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Effects of Protein Intervention on Microbial Diversity

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List of Abbreviations

RCT	randomized controlled trial
DNA	deoxyribonucleic acid
g/kg BW/d	gram per kilogram bodyweight per day
CI	confidence interval
mTORC1	target of rapamycin in complex 1
EAA	essential amino acids
BCAA	branched chained amino acids
4E-BP1	eukaryotic initiation factor 4E binding protein
p70S6K	ribosomal protein S6 kinase
RTK	receptor tyrosine kinase
INSR	insulin receptor
PKC	protein kinase C
ERK	extracellular signal regulated kinase
AKT	protein kinase b
CKD	chronic kidney disease
IGF-1	insulin like growth factor 1
GSK-3β	glycogen like growth factor 1 β
TSC	tuberous sclerosis protein
RAG	recombination activating gene
elF2 β	eukaryotic initiation factor 2 β
TEE	total energy expenditure
BMR	basal metabolic rate
SCFA	short chain fatty acids
Meta-HIT	Metagenomics of the Human Intestinal Tract
HMP	Human Microbiome Project
rRNA	ribosomal ribonucleic acid
NGS	next generation sequencing
PCR	polymerase chain reaction
OTU	operational taxonomic unit
ASV	amplicon sequence variant
MMS	mini mental status
BMI	body mass index
PCoA	principal coordinates analysis
RDA	redundancy analysis

Introduction and Focus of Research

Austria's population experiences significant changes concerning its age-structure. Several independent and co-operating factors lead to a rise in life expectancy and consequently to an increase in the number of senior citizens, aged 65 years and older. At the moment, at 2020, 19% of Austria's population is 65 years or older. While the middle age group, 20 to 64 years, remained relatively stable in the past 10 years, a decline in the youngest age group (up to 20 years), and a simultaneous growth in the oldest group could be observed. Following that trend, predictions state that the oldest group will account for 25% of the total population by the year 2030 and 30% by 2060. (Statistik Austria, 2020) This is a challenge for numerous parts of the public sector, including public health systems or employment market.

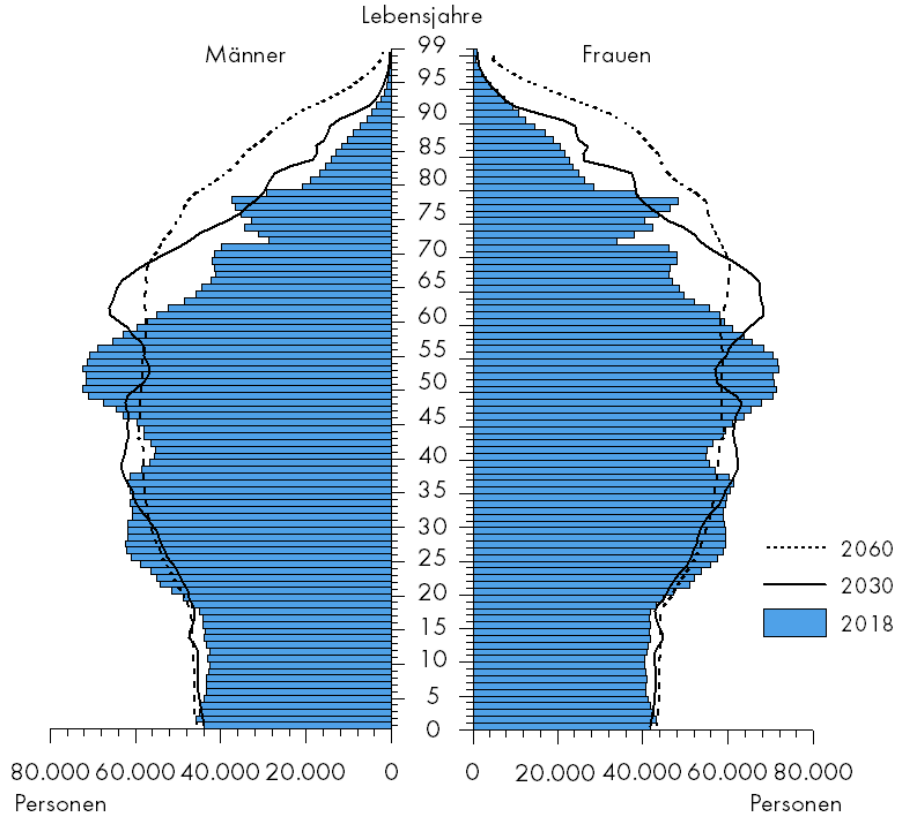

Figure 1: Age-distribution in Austria 2018 and prediction for 2030 and 2060 (Statistik Austria, 2019)

Age alone is not considering the element of well-being, which is an essential factor best described by the years spent in health. Considering the rise in elderly adults, it is of great importance to investigate their special needs in order to counteract preventable, age-related diseases and rise not only of life- but health expectancy. To meet that goal, lifestyle, nutrition and exercise are fundamental aspects. This thesis was composed in the course of the NutriAging study, an EU-project taking place at the Department of Nutritional Sciences at the University of Vienna, which addresses the numerous changes and requirements that come with ageing. Intention of the study is to find effects of protein and strength training on several parameters of well-being and health in elderly adults, while this thesis focuses especially on its outcome on the human microbiome.

Microbiota inhabiting the intestine have shifted into our interest, not only because of progression in technical potential of newly developed sequencing techniques, but due to increasing evidence of its influence on our metabolism. The effects of nutrition, lifestyle, health- and disease status on microbiota and vice versa have been massively underrated and only recently got into the focus of research. The huge number of prokaryotic creatures inhabiting the surface of our body, to which the gastrointestinal tract belongs, rises the question about who is driven by whom, considering there are more bacterial cells in our intestine than there are somatic cells in our body. (Turnbaugh et al. 2007)

To investigate the effect of different amounts of protein in the diet of elderly adults on their gut bacteria, the study population was allocated to one of three groups (control, low and high protein), receiving different amounts of protein and muscle strength training. At three timepoints (T1, T2 and T3) faecal samples were collected, to investigate bacterial DNA and deduce the composition of the microbiome in the large intestine. Ecological metrics representing that composition were calculated and compared between and within the intervention groups and timepoints. For that purpose, the research emphasis of this thesis focuses on the following:

- To give an overview of the current research state of protein recommendations and muscle strength training for elderly adults and its effects on healthy ageing, the human microbiome and the interaction between the factors, a literature research from September to December 2019 was conducted. Common databases such as "PubMed-NCBI" ((https://www.ncbi.nlm.nih.gov/pubmed/), "Science Direct" (http://www.sciencedirect.com/) and "Google Scholar" (https://scholar.google.at/)

were used. Chosen studies included reviews, RCTs (randomised controlled trials) and cohort studies and were selected by quality of research and year of publication. Studies published after 2005 were preferred.

- Assessment of the sufficiency of the chosen method of DNA sequencing for the detection of changes in the microbiota.
- The diversity of the participant's microbiome was described using certain ecological indices of microbial species richness (observed species richness: Simpson Index, Shannon Index; estimated species richness: Chao1 Estimator and ACE Estimator).
- And finally, to answer the question to what extent these indices are driven by the intervention of protein intake and muscle strength training.

Current literature suggested an impact of nutrition on the microbiome, which has been extensively investigated for carbohydrates and nutrient independent diet compositions. However, there are no investigations of the effect of protein in elderly adults.

Literature Research

Protein Intake in the Elderly Population

Recommendations

The ideal protein intake for elderly adults has been discussed extensively in the past years. There is evidence from epidemiological studies and RCTs that a higher amount of protein, compared to the recommendations for younger adults, could be beneficial for the elderly population. (Baum et al. 2016; Franzke et al. 2018)

The Austrian national recommendation for adults younger than 65 years is 0.8 g protein per kg bodyweight per day (g/kg BW/d), while the estimation for elderly adults was recently changed to 1 g/kg BW/d (table 1). Unlike the recommendation for younger adults, the value for the elderly had to be estimated since no recommendation could be made with sufficient accuracy with currently available data. Previous protein recommendations were based on studies that estimated the minimum intake to maintain physiological nitrogen balance, but failed to measure relevant endpoints for healthy aging, such as muscle strength and prevention of chronic diseases. (DACH, 2019)

	Protein			
	g/kg bodyweight/day[a]		**g/day[b]**	
Age	m	f	m	f
Adults				
19- <25	0.8	0.8	57	48
25- <51	0.8	0.8	57	48
51- <65	0.8	0.8	55	47
>65	**1.0**	**1.0**	67	57
a) for BMI 18-25kg/m^2 for adults b) reference person as calculated based on data from Gößwald, et al., 2012				

Table 1: Recommendations for Protein Intake (DACH, 2019)
In German-speaking countries the value for protein for people over the age of 65 is an estimated value; the requirement for elderly adults cannot be assessed with sufficient accuracy.

A vast amount of data supports the positive effects of a higher protein intake for elderly people and is the basis for the rise of protein recommendations in the DACH-region in 2017. (DACH, 2017) Positive outcomes of an elevated protein intake include preservation

of muscle mass and strength, prevention of injuries and following hospitalization and maintenance of mobility. This allows implementation of an active lifestyle and lowers risk factors for secondary diseases such as overweight, diabetes and cancer, and neurological diseases including Alzheimer's or dementia, which can be consequences of an inactive lifestyle or inadequate nutrition. (Baum et al. 2016)

Protein recommendations for elderly adults vary between 0.8 g/kg BW/d, as recommended e.g. by the WHO (World Health Organisation), to 1.0g/kg BW/d, as it is the case in German-speaking countries, up to 1.2g/kg BW/d in Nordic countries, among others. (DACH, 2017; WHO, 2007) Suggestions on protein intake in elderly go as far as 1.5 (Mitchell et al. 2017) to 3.0 g/kg BW/d (Traylor et al. 2018), under certain circumstances, which can be achieved without the occurrence of side effects and showed positive outcomes for people of high age. These high amounts may be difficult to meet due to practical limitations but could counteract the fact that elderly adult's muscle tissue reacts less sensitive to anabolic stimuli. According to Moore and colleagues, the optimum amount of protein per meal is suggested to be 0.40 g/kg/meal, which translates to around 35 g of high-quality protein per day or 1.2 g/kg BW/d, which is, again, higher than the recommended amount at the moment (Moore et al. 2015)

Beneficial Effects of Higher Protein Intake in Elderly

Several studies, as reviewed by Liao and colleagues in 2019, suggest that the loss of muscle mass that comes with aging can be effectively prevented by protein supplementation and strength training. 19 RCTs from 1994 to 2019 were analysed, including 1888 participants aged 64 to 89 years. Effects of protein supplements, in different amounts, in combination with muscle strength training (738 participants) or either one of the interventions (556 received muscle strength training, 209 received protein supplementation) were investigated. 385 participants had no intervention and represented the control group. The examined outcomes included changes of body composition, muscle mass and physical functions. The amount of protein varied greatly between the examined studies, ranging from 3.0-40 g/d. Exercise duration was a medium of 12-24 weeks of either resistance, aerobic or multi-component exercise. The outcome showed highly significant results in favour of the interventions (figure 2). (Liao et al. 2019)

Maintaining lean body mass can reduce the risk of sarcopenia, which is the loss of skeletal muscle mass occurring with age, and frailty. Any intervention, protein supplementation of

any composition, and muscle strength training, regardless of the type, was beneficial. Physical activity and protein supplementation exceeding the recommended value by the WHO of 0.8 g/kg BW/d, have significant positive effects on several outcomes associated with healthy ageing. These have, in turn, beneficial effects on physical outcomes, such as leg strength and walking capability. The prevention of sarcopenia, a main target of these interventions, plays a key role in maintaining health during ageing. The combination of strength training and protein supplementation had greater effects than only one intervention. These effects are better for people at high risk of sarcopenia and frailty than for their healthy contemporaries. (Liao et al. 2019)

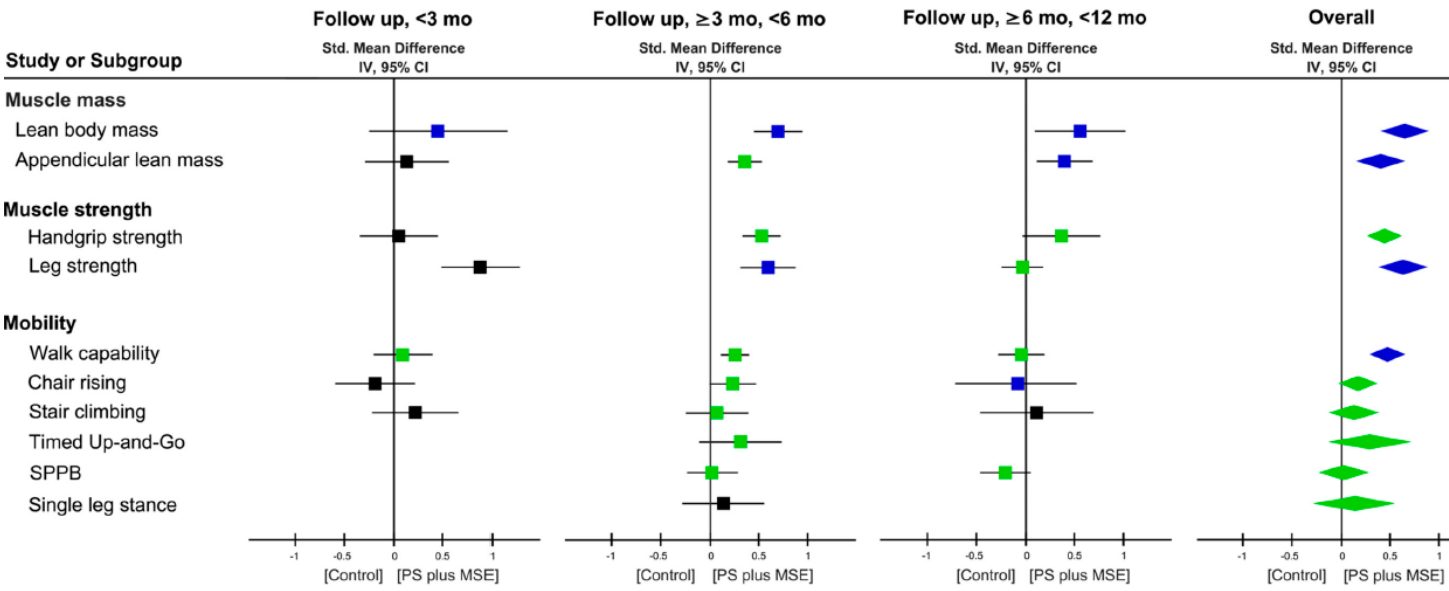

Figure 2: Effects of protein supplementation of different amounts in combination with muscle strength training on muscle mass, strength and mobility of elderly adults. Combined effects of outcomes are shown with a 95% CI (confidence interval). Any intervention investigated in the review, regardless of the type of protein intake or training, was beneficial for participants. (Liao et al. 2019)

Protein seems to be one of the key nutrients for elderly adults and plays an important role in prevention of age-related diseases. Optimized recommendations for crucial nutrients, such as protein, can lead to an improvement of muscle strength and prevention of chronic diseases which allows elderly people to spend more years in health, maintain independence and quality of life. A lack in protein and exercise, on the other hand, leads to the development of sarcopenia, which increases morbidity and mortality directly or via greater risks for the development of cardiovascular diseases, obesity or diabetes, among others. Furthermore, sarcopenia and secondary diseases, especially obesity, act synergistically. (Baum et al. 2016)

Interaction between Protein Intake and Muscle Response

The synergy between protein, or amino acids, as an essential nutrient for the maintenance of muscle function, and its effect on muscle tissue on a biochemical level, is crucial for understanding the effects of an elevated protein intake.

