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MASTERARBEIT

Titel der Masterarbeit

The effect of Colon-hydrotherapy with probiotic intervention on gastrointestinal microbiotic diversity

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Vorwort

Die Studie, über die diese Masterarbeit verfasst wurde, wurde in Absprache mit Herrn Univ.Doz. Dr. Alexander Haslberger, von meiner Kollegien, Frau Isabella Geretschläger, und mir bearbeitet. Zur Analyse und Auswertung der Stuhlproben wurden die gleichen Methoden verwendet. Der Fokus der Arbeiten lag jedoch auf verschiedenen bakteriellen Gruppen. Meine Kollegin übernahm die Analyse von 5 Gruppen (*Bifidobakterien, Akkermansia, F.prausnitzii, Enterobakterien, Archaea*) und ich konzentrierte mich auf 6 Gruppen (*Gesamtbakterien, Laktobazillen, Clostridium Cluster IV und XIVa, Prevotella, Bakteroidetes*). Die bakterielle Diversität wurde gemeinsam analysiert.

Prozentuelle Auflistung der Tätigkeiten:

	Anteil am Arbeitsaufwand	
Methode	**Isabella Geretschläger**	**Sonja Stegmayer**
Stuhlextraktion	50%	50%
qPCR	40% (5 Bakteriengruppen)	60% (6 Bakteriengruppen)
DGGE	50%	50%
statistische Auswertung	100% der selbst analysierten Daten	100% der selbst analysierten Daten
Fragebogenauswertung	60%	40%

Content

1 List of Abbreviotaions

°C	degree Celsius
µl	microliter
BMI	body mass index
Bp	base pair
BSA	bovine serum albumin
C. leptum	Clostridium leptum
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
e.g.	exemplī grātiā
FFQ	food frequency questionnaire
g	gramm
GC	guanine-cytosine
IBS	Irritable bowel syndrome
IL	Interleukin
min	minute
ml	milliliter
NFW	nuclease free water
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
SCFA	short-chain fatty acid
Sec	second
spp.	subspecies
TAE-buffer	tris-acetat-ethylendiamintetraacetat-buffer
TEMED	tetramethylethylendiamin
T	time point
UV/VIS	ultraviolet-visible spectrophotometry
TNF-alpha	tumor necrosis factor gamma
C. cluster IV	Clostridium cluster IV
C. cluster XIVa	Clostridium cluster XIVa
F. prausnitzii	Faecalibacterium prausnitzii
B. thetaiotaomicron	Bacteroides thetaiotaomicron
B. ovatus	Bacteroides ovatus
IgE	Immunglobulin
EAACI	European Academy of Allergy and clinical Immunology
L	liter
d	day
kJ	kilojoule
kcal	kilocalorie
rDNA	ribosomal deoxyribonucleic acid
DACH	Deutschland-Österreich-Schweiz (Germany-Austria-Switzerland)

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4 Zusammenfassung

Ein Großteil an Personen leidet an Magen-Darm-Erkrankungen, häufig verursacht durch eine unausgewogene Ernährung oder Nebenwirkungen von Lebensmittelbestandteilen (Mackenzie and Dean 2011). Nicht immer spielen dabei pathogene Mikroorganismen eine Rolle, sonder eher eine Ungleichgewicht der kommensalen Mikrobiota (Ross, Mills et al. 2009). Interventionen, wie Fasten, Probiotika oder Darmspühlungen können helfen, dieses Ungleichgewicht zu modulieren.

Stuhlproben von 6 Personen eines Fastenprogramms und Proben von 49 Patienten mit Verdauungsstörungen, welche zusätzlich eine Colon-Hydrotherapy erhielten, wurden untersucht. Weiters, wurden die 49 Patienten in zwei Untergruppen eingeteilt. Davon erhielten 29 Personen eine 6-wöchige Behandlung mit einem Probiotikum und 20 ein Vitamin B-Supplement. Personen aus dem Fastenprogramm erhielten ebenfalls die Probiotakatherapie.

In der Colon-Hydrotherapiegruppe konnte ein signifikanter Unterschied in der Mikrobiotadiversität zwischen T1 und T3 der Probiotikgruppe festgestellt werden. Bei Personen der Fastengruppe zeigte sich eine Zunahme der Gesamtbakterienzahl nach dem Fasten. Jedoch kam es nach 6-wöchiger Intervention wieder zu einem Rückgang. Es gab keinen signifikanten Anstieg der Gesamtbakterienzahl in beiden Gruppen der Colon-Hydrotherapiegruppe. Das Verhältnis von *Firmicutes* zu *Bakteroidetes*, sowie *C. Cluster IV, C. Cluster XIVa, Bakteroidetes* und *Prevotella* zeigten keine signifikanten Veränderungen in beiden Gruppen. Interessanterweise zeigte sich bei den Laktobazillen eine Steigerung über alle drei Zeitpunkte in Fastengruppe, sowie in der Probiotikumgruppe nach Colon-Hydrotherapie.

Zusammenfassend zeigen die Ergebnisse keine eindeutigen Unterschiede in der intestinalen Mikrobiota vor und nach der Fastenwoche, sowie nach Colon-Hydrotherapie und probiotischer Intervention. Einige Personen zeigten Veränderungen in der bakteriellen Zusammensetzung. Schließlich kann Colon-Hydrotherapie und/oder Fasten, in Kombination mit weiteren dietätischen oder probiotischen Interventionen, eine Möglichkeit zur Verbesserung der Mikrobiotaanzahl und -diversität sein.

5 Summary

A large number of individuals are suffering from gastrointestinal disorders often caused by an unbalanced diet or adverse reactions to food (Mackenzie and Dean 2011). The gut microbiota plays a crucial role although not always pathogenic microorganisms play a role, rather an imbalance of the commensal microbiota have an influence (Ross, Mills et al. 2009). Interventions, such as fasting, probiotics or bowel cleansing, can help to modulate this imbalance.

Stool samples of 6 individuals from a fasting program and samples from 49 patients with digestive disorders, receiving an implementation of colon-hydrotherapy were examined. Furthermore, the 49 patients were divided into a subgroup of 29 individuals receiving a six week probiotic treatment and 20 a vitamin B supplementation. Individuals from the fasting program received also the probiotic supplement.

In the colon hydrotherapy group gut microbial diversity showed a significant difference in probiotic intervention group between T1 and T3. Individuals from the fasting group showed an observable increase of total bacterial abundance after fasting week but a decline after 6 weeks of probiotic intervention. There was no significant increase in total bacterial abundance in both intervention groups of the colonic hydrotherapy group. The ratio of *Firmicutes/Bacteroidetes,* the abundance of *C. cluster IV* and *C. cluster XIVa, Bacteroidetes* and *Prevotella* indicated no significant changes over the study period in both groups.

Interestingly, *Lactobacilli* showed a trend of an increase over all three time points in fasting individuals and in the probiotic intervention group after colonic hydrotherapy.

Summing up, the results show no clear differences in the gut microbiota subpopulations before and after fasting week, as well as colonic hydrotherapy and probiotic intervention. In some individuals we could observe changes in bacterial composition. In conclusion, colonic hydrotherapy and/or fasting in combination with further nutritional or probiotic intervention may be an opportunity to enhance abundance and diversity of gut microbiota.

6 Introduction

6.1 The human gut microbiota

The colonization of the gut begins during delivery by exposure to the external environment, such as maternal vaginal, skin and fecal microbiota (König and Brummer 2013). This process is influenced by many factors, such as mode of delivery (vaginal or by caesarean section), infant diet (breast or formula feeding) and the use of antibiotics by mother and child. All factors have an impact on microbiota composition and the development of the intestinal immune system, as well as long-term effects on adults‘ microbiota and health status. In the first month of life, the microbial composition is continuously increasing, but there are great individual variations. After one year the microbiota has stabilized to a more adult form (Ross, Mills et al. 2009) (König and Brummer 2013).The gut microbiota includes a population of about 10^{14} bacterial cells, which is 10 times more than the total number of human cells in our bodies (Hayes, Fraher et al. 2014). It is classified according to the classic biological nomenclature (phylum-class-order-family-genus-species) and dominated by two phyla: the *Firmicutes* and *Bacteroidetes* (90%). Moreover, *Proteobacteria*, *Actinobacteria* and *Euryarchaeota* are present in the gut. Different studies showed that the gut can be colonized by more than 1000 different bacterial species. 18 species were observed in all individual, an 57 were observed in 90% of them (Hong and Rhee 2014). In later life, age, diet, environment, and ethical background influence the gut microbiota composition and are often responsible for differences between individuals (Shoaie and Nielsen 2014). The microbiota is responsible for a variety of functions, such as the protection from pathogens, development of the immune system, extraction of nutrients and synthesis of vitamins (Hayes, Fraher et al. 2014) (Erejuwa, Sulaiman et al.). The composition of the gut is significantly affected by the diet of the host. Individuals with similar diets often have more related bacterial strains (Ross, Mills et al. 2009). In general, overweight persons show an increase in *Firmicutes* and a decrease in *Bacteriodetes*, this probably comes from differences in their diets (Hong and Rhee 2014).

6.2 Diversity of the gut microbiota

Several studies demonstrate that changes in the gut microbiota, named dysbiosis, are prevalent in several gastrointestinal disorders. These changes are often characterized by a reduction of total microbial diversity, an increased number of *Enterobacteriaceae*, an increased abundance of *Bacteroides-Prevotella*, a decrease in *Bifidobacteria*, and a decline in the number of *Firmicutes* (especially *Clostridium coccoides*, *Eubacterium rectale* and *Faecalibacterium prausnitzii*) (Kabeerdoss, Sankaran et al. 2013).

6.2.1 Firmicutes

About 60% of the gut microbiota belongs to the phylum of *Firmicutes* which includes 3 classes: *Bacilli*, *Clostridia* and *Mollicutes*. These three classes contain a number of 235 genera and all species of lactic acid bacteria. Members of the *Firmicutes* are very different in morphology, physiolgy and gram-straining characteristics. This phylum has a variety of functions, which can be beneficial, for example antioxidative or immunmodulating activities, for human health (Haakensen, Dobson et al. 2008) (Tuovinen, Keto et al. 2012).

6.2.2 Lactobacilli

Lactobacilli occur naturally in the human gastrointestinal tract and generally regard as safe (GRAS). This important group is often used in probiotic supplements for treating or preventing diverse functional gastrointestinal disorders, for stimulating the immune system and for supporting the colonization of beneficial bacteria. *Lactobacilli* have special surface properties, such as hydrophobicity and extracellular protein profiles, which are beneficial for the gut colonization. Studies observed that many strains have antioxidative and immunmodulatory activities. The microbiota of the host has to tolerate endogenous and exogenous oxidative stress. The antioxidative properties protects the microbiota from free radicals and play a major role in the prevention of different disorders such as metabolic and cardiovascular diseases as well as functional gastrointestinal disorders (Ren, Li et al. 2014). Many investigations show that some *Lactobacilli*

strains can reduce symptoms of patients with chronic gastrointestinal diseases. Studies show a significant decline in the number of *Lactobacilli* in such patients (Zhang, Liu et al. 2006).

6.2.3 Clostridium

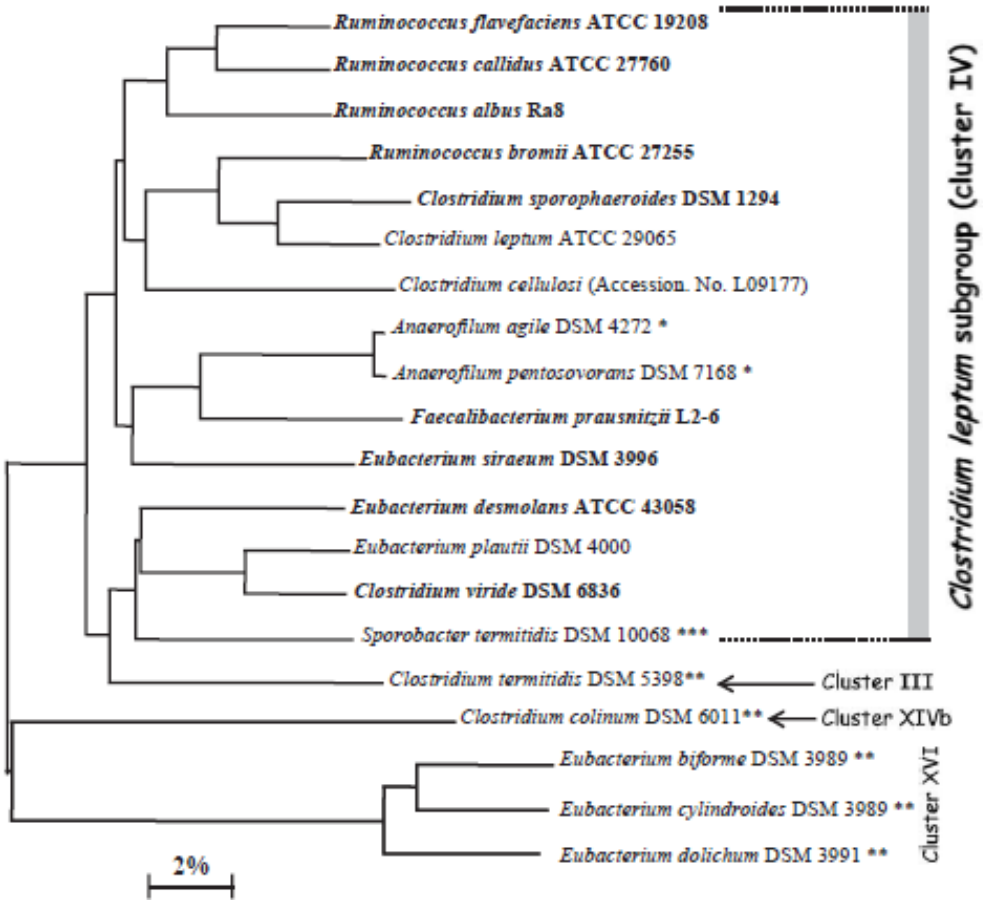
The genus *Clostridium* is among the largest and most diverse bacterial group within the *Firmicutes*. *Clostridia* are a group of anaerobic, gram-positive, spore-forming, rod-shaped bacteria and are divided into nineteen phylogenetic clusters. The main clusters in the human intestine are cluster I, XIVa, XI and IV. *Clostridia* include both beneficial as well as pathogenic organisms. It is suspected that *Clostridia* play an important role in the regulation of the immunological balance. A significant proportion of immune cells are located in the human gut. There is a permanent interaction of the microbiota with the intestinal immune system. Changes of the microbiota are associated with different immunological disorders

In a study from Tuovinen et al. they examined cytokine responses of human mononuclear cells to a panel of six type strains representing common intestinal *Clostridial species* from four clusters. The results demonstrate that several *Clostridial species* cause obvious TNF-alpha, IL-10 and IL-8 responses in human mononuclear cells. Further, the outcomes support that the quality and quantity of cellular cytokine response occurs from the interaction of host and microbial factors. This leads to the assumption that the relative proportion of *Clostridial species* has an influence on gastrointestinal inflammatory disorders (Tuovinen, Keto et al. 2012).

6.2.3.1 Clostridium leptum (Cluster IV)

C. leptum is one of the most dominant subgroups of human microbiota, representing 16-25% of fecal microbiota. This cluster encloses species of *Eubacterium* and *Ruminococcus*, as well as the main butyrate-producer *F. prausnitzii.* The members of this group are described by fermenting undigested dietary carbohydrate to short chain fatty acids (SCFA). Butyrate is the main produced SCFA and represents an important energy source for the colonic epithelium by affecting the intestinal epithelial function (Saunier, Rouge et al. 2005) (Kabeerdoss, Sankaran et al. 2013).

Figure 1 Clostridium leptum subgroup



(Saunier, Rouge et al. 2005)

6.2.3.2 Clostridium coccoides (Cluster XIVa)

The *Clostridium coccoides* subgroup represents 25-60% of total clones, and the human gut is colonized by 10^{10} to 10^{11} bacteria cells per gram of fecal matter. This cluster encloses species of *Butyrivibrio*, *Clostridium*, *Coprococcus*, *Dorea*, *Eubacterium*, *Lachnospira*, *Roseburia* and *Ruminococcus*, which are all high oxygen-sensitive anaerobes. Most of this species are butyrate-producing bacteria and therefore contribute to important regulatory processes in the colon (Hayashi, Sakamoto et al. 2006).

6.2.4 Bacteroidetes

The phylum of *Bacteriodetes* is gram-negative, anaerobic bacteria which includes species such as *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*. This group has got a large amount of glycosidase activities (Phansopa, Roy et al. 2014) and variety of complex glycans is demoted by this bacteria. Species like *B. thetaiotaomicron* and *B. ovatus* own more than twice the number of glycosidase and lyase genes compared to the human genome.
During the weight loss *Bacteroidetes* levels are enhanced, notably by fat- or carbohydrate restricted diets. This suggests that *Bacteroidetes* are responsible for calorie intake. High amount of *Bacteroides* or *Prevotella* are associated with a diet rich in animal protein and animal fat (Tremaroli and Backhed 2012).

6.2.5 Prevotella

Prevotella are obligately anaerobic, gram-negative, coccoid, moderately saccharolytic and bile-sensitive strains (Boyanova, Kolarov et al. 2010).
There is an association between the number of *Prevotella* and long-term nutritional behavior. A study showed that children living in rural African village of Burkina Faso, consuming a plant polysaccharide diet, have lower levels of *Bacteroidetes*, specially *Prevotella* and *Xylanibacter* compared with Italina children. *Prevotella* and *Xylanibacter* have the properties to reduce cellulose and xylans. Furthermore, they are connected with enhanced fecal SCFAs (short chain fatty acids). This indicates that the microbiota of the African children had

adapted to intensify the energy extraction from a fibre-rich diet (Tremaroli and Backhed 2012).

6.2.6 Firmicutes/Bacteroidetes ratio

In the composition of human gut microbiota the *Firmicutes* to *Bacteroidetes* ratio is of great importance. On the one hand, in obese individuals an increased number of *Firmicutes* and a decreases population of *Bacteroidetes* can be observed. On the other hand, the reduction of body weight leads to a decreased *Firmicutes* to *Bacteroidetes* ratio.

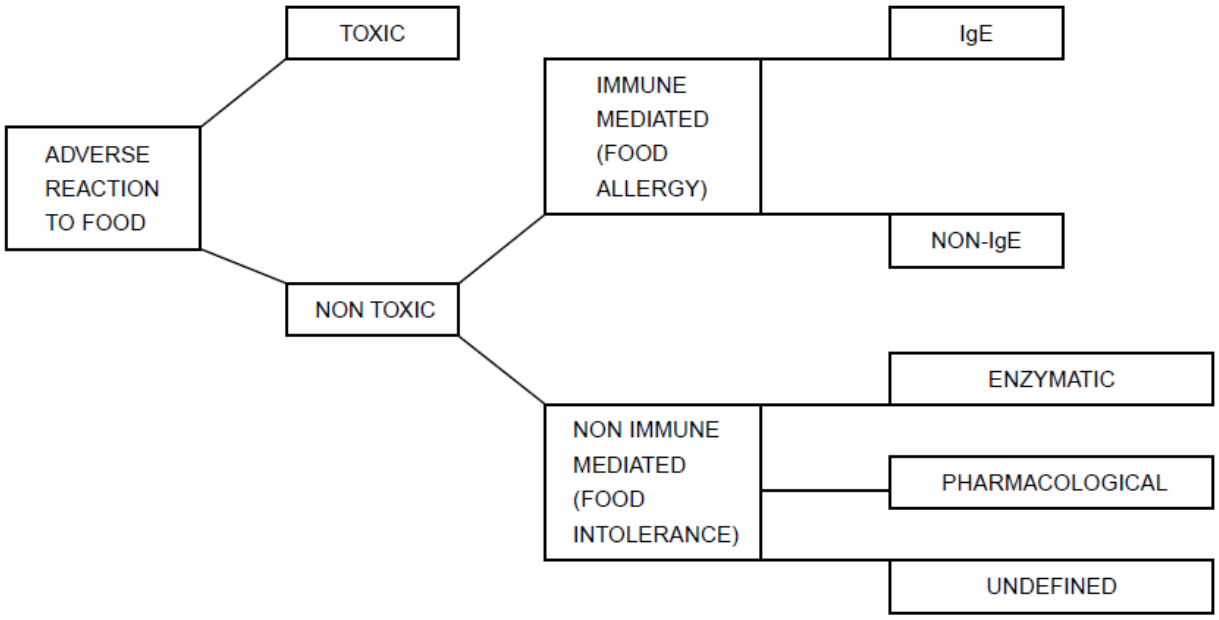
It is already known, that this ratio is an important marker of the human gut microbiota status. After birth, there is a constant increase of the ratio until adulthood. With increasing age there is a decline in transit and digestive secretion in comparison with younger adults. These observations may explain alterations in the human gut microbiota in elderly people.

