universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Effects of probiotics on antibiotic disturbed gastrointestinal microbiota

Verfasserin

Angelika Pirker

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2012

Studienkennzahl lt. Studienblatt:	A 474
Studienrichtung lt. Studienblatt:	Diplomstudium Ernährungswissenschaften
Betreuer:	Univ. Doz. Dr. Alexander G. Haslberger

Ich erkläre hiermit, dass ich die vorliegende Diplomarbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benützt habe.

Angelika Pirker

Danksagung

Während meines Studiums und der Erarbeitung dieser Diplomarbeit, wurde ich von vielen Menschen tatkräftig unterstützt und begleitet. Dafür möchte ich mich an dieser Stelle ganz herzlich bedanken!

Vielen Dank an meinen Diplomarbeitsbetreuer Herrn Univ. Doz. Dr. Alexander Haslberger, der mir diese Arbeit überhaupt erst ermöglicht hat und dessen Unterstützung ich mir immer sicher sein konnte. Ich möchte auch Frau Mag. Berit Hippe und Frau Mag. Eva Aumüller, sowie dem gesamten Laborteam danken, welches mir immer mit Rat und Tat zur Seite gestanden und für ein außerordentlich gutes Arbeitsklima gesorgt hat.

Besonderer Dank gilt meinem Ehemann Stefan, der die ganze Studienzeit mit mir durchlebt und mich dabei stets bestärkt hat, sowie meinen Eltern und Freunden, die mir eine schöne Studienzeit beschert haben.

Danke!

The results of this diploma thesis have been presented at

- the 6th International Yakult Symposium 2011: The Gut and its Role in Health Maintenance (Vienna),
- the 6th Probiotics, Prebiotics & New Foods meeting 2011 (Rome) and
- the 2nd Internal Symposium microbes for health 2011 (Paris).

Furthermore, the results have been published in the following article:

Pirker A., Stockenhuber A., Remely M., Harrant A., Hippe B., Kamhuber C., Stockenhuber F., Haslberger A.G. (2011) Effects of Antibiotic Therapy on the Gastrointestinal Microbiota and the Influence of *Lactobacillus casei*

The abstracts and publications can be found in the appendix of this thesis.

TABLE OF CONTENTS

1. LIST OF FIGURES

2. LIST OF TABLES

3. ABBREVIATIONS

AAD	antibiotic-associated diarrhea
ANOVA	Analysis of Variance
bp	base pairs
C	cytosine
c	concentration
°C	degree centigrade
CDAD	*Clostridium difficile* associated Diarrhoe
CDI	*Clostridium difficile* infection
ct	cycle time
DNA	deoxyribonucleic acid
e.g.	exempli gratia
G	guanine
GI	gastro intestinal
$MgCl_2$	Magnesium chloride
mL	milliliter
µL	mycroliter
ng	nanogram
no.	numero
p	statistical power
PCR	Polymerase Chain Reaction
pH	pondus Hydrogenii
pmol	picomol
qPCR	quantitative Polymerase Chain Reaction
rRNA	ribosomal ribonucleic acid
SCFAs	short-chain fatty acids
UDP	Uridindiphosphat
´	minute/s
´´	second/s
%	percent

4. OBJECTIVES

The human body is a super-organism, which contains ten times more microbial cells than its own body cells (Zhu et al., 2010). All epithelial cells, which are in contact with the environment, are colonized by microorganisms. Most of these exogenous cells are localized in the gastrointestinal tract (Backhed et al., 2005), where they form an ecosystem. This ecosystem performs several functions, including gut maturation, host nutrition, pathogen resistance, regulation of the intestinal epithelial proliferation, host energy metabolism and inflammatory immune response (Dethlefsen et al., 2006).

Many different factors influence the microbiota stability as well as shifts in populations, comprising intestinal pH, microbial interactions, environmental temperature, physiologic factors, peristalsis, bile acids, host secretions, immune responses, drug therapy and bacterial mucosal receptors (Thompson-Chagoyan et al., 2007).

Antibiotic treatment causes a loss of stability, by decreasing the bacterial diversity (Sommer and Dantas, 2011), so pathogens are being enabled to come up and cause harm. A particularly dangerous complication of antibiotic intake is the overgrowth of *Clostridium difficile*. The bacterium causes severe diarrhea and pseudomembranous colitis, entities associated with outbreaks with a high rate of mortality in hospitalized patients. Furthermore an increase in yeast and *Escherichia coli* is described in numerous studies (Thompson-Chagoyan et al., 2007). As the therapeutic options for *C.difficile* infection are limited (McFarland et al., 2007), it is very desirable to find alternative treatments or preventative measures.

There are indications that some probiotic strains are able to reduce the incidence of antibiotic-associated diarrhea (AAD) and *C.difficile* associated diarrhoe (CDAD). Especially *Lactobacillus* strains have the reputation of positive effects on antibiotic-caused complications (Hickson et al., 2007, Gao et al., 2010, Wenus et al., 2008, Stockenhuber et al., 2008), although the greatest evidence exists for *Saccharomyces boulardii* (Breves et al., 2000, McFarland, 2010). Probiotics may competitively inhibit pathogens, and show antimicrobial activity (Ng et al., 2009).

McFarland (2006) defined the hypothesis, that in a combined antibiotic/probiotic therapy, the antibiotic kills vegetative *C.difficile* organisms in the intestine, which would

clear the pathogenic toxins. The probiotic would assist in reestablishing the protective intestinal microbiota (McFarland, 2006).

The aim of this diploma thesis was to investigate the qualitative and quantitative changes in human gastrointestinal microbiota, caused by antibiotic treatment and the effects of a probiotic drink containing *Lactobacillus casei* Shirota on the antibiotic disturbed microbiota. Furthermore, the influence of a combined antibiotic/*L.casei* Shirota therapy on the occurrence of *C.difficile* infection (CDI) should be explored.

In detail, the abundances of total Bacteria and the bacterial subgroups *Clostridium* Cluster IV (*Clostridium leptum* subgroup), *Clostridium* cluster XIVa (Lachnospiraceae subgroup), *Clostridium* cluster XI*, Clostridium difficile*, *Lactobacillus* spp., *L.casei*, *Bacteroides* spp., *Bifidobacterium* spp., Enterobacteriaceae and *Salmonella* spp. were determined by 16S rRNA based qPCR.

5. INTRODUCTION AND LITERATURE REVIEW

The human body contains approximately ten times more microbial than somatic cells and 150 times more genes than its own genome (Backhed et al., 2005). Overall more than 1000 bacterial species are living in and on the human body (Zhu et al., 2010). The human gut harbors most of these microbial cells, with ten to 100 trillion organisms (Turnbaugh and Gordon, 2009, Zhu et al., 2010), where all three domains of life are represented: Bacteria, Archaea and Eukarya (Backhed et al., 2005). Thereby the bacteria concentration increases towards the colon and reaches its maximum value (See Figure 1) (Walter and Ley, 2011).

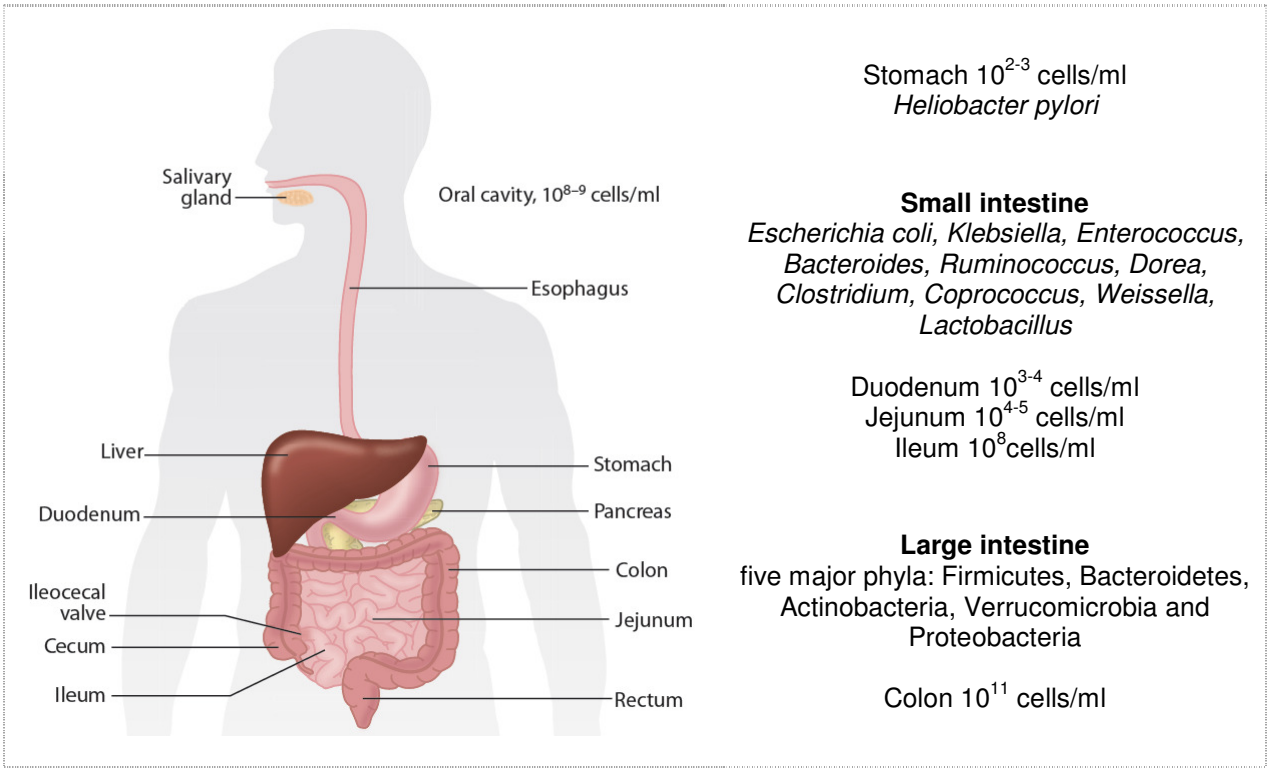


Figure 1 Qualitative and quantitative colonization of gastrointestinal sections (Walter and Ley, 2011).

5.1. The human gastrointestinal tract and its microbiota

The gastrointestinal tract is a very complex ecosystem, involving interplay between food, host cells and microbes (Zoetendal et al., 2006). The human as a host for microbial communities, represents a superorganism, where the survival of both is

interdependent (Lederberg, 2000, Ley et al., 2006) and essential to the health of the host (Dethlefsen and Relman, 2011).

At the present many effects of the intestinal microbiota are still unidentified, known functions are namely (1) the microbial degradation of indigestible polysaccharides of human diet (Flint et al., 2008), (2) the fermentation of monosaccharides to short-chain fatty acids (Backhed et al., 2005), (3) the regulation of host fat storage (Backhed et al., 2004), (4) vitamin synthesis, (5) cholesterol reduction, (6) immunostimulation (Wallace et al., 2011), (7) the supportive resistance to epithelial injury and protection from direct epithelial injury as well as the maintenance of epithelial homeostasis (Rakoff-Nahoum et al., 2004) and (8) the prevention against pathogen colonization in the gastrointestinal tract (De La Cochetiere et al., 2005). In summary, the microbiota and its microbiome provides us with genetic and metabolic attributes, we have not been required to evolve on our own, including the ability to harvest otherwise inaccessible nutrients (Backhed et al., 2005).

Until recently, the knowledge about human intestinal microbiota was examined by culture-based studies. However, these methods are very insensitive, consequently nowadays culture independent molecular fingerprinting methods and sequence analysis of cloned microbial small subunit ribosomal RNA genes (16S rRNA) are preferred for such surveys (Eckburg et al., 2005) (Backhed et al., 2005).

5.2. Health and harm

Gut bacteria can be categorized as either beneficial or potentially pathogenic, because of their metabolic activities and fermentation end products (Wallace et al., 2011).

Health-promoting factors such as vitamin synthesis and immunostimulation have already been mentioned above. Unfortunately harmful effects may also occur such as carcinogen production, intestinal putrefaction, toxin production, diarrhea/constipation, liver damage and intestinal infections (Wallace et al., 2011). There are some pathogens such as *Salmonella* and *Listeria*, which succeed by accessing unoccupied niches and escaping the lumen by entering epithelial cells. Another class of pathogen that can persist in the lumen without becoming abundant or causing disease (unless the existing community is perturbed e.g. by antibiotics), is represented by *C.difficile*. Such

perturbations of the microbiota are also associated with chronic health conditions. At the same time, commensal *Lactobacillus* and *Bifidobacterium* strains appear to block physically pathogen access to host cells (Dethlefsen et al., 2006, Ozaki et al., 2004). A large population of beneficial bacteria is able to produce antimicrobial agents and to competitively exclude pathogens by occupying receptor sites and competing for space, nutrients, etcetera (Rastall, 2004).

5.3. The microbiota

The gut microbiota is dominated by two divisions of bacteria: Bacteroidetes and Firmicutes (Backhed et al., 2005, Wang et al., 2003); and one single phylotype of Archaea: *Methanobrevibacter smithii* (Eckburg et al., 2005). Eckburg et al. (2005) pictured, that 95 % of the Firmicutes sequences were members of the *Clostridia* class. Among the Bacteroidetes phylotypes large variations can be found (Eckburg et al., 2005). Further bacterial phyla belonging to the phylogenetic core of a healthy adult gut microbiota are Actinobacteria, Proteobacteria and Verrucomicrobia (Mariat et al., 2009, Tap et al., 2009).

The bacterial diversity in human gut is highly dependent on diet, geographic location, health and other environmental factors. Nonetheless, many studies suggest that human share a core microbiota within a population of defined size without considering the abundance frequency (Zhu et al., 2010, Kurokawa et al., 2007, Qin et al., 2010, Zwielehner et al., 2009). If an abundance frequency is being taken into account, the core microbiota may disappear (Turnbaugh et al., 2009). Turnbaugh et al. (2009) suggest that a core gut microbiome exists at the level of metabolic functions (Turnbaugh et al., 2009). Even an inter-individual core microbiota is controversial. There are both, studies indicating that the composition of the predominant bacterial community is host-specific and stable for longer periods (Caporaso et al., 2011, Seksik et al., 2003, Zoetendal et al., 2006) , as well as studies that state the complete opposite (Dethlefsen and Relman, 2010). Qin et al. (2010), for example, performed illumina-based metagenomic sequencing on the gastro intestinal microbiota of 124 European individuals. At a 1 % coverage (corresponds to an average length of about 40 kb in a typical gut bacterial genome) 18 species in all individuals, 57 in ≥ 90% and 75 in ≥ 50% of individuals were detected (Qin et al., 2010). Arumugam et al. (2011) identified in a

large-scaled study three robust bacterial clusters that are not nation or continent specific. These functional subtypes are called enterotypes (Arumugam et al., 2011).

For ecosystem stability diversity is an important factor and can confer resilience in the intestine as well as generally in ecosystems. A stable gut microbiota provides protection against invading pathogenic organisms. Furthermore, by functional redundancy of microbial community it is possible that key processes are independent of changes in diversity (Backhed et al., 2005).

5.3.1. Firmicutes

The genera *Peptococcus*, *Peptostreptococcus* and *Clostridium* are the predominant proteolytic and amino acid-fermenting organisms in the colon; some of them also ferment sugars (Dethlefsen et al., 2006). The most abundant Firmicutes belong to *Clostridium* cluster IV (*C.leptum* subgroup) and XIVa (Lachnospiraceae subgroup). Together with *Clostridium* cluster IX they can comprise up to 60 % of colonic microbiota (Louis et al., 2007).

***Clostridium* cluster IV** includes certain members of the genera *Clostridium*, *Ruminococcus*, *Eubacterium* and *Faecalibacterium* (Matsuki et al., 2004). This subgroup represented 22 % of the total faecal bacteria, where *Faecalibacterium prausnitzii* strains are the most abundant (Lay et al., 2005). Faecalibacter are frequent fermenters of starch and inulin to butyrate and lactate (Dethlefsen et al., 2006).

The second dominant subgroup ***Clostridium* cluster XIVa**, contains many butyrate-producing strains. *Roseburia* and its relatives, for example, can degrade starch and inulin; a cluster related to *Eubacterium halii* ferments lactate and acetate to butyrate and hydrogen. Important non-butyrate producing members of cluster XIVa are *Ruminococcus torques* and *Ruminococcus gnavus*, which are among the primary mucin-degrading organisms (Dethlefsen et al., 2006).

Clostridium cluster XIVa and IV contain the main butyrate-producing Bacteria in the human gut. Strains related to *F.prausnitzii* produce very high levels of butyrate in vitro. Butyrate, arising from microbial fermentation, is important for the energy metabolism and normal development of colonic epithelial cells and has a mainly protective role in

relation to colonic disease (e.g. prevention of colitis or colorectal cancer) (Pryde et al., 2002). Together with acetate and propionate, butyrate belongs to the main short-chain fatty acids (SCFAs), stimulating colonic blood flow and fluid and electrolyte uptake (Topping and Clifton, 2001). Furthermore butyrate is a preferred substrate for colonocytes (Topping and Clifton, 2001) and may have an anti-carcinogenic and anti-inflammatory potential, affects the intestinal barrier and plays an important role in satiety and oxidative stress (Hamer et al., 2008).

***Clostridium* cluster XI** is a heterogeneous phylogenetic cluster comprising opportunistic pathogens, such as *Clostridium difficile* (Nadal et al., 2009).

C.difficile is a gram-positive, spore-forming, anaerobic bacterium that can reside asymptomatically in the human intestine. If antimicrobial agents such as broad-spectrum antibiotics disturb the normal gut microbiota, *C.difficile* can proliferate and cause intestinal damage, inflammation and clinical disease (Lawley et al., 2009). McFarland et al. (2007) summarized the pathogenesis of *C.difficile* associated disease as a triad of factors: (1) disruption of normal intestinal flora, (2) exposure to *C.difficile* and (3) host factors (comorbidity and advanced age or impaired immune status). The main virulence determinants produced by *C.difficile* are the enterotoxin “toxin A” and the cytotoxin “toxin B” (McFarland et al., 2007). Because of controversy concerning the essentiality of the two toxins for the virulence of *C.difficile*, Kuehne et al. (2010) tested the responsibility *in vitro* and in the hamster model. Both investigations indicate that toxin A, as well as toxin B, contribute to virulence (Kuehne et al., 2010). Both toxins disrupt the actin cytoskeleton of intestinal epithelial cells by the UDP-glucose-dependent glycosylation of Rho and Ras proteins. Even nontoxigenic or atypical toxin strains may cause symptoms. In addition to toxin A and B, a binary toxin, namely “CDT”, was described in 1988. The association between binary toxin and pathogenicity is still unclear (McFarland et al., 2007).

Lactobacilli are facultative anaerobic Bacteria. Hence, they represent one of the less-dominant Bacteria of the gut microbiota. Lactobacilli are health positive and produce a range of antimicrobial agents (Rastall, 2004).

Reuter (2001) argues that there exists an autochthonous *Lactobacillus* spp. and *Bifidobacterium* spp. microbiota, which remains stable in human life-long. In case of

Lactobacilli, these species are *L.gasseri*, *L.reuteri*, *L.ruminis* and to some degree *L.salivarius*. Some successions may be caused by transient species derived from food or from the oral cavity, thus giving the impression of an altered microflora (Reuter, 2001). The number of naturally occurring Lactobacilli in the intestine can be evidently increased by Galacto-oligosaccharides (Walton et al., 2011).

5.3.2. Bacteroidetes

Bacteroides are among the main inhabitants of the human gut. Members of the subgroup can degrade starch and many strains are also able to degrade some types of structural polysaccharides. Furthermore, they can import oligosaccharides into their periplasmic space for further hydrolysis. *Bacteroides* are primarily responsible for removing the sulfate ester-linked substituents of mucin (Dethlefsen et al., 2006).

As already mentioned, there are large variations among the Bacteroidetes phylotypes. But *Bacteroides thetaiotaomicron* occurs ubiquitously and fulfills beneficial functions, including nutrient absorption and epithelial cell maturation and maintenance (Eckburg et al., 2005).

5.3.3. Actinobacteria

The genus ***Bifidobacterium*** includes gram-negative anaerobic species, which are common inulin and starch degraders and some of them also mucin degraders (Dethlefsen et al., 2006). Further metabolic activities are conjugated linoleic acid production, short chain fatty acid production, exopolysaccharide production and immune-mofulating effects on host mucosal cells (Russell et al., 2011). Bifidobacteria are the major component of the microbial barrier to infection. They produce a range of antimicrobial agents, which are active against gram-positive and gram-negative organisms (Rastall, 2004).

Several strains of Bifidobacteria are often used as probiotics (e.g. in milk and dairy products, infant formula and dietary supplements).

5.3.4. Proteobacteria

Proteobacteria belong to the less represented phyla in the intestine. The facultative species may represent about 0.1 % of the Bacteria in the strict anaerobic environment of the colon (Eckburg et al., 2005).

Bacteria of the **Enterobacteriaceae** family are among the sub-dominant ones in the human gut, especially *E.coli* (Mariat et al., 2009, Rastall, 2004), which is part of the normal gastrointestinal microbiota, performing a barrier effect against enteropathogens. Several *E.coli* strains develop a protective effect against other Enterobacteriaceae (Hudault et al., 2001), but there also exist strains with different pathogenicity, including EHEC (enterohaemorrhagic *E.coli*), EPEC (enteropathogenic *E.coli*) and ETEC (enterotoxic *E.coli*) (Mahajan and Gally, 2011).

Salmonella spp. is the major cause of human morbidity and mortality worldwide. These species are able to overcome the mucosal colonization resistance and inducing inflammation (Ahmer and Gunn, 2011). *Salmonella enteric*a serovar Typhimurium is a common cause of food-borne illness and causes gastroenteritis in human (Barman et al., 2008).

5.4. Antibiotics

Antibiotic compounds work to either stop bacteria from growing or to kill them. Thereby, they are quite likely to impart collateral damage to the bacterial community which shares the environment of the intended target organism. A treatment with antibiotics causes both, short- and long-term effects on human gut microbiota. The relative proportions of different species can be changed; on the one hand new species can be introduced and on the other hand existing species can be completely eradicated (Sommer and Dantas, 2011). During treatment with antibiotics, the bacterial diversity is generally reduced and the normally low-abundant Proteobacteria are increased at the expense of the normally dominant Firmicutes and Bacteroidetes. These are drug resistant strains of human pathogens, including *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and certain Enterobacteriaceae (Sommer and Dantas, 2011). Such changes in the microbiota cause a loss of stability and, as already mentioned above, an unstable system can allow pathogens to penetrate.

De La Cochetire et al. (2005) demonstrated that the dominant fecal microbiota needs up to 60 days following a short-course antibiotic challenge (5 days), to return to its initial profile. This fact can help to understand the occasional occurrence of chronic disorders after the treatment with antibiotics (De La Cochetiere et al., 2005).

5.4.1. Antibiotic-Associated Diarrhea (AAD)

Diarrhea is a common side effect of antibiotics. Nearly all types of antibiotics are reported to induce AAD, but those with a spectrum of activity including anaerobic bacteria have been associated with higher rates of AAD (Breves et al., 2000). Mild clinical AAD are mostly caused by functional disturbances of intestinal carbohydrate or bile acid metabolism, allergic and toxic effects of antibiotics on intestinal mucosa or pharmacological effects on motility. More severe AAD are caused by infection with *Clostridium difficile* or other infectious agents, including *Clostridium perfringens*, *Staphylococcus aureus*, *Klebsiella oxytoca*, *Candida species* and *Salmonella species* (Hogenauer et al., 1998).

C.difficile is believed to be responsible for 5 % to 20 % (Hogenauer et al., 1998) of all cases of AAD, depending on epidemiology of CDI and for virtually all cases of pseudomembranous colitis (Bartlett, 1987). In endemic outbreaks *C.difficile* is even more often responsible for AAD (Arumugam et al., 2011, Hensgens et al., 2011, McFarland et al., 2007).

The almost complete inhibition of butyrate fermentation, is described as the most important pathophysiological factor for the development of AAD. Butyrate, as well as other short-chain fatty acids are essential for sodium and water uptake in the intestine (Breves et al., 2000), beside their role as an important energy source for intestinal epithelial cells (Hamer et al., 2008).

For *C.difficile* infection, there are only two standard antibiotic treatments: vancomycin and metronidazole. The response rate for metronidazole is declining (McFarland et al., 2007). Therefore, it is very desirable to find alternative treatments or preventative measures.

5.5. Probiotics

Probiotics are defined as *"Live microorganisms which when administered in adequate amounts confer a health benefit on the host' but restricted its scope to discussion of 'Live microorganisms which when consumed in adequate amounts as part of food confer a health benefit on the host."* (FAO/WHO, 2001)

It is important to note that effects of any probiotic bacteria are strain specific, meaning that results cannot be extrapolated to other species or strains (Hickson, 2011).

Common types of probiotics include Bacteria such as lactic-acid bacteria (LAB) and *E.coli* strains (e. g. *E.coli* Nissle 1917) or yeast species such as *S.boulardii* (Table 1). In contrast, prebiotics such as lactulose, inulin, psyllium and other oligosaccharides are nondigestible food ingredients that stimulate the growth or activity of bacteria in the GI tract which are beneficial to the health of the body (Verna and Lucak, 2010); e.g. *Bifidobacterium* spp. or *Lactobacillus* spp. (Rastall et al., 2005).

Single-organism probiotics	*Escherichia coli* 1917 Nissle *Lactobacillus salivarius* UCC4331 *Lactobacillus reuteri* *Lactobacillus casei* *Lactobacillus plantarus* 299v *Lactobacillus rhamnosus* GG *Bifidobacterium infantis* 35624 *Bifidobacterium animalis* DN-173010 *Bifidobacterium longum* *Saccharomyces boulardii*
Composite probiotics	VSL #3: *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus bulgaricus* Lacteol Fort: *L.acidophilus*, lactose monohydrate, anhydrous lactose

Table 1 Common probiotic formulations (Verna and Lucak, 2010).

5.5.1. Probiotic actions

Hickson (2011) summarized the three broad areas of antibiotic benefits as follows: modulation of the hosts' immune system, antimicrobial activity and other mechanisms relating to indirect action on pathogens, the host or food components (Hickson, 2011). Immunomodulation is performed by effects on epithelial cells, dendritic cells, monocytes, macrophages and lymphocytes (Ng et al., 2009). Immune modulatory effects might even be achieved with dead probiotic bacteria or just probiotics-derived components like peptidoglycan fragments or DNA (Oelschlaeger, 2010).

Antimicrobial activities of probiotics include the competitive inhibition with pathogenic bacteria, the secretion of bacteriocins/defensins, the inhibition of bacterial adherence or translocation and the reduction of luminal pH. Probiotic bacteria can also enhance intestinal barrier function by increasing mucus production (Ng et al., 2009).

There is evidence that some probiotic strains are able to reduce the incidence of AAD and CDAD. By a metaanalysis of McFaraland (2010), a significant therapeutic efficacy of *S.boulardii* in the prevention of AAD has been found (McFarland, 2010). The effect of decreasing SCFAs production and associated diarrhea could be compensated partly by *S.boulardii*. However, only the acetate and propionate fermentation could be increased to control levels, butyrate fermentation could not be reconstituted (Breves et al., 2000).

Several studies indicate as well that *Lactobacillus* spp. stains are very efficient in AAD prevention (Hickson, 2011). Declined incidence of AAD and CDAD could be detected for example by the strains *L.casei* Shirota (Stockenhuber et al., 2008), *L.acidophilus* CL1285 and *L.casei* LBC80R (Gao et al., 2010), *L.rhamnosus* GG, *L.acidophilus* La-5 and *Bifidobacterium* Bb-12 (Wenus et al., 2008) and *L.casei* DN-114 001, *L.delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Hickson et al., 2007).

6. MATERIAL AND METHODS

6.1. Study design and participants

Figure 2 shows an overview of the study design and the used methods of this diploma thesis.

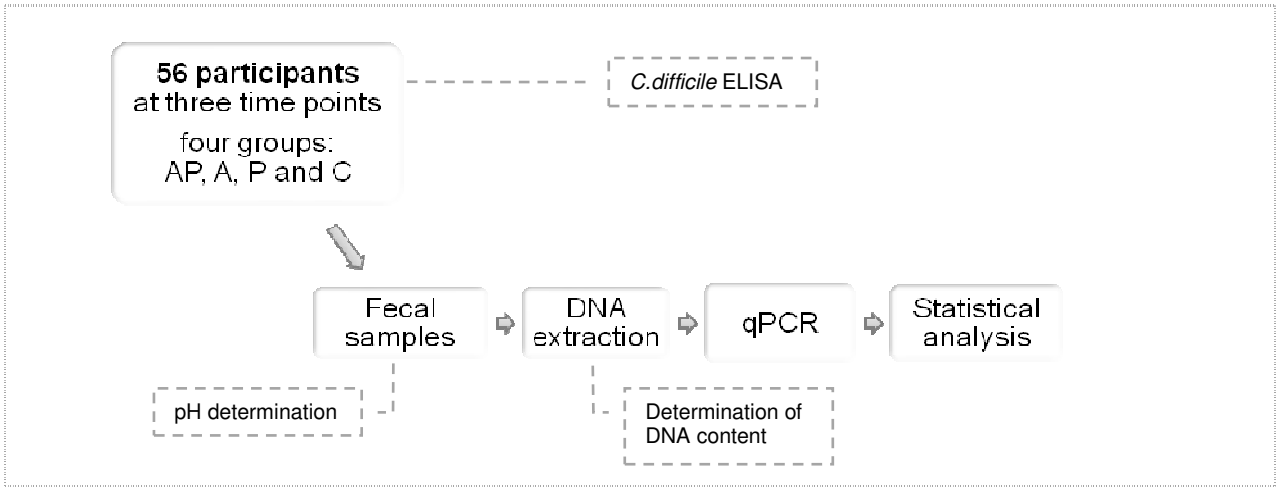


Figure 2 Overview of the study design and used methods.

The subjects were patients of the General Hospital Oberpullendorf (Burgenland, Austria). In hospital *C.difficile* toxin A and toxin B were detected in feces samples by immunoassay (TOX A/B QUICK CHEK®, Wampole®).

The participants (aged 60 ± 22 years) were divided into four groups (Table 2) of each 15 (group AP, P and C) respectively 11 (group A) individuals.

Group	characterization
AP	antibiotic treatment and intake of a probiotic drink containing *Lactobacillus casei* Shirota
A	antibiotic treatment
P	intake of a probiotic drink containing *Lactobacillus casei* Shirota
C	control group

Table 2 Characterization of participant groups.

6.2. Sampling and DNA extraction

Stool samples were taken before antibiotic treatment and/or intake of a probiotic drink containing *L.casei* Shirota (day 0) and two times during antibiotic treatment and/or intake of a probiotic drink containing *L.casei* Shirota (day 3 and day 5). Samples of the control group were taken at similar time points. After collection, stool samples were immediately frozen at -70 °C.

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

6.3. Quantitative analysis by real time PCR

Real time PCR, also called qPCR, was used to determine the abundance of the analyzed bacterial groups. TaqMan® and SYBR® Green method were used for this diploma thesis. The DNA concentration of the stool samples and type strains were measured by nano drop method, which enables an absolute quantification.

For a reaction mix, with the total volume of 10 µL, 5 µL SensiMix™ Probe Kit (TaqMan®) or SensiMix™ SYBR No-ROX Kit (SYBR® Green), 1 µL of each primer, 1 µL of TaqMan®-probe (only TaqMan®) and 10 ng template were used. Primers and TaqMan®-Probes targeting 16S rRNA coding regions of total Bacteria*, Clostridium* cluster IV, *Clostridium* cluster XIVa, *Clostridium difficile,* all *Bacteroides* spp. and *Bifidobacterium* spp., as well as the concentration of the primers and probes and the expected fragment length, are listed in Table 3. For SYBR® Green method, primers targeting 16rRNA coding regions of *Clostridium* cluster XI*, Lactobacillus* spp., *Lactobacillus casei* subgroup, Enterobacteriaceae and *Salmonella* spp., as well as the primer concentrations and the expected fragment length, are listed in Table 4.

For the PCR of the Enterobacteriaceae subgroup, $MgCl_2$ concentration of 4 mM was used. For all the other investigated groups, the $MgCl_2$ content of the SensiMix™ Probe Kit (TaqMan®) or SensiMix™ SYBR No-ROX Kit (SYBR® Green) (3 mM) was sufficient.

Target organism	Primer/Probe	Sequence (5' - 3')	Size [bp]	Conc. [pmol/µL]	Reference
All Bacteria	BAC-338-F	ACT CCT ACG GGA GGC AG	468	10	(Yu et al., 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	
***Clostridium* cluster IV (*Clostridium leptum* subgroup)**	sg-Clept-F	GCA CAA GCA GTG GAG T	239	4	(Matsuki et al., 2004)
	sg-Clept-R	CTT CCT CCG TTT TGT CAA		4	
	Clept-Pa	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		2	(Zwielehner et al., 2009)
***Clostridium* cluster XIVa (Lachnospiraceae subgroup)**	195-F	GCA GTG GGG AAT ATT GCA	538	7	(Meier et al., 1999)
	Ccocc-R	CTT TGA GTT TCA TTC TTG CGA A		7	(Matsuki et al., 2004)
	Ccocc-P	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)		1.5	
Clostridium difficile	Cdiff-F	TTG AGC GAT TTA CTT CGG TAA AGA	151	10	(Penders et al., 2005)
	Cdiff-R	TGT ACT GGC TCA CCT TTG ATA TTC A		10	
	Cdiff-P	(6-FAM)-CCA CGC GTT ACT CAC CCG TCC G-(BHQ-1)		2	
***Bacteroides* spp.**	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	
***Bifidobacterium* 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	

Table 3 Primers and TaqMan®-probes targeting 16rRNA coding regions of bacteria.

Target organism	Primer/Probe	Sequence (5' - 3')	Size [bp]	c [pmol]	Reference
***Clostridium* cluster XI**	C-XI F C-XI R	ACG CTA CTT GAG GAG GA GAG CCG TAG CCT TTC ACT	180	3	(Song et al., 2004)
***Lactobacillus* spp.**	Lac1 Lac2	AGC AGT SGG GAA TCT TCC A ATT YCA CCG CTA CAC ATG	352-700	4 4	(Walter et al., 2001)
Lactobacillus casei	sg-Lcas-F sg-Lcas-R	ACC GCA TGG TTC TTG GC CCG ACA ACA GTT ACT CTG CC	296	4 4	(Matsuda et al., 2009)
Enterobacteriaceae	LPW69	AGC ACC GGC TAA CTC CGT	492-509	3	(Woo et al., 2000)
	pB-00608 r	GAA GCC ACG CCT CAA GGG CAC AA	834 - 856	3	(Ootsubo et al., 2002)
***Salmonella* spp.**	16SIII	CAC AAA TCC ATC TCT GGA	1025-1008	2	(Lin and Tsen, 1996)
	16S-Sal	GTG TTG TGG TTA ATA ACC GCA GCA	453-477	2	(Lin et al., 2004)

Table 4 Primers targeting 16rRNA coding regions of bacteria (SYBR® Green).