Protein intake in general stimulates muscle protein synthesis in a dose-dependent manner. Especially essential amino acids (EAA; phenylalanine, tyrosine, tryptophan, valine, isoleucine, leucine, lysine, methionine) play a key role in generation of muscle response through stimulation of muscle protein synthesis and suppression of protein breakdown. While small to medium doses of EAA (up to 10 g/d) increase myofibrillar protein synthesis, and this effect rises with the quantity of EAAs, a very large dose (20-40 g/d of EAA) does not provide additional effects. There seems to be a threshold, both at minimum and maximum level, concerning EAA intake to generate and increase muscle response, that reaches a plateau, which value varies. (Baum et al. 2016)

Even though the exact amount to reach that plateau remains unclear, or varies between individuals, it certainly rises with age. When we consider the optimal protein intake to be the minimum required protein to induce the maximum anabolic response, in combination with the decreased anabolic sensitivity in elderly, the higher protein requirement is supported. (Moore et al. 2015)

Anabolic muscle response is partly provoked by anabolic signalling pathways in muscle tissues. Protein synthesis is regulated partly through the protein mTORC1 (mechanistic target of rapamycin in complex 1), which initiates the process as described in figures 3 and 4. EAAs and especially branched chained amino acids (BCAAs, leucine, isoleucine and valin) are required for an activation of the mTORC1-pathway, which leads to initiation of the factors 4E-BP1 (eukaryotic initiation factor 4E binding protein) and p70S6K (ribosomal protein S6 kinase). Three main reasons contribute to an oppression of the mTORC pathway and following resistance of muscle tissue to leucine activation in elderly: a reduced sensitivity to leucine, a less efficient absorption of nutrients in general, and the decrease of food and protein intake with age. The reduced phosphorylation of mTORC1 and p70S6k are thought to be another reason for a decreased response to essential amino acids and consequently for sarcopenia. (Gordon et al. 2013)

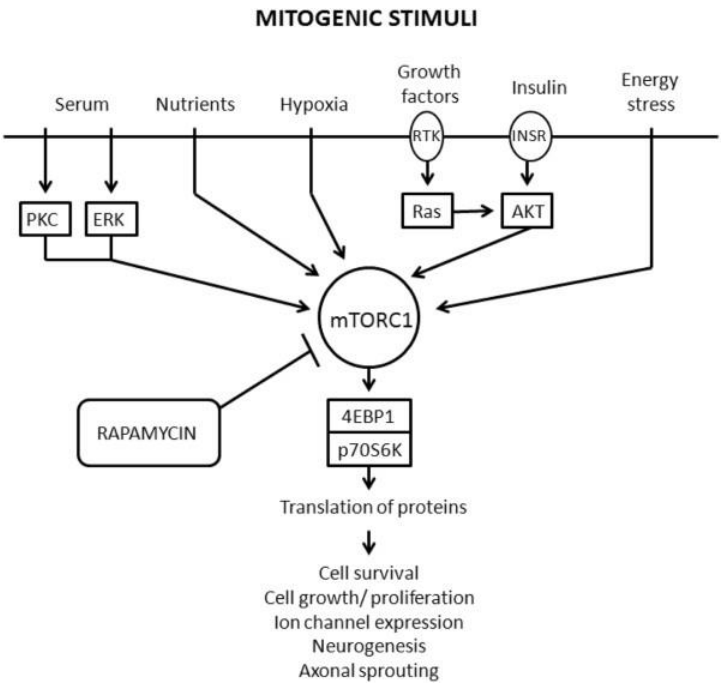

Figure 3: Schematic presentation of influencing factors on the mTORC1 signalling pathway. mTORC1 is influenced by upstream pathways, including nutrients, among others, that increase cell growth and differentiation, while Rapamycin inhibits mTORC1. Nutrients in general, but EAAs especially, impact this signalling pathway. Abbreviations: RTK: receptor tyrosine kinase; INSR: insulin receptor; PKC: protein kinase C; ERK: extracellular signal regulated kinase; AKT: protein kinase B; (Yates et al. 2013)

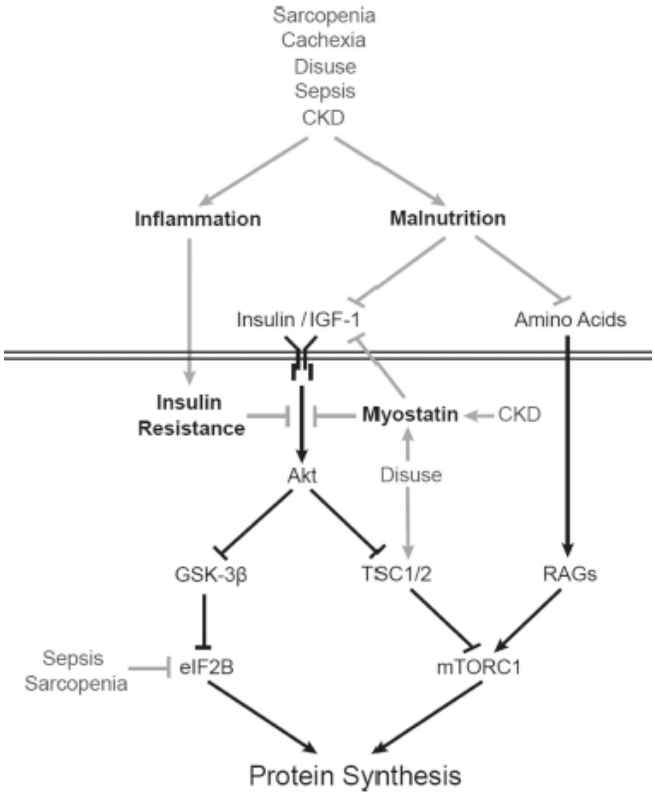

Figure 4: Signalling pathways of cells which affect protein synthesis. Sarcopenia and other muscle wasting diseases have a large impact on metabolic outcomes such as malnutrition, inflammation and the development of diabetes. The mTORC1-pathway is activated by amino acids via Rag proteins and a sufficient amount of protein can reduce the negative effects. Arrows show stimulation, blunt ends show inhibition. Abbreviations: CKD: Chronic kidney disease; IGF-1: insulin like growth factor 1; GSK-3β: glycogen synthase kinase 3β; TSC: tuberous sclerosis protein; RAG: recombination activating gene; elF2B: eukaryotic initiation factor 2β; (Gordon et al. 2013)

Another factor with influence on muscle metabolism is extensive bed rest or hospitalization, which can have serious impacts on muscle tissue and lead to muscle loss and sarcopenia. As described by Tanner and colleagues after 5 days of bed rest elderly, but not younger adults, experienced a reduction in leg lean mass and strength, reduced sensitivity to anabolic stimulation by amino acids, which resulted in decreased muscle protein synthesis and enhanced markers of muscle proteolysis. Simultaneously, an increase in muscle protein breakdown could be assessed, supporting the decrease of muscle function by injuries or hospitalization. Neither of those appeared in younger study participants. The study suggests changes in sensitivity of mTORC1 and related pathways and factors in the anabolic response and anabolic resistance in elderly adults, which is supported by previously mentioned studies. The loss in muscle mass after bed rest is significantly age-dependent, as is rehabilitation time. (Tanner et al. 2015)

Energy Metabolism and Protein Quality

When speaking of protein intake, quality varies significantly, and not only the amount, but protein quality must be considered. In combination with muscle training and an active lifestyle, health and independency can be prolonged. (Baum et al. 2016)

Due to the reduced energy requirement, protein quality gains importance with age. One of the largest components of total energy expenditure (TEE) is basal metabolic rate (BMR). Based on BMR measurements, energy requirement declines by 1-2% per decade after the age of 20, based on longitudinal investigations. This effect is partially due to the loss of fat free mass in favour of metabolic inactive fat mass. This decline is not linear but occurs more rapidly after the age of 40 for men and 50 for women (Fig. 5). Even if BMR is adjusted for the changes in body composition, it is still around 5% lower than expected in elderly compared to younger adults. Studies explain this remaining percentage by the loss of extremely active organ tissue and additionally a decrease in metabolic rate per unit in lean tissues. (Roberts et al. 2005)

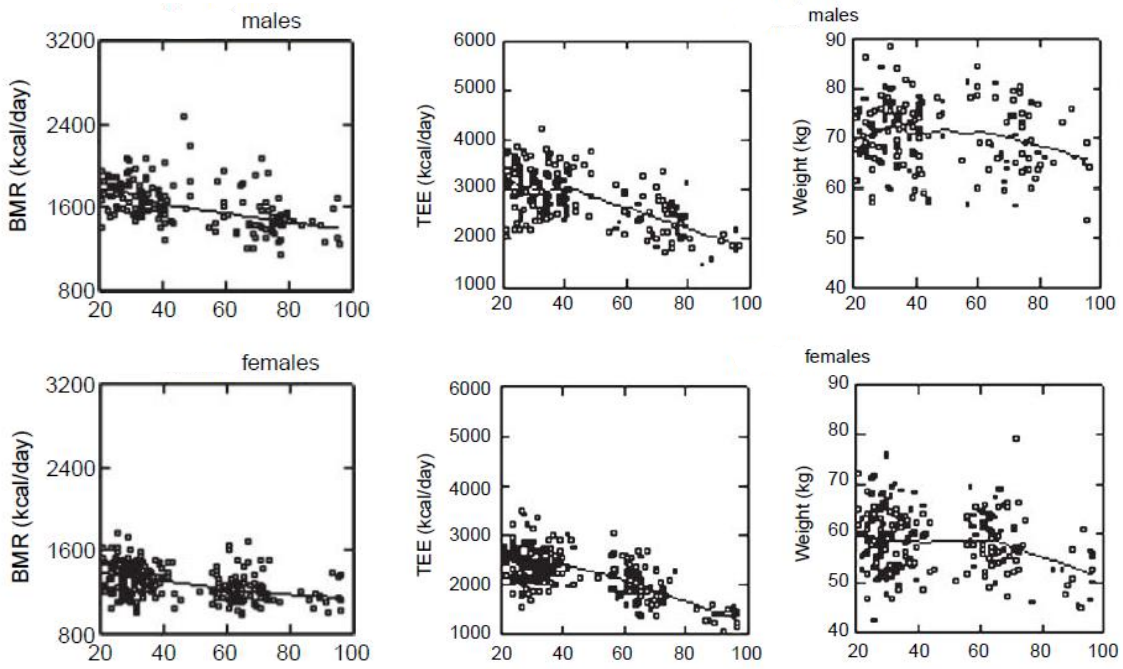

Figure 5: Effect of aging on BMR and TEE and weight distribution for normal weight men and women. Between the age of 20 and 96 a 2% decrease of BMR in women and 2.9% in men was observed. The degree of decline remained significantly steady in all age groups. For TEE the decline was 150kcal per decade between 20-90 years of age. (figure adapted from figures 1 and 2 from Roberts et al. 2005)

The question whether plant or animal sources for protein are more beneficial, cannot easily be answered. In the Health, Aging and Body Composition study from 2008 animal protein turned out to prevent loss of muscle mass more effectively than plant protein sources. This included meat and dairy foods. A 40% minor loss could be observed in the highest protein intake group compared to the lowest group over a period of three years. (Houston et al. 2008)

Animal sources should be considered, not only because of protein quality and amino acid profiles, but due to their higher protein content per total energy, which decreases the caloric intake while providing a sufficient amount of protein. This is of special interest, since on one hand obesity is one of the main reasons for limited mobility in later years of life, but on the other hand the elderly struggle to meet their energy needs due to a decrease in general food intake. (Montero-Fernandez et al. 2013)

It is unclear if the thermic effect of food intake also changes with age. Generally, it contributes around 10% to TEE. Studies show different effects of age from no change to a

non-significant increase. Limited nutrient absorption affects the thermic effect, which might explain the mixed results. (Roberts et al. 2005)

Inadequate protein intake in combination with an inactive lifestyle, among other factors, can lead to the development of sarcopenia, a main cause for impaired mobility and risk factor for injuries (e.g. by falls). Eventually sarcopenia increases the risk of mortality and morbidity. Muscle strength training can be seen as a factor to alter the risk both as treatment and prevention. Additionally, sarcopenia can effectively be modulated by adequate diet, with a sufficient amount of protein and total energy intake, in combination with muscle training. It is to a large extent driven by the inability to stimulate protein synthesis after dietary protein ingestion. (Moore et al. 2015)

The Human Gut Microbiota

The human microbiome, the entirety of microorganisms inhabiting the gastrointestinal tract, consists of numerous microbes, which influence physiology and disease and consist of a complex ecological community. Most of the intestinal dwellers are harmless or even beneficial for the host. Especially nutrient and energy metabolism, immune function and protection against enteropathogens are determined by microbiota. Disruption of the microbial balance, dysbiosis, is associated with various malfunctions including obesity, neurological disorders, malnutrition, inflammatory bowel disease and cancer. The understanding of microbiota as an ecological unit, and the differences between healthy microbiota and those associated with disease, is a challenge that could affect the definition of what a desired state may look like for a population and how to achieve that individually. The complexity and individual variability complicate the definition of a "healthy" microbiome. (Lozupone et al. 2012)

Intestinal microbiota are classified in pathogenic and symbiotic microorganisms. The microbiome consists of more than 90% of symbiotic phyla, which are mainly anaerobic and beneficial for physiological functions of the host. Pathogenic microorganisms are mainly facultative anaerobes and account only for a small part of the intestinal microbiome. They are not harmful in an equilibrium state, but only when the balance in the gut is altered in favour of the pathogens. It is well known that the microbiome of the gastrointestinal tract is to a large extent influenced by several endogenous and exogenous factor and varies greatly between and within individuals. Identification of the factors that drive that variation could be used for therapeutic purposes. Recent studies clarified the role of certain exogenous effects on microbiota, such as nutrition, age, pregnancy, genetics, diseases, lifestyle etc. Therefore, nutrients and diet interventions can be an important tool in maintaining intestinal microbiota balance for disease prevention or even treatment. However, there seems to be a vast number of influences still to be detected. (Ma, et al., 2017)

The role of genetics and environment, including nutrition is still partly unclear because of various confounders. Familial similarities, as examined in twins, and mother-daughter pairs for instance, can be explained by genetics, and the often shared environment. (Dicksved et al. 2008)

Crucial exogenous factors shaping gut microbiota are environmental exposure, hygiene, diet, lifestyle and antibiotic use. The ratio of two genera, *Prevotella* and *Bacteriodes*, correlates well with overall diversity. While *Prevotella* are more abundant in diets dominated by plant-derived polysaccharides and simple sugars, *Bacteriodes* are associated with a diet high in animal protein, several amino acids and saturated fats. The impact and significance of the diet in comparison with other factors on microbiota remains to be investigated. (Wu et al. 2011)

Different physiological states result in significant changes in abundances of taxa and functional genes. Lean and obese people for example can be classified solely on their gut microbiota composition with 90% accuracy. (Knights et al. 2011)

The ratio of the two phyla *Firmicutes* and *Bacteroidetes* (F/B ratio), which represent the two most abundant organisms, correlates well with body composition. A higher content of *Firmicutes*, and a simultaneous decrease in *Bacteroidetes* is a characteristic for obese subjects, as confirmed by numerous human and animal studies. (Koliada et al. 2017)

Microbial differences can also directly affect disease development, as seen in mice studies. Germ-free mice introduced to microbiota from an obese mouse gained obesity more rapidly than those introduced to lean mice's microbiota. (Turnbaugh et al. 2006) This effect is the result of several impacts of the microbiome on metabolism, such as enhanced hepatic lipogenesis, cellular uptake and storage of fatty acids and triglycerides, decrease in fatty acid oxidation in skeletal muscles, decline in motility through an interaction between short chain fatty acids (SCFA), fermentation products of bacteria and G-protein coupled receptor 41. Microbial dysbiosis leads to subliminal inflammation promoting several health outcomes including obesity. (Koliada et al. 2017)

One of the main functions of intestinal bacteria is the metabolism of non-digestible dietary compounds, mainly resistant starch and dietary fibre through fermentation. Main products are the SCFA butyrate, propionate and acetate, which are then used in different metabolic pathways as an energy source. Acetate is used for cholesterol-synthesis, butyrate plays an important role as an energy substrate for colonic epithelial cells and propionate works as a precursor for liponeogenesis, gluconeogenesis and protein-synthesis in the liver. (Riva et al. 2012)

Variability in one individual is limited, but not axiomatic. The extent to which certain influencing factors have to operate, temporal and quantitative, until the microbiome

responds to this change, is of great interest. Changing an equilibrium state in a certain direction, for example by nutritional interventions or medication, holds great potential for therapeutic purposes. Availability of new substrates or different concentration of the same substrates provide a selective advantage to certain species, raising their abundance, while others are supressed and decline. Whether an intervention leads to taxonomic and functional changes is linked to the systems resilience to a certain interruption. This is well investigated for different amounts of carbohydrates and fibre in the diet, while studies on the effects of protein showed inconsistent results. (Lozupone et al. 2012)

Changes in Microbial Composition Throughout Life

The most significant changes occur during early stages of life. In the first three years, diversity and stability rise, due to compositional and functional changes after initial colonization. In infants, the colonization of the gut starts with facultative anaerobes, that shape the oxygen environment for strict anaerobe bacteria. The infant microbiome is influenced by mode of delivery (section or vaginal), feeding type (breast feeding or formula), gestational age and antibiotic use. After 2-3 years of age, the colonization is completed and reaches a more or less stable state, that remains throughout life. This initial colonization has crucial impacts on several factors of adult life, such as well-being, epigenetic programming, immune function and disease susceptibility. The extent to which the composition of an infant microbiome influences adult microbiota composition is not yet fully clarified. (Dicksved et al. 2008)

Around adolescence the microbial complexity reaches its peak, but the phylogenetic diversity continues to increase during adulthood. The number of bacterial individuals in a healthy adult can be up to 10^{14}, which is greater than the number of somatic cells in the human body. It is dominantly colonized by *Bacteroidetes* and *Firmicutes*, while *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* appear to a smaller extent. (Salazar et al. 2014)

