} blood level was determined at laboratory Health Center South in Vienna. The levels were between 7-8 % in our type 2 diabetes group. 19 out of 24 type 2 diabetes patients showed an average of 3.5 % ($\pm$ 1.9) of weight reduction.

Discussion

This study focused on the characteristics of fecal microbiota in type 2 diabetic patients, before and after intervention with nutritional counseling and therapy with GLP-1-Agonist, comparing to a group of obese volunteers receiving nutritional counseling and a lean group.

Similar counts of total bacterial abundance

In this study we did not observe any significant differences in total bacterial abundance between type 2 diabetes, obese and lean controls, as a possible result of the intervention. Accordingly Larsen et al detected similar counts of total bacterial abundance in type 2 diabetics and the control group (Larsen *et al.*, 2010). Bifidobacteria showed no significant differences between the three groups and intervention, possibly reflecting no significant differences in food composition or as an indicator for the well-adjusted HbA_{1c} values. Wu et al 2010 reports a reduced abundance of bifidobacteria in type 2 diabetics in qPCR as well as in sequencing results as a possible characteristic of diabetes (Wu *et al.*, 2010). In mice a significant negative correlation has been observed between endotoxemia and *Bifidobacterium spp.*, but no relationship with any other bacterial group during treatment with high fat diet and oligofructose (Cani *et al.*, 2007). A treatment with prebiotics may improve or prevent disruption of intestinal epithelial barrier function, reduce intestinal permeability and consequently improve endotoxemia (Cani & Delzenne, 2009).

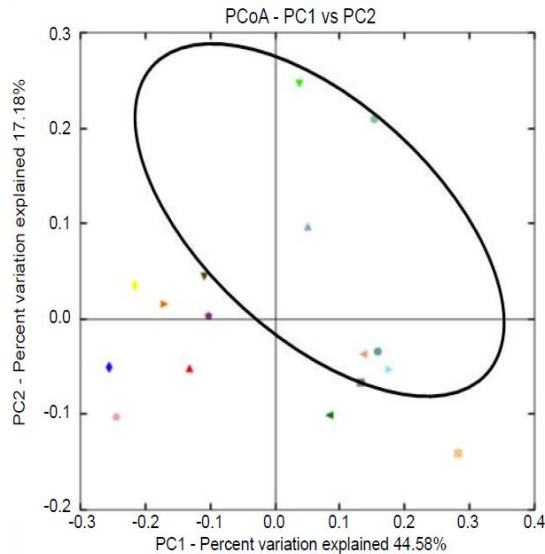


Figure 22

Figure 4: PCA based on dominant bacteria of PC1 (*Firmicutes* and *Verrumicrobia*) and of PC2 (*Proteobacteria*, *Actinobacteria* and *Bacteroides*) illustrating grouping of type 2 diabetic samples (ellipse), as a result of a higher ratio of *Firmicutes*:*Bacteroidetes* in type 2 diabetics, lower abundance of *Verrumicrobia* and higher abundance of *Proteobacteria*.

Moderate increase of diversity in type 2 diabetics with intervention

More than the abundance of total bacteria or certain bacterial groups the diversity of microbiota indicated by richness of species or gene counts is now seen as an important indicator of microbiota resilience and gut health (Turnbaugh *et al.*, 2009). Shannon's diversity index showed no significant differences between the groups, whereas band pattern analysis showed significant differences between the lean control group and type 2 diabetics. In type 2 diabetics an increase of bands was seen with intervention whereas the values of lean controls remained unaffected. Wu *et al.*, 2010 does not observe significant differences by comparing diversity profiles of type 2 diabetics and non-diabetics, which indicates an association of health and disease with a shift in the balance of gut microbiota rather than an action of a single microbe affecting the diversity (Wu *et al.*, 2010). Ley *et al.*, 2005 observes a lower diversity in ob/ob animals with lower abundance of *Bacteroidetes* and a proportional increase in *Firmicutes* compared to lean mice. In this model the diversity is affected by obesity (Ley *et al.*, 2005), however we indicated a lower diversity in type 2 diabetics, but not in obese volunteers. In agreement to our observed PCs, PC1 *Firmicutes* and *Verrumicrobia*, PC2 *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*, Larsen *et al.*, 2010 shows also a higher separation between the groups attributed to *Bacteroides*, *Firmicutes* and *Verrumicrobia* (45 %) and in second direction the combination of *Proteobacteria* and *Actinobacteria* (28 %) (Larsen *et al.*, 2010). More than changes in the abundance of some microbiota groups or the small changes in body weight in some patients this increase in diversity could indicate the success of the nutritional/ GLP-1-Agonist intervention.

Higher ratio of *Firmicutes* versus *Bacteroidetes* in type 2 diabetes patients

We detected a higher abundance of *Firmicutes* in type 2 diabetics. *Firmicutes* (60 % at T3) increased and *Bacteroidetes* (35 % at T3) decreased during intervention. The phylum *Firmicutes* harbours many butyrate and acetate producers. According to previous studies with higher concentration of fermentation end products in tissue and lower energy content in feces, *butyryl-CoA: acetate CoA-transferase* genes tended to be more abundant in type 2 diabetics, but this difference was not significant. Obese patients on the contrary showed a decreasing ratio of *Firmicutes* (55 %) to *Bacteroidetes* (45 %). Intervention here resulted in a weight loss of $(4,87 \pm 3,68 \text{ kg})$. Weight loss was highly diverse between individuals.

Consequences of these shifts remain under discussion for their effects on endotoxin mediated low grade inflammation, but also for consequences on energy extraction and body weight. An increased *Firmicutes* to *Bacteroidetes* ratio is previously associated with increased body weight (Turnbaugh *et al.*, 2006), but also controversial data exist, e.g. by higher Clostridia in association with higher plasma glucose (Larsen *et al.*, 2010). A higher ratio of *Firmicutes* versus *Bacteroidetes* increases the energy harvest and the content of SCFA and as such the lipogenesis and accumulation in adipocytes (Diamant *et al.*, 2011). Schwartz *et al.* on the contrary reports a lower *Firmicutes* to *Bacteroidetes* ratio in overweight human adults compared to lean controls (Schwartz *et al.*, 2010) whereas other working groups do not find an association between the *Bacteroidetes* to *Firmicutes* ratio and BMI (Abdolrasulnia *et al.*, 2008, Duncan *et al.*, 2008, Cani & Delzenne, 2009, Arumugam *et al.*, 2011).

Not only obesity linked microbiota characteristics, but also changes in microbiota composition during weight loss are under discussion. It has been suggested that the abundance of *Bacteroidetes* responds to the weight loss whereas the *Firmicutes* remain unaffected (Turnbaugh & Gordon, 2009). Recently published data show that especially *Firmicutes* promote caloric extraction from the diet in zebra-fish. Eating encourages the growth of *Firmicutes* and the fat storage in intestinal cells. *Firmicutes* decrease during hypocaloric diets and *Bacteroidetes* get the chance to proliferate (Semova *et al.*, 2012). Larsen *et al.* shows a positive correlation between plasma glucose levels and *Bacteroidetes* to *Firmicutes* ratio (Larsen *et al.*, 2010). As we approved the HbA_{1c} blood levels of our type 2 diabetic patients, we had no possibility to approve this correlation. After the start of intervention with Victoza® all type 2 diabetics had well-adjusted HbA_{1c} blood levels of 7-8 %, a value that according to American Diabetes Association protects against long-term consequences (Association, 2010).

Table 4: Number of bands observed in PCR-DGGE fingerprinting in lean controls, obese patients and type 2 diabetics at all three time points.

Table 14

time point	LC	OC	D
T1	12.1 ± 3.5	10.5 ± 3.6	8.5 ± 4.4
T2	13.3 ± 4.9	9.0 ± 1.6	9.4 ± 3.4
T3	14.0 ± 4.0	10.3 ± 5.6	9.6 ± 3.6

LC: Lean Controls; OC: Obese Participants; D: Type 2 Diabetics; T: Time Point

Higher Lactic Acid Bacteria (LAB) may contribute to chronic inflammation?

Our type 2 diabetes patients harbored higher proportions of LAB according to qPCR analysis. This was confirmed by sequencing for higher abundance of *Enterococcus*, *Streptococcus* and *Lactobacillus*. Furthermore, LAB increased in type 2 diabetics during the study period.

LAB are very well known, but also subject of controversy (Delzenne & Cani, 2011), they represent a very heterogeneous group with well documented immune modulating qualities, which may potentially contribute to chronic inflammation in type 2 diabetes (Larsen *et al.*, 2010). The relative abundance of *Bacilli* is increased in diabetics; especially the *Lactobacillus* group is enriched in type 2 diabetic mice models. Many bacterial isolates received from diabetic patients are able to produce toxins, indicating the necessity for virulence of these bacteria in blood stream infections (Hancock IE & MS, 2000). On the other hand several strains of *Lactobacillus* are tested as probiotics, e.g. *L. paracasei ssp. paracasei* F19 and *L. gasseri* SBT2055 to decrease fat mass (visceral and subcutaneous).

Therefore, increases in LAB can be interpreted as immune modulating, even inflammatory process or an immune mechanisms underlying body-weight management.

Higher enterobacteria in type 2 diabetics - contributors to bacteremia?

Type 2 diabetics and to a lesser extent also obese volunteers showed a higher abundance of enterobacteria, especially *E. coli*. Enterobacteria are of special interest in type 2 diabetes as enterobacteria are a major cause of morbidity and mortality in type 2 diabetics (Graff *et al.*, 2002). Thomsen *et al.* observed a 3-fold increased risk to *E. coli* and other enterobacteria. Diabetic patients aged <45 years have a 15-fold higher risk for acute pyelonephritis and diabetics aged >65 had 3-6 times higher risk compared to non-diabetics. A diabetes prevalence of 20-30 % was previously reported among patients with enterobacterial bacteremia (Thomsen *et al.*, 2005).

Caloric restriction increases abundance of *Akkermansia* and *Archaea*?

We report an enhanced abundance of *Akkermansia* and a higher appearance of *Archaea* in the type 2 diabetes group. Only few lean controls harbored *Archaea*.

On the basis of mucin-degradation *Akkermansia* do not compete with the other microbiota and are not dependent on nutrients deriving from host food consumption (Derrien *et al.*, 2011). The observed higher abundance of *Akkermansia* in type 2 diabetics in our study may reflect a better adaption to caloric restriction compared to the rest of intestinal microbiota as they do not depend on host diet. An increased abundance of *A. muciniphila* has also been observed in hamsters lacking food-derived enteral nutrients (Sonoyama *et al.*, 2009).

Archaea have been reported to be slightly more abundant in obesity and anorexic patients and were discussed to adapt towards optimal exploitation of hypocaloric diet (Arumugam *et al.*, 2011). Therefore, *Archaea* are indirect promoters of caloric intake by the colon and further fat accumulation

(Dridi *et al.*, 2011). Studies in humans show that removal of H₂ by the synohtropic relationship with bacteria improves fermentation efficiency (Gill *et al.*, 2006, DiBaise *et al.*, 2008). Moreover less energy has been measured in obese feces in mice relative to lean littermates (Gill *et al.*, 2006). The symbiotic relationship might maximize the microbiota's ability to generate energy from otherwise non-digestible food components, thereby they get enriched during caloric restriction to guarantee an adequate caloric intake of the host and as such complicate or even prevent weight reduction over time.

Prevotella reflect same compositional diet between the three groups

Our study participants showed no differences in *Prevotella* abundance over time, as our type 2 diabetics were well adjusted to HbA_{1c} blood levels. In contrast Larsen et al. reports significant higher ratios of *Bacteroides-Prevotella* group versus class *Clostrida* and *C. coccoides- E. rectale* group in type 2 diabetics. *Clostridium ssp.*, *C. coccoides* decrease and *Bacteroides-Prevotella* group increase with body weight loss (Larsen *et al.*, 2010).

Prevotella are also of special interest because of their role in the formation of discussed gut enterotypes. First of all we made the distribution into the nowadays confirmed two enterotypes (*Bacteroides-Akkermansia* or *Prevotella*) with no observable difference. However, if we have a detailed view with the distribution of *Bacteroides* and *Akkermansia* in each enterotype, type 2 diabetics are more related to enterotype 3 and obese participants and the lean control group to enterotype 1, which might reflect the dependence of an enterotype on caloric restriction although both are related to a Western diet.

On the other hand Oluf Pedersen indicates that obese and “metabolically unhealthy” people tend to have the *Bacteroides* enterotype, so the classification may get very important to ensure an individual therapy and treatment (Yong, 2012).

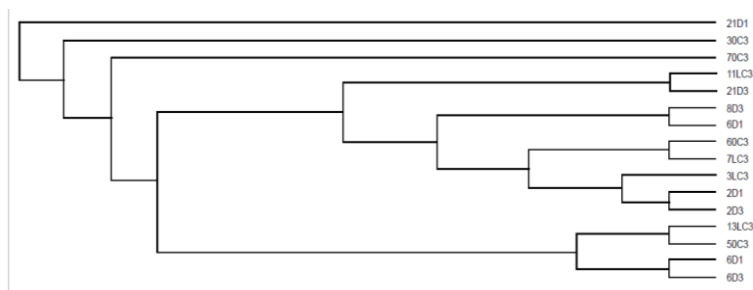


Figure 23

Figure 5: Jackknifing did not affect UPGMA clustering results as groups and patients are less different compared to differences between the three groups (LC, lean control group; OC, obese patients). Type 2 diabetics (D) are grouped between the two time points (T1, samples taken before intervention; T3, samples taken after 4 month of intervention).

Conclusion

Gut microbiota composition and activity is a key element in controlling vital functions of the host and also the nutritional status. Type 2 diabetes and obesity are associated with changes in its abundance and diversity. Microbiota interact with functions underlying inflammation, energy extraction as well as the gut barrier. If changes to its characteristics in obese and type 2 diabetics reflect functional differences or more the progression of the disease is unclear.

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Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity

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Abstract

The human gut microbiota and microbial influences on lipid and glucose metabolism, satiety, and chronic low-grade inflammation are known to be involved in metabolic syndrome. Fermentation end products, especially short chain fatty acids, are believed to engage the epigenetic regulation of inflammatory reactions via FFARs (free fatty acid receptor) and other short chain fatty acid receptors.

We studied a potential interaction of the microbiota with epigenetic regulation in obese and type 2 diabetes patients compared to a lean control group over a four month intervention period.

Intervention comprised a GLP-1 agonist (glucagon-like peptide 1) for type 2 diabetics and nutritional counseling for both intervention groups. Microbiota was analyzed for abundance, *butyryl-CoA:acetate CoA-transferase* gene and for diversity by polymerase chain reaction and 454 high-throughput sequencing. Epigenetic methylation of the promoter region of FFAR3 and LINE1 (long interspersed nuclear element 1) was analyzed using bisulfite conversion and pyrosequencing.

The diversity of the microbiota as well as the abundance of *Faecalibacterium prausnitzii* were significantly lower in obese and type 2 diabetic patients compared to lean individuals. Results from *Clostridium cluster IV* and *Clostridium cluster XIVa* showed a decreasing trend in type 2 diabetics in comparison to the *butyryl-CoA:acetate CoA-transferase* gene and according to melt curve analysis. During intervention no significant changes were observed in either intervention group.

The analysis of five CpGs in the promoter region of FFAR3 showed a significant lower methylation in obese and type 2 diabetics with an increase in obese patients over the intervention period. These results disclosed a significant correlation between a higher body mass index and lower methylation of FFAR3. LINE-1, a marker of methylation, indicated no significant differences between the three groups or the time points, although methylation of type 2 diabetics tended to increase over time.

Our results provide evidence that a different composition of gut microbiota in obesity and type 2 diabetes affect the epigenetic regulation of genes. Interactions between the microbiota and epigenetic regulation may involve not only short chain fatty acids binding to FFARs but also NF-κB signaling by bacterial cell wall components or DNA.

Keywords

butyryl-CoA:acetate CoA-transferase gene – *Faecalibacterium prausnitzii* – FFAR3 – LINE-1- GLP-1 agonist

Abbreviations

B.D.I.	lat. bis in die (two times per day)
BMI	body mass index
DGGE	denaturing gradient gel electrophoresis
FFAR	free fatty acid receptor
FFQ	food frequency questionnaire
GLP-1	glucagon like peptide-1
GPR	G-protein coupled receptor
HbA _{1c}	glycol hemoglobin
HDAC	histone deacetylase
IFN- γ	interferon- γ
IL	Interleukin
LCFA	long chain fatty acid
LINE-1	long interspersed nuclear element 1
LPS	lipopolysaccharide
OEGE	Austrian association for nutrition
O.D.	drug overdose
NF- κ B	nuclear factor κ B
PCA	principal component analysis
PPAR- γ	peroxisome proliferator-activated receptor- γ
PYY	Peptide YY
SCFA	short chain fatty acid
T	time point

Introduction

The gut microbiota plays a critical role in the establishment and maintenance of human health. A wide range of inflammatory and metabolic diseases is associated with microbial imbalance (Larsen *et al.*, 2010, Vrieze *et al.*, 2010). Obesity is associated with changes in abundance, diversity, and metabolic function of the gut microbiota (Vrieze *et al.*, 2010). These compositional changes are indicated by a higher abundance of *Firmicutes* and a decreased abundance of *Bacteroidetes* in animal studies (Turnbaugh *et al.*, 2006), but also a relation between a higher *Firmicutes*/*Bacteroidetes* ratio

and obesity is mentioned in human studies (Larsen *et al.*, 2010, Schwartz *et al.*, 2010). Duncan *et al.* (2008) showed no correlation between the proportion of the ratio and human obesity (Duncan *et al.*, 2008). A higher *Firmicutes*/*Bacteroidetes* ratio is hypothesized to increase the fermentation end-products butyrate and acetate, as many *Firmicutes* are butyrate producers (Turnbaugh & Gordon, 2009). *Roseburia* and *Faecalibacterium prausnitzii* are highly discriminant in type 2 diabetes. They are well known for butyrate synthesis and are associated with improved insulin sensitivity (Karlsson *et al.*, 2013). *Faecalibacterium prausnitzii* is also established for its anti-inflammatory activity, decreasing the rates of NF- κ B (nuclear factor- κ B) activation and IL-8 (interleukin) secretion, increasing IL-12, IL-10, and IFN- γ (interferon- γ), and also as an indicator of microbial diversity (Furet *et al.*, 2010). Evidence is accumulating that a higher diversity is related to health, whereas a lower diversity is observed in relation to various diseases (Sokol *et al.*, 2008), but also no association has been observed (Wu *et al.*, 2010). In addition the gut microbiota is involved in obesity-associated metabolic disorders, type 2 diabetes, metabolic endotoxemia, and low-grade inflammation (Gerritsen *et al.*, 2011). Type 2 diabetes has often been associated with lower abundance of *Firmicutes*, while *Bacteroidetes* and *Proteobacteria* tended to be more abundant (Larsen *et al.*, 2010). Other data showed lower abundance of *Bacteroidetes vulgatus* and *Bifidobacterium spp.* and a higher one of *Clostridium leptum cluster* in diabetics compared to healthy volunteers (Wu *et al.*, 2010). The relative abundance of *Bacilli* is increased in diabetic mouse models (Larsen *et al.*, 2010). Our recently published data support these findings of a higher *Firmicutes*/*Bacteroidetes* ratio with the highest contribution coming from lactic acid bacteria. *Lactobacilli* together with *Enterobacteria* may contribute to the associated systemic low-grade inflammation of obesity and type 2 diabetes. Both species and especially LPS (lipopolysaccharide) are suggested to influence inflammation via their immune-modulating properties and to provoke the macrophage-mediated adipose tissue inflammation (Remely *et al.*, 2013) and also gut permeability (de Kort *et al.*, 2010). Karlsson *et al.* (2013) observed a positive correlation between *Lactobacillus gasseri* and HbA_{1c} values and a negative one between *Clostridium* species and HbA_{1c} values. However, neither species correlated with body mass index (BMI), waist circumference, or waist-to-hip ratio (Karlsson *et al.*, 2013).

SCFA-producing bacteria are known to be involved in the fermentation of dietary fibers by partially converting sugars (Louis & Flint, 2007), proteins, and peptides to short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate (Macfarlane & Macfarlane, 2003). These SCFAs may help to explain mechanisms by which gut microbes affect obesity and chronic diseases. All three SCFAs are considered to protect against diet-induced obesity, with butyrate and propionate being more efficient than acetate (Lin *et al.*, 2012). They also provide additional energy (5-15 % of the total caloric requirements), not only for gut epithelial cells but also via absorption by diffusion. Furthermore, SCFAs play a role in immunity (anti-carcinogenic and anti-inflammatory potential) (Hartung *et al.*, 2008) and adipose tissue expansion (Delzenne & Cani, 2011). The majority of butyrate is metabolized by the colonic epithelium and the remainder is most likely extracted by the liver. Concentrations in the venous systemic serum range from 0.5 to 3.3 μ mol (Hartung *et al.*, 2008), where butyrate acts as signaling molecule (Brown *et al.*, 2003) via G-protein coupled receptors (GPRs). The affinity of SCFAs to GPR41 (FFAR3; free fatty acid receptor) and GPR43 (FFAR2) differs with the length of the fatty

acids. FFAR3 prefers the ligands butyrate and propionate, whereas FFAR2 is activated by acetate and propionate (Layden *et al.*, 2012, Lin *et al.*, 2012). Additionally, GPR40 does not bind SCFAs but LCFAs (long chain fatty acid) and is located upstream close to the GPR41 gene. Halpern *et al.* (2012) hypothesized that GPR41 may have no promoter region and thus is transcribed by the promoter of GPR40 (Halpern *et al.*, 2012). Biological functions of FFARs are the modulation of lipid metabolism (Kebede *et al.*, 2009), influences on leptin production (Kebede *et al.*, 2009, Talukdar *et al.*, 2011) and other satiety hormones (Samuel *et al.*, 2008), and control of host energy metabolism (Talukdar *et al.*, 2011).

Effects of SCFAs are also believed to include epigenetic control of gene expression (Pegorier *et al.*, 2004). Butyrate is well-known for its epigenetic activity as a histone deacetylase inhibitor (HDACi), and activities of histone deacetylases and methyl CpG binding proteins may influence DNA-methylation. Modulation of histone acetylation by SCFAs may also prevent obesity through anti-inflammatory effects (Duranton *et al.*, 1998). Folate is the best known methyl group donor; however, other substances such as methionine, choline, and butyrate are also able to influence the DNA methylation status. Changes in the CpG methylation of genes or promoter regions regulate the mRNA expression. Highly methylated promoter regions are proposed to decrease transcriptional activity of the downstream gene (Canani *et al.*, 2011). Global DNA methylation, often analyzed by the methylation of long interspersed nuclear element 1 (LINE-1) is used for DNA stability. LINE-1 elements are usually highly methylated and their variability can be associated with cancer, age, ischemic heart disease, and stroke as well as with plasma fasting glucose and plasma lipid levels (Turcot *et al.*, 2012).

On this basis we hypothesized that compositional differences in gut microbiota and therefore different metabolic synthesis of short chain fatty acids, especially butyrate influence epigenetic methylation of SCFA-receptors, especially FFAR3 promoter region, in type 2 diabetes, obese, and normal weight individuals. These changes in the epigenetic regulation would cause changes in expression and signaling of FFARs and influence hunger and satiety cycles as shown by Schloegl *et al.* (2010) (Schloegl *et al.*, 2010). We investigate differences in methylation of the promoter region of FFAR3 and LINE-1 in human blood samples, *butyryl-CoA:acetate CoA-transferase* gene and compositional changes in *Firmicutes* of microbial DNA from feces of obese patients without established insulin resistance, type 2 diabetics under GLP-1 agonist therapy (glucagon like peptide-1 agonists), and a lean control group. The GLP-1 agonist increases the glucose-dependent insulin secretion in beta cells and suppresses the glucagon secretion in the pancreas. It also slows the gut passage in order to decrease the postprandial spike in blood glucose (Nauck *et al.*, 2009). In addition, we investigated the response of gut microbiota and according epigenetic consequences to intervention and its potential role in controlling metabolic syndrome.

Materials and methods

Study participants and study design

The study “Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity” was approved by the Viennese Human Ethics committee. All study

participants gave written consent for using blood and stool samples and data obtained from food frequency questionnaires (FFQ) for the analyses presented here.

We studied fourteen obese individuals (OC) with no established insulin resistance (aged 38 +/- 14 years) and twenty-four insulin-dependent type 2 diabetes (D) patients (aged 56 +/- 9 years) over a four month intervention period compared to a lean control (LC) group (n = 18; aged 30 +/- 8 years). Intervention comprises GLP-1 agonists for type 2 diabetics and nutritional counseling with regards to a balanced diet according to OEGE (Austrian association for nutrition) for both intervention groups (Table 1). An oral glucose tolerance test was used to determine insulin resistance, diabetes. HbA_{1c} (glycosylated hemoglobin) blood levels were assessed at Health Center South in Vienna. The levels were between 7 and 8 % hemoglobin in our type 2 diabetes group, a value that is protective against long-term consequences according to American Diabetes Association (Association, 2010). As GLP-1 agonist we used primarily Liraglutide (Victoza®), which was dosed o.d. 0.6 mg for the first month, then o.d. 1.2 mg. Exenatide (Byetta®) was dosed b.i.d. 5 µg for the first month, then 10 µg b.i.d. All GLP-1 agonists were injected subcutaneously by the patient, after carefully teaching him/her how to do so. Blood and fecal samples were collected at three time points: before (T1), during (T2), and after treatment (T3), with an interval of 1 month between the first two time points and 3 months between the second and third time point. The FFQ asked participants to report the frequency of consumption and portion size over a defined period (last three months). Additional to dietary habits, the FFQ included questions about lifestyle (i.e. smoking, alcohol consumption, physical activity), medical relevant influences (i.e. vitamin and other supplements), BMI and age at the enrollment. To ensure comparable data, patients were interviewed for their history of gastrointestinal diseases, use of antibiotics, probiotics, or prebiotics.

Table 1: Characterization of study participants.

Table 15

Group	Number	Sex	Age ± SD (years)	Time point	BMI ± SD (kg/m ²)
D	24	♀ 10 ♂ 14	58.36 ± 9.35	1	38.44 ± 5.14
				3	37.63 ± 5.58
OC	14	♀ 7 ♂ 7	39.63 ± 15.18	1	33.71 ± 4.24
				3	32.66 ± 3.75
LC	18	♀ 15 ♂ 3	25.67 ± 3.09	1	21.19 ± 1.93
				3	20.63 ± 1.87

Fecal sample collection, processing, and analysis

Stool samples were stored at -70°C after collection. Bacterial DNA was extracted from fecal samples using the QIAamp® DNA Stool mini kit (Qiagen, Germany) according to the manufacturer's protocol. In addition samples were treated in FastPrep™ Lysing Matrix E tubes (MP Biomedicals, USA) twice for 45 sec in a bead-beater (Mini-Beadbeater 8 Bio-Spec Products, USA) with an intervening minute on

ice. DNA concentration and quality was determined with a Pico100 (Picodrop, UK) and agarose gel electrophoresis.

The total bacterial diversity was measured by DGGE (denaturing gradient gel electrophoresis) using the primer set 341f-GC 5'-CCT ACG GGA GGC AGC AG-3' (Muyzer *et al.*, 1993) and 518r 5'-ATT ACC GCG GCT GCT GG-3' (Neefs *et al.*, 1991) according to Remely *et al.* (2013) (Remely *et al.*, 2013).

For further analysis in qPCR, clone libraries were created from members of *Faecalibacterium prausnitzii* in stool samples of healthy volunteers. Cloning was done following Remely *et al.* 2013 (Remely *et al.*, 2013). Bacterial abundances were quantified with TaqMan qPCR and SYBR Green qPCR in a Rotorgene 3000 (Corbett Life Science, Australia) using 16S rDNA group specific primers (Table 2). *Butyryl-CoA:acetate CoA-transferase* gene was amplified and thermal denatured with SYBR Green qPCR analysis. The amplicons were divided into three areas, as described by Louis & Flint (2009). These peaks are assigned to represent bacteria related to *Eubacterium hallii* and *Anaerostipes coli* (82.5–85.0 1C), *Roseburia/E. rectale spp.* (85.5–89.0 1C) and *F. prausnitzii* (89.5–92.5 1C).

The specificity of primers and probes was checked with the ProbeMatch function of the ribosomal database project 10 (<http://rdp.cme.msu.edu/>). The PCR reactions mixture and serial DNA dilution of typically strains were prepared according to Pirker *et al.* 2012 (Pirker *et al.*, 2012).

Sixteen samples were selected on basis of qPCR results for Roche GS FLX + Titanium next generation sequencing (LGC sequencing GmbH, Germany) to facilitate a high diversity and comparison although not all samples were sequenced. For alignment and further analyses the pyro pipeline of the ribosomal database project 10 (<http://rdp.cme.mus.edu/>) and qiime were used. Significance tests and principal coordinate analysis were then performed in qiime.

Table 2: Primers and TaqMan®-probes targeting 16S rDNA coding regions of bacteria, BCoAT-gene and for CpG-Methylation analysis.

Table 16

Target	Primer/Probe	Sequence (5'-3')	Conc. [pmol/μL]	Reference
<i>Clostridium</i> cluster IV (Ruminococcaceae)	Fwd primer	GCA CAA GCA GTG GAG T	4	(Ichimura <i>et al.</i> , 2009)
	Rev primer	CTT CCT CCG TTT TGT CAA	4	
	Probe	(6-FAM)-AGG GTT GCG CTC GTT-(BHQ-1)	2	
<i>Clostridium</i> cluster XIVa (Lachnospiraceae)	Fwd primer	GCA GTG GGG AAT ATT GCA	5	(Wu <i>et al.</i> , 2012)
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A	5	
	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)	1.5	
Lactic Acid Bacteria (LAB)	Fwd primer	AGC AGT SGG GAA TCT TCC A	4	(Musso <i>et al.</i> , 2010)
	Rev primer	ATT YCA CCG CTA CAC ATG	4	
BCoAT gene	Fwd primer	GCI GAI CAT TTC ACI TGG AAY WSI TGG CAY ATG	27	(Sokol <i>et al.</i> , 2008)
	Rev primer	CCT GCC TTT GCA ATR TCI ACR AAN GC	27	
<i>Faecalibacterium prausnitzii</i>	Fwd primer	GGA GGA AGA AGG TCT TCG G	0.25	(Baccarelli <i>et al.</i> , 2010)
	Rev primer	AAT TCC GCC TAC CTC TGC ACT	0.25	
FFAR3	Fwd primer	GTG ATA GGG AAA ATA GTA GTT GGT A	0.5	
	Rev primer	Biotin-CCC TAC AAA ACA AAA CTC ATC ATA AT	0.5	
	Seq primer	GGG AAA ATA GTA GTT GGT AT	0.4	
LINE-1	Fwd primer	TTT TGA GTT AGG TGT GGG ATA TA	1	(Di <i>et al.</i> , 2011)
	Rev primer	Biotin-AAAATC AAA AAA TTC CCT TTC	1	
	Seq primer	AGT TAG GTG TGG GAT ATA GT	0.4	

Blood sample collection, processing, and analysis

Blood samples were collected in PAXgene Blood DNA Tubes (Qiagen, Germany) and stored at -20°C. For extraction of DNA, they were treated with the PAXgene Blood DNA Kit (Qiagen, Germany). For

epigenetic analysis the DNA was converted with the EpiTect Bisulfite Kit (Qiagen, Germany) according to manufacturer's protocol. The DNA was measured and approved for purity with a Pico100 (Picodrop, UK) and agarose gel-electrophoresis. Suitable primers for the five selected CpGs in the promoter regions of FFAR3 (Table 2-3) were designed using the PyroMark Assay Design Software 2.0 (Qiagen, Germany). The PCR was carried out in a total reaction volume of 25 µl, which contains 12.5 µl PyroMark 2X PCR master mix (Qiagen, Germany), 5 pmol (FFAR3) or 1 pmol (LINE-1) of each primer, 2.5 µl CoralLoad Concentrate 10X (Qiagen, Germany), and 5 ng (FFAR3) or 10 ng (LINE-1) bisulfite converted DNA. PCR product quality was investigated with agarose gel-electrophoresis. Analysis of CpG methylation was performed with a PyroMark Q24 MDx (Qiagen, Germany).