The temperature program for StepOnePlus™ (96 wells) Real-Time PCR System Version 2.1 (Applied Biosystems) depends on the particular group of bacteria (Table 5). It includes initial denaturation followed a cycling phase (40 cycles) – the exponential doubling of the DNA.

Target organism	Temperature [°C]		Period	
	Initial denaturation	cycling	Initial denaturation	cycling
All Bacteria	95	95/55/72	10´	30´´/30´´/50´´
***Clostridium* cluster IV (*Clostridium leptum* subgroup)**	95	95/55/72	10´	30´´/30´´/50´´
***Clostridium* cluster XIVa (Lachnospiraceae subgroup)**	95	95/56/72	10´	15´´/15´´/45´´
***Clostridium* cluster XI**	95	95/62/72	5´	20´´/1´/1´
Clostridium difficile	95	95/58/72	10´	30´´/30´´/50´´
***Bacteroides* spp.**	95	95/60/72	10´	30´´/30´´/50´´
***Bifidobacterium* spp.**	95	95/60/72	10´	30´´/30´´/50´´
***Lactobacillus* spp.**	95	95/61/72	10´	30´´/1´/50´´
Lactobacillus casei	95	94/55/72	10´	20´´/20´´/50´´
Enterobacteriaceae	95	95/59/72	10´	20´´/1´/1´
***Salmonella* spp.**	95	94/66/72	5´	20´´/30´´/30´´

Table 5 Programs for qPCR.

Each real time PCR run contained a tenfold series of DNA dilutions of a type strain (Table 6) to construct a standard curve and one stool sample that runs through all the experiments for efficiency comparison. All templates were determined in duplicate and for calculation the average was used.

Target organism	Type strain	Size [bp]	GC-content [%]	Initial c [ng/µL]
All Bacteria	Stool sample	468	50	86
***Clostridium* cluster IV (*Clostridium leptum* subgroup)**	*Clostridium leptum* DSM 753	3270109	50	40
***Clostridium* cluster XIVa (Lachnospiraceae subgroup)**	*Clostridium blautia*	538	41	16,7
Clostridium difficile	*Clostridium difficile* 301968 DNA	4298133	28	4
***Bacteroides* spp.**	*Bacteroides thetaiotaomicron* DSM 2079	6260361	43	40
***Bifidobacterium* spp.**	*Bifidobacterium longum* DSM 20211	2260000	60	40
***Lactobacillus* spp.**	Lactobacillus *casei*	3079196	46	40
***Lactobacillus casei* subgroup**	*Lactobacillus casei*	3079196	46	40
Enterobacteriaceae	*Escherichia coli* 1029	5634850	50	40
***Salmonella* spp.**	*Salmonella* atcl 14028	101461	50	40

Table 6 Overview of type strains.

6.4. Statistical analysis

The respective standard curves were created by using serial dilutions of known concentrations. From these standard curves, the respective linear equations were derived for calculating the sample concentration (ng/µL). Subsequently, the copy numbers for the standards of known concentration were calculated, using the GC contents of the respective type strains and the molecular masses of guanine, cytosine, adenine and thymine. The obtained values were applied to the samples by factors, so that the results could be compared in units of copies per gram. For preparatory calculations Microsoft® Excel was used.

Determined data were statistically analyzed using the program OriginPro 8 (OriginLab®). To check whether the values are normally distributed the normality test was applied.
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 was used. As statistically significant, p-values less than 0.05 were defined.

7. RESULTS

In the following, the results of the gastrointestinal microbial composition of the examined groups are listed. The results are arranged into subgroups of Bacteria.

All values have been determined by qPCR and then analyzed statistically.

7.1. Relative amount of bacterial subgroups

Figure 3 shows the mean percentage of bacterial subgroups in relation to the analyzed Bacteria. The individual values for this figure are mentioned in the interpretation of the respective bacterial groups.

In patients receiving antibiotics, the *Clostridium* Cluster IV and XIV is clearly reduced compared to healthy controls and Enterobacteriaceae are increased at the first time point. Furthermore, in patients under antibiotic treatment, there were more bacteria which cannot be identified by the primers used in this study.

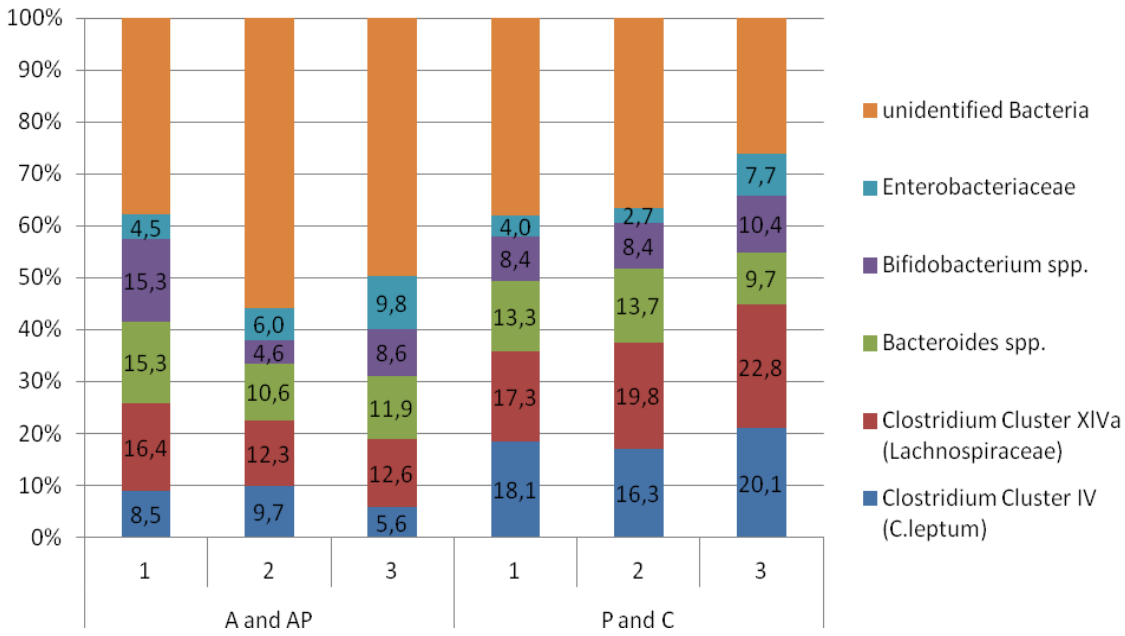


Figure 3 Percentage of bacterial subgroups in relation to the analyzed Bacteria (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group; 1: day 0, 2: day 5, 3: day 5).

7.2. Total Bacteria

In Table 7 the results of 16S rRNA based qPCR of total Bacteria of all patient groups and time points are listed, including mean values and standard variations.

Antibiotic treated groups (AP and A) had a significant lower number of copies than the control groups (P and C) ($p = 8{,}4 \times 10^{-4}$); especially at time point two (AP: $p = 0.02$; A: $p = 0.03$) and three (AP: $p = 0.003$; A: $p = 0.02$) of group AP and A, compared to group C as a whole. Within the antibiotic treated groups, a mean decrease of number of copies could be observed. In group AP the decrease from time point 1 to 3 was significant ($p = 0.054$). The values of group P as a whole did not differ from those of group C; the same applied to group AP and A (Figure 4).

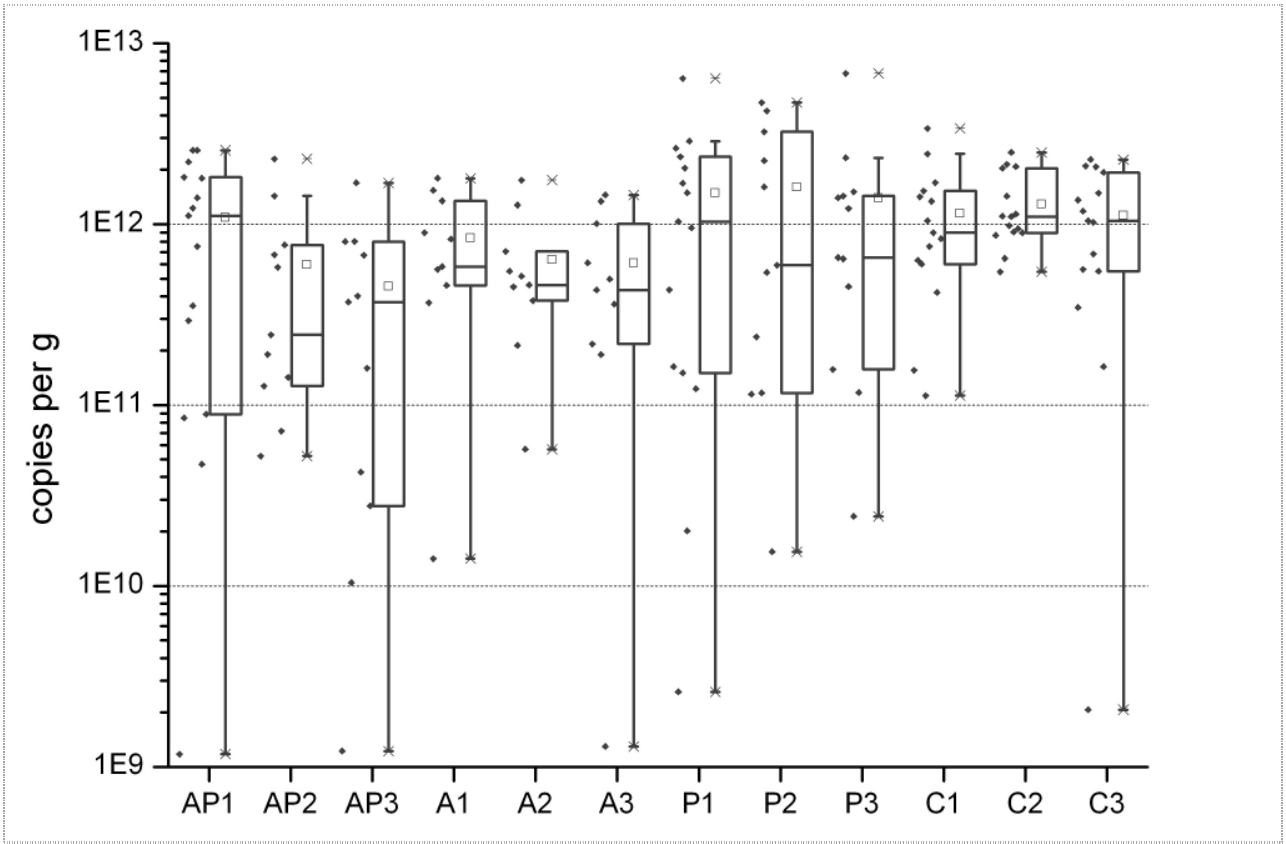


Figure 4 16S rRNA based qPCR of all Bacteria (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group; 1: day 0, 2: day 5, 3: day 5)

participant	time point 1 [copies per gram]	time point 2 [copies per gram]	time point 3 [copies per gram]
	group AP		
N21	1 227 939 074 294	676 963 534 853	400 046 587 182
N28	2 566 184 877 255	244 880 190 170	803 698 515 820
N29	353 630 702 992	579 113 342 084	
N30	293 390 379 947	1 432 295 157 691	1 691 355 884 077
N31	752 398 691 218	190 581 111 361	42 516 108 707
N32	2 206 391 732 380		10 426 379 545
N33	84 728 999 235		
N34	1 114 144 610 224	71 814 174 616	673 091 338 222
N35	47 063 951 833	2 298 856 090 132	370 733 306 327
N36	1 395 436 447 603	127 329 232 678	159 975 733 375
N37	1 173 016 497	768 179 283 124	800 189 188 195
N38	1 820 421 339 357	1 482 620 546 285	27 667 095 578
N39	1 795 485 643 464	52 191 929 087	1 220 176 962
N40	2 563 449 580 975	142 418 430 072	
N41	89 047 764 215		
mean value	1 087 392 454 099	672 270 251 846	452 810 937 635
standard variance	937 971 485 485	715 759 019 431	516 750 568 192
	group A		
N3	563 179 751 103	516 083 578 945	190 083 362 556
N7	14 160 593 502	213 875 268 359	432 938 443 649
N9	1 794 608 063 220	56 983 128 828	1 293 837 789
N13	583 509 517 264	1 752 787 366 543	217 577 377 128
N14	367 364 358 978	1 274 501 491 111	1 337 318 125 352
N17	458 636 680 964	450 848 726 180	497 218 678 806
N42	1 536 061 243 091	461 638 468 163	611 598 854 213
N43	1 345 360 282 854	549 894 154 445	1 007 871 218 305
N45	897 870 441 264	377 285 589 437	1 448 865 019 931
N47	827 328 025 596	705 874 587 714	360 886 819 700
mean value	838 807 895 784	635 977 235 972	610 565 173 743
standard variance	562 525 273 832	508 444 327 129	494 184 348 488
	group P		
N5	1 683 452 486 345	2 241 659 662 807	451 984 163 185
N6	2 042 139 489 739	4 555 557 578	
N8	150 747 067 112	541 889 426 931	1 221 234 866 318
N10	2 592 189 262		
N11	20 139 286 444	911 216 319 720	642 896 194 869
N15	162 988 547 056	116 580 987 875	24 247 896 180
N19	2 357 653 455 488	15 452 699 139	1 431 959 149 924
N20	1 034 887 116 468	238 488 494 238	1 510 026 278 840
N22	2 872 577 376 326	594 312 813 074	2 332 420 927 299
N23	6 397 672 896 933	4 229 247 702 942	6 809 975 669 572
N26	955 576 650 587	4 711 559 216 802	654 744 164 196
N27	433 710 249 420		
N44	2 629 706 361 548	3 239 688 866 773	117 406 171 514
N46	122 936 798 441	114 815 105 592	157 250 686 778
mean value	1 490 484 283 655	1 413 288 904 456	1 395 831 469 879
standard variance	1 749 628 122 816	1 737 249 629 897	1 931 104 648 257
	group C		
N1	753 858 465 924	2 498 006 851 240	2 283 299 096 259
N2	2 451 784 028 561	2 142 993 236 599	683 546 717 588
N4	1 043 689 607 431	982 692 560 867	2 069 985 382
N12	1 524 157 208 834	646 274 078 764	1 021 729 978 070
N16	112 813 180 585	905 161 715 810	549 574 665 425
N18	897 626 394 418	546 102 259 287	1 043 607 618 596
N24	602 598 761 044	2 081 340 371 285	2 100 246 322 948
N25	418 981 412 484	1 102 070 025 180	1 483 496 748 765
N48	1 338 348 840 808	944 939 614 853	1 179 670 535 303
N49	1 413 965 694 269	867 364 097 843	1 934 513 525 697
N50	3 384 141 280 408	894 850 730 595	562 729 239 778
N51	630 911 190 834	2 039 950 495 380	1 360 992 031 587
N52	1 697 821 022 485	1 428 148 727 054	162 733 633 903
N53	830 189 520 838	1 137 862 383 984	346 270 197 953
N54	155 810 055 790	1 107 275 092 814	2 076 912 653 396
mean value	1 150 446 444 314	1 288 335 482 770	1 119 426 196 710
standard variance	875 587 207 550	606 544 944 376	741 317 778 095

Table 7 Results of 16S rRNA based qPCR of all Bacteria.

7.3. *Clostridium* Cluster IV (*Clostridium leptum* subgroup)

In Table 8 the results of 16S rRNA based qPCR of *Clostridium* Cluster IV of all patient groups and time points are listed, including mean values and standard variations.

The analysis of the *Clostridium* Cluster IV showed, that the values of the groups receiving antibiotics (AP and A) are on average lower by a half-power than the values of the two control groups (P and C) (Figure 5). The difference was statistically significant ($p = 6.31 \times 10^{-7}$).

Group AP and A, and group P and C did not differ from each other.

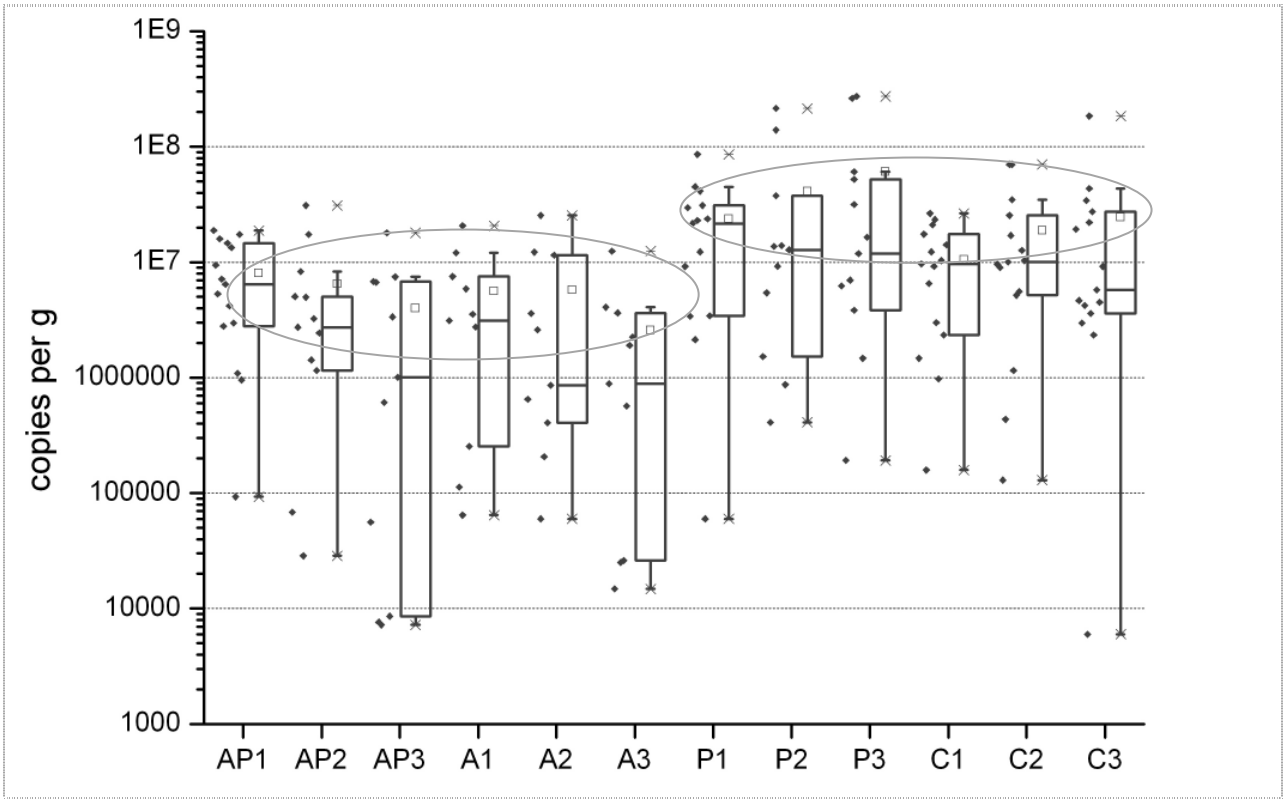


Figure 5 16S rRNA based qPCR of *Clostridium* Cluster IV (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group; 1: day 0, 2: day 5, 3: day 5).

participant	time point 1 [copies per gram]	time point 2 [copies per gram]	time point 3 [copies per gram]
	group AP		
N21	14 612 600	4 969 630	613 064
N28	6 440 980	28 699	7 196
N29	4 193 900	17 388 200	17 906 300
N30	2 790 440	8 275 630	7 598
N31	13 357 400	1 425 460	8 602
N32	7 108 450		6 713 420
N33	2 977 930	2 733 920	3 366 250
N34	15 905 700	31 069 100	6 777 500
N35	92 978	3 238 550	7 460 990
N36	5 306 740	5 024 720	55 948
N37	1 093 580	1 148 260	1 005 270
N38	9 437 490	68 480	
N39	17 372 800	2 440 010	
N40	18 840 100		
N41	956 100		
mean value	8 032 479	6 484 222	3 992 922
standard variance	6 433 719	9 089 455	5 519 512
	group A		
N3	64 436	60 069	24 997
N7	112 426	2 594 580	3 631 970
N9	5 886 010	206 089	26 106
N13	12 026 800	12 212 600	14 820
N14	254 476	408 350	567 910
N17	7 515 130	25 436 900	12 434 000
N42	3 543 680	3 578 410	1 906 280
N43	3 130 780	857 139	889 860
N45	2 746 500	646 928	2 249 890
N47	20 721 800	11 476 100	4 061 260
mean value	5 600 204	5 747 717	2 580 709
standard variance	6 510 851	8 289 383	3 763 872
	group P		
N5	12 317 300	9 260 290	3 841 660
N6	22 997 500		6 990 060
N8	2 135 220	13 672 500	11 865 400
N10		13 909 200	192 202
N11	59 823	410 443	31 676 200
N15	3 410 130	872 053	51 993 900
N19	21 662 500	5 404 650	1 474 490
N20	41 317 200		273 700 000
N22	3 435 320	139 832 000	262 662 000
N23	85 891 300	215 226 000	6 217 880
N26	44 866 800	12 785 400	16 439 400
N27	31 096 500	1 518 250	
N44	29 635 800		
N46	9 160 950		
mean value	23 691 257	41 289 079	60 641 199
standard variance	23 992 927	74 154 048	103 766 272
	group C		
N1	9 230 480	17 048 200	22 021 100
N2	12 264 500	10 099 500	3 582 060
N4	3 007 800	1 149 160	5 991
N12	6 511 190	436 471	2 343 150
N16	973 146	5 160 930	4 203 260
N18	158 718	129 436	5 738 530
N24	21 014 300	70 090 200	43 471 000
N25	10 337 700	70 440 800	184 649 000
N48	17 534 200	34 811 900	34 285 200
N49	2 348 320	5 561 880	2 974 370
N50	26 424 900	8 997 230	4 495 470
N51	9 663 080	12 610 300	27 294 600
N52	14 094 900	9 633 070	4 654 740
N53	23 470 400	25 339 300	9 196 480
N54	1 470 230	10 360 600	19 330 300
mean value	10 566 924	18 791 265	24 549 683
standard variance	8 526 872	22 871 790	46 235 387

Table 8 Results of 16S rRNA based qPCR of *Clostridium* Cluster IV.

7.4. *Clostridium* Cluster XIVa (Lachnospiraceae subgroup)

In Table 9 the results of 16S rRNA based qPCR of *Clostridium* Cluster XIVa of all patient groups and time points are listed, including mean values and standard variations.

As shown in Figure 6, the numbers of copies were spread from about 10^6 to 10^{12}. However, within the groups the values of the three time points did not differ significantly from each other. By contrast, the difference between all groups was statistically significant ($p = 1.21 \times 10^{-6}$). Overall, the values of antibiotic groups were significantly lower than those of the control groups ($p = 1.94 \times 10^{-4}$).

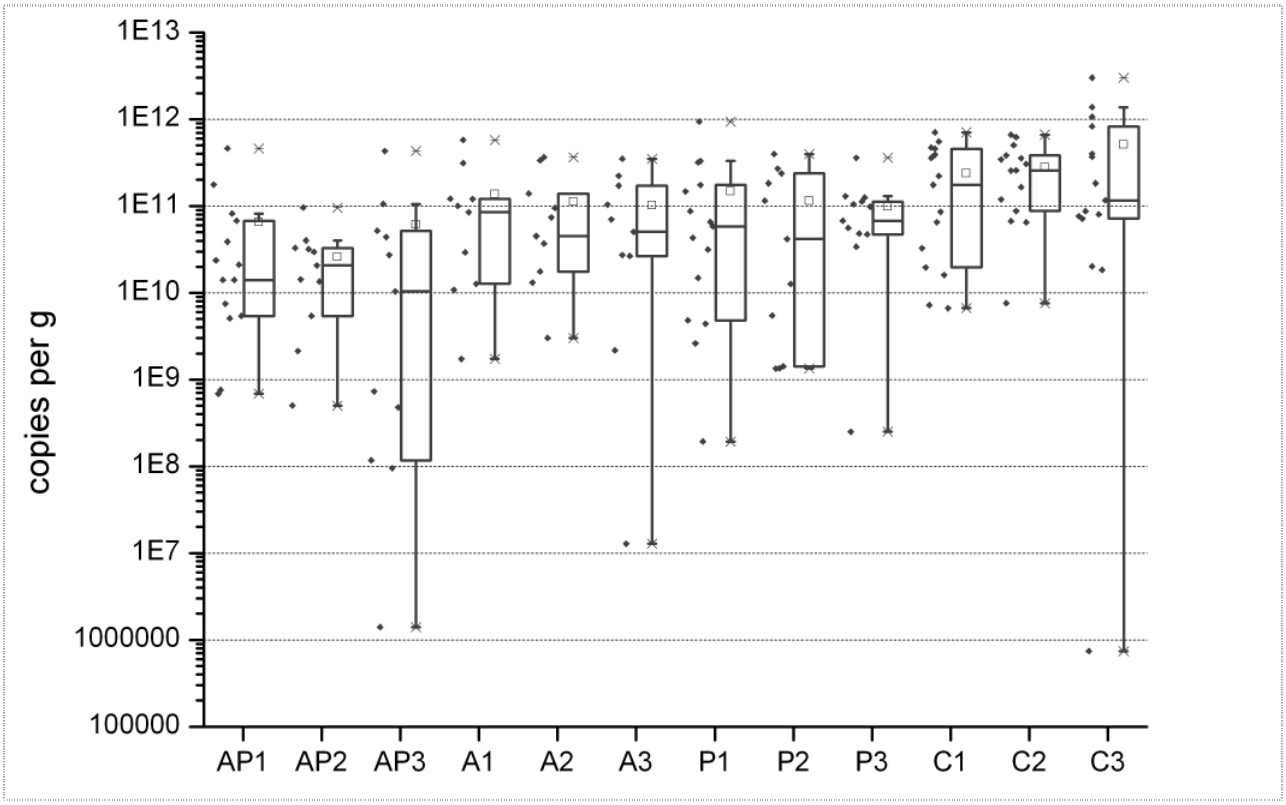


Figure 6 16S rRNA based qPCR of *Clostridium* Cluster XIVa (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group; 1: day 0, 2: day 5, 3: day 5).

participant	time point 1 [copies per gram]	time point 2 [copies per gram]	time point 3 [copies per gram]
	group AP		
N21	38 930 400 000	40 117 100 000	43 965 900 000
N28		96 160 900 000	105 840 000 000
N29	7 480 370 000	31 819 200 000	27 260 400 000
N30	5 085 930 000	14 243 600 000	1 407 180
N31	14 079 900 000	5 417 370 000	95 201 900
N32	81 924 000 000		51 721 300 000
N33	757 482 000	2 137 790 000	10 381 700 000
N34	14 040 500 000	29 645 800 000	729 189 000
N35	686 867 000	32 855 700 000	431 093 000 000
N36	67 493 300 000	--	478 609 000
N37	461 954 000 000	20 675 200 000	117 740 000
N38	23 618 300 000	502 456 000	
N39	21 084 100 000	13 465 100 000	
N40	176 482 000 000		
N41	5 410 460 000		
mean value	65 644 829 214	26 094 565 091	61 062 222 462
standard variance	123 571 944 545	26 800 683 256	127 078 133 905
	group A		
N3	29 244 600 000	36 932 900 000	27 263 300 000
N7	1 727 740 000	17 593 500 000	171 578 000 000
N9	84 779 800 000	3 014 220 000	12 799 300
N13	100 195 000 000	366 014 000 000	2 182 240 000
N14	120 827 000 000	45 083 900 000	26 643 600 000
N17	10 833 000 000	73 864 800 000	70 225 900 000
N42	12 725 700 000	13 072 100 000	50 301 700 000
N43	578 541 000 000	335 896 000 000	350 751 000 000
N45	312 002 000 000	95 316 200 000	222 643 000 000
N47	121 482 000 000	139 339 000 000	104 310 000 000
mean value	137 235 784 000	112 612 662 000	102 591 153 930
standard variance	179 950 472 938	132 421 419 831	113 815 726 468
	group P		
N5	331 148 000 000	1 352 010 000	34 072 000 000
N6	174 199 000 000	1 337 480 000	105 327 000 000
N8	14 818 700 000	269 404 000 000	47 991 000 000
N10	193 191 000		250 356 000
N11	2 613 440 000	1 419 630 000	113 025 000 000
N15	4 402 330 000		55 734 100 000
N19	42 885 500 000	5 473 350 000	125 582 000 000
N20	31 614 200 000	41 670 700 000	360 980 000 000
N22	87 183 000 000	398 233 000 000	130 477 000 000
N23	319 028 000 000	183 447 000 000	47 046 400 000
N26	65 340 600 000	237 973 000 000	67 537 600 000
N27	4 807 940 000	12 596 900 000	
N44	937 967 000 000		
N46	58 309 700 000		
mean value	148 179 328 643	115 290 707 000	98 911 132 364
standard variance	253 064 833 845	145 473 646 204	96 279 604 309
	group C		
N1	65 232 400 000	258 061 000 000	400 923 000 000
N2	174 974 000 000	87 857 200 000	20 142 000 000
N4	85 870 900 000	66 832 100 000	742 709
N12	392 122 000 000	256 560 000 000	183 409 000 000
N16	7 216 620 000	165 646 000 000	87 447 500 000
N18	16 079 500 000	7 591 970 000	80 375 600 000
N24	19 652 800 000	64 933 800 000	71 630 300 000
N25	6 660 430 000	119 281 000 000	18 325 000 000
N48	458 143 000 000	623 389 000 000	826 433 000 000
N49	472 872 000 000	663 318 000 000	3 009 220 000 000
N50	707 562 000 000	355 644 000 000	370 131 000 000
N51	554 903 000 000	383 101 000 000	1 066 210 000 000
N52	357 965 000 000	304 771 000 000	76 286 200 000
N53	222 136 000 000	345 713 000 000	116 634 000 000
N54	32 838 100 000	503 393 000 000	1 377 270 000 000
mean value	238 281 850 000	280 406 138 000	513 629 156 181
standard variance	233 864 766 916	203 327 934 696	810 010 014 504

Table 9 Results of 16S rRNA based qPCR of *Clostridium* Cluster XIVa.

7.5. *Clostridium* Cluster XI

In Table 10 the results of 16S rRNA based qPCR of *Clostridium* Cluster XI of all patient groups and time points are listed in form of four ranges: < 250 000, 250 000-10^6, 10^6-10^7 and >10^7 copies per g. For the graphical view (Figure 7) values < 250 000 copies per g were set to 100 000 copies per g.

After commencement of the antibiotic treatment, the abundance of Clostridium clusters XI decreased, whereas in the control groups values remained constant.

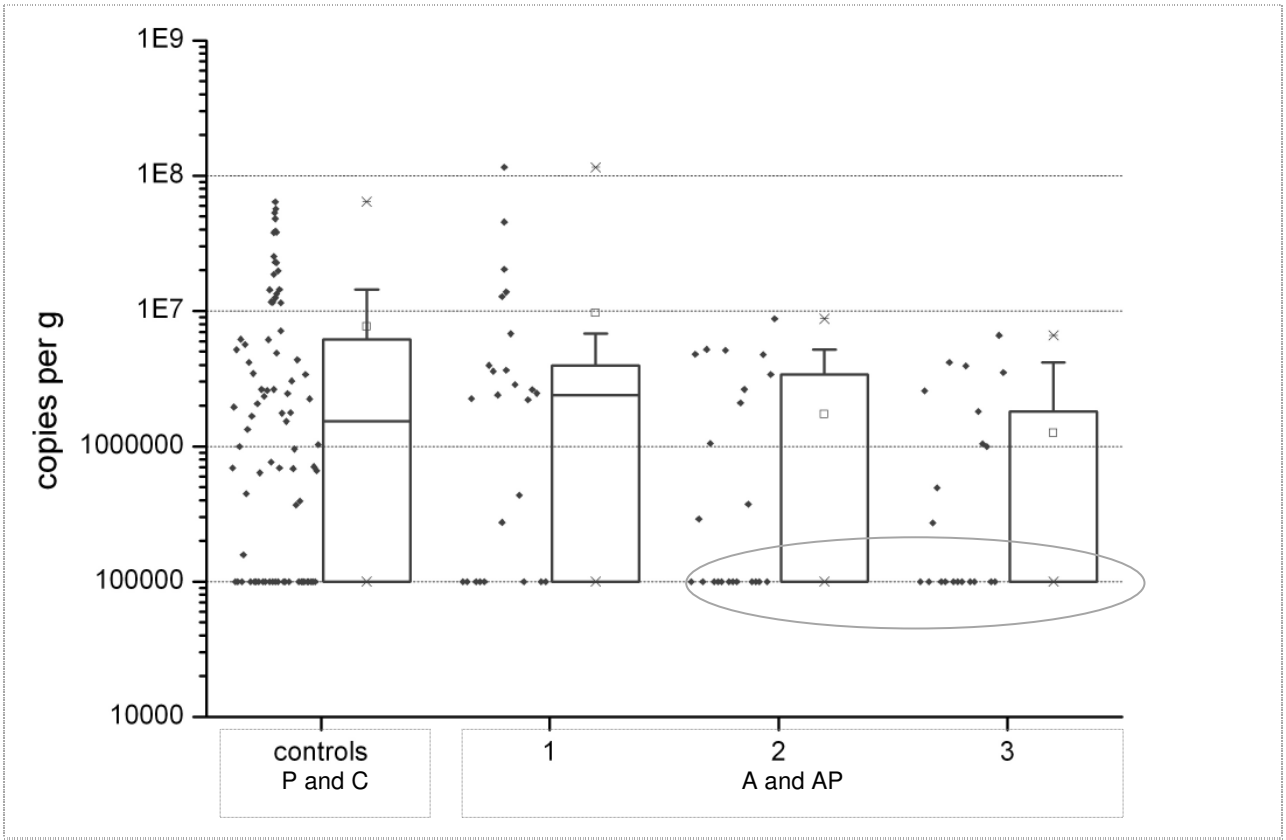


Figure 7 Absolute quantification of *Clostridium* Cluster XI by 16S rRNA based qPCR (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group; 1: day 0, 2: day 5, 3: day 5).

participant	time point 1 [copies per gram]	time point 2 [copies per gram]	time point 3 [copies per gram]
	group AP		
N21	10^6 - 10^7	< 250 000	< 250 000
N28	250 000 - 10^6	< 250 000	< 250 000
N29	10^6 - 10^7	< 250 000	
N30	10^6 - 10^7	10^6 - 10^7	10^6 - 10^7
N31	10^6 - 10^7	10^6 - 10^7	< 250 000
N32	10^6 - 10^7	< 250 000	< 250 000
N33	250 000 - 10^6		
N34	10^6 - 10^7	10^6 - 10^7	10^6 - 10^7
N35	< 250 000	< 250 000	< 250 000
N36	< 250 000	250 000 - 10^6	< 250 000
N37	10^6 - 10^7	< 250 000	10^6 - 10^7
N38	< 250 000	< 250 000	< 250 000
N39	> 10^7	10^6 - 10^7	10^6 - 10^7
N40	> 10^7	< 250 000	
N41	10^6 - 10^7	10^6 - 10^7	
	group A		
N3	> 10^7	10^6 - 10^7	250 000 - 10^6
N7	< 250 000	< 250 000	250 000 - 10^6
N9	10^6 - 10^7	< 250 000	250 000 - 10^6
N13	> 10^7	10^6 - 10^7	< 250 000
N14	10^6 - 10^7	250 000 - 10^6	< 250 000
N17	< 250 000	10^6 - 10^7	< 250 000
N42	> 10^7	< 250 000	10^6 - 10^7
N43	< 250 000	10^6 - 10^7	10^6 - 10^7
N45	< 250 000	< 250 000	< 250 000
N47	< 250 000	10^6 - 10^7	10^6 - 10^7
	group P		
N5	< 250 000	< 250 000	< 250 000
N6	10^6 - 10^7	< 250 000	> 10^7
N8	10^6 - 10^7	> 10^7	
N10	< 250 000		
N11	< 250 000	> 10^7	> 10^7
N15	250 000 - 10^6	< 250 000	250 000 - 10^6
N19	> 10^7	10^6 - 10^7	> 10^7
N20	250 000 - 10^6	10^6 - 10^7	10^6 - 10^7
N22	< 250 000	< 250 000	< 250 000
N23	> 10^7	> 10^7	> 10^7
N26	10^6 - 10^7	> 10^7	10^6 - 10^7
N27	10^6 - 10^7		
N44	10^6 - 10^7	10^6 - 10^7	10^6 - 10^7
N46	10^6 - 10^7	10^6 - 10^7	> 10^7
	group C		
N1	> 10^7	> 10^7	> 10^7
N2	250 000 - 10^6	< 250 000	< 250 000
N4	< 250 000	< 250 000	< 250 000
N12	250 000 - 10^6	250 000 - 10^6	< 250 000
N16	< 250 000	< 250 000	< 250 000
N18	> 10^7	10^6 - 10^7	10^6 - 10^7
N24	250 000 - 10^6	10^6 - 10^7	250 000 - 10^6
N25	10^6 - 10^7	> 10^7	> 10^7
N48	10^6 - 10^7	> 10^7	> 10^7
N49	10^6 - 10^7	< 250 000	< 250 000
N50	< 250 000	< 250 000	< 250 000
N51	< 250 000	< 250 000	10^6 - 10^7
N52	250 000 - 10^6	< 250 000	250 000 - 10^6
N53	10^6 - 10^7	10^6 - 10^7	250 000 - 10^6
N54	< 250 000	10^6 - 10^7	10^6 - 10^7

Table 10 Results of 16S rRNA based qPCR of *Clostridium* Cluster XI.