While the ecosystem is relatively stable in adult life, dysbiosis can appear with old age. With aging come several physiological changes, affecting the immune system, physiology of the gastrointestinal tract, dietary and lifestyle patterns. Modifications in the immune system lead to an elevated susceptibility to infectious diseases. Elderly people have a reduced stability of the microbial ecosystem and the core composition differs significantly from the one of healthy younger adults. The abundance of *Firmicutes* and *Bacteroidetes* in

elderly highly differs in various populations and countries. Generally, changes include a reduction in species diversity of most groups, which makes the ecosystem less resistant to changes due to the environment. Other shifts may be found in dominant species, declines in beneficial groups, increasement of facultative anaerobic bacteria and decreased availability of short chain fatty acids. All these alterations might contribute to disease susceptibility. Specific changes that occur with age regarding microbial composition on species level occur with a high interindividual variation. Even though microbiota composition seems to be stable throughout adulthood, it reacts more to physiological and environmental changes in the very old (and very young) stages of life. This instability seems to make certain age-groups targets for alterations in microbiota composition, as interventions may have a bigger impact at unstable periods. (Salazar et al. 2014)

While mean anaerobe counts from faecal samples remain stable, declines in *Bacteroides* can be found when compared to healthy younger adults. Due to a complex cross-feeding network in the gut, changes in one species lead to further changes in others and to consequences for the host because of alterations in metabolic activities. Proteolytic bacteria, that ferment amino acids, rise with age, including *Fusobacteria*, *Propionibacteria* and *Clostridia*. While species diversity declines, the genus *Clostridium* rises. *Eubacteria*, that are partly phylogenetically related to *Clostridia*, increase with age, which can lead to potentially harmful health consequences for the host. (Woodmansey et al. 2007)

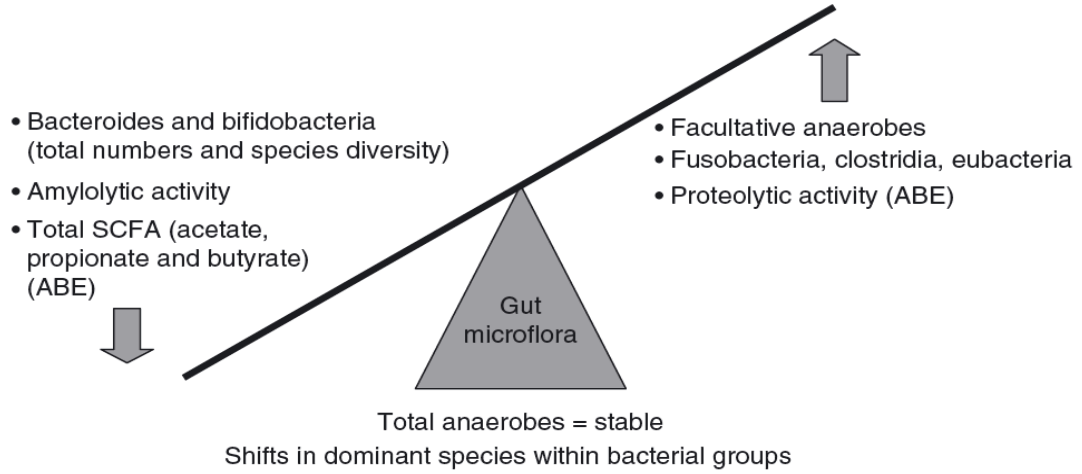

Figure 6: Changes of intestinal microbiota in elderly. Main alterations concern Bacteroides *and* Bifidobacteria. *Total SCFA-production declines, while facultative anaerobes, such as* Fusobacteria, Clostridia *and* Eubacteria *rise. While the total amount of anaerobes is relatively stable, there are shifts in dominant species. (Woodmansey et al. 2007)*

The first step of understanding the link between the human microbiome and host health, is to identify a desired state of microbiota composition. Large effort was made by researchers of the Metagenomics of the Human Intestinal Tract (Meta-HIT) and the Human Microbiome Project (HMP). Once the healthy state is clear, we can investigate different influences on disruption and dysbiosis, but also identify beneficial effects of lifestyle changes or even medication. However, the great variation, inter- and intra-individually, the complexity of the microbiome and its influencing factors and the elaborate identification complicate the definition of a healthy state on individual or population level. (Turnbaugh et al. 2007)

Diversity of the Microbiota

The average mammalian gut is composed of approximately 500-1000 bacterial species, with a relatively stable community individually over time. Gut microbiota can be classified roughly into six phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia* and *Fusobacteria*, with *Firmicutes* and *Bacteroidetes* being the dominant phyla, contributing up to 90% of the community. (Eckburg, et al. 2005)

To understand the diversity of the microbiota, composition alone is insufficient to explain community function. Sequencing DNA helps to understand functional information of communities. Besides taxonomic diversity microbiota can be classified by functional diversity, which seems to be much more similar between individuals, as confirmed in large populations by MetaHIT and HMP. Despite the high variation in microbiota composition, functional gene profiles are similar. These functions include central metabolic pathways, carbohydrate and amino acid metabolism and variable functions, restricted to certain species, like vitamin or drug catabolism, motility, nutrient transport or pathogenicity islands. It seems that we share a functional core, but not core microbiota. (Yatsunenko et al. 2012)

That functional core consists of several independently evolved species, that altogether form the similarity in activity and metabolism. This concept is explained by redundancy, which describes the functional niches and which members of these niches can substitute for one another. An example of functional microbiota is a group of bacteria, including *Clostridiales*, such as *Anaerostipes caccae*, *Eubacterium hallii* and *Roseburia intestinalis*, which all produce butyrate, but differ in ecological requirements, like oxygen tolerance or substrate specificity. Yet, samples of one individual over time are more similar than samples of different individuals, suggesting an individual diversity core. This stability can

be seen as an equilibrium state, that undergoes individual temporal dynamics. However, the number of configurations of such stable communities may be quite large and difficult to classify into different types. Better understanding of the microbial ecology in the large intestine will help to explain the effects of nutrition, pro- and prebiotics, medication and lifestyle changes and can support interventions and predict effects of interventions on microbiota composition. (Lozupone et al. 2012)

Although several studies suggest that certain bacterial species are shared by most healthy adults, other, culture-independent, studies showed a broad diversity over time and populations. Every human harbours between 500 and 1000 species in the intestine, but most of these belong to just a limited number of phyla. Besides bacterial dwellers, the intestine also accommodates other domains, such as archaea, eucarya (yeasts) and viruses, whose abundance varies significantly. (Lozupone et al. 2012)

To classify the microbiome, or break it down to a more simple approach, the concept of enterotypes is used. The idea to classify the microbiome into a few, differentiable, relatively stable states could be used to divide a given population by abundance of certain species or functions. This could be especially useful in disease detection and treatment or to identify risk factors. (Clayton et al. 2009)

It is not yet discovered how to classify enterotypes, and there seems to be a large number of unique configurations building stable communities. The number of exogenous and endogenous factors that influence the composition hinders the concept of enterotypes. If clustering into certain types is necessary, all these factors must be considered and microbiota composition from different age and ethnic groups, cultures, nutritional, physiological and pathological states must be investigated. To quantify the current composition of the microbiome, several diversity metrics can be calculated. They can then be used to detect changes after interventions. Composition on species level solely is insufficient to describe the current state. Several factors need to be considered to characterize microbial diversity in an ecosystem. The characterization of the microbiome is summarized in figure 7. (Lozupone et al. 2012)

Figure 7: While small subunit rRNA gene sequencing enables estimation of compositional diversity, Shotgun Metagenomic sequencing reveals functional diversity of the microbiome. Combined, they can link species to functions via the sequenced genes and help to understand the role of the human intestinal genome on different levels (α-, β- diversity, etc.) Figure adapted from figure 2 from Lozupone et al. 2012

Species richness describes the number of species in a system. In culture-independent studies, the richness depends on the sampling depth. Rich communities are less prone to environmental changes. Their use of resources, such as substrate, is more efficient, with each species specialized to a certain resource. When it comes to excessive nutrient availability, or eutrophication, a small number of bacterial taxa tend to grow to a large extent, and suppress other species, leading to a decline in richness. Low species richness is linked to obesity, and a diet high in fat and simple sugars. (Yatsunenko et al. 2012)

Effects of Protein Intake on Microbiota Composition

Growing evidence suggests an interaction between protein intake and gut microbiota. Sources and amount of protein seem to alter the composition and activity of intestinal bacteria, and reciprocally protein metabolism is influenced by the composition of the microflora. All macro-nutrients, also protein, play a role as energy sources and amino acids act as a substrate for fermentation. To what extent protein affects the environment in the colon depends on several factors, including the amount of protein and fermentation metabolites to reach the colon, which in turn depends on digestibility, source and amount of dietary protein. (Ma, et al. 2017)

Most of the dietary protein is digested in the small intestine, rather than the colon. When high amounts are ingested, it reaches the colon, where bacterial hydrolysis breaks protein into amino acids or smaller peptides. These elevate intestinal pH and are further metabolized. The higher pH favours pathogen microorganisms and can be harmful for the gut environment. Protein fermentation is also under discussion of favouring enteric disease development. The negative effects of high amounts of protein, and protein reaching the colon, are controversial. (Louis et al. 2007; Ma et al. 2017)

Alterations in diet composition result in qualitative and quantitative changes of the substrate availability resulting most likely in changes in microbiota composition. The impact dietary changes have on microbial ecology occurs through different, partly related, mechanisms. The same substrates can be processed via different routes, depending on several factors, like supply, environment or bacterial cells. This is well investigated with non-digestible carbohydrates. Changes in the gut environment, including local conditions of oxygen and hydrogen, pH, metabolites, transit time, host secretions and bacterial metabolites, are highly associated with changes in bacterial composition. (Louis et al. 2007)

Identification of Intestinal Microbiota

The human gut microbiome consists of a complex ecological community. Its influence on host physiology has moved into the focus of researchers, not only because of raising the understanding of its relevance, but also because of newly achieved technologies that allows us to identify these unculturable organisms. Next generation sequencing (NGS) facilitates the identification of intestinal bacteria and showed the importance of diversity and balance of microbiota composition for host health. This newly gained information can help to develop diet or lifestyle interventions to maintain a healthy symbiosis. To investigate the composition and how the presence and absence of certain species affect our physiology, microbiota studies use 16S rRNA-PCR (ribosomal ribonucleic acid, polymerase chain reaction) methods coupled to sequencing. The results of NGS methods are millions of reads, that require bioinformatic effort for data handling and analysation. (Salazar et al. 2014)

The gained sequences are then merged into clusters, Operational Taxonomic Units (OTUs) based on a 97% similarity threshold, which can then be used to calculate microbial diversity and be compared to detailed metadata, containing information about subjects, samples and sequences. Another way to depict the gained data is the use of amplicon sequence variants (ASVs) basically describing individual sequences in a sample. (Nguyen et al. 2016)

This method enables to capture all the genetic information contained in an environmental, in our case faecal, sample. 16S rRNA is used as a marker gene to generate a community profile. This special rRNA gene is present in all taxa which makes it especially suitable as a marker gene. Slowly and fast evolving regions can be used in two crucial ways for this method. Slowly evolving regions as broad PCR primers to detect largely different taxa, while fast evolving regions to group organisms at closer taxonomic levels. While sufficient for family or genus level, the information from sequencing alone might not be sufficient for the detection of taxa on species level, since a 97% similarity threshold is more suitable to distinguish on genus or family rather than on species level. (Kuczynski et al. 2011)

Description of Microbial Diversity

As species richness is not a simple number, its measurement turns out to come with certain difficulties. To describe the actual richness of an environmental sample through observation of a limited number of species, thus creating a random sample, is biased by a multitude of confounders and therefore can be neither measured nor estimated with

sufficient accuracy. Approaches to that effect use tools that proofed expedient and correct the occurrent bias in the estimation of species richness. (Gotelli et al. 2011)

Presence or absence alone do not describe microbiota composition sufficiently. The absence of a species can either describe the species simply does not appear in a sample, or it was not detected by the chosen method. Diversity methods rely on the assumption of a closed system (e.g. intestinal tract), describing a stable, unaltering state of the distribution of proportional abundance of species. In an open system, where the number of species and their relation changes over time, we can picture a snapshot of the state at one specific timepoint. The concept of a closed system remains theoretically, since no biological system is truly isolated from exogenous factors. Sampling in a microbial community always assumes the statistical concept of "sampling with replacement", which describes the data remaining unchanged by the sampling process (either the individual is not removed from the sample or if they are removed, this does not change the relation of remaining individuals). (Gotelli et al. 2011)

Materials and Methods

Study Design

This thesis is part of the NutriAging study, a blinded, randomised, controlled trial with two intervention and one control group that occurred from July to December 2018 at the Department of Nutritional Sciences in the working group around Univ.-Prof. Mag. Dr Karl-Heinz Wagner at the University of Vienna. The aim of the study was to investigate effects of optimized protein intake and muscle strength training on health and well-being of the elderly population and to use the results to frame recommendations concerning lifestyle and the diet. The focus of this investigation was protein intake above the recommended amount and muscle strength training, while this thesis focuses especially on their effect on the intestinal microbiome by investigating the results of bacterial DNA sequencing from stool samples. 117 subjects, male and female, aged 65-85 finished the study. Other factors, apart from age, considered in choosing participants were mental health, tested with the MMS (mini mental status) test and successful completion of pre-study exams. Several criteria for exclusion were defined: predominant coronary heart disease, including bypass, cardiac pulse generator, defibrillator or aortic aneurism. Further cancer, diseases of the eye and antibiotic use six months prior the study lead to exclusion, as well as regular muscle strength training in the last six months prior the study.

The three groups were randomised, matched by age and sex by a computer program to minimize bias. After one week of physiological tests, the intervention lasted for 15 weeks, as described in figure 8.

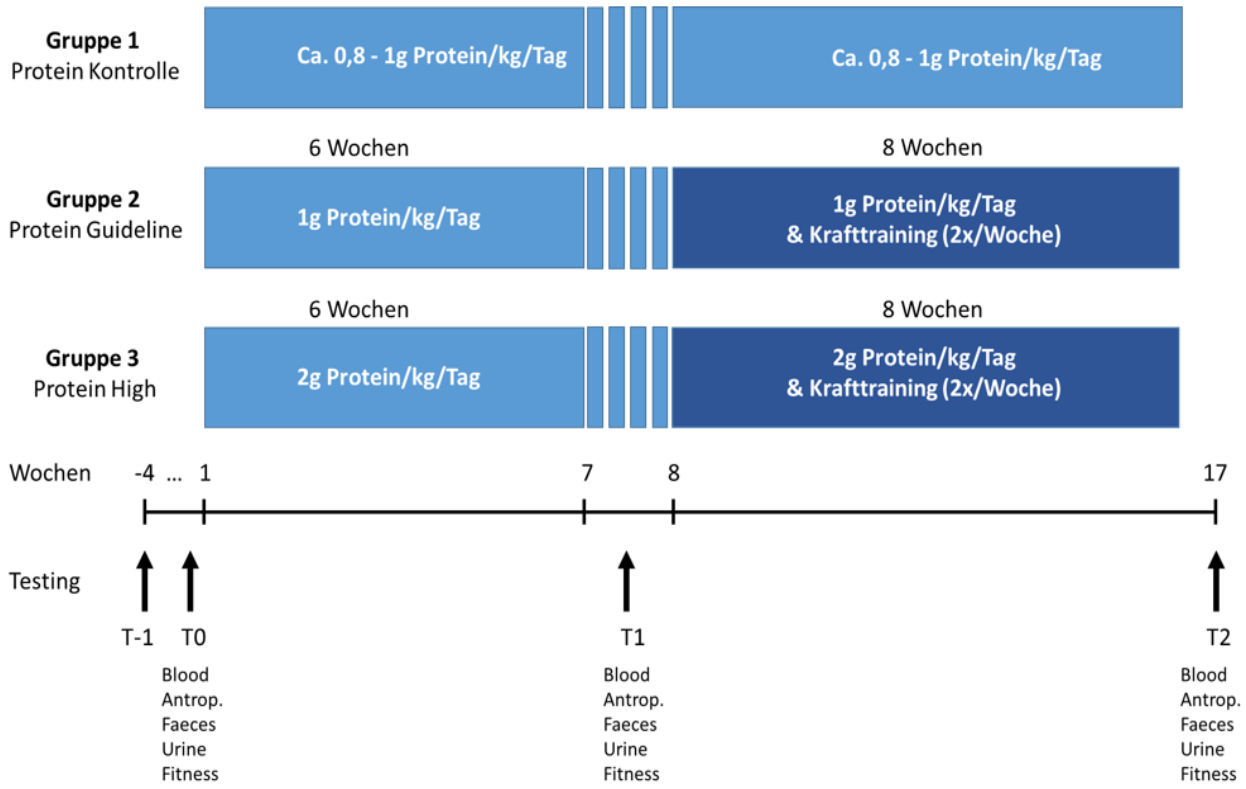

Figure 8: Study protocol of the NutriAging study

After assignment to one of the three groups, intervention lasted for 15 weeks. The control group received 0.8-1.0 g of protein /kg BW/d throughout the whole study duration. P1 (low protein group) was adjusted to 1g protein/kg BW/d and an additional muscle strength training after 7 weeks. P2 (high protein group) received twice the amount of protein and the same training procedure than P1. (Figure 8). Faecal samples were collected at timepoints T0, T1 and T2. Before the intervention, the ideal, effective amount of protein was assessed. This amount was then used to provide several protein-rich foods to the P2 group. P1 group received similar, carbohydrate rich foods. 24-hour recalls were performed every 10 to 14 days to monitor nutrient intake and were used to adjust the protein- or carbohydrate intake accordingly.