Summing up, the *Firmicutes* to *Bacteroidetes* ratio is a possible marker for alterations in bacterial profiles at different stages of life (Mariat, Firmesse et al. 2009).

6.3 Intolerances and allergies

Nowadays, a large number of individuals are suffering from food allergies or intolerances. The most common allergies or intolerances among individuals are lactose-, fructose- and histamin-intolerance as well as cow milk protein allergy or wheat protein allergy. Adverse reactions to food can cause a variety of different symptoms, and although the terms “food allergy“ and “food intolerance“ are often mixed up, but they refer to quite different illnesses. The term “food hypersensitivity“ means a non-toxic reaction to food, this reactions are divided into immune mediated and non-immune mediated. Food allergies are immune mediated reactions by the antibody IgE or occur via other immune mechanisms. Reactions that are not immune mediated are called „food intolerances“, these can appear via a variety of mechanisms, such as enzyme deficiencies or pharmacological effects. Symptoms range from mild to serious and affect one or more organs, typically the skin, the gastrointestinal tract or the respiratory system (Mackenzie and Dean 2011). In general, to avoid specific foods can alleviate symptoms. (Wilder-Smith, Materna et al. 2013)

Figure 2 Adverse reactions to Food EAACI classification



(Ortolani and Pastorello 2006)

The IgE antibodies play the main role in allergy. In theory, all foods can cause allergic reactions, but in reality a small part is responsible for food allergies: milk, eggs, wheat, fish, soy and peanuts and some fruits, especially cherries, peaches, plums and apricots. Many of the symptoms occurring in food allergies are also present in food intolerances but have other causes. (Zukiewicz-Sobczak, Wroblewska et al. 2013)

As already mentioned above, various food allergies and intolerances can affect the development and the symptoms of gastrointestinal disorder. Therefore, the treatment of food allergies and intolerances should be considered and included in the treatment of gastrointestinal diseases. Some studies examined, consuming an exclusion diet, by avoiding foods which promote increased IgE antibodies, a significant decline of IBS symptoms could be shown (Hayes, Fraher et al. 2014).

6.4 Gastrointestinal diseases and gut microbiota

Different disorders are associated with changes in the microbiota as well as with host-microbiota interactions, ranging from metabolic disorders, such as obesity or diabetes to gastrointestinal disorders such as IBS (irritable bowel syndrome) (Hong and Rhee 2014). Not always pathogenic microorganisms play a role in these conditions, rather components of the normal microbiota have an influence on these diseases (Ross, Mills et al. 2009). These problems can play a role in digestive disorders such as IBS, typically symptoms are often caused by an altered intestinal microbiota composiotion and bacterial overgrowth (Owen 2011). A recent study described decreased fecal *Lactobacilli* and *Bifidobacteria* and increased *Streptococci*, *Escherichia coli* and anaerobic organisms such as *Clostridium* in patients suffering from IBS (Hong and Rhee 2014). Symptoms may be due to the characteristics of these bacteria. Some observations show that increased numbers of *Firmicutes* may be responsible for abdominal pain, because they produce proteases, which stimulate sensory afferents in the intestine. *Lactobacilli* and *Bifidobacteria* have anti-inflammatory effects, decreased

numbers of these bacteria could lead to low-grade inflammation (Hayes, Fraher et al. 2014).

6.4.1 Irritable bowel syndrome (IBS)

About 10 to 20% of the European population is affected by irritable bowel syndrome (IBS). This global prevalence can underlie individual variations. Besides, it is 2-3 times more common in women than in men. IBS is described as a functional gastrointestinal disorder and patients often suffer from abdominal pain or discomfort due to bloating, problems with defecation, changes in stool frequency and consistency. Because of the difficult diagnosis of IBS, patients are divided according to their predominant symptoms. There are 3 subtypes of IBS: constipation-predominant IBS (IBS-C), diarrhea-predominant IBS (IBS-D), mixed IBS (IBS-M) and unsubtyped IBS. However, there is the possibility that a patient will vary during its life between several types. In addition, patients often suffer from psychosocial or mental problems and complain about a reduced quality of life and work productivity, summarized in an affected gut-brain axis (Dupont 2014). Few studies investigated the influence of diet on IBS. Intolerances or food allergies may intensify symptoms (Dai, Zheng et al. 2013) (Hayes, Fraher et al. 2014)

Patients mostly suffer from psychological disorders like anxiety and depression. In addition, stress is often associated with an intensification of IBS symptoms. Many patients stay away from social events to avoid embarrassment due to postprandial exacerbation of symptoms (flatulence and distension) and lack of access to toilet facilities. In many cases this behaviour leads to social isolation (Hayes, Fraher et al. 2014).

6.5 Modulation of the gut microbiota

6.5.1 Probiotics

One possibility to influence the gut microbiota is the intake of probiotics. The word “probiotic“ comes from the Greek language and means “for-life“. Probiotics are live organisms that, in adequate amounts, can have positive effects on the health of human body, by ameliorating intestinal microbial balance. The market of such commercially available supplements, containing different microorganisms, is enormous. For the production of probiotics the mainly used bacterial strains are *Lactobacilli* and *Bifidobacteria*. In general probiotics apply to the host as safe, with less or no adverse side effects (Erejuwa, Sulaiman et al. 2014), (Quigley 2010). Still, not all positive effects of probiotics are observed and completely understood. Some have the ability to be effective as antagonists against pathogenic species by replacing them or by inhibiting their occurrence. Furthermore, they have also the ability to induce beneficial immune response, by direct interaction with immune or epithelial cells, or by secreted molecules (König and Brummer 2013). Different studies suggest that the supplementation of probiotics is a beneficial strategy in the treatment of several metabolic and gastrointestinal disorders (Erejuwa, Sulaiman et al. 2014), (Quigley 2010).

6.5.2 Vitamins

The microbiota has the ability to synthesize different vitamins, which are involved in several metabolic pathways of the host. These include cobalamin (B_{12}), pyridoxal phosphate (B_6), pantothenic acid (B_5), niacin (B_3), biotin, tetrahydrofolate and vitamin K. (Kau, Ahern et al. 2011). Unfortunately, there is a lack of studies showing and describing possible advantages or disatvantages of a vitamin supplementation on the human gut microbiota in more detail.

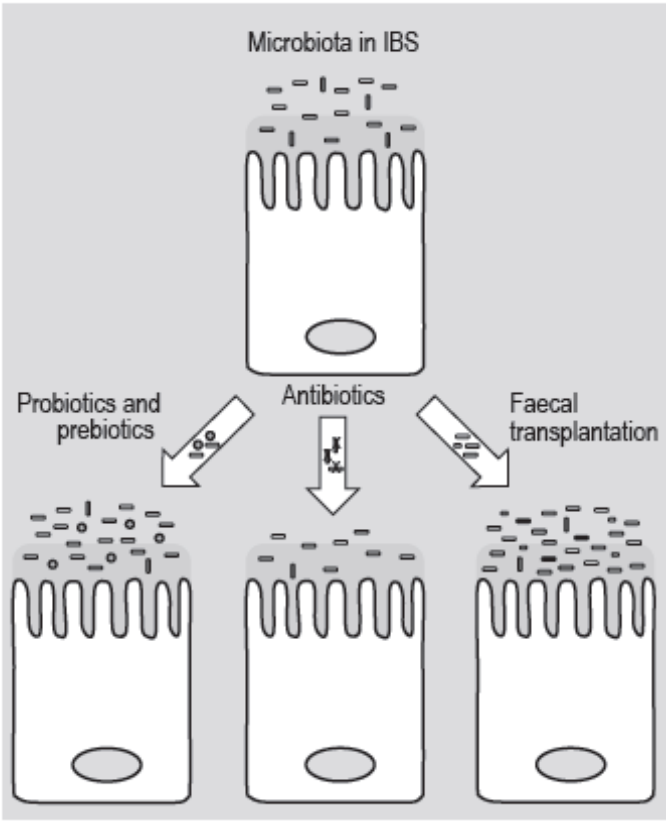
6.5.3 Probiotics and gastrointestinal disorders

Possible positive effects of probiotics in patients with gastrointestinal disorders could be a reduction of bacterial overgrowth, increasing number of beneficial bacteria, as well as compensating the imbalance of anti- and pro-inflammatory cytokines. Moreover, there could be a normalization of the digestive tract motility and a reinforcement of the mucosal barrier. Recent studies showed that there could be a modulating effect of some *Lactobacilli* strains on intestinal pain attacks by an inclusion of the expression of μ-opioid and cannabinoid receptors in the gastrointestinal epithelial cells.(Dai, Zheng et al. 2013)

In Europe, probiotics, as dietary supplement, become more and more important in the treatment of patients with IBS. Qualitative and quantitative changes in the microbiota and immune dysfunction may be prevalent in IBS. Studies have shown, that, a number of organisms, *Lactobacilli* and *Bifidobacteria* mitigate patients IBS symptoms, such as bloating, flatulence and constipation, but only a few products have been shown to affect pain and other symptoms in IBS (Quigley and Flourie 2007). Some probiotics might be able to modulate the perception of visceral pain, by inducing the expression of opioid and cannabinoid receptors (König and Brummer 2013). Benefits of specific probiotic strains can also be a reduction in gas production, changes in bile salt conjugation, antibacterial or –viral effects, effects on mucus secretion, or even anti-inflammatory effects (Quigley and Flourie 2007).

In the figure below, potential strategies for the re-establishment of a healthy microbiota in IBS patients are shown. In IBS, the microbiota indicates a poor diversity and is characterized by a small number of beneficial bacteria (horizontal rods). An increase of harmful bacteria (vertical rods) could be reported. Probiotics might act by enhancing the number of beneficial bacteria, while antibiotics destroy harmful ones (König and Brummer 2013).

Figure 3 Microbiota in IBS



(König and Brummer 2013)

In general, diet has a significant impact on the composition of the gut microbiota. Mainly the microbiota is influenced of the following macronutrients: carbohydrates, proteins and fats. Indigestible carbohydrates for the host, such as resistant starch that is mainly used by *Ruminococcus bromii*, are fermented by the microbiota. Every day about 40g of carbohydrates, consisting of resistand starch, non-starch polysaccharides and oligosaccharides, reach the colon. *Bacteroides* and *Clostridium* species are important for protein fermentation. Proteins are used for the production of short chain fatty acids (SCFAs), ammonia, phenols, amines and sulfide. Fat is usually absorbed through the small intestine, only a small fraction is excreted in the feces. As a result of a low fat diet the production of SCFAs are reduced. In several animal studies they observed that, the microbiota alterations for different fat diets are not connected to the host phenotype. 60-90% of SCFAs are absorbed by the epithelial cells and may have an influence on the host physiology. SCFAs have a variety of functions: regulation of the energy supply for epithelial cells, influence on the pH in the colon and prevention of the growth of pathogens. Different disorders such as obesity, type2 diabetes and colorectal cancer may be affected by disturbances in the metabolism of SCFAs (Shoaie and Nielsen 2014).

6.5.4 Fasting and caloric restriction

Fasting is a limited period of time with an extreme shortage or complete absence of food. The objective of this therapy is to use the body's energy reserves, without endangering health. The energy which is normally required for digestion, resorption, transport and storage of nutrients, is saved during the fasting period. Aging pathways are reduced and the cell switches to a protected mode. Due to, the lack of nutrients, diseased cells are weakened by this nutrient deficiency.

Earlier fasting had religious or spiritual reasons, and sometimes it was used as medical or therapeutic treatment. In Germany, fasting has a long tradition and two methods are most commonly used: The fasting cure (Heilfasten) by Otto Buchinger and the medical fasting cure by Franz-Xaver Mayr. Buchinger devel-

oped a multidisciplinary treatment concept in which physiotherapy, nutrition, psychotherapy and physical activity are combined. Additionally, to the physical component of fasting there is also a psychosocial component caused by group support and group dynamics during the fasting program.

A fasting program involves body, soul and spirit of all individuals. It is important that during the fasting period one takes up about 2.5l/d of calorie-free liquid (water or herbal tea.). Furthermore, the diet consists of vegetable broth, fruit or vegetable juices and honey. Summing up, the intake includes not more than 1.500-2.100 kJ (250-500 kcal) per day. Fasting releases cardiovascular, metabolic and psychological changes which should be observed during the fasting period. After fasting the participants should reach a good level of vitality and absence of hunger. Moreover, a stepwise reintroduction of solid food intake is of importance to have the maximal benefit after the end of the fasting period.

Some reported positive effects of fasting are: improvement of metabolic syndrome, chronic inflammatory diseases, chronic cardiovascular diseases, chronic pain syndromes, atopic disease and psychosomatic disorders (de Toledo, Buchinger et al. 2013).

6.5.5 Colonic hydrotherapy - Bowel cleansing

For one treatment about 60l of water are pumped through the rectum by a tube, to stimulate the emptying of the intestine by extending the lower bowel. During the treatment the patient lies on a table and often herbal infusions or coffee are used. Another tube removes fecal substances and fluids. One therapy lasts about 30 to 45 minutes and can be repeated several times. A considerable percentage of hydrotherapists have a medical education. The treatment will be attributed to a number of beneficial effects. Many hydrotherpists claim that during life a thick layer of fecal substances accumulates in the colon. This wall disturbes other organs, the nervous system, as well as the absorption of essential nutrients from the intestine. This may cause autointoxication processes, by absorbing toxins from this fecal matter into the bloodstream, which leads to different immune system related disorders. Moreover, this malfunction will lead to

weight gain, constipation, diarrhea and other symptoms. There is also the assumption that herbal and coffee consist of ingredients that may enhance the function of the gut, improve the microbiota compositon, improve repair mechanismen and influence intestinal muscle contractions. Furthermore, they claim that colonic hydrotherapy leads to a better weight regulation and also an improvement of several immune and inflammatory disorders. After the treatment the absorption of nutrients from the intestine will be improved and the absorption of enterotoxins deteriorated (Mishori, Otubu et al. 2011) (Seow-Choen 2009).

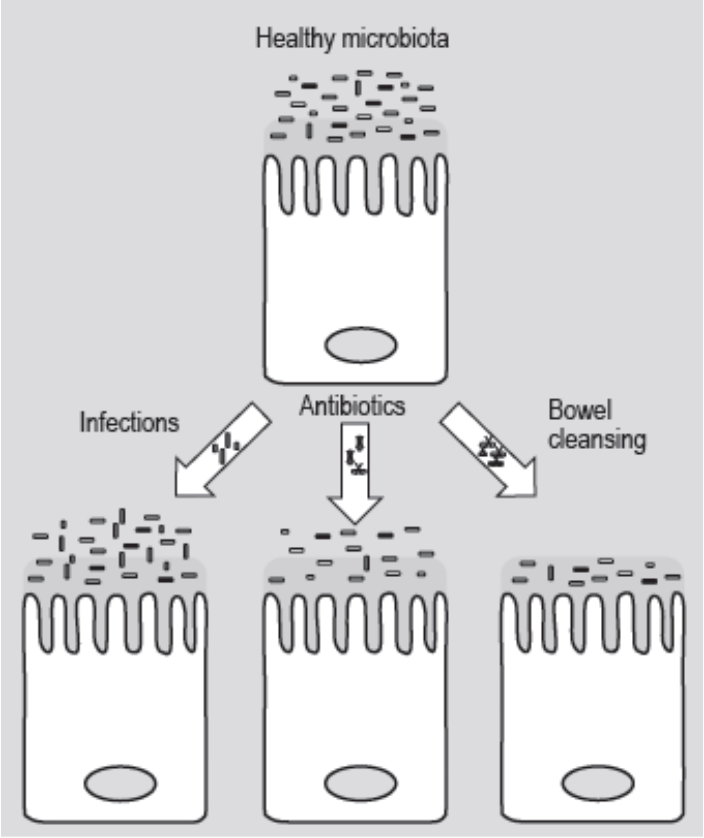
The main essential nutrients are absorbed during passage through the small intestine and not in the colon. Fecal substances from the left colon are solid and consist of indigestible components of food, bacteria and dead cells. By using colonic hydrotherapy this solid components will be liquefied and can be easier absorbed than the solid ones. But to absorbe this matters would not always be a benefit for the body (Seow-Choen 2009).

Summing up, scientifically studies are missing to confirm all these assumptions, but probably some of them are untrue.

The influence of a routine or self-administrated bowel cleansing on the symptoms of IBS has never been examined and compared to antibiotic use, also positive effects can occur. In IBS patients, it might be that bowel cleansing leads to a reduction in the total bacteria abundance and thereby gives the microbiota an opportunity to re-establish a healthy balance. This effect could be enforced by the intake of probiotic supplements (König and Brummer 2013).

The figure below shows interferences of the human microbiota by supporting the development of irritable bowel syndrome. Infections lead to an overgrowth of harmful bacteria, while bowel cleansing and antibiotics are able to decrease the diversity of the microbiota by reducing both pathogenic and beneficial bacterial strains. Horizontal rods picture beneficial bacteria, such as *Bifidobacteria*, *Lactobacilli* and butyrate producers. Vertical rods picture harmful bacteria, such as *Ruminococcus torques* (König and Brummer 2013).

Figure 4 Interferences of the human microbiota



(König and Brummer 2013)

7 Objectives

Gut microbiota and its interactions with the human metabolism play an important role in the development of several disorders. Most of the time a dysbiosis of gut microbiota subpopulations, or/ and a reduction of total microbial diversity are causative. Thus different strategies can modulate the human gut microbiota composition and mitigate symptoms. The microbiota can be influenced by the intake of probiotics and vitamins, short-term changes in the diet, such as fasting or caloric restriction, as well as the implementation of a colon hydrotherapy.

The aim of the present study was to determine differences of the microbial composition and diversity in individuals suffering from digestive problems receiving an intervention over three timepoints. The impact of a one week fasting therapy in 6 individuals and the influence of colonic hydrotherapy in 49 patients with a subsequent intervention of probiotics or a vitamin B control were investigated. Results between each group were compared before, during and 6 weeks after intervention period. Moreover, changes of dietary habits were examined by food frequency questionnaire. Bacterial groups were analyzed using quantitative real time polymerase chain reaction of 16s rDNA and PCR-DGGE.

The results should help to better understand the complex interplay between human gut microbiota, digestive disorders and different therapeutic approaches to treat gastrointestinal disorders and to mitigate symptoms. Although further investigations shall focus on a more individual treatment in order to achieve the best results.

8 Material and Methods

8.1 Study participants and study design

Stool samples of 6 individuals from a fasting program and samples from 49 patients with digestive disorders, who received an implementation of colon-hydrotherapy were examined.