7.5.1. *Clostridium difficile*

Table 11 lists the number of patients in whose samples *C.difficile* was detected by a 16S rRNA based *C.difficile* specific qPCR or by a positive ELISA test at the General Hospital Oberpullendorf.

Group A patients no. 7, 9, 13, 14 and 17, group P patient no. 8 and group C patient no. 2 had a positive result in *C.difficile* ELISA testing (TOX A/B QUICK CHEK®, Wampole®) at the beginning of the therapy. In group AP in patient no. 40 at time point 1, in group A in patient no. 42 at time point 2 and 3 and in group P in patient no. 32 at time point 2, no. 8 at time point 3 and no. 3 at all time points, *C.difficile* was detected by 16S rRNA based *C.difficile* specific qPCR. So there was no patient who was positive for both of the two checks on *C.difficile*.

	***C.difficile* ELISA**	**time point 1**	**time point 2**	**time point 3**
AP (n = 15)	-	1	-	-
A (n = 10)	5	1	2	2
P (n = 15)	1	-	1	1
C (n = 15)	1	-	-	-

Table 11 Number of patients in whose samples *C.difficile* was detected by 16S rRNA based *C.difficile* specific qPCR and positive ELISA test (TOX A/B QUICK CHEK®, Wampole®) performed at the beginning of the therapy in the hospital Oberpullendorf (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group).

7.6. *Lactobacillus* spp.

In Table 12 the results of 16S rRNA based qPCR of *Lactobacillus* spp. of all patient groups and time points are listed, including mean values and standard variations.

Within group P the values of the three time points differed significantly from each other ($p = 0.01$) and there was a significant increase of number of copies 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 was significantly higher in group P than in group C ($p = 0.04$) (Figure 8).

Overall, group A and AP were significantly different from group C (A: $p = 0.01$ and AP $p = 0{,}002$). Between group A and AP, no statistically detectable varieties could be found.

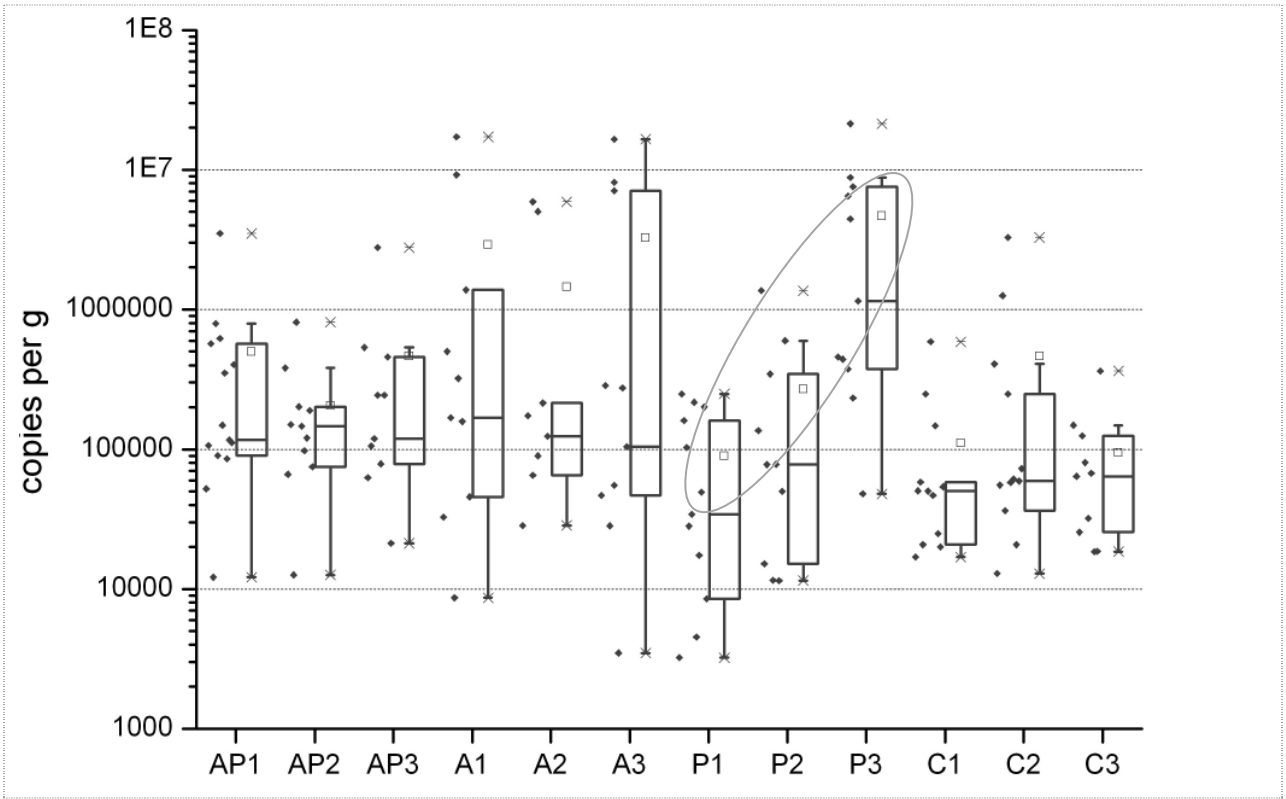


Figure 8 16S rRNA based qPCR of *Lactobacillus* spp. (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group; 1: day 0, 2: day 5, 3: day 5).

participant	time point 1 [copies per gram]	time point 2 [copies per gram]	time point 3 [copies per gram]
	group AP		
N21	621 710	201 484	
N28	90 524	811 348	243 529
N29	148 947	146 326	119 179
N30	792 152	12 629	78 759
N31	351 392	97 356	105 557
N32	12 102	150 417	243 132
N33	85 385	120 660	2 773 510
N34	568 540		62 620
N35	116 415	66 081	459 595
N36	106 737	190 208	534 658
N37	111 675	382 826	21 241
N38	52 144		
N39	3 509 920	74 791	
N41	402 942		
mean value	497 899	204 921	464 178
standard variance	900 852	223 135	829 268
	group A		
N3	321 352	89 682	55 048
N7	8 638	64 983	28 320
N9	157 722		3 484
N13	167 927	213 948	285 405
N14	1 382 060	5 036 060	7 096 270
N17	17 206 200		8 176 170
N42	500 636	173 546	275 604
N43	45 651	123 649	46 715
N45	9 202 930	5 907 940	16 557 100
N47	32 774	28 539	104 034
mean value	2 902 589	1 454 793	3 262 815
standard variance	5 762 111	2 491 085	5 627 897
	group P		
N5			7 568 820
N6	217 338	11 518	233 314
N8	34 294	346 357	375 253
N10	4 521	78 147	1 149 210
N11	28 125	77 721	6 500 000
N15	17 431	11 455	441 406
N19		15 099	47 981
N20	103 067	49 953	8 826 820
N22	49 205	1 367 170	21 393 200
N23	160 461	597 735	457 334
N26	200 273	136 462	4 457 280
N27	249 125		
N44	8 491		
N46	3 226		
mean value	89 630	269 162	4 677 329
standard variance	92 526	428 911	6 451 797
	group C		
N1	588 039	249 209	
N2	50 129	36 333	31 962
N4	46 775	57 856	
N12	249 487	1 258 540	80 263
N18	147 343	3 279 710	
N24		61 366	67 349
N48	20 757	55 510	124 888
N49	24 884		18 439
N50	58 125	20 751	25 548
N51	19 999	12 919	18 570
N52	50 355	59 395	64 179
N53	53 958	407 882	364 075
N54	16 913	72 655	148 591
mean value	110 564	464 344	94 386
standard variance	164 680	953 578	104 666

Table 12 Results of 16S rRNA based qPCR of *Lactobacillus* spp.

7.6.1. *Lactobacillus casei*

In Table 13 the results of 16S rRNA based qPCR of *L.casei* of all patient groups and time points are listed, including mean values and standard variations.

In group AP and P there was a rising abundance of number of copies. However, this increase was within the natural range of variation, which was also present in group A and C (Figure 9).

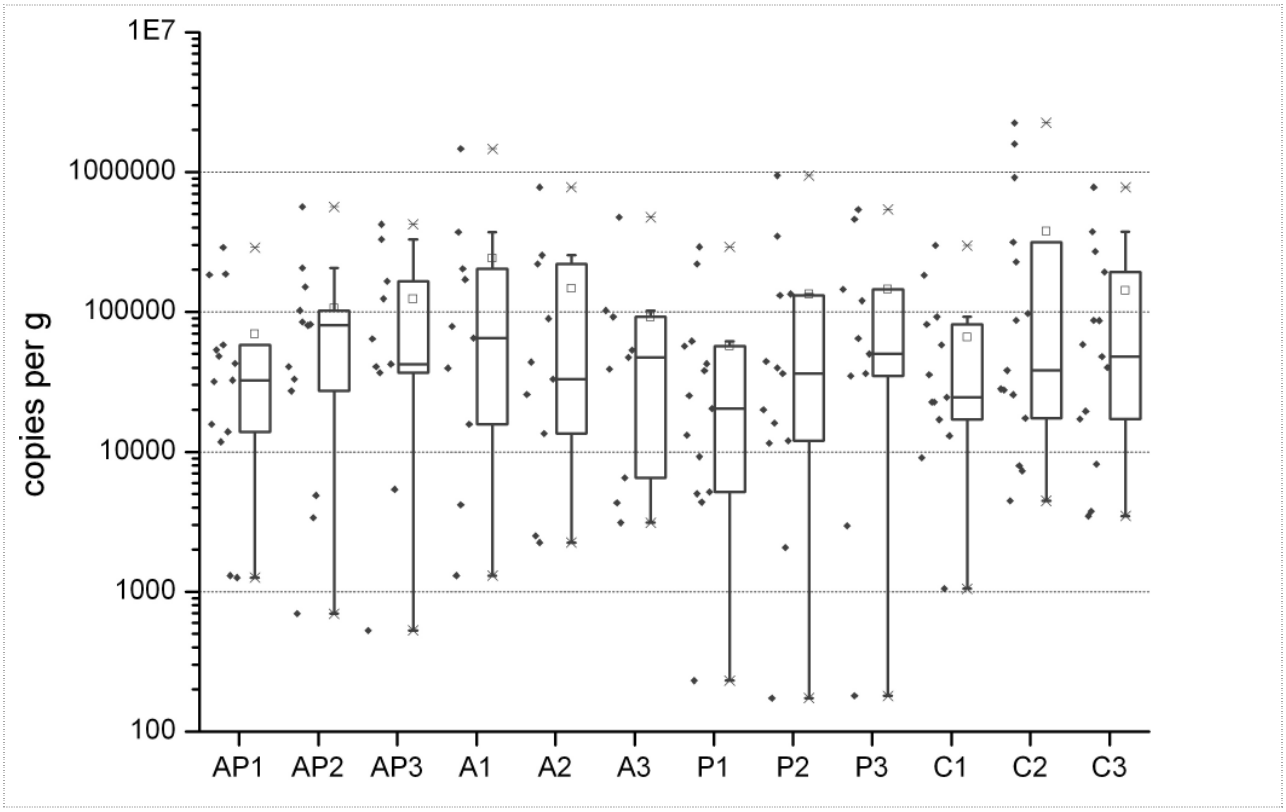


Figure 9 16S rRNA based qPCR of *L.casei* (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group; 1: day 0, 2: day 5, 3: day 5).

participant	time point 1 [copies per gram]	time point 2 [copies per gram]	time point 3 [copies per gram]
	group AP		
N21	58 241	84 738	124 530
N28	288 359	102 525	36 818
N29	11 770	150 748	166 103
N30	186 662	695	40 511
N31	48 106	80 175	424 222
N32	13 886	565 454	
N33	53 556	33 133	42 356
N34		206 282	64 447
N35	1 300	81 729	331 013
N36	31 874	27 261	5 395
N37	32 536	3 376	527
N38	15 763	40 649	
N39	42 932	4 870	
N40	184 964		
N41	1 260		
mean value	69 372	106 280	123 592
standard variance	86 782	150 694	144 858
	group A		
N3	4 174	2 249	3 113
N7	1 307	2 515	4 330
N9	170 495	13 531	6 498
N13	78 793	254 187	475 915
N14	15 699	43 910	92 149
N17	39 710	89 929	47 473
N42	203 426	25 736	38 988
N43	65 143	32 970	53 190
N45	1 463 940	780 429	--
N47	372 921	220 373	102 412
mean value	241 561	146 583	91 563
standard variance	444 899	240 217	148 616
	group P		
N5	292 678	948 722	539 966
N6	9 226	39 823	459 899
N8	5 005	16 105	64 748
N10	4 363	131 651	180
N11	231	173	
N15		36 318	120 378
N19	37 994	11 553	34 932
N20	61 934	2 064	
N22	42 769	349 257	36 297
N23	220 561	44 250	2 965
N26	25 136	11 979	50 128
N27	5 163	19 978	
N44	13 186		
N46	20 456		
mean value	56 823	134 323	145 499
standard variance	91 661	274 471	205 025
	group C		
N1	92 262	87 122	87 367
N2	22 708	25 630	3 763
N4	17 031	7 937	
N12	22 657	4 454	8 173
N16	58 112	7 321	3 487
N18	35 550	38 240	86 781
N24	1 051	17 390	19 532
N25	81 444	227 310	270 305
N48	24 446	27 692	47 945
N49	183 020	316 689	374 148
N50		915 997	58 687
N51	13 048	97 652	192 311
N52		1 597 470	17 194
N53	298 757	2 244 750	778 758
N54	9 077	28 147	40 098
mean value	66 089	376 253	142.039
standard variance	85 599	680 313	214.356

Table 13 Results of 16S rRNA based qPCR of *L.casei*.

7.7. *Bacteroides* spp.

In Table 14 the results of 16S rRNA based qPCR of *Bacteroides* of all patient groups and time points are listed, including mean values and standard variations. In Figure 10 these findings are illustrated.

Neither within the groups, nor between the groups could be observed significant differences.

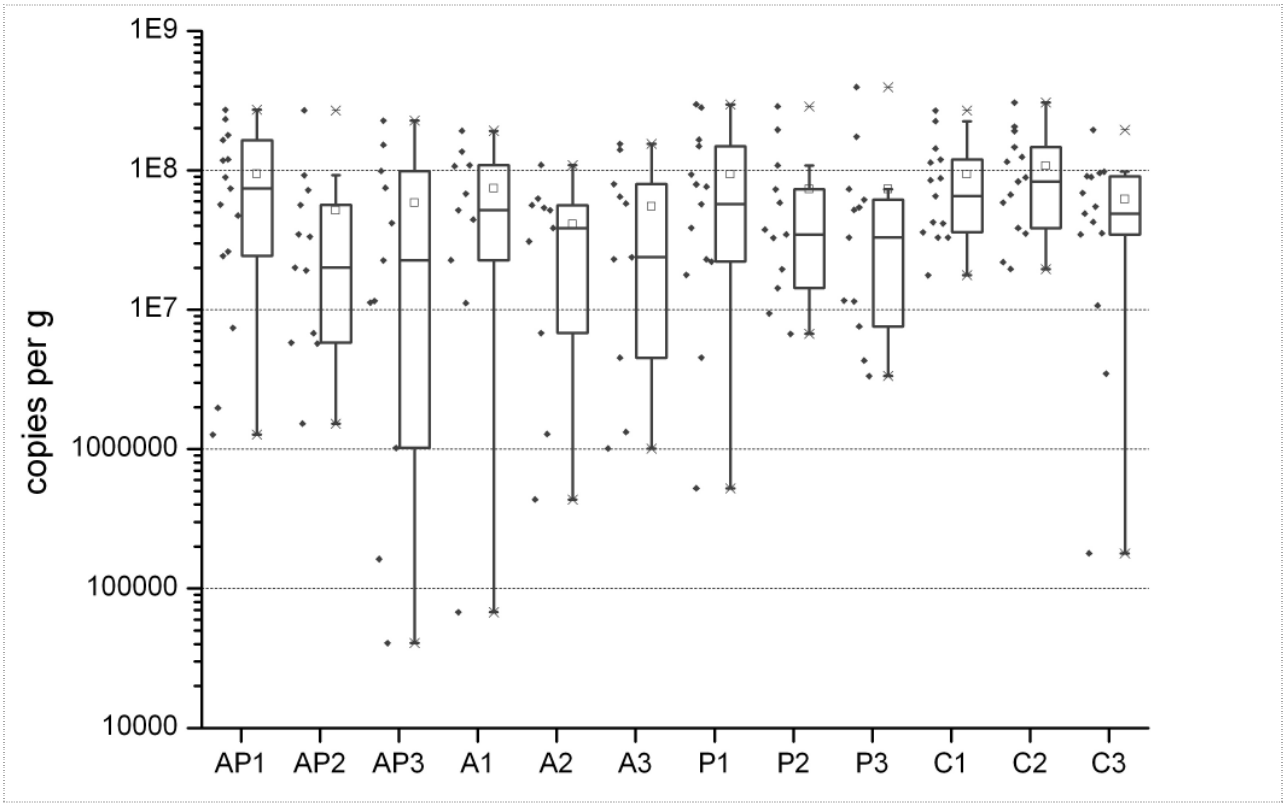


Figure 10 16S rRNA based qPCR of *Bacteroides* spp. (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group; 1: day 0, 2: day 5, 3: day 5).

participant	time point 1 [copies per gram]	time point 2 [copies per gram]	time point 3 [copies per gram]
	group AP		
N21	89 251 237	19 131 327	22 628 554
N28	232 722 741	1 527 448	152 394 473
N29	26 191 503	33 448 623	
N30	56 941 377	92 268 736	227 466 971
N31	24 376 017	34 786 334	162 982
N32	271 705 271		40 612
N33	7 420 423		
N34	119 863 852	6 778 510	74 939 063
N35	1 976 249	56 601 473	11 590 407
N36	47 390 812	20 034 203	41 903 010
N37	117 802 721	72 253 643	98 787 875
N38	179 506 751	269 711 396	11 262 578
N39	74 271 746	5 726 199	1 019 914
N40	165 111 156	5 787 477	
N41	1 269 515		
mean value	94 386 758	51 504 614	58 381 495
standard variance	85 519 826	74 504 552	74 416 385
	group A		
N3	11 176 568	6 803 353	4 523 837
N7	67 533	434 041	23 060 948
N9	68 027 786	1 281 642	1 327 690
N13	51 794 161	109 280 209	1 009 749
N14	44 361 483	54 008 827	64 720 832
N17	22 698 570	30 973 440	23 852 521
N42	136 577 333	62 882 834	140 610 342
N43	107 019 582	38 584 710	79 602 013
N45	108 956 386	51 760 593	154 899 080
N47	192 393 377	56 249 488	57 869 994
mean value	74 307 278	41 225 914	55 147 701
standard variance	61 188 280	33 594 019	56 125 360
	group P		
N5	57 262 495	58 682 529	7 580 168
N6	79 500 584		
N8	4 545 146	14 289 078	54 292 696
N10			
N11	524 572	32 711 164	11 512 059
N15	22 965 422	19 567 412	4 321 272
N19	149 163 770	9 421 356	32 998 973
N20	38 663 284	34 679 688	52 067 559
N22	282 013 247	37 513 257	174 614 124
N23	297 518 372	195 884 402	395 696 259
N26	76 418 526	287 747 877	61 629 160
N27	22 177 433		
N44	166 201 804	108 406 416	3 347 077
N46	17 829 962	6 702 769	11 671 376
mean value	93 444 971	73 236 904	73 611 884
standard variance	101 117 950	90 424 410	117 627 256
	group C		
N1	32 923 776	38 516 723	55 026 209
N2	268 122 539	306 195 620	42 597 615
N4	65 470 822	83 035 714	178 893
N12	143 705 707	19 630 593	89 469 616
N16	42 475 951	66 935 521	10 735 886
N18	113 533 050	88 823 597	95 580 963
N24	41 664 910	147 090 028	194 803 043
N25	17 667 502	35 206 299	48 910 169
N48	32 951 615	21 960 917	35 339 840
N49	84 931 854	58 837 651	90 543 548
N50	224 662 482	115 343 932	34 644 766
N51	35 898 815	124 713 076	97 995 471
N52	119 449 111	205 849 706	3 482 616
N53	87 581 564	192 068 394	69 102 106
N54	22 961 247	76 686 594	153 250 231
mean value	88 933 396	105 392 958	68 110 732
standard variance	74 901 499	79 774 833	54 363 782

Table 14 Results of 16S rRNA based qPCR of *Bacteroides* spp..

7.8. *Bifidobacterium* spp.

In Table 15 the results of 16S rRNA based qPCR of *Bifidobacterium* spp. of all patient groups and time points are listed, including mean values and standard variations.

Figure 11 shows that the mean number of copies per g of both antibiotic treated groups (AP and A) were lower than those of the control groups (P and C) ($p = 0{,}051$). On average, the numbers of copies fell in group AP and A, but in group AP the decrease stopped at time point 2 and raised again slightly.

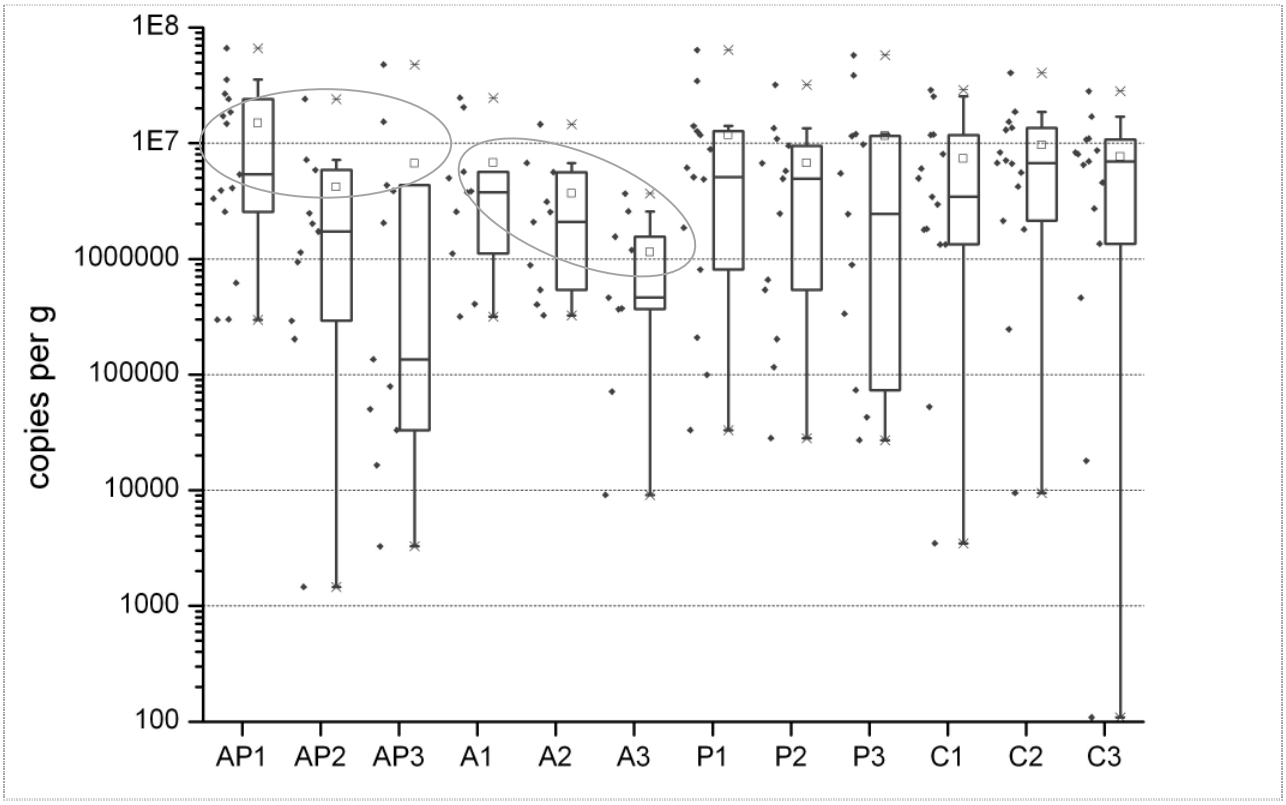


Figure 11 16S rRNA based qPCR of *Bifidobacterium* spp. (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group; 1: day 0, 2: day 5, 3: day 5).

participant	time point 1 [copies per gram]	time point 2 [copies per gram]	time point 3 [copies per gram]
	group AP		
N21	24 117 279	7 193 838	2 043 239
N28	300 184	1 461	3 266
N29	14 763 289	24 085 388	
N30	2 550 763	2 485 486	4 345 478
N31	4 112 969	1 140 196	16 477
N32	3 920 843		79 014
N33	617 614		
N34	35 567 192	2 014 524	47 825 930
N35	297 369	165 133 257	15 267 315
N36	5 371 472	939 648	135 548
N37	3 328 445	5 868 997	3 867 789
N38	17 154 095	202 592	50 291
N39	18 610 141	1 727 399	33 017
N40	66 289 804	292 168	
N41	26 776 948		
mean value	14 918 560	17 590 413	6 697 033
standard variance	18 042 414	46 940 729	14 370 470
	group A		
N3	5 665 067	539 272	368 474
N7	316 886	403 148	1 556 019
N9	3 766 955	324 750	373 699
N13	2 553 275	2 085 775	71 296
N14	3 857 586	3 112 168	3 669 240
N17	1 114 320	883 458	463 307
N42	20 472 479	14 564 824	2 578 603
N43	408 590	2 544 416	9 093
N45	24 747 223	6 733 904	50 581 614
N47	5 001 907	5 604 558	1 196 379
mean value	6 790 429	3 679 627	6 086 772
standard variance	8 593 664	4 413 911	15 678 974
	group P		
N5	208 223	202 586	73 142
N6	5 100 195	116 024	
N8	808 171	2 460 392	889 916
N10	33 034		
N11		28 186	27 036
N15	12 736 949	4 942 335	2 440 747
N19	4 875 066	658 613	9 761 588
N20	6 122 822	10 926 845	335 802
N22	14 053 624	5 750 985	12 051 224
N23	63 951 084	32 070 926	57 626 141
N26	99 637	540 644	42 804
N27	1 853 779		
N44	8 890 846	9 519 379	5 475 804
N46	34 546 909	13 512 513	38 587 336
mean value	11 790 795	6 727 452	11 573 776
standard variance	18 271 393	9 255 852	19 009 614
	group C		
N1	3 449 588	6 668 043	6 965 380
N2	52 704	246 920	18 043
N4	3 474	9 470	109
N12	25 414 798	13 602 756	6 496 129
N16	1 807 544	7 107 051	10 992 637
N18	2 955 519	4 229 162	2 718 342
N24	1 779 460	2 132 781	462 080
N25	1 329 330	5 563 158	8 658 291
N48	28 896 066	40 608 331	28 212 206
N49	6 020 844	8 332 934	8 038 215
N50	11 893 121	1 796 429	1 351 354
N51	8 065 795	15 306 765	8 328 612
N52	11 807 046	18 710 738	10 778 297
N53	4 973 910	6 731 735	4 569 133
N54	1 336 356	13 008 973	16 970 503
mean value	7 319 037	9 603 683	7 637 289
standard variance	8 923 862	10 249 151	7 444 945

Table 15 16S rRNA based qPCR of *Bifidobacterium* spp..

7.9. Enterobacteriaceae

In Table 16 the results of 16S rRNA based qPCR of Enterobacteriaceae of all patient groups and time points are listed, including mean values and standard variations.

Figure 12 points out, that the number of copies in the antibiotic treated groups (AP and A) were higher by a power than those of both groups without antibiotic treatment (P and C) ($p = 0.06$). The values of group P were significantly higher than those of group C ($p = 1.7 \times 10^{-4}$). Between group AP and A there was no statistically significant difference.

Furthermore, it was apparent that the individual values were widely spread. There was a statistically significant difference between all four groups ($p = 6.9 \times 10^{-5}$) but within the groups there were no statistically differences between the three time points.

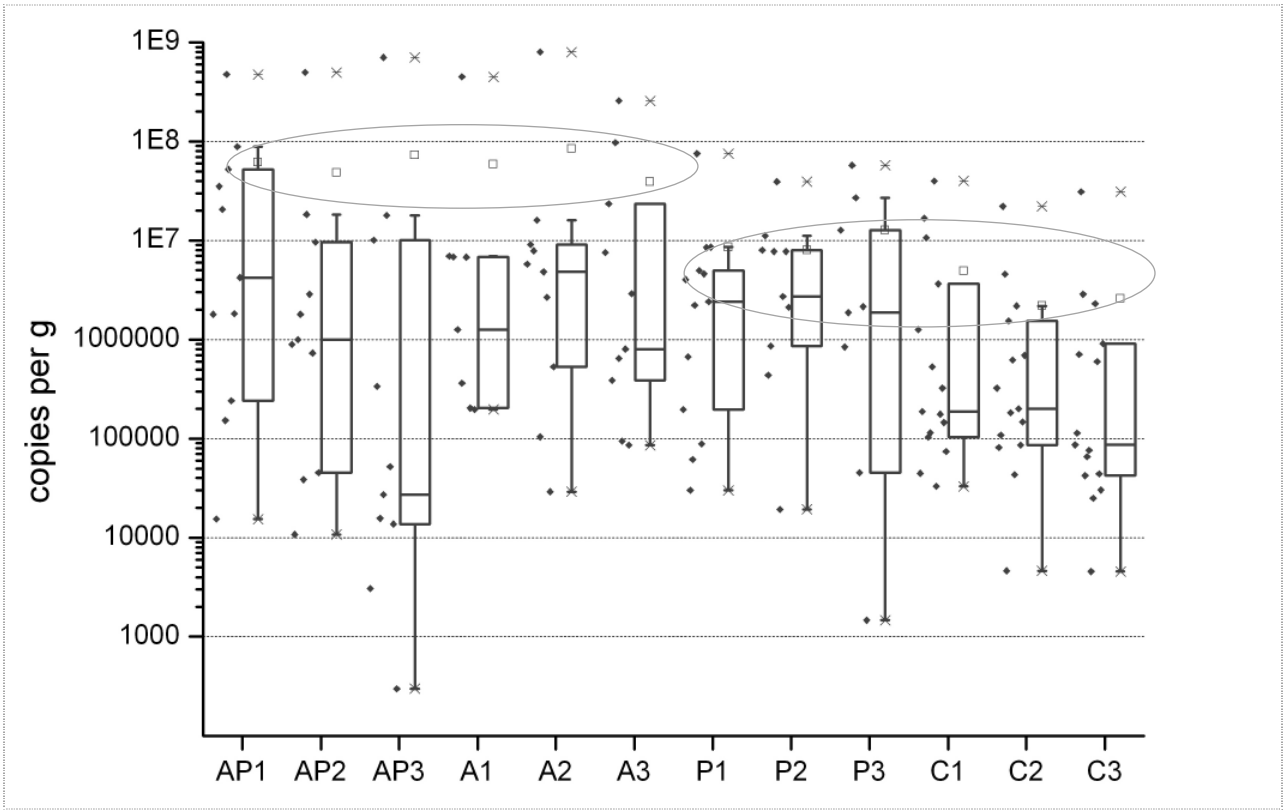


Figure 12 16S rRNA based qPCR of Enterobacteriaceae (A: antibiotic treatment, AP: antibiotic treatment and intake *L.casei* Shirota, P: intake of *L.casei* Shirota, C: control group; 1: day 0, 2: day 5, 3: day 5).

participant	time point 1 [copies per gram]	time point 2 [copies per gram]	time point 3 [copies per gram]
	group AP		
N21	52 188 000	18 294 200	27 249
N28	152 760	38 562	15 710
N29	472 852 000	2 860 840	17 963 500
N30	241 292	1 796 070	336 336
N31	20 626 600	729 125	52 395
N33	1 825 990	997 234	703 422 000
N34		495 436 000	10 095 500
N35	35 133 200	9 608 970	13 764
N36	88 721 200	10 798	3 076
N37	15 478	45 501	299
N39	4 236 050	895 319	
N41	1 805 950		
mean value	61 618 047	48 246 602	73 192 983
standard variance	139 321 158	148 422 539	221 523 790
	group A		
N3	362 907	104 036	644 370
N7		16 115 000	97 151 100
N9	448 694 000	4 821 360	94 540
N13	1 261 080	7 877 010	386 353
N14		799 100 000	257 071 000
N17	6 792 060	2 674 830	801 550
N42	6 846 970	9 090 520	23 426 900
N43	203 852	29 088	85 752
N45	6 974 090	5 766 610	7 545 460
N47	197 529	531 577	2 904 060
mean value	58 916 561	84 611 003	39 011 108
standard variance	157 525 721	251 095 139	82 273 649
	group P		
N5	75 173 600	39 232 500	26 942 000
N6	2 217 090		57 343 100
N8	4 959 930	7 718 430	
N11	61 314	19 349	
N15	88 254	861 916	45 253
N20	29 988	2 721 490	1 873 300
N22	4 621 290	439 825	2 160 090
N23	672 842	7 730 990	839 650
N26	8 492 850	11 148 900	1 468
N27	4 026 270	2 125 530	
N44	2 412 290		
N46	197 057		
mean value	8 579 398	7 999 881	12 743 551
standard variance	21 134 488	12 347 609	21 932 465
	group C		
N1	531 182	182 654	76 128
N2	114 796	1 535 360	65 853
N4	39 876 800	623 543	4 548
N12	103 111	4 646	42 243
N16	33 021	43 493	24 991
N18	10 690 700	4 587 650	2 862 030
N24	3 636 210	2 188 640	2 312 340
N25	16 907 300	22 116 200	31 083 600
N48	176 912	200 694	598 252
N49	188 131	108 659	707 844
N50	323 753	85 718	44 079
N51	44 567	81 560	113 138
N52	145 075	147 209	30 236
N53	1 260 190	323 095	86 389
N54	74 264	694 726	908 740
mean value	4 940 401	2 194 923	2 597 361
standard variance	10 821 641	5 645 187	7 929 396

Table 16 Results of 16S rRNA based qPCR of Enterobacteriaceae.

