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Changes of gut microbiome in patients with inflammatory bowel diseases treated with anti-TNF-α antibody

Změny střevního mikrobiomu u pacientů s idiotypickými střevními záněty léčenými pomocí anti-TNF-α

Master thesis

Supervisor: Ing. Jakub Mrázek, PhD.

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Prohlašuji, že jsem tuto diplomovou práci vypracovala samostatně s využitím uvedené literatury a informací, na něž odkazuji. Svoluji k jejímu zapůjčení s tím, že veškeré (i přejaté informace) budou řádně citovány.

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Abstrakt česky

Crohnova choroba spadá společně s ulcerózní kolitidou do skupiny idiopatických střevních zánětů označovaných jako IBD (inflammatory bowel disease). Vyskytuje se především ve vyspělých zemích, kde pacientů s touto nemocí neustále přibývá. IBD je imunologicky podmíněné multifaktoriální onemocnění, jehož mechanismus vzniku stále není znám. Zavedeným léčebným postupem je léčba zánětu pomocí kortikosteriodů a imunosupresiv. Kromě střevního zánětu, který je primárním cílem léčby, je u pacientů prokázaná i střevní dysbióza. Právě mikrobiota může být jedním z možných terapeutických cílů a léčebným postupem adjuvantní nebo biologická terapie. Adjuvantní terapie přímo cílí střevní mikrobiotu za pomoci probiotik, kdežto cílem biologické léčby je TNF-α, prozánětlivý cytokin, produkovaný ve velké míře makrofágy. Cílem této diplomové práce je zhodnotit změny střevní mikrobioty u IBD pacientů ve vztahu k adjuvantní a biologickou terapii.

Pro analýzu bakteriální diverzity byly vyzkoušeny tři možné metody izolace DNA. Rapid beat beating + column (RBB+C) byla vybraná pro analýzu vzorků pacientů, protože vykazovala jak největší výtěžek DNA, tak nejvyšší čistotu DNA. Nejprve byla diverzita zkoumána kvalitativně pomocí degradační gradientové gelové elektroforézy (DGGE) s následnou sekvenací zajímavých bandů. Dále byla použita metoda NGS, která poskytuje více kvantitativních údajů. Výsledky získané výše zmíněnými metodami byly v souladu.

U pacientů v remisi, kteří podstoupili adjuvantní terapii s VSL#3 probiotickou směsí, nebyly prokázány žádné signifikantní změny v diverzitě střevní mikrobioty, zatímco u pacientů, kteří podstoupili biologickou léčbu za pomoci infliximabu, byly pozorovány signifikantní změny v diverzitě střevní mikrobioty. S nástupem biologické léčby jsem identifikovala několik bakterií (*Prevotella copri* a *Megamonas funiformis*) nebo jsem naopak zaznamenala ztrátu (*Streptococcus salivarius*). U jednoho pacienta korespondoval nárůst skupiny Bacteroidetes se zmírněním aktivity nemoci. U jiného pacienta byl prokázán nárůst skupiny Firmicutes.

Výsledky studií zabývajících se složením střevního mikrobiomu mohou být ovlivněny mnoha faktory zahrnující selekci pacientů, zdroj DNA, DNA extrakční techniku, cílový gen pro PCR amplifikaci, věrohodnost H indexu, definici OTU a náhled na taxonomii. Tato variabilita a nekonsistence si žádá standardizaci budoucích studií a obezřetnou interpretaci při porovnávání výsledků.

Klíčová slova: idiopatické střevní záněty (IBD), biologická léčba, anti-TNF preparáty, mikrobiom

English Abstract

Crohn's disease together with ulcerative colitis, is a type of inflammatory bowel disease (IBD) with increasing incidence and prevalence in developed countries. IBD is an immunologically mediated multifactorial disease and it´s mechanism of action is still unknown. Current well-established treatment targets the inflammation with corticosteroids and immunosuppressive drugs. Apart from the intestinal inflammation, which is the primary target of the treatment, patients are characteristically afflicted with intestinal dysbiosis. Therefore, possible interventions might be an adjuvant or biological therapy. Adjuvant therapy directly aims the microbiota with probiotics, whereas the target of biological therapy is TNF-α, a pro-inflammatory cytokine excessively secreted by macrophages. The aim of this thesis is to evaluate intestinal microbiota composition changes in IBD patients with regard to courses of adjuvant and biological therapy.

Bacterial diversity was analyzed using three different DNA extraction techniques. Rapid beat beating + column (RBB+C) was chosen for analyzing patient samples, as it showed the highest DNA yield and the highest DNA purity. Primarily the bacterial diversity was analyzed using degradation gradient gel electrophoresis (DGGE) with subsequent sequencing of bands of interest. In addition, the NGS approach was applied to assess more detailed and quantitative data. Results from both methods were in agreement.

Patients in remission on adjuvant therapy with VSL#3 probiotic mixture showed no significant differences in overall bacterial diversity, whereas patients on biological therapy with infliximab showed significant changes in intestinal microbiota diversity. Several bacteria appeared (such as *Prevotella copri* and *Megamonas funiformis*) or disappeared (*Streptococcus salivarius*) with the onset of the biological therapy. In one patient, the levels of Bacteroidetes increased during the course of the biological therapy which corresponded to the disease activity (severe to moderate). In another patient, an increase in Firmicutes was detected.

There are many factors which can influence the outcomes of studies addressing microbiome composition: patient selection, DNA source, DNA extraction technique, target gene for PCR amplification to H index difference credibility, OTU definition and taxonomical view. This variability and inconsistency calls for standardization of the procedures in future studies and cautious interpretation when cross-comparing results.

Key words: inflammatory bowel diseases (IBD), biological therapy, anti-TNF drugs, microbiome

Table of contents

Abbreviations

bp	base pairs
CD	Crohn´s disease
DGGE	degradation gradient gel electrophoresis
E.coli	*Escherichia coli*
GI	gastrointestinal tract
H index	Shannon index
HMP	Human Microbiome Project
IBD	inflammatory bowel disease
MM	magnetic microspheres
OTU	operational taxonomical unit
QIA kit	QIAamp DNA Stool Mini Kit
RBB+C	Zr beads beating combined with subsequent QIAamp column purification
ROS	reactive oxygen species
TNF-α	tumor necrosis factor alpha
UC	ulcerative colitis

1 Introduction

Crohn's disease together with ulcerative colitis, is a type of inflammatory bowel disease (IBD) which is increasingly prevalent in developed countries. IBD is an immunologically mediated multifactorial disease and it´s mechanism of action is still unknown. Current well-established treatment targets the inflammation with corticosteroids and immunosuppressive drugs. Apart from the intestinal inflammation, one well-known characteristic of IBD is intestinal dysbiosis. Therefore, in this thesis I decided to investigate the changes in microbiota composition during the course of two therapies that are currently used to treat the disease.

The first therapy to be investigated is adjuvant therapy which uses VSL#3 probiotic mixture, thought to directly affect microbiota composition.

The second therapy to be investigated is biological therapy, which targets the pro-inflammatory cytokine TNF-α, which is responsible for the pathology of this disease. This therapy does not directly act on microbiota; but that makes it even more interesting to researchers to investigate possible connections to the microbiome composition alteration.

1.1 Aims of the Study

The aim of this thesis is to investigate changes in microbiota composition in IBD patients during therapeutic interventions, including adjuvant and biological therapy. A crucial step in assessment of microbiota composition is bacterial DNA isolation. Therefore, the initial step is to compare three available DNA extraction techniques. Based on the purity and yield of DNA, one technique is chosen to investigate the microbiota changes in both of the aforementioned therapies. Qualitative information about microbiota composition will be acquired by PCR-DGGE approach for both adjuvant and biological therapy. In addition, quantitative data will be acquired by a high throughput NGS approach for the biological therapy group.

The aims of this thesis can be summarized in 3 major points:

- Comparison of three available bacterial DNA extraction techniques
- Influence of adjuvant therapy on human microbiota composition
- Influence of biological therapy on human microbiota composition

The outcome should contribute to understanding the mechanism of the disease's development, as well as evaluating currently-available methods for studies of human microbiota.
This work is just a pilot study for much broader project addressing non-responsiveness to biological therapy which is currently a serious clinical problem. The hypothesis is that there are differences in microbiome composition among those two groups of IBD patients.

2 Literature Review

2.1 Human gut microbiome

Human gut microbiota create a vast ecosystem, which varies greatly across sites in the body. The majority of microbial cells are located in the intestine and form an entire organ. The human gut microbiome comprises a vast number of bacteria; yet despite, previous estimations (Bäckhed et al. 2005; Gill et al. 2011) that microbial cells outnumber human cells by 10:1, a more recent article (Sender, Fuchs, and Milo 2016) revised the cell counts, showing a ratio close to 1:1 with red blood cells comprising 70 % of the total amount. In terms of microbial genes, There are over three millions (3×10^6) microbial genes: that is,150 times more than there are human genes (Qin et al. 2010). Microbes are largely beneficial for the body, as they help with digestion and healthy immune system functions. There is a delicate balance between microbiota in health and disease. Such equilibrium is dependent on the environment, in which microbes live, which is undoubtedly affected by our behavior. From that perspective, one can no longer look at the human body as a self-sustaining system but rather a niche in which microbial cells cooperate, often referred to as a supraorganism. Health can be viewed as a symbiotic state between host and microbes; this has to be taken into account when treating diseases.

The human microbiome is plastic and can adapt to multiple environmental changes. Although, one can find some common species among human microbiomes and assign them to certain enterotypes (Arumugam et al. 2011), the overall picture remains unique to each person. This fact emphasizes the need for an individual approach to each patient by means of personalized medicine.

There has been extensive effort in the scientific community recently to sequence the human microbiome. Not surprisingly, it was a logical follow-up to completing the Human Genome Project. Two projects were launched: MetaHIT in Europe and the Human Microbiome Project (HMP) in USA. The MetaHIT project showed that there are 150 more microbial genes than human genes and that 40% of one person‘s genes were shared with at least half of the people from the cohort (124 individuals). There were several outcomes of the HMP, one of which was the confirmation of the absence of the core microbiome in the human body (Caporaso et al. 2011). In this study, they tracked two individuals (one male, one female) for 15 and 6 months respectively which is quite extensive time period. They have come with a conclusion that the microbiomes of individuals are quite different and vary during time but each person has body specific niches which remain of similar patterns. However, taken together we are still missing reliable complex reference data sets which would allow for better interpretation of results. The

studies so far covered mainly Caucasian population hence we are still lacking quite a big piece with regards to variability in human race.

Contrary to the outcome of the large sequencing projects described above, the commonalities among the microbiomes were described as three dominant enterotypes in the human population. Those enterotypes are represented by microbial communities predominantly comprising *Bacteriodes*, *Prevotella* and *Ruminococcus* (Arumugam et al. 2011). However, others point out, that using certain analytical methods, such groups could be clustered only by chance and in addition only small number of samples from European population were used which could also lead to a biased results (Wu et al. 2012). But Arumugam et al., (2013) claim that the enterotypes appear quite complex and are probably not dependent on diet, sex, age, BMI, and other host properties (Arumugam et al. 2011). Those results are opposed by Wu et al. (2012), who claims those three enterotypes in their study groups could be attributed to certain nutritional habits, particularly protein and fat (Bacteroidetes) versus carbohydrates (*Prevotella*). This nutritionally biased hypothesis is supported by De Filippo et al. (2010), who compared European and African children, and associated them with particular enterotypes with respect to high protein and fat consumtion (Bacteroidetes), and low protein, and high carbohydrate consumption (*Prevotella*). Interestingly, microbiome composition could change short term according to a dietary intervention, however even after 10 days there was not an stable enterotype switching (Wu et al, 2011). On the other hand long term shifts in microbiota composition were observed in correlation with age. For example a difference has been documanted in maturing children (Yatsunenko et al. 2012) or aging adults (Mariat et al. 2009). In infancy and old age the changes in microbiota composition were associated with low Firmicutes/Bacteroidetes ratio compared to that of adulthood (Mariat et al. 2009).

2.2 IBD

Inflammatory bowel diseases (IBD) are autoimmune diseases of gastrointestinal tract with growing incidence and prevalence today (Molodecky et al. 2012). IBD could be defined as an inappropriate immune response to the commensal microbiota. There are two forms with clinically different manifestations: ulcerative colitis (UC), and Crohn´s disease (CD). The onset of the disease is dependent on genetic factors, as well as environmental factors, state of immune system, and intestinal microbiota. Nowadays it is generally accepted that pathological inflammation in caused by aberrant immune response to luminal antigens generated by gut microbiota in genetically susceptible individuals (Sartor 2008).

Ulcerative colitis is localized to large intestine, and is typical of intestinal wall thinning, ulceration, and loss of haustra, and crypts. Crohn´s disease could be distributed through the entire gastrointestinal tract, and is characterized by gut wall thickening and cobblestoning with fissures.

Genetic susceptibility to IBD is connected to genes with immunological functions. For instance ATG16L1, an autophagy gene (Hampe et al. 2007) or NOD2, a gene for NOD-like receptors (Hugot et al. 2001) are related to CD, whereas IL10, a regulatory gene (Franke et al. 2008) or ECM1 (Fisher et al. 2008), a gene involved in epithelial cell differentiation, claim to be specific for UC. In identical twins there is 50-75% probability that the other twin will also suffer from CD. In UC the heritability is lower with the concordance of 10-20% (Halme et al. 2006).

Environmental factors which contribute to development of IBD, ranging from diet, antibiotics and pathological infections all have the same denominator, effect on the microbiota. Dysbiosis, so peculiar of these diseases, is far the major driver of IBD hence it is also in the main focus of my thesis.

As dysbiosis is the root of inappropriate immune response, immune mechanisms cannot be omitted. The inappropriate response is represented by intestinal inflammation which causes the pathogenesis of this debilitating disease. The number one mediator of the inflammation is a cytokine called tumor necrosis factor alpha (TNF-α). It is produced in large amounts by macrophages and it´s level is commonly elevated in the patient´s serum (Komatsu et al. 2001). Therefore, one of the successful cures for IBD is a biological therapy. It takes advantage of the neutralizing effect of anti-TNF-α antibody, hence diminishing the pathological inflammation. But the question remains whether this immunotherapy has any effect on the state of intestinal microbiota what so ever. Answering this question is the main purpose of my thesis.

2.3 IBD and microbiome

The human microbiome is a large ecosystem: there are over 1100 microbial species, with at least 160 per individual, living in a mutualistic relationship with their host (Qin et al. 2010). If the symbiotic equilibrium is broken, then a state of dysbiosis follows. Dysbiosis is a long-term shift from healthy microbiota to harmful pathogenic microbiota, characterized by decreased diversity, and has been well documented in IBD (Tamboli et al. 2004). Dysbiosis does not mean that a single pathogen causes the initial inflammation, as thought previously, but that the microbiome is altered as a whole (Tamboli et al. 2004). Regarding the diversity, a large metagenomics analysis revealed, that IBD patients have, on average, 25% fewer genes than healthy individuals (Qin et al. 2010).

Certain taxonomical groups were identified with respect to intestinal dysbiosis and IBD: some of them protective, others pathogenic. Evidence that Proteobacteria contribute to the pathogenicity of IBD was found in studies in the 70s (Keighley et al. 1978). In particular the adhesive *E. coli* has been documented sticking to the ileal walls of diseased patients (Darfeuille–Michaud et al. 1998). Despite the pathogenic associations of Proteobacteria *E. coli* Nissle 1917 was documented to induce production of antimicrobial peptide by epithelial cells as a response to flagellin (Schlee et al. 2007). Bacteriodetes counts differ across studies: some claim lower levels in IBD patients (Frank et al. 2007; Seksik et al. 2003) others higher levels (Giaffer, 1991). *Bacteroides fragilis* has been associated with the inflammation present in the patients with the active disease (Prindiville et al. 2000). Contrary to this polysaccharide A from *Bacteroides fragilis* has been reported to induce $Foxp3^{+}$ regulatory T-cells which secrete suppressive cytokines such as IL-10 and TGFβ and therefore inducing tolerance at the intestinal mucosa (Round and Mazmanian 2010). Firmicutes have been linked to have beneficial effect with regards to inflammation and, correspondingly, levels of Firmicutes were diminished in IBD patients (Frank et al. 2007). In particular, *Faecalibacterium prausnitzii*, which has a protective effect, was decreased (Sokol et al. 2008). Additionaly, whole genera of phylum Firmicutes were associated with a beneficial probiotic effect, including *Lactobacilli* and *Bifidobacteria* of phylum Actinobacteria (Jonkers and Stockbrugger 2003).

2.3.1 Mucosal immunity

Since epithelia of human gut are exposed to various micro-organisms right after birth, good defense mechanisms need to be employed. Apart from mechanical and chemical agents it is protected by highly effective immune system whose task is to protect from pathogens and induce tolerance to foreign antigens (Tlaskalová-Hogenová et al. 2004). Commensal microbiota

is a part of this complex defense mechanism at mucosa surfaces and is capable of modulating the immune response.