8.1.1 Fasting group

Six individuals (aged 53.33±6.55 ages, BMI 28.10±3.50 kg/m^2) were recruited in cooperation with health trainer Mrs Ingrid Höfinger and Dr. Georg Wögerbauer. All participants joined a one-week fasting program in Pernegg Monastery, defined and supervised by Mrs. Höfinger (Table 1). On the second day of the fasting program all participants received Glauber's salt (sodium sulfate), which was used as a laxative for full defecation.

After a one-week fasting the participants get a probiotic supplement for 6 weeks. The probiotic supplement “Progutic® LactoVitamin BALANCE” includes per capsule 7 different DUOLAC® bacterial strains: *Lactobacillus plantarum, Streptococus thermophiles, Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium breve.* Moreover, one capsule consists of fructooligosaccharides, 200 µg folic acid, 2.50 µg vitamin B12 and 55 µg selenium (all amounts corresponding to 100% of daily demand).

Table 1 Details of the fasting program supervised by health trainer Mrs Ingrid Höfinger

Day of Arrival	dinner: vegetable soup and crispbread
First day	breakfast: Pernegg muesli (prunes, dates, raisins, flaxseed, water) lunch: potatoes and vegetables dinner: vegetable soup
Second day	breakfast: herbal tea, Glauber's salt lunch: fresh squeezed fruit and vegetable juice dinner: fasting soup
All other fasting days	breakfast: herbal tea lunch: fresh squeezed fruit and vegetable juice dinner: fasting soup
Day before departure	breakfast: herbal tea lunch: apple dinner: steamed potato with vegetables and herbs
Day of Departure	breakfast: Pernegg muesli, porridge, fruit salad

Table 2 Characterization of study participants

Group		**Fasting patients**
Number		6
Sex	Female	3
	Male	3
Age ± SD (years)		53.33 ± 6.55
BMI ± SD (kg/m^2)		28.10 ± 3.50

8.1.2 Colonic hydrotherapy group

49 individuals (aged 45±13 ages, BMI 25.31±6.91 kg/m^2) with gastrointestinal problems, IBS, were enrolled for this study. The participanting individuals obtained a colon-hydrotherapy (between 3-5 repetitions) to mitigate symptoms. After colon-hydrotherapy the participants were subdivided into two groups: a probiotic group, who also received the above mentioned Progutic® LactoVitamin BALANCE for six weeks. The other group received an equivalent capsule of a vitamin B supplement (Vitamin B complex includes per capsule: 10µg cobalamin, 450µg folic acid, 55 µg selenium) for upcoming six weeks.

Table 3 Characterization of study participants

Group	**Probiotic**	**Vitamin**
Number	29	20
Sex	♀ 22 ♂ 7	♀ 13 ♂ 7
Age ± SD (years)	43,72±11,55	48,85±14,47
BMI ± SD (kg/m^2)	25,91±8,08	24,21±5,32

Fecal samples were collected at three time points: before fasting and colon-hydrotherapy (T1), during fasting (after sodium sulfate intake) and direct afer colon-hydrotherapy (first consistent stool) (T2) and 6 weeks after probiotic intervention, respectively after vitamin B supplementation (T3). Additionally, all participants were asked a FFQ at the beginning (T1) and at the end of the intervention (T3). The FFQ reported the frequency of consumption and portion size, as well as questions about lifestyle (i.e. smoking, alcohol consumption, physical activity), medical relevant influences (i.e. vitamin and other supplements), body mass index (BMI), and age.

8.2 Fecal sample collection, processing, and analysis

Stool samples were collected and immediately stored at -18°C until further processing. According to the manufacturer´s protocol bacterial DNA was extracted from fecal samples using the QIAamp® DNA Stool mini kit (Qiagen, Germany). Additionally, samples were treated in FastPrep™ Lysing Matrix E tubes (MP Biomedicals, USA) twice for 45 sec in a bead-beater (Mini-Beadbeater 8 Bio-Spec Products, USA) with an intervening minute on ice. DNA concentration and quality of extraction was analysed by Pico100 (Picodrop, UK) and gel electrophoresis. Picotrop is a UV/VIS Spectrophotometer for DNA, RNA and other nucleic acid concentration measurements and protein analysis.

8.2.1 Denaturing gradient gel electrophoresis

The total bacterial diversity was measured by DGGE (denaturing gradient gel electrophoresis). DGGE is a type of electrophoresis that uses a chemical gradient for denaturing the samples. The PCR products are based on the GC content (melting behavior) separated in denaturing gels. A separation with only one base difference is possible. This method can be used for DNA, RNA and proteins.

8.2.2 Real time quantitative polymerase chain reaction

Bacterial abundance was quantified with TaqMan qPCR and SYBR Green qPCR in a Rotorgene 3000 (Corbett Life Science, Australia) using 16S rDNA group specific primers (Table 1-2). The PCR reactions mixture and serial DNA dilution of typically strains were prepared according to Pirker et al. 2012 (Pirker, Stockenhuber et al. 2012).

Table 4 Primers and TaqMan®-probes targeting 16rRNA coding regions of bacteria and archaea

Target organism	Primer/Probe	Sequence (5' - 3')	Size (bp)	Conc. [pmol/µL]	Reference
All *Bacteria*	Fwd primer	ACT CCT ACG GGA GGC AG	468	10	(Yu, Lee et al. 2005)
	Rev primer	GAC TAC CAG GGT ATC TAA TCC		10	
	Probe	(6-FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		2	
Clostridium* cluster IV *(Ruminococcaceae)	Fwd primer	GCA CAA GCA GTG GAG T	239	4	(Matsuki, Watanabe et al. 2004)
	Rev primer	CTT CCT CCG TTT TGT CAA		4	
	Probe	(Louis, Guerineau et al.)-AGG GTT GCG CTC GTT-(BHQ-1)		2	
Cluster XIVa (Lachnospiraceae)	Fwd primer	GCA GTG GGG AAT ATT GCA	477	5	(Matsuki, Watanabe et al. 2004)
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A		5	
	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)		1,5	
Bacteroidetes	Fwd primer	GAG AGG AAG GTC CCC CAC	106	3	(Layton, McKay et al. 2006)
	Rev primer	CGC TAC TTG GCT GGT TCA G		3	
	Probe	(6-FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)		1	

Table 5 Primers (SYBR® Green) targeting 16rRNA coding regions of bacteria

Target organism	Primer	Sequence (5' - 3')	Size (bp)	Conc. [pmol/µL]	Reference
Lactobacilli	Fwd primer	AGC AGT SGG GAA TCT TCC A	352-700	4	(Walter, Hertel et al. 2001)
	Rev primer	ATT YCA CCG CTA CAC ATG		4	
Prevotella	Fwd primer	CACCAAGGCGACG ATCA	1458	2,5	(Larsen, Kondo et al. 2008)
	Rev primer	GGATAACGCCYGG ACCT		2,5	

The detailed procedure prodocol of all methods, see appendix.

8.3 Statistical analysis

For statistical analyzes we used the OriginPro version 8 (OriginLab, USA). With the non-parametric Kruskal Wallis ANOVA and the non-parametric Mann-Whitney U-Test we compared the groups. Results were defined as statistically significant at a p-values < 0.05.

9 Results

9.1 Fasting group

9.1.1 Analyses of the retrospective FFQ

Evaluation of the FFQ showed that only one out of six participants (16.67%) consumed vegetables 5-10 times per week and two out of six (33.33%) consumed fruits 5-10 times. In comparison DACH guidelines recommend five portions of fruits and vegetables per day. According to DACH guidelines meat and sausage are advised for 2-3 times per week, 50% of participants comply with recommendations, 33.33% eat meat and sausages nearly every day. 83.33% consume fish 1-3 times per week and only one 5-10 times per week.

Only one third of participants meet the recommendations of wheat and whole grain products consumption with a daily intake, 50% eat less than 4 portions per week. 33.33% consume dairy products nearly daily, 50% of participants eat less than 5 portions and only one out of six eat more than 15 portions per week. One third consumes sweets 1-3 times per week, another third 3-5 times and another third more than 10 times per week. In addition to normal diet 33.33% take nutritional supplements (i.e. ascorbic acid, vitamin D, calcium). Additionally questions to physical activity disclosed only one participant practicing daily movement, but 66.67% do sport regularly 1-3 times per week. Questions about stool behavior show that 83.33% of participants documented no conscious problems with defecation.

The FFQ asked after probiotic intervention showed no significant differences in the dairy product consumption, as well as in the intake of meat, sausages and fish compared to the first FFQ. There is no observable increased intake of fruits and vegetables. Furthermore, the evaluation shows no increased or decreased uptake of wheat, whole grain products and sweets. There was a noticeable increase in daily movement in all participants from 16.67% to 33.33%, also regular physical activity improved from 66.67% to 83.33% of participants. However, after fasting week and probiotic intervention 100% documented no conscious problems with defecation.

9.1.2 Compositional evaluation of gut microbiota

9.1.2.1 Total bacterial abundance

There was no significant increase in total bacterial abundance between all three time points (p=0.75). However, there was an observable increase between T1 and T2 (p=0.47) and a decline from T2 to T3 (p=0.81).

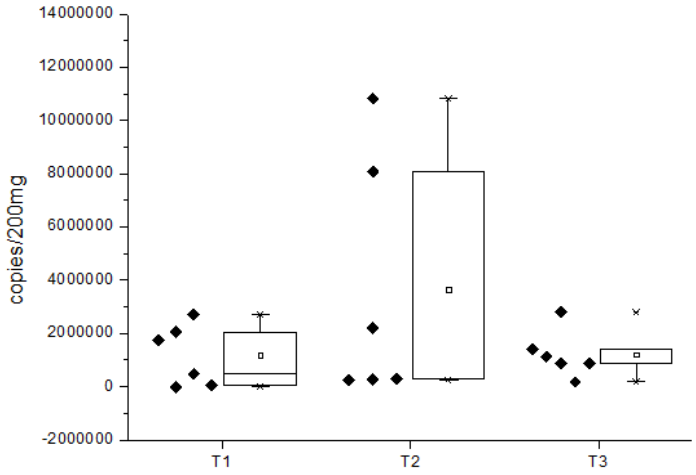


Figure 5 The boxplot diagram shows results of *total bacterial abundance;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

9.1.2.2 Firmicutes/Bacteroidetes ratio

We observed no significant changes in the ratio of *Firmicutes/Bacteroidetes* between the three time points (p=0.46).

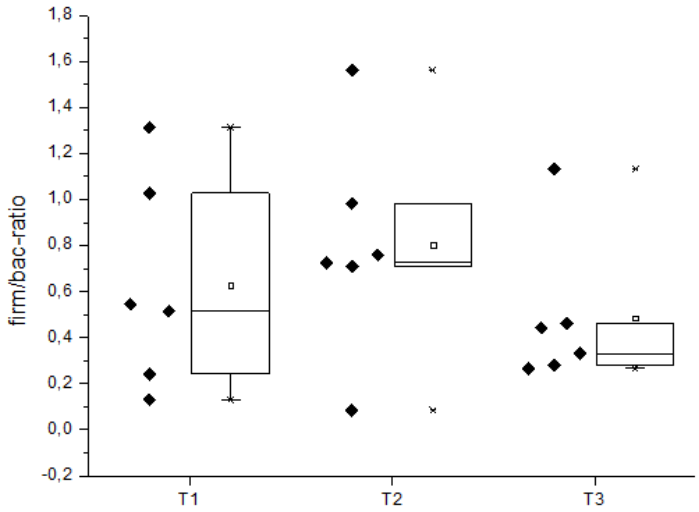


Figure 6 The boxplot diagram shows results of firmicutes/bacteroidetes ratio; Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

9.1.2.3 Clostridium cluster IV and Clostridium cluster XIVa

Furthermore, we detected no significant changes in the abundance of *Clostridium cluster IV* (p=0.74), as well as in the abundance of *Clostridium cluster XIVa* (p=0.71) between the three time points.

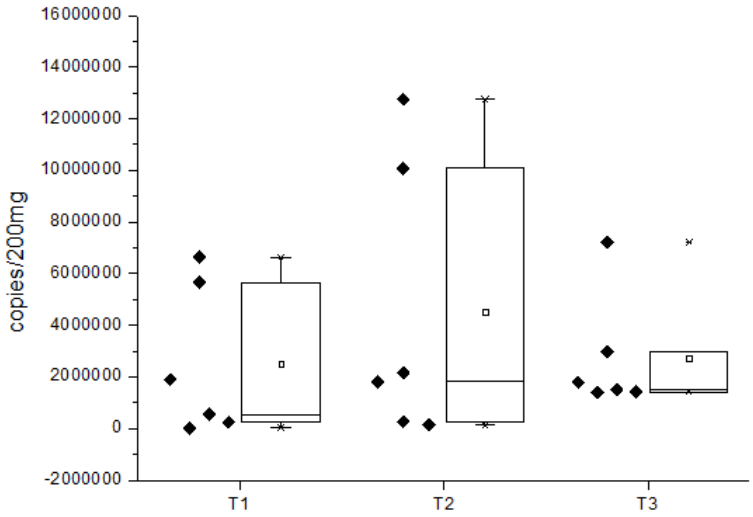


Figure 7 The boxplot diagram shows results of *Clostridium cluster IV;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

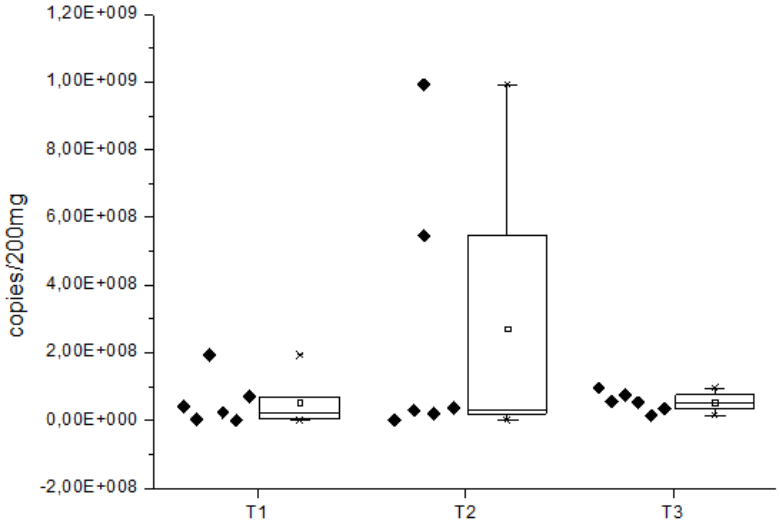


Figure 8 The boxplot diagram shows results of *Clostridium cluster XIVa;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

9.1.2.4 Lactobacilli

Lactobacilli show a trend of an increase from the first to the second time point (p=0.47) and also from the first to the third time point (p=0.14, Figure 2).

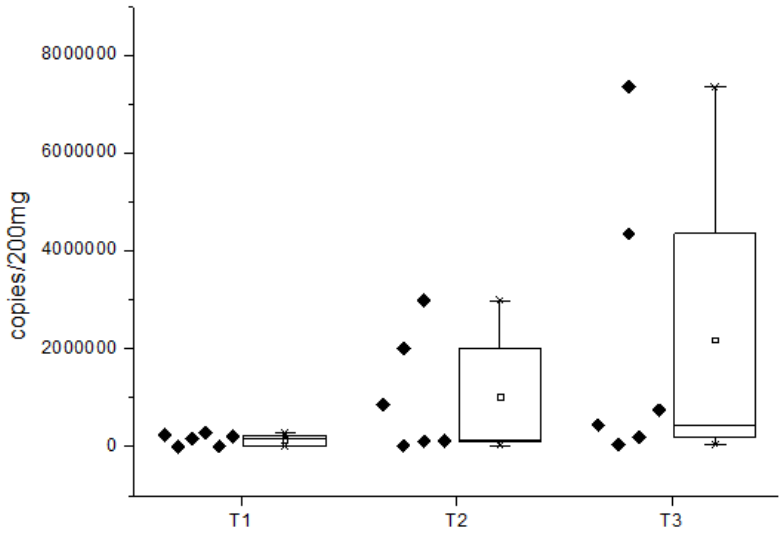


Figure 9 Quantification of *Lactobacilli* showing an increase over intervention period (T1-T3: p=0.14) Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

9.1.2.5 Bacteroidetes and Prevotella

There were no significant alterations in the abundance of *Bacteroidetes* (p=0.59) and *Prevotella* (p=0.81) over the study period.

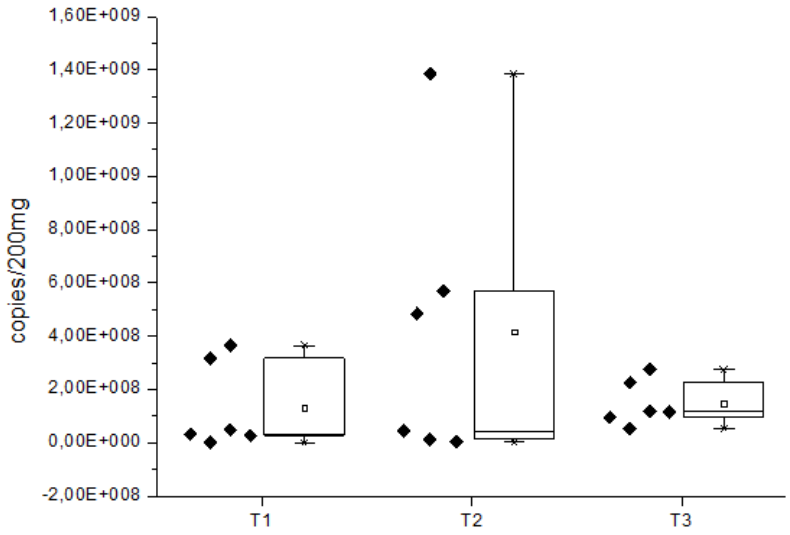


Figure 10 The boxplot diagram shows results of *Bacteroidetes;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

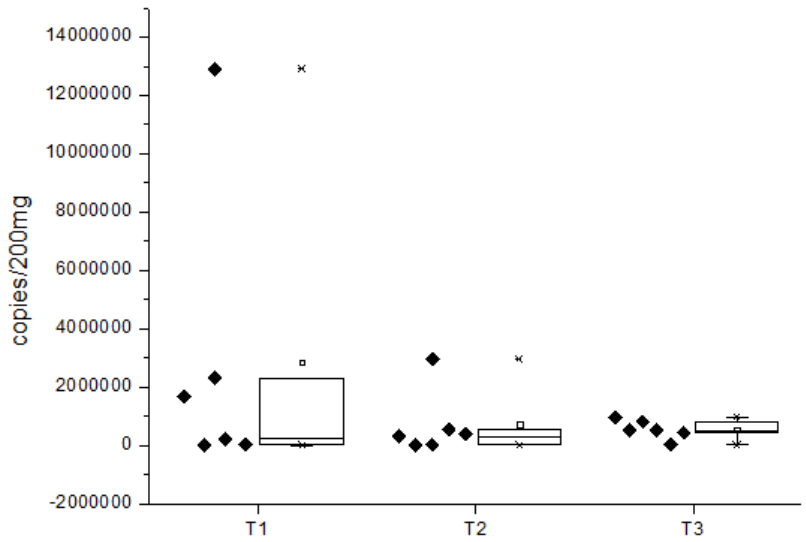


Figure 11 The boxplot diagram shows results of *Prevotella;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

9.2 Colonic hydrotherapy group

9.2.1 Analyses of the retrospective FFQ

Most of the participating individuals (T1:31%, T3:33%) ate fruits and vegetables 5-10 times per week. At both dates 25% ate this food group more than 10 times per week. DACH guidelines recommend five portions of fruits and vegetables per day. About 50% of participants met the recommendation of the DACH guidelines consuming meat and sausages only 2-3 times weekly. The first FFQ showed that 36% of them consumed meat almost daily and this number rose compared to the second FFQ to 38%.