Sampling and Aliquots

For the investigations on changes in microbial diversity and composition of microbiota in the colon investigated from faecal samples due to the intervention, faecal samples were collected at three timepoints, at the beginning of the study (T1), after the protein intervention (T2) and after the additional training intervention (T3) and used for sequencing of bacterial DNA (see figure 8).

The faecal samples were collected by the study participants, following an instruction sheet, and stored in sterile plastic cups until arrival at the study centre. To ensure consistent temperature and to avoid modification due to temperature changes, the samples were transported with thermal packs and stored in isolated boxes for transportation. After receipt of the samples, they were subdivided into aliquots of 200 (±10) mg and frozen at -80°C immediately. The remaining samples were disposed, after collection of a retail sample of approximately 2-3g, which was also frozen at -80°C. A maximum of 48 hours was allowed between sample collection and freezing.

DNA Sequencing

Microbial composition at different phylogenetic levels requires identification of total bacterial DNA through quantitative real-time PCR with target gene primers. DNA was extracted from the frozen faecal samples, subjected to multiplex Illumina sequencing of 16S rRNA genes and analysed via PCR, as described by Herbold et al. and the team of the Division on Microbial Ecology at the University of Vienna. (Herbold et al. 2015)

The approach of high throughput NGS with 16S rRNA and functional marker genes is the method of choice for the evaluation of environmental samples. 16S rRNA is used as a marker gene to generate a community profile. The results of this effective technique are millions of reads per run. It is based on the interaction between genomic bacterial DNA with primers. Nucleotides act as a starting point for DNA-replicating enzymes. The PCR consisted of two steps. First, a head-sequence, as a diagnostic primer, was introduced into target genes. The second step consisted of the amplicon tagged with a primer. After PCR, the amplicons were purified, quantitatively assessed and finally sequenced on a MiSeq sequencing system. The result of MiSeq (Illumina) sequencing were paired reads, that could be represented by ASVs. (Herbold et al. 2015)

Figure 9 summarizes the steps from the sample to sequencing: It shows the two steps of the PCR (gene-target head primers and barcode primers). Figure 10 describes the steps from “raw” reads, as a result of the sequencing method, to OTU tables.

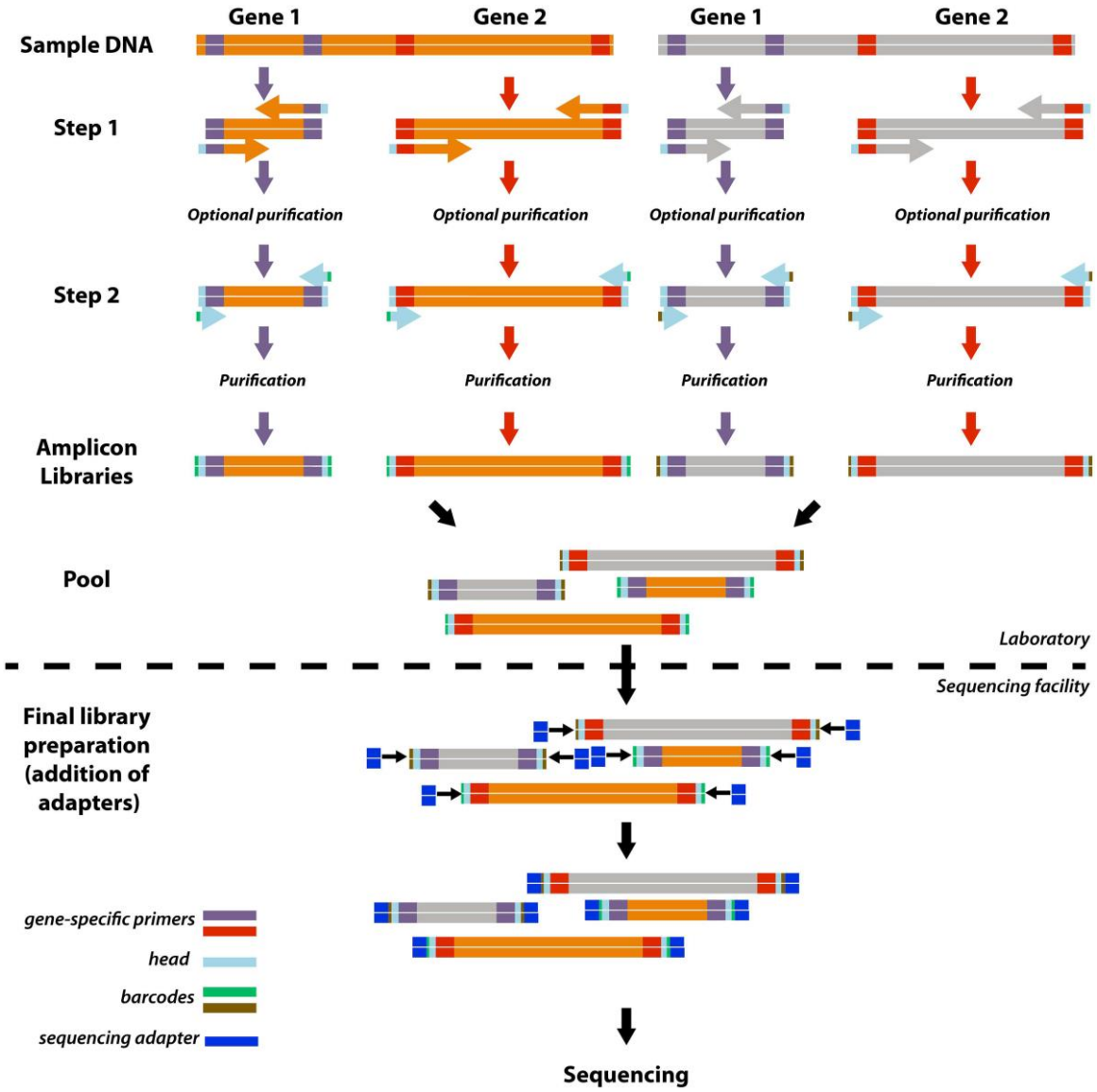

Figure 9: Schematic display of the way from DNA samples to raw reads. Production of amplicon libraries from two gene targets and samples respectively. In a first step, gene target-head primers and in a second step head-barcode primers are used to generate the amplicon libraries with a unique combination of barcode and primer to produce a pooled library for sequencing. (Herbold et al. 2015)

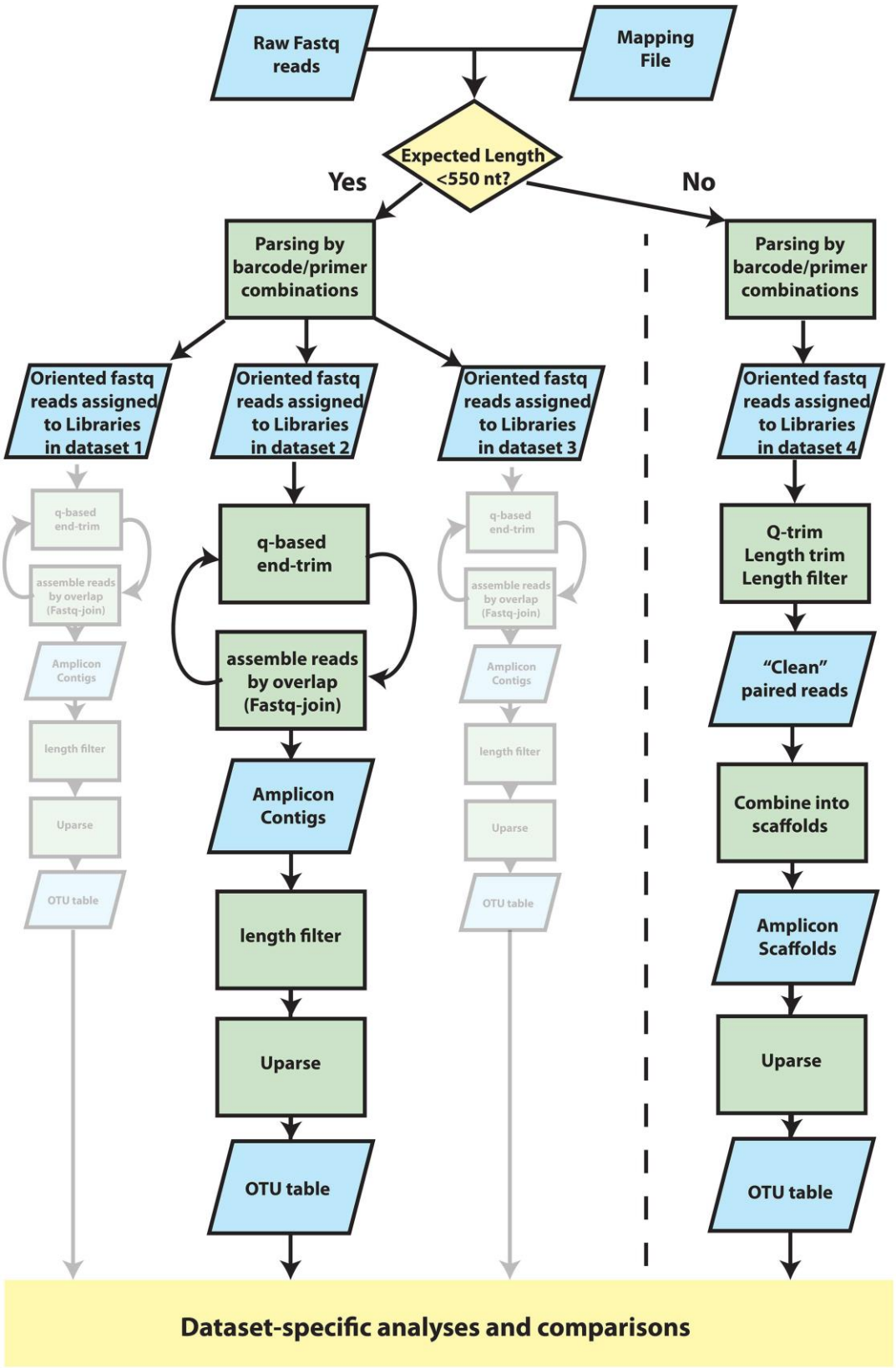

Figure 10: Raw reads are clustered into OTUs. The raw reads are separated by a 550 nucleotide-threshold for amplicon length, with each dataset being filtered and processed. Trimming resulted in individual reads with a standard length, which were then clustered and used for data analysis. (Herbold, et al., 2015)

The results of sequencing required bioinformatic data handling, which was processed by the Division of Microbial Ecology at the University of Vienna. The results of sequencing and data processing were individual sequences in a sample, ASVs, which were then used to identify microbiota on different taxonomic levels by comparison with a data base. Further details are described in the paper of Herbold and colleagues from 2015. (Herbold et al. 2015)

Statistics

After bioinformatic data generation from results of the sequencing method by the Division of Microbial Ecology, data was analysed and interpreted. To analyse impacts of the intervention on diversity of colonic microbiota, statistical analysis of the provided data was conducted using the computational environment R version 3.6.0. (R Core Team, 2019)

Shapiro Wilko normality test ensured normal distribution for metric variables. For variables with a non-normal distribution, non-parametric methods were used for data analysis. To identify statistical correlation of various factors on microbial diversity, three main data matrices were created. The meta-data included general information about study participants, nutritional behaviour, anthropometric data and faecal samples. ASVs and phylogenetic allocation of ASVs were summarized in separate data files, respectively.

Several packages were installed to perform data analysis. Vegan package was used for ecological metrics. For plots and figures the package ggplot2 provided useful support. A list of used packages and citation can be found in the list of references. (Oksanen, et al., 2019; Wickham, 2016)

The result of sequencing were 6.61 Mio. sequences, which represent high dimensional data, for which multivariate statistical analysis is necessary. After subsequent bioinformatic data processing, amplicon sequence variants were created. These can be assigned to further data sets, containing taxonomic data, resulting in taxonomic orders. After sub-setting due to dropout, ecological diversity metrics could be calculated and added to the data and could be compared between and within the individuals and to information of the metadata file. Before calculation, rarefaction has to be performed. Species richness is highly dependent on sample size. Rarefaction prevents false assumptions of diverse samples due to high sample sizes. The sample with the smallest number of reads is being used and multiplied by 0.95. The smallest sample was below 1000 reads (981) and was excluded, so the originally second smallest sample is being chosen. After rarefaction, some ASVs have a

value of 0 and can be excluded. Thereafter data is ready for estimation of ecological metrics. (Oksanen et al. 2013)

While α-diversity describes a microbial community at one time and place, e.g. the microbial composition of a study participant at timepoint T1, β-diversity characterizes the variation in species composition among studied sites. The turnover in space and time or the community variation is being summarized by β-diversity metrics. γ-diversity describes the diversity of the whole study unit by describing pooled sample units. α-, β -and γ-diversity are represented by the same metrics. Several metrics for the estimation of diversity can be applied, that can be divided into two main methods: Observed species richness (S_{Obs}) describes the number of different species (or other phylogenetic orders) in a sample, while estimated species richness (S_{Est}) estimates the number of species in the population represented by the available data (elderly adults in Vienna). Observed species richness is quantified by Shannon Index (H) and Simpson Index (D_1), while the estimated species richness uses the ACE Estimator (S_{est}ACE) and Chao1 Estimator (S_{est}Chao1). (Oksanen et al. 2013)

Results

Study Population

Of 137 recruited participants, 117 finished the study. The microbiota composition from stool samples could be assessed in 114 individuals. 40 participants were included in the control group, 35 in the low protein and 39 in the high protein group. The number of male and female participants did not differ between the groups. Table 2 describes the study participants at timepoint T1.

Intervention Group	**Control** mean ± SD		**P1** mean ± SD		**P2** mean ± SD		**p-value**
Sex	male	female	male	female	male	female	-
n	19	21	17	18	19	20	-
Age [years]	72±5	73±5	72±5	73±4	73±5	73±4	0.45
BMI [kg/m^2]	25.6±3.4	26.2±4.5	27.4±4.6	25.6±3.9	26.4±2.7	25.2±4.4	0.31

Table 2: Description of the Study Population. No significant differences could be found regarding age or BMI between the intervention groups. Matching for sex was successful, the number of male and female participants did not differ in the intervention groups.

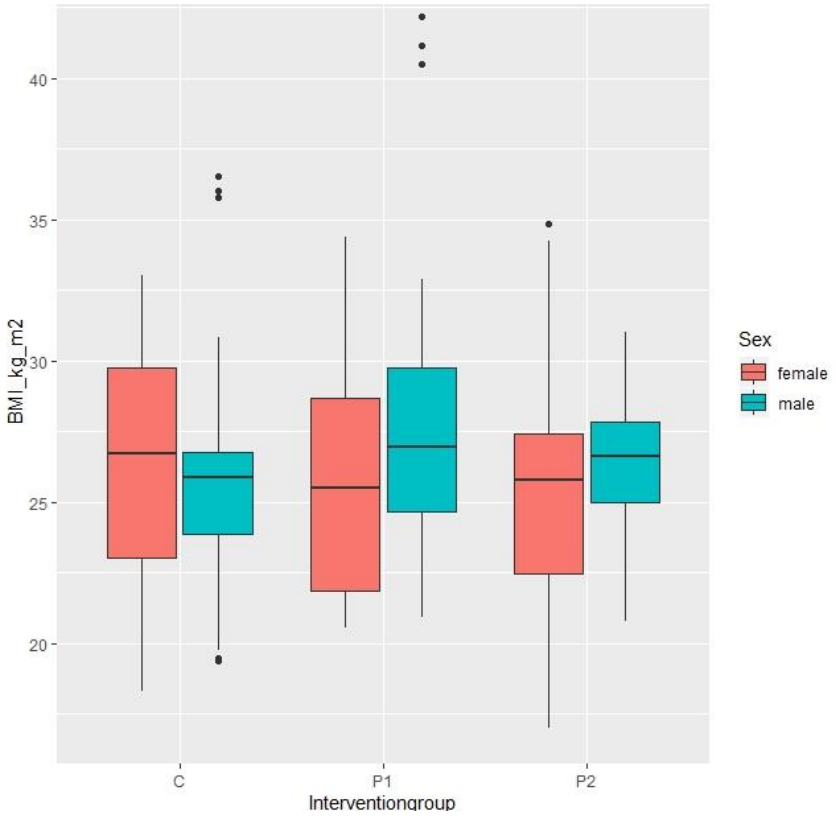

Figure 11: Boxplot for BMI [kg/m2] for men and women in intervention groups. BMI did not depend on sex or intervention group. (p>0.05).