The normal immunological response to foreign antigens in the mucosa is tolerance. Prevention of unfavorable inflammation is ensured by the production of anti-inflammatory cytokines such as IL-10 and TGFβ. These cytokines are produced chiefly by Tregs, which suppress the pro-inflammatory branches of immune response (Coombes et al. 2005). For instance, polysaccharide A from *Bacteroides fragilis* has been shown to be responsible for inducing development of $CD4^+$ $Foxp3^+$ Tregs via TLR2 signaling. Mice treated with polysaccharide A were able to escape experimentally induced colitis. (Round and Mazmanian 2010). This provides an example of the importance of commensal bacteria for the proper functioning of our immune system, as well as a potential treatment approach. Other mechanisms, such as the absence of antibodies activating a complement, contribute to preventing the development of inflammation at the mucosal site.

NOD2 is another example illustrating the tight cooperation of commensal microbiota with immune system. NOD2 expressioin was shown to influence commensal microbiota and vice versa. This finding demonstrates how homeostatsis is mainted by creation of regulatory feedback loop between host (via NOD2) with the commensal microbiota (Petnicki-Ocwieja et al. 2009).

2.4 IBD mechanism of disease development

So far there has not been a single obvious trigger responsible for the disease development identified. Rather, it seems that the cause is an interplay of various factors which contribute to the resulting condition, both genetic and environmental. There are several hypotheses, each taking into account and emphasizing different factors.

2.4.1 Not a single pathogen but dysbiosis

The disruption of intestinal microbiota does not involve only a single pathogen but alters a microbiota as a whole causing a state of dysbiosis. It would be quite naïve to think there is a single common factor responsible for the pathology. So far, all sources point to the fact, that the microbiome is a complex ecosystem which can be affected by many different conditions.

2.4.2 Autoimmune or an immunodeficient condition

One of the hypotheses is that IBD is not an autoimmune disorder, as commonly accepted, but rather an immunodeficiency. This idea is supported by studies on patients with immunodeficiencies such as Wiskott–Aldrich syndrome, immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) and chronic granulomatous disease, which all share an IBD phenotype. The premise is that the primary immunodeficiency causes an altered response to luminal bacteria which, in the end, result in inflammation of the bowel (Glocker and Grimbacher 2012). From this point of view, one can look at IBD as a complex of immune irregularities which cause the same symptoms.

2.4.3 Oxygen hypothesis

Another hypothesis which may lie behind the development of dysbiosis in IBD is the oxygen hypothesis. The assumption is that the healthy intestine is characterized by a low oxygen abundance while in IBD patients there has been a decrease in Firmicutes (obligate anaerobes) and an increase in Enterobacteriaceae (facultative anaerobes) (Rigottier-Gois 2013). Could that mean that dysbiosis is caused by dysanaerobiosis? We can find several examples supporting this possibility. One of these is reactive oxygen species (ROS) created during the inflammatory process reacting with an endogenous compound and creating a new electron acceptor. *Salmonella* have genes which enable the use of such compounds, which gives it the growth advantage over other microbiota that reside in the inflamed gut (Winter et al. 2011). Another example could be that increased oxygen levels result from the passage of blood to the GI tract during chronic inflammation, and the subsequent release of oxygen from hemoglobin allows the overgrowth of facultative anaerobes such as Enterobacteriaceae (Lupp et al. 2007). Also,

one bacterium commonly associated with the shift to a healthy microbiome is *Faecalibacterium prausnitzii*, which is extremely sensitive to oxygen levels (Rigottier-Gois 2013).

2.4.4 Chicken and egg question

In this chapter I have presented several different views on IBD and it´s mechanisms of development. The fact that there are so many different angles from which it may be viewed is a typical example of chicken and egg question: of the various phenomena described, no one knows what comes first; that is, the root cause of the disease.

It is also possible that IBD is not a disease per se, but rather a group of symptoms resulting from different conditions different triggers.

2.5 IBD and Biological Therapy

Biological therapy is a kind of treatment which uses the body´s natural substances, or laboratory-produced versions of such substances, to treat certain conditions such as cancer or chronic inflammatory disorders. In the case of IBD, biological therapy means the involvement of monoclonal antibodies, which have a neutralizing effect on cytokine TNF-α.

TNF-α is a pro-inflammatory cytokine predominantly produced by activated macrophages. It was first described in 1975 by Lloyd Old as a protein released from host cells while he was studying the hemorrhagic necrosis of tumors by gram-negative bacteria (Carswell et al. 1975). A decade later, the first evidence about involvement of TNF-α in pathogenesis caused by LPS appeared (Beutler, Milsark, and Cerami 1985). Then, in the early 1990s, the first chimeric monoclonal antibodies were shown to inhibit the biological effect of TNF both in vitro and in vivo (Siegel 1995). In this era, a number of clinical studies proved a beneficial anti-TNF effect in the treatment of rheumatoid arthritis and Crohn´s disease (Targan et al. 1997). Former Czechoslovakian scientist Jan Vilček had an active role in the invention of this monoclonal antibody, which was named infliximab and commercially sold as Remicade (Vilcek 2009). This discovery was breaking in the treatment of inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis, ankylosing spondylitis, psoriatic arthritis, plaque psoriasis and others. Nowadays, there is variety of other TNF-blocking agents approved, such as adalimumab-Humira, etanercept-Enbrel, golimumab-Simponi and certolizumab pegol-Cimzia. According to European Medicines Agency quite recently, the biosimilar versions Inflectra and Remsima were released (http://www.ema.europa.eu/ema/).

Infliximab is manufactured as a powder which is suspended and administered to the patients intravenously. Infusion takes about an hour and patient come after two and then six weeks for the next doses; if there is a good response, the maintenance regimen is set every eight weeks. Patients are carefully chosen for such therapy, since its cost can reach up to $28,519 annually (Schabert et al. 2013).

Infliximab is a chimeric monoclonal IgG antibody with a molecular weight of 150 kDa. It consists of a mouse variable Fab region attached to a human constant Fc region. In contrast to that adalimumab´s variable region is of human origin (Tracey et al. 2008). The mechanisms of action involve two major effects: (1) antagonist, a blockade of TNFR- mediated mechanisms and (2) agonist, the induction of tmTNF-mediated mechanisms (Tracey et al. 2008). Infliximab is capable of binding both forms of TNF, secretory and transmembrane, thereby causing the two diverse effects mentioned above. The pleiotropic effects range from inflammation to apoptosis control.

The antagonistic action of Infliximab involve blocking the NF-κB pathway by neutralizing sTNF-α, and hence diminishing inflammation (Ware 2005). Apart from that, inflammation can be also inhibited by action upon the TNF-α-mediated release of CD40 and VCAM in human intestinal microvascular endothelial cells (Danese et al. 2006).

The agonistic action of Infliximab involves binding to tmTNF-α, thereby triggering apoptotic pathways (Lügering et al. 2001). Apart from those straight-forward mechanisms, there is also a phenomenon called reverse signaling. It involves tmTNF-α, which can act either as a ligand of TNFR or as a receptor and result in the induction of intersecting pathways. This means TNF antagonists can act in both blocking (Eissner, Kolch, and Scheurich 2004) and initiating apoptosis (Monastra et al. 1996).

2.6 Adjuvant therapy with probiotics

As there is a strong link between disrupted microbiome and IBD, it seems a logical step to use therapy whereby beneficial microbiota are directly introduced into the patient. This idea was previously discussed by Metchnikoff (1908) in his optimistic studies on life prolongation. He emphasizes the beneficial effect of lactate-producing bacteria in fermented milk products such as yogurt, which is commonly consumed in the states of Eastern Europe where there are high numbers of centenarians. There are currently plenty of studies which address this adjuvant treatment; however, they vary greatly in size, duration, dose, strains used and techniques employed.

Regarding the adjuvant therapy with probiotics, the main focus is put on VSL#3 food supplement, which has been marketed in Europe since 2002. VSL#3 is a probiotic mixture of 8 probiotic strains: namely, *Streptococcus thermophilus*, *Bididobacteria* (*B. breve, B. longum, B. infantis*) and *Lactobacilli* (*L. paracasei, L. acidophilus, L. delbrueckii subsp bulgaricus, L. plantarum*).

Comparing CD with UC, there is more data about the induction or maintaining of remission by probiotic treatment in UC than there is for CD. In UC VSL#3 was evidenced to both induce (Sood et al. 2009) and maintain remission (Miele et al. 2009). In CD a much lesser effect was observed, perhaps partially due to a lack of studies having been done (Veerappan, Betteridge, and Young 2012).

It is not entirely clear how probiotics work; however, several plausible immunomodulatory mechanisms were suggested for certain bacteria, such as inducing TGFβ or IL-10 production, suppressing the NF-κB inflammatory pathway and improving the barrier function of intestine (Preidis and Versalovic 2009).

The advantages of the probiotic treatment are that it is generally well-tolerated and has no significant side effects. However, one should be extremely cautious in evaluating the results, since there are numerous factors which can influence the process as a whole.

2.7 Molecular Methods of Bacteria Diversity Estimation

There are various methods which are currently used for bacteria diversity detection. Firstly, there are numerous DNA extraction techniques; secondly, there are different techniques for comparing DNA sequences; and finally, there are several methods used for the evaluation of the latter techniques.

We already have many tools how for isolating bacterial DNA. There are three major principles which are used for DNA extraction: (a) isopropanol precipitation, (b) phenol chloroform extraction and (c) column purification.

In this study we chose three of the available methods of bacterial DNA extraction: (1) QIAamp® DNA stool mini kit, Qiagen, (2) DNA extraction using Zr bead beating, combined with subsequent QIAamp column purification (Yu and Morrison 2004) and (3) DNA extraction with magnetic microspheres (Horák, Rittich, and Španová 2007) followed by comparing the DNA yield.

The QIAamp® DNA stool mini kit, Qiagen is based on DNA´s ability to adhere under particular pH and salt concentration to a solid phase in the column. The second method, DNA extraction using Zr beads beating combined with subsequent QIAamp column purification, works the same as the preceding method but is enriched for the mechanical disruption step with Zr beads and precipitation step by isopropanol and ammonium acetate. Magnetic Hydrophilic Poly(2-Hydroxyethyl Methacrylate-co-Glycidyl Methacrylate) Microspheres work as functional carriers containing carboxyl groups with the capacity to bind bacterial DNA.

Many methods of microbial diversity estimation are known in the field. They might be fingerprinting techniques (TGGE/DGGE, T-RFLP...), be based on the quantification of selected bacterial groups by real-time PCR or fluorescent in-situ hybridisation (FISH), or might even be a direct sequencing of microbial DNA via cloning or a next-generation sequencing approach (Inglis et al. 2012). PCR-DGGE operates on 16S rDNA amplicons and gives a unique pattern based on the different melting stages of DNA in a denaturing gradient. According to the sequence, DNA melts at different sites, giving fragments of various length which are concurrently divided in the gel according to their size. The resulting pattern is unique for each sample. The advantage of this method is that it gives a fast and relatively cheap screen for comparing bacterial composition.

For the acquisition of specific data about bacterial composition, sequencing techniques are the most appropriate choice. For the purpose of this thesis, I used 2 different sequencing techniques: Sanger sequencing and Ion Torrent sequencing. Commercial Sanger sequencing was used to identify excised bands in DGGE gel if an interesting trend or pattern was observed. Ion Torrent

sequencing was employed to obtain complete V4-V5 16S rDNA sequence from the samples analyzed. Sequencing techniques give more specific data about bacteria composition than does DGGE; however, they are laborious, time-consuming and more expensive.

3 Materials and Methods

3.1 Study groups

The fecal samples for comparing the DNA extraction techniques were collected from healthy donors. These fecal samples were transferred on ice to the Institute of Animal Physiology and Genetics and stored at - 20 °C until they were analyzed. The group of donors was diverse in terms of age and gender: two children (5 and 8), two young adults (23 and 26) and two adults (54 and 54), with two being female and four male.

Fecal samples were collected from patients with IBD who had been put on adjuvant therapy. The inclusion criteria were: diagnosis of IBD (CD and UC) with no clinical, laboratory or endoscopic signs of disease activity. The exclusion criteria were: administration of bile acids, bile acid sequestrants or FXR agonists/antagonists (guggulsteron etc.). Patients were not included sooner than 1 month after colonoscopy.

The therapeutic regimen consisted of administration of one 2.7 g sachet of VSL#3 probiotic mixture, each with the dose of 900 billion live bacteria, two times a day for a period of 42 days; in total, 84 packs. This VSL#3 was a mixture of 8 probiotic strains: *Streptococcus thermophilus*, the B*ifidobacteria* (*B. breve, B. longum, B. infantis*) and the *Lactobacilli* (*L. paracasei, L. acidophilus, L. delbrueckii subsp bulgaricus and L. plantarum*).

The fecal samples were also collected from patients with IBD who had been put on biological therapy. The inclusion criteria were: diagnosis of IBD (CD and UC) with a consecutive treatment with TNF-α drug (infliximab) and a therapeutic regimen of induction according to the standards of the Czech gastroenterological society J.E.Purkyně (ČGS JEP). The exclusion criteria were unconfirmed diagnosis of IBD, other organ or systemic autoimmunity and episodic treatment with anti-TNF- α drugs or antibiotics.

3.2 Sample collection

The fecal samples were collected at the IBD Clinical and Research Centre, ISCARE, Prague. Fecal samples were kept at -20 °C and transferred on ice to the Institute of Animal Physiology and Genetics and stored at -20 °C until analyzed.

Stool samples were collected at different points during the treatment in order to catch the window of microbiota shift. Fecal samples for the biological therapy group were collected before and after 2, 6, 14 and, if possible, 22, 30, 38 weeks of therapy (Table 1). For adjuvant therapy samples were collected only at the start and end of the therapy.

In addition to the fecal samples, data for each patient´s clinical status was obtained (Table 1).

Table 1: Algorithm of fecal sample collection, in patients undergoing biological therapy, fecal sample collection before colonoscopy preceding biological therapy (C)

Weeks of treatment	**C**	**0 (1. dose)**	**1**	**2 (2. dose)**	**3**	**4**	**5**	**6 (3. dose)**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14 (4. dose)**
stool	x	x		x				x								x
disease activity	x	x		x				x								x
clinical response								x								x

3.3 Bacterial DNA isolation

Three techniques were used for the extraction of bacterial DNA: (1) QIAamp® DNA stool mini kit, Qiagen, (2) DNA extraction using Zr beads beating combined with subsequent QIAamp column purification (Yu and Morrison 2004) and (3) DNA extraction using magnetic microspheres (Horák, Rittich, and Španová 2007).

When extracting DNA with the kit, the procedure was followed according to the manufacturer´s protocol. The DNA extraction using Zr beads combined with column purification was performed according to Protocol of repeated bead beating plus column method (Yu and Morrison 2004). DNA extraction with magnetic microspheres was performed according to the

protocol for DNA isolation from dairy products with an increased elution time of 60 min (Horák, Rittich, and Španová 2007). DNA was isolated from 100 mg of each stool sample.

PCR amplification

PCR amplification of bacterial 16S rDNA was performed using universal bacterial primers of the V3 region: 338GC (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCG CCG CCG CAC TCC TAC GGG AGG CAG CAG - 3') and RP534 (5'- ATT ACC GCG GCT GCT GG - 3'). The procedure was: 3 min step at 94°C to denature the template which was followed by 35 cycles consisting of 1 min at 94°C, 30 s at 55°C, 1 min at 72°C, and the final primer extension step at 72°C for 10 min (Muyzer, De Waal, and Uitterlinden 1993). The PCR mixture contained 1 µl of DNA template, 1 µl of each primer (10 µM), 15 µl of OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (BioLabs, New England), and 12 µl of sterile H_2O to the total volume of 30 µl. PCR products of expected 300 bp length were checked using agarose gel electrophoresis.

DGGE

The obtained PCR products were used for DGGE analysis. The gel consisted of 40% polyacrylamide (38:2, acrylamide:bisacrylamide) with a urea/formamide denaturing gradient ranging from 35% to 60%. The gel recipe is shown in Table 2.

Table 2: Recipe for DGGE gel preparation

Rate of denaturation	**35%**	**60%**
Urea (g)	3,68	6,30
40% Acrylamide (ml)	5,56	5,56
50x TAE (ml)	0,50	0,50
Formamide (ml)	3,50	6,00
H_2O	12,25	9,50
Tetramethylethylenediamine (µl)	20	20
Ammonium persulfate (µl)	200	200

Electrophoresis was run in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) for 18 hours at 55 V and 60°C (Fischer and Lerman 1983). Gels were stained in 1x TAE with 0.001% SYBRr Green I for 30 min and subsequently visualized using Vilber Lourmat System

(France) with exposure to UV light. The DGGE standard ladder was prepared by using the following gut anaerobic microorganisms available at the Laboratory of Anaerobic Microbiology: *Bacteroides sp.* AR20, *Lachnospira multipara* ATCC 19204, *Ruminococcus albus* SY3, *Pseadobutyrivibrio sp*. JK 618, *Treponema sp.* 704, *Ruminococcus flavefaciens* 627, *Faecalibacterium prausnitzii* A2 165, *Butyrivibrio fibrisolvens* ATCC 19171, *Clostridium proteoclasticum* X2, *Escherichia coli* JM 109 and *Megasphera elsdenii* AW 106. The standard was only used for the visual comparison of DGGE gels and not for the identification of DNA bands.