Question about fish intake demonstrated that the majority of participants (46% at T1 and 48% at T3) consumed fish 1-3 times weekly. 34% (T2) of individuals reported a fish intake below the DACH recommendation of 1-3 portions. 25% (T1) of participants ate dairy products every day. We observed a reduction of the dairy products intake in the second FFQ (16%). The recommendation for wheat and whole grain products is a daily intake. Almost one half of individuals reached this recommendation. Individuals reported eating sweets between 1 and 5 times weekly (T1:62%, T3:60%). 18% (T1) and 20% (T3) of individuals ate sweets less than once per week. Only 15% consumed sweets daily. Only 32% (T1) and 40% (T3) of participants practice daily movement. But 42% did sport 2-3 times weekly. Questions about stool behavior indicated that 42% had no conscious problems with defecation at T1. 50% (T3) documented no conscious problems with defecation.

9.2.2 Compositional evaluation of gut microbiota

We observed a significant difference of microbial diversity in the probiotic group between T1 and T3 (p=0.003) with a mean at T1 of 12 +/- 5.5 and at T3 of 17+/- 4.6 showing a correlation between the time-points (R=0.65, p=0.02).

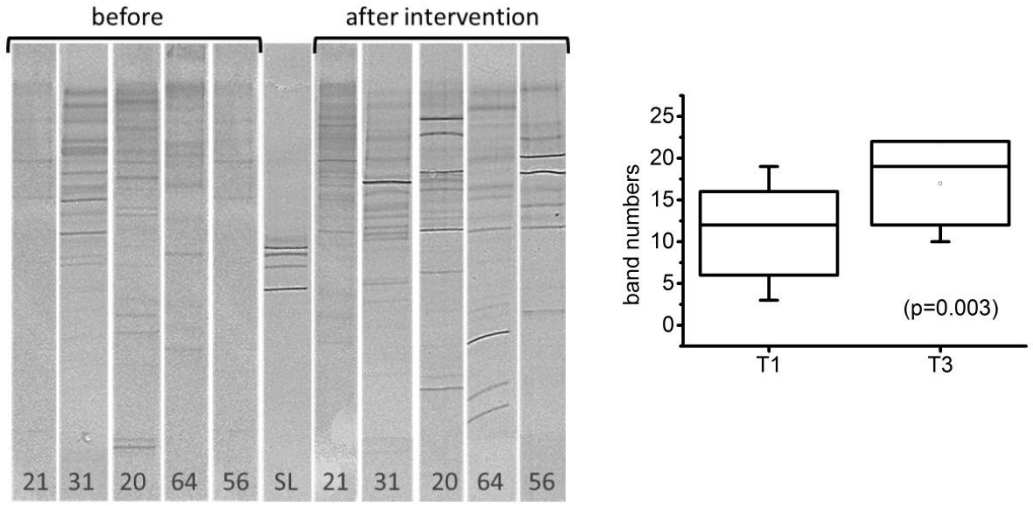


Figure 12 Diversity analysis. A PCR-DGGE fingerprinting of 16S rDNA coding regions of dominant bacteria over time indicating a lower number of bands in the probiotic intervention group at T1 in comparison to T3 B Quantification of number of bands showing an increase of diversity in the probiotic intervention group between T1 and T3 (p=0.003) Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range(T1: before colon-hydrotherapy, T3: after six weeks of probiotic or vitamin intervention, SL: standard lane)

9.2.2.1 Total bacterial abundance

There was no significant increase in total bacterial abundance in the probiotic group (p=0.83), as well as in the vitamin group (p=0.91) at all three time points. Comparing time point three of the two groups we could also show no significant difference (p=0.94).

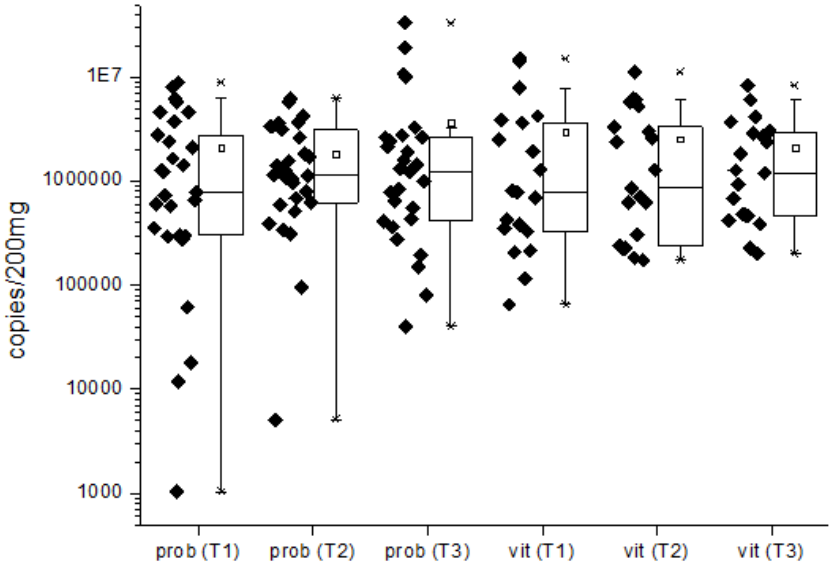


Figure 13 The boxplot diagram shows results of *total bacterial abundance* in both groups (prob=probioticum; vit=vitamin)*;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before colonic hydrotherapy (T1), after colonic hydrotherapy (T2), and 6 weeks after probiotic or vitamin intervention (T3)).

9.2.2.2 Firmicutes/Bacteroidetes ratio

The ratio of *Firmicutes/Bacteroidetes* showed no significant change between the groups neither between the time points ($p_{(prob)}$=0.59, $p_{(vit)}$=0.45).

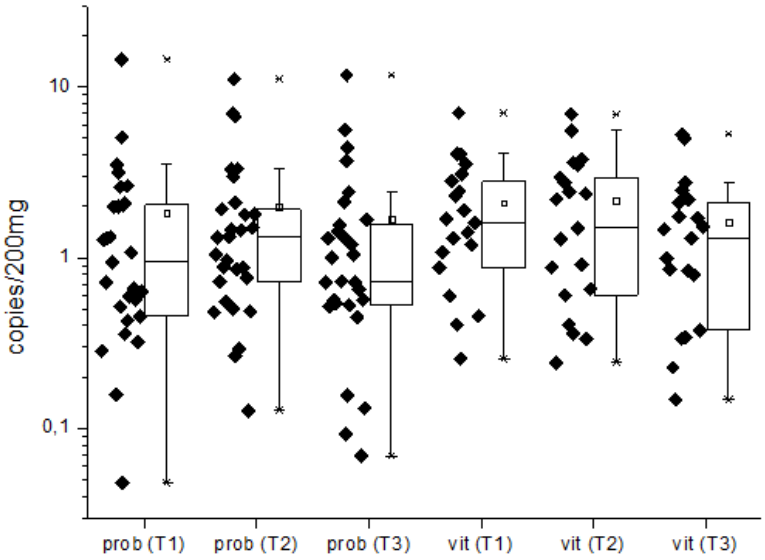


Figure 14 The boxplot diagram shows results of *bacteroidetes/firmicutes ratio* in both groups (prob=probioticum; vit=vitamin)*;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before colonic hydrotherapy (T1), after colonic hydrotherapy (T2), and 6 weeks after probiotic or vitamin intervention (T3)).

9.2.2.3 Clostridium cluster IV and Clostridium cluster XIVa

Furthermore, no significant changes in the abundance of *Clostridium cluster IV* of both groups ($p_{(prob)}$=0.63; $p_{(vit)}$=0.93) between the three time points has been observed, as well as in the abundance of *Clostridium cluster XIVa* ($p_{(prob)}$=0.85; $p_{(vit)}$=.0.43).

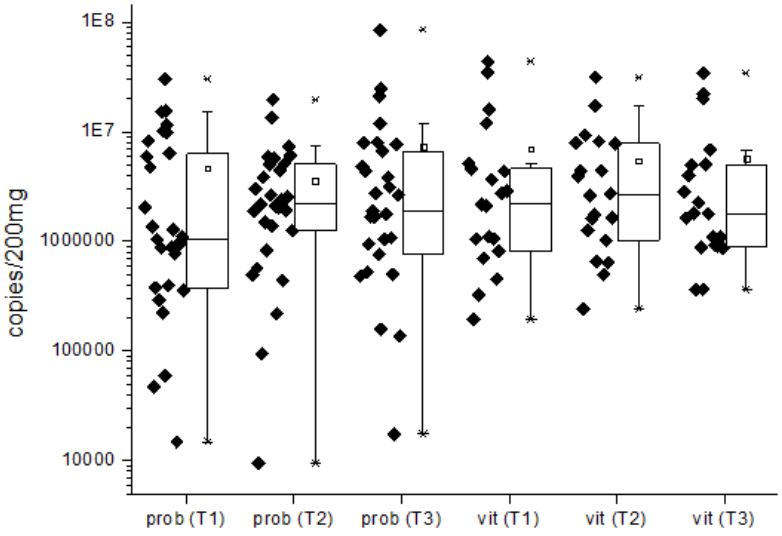


Figure 15 The boxplot diagram shows results of *Clostridium cluster IV* in both groups (prob=probioticum; vit=vitamin)*;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before colonic hydrotherapy (T1), after colonic hydrotherapy (T2), and 6 weeks after probiotic or vitamin intervention (T3)).

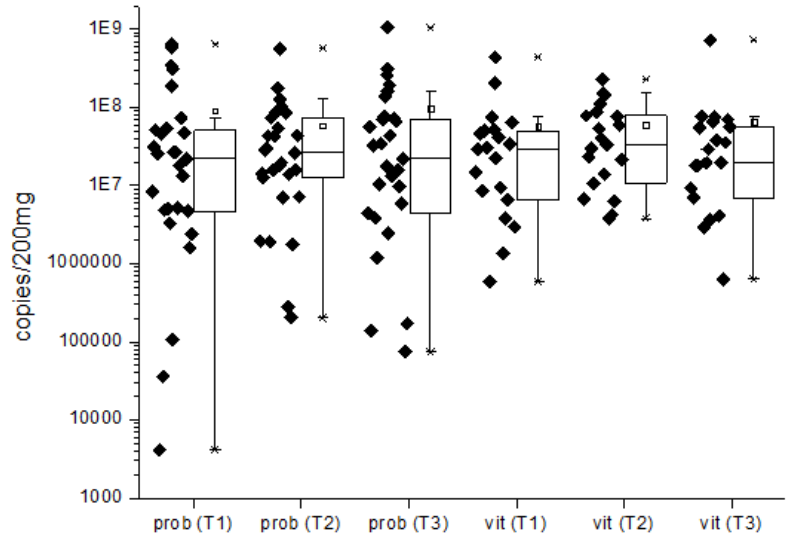


Figure 16 The boxplot diagram shows results of *Clostridium cluster XIVa* in both groups (prob=probioticum; vit=vitamin)*;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before colonic hydrotherapy (T1), after colonic hydrotherapy (T2), and 6 weeks after probiotic or vitamin intervention (T3)).

9.2.2.4 Lactobacilli

Lactobacilli show a trend of an increase in the probiotic group from the first to the second time point (p=0.44) but a decline from T2 to T3 (p=0.35). There were no significant differences between the three time points in the vitamin group (p=0.74). Comparing T3 of the two groups we could also detect no significant difference (p=0.43). However, the mean values of the probiotic group were higher in comparison to the vitamin group.

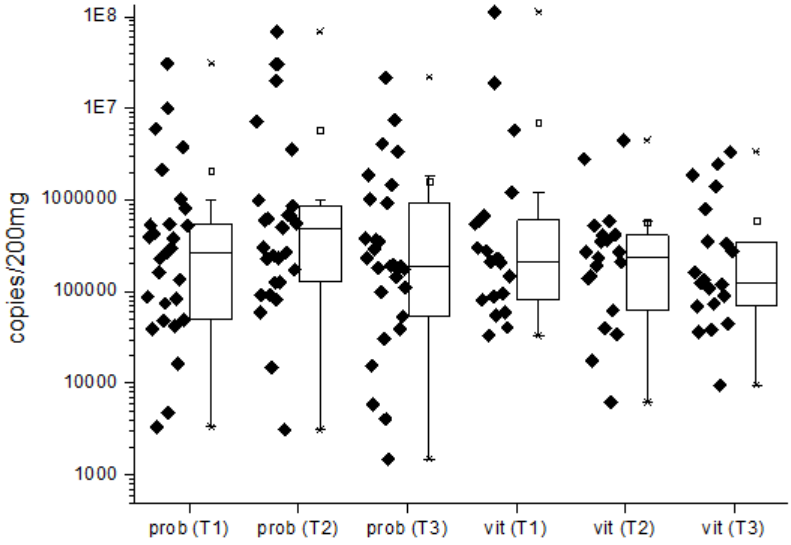


Figure 17 The boxplot diagram shows results of *Lactobacilli* in both groups (prob=probioticum; vit=vitamin)*;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before colonic hydrotherapy (T1), after colonic hydrotherapy (T2), and 6 weeks after probiotic or vitamin intervention (T3)).

9.2.2.5 Bacteroidetes and Prevotella

There were no significant alterations in the abundance of *Bacteroidetes* in both groups ($p_{(prob)}$=0.64; $p_{(vit)}$=0.87), as well as in the abundance of *Prevotella* ($p_{(prob)}$=0.73; $p_{(vit)}$=0.49) over the study period.

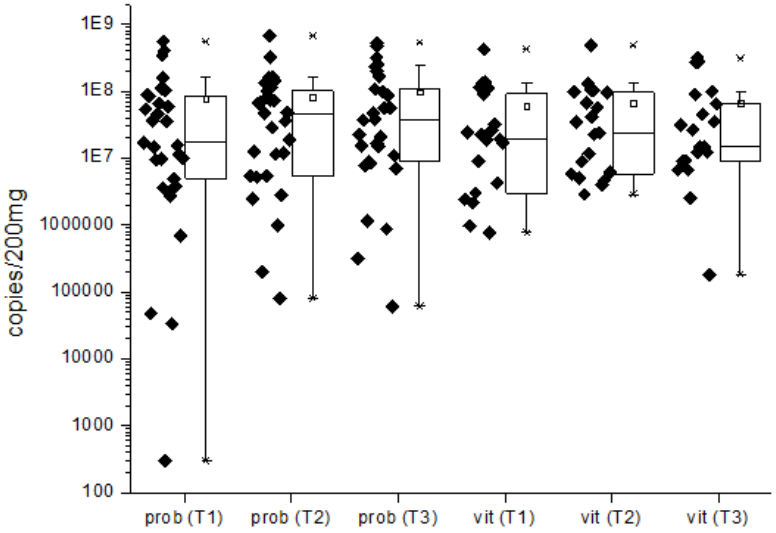


Figure 18 The boxplot diagram shows results of *Bacteroidetes* in both groups (prob=probioticum; vit=vitamin)*;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before colonic hydrotherapy (T1), after colonic hydrotherapy (T2), and 6 weeks after probiotic or vitamin intervention (T3)).

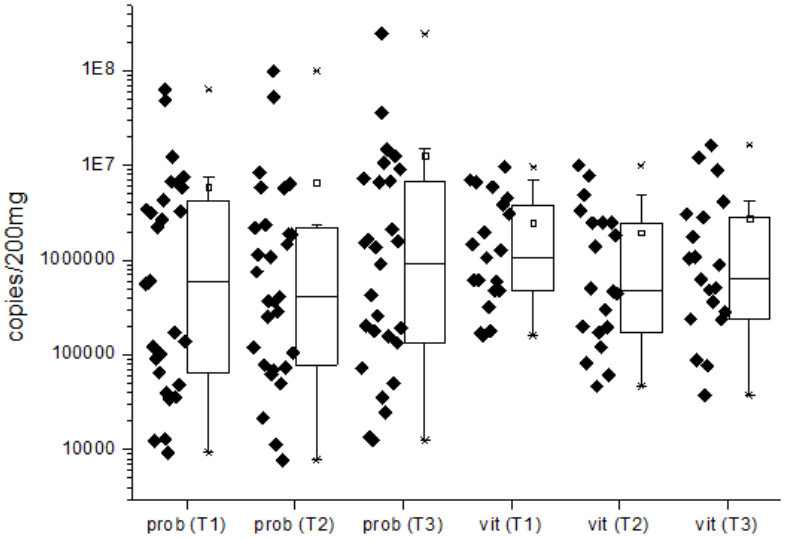


Figure 19 The boxplot diagram shows results of *Prevotella* in both groups (prob=probioticum; vit=vitamin)*;* Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before colonic hydrotherapy (T1), after colonic hydrotherapy (T2), and 6 weeks after probiotic or vitamin intervention (T3)).

10 Discussion

On the one hand we focused on a small group of individuals participating in a fasting week, receiving a treatment with Glauber's salt and a 6-week probiotic intervention. On the other hand we investigated patients suffering from digestive disorders recieving a colon-hydrotherapy and divided in to two subgroups intervented with a probiotic treatment or a vitamin B-supplement for the duration of 6 weeks.

Gastrointestinal problems belong to a wide field of disorders, which causes are often not clear and in some cases a wrong diagnosis following an unadequate treatment leads to a worsening of symptoms. Unfortunately, such fail diagnoses can not always be avoided, since not all patients with the same diseases are suffering from the same symptoms. Symptoms range from incomplete defecation, abdominal bloating and extragastrointestinal symtoms such as headache, dizziness, sleep disorders and neck pain. Many IBS patients indicate that abdominal discomfort and mental problems are greater than abdominal pain and that is not always a reason to go to the doctor. In some cases, it takes years until consulting a specialist (Dai, Zheng et al. 2013).

Overall acceptet is the influence of the intestinal microbiota in disease development (Noor, Ridgway et al. 2010). Intestinal disorders and symptoms are often caused by altered intestinal microbiota composition and bacterial overgrowth (Owen 2011). This is often due to a decrease in the abundance of *Lactobacilli*, *Bifidobacteria, Bacteroidetes* and diversity and an increase in *Streptococci*, *Escherichia coli* and *Clostridium* species (Ponnusamy, Choi et al. 2011; Hong and Rhee 2014). In our study we observed no abnormalities in gut microbiota in examined persons compared to normal microbiota composition. The above mentioned decrease of *Bacteroidetes* was not indicated in the phylum analysis or in the *Bacteroidetes/Firmicutes* ratio. Fasting as well as colon hydrotherapy showed no adverse effects in all individuals rather positive effects were documented from the patients. In the majority of individuals an allevation of symptoms and an improved defecation was the result. Previous mentioned negative effects of colon-hydrotherapy could not be confirmed (Mishori, Otubu et al. 2011) (Seow-Choen 2009). After the implemention of the colon-

hydrotherapy or the intervention with Glauber's salt we expected an overall decrease of microbiota diversity and total bacteria abundance. However, only a view patients show this expected effect. These differences might be explained by variations in the sampling of the second stool sample. Probably not all patients take their first consistent stool as from our side descriped and required. Unfortunately, there is a lack of studies documenting the beneficial effects of bowel cleansing. Generall a crucial property of probiotics that affect different gastrointestinal disorders consists in improving the gut microbiota composition and keeping its stability. Further, functions of probiotics are the stabilization of the immune system by stimulating immune mechanisms, helping to regulate the gut motiltity, and act as anti-inflammatory compounds (Ivanov and Honda 2012). The probiotic treatment significantly increases diversity but had no impact on residual gut microbiota. As the probioticum contains a high amount of *Lactobacilli* strains, we expected an elevation in the abundance of *Lactobacilli* in patient with probiotic intervention. A high amount of Lactobacilli is mentioned to induce the expression of the immune suppressive cytokine IL-10 in Treg cells (Mazmanian, Round et al. 2008; Ivanov and Honda 2012). After colon hydrotherapy our findings show a trend of an increase of Lactobacilli which might indicate the disturbance of mucosa and induced endotoxemia due to colon hydrotherapy. Some patients maybe started not direct after bowel cleansing with the probiotic supplementation or occasionally forgot the intake over the period of 6-weeks. Mentioned limitations could lead to diffenences in the results.