7.9.1. *Salmonella* spp.

In the Table 17 those patients are listed, in which stool samples *Salmonella* was detected by 16S rRNA based *Salmonella* spp. specific qPCR. The groups did not differ significantly from each other.

participant	1 (day 0)	2 (day 3)	3 (day 5)
group P			
N5			
N6	✓	✓	
N8		✓	✓
N10			✓
N11			
N15			✓
N19	✓		
N20			
N22		✓	
N23			
N26	✓	✓	✓
N27	✓		
N44			
N46			
group C			
N1			
N2		✓	
N4			
N12			
N16	✓	✓	✓
N18		✓	
N24	✓		✓
N25	✓	✓	✓
N48			
N49			
N50			
N51			
N52			
N53			
N54		✓	✓

participant	1 (day 0)	2 (day 3)	3 (day 5)
group AP			
N21	✓		✓
N28		✓	✓
N29			
N30		✓	
N31		✓	
N32		✓	
N33			✓
N34	✓	✓	✓
N35	✓	✓	✓
N36			
N37			
N38	✓	✓	✓
N39	✓		
N40	✓	✓	
N41	✓		
group A			
N3	✓	✓	✓
N7		✓	✓
N9			
N13			
N14		✓	
N17			
N42	✓		✓
N43			
N45			
N47			

Table 17 Results of 16S based rRNA qPCR of *Salmonella* spp..

8. DISCUSSION

8.1. Methods

8.1.1. Fecal samples

Using stool samples for analysis of changes in gastrointestinal microbiota has been controversially discussed. On the one hand, studies suggested that fecal samples do not necessarily represent the bacterial community in other parts of the gastrointestinal tract (Zoetendal et al., 2006), on the other hand it is referred that the composition of the bacterial microbiota in the feces was similar to that at the mucus layer of the terminal ileum and colon regions (van der Waaij et al., 2005). However, for this study fecal samples were used to investigate the quantitative changes of total Bacteria and bacterial subgroups, because they are easily collected and do not involve any ethical issues (Turroni et al., 2008).

8.1.2. Quantification by real time PCR (qPCR)

qPCR is a well established and reliable method to detect quantitative differences within bacterial subgroups of fecal samples by 16S rRNA coding regions. The 16S rRNA gene can be found in all microorganisms. It has enough sequence conservation for accurate alignment and enough variation for phylogenetic analyses (Turnbaugh et al., 2007). Therefore, this method has been chosen to investigate the impact *of L.casei* Shirota on antibiotic disturbed microbiota.

8.2. Results

Antibiotics changed the composition of the gastrointestinal microbiota just as described in recent studies (De La Cochetiere et al., 2005, Dethlefsen and Relman, 2011, Jakobsson et al., 2010, Sommer and Dantas, 2011). The results have shown that antibiotic intake causes group specific shifts and in general a reduction of bacterial abundance. Disease on its own as well, affected the microbiota.

There are indications that the additional intake of *L.casei* Shirota implicates positive characteristics, which are discussed in the following.

8.2.1. Total Bacteria

The quantitative analysis by 16S rRNA based qPCR revealed, that the total amount of Bacteria decreases under antibiotic treatment, either with or without probiotic intake. Total bacteria are reduced by disease itself, but antibiotic administration decreases the abundance additionally.

Considering that antibiotics have the task of inactivating or killing harmful bacteria, accordingly beneficial bacteria are affected by this action as well. Well, a probiotic drink containing *L.casei* Shirota cannot offset this effect, but further qualitative analysis using PCR/DGGE indicated, that diversity remains slightly higher, when a combined therapy of antibiotics and *L.casei* Shirota is being performed (Pirker et al., 2012).

8.2.2. Bacterial subgroups

During antibiotic administration the abundances of the bacterial subgroups *Bifidobacteria* spp., *Clostridium* Cluster IV, XIVa and XI are being reduced and Enterobacteriaceae are increasing.

8.2.2.1. Clostridiales

The abundance of the investigated subgroups *Clostridium* Cluster IV, XIVa and XI is being reduced in the patient groups of antibiotic treatment.

The percentage in relation to the analyzed bacteria of *Clostridium* Cluster IV and XIVa is lower in participants with disease than in healthy controls at all time points. The same result applies to absolute values. In *Clostridium* cluster IV the number of copies is even lower by a half-power in patients under antibiotic treatment. As already mentioned above, *Clostridium* cluster IV and XIVa comprise up to 60 % of colonic microbiota (Louis et al., 2007) and contain the main butyrate producing bacteria (Pryde et al., 2002). With the decline of *Clostridium* Cluster IV and XIVa also important SCFAs producing groups are being decreased.

The abundance of *Clostridium* cluster XI is being lowered whilst a disease and continues to decrease during antibiotic treatment. This heterogeneous phylogenetic cluster includes opportunistic pathogens like *C.difficile* (Nadal et al., 2009). The

C.difficile specific primers used for this diploma thesis also very probably amplify non-toxin-producing *C.difficile*. The results obtained therefore cannot be used to prove the occurrence of *C.difficile* toxins. Also the ELISA test (TOX A/B QUICK CHEK®, Wampole®) has to be considered critically. Problems and different outcomes in the detection of *C.difficile* using different test systems are discussed broadly (Alcala et al., 2008, Eastwood et al., 2009, Luna et al., 2011).

However, results of a clinical study suggest, that the consumption of *L.casei* Shirota, prevents the development of CDI and reduces the development of AAD efficiently (Stockenhuber et al., 2008). The results of this thesis cannot demonstrate this positive effect of *L.casei* Shirota on its own.

8.2.2.2. *Lactobacillus* spp.

Results clearly demonstrate, that the intake of a probiotic drink containing *L.casei* Shirota, increases *Lactobacillus* spp. in the human gut microbiota of healthy individuals rapidly. In patients, receiving antibiotic therapy and *L.casei* Shirota, there is no increase statistically detectable.

Contrary to the expectations, the quantification using a 16S rRNA specific *L.casei* primer pair cannot detect a significant increase of the *L.casei* content. In patients receiving the probiotic drink containing *L.casei* Shirota, as well as in patients receiving the combined antibiotic/*L.casei* Shirota therapy, an increase in the abundance of *L.casei* can be observed. But the values have not increased compared to controls.

Since the results, using the universal *Lactobacillus* spp. primer pair, have proven for an evident increase of the abundance, it is reasonable to affiliate the lack of success of specific *L.casei* analysis to less specific or less amplifying primers.

8.2.2.3. *Bacteroides* spp.

Although no remarkable changes occur in the absolute quantification, in the relative quantification the percentage of patients receiving antibiotic therapy or a combined antibiotic/*L.casei* Shirota therapy is being increased before and decreased during antibiotic treatment.

8.2.2.4. *Bifidobacterium* spp.

A dramatic decline in Actinobacteria phylum by antibiotic intake, which includes *Bifidobacterium* spp., has already been described previously (Jakobsson et al., 2010). Bifidobacteria are important inulin and starch degraders (Dethlefsen et al., 2006). With the decline of Bifidobacteria, also their SCFAs-production is being decreased. A decrease in *Clostridium* Cluster XIVa and IV raises the risk of AAD as well.

Due to combined antibiotic/*L.casei* Shirota therapy, the drop of *Bifidobacterium* spp. is being stopped. This treatment can be assumed as a positive influence on the production of SCFAs and hence on the diarrheal probability.

8.2.2.5. Enterobacteriaceae

Whereas the dominant bacterial groups are decreasing, the abundance of sub-dominant Enterobacteriaceae is increasing by a power, both by disease and by antibiotic treatment. These results are consistent with the statement of Sommer and Dantas (2011), indicating that the low-abundant Proteobacteria containing drug resistant strains, including Enterobacteriaceae, increased under antibiotic treatment.

The results of this thesis suggest, that not every patient responds equally to antibiotic therapy or a combined antibiotic/*L.casei* Shirota therapy. In some patients, Enterobacteriaceae increase strongly, while in others there are hardly any changes. A combined therapy apparently reduces the abundance of Enterobacteriaceae.

9. CONCLUSIONS

The results of this diploma thesis points out that disease as well as antibiotic treatment are mainly associated with changes in the composition of the gastrointestinal microbiota and group specific shifts. During antibiotic administration, the abundance of total Bacteria and of the bacterial subgroups *Bifidobacterium* spp., *Clostridium* Cluster IV, XIVa and XI is being reduced. However, the abundance of Enterobacteriaceae subgroup increases.

An intervention with *L.casei* Shirota seems to antagonize the decline of *Bifidobacterium* spp. in antibiotic treated patients. In healthy individuals the intake of *L.casei* Shirota results in increasing *Lactobacillus* spp. abundance.

10. SUMMARY

Background: A healthy, divers microbiota maintains a stable and preserved ecosystem in the human gut (Backhed et al., 2005). Antibiotic treatment can disturb this ecosystem, whereby a loss of stability may be caused by decreasing the bacterial diversity or group specific shifts (Sommer and Dantas, 2011). Thereby pathogens such as *C.difficile* can come up and cause harm (Thompson-Chagoyan et al., 2007).

There are indications that some probiotic strains are able to reduce the incidence of antibiotic-associated diarrhea and *Clostridium difficile* associated diarrhea, especially *Lactobacillus* strains (Hickson et al., 2007, Gao et al., 2010, Wenus et al., 2008, Stockenhuber et al., 2008).

Aims: This diploma thesis investigated the qualitative and quantitative changes in human gastrointestinal microbiota, caused by antibiotic treatment and the effects of a probiotic drink containing *Lactobacillus casei* Shirota on the antibiotic disturbed microbiota. Furthermore, the influence of a combined antibiotic/*L.casei* Shirota therapy on the occurrence of *C.difficile* Infection should be explored.

Study design: Stool samples from four groups (group AP: antibiotic treatment and intake of a probiotic drink containing L.casei Shirota; group A: antibiotic treatment; group P: intake of a probiotic drink containing L.casei Shirota; and group C: control group) of each 15 (group AP, P and C) respectively 11 (group A) patients were taken at one before, and two time points after antibiotic treatment and/or intake of *L.casei* Shirota. Samples of control group were taken at similar time points.

Bacterial DNA was extracted and the abundances of total Bacteria and the bacterial subgroups *Clostridium* Cluster IV (*Clostridium leptum* subgroup), *Clostridium* cluster XIVa (Lachnospiraceae subgroup), *Clostridium* cluster XI, *Clostridium difficile*, *Lactobacillus* spp., *L.casei*, *Bacteroides* spp., *Bifidobacterium* spp., Enterobacteriaceae and *Salmonella* spp. were determined by 16s rRNA based qPCR.

Results: In the antibiotic treated groups (A and AP) a significant decrease of total bacteria, *Bifidobacterium* spp., *Clostridium* Cluster IV, XIVa and XI could be observed, whereas Enterobacteriaceae were increased. In healthy individuals, the abundance of

Lactobacillus spp. increased rapidly after intake of the probiotic drink containing *L.casei* Shirota.

Investigations of *Bacteroides* spp., *Salmonella* spp., *L.casei* and *C.difficile* have not produced clear results.

Conclusions: The results of this diploma thesis points out that disease and antibiotic treatment are mainly associated with changes in the composition of the gastrointestinal microbiota and group specific shifts. During antibiotic administration both, the abundance of total Bacteria and of the bacterial subgroups *Bifidobacterium* spp., *Clostridium* Cluster IV, XIVa and XI is reduced. However, the abundance of the Enterobacteriaceae subgroup is increasing.

An intervention with *L.casei* Shirota seems to antagonize the *Bifidobacterium* spp. decrease in antibiotic treated patients. In healthy individuals the intake of *L.casei* Shirota results in increasing *Lactobacillus* spp. abundance.

11. ZUSAMMENFASSUNG

Hintergrund: Eine gesunde und diverse Microbiota im Darm ermöglicht die Aufrechterhaltung eines stabilen und geschützten Ökosystems (Backhed et al., 2005). Durch die Behandlung mit Antibiotika kann es zum Verlust dieser Stabilität kommen; induziert durch eine Verringerung der bakteriellen Vielfalt oder gruppenspezifischen Verschiebungen (Sommer und Dantas, 2011). Dabei können Pathogene, wie zum Beispiel *C.difficile*, aufkommen und dem Wirt schaden (Thompson-Chagoyan et al. 2007).

Es gibt bereits Hinweise dafür, dass einige probiotische Stämme in der Lage sind, die Inzidenz von Antibiotika-assoziierter Diarrhoe und *C.difficile* assoziierter Diarrhoe zu reduzieren; insbesondere *Lactobacillus* Stämme (Hickson et al., 2007, Gao et al., 2010, Wenus et al., 2008, Stockenhuber et al., 2008).

Zielsetzung: Im Rahmen der vorliegenden Diplomarbeit wurden die qualitative und quantitative Veränderungen der menschlichen gastrointestinalen Microbiota, durch die Behandlung mit Antibiotika untersucht, sowie die Wirkung eines *L.casei* Shirota-enthaltenden probiotischen Getränks, auf die durch Antibiotika zerstörte Microbiota. Darüber hinaus sollte der Einfluss einer kombinierten Antibiotika/*L.casei* Shirota Therapie, auf das Auftreten von *C.difficile*-Infektion untersucht werden.

Studiendesign: Es wurden Stuhlproben von vier Gruppen (Gruppe AP: Behandlung mit Antibiotika und Einnahme eines *L.casei* Shirota-enthaltenden probiotischen Getränks; Gruppe A: Behandlung mit Antibiotika; Gruppe P: Einnahme eines *L.casei* Shirota-enthaltenden probiotischen Getränks; und Gruppe C: Kontrollgruppe) zu jeweils 15 (Gruppe AP, P und C) beziehungsweise 11 Patienten (Gruppe A) genommen. Dabei wurde jeweils eine Probe vor der Behandlung mit Antibiotika und/oder der Einnahme eines *L.casei* Shirota-enthaltenden probiotischen Getränks genommen und zwei weitere währenddessen. Die Proben der Kontrollgruppe stammen von vergleichbaren Zeitpunkten.

Die bakterielle DNA wurde extrahiert und mittels 16s rRNA basierter qPCR die Abundanzen der gesamten Bakterien und der Subgruppen *Clostridium* Cluster IV (*Clostridium leptum* subgroup), *Clostridium* cluster XIVa (Lachnospiraceae subgroup),

Clostridium cluster XI*, Clostridium difficile*, *Lactobacillus* spp., *L.casei*, *Bacteroides* spp., *Bifidobacterium* spp., Enterobacteriaceae und *Salmonella* spp. bestimmt.

Ergebnisse: Durch die Behandlung mit Antibiotika, konnte ein signifikantes Absinken der gesamten Bakterien, *Bifidobacterium* spp., *Clostridium* Cluster IV, XIVa and XI beobachtet werden. Gleichzeitig war ein Ansteigen von Enterobacteriaceae festzustellen. In den gesunden Probanden die das *L.casei* Shirota-enthaltenden probiotischen Getränk konsumierten, stieg die Abundanz von *Lactobacillus* spp. rasant an.

Die Untersuchungen von *Bacteroides* spp., *Salmonella* spp., *L.casei* und *C.difficile* haben keine klaren Ergebnisse erbracht.

Schlussbetrachtung: Die Ergebnisse diese Diplomarbeit zeigt, dass sowohl Krankheit als auch Antibiotika-Therapie, mit Veränderungen in der Zusammensetzung der gastrointestinalen Microbiota assoziiert sind. Während der Behandlung mit Antibiotika sinkt sowohl die Abundanz der gesamten Bakterien, als auch jene der bakteriellen Subgruppen *Bifidobacterium* spp., *Clostridium* Cluster IV, XIVa und XI, während die Abundanz der Enterobacteriaceae ansteigt.

Die Intervention mit *L.casei* Shirota scheint dem Absinken von *Bifidobacterium* spp. in Antibibiotika-behandelten Patienten entgegenzuwirken. Die Aufnahme von *L.casei* Shirota von gesunden Probanden, verursacht einen Anstieg der Abundanz von *Lactobacillus* spp..

12. REFERENCES

AHMER, B. M. & GUNN, J. S. (2011) Interaction of Salmonella spp. with the Intestinal Microbiota. *Front Microbiol,* 2**,** 101.

ALCALA, L., SANCHEZ-CAMBRONERO, L., CATALAN, M. P., SANCHEZ-SOMOLINOS, M., PELAEZ, M. T., MARIN, M. & BOUZA, E. (2008) Comparison of three commercial methods for rapid detection of Clostridium difficile toxins A and B from fecal specimens. *J Clin Microbiol,* 46**,** 3833-5.

ARUMUGAM, M., RAES, J., PELLETIER, E., LE PASLIER, D., YAMADA, T., MENDE, D. R., FERNANDES, G. R., TAP, J., BRULS, T., BATTO, J. M., BERTALAN, M., BORRUEL, N., CASELLAS, F., FERNANDEZ, L., GAUTIER, L., HANSEN, T., HATTORI, M., HAYASHI, T., KLEEREBEZEM, M., KUROKAWA, K., LECLERC, M., LEVENEZ, F., MANICHANH, C., NIELSEN, H. B., NIELSEN, T., PONS, N., POULAIN, J., QIN, J., SICHERITZ-PONTEN, T., TIMS, S., TORRENTS, D., UGARTE, E., ZOETENDAL, E. G., WANG, J., GUARNER, F., PEDERSEN, O., DE VOS, W. M., BRUNAK, S., DORE, J., ANTOLIN, M., ARTIGUENAVE, F., BLOTTIERE, H. M., ALMEIDA, M., BRECHOT, C., CARA, C., CHERVAUX, C., CULTRONE, A., DELORME, C., DENARIAZ, G., DERVYN, R., FOERSTNER, K. U., FRISS, C., VAN DE GUCHTE, M., GUEDON, E., HAIMET, F., HUBER, W., VAN HYLCKAMA-VLIEG, J., JAMET, A., JUSTE, C., KACI, G., KNOL, J., LAKHDARI, O., LAYEC, S., LE ROUX, K., MAGUIN, E., MERIEUX, A., MELO MINARDI, R., M'RINI, C., MULLER, J., OOZEER, R., PARKHILL, J., RENAULT, P., RESCIGNO, M., SANCHEZ, N., SUNAGAWA, S., TORREJON, A., TURNER, K., VANDEMEULEBROUCK, G., VARELA, E., WINOGRADSKY, Y., ZELLER, G., WEISSENBACH, J., EHRLICH, S. D. & BORK, P. (2011) Enterotypes of the human gut microbiome. *Nature,* 473**,** 174-80.

BACKHED, F., DING, H., WANG, T., HOOPER, L. V., KOH, G. Y., NAGY, A., SEMENKOVICH, C. F. & GORDON, J. I. (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A,* 101**,** 15718-23.

BACKHED, F., LEY, R. E., SONNENBURG, J. L., PETERSON, D. A. & GORDON, J. I. (2005) Host-bacterial mutualism in the human intestine. *Science,* 307**,** 1915-20.

BARMAN, M., UNOLD, D., SHIFLEY, K., AMIR, E., HUNG, K., BOS, N. & SALZMAN, N. (2008) Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infect Immun,* 76**,** 907-15.

BARTLETT, C. L. (1987) Efficacy of different surveillance systems in detecting hospital-acquired infections. *Chemioterapia,* 6**,** 152-5.

BIAN, L., NAGATA, S., ASAHARA, T., RAHMAN, M. S., OHTA, T., YUKI, N., WANG, C., TAKANO, K., DAIBO, M., NOMOTO, K. & YAMASHIRO, Y. (2011) Effects of the continuous intake of Lactobacillus casei strain Shirota-fermented milk on risk management of long-term inpatients at health service facilities for the elderly. *International Journal of Probiotics and Prebiotics,* 6, 2**,** 123-132.

BREVES, G., FAUL, K., SCHRODER, B., HOLST, H., CASPARY, W. F. & STEIN, J. (2000) Application of the colon-simulation technique for studying the effects of Saccharomyces boulardii on basic parameters of porcine cecal microbial metabolism disturbed by clindamycin. *Digestion,* 61**,** 193-200.

CANANI, R. B., COSTANZO, M. D., LEONE, L., PEDATA, M., MELI, R. & CALIGNANO, A. (2011) Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J Gastroenterol,* 17**,** 1519-28.

CAPORASO, J. G., LAUBER, C. L., COSTELLO, E. K., BERG-LYONS, D., GONZALEZ, A., STOMBAUGH, J., KNIGHTS, D., GAJER, P., RAVEL, J., FIERER, N., GORDON, J. I. & KNIGHT, R. (2011) Moving pictures of the human microbiome. *Genome Biol,* 12**,** R50.

DE LA COCHETIERE, M. F., DURAND, T., LEPAGE, P., BOURREILLE, A., GALMICHE, J. P. & DORE, J. (2005) Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. *J Clin Microbiol,* 43**,** 5588-92.

DETHLEFSEN, L., ECKBURG, P. B., BIK, E. M. & RELMAN, D. A. (2006) Assembly of the human intestinal microbiota. *Trends Ecol Evol,* 21**,** 517-23.

DETHLEFSEN, L. & RELMAN, D. A. (2010) Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad Sci U S A,* 108 Suppl 1**,** 4554-61.

DETHLEFSEN, L. & RELMAN, D. A. (2011) Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad Sci U S A,* 108 Suppl 1**,** 4554-61.

EASTWOOD, K., ELSE, P., CHARLETT, A. & WILCOX, M. (2009) Comparison of nine commercially available Clostridium difficile toxin detection assays, a real-time PCR assay for C. difficile tcdB, and a glutamate dehydrogenase detection assay to cytotoxin testing and cytotoxigenic culture methods. *J Clin Microbiol,* 47**,** 3211-7.

ECKBURG, P. B., BIK, E. M., BERNSTEIN, C. N., PURDOM, E., DETHLEFSEN, L., SARGENT, M., GILL, S. R., NELSON, K. E. & RELMAN, D. A. (2005) Diversity of the human intestinal microbial flora. *Science,* 308**,** 1635-8.

FAO/WHO (2001) Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. . *Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria.* Córdoba, Argentina, Food and Agriculture Organization of the United Nations and World Health Organization.

FLINT, H. J., BAYER, E. A., RINCON, M. T., LAMED, R. & WHITE, B. A. (2008) Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Microbiol,* 6**,** 121-31.

FREDSTROM, S. B., LAMPE, J. W., JUNG, H. J. & SLAVIN, J. L. (1994) Apparent fiber digestibility and fecal short-chain fatty acid concentrations with ingestion of two types of dietary fiber. *JPEN J Parenter Enteral Nutr,* 18**,** 14-9.

GAO, X. W., MUBASHER, M., FANG, C. Y., REIFER, C. & MILLER, L. E. (2010) Dose-response efficacy of a proprietary probiotic formula of Lactobacillus acidophilus CL1285 and Lactobacillus casei LBC80R for antibiotic-associated diarrhea and Clostridium difficile-associated diarrhea prophylaxis in adult patients. *Am J Gastroenterol,* 105**,** 1636-41.

GUILLOTEAU, P., MARTIN, L., EECKHAUT, V., DUCATELLE, R., ZABIELSKI, R. & VAN IMMERSEEL, F. (2010) From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutr Res Rev,* 23**,** 366-84.

HAMER, H. M., JONKERS, D., VENEMA, K., VANHOUTVIN, S., TROOST, F. J. & BRUMMER, R. J. (2008) Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther,* 27**,** 104-19.

HENSGENS, M. P., GOORHUIS, A., VAN KINSCHOT, C. M., CROBACH, M. J., HARMANUS, C. & KUIJPER, E. J. (2011) Clostridium difficile infection in an endemic setting in the Netherlands. *Eur J Clin Microbiol Infect Dis,* 30**,** 587-93.

HICKSON, M. (2011) Probiotics in the prevention of antibiotic-associated diarrhoea and Clostridium difficile infection. *Therap Adv Gastroenterol,* 4**,** 185-97.

HICKSON, M., D'SOUZA, A. L., MUTHU, N., ROGERS, T. R., WANT, S., RAJKUMAR, C. & BULPITT, C. J. (2007) Use of probiotic Lactobacillus preparation to prevent diarrhoea associated with antibiotics: randomised double blind placebo controlled trial. *BMJ,* 335**,** 80.

HIPPE, B., ZWIELEHNER, J., LISZT, K., LASSL, C., UNGER, F. & HASLBERGER, A. G. (2011) Quantification of butyryl CoA:acetate CoA-transferase genes reveals different butyrate production capacity in individuals according to diet and age. *FEMS Microbiol Lett,* 316**,** 130-5.

HOGENAUER, C., HAMMER, H. F., KREJS, G. J. & REISINGER, E. C. (1998) Mechanisms and management of antibiotic-associated diarrhea. *Clin Infect Dis,* 27**,** 702-10.

HUDAULT, S., GUIGNOT, J. & SERVIN, A. L. (2001) Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection. *Gut,* 49**,** 47-55.

JAKOBSSON, H. E., JERNBERG, C., ANDERSSON, A. F., SJOLUND-KARLSSON, M., JANSSON, J. K. & ENGSTRAND, L. (2010) Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One,* 5**,** e9836.

KUEHNE, S. A., CARTMAN, S. T., HEAP, J. T., KELLY, M. L., COCKAYNE, A. & MINTON, N. P. (2010) The role of toxin A and toxin B in Clostridium difficile infection. *Nature,* 467**,** 711-3.

KUROKAWA, K., ITOH, T., KUWAHARA, T., OSHIMA, K., TOH, H., TOYODA, A., TAKAMI, H., MORITA, H., SHARMA, V. K., SRIVASTAVA, T. P., TAYLOR, T. D., NOGUCHI, H., MORI, H., OGURA, Y., EHRLICH, D. S., ITOH, K., TAKAGI, T., SAKAKI, Y., HAYASHI, T. & HATTORI, M. (2007) Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. *DNA Res,* 14**,** 169-81.

LAWLEY, T. D., CLARE, S., WALKER, A. W., GOULDING, D., STABLER, R. A., CROUCHER, N., MASTROENI, P., SCOTT, P., RAISEN, C., MOTTRAM, L., FAIRWEATHER, N. F., WREN, B. W., PARKHILL, J. & DOUGAN, G. (2009) Antibiotic treatment of clostridium difficile carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect Immun,* 77**,** 3661-9.

LAY, C., SUTREN, M., ROCHET, V., SAUNIER, K., DORE, J. & RIGOTTIER-GOIS, L. (2005) Design and validation of 16S rRNA probes to enumerate members of the Clostridium leptum subgroup in human faecal microbiota. *Environ Microbiol,* 7**,** 933-46.

LAYTON, A., MCKAY, L., WILLIAMS, D., GARRETT, V., GENTRY, R. & SAYLER, G. (2006) Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. *Appl Environ Microbiol,* 72**,** 4214-24.

LEDERBERG, J. (2000) Infectious history. *Science,* 288**,** 287-93.

LEWIS, J. D. N., THOMAS, L. V. & WEIR, W. (2009) The potential of probiotic fermented milk products in reducing risk of antibiotic-associated diarrhoea and Clostridium difficile disease. *Int J Dairy Technol,* 62 (4)**,** 461-471.

LEY, R. E., PETERSON, D. A. & GORDON, J. I. (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell,* 124**,** 837-48.

LIN, C. K., HUNG, C. L., CHIANG, Y. C., LIN, C. M. & TSEN, H. Y. (2004) The sequence heterogenicities among 16S rRNA genes of Salmonella serovars and the effects on the specificity of the primers designed. *Int J Food Microbiol,* 96**,** 205-14.

LIN, C. K. & TSEN, H. Y. (1996) Use of two 16S DNA targeted oligonucleotide as PCR primers for the specific detection of Salmonella in foods. *J. Appl. Microbiol.,* 80**,** 659-666.

LOUIS, P. & FLINT, H. J. (2007) Development of a semiquantitative degenerate real-time pcr-based assay for estimation of numbers of butyryl-coenzyme A (CoA) CoA transferase genes in complex bacterial samples. *Appl Environ Microbiol,* 73**,** 2009-12.

LOUIS, P., SCOTT, K. P., DUNCAN, S. H. & FLINT, H. J. (2007) Understanding the effects of diet on bacterial metabolism in the large intestine. *J Appl Microbiol,* 102**,** 1197-208.

LUNA, R. A., BOYANTON, B. L., JR., MEHTA, S., COURTNEY, E. M., WEBB, C. R., REVELL, P. A. & VERSALOVIC, J. (2011) Rapid stool-based diagnosis of Clostridium difficile infection by real-time PCR in a children's hospital. *J Clin Microbiol,* 49**,** 851-7.

MAHAJAN, A. & GALLY, D. (2011) Escherichia coli and the mucosal immune system. *Expert Rev Clin Immunol,* 7**,** 743-5.

MARIAT, D., FIRMESSE, O., LEVENEZ, F., GUIMARAES, V., SOKOL, H., DORE, J., CORTHIER, G. & FURET, J. P. (2009) The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol,* 9**,** 123.

MARTINEZ, C. O., PAZ, V. S. & O'DONNELL, A. (2003) Lactobacillus Casei Shirota Prevention Approach for Healthy Children with Mild Acute Infection Treated with Antibiotics. *Ped Res,* 4(2) S**,** 174A-174A.

MATSUDA, K., TSUJI, H., ASAHARA, T., MATSUMOTO, K., TAKADA, T. & NOMOTO, K. (2009) Establishment of an Analytical System for the Human Fecal Microbiota, Based on Reverse Transcription-Quantitative PCR Targeting of Multicopy rRNA Molecules. *Applied and Environmental Microbiology,* 75**,** 1961-1969.

MATSUKI, T., WATANABE, K., FUJIMOTO, J., TAKADA, T. & TANAKA, R. (2004) Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl Environ Microbiol,* 70**,** 7220-8.

MCFARLAND, L. V. (2006) Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of Clostridium difficile disease. *Am J Gastroenterol,* 101**,** 812-22.

MCFARLAND, L. V. (2010) Systematic review and meta-analysis of Saccharomyces boulardii in adult patients. *World J Gastroenterol,* 16**,** 2202-22.

MCFARLAND, L. V., BENEDA, H. W., CLARRIDGE, J. E. & RAUGI, G. J. (2007) Implications of the changing face of Clostridium difficile disease for health care practitioners. *Am J Infect Control,* 35**,** 237-53.

MEIER, H., AMANN, R., LUDWIG, W. & SCHLEIFER, K. H. (1999) Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G + C content. *Syst Appl Microbiol,* 22**,** 186-96.

MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol,* 59**,** 695-700.

MUYZER, G. & SMALLA, K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek,* 73**,** 127-41.

NADAL, I., SANTACRUZ, A., MARCOS, A., WARNBERG, J., GARAGORRI, M., MORENO, L. A., MARTIN-MATILLAS, M., CAMPOY, C., MARTI, A., MOLERES, A., DELGADO, M., VEIGA, O. L., GARCIA-FUENTES, M., REDONDO, C. G. & SANZ, Y. (2009) Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents. *Int J Obes (Lond),* 33**,** 758-67.

NAGATA, S., ASAHARA, T., OHTA, T., YAMADA, T., KONDO, S., BIAN, L., WANG, C., YAMASHIRO, Y. & NOMOTO, K. (2011) Effect of the continuous intake of probiotic-fermented milk containing Lactobacillus casei strain Shirota on fever in a mass outbreak of norovirus gastroenteritis and the faecal microflora in a health service facility for the aged. *Br J Nutr,* 106**,** 549-56.

NEEFS, J. M., VAN DE PEER, Y., DE RIJK, P., GORIS, A. & DE WACHTER, R. (1991) Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Res,* 19 Suppl**,** 1987-2015.

NG, S. C., HART, A. L., KAMM, M. A., STAGG, A. J. & KNIGHT, S. C. (2009) Mechanisms of action of probiotics: recent advances. *Inflamm Bowel Dis,* 15**,** 300-10.

OELSCHLAEGER, T. A. (2010) Mechanisms of probiotic actions - A review. *Int J Med Microbiol,* 300**,** 57-62.

OOTSUBO, M., SHIMIZU, T., TANAKA, R., SAWABE, T., TAJIMA, K., YOSHIMIZU, M., EZURA, Y., EZAKI, T. & OYAIZU, H. (2002) Oligonucleotide probe for detecting Enterobacteriaceae by in situ hybridization. *J Appl Microbiol,* 93**,** 60-8.

OZAKI, E., KATO, H., KITA, H., KARASAWA, T., MAEGAWA, T., KOINO, Y., MATSUMOTO, K., TAKADA, T., NOMOTO, K., TANAKA, R. & NAKAMURA, S. (2004) Clostridium difficile colonization in healthy adults: transient colonization and correlation with enterococcal colonization. *J Med Microbiol,* 53**,** 167-72.

PENDERS, J., VINK, C., DRIESSEN, C., LONDON, N., THIJS, C. & STOBBERINGH, E. E. (2005) Quantification of Bifidobacterium spp., Escherichia coli and Clostridium difficile in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett,* 243**,** 141-7.