3.4 Sanger sequencing

Amplicons of interest were excised from the DGGE gel on a UV box in a dark room using a sterile scalpel. 100 µl of sterile dH_2O was added, the mixture was vortexed and centrifuged (10 min, 10 000 rpm) to elute DNA from the gel blocks. Additionally, to increase the DNA yield from the DNA blocks, 10 µl of TE buffer (10mM Tris, 1mM EDTA) was added and kept overnight at 8°C. Eluted DNA was amplified by PCR, using FP341 (CCT ACG GGA GGC AGC AG) and RP534 (see above) primers. The PCR mixture contained 4 µl of a DNA template (H_2O with gel block), 0,5 µl of each primer (10 µM), 15 µl of OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (BioLabs, New England), and 10 µl of sterile H_2O to the total volume of 30 µl. The PCR program was the same as that of DGGE. The resulting amplicons were purified by UltraClean® PCR Clean-Up Kit (Mo Bio Laboratories) in order to remove PCR primers, dNTPs and reaction components, as well as with purify the PCR product within the range 60 to 10 000 bp. For commercial sequencing, samples were prepared according to web protocol: 50 ng of DNA was mixed with FP341 or RP534 primer and added with H_2O to yield a final volume of 10 µl (https://www.seqme.eu). The DNA concentration was checked using a NanoDrop™ One Micro-UV/Vis Spectrophotometer (Thermo Fisher Scientific).

3.5 Ion Torrent sequencing

In order to perform semi-conductor sequencing, several preparation steps should precede it: (1) DNA isolation, (2) PCR amplicon preparation, (3) library preparation, (4) template preparation, (5) sequencing itself and (6) data analysis. The protocol from Milani et al. (2013) was adopted.
(1) DNA was isolated by the bead beating method (Yu and Morrison 2004).
(2) Bacterial V4-V5 16S rRNA region was amplified according to Fliegerova et al. (2014) with the primers BactBF (GGATTAGATACCCTGGTAGT) and BactBR

(CACGACACGAGCTGACG). 1 µl of DNA, 1 µl of forward (10 µM) and 1 µl of reverse primer (10 µM), 20 µl of KAPA2G Fast ReadyMix with dye (2X) (Kapa Biosystems) and 5.7 µl dH_2O were added to the reaction mixture giving a final volume of 40 µl. The PCR program was as follows: 95°C for 10 min, 45 cycles (95°C 30 s, 57°C 30 s, 72°C 30s). The PCR reaction was checked by agarose electrophoresis (2 ul), where 300 bp product was expected.

(3) Next, library preparation was carried out, this is a process, where sequencing adaptors (short oligonucleotides) are ligated to PCR products. The PCR amplicons from the previous step were purified by UltraClean® PCR Clean-Up Kit (Mo Bio Laboratories) in order to remove PCR primers, dNTPs and reaction components; the PCR product was purified within the range 60 to 10 000 bp. DNA concentration was checked using NanoDrop™ One Micro-UV/Vis Spectrophotometer (Thermo Fisher Scientific). Approximately 100 ng of DNA was used for library preparation with the 'NEBNext® Fast DNA Library Prep Set for Ion Torrent (50)', according to the manufacturer´s protocol. The 'Ion Xpress Barcode Adapters 1 - 32' were used to label each sample specifically. The libraries were then quantified using qPCR approach with the 'Ion Library Quantitation Kit' and mixed to obtain 25 µl of mixture with an equimolar concentration of 26 pM for each sample.

(4) An amplified library was used to prepare a sequencing template by using the 'Ion PGM Template OT2 Hi-Q Kit' on OneTouch 2 equipment.

(5) The sequencing template was prepared on the Ion OneTouch 2 system and sequenced on a PGM platform (Thermo Fisher Scientific) by using the Ion PGM™ Hi-Q™ Sequencing Kit and the Ion 316™ v2 chip, both according to manufacturer´s protocols.

(6) The data were processed by the open-source software package QIIME 1.9.1, revealing alpha and beta diversities (Caporaso et al. 2010).

Data from Ion Torrent PGM (Life Technologies) were acquired in fastq format which combines qualitative information (q) with sequences (fasta). This file was split into a fasta file and a qual file using the command '*convert_fastaqual_fastq.py*'.

A map file was created for each sample, containing: SampleID, BarcodeSequence, LinkerPrimerSequence and additional information such as Study, Sample, Patient, Week and Description. The map files were validated using the QIIME script '*validate_mapping_file.py*'.

In the next step, the different samples were sorted according to their barcodes using '*split_libraries.py*'. The outcome are fna files: fasta files containing all sequences that meet the user-defined parameters, where each sequence identifier contains its corresponding sample id from the map file.

All files were combined into a single file , using the '*cat*' command to concatenate them.

By choosing the '*pick_open_reference_otus.py*' script, OTUs were picked from open references. This script can be broken down into six steps: four OTU picking steps and two steps for creating OTU trees and tables. First, OTUs are picked from a closed reference such as a supplied reference database. Second, the sequences which fail to be assigned to a reference database are clustered de novo, which serve as references in step three. Third, the failed sequences are picked from a new reference database created in step 2. Fourth, more OTUs are additionally picked in order for all sequences to be assigned to OTUs. Fifth, OTU maps may then be created from steps 1, 3 and 4. If the minimal OTU size parameter is reached (which is 2 in this case), a final OTU map is created. Sixth, OTU tables and trees are created.

To find the sample with minimal counts, the '*biom summarize-table*' command was used.

Beta diversity plots, alpha diversity plots, taxa plots, and group significance were generated using the script '*core_diversity_analyses.py*' from the biom table, map file, and phylogenetic tree.

Lastly, different alpha diversity metrics may be generated from the OTU table by using the '*alpha_diversity.py*' script.

3.6 Software tools

Image Lab software was used for analyzing the gel pictures obtained using DGGE.

MXpro software was used to manage the data acquired using qPCR.

MEGA and Geneious software packages were used for Sanger sequence data processing.

Quiime software was used to analyze the NGS data.

4 Results

4.1 DNA extraction techniques comparison

In order to estimate the bacterial diversity of a patient, one must proceed in a stepwise manner employing several methods. The first step is to isolate DNA from fecal samples. To obtain the highest DNA yield from patient samples, different extraction techniques on samples from healthy donors were compared first of all.

Three DNA extraction techniques were tested: (1) the QIAamp DNA Stool Mini Kit, (2) DNA extraction using Zr beads beating combined with subsequent QIAamp column purification (Yu and Morrison 2004) and (3) DNA extraction with magnetic microspheres (Horák, Rittich, and Španová 2007).

4.1.1 DNA yield and purity

The highest DNA yield was obtained by using the repeated bead beating method combined with columns, where the average DNA concentration extracted from 100 mg of stool reached 220.7 ng/µl. The next best method in terms of DNA concentration was QIA kit which yielded 52.4 ng/µl from 100 mg stool. The lowest DNA yield of 11.8 ng/µl was isolated by using magnetic microspheres. The DNA concentrations from tested methods are summarized in Table 3 and Figure 1.

Based on these results, the repeated bead beating method combined with columns was identified as superior to the other methods. Not only did RBB+C give the highest DNA yield, it also showed the highest DNA purity and was therefore chosen as the method for DNA isolation from patient samples.

Table 3: DNA yield and purity acquired by three different extraction techniques from 100 mg stool, QIAamp DNA Stool Mini Kit (QIA kit), Zr beads beating combined with subsequent QIAamp column purification (RBB+C), magnetic microspheres (MM), standard deviation (SD)

	QIA kit		**RBB+C**		**MM**	
donor	**conc. ng/µl**	**A260/A280**	**conc. ng/µl**	**A260/A280**	**conc. ng/µl**	**A260/A280**
1	63.2	2.23	159.4	1.86	13.0	1.99
2	22.2	2.12	192.4	1.72	16.8	2.07
3	58.1	2.11	189.4	1.71	8.9	1.74
4	73.1	2.15	206.4	1.92	8.6	2.46
5	72.1	2.25	307.9	1.84	13.1	2.08
6	25.7	2.35	268.8	1.84	10.1	1.76
average	52.4	2.20	220.7	1.81	11.8	2.01
SD	20.78	0.09	51.09	0.08	2.88	0.24

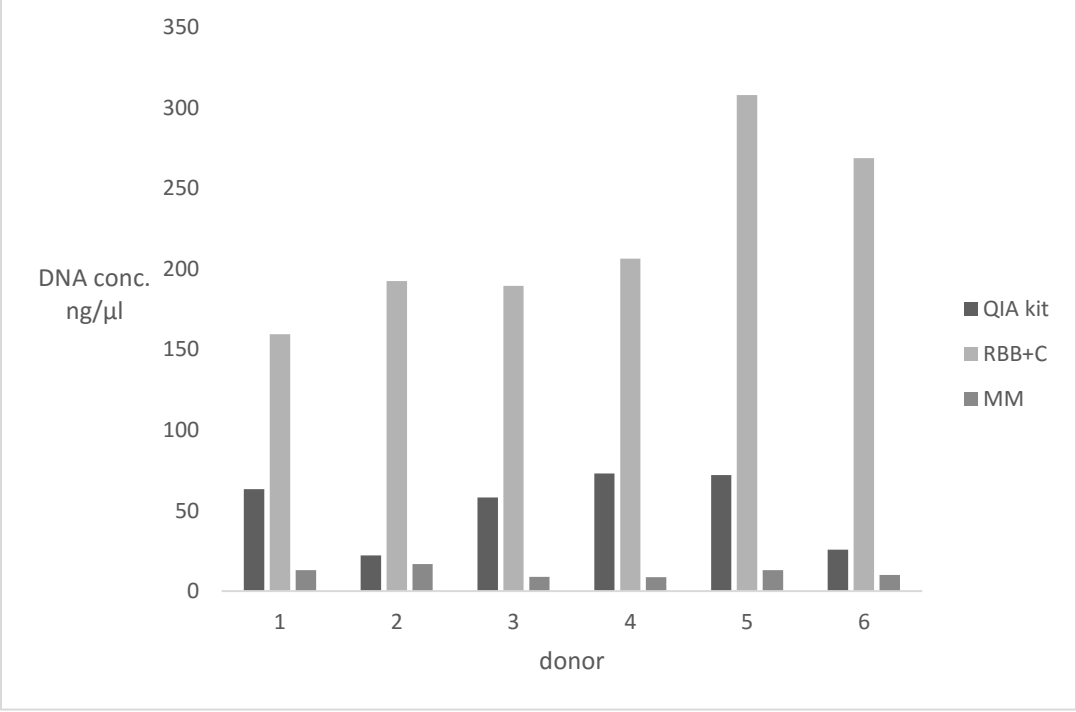

Figure 1: DNA concentrations acquired by different DNA extraction techniques from 100 mg stool: QIAamp DNA Stool Mini Kit (QIA kit), Zr beads beating combined with subsequent QIAamp column purification (RBB+C), magnetic microspheres (MM)

4.1.2 PCR-DGGE

To see whether there are any differences between bacteria groups isolated by different DNA extraction techniques, DGGE patterns were acquired. Isolated DNA was amplified using primers for the 16S rDNA region and the resulting amplicon was utilized for DGGE analysis. From Figure 2, one can see that DGGE patterns differ within an individual. This means that different extraction techniques were efficient for the isolation of different bacteria groups. It therefore, leads to the conclusion that the bacteria identified heavily depend on the DNA extraction technique used.

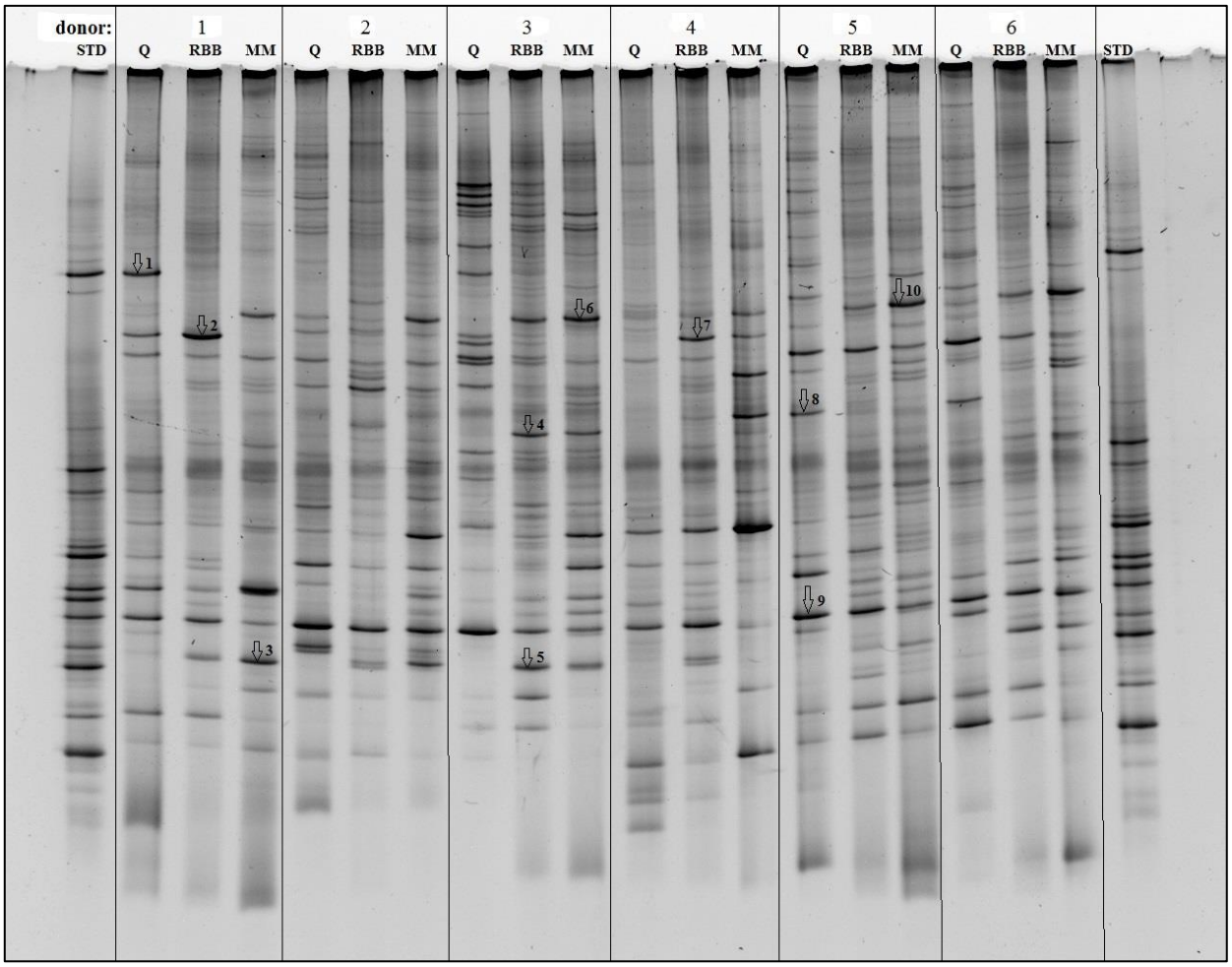

Figure 2: DGGE patterns from healthy donors acquired by different DNA extraction techniques. Arrows indicate bands which were excised and sequenced; standard (STD), QIAamp DNA Stool Mini Kit (Q), Zr beads beating combined with subsequent QIAamp column purification (RBB+C), magnetic microspheres (MM)

To identify some bacteria groups, bands were excised from the gel and sent for commercial sequencing. Sequences were blasted to the ncbi library. The results are summarized in Table 4.

Table 4: Bands 1-5 selected from DGGE gel (Figure 2), identification by ncbi blastn

band number	similarity %	organism	phylogenetic affiliation	isolation source	accession number
1	overlapping sequences - no match				
2	100	*Ruminococcus bromii*	Firmicutes; Clostridia	unknown	X85099.1
3	100	*Blautia luti*	Firmicutes; Clostridia	human feces	NR_114315.1
	100	*Ruminococcus obeum*	Firmicutes; Clostridia	unknown	NR_119185.1
	99	*Coprococcus catus*	Firmicutes; Clostridia	unknown	NR_024750.1
4	100	*Bacillaceae bacterium*	Firmicutes; Bacilli	calcium carbonate-cementitious materials	KJ882413.1
	100	*Bacillus licheniformis*	Firmicutes; Bacilli	Hami-melon juice	FJ907196.1
5	98	*Fusicatenibacter saccharivorans*	Firmicutes; Clostridia	human feces	NR_114326.1
	98	*Ruminococcus gauvreauii*	Firmicutes; Clostridia	human feces	NR_044265.1
	97	*Roseburia hominis*	Firmicutes; Clostridia	human gut	NR_074809.1
	97	*Ruminococcus faecis*	Firmicutes; Clostridia	human feces	NR_116747.1
	95	*Clostridium asparagiforme*	Firmicutes; Clostridia	human feces	NR_042200.1

From the sequencing results, one can see that in Donor 1, *Ruminococcus bromii* (band 2) was detectable using the QIA kit and RBB+C, yet failed to be detected by MM, whereas *Blautia luti* (band 3) was detectable by RBB+C and MM but failed to be detected by QIA kit. In Donor 3, both bands 4 and 5, belonging to *Bacillaceae bacterium* (band 4), and some Clostridia from band 5 (specified in Table 4) were detected by RBB+C and MM but failed to be detected by the QIA kit. Based on the sequencing of particular bands, one can conclude that different bacteria groups were identified depending on the DNA extraction technique used. Therefore, one should take note of the DNA extraction technique used when comparing results from different studies, as there might be some discrepancies. To specify which groups of bacteria are prevalent using a particular DNA extraction method, deeper investigation must be undertaken.