Table 3: Sequence to analyze: FFAR3 (geneID (NCBI) 2865) and LINE-1.

Table 17

Assay	Sequence 5'-3'	Number of CpGs	Size
LINE1	TTC GTG GTG CGT CGT TT	3	17
FFAR3	CCG CTG CCT GCT CAC GAC CAC ACG CCA GGC TTC CGT CAA ACC ACT CAA CAT GTA TTA G	5	58

Statistical analysis

The normality of distribution was tested with the Kolmogorov-Smirnov test. The comparison of gut microbiota was performed using the non-parametric Mann-Whitney U test, and for three unpaired groups the non-parametric Kruskal Wallis ANOVA was applied. Differences in the percent of methylation of LINE-1 and FFAR3 were determined using Student's two-tailed T-test, one-way analysis of variance (ANOVA) with the Tukey post hoc correction test. For correlation analysis we used the two-tailed Pearson test. P values < 0.05 were taken as statistically significant. All data shown are mean ± sd.

Results

FFQ analysis

Analysis of the FFQ revealed differences in life style patterns and in the consumption of sugary products. At all three time points the total sweets consumption was the lowest in type 2 diabetics. In contrast obese patients showed the highest intake: 50 % of all participants consumed sweets one to three times per week. No differences were observed in the consumption of grain, vegetables, fruits, meat, dairy products, and fish. Also no confounding errors with regard to dietary supplementation – for example probiotics, prebiotics – were indicated. In life style patterns, type 2 diabetics showed the lowest physical activity (64.0 % at T1 and 52.0 % at T3 never exercise), followed by obese participants (37.5 % at T1 and 12.5% at T3 never do sports), whereas 44.0 % of lean controls exercised 1 to 3 times per week. With the dietary intervention, 19 out of 24 type 2 diabetes patients showed an average weight reduction of 3.1 ± 2.1 kg, and obese patients lost about 4.9 ± 3.7 kg (Table 1).

Analysis of microbial diversity

DGGE fingerprinting analysis of total bacteria revealed significant differences between type 2 diabetics and lean controls at T2 ($p = 0.03$) and T3 ($p = 0.01$). Lean controls showed the highest diversity, followed by obese participants and by type 2 diabetics showing the lowest diversity.

Lean controls had a higher Shannon's diversity index (6.48 ± 0.47) of 454 sequencing results compared to obese controls (4.51 ± 2.60) and type 2 diabetics (5.19 ± 2.62). A clustering of the participants in the PCA revealed grouping of type 2 diabetes subjects. The first three calculated PCs of 454 sequencing results explained 44.6 %, 17.2 %, and 8.3 % of the variance in the data. *Firmicutes* and *Verrumicrobia* contributed most to the separation along PC1, and *Proteobacteria*, *Actinobacteria* and *Bacteroides* contributed to the clustering along PC2 (Figure 1).

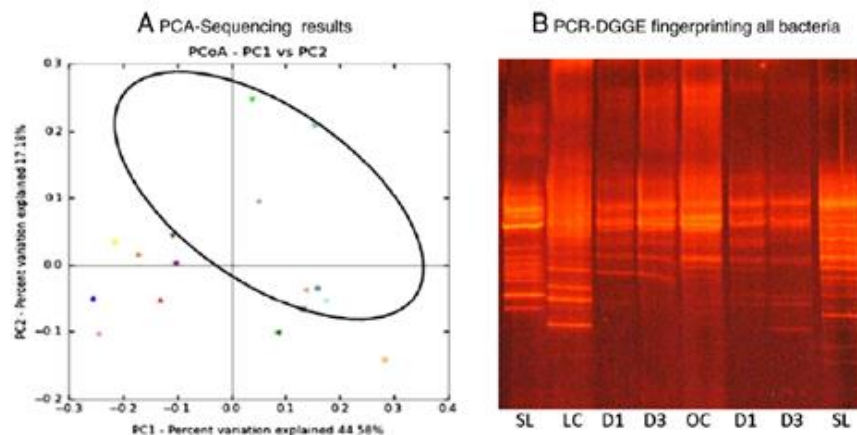


Figure 24

Fig. 1. Diversity analysis. A PCA based on dominant bacteria of PC1 (*Firmicutes* and *Verrumicrobia*) and of PC2 (*Proteobacteria*, *Actinobacteria* and *Bacteroides*) illustrating grouping of type 2 diabetic samples (ellipse). B PCR-DGGE fingerprinting of 16S rDNA coding regions of dominant bacteria over time indicating a lower number of bands in type 2 diabetes and obesity (LC—lean control, D1—type 2 diabetes, samples taken before intervention, D3—type 2 diabetes, samples taken after 4 month of intervention, OC—obese patients; SL—standard lane). Analysis revealed a higher diversity in lean controls compared to obese patients and also in comparison to type 2 diabetics. No significant differences in type 2 diabetics over study period can be observed (D1–D3).

Analysis of SCFA-producing bacteria

Analysis of total bacterial abundance did not show significant differences between the groups over the study period (Remely *et al.*, 2013). Therefore we focused on qPCR results from groups of *Firmicutes*, known for their high number of SCFA-producing bacteria. *Clostridium Cluster IV* and

Clostridium Cluster XIVa abundances were not significantly different between the groups or over the intervention period but tended to decrease with intervention in type 2 diabetics. High-throughput sequencing confirmed these findings as type 2 diabetics were enriched in *Firmicutes*, especially *Clostridiales* and *Bacilli*, in particular *Lactobacillales* as previously reported (Remely *et al.*, 2013). Quantitative PCR analyses of *Faecalibacterium prausnitzii* were consistent with high throughput sequencing analysis. Type 2 diabetics showed a significantly lower abundance at all three time points with an increasing trend at the third time point. The lean control group showed the highest abundance. These findings were significant at all three time points between lean control group, type 2 diabetics, and obese patients (T1: $p < 0.001$; T2: $p < 0.001$; T3: $p < 0.001$). In addition, obese participants showed a higher abundance compared to type 2 diabetics. No significant changes within a group were observed over the study period (Figure 2).

The *butyryl-CoA:acetate CoA-transferase* gene was used as a marker for butyrate production as the direct analysis of SCFAs in feces is difficult because of rapid uptake by the mucosa, making fecal SCFA concentrations an unreliable indicator of production rates. *Butyryl-CoA:acetate CoA-transferase* gene tended to increase in type 2 diabetics over the intervention period from an already lower level compared to the lean control group. Dominant butyrate producers differ in their DNA G+C content, as was shown by the analysis of the melt curves obtained from PCR amplicons of the *butyryl-CoA:acetate CoA-transferase* gene. Detailed analysis allowed us to differentiate between *Faecalibacterium prausnitzii*, *Eubacterium hallii*, and *Roseburia* (Hippe *et al.*, 2011). We observed no significant differences in our study population, although *Faecalibacterium prausnitzii* showed a trend to increase in type 2 diabetics with intervention (data not shown).

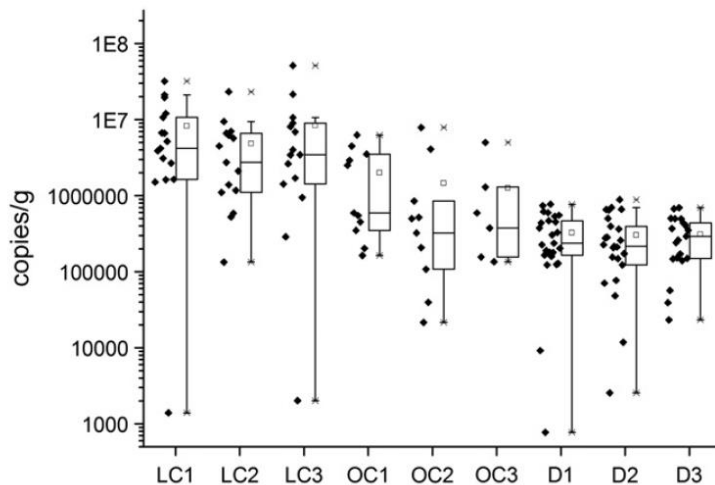


Figure 25

Fig. 2. 16S rDNA qPCR quantification with primer pair for *Faecalibacterium prausnitzii*. A significant ($p < 0.01$) lower abundance of *Faecalibacterium prausnitzii* was shown in type 2 diabetics (D) compared to obese patients (OC) and lean controls (LC) at all three time points: T1, samples taken before

intervention; T2, samples taken after 1 month of intervention; T3, samples taken after 4 month of intervention.

Methylation analysis of FFAR3 and LINE-1

Analysis of five CpGs in the promoter region of the FFAR3 gene in blood showed a significantly higher methylation in the lean control group compared to type 2 diabetics at all three time points (T1: $p = 0.003$; T2: $p = 0.011$; T3: $p = 0.000$) and to obese participants at the first time point ($p = 0.031$, Figure 3). In type 2 diabetics, we observed no significant changes in the methylation during the intervention period (T1: $31.46 \pm 5.74\%$; T2: $32.53 \pm 7.17\%$; T3: $31.25 \pm 6.85\%$). In comparison, obese patients showed an increase of methylation with nutritional counseling (T1: $30.69 \pm 6.46\%$; T2: $32.34 \pm 7.13\%$; T3: $34.51 \pm 2.93\%$), although they do not reach the methylation status of the lean control group (mean over all three time points: $39.23 \pm 0.9\%$). CpG4 shows the highest methylation and CpG2 the least at all three time points. At T1 comparing lean controls to type 2 diabetics and obese (Table 4), a significantly higher methylation is observed only at CpG4 ($p = 0.001$) and CpG5 ($p = 0.001$). At T2 significant differences were also observed at CpG2, CpG3, CpG4 and CpG5. At T3 lean controls are significantly higher methylated at all five CpGs compared to type 2 diabetics (CpG1: $p = 0.025$; CpG2: $p = 0.009$; CpG3: $p = 0.013$; CpG4: $p = 0.000$; CpG5: $p = 0.000$), and there is no longer any significant difference between lean controls and obese (data not shown). The mean methylation of LINE-1 indicates no significant differences between the groups or the time points, but obese individuals had the lowest methylation. We found a significant negative correlation between the BMI and methylation of FFAR3 ($R = -0.474$; $p = 0.001$; Figure 4), whereas LINE-1 showed a tendency of a positive correlation. We did not observe a correlation between methylation of FFAR3 or LINE-1 and age.

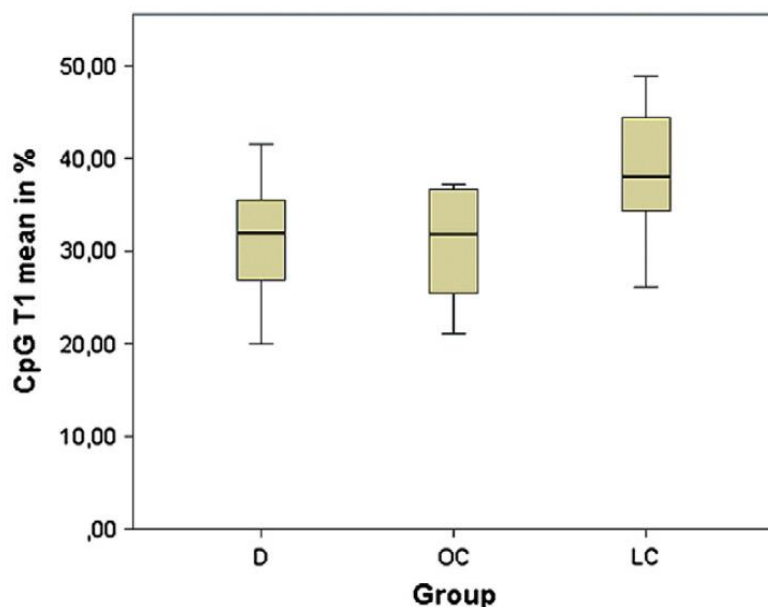


Figure 26

Fig. 3. Meanmethylation of FFAR3 promoter region between the three groups at the first time point. Lean controls (LC) showed highestmethylation compared to both intervention groups. These differences were significant between lean controls and type 2 diabetics (D, $p = 0.003$) and between obese participants (OC) and lean ($p = 0.031$).

Table 4

Methylation of 5 CpGs in the promoter region of FFAR3. At the first time-point (T1) lean controls (LC) showed highest methylation over all 5 CpGs, the lowest methylation has been observed in type 2 diabetics (D) and obese patients (OC).

Table 18

GroupT1	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	Σ: CpGs
D	37.29 ± 12.10	20.47 ± 5.22	24.48 ± 5.71	41.27 ± 7.85	33.82 ± 7.95	31.46 ± 5.74
OC	37.80 ± 14.32	18.78 ± 5.09	24.89 ± 3.78	37.13 ± 9.16	34.84 ± 6.31	30.69 ± 6.46
LC	42.86 ± 13.25	23.66 ± 3.95	27.21 ± 3.61	51.70 ± 6.46	46.13 ± 11.13	38.19 ± 6.19

Discussion

We analyzed subgroups of the phylum *Firmicutes*, with focus on microbial diversity, *Faecalibacterium prausnitzii* in particular, and the *butyryl-CoA:acetate CoA-transferase* gene as a proxy for butyrate producers. Epigenetic methylation analysis included five CpGs in the promoter region of FFAR3 and LINE-1 as an indicator of the global methylation. The results are discussed with regard to the influence of SCFAs on epigenetic control of gene expression, especially of FFAR3.

DGGE fingerprinting showed a lower diversity in type 2 diabetics compared to obese patients and lean controls. These differences were significant between lean controls and type 2 diabetics.

However, we did not observe significant differences in Shannon's diversity index of our selected subgroup in 454 sequencing results. Diversity as an indicator of health and disease is still under discussion, as other research groups do not observe significant differences between type 2 diabetics and non-diabetics (Wu *et al.*, 2010), or even higher diversity and richness in diabetics with higher BMI (BMI > 31) compared to lean diabetics. In agreement with our derived PCA (PC1 *Firmicutes* and *Verrumicrobia*, PC2 *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* (Figure 1)), Larsen et al. (2010) also show a separation between the groups attributed to *Bacteroides*, *Firmicutes*, and *Verrumicrobia* (45 %), and in the second direction the combination of *Proteobacteria* and *Actinobacteria* (28 %) (Larsen *et al.*, 2010).

The analysis of one of the major butyrate producers (Louis & Flint, 2009) showed a lower abundance of *Faecalibacterium prausnitzii* in type 2 diabetics, followed by obese participants (Figure 2). *Faecalibacterium prausnitzii* is identified as a dominant and conserved species in the healthy human gut microbiota and is described to play a role in the pathology of low-grade inflammation (Tremaroli & Backhed, 2012). A negative relationship between *Faecalibacterium prausnitzii* and inflammatory markers was observed in obese and diabetic patients and remained after adjustment for BMI. The proportions of *Faecalibacterium prausnitzii* were lower in type 2 diabetic subjects indicating a higher incidence of low-grade inflammation (Furet *et al.*, 2010). *Butyryl-CoA:acetate CoA transferase* gene analysis showed neither significant differences between the groups nor a correlation with qPCR analysis of *Faecalibacterium prausnitzii*. In addition, analysis of *Clostridium cluster IV* and *Clostridium cluster XIVa* are in line with analysis of *butyryl-CoA:acetate CoA-transferase* gene. One cause might be that we only observed bacteria that use the *butyryl-CoA:acetate CoA-transferase* gene, but not the other described pathways of butyrate production (*butyrate kinase*, *phosphotransbutyrylase*) (Hippe *et al.*, 2011). In addition butyrate producers do not form a homogenous phylogenetic group as butyrate producers and non-butyrate producers are in the same phylogenetic cluster, and although they are carriers of the gene there is no evidence for its expression (Turnbaugh *et al.*, 2006). Significant correlations are observed between butyrate and dietary factors, especially resistant starch. For example the consumption of inulin stimulates the growth of *F. prausnitzii* and *E. rectale* (Louis & Flint, 2009). Duncan et al. (2008) reported a relationship of butyrate-producing bacteria on weight loss, dependent on diet. A reduction in these species was observed during low-carb diets, but no changes are shown during moderate-carbohydrate intake (Duncan *et al.*, 2008). Hippe et al. (2011) also showed the important influence of diet on the butyrate production in the GI tract. The microbiota of elderly study participants is characterized by decreased butyrate production capacity regarding a lower fiber intake. In contrast, a vegetarian diet leads to an increase of *butyryl-CoA:acetate CoA-transferase* gene copy number (Hippe *et al.*, 2011). These findings are in line with our reported FFQ results and analysis of *butyryl-CoA:acetate CoA-transferase* copy number. Additionally, the relationship between dietary composition, intestinal microbiota, epithelial integrity, and obesity has been investigated (de Kort *et al.*, 2010). Type 2 diabetes is associated with increased gut permeability, and this alteration includes reduced gastrointestinal motility (a consequence of diabetic autonomic neuropathy), intestinal enteropathy, and increased mucosal surface. These disturbances can lead to bacterial overgrowth thus possibly affecting gut permeability (de Kort *et al.*, 2010).

The absorption of SCFAs by diffusion or SCFA-transporters (Hartung *et al.*, 2008) together with their conversion to fat in the liver and the regulation of host genes that promote deposition of fat in lipocytes can cause an increase of adipose tissue (Tsukumo *et al.*, 2009). Butyrate, in particular, has an important impact on promoting body energy expenditure via a modulation of mitochondrial activity. These results are consistent with the role of SCFAs as an additional energy source promoting adiposity. Research of human SCFA content in feces indicated a higher proportion of SCFAs in overweight and obese participants compared to lean controls, although the ratio of *Firmicutes*/*Bacteroidetes* is lower in overweight and obese individuals (Schwiertz *et al.*, 2010). A higher concentration of butyrate and acetate was measured in the caeca of obese mice, while propionate was not significantly different between the lean or obese group (Turnbaugh *et al.*, 2006). A higher ratio of *Firmicutes*/*Bacteroidetes* increases the energy harvest and the content of SCFAs and as such the lipogenesis and accumulation in adipocytes (Diamant *et al.*, 2011). In contrast, high-fat diets supplemented with butyrate prevent and even reverse insulin resistance in obese mice (Gao *et al.*, 2009, Kootte *et al.*, 2012). Besides being an energy source for the epithelial cells, butyrate also influences a wide array of cellular functions. It may have an anti-carcinogenic and anti-inflammatory potential and play a role in satiety and oxidative stress (Hartung *et al.*, 2008). These observations support the impact of SCFAs on host inflammatory signaling processes via epigenetic control of gene expression (Qin *et al.*, 2010), although the precise role of SCFAs in signaling pathways is still unclear (Kootte *et al.*, 2012), and currently insufficient data prevent an estimation of optimal levels of fecal SCFAs for the promotion of colonic health.

The signaling actions of SCFAs are mediated by the G-protein coupled receptors (FFARs). We observed a higher methylation in the promoter region of the FFAR3 gene in blood of the lean control group. Both intervention groups had a lower methylation with the lowest in type 2 diabetics (Table 4, Figure 3). These differences of methylation between the groups result in a negative correlation between methylation and BMI (Figure 4). Type 2 diabetics showed no significant differences over the intervention period, whereas obese patients showed an increase of methylation after nutritional counseling, although they do not reach the methylation status of the lean control group. Since there are no other available investigations on the methylation of FFAR3 promoter region and expression, we expected a lower expression of FFAR3 receptor in the lean control group according to Canani *et al.* (2011) (Canani *et al.*, 2011). In mice, an increase of leptin production caused by an overexpression of FFAR3 was observed after oral doses of propionate (Ichimura *et al.*, 2009). A knockdown of FFAR3 by siRNA inhibits the ability of propionate to induce leptin expression, mainly in adipocytes (Wu *et al.*, 2012). Leptin, exclusively produced in adipose tissue, takes influence on the hunger-satiety cycle by suppression of the appetite and contribution to the fat metabolism. Although obese patients harbor many fat cells with according higher leptin levels, leptin is no longer able to contribute to satiety. Studies have demonstrated a positive correlation of leptin plasma levels and body fat mass. It was discussed that high leptin levels over time make the satiety cells insensitive to leptin, whereas a supplementation with SCFAs induces sensitivity or the production of leptin. Only few obese patients are affected from leptin deficiency, so therapies using recombinant leptin have not been successful (Schloegl *et al.*, 2010) and might affect therapies with SCFAs. These results are consistent with our

results of significant negative correlation of BMI and methylation levels found in FFAR3 promoter region. On the other hand FFARs are suggested to stimulate the secretion of satiety hormone PYY (Peptide YY), which inhibits gut motility, slows intestinal transit time, and enhances nutrient absorption. These results are underlined by a study with Gpr41-deficient mice and conventionally raised mice. A deficiency of the receptor is associated with a lower expression of PYY and as a consequence with a higher energy harvest from diet. Inhibition of Gpr41 activation may result in decreased extraction of energy from the diet and leaner individuals (Samuel *et al.*, 2008). However, current researches indicate a FFAR3-independent influence of butyrate and propionate on body weight and food intake. In addition FFAR3 is mentioned to play a minor role in butyrate stimulation of GLP-1 secretion. SCFAs increase the reduced GLP-1 secretion of obese and type 2 diabetics and thus promote insulin secretion (Lin *et al.*, 2012). Therefore, a different microbial composition may contribute to reduced GLP-1 release. The main production of GLP-1 is located in the mucosal L cells, in the ileum and colon but also in pancreatic α -cells and in neurons (Vrieze *et al.*, 2010). Prebiotic treatment induced GLP-1 secretion in ob/ob mice, whereas a deletion of GLP-1 prevented the beneficial effects of prebiotics on weight gain, glucose metabolism and inflammatory pathway activation (Musso *et al.*, 2010). However, there is no evidence that an intervention with GLP-1 agonists for adjustment of blood glucose levels influences epigenetic methylation. Together, these findings assume an implication of FFAR3 in microbiota-host interaction with host adiposity via circulating levels of enteroendocrine hormones that reduce gut motility and thus increase absorption of SCFAs, which may contribute to lipogenesis.

Changes in global methylation can affect gene expression, genomic stability, and chromosomal structure. In the case of LINE-1, our type 2 diabetics and lean controls showed the same methylation status, whereas obese individuals had the lowest methylation. Baccarelli *et al.* (2010) associated blood DNA hypomethylation with higher risk of cardiovascular disease caused by older age, smoking, exposure to air pollution, folate deficiency, hyperhomocysteinemia, and end-stage renal disease (Baccarelli *et al.*, 2010). Turcot *et al.* (2012) revealed lower LINE-1 methylation levels in visceral adipose tissue, especially in obese subjects without established insulin resistance, with an association to higher plasma glucose level, lower HDL-cholesterol level, and greater risk to be affected by metabolic syndrome (Turcot *et al.*, 2012). The lower global methylation in our obese study population compared with our type 2 diabetics might be related to the well-adjusted HbA_{1c} value as a result of GLP-1 agonists, whereas a higher blood glucose level in obese participants could not be excluded.

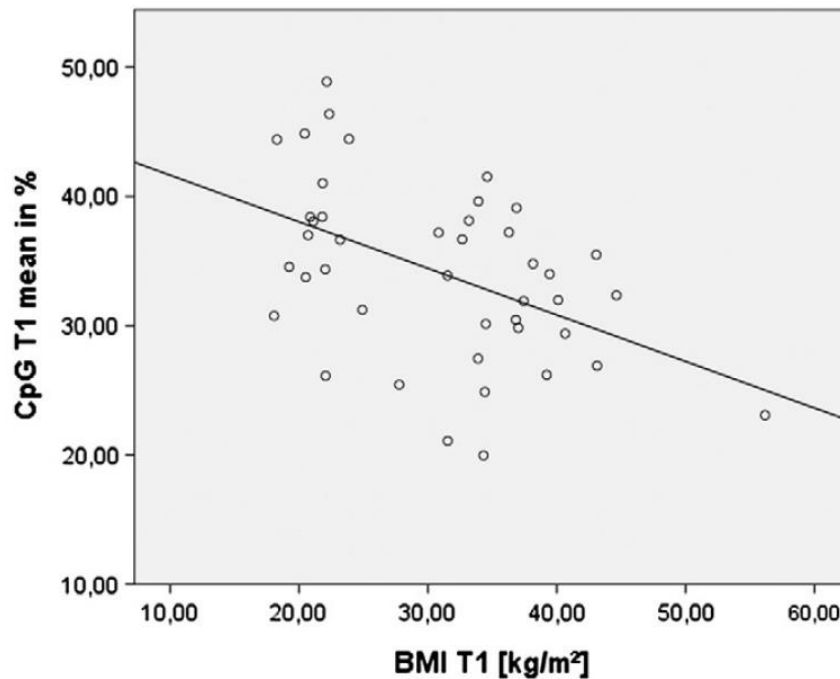


Figure 27

Fig. 4. Correlation between BMI and FFAR3methylation%. Significant correlation between a higher BMI of all study participants and lower FFAR3methylation% at time point 1 (T1), before intervention ($R = -0.474$; $p = 0.001$).

Conclusion

Our results suggest that a different composition of gut microbiota in obese individuals and type 2 diabetics affects the epigenetic regulation of FFARs. These interactions between microbiota and epigenetic regulation may influence the satiety and hunger cycle. Especially butyrate may impact strategies for regenerative medicine by promoting epigenetic remodeling and the expression of associated genes. Our results highlight the necessity to concentrate on gene-specific methylation to better understand the involvement of epigenetic regulation in the development of obesity and type 2 diabetes. However, other data from animal tissues will be desirable.

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Abundance and diversity of GI microbiota rather than IgG₄ levels correlate with abdominal inconvenience and gut permeability in consumers claiming food intolerances

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Abstract

Food intolerances are an increasing global health problem. Interactions between genetics and environmental changes such as microbial- and stress factors remain poorly understood. Whereas the analyses of IgE mediated allergic responses is based on solid concepts, the roles of microbiota, gut permeability, and IgG antibodies remain widely unclear and are under fierce discussion for scientific relevance. The present pilot study analyzes forty participants, under consultation of nutritional health professionals, for gastrointestinal discomfort and claimed food intolerances. Food frequency questionnaire addresses nutrition, lifestyle and present discomfort. Feces samples are analyzed for dominant microbiota using 16S rDNA based methods and the fecal marker Calprotectin. Blood samples are analyzed for IgG₄ levels. The total microbial abundance significantly correlates with claimed discomfort ($R=-0.37$; $p=0.02$). The abundance and diversity of microbiota significantly correlates with low Calprotectin values ($R=-0.35$; $p=0.01$) and with higher abundance of *Faecalibacterium prausnitzii* ($R=0.78$; $p<0.01$) and *Akkermansia* ($R=0.82$; $p<0.01$). Participants with low discomfort show enhanced *Clostridium Cluster XIVa* ($p=0.008$). An increased diversity is also correlating with reduced antibodies against IgG₄ of egg white ($R=0.68$; $p<0.01$).

Data suggest an interaction of low gut permeability and reduced inflammation with an established microbial equilibrium. Self-reported abdominal inconvenience of participants relates mainly to characteristics of microbiota and gut permeability. Anti-inflammatory effects of *Faecalibacterium prausnitzii* or *Lactobacilli* and gut barrier functions of *Akkermansia* may have a key role in food intolerances. The role of IgG₄ linking food immune responses with intolerances remains unclear.

Keywords

Abdominal pain; Akkermansia; Calprotectin; DGGE; *Faecalibacterium prausnitzii*; IgG₄ ELISA; Lactobacilli

Introduction

Food intolerances and food allergies are defined as “adverse reactions to food” (Montalto *et al.*, 2008). They are a rapidly increasing problem in the western world and according to the World Allergy Organization (WAO) 220-250 million people are affected. The trend continues to rise, especially in children (Dorsano & Chaudary, 2013). In Europe, 2-5% of people suffer from a food allergy, while intolerances are estimated at 20% (Oestmann & Zuberbier, 2013). Genetic aspects such as single nucleotide polymorphisms (Mattar *et al.*, 2013), environmental influences such as the discussed “hygiene hypothesis” (Gigante *et al.*, 2011), and stress from the social environment (Konturek *et al.*, 2011, Cortes *et al.*, 2013) have been linked to intolerances. Recently aspects of gut microbiota and gut permeability are in discussion for their role in food intolerance (Konturek *et al.*, 2011).

An inappropriate tolerance to food antigen has been associated with mechanisms of apoptosis (Loser *et al.*, 2009) or paralysis of T-cells (Hoyne *et al.*, 2000) and failure in the production of regulatory T-cells (Bellanti *et al.*, 2003, Ortolani & Pastorello, 2006, Morita *et al.*, 2013). Among the food intolerances, an IgE-mediated allergy is well understood. Also mechanisms of lactose intolerance (Scrimshaw & Murray, 1988, Suarez *et al.*, 1995), histamine intolerance (Maintz & Novak, 2007), and fructose intolerance are well described (Jones *et al.*, 2011). In contrast many aspects (Schmid-Grendelmeier *et al.*, 2001) in the area of non IgE-mediated intolerances remain poorly understood (Sabra *et al.*, 2003, Chehade, 2007, Jyonouchi, 2012). Various food components, but also a number of natural and artificial food additives have been discussed for non-IgE mediated adverse reactions (Turner & Kemp, 2012, Canonica *et al.*, 2013). Furthermore, the role of IgG antibodies in these intolerances remains highly controversial. IgG₁ is formed as the initial response after contact with a new food antigen. With further exposure to the antigen, a change in the formation of IgG₄ antibodies seems to take place (Snapper, 1999). Following this change the amount of IgG₄ rises from 3% up to 50%. High IgG₄ concentrations may indicate an intense conflict of the immune system with food antigens. The synthesized IgG₄ antibodies are suspected to sensitise cells, but may also block the IgE mediated allergic response (Strait *et al.*, 2006). Actual untreated allergies show increased IgG₄ levels in 50% of cases. However, the identification of the triggering food is difficult as the symptoms often stop after hours or days. In many of these IgG₄ mediated immune responses a disturbed intestinal permeability, leading to a constant exposure of the immune system to antigenic structures, has been postulated as a reason of the food intolerance.

An important role was assigned for IgG₄ antibodies in immune-mediated pathological mechanisms in patients with a delayed non-IgE cow's milk allergy. These patients show a significantly higher concentration of beta-lactoglobulin-specific IgG₄ antibodies compared to cow's milk tolerant (asymptomatic) patients (Sletten *et al.*, 2006). Obese children show significantly higher IgG antibody levels against food antigens than children of normal weight. In overweight people IgG antibodies may be associated with increased atherosclerosis risk and concomitantly increased CRP (C-reactive

protein) levels (Wilders-Truschnig *et al.*, 2008). Other publications indicate high IgG4 values testing an elimination diet on patients with irritable bowel symptoms. The work of Zar *et al.* (2005) also shows a positive influence on psychological symptoms (Atkinson *et al.*, 2004, Zar *et al.*, 2005, Zar *et al.*, 2005). It is suggested that the determination of IgG or IgG₄ antibodies can be used as marker for distinction between healthy and sick patients, for both food intolerance and allergy (Kleine-Tebbe & Herold, 2010). Despite this, the presence of food specific IgG/IgG₄ antibodies shall always be considered when common causes such as lactose intolerance, fructose malabsorption, celiac disease, or IgE mediated food allergies can be excluded (Sletten *et al.*, 2006).