Changes in the diet and antibiotic use have an influence on the development of digestive problems or even intensify symptoms. An unbalanced diet which is poor in salads, fuits and vegetables and therefore also poor in vitamins could have negative effects on digestive health (Simren, Barbara et al. 2012). However, the additional supplementation of a vitamin B-complex did not show effects on the intestinal microbiota composition.

While the mechanisms of probiotics have been widely studied, the potential benefits of prebiotics may also have an impact on the modulation of the gut microbiota. They can have beneficial health effects on the host by stimulating the metabolism and growth of beneficial bacteria (König and Brummer 2013). In

some studies a prebiotic treatment showed qualitative changes in fecal microbiota compared to a placebo group as well as significant improvements in terms of stool consistency and flatulances (Dupont 2014). The most administered prebiotics have an influence on the abundance of *Bifidobacteria* and *Lactobacilli*. Another study including 44 IBS patients using two differenct doses of prebiotics (3.5 and 7 g/d) showed significant increased *Bifidobacteria* and *Lactobacilli* numbers, but better improvements were shown with the lower dose. *Bifidobacteria* and *Lactobacilli* strains do not produce gases as part of their metabolism. The fast fermentation of prebiotics in the proximal bowel often leads to an elevation of the intestinal gas production. This mechanism increases flatulences and bloating, which would be not beneficial in patients suffering from digestive problems. Ideal would be a slowly fermented prebiotic throughout the entire colon, so that the gases are equally distributed, which causes less complaints (König and Brummer 2013).

Earlier fasting had religious or spiritual reasons, and sometimes it was used as medical or therapeutic treatment. During a fasting week the intake includes not more than 250-500 kcal and the diet consists mainly of vegetables, fruits and water (de Toledo, Buchinger et al. 2013). Most studies investigate the long-term effect of caloric restriction and fasting on health and several disorders. Our findings documented that already short-term fasting could have an impact on human health and gut microbiota composition. The results show a trend of an increase in the *Lactobacilli* abundance in fasting individuals from T1 to T3. Weight loss played no significant role in our individuals: the average weight loss was only 1 kg. Maybe this is the reason why no significant changes in the *Firmicutes/Bacteriodetes* ratio could be observed. After fasting participants reported improvements in defecation and gastrointestinal comfort. Investigations reported beneficial effects ranging from improvements of metabolic syndrome and inflammatory diseases to psychosomatic disorders (de Toledo, Buchinger et al. 2013). The improvement of gastrointestinal symtomps could be also due to the reduction of psychologic disorders such as anxiety and depression. In many cases stress has been associated with a reinforcement of gastrointestinal

symptoms (Hayes, Fraher et al. 2014). After fasting individuals often reach a level of improved mental health and vitality.

11 Conclusion

A disturbed microbiota composition can have many causes – use of anticiotics, pathogens, colon hydrotherapy – among them the impact of the daily food intake and lifestyle must be taken under contemplation. In summary, our study shows that gut microbiota modification due to fasting and bowel cleansing increases gut microbiota composition. Unfortunately, gastrointestinal treatment only affects gut microbiota composition during intervention with short-term effects but long-term impact needs complete changes in eating habits and lifestyle. An additional probiotic intervention increased probiotic administered gut microbial populations. Our findings should help to better understand the complex interplay between human gut microbiota, digestive disorders and different therapeutic approaches to treat gastrointestinal disorders by preventing dysbiosis.

12 Appendix

12.1 Paper (draft)

12.1.1 Colon-hydrotherapy and probiotic intervention impact digestive problems

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Abstract
Keywords
Abbreviations
Introduction

The distress of digestive problems is getting increasingly popular, alterations of bowel habits ranges from bloating, diarrhea, too constipation. Meanwhile conventional therapies primarily target mucosal inflammatory responses, but the cause often remains untreated although the contribution of the gut microbiota in certain clinical manifestations underpins the use of probiotics. Recent research engages quantitative and qualitative changes of mucosal and fecal microbiota but also their impact on mucosal innate immune responses by increasing the epithelial permeability, activating nociceptive sensory pathways and dysregulation of the enteric nervous system in IBS. Moreover the treatment with probiotics seems promising; several studies show an improvement after intake (Simren, Barbara et al. 2012).

The pathophysiology of IBS is incompletely understood, an interaction of various mechanisms has been proposed: abnormal gastrointestinal motility, visceral hypersensitivity, altered brain-gut barrier, low-grade inflammation, psychosocial disturbance and intestinal microbes might contribute. Three predominate phyla (*Firmicutes, Bacteroidetes, Actinobacteria*) mainly represent the human gut microbiota (Eckburg, Bik et al. 2005; Karlsson, Ussery et al. 2010; Remely, Dworzak et al. 2013) although varying in abundance (Suau, Bonnet et al. 1999). IBS was reported to determine reduced numbers of *Lactobacilli*, *Bifidobacteria* (Lyra, Rinttila et al. 2009), *Bacteroidetes*, and a decline in diversity (Ponnusamy, Choi et al. 2011). *Enterobacteria* have been shown to have a higher abundance (Si, Yu et al. 2004). Probiotic interventions too the relieve of symptoms are promising as they address visceral hypersensitivity, GI

dysmotility, intestinal permeability, the intestinal microbiota, and immune function although the effects significantly differ between organisms and depend on interactions of strains (Simren, Barbara et al. 2012). The most promising results are shown for *Bifidobacterium infantis* 35624 at a dose of 13108 cfu/day taken for at least 4 weeks (Whorwell, Altringer et al. 2006). In addition dietary composition is known to profoundly alter gut microbiota. A reduction in fibre intake can improve bloating and diarrhea by altering the intestinal microbiota. Prebiotic administered oligosaccharides, e.g. inulin, increase fecal concentration of *Bifidobacterium* spp. but IBS patients suffer from increased flatulence due to fermentation (Simren, Barbara et al. 2012). Thus, an additional treatment with colon-hydrotherapy (colonic irrigation or colon cleansing) might improve the probiotic impact by depletion of persistent gut microbiota and clearing space for mucosal adherence of probiotic administered strains. According to Gail Naas (I-ACT President) "Colon hydrotherapy is a safe, method of removing waste from the large intestine, without the use of drugs." For the implementation of a colon-hydrotherapy about 60 liters of warm, filtered water (often with additional compounds like for example herbs or coffee) are pumped via a tube trough the patient's rectum in several cycles. Additionally the patients get an abdominal massage. In this way the bowel is stimulated to get rid of long-term depositional fluids and waste. The duration of one treatment lasts about 40 minutes (Seow-Choen 2009).

In the early 1900´s evidences for the use of colon-hydrotherapy were mainly to avoid autointoxication, a poisoned body by toxins having their origin from the intestine (Mishori, Otubu et al.). The reasons why people use it nowadays are different: Improvement of gastrointestinal symptoms like bloating, constipation and diarrhea, but it is also used as treatment of allergies or skin problems or simple to enhance the personal well-being (Harrell, Wang et al. 2012).

Thus, we investigated patients under colon-hydrotherapy with a subsequent intervention with probiotics or an adequate vitamin product. We examined the gut microbial diversity using DGGE and the relative abundance of microorganism in the gastrointestinal tract using qPCR of the 16S rDNA.

Materials and Methods

Study participants and study design

Individuals (n=55, aged 45±13 ages, BMI 25.31±6.91) suffering from digestive problems, IBS, or food intolerances have been recruited for this study. All participants received an implementation of colon-hydrotherapy to relieve of symptoms. Afterwards the participants were divided into two subgroups: one receiving an probiotic intervention (Progutic® LactoVitamin BALANCE contains 7 different DUOLAC® bacterial strains per capsule: Lactobacillus plantarum, Streptococus thermophiles, Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium breve. In addition, a capsule contains fructooligosaccharides, 200 µg folic acid, 2.50 µg vitamin B12 and 55 µg selenium; all amounts corresponding to 100% of daily demand) another group ingesting an equivalent capsule of a vitamin B supplement (Vitamin B-Komplex-ratiopharm® contains per capsula: 15mg thiamin, 10µg cobalamin, 15mg riboflavin, 15mg niacin, 25 mg panthotenic acid, 10mg pyridoxine, 150µg biotin, 450µg folic acid) for the following six weeks. Stool samples were taken at three different time points: T1: before colon-hydrotherapy, T2: immediately after colon-hydrotherapy (first hard stool), and T3: after six weeks of probiotic or vitamin intervention. Furthermore a food frequency questionnaire (FFQ) has been asked at time point one and three. The FFQ reported the frequency of consumption and portion size but also included questions about lifestyle, medical relevant influences, stool frequency, kind of gastrointestinal pain, BMI, and age to ensure comparable data.

Table 1: Characterization of study participants

Group	**Probiotic**	**Vitamin**
Number	29	20
Sex	♀ 22 ♂ 7	♀ 13 ♂ 7
Age ± SD (years)	43,72±11,55	48,85±14,47
BMI ± SD (kg/m^2)	25,91±8,08	24,21±5,32

Fecal sample collection, processing, and analysis

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

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

Bacterial abundance was quantified with TaqMan qPCR and SYBR Green qPCR in a Rotorgene 3000 (Corbett Life Science, Australia) using 16S rDNA group specific primers (Table 1-2). The specificity of primer and probes was checked with the ProbeMatch function of the ribosomal database project 10 (http://rdp.cme.msu.edu/). The PCR reactions mixture and serial DNA dilution of typically strains were prepared according to Pirker et al. 2012 (Pirker, Stockenhuber et al. 2012).

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

Target organism	Primer/Probe	Sequence (5' - 3')	Size (bp)	Conc. [pmol/µL]	Reference
All *Bacteria*	Fwd primer	ACT CCT ACG GGA GGC AG	468	10	(Yu, Lee et al. 2005)
	Rev primer	GAC TAC CAG GGT ATC TAA TCC		10	
	Probe	(6-FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		2	
Clostridium* cluster IV *(Ruminococcaceae)	Fwd primer	GCA CAA GCA GTG GAG T	239	4	(Matsuki, Watanabe et al. 2004)
	Rev primer	CTT CCT CCG TTT TGT CAA		4	
	Probe	(6-FAM)-AGG GTT GCG CTC GTT-(BHQ-1)		2	
Cluster XIVa (Lachnospiraceae)	Fwd primer	GCA GTG GGG AAT ATT GCA	477	5	(Matsuki, Watanabe et al. 2004)
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A		5	

	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)		1,5	
Bacteroidetes	Fwd primer	GAG AGG AAG GTC CCC CAC	106	3	(Layton, McKay et al. 2006)
	Rev primer	CGC TAC TTG GCT GGT TCA G		3	
	Probe	(6-FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)		1	
***Bifidobacterium* spp.**	Fwd primer	GCG TGC TTA ACA CAT GCA AGT C	125	3	(Penders, Vink 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	
Archaea	Fwd primer	ATT AGA TAC CCG GGT AGT CC		4	(Raskin, Stromley et al. 1994)
	Rev primer	GCC ATG CAC CWC CTC T	1044–1059	4	(Yu, Lee et al. 2005)
	Probe	(6-FAM)-AGG AAT TGG CGG GGG AGC AC(BHQ-1)	915–934	4	

Table 3: Primers (SYBR® Green) targeting 16rRNA coding regions of bacteria

Target organism	**Primer**	**Sequence (5' - 3')**	**Size (bp)**	**Conc. [pmol/µL]**	**Reference**
Lactobacilli	Fwd primer	AGC AGT SGG GAA TCT TCC A	352-700	4	(Walter, Hertel et al. 2001)
	Rev primer	ATT YCA CCG CTA CAC ATG		4	
Enterobacteria	Fwd primer	AGC ACC GGC TAA CTC CGT	492-509	3	(Woo, Leung et al. 2000)
	Rev primer	GAA GCC ACG CCT CAA GGG CAC AA	834 - 856	3	(Ootsubo, Shimizu et al. 2002)
Prevotella	Fwd primer	CACCAAGGCGACGATCA	1458	2,5	(Larsen, Kondo et al. 2008)
	Rev primer	GGATAACGCCYGGACCT		2,5	
Akkermansia	Fwd primer	CAGCACGTGAAGGTGGGGAC	1505	2,5	(Collado M.C. 2007)
	Rev primer	CCTTGCGGTTGGCTTCAGAT		2,5	

Results

Analyses of the retrospective FFQ

Evaluation of the FFQ showed, that the majority of the study participants (T1:31%, T3:33%) consumed fruits and vegetables 5-10 times per week. At both dates about one fourth consumed this food group even more than 10 times weekly. In comparison, DACH guidelines recommend five portions of fruits and vegetables per day. The recommendation of the DACH guidelines eating meat and sausage only 2-3 times per week met about 50% of the individuals. At time point one 36% of them ate meat nearly daily and this number increased at time point three to 38%.

The question about the fish intake showed that the majority of the participating persons (46% at T1 and 48% at T3) ate fish 1-3 times per week which is consistent with the DACH guidelines. In the first FFQ 38% (34% in the second) stated that their intake of this food group is too low. At the beginning about one fourth of the study participants consumed dairy products every day. In the second questionnaire only about 16% reported a daily intake of this food group. The majority of the individuals (T1:46%, T3:52%) consume milk- and milkproducts only 1-3 times weekly or less. Wheat and whole grain products are recommended daily. This recommendation comply approximately one half of the participants at every time point. When asked how often they eat sweets the most common response was between 1 and 5 times per week (T1:62%, T3:60%). 18% of the participating individuals at T1 and at T3 20% consumed sweets less than once per week and at both time points only about 15% ate them every day. Questions about physical activity showed that only 32% (at time point one) and 40% (at time point three) practice daily movement. But 42% stated that they did sport 2-3 times per week). Questions about stool behavior showed that 42% documented no conscious problems with defecation at the first time point. At time point three 50% reported a stable and untroubled digestion.

Compositional evaluation of gut microbiota

Gut microbial diversity showed a significant difference in probiotic intervention group between T1 and T3 ($p=0.003$) with a mean at T1 of 12 +/- 5.5 and at T3 of 17+/- 4.6 showing a correlation between the time-points ($R=0.65$, $p=0.02$).
There was no significant increase in total bacterial abundance in the probiotic group ($p=0.83$), as well as in the vitamin group ($p=0.91$) at all three time points. Comparing time point three of the two groups we could also show no significant difference ($p=0.94$). The ratio of *Firmicutes/Bacteroidetes* showed no significant between the groups neither between the the time points ($p_{(prob)}==0.59$, $p_{(vit)}=0.45$). Furthermore, no significant changes in the abundance of *Clostridium Cluster IV* of both groups ($p_{(prob)}=0.63$; $p_{(vit)}=0.93$) between three time points has been observed, as well as in the abundance of *Clostridium Cluster XIVa* ($p_{(prob)}=0.85$; $p_{(vit)}=.0.43$). The abundance of *Faecalibacterium prausnitzii* increased between T1 and T2 ($p=0.28$) in the probiotic group, whereas between T2 and T3 there is a small decline ($p=0.95$). In the vitamin interventiongroup no clear differences could be observed between all three time points ($p=0.67$). *Lactobacilli* show a trend of an increase in the probiotic group from the first to the second time point ($p=0.44$) but a decline from T2 to T3 ($p=0.35$). There were no significant difference between the three time points in the vitamin group ($p=0.74$). Comparing T3 of the two groups we could also find no significant difference ($p=0.43$). However, the mean values of the probiotic group were higher in comparison to the vitamin group. There were no significant alterations in the abundance of *Bacteroidetes* in both groups ($p_{(prob)}=0.64$; $p_{(vit)}=0.87$), as well as in the abundance of *Prevotella* ($p_{(prob)}=0.73$; $p_{(vit)}=0.49$) over the study period.
Regarding B*ifidobacteria* in the probiotic group an increase between the individual time points has been observed (T1-T2: $p=0.21$, T2-T3: $p=0.11$). This increase was significant between T1 and T3 ($p<0.05$). The levels of the vitamin group showed no remarkable differences over the three time points ($p=0.79$). As we compared the third time point of the two various intervention groups, a clear but not significant difference could be detected ($p=0.13$).
Concerning the *Enterobacteri*a our analysis showed no remarkable changes, the level remained unaffected in the probiotic group as well as in the vitamin

group over study period ($p_{(prob)}$=0.37; $p_{(vit)}$=0.81). *Akkermansia* levels showed an increase in the probiotic intervention group between T1 and T2 (p=0.57), between T2 and T3 (p=0.37). Whereas in the vitamin intervention group we observed a trend of a decrease (T1-T2: p=0.58, T2-T3: p=0.95). The number of individuals owing *Archaea* decreased in the probiotic group from 27.6% at T1 to 17% at T3. In the vitamin group 42% of the participants harbor *Archaea* at T1 and only 28.9% at T3.

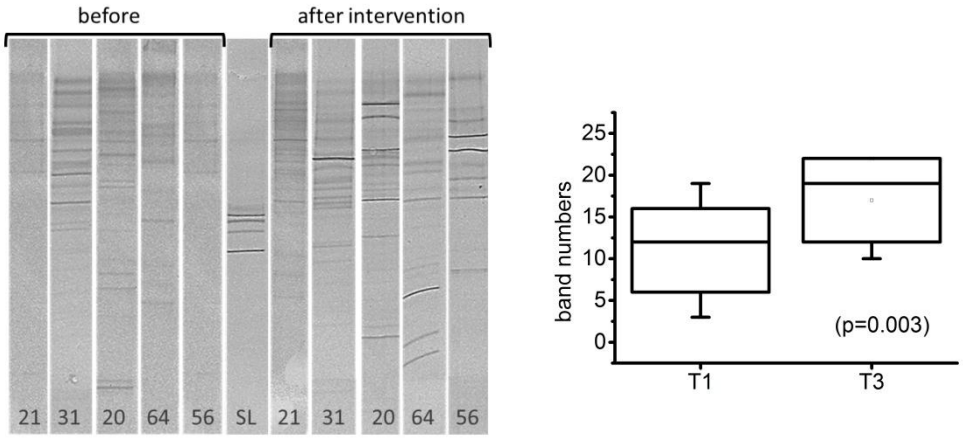

Figure 1: Diversity analysis. A PCR-DGGE fingerprinting of 16S rDNA coding regions of dominant bacteria over time indicating a lower number of bands in the probiotic intervention group at T1 in comparison to T3 B Quantification of number of bands showing an increase of diversity in the probiotic intervention group between T1 and T3 (p=0.003) Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range(T1: before colon-hydrotherapy, T3: after six weeks of probiotic or vitamin intervention, SL: standard lane)

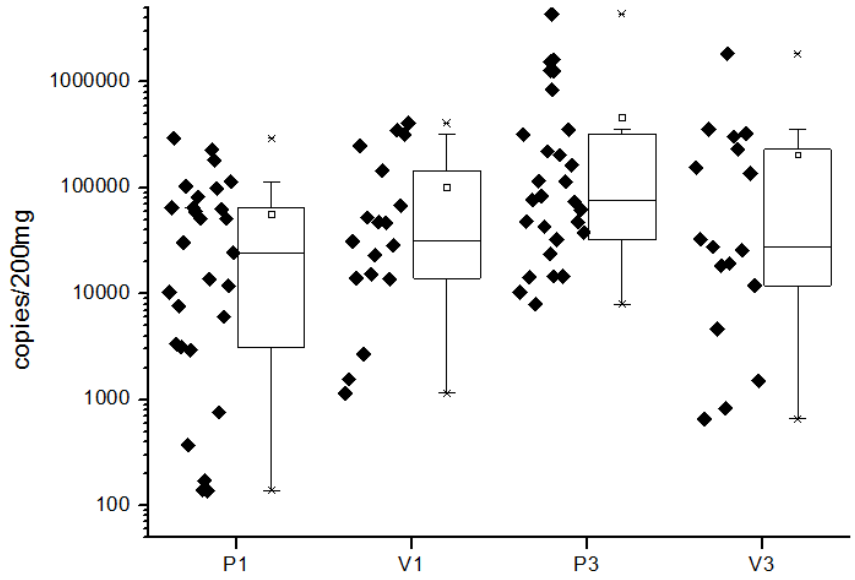

Figure 2: Quantification of *Bifidobacteria* showing a clear difference at T3 between the probiotic (P) and vitamin group (V; p=0.13) as well as a significant increase in the probiotic intervention group between T1 and T3 ($p<0.05$). Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range. (T1: before colon-hydrotherapy, T3: after six weeks of probiotic or vitamin intervention)

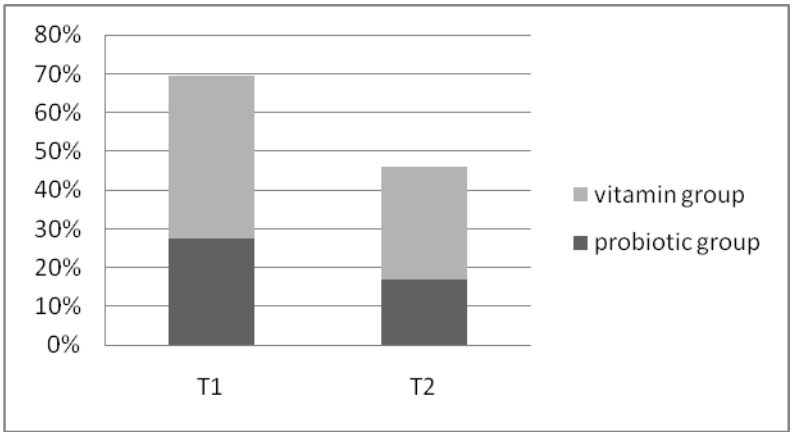

Figure 3: Percentage of individuals harboring Archaea decrease due to probiotic and vitamin B intervention of about 10.6 and 13.1 %. (T1: before colon-hydrotherapy, T3: after six weeks of probiotic or vitamin intervention)

Discussion

We have focused on patients suffering from digestive problems intervened with a colon-hydrotherapy followed by an intervention divided into the two subgroups receiving a probiotic intervention or a vitamin D complex afterwards.