4.1.3 H index

For bacterial diversity estimation extracted from a DGGE gel Shannon (H) index was used (Eichner et al. 1999). For extraction technique comparison, the H indices of three different extraction techniques were compared (Table 5). The results from the ANOVA indicate a significant difference in bacteria diversity using different extraction techniques, Wilks´ lambda = 0.216; F = 7.259; $p < 0.05$; $\eta^2 = 0.78$. Follow-up comparisons indicate that not all pairwise differences were significant. The same was demonstrated by comparing two sectional techniques by paired T test. There were no significant differences in H index between RBB+C and Q ($p = 0.051$; $t = 2.57$), or between RBB+C and MM ($p = 0.354$; $t = 2.57$). A significant difference was found between Q and MM techniques ($p = 0,014$; $t = 2.57$). The corresponding H indices of different DNA extraction techniques are illustrated in Figure 3.

Table 5: H indexes of healthy donors 1-6 using different DNA extraction techniques, QIAamp DNA Stool Mini Kit (QIA kit), Zr beads beating combined with subsequent QIAamp column purification (RBB+C), magnetic microspheres (MM)

sample	**Q**	**RBB**	**MM**
1	1,077367	1,110816	1,206226
2	1,115308	1,118069	1,218181
3	1,214576	1,242843	1,246472
4	1,05975	1,202835	1,09136
5	1,149806	1,271039	1,341134
6	1,216163	1,245875	1,28677
mean	1,138828	1,19858	1,23169
SD	0,066967	0,068773	0,084562

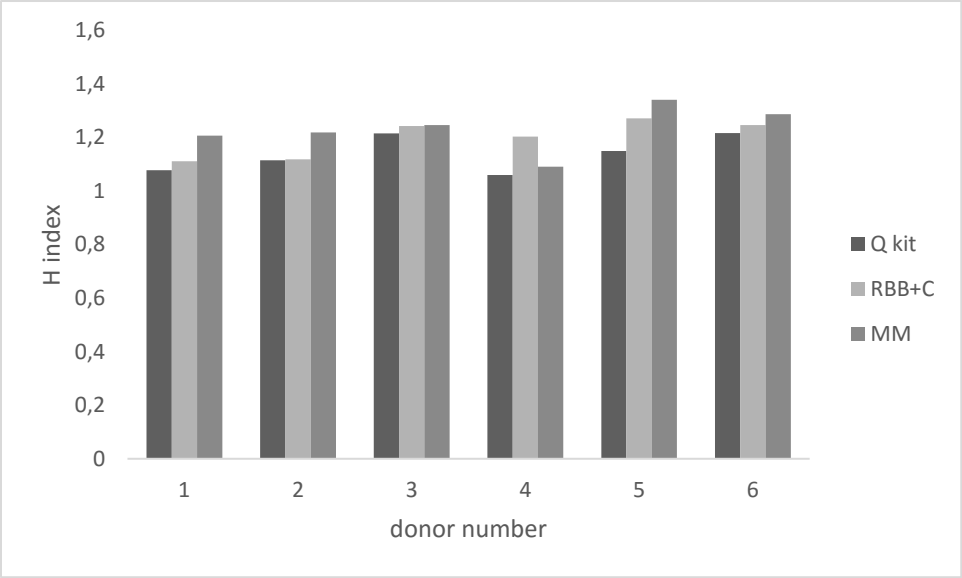

Figure 3: H indices of healthy donors 1-6 using different DNA extraction techniques: the QIAamp DNA Stool Mini Kit (QIA kit), Zr beads beating combined with subsequent QIAamp column purification (RBB+C), magnetic microspheres (MM)

We can therefore conclude from the H index of diversity that different extraction techniques yielded significantly different results.

4.2 Adjuvant therapy induced no changes in gut microbiome of IBD remission patients

4.2.1 PCR-DGGE

Isolated DNA was amplified using primers for the 16S rDNA region and the resulting amplicon was used for DGGE analysis. From Figure 4 and Figure 5 one can see the bacteria profiles of the patients on adjuvant therapy look almost the same. However, on closer inspection, one can find subtle differences. The outlying bands were of interest and so they were excised and sent for commercial sequencing.

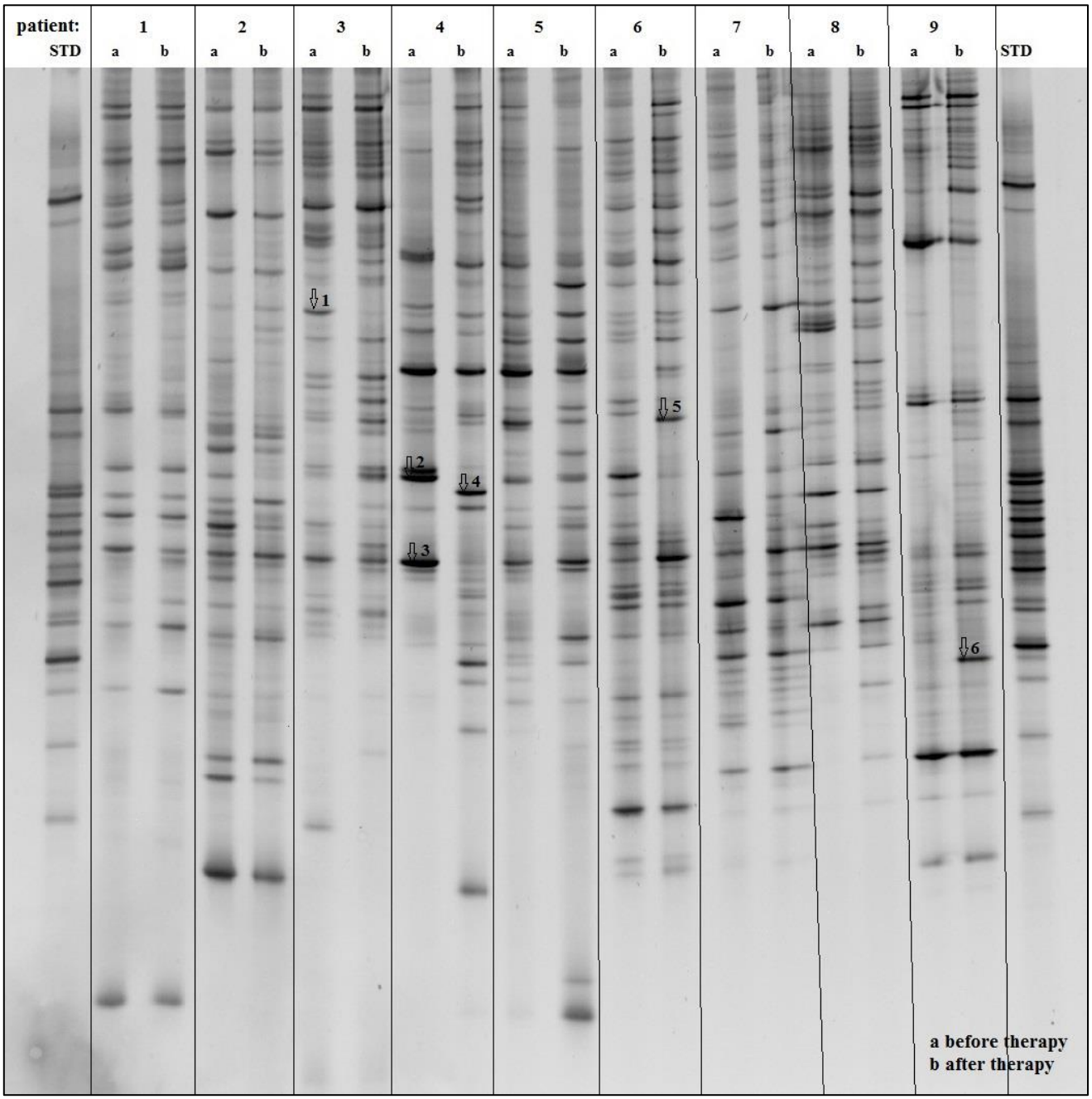

Figure 4: DGGE pattern of patients 1-9 on adjuvant therapy. Arrows indicate bands which were excised and sequenced, (STD) standard, (a) before therapy, (b) after therapy

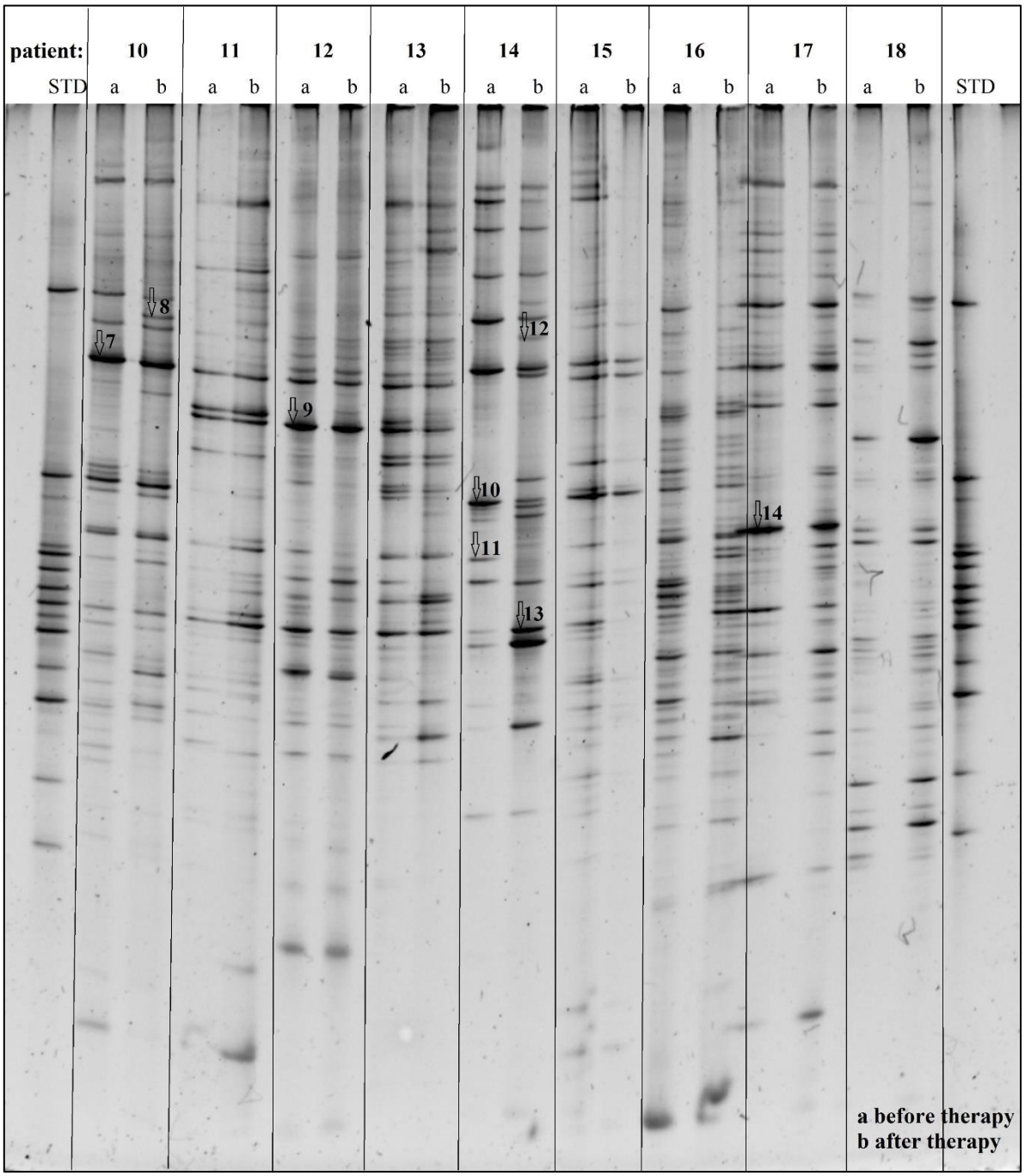

Figure 5: DGGE pattern of patients 10-18 on adjuvant therapy. Arrows indicate bands which were excised and sequenced, (STD) standard, (a) before therapy, (b) after therapy

To identify bacteria groups in patients whose bacteria DGGE profiles differed, bands were excised from the gel and sent for commercial sequencing. Sequences were blasted to the ncbi library. The results are summarized in Table 6.

Table 6: Bands 1-14 selected from DGGE gel (Figure 4, Figure 5), identification by ncbi blastn

band number	similarity %	organism	phylogenetic affiliation	isolation source	accession number
1	94	*Ruminococcus sp.*	Firmicutes; Clostridia	marine sediments	FJ889653.1
1	95	*Clostridiaceae bacterium*	Firmicutes; Clostridia	porcine intestine	EU728793.1
1	93	*Acetivibrio cellulolyticus*	Firmicutes; Clostridia		NR_025917.1
1	92	*Clostridium aldrichii*	Firmicutes; Clostridia		NR_026099.2
2	98	*Eubacterium eligens*	Firmicutes; Clostridia	human feces	NR_074613.1
3	96	*Faecalibacterium prausnitzii*	Firmicutes; Clostridia	human feces	NR_028961.1
4	95	*Clostridium sp.*	Firmicutes; Clostridia	human feces	AB491207.1
5		*several species in selected bend - no match*			
6		*overlapping sequences - no match*			
7	99	*Bacteroides dorei*	Bacteroidetes; Bacteroidia	human feces	NR_041351.1
7	99	*Bacteroides vulgatus*	Bacteroidetes; Bacteroidia	human feces	NR_112143.1
8	96	*Bacteroides ovatus*	Bacteroidetes; Bacteroidia	human feces	NR_040865.1
9	97	*Prevotella copri*	Bacteroidetes; Bacteroidia	human feces	NR_040877.1
10	99	*Bacteroides uniformis*	Bacteroidetes; Bacteroidia	human feces	AB908393.1
10	97	*Bacteroides intestinalis*	Bacteroidetes; Bacteroidia	human feces	NR_041307.1
11		*overlapping sequences - no match*			
12	92	*Veillonella ratti*	Firmicutes; Negativicutes	human clinical sample	NR_029101.1
13	94	*Veillonella ratti*	Firmicutes; Negativicutes	human sample	NR_029101.1
13	94	*Veillonella parvula*	Firmicutes; Negativicutes	human intestine	NR_117759.1

From the sequencing results, one can see that in Patient 3, there were some Clostridia (band 1) present before the therapy. In Patient 4, *Eubacterium eligens* (band 2) and *Faecalibacterium prausnitzii* (band 3) were present before adjuvant therapy, whereas some non-specific Clostridia (band 5) occurred after the therapy. In Patient 10, *Bacteroides dorei/vulgatus* (band 7) was present before adjuvant therapy, whereas *Bacteroides ovatus* (band 8) appeared afterwards. In Patient 14, *Bacteroides uniformis/ intestinalis* (band 10) was present before the adjuvant therapy, whereas *Veillonella ratti* (band 12) and *Veillonella ratti/ parvula* (band 13) appeared afterwards. *Prevotella copri* (band 9) was present Patient 12 both before and after adjuvant therapy. Across all patients, the presence or absence of several Bacteroidetes and Firmicutes was documented.

By simple observation, one can assume that the DGGE patterns of patients before and after adjuvant therapy are similar; however, in order to confirm such a conclusion precise data need to be extracted, which was done by comparing diversity (H index).

4.2.2 H index

For the group on adjuvant therapy, the H indices of patients before and after the therapy were compared. At a level of significance of 0.05, there were no differences in H indices of patients; t = 2.31; p = 0.07. The H indices for this group are shown in Figure 6.