PIRKER, A., STOCKENHUBER, A., REMELY, M., HARRANT, A., HIPPE, B., KAMHUBER, C., STOCKENHUBER, F. & HASLBERGER, A. G. (2012) Effects of antibiotic therapy on the gastrointestinal microbiota and the influence of Lactobacillus casei. Vienna.

PRYDE, S. E., DUNCAN, S. H., HOLD, G. L., STEWART, C. S. & FLINT, H. J. (2002) The microbiology of butyrate formation in the human colon. *FEMS Microbiol Lett,* 217**,** 133-9.

QIN, J., LI, R., RAES, J., ARUMUGAM, M., BURGDORF, K. S., MANICHANH, C., NIELSEN, T., PONS, N., LEVENEZ, F., YAMADA, T., MENDE, D. R., LI, J., XU, J., LI, S., LI, D., CAO, J., WANG, B., LIANG, H., ZHENG, H., XIE, Y., TAP, J., LEPAGE, P., BERTALAN, M., BATTO, J. M., HANSEN, T., LE PASLIER, D., LINNEBERG, A., NIELSEN, H. B., PELLETIER, E., RENAULT, P., SICHERITZ-PONTEN, T., TURNER, K., ZHU, H., YU, C., JIAN, M., ZHOU, Y., LI, Y., ZHANG, X., QIN, N., YANG, H., WANG, J., BRUNAK, S., DORE, J., GUARNER, F., KRISTIANSEN, K., PEDERSEN, O., PARKHILL, J., WEISSENBACH, J., BORK, P. & EHRLICH, S. D. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. *Nature,* 464**,** 59-65.

RAKOFF-NAHOUM, S., PAGLINO, J., ESLAMI-VARZANEH, F., EDBERG, S. & MEDZHITOV, R. (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell,* 118**,** 229-41.

RASTALL, R. A. (2004) Bacteria in the gut: friends and foes and how to alter the balance. *J Nutr,* 134**,** 2022S-2026S.

RASTALL, R. A., GIBSON, G. R., GILL, H. S., GUARNER, F., KLAENHAMMER, T. R., POT, B., REID, G., ROWLAND, I. R. & SANDERS, M. E. (2005) Modulation of

the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: an overview of enabling science and potential applications. *FEMS Microbiol Ecol,* 52**,** 145-52.

REUTER, G. (2001) The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and succession. *Curr Issues Intest Microbiol,* 2**,** 43-53.

RUSSELL, D. A., ROSS, R. P., FITZGERALD, G. F. & STANTON, C. (2011) Metabolic activities and probiotic potential of bifidobacteria. *Int J Food Microbiol,* 149**,** 88-105.

SEKSIK, P., RIGOTTIER-GOIS, L., GRAMET, G., SUTREN, M., POCHART, P., MARTEAU, P., JIAN, R. & DORE, J. (2003) Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. *Gut,* 52**,** 237-42.

SNYDMAN, D. R. (2008) The safety of probiotics. *Clin Infect Dis,* 46 Suppl 2**,** S104-11; discussion S144-51.

SOMMER, M. O. & DANTAS, G. (2011) Antibiotics and the resistant microbiome. *Curr Opin Microbiol*.

SONG, Y., LIU, C. & FINEGOLD, S. M. (2004) Real-time PCR quantitation of clostridia in feces of autistic children. *Appl Environ Microbiol,* 70**,** 6459-65.

STOCKENHUBER, A., ADELMANN, K., KAMHUBER, C., HÖRIST-KOLLMANN, S., REK, A., ECKHARDT, G., G., L. & STOCKENHUBER, F. (2008) Preventing diarrhoea associated with antibiotics using a probiotic Lactobacillus casei preparation. *Gut,* 57.

TAP, J., MONDOT, S., LEVENEZ, F., PELLETIER, E., CARON, C., FURET, J. P., UGARTE, E., MUNOZ-TAMAYO, R., PASLIER, D. L., NALIN, R., DORE, J. & LECLERC, M. (2009) Towards the human intestinal microbiota phylogenetic core. *Environ Microbiol,* 11**,** 2574-84.

THOMPSON-CHAGOYAN, O. C., MALDONADO, J. & GIL, A. (2007) Colonization and impact of disease and other factors on intestinal microbiota. *Dig Dis Sci,* 52**,** 2069-77.

TOPPING, D. L. & CLIFTON, P. M. (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol Rev,* 81**,** 1031-64.

TURNBAUGH, P. J. & GORDON, J. I. (2009) The core gut microbiome, energy balance and obesity. *J Physiol,* 587**,** 4153-8.

TURNBAUGH, P. J., HAMADY, M., YATSUNENKO, T., CANTAREL, B. L., DUNCAN, A., LEY, R. E., SOGIN, M. L., JONES, W. J., ROE, B. A., AFFOURTIT, J. P., EGHOLM, M., HENRISSAT, B., HEATH, A. C., KNIGHT, R. & GORDON, J. I. (2009) A core gut microbiome in obese and lean twins. *Nature,* 457**,** 480-4.

TURNBAUGH, P. J., LEY, R. E., HAMADY, M., FRASER-LIGGETT, C. M., KNIGHT, R. & GORDON, J. I. (2007) The human microbiome project. *Nature,* 449**,** 804-10.

TURRONI, F., RIBBERA, A., FORONI, E., VAN SINDEREN, D. & VENTURA, M. (2008) Human gut microbiota and bifidobacteria: from composition to functionality. *Antonie Van Leeuwenhoek,* 94**,** 35-50.

VAN DER WAAIJ, L. A., HARMSEN, H. J., MADJIPOUR, M., KROESE, F. G., ZWIERS, M., VAN DULLEMEN, H. M., DE BOER, N. K., WELLING, G. W. & JANSEN, P. L. (2005) Bacterial population analysis of human colon and terminal ileum biopsies with 16S rRNA-based fluorescent probes: commensal bacteria live in suspension and have no direct contact with epithelial cells. *Inflamm Bowel Dis,* 11**,** 865-71.

VERNA, E. C. & LUCAK, S. (2010) Use of probiotics in gastrointestinal disorders: what to recommend? *Therap Adv Gastroenterol,* 3(5)**,** 307-19.

WALLACE, T. C., GUARNER, F., MADSEN, K., CABANA, M. D., GIBSON, G., HENTGES, E. & SANDERS, M. E. (2011) Human gut microbiota and its relationship to health and disease. *Nutr Rev,* 69**,** 392-403.

WALTER, J., HERTEL, C., TANNOCK, G. W., LIS, C. M., MUNRO, K. & HAMMES, W. P. (2001) Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl Environ Microbiol,* 67**,** 2578-85.

WALTER, J. & LEY, R. E. (2011) The Human Gut Microbiome: Ecology and Recent Evolutionary Changes. *Annu Rev Microbiol*.

WALTON, G. E., VAN DEN HEUVEL, E. G., KOSTERS, M. H., RASTALL, R. A., TUOHY, K. M. & GIBSON, G. R. (2011) A randomised crossover study investigating the effects of galacto-oligosaccharides on the faecal microbiota in men and women over 50 years of age. *Br J Nutr***,** 1-10.

WANG, X., HEAZLEWOOD, S. P., KRAUSE, D. O. & FLORIN, T. H. (2003) Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. *J Appl Microbiol,* 95**,** 508-20.

WENUS, C., GOLL, R., LOKEN, E. B., BIONG, A. S., HALVORSEN, D. S. & FLORHOLMEN, J. (2008) Prevention of antibiotic-associated diarrhoea by a fermented probiotic milk drink. *Eur J Clin Nutr,* 62**,** 299-301.

WHELAN, K. & MYERS, C. E. (2010) Safety of probiotics in patients receiving nutritional support: a systematic review of case reports, randomized controlled trials, and nonrandomized trials. *Am J Clin Nutr,* 91**,** 687-703.

WOO, P. C., LEUNG, P. K., LEUNG, K. W. & YUEN, K. Y. (2000) Identification by 16S ribosomal RNA gene sequencing of an Enterobacteriaceae species from a bone marrow transplant recipient. *Mol Pathol,* 53**,** 211-5.

YU, Y., LEE, C., KIM, J. & HWANG, S. (2005) Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng,* 89**,** 670-9.

ZHU, B., WANG, X. & LI, L. (2010) Human gut microbiome: the second genome of human body. *Protein Cell,* 1**,** 718-25.

ZOETENDAL, E. G., VAUGHAN, E. E. & DE VOS, W. M. (2006) A microbial world within us. *Mol Microbiol,* 59**,** 1639-50.

ZWIELEHNER, J., LISZT, K., HANDSCHUR, M., LASSL, C., LAPIN, A. & HASLBERGER, A. G. (2009) Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and Clostridium cluster IV in institutionalized elderly. *Exp Gerontol,* 44**,** 440-6.

13. PUBLICATIONS

13.1. Manuscript: "Effects of Antibiotic Therapy on the Gastrointestinal Microbiota and the Influence of *Lactobacillus casei*"

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

Angelika Pirker[1], Alexander Stockenhuber[2], Marlene Remely[1], Anna Harrant[1], Berit Hippe[1], Christoph Kamhuber[2], Felix Stockenhuber[2], Alexander G. Haslberger[1]

[1] Department for Nutritional Sciences, University of Vienna, Vienna, Austria
[2] Krankenhaus Oberpullendorf, Vienna, Austria

Univ.-Doz. Dr. Alexander Haslberger'
Department of Nutritional Sciences
Althanstrasse 14, UZAII; 2D541
1090 Vienna
phone: +43 1 42 77 54 996
mail: alexander.haslberger@univie.ac.at

Keywords:

Antibiotics, antibiotic-associated diarrhoea, Bacterial, *Clostridium difficile* infection, Gastrointestinal microbiota, *Lactobacillus casei*

Abstract

Aims and Methods: 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 PCR/DGGE (56 patients).

Results: AAD developed in 18.5% group A and 5% group AP patients. Following antibiotic therapy, a decrease of 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..

Conclusions: Antibiotic treatment effects diversity and composition of the microbiota impairing short-chain fatty acids (SCFAs) production. Intervention with certain Lactobacillus strains may antagonise some of these changes and more potent SCFA-stimulating probiotics are desirable for intervention in AAD.

1. Introduction

The human gut contains approximately 10-100 trillion organisms (Qin et al., 2010), including all three domains of life: Bacteria, Archaea and Eukarya (Backhed et al., 2005); and 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 et al., 2003). 95% 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 et al., 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 (Sommer and Dantas, 2011, Dethlefsen and Relman, 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 and Dantas, 2011), all of which makes it important that antibiotic-associated changes to the intestinal microbiota are characterized 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 et al., 1998), accompanied by a decrease of 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 (Hogenauer et al., 1998, Breves et al., 2000).

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 hospitalized 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 et al., 2007). *C.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 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 SCFAs 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).

S.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 et al., 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 (Martinez et al., 2003, Lewis et al., 2009). The safety of *Lactobacillus casei* Shirtoa (LcS) has been confirmed in several studies (Snydman, 2008, Whelan and 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 colonization 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 hospitalized 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 feacal microbiota were analysed in a subgroup of 56 subjects. The results suggest an important role of LcS in antagonizing antibiotic-induced changes in the intestinal microbiota with regard to its abundance, diversity and SCFA-producing capacity.

2. Material and methods

2.1. Subjects

The study was performed at the department of Internal Medicine of the General Hospital Oberpullendorf (Austria). 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 $6.5x10^9$ 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, cephaolosporins, 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.

Feacal samples were taken for further laboratory study from sub-set of 56 subjects (aged 60 ± 22 years), divided into four groups (Table 1). Patients of group 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 (C) consisted of healthy adults receiving neither antibiotics nor probiotics.

2.2. 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®).

2.3. Sampling and DNA extraction from stool

Feacal 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 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.

2.4. 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 $MgCl_2$), 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 $MgCl_2$) and *Clostridium* cluster XI, a reaction mix with the total volume of 10 µL, 5µL SensiMix™ SYBR No-ROX Kit (SYBR® Green) (3 mM $MgCl_2$), 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).

For comparison of PCR reaction efficiencies among different experiments, one feacal sample and tenfold series of DNA dilutions of following type strains were used: *Clostridium leptum* DSM 753, *Clostridium blautia*, *Bifidobacterium longum* DSM 20211, *Lactobacillus casei*, *Escherichia coli* 1029 and a bacterial mix containing *Clostridium leptum* DSM 735, *Clostridium blautia*, *Clostridium difficile* 301968DNA, *Staphylococcus aureus*, *Enterococcus faecium* DSM 20477, *Listeria monocytogenes*, *Lactobacillus casei*, *Bacteroides thetaiotaomicron* DSM 2079, *Escherichia coli* 1029, *Citrobacter caseri*, *Enterobacter cloacae* 1252, *Salmonella typhimurium* SL7207, *Bifidobacterium 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 and Flint, 2007) (Table 3).

2.5. PCR/DGGE

Polymerase chain reaction (PCR) was used to amplify 16S rRNA gene sequences from total Bacteria in feacal 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 et al., 1993) and 518r 5'-ATT ACC GCG GCT GCT GG-3' (Neefs et al., 1991). The DGGE gel was prepared as described previously (Muyzer and 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 feacal material: *Bacteroides thetaiotaomicron, Enterococcus faecium, Clostridium leptum 16, Escherichia coli, Clostridium coccoides 43, Lactobacillus reuteri* and *Bifidobacterium longum*.

2.6. Statistical analysis

Fisher's exact test was performed to compare the rates of AAD and CDI.

Feacal 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 was used. P-values less than 0.05 were defined as statistically significant.

3. Results

3.1. Antibiotic-associated diarrhoea 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 feacal 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.

3.2. Total bacteria

In the feacal analysis of the subset of subjects, both antibiotic-treated groups had a significant lower number of copies per g 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 g was observed (Figure 1 A).

DGGE 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.

3.2.1. Relative amount of bacteria.

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

3.3. Bacterial subgroups

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

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

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

***Bifidobacterium* spp.:** The mean number of copies per g of both antibiotic groups were 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 LcS intake was begun and levels started to rise again slightly. (Figure 1 D)

***Lactobacillus* spp:** In group P the values of each time point differed significantly ($p = 0.01$) and the number of copies per g 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 g was significantly higher in group P than in group C ($p = 0.04$). (Figure 1 F)

Enterobacteriaceae: In the antibiotic groups AP and A, the number of copies per g were 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 1 E)

3.4. Butyrate

In patients receiving antibiotics (group AP and A), the amount of butyryl-CoA CoA transferase genes was lower than 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)

4. 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 feacal 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 and Relman, 2011, Jakobsson et al., 2010, Sommer and 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* cluster 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 et al., 2006) thus a decline in Bifidobacteria represents a decrease in an important SCFA-producing group.

SCFAs result from anaerobic microbial fermentation of indigestible carbohydrate or dietary fibre (Fredstrom et al., 1994). The main SCFAs are acetate, propionate and butyrate, which stimulate the colonic blood flow and fluid and electrolyte uptake (Topping and Clifton, 2001). Furthermore, indirect actions of butyrate are believed to involve the hormono–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 diarrhea. Therefore, the observation that combined use of *L.casei* Shirota antagonizes 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 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 feacal 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 et al., 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 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.

The abundance of *Clostridium* cluster XI was decreased in patients 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). *C.difficile* is a Gram-positive, spore-forming, anaerobe that can be carried asymptomatically 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 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 et al., 2009, Luna et al., 2011).

5. Conclusions

This study also confirmed that antibiotic treatment as well as the underlying disease requiring this therapy, were 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 colonize 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 antagonizes some of these changes. Successful probiotic intervention in AAD might therefore be directed at the problem of reduced production of short-chain fatty acids that occurs after antibiotic treatment.

Acknowledgements

We thank the staff of the General Hospital Oberpullendorf. The project was funded by the Austrian Science Fund FWF.

References

AHMER, B. M. & GUNN, J. S. (2011) Interaction of Salmonella spp. with the Intestinal Microbiota. *Front Microbiol,* 2**,** 101.

ALCALA, L., SANCHEZ-CAMBRONERO, L., CATALAN, M. P., SANCHEZ-SOMOLINOS, M., PELAEZ, M. T., MARIN, M. & BOUZA, E. (2008) Comparison

of three commercial methods for rapid detection of Clostridium difficile toxins A and B from fecal specimens. *J Clin Microbiol,* 46**,** 3833-5.

ARUMUGAM, M., RAES, J., PELLETIER, E., LE PASLIER, D., YAMADA, T., MENDE, D. R., FERNANDES, G. R., TAP, J., BRULS, T., BATTO, J. M., BERTALAN, M., BORRUEL, N., CASELLAS, F., FERNANDEZ, L., GAUTIER, L., HANSEN, T., HATTORI, M., HAYASHI, T., KLEEREBEZEM, M., KUROKAWA, K., LECLERC, M., LEVENEZ, F., MANICHANH, C., NIELSEN, H. B., NIELSEN, T., PONS, N., POULAIN, J., QIN, J., SICHERITZ-PONTEN, T., TIMS, S., TORRENTS, D., UGARTE, E., ZOETENDAL, E. G., WANG, J., GUARNER, F., PEDERSEN, O., DE VOS, W. M., BRUNAK, S., DORE, J., ANTOLIN, M., ARTIGUENAVE, F., BLOTTIERE, H. M., ALMEIDA, M., BRECHOT, C., CARA, C., CHERVAUX, C., CULTRONE, A., DELORME, C., DENARIAZ, G., DERVYN, R., FOERSTNER, K. U., FRISS, C., VAN DE GUCHTE, M., GUEDON, E., HAIMET, F., HUBER, W., VAN HYLCKAMA-VLIEG, J., JAMET, A., JUSTE, C., KACI, G., KNOL, J., LAKHDARI, O., LAYEC, S., LE ROUX, K., MAGUIN, E., MERIEUX, A., MELO MINARDI, R., M'RINI, C., MULLER, J., OOZEER, R., PARKHILL, J., RENAULT, P., RESCIGNO, M., SANCHEZ, N., SUNAGAWA, S., TORREJON, A., TURNER, K., VANDEMEULEBROUCK, G., VARELA, E., WINOGRADSKY, Y., ZELLER, G., WEISSENBACH, J., EHRLICH, S. D. & BORK, P. (2011) Enterotypes of the human gut microbiome. *Nature,* 473**,** 174-80.

BACKHED, F., DING, H., WANG, T., HOOPER, L. V., KOH, G. Y., NAGY, A., SEMENKOVICH, C. F. & GORDON, J. I. (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A,* 101**,** 15718-23.

BACKHED, F., LEY, R. E., SONNENBURG, J. L., PETERSON, D. A. & GORDON, J. I. (2005) Host-bacterial mutualism in the human intestine. *Science,* 307**,** 1915-20.

BARMAN, M., UNOLD, D., SHIFLEY, K., AMIR, E., HUNG, K., BOS, N. & SALZMAN, N. (2008) Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infect Immun,* 76**,** 907-15.

BARTLETT, C. L. (1987) Efficacy of different surveillance systems in detecting hospital-acquired infections. *Chemioterapia,* 6**,** 152-5.

BIAN, L., NAGATA, S., ASAHARA, T., RAHMAN, M. S., OHTA, T., YUKI, N., WANG, C., TAKANO, K., DAIBO, M., NOMOTO, K. & YAMASHIRO, Y. (2011) Effects of the continuous intake of Lactobacillus casei strain Shirota-fermented milk on risk management of long-term inpatients at health service facilities for the elderly. *International Journal of Probiotics and Prebiotics,* 6, 2**,** 123-132.

BREVES, G., FAUL, K., SCHRODER, B., HOLST, H., CASPARY, W. F. & STEIN, J. (2000) Application of the colon-simulation technique for studying the effects of Saccharomyces boulardii on basic parameters of porcine cecal microbial metabolism disturbed by clindamycin. *Digestion,* 61**,** 193-200.

CANANI, R. B., COSTANZO, M. D., LEONE, L., PEDATA, M., MELI, R. & CALIGNANO, A. (2011) Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J Gastroenterol,* 17**,** 1519-28.

CAPORASO, J. G., LAUBER, C. L., COSTELLO, E. K., BERG-LYONS, D., GONZALEZ, A., STOMBAUGH, J., KNIGHTS, D., GAJER, P., RAVEL, J., FIERER, N., GORDON, J. I. & KNIGHT, R. (2011) Moving pictures of the human microbiome. *Genome Biol,* 12**,** R50.

DE LA COCHETIERE, M. F., DURAND, T., LEPAGE, P., BOURREILLE, A., GALMICHE, J. P. & DORE, J. (2005) Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. *J Clin Microbiol,* 43**,** 5588-92.

DETHLEFSEN, L., ECKBURG, P. B., BIK, E. M. & RELMAN, D. A. (2006) Assembly of the human intestinal microbiota. *Trends Ecol Evol,* 21**,** 517-23.

DETHLEFSEN, L. & RELMAN, D. A. (2010) Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad Sci U S A,* 108 Suppl 1**,** 4554-61.

DETHLEFSEN, L. & RELMAN, D. A. (2011) Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad Sci U S A,* 108 Suppl 1**,** 4554-61.

EASTWOOD, K., ELSE, P., CHARLETT, A. & WILCOX, M. (2009) Comparison of nine commercially available Clostridium difficile toxin detection assays, a real-time PCR assay for C. difficile tcdB, and a glutamate dehydrogenase detection assay to cytotoxin testing and cytotoxigenic culture methods. *J Clin Microbiol,* 47**,** 3211-7.

ECKBURG, P. B., BIK, E. M., BERNSTEIN, C. N., PURDOM, E., DETHLEFSEN, L., SARGENT, M., GILL, S. R., NELSON, K. E. & RELMAN, D. A. (2005) Diversity of the human intestinal microbial flora. *Science,* 308**,** 1635-8.

FAO/WHO (2001) Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. . *Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria.* Córdoba, Argentina, Food and Agriculture Organization of the United Nations and World Health Organization.

FLINT, H. J., BAYER, E. A., RINCON, M. T., LAMED, R. & WHITE, B. A. (2008) Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat Rev Microbiol,* 6**,** 121-31.

FREDSTROM, S. B., LAMPE, J. W., JUNG, H. J. & SLAVIN, J. L. (1994) Apparent fiber digestibility and fecal short-chain fatty acid concentrations with ingestion of two types of dietary fiber. *JPEN J Parenter Enteral Nutr,* 18**,** 14-9.

GAO, X. W., MUBASHER, M., FANG, C. Y., REIFER, C. & MILLER, L. E. (2010) Dose-response efficacy of a proprietary probiotic formula of Lactobacillus acidophilus CL1285 and Lactobacillus casei LBC80R for antibiotic-associated diarrhea and Clostridium difficile-associated diarrhea prophylaxis in adult patients. *Am J Gastroenterol,* 105**,** 1636-41.

GUILLOTEAU, P., MARTIN, L., EECKHAUT, V., DUCATELLE, R., ZABIELSKI, R. & VAN IMMERSEEL, F. (2010) From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutr Res Rev,* 23**,** 366-84.

HAMER, H. M., JONKERS, D., VENEMA, K., VANHOUTVIN, S., TROOST, F. J. & BRUMMER, R. J. (2008) Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther,* 27**,** 104-19.

HENSGENS, M. P., GOORHUIS, A., VAN KINSCHOT, C. M., CROBACH, M. J., HARMANUS, C. & KUIJPER, E. J. (2011) Clostridium difficile infection in an endemic setting in the Netherlands. *Eur J Clin Microbiol Infect Dis,* 30**,** 587-93.

HICKSON, M. (2011) Probiotics in the prevention of antibiotic-associated diarrhoea and Clostridium difficile infection. *Therap Adv Gastroenterol,* 4**,** 185-97.

HICKSON, M., D'SOUZA, A. L., MUTHU, N., ROGERS, T. R., WANT, S., RAJKUMAR, C. & BULPITT, C. J. (2007) Use of probiotic Lactobacillus preparation to prevent

diarrhoea associated with antibiotics: randomised double blind placebo controlled trial. *BMJ,* 335**,** 80.

HIPPE, B., ZWIELEHNER, J., LISZT, K., LASSL, C., UNGER, F. & HASLBERGER, A. G. (2011) Quantification of butyryl CoA:acetate CoA-transferase genes reveals different butyrate production capacity in individuals according to diet and age. *FEMS Microbiol Lett,* 316**,** 130-5.

HOGENAUER, C., HAMMER, H. F., KREJS, G. J. & REISINGER, E. C. (1998) Mechanisms and management of antibiotic-associated diarrhea. *Clin Infect Dis,* 27**,** 702-10.

HUDAULT, S., GUIGNOT, J. & SERVIN, A. L. (2001) Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection. *Gut,* 49**,** 47-55.

JAKOBSSON, H. E., JERNBERG, C., ANDERSSON, A. F., SJOLUND-KARLSSON, M., JANSSON, J. K. & ENGSTRAND, L. (2010) Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One,* 5**,** e9836.

KUEHNE, S. A., CARTMAN, S. T., HEAP, J. T., KELLY, M. L., COCKAYNE, A. & MINTON, N. P. (2010) The role of toxin A and toxin B in Clostridium difficile infection. *Nature,* 467**,** 711-3.

KUROKAWA, K., ITOH, T., KUWAHARA, T., OSHIMA, K., TOH, H., TOYODA, A., TAKAMI, H., MORITA, H., SHARMA, V. K., SRIVASTAVA, T. P., TAYLOR, T. D., NOGUCHI, H., MORI, H., OGURA, Y., EHRLICH, D. S., ITOH, K., TAKAGI, T., SAKAKI, Y., HAYASHI, T. & HATTORI, M. (2007) Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. *DNA Res,* 14**,** 169-81.

LAWLEY, T. D., CLARE, S., WALKER, A. W., GOULDING, D., STABLER, R. A., CROUCHER, N., MASTROENI, P., SCOTT, P., RAISEN, C., MOTTRAM, L., FAIRWEATHER, N. F., WREN, B. W., PARKHILL, J. & DOUGAN, G. (2009) Antibiotic treatment of clostridium difficile carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect Immun,* 77**,** 3661-9.

LAY, C., SUTREN, M., ROCHET, V., SAUNIER, K., DORE, J. & RIGOTTIER-GOIS, L. (2005) Design and validation of 16S rRNA probes to enumerate members of the Clostridium leptum subgroup in human faecal microbiota. *Environ Microbiol,* 7**,** 933-46.

LAYTON, A., MCKAY, L., WILLIAMS, D., GARRETT, V., GENTRY, R. & SAYLER, G. (2006) Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. *Appl Environ Microbiol,* 72**,** 4214-24.

LEDERBERG, J. (2000) Infectious history. *Science,* 288**,** 287-93.

LEWIS, J. D. N., THOMAS, L. V. & WEIR, W. (2009) The potential of probiotic fermented milk products in reducing risk of antibiotic-associated diarrhoea and Clostridium difficile disease. *Int J Dairy Technol,* 62 (4)**,** 461-471.

LEY, R. E., PETERSON, D. A. & GORDON, J. I. (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell,* 124**,** 837-48.

LIN, C. K., HUNG, C. L., CHIANG, Y. C., LIN, C. M. & TSEN, H. Y. (2004) The sequence heterogenicities among 16S rRNA genes of Salmonella serovars and the effects on the specificity of the primers designed. *Int J Food Microbiol,* 96**,** 205-14.

LIN, C. K. & TSEN, H. Y. (1996) Use of two 16S DNA targeted oligonucleotide as PCR primers for the specific detection of Salmonella in foods. *J. Appl. Microbiol.,* 80**,** 659-666.

LOUIS, P. & FLINT, H. J. (2007) Development of a semiquantitative degenerate real-time pcr-based assay for estimation of numbers of butyryl-coenzyme A (CoA) CoA transferase genes in complex bacterial samples. *Appl Environ Microbiol,* 73**,** 2009-12.

LOUIS, P., SCOTT, K. P., DUNCAN, S. H. & FLINT, H. J. (2007) Understanding the effects of diet on bacterial metabolism in the large intestine. *J Appl Microbiol,* 102**,** 1197-208.

LUNA, R. A., BOYANTON, B. L., JR., MEHTA, S., COURTNEY, E. M., WEBB, C. R., REVELL, P. A. & VERSALOVIC, J. (2011) Rapid stool-based diagnosis of Clostridium difficile infection by real-time PCR in a children's hospital. *J Clin Microbiol,* 49**,** 851-7.

MAHAJAN, A. & GALLY, D. (2011) Escherichia coli and the mucosal immune system. *Expert Rev Clin Immunol,* 7**,** 743-5.

MARIAT, D., FIRMESSE, O., LEVENEZ, F., GUIMARAES, V., SOKOL, H., DORE, J., CORTHIER, G. & FURET, J. P. (2009) The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol,* 9**,** 123.

MARTINEZ, C. O., PAZ, V. S. & O'DONNELL, A. (2003) Lactobacillus Casei Shirota Prevention Approach for Healthy Children with Mild Acute Infection Treated with Antibiotics. *Ped Res,* 4(2) S**,** 174A-174A.

MATSUDA, K., TSUJI, H., ASAHARA, T., MATSUMOTO, K., TAKADA, T. & NOMOTO, K. (2009) Establishment of an Analytical System for the Human Fecal Microbiota, Based on Reverse Transcription-Quantitative PCR Targeting of Multicopy rRNA Molecules. *Applied and Environmental Microbiology,* 75**,** 1961-1969.

MATSUKI, T., WATANABE, K., FUJIMOTO, J., TAKADA, T. & TANAKA, R. (2004) Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl Environ Microbiol,* 70**,** 7220-8.

MCFARLAND, L. V. (2006) Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of Clostridium difficile disease. *Am J Gastroenterol,* 101**,** 812-22.

MCFARLAND, L. V. (2010) Systematic review and meta-analysis of Saccharomyces boulardii in adult patients. *World J Gastroenterol,* 16**,** 2202-22.

MCFARLAND, L. V., BENEDA, H. W., CLARRIDGE, J. E. & RAUGI, G. J. (2007) Implications of the changing face of Clostridium difficile disease for health care practitioners. *Am J Infect Control,* 35**,** 237-53.

MEIER, H., AMANN, R., LUDWIG, W. & SCHLEIFER, K. H. (1999) Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G + C content. *Syst Appl Microbiol,* 22**,** 186-96.

MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol,* 59**,** 695-700.

MUYZER, G. & SMALLA, K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek,* 73**,** 127-41.

NADAL, I., SANTACRUZ, A., MARCOS, A., WARNBERG, J., GARAGORRI, M., MORENO, L. A., MARTIN-MATILLAS, M., CAMPOY, C., MARTI, A., MOLERES, A., DELGADO, M., VEIGA, O. L., GARCIA-FUENTES, M., REDONDO, C. G. & SANZ, Y. (2009) Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents. *Int J Obes (Lond),* 33**,** 758-67.

NAGATA, S., ASAHARA, T., OHTA, T., YAMADA, T., KONDO, S., BIAN, L., WANG, C., YAMASHIRO, Y. & NOMOTO, K. (2011) Effect of the continuous intake of probiotic-fermented milk containing Lactobacillus casei strain Shirota on fever in a mass outbreak of norovirus gastroenteritis and the faecal microflora in a health service facility for the aged. *Br J Nutr,* 106**,** 549-56.

NEEFS, J. M., VAN DE PEER, Y., DE RIJK, P., GORIS, A. & DE WACHTER, R. (1991) Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Res,* 19 Suppl**,** 1987-2015.

NG, S. C., HART, A. L., KAMM, M. A., STAGG, A. J. & KNIGHT, S. C. (2009) Mechanisms of action of probiotics: recent advances. *Inflamm Bowel Dis,* 15**,** 300-10.

OELSCHLAEGER, T. A. (2010) Mechanisms of probiotic actions - A review. *Int J Med Microbiol,* 300**,** 57-62.

OOTSUBO, M., SHIMIZU, T., TANAKA, R., SAWABE, T., TAJIMA, K., YOSHIMIZU, M., EZURA, Y., EZAKI, T. & OYAIZU, H. (2002) Oligonucleotide probe for detecting Enterobacteriaceae by in situ hybridization. *J Appl Microbiol,* 93**,** 60-8.

OZAKI, E., KATO, H., KITA, H., KARASAWA, T., MAEGAWA, T., KOINO, Y., MATSUMOTO, K., TAKADA, T., NOMOTO, K., TANAKA, R. & NAKAMURA, S. (2004) Clostridium difficile colonization in healthy adults: transient colonization and correlation with enterococcal colonization. *J Med Microbiol,* 53**,** 167-72.

PENDERS, J., VINK, C., DRIESSEN, C., LONDON, N., THIJS, C. & STOBBERINGH, E. E. (2005) Quantification of Bifidobacterium spp., Escherichia coli and

Clostridium difficile in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett,* 243**,** 141-7.

PRYDE, S. E., DUNCAN, S. H., HOLD, G. L., STEWART, C. S. & FLINT, H. J. (2002) The microbiology of butyrate formation in the human colon. *FEMS Microbiol Lett,* 217**,** 133-9.

QIN, J., LI, R., RAES, J., ARUMUGAM, M., BURGDORF, K. S., MANICHANH, C., NIELSEN, T., PONS, N., LEVENEZ, F., YAMADA, T., MENDE, D. R., LI, J., XU, J., LI, S., LI, D., CAO, J., WANG, B., LIANG, H., ZHENG, H., XIE, Y., TAP, J., LEPAGE, P., BERTALAN, M., BATTO, J. M., HANSEN, T., LE PASLIER, D., LINNEBERG, A., NIELSEN, H. B., PELLETIER, E., RENAULT, P., SICHERITZ-PONTEN, T., TURNER, K., ZHU, H., YU, C., JIAN, M., ZHOU, Y., LI, Y., ZHANG, X., QIN, N., YANG, H., WANG, J., BRUNAK, S., DORE, J., GUARNER, F., KRISTIANSEN, K., PEDERSEN, O., PARKHILL, J., WEISSENBACH, J., BORK, P. & EHRLICH, S. D. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. *Nature,* 464**,** 59-65.

RAKOFF-NAHOUM, S., PAGLINO, J., ESLAMI-VARZANEH, F., EDBERG, S. & MEDZHITOV, R. (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell,* 118**,** 229-41.

RASTALL, R. A. (2004) Bacteria in the gut: friends and foes and how to alter the balance. *J Nutr,* 134**,** 2022S-2026S.

RASTALL, R. A., GIBSON, G. R., GILL, H. S., GUARNER, F., KLAENHAMMER, T. R., POT, B., REID, G., ROWLAND, I. R. & SANDERS, M. E. (2005) Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: an overview of enabling science and potential applications. *FEMS Microbiol Ecol,* 52**,** 145-52.

REUTER, G. (2001) The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and succession. *Curr Issues Intest Microbiol,* 2**,** 43-53.

RUSSELL, D. A., ROSS, R. P., FITZGERALD, G. F. & STANTON, C. (2011) Metabolic activities and probiotic potential of bifidobacteria. *Int J Food Microbiol,* 149**,** 88-105.