In addition to gut permeability, individual gastrointestinal microbiota and their involvement in inflammatory and metabolic reactions of the gut may play a role in intolerances. The groups of *Faecalibacterium prausnitzii*, *Akkermansia* and *Lactobacilli* are associated with inflammatory immune regulation and the gut barrier (Louis & Flint, 2007, Furet *et al.*, 2010, Everard *et al.*, 2013, Kim *et al.*, 2013, Le Chatelier *et al.*, 2013). *Faecalibacterium prausnitzii* is named as one of the main butyrate producers (Louis & Flint, 2007), often seen in conjunction with other bacteria producing acetate or propionate as substrates for butyrate production (Duncan *et al.*, 2002). Also epigenetic effects of butyrate producing microbiota in the regulation of inflammatory genes for example Toll-like receptors (TLR) and short chain fatty acid (SCFA) receptors have been described, also by our group (Macfarlane & Macfarlane, 2003, Remely *et al.*, 2013). *Lactobacilli* are associated with anti-inflammatory effects (Tien *et al.*, 2006, Jones & Versalovic, 2009). Other researchers describe *Lactobacilli* as pro-inflammatory for example in type 2 diabetes (Andreasen *et al.*, 2010, Remely *et al.*, 2013, Remely *et al.*, 2013). *Akkermansia* are known to feed on mucus and might indicate inflammatory processes (Everard *et al.*, 2013).

The present study was designed as a pilot study to establish parameters and inclusion criteria for a subsequent clinical study. Because of the broad nature of factors for food intolerance or gut inconvenience, inclusion criteria for the pilot study was self-reported abdominal discomfort of participants consulting with health nutritional experts. The present study aims to analyze possible interactions between questionnaire based symptoms, gut microbiota, Calprotectin as inflammatory and gut permeability parameter and IgG4 levels. Convincing evidence for interactions between gut microbiota and claimed food intolerances and a possible involvement of specific IgG levels in certain food ingredients is discussed.

Methods

Study participants; in- and exclusion criteria

All study participants gave written consent for using blood and stool samples and data obtained from food frequency questionnaires (FFQ) for the analyzes presented here. Clearance was given by the local ethical committee with acceptance number 31/2009 Top 8.

We studied a population of 40 participants, visiting health nutritional experts for gastrointestinal inconvenience. Inclusion criteria were: 21 years of age or more, both genders, present abdominal

inconvenience with a suspicion of food intolerance, currently consulting with health nutritional experts. Exclusion criteria were: diagnosed inflammatory bowel disease, established IgE mediated food allergies, lactose intolerance, pregnancy, BMI>30 kg/m² and diagnosed metabolic- and malignant disease. 37 participants (aged 46±14.2 years; 29 female, 8 male) fulfil all criteria for an inclusion in the pilot study.

The FFQ, was adapted from EPIC questionnaires and forms addressing food intolerances (Kroke *et al.*, 1999) and focuses on the consumption frequency of foods and portion size. In addition to dietary habits, questions about lifestyle (i.e. smoking, alcohol consumption, and physical activity), medical relevant influences (i.e. vitamin and other supplements), BMI and age at enrolment were included. The questionnaire also asks for any history of potential gastrointestinal diseases, use of antibiotics, probiotics, prebiotics, as well as discomfort and proven or even self-reported (non-established) intolerances and allergies. Inconvenience is assessed in self-reporting, addressing present abdominal pain. The evaluation of the nutritional habits follows the recommendations of the OEGE (Austrian Society for Nutrition).

Fecal sample collection, processing, and analysis

After collection, study participants immediately stored their stool samples at -20°C. Fecal samples were transported frozen to the laboratory and immediately restored at -20°C. The DNA of 200mg stool sample was extracted using the QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol. DNA concentration and quality was determined with a Pico100 (Picodrop, UK) and agarose gel-electrophoresis. DNA was stored at -20°C until analysis.

The total bacterial diversity was assessed by DGGE (denaturing gradient gel electrophoresis) using the primer set 341f-GC 5'-CCT ACG GGA GGC AGC AG-3' and 518r 5'-ATT ACC GCG GCT GCT GG-3' according to Remely *et al.* (2013) (Remely *et al.*, 2013), Primers for all bacteria with GC clamp are used on a 30% to 55% Gel.

Microbiota is analyzed using 16S rDNA based TaqMan qPCR and SYBR Green qPCR in StepOnePlus™ Real-Time PCR System (Applied Biosystems®) focusing on dominant bacterial groups: *all* bacteria, *Clostridium cluster XIVa*, Archaea, Bacteroides, Bifidobacteria, Akkermansia, Lactobacilli and *Faecalibacterium prausnitzii* (Table 1).

Primer and probes are checked for specificity with the Probematch function of the ribosomal database project 10 (<http://rdp.cme.msu.edu/>). The mixture of PCR reactions and serial DNA dilution of typically strains are prepared according to Pirker *et al.* 2012 (Pirker *et al.*, 2012).

Primer sequences of qPCR and conditions

Table 19

Target organism	Primer/ Probe	Sequence (5' - 3')	Conc. [pmol/μL]	Reference
All Bacteria	Fwd primer	ACT CCT ACG GGA GGC AG	10	(Yu <i>et al.</i> , 2005)
	Rev primer	GAC TAC CAG GGT ATC TAA TCC	10	
	Probe	(6-FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)	2	
Clostridium cluster IV (Ruminococcaceae)	Fwd primer	GCA CAA GCA GTG GAG T	4	(Matsuki <i>et al.</i> , 2004)
	Rev primer	CTT CCT CCG TTT TGT CAA	4	
	Probe	(6-FAM)-AGG GTT GCG CTC GTT- (BHQ-1)	2	
Cluster XIVa (Lachnospiraceae)	Fwd primer	GCA GTG GGG AAT ATT GCA	5	(Matsuki <i>et al.</i> , 2004)
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A	5	
	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)	1,5	
Bacteroidetes	Fwd primer	GAG AGG AAG GTC CCC CAC	3	(Layton <i>et al.</i> , 2006)
	Rev primer	CGC TAC TTG GCT GGT TCA G	3	

	Probe	(6-FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)	1	
Lactobacilli	Fwd primer	AGC AGT SGG GAA TCT TCC A	4	(Walter <i>et al.</i> , 2001)
	Rev primer	ATT YCA CCG CTA CAC ATG	4	
Akkermansia	Fwd primer	CAGCACGTGAAGGTGGGGAC	2,5	(Collado M.C., 2007)
	Rev primer	CCTTGCGGTTGGCTTCAGAT	2,5	
Faecalibacterium prausnitzii	Fwd primer	GGA GGA AGA AGG TCT TCG G	0.25	(Ramirez-Farias <i>et al.</i> , 2009)
	Rev primer	AAT TCC GCC TAC CTC TGC ACT	0.25	

The Bühlmann Calprotectin ELISA kit is used for quantitative determination of human Calprotectin (MRP8/14; S100A8/S100A9) extracted from stool samples. After a brief extraction step using a stool sample volume fraction and 49 volume fractions of extraction buffer, Calprotectin is selectively determined by sandwich ELISA. The wells of the microtiter plate are coated with a capture monoclonal antibody (mAb), which is highly specific to polymeric and heterodimeric Calprotectin.

The actual concentrations of the standards A-E are 4, 12, 40, 120, and 240 ng/µl Calprotectin. During extraction the sample is diluted 1:50 and the extract diluted again 1:50, resulting in a total dilution of 1:2500. Both dilution steps were included in the standard concentration and are given as follows: 10, 30, 100, 300 and 600 µl/g Calprotectin.

The optical density of the solution, measured at a wavelength of 450 nm using FLUOstar Optima, is directly proportional to the Calprotectin concentration of the sample. After entering the standard concentrations, the FLUOstar Optima software (version 1.30R2) automatically calculates the concentrations of Calprotectin. (BUEHLMANN, 2012)

Blood sample processing, IgG

Blood samples were collected in Li-Heparin tubes (Sarstedt, Germany). The blood samples were stored at -8°C. IgG4 levels were also analyzed according ELISA following the manufacturer's protocol. The Immunolab IgG4 Screen Nutritional 20 ELISA® test kit is based on the principle of the enzyme immunoassay (EIA). After spectrophotometric analysis, Immunolab offers a simple evaluation of all its

food intolerance screening tests. The definition of intolerance classes 1-4 follows the standard concentrations suggested by the manufacturer: Standard 1: 0.35 U/ml, Standard 2: 0.70 U/ml, Standard 3: 3.5 U/ml and Standard 4: 17.5 U/ml (IMMUNOLAB, 2010).

Statistical Analysis

Normal distribution (Kolmogorov-Smirnov-test) and descriptive statistics were tested with IBM SPSS Statistics 20. All correlations (Spearman), 2 sample t-test and linear fit were analyzed using OriginPro 8. P-values <0.05 were taken as statistically significant. All data is shown as mean $\pm$ SD.

Results

Nutrition

For 37 participants all data could be obtained. The average of age is 46 ± 14.2 years. 29 of the participants are female, 8 are male. The evaluation of nutritional habits follows the recommendations of the OEGE (Austrian Society for Nutrition) and where the diet is arranged in the 4 major food groups: milk and dairy products, fruit and vegetables, meat and meat products, and cereals.

With regards to milk and milk products 32% of the participants consume the recommended 2-3 servings per day. 67% under eat milk and dairy products. Those participants show a tendency of reduced levels of Lactobacilli and Bifidobacteria in stool ($p=0.07$). 40% of these participants showed high class of IgG₄ levels for “cow’s milk” without diagnosed intolerance, and stated an inconvenience after dairy product consumption. Even in absence of a diagnosed milk intolerance, discomfort increased, if the IgG₄ levels are high or Lactobacilli and Bifidobacteria levels are low. Participants reporting a proven lactose intolerance were not included in the study.

In fruit and vegetables, 54% comply with the recommended “5 a day”. Within these 54%, 10% reported a fructose intolerance when asked. It is remarkable that these participants consume even more vegetables, to reach the “5 a day”. From those who do not achieve the “5 a day” 71% indicate discomfort after the consumption of fruit.

Cereals should be consumed up to 4-5 times a day, more than half of them in the form of whole grains. Only 27% of all 37 participants reach the recommendations and report no intolerance to cereals. 10% show intolerances (class 4) against the antigen of wheat flour and rye flour according to the IgG₄ ELISA. From the remaining 73% only 11% show an intolerance.

Up to 3 servings (300-450g) meat or meat products are recommended per week. Only 30% report eating meat or meat products 3 times a week or less. 36% of them have no intolerance against pork meat, according to the IgG₄ ELISA. The remaining 70% consume meat more than three3 times a week. 7% of them are revealed in IgG4 class three.

76% of all participants reported consumption of dietary supplements. The most frequently mentioned food supplements are: vitamin C, vitamin D, calcium, magnesium, and multivitamins. In conclusion the nutrition of all participants showed typical characteristics of a central European diet.

When asked about physical exercise, 60% replied with frequencies between once a day to several times a day, 11% exercise 4-6 times a week, 24% 1-3 times a week, and 5% only one to two times a month. In sports (exercise with sweating over 30 minutes) 54% follow the recommended two to three sport units per week or more. 16% practice sports only one or two times a month and 30% never do any sports.

Discomfort

46% of all participants address abdominal gastrointestinal discomfort in the FFQ, remaining 54% report no present discomfort, but abdominal pain in the last year. 19% of the study participants state fructose intolerance, 8% gluten intolerance, and 22% of participants state histamine intolerance in the FFQ. The absence of inconvenience is correlated with higher abundance of total microbiota ($R=-0.37$; $p=0.02$) as well as with diversity of microbiota.

No correlations are observed between groups reporting discomfort and no discomfort and IgG4 levels. However, participants reporting comfort/ discomfort convenience or inconvenience differ significantly in abundance of main butyrate producers *Faecalibacterium prausnitzii* ($p=0.049$) and *Clostridium cluster XIVa* ($p=0.008$; figure 1).

Figure 1: 3D correlation model of *Faecalibacterium prausnitzii* in copies, IgG₄ in classes and convenience/inconvenience.

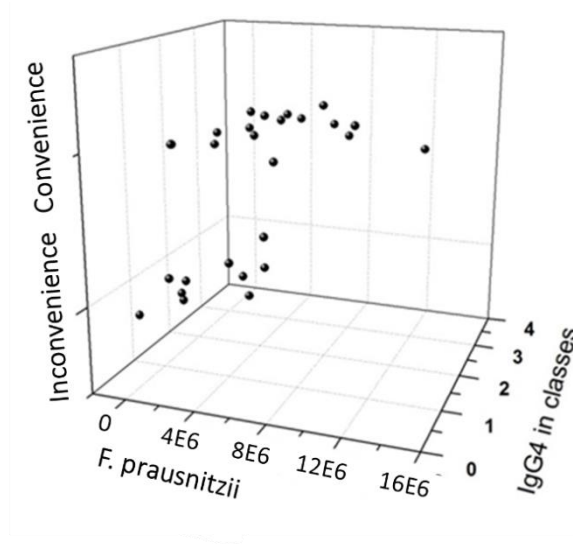


Figure 28

Analysis of GI microbiota

Fecal microbiota abundance is analyzed with 16S rDNA for all bacteria, *Clostridium cluster XIVa*, *Archaea*, *Bacteroides*, *Bifidobacteria*, *Akkermansia*, *Lactobacilli*, and *Faecalibacterium prausnitzii*. Participants with high all bacterial abundance show reduced values of Calprotectin ($R=-0.35$; $p=0.01$; figure 2). The total bacterial abundance is correlated with *Faecalibacterium prausnitzii* ($R=0.78$; $p<0.01$) as well as with *Akkermansia* ($R=0.82$; $p<0.01$) and *Lactobacilli* ($R=0.80$; $p<0.01$). In addition participants with higher abundance of all bacteria report decreased inconvenience ($R=-0.37$; $p=0.02$; figure 3). Therefore, a higher abundance of all bacteria lowers the abdominal pain in comparison to participants with low total bacterial amount.

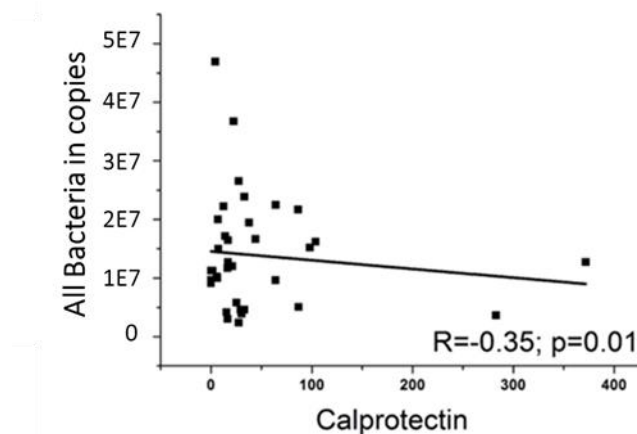


Figure 29

Figure 2: Regression chart with linear fit between Calprotectin in $\mu\text{g/g}$ feces and all bacteria in copies ($R=-0.35$; $p=0.01$)

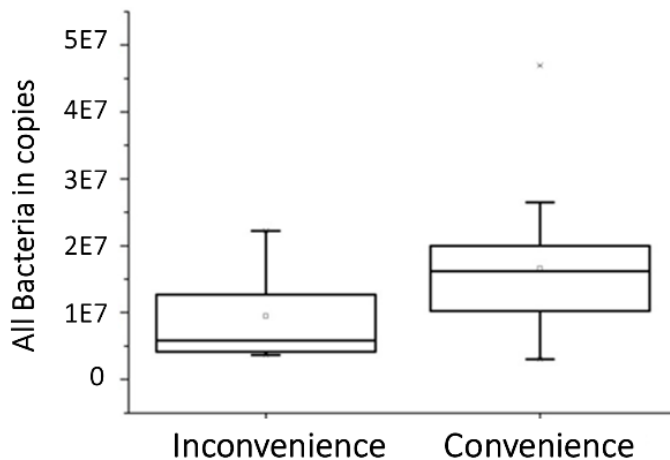


Figure 30

Figure 3: Boxplot of self-reported convenience and inconvenience compared to all bacteria in copies; Box range 25, 27 Perc; Whisker indicates outliers; □ indicates mean; x indicates maximum and minimum data range

Diversity is assessed by PCR-DGGE analyzes. As shown in figure 4, DGGE diversity has a band variety of 18.65 ± 4.04 bands, with a minimum of 12 and a maximum of 27 bands. In previous reports from our group, the analysis of dominant bacteria and DGGE correlates significantly with 454 pyrosequencing and the abundance of *Faecalibacterium prausnitzii* (Sokol *et al.*, 2008). In accordance with the report we show a correlation between *Faecalibacterium prausnitzii* and diversity of DGGE bands ($R=0.37$; $p=0.02$, figure 5).

Figure 4: DGGE image of PCR amplified all bacteria in a 30-50% gel; comparison of 8 band patterns from 4 participants with low (lane 36, 37, 14, 26) and 4 participants with high (lane 27, 30, 32, 35) diversity; SL: standard lane with 9 common commensal bacteria

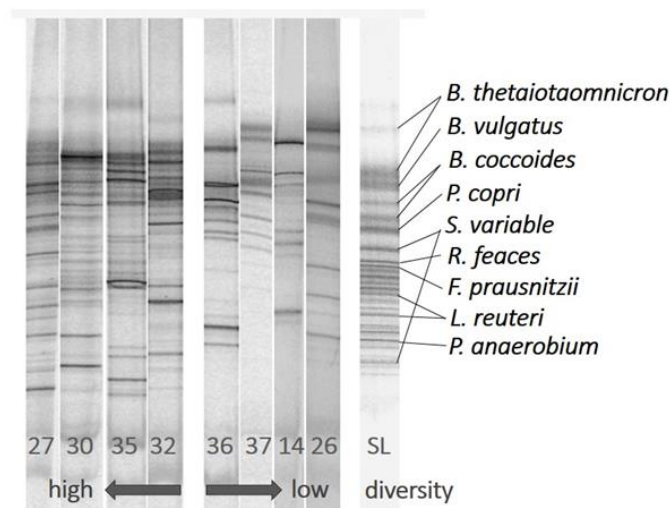


Figure 31

Figure 5: Regression chart with linear correlation between *F. prausnitzii* in copies and DGGE diversity in band counts (R=0.37; p=0.02)

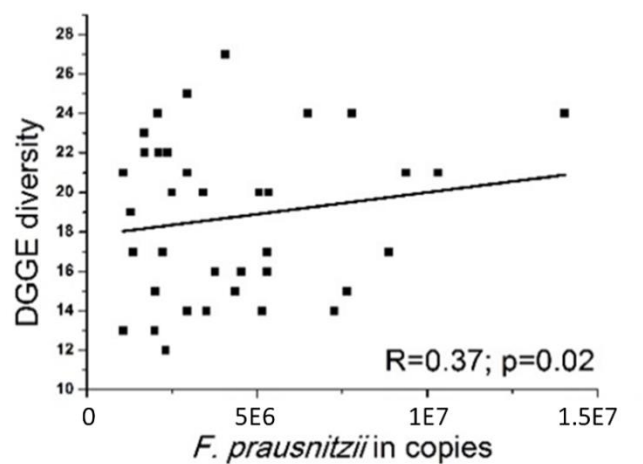


Figure 32

Calprotectin

Calprotectin is analyzed in feces as a marker for inflammation but also as cause for possible changes in gut permeability and concurrent minor inflammation. Calprotectin values vary between 3 and 371.7 µg Calprotectin/g feces, with a mean level of 45.7 ± 74.3 µg Calprotectin/g feces, and a median of 25.3 µg Calprotectin/g feces. A negative correlation is shown between the general abundance of all bacteria and Calprotectin ($R = -0.35$; $p = 0.01$, figure 1). However, no correlation is indicated for Calprotectin values between specific aspects of microbiota or IgG4 values, but those patients with a high diversity and bacterial abundance, often addressed as balanced microbial equilibrium, show low Calprotectin values.

IgG₄ levels

IgG₄ levels are analyzed against egg white, cow's milk, cod, wheat flour, rye flour, barley flour, orange, banana, kiwi, strawberry, celery, soy bean, carrot, tomato, hazelnut, peanut, curry, pepper, sesame, and pork (meat). These antigens are classified into four categories (class 1-4) according to method section (IMMUNOLAB, 2010).

5% of tested participants were grouped in class 1, 17% class 2, 36% class 3 and 42% class 4 as highest detected class. The foods with the highest IgG₄ class 4 are: egg white, cow's milk, wheat flour, rye flour, barley flour, banana, kiwi, and hazelnut. For egg white 27% are grouped in class 4 and 22% had no IgG levels. For cow's milk 38% show class 4 and 33% were not classified in one class. For wheat flour and rye flour 8% of the participants are classified in class 4, and 35% of the participants were

grouped in no class in both. For barley flour 3% of the participants show the highest class 4. For banana 5%, for kiwi and hazelnut 11% of the participants are classified in class 4. The lowest IgG₄ classes have been detected for strawberry, celery, and soy bean. There has been no food class in which no IgG₄ values have been measured.

A correlation can be seen between egg white and wheat flour ($R=0.43$; $p<0.01$), as well as rye- ($R=0.40$; $p=0.014$) and barley flour ($R=0.60$; $p<0.01$). In addition a positive correlation is found between cow's milk and the collective of grain flours ($R=0.41$; $p=0.012$). No correlation is seen for general IgG₄ levels with abundance or diversity of microbiota or Calprotectin values, however IgG₄ levels for egg white decrease with increasing DGGE bands ($R=0.68$; $p<0.01$) (figure 6). Wheat flour, rye flour, and barley flour correlate well: The p-value for the correlation between wheat flour and rye flour is <0.01 ($R=0.73$). For the correlation between wheat flour and barley flour the p-value is <0.01 ($R=0.48$) (figure 7).

Figure 6: Regression chart with linear fit between DGGE diversity in band counts and IgG₄ egg white in U/ml ($R=0.68$; $p<0.01$)

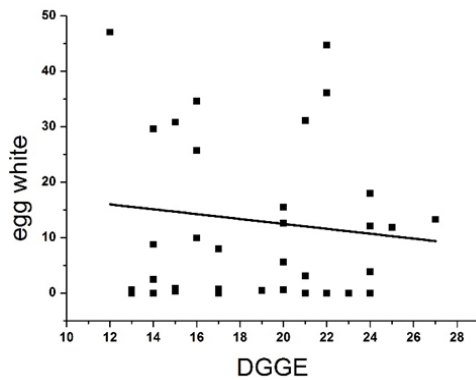


Figure 33

Figure 7: 3D correlation model of wheat, rye, and barley flour IgG₄ in classes

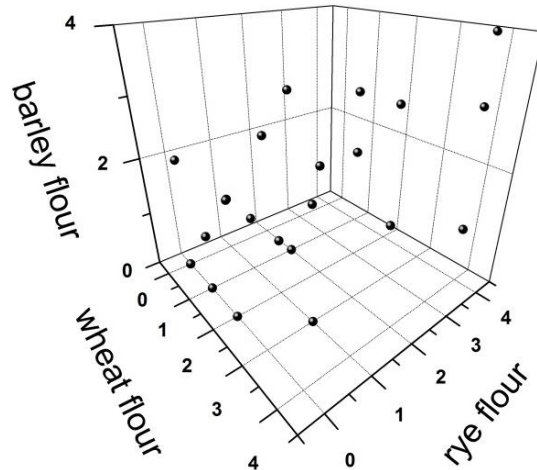


Figure 34

Discussion

The present pilot study analyses interactions of gastrointestinal microbiota, gut permeability, nutrition, IgG₄ levels, and their impact or correlation on food intolerances and well-being. High total bacteria abundance is correlated with high amounts of *F. prausnitzii*, *Akkermansia*, low Calprotectin values, decreased inconvenience, and less abdominal pain. Bacterial diversity and *F. prausnitzii* abundance are positively correlated.

67% of participants did not reach recommended intake of milk and dairy products. These participants show a tendency of reduced *Lactobacilli* and *Bifidobacteria* levels in their stool ($p=0.07$). 40% of these participants showed high class of IgG₄ levels for “cow’s milk” and stated inconvenience after dairy product consumption. Even in absence of diagnosed milk intolerance, discomfort increased, if the IgG₄ levels are high or *Lactobacilli* and *Bifidobacteria* levels are low.

Post consumption discomfort was not the reason to consume fewer vegetables or whole grain products. The group with a low fruit consumption had a 3-fold higher self-reported fructose intolerance rate. 11% of participants had IgG₄ Class 4 levels for wheat-, flour-, and rye flour.

A population of 40 participants, visiting dietary health experts, was included in the pilot study with the objectives to find improved criteria and markers for a future study.

The analytical strategy to include the analysis of major commensal gastro intestinal microbiota groups in addition to food intolerances, IgG₄ levels and inconvenience, resulted in significant correlations: Several studies show that focusing only on IgG₄ levels and nutritional behavior does not explain intolerances (Verdu *et al.*, 2009, Gibson & Shepherd, 2012).

Strategies to lower in particular inconvenience in gluten sensitive persons, are following a prebiotic approach, addressing gastro intestinal microbiota in intolerances (Holmes *et al.*, 2013). This prebiotic intervention may increase abundance of SCFA producers such as *F. prausnitzii*, and decreases

inconvenience. Lopez-Siles *et al.* (2012) showed the importance of strain specificity in analyzing the role of *F. prausnitzii*. To show correlations of microbiota and intolerances including non-dominant microbiota or specific strains, 454 analysis may be important in future studies.

The inflammatory and intestine permeability marker Calprotectin did not correlate with IgG₄ levels, but correlated significantly with microbiota abundance and diversity in this study. To improve analysis of intestinal permeability in the different subareas of the intestine the sucrose, lactulose/mannitol, sucralose diffusion test should be considered as an additional important tool (Arrieta *et al.*, 2006) for gut permeability.

The questionnaire used in the present study was carefully adapted from the EPIC study, multiple related questions were used to validate responses (Kroke *et al.*, 1999). Health experts interviewed every participant personally in a face-to-face meeting. However an international validation is desirable.

Whereas correlations between microbiota and discomfort and Calprotectin were found in the present study the use of IgG₄ antibodies as marker for food intolerance could not be encouraged, as also discussed before by Steve n O. Stapel 2008 (Stapel *et al.*, 2008). Significant correlation is seen between IgG₄ titers of egg white and wheat, rye and barley flour (R=0.43; p<0.01; R=0.40; p=0.014; R=0.60; p<0.01). Zar *et al.* (Zar *et al.*, 2005) reported significantly IgG₄ titers correlation between wheat, beef, and lamb in IBS patients before. IgG₄ levels for egg white decrease with increasing DGGE bands (R=0.68; p<0.01). The diversity analyzed by DGGE shows a band variety of 18.65±4.04 bands. This method has been proven to show correlations with 454 based pyrosequencing results [36,41]. As reported before, the gut microbiota shows individual characteristics depending on lifestyle, ethnicity, and nutrition [40,54,55]. In accordance with recent publications [53,56] patients with a high abundance of all bacteria also show a significant higher abundance of *Faecalibacterium prausnitzii* (R=0.78; p<0.01). A tendency can be observed between the number of *Lactobacilli* and *Bifidobacteria* and the consumption of milk products (p=0.07) as already reported by Shiby *et al.* (2013) [57].

An important aspect may be the high correlations between IgG₄ titers in the groups of wheat, rye, and barley flour (R=0.72; p<0.01; R=0.48; p<0.01) as this may indicating an interaction of their protein structures with immune responses. For Gliadin both IgE and IgA antibodies are known [59,60]. The study by Constantin *et al.* (2005) suggests that wheat includes antigens / epitopes which are mainly recognized by CD patients. These results suggest that food antigens affect the quality of the pathological immune response in the gut [61].

In subjects with high abundance of butyrate producing bacteria (*Faecalibacterium prausnitzii* (p=0.049), *Clostridium Cluster XIVa* (p=0.008)) absence of discomfort convenience was significant higher, despite high IgG₄ levels or nutrition.

Faecalibacterium prausnitzii, one of the main butyrate producers [32] with effect on epigenetic regulation of inflammatory genes [36], is discussed as an indicator for high gene counts (Le Chatelier *et al.*, 2013). The groups of *Lactobacilli*, *Faecalibacterium prausnitzii*, and *Akkermansia* have been widely discussed for their role in inflammatory immune regulation as well as in mucus and gut barrier

associated aspects of microbiota [31, 32, 33, and 34]. *Akkermansia*, a mucin consuming and degrading species [33], discussed as a marker for gut wall permeability, shows also a trend for a correlation with convenience. Calprotectin, a marker for inflammation, discussed for gut permeability [62] correlates with the general abundance of all bacteria ($R=-0.35$; $p=0.01$). Participants with present pain (46%) show significant higher Calprotectin values ($R=0.89$; $p>0.01$). However, those patients with a high diversity and abundance, seen as patients with balanced microbial equilibrium, showed typically low Calprotectin values (not significant). Recently an association between changes in microbiota, influences on the expression of γ -Aminobutyric acid receptors and pain was reported. For specific *Lactobacilli* strains increased expression of the receptor and reduced pain was mentioned. γ -Aminobutyric acid has a relaxing effect on gut smooth muscles [58]. In our results we observe a trend between enhanced abundance of IgG₄ and increased abundance of *Lactobacilli* ($p=0.05$). It could be hypothesized that characteristics of microbiota decide on the perception of abdominal inconvenience independently from immune reactivity addressing food compounds.

In conclusion an interaction between IgG₄ levels and single aspects of gastrointestinal microbiota and gut permeability can be suggested by the present results.

Conclusion

An interaction between IgG₄ levels and single aspects of gastrointestinal microbiota and gut permeability can be suggested by the present results.

The correlation of gut permeability and enhanced immune system responds to food antigens needs to be observed. Presumably analysis of GI- microbiota can be a way to an individualized intervention in situations of gastro intestinal discomfort linked to intolerances.

Acknowledgements

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List of Abbreviation

FFQ	food frequency questionnaire
F. prausnitzii	Faecalibacterium prausnitzii
CRP	C-reactive protein
ÖGE	Austrian Society for nutrition
SCFA	short-chain fatty acid
TLR	toll-like receptors

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Microbiota and epigenetic regulation of inflammatory mediators in type 2 diabetes and obesity

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Abstract

Metabolic syndrome is associated with alterations in the structure of the gut microbiota leading to low-grade inflammatory responses. An increased penetration of the impaired gut membrane by bacterial components is believed to induce this inflammation, possibly involving epigenetic alteration of inflammatory molecules such as Toll-like receptors (TLRs). We evaluated changes of the gut microbiota and epigenetic DNA methylation of TLR2 and TLR4 in three groups of subjects: type 2 diabetics under GLP1-Agonist therapy, obese individuals without established insulin resistance, and a lean control group. *Clostridium cluster IV*, *Clostridium cluster XIVa*, lactic acid bacteria, *Faecalibacterium prausnitzii*, and *Bacteroidetes* abundances were analyzed by polymerase chain reaction and 454 high-throughput sequencing. The epigenetic methylation in the regulatory region of TLR4 and TLR2 was analyzed using bisulfite conversion and pyrosequencing.