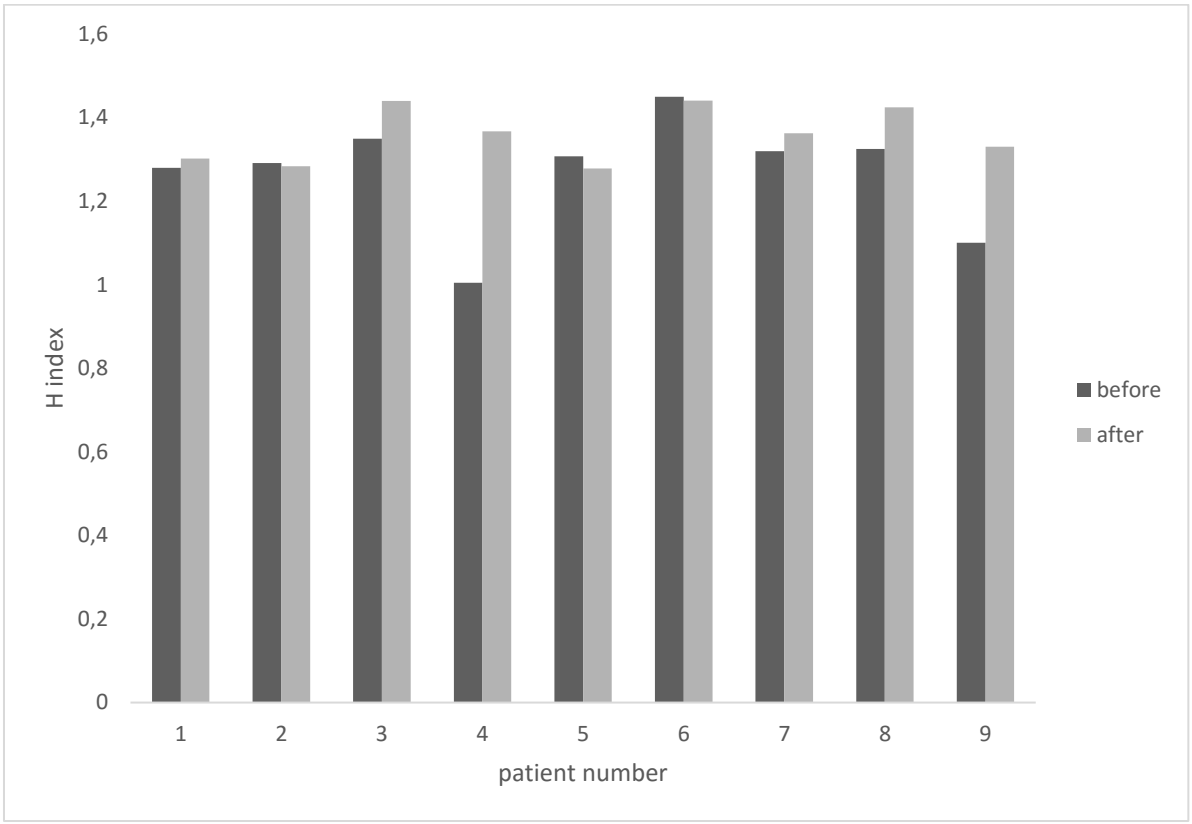

Figure 6: H indices of Patients 1-9 before and after adjuvant therapy

4.3 Biological therapy-induced changes of human gut microbiome

4.3.1 PCR-DGGE

Isolated DNA was amplified using primers for the 16S rDNA region and the resulting amplicon was utilized for DGGE analysis. In Figure 7 and Figure 8, one can see how the bacteria profiles of the patients on biological therapy changed over the course of the therapy.

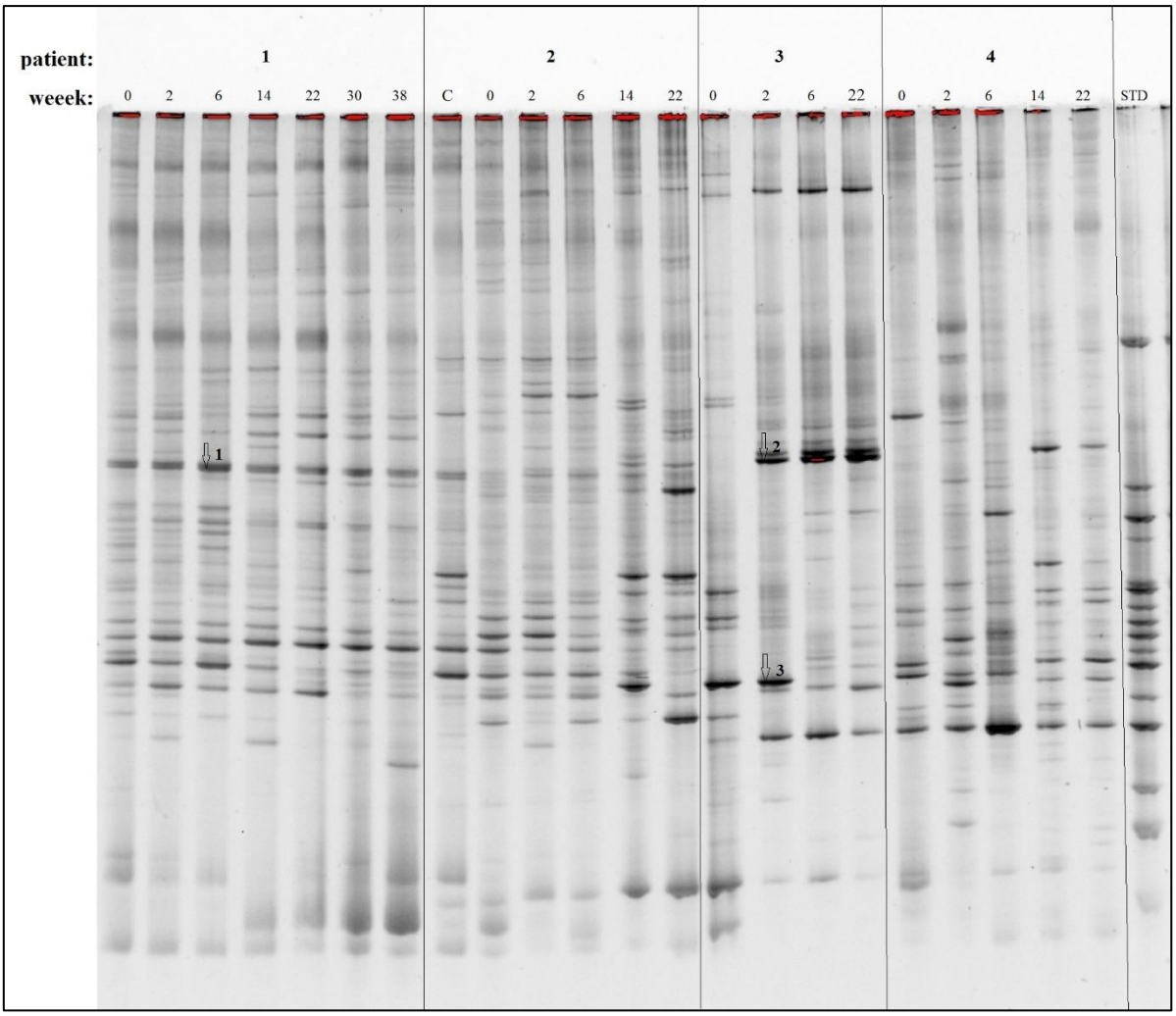

Figure 7: DGGE pattern of patients 1-4 on biological therapy. Samples were taken in certain weeks of the therapy. Arrows indicate bands which were excised and sequenced. (C) fecal sample was collected before colonoscopy preceding biological therapy, (STD) standard

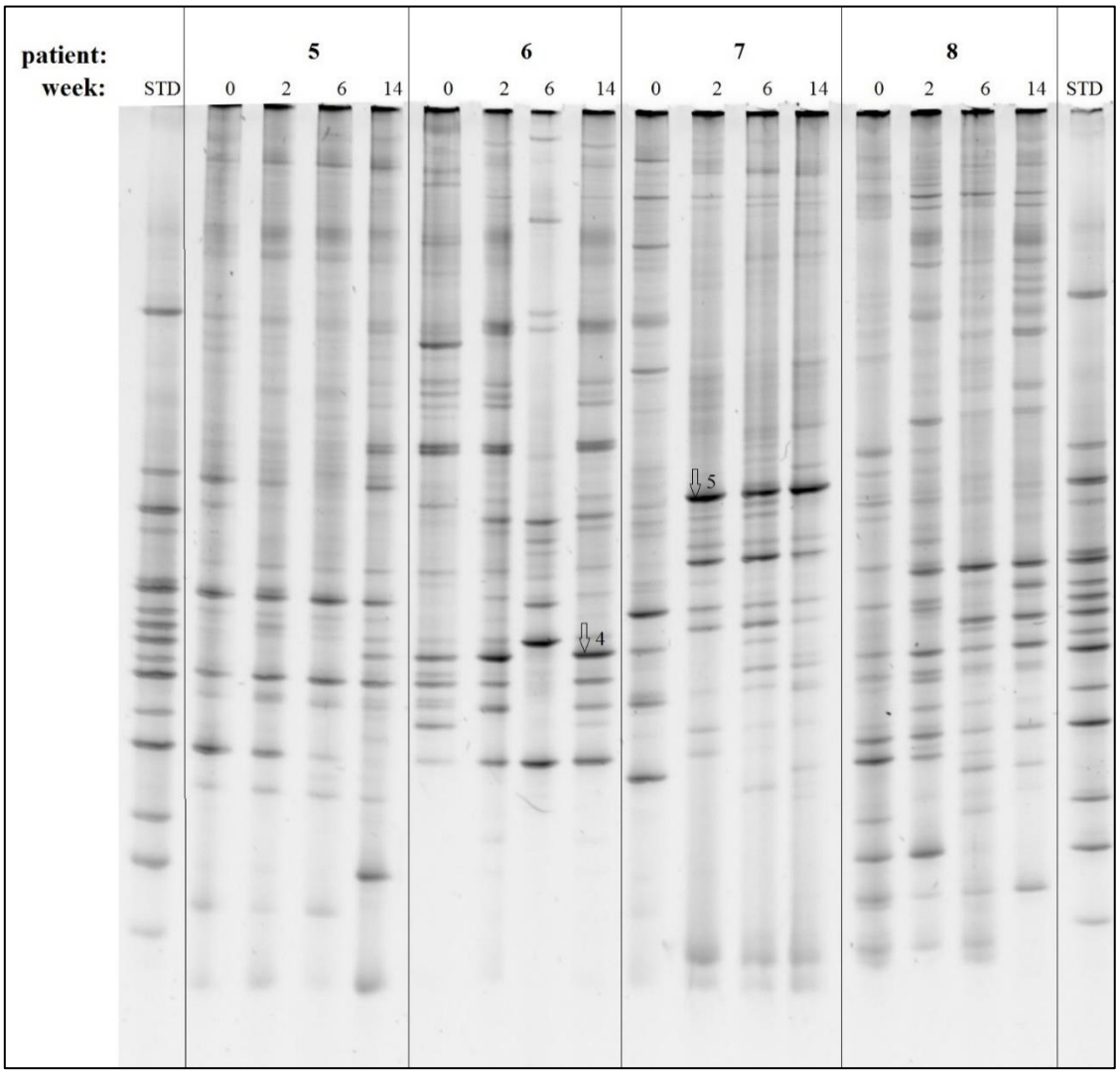

Figure 8: DGGE pattern of patients 5-8 on biological therapy. Samples were taken in certain weeks of the therapy. Arrows indicate bands which were excised and sequenced. (STD) standard

To identify bacteria groups in patients whose bacteria DGGE profiles differed, bands were excised from the gel and sent for commercial sequencing. Sequences were blasted to the ncbi library. The results are summarized in Table 7.

Table 7: Bands 1-5 selected from DGGE gel (Figure 7, Figure 8), identification by ncbi blastn

band number	similarity %	organism	phylogenetic affiliation	isolation source	accession number
1	98	*Blautia luti*	Firmicutes; Clostridia	human feces	NR_114315.1|
1	98	*Coprococcus catus*	Firmicutes; Clostridia	culture collection	NR_024750.1|
2	96	*Prevotella copri*	Bacteroidetes; Bacteroidia	human feces	NR_040877.1|
3	100	*Streptococcus salivarius*	Firmicutes; Bacilli	unknown	NR_102816.1|
4	100	*Blautia luti*	Firmicutes; Clostridia	human feces	NR_041960.1|
5	95	*Megamonas funiformis*	Firmicutes; Negativicutes	human feces	NR_041590.1|

From the sequencing results, one can see that in Patient 1, there is stable occurrence of band 1, which corresponds to *Blautia luti/ Coprococcus catus*. In Patient 3, *Prevotella copri* (band 2) occurred after initiation of the biological therapy and persisted. Similarly, in Patient 7, *Megamonas funiformis* (band 5) occurred after initiation of the biological therapy and persisted. In contrast, in Patient 3, *Streptococcus salivarius* (band 3) disappeared after the second week of biological therapy. Lastly, in Patient 6, *Blautia luti* (band 4) was not detected in the sixth week of the therapy, whereas in the preceding and following weeks it was present. These interesting results could reflect the dynamics of the microbiota change, which could be dependent on many diverse factors. One can only speculate as to whether dietary changes or disease status was the true cause.

By simple observation, one can see how the DGGE patterns of patients changed during the course of biological therapy; in some patients, we can see striking changes; in others, rather more subtle differences. However, in order to reach this conclusion, precise data have to be extracted, which was done by comparing diversity (H index).

4.3.2 H index

The H indexes of patients on biological therapy were compared at various points during their therapy. At a significance level of 0.05, where H index before biological therapy and mean H index post-therapy, were compared, one can conclude that the H indices are different: $t = 1.89$; $p = 0.07$. The H indices are shown in Figure 9.

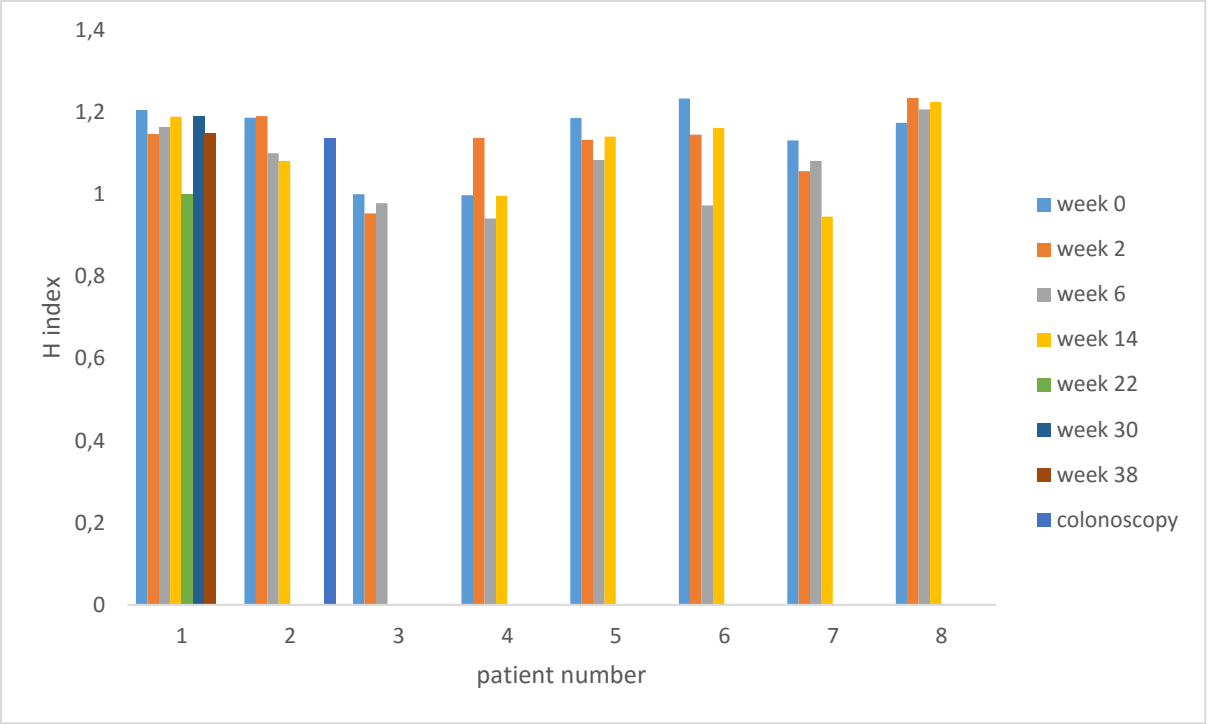

Figure 9: H indices of patients 1-8 at different points during biological therapy, fecal sample collection before colonoscopy preceding biological therapy (colonoscopy)

In conclusion, bacteria diversity of patients undergoing biological therapy with infliximab was significantly different.

4.3.3 NGS Sequencing

The following taxonomical data were acquired by high throughput NGS analyses. The data were processed by the Qiime software package. The core diversity was established by picking open reference OTUs. The minimal counts per sample were and it was chosen as analysis depth. Figure 10 shows a relative abundance of phylum-level bacteria groups in all eight patients. This figure gives less biased data than the following figures (Figure 11, Figure 12 and Figure 13). However, by simple visual analysis, there is no observable trend in taxonomic profiles common to all patients. In Patient 3, one can see an increase in Bacteroidetes and in Patient 8, an increase in Firmicutes during the course of therapy. Bacteroides increase correlated with the disease activity (Supplementary Figure 1).