SEKSIK, P., RIGOTTIER-GOIS, L., GRAMET, G., SUTREN, M., POCHART, P., MARTEAU, P., JIAN, R. & DORE, J. (2003) Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. *Gut,* 52**,** 237-42.

SNYDMAN, D. R. (2008) The safety of probiotics. *Clin Infect Dis,* 46 Suppl 2**,** S104-11; discussion S144-51.

SOMMER, M. O. & DANTAS, G. (2011) Antibiotics and the resistant microbiome. *Curr Opin Microbiol*.

SONG, Y., LIU, C. & FINEGOLD, S. M. (2004) Real-time PCR quantitation of clostridia in feces of autistic children. *Appl Environ Microbiol,* 70**,** 6459-65.

STOCKENHUBER, A., ADELMANN, K., KAMHUBER, C., HÖRIST-KOLLMANN, S., REK, A., ECKHARDT, G., G., L. & STOCKENHUBER, F. (2008) Preventing diarrhoea associated with antibiotics using a probiotic Lactobacillus casei preparation. *Gut,* 57.

TAP, J., MONDOT, S., LEVENEZ, F., PELLETIER, E., CARON, C., FURET, J. P., UGARTE, E., MUNOZ-TAMAYO, R., PASLIER, D. L., NALIN, R., DORE, J. & LECLERC, M. (2009) Towards the human intestinal microbiota phylogenetic core. *Environ Microbiol,* 11**,** 2574-84.

THOMPSON-CHAGOYAN, O. C., MALDONADO, J. & GIL, A. (2007) Colonization and impact of disease and other factors on intestinal microbiota. *Dig Dis Sci,* 52**,** 2069-77.

TOPPING, D. L. & CLIFTON, P. M. (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol Rev,* 81**,** 1031-64.

TURNBAUGH, P. J. & GORDON, J. I. (2009) The core gut microbiome, energy balance and obesity. *J Physiol,* 587**,** 4153-8.

TURNBAUGH, P. J., HAMADY, M., YATSUNENKO, T., CANTAREL, B. L., DUNCAN, A., LEY, R. E., SOGIN, M. L., JONES, W. J., ROE, B. A., AFFOURTIT, J. P., EGHOLM, M., HENRISSAT, B., HEATH, A. C., KNIGHT, R. & GORDON, J. I. (2009) A core gut microbiome in obese and lean twins. *Nature,* 457**,** 480-4.

TURNBAUGH, P. J., LEY, R. E., HAMADY, M., FRASER-LIGGETT, C. M., KNIGHT, R. & GORDON, J. I. (2007) The human microbiome project. *Nature,* 449**,** 804-10.

TURRONI, F., RIBBERA, A., FORONI, E., VAN SINDEREN, D. & VENTURA, M. (2008) Human gut microbiota and bifidobacteria: from composition to functionality. *Antonie Van Leeuwenhoek,* 94**,** 35-50.

VAN DER WAAIJ, L. A., HARMSEN, H. J., MADJIPOUR, M., KROESE, F. G., ZWIERS, M., VAN DULLEMEN, H. M., DE BOER, N. K., WELLING, G. W. & JANSEN, P. L. (2005) Bacterial population analysis of human colon and terminal ileum biopsies with 16S rRNA-based fluorescent probes: commensal bacteria live in suspension and have no direct contact with epithelial cells. *Inflamm Bowel Dis,* 11**,** 865-71.

VERNA, E. C. & LUCAK, S. (2010) Use of probiotics in gastrointestinal disorders: what to recommend? *Therap Adv Gastroenterol,* 3(5)**,** 307-19.

WALLACE, T. C., GUARNER, F., MADSEN, K., CABANA, M. D., GIBSON, G., HENTGES, E. & SANDERS, M. E. (2011) Human gut microbiota and its relationship to health and disease. *Nutr Rev,* 69**,** 392-403.

WALTER, J., HERTEL, C., TANNOCK, G. W., LIS, C. M., MUNRO, K. & HAMMES, W. P. (2001) Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl Environ Microbiol,* 67**,** 2578-85.

WALTER, J. & LEY, R. E. (2011) The Human Gut Microbiome: Ecology and Recent Evolutionary Changes. *Annu Rev Microbiol*.

WALTON, G. E., VAN DEN HEUVEL, E. G., KOSTERS, M. H., RASTALL, R. A., TUOHY, K. M. & GIBSON, G. R. (2011) A randomised crossover study investigating the effects of galacto-oligosaccharides on the faecal microbiota in men and women over 50 years of age. *Br J Nutr***,** 1-10.

WANG, X., HEAZLEWOOD, S. P., KRAUSE, D. O. & FLORIN, T. H. (2003) Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. *J Appl Microbiol,* 95**,** 508-20.

WENUS, C., GOLL, R., LOKEN, E. B., BIONG, A. S., HALVORSEN, D. S. & FLORHOLMEN, J. (2008) Prevention of antibiotic-associated diarrhoea by a fermented probiotic milk drink. *Eur J Clin Nutr,* 62**,** 299-301.

WHELAN, K. & MYERS, C. E. (2010) Safety of probiotics in patients receiving nutritional support: a systematic review of case reports, randomized controlled trials, and nonrandomized trials. *Am J Clin Nutr,* 91**,** 687-703.

WOO, P. C., LEUNG, P. K., LEUNG, K. W. & YUEN, K. Y. (2000) Identification by 16S ribosomal RNA gene sequencing of an Enterobacteriaceae species from a bone marrow transplant recipient. *Mol Pathol,* 53**,** 211-5.

YU, Y., LEE, C., KIM, J. & HWANG, S. (2005) Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng,* 89**,** 670-9.

ZHU, B., WANG, X. & LI, L. (2010) Human gut microbiome: the second genome of human body. *Protein Cell,* 1**,** 718-25.

ZOETENDAL, E. G., VAUGHAN, E. E. & DE VOS, W. M. (2006) A microbial world within us. *Mol Microbiol,* 59**,** 1639-50.

ZWIELEHNER, J., LISZT, K., HANDSCHUR, M., LASSL, C., LAPIN, A. & HASLBERGER, A. G. (2009) Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and Clostridium cluster IV in institutionalized elderly. *Exp Gerontol,* 44**,** 440-6.

Group	Treatment and/or intervention
AP	Antibiotic treatment and intake of a probiotic drink containing *Lactobacillus casei* Shirota
A	Antibiotic treatment only
P	intake of a probiotic drink containing *Lactobacillus casei* Shirota
C	control group

Table 1 Characterization of participant groups.

Target organism	Primer/Probe	Sequence (5' - 3')	Size (bp)	Conc. [pmol/µL]	Reference
All Bacteria	BAC-338-F	ACT CCT ACG GGA GGC AG	468	10	(Yu et al., 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	
***Clostridium* cluster IV (*Clostridium leptum*)**	sg-Clept-F	GCA CAA GCA GTG GAG T	239	4	(Matsuki et al., 2004)
	sg-Clept-R	CTT CCT CCG TTT TGT CAA		4	
	Clept-Pa	(FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		2	(Zwielehner et al., 2009)
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	
***Bifidobacterium* 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	
***Clostridium* cluster XIVa (Lachnospiraceae)**	195-F	GCA GTG GGG AAT ATT GCA	538	7	(Meier et al., 1999)
	Ccocc-R	CTT TGA GTT TCA TTC TTG CGA A		7	(Matsuki et al., 2004)
	Ccocc-P	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)		1.5	

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

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

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

	A (no intervention)	AP (intervention)
Euroclone® *C. difficile* 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)	

Table 4 Group distribution concerning AAD and CDI (n = sample size).

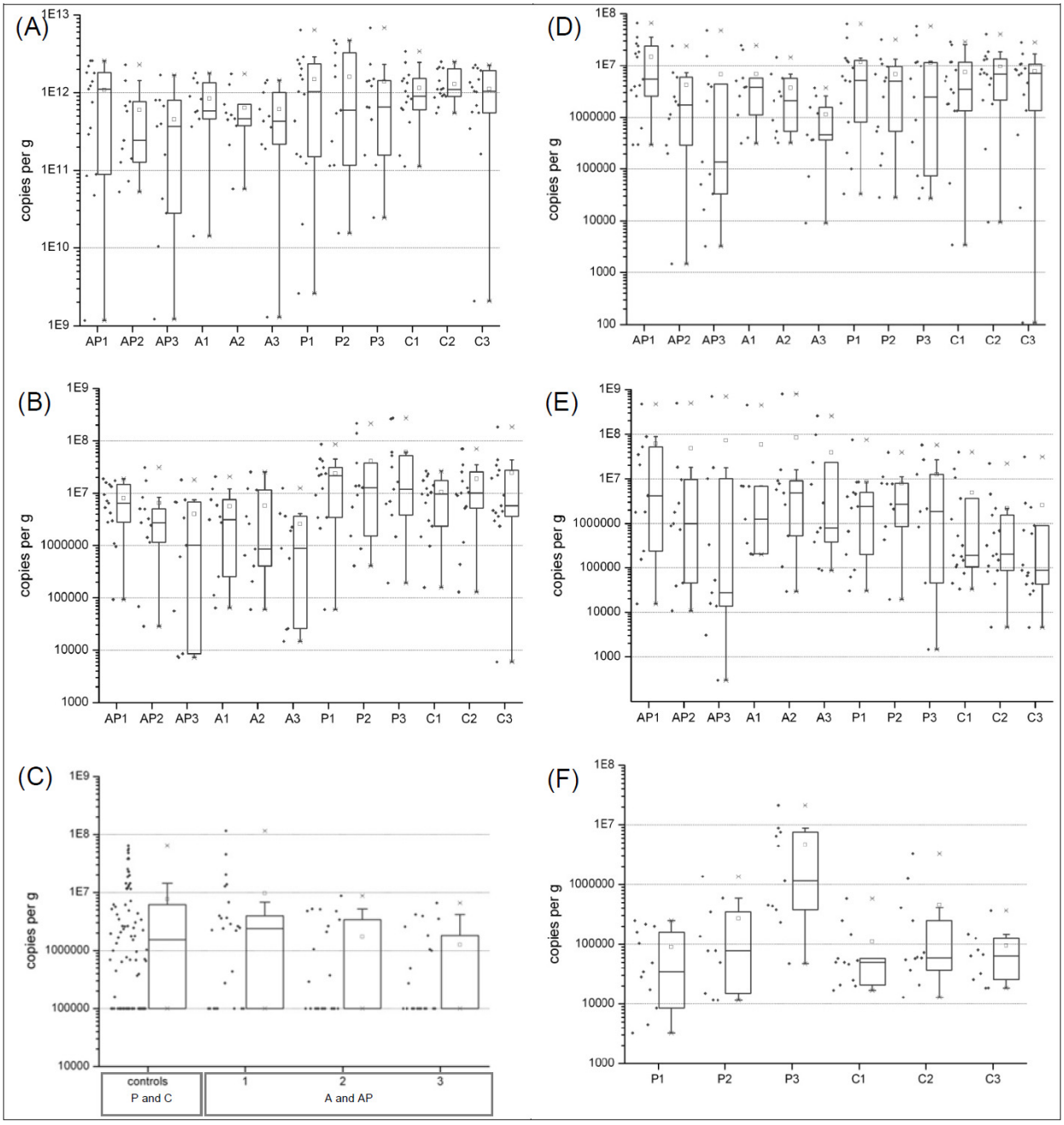


Figure 1 16S rRNA qPCR of (A) total Bacteria, (B) *Clostridium* Cluster IV, (C) *Clostridium* cluster XI, (D) *Bifidobacterium* spp., (E) Enterobacteriaceae, (F) *Lactobacillus* spp. (A: antibiotic treatment, AP: antibiotic treatment and intake of a probiotic drink containing *L.casei* Shirota, P: intake of a probiotic drink containing *L.casei* Shirota, C: control group; 1: day 0, 2: day 3, 3: day 5).

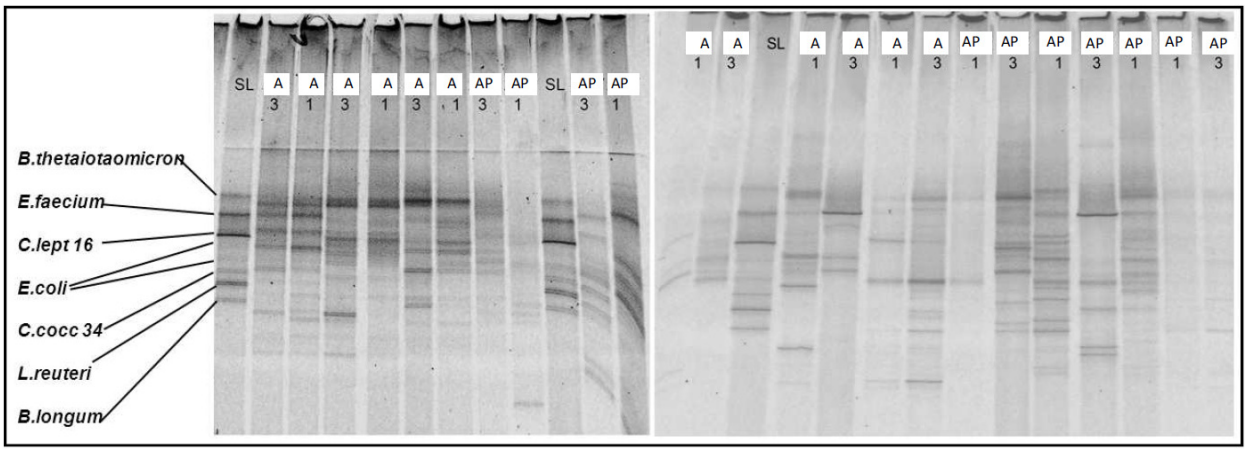


Figure 2 PCR-DGGE fingerprinting of16S rRNA coding regions amplified with primer pair 341GC-518. (A: antibiotic treatment, AP: antibiotic treatment and intake of a probiotic drink containing *L.casei* Shirota, P: intake of a probiotic drink containing *L.casei* Shirota, C: control group; 1: day 0, 3: day 5).

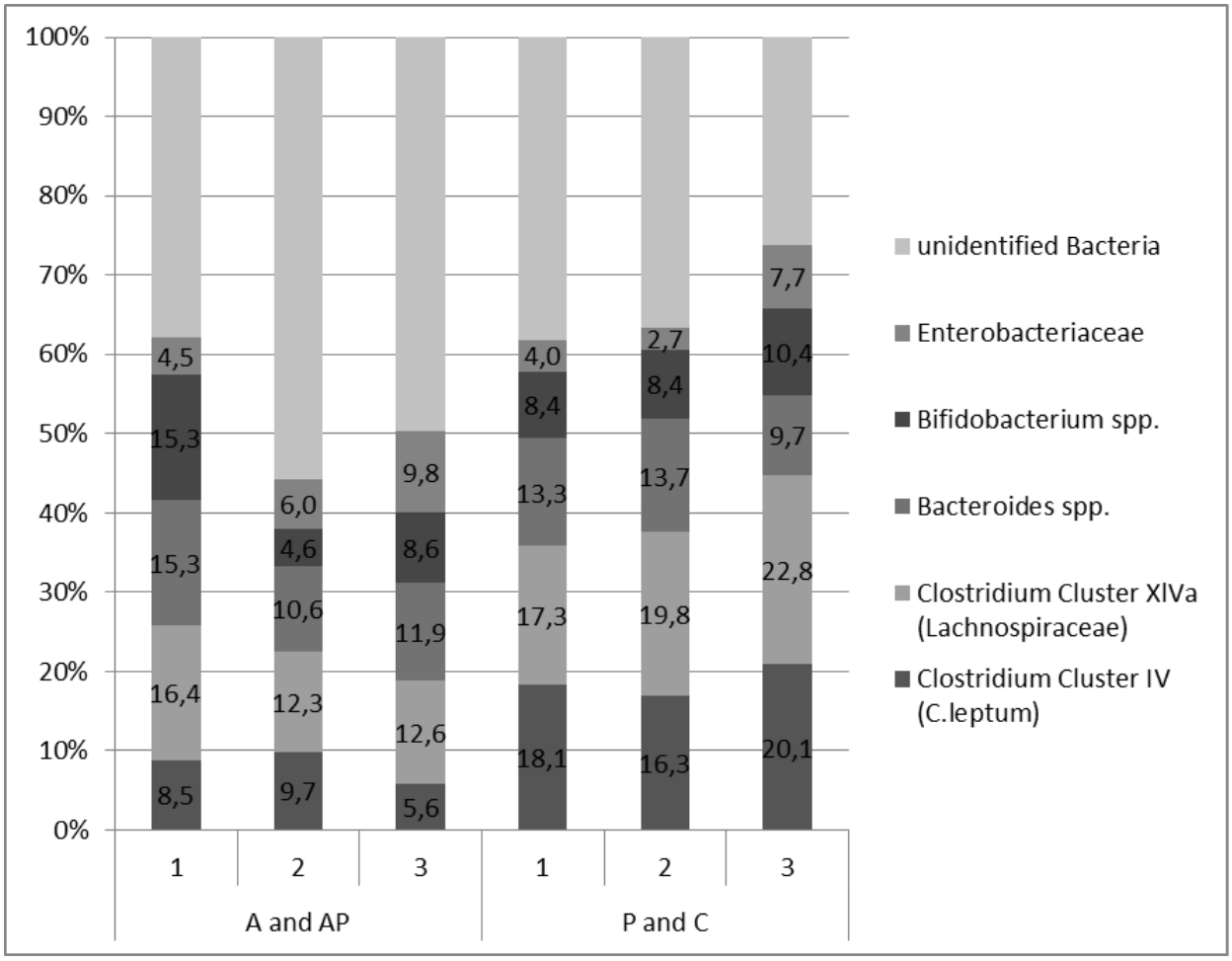


Figure 3 Percentage of bacterial subgroups in relation to the analysed Bacteria. In patients under antibiotic treatment, there were more bacteria which cannot be identified by the primers used in this study. (A: antibiotic treatment, AP: antibiotic treatment and intake of a probiotic drink containing *L.casei* Shirota, P: intake of a probiotic drink containing *L.casei* Shirota, C: control group; 1: day 0, 2: day 3, 3: day 5).

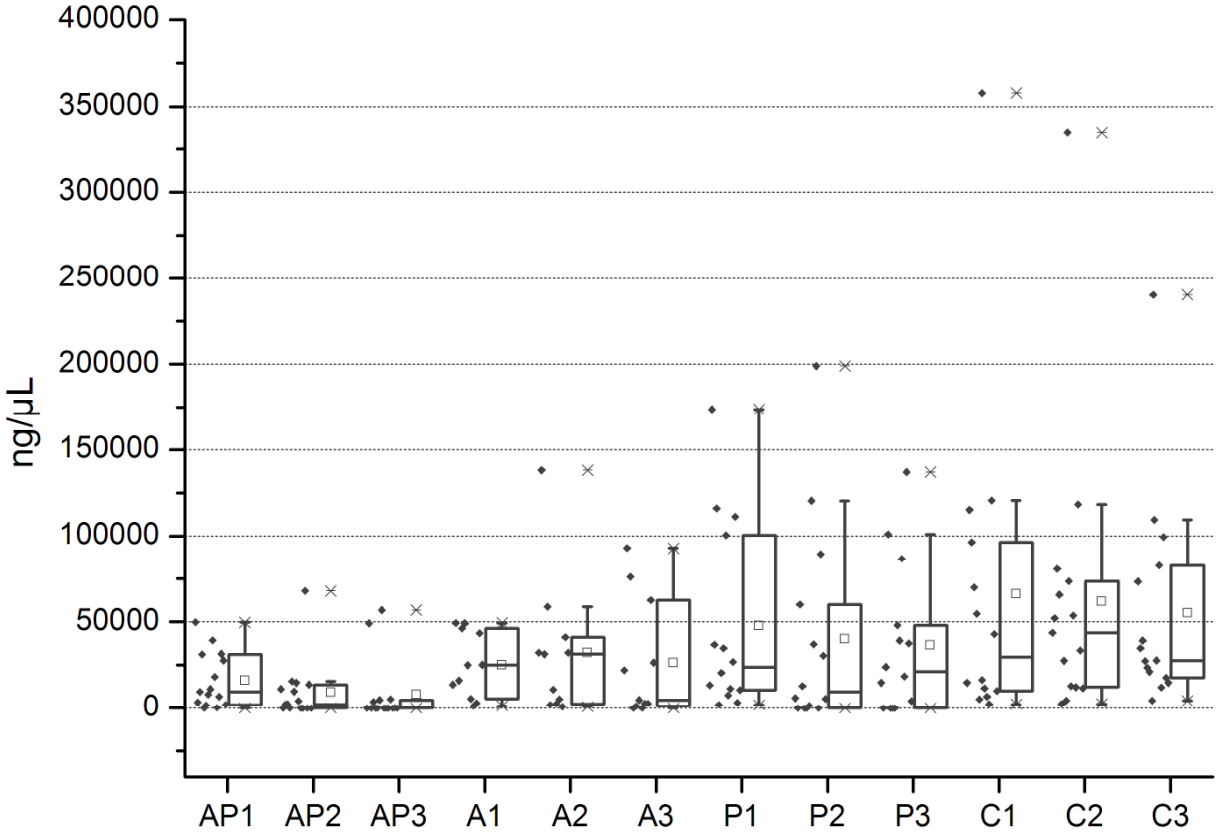


Figure 4 Abundance of butyryl-CoA CoA transferase genes in group A (antibiotic treatment), AP (antibiotic treatment and intake of a probiotic drink containing *L.casei* Shirota), P (intake of a probiotic drink containing *L.casei* Shirota) and C (control group) (1: day 0, 2: day 5, 3: day 5). Amplification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

13.2. **Poster presentation:** 6th International Yakult Symposium 2011: The Gut and its Role in Health Maintenance (Vienna)

universität wien

Effects of *L.casei* Shirota on gastrointestinal microbiota during antibiotic therapy

Pirker A., Hippe B., Remely M., Harrant A., Kamhuber C., Stockenhuber F., Haslberger A.G.
alexander.haslberger@univie.ac.at Department of Nutritional Science, Vienna, Austria

Recently, results from a clinical study reported a reduced appearance of antibiotic associated diarrhoea and immune diagnosed *C.difficile* infection by intervention with a *L.casei* Shirota based product (Stockenhuber et al., 2008).

The hypothesis for a combined antibiotic/probiotic therapy is "that the antibiotic kills vegetative *C.difficile* organisms in the GI tract, which would clear the pathogenic toxins, and the probiotics would assist in reestablishing the protective intestinal microbiota so that residual spores may germinate, colonization is rebuffed by the newly restored microbiota barrier" (McFarland and Dublin, 2008).

Two potent toxins released by *C.difficile* are suspected as main cause of antibiotic associated diarrhea.

Objectives: In the present study effects of a combination therapy antibiotic/*L.casei* Shirota and controls should be tested for AAD, *C.difficile* and changes of fecal microbiota in a small group of 55 patients.

Methods: Stool samples from 4 groups (Table 1) of each 15 resp. 11 patients (aged 60 ± 22 years) were taken at three time points: before antibiotic treatment and/or intake of a probiotic drink containing *L.casei* Shirota (day 0) and two times during antibiotic treatment and/or intake of a probiotic drink containing *L.casei* Shirota (after 3 and 5 days). Samples of the control group are taken at similar time points.

Study design: GI microbiota, including *C.difficile*, were analyzed by real time PCR using 16S rRNA group specific primers, Euroclone® *C.difficile* A/B kit (showing 2 of 2 positive controls provided by AGES as positive), PCR/DGGE and a *C.difficile* ELISA test.

Group	characterization
AB	antibiotic treatment
ABY	antibiotic treatment and intake of L.casei Shirota
Y	intake of L. casei Shirota
C	control group

Table 1 characterization of participant groups.

Clostridium difficile

	AB	ABY
Euroclone® *C.difficile* A/B kit	1 (n=15)	0 (n=10)
16S rRNA *C.difficile* specific qPCR	2 (n=15)	1 (n=10)
***C.difficile* ELISA (CDI present study)**	5 (n=15)	0 (n=10)
Stockenhuber et al. (2008)	AAD: 63 (n=338) (18 %) CDI: 21	AAD: 17 (n=340) (5 %) CDI: 1

Table 2 Overview of investigations into *C.difficile*. (AAD (Antibiotic associated diarrhoea); CDI (*Clostridium Difficile* Infection))

Discussion:
C.difficile ELISA test is discussed as rather unspecific. In previous studies *C.difficile* ELISA have only been performed at the beginning of the therapy.
Previous results of our group suggest that 16S rRNA *C.difficile* specific qPCR is also detecting non toxin forming *C.difficile*.
Euroclone® *C.difficile* A/B kit specifically detects toxin producing *C.difficile*.

Effects on microbiota subgroups

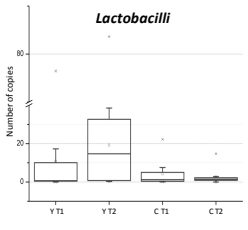


Figure 1 qPCR (SYBR® Green) using Primers sg-Lcas-F and sg-Lcas-R targeting 16S rRNA coding regions of *Lactobacilli*. (T1 = Δ timepoint 1 and 2; T2 = Δ timepoint 2 and 3)

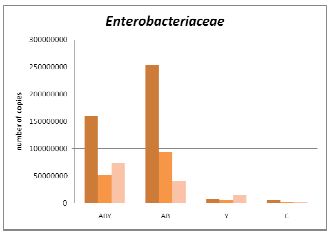


Figure 2 qPCR (SYBR® Green) using primers LPW69 and pB-00608r targeting 16S rRNA coding regions of *Entoerobacteriaceae*.

Discussion:
All bacteria: antibiotic therapy decreases total amount of bacteria.

Lactobacilli: The increase of copie numbers from timepoint 2 to 3 is significantly higher in group Y than in group C.

Enterobacteriaceae: Patients show a higher abundance of *Enterobacteriaceae* compared with controls, decreasing under therapy.

Diversity

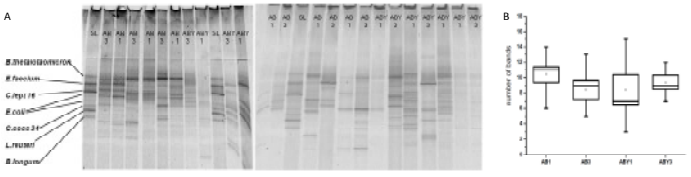


Figure 2 (A) PCR-DGGE fingerprinting of 16S rRNA coding regions amplified with primer pair 341GC-518 **(B)** number of bands of DGGE fingerprinting (SL (standard lane); 1 (day 0); 3 (day 5))

Discussion:
In group ABY in average two more bands can be seen as an increase from timepoint 1 to 3, in comparison to group AB. This suggestes a high diversity in the ABY group.

Conclusio: There are many evidences that *L.casei* Shirota has a protective effect on the emergence of antibiotic induced diarrhea AAD. For a significant evaluation of decreases of *C.difficile* with combination therapy at least 300 persons need to be analyzed using a A/B toxin specific qPCR addressing the emergence of *C.difficile* in AAD.

References:
KAZUNORI, M., HIROKAZU, T., TAKASHI, A., KAZUMASA, M., TOSHIHIKO, T. & KOJI, N. (2008) Establishment of an Analytical System for the Human FecalMicrobiota, Based on Reverse Transcription-QuantitativePCR Targeting of Multicopy rRNA Molecules. Yakult Central Institute for Microbiological Research. Tokyo 186-8650.
MCFARLAND, L. V., BENEDA, H. W., CLARRIDGE, J. E. & RAUGI, G. J. 2007. Implications of the changing face of Clostridium difficile disease for health care practitioners. *Am J Infect Control*, 35, 237-53.
MUYZER, G. & DE WAAL E.C. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol. 59, 695-700
OOTSUBO, M., SHIMIZU, T., TANAKA, R., SAWABE, T., TAJIMA, K., YOSHIMIZU, M., EZURA, Y., EZAKI, T. & OYAIZU, H. (2002) Oligonucleotide probe for detecting Enterobacteriaceae by in situ hybridization. J Appl Microbiol, 93, 60-8.
STOCKENHUBER, A., ADELMANN, K., KAMHUBER, C., HÖRIST-KOLLMANN, S., REK, A., ECKHARDT, G., LEEB, G., & STOCKENHUBER, F. (2008). Preventing antibiotic associated diarrhea using a probiotic Lactobacillus casei preparation. Gut 57 Suppl II: A-20
WOO, P. C., LEUNG, P. K., LEUNG, K. W. & YUEN, K. Y. (2000) Identification by 16S ribosomal RNA gene sequencing of an Enterobacteriaceae species from a bone marrow transplant recipient. *Mol Pathol*, 53, 211-5.

Abstract:

Changes in fecal microbiota underlie reduced C.difficile after intervention with L.casei Shirota in antibiotic diarrhea.

Angelika Kolle, Berit Hippe, Felix Stockenhuber, Alexander G. Haslberger

Department for Nutrition Sciences, University of Vienna, KH Oberpullendorf, Austria. alexander.haslberger@univie.ac.at

Introduction

Recently, results from a clinical study reported a reduced appearance of antibiotic associated diarrhea (AAD, 18 % : 5 %) and immune diagnosed *C.difficile* infection (CDI, 21:1 patient) by intervention with a *L.casei* Shirota based product (Y).

Methods

To investigate changes of GI microbiota underlying the reduced development of *C.difficile*, 4 groups of each 15 patients each were investigated for *C.difficile* toxin and changes of bacterial groups in fecal microbiota. The abundance of total bacteria, *Lactobacillus*, *Bifidobacteria*, *Bacteroides*, *C.* cluster IV, *C.* cluster XIV, *C.difficile*, and *Enterobacteriaceae* using qPCR with 16S group specific primers was analyzed. Samples were taken before, and 3 and 5 days after start of the antibiotic treatment. Groups consisted of controls, controls receiving the *L.casei* product (Y), patients receiving various antibiotic treatments and patients receiving various antibiotic treatments in combination with Y.

Results

Comparable to the previous clinical study, about 20 % of the antibiotic group (AB) patients were positive for *C.difficile* toxin. Furthermore, one patient of each control group showed *C.difficile* toxin. qPCR analysis detected *C.difficile* in 3 out of15 patients receiving AB. In one patient who showed *C.difficile* at the start of AB therapy, no *C.difficile* could be detected after AB plus Y therapy. Abundance of *Lactobacillus* increase in groups receiving Y (Y, AB+Y: >200 %, sign). In general, patients receiving AB showed an impaired abundance of bacteria and *Lactobacillus* at the start of the therapy. AB therapy further decrease the abundance of total bacteria (- 40 %) compared

to the abundance before AB therapy and compared to healthy controls. Patients receiving AB therapy, showed already before begin of the therapy a significant increased abundance of *Enterobacteriaceae* compared to healthy controls (73 %). In the AB plus Y group increased *Enterobacteriaceae* were strongly decreased due to Y therapy.

Discussion

Four groups of 15 patients each were analyzed for changes in fecal microbiota at time points before and after AB using 16S based qPCR. Results confirm previous clinical results on the occurrence of *C.difficile* in patients receiving AB because of gastrointestinal disturbance. However, the significance of the detection of *C.difficile* might be biased by low patient numbers. Furthermore, *C.difficile* toxin test can be biased for toxin production. The *C.difficile* qPCR using group specific primers is discussed for detection limits and might also detect non-toxic strains. Pyrosequencing of cloned samples should therefore be done. 16S based qPCR of fecal samples can detect changes in fecal microbiota as indicated by decreased bacteria and increased abundance of *Lactobacillus* in Y groups. However, quantitative assessment with qPCR should be assessed by analysis of the bacterial group diversity. Especially the striking changes of *Enterobacteriaceae* need a more specific analysis addressing diversity and subgroups of *Enterobacteriaceae*.

Conclusion

Clinical results with AB and AB plus Y are reflected by changes in GI microbiota.

Reference

Stockenhuber A. et al. (2008). Preventing antibiotic associated diarrhea using a probiotic *Lactobacillus casei* preparation. Gut 57 Suppl II:A2

13.3. **Poster presentation:** 2nd Internal Symposium microbes for health 2011 (Paris)

universität wien

Effects of antibiotic therapy on the gastrointestinal microbiota and the intervention with *L.casei*

Pirker A., Hippe B., Remely M., Harrant A., Kamhuber C., Stockenhuber F., Haslberger A.G.
alexander.haslberger@univie.ac.at Department of Nutritional Science, Vienna, Austria

Recently, results from a clinical study reported a reduced appearance of antibiotic associated diarrhoea and immune diagnosed *C.difficile* infection by intervention with a *L.casei* Shirota based product (Stockenhuber et al., 2008).

The hypothesis for a combined antibiotic/probiotic therapy is "that the antibiotic kills vegetative *C.difficile* organisms in the GI tract, which would clear the pathogenic toxins, and the probiotics would assist in reestablishing the protective intestinal microbiota so that residual spores may germinate, colonization is rebuffed by the newly restored microbiota barrier" (McFarland, 2006).

Two potent toxins released by *C.difficile* are suspected as main cause of antibiotic associated diarrhea.

Objectives: In the present study effects of a combined antibiotic/*L.casei* Shirota therapy and controls should be tested for Antibiotic Associated Diarrhea (AAD), *C.difficile* and changes of fecal.

Methods: Stool samples from 4 groups (Table 1) of each 15 resp. 11 patients (aged 60 ± 22 years) were taken at three time points: before antibiotic treatment and/or intake of a probiotic drink containing *L.casei* Shirota (day 0) and two times during antibiotic treatment and/or intake of a probiotic drink containing *L.casei* Shirota (after 3 and 5 days). Samples of the control group are taken at similar time points.

Study design: GI microbiota, including *C.difficile*, were analyzed by real time PCR using 16S rRNA group specific primers, Euroclone® *C.difficile* A/B kit (showing 2 of 2 positive controls provided by AGES as positive), PCR/DGGE and a *C.difficile* ELISA test.

Group	characterization
AB	antibiotic treatment
ABY	antibiotic treatment and intake of *L.casei* Shirota
Y	intake of *L. casei* Shirota
C	control group

Table 1 characterization of participant groups.

Clostridium difficile

	AB	ABY
Euroclone® *C.difficile* A/B kit	1 (n=15)	0 (n=11)
C.difficile ELISA (CDI)	5 (n=15)	0 (n=11)
Stockenhuber et al. (2008)	AAD: 63 (n=338) CDI: 21	AAD: 17 (n=340) CDI: 1

Table 2 Overview of investigations into *C.difficile*. (AAD (Antibiotic Associated Diarrhoea); CDI (Clostridium Difficile Infection))

Result and discussion:

Results suggest that consumption of *L.casei* Shirota, efficiently prevent the development of CDI and reduce the development of AAD.