Protein Intervention

To test if the dietary intervention was successfully performed by study participants, protein intake in the different intervention groups at timepoints using data from the routine 24-hour recalls, was compared.

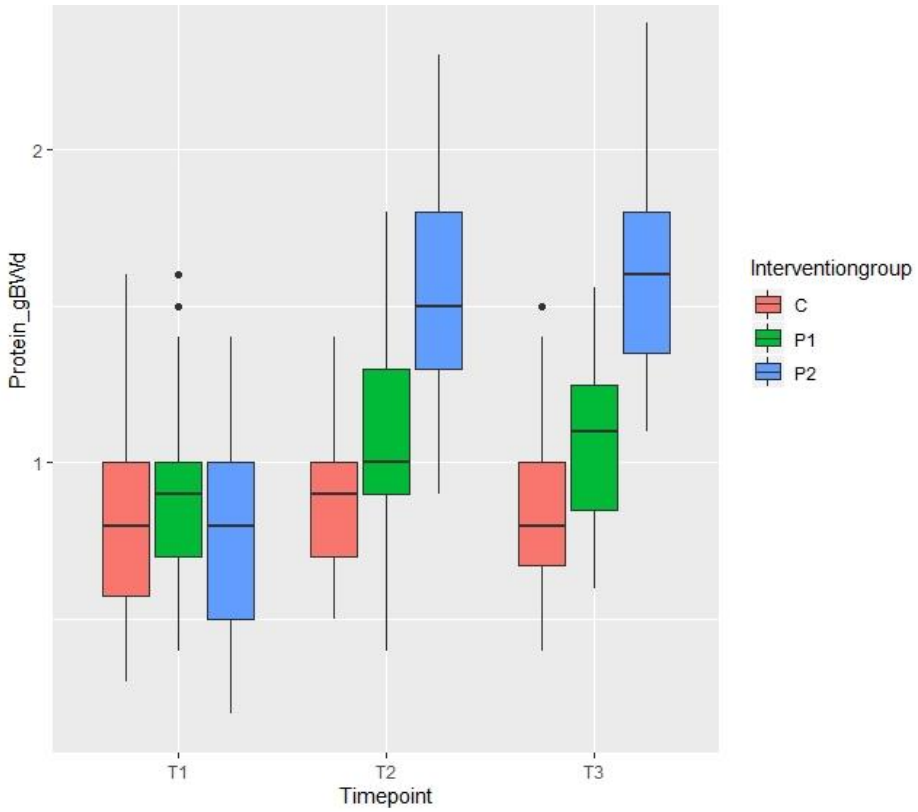

Figure 13: Protein intake [g/kg BW/d] in the intervention groups at timepoints T1, T2, T3. The difference in protein intake was statistically significant for the variable intervention group (ANOVA; p<0.01). This effect was time-dependent, as intended by the study (p<0.01).

	T1 mean ± SD	**T2** mean ± SD	**T3** mean ± SD
C	0.84±0.41	0.91±0.36	0.85±0.26
P1	0.90±0.28	1.1±0.34	1.1±0.25
P2	0.80±0.32	1.5±0.37	1.6±0.37

Table 3: Protein intake in intervention groups; mean ± SD, [g/kg BW/d]

Microbial Diversity

Sequencing Depth

Sequencing depth is an important metric to test successful method procedure. The sample with the smallest depth (P092 from timepoint 2) counted less than 1000 reads and was excluded for further calculations to avoid bias.

Min.	1st Qu.	Median	3rd Qu.	Max
1658	12829	17832	17420	39731

Table 4: Statistical description of sequencing depth [number of reads]

For further calculations, normal distribution of sequencing depth is necessary, which is confirmed by Kolmogorov-Smirnov-Test ($p<0.05$) and visualized by a histogram and a normal QQ-plot.

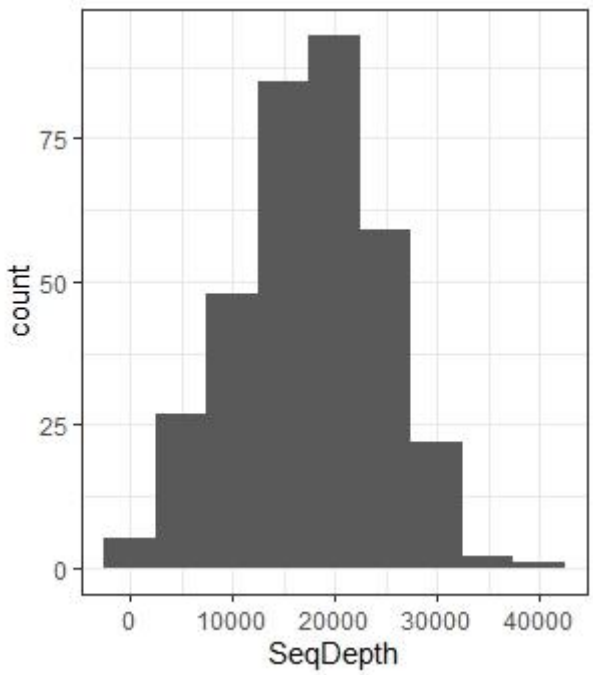

Figure 14: The histogram of sequencing depth leads to the assumption of normal distribution of the variable.

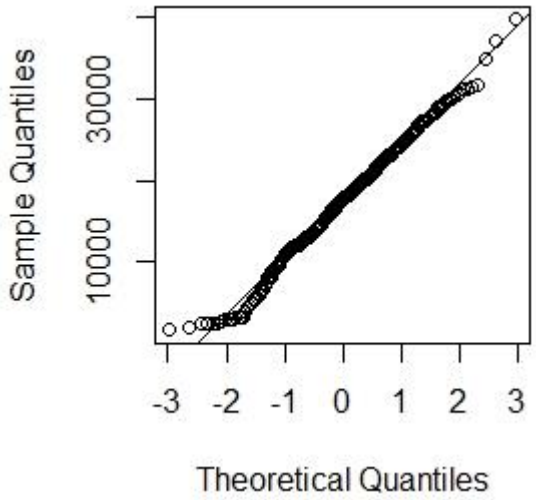

Figure 15: The assumption of normal distribution was confirmed by a normal QQ-plot of sequencing depth.

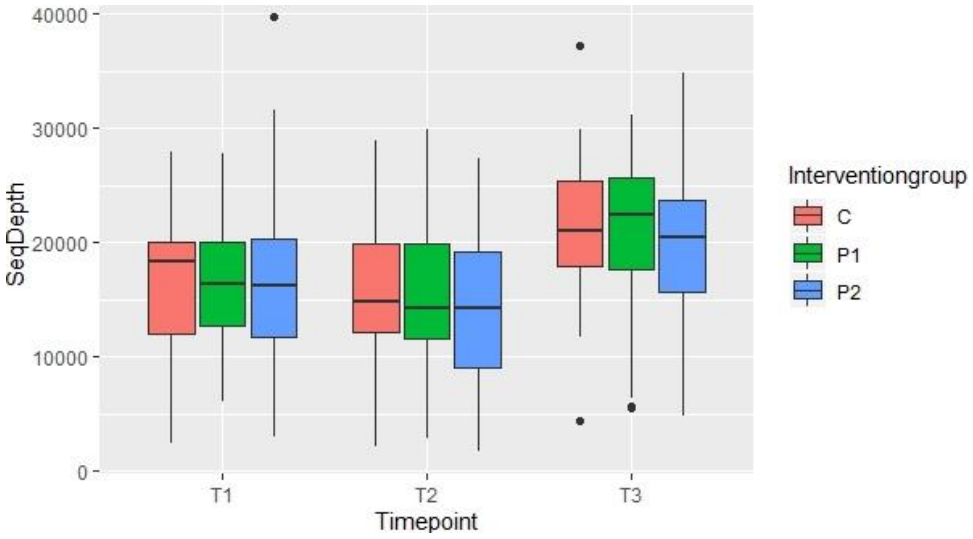

Figure 16: Boxplot of sequencing depth [number of reads] for intervention groups and timepoints. Sequencing depth did not differ within the intervention groups (p=0.6) but was significantly higher at timepoint three for all groups (p<0.01).

Rarefaction

The samples must be rarefied to the same number of individuals with the following formula. (Oksanen et al. 2013)

$$\hat{S}_n = \sum_{i=1}^{S} (1 - q_i), \quad \text{where } q_i = \frac{\binom{N-x_i}{n}}{\binom{N}{n}}.$$

x_i… counts of species i

$\binom{N}{n}$ ... binomial coefficient

q_i… probability that species *i* does not occur in a sample size of *n*

(Oksanen et al. 2013)

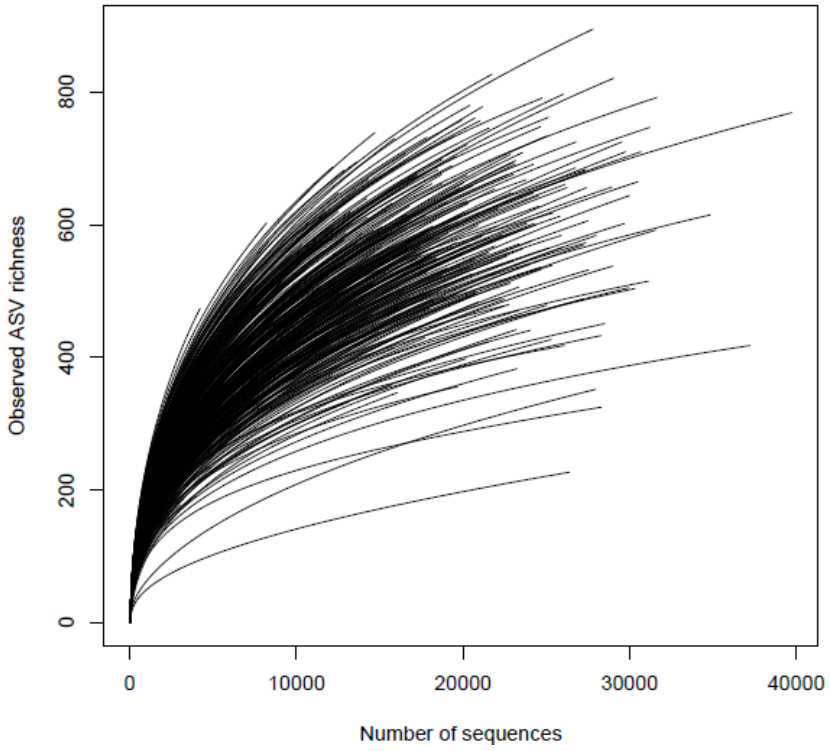

Figure 17: To evaluate the integrity of observed ASV richness, rarefaction has to be performed. It shows the expected species accumulation and estimates the number of sequences in one sample. (Oksanen et al. 2013)

Observed Species Richness

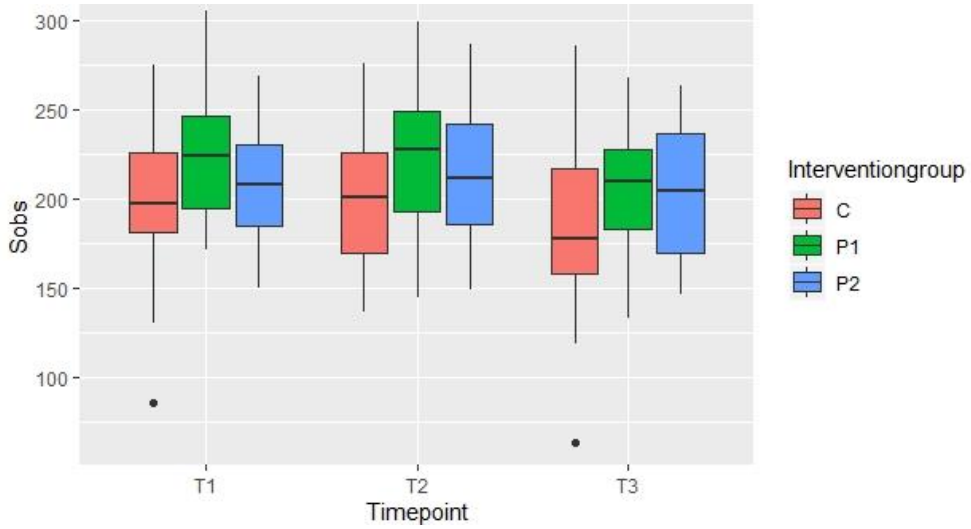

Figure 18: Boxplot of observed species richness, the total number of different species per sample, for intervention groups and timepoints. S_{Obs} was significantly different between the intervention groups at timepoint T2 and T3 ($p<0.05$). The S_{Obs}-value did not change significantly for any of the intervention groups throughout the study.

S_{Obs}	T1	T2	T3	p-value
C	202±42	202±45	184±45	>0.05
P1	220±34	224±40	210±33	>0.05
P2	206±31	211±34	204±37	>0.05
p-value	>0.05	**<0.05***	**<0.05***	

Table 5: Description of observed species richness [n/sample]; mean ± SD, p-value for differences between (horizontal) and within (vertical) the groups calculated by ANOVA and post hoc test.

Observed species richness was normally distributed (Shapiro-Wilk normality test, p>0.05 for all three intervention groups) and did not differ in the intervention groups at timepoint T1 (ANOVA p>0.05). A significant difference in observed species could be assessed at timepoint three between control and P1 group (p<0.05). S_{Obs} simply describes the number of present species in a sample, which makes it very sensitive to rare species and less robust than other diversity metrics. None of the groups experienced changes in the number of species over the duration of the study. The diversity does not seem to change.

Simpson Index

Simpson index measures the probability that two individuals selected randomly from a community belong to the same species, so a high Simpson Index means low diversity and vice versa. It is calculated by the following equation: (Simpson, 1949, Hill, 1973)

$$D_1 = 1 - \sum_{i=1}^{S} p_i^2$$

i… species

p_i… proportion of species

S… number of species so that the sum of proportions of species = 1

b… base of the logarithm

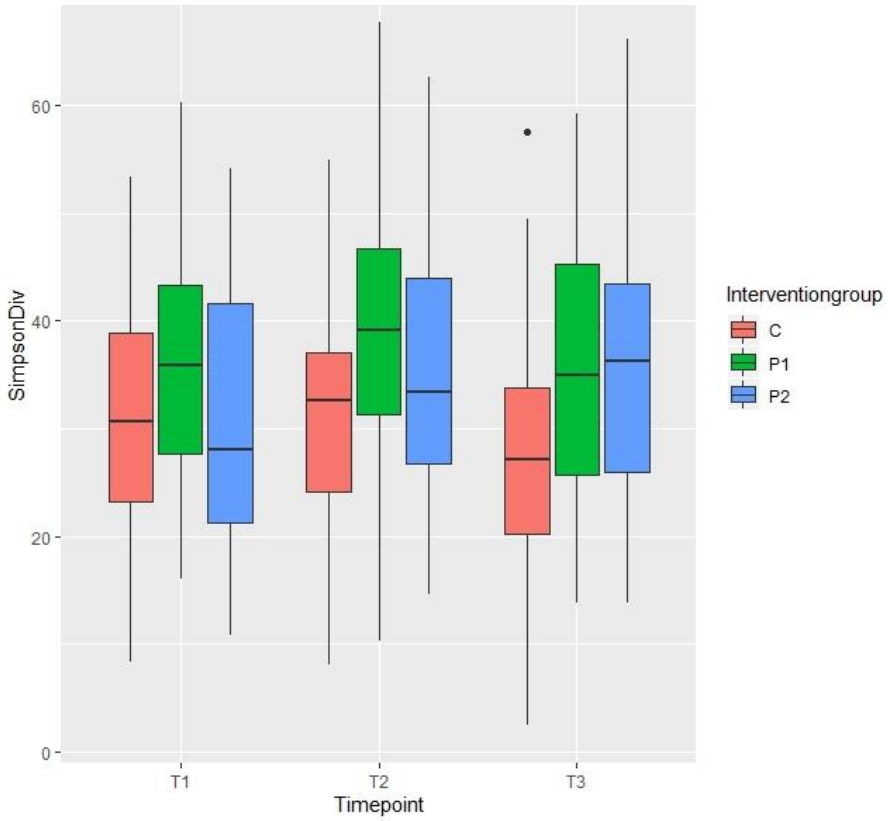

Figure 19: Boxplot of Simpson diversity for intervention groups and timepoints. Simpson diversity was significantly different between the intervention groups at T2 and T3 ($p<0.05$). It seems that P1 and P2 converged to each other, while the control group had a non-significant drop in Simpson diversity at timepoint T3.

Simpson D_1	**T1**	**T2**	**T3**	**p-value**
C	31±12	31±11	28±12	0.30
P1	36±13	39±13	36±12	0.73
P2	30±12	35±11	36±13	0.075
p-value	0.063	**<0.05***	**<0.05***	

Table 6: Description of Simpson Index; mean ± SD. p-value for differences between the groups (horizontal) and timepoints (vertical) calculated by ANOVA and post hoc test.