We observed a significantly higher ratio of *Firmicutes/Bacteroidetes* in type 2 diabetics compared to lean controls and obese. Major differences were shown in lactic acid bacteria, with the highest abundance in type 2 diabetics, followed by obese and lean participants. In comparison *Faecalibacterium prausnitzii* was least abundant in type 2 diabetics, and most abundant in lean controls. Methylation analysis of four CpGs in the first exon of TLR4 showed significantly lower methylation in obese individuals, but no significant difference between type 2 diabetics and lean controls. Methylation of seven CpGs in the promoter region of TLR2 was significantly lower in type 2 diabetics compared to obese subjects and lean controls. The methylation levels of both TLRs were significantly correlated with body mass index. Our data suggest that changes in gut microbiota and thus cell wall components are involved in the epigenetic regulation of inflammatory reactions. An improved diet targeted to induce gut microbial balance and in the following even epigenetic changes of pro-inflammatory genes may be effective in the prevention of metabolic syndrome.

Keywords

Firmicutes/Bacteroidetes ratio, *Faecalibacterium prausnitzii*, toll-like receptors, LINE-1, metabolic syndrome

Abbreviations

B. I. D.	lat. bis in die (two times per day)
BMI	body mass index
CD14	cluster of differentiation 14
FFAs	free fatty acids
GLP-1	glucagon like peptide-1
HbA1c	glycol hemoglobin
HDL	high density lipoprotein
IECs	intestinal epithelial cells
IFN- γ	interferon- γ
IL	Interleukin
LAB	lactic acid bacteria
LINE-1	long interspersed nuclear element 1
LDL	low density lipoprotein
LPS	lipopolysaccharide
O.D.	drug overdose
NF- κ B	nuclear factor κ B
SFA	saturated fatty acid
TIR	Toll/IL-1R homology domain
TLR	toll-like receptor
TNF- α	tumor necrosis factor α
VLDL	very low density lipoprotein
WT	wild type
ZNF	zinc finger protein

Introduction

The human gut harbors over 10^{14} microorganisms from 500–1,000 different species (Sommer & Backhed, 2012). This symbiosis between the host and the commensals is required for intestinal homeostasis, with disorders in the system leading to increased risk of various diseases, including metabolic syndrome (Takahashi *et al.*, 2011). Germ-free mice illustrate the role of the gut microbiota in the harvest and storage of energy: colonization with the microbiota of conventionally raised mice resulted in a 60 % increase in body fat and insulin resistance despite reduced chow consumption and increased activity compared with germ-free mice (Backhed *et al.*, 2004). A transfer of obese gut microbiota showed even more profound results (Turnbaugh *et al.*, 2006). The data suggest differences in the gut microbiota between obese, lean, and type 2 diabetic individuals. An established indicator is the ratio of *Firmicutes* to *Bacteroidetes*, which together make up about 95 % of gut microbiota, with varying relative abundance (*Firmicutes* 17-60 %; *Bacteroidetes* 35-80 %) (Karlsson *et al.*, 2010, Remely *et al.*, 2013).

These disparities in gut microbiota remain under discussion for the efficiency of food-derived energy extraction, body weight, and effects on endotoxin-mediated low-grade inflammation or even insulin resistance (Turnbaugh *et al.*, 2006). Intestinal epithelial cells (IECs) contribute especially to the symbiotic system by partially tolerating commensals and regulating mucosal inflammation (Takahashi *et al.*, 2009, Takahashi *et al.*, 2011). The higher expression of pro-inflammatory cytokines is coordinated mainly through distribution patterns of toll-like receptor (TLR) and NF(nuclear factor)- κ B, a key transcription factor in the inflammatory cascade. Toll-like receptors are common transmembrane proteins in the immune system, recognizing structural molecules derived from microbes (Creely *et al.*, 2007). Each TLR recognizes specific ligands; however all are competent to initiate activation of NF- κ B (Himes & Smith, 2010). The recognition of specific ligands, for example LPS (lipopolysaccharide), promotes the transcription of pro-inflammatory molecules and thus induces an inflammatory signalling cascade dependent on the stimulated TLR and TIR (Toll/IL-1R homology) domain. Phosphorylation and thus activation of intracellular kinases conducts the activation of the transcription factor NF- κ B, which in turn induces expression of pro-inflammatory genes (TNF- α (tumor necrosis factor α), IL-1 (interleukin), IL-12) in the cell nucleus. These inflammatory markers initialize Th17 (CD4+ T) and Th1 cells and promote an increased generation of chemokines and cytokines (Lee *et al.*, 2010, Sommer & Backhed, 2012).

While optimal TLR-signalling is required for the protection against microorganisms, inappropriate TLR expression and signalling can lead to hyper-responsiveness against bacterial ligands and thus enhance susceptibility to chronic inflammation (Furuta *et al.*, 2008). It can also lead to host-destructive pathology, for example inflammatory bowel disease, diabetes, and multiple sclerosis (Lee *et al.*, 2010, Sommer & Backhed, 2012). Metabolic endotoxemia correlates significantly with oxidative stress, macrophage infiltration markers, and all inflammatory markers triggering insulin resistance (PAI-1, IL-1, IL-6 and TNF- α) (Cani *et al.*, 2008, Cani & Delzenne, 2009, Caesar *et al.*, 2010, Musso *et al.*, 2010).

TLR4 senses bacterial LPS and saturated fatty acids (SFA), and also endogenous lipids that may contribute to the pathogenesis of lipid-induced insulin resistance (Shi *et al.*, 2006). In vivo, a high-fat diet induced the expression of TNF- α , IL-6, SOCS3, and MCP-1. In particular, a medium-chain fatty acid component of LPS, lauric acid, is thought to initiate TLR4 signaling. Thus it is hypothesized that increased fatty acid levels in obese individuals also promote TLR4 signaling in adipocytes and macrophages to induce inflammatory signaling and suppress insulin-mediated regulation of glucose metabolism - insulin resistance (Shi *et al.*, 2006). Mice lacking the co-receptor CD14 (cluster of differentiation 14) (Cani *et al.*, 2008, DiBaise *et al.*, 2008, Cani & Delzenne, 2009, Blaut & Bischoff, 2010), or TLR 4 deficiency itself or antibiotic treatment, a reduction of LPS, are protected from systemic lipid infusion and relieved of metabolic syndrome symptoms (Diamant *et al.*, 2011). A repression of TLR4 gene transcription is mediated by epigenetic mechanisms including DNA methylation and histone deacetylation (Takahashi *et al.*, 2011).

TLR2 senses lipoteichoic acid and FFAs (free fatty acids) (Bayarsaihan). Shuto *et al.* (2006) showed elevated TLR2 expression with hypo-methylation of the TLR2 promoter due to cystic fibrosis in bronchial epithelial cells (Shuto *et al.*, 2006). Additional cell line studies demonstrated that a high CpG methylation of the TLR2 promoter correlates with a lower TLR2 expression (Haehnel *et al.*, 2002).

However, on the basis of methylation patterns and their effect on the transcription level of TLR2, it is suggested that TLR2 regulation differs in cells from different origins (Diesel *et al.*, 2012). Mice lacking the receptor are associated with lower body weight, lower serum glucose, and improved insulin sensitivity (Caricilli *et al.*, 2008, Kellermayer *et al.*, 2011). Furthermore, an involvement of TLR2 in the regulation of serum lipids is indicated (Himes & Smith, 2010).

We hypothesize that gut microbes play a role in the regulation of intestinal inflammation through epigenetic modification of host genes, thereby providing a mechanism for the maintenance of the intestinal symbiotic system. We analyzed DNA methylation in the regulator regions of TLR4, TLR2, and LINE-1 in human whole blood samples, to determine the involvement of the intestinal gut microbiota. The *Firmicutes/Bacteroidetes* ratio, comprising Gram⁻ and Gram⁺ bacteria, and *Faecalibacterium prausnitzii*, as a marker of inflammation, were analyzed in feces of obese patients without established insulin resistance, insulin dependent type 2 diabetics under GLP-1 (glucagon like peptide-1) agonist therapy, and a lean control group.

Materials and methods

Study participants and study design

In accordance with the declaration of the Viennese Human Ethics committee, all study participants gave written consent for use of stool and blood samples. We conducted 24 insulin-dependent type 2 diabetes (D) patients under GLP-1 Agonist therapy (aged 58 ± 9 years; BMI 38.44 ± 5.14 kg/m²), fourteen obese participants (OC) with no established insulin resistance (aged 40 ± 15 years; BMI 33.71 ± 4.24 kg/m²), and a lean control (LC) group (n = 18; aged 26 ± 3 years; BMI 21.19 ± 1.93 kg/m²; Table 1). An oral glucose tolerance test was used to determine insulin resistance and diabetes. HbA_{1c} (glycol hemoglobin) blood levels were determined at laboratory Health Center South in Vienna. The levels were between 7-8 % hemoglobin in our type 2 diabetes group, a value that is protective against long-term consequences according to American Diabetes Association. As GLP-1 Agonist we used primarily Liraglutide (Victoza®), which was dosed o.d. 0.6 mg. Exenatide (Byetta®) was dosed b.i.d. 5 µg. All GLP-1 Agonists were injected subcutaneously by the patient himself, after teaching him/her carefully how to do so. To ensure comparable data patients were interviewed for their history of gastrointestinal diseases, use of antibiotics, probiotics or prebiotics.

Table 20

Table 1: Characterization of study participants

Group	number	sex		Age ± SD (years)	BMI ± SD (kg/m ²)
D	24	♀ 10	♂ 14	58 ± 9	38,44 ± 5,14
OC	14	♀ 7	♂ 7	40 ± 15	33,71 ± 4,24
LC	18	♀ 15	♂ 3	26 ± 3	21,19 ± 1,93

Fecal sample collection, processing, and analysis

Stool samples were stored at -70 C after collection. Bacterial DNA was extracted from fecal samples using the QIAamp® DNA Stool mini kit (Qiagen, Germany) according to the manufacturer's protocol. Additional samples were treated in FastPrep™ Lysing Matrix E tubes (MP Biomedicals, USA) twice for 45 sec in a bead-beater (Mini-Beadbeater 8 Bio-Spec Products, USA) with an intervening minute on ice. DNA concentration and quality was determined with a Pico100 (Picodrop, UK) and agarose gel-electrophoresis.

For qPCR analysis, we created clone libraries from dominant members of *Faecalibacterium prausnitzii* from two stool samples of healthy volunteers. Cloning was done following Remely et al. (2013) (Remely *et al.*, 2013). Bacterial abundance was quantified with TaqMan qPCR and SYBR Green qPCR in a Rotorgene 3000 (Corbett Life Science, Australia) using 16S rDNA group specific primers (Table 2). Primer and probes were checked for specificity with the Probematch function of the ribosomal database project 10 (<http://rdp.cme.msu.edu>). The mixture of PCR reactions and serial DNA dilution of typically strains were prepared according to Pirker et al. () (Pirker *et al.*, 2012).

On the basis of qPCR results we selected twelve samples for Roche GS FLX + Titanium next generation sequencing (LGC sequencing GmbH, Germany) to facilitate a comparison, although not all study samples were sequenced. Alignment and further analysis was done with the pyro pipeline of the ribosomal database project 10 (<http://rdp.cme.mus.edu/>) and qiime (Caporaso *et al.*, 2010).

Table 2: Primers and TaqMan®-probes targeting 16S rDNA coding regions of bacteria and for CpG-Methylation analysis

Table 21

Target	Primer/P robe	Sequence (5' - 3')	Conc. [pmol/μL]	Reference
<i>Clostridium cluster IV</i> (<i>Ruminococcaceae</i>)	Fwd primer	GCA CAA GCA GTG GAG T	4	(Matsuki <i>et al.</i> , 2004)
	Rev primer	CTT CCT CCG TTT TGT CAA	4	
	Probe	(6-FAM)-AGG GTT GCG CTC GTT-(BHQ-1)	2	
<i>Clostridium cluster XIVa</i> (<i>Lachnospiraceae</i>)	Fwd primer	GCA GTG GGG AAT ATT GCA	5	(Matsuki <i>et al.</i> , 2004)
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A	5	

	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)	1.5	
Lactic Acid Bacteria (LAB)	Fwd primer	AGC AGT SGG GAA TCT TCC A	4	(Walter <i>et al.</i> , 2001)
	Rev primer	ATT YCA CCG CTA CAC ATG	4	
<i>Faecalibacterium prausnitzii</i>	Fwd primer	GGA GGA AGA AGG TCT TCG G	0.25	(Ramirez- Farias <i>et al.</i> , 2009)
	Rev primer	AAT TCC GCC TAC CTC TGC ACT	0.25	
Bacteroidetes	Fwd primer	GAG AGG AAG GTC CCC CAC	3	(Layton <i>et al.</i> , 2006)
	Rev primer	CGC TAC TTG GCT GGT TCA G	3	
	Probe	(6-FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)	1	
TLR4	Fwd primer	GGTTAGAGGAAGAGATATTAGT GT	0,5	
	Rev primer	Biotin- TAACTCCAACCACATACCTCCA	0,5	
	Seq primer	ATATTAGTGTTTTAGAAATTG	0,5	
TLR2	Fwd primer	GGGAGTTTGTGGGAAGT	0,2	
	Rev primer	Biotin-ACCCCAAAAAAAAAA TACAAAAAACC	0,2	
	Seq primer	GTTTTAGTATTTTAGAAGGAGG G	0,2	

LINE-1	Fwd	TTT TGA GTT AGG TGT GGG	1	(Di <i>et al.</i> , 2011)
	primer	ATA TA		
	Rev	Biotin-AAA ATC AAA AAA TTC		
	primer	CCT TTC		
	Seq	AGT TAG GTG TGG GAT ATA	0.4	
	primer	GT		

Blood sample collection, processing and analysis

Because of missing understanding in the area of interactions between gut microbiota and blood cells we have used whole blood for epigenetic analysis. The samples were collected with PAXgene Blood DNA Tubes (Qiagen, Germany) and stored for a maximum of 14 days at 4°C. DNA was extracted using the PAXgene Blood DNA Kit (Qiagen, Germany) and bisulfite converted for epigenetic analysis with the EpiTect Bisulfite Kit (Qiagen, Germany) according to manufacturer's protocols. The DNA was measured and approved for purity with Pico100 (Picotrup, UK) and agarose gel-electrophoresis.

Suitable primers for the selected 4 (TLR4) and 7 (TLR2) CpGs in the regulatory regions of TLR4 and TLR2 (Table 2-3) were designed using the PyroMark Assay Design Software 2.0 (Qiagen, Germany). The selected TLR2 region, already described in the literature before, is a binding site of the transcription factor NF-κB which is known to regulate the TLR2 expression (Johnson & Tapping, 2007). To our knowledge the chosen region on the TLR4 gene was not analyzed before. There are binding sites for the transcription factors: GATA2, GATA3 and ZNF354C (zinc finger protein) within this region. Primers for LINE-1 are used to verify global methylation (Table 2-3). The PCR was carried out in a total reaction volume of 25 µL, containing 12.5 µL Pyromark 2X PCR master mix, 12.5 pmol (TLR4), 5 pmol (TLR2) or 1 pmol (LINE-1; long interspersed nuclear element 1) of each primer, 2.5 µL Coralload Concentrate 10X (Qiagen, Germany), and 5 ng (TLR4) or 10 ng (TLR2 and LINE-1) bisulfite converted DNA. PCR product quality was investigated with agarose gel-electrophoresis. Analysis of CpG methylation was performed with a Pyromark Q24 MDx (Qiagen, Germany).

Table 3: Sequence to analyze: TLR4 (geneID (NCBI): 7099), TLR2 (geneID (NCBI): 7097) and LINE-1

Table 22

Assay	Sequence 5'-3'	Number of CpGs	Size
TLR4	CTCGGTCAGACGGTGATAGCGAGCCACGCAT	4	31
TLR2	GGCGCCCGGCCGCGCGGACTTTCCTTTTGCTTACTTCCTAGTCCCG GGCAGGCCGGCT	7	59
LINE-1	TTCGTGGTGCGTCGTTT	3	17

Statistical analysis

The normality of distribution of the data was confirmed with the Kolmogorov-Smirnov test. The comparison of gut microbe abundances was performed using the non-parametric Mann-Whitney U test and for three unpaired groups the non-parametric Kruskal Wallis ANOVA. Differences in the percentages of methylation of TLR4 and TLR2 were tested for statistical significance using the student's two tailed t-test, one-way analysis of variance (ANOVA) and with the Tukey post hoc correction test. For correlation analysis we used the two-tailed Pearson test. P values <0.05 were taken as statistically significant. All data are shown as mean $\pm$ standard deviation.

Results

To evaluate possible effects of TLRs on epigenetic regulation we focused on the Gram⁺ bacteria *Clostridium Cluster IV*, *Clostridium Cluster XIVa*, and *Lactobacilli*, known for lipoteichoic acid, and on the Gram⁻ *Bacteroidetes*, known to include LPS in bacterial cell walls. These two divisions represent the majority of bacterial abundance (aprox. 95 %). Analysis of total bacterial abundance revealed no significant differences between the three groups. The measured *Firmicutes/Bacteroidetes* ratio was significantly different between the three groups, type 2 diabetics, obese patients, and lean controls. The *Firmicutes/Bacteroidetes* ratio was significantly higher in type 2 diabetics ($p < 0.01$). Between the obese and lean group a lower *Firmicutes/Bacteroidetes* ratio correlated with a lower BMI. Significantly higher abundance of lactic acid bacteria (LAB) was observed in type 2 diabetics compared to the other groups ($p < 0.01$; Figure 1), although both clusters of *Clostrida* showed no significant differences. These qPCR results were confirmed by high throughput sequencing analysis (Figure 2). Type 2 diabetics were enriched in *Firmicutes*, especially *Clostridiales* and *Bacilli*, in particular *Lactobacillales*. Accordingly *Bacteroidetes* were less abundant in type 2 diabetics, resulting in a higher *Firmicutes/Bacteroidetes* ratio. 454 sequencing and qPCR both indicated a *Firmicutes/Bacteroidetes* ratio of 1 in lean controls. *Faecalibacterium prausnitzii* showed a significantly lower abundance in type 2 diabetics compared to obese patients and lean controls ($p < 0.01$). In addition, obese participants had a lower abundance compared to lean controls.

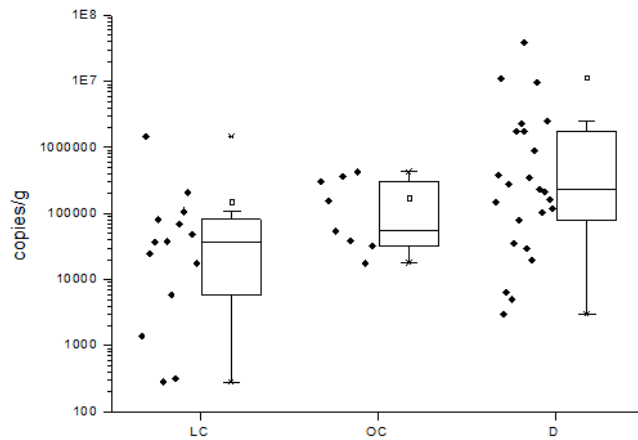


Figure 35

Figure 1

Quantification of lactic acid bacteria showing significant higher abundance in type 2 diabetics (D) than obese patients (OC) and lean controls (LC). Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range.

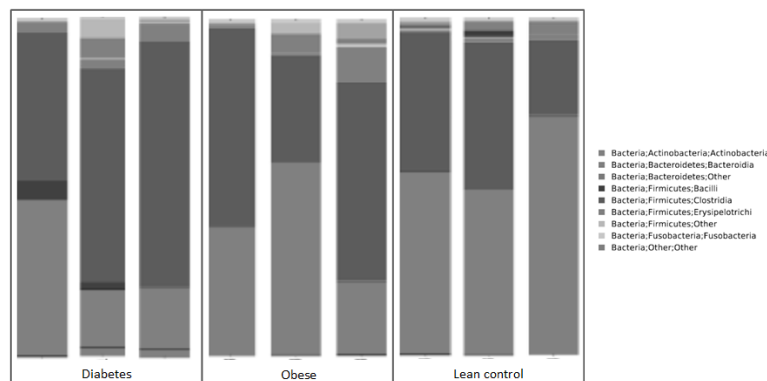


Figure 36

Figure 2

Bar Charts of 454 sequencing results reflect qPCR analysis with increased abundance of *Firmicutes* especially *Clostridiales* and *Bacilli* in type 2 diabetics (D) resulting in a higher ratio of *Firmicutes/Bacteroidetes*. Lean controls (LC) showed an according lower abundance of named bacterial groups and higher abundance of *Bacteroidetes*. Obese patients (OC).

The mean methylation of four CpGs in the first exon of TLR4 gene was significantly lower in the obese group compared to type 2 diabetics ($p < 0.01$; OC: 3.52 ± 1.17 %; D: 5.45 ± 1.33 %, Figure 2) and to the lean control group ($p < 0.01$; LC: 5.32 ± 1.17 %). We observed no significant differences between type 2 diabetics and the lean control group ($p = 0.95$). Single CpGs showed no differences in

methylation, and all four CpGs were significantly lower in obese participants than in lean controls and diabetics (Table 4). We observed a negative correlation between BMI and methylation in all study participants ($R = -0.288$; $p = 0.05$, Figure 3).

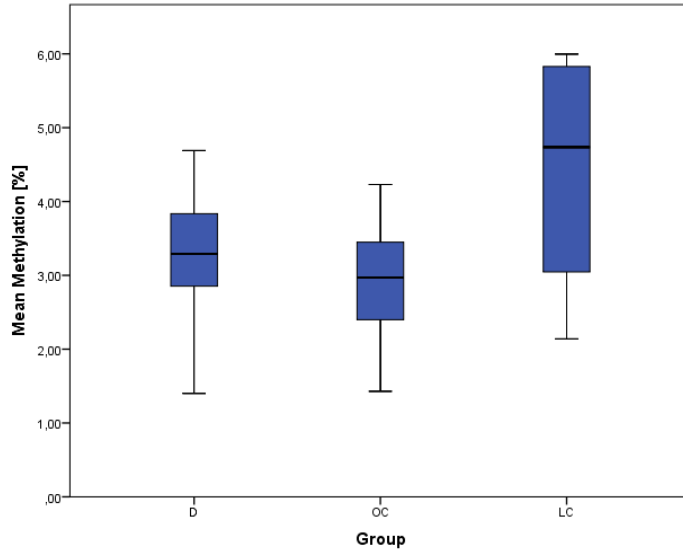


Figure 37

Figure 3

Mean methylation of TLR 4 of each group. Obese patients (OC) were significantly less methylated compared to type 2 diabetics (D; $p < 0.01$) and lean controls (LC; $p < 0.01$), but no significant differences between type 2 diabetics (D) and lean controls (LC) were observed ($p = 0.95$). Box range 25, 75 Perc; Whiskers indicate outliers; $\square$ indicates mean; x indicates maximum and minimum data range.

Table 4: Methylation % of 4 CpGs in the first exon of TLR 4

Table 23

Group	CpG1	CpG2	CpG3	CpG4
D	6.80	5.27	5.23	4.50
	± 1.58	± 1.50	± 1.51	± 1.19
OC	4.50	3.14	3.50	2.93
	± 1.62	± 1.31	± 1.02	± 1.39

LC	6.44 ± 1.34	5.39 ± 1.83	5.01 ± 0.90	4.43 ± 1.18
Significance				
LC – OC	0.005*	0.001*	0.006*	0.007*
LC – D	0.791	0.971	0.876	0.989
OC – D	0.000*	0.000*	0.000*	0.001*

* $p < 0.05$

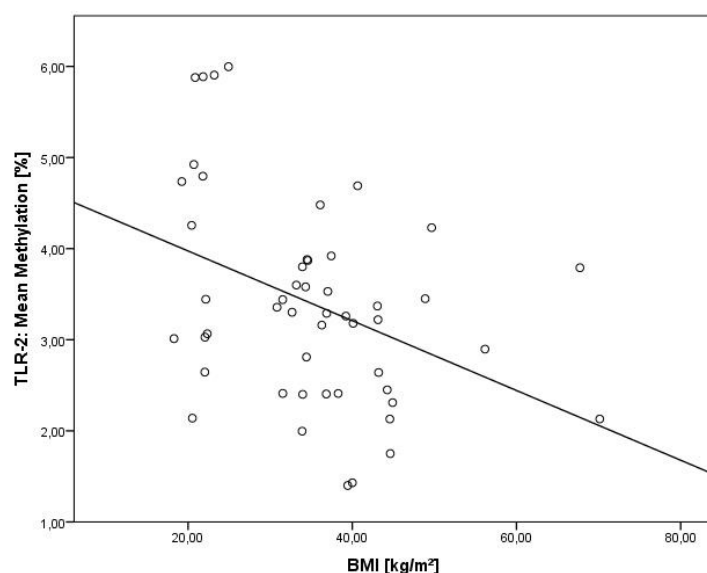


Figure 38

Figure 4

Correlation between TLR4 methylation % of mean methylation and BMI. Significant negative correlation between BMI and methylation of all study participants ($R = -0.288$; $p = 0.05$).

Analysis of seven CpGs in the promoter region of TLR2 gene showed a significantly higher methylation of all CpGs in the lean control group compared to both intervention groups ($p < 0.01$; LC: 4.37 ± 1.37 %; OC: 2.91 ± 0.74 %; D: 3.24 ± 0.83 %). No difference in mean methylation could be observed between obese and diabetic participants ($p = 0.521$, Figure 4). Different CpGs were not equally methylated: CpG 6 showed the highest methylation in all groups, while CpG 4 showed lowest methylation. We observed a significant difference in all single CpGs between lean controls and obese

controls, and also between lean controls and diabetics. Consistent with our mean methylation results, we observed no difference between obese controls and diabetic patients at all single CpGs (Table 5). Our results show a negative correlation of the mean methylation of all CpGs to BMI for all study participants ($R = -0.391$; $p < 0.01$; Figure 5).

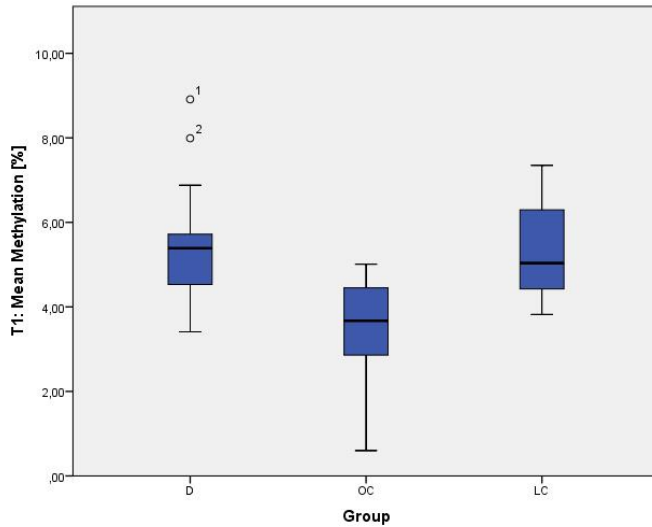


Figure 39

Figure 5

Mean methylation of TLR 2 promoter region between the three groups. Lean controls (LC) showed a significant higher methylation of all CpGs compared to obese and type 2 diabetics ($p < 0.01$; LC: 4.37 ± 1.37 %; OC: 2.91 ± 0.74 %; D: 3.24 ± 0.83 %). Type 2 diabetics (D) and obese patients (OC) showed no significant difference ($p = 0.52$). Box range 25, 75 Perc; Whiskers indicate outliers; $\square$ indicates mean; x indicates maximum and minimum data range.

Table 5: Methylation % of 7 CpGs in the promoter region of TLR 2.

Table 24

Group	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7
OD	1.90	3.85	3.23	1.53	2.05	6.72	3.41
	± 0.47	± 1.40	± 1.06	± 0.54	± 0.59	± 2.01	± 1.04
OC	1.77	3.60	3.04	1.53	1.77	5.62	3.00
	± 0.47	± 1.01	± 1.05	± 0.64	± 0.78	± 1.65	± 0.99
LC	2.69	5.11	4.25	2.49	2.39	9.10	4.52

	±1.16	±1.93	±1.26	±0.83	±0.69	±3.39	±1.73
Significance							
LC – OC	0.002*	0.013*	0.008*	0.000*	0.031*	0.000*	0.003*
LC – D	0.005*	0.031*	0.020*	0.000*	0.279	0.010*	0.027*
OC – D	0.818	0.860	0.857	1.000	0.419	0.308	0.546

* $p < 0.05$

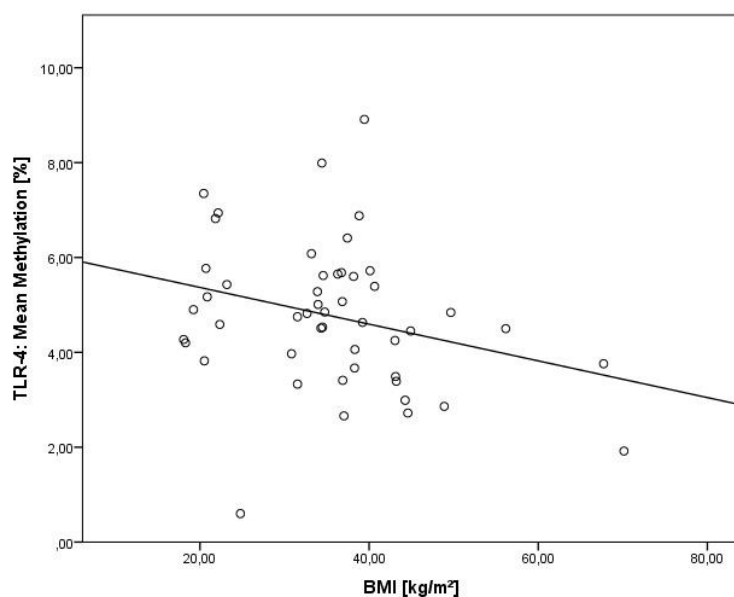


Figure 40

Figure 6

Correlation between TLR2 methylation % of mean methylation and BMI. Significant correlation between a higher BMI of all study participants and lower TLR2 methylation% ($R = -0.391$; $p = 0.005$).

The mean methylation of LINE-1 showed no significant differences between the groups. No correlations between gender or age and methylation were indicated in the TLR4 or TLR2 regulatory region or LINE-1 (data not shown) as well as in gut microbiota composition.

Discussion

Our study was designed to verify whether changes in gut microbiota composition correlate with epigenetic methylation patterns of TLRs associated with low-grade inflammation in metabolic syndrome (Turnbaugh *et al.*, 2006, Kim & Sears, 2010).

Gut microbiota composition

We observed a different *Firmicutes/Bacteroidetes* ratio between type 2 diabetics, obese, and lean controls, with significant differences particularly in *Lactobacilli*, but also for the species *Faecalibacterium prausnitzii*. LAB showed an increased abundance in type 2 diabetics (Figure 1), while *Faecalibacterium prausnitzii* was less abundant in comparison.