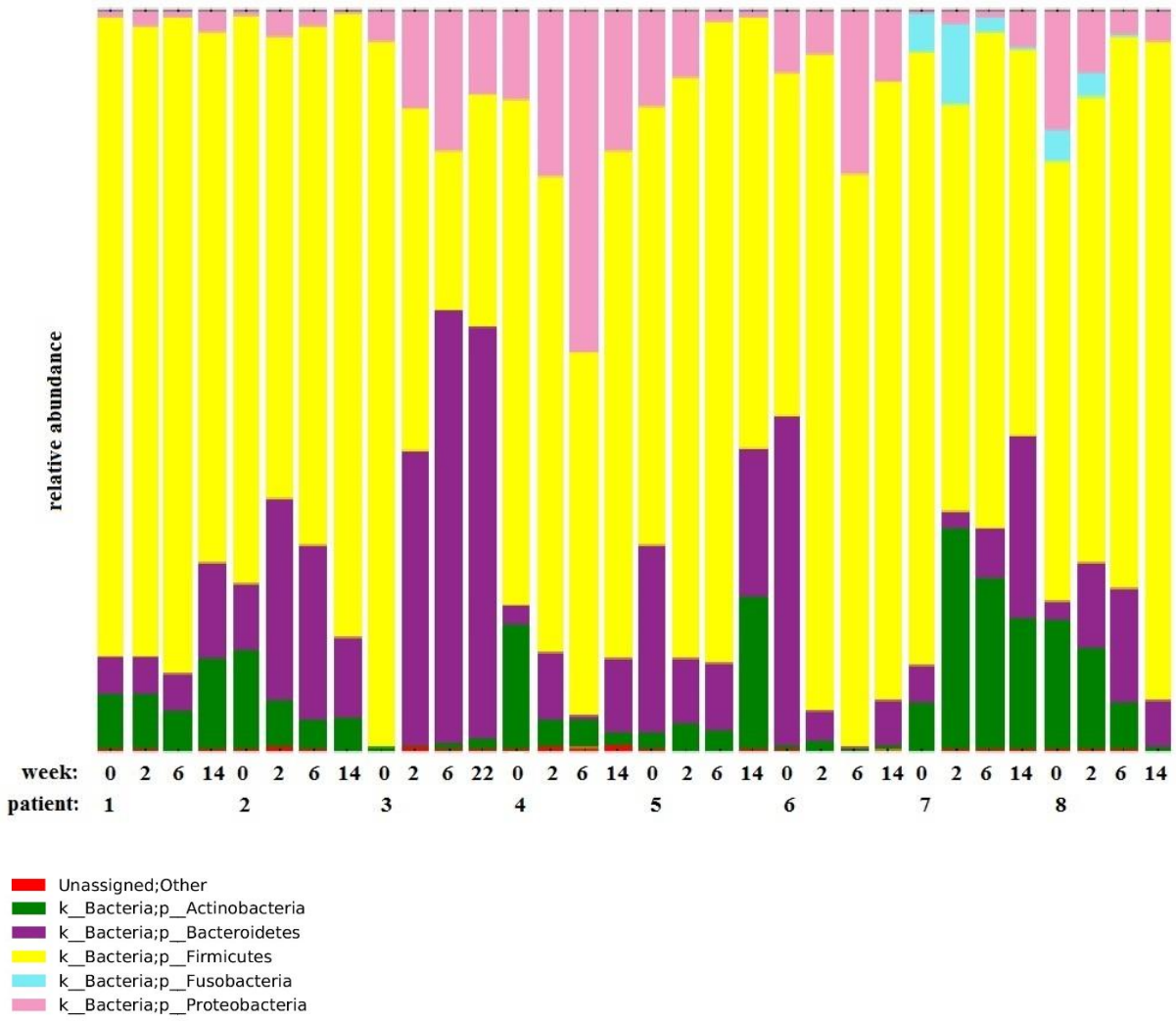

Figure 10: Relative abundance of phylum-level taxonomy summary of gut microbiome from all samples examined (8 patients, 4 weeks each).

Taking into account the bacteria groups commonly associated with IBD dysbiosis, I looked at the lower taxonomical level (Supplementary Figure 2). There were no bacteria of genus *Escherichia* detected. *Bifidobacterium* was detected as increasing in Patients 3 and 5 but decreasing in Patients 4 and 8. *Lactobacillus* was decreased and so was *Streptococcus* in Patient 3 and 4. *Faecalibacterium* decreased in Patient 2, but it increased in Patient 8.

Figure 11 shows the average values for each patient. We can see that all patients have different taxonomical profiles. This result highlights the fact that each person is unique with the respect to his microbiome composition.

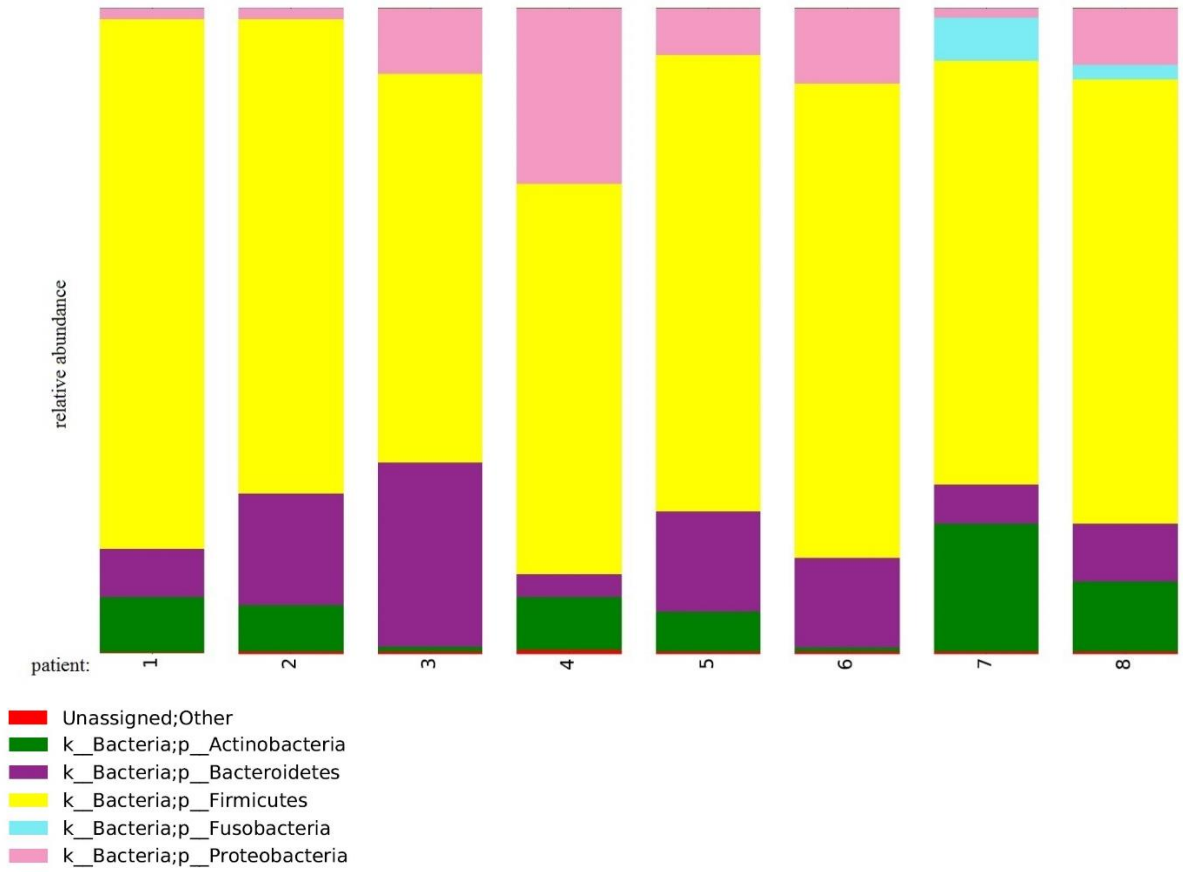

Figure 11: Relative abundance of phylum level taxonomy summary of patients gut microbiome from all weeks together

In Figure 12, one can see a taxonomic summary by weeks. From this figure, one can conclude that there is a difference in taxonomic profiles across the weeks, especially in Week 22, where there is a large increase in Bacteroidetes, particularly *Prevotella*. However, this phenomenon is observed by measuring the microbiota in Week 22 of only one patient, whereas for the other weeks there is a mean from all of the other participating patients.

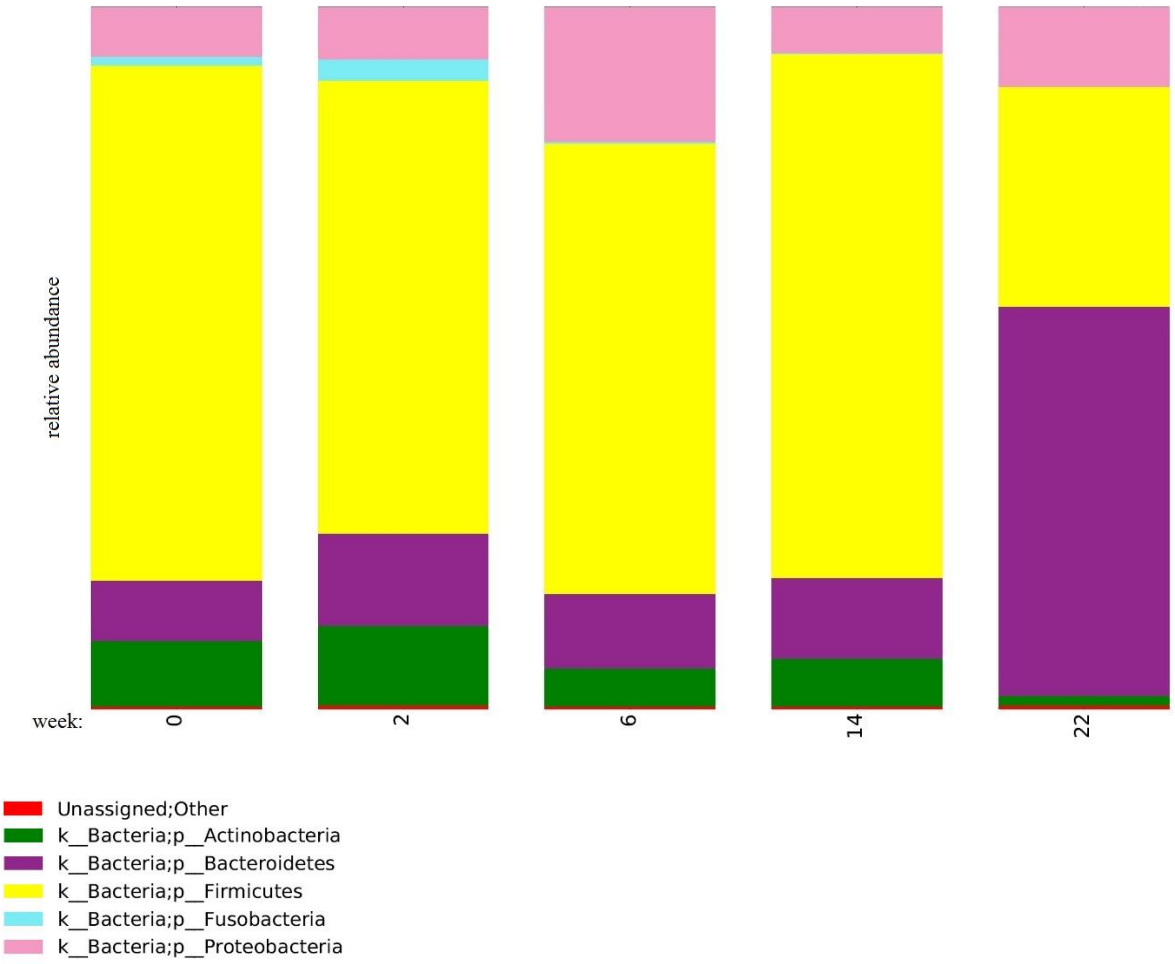

Figure 12: Relative abundance of phylum level taxonomy summary of gut microbiome, sorted by week for all patients

In Figure 13, one can see the taxonomic profiles sorted by category, at the starting point of biological therapy and during the treatment. On this taxonomic level, it is apparent that there are no major differences before and during the therapy. However, this is most likely caused by large differences between individuals which are much greater than the differences within an individual.

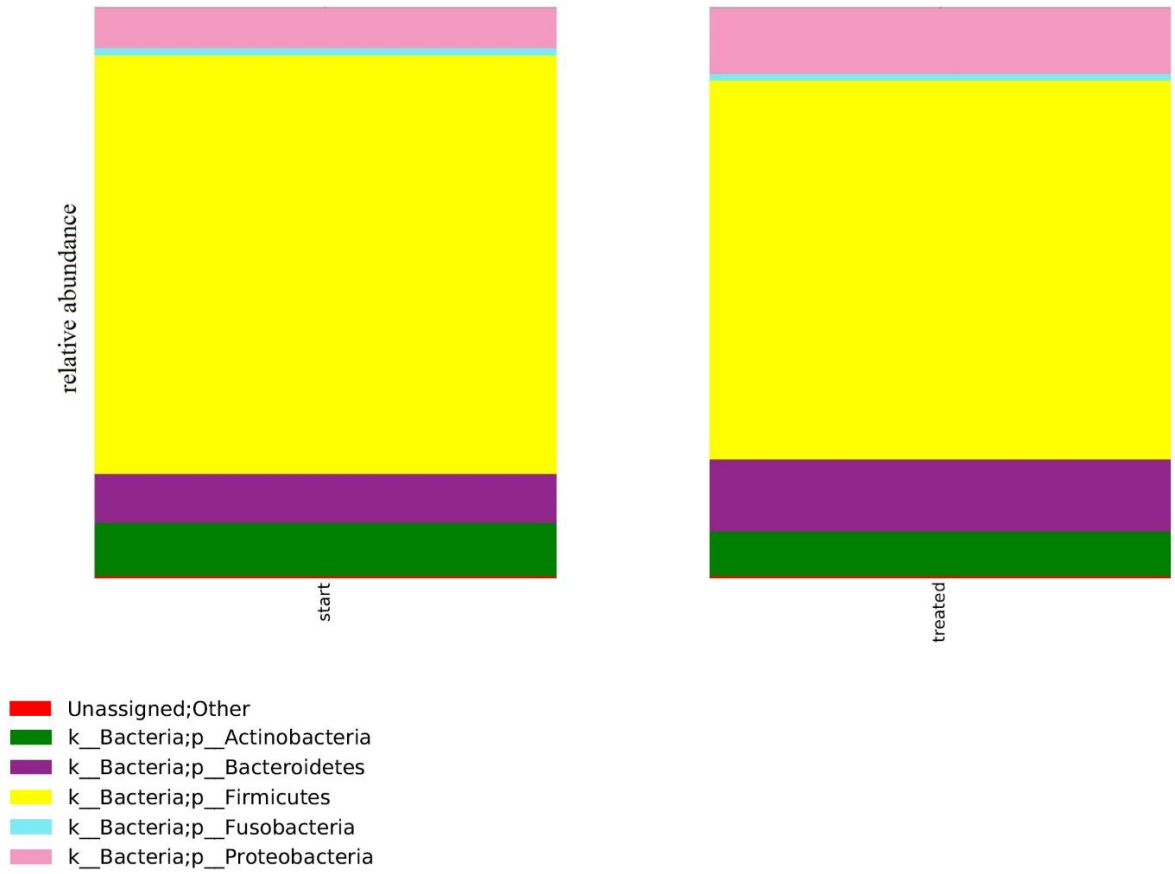

Figure 13: Relative abundance of phylum level taxonomy summary of gut microbiome sorted by category in all samples

Principal coordinate analysis (see Figure 14) revealed clustering according to patient, which further emphasized the differences across individuals. There were no observable clusters when sorted by week or category (start, treated).

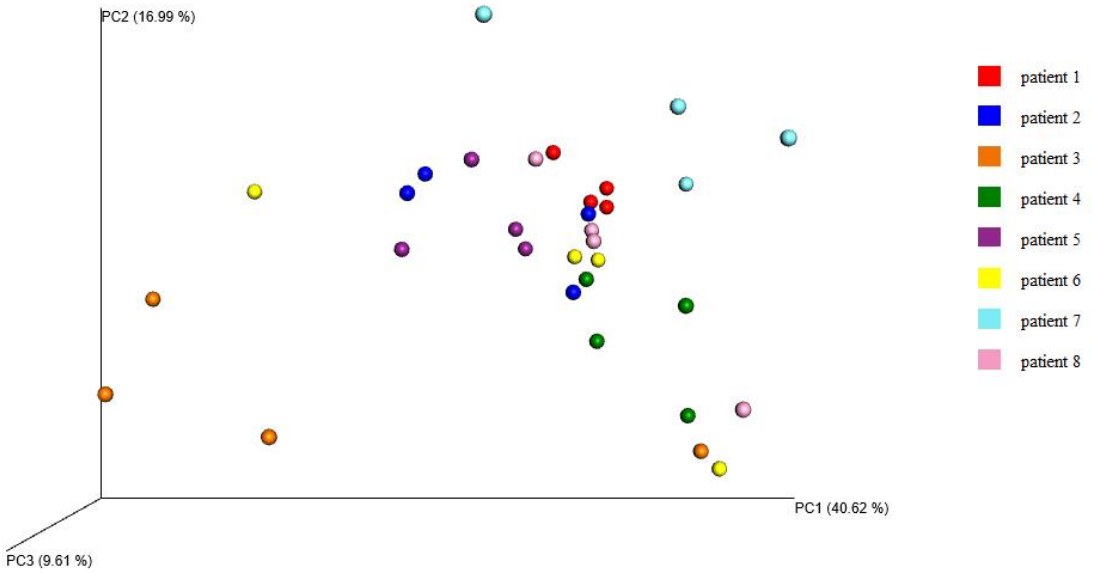

Figure 14: Principal coordinate analysis generated by the QIIME software package from eight patients at four different time points

5 Discussion

The aim of this thesis was to investigate the changes in microbiota composition of IBD patients undergoing adjuvant or biological therapy. Since the crucial step in estimating microbiota diversity is the isolation of DNA, three available DNA extraction techniques were compared.