Simpson Index was normally distributed (Shapiro-Wilk normality test, $p>0.05$) for all three intervention groups and did not differ in intervention groups at timepoint T1. At T2, a difference could be observed between control and P1 group (ANOVA and post hoc test, $p<0.05$). At timepoint T3 the control group differed even more and not only to P1 but to both intervention groups (ANOVA and post hoc test, $p<0.01$ respectively), even though it did not significantly change over time.

Shannon Index

Shannon diversity index includes both richness and evenness of a sample and is at its minimum when there is only one species in a sample and at its maximum when all species are equally abundant. The higher the Shannon Index, the higher the overall diversity. (Hill, 1973, Oksanen, et al. 2019)

$$H = -\sum_{i=1}^{S} p_i \log_b p_i$$

i… species

p_i… proportion of species

S… number of species so that the sum of proportions of species = 1

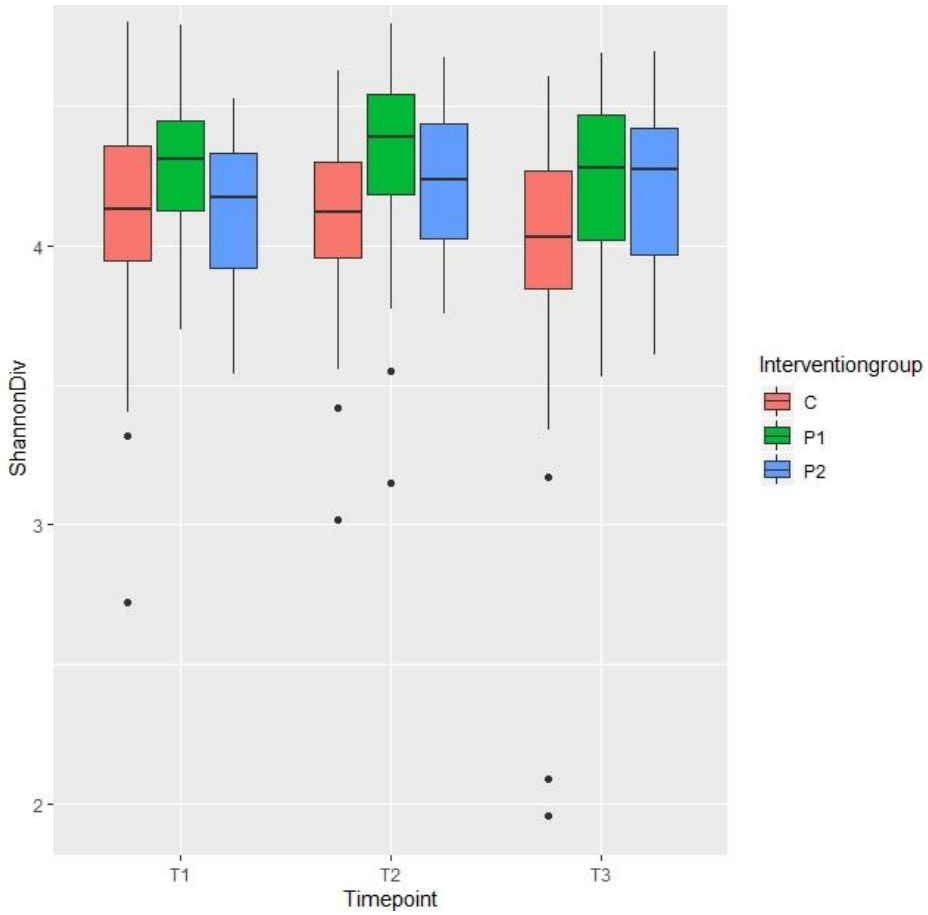

Figure 20: Boxplot of Shannon diversity for intervention groups and timepoints. Shannon Index was significantly different between the intervention groups at timepoint T1 ($p<0.05$). The value changed for the control group, while P1 and P2 adjusted.

Shannon H	T1	T2	T3	p-value
C	4.1±0.38	4.1±0.34	3.9±0.55	0.09
P1	4.3±0.27	4.3±0.35	4.3±0.29	0.70
P2	4.1±0.28	4.2±0.26	4.2±0.29	0.19
p-value	**<0.05***	**<0.05***	**<0.05***	

Table 7: Description of Shannon diversity; mean ± SD, p-value for differences between the groups (horizontal) and timepoints (vertical) calculated by ANOVA and post hoc test.

Shannon diversity was normally distributed (Shapiro-Wilk normality test, $p>0.05$ for all three intervention groups). There was a significant difference between the intervention groups at all timepoints (ANOVA post hoc test, $p<0.05$ respectively).

At timepoint T3 the control group differed even more from the other two groups. While P1 and P2 did not change, the Shannon diversity in the control group remained low.

Estimated Species Richness

S_{est}Chao1

Chao1 is an estimator of minimum richness per sample, based on abundance. It takes into account the rare species, which are only represented by either one (singletons) or two (doubletons) individual bacteria per sample. Although data has been rarefied, this estimator extrapolates to find what the true number of species may have been by estimating the likelihood to discover further species in a sample. Additionally, Chao 1 allows the conclusion, that no further species are to be found, when all species are represented by two or more individuals.

$$S_1 = S_{obs} + \frac{F_1^2}{2F_2}$$

where

S_{obs}… Number of species

F_1… Number of singletons

F_2… Number of doubletons

(Chao et al. 2014)

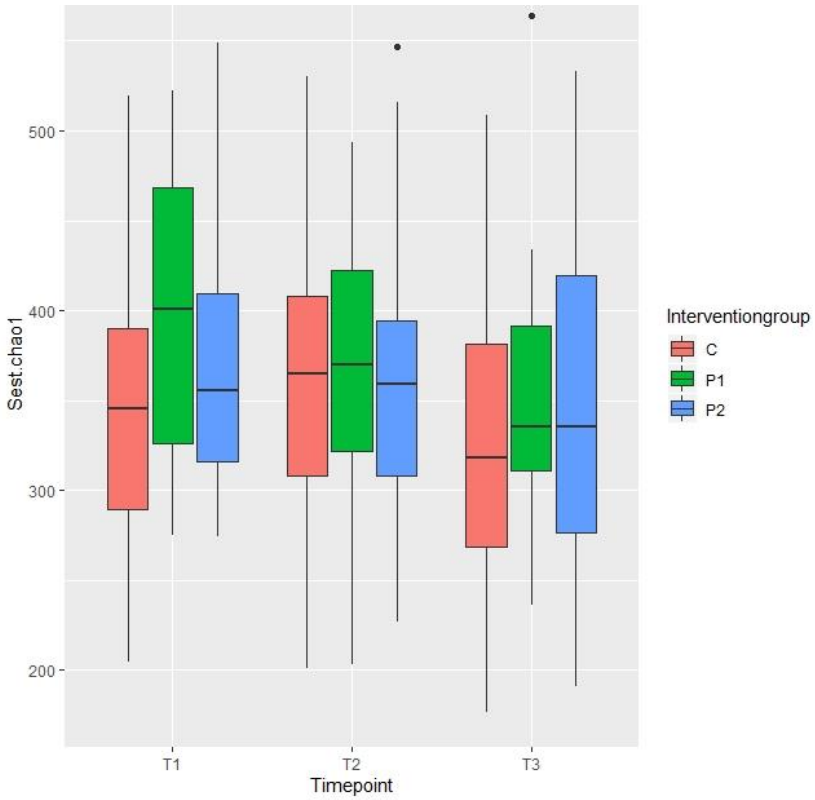

Figure 21: Boxplot of S_{est} Chao1 for intervention group and timepoint. Control group was slightly lower initially; at timepoint three no significant difference could be found.

S_{est}Chao1	**T1**	**T2**	**T3**	**p-value**
C	344±76	362±80	318±82	0.51
P1	396±75	371±71	348±65	0.16
P2	368±63	365±71	347±91	0.28
p-value	0.07	0.13	0.35	

Table 8: Description of S_{est} Chao1; mean ± SD; p-value for differences between the groups (horizontal) and timepoints (vertical) calculated by ANOVA and post hoc test.

The species richness estimator Chao1 did not change over the study period, nor did it differ between the intervention groups. This may be due to the high variation.

S_{est}ACE

The Abundance-based Coverage Estimator (*S_{est}ACE*) is defined as:

$$S_p = S_{\text{abund}} + \frac{S_{\text{rare}}}{C_{\text{ACE}}} + \frac{a_1}{C_{\text{ACE}}}\gamma^2, \quad \text{where}$$

$$C_{\text{ACE}} = 1 - \frac{a_1}{N_{\text{rare}}}$$

$$\gamma^2 = \frac{S_{\text{rare}}}{C_{\text{ACE}}} \sum_{i=1}^{10} i(i-1)a_1 \frac{N_{\text{rare}} - 1}{N_{\text{rare}}}.$$

α_1… number of species with only one individual

S_{abund}, S_{rare} … numbers of species of abundant and rare species

(O'Hara, 2005)

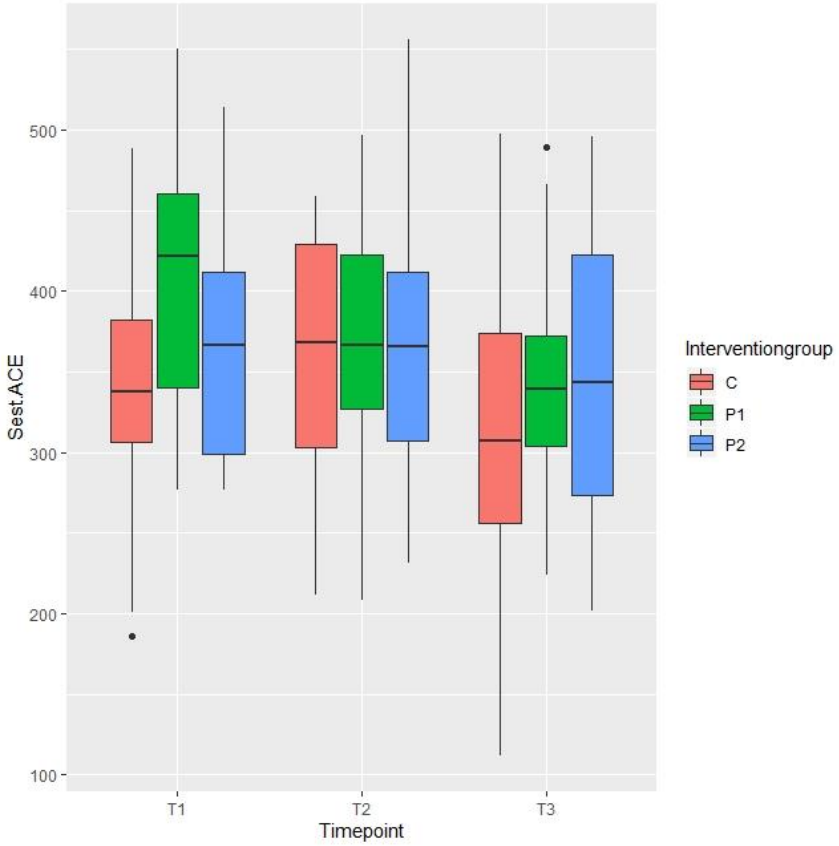

Figure 23: Boxplot of S_{est} ACE for intervention groups and timepoints. The groups predominantly differed in ACE (p=0.01) and at T3. P1 and control group changed significantly over the study period, while P2 group remained the same.

S_{est}ACE	T1	T2	T3	p-value
C	347±75	358±74	308±84	**<0.05***
P1	398±71	374±69	343±63	**<0.05***
P2	369±64	371±76	348±84	0.35
p-value	**<0.05***	0.54	**<0.05***	

Table 9: Description of S_{est} ACE; mean± SD; p-value for differences between the groups (horizontal) and timepoints (vertical) calculated by ANOVA

The ACE estimator was normally distributed (Shapiro-Wilk normality test, p>0.05 for all three intervention groups). The value was different between all intervention groups at T1 and T3 (ANOVA and post hoc p<0.05), even though the variation of the estimated richness is high. Both control and P1 group changed in the course of the study, there were significant differences within these two groups (p<0.05). The ACE estimator of the high protein group did not change significantly over the course of the study duration.

Species Accumulation Curve

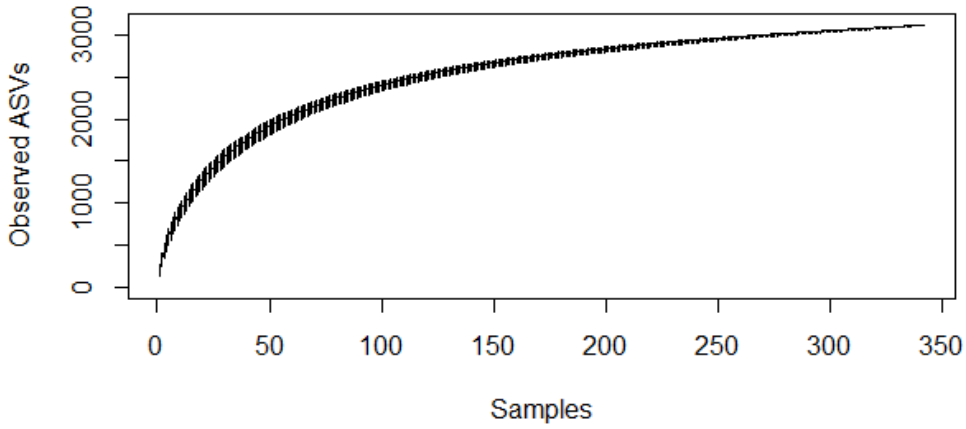

Figure 23: The species accumulation curve estimates the number of species in all sequenced samples and is an indicator for adequacy of the sequencing process. In this study, after 350 samples, approximately 3000 ASVs have been found. The plateau indicates that sequencing was successful and further investigation would not lead to a significant rise in observed ASVs. (Gotelli et al. 2011)

Principal coordinates analysis (PCoA)

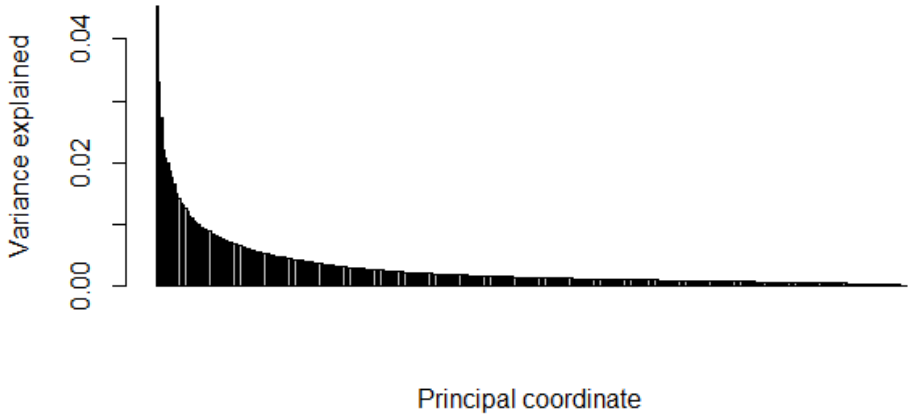

Figure 24: Variance explained by the principal coordinates. Only 4% of variance can be explained by PCoA.

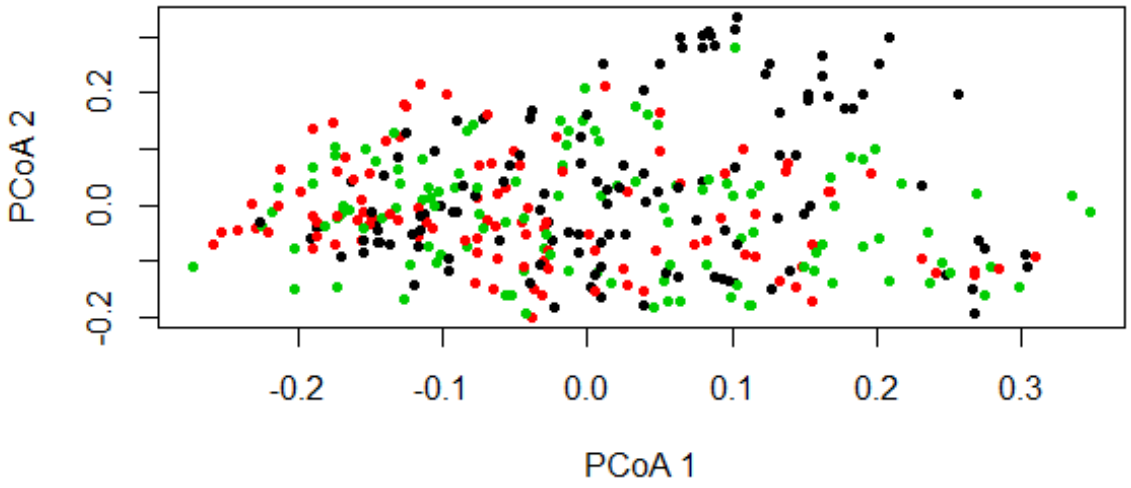

Figure 25: PCoA plot of Bray-Curtis distances of microbiota abundance for principal components 1 and 2. Black: control group, red: P1, green: P2. perMANOVA shows no significant correlation ($p>0.05$).