Previous studies reported obesity to be associated with a higher abundance of *Firmicutes* and reduced *Bacteroides* (Ley *et al.*, 2006, Turnbaugh *et al.*, 2006, DiBaise *et al.*, 2008, Musso *et al.*, 2010), but with no correlation to BMI (Abdolrasulnia *et al.*, 2008, Duncan *et al.*, 2008, Cani & Delzenne, 2009, Arumugam *et al.*, 2011). Enhanced nutritional intake may support the growth of *Firmicutes*, correlating with a higher number of genes coding for enzymes for the breakdown of dietary fiber (Turnbaugh *et al.*, 2006), and the fat storage in intestinal cells (Semova *et al.*, 2012). Weight loss reversed the disparity in the ratio by proliferation in the number of *Bacteroides* (Cani *et al.*, 2008, DiBaise *et al.*, 2008, Duncan *et al.*, 2008, Musso *et al.*, 2010, Schwartz *et al.*, 2010, Diamant *et al.*, 2011) rather than in the *Firmicutes* group (Cani *et al.*, 2008).

Faecalibacterium prausnitzii, a member of *Clostridium* Cluster IV, is known for its anti-inflammatory activity, decreasing the rates of NF- κ B activation and IL-8 secretion, increasing IL-12, IL-10, and IFN- γ (interferon- γ) (Furet *et al.*, 2010), and is also associated with improved insulin sensitivity (Karlsson *et al.*, 2013). A negative relationship between *Faecalibacterium prausnitzii* and inflammatory markers was shown in obese and diabetic patients after adjustment for BMI (Furet *et al.*, 2010). It is also associated with improved insulin sensitivity and diversity (Karlsson *et al.*, 2013). A negative relationship between *Faecalibacterium prausnitzii* and inflammatory markers was shown in obese and diabetic patients and remained after adjustment for BMI (Furet *et al.*, 2010). The proportions of *Faecalibacterium prausnitzii* were lower in type 2 diabetic patients, suggesting a higher incidence of low-grade inflammation, but also reduced microbial diversity (Furet *et al.*, 2010). Type 2 diabetics showed a significantly lower abundance of *Firmicutes*, while *Bacteroidetes* and *Proteobacteria* were more abundant, indicating a higher risk of endotoxemia by accumulation of Gram⁻ bacteria (Larsen *et al.*, 2010), especially plasma LPS (Cani *et al.*, 2008, Cani & Delzenne, 2009, Tsukumo *et al.*, 2009, Larsen *et al.*, 2010, Luoto *et al.*, 2010, Diamant *et al.*, 2011). However, the abundance of *Bacilli* is increased in diabetic mice and humans (Larsen *et al.*, 2010). Recently published data showed a positive correlation between *Lactobacillus gasseri* and HbA_{1c} values and a negative one between *Clostridium* species and HbA_{1c} values, but both species did not correlate with BMI, waist circumference, or waist-to-hip ratio (Karlsson *et al.*, 2013).

Consequences of differences in gut microbiota are under discussion and include increased energy expenditure (Turnbaugh *et al.*, 2006, Delzenne & Cani, 2011, Diamant *et al.*, 2011), as well as a contribution to various physiological functions and immune responses. Experiments comparing germ-free and colonized animals have shown that the host metabolism is shaped by the presence of gut microbiota (Backhed *et al.*, 2004, Turnbaugh *et al.*, 2006). Germ-free animals have a reduced expression of inflammatory agents, decreased abundance of macrophages in adipose tissue and an improved insulin tolerance (Diamant *et al.*, 2011). The homeostasis is maintained by stimulation of

the microbiota through IECs, TLRs, and NF- κ B, but also through nucleotide binding oligomerization domain (NOD)-like receptors (Takahashi *et al.*, 2011). A constant exposure to gut bacteria results in a partial toleration of the commensals and no longer affects TLRs stimulation by regulating mucosal inflammation through inhibitory molecules (toll interacting protein, COX-2 inhibitors, IL-10) (Takahashi *et al.*, 2011).

Epigenetic regulation of TLR4

TLR4, binding LPS, is indicated to play an important role in low-grade inflammation observed in metabolic syndrome (Kim & Sears, 2010). We observed a significantly lower methylation of TLR4 in the obese group compared to type 2 diabetics and lean controls. We also showed a negative correlation between BMI and methylation. Takashi *et al.* (2009) indicated a down-regulation of TLR4 gene transcription in IECs by DNA methylation and histone deacetylation (Takahashi *et al.*, 2009). Therefore, we assumed a higher expression of TLR4 receptors in obese participants according to lower methylation levels. However we observed no differences in the methylation of TLR4 gene between lean controls or type 2 diabetics (Figure 2).

Cani *et al.* (2007) observed changes in gut microbiota and an increase of LPS levels due to high-fat feeding after 4 weeks. The same metabolic endotoxemia was reproduced by subcutaneous infusion of LPS, while CD14 knock-out mice were resistant during both treatments. These knock-out mice were hypersensitive to insulin even during normal diet, suggesting an involvement of CD14 in the modulation of insulin sensitivity (Cani *et al.*, 2007, Kim & Sears, 2010). Antibiotic treatment reduced metabolic endotoxemia and the cecal content of LPS (Cani *et al.*, 2008). A higher expression of TLR4 and a hyper-responsiveness to commensal bacteria are also observed in patients with inflammatory bowel diseases (Vamadevan *et al.*, 2010, Takahashi *et al.*, 2011). Mice with a loss-of-function mutation in TLR4 are protected against diet-induced obesity; they showed a decreased adiposity, improved insulin sensitivity, and enhanced insulin-signaling capacity in adipose tissue, muscle, and liver compared to control mice on high-fat feeding (Tsukumo *et al.*, 2007).

Additional TLR4 is attributed to the regulation of intestinal mucosal repair by enhancing enterocyte migration and proliferation as well as increased apoptosis (Leaphart *et al.*, 2007). These mechanisms may also promote bacterial translocation, resulting in intestinal inflammation and systemic sepsis (Gribar *et al.*, 2008). Impairment, like a leaky gut, especially in type 2 diabetes is associated with reduced gastrointestinal motility as consequence of diabetic autonomic neuropathy, intestinal enteropathy, increased mucosal surface, and further bacterial overgrowth (de Kort *et al.*, 2010). A high-fat diet promotes the LPS incorporation into chylomicrons and the TLR4 induced phagocytosis by gut enterocytes, contributing to postprandial endotoxemia (Kim & Sears, 2010).

However, healthy IECs express low levels of TLR4 and are hyporesponsive to LPS and thus are able to tolerate the exposure to large quantities of luminal bacteria. The expressed low levels of TLR4 in the intestinal epithelial cells are associated with a higher methylation of the TLR4 gene (Takahashi *et al.*, 2009). Although, the *in vivo* methylation pattern of the TLR4 was dependent on the IECs differentiation state. TLR4 shows higher methylation and lower expression expressed in differentiated villus IECs than in undifferentiated crypt IECs. Thus, it is hypothesized that IECs are able to respond to

commensal and pathogenic bacteria in a different way. While TLR4 gene expression is low in villus IECs to prevent excessive inflammatory reaction, undifferentiated crypt IECs express high levels of TLR4, as a necessary reaction against pathogenic microbes (Takahashi *et al.*, 2011). Due to these differences between villus and crypt and a leaky gut with increased penetration, we supposed whole blood samples as sufficient to eliminate these differences between body tissues in analysis. Furthermore, the methylation level of the TLR4 region is significantly lower in IECs of the large intestine of germ-free mice than in those of conventional mice. Thus, Takahashi *et al.* (2011) reported a contribution of commensal bacteria to intestinal symbiosis by regulation of host gene expression via epigenetic modification (Takahashi *et al.*, 2011), although they did not differentiate between different gut microbiota compositions.

We are able to give evidence for a role of the *Firmicutes*/*Bacteroidetes* ratio, in TLRs methylation and inflammation. The *Firmicutes*/*Bacteroidetes* ratio indicates a shift towards Gram⁺ bacteria mainly in type 2 diabetics, but also in obese patients. These disparities in microbiota may result in the indicated changes of methylation status in the promoter region of TLR2, sensing lipoteichoic acid and fatty acids.

Epigenetic regulation of TLR2

We observed a significantly higher methylation of all CpGs in the TLR2 promoter region in the lean control group compared to both intervention groups resulting in a negative correlation between BMI and mean methylation (Figure 4, Figure 5). According to Nagy-Szakal and Kellermayer (2011) a higher DNA methylation is correlating with transcriptional down-regulation of associated genes (Nagy-Szakal & Kellermayer, 2011). In C57BL/6 TLR 2^{-/-} mice a genome wide colonic mucosal DNA methylation and changes in gene expression were observed compared to wild-type mice. This TLR2 deficiency resulted in a resistance to high fat and carbohydrate induced obesity. Additional TLR2 knock-out mice did not increase total serum cholesterol, VLDL (very low density lipoprotein) and LDL (low density lipoprotein) cholesterol, indicating an involvement of TLR2 also in the regulation of serum lipids (Himes & Smith, 2010). They had a lower body weight, lower serum glucose and improved insulin sensitivity, which may be explained by failed up-regulation of IL-6 in adipose tissue due to high-fat diet (Caricilli *et al.*, 2008, Kellermayer *et al.*, 2011). Improved insulin sensitivity was only observed during high-fat diet, but there was no evidence that TLR2 has a role in insulin resistance under regular chow, as it has no physiological role in the control of insulin sensitivity and signalling. Cell culture analysis provide convincing evidence that fatty acids lead to insulin resistance at least in part through TLR2 (Caricilli *et al.*, 2008). Caricilli *et al.* (2011) showed increased LPS absorption accompanied by subclinical inflammation, insulin resistance, glucose tolerance and obesity in TLR2 knock-out mice under regular chow after four months (Caricilli *et al.*, 2011).

The TLR2 knock-out correlated with a reduced *Firmicutes*/*Bacteroidetes* ratio on regular chow. The ratio was ~8.1 in WT (wild type) and ~4.3 in TLR 2^{-/-} animals (Kellermayer *et al.*, 2011). In addition Caricilli *et al.* (2011) indicated a 3-fold increase in *Firmicutes* and a slight increase in *Bacteroidetes* (Caricilli *et al.*, 2011). As already mentioned above a lower proportion of *Bacteroidetes* and an increase of *Firmicutes* (increased *Firmicutes*/*Bacteroidetes* ratio) in humans and animals is associated

with obesity (Ley *et al.*, 2006, Turnbaugh *et al.*, 2006, DiBaise *et al.*, 2008, Musso *et al.*, 2010) and type 2 diabetes (Remely *et al.*, 2013). In comparison the TLR 2-/- mice (Himes & Smith, 2010) as well as the lean controls in our study showed a lower *Firmicutes/Bacteroidetes* ratio. We assumed that the lean phenotype might be a result of reduced stimulation of inflammatory pathways (Diamant *et al.*, 2011). In healthy lean participants a base-line physiological level of inflammation is hypothesized as a result of the continuous exposure to gut microbiota, indicating that inflammatory reactions are regulated by specific mechanisms in the healthy intestine (Takahashi *et al.*, 2011).

Epigenetic regulation of LINE-1

Changes in global methylation can affect gene expression, genomic stability, and chromosomal structure. LINE-1 is a well described marker for global methylation (Di *et al.*, 2011). In the case of LINE-1 methylation, we observed no significant differences in our study population, although a hypomethylation in blood is associated with a higher risk of cardiovascular disease (Baccarelli *et al.*, 2010), visceral adipose tissue, higher plasma glucose level, lower HDL (high density lipoprotein) cholesterol level and greater risk for metabolic syndrome (Turcot *et al.*, 2012). Summarized LINE-1 elements are usually highly methylated and their variability can be associated with older age (Turcot *et al.*, 2012). In the distribution of age in our study groups, type 2 diabetics show a higher age median, reflecting the typical incidence of metabolic syndrome with aging due to western diet/lifestyle. However, LINE-1 methylation suggests no difference in the global methylation in our study population due to age. In addition we used whole blood analysis to include all type of cells providing a blood methylation overview although methylation pattern can be different between different types of white blood cells but may cause the danger to oversee relevant changes on other cell types.

Conclusion

Our results indicate that the gut microbiota, especially the *Firmicutes/Bacteroides* ratio, influences epigenetic modifications of host genes and thus helps maintain intestinal symbiosis. We provide evidence that different gene-specific methylation patterns are associated with gut microbiota composition, resulting in different states of disease, obesity or type 2 diabetes. Additional data from animal tissue would be desirable to verify the dependency of DNA-methylation on bacterial groups.

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Book: Detection and Identification Of Probiotic Microorganisms And Other Beneficial Organisms From The Human GI Tract.

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Abstract

Molecular microbiological analysis has increased the understanding of the diversity and phylogeny of beneficial strains and their functions. This modern techniques, including genotyping methods, become increasingly important for species identification and for the differentiation of probiotic strains. The precise classification and identification of probiotic strains give a strong indication of its typical habitat and origin, safety and technical applicability and provides possibilities for monitoring and product quality.

This chapter provides an overview of probiotic strain characterization, gut metagenomics and the analytical methods (FISH, PCR, RAPD, DGGE, repPCR, PFGE, RFLP, microarray, high throughput sequencing) required for their study.

Introduction

Experts have debated how to define probiotics. One widely used definition, developed by the World Health Organization and the Food and Agriculture Organization of the United Nations, is that probiotics are “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host.” Most probiotics are similar to microorganisms naturally found in the human gastrointestinal tract. Most often, these organisms are *Lactobacillus* or *Bifidobacterium* species. Relatively few probiotics, such as *Saccharomyces boulardii*, are yeasts (NIH, <http://nccam.nih.gov/health/probiotics>). Until recently, mostly only the lactic acid bacteria (LAB) were considered of relevance for food and nutrition. LAB are gram-positive, non-spore forming, catalase-negative, aerotolerant anaerobes. They are fastidious, acid-tolerant and strictly fermentative, producing lactic acid as the major end product of sugar fermentation (Axelsson, 1998). However, exceptions from this general description do occur.

According to this view probiotics relate to the beneficial microbiota in the gut. In contrast to the high diversity of gut microbiota, probiotic bacteria historically comprise only a few groups of this diversity. This has been discussed in a previous critical review: “Much effort has been devoted to screening bacterial isolates for properties deemed appropriate for a “probiotic” strain, mostly characteristics that might enable the microbes to at least survive passage through the digestive tract. There must be millions of such strains to choose from, because the intestinal milieu of humans is already the home to bacteria with these properties. The concept concentrates essentially on two groups of bacteria, lactobacilli and bifidobacteria, while practically ignoring the vast array of other species that inhabit the intestinal tract of humans. It is the impact of probiotics on the composition of the intestinal microbiota, nevertheless, that forms the basis for the probiotic concept” (Tannock *et al.*, 1999).

Further research into the mechanisms of probiotic action has extended to the ability of cell walls or particles derived from probiotic microorganisms to exert probiotic functions. These non-living probiotic derivatives are proposed to act by receptor mediated functions in immune or intestinal cells (Probiotics, Markowitz *et al.*, 2012). Many of the effects obtained from viable cells of probiotics are also obtained from populations of dead cells. Heat-killed cells of *Enterococcus faecalis* stimulate the gastrointestinal immune system in chickens. Likewise, dead bifidobacteria induce significant increases in TNF- α production. Administration of heat-killed *E. faecalis* to healthy dogs has also been shown to increase neutrophil phagocytes. The probiotic paradox is that both live and dead cells in probiotic products can generate beneficial biological responses. Live probiotic cells influence both the gastrointestinal microbiota and the immune response whilst the components of dead cells exert an anti-inflammatory response in the gastrointestinal tract (Adams, 2010).

This interaction between probiotic microorganisms or their derivatives with the gut microbiota is now a focal point of probiotics research. This requires the characterization and enumeration of all microorganisms colonizing the gut. Modern molecular techniques, including genotyping methods, have become increasingly important for species identification and for the differentiation of probiotic strains. The precise classification and identification of a probiotic strain gives a strong indication of its typical habitat and origin, indicate the strain’s safety and technical applicability, provides possibilities

for monitoring and product quality and is a basis for patenting and commercial use. Current genotyping methods include denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), pulse field gel electrophoresis (PFGE) and others. These methods all require previously isolated pure cultures of gut microorganisms.

In gut metagenomics methods are applied which do not require the isolation of pure cultures. It enables the analysis and characterization of species and groups of microorganisms with possibly probiotic functions, their diversity, ecology and interaction as well as possible consequences of probiotic intervention. Clearly, methods for identification and classification of strains and metagenomic strategies follow different objectives and face specific restrictions. Compared with Sanger sequencing-based methods, new high-throughput pyrosequencing methods provide orders of magnitude more data on the diversity of organisms in their natural habitat, but technological biases and relative accuracy remain poorly understood (Tedesoo *et al.*, 2010)

This chapter provides an overview (Fig. 12) of probiotic strain characterization, gut metagenomics and the analytical methods required for their study.

Bacteriophage And Transposon Infections Of Probiotic Strains

Bacteriophage infections of lactic acid bacteria (LAB) pose a serious risk to the industrial production of dairy foodstuffs. Dairy fermentations are susceptible to phage infection since the starting material (mainly, raw milk) is not sterile and, in general, pasteurization processes are not adequate to deactivate viral particles. In addition, the continued use of the same starter cultures provides a constant host for phage proliferation which, consequently, can lead to slow lactic acid production or even the complete failure of fermentation, which allows the proliferation of pathogenic bacteria and can cause significant economic losses due to product rejection. Ways to improve resistances against phage infections have been described including the triggering of suicide systems ((Djordjevic *et al.*, 1997, Moineau, 1999).

The available knowledge of *Lactobacillus* phages is limited when compared with that of *Lactococcus* and *Streptococcus* bacteriophages. Only a small number of *Lactobacillus* phages have been studied in detail (Capra *et al.*, 2006, Alemayehu *et al.*, 2009). PCR methods have been described for specific identification of strains based on phage-related sequences for probiotic *Lactobacillus rhamnosus* strains (Brandt & Alatossava, 2003) and for the detection and identification of bacteriophages infecting *Lactobacillus casei/paracasei*, based on highly conserved regions (Binetti *et al.*, 2008).

Complete genome sequencing of several probiotic strains provides insight into their adaptation to the gut environment and also highlights the abundance of transposable elements present in these bacteria. Transposable elements can promote genome plasticity, phenotypic changes and contribute to bacterial adaptability. Identified phages, tempered phages or prophages contribute to mechanisms of a horizontal gene transfer among bacteria in the human gut (Falentin *et al.*). This genetic transfer is a serious aspect of the risk assessment required for approval of probiotic products, especially those probiotic strains that are genetically modified.

Identification Of Gut Microorganisms

Historically, bacteria have been identified by phenotypic characterization, although more recently a number of molecular methods have been developed for the identification of a microorganism, i.e. the assignment of an unknown micro-organism to a known taxon at the species or subspecies level. Depending on the objectives, a multilevel approach is recommended. The appropriate molecular methods to be used depends on the level of identification required (genus, species or subspecies) and the target organism. The methods differ significantly in labor and personnel expenses, in the degree of discrimination and in reproducibility within and between laboratories.

One of the first molecular methods for species differentiation is DNA-DNA hybridization (Sibley *et al.*, 1984). The technique compares the whole genomes of two organisms. A DNA mixture is incubated so that the DNA strands can separate and re-associate. Reassociation leads to the formation of hybrid DNA doublestrands. Hybrid DNA can only be formed when the sequences are at least 80% complementarity. The smaller the genetic distance between the two organisms is, the more closely will the hybrids bind together and the more energy will be required to separate the two strands again. Two individuals belong to one species if the difference of the melting temperature of the DNA hybrids is less than 5% or their similarity is at least 97.5 over their whole genome. (Shneyer, 2007, Krogus-Kurikka *et al.*, 2009)

The 16S rRNA gene is often used for differentiation and classification of microorganisms. The degree of similarity of the 16S rRNA genes from different individuals is representative of the variation in the whole genome of different individuals.

The first crucial step for successful identification of bacteria from faeces using 16S rDNA sequence comparison is to isolate DNA from the appropriate sample. DNA from Gram-positive bacteria, including LAB and bifidobacteria (CYPIONKA, 2010), is more difficult to extract as from Gram-negative bacteria. This is due to their thick peptidoglycan cell wall which requires additional enzymatic or mechanical lysis steps prior to kit purification to yield useful quantities of pure DNA.

LAB colonize the small intestine at cell numbers of 10^2 /g gut content. LAB that migrate through the gastro-intestinal (GI) tract encounter high numbers of anaerobic bacteria in the colon at cell densities of about 10^{12} - 10^{13} cells/g fecal content (Fujimura *et al.*, 2010). This considerable dilution of LAB in the fecal content renders their detection with PCR based methods difficult and in some cases molecular methods will even fail to detect LAB in fecal samples.

The colon is a strictly anaerobic environment and is most suitable for establishment and proliferation of bifidobacteria (which comprise only 3-6% of adult GI microbiota). Their detection from fecal content will be possible in most cases, but the fact that they occur at relatively low abundance among the densely populated colon environment does pose a challenge for the molecular biologist (Fujimura *et al.*, 2010)

Each of the methods used for the metagenomic detection and identification of beneficial microbes has its advantages and disadvantages.

16S rRNA Sequencing

DNA sequencing, developed by Frederick Sanger, has brought about a revolution in molecular biology. DNA sequence data can be compared to determine the degree of relatedness between the two sequences of DNA. This can be translated to the degree of relatedness of organisms that the DNA originates from.

During sanger sequencing, the target sequence is amplified in a PCR reaction using a mixture of nucleotides and fluorescently labeled dideoxy-nucleotides. Incorporation of a dideoxy-nucleotide in the newly synthesized DNA strand causes strand termination. Statistically, DNA synthesis is terminated at each possible nucleotide position in each reaction. Terminated DNA molecules of different lengths can be separated in an agarose gel according to size. The nucleotide sequence can then be determined (LÖFFLER, 2004).

It has been well documented that the different variable regions within the 16s rRNA gene are more or less suitable for phylogenetic identification of bacteria. There are 9 of variable regions within the 16S rRNA gene. They differ in information content, and some bacteria, such as *Escherichia coli* and *Shigella*, even do not differ at all within the 16S rRNA gene. 16S rRNA gene sequencing allows species assignment within the *Bifidobacteria*, but is not suitable for strain differentiation (Bottacini *et al.*, 2010). A major advantage of 16S rRNA gene sequencing is that it is possible to obtain sequence data from uncultured microbes originating from environmental samples (i.e. stool samples), however 16S rRNA gene sequencing can be costly and laborious (Maukonen *et al.*, 2008).

Alternative Targets To 16S rRNA Coding Regions

The simultaneous use of several housekeeping genes in bacterial taxonomy offers a higher resolution than 16S rRNA gene sequence data at the species level as it integrates information from different molecular markers from throughout the bacterial chromosome {Stackebrandt *et al.*, 2002} {Zeigler, 2003}. Common genes used as an alternative to 16S rRNA are those encoding the alpha subunit of the ATP synthase (*atpA*), RNA polymerase alpha subunit (*rpoA*) and the phenylalanine t-RNA synthase alpha subunit (*pheS*) can be used to differentiate the relatedness between strains of the same species. These genes are often more discriminatory among closely related species and strains than the sequence of the 16S rRNA gene (Naser *et al.*, 2006).

Bacterial genomes often contain a significant proportion of sequences originating from prophages (Chopin *et al.*, 2001, Hayashi *et al.*, 2001). Such phage-related sequences have been targeted for strain-specific differentiation, termed phi-sequencing. Nucleotide primers for targeting phage-related sequences in probiotic *Lactobacillus rhamnosus* strains have been successfully developed (Brandt & Alatossava, 2003). The authors found their assay could discriminate well between strains of *L. rhamnosus* but described amplification of phylogenetically unrelated non-target strains, pointing out that phi-sequencing should be preferentially used in combination with other molecular methods.

Quantitative PCR (qPCR)

Quantitative PCR is used for absolute or relative quantification of microbial DNA. The specificity of primers determines which bacteria can be identified and their accurate design is essential for

sensitive and specific amplification. A lack of specificity can lead to false-positive amplification of species or strains and overestimation of their abundance. In probiotic research, it is important to differentiate bacteria at the strain level because only certain strains of a species possess the desired properties (Felis & Dellaglio, 2007).

Bacterial enumeration using qPCR is dependent on acquiring DNA concentration in each sample used as a template by spectrophotometric measurement e.g. nanodrop. To avoid PCR bias and to allow the linearity of measurement, the same DNA concentration for all templates should be used. In addition, impurities in the DNA suspension can be identified by A_{260}/A_{280} ratio for protein contamination and A_{260}/A_{230} ratio for salt contamination. Impurities of the DNA template can have adverse effects on the efficiency of PCR and significantly affect the accuracy of quantification.

Absolute quantification is calculated by comparing the cycle threshold value of a test sample to a standard curve generated from cycle threshold values samples of known bacterial concentrations. Relative quantification is calculated by comparing the cycle threshold value of a test sample with the cycle threshold value of control DNA. Cycle threshold refers to the PCR cycle at which the fluorescence raises over threshold (Carey *et al.*, 2007, Louis & Flint, 2009). One commonly used fluorescent dye is SYBR green, which intercalates with double stranded DNA. For reliable quantification, the concentration range of dilutions should be chosen to cover the whole concentration spectrum of the samples. If using broad specificity primers targeting phylum or class levels, a mixed standard is recommended. For comparison of different runs the use of control sample in each run is recommended (Carey *et al.*, 2007).

Following amplification in qPCR, the PCR products can be analysed in melt curve analysis. PCR products are subject to an increasing temperature gradient which results in denaturation of double stranded DNA to single stranded DNA. As SYBR green only generates fluorescence when intertwined with double stranded DNA, the rate of fluorescence decrease can be measured generating melt curves. Melt curve analysis can be used as a quality control, as primer dimers and unspecific PCR products might melt at a different temperature. In a special application of melt curve analysis, different isoforms of a gene involved in butyrate production were analyzed. Different peaks were shown to be representative of a different phylogenetic lineage of bacteria (Louis & Flint, 2009). The 16S rRNA gene is often targeted in this technique although other house-keeping genes can be used. This method requires DNA from pure, previously characterized bacterial isolates for generation of PCR products and melt curves for comparison with unknown samples.

High Resolution Melt (HRM) Analysis

HRM analysis is used for the detection of mutations, polymorphisms and epigenetic differences in PCR amplicons by measuring the thermal denaturation of double-stranded DNA. In microbiology, HRM analysis is used for species- and strain characterization. It characterizes nucleic acid samples based on their disassociation (melting) behavior in a similar fashion as a basic melt analysis. HRM employs a slower increase in temperature for denaturation and acquires many times more data in a single run allowing for discrimination of highly similar sequences. Samples can be discriminated according to their sequence by a combination of length and G-C content (Wittwer, 2009). Detection

and differentiation of samples containing sequence variants rely on a change in the amplicon T_m and/or shape of the amplicon melting curve. Samples containing the same sequence variants are identified as groups that exhibit similar melting profiles (Radvansky *et al.*). Even single base changes such as SNPs (single nucleotide polymorphisms) can be readily identified (Wojdacz *et al.*, 2008). Depending on primer design and the gene targeted, bacteria can be identified and differentiated at the strain level (Ereqat *et al.*).

Fluorescence In Situ Hybridization (FISH)

By this technique it is possible to identify bacteria directly in their habitat with high-resolution microscopic techniques such as confocal laser scanning microscopy. It provides, information on the number and spatial distribution of microorganisms (Amann *et al.*, 2001). FISH employs fluorescence-labeled DNA probes to detect or confirm genes within chromosomes or gene expression. It is a method to localize a specific DNA or RNA sequence, prove genetic changes in tissues. It can be carried out at the single cell scale in intact cells -, in situ.

The hybridization is generally described as a fusion of two complementary, exactly matching, single-stranded nucleic acid molecules. For detection of a specific DNA or RNA molecule, a complementary gene probe typically labeled with a fluorochrome is required for hybridization. Typical labels include cyanine (e. g. Cy3 and Cy5) and fluorescein molecules (Michalet *et al.*, 2005). FISH experiments often employ several differently labeled probes of different specificity (from phylum to species) allowing for reliable identification of bacteria in complex environmental habitats as demonstrated in Fig. 1.

Commonly used probes have a length of 15 - 30 nucleotides and are covalently linked at the 5'-end to a single fluorescent dye molecule (table 1). Sequence signatures serving as suitable target site for nucleic acid probing can be conveniently and automatically identified using a probe design tool (Ludwig *et al.*, 2004). Short probes have easier access to their target, but they might carry fewer labels and are not so selective. Not all bacterial and archaeal cells can be permeabilized by oligonucleotide probes using standard fixation protocols (Bottari *et al.*, 2006). The accessibility of selected target sites for oligonucleotide probes can be increased by adding unlabelled oligonucleotide probes that bind adjacent to the probe target site. The aim is to unfold the nucleic acid and thus facilitate probe hybridization. These so-called helper probes need to be designed carefully because of their specificity to the respective probe and must have a differentiation temperature (T_d) at least as high as the temperature of the probe to prevent dissociation of the helper at stringent hybridization conditions (Fuchs *et al.*, 2000).

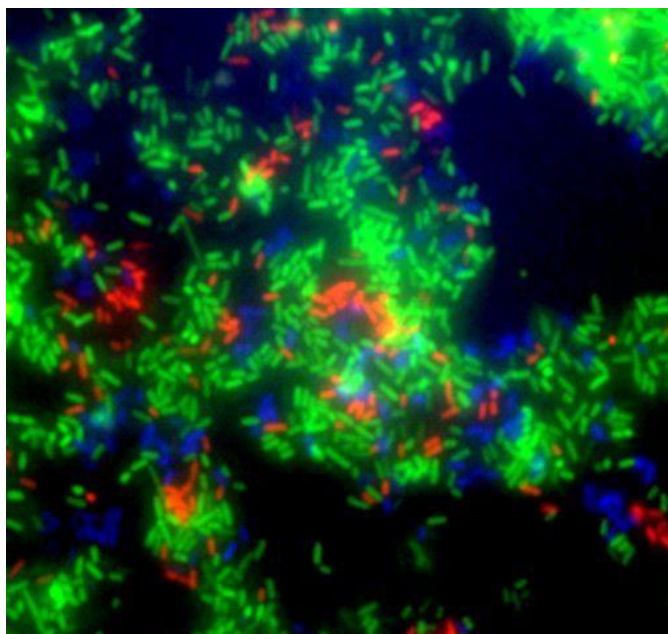


Figure 41

Fig. 1 Three-color fluorescent in situ hybridisation image of bacteria. Three FISH probes labelled with different fluorescent dyes have been used to simultaneously identify the structure of microbial community. Each labelled single bacterial cell can be measured by Raman microspectroscopy to examine if the cell has special ecological function.

A typical FISH protocol (Fig. 2) includes four steps: fixation and permeabilization of the sample, hybridization, washing steps to remove unbound probe and detection of labelled cells by microscopy or flow cytometry (Amann *et al.*, 2001). Prior to hybridization, bacteria must be fixed and permeabilized in order to allow penetration of the fluorescent probes into the cell and to protect the RNA from degradation by endogenous RNases (Moter & Gobel, 2000). The sample is either settled on membrane filters and covered with the fixing agent (Glockner *et al.*, 1999), or mixed with the fixing agent, incubated, sedimented by centrifugation, resuspended, transferred to glass slides and dried (Amann *et al.*, 1990).