Gastrointestinal pain and changed bowel habits are often mentioned although the exact cause are not clear and range from previous antibiotic use too travelers' diarrhea etc. Generally accepted is the importance of gut microbiota popu-

lations in disease development (Noor, Ridgway et al. 2010). We observed no abnormalities in gut microbiota in diseased participants compared to common gut microbiota composition; even the previous mentioned decline of *Bacteroidetes* (Ponnusamy, Choi et al. 2011) was neither shown in phylum analysis itself nor in the *Firmicutes/Bacteroidetes* ratio. Colon-hydrotherapy also did not affect gut microbiota composition, although more evidences in regard to adverse effects of colon-hydrotherapy on gut microbiota, depletion of the human gut microbiota, are known (Harrell, Wang et al. 2012). Despite a long history and current popularity, there is no scientific literature available which supports the benefits of cleansing. In contrast, a variety of adverse effects ranging from mild (e.g. cramping, abdominal pain, fullness, bloating, nausea, vomiting, perianal irritation, and soreness) to severe (e.g. electrolyte imbalance and renal failure) are mentioned (Harrell, Wang et al. 2012). Under consideration that participants had to take their first consistent stool after colon hydrotherapy intervention, this lack of differences might be explainable. In addition, Harrell et al. (2012) mentioned obvious effects of colonic lavage in some individuals although in general they can be unpredictable (Harrell, Wang et al. 2012). However, a probiotic intervention significantly increases diversity and *Bifidobacteria*, but did not affect residual investigated gut microbiota. Even a correlation of diversity between the time-point can be shown, thus a balanced, divers microbiota improves the impact of intervention. A generally important ability of probiotics that affects various digestive disorders consists in improving the gut's microbial composition and preserving its stability. The absence of an additional improvement of dietary intake considering the whole study population might affect these results. Although a diet rich in vegetables, salads and fruits has been proven to be beneficial to digestive health under normal circumstances but polysaccharides might affect digestive problems depending on their application (Simren, Barbara et al. 2012). In accordance to diet different fermentation end-products and vitamins can be generated (Kau, Ahern et al. 2011) although a vitamin B intervention did not show an influence on human gut microbiota composition.

In addition, probiotics strengthen the immune system by stimulating immune mechanisms, they help to regulate the gut motility, and act as anti-inflammatory

compounds. The effects include immunostimulatory, and immunomodulatory effects (Ivanov and Honda 2012). On the one hand the recruitment of immune cells to the mucosa, generation and maturation of organized gut associated lymphoid tissues and stimulation of protective epithelial cell functions, but also reversible changes in differentiation or effector function of host immune cells are mentioned (Ivanov and Honda 2012). Unfortunately, data on specific probiotic implications are rare. *Bifidobacteria* are considered to protect against gut barrier dysfunction, metabolic endotoxemia, insulin resistance, obesity, reduce gastrointestinal disorders, and correlate with inflammatory markers (Furet, Kong et al. 2010; Luoto, Kalliomaki et al. 2010). Prebiotic inulin has been shown to significantly increase the levels of *Bifidobacteria* but also of *F. prausnitzii* (Duncan, Belenguer et al. 2007; Ramirez-Farias, Slezak et al. 2009). An increased abundance of *Lactobacilli* is mentioned to induce the expression of the immune suppressive cytokine IL-10 in Treg cells (Mazmanian, Round et al. 2008; Ivanov and Honda 2012). Although the results are not consistent yet, *Lactobacilli* are suggested to have a role in “low-grade” inflammation (Bervoets, Van Hoorenbeeck et al. 2013) with a potential species dependence. Thus, the trend of an increase of Lactobacilli between the first two time points might indicate the disturbance of mucosa and induced endotoxemia due to colon-hydrotherapy although *Enterobacteria* showed no remarkable changes.

Conclusion

A disrupted microbial equilibrium can have many causes -- infectious pathogens, use of antibiotics, colon-hydrotherapy - among them the influence of our daily food intake and lifestyle must be taken under consideration. In particular it is shown, that gastrointestinal treatment only affects gut microbiota composition during intervention with short-term effects but long-term impact needs profound changes in eating habits and lifestyle.

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12.1.2 Mucin-degrading gut microbiota proliferates due to caloric restriction

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Abstract

An impaired gut microbiota has been reported as an important element in the pathogenesis of obesity. A therapy by weight reduction has already been mentioned to improve subpopulations involved in inflammatory processes although others may need further treatment. Thus, weight reduction in the context of a fasting program together with a probiotic intervention may improve abundance and diversity of gut microbiota.

Overweight people underwent a fasting program with a laxative for one week followed by a six week intervention with a probiotic formula. Gut microbiota was analyzed on the basis of 16s rDNA with quantitative real time polymerase chain reaction. Additionally a food frequency questionnaire with questions to nutritional behavior, lifestyle, and physical activity has been administered before and after intervention.

We observed no significant differences in total bacterial abundance, *Bacteroidetes*, *Prevotella*, *Clostridium cluster XIVa, Clostridium cluster IV* although *Faecalibacterium prausnitzii* showed a significant increase over study period. In addition, *Akkermanisa* and *Bifidobacteria* increased significantly due to intervention. The inflammation-associated gut microbiota, *Enterobacteria* and *Lactobacilli*, increased between the first two time points and declined from the second time point to the third time point. 66.7% of study participants harbored

Archaea. No significant improvements of eating habits were reported although, physical activity and conscious problems improved due to intervention.
Our results show that caloric restriction affects gut microbiota by proliferating mucin-degrading microbial subpopulations. An additional intervention with a probiotic formula significantly increased probiotic administered gut microbial populations.

Key words
Akkermansia; fasting; laxative; probiotic;

Abbrevations

BMI	body mass index
FFQ	food frequency questionnaire
IBS	irritable bowel syndrome
SD	standard deviation
T	time point

Introduction

Different disorders, such as metabolic disorders (obesity, diabetes) are associated with changes in the microbiota as well as with host-microbiota interactions (Hong and Rhee 2014). Not always pathogenic microorganisms play a role, rather members of the “healthy” microbiota have an influence on diseases state (Ross, Mills et al. 2009). An altered intestinal composition or bacterial overgrowth affect host health (Owen 2011).
Dietary composition has a significant impact on the gut microbiota composition and digestive symptoms like bloating, constipation, or diarrhea. Adverse reactions to food components are very common and about 67% of individuals reporting functional gastrointestinal disorders suffer from one or more intolerance (Wilder-Smith, Materna et al. 2013). More recently a link between food intolerances without involvement of IgE based mechanisms on gut microbiota as well as gut barrier disturbances were discussed (Hippe, Remely et al. 2014). Avoidance of food groups helps to alleviate symptoms (Wilder-Smith, Materna et al. 2013).

Thus, fasting, the ability of accomplishment to meet the macro- and micronutrient requirements of the body by limited or absence of food due to make use of body´s energy reserves without endangering health, might succeed in relief. For a limited period the abstinence of solid food and natural stimulants is practiced. In demand is the uptake of 2.5 L/day of calorie-free liquid (water, herbal tea) or vegetable broth (600–800 kcal/day) stimulating the excretory systems (liver, kidneys). The decreased use of energy suggested for digestion and storage and energy-sparing mechanisms, e.g. the ability to guarantee adequate energy administration by changing from glucose to fat oxidation, allow sparing of essential proteins to maintain organs and cellular functions in the long-term. Although since the biological necessity evolved into voluntary, also religious/spiritual contexts or medical therapeutic purposes developed. One school of fasting developed after Dr. Otto Buchinger, a multidisciplinary and multimodal treatment implicating physiotherapy, nutrition, mind-body methods, and psychotherapy as well as physical activity. Indications for fasting are for example: metabolic syndrome and diseases, chronic inflammatory diseases, chronic cardiovascular diseases, chronic pain syndromes, atopic diseases, and psychosomatic disorders (Toledo, A.Buchinger et al. 2013), but effects on gut microbiota might also be of interest. However, cardiovascular, metabolic and psychological changes must be considered and monitored, thus contra indicatory are cachexia, anorexia nervosa and eating disorders, uncontrolled hyperthyroidism, advanced cerebrovascular insufficiency or dementia, advanced liver or kidney insufficiency pregnancy and nursing (Toledo, A.Buchinger et al. 2013). Although until now there are hardly any studies about the influence of fasting on gut microbiota. One animal study showed that caloric restriction results in a change of the intestinal microbiota, an increase of *Lactobacillus* spp., supposed to protect against invading pathogens and to reduce inflammatory cytokines, have been shown. *Streptococcacae*, inductors of mild inflammation, are mentioned to be reduced abundant (Zhang, Li et al. 2013). An additional treatment with probiotics might improve the impact of fasting; mucosal clearing of persistent gut microbiota facilitates the adherence of probiotic administered strains and improves the release of gastrointestinal symptoms.

In our study we examined if a participation of a one week supervised fasting program in combination with a probiotic intervention modulates the relative abundance, composition of microorganism in the gastrointestinal tract measured by qPCR of the 16S rDNA.

Materials and Methods

Study participants and study design

In accordance with the declaration of the Viennese Human Ethics committee, all study participants gave written consent for use of data obtained from food frequency questionnaire (FFQ) and stool samples. Six individuals (aged 53.33 ± 6.55 ages, BMI 28.10 ± 3.50 kg/m^2) were recruited in cooperation with health trainer Mrs Ingrid Höfinger and Dr. Georg Wögerbauer. All participants joined a one-week fasting program according to Dr. Buchinger in Pernegg Monastery, defined and supervised by Mrs. Höfinger (Table 1). On the second day of the fasting program all participants received Glauber's salt (sodium sulfate), which was used as a laxative for full defecation.

After a one-week fasting all individuals received a probiotic intervention for 6 weeks. Progutic® LactoVitamin BALANCE contains 7 different DUOLAC® bacterial strains per capsule: Lactobacillus plantarum, Streptococus thermophiles, Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium breve. In addition, a capsule contains fructooligosaccharides, 200 µg folic acid, 2.50 µg vitamin B12 and 55 µg selenium (all amounts corresponding to 100% of daily demand).

Fecal samples were collected at three time points: before (T1), during (T2) fasting (after sodium sulfate intake), and 6 weeks after probiotic intervention (T3). Additionally, all participants were asked a FFQ at the beginning (T1) and at the end of the intervention (T3). The FFQ reported the frequency of consumption and portion size, as well as questions about lifestyle (i.e. smoking, alcohol consumption, physical activity), medical relevant influences (i.e. vitamin and other supplements), body mass index (BMI), and age to ensure comparable data.

Table 1: Details of the fasting program supervised by health trainer Mrs Ingrid Höfinger

Day of Arrival	dinner: vegetable soup and crispbread
First day	breakfast: Pernegg muesli (prunes, dates, raisins, flax-seed, water) lunch: potatoes and vegetables dinner: vegetable soup
Second day	breakfast: herbal tea, Glauber's salt lunch: fresh squeezed fruit and vegetable juice dinner: fasting soup
All other fasting days	breakfast: herbal tea lunch: fresh squeezed fruit and vegetable juice dinner: fasting soup
Day before departure	breakfast: herbal tea lunch: apple dinner: steamed potato with vegetables and herbs
Day of Departure	breakfast: Pernegg muesli, porridge, fruit salad

Table 2: Characterization of study participants

Group		**Fasting patients**
Number		6
Sex	Female Male	3 3
Age ± SD (years)		53.33 ± 6.55
BMI ± SD (kg/m^2)		28.10 ± 3.50

Stool sample processing and extraction

Stool samples were collected and immediately stored at -18°C until extraction. According to the manufacturer´s protocol bacterial DNA was extracted from fecal samples using the QIAamp® DNA Stool mini kit (Qiagen, Germany). In addition samples were treated in FastPrep™ Lysing Matrix E tubes (MP Biomedi-

cals, USA) twice for 45 sec in a bead-beater (Mini-Beadbeater 8 Bio-Spec Products, USA) with an intervening minute on ice. DNA concentration and quality of extraction was determined by Pico100 (Picodrop, UK) and agarose gel electrophoresis.

Real-time qPCR

The abundance of bacteria and bacterial subgroups was measured by 16S rDNA gene using TaqMan qPCR and SYBR Green qPCR in a Rotorgene 3000 (Corbett Life Science, Australia) with group specific primers (Table 1-2). Primer and probes were checked for specificity with the Probematch function of the Ribosomal Database Project release10 (http://rdp.cme.msu.edu/) (Cole, Wang et al. 2014). The PCR reaction mixtures and serial DNA dilution of typical strains were prepared according to Pirker et al. (2012) (Pirker, Stockenhuber et al. 2012).

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

Target organism	Primer/Probe	Sequence (5' - 3')	Size (bp)	Conc. [pmol/µL]	Reference
All *Bacteria*	Fwd primer	ACT CCT ACG GGA GGC AG	468	10	(Yu, Lee et al. 2005)
	Rev primer	GAC TAC CAG GGT ATC TAA TCC		10	
	Probe	(6-FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)		2	
Clostridium* cluster IV *(Ruminococcaceae)	Fwd primer	GCA CAA GCA GTG GAG T	239	4	(Matsuki, Watanabe et al. 2004)
	Rev primer	CTT CCT CCG TTT TGT CAA		4	
	Probe	(Louis, Guerineau et al.)-AGG GTT GCG CTC GTT-(BHQ-1)		2	
Cluster XIVa (Lachnospiraceae)	Fwd primer	GCA GTG GGG AAT ATT GCA	477	5	(Matsuki, Watanabe et al. 2004)
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A		5	
	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)		1,5	
Bacteroidetes	Fwd primer	GAG AGG AAG GTC CCC CAC	106	3	(Layton, McKay et al.
	Rev primer	CGC TAC TTG GCT GGT TCA G		3	
	Probe	(6-FAM)-CCA TTG ACC		1	

		AAT ATT CCT CAC TGC TGC CT-(BHQ-1)			2006)
***Bifidobacterium* spp.**	Fwd primer	GCG TGC TTA ACA CAT GCA AGT C	125	3	(Penders, Vink 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	
Archaea	Fwd primer	ATT AGA TAC CCG GGT AGT CC		4	(Raskin, Stromley et al. 1994)
	Rev primer	GCC ATG CAC CWC CTC T	1044–1059	4	(Yu, Lee et al. 2005)
	Probe	(6-FAM)-AGG AAT TGG CGG GGG AGC AC(BHQ-1)	915– 934	4	

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

Target organism	**Primer**	**Sequence (5' - 3')**	**Size (bp)**	**Conc. [pmol/µL]**	**Reference**
Lactobacilli	Fwd primer	AGC AGT SGG GAA TCT TCC A	352-700	4	(Walter, Hertel et al. 2001)
	Rev primer	ATT YCA CCG CTA CAC ATG		4	
Enterobacteria	Fwd primer	AGC ACC GGC TAA CTC CGT	492-509	3	(Woo, Leung et al. 2000)
	Rev primer	GAA GCC ACG CCT CAA GGG CAC AA	834 - 856	3	(Ootsubo, Shimizu et al. 2002)
Prevotella	Fwd primer	CACCAAGGCGACGATCA	1458	2,5	(Larsen, Kondo et al. 2008)
	Rev primer	GGATAACGCCYGGACCT		2,5	
Akkermansia	Fwd primer	CAGCACGTGAAGGTGGGGAC	1505	2,5	(Collado M.C. 2007)
	Rev primer	CCTTGCGGTTGGCTTCAGAT		2,5	

Statistical analysis

Differences between and within the groups were analyzed statistically with the OriginPro version 8 (OriginLab, USA). All data were tested for normal distribution using descriptive statistic, normality test. For comparison of three unpaired groups the non-parametric Kruskal Wallis ANOVA and for comparison of two non-parametric unpaired values the non-parametric Mann-Whitney U-Test were used. Correlations were determined using Spearman correlation. P-values < 0.05 were determined as statistically significant.

Results

Analyses of the retrospective FFQ

Evaluation of the FFQ showed that only one out of six participants (16.67%) consumed vegetables 5-10 times per week and two out of six (33.33%) consumed fruits 5-10 times. In comparison DACH guidelines recommend five portions of fruits and vegetables per day. According to DACH guidelines meat and sausage are advised for 2-3 times per week, 50% of participants comply with recommendations, 33.33% eat meat and sausages nearly every day. 83.33% consume fish 1-3 times per week and only one 5-10 times per week.

Only one third of participants meet the recommendations of wheat and whole grain products consumption with a daily intake, 50% eat less than 4 portions per week. 33.33% consume dairy products nearly daily, 50% of participants eat less than 5 portions and only one out of six eat more than 15 portions per week. One third consumes sweets 1-3 times per week, another third 3-5 times and another third more than 10 times per week. In addition to normal diet 33.33% take nutritional supplements (i.e. ascorbic acid, vitamin D, calcium). Additionally questions to physical activity disclosed only one participant practicing daily movement, but 66.67% do sport regularly 1-3 times per week. Questions about stool behavior show that 83.33% of participants documented no conscious problems with defecation.

The FFQ asked after probiotic intervention showed no significant differences in the dairy product consumption, as well as in the intake of meat, sausages and fish compared to the first FFQ. There is no observable increased intake of fruits

and vegetables. Furthermore, the evaluation shows no increased or decreased uptake of wheat, whole grain products and sweets. There was a noticeable increase in daily movement in all participants from 16.67% to 33.33%, also regular physical activity improved from 66.67% to 83.33% of participants. However, after fasting week and probiotic intervention 100% documented no conscious problems with defecation. BMI did not change due to fasting (T1: 28.1±3.8; T3: 28.01±3.5).