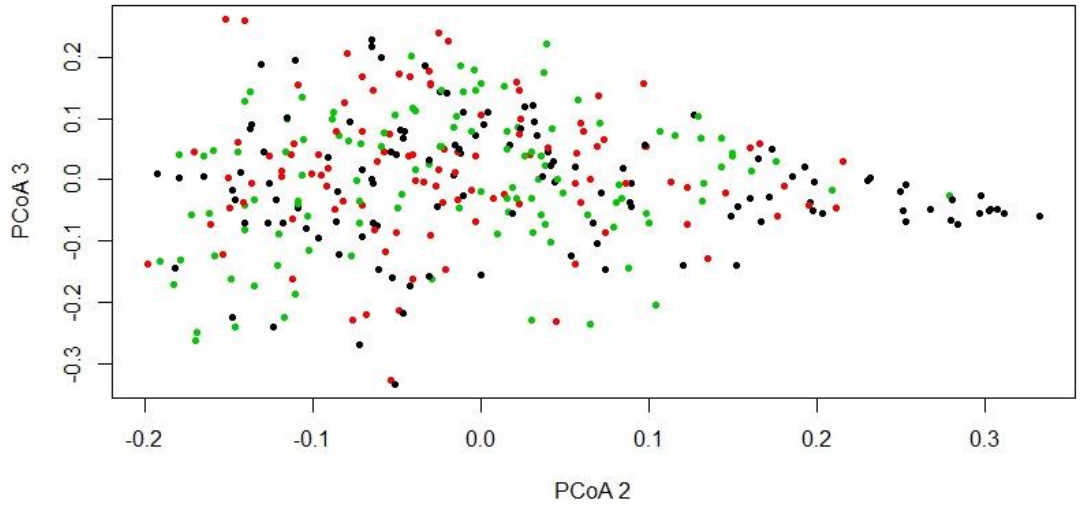

Figure 26: PCoA plot of Bray-Curtis distances of microbiota abundance for principal components 2 and 3. Black: control group, red: P1, green: P2. perMANOVA shows no significant correlation ($p>0.05$).

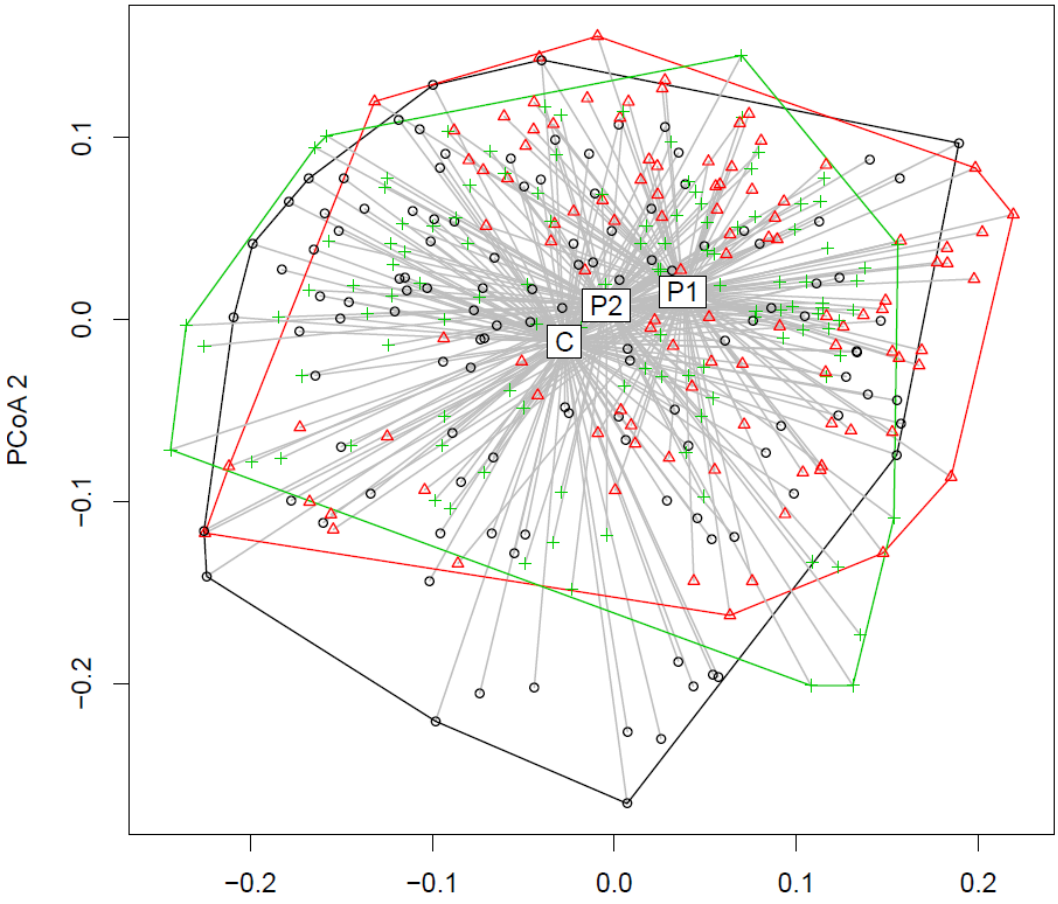

Figure 27: Homogeneity of groups. Permutation test for homogeneity (999 permutations) revealed no significant differences in variance for the intervention groups.

```
Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

                                          Df SumsOfSqs MeanSqs F.Model      R2
meta_sub$Interventiongroup                 2     1.740 0.86975 2.97286 0.01740
meta_sub$Timepoint                         2     0.405 0.20271 0.69287 0.00406
meta_sub$Interventiongroup:meta_sub$Timepoint  4     0.404 0.10099 0.34519 0.00404
Residuals                                333    97.424 0.29256         0.97450
Total                                    341    99.973                 1.00000
                                          Pr(>F)
meta_sub$Interventiongroup                 0.001 ***
meta_sub$Timepoint                         0.999
meta_sub$Interventiongroup:meta_sub$Timepoint  1.000
Residuals
Total
```

Table 10: A permuted ANOVA of β-diversity showed a significant influence of intervention group. This effect was not time dependant, and no interaction could be found.

```
Pairwise comparisons:
(Observed p-value below diagonal, permuted p-value above diagonal)
          C       P1   P2
C           0.010000 0.01
P1 0.004272          0.40
P2 0.022435 0.437094
```

Table 11: The pairwise comparison of the perMANOVA above shows a significant difference for β-diversity between control and P1 and control and P2 group ($p<0.05$). P1 and P2 did not differ ($p>0.05$).

Taxonomic diversity

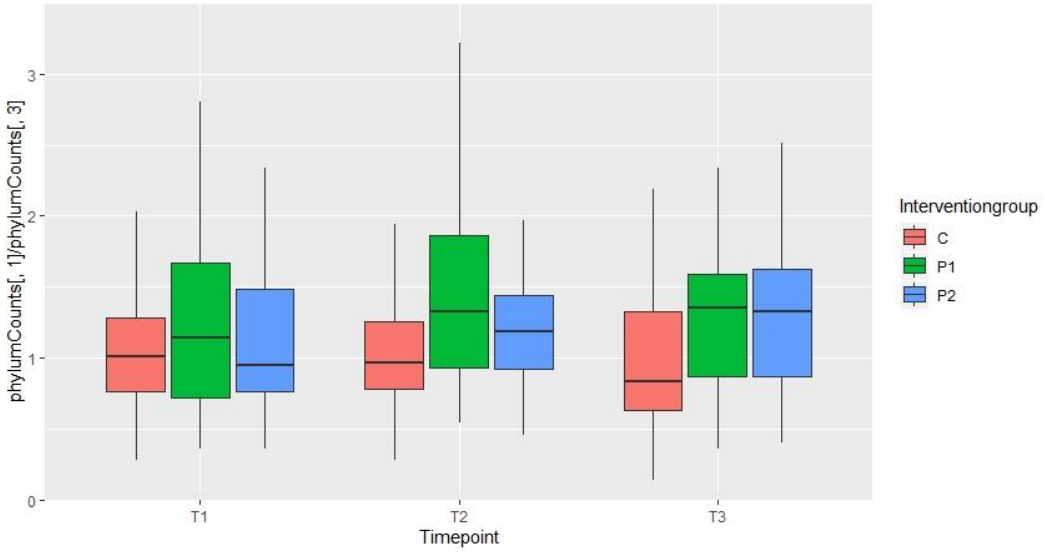

Figure 28: Ratio of Firmicutes *to* Bacteroidetes*. While the ratio did not change in the control group, and a trend showed a lower ratio at timepoint T3 in this group, there was a non-significant rise in both intervention groups.*

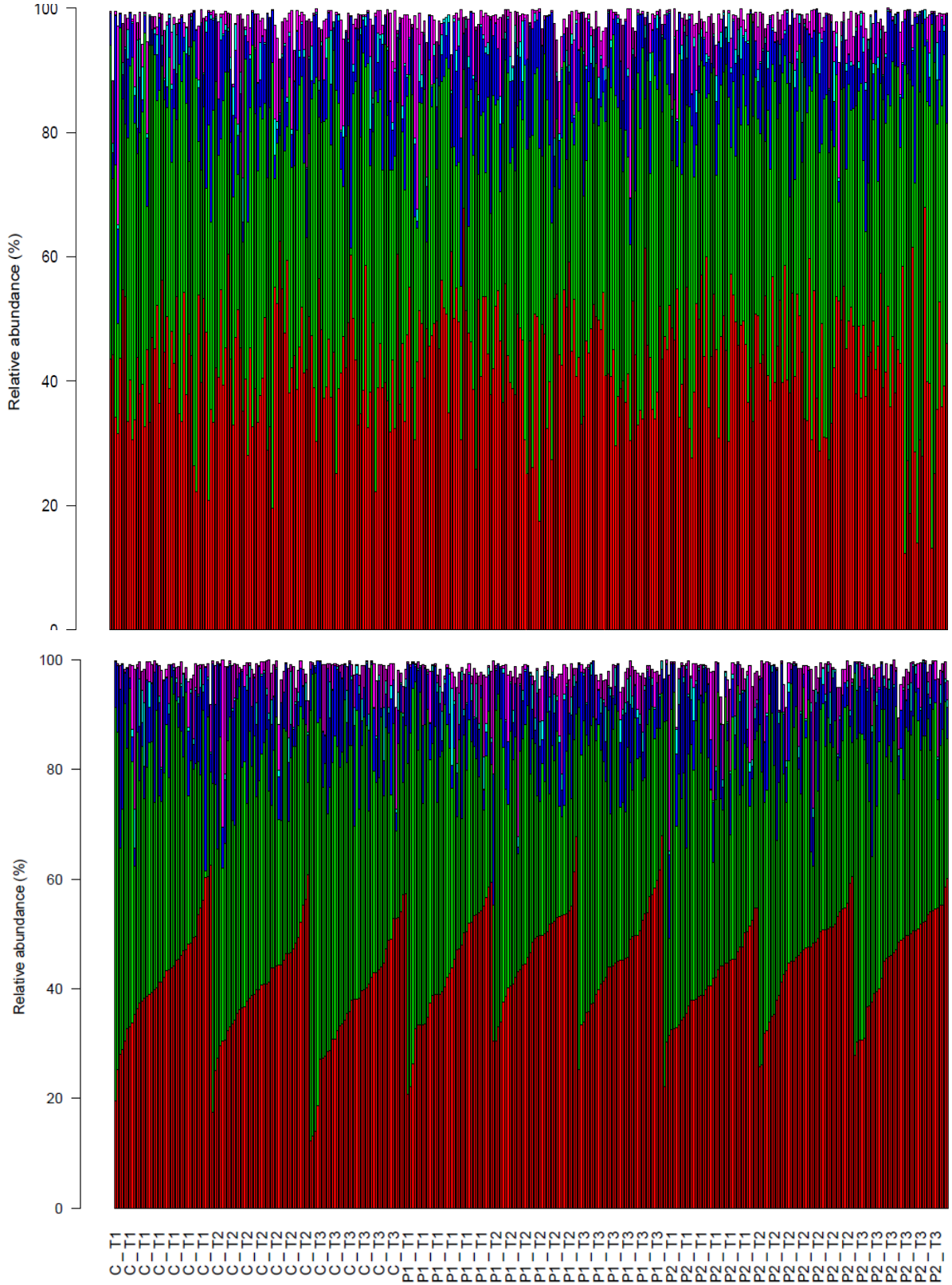

Figure 29: Barplot of phyla with more than 1% abundance. Red: Firmicutes, *green:* Bacteroidetes, *blue:* Proteobacteria, *light blue:* unclassified, *pink:* Verrucomicrobia. *The latter barplot is sorted by timepoint and intervention group and displays the individual stability over the study period.*

Redundancy Analysis

```
                              Df     AIC      F Pr(>F)
+ meta_sub$Interventiongroup   2 -1215.9 2.3807  0.005 **
+ meta_sub$Timepoint           2 -1212.5 0.6968  0.990
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Step: data_sub_RA ~ meta_sub$Interventiongroup

                      Df     AIC      F Pr(>F)
+ meta_sub$Timepoint   2 -1213.3 0.7025  0.985

Call: rda(formula = data_sub_RA ~ meta_sub$Interventiongroup)

               Inertia Proportion Rank
Total         0.0285565  1.0000000
Constrained   0.0003955  0.0138508    2
Unconstrained 0.0281610  0.9861492  339
Inertia is variance

Eigenvalues for constrained axes:
      RDA1       RDA2
0.00025750 0.00013803

Eigenvalues for unconstrained axes:
      PC1       PC2       PC3       PC4       PC5       PC6       PC7       PC8
0.0025541 0.0022169 0.0021787 0.0010835 0.0010688 0.0008713 0.0008010 0.0007818
(Showing 8 of 339 unconstrained eigenvalues)
```

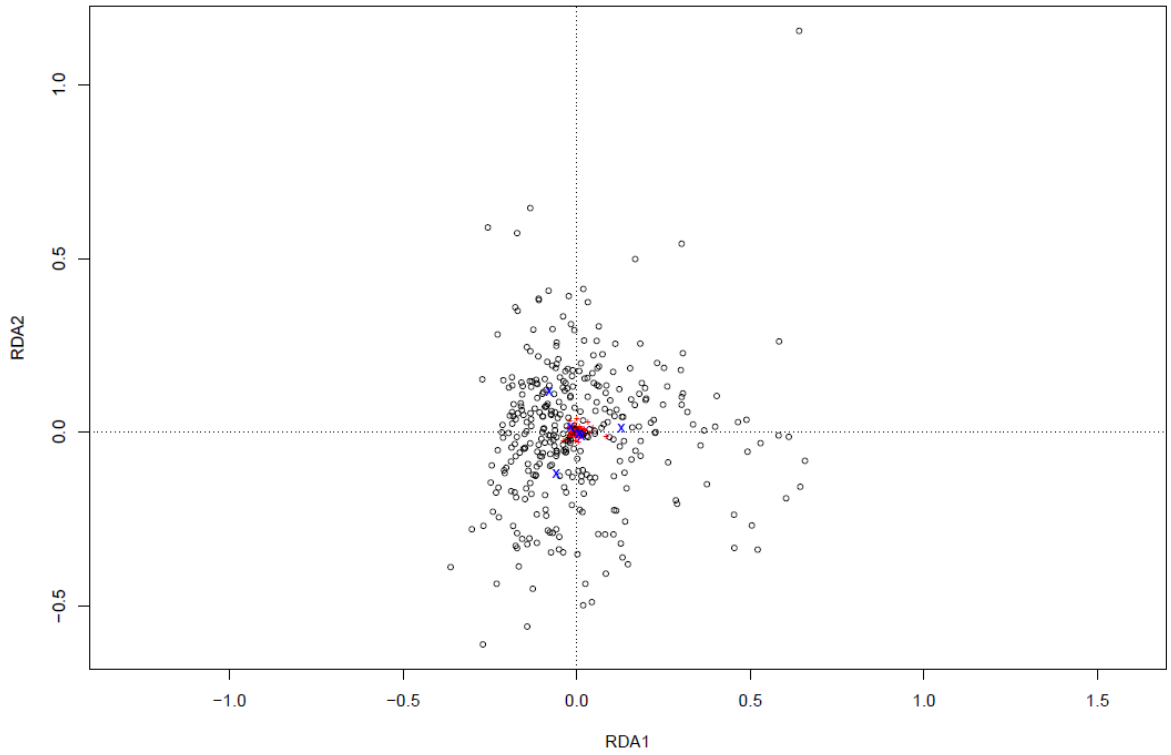

Figure 30: The redundancy analysis reduces the data on principal components. There is a large proportion of unconstrained variation, indicating only a small part of the variation can be explained by the principal components. This is confirmed by the RDA plot.