Table 25

Fluorochrome	Colour	Max. excitation λ (nm)	Max. emission λ (nm)
Alexa488	Green	493	517
AMCA	Blue	399	446
CY3	Red	552	565
CY5	Red	649	670
CY7	Violet	743	767
DAPI	Blue	350	456
Fluorescein	Green	494	523
Rodamine	Red	555	580
TAMRA	Red	543	575
Texas red	Red	590	615
TRITC	Red-orange	550	580

AMCA methyl cumarinic acetic acid, *CY* carbocyanine, *DAPI* 4'6-diamidino-2-phenylindole dihydrochloride, *TAMRA* tetramethyl rhodamine, *TRITC* tetramethylrodamine-isotiocyanate

Table 1: Most commonly employed fluorescent dyes to label oligonucleotides for FISH analysis.

From :	Application of FISH technology for microbiological analysis: (Bottari <i>et al.</i> , 2006)
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Probes and the stained preparations must always be stored in the dark as they are very sensitive to degradation by light. FITC (green) fades the fastest, whereas red and DAPI are very robust. When taking photos, always begin with green and use DAPI last. For a third colour, mix the probes red and green to create yellow. This technique is a relatively rapid method to evaluate the presence and activity of probiotics and other gut microorganisms, directly in the sample or the process studied (Bottari *et al.*, 2006). FISH is an *in situ* technique and has the potential to reveal the composition of complex microbial associations in natural systems.

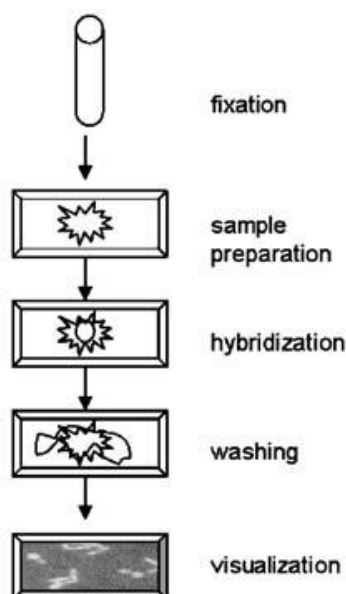


Figure 42

Fig. 2: Flow chart of a typical FISH. The procedure includes the following steps: (1) fixation of the specimen directly in sample tubes; (2) transfer of the sample on a glass slide and preparation of the sample, including specific pretreatment steps; (3) hybridization with the respective probes for detecting the respective target sequences; (4) washing steps to remove unbound probes; (5) mounting, visualization and documentation of results.

Fingerprinting Techniques

Denaturing DNA Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis is used to determine microbial structural differences between DNA, and also to investigate broad phylogenies or specific target organisms such as pathogens or xenobiotics degraders (Sigler et al., 2004). DGGE separates bacterial sequences by electrophoresis of PCR-amplified 16S rRNA gene fragments in a polyacrylamide gel with constantly increasing concentration of denaturants (Fischer & Lerman, 1979). These denaturants are usually formamide and urea (Felske & Osborn, 2005). Group-specific primers, which target a groups of bacteria, such as lactobacilli (Walter *et al.*, 2001) or bifidobacteria (Satokari *et al.*, 2001) are available.

The oligonucleotide primers used to amplify DNA fragments for DGGE contain a GC-clamp, an approximately 40bp region consisting mainly of guanine and cytosine. This GC-clamp prevents complete melting of the DNA fragments so that each double stranded DNA PCR product stops migrating at a unique denaturant concentration. DNA melting depends on GC content of the fragments in contrast to agarose gel electrophoresis, which separates DNA according to length. Occasionally, some microorganisms such as *Bacteroides thetaiotaomicron*, *Lactobacilli* and a pair of *Fungi* produce several bands because of their multiple 16S rRNA operons. As a result the gel contains

a pattern of bands and the diversity of the sample can be estimated (Sigler, 2004) (Blume *et al.*, 2010). For *Bifidobacteria* the identification at the species level only is reliable (Ventura *et al.*, 2004). The resulting bands on the DGGE gel can be compared with references amplified from known bacteria.

DGGE allows a rapid diversity assessment and comparative analysis as seen in Fig. 3. Analysis software is available for closely grouped bands at the same height to determine the degree of relatedness (Vitali *et al.*). Further identification of fragments can be carried out by sequencing (Ventura *et al.*, 2004).

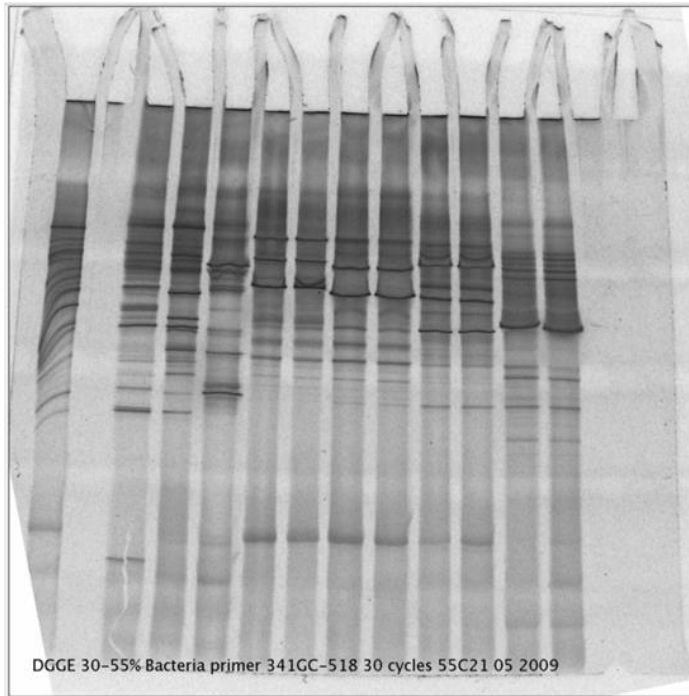


Figure 43

Fig. 3: PCR-DGGE band patterns of 16S rRNA coding regions of Bacteria amplified with primers 341GC and 518.

Pulsed Field Gel Electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) enables separation of high-molecular-weight fragments which is not possible using conventional gel electrophoresis methods. During electrophoresis, the direction of the electric field changes periodically. For PFGE rare-cutting restriction endonucleases can be used to produce a small number of large fragments from DNA. This creates an easily interpretable pattern of bands (Holzapfel *et al.*, 2001).

PFGE is a useful tool for subtyping clinical isolates from different geographic regions and different hosts and for recognizing potentially new serovars (Galloway and Levett). It is a very useful method for highlighting isolates which may represent new species or serovars (Galloway and Levett, 2008). The method has high reproducibility and a very good selectivity at the strain level but can be

laborious and time consuming (Ventura *et al.*, 2004) (Kneifel & Domig, 2009). Therefore, only a limited number of samples can be readily processed. PFGE is a frequently used method to differentiate strains of *Bifidobacteria* (Wall *et al.*, 2008) (Roy *et al.*, 1996) (Ventura *et al.*, 2004), *Lactobacilli* at the strain level (Ventura & Zink, 2002) but not at the genus or species level (Huys *et al.*, 2006).

Randomly Amplified Polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA (RAPD) is a special form of PCR in which a short random primer at low stringency. This provides randomly sized DNA fragments (Holzapfel *et al.*, 2001). Different species and strains can be distinguished according to the generated bandpattern.

An advantage of the method is that it is simple, fast and cheap. The disadvantage is that experiments are particularly difficult to standardize (Kneifel & Domig, 2009) (Ventura *et al.*, 2004). This method makes it possible to identify lactobacilli at the strain level (Mahenthiralingam *et al.*, 2009) (Booysena *et al.*, 2002); but comparison of whole genus or species levels is not possible (Huys *et al.*, 2006). In contrast, *Bifidobacteria* can be identified at species as well as strain level (Ventura *et al.*, 2004).

Repetitive Genomic Element PCR (repPCR)

All living things harbour repetitive DNA sequences in multiple sites. These sequences form the basis for several powerful tools in molecular diagnostics, medical microbiology, epidemiological analyses and environmental microbiology (Ishii and Sadowsky, 2009).

RepPCR is a simple PCR-based technique with a high discriminatory power. Furthermore repPCR is suitable for a high-throughput of strains at low cost and makes it possible to type a wide range of bacteria (Gevers *et al.*, 2001) (Olive & Bean, 1999). The discriminatory power for LAB (Gevers *et al.*, 2001) and *Bifidobacteria* for example, reaches from species to strain level (Huys *et al.*, 2006).

Restriction Fragment Length Polymorphism (RFLP) And Terminal Restriction Fragment Length Polymorphism (T-RFLP)

In RFLP, a PCR product is digested with one or several restriction endonucleases in order to obtain a pattern of nucleotide fragments capable of distinguishing different species or strains. Restriction enzymes cleave DNA at or around a distinct recognition site. Some restriction enzymes in nature cut DNA at random far from their recognition sites and are of little value to the molecular biologist. Other restriction enzymes recognize modified, typically methylated DNA and are exemplified by the McrBC and Mrr system of *E. coli* (BioLabs, 2010, Duncan *et al.*, 2011).

The most common restriction enzymes used in RFLP are HhaI, HindII and NotI that cleave DNA within their recognition sequences. Enzymes of this kind are readily available commercially. Most recognize DNA sequences that are symmetric because they bind to DNA as homodimers, but a few, (e.g., BbvCI: CCTCAGC) recognize asymmetric DNA sequences because they bind as heterodimers. Some enzymes recognize continuous sequences (e.g., EcoRI: GAATTC) in which the two half-sites of the recognition sequence are adjacent, while others recognize discontinuous sequences (e.g., BglII: GCCNNNNNGGC) in which the half-sites are separated. Cleavage leaves a 3'-hydroxyl on one side of each cut and a 5'-phosphate on the other. They require only magnesium for activity and the corresponding

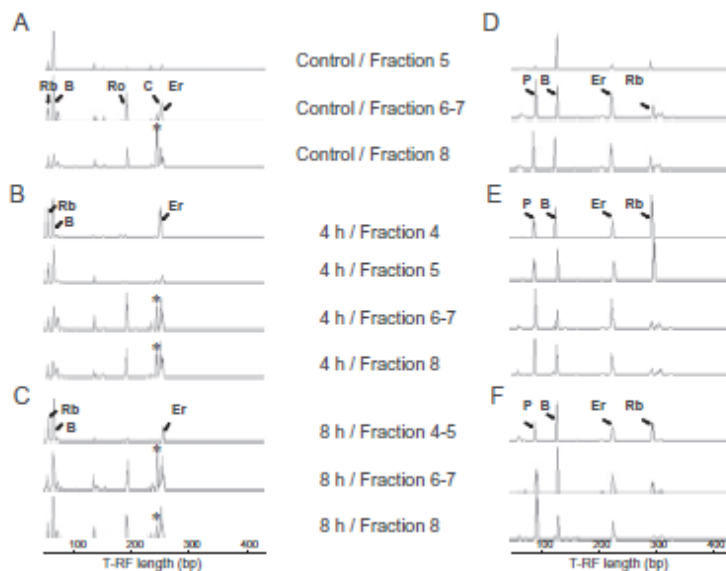


Figure 45

Fig. 5: T-RFLP profiles of bacterial 16S rRNA obtained from fractions of the control (A), 4h gradient (B) and 8h gradient (C) after applying *Alul* as restriction endonuclease; control (D), 4h gradient (E) and 8h gradient (F), after applying *MspI* as restriction endonuclease. Assignment of T-RF peaks to bacteria phylotypes (Rb, *Ruminococcus bromii*, B, *Bifidobacterium adolescentis*; Ro, *Ruminococcus obeum*; C, uncultured *Clostridium* spp.; Er, *Eubacterium rectale*; P, *Prevotella* spp.) from (Kovatcheva-Datchary *et al.*).

High- Throughput Sequencing

The rapid development of next-generation sequencing technologies has allowed vast numbers of partial 16S rRNA genes from uncultured bacteria to be sequenced, at a much lower cost than Sanger sequencing. In addition to bypassing previously needed cloning and/or cultivation procedures, with their associated biases, community structures can now be investigated at much higher resolution by revealing taxa that are much less abundant. However, this may be at the expense of lower taxonomic certainty due to the shorter read lengths of sometimes poorer quality (claesson, Sullivan, .2009). The pyrosequencing technology introduced by 454 Life Science (Margulies, Egholm, Altman, attiya 2005), now part of Roche, uses microscopic beads to bind individual DNA fragments. These beads with attached DNA sit in nanotiter plates and the nucleotide sequences are amplified using emulsion PCR. 454 sequencing produces about 400 000 reads of 250 - 450 bp in length and an average quality score of greater than 99.5% accuracy rate (droege, hill 2008). Pyrosequencing has been applied to a wider range of microbial communities and variable regions of the 16S rRNA gene, such as the V6 region in microbial communities of deep-sea vents (Huber, Welch, Morrison, Huse 2007); V1,V2, V6 and V3 in human (andersson, Lindberg, jakobsson backed 2008)(Dethlefsen, Huse, Sogin, Relman 2008)(Turnbaugh, Hamady, Yatsunenkov2008)(Zhang, Diabise, Zuccolo, Kudrna 2009) and in macaque (McKenna, Hoffmann, Minkah 2008) GI tract; as well as V9 in soil-derived microbial DNA (Roesch, Fulthorpe, Riva, casella, Hadwin 2007).

The choice of variable regions of the 16S rRNA gene leads to considerably different reliability of sequence assignments due to differences in information content within the 16S rRNA gene as shown in Table 2. Claesson and coworkers 2009 used sequences from human GI tract samples to investigate which region of the 16S rRNA gene would be best suited for high-throughput sequencing for this particular microbial habitat: 82.3% of sequences from the variable region V3 could be classified to genus level at a reliability of 80%, whereas only 40.4% of sequences from the V6 region could be classified to a genus at this level of reliability. The variable region V4 appeared to carry the most reliable information content, with 87.9% of sequences being assigned to genus at 80% bootstrap support (Claesson *et al.*, 2009)

Table 26

Variable region	V3			V6			V4		
Bootstrap cutoff ($\geq$)	0%	50%	80%	0%	50%	80%	0%	50%	80%
Fraction of sequences classified to genus	100%	92.4%	82.3%	100%	73.5%	40.4%	100%	97.0%	87.9%
Fraction of sequences correctly classified to genus	92.0%	95.0%	98.1%	79.0%	96.5%	98.7%	92.8%	94.5%	95.7%

Table 2: Of 7208 full-length 16S reference sequences from the human gut, 6054 were classified at genus-level with 80% bootstrap support. For each of the three extracted variable regions fragments were classified at three different bootstrap thresholds, and compared with the full-length classifications (last row). From (Claesson *et al.*, 2009)

Differences in information content within the variable regions of the 16S rRNA gene lead to great differences as to which phylogenetic depth sequences can be assigned (Fig. 6). Again, reads from the variable region V4 is shown to have superior classification efficiency over the V6 region.

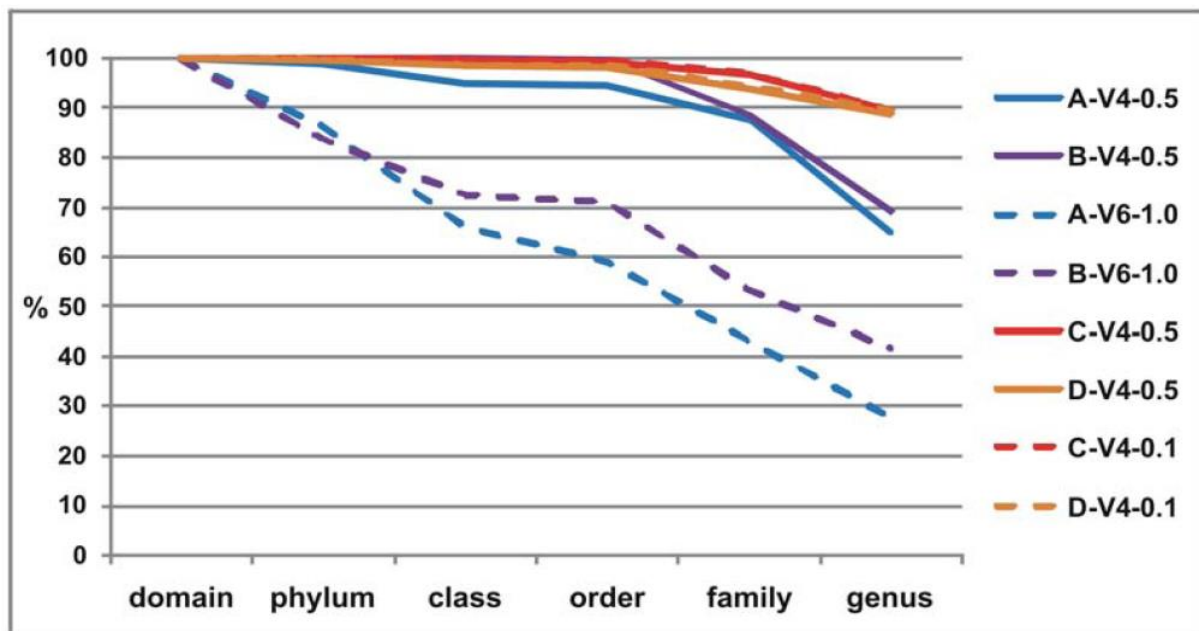


Figure 46

Fig. 6: Classification efficiencies at six taxonomic ranks for eight sets of sequences from four samples. The blue and purple colored dashed lines represent V6 amplicon reads, which have very poor classification efficiencies compared to all V4 amplicon reads, especially at the genus level. The red and orange colored dashed lines, representing V4-0.1 amplicon reads, show nearly identical classification efficiencies as the corresponding V4-0.5 amplicon reads. From (Claesson *et al.*, 2009).

In addition to variable reliability and depth of sequence classification, the choice of variable region also influences the information on microbiota composition obtained from a high-throughput sequencing experiment (Fig. 7).

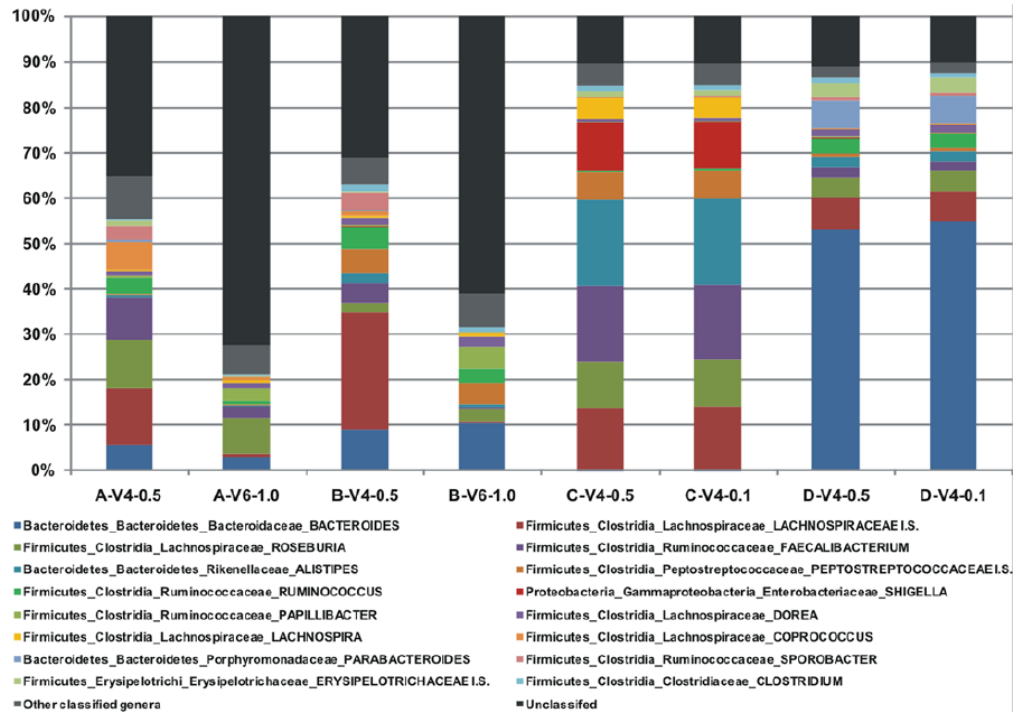


Figure 47

Fig. 7: : Relative abundance of the 16 most abundant genera classified with at least 50% bootstrap support. Genera are labelled according to phylum_class_family_GENUS. From (Claesson *et al.*, 2009)

The analysis by Claesson and co-workers (2009) gives a good insight into the difficulties arising with the use of high-throughput sequencing technologies. Although considered the most accurate and exhaustive technology for the characterization of microbial communities, high-throughput sequencing is not free of biases and cannot overcome the limitations in information content of the 16S rRNA gene. The application of high-throughput sequencing technologies is not limited to 16S rRNA coding regions. 454 sequencing has also been applied for sequencing an entire bacterial or viral genome in one day (Chen *et al.*, 2010, Monger *et al.*, 2010)

Microarray

Microarray applications allow the simultaneous comparison of the entire genome content of microorganisms by hybridisation with hundreds and thousands of DNA probes. Furthermore, when

targeting mRNA, microarrays can reveal which genes are differentially expressed under specific test conditions e.g. in the food matrix or in the host. Microarrays have also been applied to characterize the strain specific properties of probiotic strains and for the subtyping of bacterial isolates.

Subtyping

Although multilocus sequence typing is now regarded by many as a good standard to determine phylogenetic relationships between and within bacterial species, it does not always reflect the true genetic diversity of members of a species. Phylogenetic trees based on multilocus sequence typing may therefore differ significantly from a tree based on whole gene content (Tettelin *et al.*, 2005).

For the construction of a microarray aiming to subtype bacterial isolates, the total number of genes potentially present in strains of a given species, the 'pan-genome' (Medini *et al.*, 2005 Massignani V, Rappuoli 2005, Tettelin *et al.*, 2005), must be known. Thus the development of such a microarray depends on the availability of a database of whole genome sequences of a large amount of strains. From such a database, the core genome can be defined (Fig. 8).

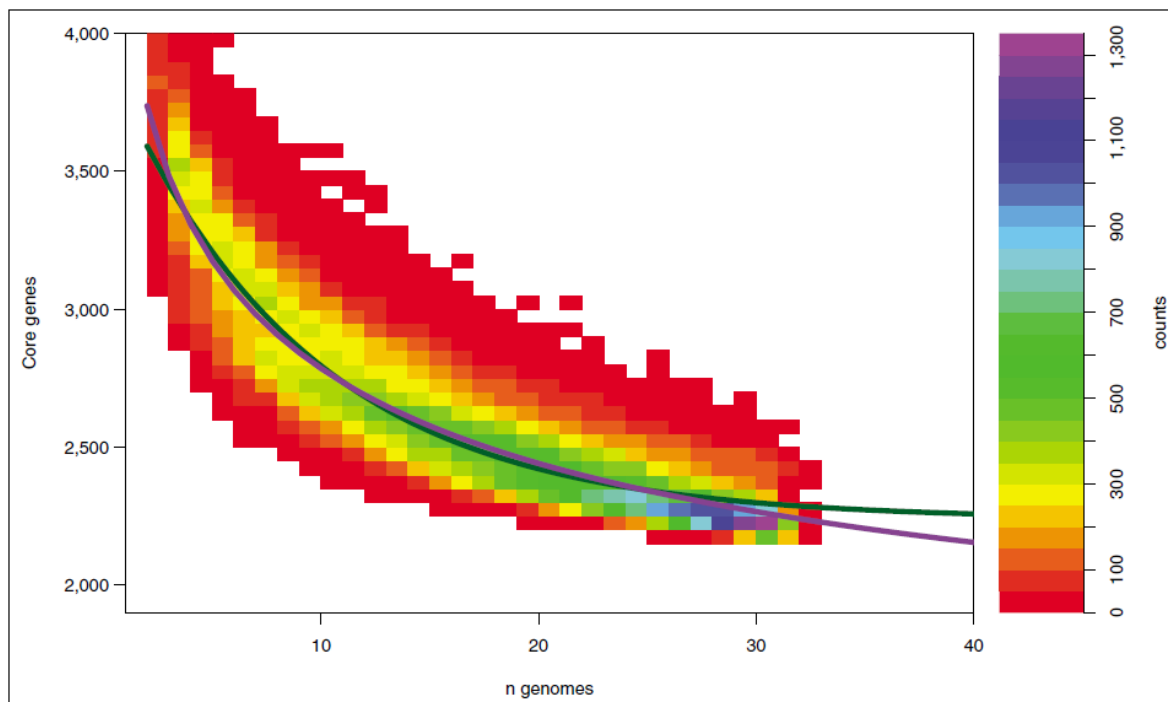


Figure 48

Fig. 8: Two-dimensional density plot of 'core genes' for the *E. coli* pan-genome. The plot illustrates the number of *E. coli* genes for $n=2$, 32 genomes based on a maximum of 3200 random combinations of genomes for each n . The density colours reflect the count of combinations giving rise to a certain number of core genes; that is for $n=3$, genome number 3 is compared to genomes 1 and 2, and the

number of core genes is the number of genome 3 genes conserved in genomes 1 and 2. The green and purple lines each indicate the fit to two slightly different exponential decay functions (Tettelin *et al.*, 2005, Willenbrock *et al.*, 2007).

Based on predicted protein sequences, the *E. coli* core genome was estimated to comprise approximately 1563 genes (Willenbrock *et al.*) for an infinite (or very large) number of *E. coli* genomes. Following the identification of the core genome, ladditional 'strain-specific' genes need to be defined (Fig. 9).

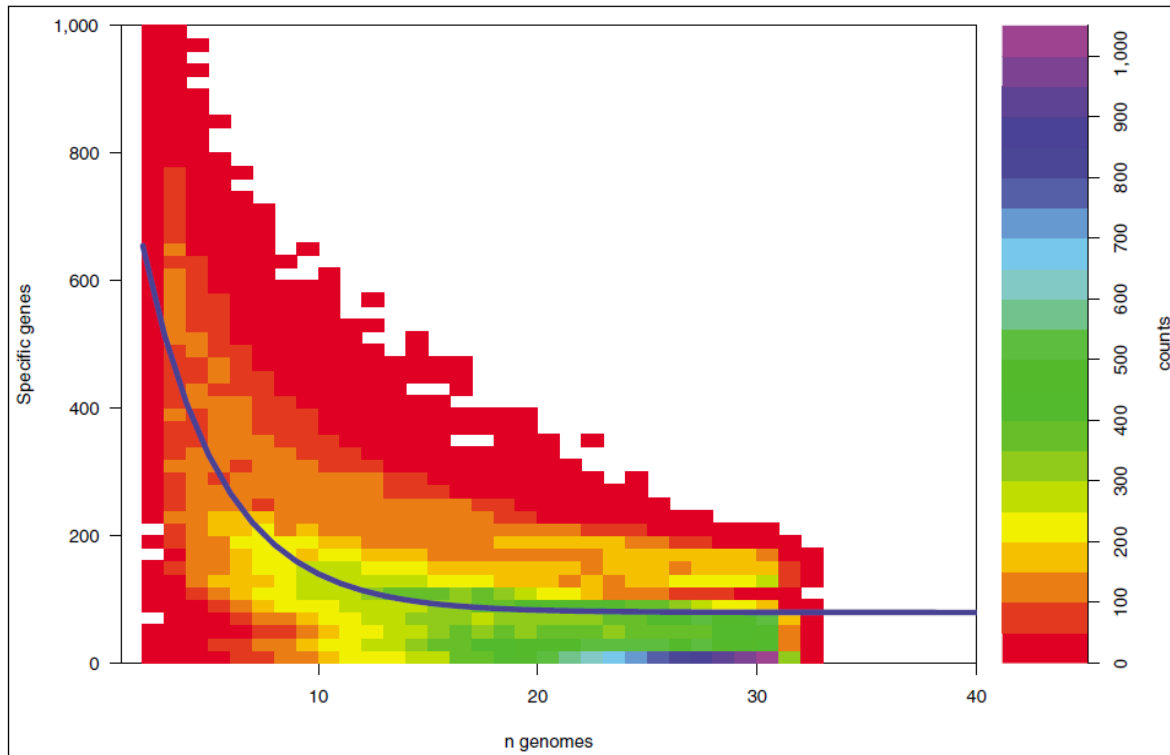


Figure 49

Fig. 9: Two-dimensional density plot of novel genome 'specific genes' for the *E. coli* pan genome. The plot illustrates the number of novel genome specific genes for the n th genome when comparing $n=2$, 32 genomes (for a maximum of 3200 random combinations at each n). The density colors reflect the count of combinations giving rise to a certain number of specific genes (y-axis) in one genome compared to $n-1$ other genomes; that is, for $n = 2$, genome number 2 is compared to genome number 1 and, on average, approximately 650 genes are found to be specific to strain 2. From (Willenbrock *et al.*, 2007).

For *E. coli* it was estimated that sequencing additional strains, approaching infinity, would add approximately 79 new genes. The pan-genome for *E. coli* is thus estimated to contain 9433 different genes in total, based on the translated protein sequence homologies. The estimated pan-genome can then serve as basis for the development of a microarray application that is capable of differentiating

every different strain of the same species. *E. coli* is an organism for whom exact strain differentiation is of particular interest, because this species comprises probiotic strains (e.g. *E. coli* Nissle) as well as highly pathogenic strains (e.g. O157:H7).

16S rDNA Microarray For Phylogenetic Typing Of Gut Communities

A number of phylogenetic arrays have been constructed that permit hybridization of nucleic acids extracted from environmental samples against probes corresponding to single-stranded full or partial 16S rRNA genes (Guschin *et al.*, 1997, Wilson *et al.*, Palmer *et al.*, 2007, Harrington *et al.*, 2008). As it is technically very difficult to include the more than 800 000 SSU sequences present in the databases (see <http://rdp.cme.msu.edu/>), microarrays with subsets of sequences specific to the ecological environment of interest are required. Recently the HIT Chip, an oligonucleotide microarray for phylogenetic profiling of human intestinal tract communities, was developed (Kovatcheva-Datchary *et al.*). The 4800 probes on this 16S rRNA gene tiling array consist of sets of three 18-30nt long overlapping oligonucleotides targeting the V1 and V6 region sequences from 1140 phylotypes, respectively. Based on 98% sequence similarity, phylotypes were defined from more than 16000 16S rRNA gene sequences identified in the human GIT. A typical work flow for phylogenetic microarray analysis is shown (Fig. 10):

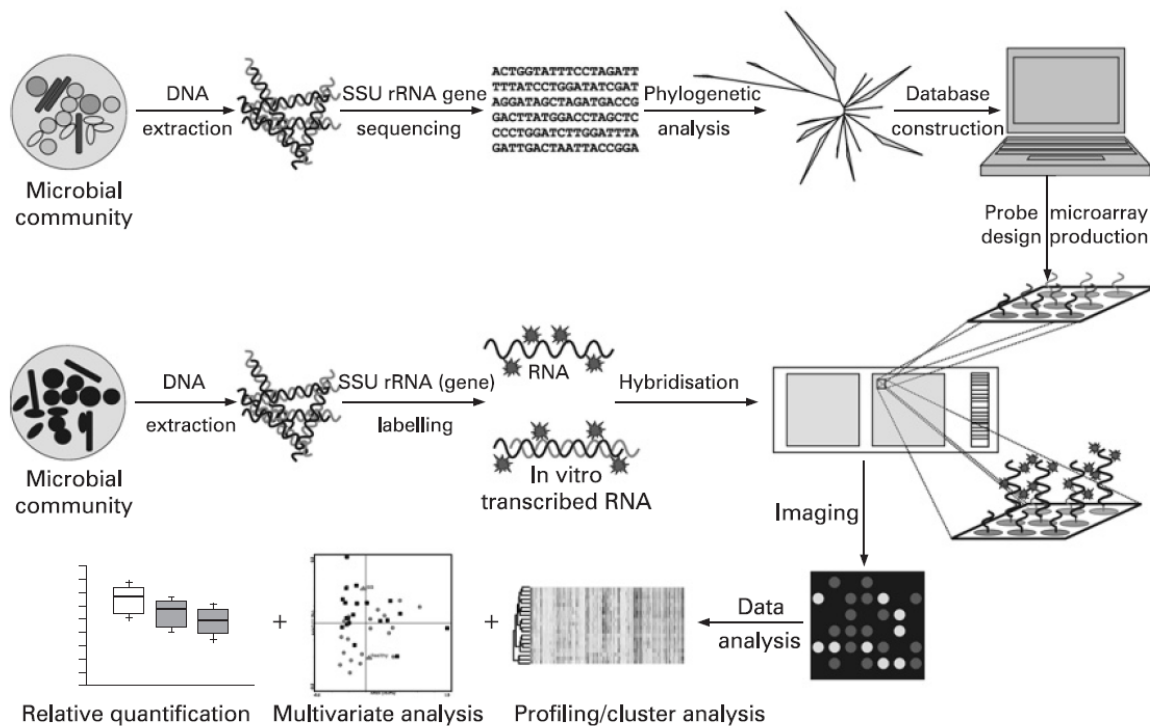


Figure 50

Fig. 10: Schematic representation of high-throughput analysis of human gastrointestinal (GI) tract microbiota via brute force sequencing and phylogenetic microarray analysis. SSU rRNA, small sub-unit ribosomal RNA. From (Zoetendal *et al.*, 2008)

With the aid of this technology it was also shown that a multispecies probiotic cocktail alleviated symptoms of irritable bowel syndrome (Kajander *et al.*, 2008), and that starch-fermenting bacteria could be identified by using RNA stable isotope probing in a human colon model with great reproducibility (Kovatcheva-Datchary *et al.*, 2009).