Compositional evaluation of gut microbiota

We detected some differences in the gut bacterial composition before fasting week and after probiotic intervention. No significant differences could be observed in total bacterial abundance between all three time points (p=0.75). However, there was an observable increase between T1 and T2 (p=0.47) and a decline from T2 to T3 (p=0.81).

We observed no significant changes in the ratio of *Firmicutes/Bacteroidetes* between the three time points (p=0.46). Furthermore, we detected no significant changes in the abundance of *Clostridium Cluster IV* (p=0.74), as well as in the abundance of *Clostridium Cluster XIVa* (p=0.71) between the three time points. Regarding *Faecalibacterium prausnitzii*, the dominant butyrate producer of *Clostridium cluster IV*, a significant increase has been observed between the second and the third time point (p=0.03), but no significant changes between T1 and T2 (p=0.93, Figure 1). *Lactobacilli* show a trend of an increase from the first to the second time point (p=0.47) and also from the first to the third time point (p=0.14, Figure 2). There were no significant alterations in the abundance of *Bacteroidetes* (p=0.59) and *Prevotella* (p=0.81) over the study period. The abundance of B*ifidobacteria* significantly increased between T1 and T3 (p=0.03) but remained constant between T1 and T2 (p=0.93). Between T2 and T3 a clear but not significant change was detected (p=0.47, Figure 3). The quantity of *Enterobacteria* increased by tendency between T1 and T2 (p=0.93) but declined between the last two time-points (p=0.47). *Akkermansia* showed a significant increase between the time points (p=0.03, T1-T2: p=0.47, T2-T3: p=0.47 Figure

4). Archaea have been detected in 66.7% of the participants' at all three time points.

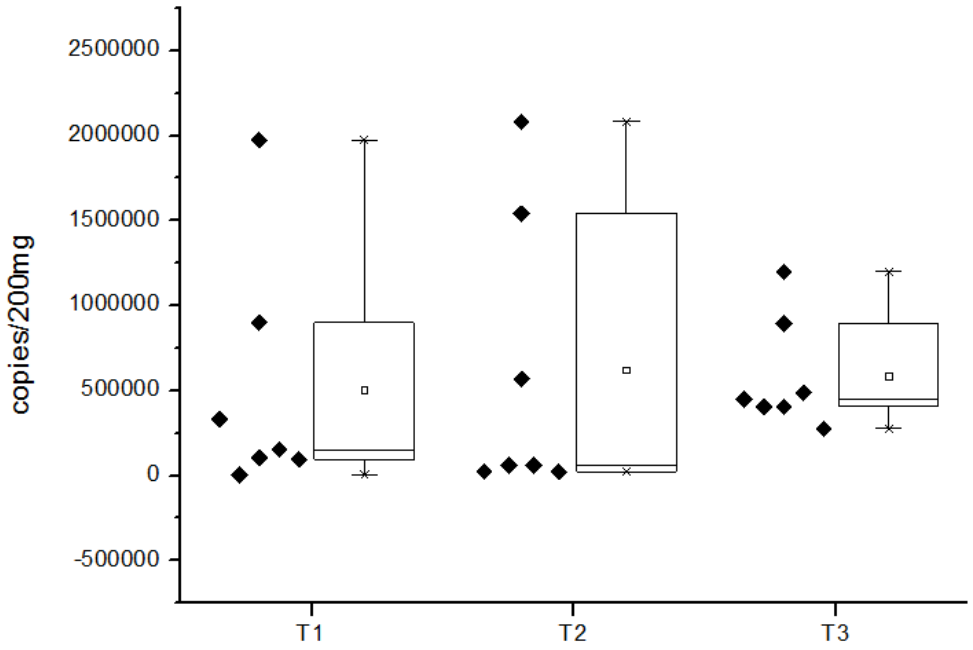

Figure 1: Quantification of *F. prausnitzii* showing an increase over intervention period (T1 to T3: p=0.05) Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

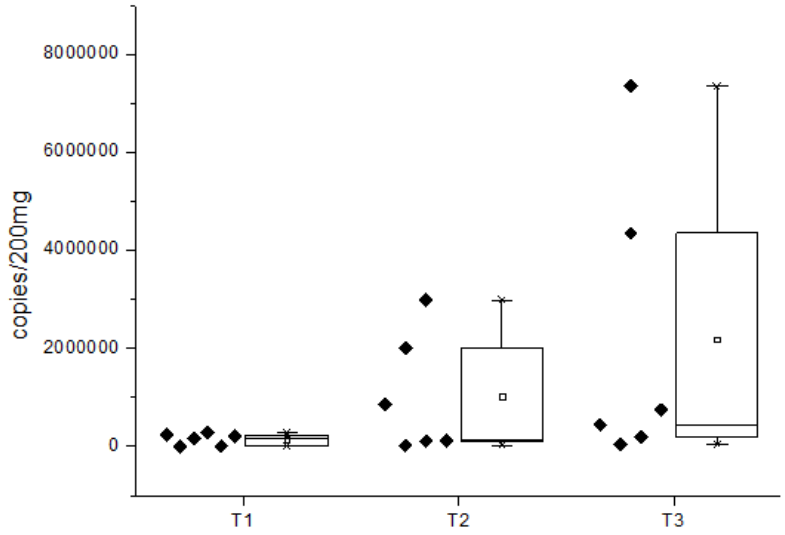

Figure 2: Quantification of *Lactobacilli* showing an increase over intervention period (T1-T3: p=0.14) Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

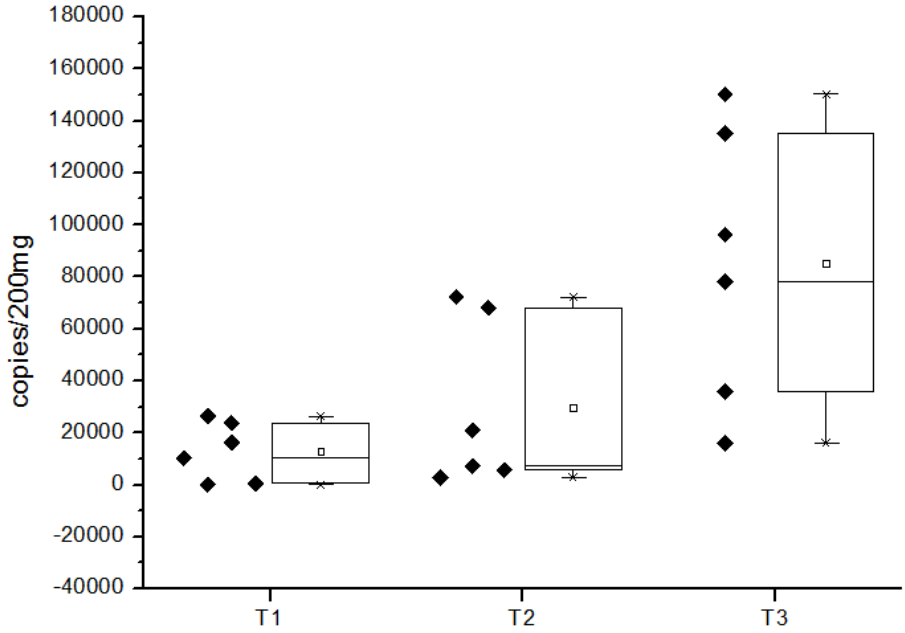

Figure 3: Quantification of *Bifidobacteria* showing a significant increase over intervention period (T1 - T3: p=0.03) Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

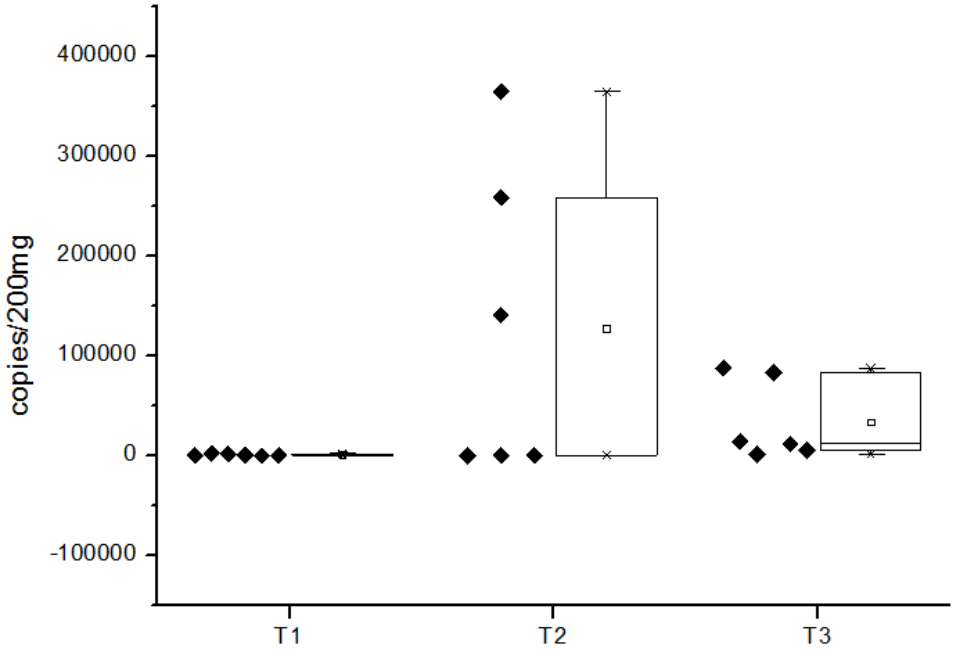

Figure 4: Quantification of *Akkermansia* showing a significant increase between the time points p=0.03; T1-T2: p=0.47, T2-T3: p=0.47). Box range 25, 75 Perc; Whiskers indicate outliers; □ indicates mean; x indicates maximum and minimum data range (before fasting (T1), during fasting (T2, after sodium sulfate intake), and 6 weeks after probiotic intervention (T3)).

Discussion

We have focused on patients suffering from obesity amd intervened with a supervised fasting program according to Dr. Otto Buchinger followed by an intervention with a probiotic formula.

According to the absence of solid food we were able to show an increase of *Lactobacilli*, *Enterobacteria*, and *Akkermansia*. Other bacterial groups remained unaffected.

On the one hand this affects may be contributed to caloric restriction, although the supervised fasting program also included the intake of Glauber´s salt at the second day of intervention, an osmotic laxative. Laxatives are commonly used to facilitate defecation by increasing intestinal passage and decreases stool consistency and increases stool weight (Gorkiewicz, Thallinger et al. 2013). Thus, also a depletion of selective gut microbiota, especially transient, maybe the result. Whereas microbial organisms with a higher mucosal adherence remain nearly unaffected or even proliferate due to provided energy source (Sonnenburg, Angenent et al. 2004), like mucin-degrading *Akkermansia* (Derrien, Vaughan et al. 2004). *Akkermansia* are suggested to be reduced abundant in diverse inflammatory disorders (e.g. ulcerative colitis, Crohns´ disease) (Png, Linden et al. 2010) and thus is associated with a healthy microbial mucosa (Png, Linden et al. 2010; Belzer and de Vos 2012). An induced osmotic diarrhea by polyethylene glycol 4000 (PEG) decreased the richness of phylotypes and showed a strong tendency to equalize the otherwise individualized microbiotas on the mucosa. *Bacteroidetes* and *Firmicutes* significantly decrease (Gorkiewicz, Thallinger et al. 2013) whereas *Proteobacteria*, *Enterobacteria*, proliferate in relative abundance of the mucosa specimens, a phenomenon also noted in several inflammatory and diarrheal gastrointestinal diseases (Gorkiewicz, Thallinger et al. 2013; Allen-Vercoe and Jobin 2014). Lactic acid bacteria also decreased on the mucosa, whereas *Faecalibacterium* showed a relative increase in stools, but a simultaneous decrease in the mucosa specimens (Gorkiewicz, Thallinger et al. 2013).

After supervised study program participants received an intervention with a probiotic formula. According changes could be observed in the gut microbiota

composition: *Bifidobacteria* and *Akkermansia* significantly increased from the first time point to the third. *Faecalibacterium prausnitzii* significantly increased from the second to the third time point. *Enterobacteria*, which increased due to fasting program, show a decrease to the third time point. Whereas *Lactobacilli* still show an increase. Total bacterial abundance, *Clostridium cluster IV, Clostridium cluster XIVa, Bacteroidetes,* and *Prevotella* remain unaffected. *Archaea* are highly abundant at all three time points.

Studies on healthy individuals show that the intake of probiotic microorganism leads in most cases to a transient colonization of the probiotic strains. Health effects are rarely studied or the outcomes are highly divers (Gerritsen, Smidt et al. 2011). Although, some studies support the use of probiotics to gain control over pain and discomfort in patients suffering of lower gastrointestinal symptoms (Simren, Barbara et al. 2012; Hungin, Mulligan et al. 2013). Positive effects of probiotics have also been reported in the treatment or prevention of gastrointestinal inflammation-associated disorder such as traveler´s diarrhea, antibiotics-associated diarrhea or pouchitis (Quigley and Flourie 2007). However, the question which organisms are the most appropriate for different symptoms remains unknown (Simren, Barbara et al. 2012).

Archaea are highly abundant and together with the non-reported weight loss, support the thesis of encouragement of caloric intake due to hypocaloric diet e.g. in anoerexic patients (Arumugam, Raes et al. 2011; Dridi, Raoult et al. 2011) but also due to fasting. *Archaea* improve bacterial fermentation efficiency through removal of H_2 by a synothropic relationship (Gill, Pop et al. 2006; DiBaise, Zhang et al. 2008). Thus, *Archaea* are discredited to complicate or even prevent weight loss (Remely, Dworzak et al. 2013).

Conclusion

In summary, our study shows that gut microbiota manipulation due to fasting with laxatives increases mucin-degrading subpopulations. An additional intervention with a probiotic formula significantly increased probiotic administered gut microbial populations. These investigations are of importance to understand the gut microbiota within an individual through time and its response to dietary

and environmental changes. The ultimate goal would be to modify the microbiota by predictive therapies and prevent dysbiosis.

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12.2 Food Frequency Questionnaire

Fragebogen zu Ihrem Ernährungs- und Lebensstil

Willkommen bei Ihrem persönlichen Fragebogen über Ihre Ess-, Verdauungs- und Bewegungs-Gewohnheiten. Voraussetzung für die Behandlung einer unzureichenden oder fehlerhaften mikrobiellen Darmbesiedelung (Dysbiose) ist eine gründliche Bestandsaufnahme möglicher Ursachen. Hierzu zählt u.a. die Beantwortung der folgenden 49 Fragen. Das Ausfüllen dauert etwa 20 Minuten.

Ihre Antworten aus dem Fragebogen werden mit Kontrollwerten hinsichtlich Geschlecht, Alter und Ethnie verglichen. Diese Werte werden von internationalen ärztlichen und wissenschaftlichen Organisationen wie die deutsche, österreichische oder Schweizer Gesellschaft für Ernährung (DGE, OeGE, SGE = DACH) festgelegt und laufend überarbeitet. Eine ausführliche Analyse Ihrer Antworten erhalten sie gemeinsam mit der Auswertung und Einschätzung Ihrer analysierten Stuhlprobe. Die daraus resultierenden Empfehlungen richten sich nach den Empfehlungen der DACH. Bei Fragen wenden Sie sich bitte an office@healthbiocare.at

Nachname:	
Vorname:	
Geburtsdatum:	
Geschlecht:	
Gewicht in kg:	
Größe in cm:	
Taillenumfang in cm:	
Ihre Adresse PLZ / Ort Strasse / Hausnummer Telefon, eMail	
Überwiesen von: Bitte geben Sie hier Ihre/n Ärztin oder Ernährungsberaterin an	
Datum	

Fragen zu Ihrem Wohlbefinden:

1. Sind Sie mit Ihrem Gewicht zufrieden?	
ja	
nein	
weiß nicht	

2. Haben Sie altersbedingte Beschwerden?	
nein	
zu starke Hautalterung	
zu viele graue Haare	
Antriebs- und Motivationsmangel	
schnelle Ermüdbarkeit	
Muskel- und Knochenschmerzen	
Gedächtnis- und Konzentrationsstörungen	
Sonstiges: _____	

3. Was sind Ihre vordringlichsten Probleme, weshalb Sie Ihren Arzt/Therapeuten konsultieren?
Welche Symptome traten auf?

4. Wie oft wurde eine Colon-Hydrotherapie in den letzten Jahren bei Ihnen durchgeführt?
Welche anderen Behandlungen, bezüglich der angegebenen Symptome, wurden außerdem unternommen?

5. Leiden Sie oft an Entzündungen und/oder Infektionen?	
ja	
nein	
weiß nicht	

5.1. Welche Art/ Wo?	
Grippale Infekte	
Entzündungen der Haut	
Entzündungen im Mundraum	
Entzündungen am Auge, Bindehautentzündungen	
Magen-/Darminfekte	

Entzündliche Erkrankungen im Magen-/Darmbereich	
Muskel-/Gelenksentzündungen	
Blasenentzündung	
Pilzinfektionen im Genitalbereich	
Pilzinfektionen von Haut oder Füssen	
Sonstiges: _____	

6. Nehmen Sie derzeit Medikamente zu sich?	
nein	
ja, folgende:	

Fragen zu Ihren Rauchgewohnheiten:

7. Rauchen Sie?	ja		nein						
8. Wie viele Zigaretten rauchen Sie?	1-6/ Woche		1-5/Tag		6-10/Tag		11-20/ Tag		>21/ Tag	
9. Seit wann rauchen Sie?	< 5 Jahren		10 Jahre		15 Jahre		20 Jahre		> 20 Jahre	
10. Seit wann rauchen Sie nicht mehr?	< 1Jahr		1-10 Jahren		11-15 Jahren		15-20 Jahren		> 20 Jahren	
11. Sind Sie Passivrauch ausgesetzt?	1-2/ Woche		2-4/ Woche		> 4/ Woche		täglich		ständig	

Fragen zu Ihren Ernährungsgewohnheiten:

12. Wie viele Portionen der angegebenen Lebensmittel konsumieren Sie wöchentlich?

Eine Portion entspricht ungefähr einer Hand voll des jeweiligen Lebensmittels.