Problems and different outcomes in the detection of *C.difficile* using different test systems are discussed broadly (Eastwood et al., 2009, Luna et al., 2011) and *C.difficile* ELISA have only been performed at the beginning of the therapy. Euroclone® *C.difficile* A/B kit specifically detects toxin producing *C.difficile*.

Total Bacteria and Diversity

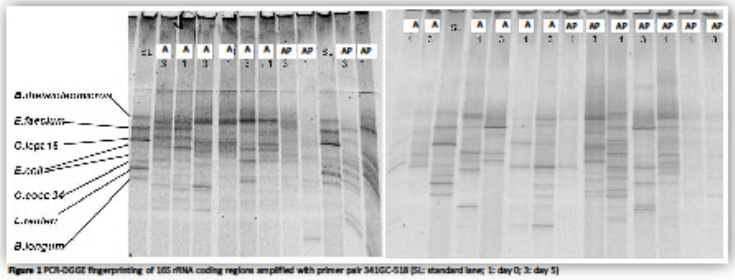


Figure 1 PCR-DGGE fingerprinting of 16S rRNA coding regions amplified with primer pair 341GC-518 (SL: standard lane; 1: day 0; 3: day 5)

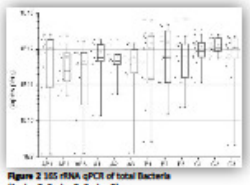


Figure 2 16S rRNA qPCR of total Bacteria (1: day 0; 2: day 5; 3: day 5)

Results and discussion:

The total amount of Bacteria was decreasing under antibiotic treatment, either with or without probiotic intake (Fig. 2). However, further qualitative analysis using DGGE indicated that diversity remains higher, when a combined antibiotic/probiotic therapy was performed: in group AP in average two more bands can be seen as an increase from timepoint 1 to 3, in comparison to group A (Fig. 1).

Effects on bacterial subgroups

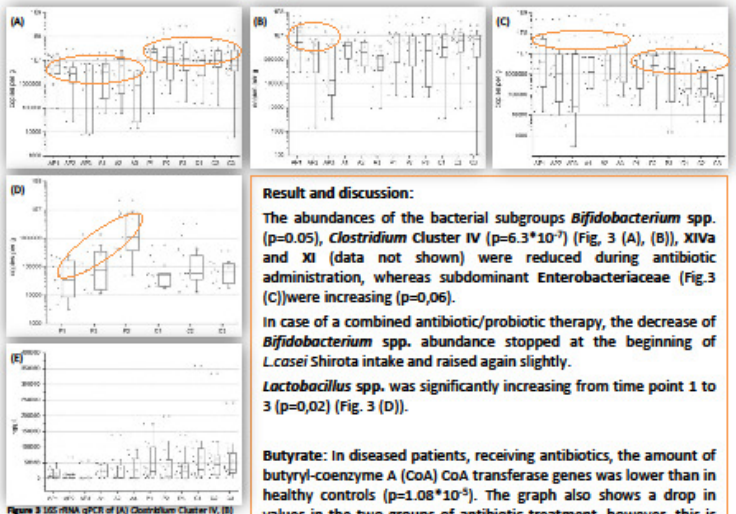


Figure 3 16S rRNA qPCR of (A) Clostridium Cluster IV, (B) Bifidobacterium spp., (C) Enterobacteriaceae, (D) Lactobacillus spp., (E) butyryl-coenzyme A (CoA) CoA transferase genes (1: day 0, 2: day 5, 3: day 5).

Result and discussion:

The abundances of the bacterial subgroups ***Bifidobacterium*** **spp.** ($p=0.05$), ***Clostridium*** **Cluster IV** ($p=6.3*10^{-7}$) (Fig, 3 (A), (B)), **XIVa and XI** (data not shown) were reduced during antibiotic administration, whereas subdominant **Enterobacteriaceae** (Fig.3 (C))were increasing ($p=0,06$).

In case of a combined antibiotic/probiotic therapy, the decrease of ***Bifidobacterium*** **spp.** abundance stopped at the beginning of *L.casei* Shirota intake and raised again slightly.

Lactobacillus **spp.** was significantly increasing from time point 1 to 3 ($p=0,02$) (Fig. 3 (D)).

Butyrate: In diseased patients, receiving antibiotics, the amount of butyryl-coenzyme A (CoA) CoA transferase genes was lower than in healthy controls ($p=1.08*10^{-5}$). The graph also shows a drop in values in the two groups of antibiotic treatment, however, this is not statistically significant (Fig. 3 (E)).

Conclusions: Antibiotic treatment as well as disease is mainly associated with changes in the diversity and composition of the gastrointestinal microbiota, accompanied by a lower occurrence of butyryl-coenzyme A (CoA) CoA transferase genes. Pathogens can obviously easier settle in the disturbed system. Reduced bacterial diversity and production of SCFAs might significantly contribute to problems following antibiotic treatment. Intervention with *L.casei* Shirota obviously antagonizes some of these changes. Successful probiotic intervention in AAD may therefore address the reduced fermentation of short-chain fatty acids.

Abstract:

Effects of antibiotic therapy on the gastrointestinal microbiota and the intervention with *L.casei*

Marlene Remely[1], Anna Harrant [1], Angelika Pirker[1], Berit Hippe [1], Christoph Kamhuber [2], Felix Stockenhuber [2], Alexander Haslberger*[1]

1Univ. Vienna, Dep for Nutritional Sciences - Vienna, Austria

2 Krankenhaus Oberpullendorf - Vienna, Austria

alexander.haslberger@univie.ac.at

Keywords: antibiotic-associated diarrhoea – microbiota- *C. difficile – L. casei Shirota*

Antibiotic-associated diarrhea (AAD) is discussed to be associated with the growth of pathogens like *C. difficile*, but also a decreased short chain fatty acid fermentation caused by impaired GI microbiota. We compared a therapy with antibiotics with a combination therapy of antibiotics and *L. casei Shirota* in 340 patients per group. Stool samples from 4 groups/ 56 patients were taken at one time-point before and 2 time-points after antibiotic treatment ± intake of *L. casei*. Fecal samples were investigated for *C. difficile* toxin, changes of bacterial groups and abundance of a crucial enzyme for butyrate production in GI microbiota by qPCR, DGGE, cloning and sequencing using 16S rRNA group specific primers.

Reduced AAD, (18% : 5%) was seen by intervention with *L. casei Shirota*. Antibiotic ± L. casei treated groups showed a decrease of total bacteria, Clostridia clusters IV, XI, XIV, bifidobacteria, the butyryl CoA:acetate CoA-transferase gene and diversity. In the antibiotic group 2 cases of C diff. were detected by toxin specific qPCR as well as a higher abundance of Enterobacteriaceae. In control groups receiving *L. casei* a significant increase of lactobacillus was seen. Combination therapy with antibiotics + *L. casei* resulted in an increase in diversity of two more bands from time point 1 to 3 and a significantly reduced decline in bifidobacteria compared to the antibiotic group. These results indicate the need of a molecular analysis of *C. difficile*. *C. difficile* growth might

only contribute to cases of AAD. Probiotic intervention could interfere with the reduced short chain fatty acid metabolism of impaired microbiota in AAD.

13.4. Lecture: 6th Probiotics, Prebiotics & New Foods 2011 (Rome)

Abstract:

Effects of antibiotic therapy on the gastrointestinal microbiota and the intervention with *L.casei*

Angelika Pirker, Berit Hippe, Christoph Kamhuber, Felix Stockenhuber, Alexander Haslberger

Univ. Vienna, Dep for Nutritional Sciences - Vienna, Austria -Krankenhaus Oberpullendorf - Vienna, Austria

Background

Results from a clinical study reported reduced antibiotic associated diarrhoea (AAD, 18%:5%) by intervention with *L.casei* Shirota (Stockenhuber et al., 2008).

Objectives

In the present pilot study effects of a combination therapy antibiotic and *L.casei* Shirota and controls were tested for AAD, C.difficile and changes of fecal microbiota in 56 patients.

Studydesign

Stool samples from 4 groups/ 56 patients were taken at one before and 2 timepoints after antibiotic treatment ± intake of *L.casei*. Samples of control group were taken at same time points. (A=antibiotic therapy; AP=antibiotic/probiotic therapy; P= control group receiving the probiotic drink; C= Control)

Methods

Feacal samples were investigated for *C.difficile* toxin and changes of bacterial groups in GI microbiota by qPCR using 16S rRNA group specific primers and probes, Euroclone®

C.difficile A/B kit, PCR/DGGE and a *C.difficile* ELISA test. Especially abundance and diversity of total bacteria, *Lactobacilli*, *Bifidobacteria*, *Bacteroides*, *Clostridium* cluster IV and XIV, *C.difficile*, and *Enterobacteriaceae* were analyzed.

Results

16S rRNA qPCR suggested some C.difficile in all groups but only one *C.difficile* positive patient could be detected by Euroclone® kit in group A. In group A and AP was a decrease of total bacteria following therapy. Also mean values of all bacteria were lower in A and AP groups compared to control groups. The results of PCR/DGGE showed a higher diversity in group AP than in group A. The abundance of *Enterobacteriaceae* was higher especially in group A than in control groups. By contrast, the abundance of *Clostridium* Cluster IV was lower in both antibiotic receiving groups than in control groups. Within group P there was a significant increase of *Lactobacilli*.

Discussion

Only qPCR tests addressing the *C.difficile* toxin may indicate CDI apropriately. CDI seems to be a rare reason for AAD. To understand other pathogenic mechanisms for AAD, group specific shifts under antibiotic treatment must be better analysed. This will also lead to the development of improved probiotic approaches.

13.5. BOOK CHAPTER: "Detection and Identification of Probiotic Microorganisms and Other Beneficial Organisms from the Human GI Tract."

Detection and Identification of Probiotic Microorganisms and Other Beneficial Organisms from the Human GI Tract.

Hippe B., Zwielehner J., Pirker A., Smith W.M., Haslberger A.G.

In: *Probiotics,* Microbiology Monographs 21. (ed.) Liong M-T. (Series ed.) Steinbüchel A. Springer-Verlag Berlin Heidelberg 2011

Detection and Identification of Probiotic Microorganisms and Other Beneficial Organisms from the Human GI Tract

Berit Hippe, Jutta Zwielehner, Angelika Pirker, William M. Smith, and Alexander G. Haslberger

Contents

B. Hippe • J. Zwielehner • A. Pirker • A.G. Haslberger (✉)
Department for Nutritional Sciences, University of Vienna, Althanstrasse 14, Vienna, Austria
e-mail: alexander.haslberger@univie.ac.at

W.M. Smith
Crop and Food Sciences, School of Land, University of Queensland, Brisbane, QLD 4072, Australia

M.-T. Liong (ed.), *Probiotics*, Microbiology Monographs 21,
DOI 10.1007/978-3-642-20838-6_3,

Abstract Most probiotics are similar to the microorganisms naturally found in the human gastrointestinal tract, and are mainly from the genera of *Lactobacillus* or *Bifidobacterium*. Conventionally, these microorganisms have been found to be fastidious, acid-tolerant, and strictly fermentative, producing lactic and acetic acids as the major end products of sugar fermentation. Exceptions from this general description have been found to occur. In addition, 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. Molecular microbiological analysis has increased the understanding of the diversity and phylogeny of beneficial strains and their functions. 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 their 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.

1 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 (GI) 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. LABs are Gram-positive, nonspore 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 nonliving probiotic derivatives are proposed to act by receptor-mediated functions in immune or intestinal cells (Probiotics). 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 GI 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 GI microbiota and the immune response whilst the components of dead cells exert an anti-inflammatory response in the GI 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 much more data on the diversity of organisms in their natural habitat, but technological biases and relative accuracy remain poorly understood (Tedersoo et al. 2010).

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

2 Bacteriophage and Transposon Infections of Probiotic Strains

Bacteriophage infections of 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 (Moineau 1999; Djordjevis et al. 1997).

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 (Alemayehu et al. 2009; Capra et al. 2006). PCR methods have been described for specific identification of strains based on phage-related sequences for probiotic *Lactobacillus rhamnosus* strains (Brandt and 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. 2010). 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.

3 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 microorganism 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 depend 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 reassociate. Reassociation leads to the formation of hybrid DNA double strands. 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 (Krogius-Kurikka et al. 2009; Shneyer 2007).

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 feces 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 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 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.

3.1 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 strain 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 (Loffler 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 nine 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).

3.2 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 α subunit of the ATP synthase (*atpA*), RNA polymerase α subunit (*rpoA*) and the phenylalanine t-RNA synthase α 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 (Hayashi et al. 2001; Chopin 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 *L. rhamnosus* strains have been successfully developed (Brandt and Alatossava 2003). The authors found their assay could discriminate well between strains of *L. rhamnosus* but described amplification of phylogenetically unrelated nontarget strains, pointing out that phi-sequencing should be preferentially used in combination with other molecular methods.

3.3 Quantitative PCR

Quantitative PCR (qPCR) 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 overestimation overestimating 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 and 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 of 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 and Flint 2009). One commonly used florescent 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 analyzed 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 and 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.

3.4 High Resolution Melt Analysis

High Resolution Melt (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 Tm 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. 2011). Even single base changes such as single nucleotide polymorphisms (SNPs) 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. 2010).

3.5 *Fluorescence In Situ Hybridization*

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). Fluorescence In Situ Hybridization (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 shown 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 unlabeled oligonucleotide probes that bind adjacent to the probe target site. The aim is to unfold the nucleic acid and thus facilitate probe hybridization. These

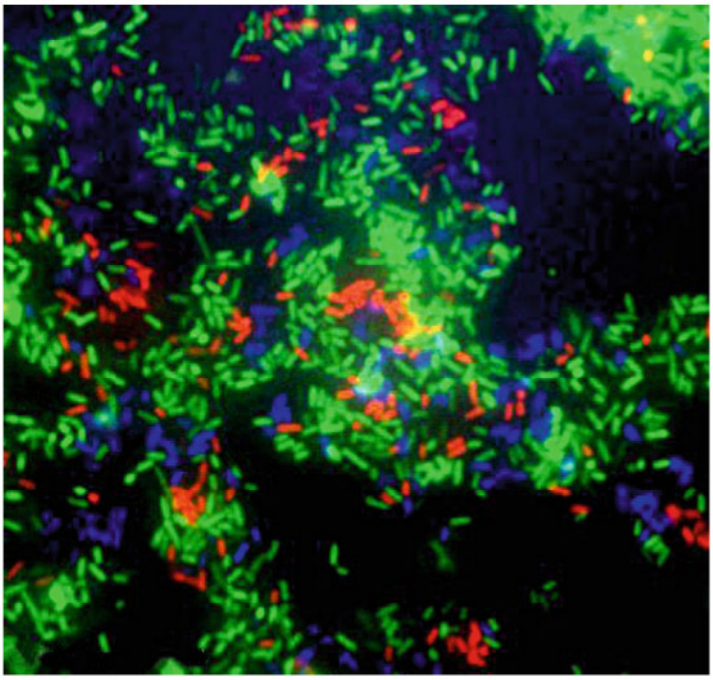


Fig. 1 Three-color fluorescent in situ hybridization image of bacteria. Three FISH probes labeled with different fluorescent dyes have been used to simultaneously identify the structure of microbial community. Each labeled single bacterial cell can be measured by Raman microspectroscopy to examine if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

Table 1 Most commonly employed fluorescent dyes to label oligonucleotides for FISH analysis (Bottari et al. 2006)

Fluorochrome	Color	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* tertramethyl rhodamine, *TRITC* tetramethylrodamine-isothiocyanate

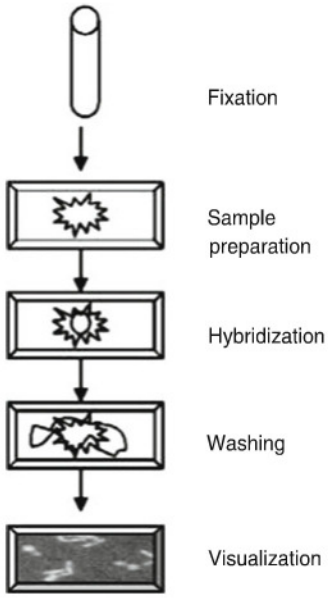


Fig. 2 Flow chart of a typical FISH. The procedure includes the following steps: (1) fixation of the specimen directly in sample tubes; (2) transfer of the sample on a glass slide and preparation of the sample, including specific pretreatment steps; (3) hybridization with the respective probes for detecting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

so-called helper probes need to be designed carefully because of their specificity to the respective probe and must have a differentiation temperature (Td) at least as high as the temperature of the probe to prevent dissociation of the helper at stringent hybridization conditions (Fuchs et al. 2000).

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 labeled 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 and 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).

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 color, 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.

4 Fingerprinting Techniques

4.1 Denaturing DNA Gradient Gel Electrophoresis

Denaturing DNA Gradient Gel Electrophoresis (DGGE) 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 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 and Lerman 1979). These denaturants are usually formamide and urea (Felske and Osborn 2005). Group-specific primers, which target 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 40-bp 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 some 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. 2010). Further identification of fragments can be carried out by sequencing (Ventura et al. 2004).

4.2 Pulsed Field Gel Electrophoresis

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 2008). It is a very useful method for highlighting isolates which may represent new species or serovars (Galloway and Levett 2008). The method has

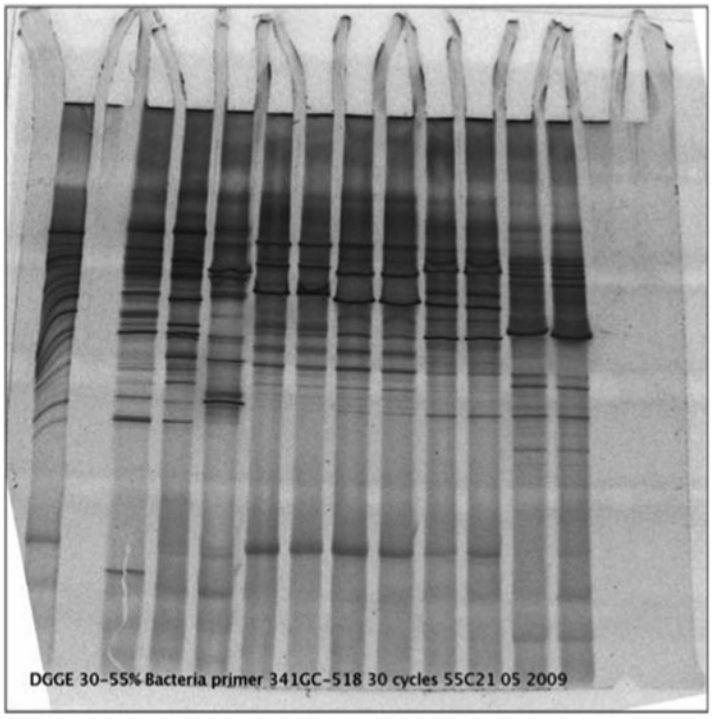


Fig. 3 PCR-DGGE band patterns of 16S rRNA coding regions of bacteria amplified with primers 341GC and 518

high reproducibility and a very good selectivity at the strain level but can be laborious and time consuming (Ventura et al. 2004; Kneifel and 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 and Zink 2002) but not at the genus or species level (Huys et al. 2006).

4.3 Randomly Amplified Polymorphic DNA

Randomly amplified polymorphic DNA (RAPD) is a special form of PCR which uses 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 band patterns.

An advantage of the method is that it is simple, fast, and cheap. The disadvantage is that experiments are particularly difficult to standardize (Kneifel and 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 genus or species levels is not possible (Huys et al. 2006). In contrast, *bifidobacteria* can be identified at species as well as at strain level (Ventura et al. 2004).

4.4 Repetitive Genomic Element PCR

All living things harbor 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).

Repetitive Genomic Element PCR (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 and 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).

4.5 Restriction Fragment Length Polymorphism and Terminal Restriction Fragment Length Polymorphism

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).

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., BgII: 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 modification enzymes require only S-adenosylmethionine. They tend to be small, with subunits in the 200–350 amino acid range (BioLabs 2010). An example for RFLP patterns is given in Fig. 4 which shows the RFLP bandpatterns of bacteria obtained from “soidon mahi” starter cultures for fermented bamboo shoot tips in

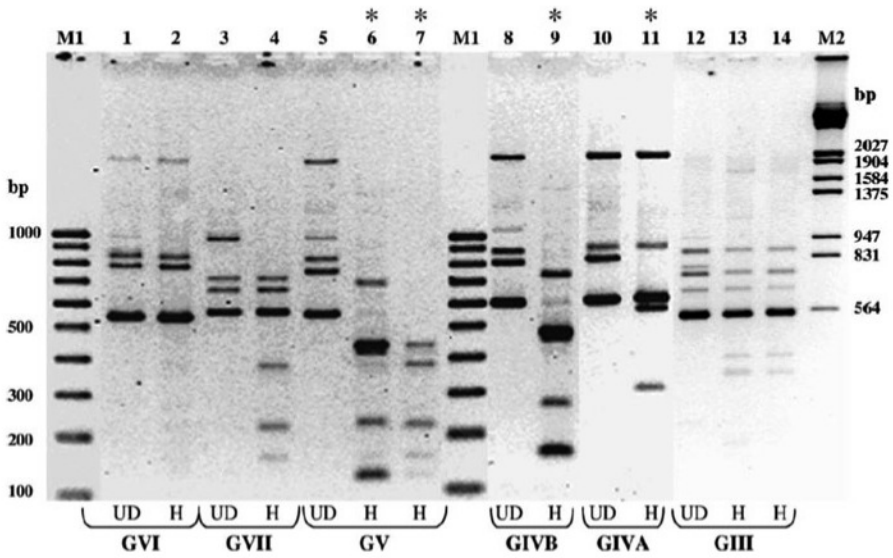


Fig. 4 ITS-PCR and HinfI-ITS-RFLP profiles of representative strains from lactic acid bacteria associated with soidon mahi starter. Lanes 1–2: *Lactobacillus plantarum* SD6B11; lanes 3–4: *Enterococcus faecium* SD3B10; lanes 5–6: *Lactobacillus brevis* SD10B10; lane 7: *L. brevis* SD10B4; lanes 8–9: *L. brevis* SD2B5; lanes 10–11: *L. brevis* SD1B8; lanes 12–13: Camobacterium sp. SD4B10; and lane 14: Carnobacterium sp. SD8B9; M1: 100 bp DNA ladder; M2: λDNA double digest (Promega). *UD* indicates undigested ITS-PCR profiles and *H* indicates HinfI digested ITS-RFLP profiles. *Asterisk* (*) indicates heterogenic HinfI-ITS-RFLP profiles of *L. brevis* isolates (Jeyaram et al. 2010)

India (Jeyaram et al. 2010). The authors found that this acidic starter was dominated by a characteristic association of *Bacillus* spp. and LAB, particularly *Lactobacillus brevis* and *Lactobacillus plantarum* (Jeyaram et al. 2010).

In terminal restriction length polymorphism analysis (T-RFLP), restriction enzymes are used on PCR products marked with a fluorescent group sitting on one primer. Thus, cleavage results in fragments of different length that can be separated using a sequencer, recognizing a fluorescent signal. Kovatcheva-Datchary et al. (2009) applied this method to analyze RNA (Fig. 5) from their RNA-stable isotope probing experiment identifying starch fermenting colonic bacteria (Kovatcheva-Datchary et al. 2009).

5 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,

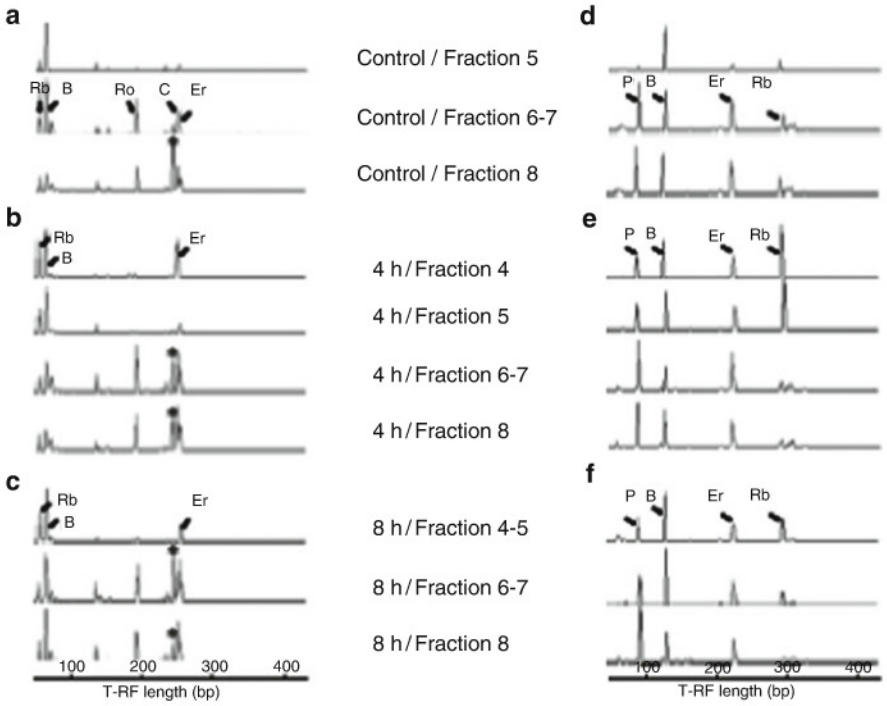


Fig. 5 T-RFLP profiles of bacterial 16S rRNA obtained from fractions of the control (**a**), 4 h gradient (**b**), and 8 h gradient (**c**), after applying AluI as restriction endonuclease; control (**d**), 4 h gradient (**e**), and 8 h gradient (**f**), after applying MspI as restriction endonuclease. Assignment of T-RF peaks to bacteria phylotypes (Rb, *Ruminococcus bromii*, B, *Bifidobacterium adolescentis*; Ro, *Ruminococcus obeum*; C, uncultured Clostridium spp.; Er, Eubacterium rectal; P, Prevotella spp.) (Kovatcheva-Datchary et al. 2009)

community structures can now be investigated at much higher resolution by revealing taxa that are much less abundant. However, this may come with lower taxonomic certainty due to the short read lengths and sometimes poorer read quality (Claesson et al. 2009). The pyrosequencing technology introduced by 454 Life Science (Margulies et al. 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. This process yields about 400,000 reads of 250–400bp length with an average quality score of greater than 99.5% accuracy rate (Droege and 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 et al. 2007); V1,V2, V6, and V3 in human (Andersson et al. 2008); (Dethlefsen et al. 2008); (Turnbaugh et al. 2008) (Zhang et al. 2009) and in macaque (McKenna et al. 2008) GI tract; as well as V9 in soil-derived microbial DNA (Roesch et al. 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 given in Table 2. Claesson et al. (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).

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.

Table 2 Of 7,208 full-length 16 S reference sequences from the human gut, 6,054 were classified at genus level with 80% bootstrap support

Variable region	V3 (%)			V6 (%)			V4 (%)		
Bootstrap cutoff (≥)	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

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*) (Claesson et al. 2009)

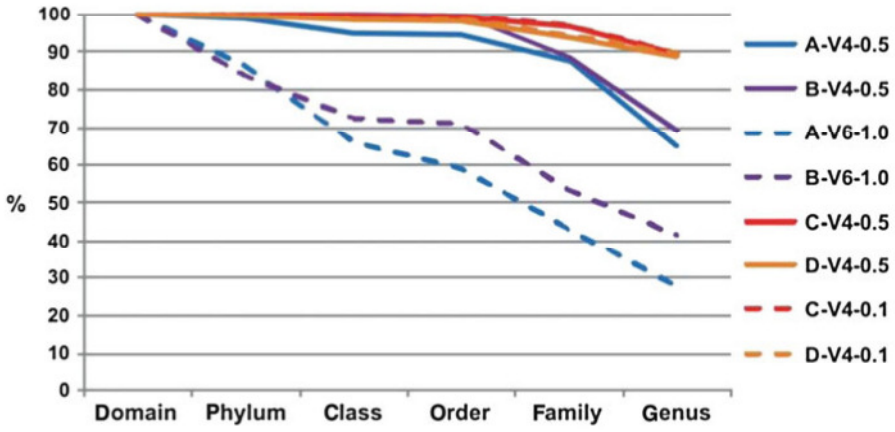


Fig. 6 Classification efficiencies at six taxonomic ranks for eight sets of sequences from four samples. The *blue* and *purple* colored *dashed lines* represent V6 amplicon reads, which have very poor classification efficiencies compared to all V4 amplicon reads, especially at the genus level. The *red*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

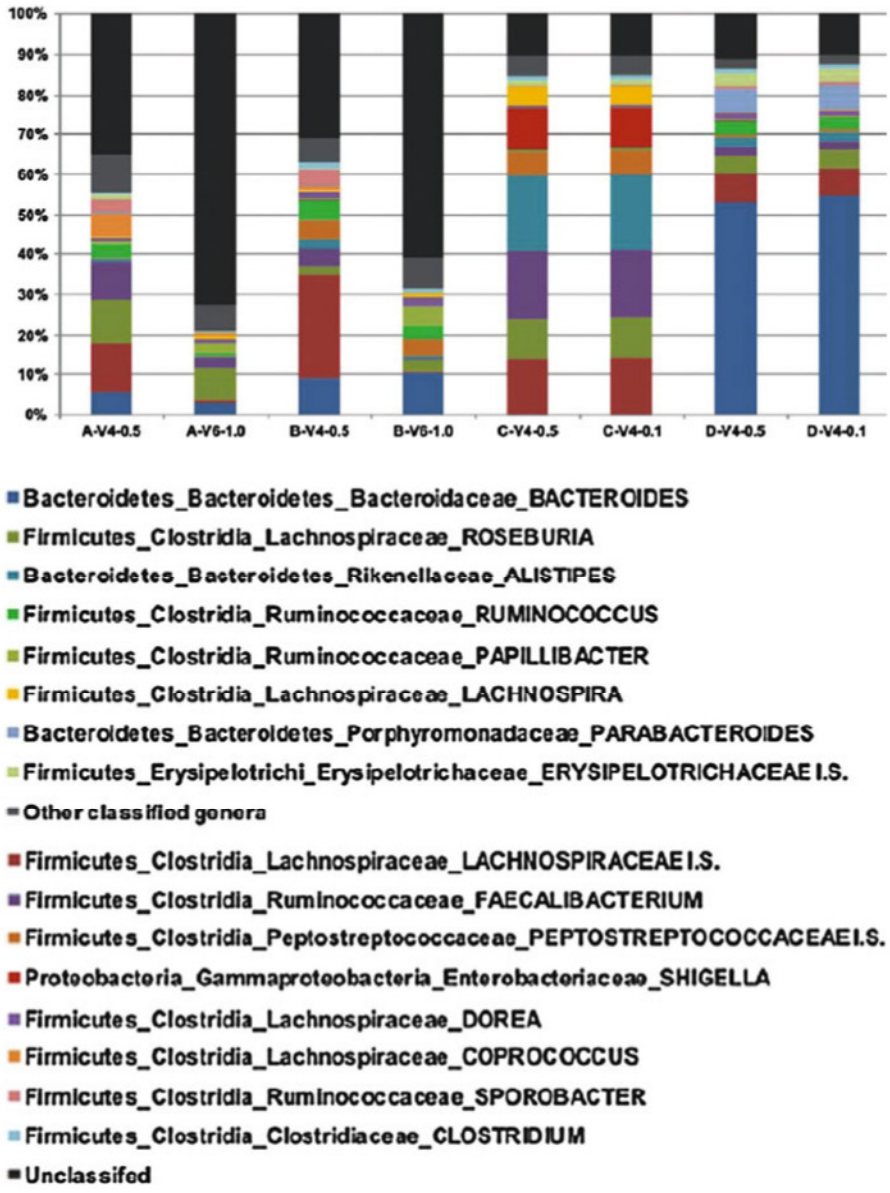


Fig. 7 Relative abundance of the 16 most abundant genera classified with at least 50% bootstrap support. Genera are labeled according to phylum_class_family_GENUS (Claesson et al. 2009)

The analysis by Claesson et al. (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 1 day (Chen et al. 2010; Monger et al. 2010).

6 Microarray

Microarray applications allow the simultaneous comparison of the entire genome content of microorganisms by hybridization 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.

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" (Tettelin et al. 2005; Medini 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).

Based on predicted protein sequences, the *E. coli* core genome was estimated to comprise approximately 1,563 genes (Willenbrock et al.) for an infinite (or very large) number of *E. coli* genomes. Following the identification of the core genome, additional "strain-specific" genes need to be defined (Fig. 9).

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 9,433 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).

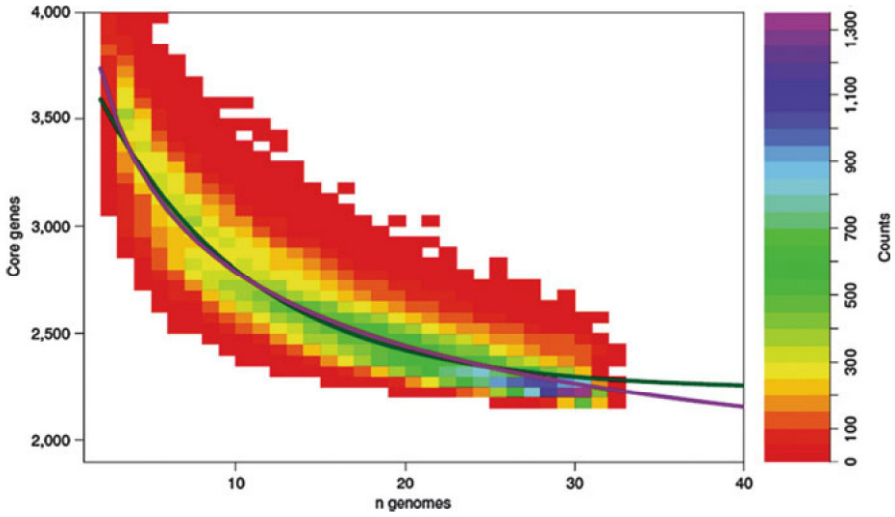


Fig. 8 Two-dimensional density plot of "core genes" for the *E. coli* pan-genome. The plot illustrates the number of *E. coli* genes for $n = 2$, 32 genomes based on a maximum of 3,200 random combinations of genomes for each n. The density colors reflect the count of combinations giving rise to a certain 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)

6.1 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; Palmer et al. 2007; Harrington et al. 2008; Wilson et al. 2002). As it is technically very difficult to include 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. 2009). The 4,800 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 1,140 phylotypes, respectively. Based on 98% sequence similarity, phylotypes were defined from more than 16,000 16S rRNA gene sequences identified in the human GIT. A typical work flow for phylogenetic microarray analysis is shown in Fig. 10.