Array Tubes

ArrayTubes (AT) platforms consist of a custom micro probe array similar to usual glass slides microarrays, integrated into a micro reaction vial. Depending on the individual assay, nucleic acid as well as protein and peptide based arrays can be manufactured. Using this method, up to 196 gene or protein sequences potentially involved in probiotic mechanisms can be analysed simultaneously to evaluate probiotic potential of food or a bacterial strain.

Microarrays For Identifying The Mechanism Of Action

An important probiotic property is the ability to adhere to the mucosa. Adherence has been linked to immune-modulation and bacterial cell retention in the gastrointestinal tract (Grangette *et al.*, 2005, Hisbergues *et al.*, 2007).

Degues and coworkers (2009) used DNA and mRNA microarrays to identify which properties of *Lactobacillus johnsohnii* NCC533 were responsible for the long gut persistence phenotype of this strain. NCC533 was detected for 12 days after administration to mice, whereas *L. johnsohnii* ATCC 33200, the type strain of this species, could only be detected for 5 days. For this purpose, all 1760 open reading frames (ORFs) of NCC533 were spotted on a microarray. The DNA of the type strain ATCC 33200 was hybridised against NCC533. As a result, 233 genes differed in the genome contents of the two strains, of which about 30% were of prophage origin. To further narrow down the number of differing genes and to identify which genes are responsible for the long gut persistence, mice were monocolonized with NCC 533 and the bacterial mRNA isolated from the jejunal mouse mucosa. Fusion of the DNA- and mRNA microarray datasets revealed that only six genes, corresponding to three genetic loci, were expressed exclusively in the long-gut-persistence phenotype of NCC 533.

The first genetic locus encodes glycosyltransferase genes involved in exopolysaccharide (EPS) synthesis of the cell wall. The second encodes genes for a membrane-bound transporter complex and a cytoplasmic protein complex of a mannose-import complex. The third gut-expressed and isolate NCC533 specific locus shared significant sequence identity with IgA (immunoglobulin A) proteases from several pathogenic bacteria that colonize human body surfaces.

Mutants were constructed, in which these three genetic loci were deleted. It became apparent that the ability to express an IgA protease was responsible for the long gut-persistence phenotype of *Lactobacillus johnsohnii* NCC 533 rather than surface characteristics of the EPS layer or sugar import ability.

A microarray was also applied to determine which genes in *Lactobacillus reuteri* ATCC 55730 are responsible for the ability to thrive under the presence of bile (Whitehead & Versalovic, 2008). Bile salts have been proposed to have a wide range of cellular effects, including cell wall or membrane damage, DNA damage, protein denaturation, oxidative stress, and low intracellular pH (Begley *et al.*,

2005). *L. reuteri* was shown to possess several multidrug resistance efflux pumps and another unknown hypothetical protein involved in bile resistance. Efflux pumps have already been shown to play important roles in the bile response of *Campylobacter jejuni*, *Listeria monocytogenes* and *Escherichia coli* (Thanassi et al., 1997, Lin et al., 2003, Sleator et al., 2005).

Detection Of GMO - Probiotics

Various genetic systems have been introduced to analyze and modify LAB including plasmid vectors, selectable markers or markers that restore an impaired function necessary for cell viability. In addition gene-expression systems which allow the controlled expression of homologous or heterologous genes, such as those based on promoters controlled by sugar, such as the lactose operon promoter, have been introduced to LAB (Ahmed, 2003).

There are no validated results for detection of legally approved genetically modified microorganisms (GMM). The plasticity of the bacterial genome, the frequency of horizontal gene transfer and the application of self-cloning complicate the detection of GMM in foods, especially when recombinant DNA is naturally present within a rich indigenous or contaminating flora. Detection usually relies on available experience on tracing of traits at the genomic and phenotypic levels. When GMMs are used for the production of food, four categories of detection can be differentiated:

1. The product is free of any DNA and impurities indicative of a GMM, for example highly purified food ingredients or additives;
2. The product contains DNA but no GMM, for example liquid products (beer or wine) that were subjected to separation processes such as filtration or centrifugation;
3. Products that contain the dead GMM with its DNA, for example a pasteurized yoghurt or baked goods; and
4. Products containing the living GMM, for example a non-pasteurized yoghurt, cheese, beer, sauerkraut, fermented sausage, etc.

Isolation of DNA is the first step in analysis for detection of the use of GMM in food by molecular methods. When the cells are not lysed, the GMMs may first be separated from the food matrix by homogenizing the food and separation of the cells by filtration and centrifugation. Detection of DNA may be hampered by the presence of inhibitors that interfere with cell lysis, degrade DNA during isolation, or interfere directly with PCR.

DNA stability in fermented foods is another concern. As long as the DNA is contained in cells, it is naturally protected against degradation. DNA released from cells into the food matrix, as occurs in the course of food processing, undergoes physical, chemical, and enzymatic degradation. Straub et al. reported that free plasmid DNA in sausages remained detectable after storage for nine weeks (Straub et al., 1999).

For an organism-specific detection of the GMM it needs to be ensured that the recombinant DNA detected is contained in the very host strain that had been used for construction of the GMM. Detection of the recombinant DNA must be combined with identification of the host organism. This

measure takes into account the possibility of horizontal transfer of recombinant DNA between different strains, species, or even less-related organisms. A combined GMM detection approach based on specific DNA probe hybridization and on diagnostic DNA in-vitro amplification can be used taking into account the contaminating or indigenous microbiota present together with the GMM in foods. Living cells are required for the combined use of probes and/or PCR primers specific for the recombinant DNA as well as the microorganism. The methods available are highly sensitive, but are applicable to living GMM exclusively.

Techniques based on the detection of strain specific DNA sequences by applying hybridization or PCR techniques with specific probes or primers are available. These unique sequences can be derived, for example, from RAPD fragments or may be obtained by the subtraction hybridization technique (Matheson *et al.*, 1997, Tilsala-Timisjarvi & Alatossava, 1998, Tilsala-Timisjarvi & Alatossava, 2001, Heller *et al.*, 2006).

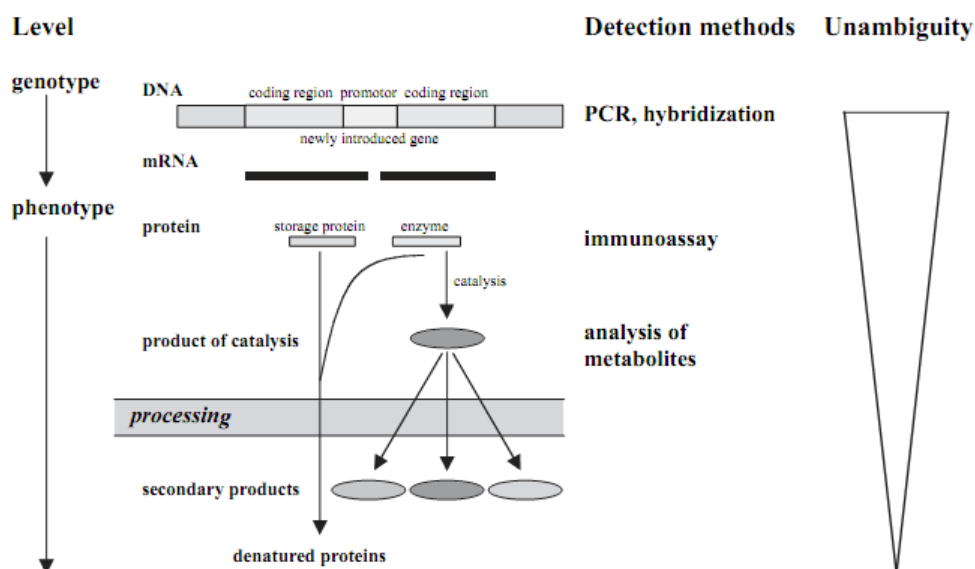


Figure 51

Fig. 11: The unambiguity of methods for the detection of GMO in food. (Heller *et al.*, 2006)

Conclusion

In conclusion there are multiple molecular methods available for the characterization of probiotic strains as well as the analysis of metagenomic background of beneficial strains in the human gut. Analysis has increased the understanding of the diversity and phylogeny of beneficial strains and their functions tremendously. However, for many aspects a combination of both molecular and culture techniques will be necessary.

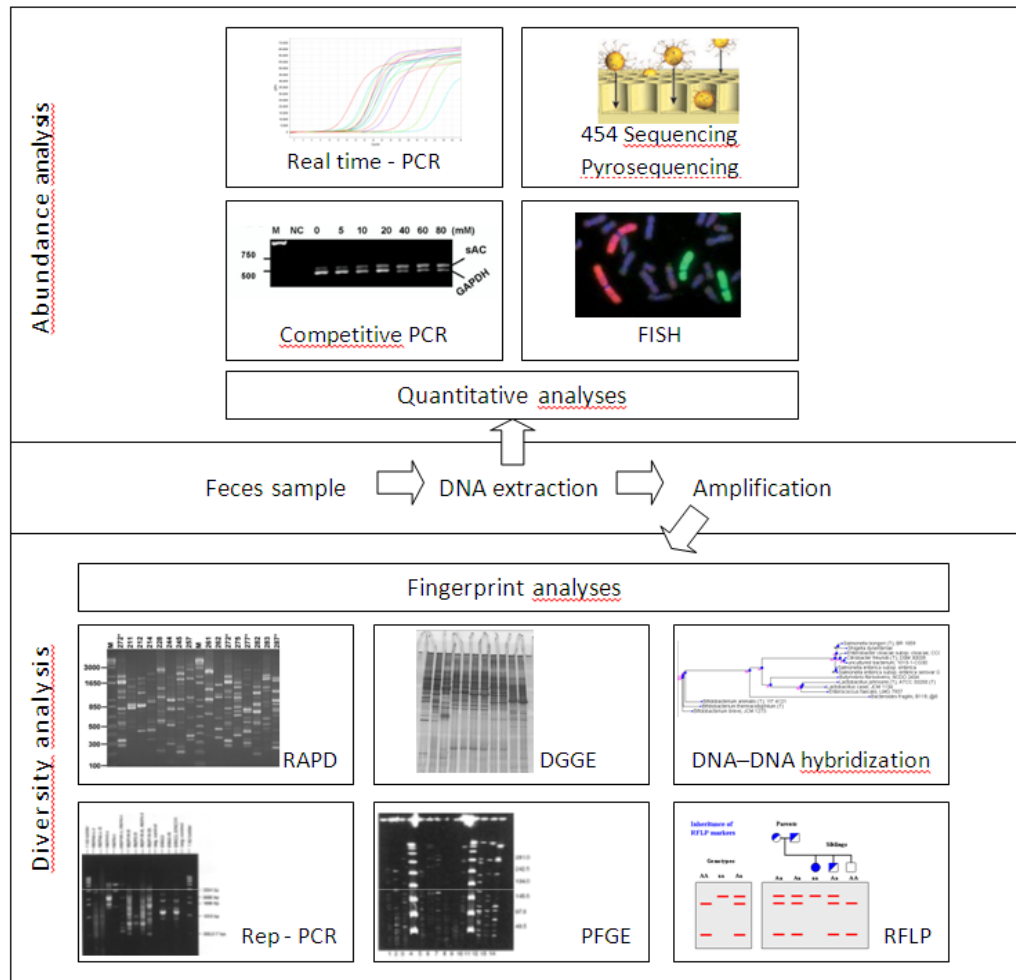


Figure 52

Fig. 12: Overview of methods for analyzing the gut microbiota out of feces sample.

Pictures from:

FISH: Flourescent In Situ Hybridization; 454 Sequencing: (Rothberg & Leamon, 2008); Competitive PCR: Competitive Polymerase Chain Reaction (Sun *et al.*, 2004); RAPD: Randomly amplified polymorphic DNA (Mahenthalingam *et al.*, 2009); DGGE: Denaturing Gradient Gel Electrophoresis; rep-PCR: Repetitive genomic element PCR (Versalovic *et al.*, 1994); PFGE: Pulsed field Gel electrophoresis; RFLP: Restriction Fragment Length Polymorphisms (Holzapfel *et al.*, 2001);

RFLP: <http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechRFLP.shtml>

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Isolation, Identification and Monitoring of Contaminant Bacteria In Iranian Kefir Type Drink By 16S rDNA Sequencing

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Abstract

Iranian kefir type drink (IKTD) is a highly consumed, traditional Iranian, fermented milk product. To improve monitoring procedures for food safety 32 industrial kefir type drinks from 4 brands and 8 different production dates as well as 32 samples from pasteurized milk of the same kefir manufacturers and air of the production sites were analyzed for contaminations.

16S rDNA extraction from Kefir samples as well as 16S rDNA obtained from samples incubated on Columbia agar were analyzed using PCR/DGGE, cloning, sequencing and phylogenetic classification. Already DGGE analysis indicated contaminations including bacillus strains.

Subsequently analysis of cultured clones indicated contaminations with *Bacillus* sp. including *B. cereus*, *B. thuringiensis* and *B. Paenibacillus* sp. in 9 (28%) from all analyzed samples. Also 38% of pasteurized milk samples were contaminated with *B. cereus*. The average count of *Bacillus cereus* was 74 ± 19 cfu/ml. *Bacillus cereus* and *Bacillus thuringiensis* were found as contaminant bacteria in the air of the all manufacturing sites. These results suggest that milk is one of the most important sources of contamination with *Bacillus* sp., especially *Bacillus cereus* for kefir products in Iran. But bacterial contamination in kefir samples might also originate from the air of the production sites. 16S rDNA analysis accelerates monitoring strategies.

Keywords: kefir, contaminant bacteria, *B. cereus*, *B. thuringiensis*, Pasteurized milk, air, manufacturing site

Introduction

Kefir is a fermented milk beverage that originated in Eastern Europe, and is enjoyed worldwide (Wouters et al. 2002). Different kinds of starters are used for fermentation of Kefir. The Kefir drink is characterized by a high acidity and, depending upon the holding time after fermentation, by either lower or higher carbon dioxide content (CO₂) and relatively low alcohol content (Duitschaeffer et al. 1987).

In industrial production, known starters with distinct characteristics are used but in traditional kefir different micro flora would be present in the product (Kwak et al. 1996; Bosch et al. 2002).

Iranian kefir type drink (IKTD) is a traditional Iranian fermented milk product and it is a very popular and highly consumed product in Iran with a considerable increasing demand for its consumption. The popularity of IKTD arises from its specific organoleptic characteristics along with its health benefits as a healthy drink based on fermented milk (FAO/WHO 2009).

However, bacterial contamination is a problem and improved methods for characterization of contaminations as well as culture independent methods are needed.

Various methods using DNA have been developed for the detection of specific microbes in foods (Lieckfeldt et al. 1993; BaleirasCouto et al. 1996; Manavathu et al. 1996; Caggia et al. 2001; Lo'pez et al. 2001; Cocolin et al. 2002). Among these methods, polymerase chain reaction-based assays (PCR-based methods) are specific, can be extremely sensitive and results are obtained in a few hours. Different genomic targets have been amplified with Polymerase Chain Reaction. Analysis of 16S rRNA (16S rDNA) based amplification and sequence analysis have been widely used to study the microbial diversity present in environmental samples (Farrelly et al. 1995; Suzuki & Giovannoni, 1996). 16S rDNA analysis has been demonstrated to be a powerful tool to investigate the biological diversity. These methods have several advantages: The structure of DNA remains stable with physiological stages, its composition does not depend on culture conditions and it has regions with different degrees of variability that allow species specific detection or the detection of wider taxonomic groups (Lieckfeldt et al. 1993; Ness et al. 1993). The detection of rRNA genes has been often used in microbial studies (Cappa & Cocconcilli, 2001). In this study 16S based rDNA PCR, cloning and sequencing was used for the characterization of contaminant bacteria of IKTD. Various strains of *Bacillus* were found as main contaminants in culture based and culture free 16S rDNA analysis.

Materials and Methods

Samples

32 industrial kefir type drinks from 4 brands (all present brands in market) and 8 different production dates were selected randomly in the Iran market and transferred in sterile condition and cold chain to the analytical laboratory. For the assessment of contaminant bacteria, 32 samples from pasteurized milk of the same kefir manufacturers were collected. In three consecutive days also the air microbial situation of each manufacturing sites were assessed.

DNA Extraction

Extraction from Kefir Drink

Considering the lower contents of both protein and fat in whey than in kefir, the DNA may be more easily extracted from the whey than from kefir (Chen et al. 2010). Extraction from Kefir samples was done by centrifugation (13 000 x g), 500 mg of pellets was subjected to lyses and beat beating (30sec, with one intervening minute on ice) and DNA was extracted using the Fast DNA spin kit for Soil (MP Biomedicals) following the instruction protocol. DNA yields were analyzed by nanodrop photometer measurement.

Extraction from Pure Culture

IKTD Samples were homogenized with a vortex (Scientific industries INC.) for 5 min. Serial dilution of suspended samples were used for microbial enumeration and isolation by culturing on Columbia agar (Mikrobiologie) before & after heat shock 75°C for 20 minutes and Worth Agar (Mikrobiologie). After inoculation the Columbia agar plates were incubated at 30°C for 2 days both in aerobic and anaerobic atmosphere. Worth Agar plates incubated at 20°C for 5days. Colonies were counted and isolated from each plate with 30-300 colonies and single colonies were purified for determination the profile of contaminant bacteria in kefir samples (Vanderzant et al. 1992; Wong et al. 1988).

Colonies obtained from cultivation of IKTD samples on Columbia agar were transferred to lysing tube of Fast DNA spin kit for Soil (MP Biomedicals) and DNA extraction was continued according to the protocol of manufacturer's instruction.

16S rDNA Amplification and DGGE Analysis:

16S rDNA was amplified in a 100µl reaction volume using a ready-to-use PCR Master Mix (Promega). Primer pairs used were 413 F-GC (5'-CCT ACG GGA GGC AGC AG-3') and 518r (5'-ATT ACC GCG GCT GCT GG-3') (3) in a 0.5 pmol concentration.

The amplification program was: 95°C for 10 min. and followed by 35 cycles at 95°C for 1 min., annealing at 55°C for 50 sec. and elongation at 72°C for 50 sec. The final elongation step was conducted at 72°C for 5 min.

The PCR product was purified with the Qiaquick PCR Purification Kit and an aliquot of 10 µl was used for quality assessment on agarose gel electrophoresis. The PCR product was then precipitated overnight at -20°C and resolved in 15µl NFW and 5µl dye for further use (Zwielehner et al. 2008). The PCR products were centrifuged for 30 minutes at 14000 rpm, the supernatant discarded completely and DNA pellets dried on a heat block either for 2 hours at 36°C or over night at 30 °C. Further 15µl water and 5µl loading dye (Lämmli, or DNA loading dye from Fermentas) were added and load to DGGE gel which prepared from 80% acrylamid-solution consisting of 40.5g urea, 48ml formamid, 40ml acrylamid to 30% gradient, 1.5ml 50x TAE buffer and dH2O ad 150ml and 0% acrylamid-solution

consisting of 40 ml acrylamid 30%, 1.5 ml 50x TAE and dH₂O ad 150 ml. Gels were running at 150V for 5.5 hours (Zwielehner et al. 2008; Garbers et al. 2004).

Cloning

To separate the different starters from kefir contaminant bacteria we constructed a clone library from kefir drink. For this purpose PCR products amplified with primers 195-F (Meier *et al.*, 1999) and Ccocc-R (Matsuki *et al.*, 2004) were inserted into a p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Nucleotide sequences were corrected for primer and vector sequences in Codon Code Aligner (www.codoncode.com) and taxonomically identified using the online tools of the ribosomal database project (<http://rdp.cme.msu.edu/>).

16S rDNA Gene Sequence Analysis

Amplicons were purified using the PCR purification kit (QIAGEN) and submitted to sequencing (DNA Confidence) according to the method mentioned by Coenye et al. (1999). The alignment was determined by BLAST program in GenBank database NCBI and ribosomal database project (Altschul et al. 1997) (<http://rdp.cme.msu.edu/>).

Cultures from Pasteurized Milk

Cultures from pasteurized milk were performed by surface inoculation on Columbia agar (Mikrobiologie) and polymyxin pyruate egg-yolk manitol bromthymol blue agar (Oxid). Inoculated plates beside the negative and positive control plates were incubated in 30°C for 2 days, selected bacteria were characterized by conventional biochemistry method (Harrigan et al. 1998).

Culture from the Air of the Production Sites

To examine the air from the production sites, plates of already poured and set contain Columbia agar (Mikrobiologie) and polymyxin pyruate egg-yolk manitol bromthymol blue agar media (Oxid) were exposed to the air of the production sites in five different of locations for 15 minutes. Inoculated plates beside the negative and positive control plates were incubated in 30°C for 2 days, and selected bacteria were characterized by conventional biochemistry method in lab (Andersen et al. 2009; Harrigan et al. 1998).

Results

Contaminations in Iranian Kefir type products were assessed using culture dependent and culture independent 16S rDNA PCR/ DGGE analysis.

Aliquots from kefir samples were extracted and purified, 16S rDNA was subjected to DGGE analysis, cloning, Sanger sequencing and phylogenetic alignment. Comparison of different extraction variants indicated highest yields of good quality DNA using beat beating and soil kit as described in methods. DGGE analysis using primers for all bacteria (413 F-GC/ 518r, including control rDNA from *lactobacilli* and other strains displayed diverse band patterns including *lactobacilli* and possible contaminating strains. 6 samples with high diversity in DGGE analysis were further cloned and run on DGGE. 10

clones (Fig.1) running differently on DGGE (band height) were subjected to Sanger sequencing. Table 1 show closest matches of clones including *Bacillus* sp.as possible contaminants. Other bacterial groups identified comprised *Halomonas* and *Alphaproteobacteria*.

Culturing of samples on Columbia agar at aerobic atmosphere resulted in 9 (28%) colonies of all kefir samples. Colonies originating from Kefir samples were sequenced and aligned. Table 2 shows the results of various *bacillus* strains as closest matches.

Corresponding to samples from IKTD products, pasteurized milk samples of the manufacturers were analyzed. In 38% of the samples *B. cereus* was detected using conventional methods (Table 3). Statistical analysis revealed, there is a high correlation ($r=0.86$) between the number of *Bacillus cereus* of pasteurized milk and contaminations in IKTD of the same manufacturing site. The average count of *Bacillus cereus* in entire examined pasteurized milk was 74 ± 19 cfu/ml.

Also the air microbial situations of the production sites were analyzed. An average number of 3 ± 2 cfu/m²/min *Bacillus cereus* and 4 ± 2 cfu/m²/min for *Bacillus thuringiensis* was detected (Table 4). Comparison of the number of contaminant bacteria in kefir samples and in the air of production sits demonstrated only low correlation ($r=0.61$).

Table 27

Table 1: Various strains originating from Kefir type samples culture independent 16S rDNA as closest matches after sequencing and alignment.

Isolated bacteria	Identity (%)
<i>Bacillus thuringiensis</i>	100
<i>Bacillus cereus</i>	97
<i>Paenibacillus</i> sp. FR1_23	100
<i>Alphaproteobacteria</i>	97
<i>Halomonas desiderata</i>	97
<i>Bacillus</i> sp. NASAb12	100
<i>Geobacillus stearothermophilus</i>	100

Discussion

PCR-based DGGE finger printing can be successfully used to typify the microbial consortium present in IKTD, as well as to distinguish between Kefir bacteria, or Kefir grains that have different origins (Garbers et al. 2004). Pasteurized milk is one of the most important materials which are used for kefir type drink production, so for identification the source of relevant contaminant bacteria, pasteurized milk of the same manufacturers were examined using culture dependent and culture independent methods.

Direct extraction of 16S rDNA from the Kefir products with beat beating and soil kit yielded high DNA concentrations and low inhibitors as indicated by nanodrop measurement. DGGE analysis, cloning and sequencing reproducibly detects expected Lactic acid bacteria strains as well as contaminant

bacteria. DGGE analysis using Lac1/2 primers discriminated different strains (data not shown). Already this fast analytical approach identified *Bacillus* as likely contaminant. Other detected bacterial groups like *Alphaproteobacteria* belongs to spoilage relevant psychotropic bacteria, *Geobacillus* and *Halomonas* were known in these samples. It is suggested that *Halomonas* would originate from the salt used in formulation of IKTD.

Table 2: Number (cfu/ml) and type of contaminant colonies isolated from culture of Kefir type samples and closest matches after sequencing and alignment.

Table 28

Sample	Worth agar	Columbia agar		Isolated bacteria	Identity (%)
		<i>B.H.S</i> ^a	<i>A.H.S</i> ^b		
A	Neg	30 ± 10	Neg	<i>Bacillus thuringiensis</i>	100
B	Neg	20 ± 10	10 ± 10	<i>Bacillus cereus</i>	97
				<i>Paenibacillus</i> sp. FR1_23	100
C	Neg	20 ± 10	Neg	<i>Bacillus cereus</i>	97
D	Neg	50 ± 20	Neg	<i>Bacillus</i> sp. NASAb12	100

Also sequencing of colonies obtained after cultivation on Columbia agar indicated *Bacillus* strains as important contaminants. However, strain identification following the sequencing of 260 bp amplified by all bacteria 16S primers can only detect on species level and not identify strains. A strain specific identification would need the further analysis addressing more 16S rDNA regions. Direct extraction of 16S rDNA method identified more different contaminant bacteria than culture based method because some bacteria such as *Halomonas* were not able to grow in usual bacteriology medium because it needs at least 5% salt for growth samples (Vanderzant et al. 1992).

8% of the samples were contaminated with *B. cereus*. According to these results, it is proposed that one of the most important sources for contamination with *Bacillus* sp., especially *Bacillus cereus* is milk, however the contamination in IKTD samples might also originate from the air of production site as *Bacillus cereus* and *Bacillus thuringiensis* were found as contaminant bacteria in the air of the manufacturing sites. Already Lin et al. (1998) suggested that *B. cereus* spores in raw milk were the major source of *B. cereus* in pasteurized milk and dairy products, and that post-pasteurization contamination along the milk processing lines was possibly a minor source of *B. cereus* in pasteurized milk. Within the dairy processing context, thermophilic bacilli are used as hygiene indicators in processed product. This is because of the ability of these strains to form endospores and biofilms. In addition, thermophiles are potential spoilage organisms, as they are capable of producing enzymes and acids that may lead to off-flavors in the final product (Burgess et al. 2010).

Table 3: Number (cfu/ml) and type of bacteria isolated from pasteurized milk.

Table 29

IKTD production site	Isolated bacteria	Positive samples	Number (cfu/ml) (mean $\pm$ SD)
Manufacturer A	<i>Bacillus cereus</i>	(11) 34.4%	95 $\pm$ 12
Manufacturer B	<i>Bacillus cereus</i>	(13) 40.6%	62 $\pm$ 9
	<i>Bacillus thuringiensis</i>		17 $\pm$ 3
Manufacturer C	<i>Bacillus cereus</i>	(9) 28.1%	46 $\pm$ 10
Manufacturer D	<i>Bacillus cereus</i>	(14) 43.8%	115 $\pm$ 17

To our knowledge, this is one of the first reports about the isolation and identification of *B.cereus* and *B. thuringiensis* strains from kefir type drink samples in Iran. The species belonging *Bacillus* genus such as *B. brevis*, *B. polymyxa*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. macerans* and *B. pumilus* were identified in tibico grains derived from Mexico (Rubio et al. 1993). Also *Bacillus cereus* in the tibico grains sample was reported by Gabriela et al. (2010), they suggested this bacteria presence may be due to contamination at some stage in the preparation.

In different kinds of dairy products *B. cereus* and *B. thuringiensis* contamination has been demonstrated before by Zhou et al. (2008) in Chinese pasteurized full fat milk packaged in cartons and also by Molva et al. (2009) in Turkish cheese.

Contamination of Dutch raw milk has been reported by Vissers and et al. (2007). The average concentration of *B. cereus* spore in raw milk was $1.2 \pm 0.05 \log_{10}$ spores/L and in none of samples the concentration was above the $3 \log_{10}$ spores/L. They concluded that *B. cereus* spores were predominantly transmitted from feeds, via feces, to raw milk. Farmers should take measures that minimize the transmission of spores via this route by ensuring low initial contamination levels in the feeds ($<3 \log_{10}$ spores/g) and by preventing growth of *B. cereus* in the farm environment.

TeGiffel and et al. (1997) studied *Bacillus cereus* in 334 pasteurized milk in household refrigerators in the Netherlands and found *B. cereus* could be isolated from 133 (40%) of the samples. In our study from 38% of pasteurized milk *Bacillus cereus* was separated so these results are very similar to results reported from Netherlands pasteurized milk contamination.

Table 4: Number (cfu/m²/min) and type of bacteria isolated from air of production sites.

Table 30

IKTD production site	Isolated bacteria	Mean $\pm$ SD (cfu/m ² /min)
Manufacturer A	<i>Bacillus cereus</i>	3 $\pm$ 2
	<i>Bacillus thuringiensis</i>	2 $\pm$ 1
	<i>Bacillus cereus</i>	2 $\pm$ 1
Manufacturer B	<i>Bacillus thuringiensis</i>	4 $\pm$ 2
	<i>Bacillus subtilis</i>	7 $\pm$ 2
Manufacturer C	<i>Bacillus cereus</i>	4 $\pm$ 2
	<i>Bacillus thuringiensis</i>	6 $\pm$ 2
Manufacturer D	<i>Bacillus subtilis</i>	3 $\pm$ 1

Larsen & Jørgensen(1997) examined 458 pasteurized milk samples in Denmark and according their report, occurrence of *Bacillus cereus* in Danish pasteurized milk was 56% which it's rate of contamination is higher than Iran and Netherlands pasteurized milk. Also they found ,the prevalence of *B. cereus* in pasteurized milk products during summer and winter was 72 % and 28%, respectively, and the mean counts of *B. cereus* was significantly higher during summer as well.