	< 1	1-3	3-5	5-10	10-15	> 15
Milch- und Milchprodukte						
Eier						
Fleisch						
Gemüse						
Obst						
Fisch						
Getreideprodukte						
Süßigkeiten						

13. Wie oft in der Woche konsumieren Sie folgende Produkte?

Eine Portion entspricht ungefähr einer "Hand voll", einem Glas oder 3 Scheiben.
Mehrfachnennungen sind möglich.

a. Milchprodukte:	Hartkäse		Joghurt	
	Weichkäse		probiotisches Joghurt	
	Sauerrahm		Buttermilch	
	Molke		Sauermilch	
	Butter		Schlagobers	
	Milch			
	Sonstiges:			
b. Obst	Weintrauben		Orangen	
	Erdbeeren		Apfel	
	Kirschen		Birne	
	Bananen		Pfirsich	
	Sonstiges			
c. Gemüse	Spinat		Zwiebel	
	Tomaten		Paprika	
	Karotten		Linsen	
	Brokkoli		Erbsen	
	Bohnen			
	Sonstiges:			
d. Brotsorten	Vollkornbrot		Weißbrot	
	Mischbrot			
	Sonstiges:			
e. Beilagen	Nudeln		Ebly (Weizen)	
	Vollkornnudeln		Kartoffeln	
	Reis		Knödel	
	Vollkornreis		Couscous	
	Sonstiges:			
f. Fleisch	Rind		Pute	
	Schwein		Lamm	
	Huhn			
	Sonstiges:			
g. Wurst- und Fleischprodukte	Extrawurst		Leberkäse	
	Salami		Kochschinken	
	Schinken		Putenwurst	
	Krakauer		Bratenaufschnitt	
	Speck		Streichwurst	
	Sonstiges:			
h. Fisch	Lachs		Dorsch	
	Kabeljau		Makrele	
	Forelle			
	Sonstiges:			

14. In welcher Form konsumieren Sie Ihr Gemüse hauptsächlich?	
Bitte ankreuzen, Mehrfachnennungen sind möglich.	
roh	
gekocht	
gebraten	
gegart	
gedünstet	
frittiert	
Sonstiges: _____	

Fragen zu Nahrungsergänzungsmittel:

15. Nehmen Sie Nahrungsergänzungsmittel zu sich?	
Bitte auch ankreuzen, wenn keine regelmäßige Einnahme (Nährstoffe in konzentrierter Form, Bsp. Vitamintabletten, Knoblauchkapseln, ...)	
ja	
nein	

15.1. Welche Nahrungsergänzungsmittel haben Sie im letzten halben Jahr eingenommen?			
Mehrfachnennungen möglich			
Vitamin C		Biotin	
Vitamin D		Knoblauchpräparate	
Calcium		Folsäure	
L-Carnitin		Cobalamin	
Magnesium		Multivitamin	
Ballaststoffe		Probiotische Kapseln	
Sonstiges:			

Fragen zu Ihrem Bewegungsverhalten

	mehr-mals/d	täglich	4-6/w	1-3/w	1-2/m	nie
16. Machen Sie regelmäßig körperliche Bewegung? (Einkäufe zu Fuß, Spazieren,...)						
17. Betreiben Sie regelmäßig Sport? (Bewegung mit Schwitzen über 30min)						

Fragen zu Ihren Trinkgewohnheiten

18. Wie viel Flüssigkeit nehmen Sie täglich zu sich?	wenig als 1 Liter		1-2 Liter		2-3 Liter		>3 Liter		
19. Wie viele Tassen Kaffee trinken Sie täglich?	< 1 Tasse		1-2 Tassen		3-5 Tassen		> 5 Tassen		
20. Wie oft trinken Sie Alkohol?	nie		nur zu Anlässen		1-2/m		2-5/m		1-2/w
	täglich		mehrmals /d						
21. Wie viel Alkohol trinken Sie durchschnittlich im Monat?	< ¼ Liter		¼-½ Liter		½-1 Liter		1-2 Liter		2-3 Liter
	> 3 Liter								

22. Welche Getränke nehmen Sie hauptsächlich zu sich?			
Mehrfachnennungen möglich			
Kaffee		**Verdünnte Säfte**	
Wasser		**Tee (schwarz, grün,..)**	
Saft		**Energiegetränke**	
Limonade		**Tee (Kräuter)**	
Cola		**Nektar**	
Dicksaft			
Sonstiges:			

Fragen zu Ihrer Mahlzeitenaufnahme

23. Welche Mahlzeiten nehmen Sie regelmäßig zu sich?			
Mehrfachnennungen möglich			
Frühstück		**Abendessen**	
Mittagessen		**Zwischenmahlzeiten**	

24. Wann nehmen Sie meistens Ihre Hauptmahlzeiten zu sich?			
Mehrfachnennungen möglich			
Frühstück		**Abend**	
Mittag		**irgendwann**	

HealthBioCare

Fragen zu Allergien, Intoleranzen (Unverträglichkeiten)

25. Gab es im letzten Jahr einen oder mehrere Stoffe auf die Sie allergisch reagiert haben?	
ja	
nein	
weiß nicht	

26. Leiden Sie oder einer Ihrer Verwandten an Allergien? Oder Neurodermitis?	
z.B. Pollen, Insektenstiche, Medikamente, Nahrungsmittel oder atopische Dermatitis	
nein	
Ich selber	
Verwandter	
Welche Allergie? Verwandschaftsverhältnis:	

27. Auf welche Substanzen reagierten Sie allergisch?
(auch noch nicht vom Arzt überprüfte Substanzen)

28. Wie reagierten Sie allergisch?
Welche Symptome traten auf?

29. Wurde bei Ihnen Lotoseintoleranz, Fruktoseintoleranz, Histaminintoleranz oder Gluten Unverträglichkeit (Zöliakie) festgestellt?
Wann ?

30. Geben Sie bitte an, welche der folgenden Beschwerden bei Ihnen häufiger als gelegentlich auftreten.		
Symptom	**Ja, wie oft**	**Nach welchen Nahrungsmitteln?**
Nasenjucken, Niesattacken		
Behinderte Nasenatmung, „Stockschnupfen"		
Heuschnupfen, wässriger Niesschnupfen		
Augentränen, Augenjucken		
Schwellung oder Brennen von Lippen oder Zunge		
Rauhe Mundschleimhaut oder Zunge		
Gaumen-, Hals-, Ohrenjucken		
Häufiges Räuspern, „beschlagene Stimme"		
Atembeklemmungen		
Übelkeit, Erbrechen, Durchfall		
Blähungen, starke Darmgeräusche		
Ekzeme, Hautausschläge, Hautrötungen		
Nesselsucht, Urtikaria, Hautjuken		
Kopfschmerzen, Migräne		
Andere Beschwerden		

Fragen zu Ihrer Milchverträglichkeit

31. Wie gut vertragen Sie Milch?	
(Ohne Joghurt oder Käse. Haben Sie nach dem Konsum von Milch Beschwerden wie Bauchschmerzen, übermäßige Blähungen, Durchfall?)	
sehr gut	
leichte Bauchschmerzen	
Durchfall	
Bauchkrämpfe	
Blähungen	
weiß nicht	

Fragen zu Ihrer Fructose Verträglichkeit

32. Wie gut vertragen Sie Fructose?	
(Haben Sie nach dem Verzehr von Obst oder Fruchtsäften Beschwerden wie Bauchschmerzen, übermäßige Blähungen, Völlegefühl oder andere Beschwerden?)	
keine Beschwerden	
Völlegefühl	
Blähungen im oberen Bauch	
Bauchkrämpfe	
Bauchschmerzen	
Übelkeit	
weiß nicht	

Fragen zu Gasbildung während der Verdauung

33. Haben Sie oft Blähungen?	
ja, den ganzen Tag über	
nur manchmal nach dem Essen	
hin und wieder (2 bis 3 mal in der Woche)	
nie	
bei Reisen	
weiß nicht	

34. Wo fühlen Sie sich aufgebläht?	
im oberen Bauch, unter den Rippenbögen	
im unteren Bauch	
im gesamten Bauch	

35. Gehen die Gase untertags ab?	
ja	
nach der Nacht flacht der Bauch wieder ab	
weiß nicht	

Fragen zu Ihrem Stuhlverhalten

36. Welche Konsistenz hat Ihr Stuhl?	
flüssig, ohne feste Bestandteile	
einzelne weiche Klümpchen mit unregelmäßigem Rand	
wurstartig mit glatter Oberfläche	
wurstartig mit rissiger Oberfläche	
wurstartig, klumpig	
einzelne, feste Kügelchen, schwer auszuscheiden	

37. Haben Sie Probleme bei oder mit der Darmentleerung?	
Nein, mein Stuhlgang ist regelmäßig und unproblematisch	
Schmerzen vor und/oder während der Entleerung	
mühsame, portionsweise Darmentleerung	
abwechselnd Durchfall und Verstopfung	
zu selten	

38. Nehmen Sie regelmäßig Abführmittel zu sich?	
ja	
nein	

Fragen zu Ihrer Verdauung

39. Hat sich Ihre Verdauung in den letzten 5 Jahren verändert?	
(Stuhlhäufigkeit, Verdauung, Verträglichkeit verschiedener Nahrungsmittel)	
ja	
nein	
weiß nicht	
Änderungen	

40. Wie äußern sich Ihre Beschwerden?
(kurze Aufzählung)

Fragen zu Ihren Cholesterinwerten

41. Sind ihre Cholesterinwerte im Normalbereich?	
ja	
nein	
weiß nicht	

42. Wie hoch ist Ihr Cholesterinspiegel?	
HDL	mg/dl
LDL	mg/dl
gesamt Cholesterin	mg/d

43. Haben Sie eine der folgenden Stoffwechselkrankheiten?	
Mehrfachnennungen möglich	
Diabetes mellitus Typ 1	
Diabetes mellitus Typ 2	
Schilddrüsenüberfunktion	
Schilddrüsenunterfunktion	
Gicht	
Pankreasleiden	
nein	
Gibt es diesbezügliche Befunde?	

Fragen zu Ihren Stressbelastungen

44. Wie hoch würden Sie Ihre derzeitige Stressbelastung einschätzen?	
null	
gering	
mäßig	
hoch	
sehr hoch	

45. Versuchen Sie den ursächlichen Anteil bei der Entstehung Ihres Stressproblems in Prozent zu schätzen.	
(z.B. Arbeit 45%, Freizeit 20%, Familie 35%)	
Arbeit	%
Freizeit	%
Familie/ Partner	%

46. Erleben Sie bei Stressbelastungen zeitweilig negative körperliche Reaktionen?					
Symptom	nein	ein wenig	mäßig	stark	sehr stark
Kopfschmerzen					
Angst					
Magen-/Darmbeschwerden					
Muskelverspannung					

Fragen zu Ihrem Angstverhalten

47. Hatten Sie schon wiederholt Panikattacken, die nicht auf eine spezifische Situation oder ein spezifisches Objekt bezogen waren, sondern ganz spontan auftraten in Situationen, wo Sie gerade keiner besonderen Belastung oder Gefahr ausgesetzt waren?	
ja	
nein	
weiß nicht	

48. Erlebten Sie einmal einen Zeitraum von mindestens sechs Monaten mit vorherrschender Anspannung, Besorgnis und Befürchtungen in Bezug auf alltägliche Ereignisse und Probleme?	
ja	
nein	
weiß nicht	

49. Traten dabei folgende Symptome auf?	
Bitte ankreuzen, Mehrfachnennungen sind möglich.	
Herzrasen, Herzklopfen oder erhöhte Herzfrequenz	
Schweißausbrüche	
Fein- oder grobmotorisches Zittern	
Mundtrockenheit	
Beklemmungsgefühl/ Atemnot	
Schmerzen in der Brust	
Übelkeit	
Gefühl von Schwindel, Unsicherheit, Schwäche oder Benommenheit	
Gefühl, dass Sie „neben sich stehen" oder die Umwelt und die Objekte unwirklich sind	
Angst vor Kontrollverlust, verrückt zu werden oder „auszuflippen"	
Angst zu sterben	
Hitzegefühle oder Kälteschauer	
Gefühllosigkeit oder Kribbelgefühle	
Muskelverspannung, akute und chronische Schmerzen	
Ruhelosigkeit und Unfähigkeit zum Entspannen	
Gefühle von Aufgedrehtsein, Nervosität und psychischer Anspannung	
Kloßgefühl im Hals oder Schluckbeschwerden	
Konzentrationsschwierigkeiten, Leeregefühl im Kopf wegen Sorgen oder Angst	
Einschlafstörungen wegen der Besorgnis	

Vielen Dank für Ihre ausführlichen Antworten!

12.3 DNA-Extraction QIAamp® DNA Stool Mini Kit (50)

- Mesure the pH-value with the strips
- Under using a spatula weight in 200-210 mg frozen stool sample in a 2 mL matrix E (soil kit) tube and supply with 1.4 mL ASL buffer.
  Take care, that the sample is homogenized to ensure maximum DNA concentration. ASL buffer provides the ability to remove inhibitory substances from the stool samples.
- Transmit the lysing matrix tubes in the beadbeater and beat for two cycles of 45 sec with one intervening minute on ice.
  The beadbeater disrupts over 90 % of the cells via cell “cracking” action rather than high shear. Also the cell wall of gram-positive bacteria gets damaged.
- Heat the suspension for 5 min at 95 °C.
  Heating increases total DNA and helps to lyse also gram-positive bacteria.
- Vortex for 15 sec and centrifuge for 1 min at 14.000 rpm to pellet stool and lysing matrix.
- Transfer 2 mL of the supernatant into a new 1.5 mL eppendorft tube and discard pellet.
  Small amounts of pellet do not affect the procedure.
- Add a half inhibitEX tablet to each sample and vortex immediately until the tablet is completely dissolved.
  To allow the inhibitiors to adsorb inhibitiors and DNA-degrading substances incubate for 1 min at room temperature.
- Centrifuge for 6 min at 14.000 rpm to pellet the tablet with bound inhibitiors.
- Pipet the supernatant into a clean 1.5 mL eppendorf tube and discard pellet.
- Centrifuge for another 3 min at 14.000 rpm.
- Pipet 25 µL proteinase K into a clean 2 mL eppendorf tube.
- Transfer approximately 600 µL supernatant to proteinase K.

- Add 600 µL buffer AL and vortex for 15 sec.
  Never change the order of the steps.
- Site the tubes at 70 °C for 10 min on the heating bloc
- Add 600 µL ethanol (96-100 %) to the lysate and vortex.
- Label the lid of a new QIAamp spin column placed in a 2 mL collection tube. Apply 600 µL lysate from the further step without moistening the rim. Centrifuge for 1 min and discard the filtrate. Place the QIAamp spin column into a new collection tube and repeat the step until all lysate has been used.
- Add 500 µL buffer AW1 and centrifuge for 1 min at 14.000 rpm. Discard the filtrate and put it into a new collection tube.
- Ad 500 µL buffer AW2 and centrifuge for 3 min at 14.000 rpm. Discard the filtrate and place it into a new collection tube.
- Centrifuge for another minute to remove redidual buffer AW2.
  For the following steps pleace work on ice:
- Place the QIAamp spin column into a new 1.5 mL eppendorf tube and elute bounded DNA with 200 µL DNAse and RNAse free water preheated to 80 °C.
- Centrifuge for 1 min. (→ Ur-solution)
- Repeat the two steps for the 1. Elution
- For storage keep the eluates at -20 °C.

12.4 Pico100 (Picotrop)

Before each sample measurement, a blanc was performed using distilled water. Before measuring, the samples were mixed using a vortex. 2 µL of each sample was taken and every sample was measured twice. The sample was removed from the upper edge, to prevent contamination, not immerse! Provided that the measurements were reproducible, the average of both measurements was used for further calculations and applications.

12.5 Real time qPCR using Rotorgene 3000

For a full run a 72-well plate was used:

Table 6 Division of the 72-well plate

Reagents	**Number**
Sample	30 (duplicate)
Standard	8
Control sample	2
DNAse and RNAse free water	2
	72

Table 7 General pipette scheme of the Mastermixmix for one sample

Reagents	**Volumes**
Mastermix	5 µL
Primer forward	1 µL
Primer reverse	1 µL
DNAse and RNAse free water	1 µL
Sample	2 µL

Procedure protocol:

- Thaw samples and reagents and prepare a box with ice.
  For the following steps pleace work on ice:
- Prepare 1.5 mL eppendorf tubes for mastermix, standards, primer and samples and label them.
- Pipette dilutions for standard series: pipette 9 µL of DNAse and RNAse free water in each of the 8 tubes. Then pipette in the first standard 1 µL of the original standard solution and vortex.
- Take 1 µL of standard 1 and pipette it into the next tube (standard 2) and vortex. Repeate this procedure for all 8 standards.
- Pipette Mastermixmix (see scheme)
- Take the PCR plate from the freezer and but the PCR strip tubes on the plate.

For the PCR a volume of 10 µL per strip tube is needed.

- Pipette 8 µL of the Mastermixmix in each of the 72 tubes and always vortex after 4 tubes.
- Pipette in the first 8 tubes 2 µL of the standards of the dilution series, in the other 60 tubes 2 µL of the samples and in the last 4 2 µL of DNAse and RNAse free water and 2 µL of the control sample.
- Cap the strip tubes with the corresponding covers and fill in the strip tubes in the Rotorgene 3000 (Start with the filling at 1).
- Run the corresponding program.

12.6 DGGE

Before starting with the DGGE a PCR has to be performed and PCR-products need to be felled and resuspended.

Procedure:

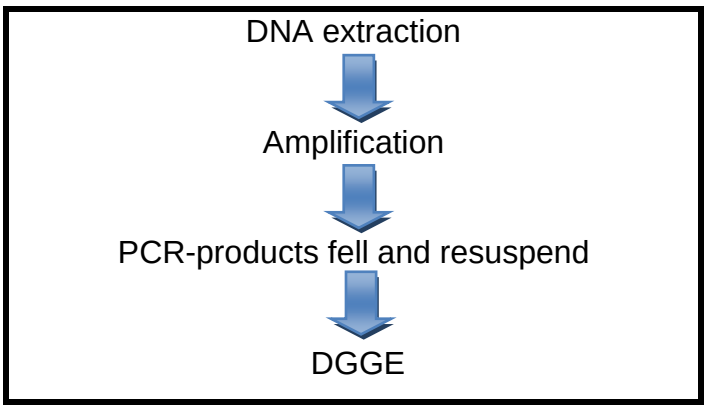

Table 8 Pipette scheme for endpoint PCR

Reagents	**Volumes**
Mastermix (ProgemaMix grün)	50 µL
341 forward GC	0.6 µL
518 reverse	0.6 µL
BSA	0.2 µL
NFW	45.6 µL
Template	3 µL

Table 9 Endpoint PCR program for DGGE

Temperature	Duration
95 °C	10 min
95 °C	1 min
55 °C	50 sec
72 °C	50 sec
72 °C	5 min

Procedure after PCR:

- Control results on a gel electrophoresis
  - 2 % agarosegel
  - Sample volume 5 µL
  - compare with a DNA ladder
- Place 1 mL of ethanol an 95 µL of a PCR sample in a 1.6 mL tube.
- Store the solution over night at -20 °C.
- Centrifugation of the felled DNA at 14.000 rpm for 30 min. Discard the supernatant and store the formed pellet.
- Place samples on a heating block with 30 °C for drying
- If the samples are completely dry, resuspend them 15 µL NFW and 5 µL loading dye.
- Now samples can be loaded on a DGGE.

Preperation of the gel solution:

Table 10 Ingredients of the gel solution

80 % solution	0 % solution
40.5 g urea	30 mL acrylamid 40 %
48 mL formamid	1.5 mL TAE
30 mL acrylamid 40 %	Needs to be filled up to 150 mL wit dest. H_2O
1.5 mL TAE	
Needs to be filled up to 150 mL wit dest. H_2O	

Table 11 APS and TEMED for preparing a DGGE-Gel

	+	-	0
0 % solution			8 mL
APS	7 mL	7 mL	4 mL
TEMED	50 mL	50 mL	35 mL

Gradient for the all bacteria primers is 30%-62.5%. The appropriate amounts of APS and TEMED need to be choosen from a list.

- Between two glass plates a spacer is positioned by using a clamp. Into the gap between the two glass plates the gel can be cast.
- Seal the bottom edge with a paraffin film.
- Clamp the glass plates on the rubber in the device.
- Clamp the pipette tip of the gradient mixer between the glass plates and select gel concentration according to the GC content.
- Now the gel can be filled.
- On the next day but the gel into the DGGE and pull out the comb.
- Preheat the DGGE to 60 °C.
- Add samples into the DGGE-slots using a Hamilton microliter syringe.
- Select volt (175 volt), temperature (60 °C) and time (4h 25min) and start the DGGE.

12.7 Curriculum Vitae

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08/2011	**AKE – Arbeitsgemeinschaft für klinische Ernährung** Praktikum im Rahmen des „nutritionDay worldwide" Höfergasse 13/1 1090 Wien Aktualisierung projektrelevanter Dokumente, Übersetzung von Projektunterlagen, Aktualisierung der Projekt-Website, Erstellung des monatlichen Newsletters
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Sprachen:

Deutsch:	Muttersprache
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Spanisch:	Maturaniveau

EDV-Kenntnisse:

Microsoft Office (Word, Power Point, Excel)	sehr gut
Adobe Photoshop	Grundkenntnisse

Freizeitaktivitäten:

Laufen, Gymnastik, Handarbeiten

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