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

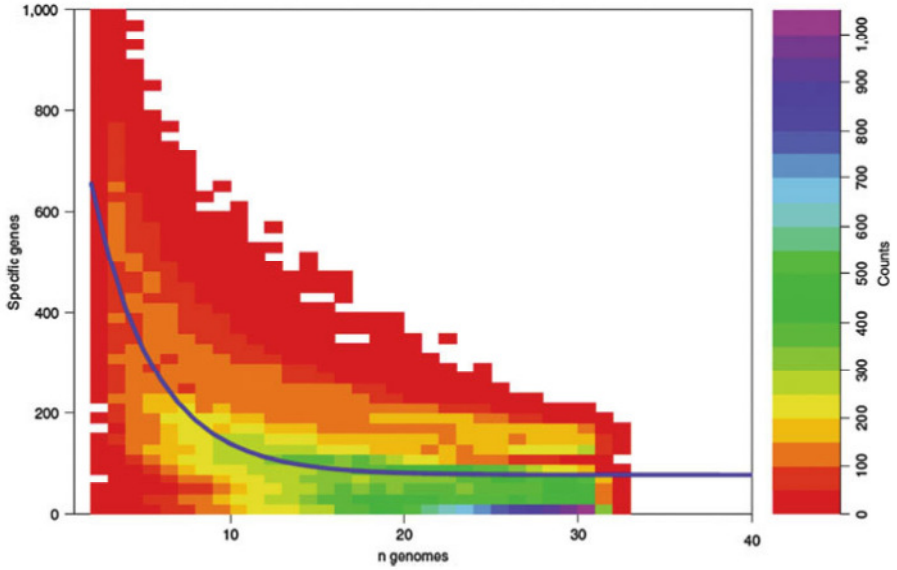


Fig. 9 Two-dimensional density plot of novel genome "specific genes" for the *E. coli* pan genome. The plot illustrates the number of novel genome specific genes for the *n*th genome when comparing $n = 2, 32$ genomes (for a maximum of 3,200 random combinations at each n). The density colors reflect 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 (Willenbrock et al. 2007)

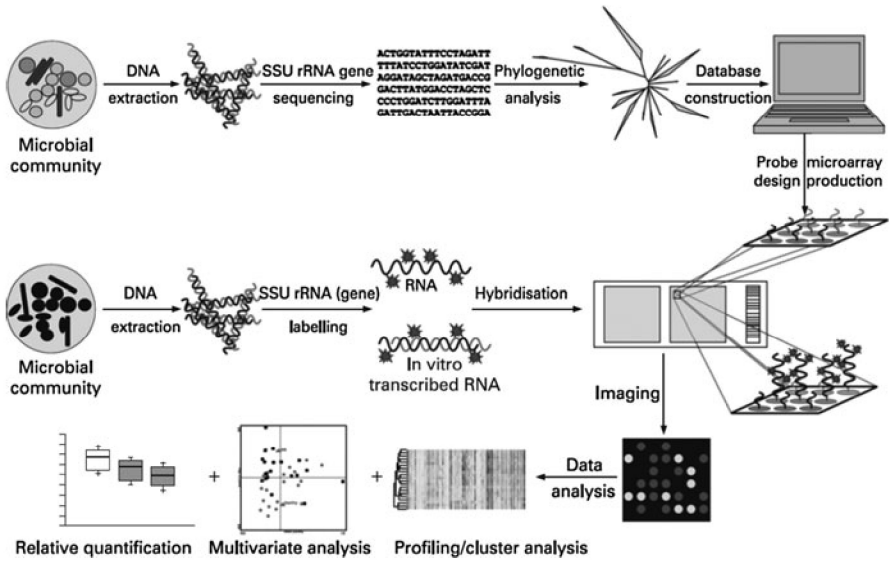


Fig. 10 Schematic representation of high-throughput analysis of human gastrointestinal (GI) tract microbiota via brute force sequencing and phylogenetic microarray analysis. *SSU rRNA* small subunit ribosomal RNA (Zoetendal et al. 2008)

probing in a human colon model with great reproducibility (Kovatcheva-Datchary et al. 2009).

6.2 Array Tubes

Array Tubes (AT) platforms consist of a custom microprobe array similar to usual glass slides microarrays, integrated into a microreaction 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 analyzed simultaneously to evaluate probiotic potential of food or a bacterial strain.

6.3 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 GI tract (Grangette et al. 2005; Hisbergues et al. 2007).

Degues et al. (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 1,760 open reading frames (ORFs) of NCC533 were spotted on a microarray. The DNA of the type strain ATCC 33200 was hybridized 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 NCC 533-specific gene locus, which was expressed in the gut, shared significant sequence identity with IgA (immune globulin 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 *L. 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 and 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 monozytogenes,* and *E. coli* (Lin et al. 2003; Thanassi et al. 1997; Sleator et al. 2005).

7 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 already 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
4. Products containing the living GMM, for example, a nonpasteurized 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 9 weeks (Straub et al. 1999).

For an organism-specific detection of the GMM it would need 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 (Tilsala-Timisjarvi and Alatossava 1998, 2001; Matheson et al. 1997; Heller et al. 2006) (Fig. 11).

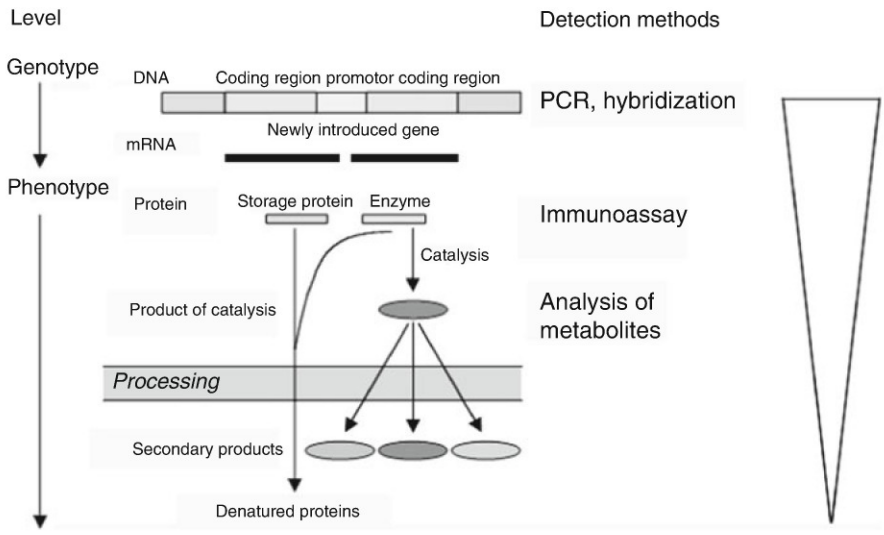


Fig. 11 The unambiguity of methods for the detection of GMO in food (Heller et al. 2006)

8 Conclusions

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 (Fig. 12).

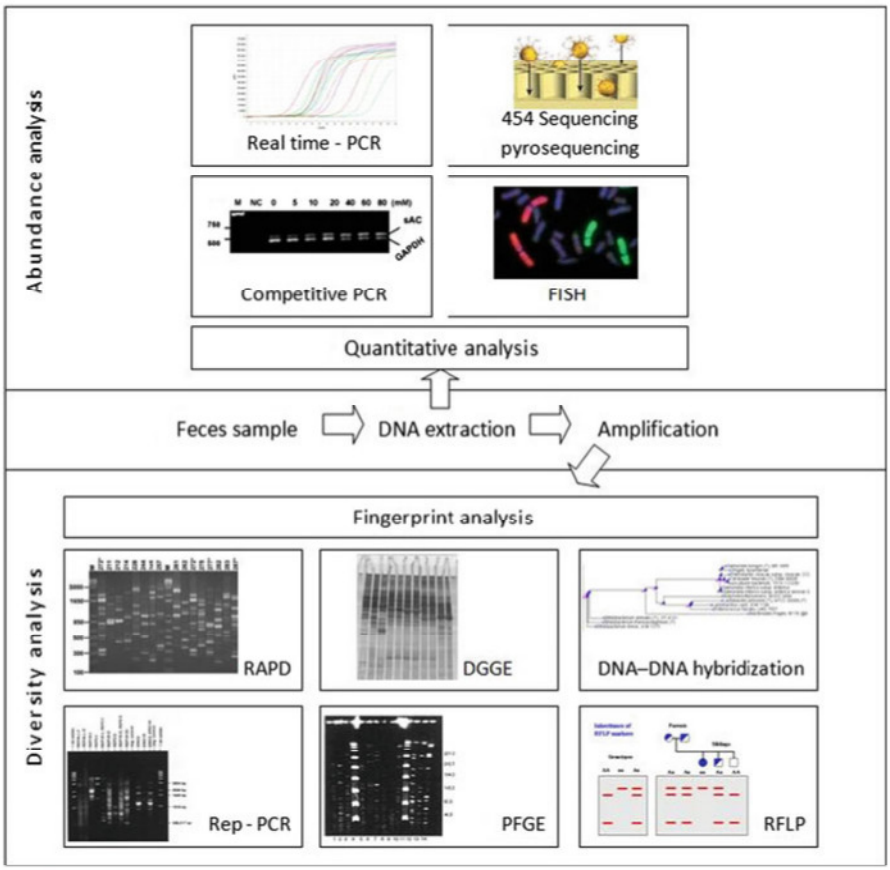


Fig. 12 Overview of methods for analyzing the gut microbiota out of feces sample (Holzapfel et al. 2001; Mahenthiralingam et al. 2009; Rothberg and Leamon 2008; Sun et al. 2004; Versalovic et al. 1994)

References

Adams C-A (2010) The probiotic paradox: live and dead cells are biological response modifiers. Nutr Res Rev 23:37–46

Ahmed F-E (2003) Genetically modified probiotics in foods. Trends Biotechnol 21:491–497

Alemayehu D, Ross R-P, O'Sullivan O, Coffey A, Stanton C, Fitzgerald G-F, Mcauliffe O (2009) Genome of a virulent bacteriophage Lb338-1 that lyses the probiotic Lactobacillus paracasei cheese strain. Gene 448:29–39

Amann R-I, Krumholz L, Stahl D-A (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172:762–770

Amann R, Fuchs B-M, Behrens S (2001) The identification of microorganisms by fluorescence in situ hybridisation. Curr Opin Biotechnol 12:231–236

Andersson AF, Lindberg M, Jakobsson H, Bäckhed F, Nyrén P, Engstrand L (2008) Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS One 3(7):e2836

Axelsson L (1998) Lactic acid bacteria: classification and physiology. Dekker, New York

Begley M, Gahan C-G, Hill C (2005) The interaction between bacteria and bile. FEMS Microbiol Rev 29:625–651

Binetti A-G, Capra M-L, Alverez M-A, Reinheimer J-A (2008) PCR method for detection and identification of Lactobacillus casei/paracasei bacteriophages in dairy products. Int J Food Microbiol 124:147–153

BIOLABS (2010) Restriction endonucleases overview [Online]. Biolabs. Available: http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/overview.asp. Accessed 28 Oct 2010

Blume H-P, Brummer G-W, Horn R, Kandeler E, Kogel-Knabner I, Kretzschmar R, Stahr K, Wilke B-M (2010) Untersuchungsmethoden. In: Scheffer FW, Schachtschabel P (eds) Lehrbuch der Bodenkunde, 16th edn. Spektrum Akademischer Verlag, Berlin

Booysena C, Dicks L-M, Meijering I, Ackermann A (2002) Isolation, identification and changes in the composition of lactic acid bacteria during the malting of two different barley cultivars. Int J Food Microbiol 76:63–73

Bottacini F, Medini D, Pavesi A, Turroni F, Foroni E, Riley D, Giubellini V, Tettelin H, Van Sinderen D, Ventura M (2010) Comparative genomics of the genus *Bifidobacterium*. Microbiology 156:3243–3254

Bottari B, Ercolini D, Gatti M, Neviani E (2006) Application of FISH technology for microbiological analysis: current state and prospects. Appl Microbiol Biotechnol 73:485–494

Brandt K, Alatossava T (2003) Specific identification of certain probiotic *Lactobacillus rhamnosus* strains with PCR primers based on phage-related sequences. Int J Food Microbiol 84:189–196

Capra M-L, Quiberoni A, Reinheimer J (2006) Phages of *Lactobacillus casei/paracasei*: response to environmental factors and interaction with collection and commercial strains. J Appl Microbiol 100:334–342

Carey C-M, Kirk J-L, Ojha S, Kostrzynska M (2007) Current and future uses of real-time polymerase chain reaction and microarrays in the study of intestinal microbiota, and probiotic use and effectiveness. Can J Microbiol 53:537–550

Chen C, Kittichotirat W, Chen W, Downey J-S, Si Y, Bumgarner R (2010) Genome sequence of naturally competent Aggregatibacter actinomycetemcomitans serotype a strain D7S-1. J Bacteriol 192:2643–2644

Chopin A, Bolotin A, Sorokin A, Ehrlich S-D, Chopin M (2001) Analysis of six prophages in *Lactococcus lactis* IL1403: different genetic structure of temperate and virulent phage populations. Nucleic Acids Res 29:644–651

Claesson M-J, O'Sullivan O, Wang Q, Nikkila J, Marchesi J-R, Smidt H, De Vos W-M, Ross R-P, O'Toole P-W (2009) Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One 4:e6669

Cypionka H (2010) Grundlagen der Mikrobiologie. Springer, Heidelberg

Dethlefsen L, Huse S, Sogin ML, Relman DA (2008) The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 6(11):e280

Djordjevis G-M, O'Sullivan D-J, Walker S-A, Conkling M-A, Klaenhammer T-R (1997) A triggered-suicide system designed as a defense against bacteriophages. J Bacteriol 179:6741–6748

Droege M, Hill B (2008) The genome sequencer FLX system – longer reads, more applications, straight forward bioinformatics and more complete data sets. J Biotechnol 136(1–2):3–10. Epub 21 June 2008

Ereqat S, Bar-Gal G-K, Nasereddin A, Azmi K, Qaddomi S-E, Greenblatt C-L, Spigelman M, Abdeen Z (2010) Rapid differentiation of *Mycobacterium tuberculosis* and *M. bovis* by high resolution melt curve analysis. J Clin Microbiol 48:4269–4272

Falentin H, Deutsch S-M, Jan G, Loux V, Thierry A, Parayre S, Maillard M-B, Dherbecourt J, Cousin F-J, Jardin J, Siguier P, Coulous A, Barbe V, Vacherie B, Wincker P, Bibrat J-F, Gaillardin C, Lortal S (2010) The complete genome of *Propionibacterium freudenreichii* CIRM-BIA1, a hardy actinobacterium with food and probiotic applications. PLoS One 5:e11748

Felis G-E, Dellaglio F (2007) Taxonomy of lactobacilli and bifidobacteria. Curr Issues Intest Microbiol 8:44–61

Felske A, Osborn A-M (2005) DNA fingerprinting of microbial communities. In: Osborn MA, Smith CJ (eds) Molecular microbial ecology. Taylor and Francis, New York

Fischer S-G, Lerman L-S (1979) Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. Cell 16:191–200

Fuchs B-M, Glockner F-O, Wulf J, Amann R (2000) Unlabeled helper oligonucleotides increase the in situ accessibility to 16 S rRNA of fluorescently labeled oligonucleotide probes. Appl Environ Microbiol 66:3603–3607

Fujimura K-E, Slusher N-A, Cabana M-D, Lynch S-V (2010) Role of the gut microbiota in defining human health. Expert Rev Anti Infect Ther 8:435–454

Galloway RL, Levett PN (2008) Evaluation of a modified pulsed-field gel electrophoresis approach for the identification of Leptospira serovars. Am J Trop Med Hyg 78(4):628–632

Gevers D, Huys G, Swings J (2001) Applicability of rep-PCR fingerprinting for identification of Lactobacillus species. FEMS Microbiol Lett 205:31–36

Glockner F-O, Fuchs B-M, Amann R (1999) Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. Appl Environ Microbiol 65:3721–3726

Grangette C, Nutten S, Palumbo E, Morath S, Hermann C, Dewulf F-J, Pot B, Hartung T, Hols P, Mercenier A (2005) Enhanced antiinflammatory capacity of a Lactobacillus plantarum mutant synthesizing modified teichoic acids. Proc Natl Acad Sci USA 102:10321–10326

Guschin D-Y, Mobarry B-K, Proudnikov D, Stahl D-A, Rittmann B-E, Mirzabekov A-D (1997) Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl Environ Microbiol 63:2397–2402

Harrington C-R, Lucchini S, Ridgway K-P, Wegmann U, Eaton T-J, Hinton J-C, Gasson M-J, Narbad A (2008) A short-oligonucleotide microarray that allows improved detection of gastrointestinal tract microbial communities. BMC Microbiol 8:195

Hayashi T, Makino K, Ohnishi M, Kurokawa K, Ishii K, Yokoyama K, Han C-G, Ohtsubo E, Nakayama K, Murata T, Tanaka M, Tobe T, Iida T, Takami H, Honda T, Sasakawa C, Ogasawara N, Yasunaga T, Kuhara S, Shiba T, Hattori M, Shinagawa H (2001) Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. DNA Res 8:11–22

Heller K-J, Hammes W-P, Hertel C, Bauer T (2006) Methods for detection of genetically modified microorganisms used in food fermentation processes. Wiley On line library

Hisbergues M, Magi M, Rigaux P, Steuve J, Garcia L, Goudercourt D, Pot B, Pestel J, Jacquet A (2007) In vivo and in vitro immunomodulation of Der p 1 allergen-specific response by *Lactobacillus plantarum* bacteria. Clin Expr Allerg 37:1286–1295

Holzapfel W-H, Harberer P, Geisen R, Bjorkroth J, Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. Am J Clin Nutr 73:365S–373S

Huse SM, Huber JA, Morrison HG, Sogin ML, Welch DM (2007) Accuracy and quality of massively parallel DNA pyrosequencing. Genome Biol 8(7):R143

Huys G, Vancanneyt M, D'haene K, Vankerckhoven V, Goosens H, Swings J (2006) Accuracy of species identity of commercial bacterial cultures intended for probiotic or nutritional use. Res Microbiol 157:803–810

Ishii S, Sadowsky MJ (2009) Applications of the rep-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution. Environ Microbiol 11(4):733–740

Jeyaram K, Romi W, Singh T-A, Devi A-R, Devi S-S (2010) Bacterial species associated with traditional starter cultures used for fermented bamboo shoot production in Manipur state of India. Int J Food Microbiol 143:1–8

Kajander K, Myllyluoma E, Rajilic-Stojanovic M, Kyronpalo S, Rasmussen M, Jarvenpaa S, Zoetendal E-G, De Vos W-M, Vapaatalo H, Korpela R (2008) Clinical trial: multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota. Aliment Pharmacol Ther 27:48–57

Kneifel W, Domig K-J (2009) Taxonomie von Milchsäurebakterienmit probiotischer Kapazität. In: Bischoff SC (ed) Probiotika, Präbiotika und Synbiotika. Thieme Verlag, Stuttgart

Kovatcheva-Datchary P, Egert M, Maathuis A, Rajilic-Stojanovic M, De Graaf A, Smidt H, De Vos W-M, Venema K (2009) Linking phylogenetic identities of bacteria to starch fermentation in an in vitro model of the large intestine by RNA-based stable isotope probing. Environ Microbiol 11:914–926

Krogius-Kurikka L, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Makivuokko H, Kajander K, Palva A (2009) Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. BMC Gastroenterol 9:95

Lin J, Sahin O, Michel L-O, Zhang Q (2003) Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of Campylobacter jejuni. Infect Immun 71:4250–4259

Loffler G (2004) Basiswissen Biochemie mit Pathobiochemie. Springer, Heidelberg

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

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüssmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32(4):1363–1371

Mahenthiralingam E, Marchbank A, Drevinek P, Garaiova I, Plummer S (2009) Use of colony-based bacterial strain typing for tracking the fate of Lactobacillus strains during human consumption. BMC Microbiol 9:251

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer ML, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437(7057):376–380. Epub 31 July 2005. Erratum in: Nature (2006);441(7089):120

Matheson V-G, Munakata-Marr J, Hopkins G-D, Mccarty P-L, Tiedje J-M, Forney L-J (1997) A novel means to develop strain-specific DNA probes for detecting bacteria in the environment. Appl Environ Microbiol 63:2863–2869

Maukonen J, Matto J, Suihko M-L, Saarela M (2008) Intra-individual diversity and similarity of salivary and faecal microbiota. J Med Microbiol 57:1560–1568

McKenna P, Hoffmann C, Minkah N, Aye PP, Lackner A, Liu Z, Lozupone CA, Hamady M, Knight R, Bushman FD (2008) The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. PLoS Pathog 4(2):e20

Medini D, Donati C, Tettelin H, Masignani V, Rappuoli R (2005) The microbial pan-genome. Curr Opin Genet Dev 15:589–594

Michalet X, Pinaud F-F, Bentolila L-A, Tsay J-M, Doose S, Li J-J, Sundaresan G, Wu A-M, Gambhir S-S, Weiss S (2005) Quantum dots for live cells, in vivo imaging, and diagnostics. Science 307:538–544

Moineau S (1999) Applications of phage resistance in lactic acid bacteria. Antonie Van Leeuwenhoek 76:377–382

Monger W-A, Alicai T, Ndunguru J, Kinyua Z-M, Potts M, Reeder R-H, Miano D-W, Adams I-P, Boonham N, Glover R-H, Smith J (2010) The complete genome sequence of the Tanzanian strain of Cassava brown streak virus and comparison with the Ugandan strain sequence. Arch Virol 155:429–433

Moter A, Gobel U-B (2000) Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. J Microbiol Methods 41:85–112

Naser N-M, Hagen K-E, Vancanneyt M, Cleenwerck I, Swings J, Tompkins P-A (2006) Lactobacillus suntoryeus Cachat and Priest 2005 is a later synonym of *Lactobacillus helveticus* (Orla-Jensen 1919) Bergey et al. 1925 (Approved Lists 1980). Int J Syst Evol Microbiol 56:355–360

Olive D-M, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol 37:1661–1669

Palmer C, Bik E-M, Digiulio D-B, Relman D-A, Brown P-O (2007) Development of the human infant intestinal microbiota. PLoS Biol 5:e177

Probiotics, DOA http://www.functionalingredientsmag.com/article/Science-Now/probiotics-dead-or-alive-.aspx. http://www.functionalingredientsmag.com/article/Science-Now/probiotics-dead-or-alive-.aspx [Online]

Radvansky J, Bazsalovicsova E, Kralova-Hromadova I, Minarik G, Kadasi L (2011) Development of high-resolution melting (HRM) analysis for population studies of Fascioloides magna (Trematoda: Fasciolidae), the giant liver fluke of ruminants. Parasitol Res 108:201–209

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

Roesch LF, Fulthorpe RR, Riva A, Casella G, Hadwin AK, Kent AD, Daroub SH, Camargo FA, Farmerie WG, Triplett EW (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J 1(4):283–290. Epub 5 July 2007

Rothberg J-M, Leamon J-H (2008) The development and impact of 454 sequencing. Nat Biotechnol 26:1117–1124

Roy D, Ward P, Champagne G (1996) Differentiation of bifidobacteria by use of pulsed-field gel electrophoresis and polymerase chain reaction. Int J Food Microbiol 29:11–29

Satokari R-M, Vaughan E-E, Akkermans A-D, Saarela M, De Vos W-M (2001) Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl Environ Microbiol 67:504–513

Shneyer V-S (2007) On the species-specificity of DNA: fifty years later. Biochemistry (Mosc) 72:1377–1384

Sibley P-E, Harper M-E, Peeling W-B, Griffiths K (1984) Growth hormone and prostatic tumours: localization using a monoclonal human growth hormone antibody. J Endocrinol 103:311–315

Sigler V (2004) Denaturing gradient gel electrophoresis (DGGE) [Online]. Laboratory for Microbial Ecology, Department of Earth, Ecological and Environmental Sciences, University of Toledo. Accessed 24 Aug 2010

Sleator R-D, Wemekamp-Kamphuis H-H, Gahan G-C, Abee T, Hill C (2005) A PrfA-regulated bile exclusion system (BilE) is a novel virulence factor in *Listeria monocytogenes*. Mol Microbiol 55:1183–1195

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PA, Kämpfer P, Maiden MC, Nesme X, Rosselló-Mora R, Swings J, Trüper HG, Vauterin L, Ward ΛC, Whitman WB (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52(Pt 3):1043–1047

Straub J-A, Hertel C, Hammes W-P (1999) A 23 S rDNA-targeted polymerase chain reaction-based system for detection of Staphylococcus aureus in meat starter cultures and dairy products. J Food Prot 62:1150–1156

Sun X-C, Cui M, Bonanno J-A (2004) [HCO3-]-regulated expression and activity of soluble adenylyl cyclase in corneal endothelial and Calu-3 cells. BMC Physiol 4:8

Tannock G-W, Tilsala-Timisjarvi A, Rodtong S, Ng J, Munro K, Alatossava T (1999) Identification of Lactobacillus isolates from the gastrointestinal tract, silage, and yoghurt by 16 S-23 S rRNA gene intergenic spacer region sequence comparisons. Appl Environ Microbiol 65:4264–4267

Tedersoo L, Nilsson R-H, Abarenkov K, Jairus T, Sadam A, Saar I, Bahram M, Bechem E, Chuyong G, Koljalg U (2010) 454 Pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. New Phytol 188:291–301

Tettelin H, Masignani V, Cieslewicz M-J, Donati C, Medini D, Ward N-L, Angiuoli S-V, Brabtree J, Jomes A-L, Durkin A-S, Deboy R-T, Davidsen T-M, Mora M, Scarselli M, Margarit Y-R, Peterson J-D, Hauser C-R, Sundaram J-P, Nelson W-C, Madupu R, Brinkac L-M, Dodson R-J, Rosovits M-J, Sullivan S-A, Daugherty S-C, Haft D-H, Selengut J, Gwinn M-L, Zhaou L, Zafar N, Khouri H, Radune D, Dimitriv G, Watkins K, O'Conner K-J, Smith S, Utterback T-R, White O, Rubens C-E, Grandi G, Madoff L-C, Kasper D-L, Telford J-L, Wessels M-R, rapuoli R, Fraser C-M (2005) Genome analysis of multiple pathogenic isolates of Streptococcus agalactiae: implications for the microbial "pan-genome". Proc Natl Acad Sci USA 102:13950–13955

Thanassi D-G, Cheng L-W, Nikaido H (1997) Active efflux of bile salts by *Escherichia coli*. J Bacteriol 179:2512–2518

Tilsala-Timisjarvi A, Alatossava T (1998) Strain-specific identification of probiotic *Lactobacillus rhamnosus* with randomly amplified polymorphic DNA-derived PCR primers. Appl Environ Microbiol 64:4816–4819

Tilsala-Timisjarvi A, Alatossava T (2001) Characterization of the 16 S-23 S and 23 S-5 S rRNA intergenic spacer regions of dairy propionibacteria and their identification with species-specific primers by PCR. Int J Food Microbiol 68:45–52

Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI (2009) A core gut microbiome in obese and lean twins. Nature 457(7228):480–484. Epub 30 Nov 2008

Ventura M, Zink R (2002) Specific identification and molecular typing analysis of *Lactobacillus johnsonii* by using PCR-based methods and pulsed-field gel electrophoresis. FEMS Microbiol Lett 217:141–154

Ventura M, Van Sinderen D, Fitzgerald G-F, Zink R (2004) Insights into the taxonomy, genetics and physiology of bifidobacteria. Antonie Van Leeuwenhoek 86:205–223

Versalovic J, Schneider M, De Bruijn F-J, Lupski J-R (1994) Genomic fngerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25–40

Vitali B, Ndagijimana M, Cruciani F, Carnevali P, Candela M, Guerzoni M-E, Brigidi P (2010) Impact of a symbiotic food on the gut microbial ecology and metabolic profiles. BMC Microbiol 10:4

Wall R, Hussey S-G, Ryan C-A, O'Neill M, Fitzgerald G, Stanton C, Ross P (2008) Presence of two Lactobacillus and Bifidobacterium probiotic strains in the neonatal ileum. ISME J 2:83–91

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

Willenbrock H, Hallin P-F, Wassenaar T-M, Ussery D-W (2007) Characterization of probiotic *Escherichia coli* isolates with a novel pan-genome microarray. Genome Biol 8:R267

Wilson K-H, Wilson W-J, Radosevich J-L, Desantis T-Z, Viswanathan V-S, Kuczmarski T-A, Andersen G-L (2002) High-density microarray of small-subunit ribosomal DNA probes. Appl Environ Microbiol 68:2535–2541

Wittwer C-T (2009) High-resolution DNA melting analysis: advancements and limitations. Hum Mutat 30:857–859

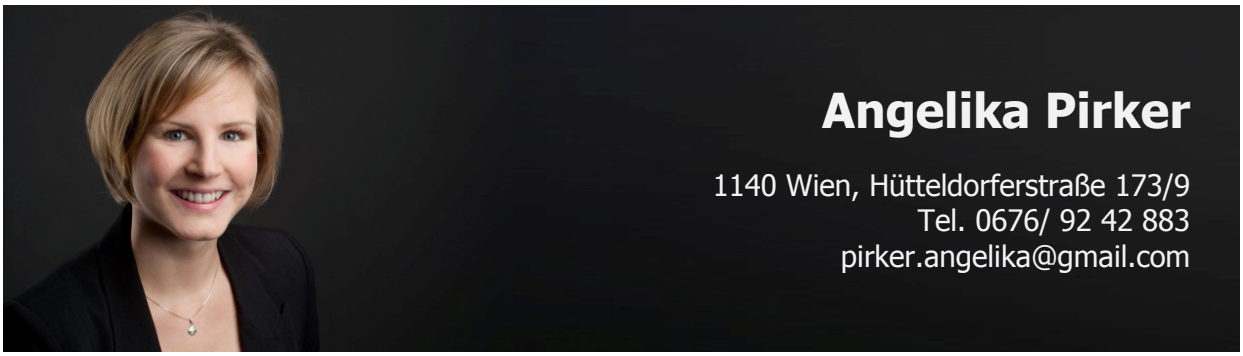
Wojdacz T-K, Dobrovic A, Algar E-M (2008) Rapid detection of methylation change at H19 in human imprinting disorders using methylation-sensitive high-resolution melting. Hum Mutat 29:1255–1260

Zeigler DR (2003) Gene sequences useful for predicting relatedness of whole genomes in bacteria. Int J Syst Evol Microbiol 53(Pt 6):1893–1900

Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, Parameswaran P, Crowell MD, Wing R, Rittmann BE, Krajmalnik-Brown R (2009) Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci USA 106(7):2365–2370. Epub 21 Jan 2009

Zoetendal E-G, Rajilic-Stojanovic M, De Vos W-M (2008) High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. Gut 57:1605–1615

14. CURRICULUM VITAE



Persönliche Daten

Name:	Angelika Pirker (geb. Kolle)
Geburtsdatum:	3. Juni 1986
Geburtsort:	Villach
Staatsbürgerschaft:	Österreich
Familienstand:	Verheiratet 04/2011

Ausbildung

Seit 06/2010	Universität Wien, Fakultät für Lebenswissenschaften Diplomarbeit: *Effects of probiotics on antibiotic disturbed gastrointestinal microbiota*
Seit 10/2005	Universität Wien, Fakultät für Lebenswissenschaften Studienrichtung: Ernährungswissenschaften Schwerpunkt: Lebensmittelproduktion und -technologie
09/2000-06/2005	Handelsakademie, Villach Abschluss mit Matura

Berufserfahrung

10/2010-02/2012	**Universität Wien, Fakultät für Lebenswissenschaften** Tutorin: UE zu mikrobiologischen und molekularbiologischen Fragen der Lebensmittel Tätigkeiten: Theoretische und praktische Betreuung der Übungen. Themen: Extraktion bakterieller DNA aus Lebensmitteln sowie deren qualitative und quantitative Bestimmunge (PCR-DGGE fingerprinting, qPCR,

Sequenzierung von DNA Fragmenten, Verwendung von online Tools)

09/2009 **Universität Wien, Fakultät für Lebenswissenschaften**
Praktikantin
Tätigkeiten: PCR-DGGE fingerprinting, cloning mit PGEM vector system (Promega), photometrische Bestimmung von DNA-Proben hinsichtlich Qualität und Konzentration, vergleichende Sequenzanalysen mittels online Tools

08/2009 **AGES – Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, PharmMed (Wien)**
Praktikantin
Tätigkeiten: Mithilfe bei der Erstellung einer pharmazeutischen Drogendatenbank

02/2009 **AGES – Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Kompetenzzentrum Ernährung und Prävention (Wien)**
Praktikantin
Tätigkeiten: Recherche und Beurteilung von guten Praxisbeispielen im Bereich der Gesundheitsförderung und Literaturrecherche, Aufbereitung relevanter Literatur für wissenschaftliche Vorträge

Seit 2004 Verschiedene Aushilfs- und Gelegenheitsarbeiten: AGES (Wien), Reed Messe (Wien), Do & Ga Stehbar (Velden), Kikeriki-Hühnergrill (Velden), PRO EVENTS Veranstaltungs GmbH (Wien), Institut für empirische Sozialforschung (Wien)

Zusatzqualifikationen

Sprachen Deutsch (Muttersprache)
Englisch (Verhandlungsfähig)
Italienisch (Grundkenntnisse)

EDV Microsoft Office Office™ (Word™, Power Point™, Excel™)

Führerschein	Klasse B
Sonstiges	Phonotypie- und Computerunterstützte Schreibtechnikprüfung (02/2003) IFS Kurs: Rhetorik I (04/2007) Peer-Mentoring-Ausbildung (09/2010)

Interessen und Hobbies

Gesundheit und Ernährung, verschiedene Sportarten wie Badminton, Tennis, Skaten, Volleyball, Schwimmen, Wandern und Klettern

Publikationen

Buchkapitel Hippe B., Zwielehner J., Pirker A., Smith W.M., Haslberger A.G. Detection and Identification of Probiotic Microorganisms and Other Beneficial Organisms from the Human GI Tract. In: *Probiotics,* Microbiology Monographs 21. (ed.) Liong M-T. (Series ed.) Steinbüchel A. Springer-Verlag Berlin Heidelberg 2011

Manuskript Pirker A., Stockenhuber A., Remely M., Harrant A., Hippe B., Kamhuber C., Stockenhuber F., Haslberger A.G. (2011) Effects of Antibiotic Therapy on the Gastrointestinal Microbiota and the Influence of *Lactobacillus casei*

Poster-präsentationen Pirker A., Hippe B., Remely M., Harrant A., Kamhuber C., Stockenhuber F., Haslberger A.G. (2011) Effects of *L.casei* Shirota on gastrointestinal microbiota during antibiotic therapy.
6th International Yakult Symposium 2011, Wien
2nd Internal Symposium microbes for health 2011, Paris

Vortrag Pirker A., Hippe B., Kamhuber C., Stockenhuber F., Haslberger A.G.* (2011) Effects of antibiotic therapy on the gastrointestinal microbiota and the intervention with *L.casei*. 6th Probiotics, Prebiotics & New Foods meeting 2011, Rome

Wien, 24. Jänner 2012