It has been supposed that antimicrobial activity of lactic acid bacteria in kefir would reduce the prevalence of *B. cereus* contamination comparison to milksamples. Røssland et al. (2003) investigated suppressive effects of different *Lactobacillus* or *Lactococcus* cultures on *B. cereus*. The inhibition patterns for the different *Lactobacillus* and *Lactococcus* cultures on *B. cereus* varied.

Noriega et al. (2003) reported that carbonated fermented bifidus milk may contribute to reduce the contamination risk of presence of *B. cereus* in bifidus milk products. According to this report the reduced contamination rate of Kefir type drink (28%) in comparison to pasteurized milk (38%) could be due to the antimicrobial activity of lactic acid bacteria.

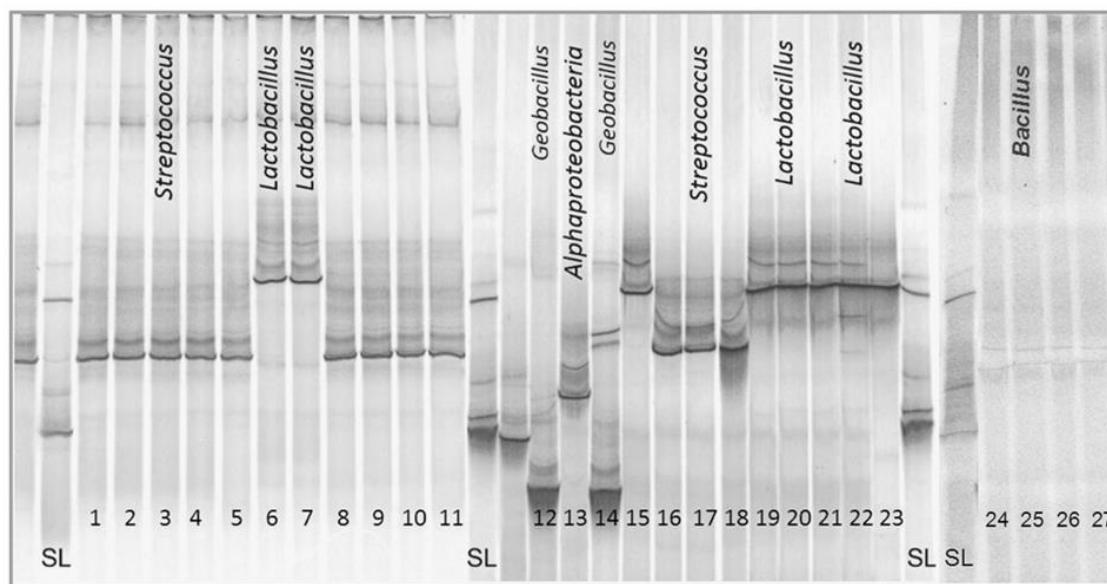


Figure 53

Fig. 1. DGGE profiles of bacterial 16S rDNA amplified from Kefir like drink samples. Legend: PCR.DGGE using primers 518r and 341f-GC of inserts derived from cloning of 16S rDNA

extracted from Kefir like drink. Results from sequencing are indicated in corresponding lanes. Standard lane composed of different Lactobacillus and lane of contaminant bacteria.

SL: standard lane. Lanes 1, 2, 3, 4, 5, 8, 9, 10, 11, 17, 18, 19: Streptococcus; lanes 6, 7, 15, 19, 20, 21, 22, 23: Lactobacillus; lanes 12, 14: Geobacillus; lane 13: Alphaproteobacteria; lanes 24,

25, 26, 27: Bacillus sp.

Conclusion

16S rDNA based method is a fast and efficient method to track contaminations in the Kefir drink. *Bacillus*, originating mainly from milk is a main contaminant in kefir.

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Conference Talks

IFB- Adiposity Diseases, Univeristy of Leipzig, Oct. 2013

Individualized Analysis of Human Microbiota in Type 2 Diabetes And Obesity

Berit Hippe

3rd Indonesian Conference on Lactic Acid Bacteria, January 2011, Yogyakarta, Indonesia

Functions of GI- microbiota and the quantification of butyryl-CoA CoA transferase genes which indicate the butyrate production capacity in individuals of different diet and age.

Berit Hippe, Jutta Zwielehner, Kathrin Liszt, Frank Unger, AG. Haslberger

Gastro-Intestinal Microbial Ecology (GME) November 2010, Kosice; Slovak Republic

Quantification of butyryl-CoA CoA transferase genes reveals different butyrate production capacity in individuals of different diet and age, Berit Hippe, Jutta Zwielehner, Kathrin Liszt, Frank Unger, AG. Haslberger

Probiotica evidence basiert, Dez. 2009, Frankfurt, Germany

Probiotica and allergy, Berit Hippe

19th International Congress of Nutrition (ICN) 2009; Bangkok

Decreased levels of butyric acid but high amounts of bacterial butyryl-CoA CoA-transferase in faecal microbiota, for vegetarians compared to omnivores; Berit Hippe, Jutta Zwielehner, Kathrin Liszt, Cornelia Lassl, AG Haslberger

PCR-DGGE and qPCR for improved characterisation of GI- microbiota and basis for probiotic intervention.

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Background

There is a stable core microbiota that is resilient to external modifications. Up to now it is not yet clear whether the core or transient populations are responsible for immune modulatory processes mediated by intestinal microbiota. We apply a strategy which combines the qualitative PCR-DGGE analysis and quantitative real-time-PCR of bacterial groups for the assessment of probands following different diets and with different age.

Methods

200 mg of feces from groups of 15 geriatric patients and young volunteers following a vegetarian or typical middle European diet have been analyzed. PCR- DGGE, cloning and sequencing, as well as real-time-PCR using universal, bifidobacteria- [1], *Clostridium* cluster IV [2,3] and *Bacteroides*- [4] specific 16S rDNA primers and corresponding 6-FAM labeled TaqMan probes [5] were used. Sensitivity and specificity of primers and probes were tested using rdp 9.52 and type strains. PCR-DGGE fingerprinting was interpreted calculating Shannon's diversity index and principal component analysis (PCA).

Results and Discussion

Elderly probands harbored less bacteria (85±33%) as compared to omnivores (100±52%); vegetarians showed slightly increased copy counts of bacteria (110±65%) but none of these differences were significant due to high inter-individual variations (Fig.1). The significant reductions of both bifidobacteria (1.1±0.9%) and members of *Clostridium* cluster IV (26.9±11.7%) in the group of elderly persisted after relative quantification despite supplementation with soluble fiber in our study population of institutionalized elderly (Fig.2). In vegetarians there tended to be a non-significant increase of bacteria of up to 10%. We found that DGGE fingerprints were composed of 20-21 bands in average (Fig.3), a value that has been described in the literature before. 13-15% of bands occurred only once and 10-13% of bands were abundant in at least 50% of probands. In our study the reduced counts of bacteria with ageing were not associated with loss of diversity in DGGE fingerprinting as suggested by Shannon's diversity indices and PCA (data not shown).

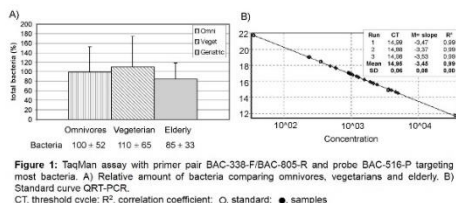


Figure 1: TaqMan assay with primer pair BAC-338-F/BAC-805-R and probe BAC-516-P targeting most bacteria. A) Relative amount of bacteria comparing omnivores, vegetarians and elderly. B) Standard curve QRT-PCR. CT, threshold cycle; R², correlation coefficient; Q, standard; ●, samples

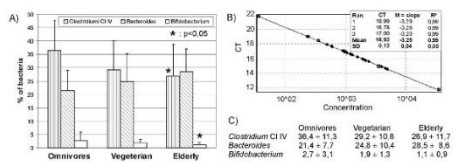


Figure 2: TaqMan assay for selected bacterial subgroups. A) Proportions of Bacteroides, Bifidobacteria and Clostridium cluster IV of the total bacterial load. B) Standard curve Clostridium cluster IV. C) Relative proportions (%) of bacterial subgroups in fecal samples. CT, threshold cycle; R², correlation coefficient standard curve

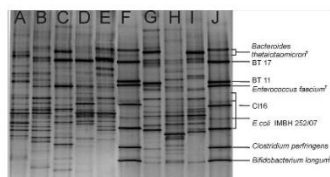


Figure 3: PCR-DGGE fingerprinting of 16S rRNA coding regions amplified with primer pair 341GC-518. A, E, I, omnivores; B, D, vegetarians; C, G, H, elderly; F, J, reference lane.

Conclusions

This polyphasic approach, together with dietary questionnaires, is a promising tool for monitoring the population dynamics of the gut microbiota in response to age and dietary behavior. We found a non significant tendency for higher counts of bacteria and *Bacteroides* in vegetarians. Ageing was associated with less overall bacteria and significantly decreased members of *Clostridium* cluster IV and bifidobacteria and an increase of *Bacteroides*.

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Consequences Of Diet And Aging For Individual SCFAs And Fecal Microbiota

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Short chain fatty acids (SCFAs) are end- and intermediate products of microbial breakdown of polysaccharides in the human gut. Especially butyric acid serves as major energy source for the gut epithelium and regulates inflammatory pathways and gene expression.

Methods: SCFAs were measured in faecal samples of elderly (n = 17; age = 86 ± 8), young omnivores (n = 17; age = 24 ± 2.5) and young vegetarians (n = 16; age = 26 ± 5) using HPLC-RI. Abundance of the Butyryl-CoA CoA-transferase gene was analysed with gene specific real-time PCR applying three degenerate primer sets. Abundance of Archaea was estimated with a TaqMan real-time PCR system.

Results: Butyric acid levels in faeces ranged between below detection limit (>0.0024 µmol/g) up to 0.095 µmol/g faeces. Vegetarians showed significantly lower butyric acid levels (68 %, p=0.06) compared to omnivores. Geriatric subject group showed lower levels of butyric acid compared to young omnivores, but not significant. The main short chain fatty acids found in faecal samples of most subjects were acetic acid, tartaric acid, lactic acid and propionic acid. Butyryl-CoA CoA transferase gene levels showed higher abundances (3 times more, p=0.0001) in vegetarians than in omnivores. The average abundance of the Butyryl-CoA CoA transferase gene was $5.26 \times 10^8 \pm 2.38 \times 10^8$ copies/µl in omnivores, $5.26 \times 10^8 \pm 2.38 \times 10^8$ copies/µl in elderly and $7.46 \times 10^8 \pm 2.29 \times 10^8$ copies/µl in vegetarians. Archaea were nine times more abundant in omnivores, and in elderly (2.8 times more often) than in vegetarians.

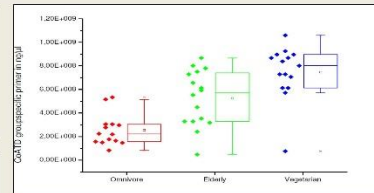


Figure 1: Scatter plot and single results: Comparison of the CoAT-Primer pair for Omnivores, Vegetarian, and Elderly subject group.

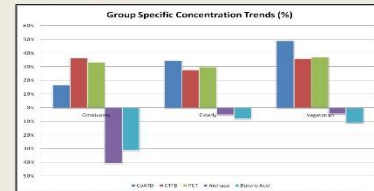


Figure 2: Relative concentration trends for CoA-transferase Butyryl-Primer Pair sets.

Group	primer pairs or probe sequence (5'-3')	Reference	
Coated Inert Organisms/Cells			
CoAT for	AAAGATCTCGGATTCATVGGGATATG	C. Charrier et al. 2006	
CoAT rev	GAGGTCTGTCGKATVTTGGATGAC		
CoA Transferase based on	CTFB for	GTAACTTGGTGGGTTTCGAC	C. Charrier et al. 2006
C. acetoabstrus (P2437)	CoAT rev	AAAGATTCATVGGGATATG	
CoA Transferase based on	PCT for	GTAGGATTAACACTVTTGATGAC	C. Charrier et al. 2006
C. perfringens (9P_2810/2)	PCT rev	TGACGACATCTGATGATGATG	
Archaea and Methanogenic Communities			
ARC181P F	ATTAGATACG CBBT AGTCC	Y. Yu et al. 2005	
ARC181P R	AGGAA TTAGC GGGG AGGAC		
ARC181P R	GGAT GCAAC TCTCTC		

Table 1: Primers and probes used for quantification of fecal bacteria protein sequences related to CoA-transferase, archaeal and Archaea using Taqman and SYBRgreen assays targeting 16S rRNA coding regions.

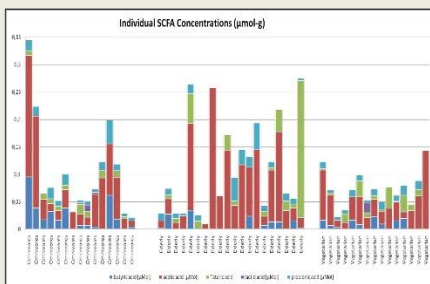
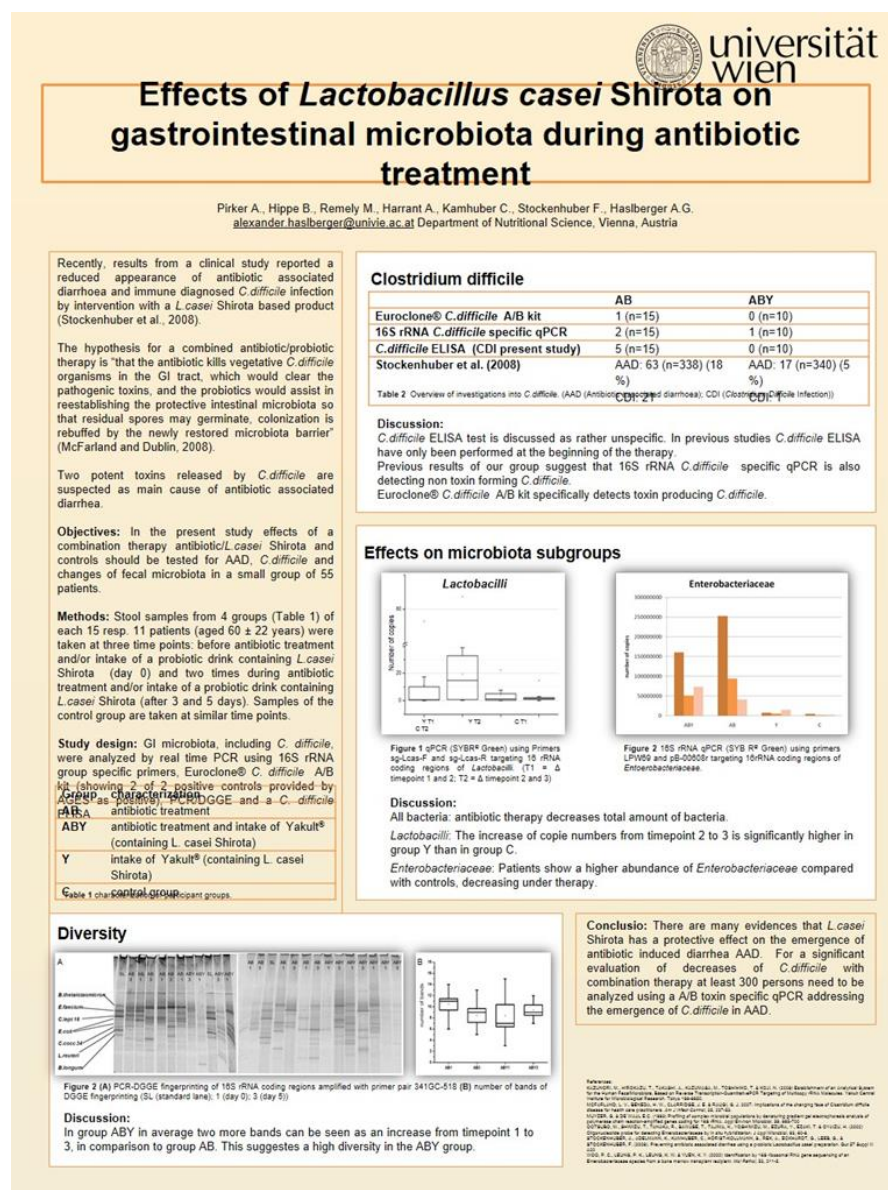


Figure 4: Individual group specific short chain fatty acid concentrations in µmol/g

Conclusion: We show that age and diet influences the microbiota and the butyric acid abundance in faeces and vegetarian diet favours growth of microbiota including SCFA producing bacteria in the gut lumen. These results demonstrate that faecal butyric acid levels do not correlate with the abundance of the butyryl-CoA CoA-transferase gene. The complexity of the colonic environment renders it difficult to use single metabolites such as butyric acid as indicators for colonic health.

References: Charrier, C., G. J. Duncan, et al. (2006). "A novel class of CoA-transferase involved in short-chain fatty acid metabolism in butyrate-producing human colonic bacteria." *Microbiology* 152(Pt 1): 179-85.
Yu, Y., C. Lee, et al. (2005). "Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction." *Biotechnol Bioeng* 89(6): 670-9.

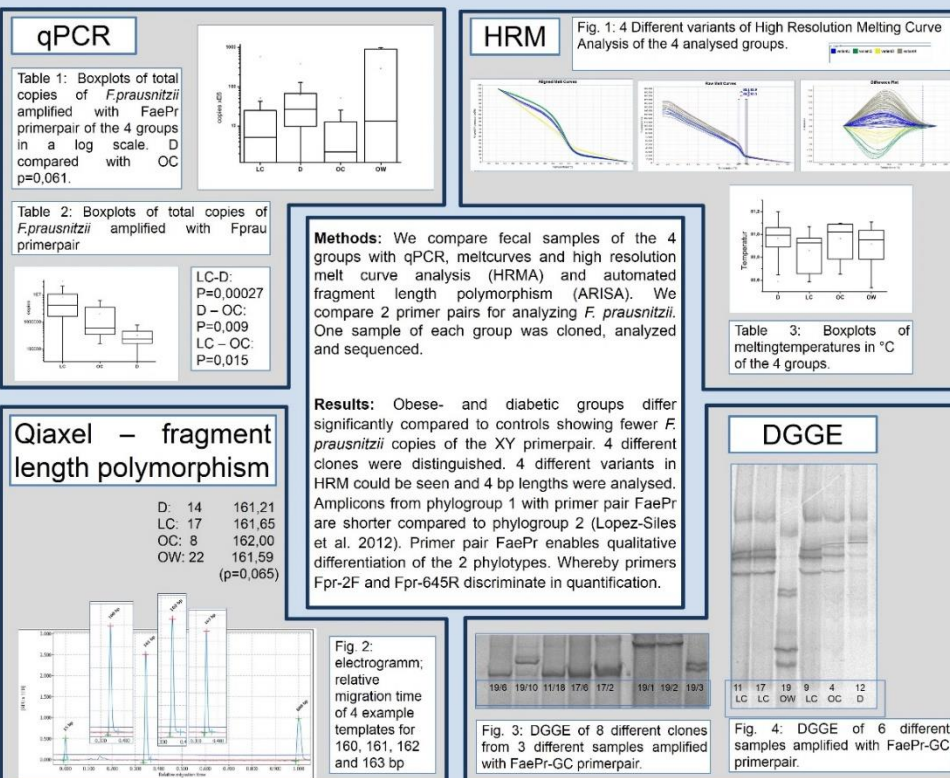


Relation of *Faecalibacterium prausnitzii* phylotypes and epigenetic regulation in obese, diabetic patients and controls

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Background: *Faecalibacterium prausnitzii* is one of the most abundant commensal bacteria and one of the main butyrate producers in the healthy human gut. Information on their genetic diversity and role in the inflammatory genesis is limited. Two genetic phylotypes could be differentiated (Lopez-Siles et al. 2012). Here we compare 4 methods analyzing *F. prausnitzii* distinguish in GC- content and fragment lengths.

Objectives: We analyze *F. prausnitzii* phylotypes, butyrate production and epigenetic regulation (here not shown) in obese (OW and OC), diabetic 2 (D)- and lean controls (LC).



References: Ramirez et al. 2009
Rinttilä et al. 2004
Lopez-Siles et al. 2012



Epigenetic results: Analysis of CpGs in the promoter regions of FFAR3, TLR2, and TLR4 show lower methylation% in obesity and type 2 diabetes. CpG methylation of LINE-1 and TNF- show no significant changes between the groups or during the time course. Negative correlation between BMI and methylation of FFAR3 was observed.

Conclusion: Different phylotypes of *F. prausnitzii* and their various functions may lead to differences in the regulation of inflammatory genesis in the host. Butyrate producers may have an influence in developing obesity and might also act as starting points for prevention and therapy of obesity.

Figure 58 Poster, FEMS, 2013

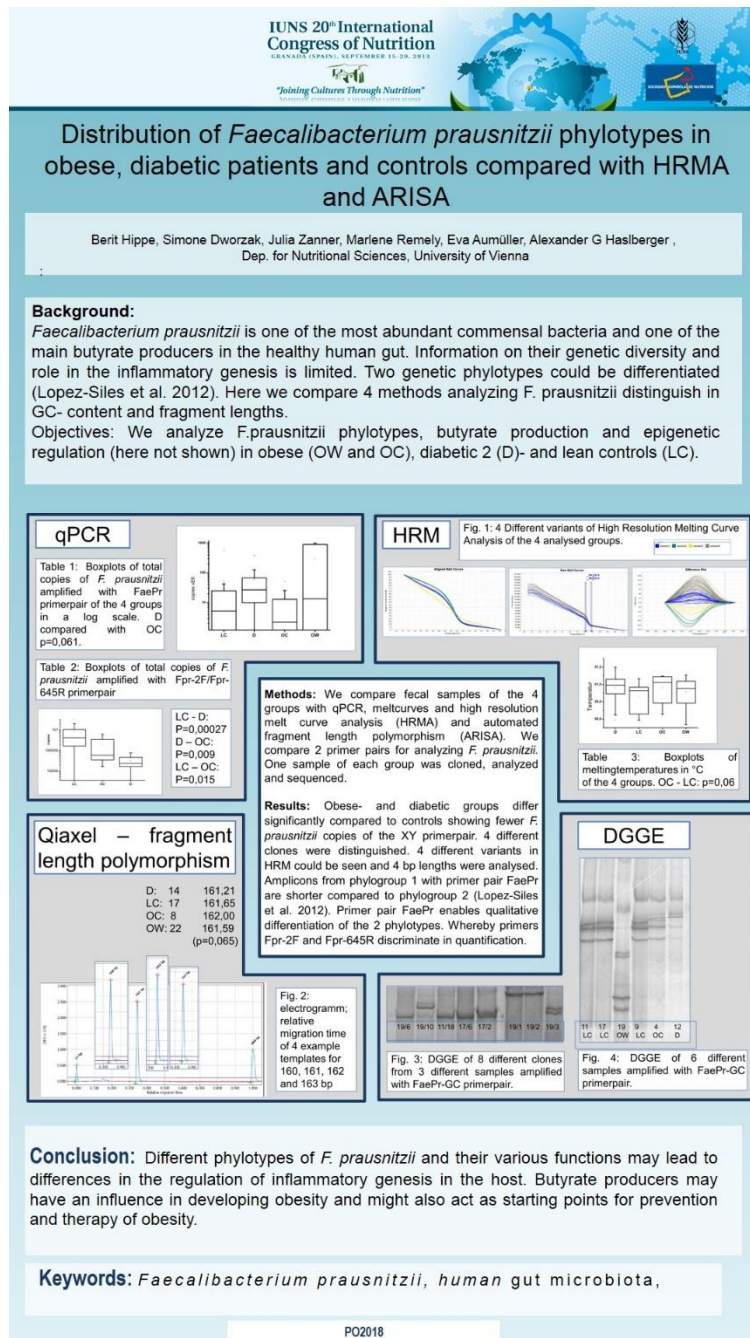


Figure 59 Poster IUNS, Granada, 2013

The Role of *Faecalibacterium prausnitzii* Phylotypes in Inflammation and Metabolic Syndrome

Background: *Faecalibacterium prausnitzii* is one of the most abundant commensal bacteria and one of the main butyrate producers in the healthy human gut. Information on their genetic diversity and role in the inflammatory genesis is limited. Two genetic phylotypes could be differentiated (Lopez-Siles et al. 2012).

Objectives: We analyze *F. prausnitzii* phylotypes, butyrate production and epigenetic regulation in obese (OW and OC), diabetic 2 (D)- and lean controls (LC).

Methods: We compare fecal samples of the 4 groups with qPCR, meltcurves and high resolution melt curve analysis (HRMA) and automated fragment length polymorphism (ARISA). We compare 2 primer pairs for analyzing *F. prausnitzii*. One sample of each group was cloned, analyzed and sequenced.

Results: Diabetic group (D) differs in almost all tests significant or shows a trend compared to results of lean group (L). L and D results are in all tests the opposite of each other. D differs significant to L in copies ($p=0.02$), *F. prausnitzii* CoA-Gen ($p=0.03$) and HRM temperature ($p=0.0058$) and variance ($p=0.0068$); and to O in *F. prausnitzii* CoA Gen ($p=0.03$) and HRM temperature ($p=0.047$) and variance ($p=0.049$). In all groups the melt curve quantity for *E. hallii*, Roseburia/ *E. rectale* and *F. prausnitzii* correlates positive to the total abundance of butyryl-CoA abundance. In the diabetic group the butyryl-CoA abundance correlates positive to the HRM temperature. Cloning results brought 43 clones for LC, 57 for OW and 12 colonies for D. 14 of the sequenced clone's align with 100% query cover and 100% identity to *F. prausnitzii* strain S4L/4 and ATCC 27768. Three of the clones cover with 100% identity with the strains L2-61, L2-15, HTF-75H, HTF-I, HTF-E, HTF-C, HTF-A. These three different clones belong to the sample from group D.

Conclusion: Intestinal MB influences host function within and beyond the GI tract. The two different phylotypes of *F. prausnitzii* may lead to differences in the inflammation in the host. In mice fecal MB transplantation with *Faecalibacterium prausnitzii* alters brain chemistry and behavior in recipient, raising the possibility of using MB to influence conditions in humans that may include diabetes. Probiotics were able to improve infection-induced memory dysfunction and diabetes-induced cognitive defects. Butyrate producers may have an influence in developing obesity and might also act as starting points for prevention and therapy of obesity and diabetes.



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- from 2011 company founding: HealthBioCare
Co-lector of ‘Biotechnology and genetic engineering in food production’
- 2011/2012 parental leave
- from 2010 Seminar at the University of Vienna ‘safety assessments of novel foods’

- from 2009 honours lecture and co-supervisor in the working group of Dr. Alexander Haslberger, University of Vienna, Austria
- Co-lector at the University of Vienna, Austria of 'Immunologically relevant aspects of foods - probiotics and Nutrigenomics' and exercises on 'microbiological and molecular biological questions of food'
- 2007-2008 Südwind Co-lector at the adult education course 'global learning', Vienna, Austria
- 1999-2000 Prison Fellowship Namibia, Windhoek, Namibia

Research Interests

Gastro intestinal Microbiota, lifestyle, nutrition

Bacteria in foods. Probiotica

BT-Foods, health, molecular epidemiology



Publications:

***Faecalibacterium prausnitzii* phylotypes in a diabetic, obese and lean control group**

Berit Hippe, Marlene Remely, Eva Aumueller, Ines Tesar, Angelika Pointner, Alexander G. Haslberger
FEMS letters, 2014, under review

Abundance and Diversity of GI Microbiota Rather than IgG₄ Levels Correlate with Abdominal Inconvenience and Gut Permeability in Consumers Claiming Food Intolerances

Berit Hippe, Marlene Remely, Natalie Bartosiewicz, Monika Riedel, Claudia Nichterl, Lulit Schatz, Sandra Pummer and Alexander Haslberger,*

Endocr Metab Immune Disord Drug Targets. 2014 Mar; 14(1):67-75.

Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity

Remely M, Aumueller E, Merold C, Dworzak S, Hippe B, Zanner J, Pointner A, Brath H, Haslberger AG
Gene. 2014 Mar 1;537(1):85-92. doi: 10.1016/j.gene.2013.11.081. Epub 2013 Dec 8.

Microbiota and epigenetic regulation of inflammatory mediators in type 2 diabetes and obesity

Remely M, Aumueller E, Jahn D, Hippe B, Brath H, Haslberger AG
Benef Microbes. 2014 Mar 1; 5(1):33-43. doi: 10.3920/BM2013.006.

Abundance and diversity of microbiota in type 2 diabetes and obesity

Marlene Remely, Simone Dworzak, Berit Hippe, Jutta Zwielerhner, Eva Aumüller, Helmut Brath, Alexander G. Haslberger

Diabetes Metab 2013, 4:3, doi.org/10.4172/2155-6156.1000253

Effects of antibiotic therapy on the gastrointestinal microbiota and the influence of *Lactobacillus casei*

Angelika Pirker, Alexander Stockenhuber, Marlene Remely, Anna Harrant, Berit Hippe, Christoph Kamhuber, Krista Adelman, Felix Stockenhuber, Alexander G. Haslberger

Food and Agricultural Immunology (2012) DOI: 10.1080/09540105.2012.689816

Isolation, Identification and Monitoring of Contaminant Bacteria In Iranian Kefir Type Drink By 16S rDNA Sequencing

Hedayat Hosseini, Berit Hippe, Ewald bm Denner, Edith Kollegger, Alexander Haslberger

Food Control, (June, 2012), p. 784-788. DOI: 10.1016/j.foodcont.2011.12.017. ISSN: 0956-7135.

Changes In Human Fecal Microbiota Due To Chemotherapy Analyzed By Taqman-PCR And PCR-DGGE Fingerprinting

Zwielehner J, Lassl C, Hippe B, Pointner A, Ruckser R, Haslberger AG

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Quantification Of butyryl CoA:acetate CoA-transferase Genes Reveals Different Butyrate Production Capacity In Individuals According To Diet And Age

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Characterization Of *Bacteria*, *Clostridia*, *Bacteroides* In Faeces Of Vegetarians Using qPCR And PCR-DGGE Fingerprinting

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Detection Of Butyrate In The GI-tract

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p. 57: Detection and Identification of Probiotic Microorganisms and Other Beneficial Organisms from the Human GI Tract; Berit Hippe, Jutta Zwielehner, Angelika Kolle, Alexander Haslberger

Individualized Analysis of Human Microbiota in Type 2 Diabetes And Obesity

Berit Hippe

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Functions of GI- microbiota and the quantification of butyryl-CoA CoA transferase genes which indicate the butyrate production capacity in individuals of different diet and age.

Berit Hippe, Jutta Zwielehner, Kathrin Liszt, Frank Unger, AG. Haslberger

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Quantification of butyryl-CoA CoA transferase genes reveals different butyrate production capacity in individuals of different diet and age

Berit Hippe, Jutta Zwielehner, Kathrin Liszt, Frank Unger, AG. Haslberger

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Probiotica and allergy, Berit Hippe

Decreased levels of butyric acid but high amounts of bacterial butyryl-CoA CoA-transferase in faecal microbiota, for vegetarians compared to omnivores

Berit Hippe, Jutta Zwielehner, Kathrin Liszt, Cornelia Lassl, AG Haslberger

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