5.1 DNA extraction technique comparison

The three DNA extraction methods which were compared were the QIA kit, RBB+C and MM. RBB+C was decided the most suitable method and was chosen for analyzing patient samples. RBB+C showed the best values for both DNA purity and yield. 100 mg of stool yielded 220.7 ng/µl of DNA. It was four times more than the QIA kit yielded and almost 20 times more than did MM. These results are according with a study which compared as many as 19 DNA extraction protocols and marked the bead beating method as the one producing the highest amount of DNA (Anderson and Lebepe-Mazur 2003). Also in favor of bead beating was a study comparing the same DNA extraction methods with and without using bead beating (Ariefdjohan, Savaiano, and Nakatsu 2010). The lowest yield was obtained by MM, which corresponds to a study comparing MM, the QIA kit and phenol chloroform extraction (Trachtová et al. 2012). The A260/A280 ratio in RBB+C was the closest to 1.8, which is considered pure DNA. It is fair to note that: (a) not all samples have equal microbial load; (b) they are extremely diverse, comprising both gram-positive and gram-negative bacteria, which require different extraction conditions due to their different cell wall architecture; (c) they contain a high amount of undigested matter; and (d) also contain compounds which could interfere with PCR.

By analyzing DGGE profiles, one cannot draw such a straight-forward conclusion. The methods compared resulted in completely different DGGE profiles using the same starting material. This result indicates that the observed composition of isolated bacteria is highly dependent on the extraction technique used. Several studies compared DGGE profiles of different commercial DNA extraction kits (Ariefdjohan, Savaiano, and Nakatsu 2010) or other commonly used extraction techniques (Carrigg et al. 2007), both coming to the conclusion that different extraction techniques result in different DGGE profiles. One cannot evaluate good or bad extraction techniques based on DGGE profiles; the only thing which can be taken from this outcome is that one should pay attention to the extraction technique used when comparing results across studies, since the discrepancies might arise from a different extraction technique being used.

Assuming that H index represents diversity of bacteria, it was concluded that the investigated techniques gave significantly different diversities; however, not all pairwise comparisons were significant. The only techniques which differed significantly were Q and MM. Neither the combination of RBB+C and Q nor that of RBB+C and MM was significant. One could say that MM gives the most diverse outcome; yet this method yielded a very low amount of DNA which was not even that pure, with its A260/A280 far from 1.8. If we compare MM to RBB+C, RBB+C had a comparable H index but had an extremely high and pure DNA yield.

Taking into account DNA yield, DNA purity and the H indices derived from the DGGE profiles, RBB+C was chosen as the most suitable technique for DNA isolation from patient samples. Since my aim is to isolate the broadest spectrum of bacteria and not a particular species, the results obtained were sufficient to decide this. However, it would be interesting to specify which groups of bacteria could potentially be omitted by using the chosen DNA extraction technique. One such article describes increased release of Firmicutes DNA by using RBB+C and high molecular weight methods (Cuiv et al. 2011).

This outcome is not only an important starting step in choosing the appropriate DNA extraction technique, but is also quite important for interpreting the results. One should consider that using a certain extraction technique, one might not gain DNA from all the bacteria present in the sample.

5.2 Adjuvant therapy induced no changes in gut microbiome of IBD remission patients

The effects of adjuvant therapy with VSL#3 probiotic on patients´ microbiomes were evaluated using the PCR-DGGE approach. Judging from visual analysis, DGGE profiles look almost the same as each other with only a few exceptional bands; H index comparison confirmed this visual impression. VSL#3 had no significant effect on changing the overall diversity between the start and end of adjuvant therapy. However, I have not looked at the particular strains which were part of the VSL#3 probiotic mixture. Only several bacteria were identified appering (*Clostridia, Bacteroides ovatus, Veillonella ratti, Veillonella parvula*) or disappearing (*Eubacterium eligens, Faecalibacterium prusnitzii, Bacteroides vulgatus/dorei, Bacteroides uniformis/intestinalis*) after the therapy. Generally speaking, several species of phyla Bacteroidetes and Firmicutes were detected to be both present and absent at the end of the adjuvant therapy. This result was expected, since the disease‘s activity is correlated with disruption of the microbiome (Gevers 2014). The patients I analyzed were all in remission, free of all signs of disease and, during the time of adjuvant therapy, remained in remission; therefore no dramatic change in the microbiome was expected. The same result was observed in UC

patients with pouchitis, who remained in remission after therapy with VSL#3 probiotic mixture. The remission maintenance was associated with increased bacterial diversity, particularly anaerobes, when compared with the placebo group (Kühbacher et al. 2006). They also suggested that the increase in bacterial diversity was independent of intestinal colonization and that the probiotic treatment could induce anti-inflammatory pathways, create a favorable growth environment, or even be secondary to the changes in microbial composition.

5.3 Biological therapy-induced changes of human gut microbiome

In assessing biological therapy effects on the human microbiome, two approaches were used: PCR-DGGE and NGS. Results from both methods indicate, that there are changes in the gut microbiome during biological therapy.

Bacterial DGGE profiles looked different and were proven to be significantly different by H index comparison. Several bacteria from DGGE identified by commercial sequencing appeared or disappeared during the course of biological therapy. In two patients, bacteria (*Prevotella copri* and *Megamonas funiformis)* occurred after the beginnig of therapy initiation and persisted throughout. Although *Prevotella copri* was not found to be associated with any particular disease, a study on pediatric patients showed high levels of *Prevotella copri* in healthy control subjects (Kaakoush et al. 2012). On the other hand, some bacteria (*Streptococcus salivarius*) disappeared after the second week of therapy. Despite the fact that *Streptococcus salivarius* natural habitat is in the oral cavity, it was also isolated from feces and documented in IBD dysbiosis (Teitelbaum and Triantafyllopoulou 2006). Nevertheless, in Teitelbaum‘s study, *Streptococcus salivarius* could be confused with *Streptococcus bovis* which was also documented to correlate with IBD dysbiosis (Ruoff et al. 1984). However, those are just anecdotal cases and no relevant conclusions can be drawn; those results were proven to be consistent with NGS analysis performed later.

Comparing the taxonomical summaries from NGS, there was no obvious trend visible among all patients. In Patient 3, there was an increase in Bacteroidetes after initiation of the therapy, which is in accordance with Frank et al. (2007); however, opinion about Bacteroidetes counts differ greatly (Giaffer, Holdsworth, and Duerden 1991). Moreover, this increase in Bacteroidetes was fairly consistent with the disease activity, which went from severe to moderate. Although the total amount of Firmicutes increased, particular genera, that is, *Lactobacillus* and *Streptococcus*, decreased during the course of biological therapy. Surprisingly, despite it‘s beneficial probiotic effect, *Lactobacillus*, as well as *Bifidobacterium*, was found to be higher in IBD patients (Wang et al. 2014; Walters, Xu, and Knight 2014). Even

more discrepant data were collected for Actinobacteria. In Patient 3, an increase in Actinobacteria as well as *Bifidobacterium* was observed, which corresponds to Willing et al. (2010). Contrary to those of Patients 2,4 and 8, counts of Actinobacteria were decreasing and the same applied to the genus *Bifidobacterium* of those patients. Despite the fact that Enterobacteriaceae are commonly associated with IBD dysbiosis (Willing et al. 2010; Gevers 2014), no *Escherichia* were detected.

To date there has only been one study addressing microbial changes after biological therapy (Rajca et al. 2014). They found that dysbiosis is characterized by low counts of Firmicutes and low counts of Bacteroidetes predicted a relapse which could correspond to our data. In addition, they have associated reduction in *Faecalibacterium prausnitzii* with relapse occurrence, confirming its anti-inflammatory property (Sokol et al. 2008). However, using NGS an increase in *Faecalibacterium* was only indicated in one of our samples and was even decreased in another. Based on the fact that certain bacteria are correlated with relpase or remission and their interaction with bile acid metabolism, antimicrobial peptide secretion and mucus glycosylation, they suggest that dysbiosis leads to gut inflammation.

Regarding the suggestion of Rajca et al. (2014) that dysbiosis leads to inflammation, I can neither prove nor validate this by the experiments presented here. In my opinion, it should rather be taken vaguely, since there are more complex studies which suggest otherwise. For example, it has been shown that dysbiosis is independent of inflammation (Haberman et al. 2014). Having identified the host gene expression profiles, Habermann suggests that the DUOX2 gene expression signature is associated with the expansion of Proteobacteria and the APOA1 gene expression signature with a reduction in Firmicutes. The shifts in those two taxonomical units were also reported by another metagenomics study (Morgan et al. 2012).

Additionally, Monast et al. (2016) using Illumina sequencing, correlate remission induced by biological therapy with Golimumab (400 mg) with increased microbial diversity. Neuman in his PhD thesis also associates severe disease activity, which improved during biological therapy (infliximab) with reduced microbial diversity.

Lastly, in order to be wholly confident with our results, it would be good to add at least one more sample before the onset of the therapy, to ensure the microbiome is stable and the changes measured could be attributed to the effects of the therapy and not to other disturbances. However, this is almost impossible with the current routine in clinics.

5.4 Methodology and study design

Finally, I have a few remarks on the methods and relevance of the study presented here.

Firstly, the patients are not easily compared to each other, since the diversity across individuals is much greater than within an individual and what is more, the Firmicutes/Bacteroidetes ratio alters with age (Mariat et al. 2009). Nor have I cansidered patient behavior such as diet or lifestyle, which could also hinder the microbiome composition. Nevertheless, this pitfall can be eliminated by studies in gnotobiotic animals, as emphasized by Kverka & Tlaskalová-Hogenová (in press).

Secondly, the DNA isolation source matters. Bacteria isolated from feces were, in this study, assumed to represent the bacteria composition residing in the intestine. However, it is known that bacteria composition found in feces or associated with the mucosa are different from the true gut ecosystem (Zoetendal et al. 2002).

Thirdly, as already mentioned, the outcomes are highly dependent on the extraction technique used and data cannot be absolutely quantified.

Fourthly, choosing the 16S rRNA gene might not be wise for diversity estimation since it was reported that different species of bacteria can have varying number of copies of this gene (Acinas et al. 2004) and could lead to overestimation of the diversity. Moreover, the bacterial primers do not have 100% coverage which means some taxa might be left out.

Fifthly, the credibility of estimating diversity by using H index values extracted from DGGE gel is highly questionable. I have experimental evidence from previous studies (not presented), that difference in H index between two samples does not corresponded with the DGGE profiles at all which makes it a poor diversity marker and the usage should be reevaluated.

Sixthly, bacterial species definition comprises both phenotypic and molecular traits. When using purely molecular data, one has to refer to operational taxonomical units (OTUs). However, there has not been a clear cut-off line drawn, so it happens that some scientists consider 97% similarity and some 98 or 99% similarity levels as one OTU (Eckburg et al. 2005).

Lastly, the taxa bias; I would like to point out one particularly interesting fact about intestinal microbiota which has to do with the taxonomic group‘s abundance. Arugumam (2011) proved that even a low-abundance group could show abundant functions. As an example, he picked low-abundance *E. coli*, which produce more than 90% of proteins involved in bacterial pili assembly. Bacterial pili are features which enable bacteria to easily colonize mucosal surfaces, play a major role in the conjugation process and therefore contribute to the prolonged survival in the human GI. Those highly beneficial traits somehow compensate for their low abundance in the overall microbial community. Hence, it is wise to take this fact into account. It is not only taxonomical groups showing high abundance in the analyzed profile which should be considered of high importance. Even though one might see some changes and interesting

patterns, it is extremely difficult to pick out which taxonomical level one should observe because it can introduce a lot of bias. The taxonomical system is an artificial system created by man, even though it is based on phylogenetical affiliation. This phenomenon is thoroughly explained in the book Jak se dělá evoluce (Zrzavý, Mihulka, and Štorch 2004).

6 Conclusion

Three available DNA extraction techniques were compared, leading to the conclusion, that the observed composition of the bacteria isolated is highly dependent on the extraction technique used. Regarding DNA yield, DNA purity and H index, RBB+C was chosen among others as the most suitable technique for analyzing patient samples.

Adjuvant therapy of IBD patients with VSL#3 probiotic mixture turned out to have no significant effect on the overall microbiome composition. Several bacteria of the phylum, Bacteroidetes and Firmicutes, were identified appearing or disappearing after the therapy; nevertheless, they did not have any significant effect on the overall microbiome composition.

Biological therapy of IBD patients with infliximab turned out to cause significant changes in the microbiome composition. Using PCR-DGGE approach, *Prevotella copri* and *Megamonas funiformis* were observed appearing after the onset of the therapy and persisted, whereas *Streptococcus salivarius* disappeared during the course of the therapy. These results were further supported by NGS data. Bacteroidetes as well as Firmicutes were found to increase during the course of therapy which, in case of Bacteroidetes, corresponded to the disease activity of the patient (from severe to moderate). However, this is just anecdotal evidence and more research needs to be done in order to draw some general conclusions.

There are many variables which could give potential bias to studies addressing microbial composition, ranging from patient selection, DNA source, DNA extraction technique, the target gene for PCR amplification, H index difference credibility, OTU definition and taxonomical view. This calls for standardization of the procedures in future studies. Taking into consideration the aforementioned reasons, one must be extremely cautious when comparing results across studies.

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8 Supplement

sample number	patient	week	age	disease	localization	disease activity	clinical response
1	1	0	50	CD	L1	2	
2	1	2	50	CD	L1	2	
3	1	6	50	CD	L1	3	1
4	1	14	50	CD	L1	2	2
5	2	0	56	CD	L2	2	
6	2	2	56	CD	L2	1	
7	2	6	56	CD	L2	0	0
8	2	14	56	CD	L2	0	0
9	3	0	55	UC	E3	8	
10	3	2	55	UC	E3	6	
11	3	6	55	UC	E3	5	1
12	3	22	55	UC	E3	4	1
13	4	0	47	UC	E2	?	
14	4	2	47	UC	E2	?	
15	4	6	47	UC	E2	?	2
16	4	14	47	UC	E2	?	2
17	5	0	21	CD	L3	3	
18	5	2	21	CD	L3	1	
19	5	6	21	CD	L3	1	2
20	5	14	21	CD	L3	1	2
21	6	0	29	CD	L3	6	
22	6	2	29	CD	L3	1	
23	6	6	29	CD	L3	3	2
24	6	14	29	CD	L3	2	2
25	7	0	22	UC	E3	8	
26	7	2	22	UC	E3	9	
27	7	6	22	UC	E3	7	2
28	7	14	22	UC	E3	2	2
29	8	0	51	CD	L1	3	
30	8	2	51	CD	L1	3	
31	8	6	51	CD	L1	1	1
32	8	14	51	CD	L1	2	2

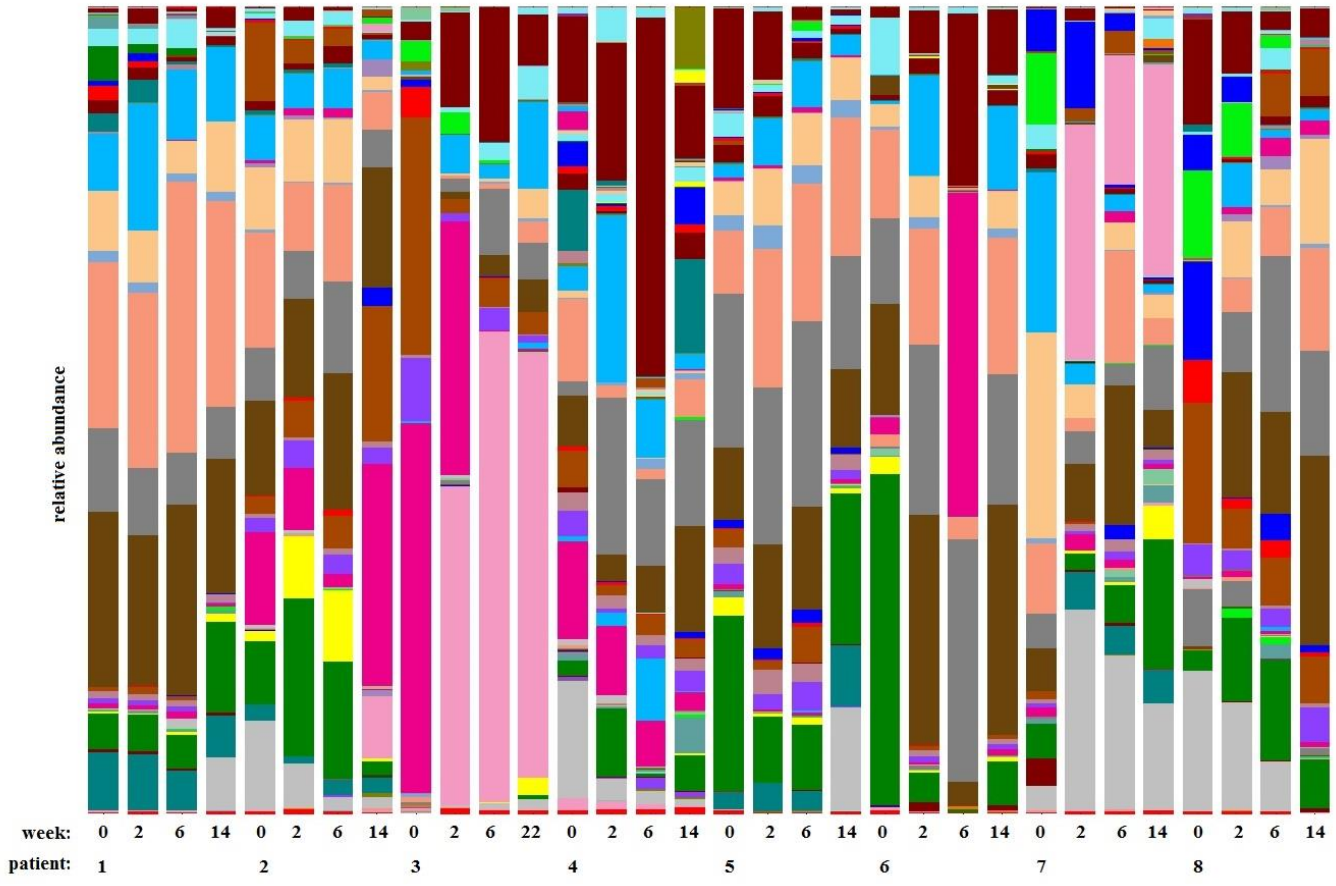
Legend:

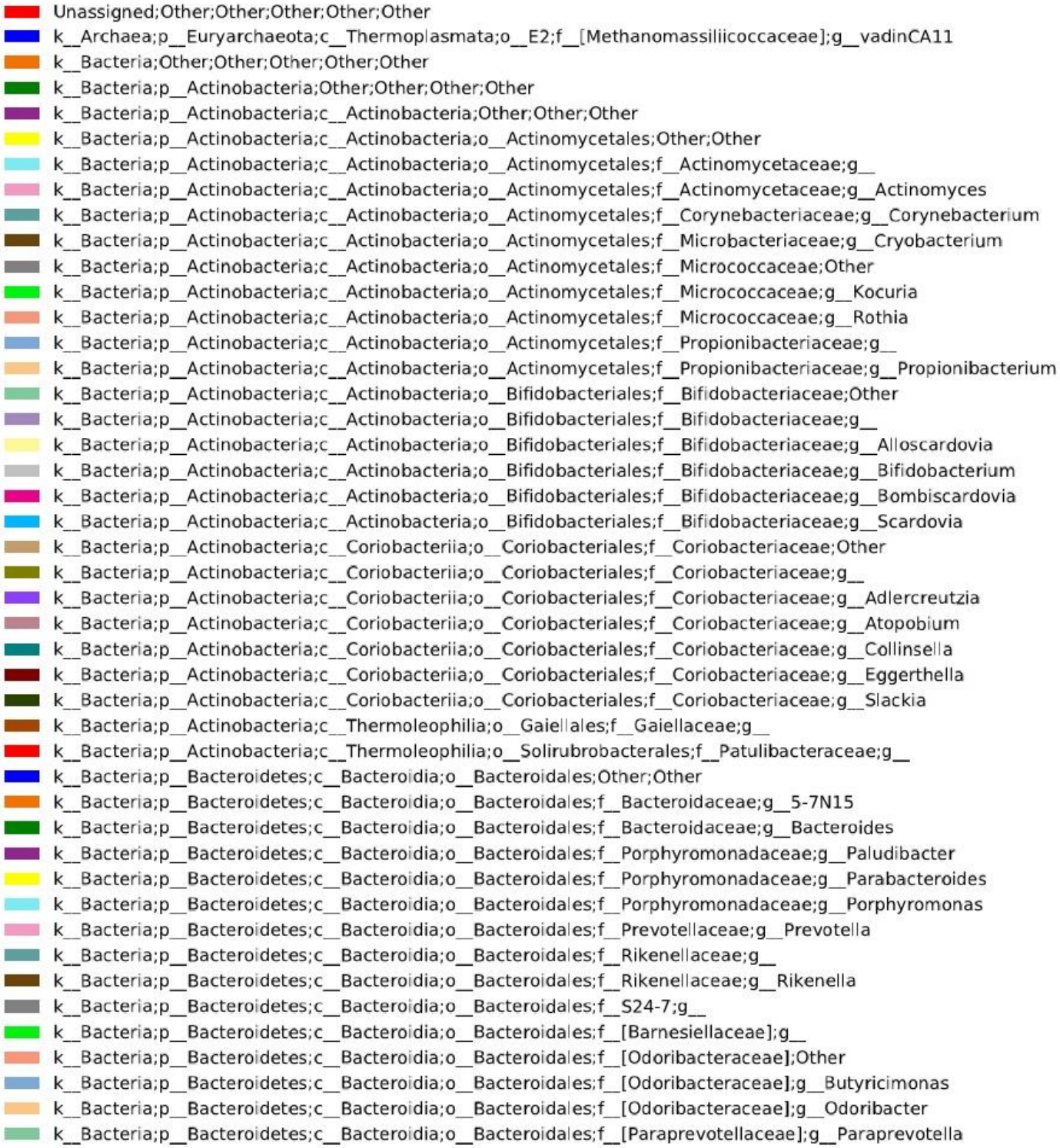
CD		UC	
L1	terminal ileum	E1	ulcerative proctitis
L2	colon	E2	left sided (distal) ulcerative colitis
L3	ileocolon	E3	extensive (pancolitis) ulcerative colitis

CD		UC	
0-4	no disease activity	0-2	no disease activity
5-7	mild	3-4	mild/moderate
8-15	moderate	5+	severe
16+	severe		

Semi-quantitative evaluation of patient´s response by clinician based on clinical and laboratory data development	
0	no response
1	partial response
2	complete response

Supplementary Figure 1: Clinical information about Patient 1-8 throughout the course of biological therapy with infliximab

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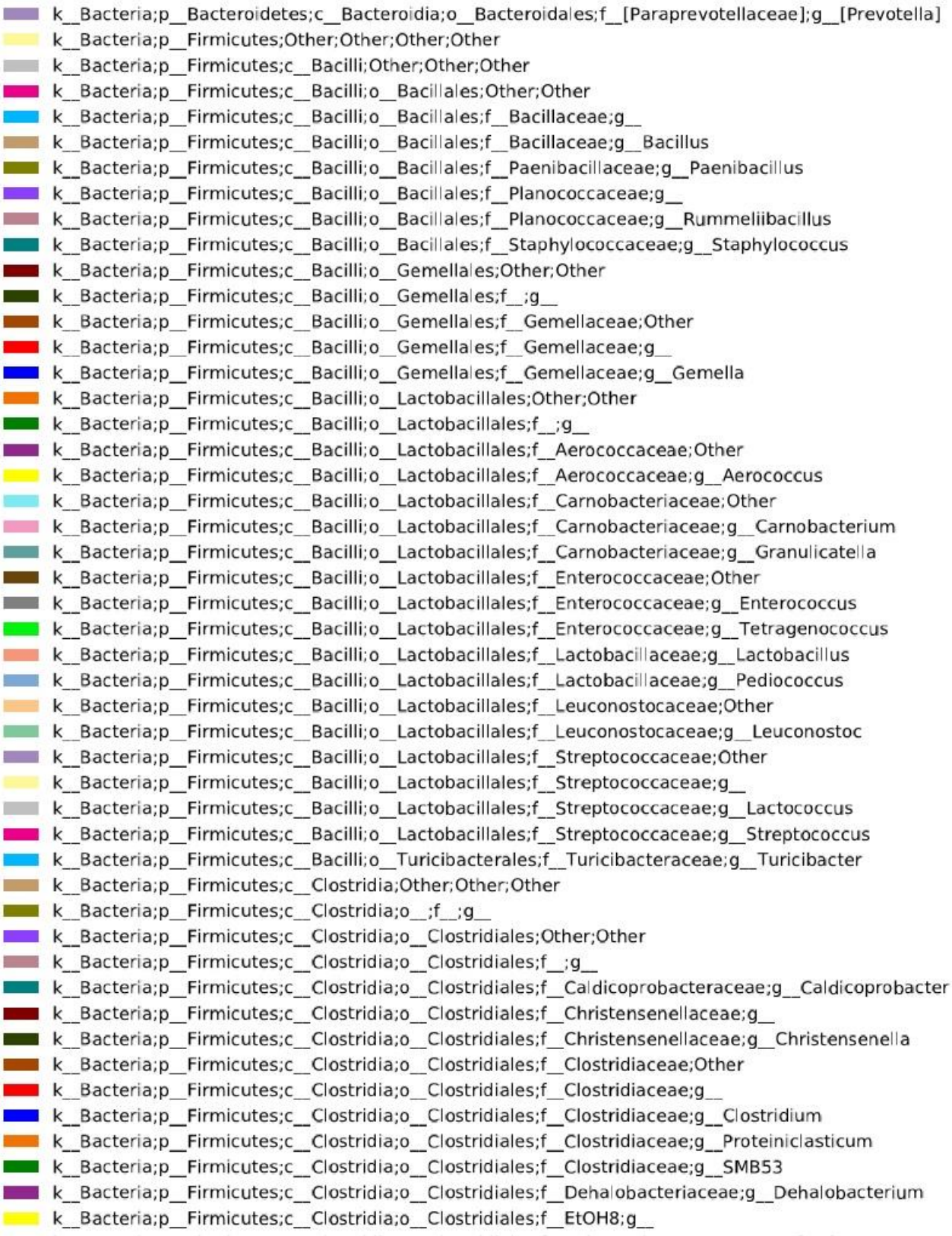
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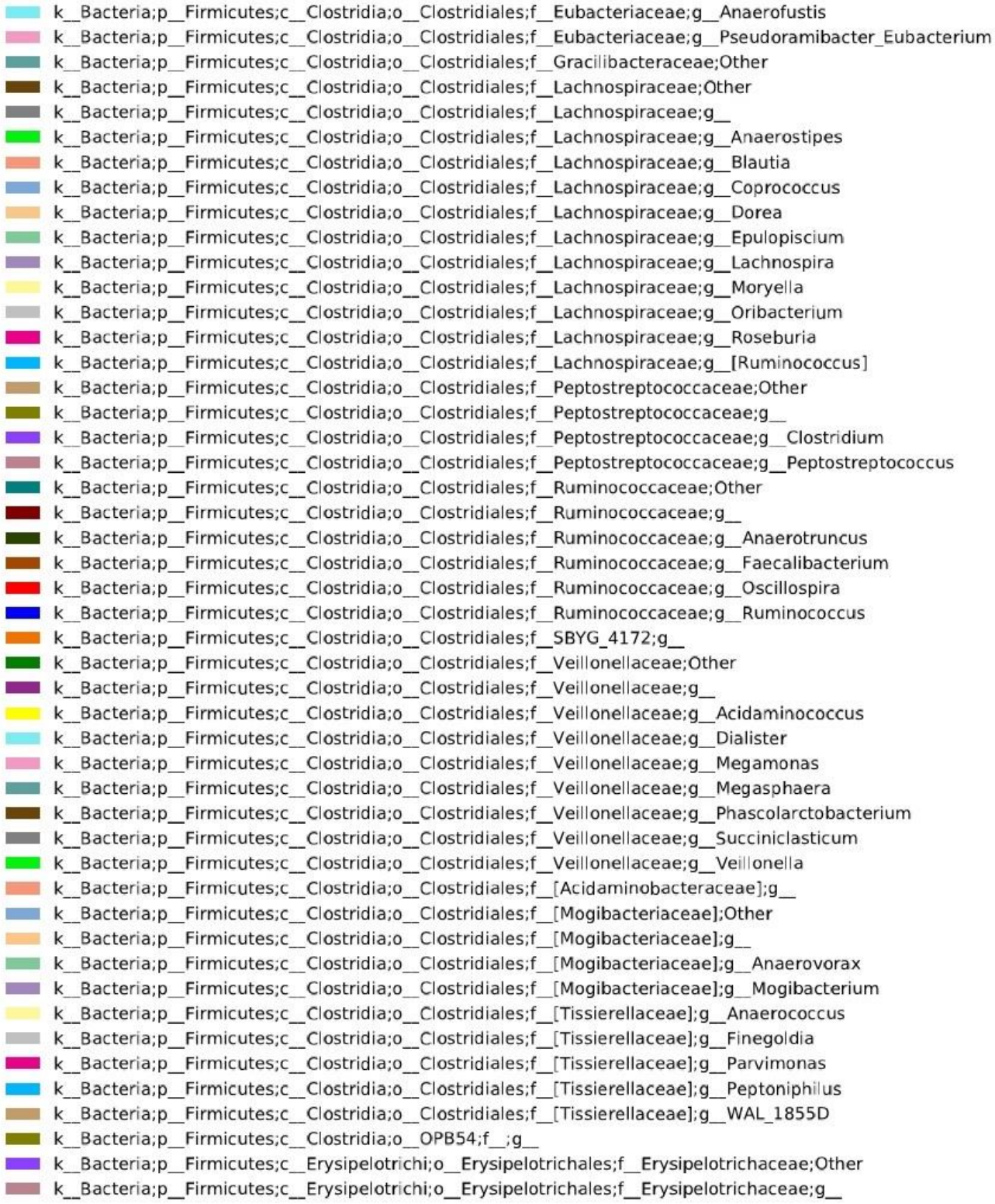
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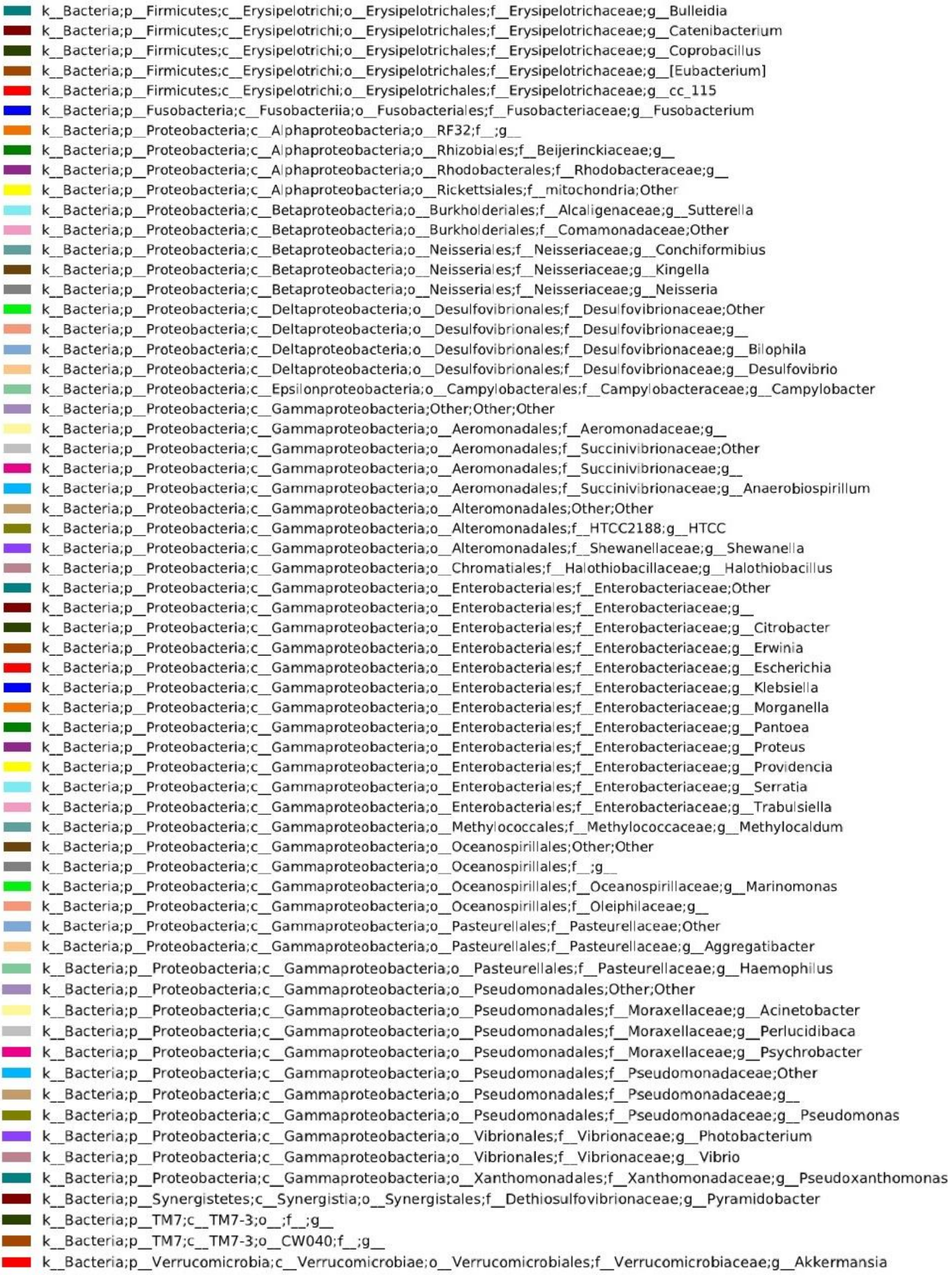
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Supplementary Figure 2: Relative abundance of genus-level taxonomy summary of gut microbiome from all samples examined (8 patients, 4 weeks each).