Analysis of Similarity

```
Call:
anosim(x = data_sub_RA, grouping = meta_sub$Interventiongroup,      distance = "bray")

Dissimilarity: bray

ANOSIM statistic R: 0.02567
      Significance: 0.001

Permutation: free
Number of permutations: 999
```

The factor of the significant principal components from the redundancy analysis turned out to be rather small, at 0.026. A significant effect on diversity can be ruled out.

Hierarchical cluster analysis:

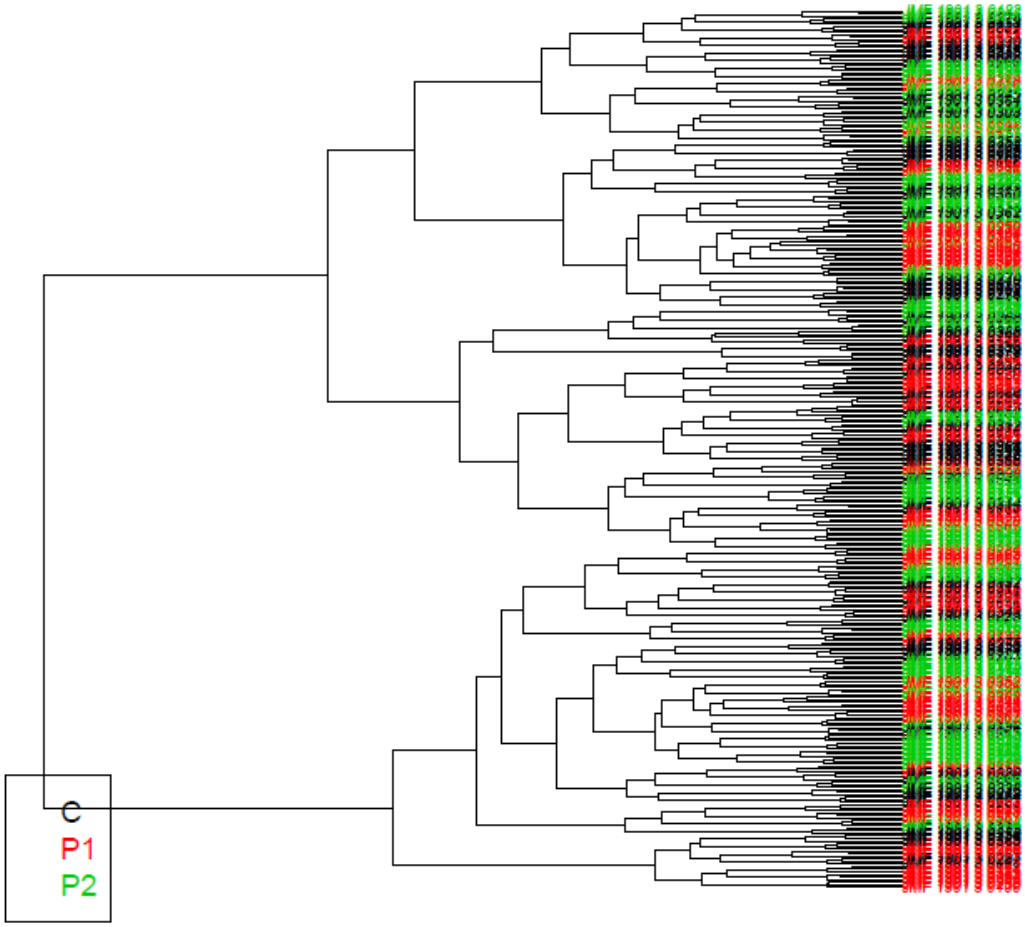

Figure 31: Cluster analysis; intervention groups are represented by colours. No apparent clusters were formed.

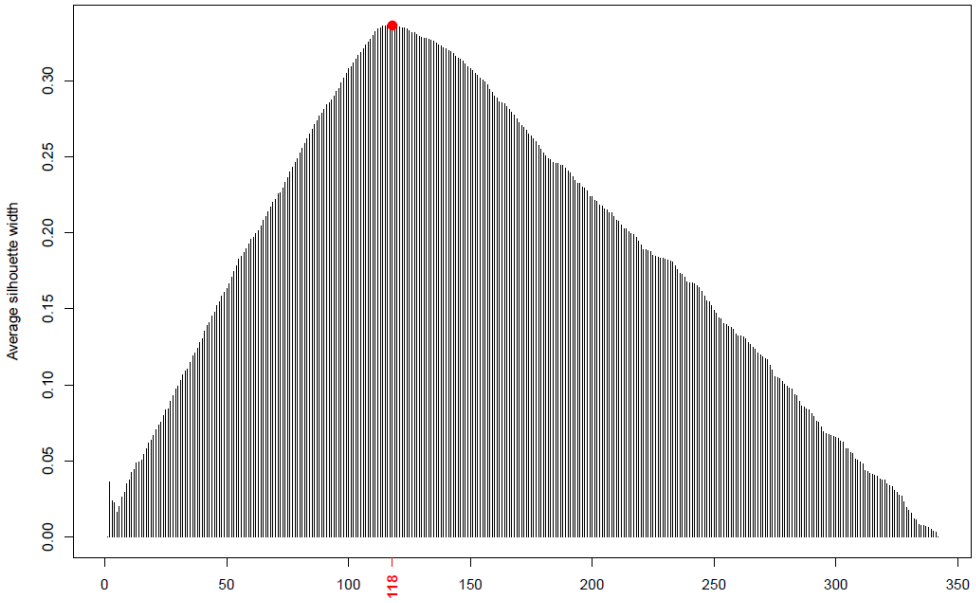

Figure 32: The absence of clusters is confirmed by the optimum calculated number of clusters, 118, calculated by the Silhouette method.

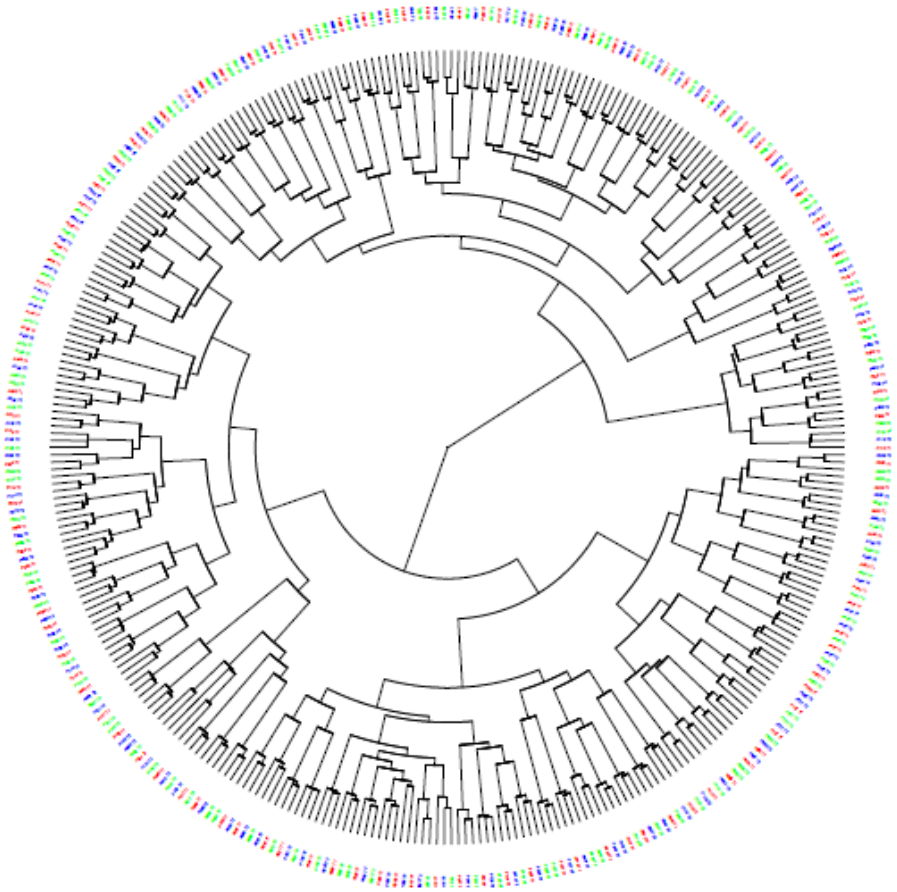

Figure 33: In a different visualization of the cluster analysis, the participants are represented by the outside circle. The conformity of the number of clusters (118) with the number of participants (117) suggests that the participants cluster with themselves at the different timepoints.

Discussion and Conclusion

The results from this thesis are part of the NutriAging study which will continue to collect data to raise understanding of elderly adult's special needs. The population investigated in this study was representative for the elderly, urban population in Austria. The allocation to the intervention groups was randomised successfully in terms of sex, age and BMI distribution (see table 2). Study participants followed the protein intervention effectively ($p<0.01$, as seen in figure 13).

The applied method for this study, next generation sequencing, proved sufficient for providing answers, as described by the variable sequencing depth (see figure 16). A rarefaction curve (figure 17) and the analysis of sequencing depth (table 4, smallest sample with 1658 reads) ensured adequate sequencing.

Diversity was measured by common diversity metrics (observed species richness: Shannon Index, Simpson Index, estimated species richness: Chao1 Index, ACE Estimator). The calculated diversity metrics for observed and estimated species richness acted similar in the course of the study.

Simpson diversity did not change significantly within the groups in the course of the study. There was a non-significant drop in the control group at timepoint T3. The difference between the intervention groups was significant at all timepoints, but that difference did not change between the timepoints (figure 19).

The same applied for Shannon Index. The groups had a different index at all three timepoints, but Shannon index did not follow a certain direction in the course of the study. They remained equally different. As for Simpson index, a non-significant drop in the control group could be assessed (figure 20)

The estimated species richness, as described by Chao1 and ACE had a high variation in all three groups, hindering predictions. Chao1 was the same for all three intervention groups at all timepoints. ACE estimator went through significant changes throughout the study period, especially for the low protein group, which was high at timepoint T1 and experienced a significant drop at T3. All three groups were lower at timepoint T3 than initially (statistically significant for control and P1 group, figures 21 and 23).

A principal coordinates analysis resulted in only 4% of explained variance, indicating numerous factors driving microbial changes (see figure 22). The intervention alone was not sufficient to explain changes (figures 24 and 25).

A permuted ANOVA with 999 permutations came to similar results. The factor intervention group had a significant influence on overall β-diversity. This was the case for the control group, which differed from P1 and P2. This effect was not time dependent, nor interactive, indicating no impact of the protein intervention or muscle strength training throughout the study period. (figure 26, table 9). The redundancy analysis confirms that result. The large proportion of unconstrained variation demonstrates that only a small part of the variation can be explained by the principal components. An R-value of 0.026 rejects a significant effect on diversity by the principal components.

F/B-ratio did rise in both intervention groups, with a simultaneous drop in the control group. Neither of the effects within the groups were significant but could lead to the assumption of a trend if a longer period of time was assessed. At all three timepoints, the differences between the groups were significant. The control group had a higher count of *Bacteroidetes* while the intervention groups counted more *Firmicutes* ($p<0.05$). A rise in the F/B-ratio would indicate a negative effect of the intervention but needs to be interpreted with caution and compared to other variables, especially BMI and overall diversity. (Koliada et al. 2017)

The hierarchical cluster analysis confirmed cited literature, stating that microbiota composition is in a stable state individually (figure 28, 30). The calculated cluster number of 118 aligns with the number of study participants, indicating that individuals cluster with themselves over the timepoints. This is not surprising, considering a stable individual state, which was clearly not weakened by the protein intervention.

By comparison of the different intervention groups over the duration of the study, an effect of high protein intake on the composition of microbiota is unlikely. The investigated diversity metrics, both observed and estimated, do not seem to follow a clear pattern. That leads to the conclusion that the microbiota are not significantly influenced by protein intake or muscle strength training, neither positively nor negatively. Comparisons with other, similar investigations would give an interesting insight in the microbiome's changes when facing different amounts of protein as a substrate. Of special interest would be an examination over a longer period of time, on one hand to exclude the effect of seasonal

changes of the microbiome, on the other hand to detect long-term changes of the microbiome. Comparison with data from younger individuals would give interesting insights in changes of the elderly adult's microbiome.

Ma and colleagues stated in a review from 2017 that an excessive amount of protein entails in several negative effects through putrefactive fermentation, which might increase the risk for infections and bowel diseases, as well as growth of pathogenic bacteria, such as *Bacteroides* or *Clostridium*. A surplus of fermentable protein reaching the colon should be avoided, according to the authors. The necessary amount of protein leading to excessive amounts of fermentation products in the colon is not clearly defined, but depends on several criteria, such as the amount of carbohydrates, digestibility and bioavailability of protein and individual differences. (Ma et al. 2017) No negative effects of a protein intake up to 2 g/kg BW/d were assessed in this thesis.

Seasonal shifts in the microbiome were examined by Davenport and colleagues in an investigation from 2014. Even though a certain individual stability could be assessed in study participants, seasonal variations were detected on population level, concerning certain taxa (especially *Bacteroidetes* and *Firmicutes*) and changes in overall diversity measured by Shannon diversity. This was investigated in a population that lived off farming and whose diet was highly dependent on seasonal harvest, thus ensuring a similar diet across individuals. (Davenport et al. 2014) The NutriAging study occurred from July to December, where seasonal differences in diet and lifestyle, e.g. outdoor activity, or physical activity in general, must be considered. As the NutriAging study is ongoing, investigations in this field, such as comparisons between study populations over the course of a year, might provide interesting results.

Considering that the individual microbiota composition has a reduced stability in elderly adults (Salazar et al. 2014), changes due to different factors (diet, physical activity, season, etc.) might be easier to detect, as they have a bigger impact at unstable conditions.

Investigation of the study participants, sequencing and bioinformatic data processing generated a vast amount of data. This thesis provides an overview of the gained material and represents a preliminary evaluation. Investigation of the microbiome by analysing data over a longer period of time, including seasonal changes and a younger study population might deliver more insights. Additionally, the difference between men and

women in combination with data from food frequency questionnaires would be further interesting factors.

The protein intervention of 1 and 2 g/kg BW/d and muscle strength training did not significantly influence the microbial diversity in the participants of this study. Considering the numerous advantages of a high protein content in the diet, especially for elderly adults, as discussed in the literature research, the results from this thesis further justify an elevation of the protein recommendations, since no negative effects could be detected.

Abstract

Aim: The significance of healthy aging rises alongside the increment of the elderly population itself. An adequate diet and an active lifestyle can prolong the years spent in health. The aim of this thesis, as part of the NutriAging study, focuses on the effects of an elevated protein intake and strength training in elderly on composition and diversity of the human gut microbiome, which has great influence on host health.

Material and Methods: A blinded, randomised, controlled trial with two intervention and one control group, including 117 subjects aged 73 ± 5years (mean ± SD), was conducted over a period of 15 weeks to observe the effects of different amounts of protein and strength training on diversity and composition of the microbiome. For that purpose, the intervention groups were provided with a diet containing either 1.0 (Protein Low) or 1.6 (Protein High) g protein/kg BW/d and received guided strength training twice a week, while the control group received no intervention and was observed only. Stool samples were collected at three timepoints to extract bacterial DNA which was then identified with next generation sequencing methods, via Illumina sequencing and PCR-analysis. After bioinformatic processing of the gained data, statistical analysis was performed using R.

Results: Diversity was measured by common diversity metrics. Neither observed species richness (Shannon Index, Simpson Index) nor estimated species richness (Chao1 Index, ACE Estimator) were significantly affected by the protein or training intervention. A principal coordinates analysis resulted in 4% of explained variance indicating microbial changes are driven by numerous factors. The factor intervention group had significant impact on β-diversity, but the effect was not time-dependent indicating no influence of the intervention. A trend was observed concerning *Firmicutes/Bacteroidetes*-ratio, which was higher in the intervention groups compared to the control group. This effect did not change over time and needs to be compared to other variables, such as BMI or overall diversity, to confirm an effect of the intervention. Overall, microbiota composition remained stable throughout the study, as confirmed by a cluster analysis.

Conclusion: Composition and diversity of the human gut microbiome have significant effects for the host, and determine factors of inflammation, metabolism, aging and wellbeing. Since the significance of the human gut microbiome rises, and the effect of a high protein intake is not fully clarified, further research on this topic could help to express recommendations, not only for the elderly, but for the adult population. The results from

this thesis indicate no effects of the amount of protein (0.8-2.0 g/kg BW/d) on the diversity of the microbiome. Neither positive nor negative impacts could be detected, further supporting the elevation of protein recommendations for elderly adults as it contributes to healthy aging.

Zusammenfassung

Ziel der Arbeit: Mit dem Anstieg der Anzahl an älteren Personen in der Bevölkerung, steigt auch die Relevanz des „gesunden Alterns“. Angemessene Ernährung und ein aktiver Lebensstil können die Lebensjahre in Gesundheit verlängern. Das Ziel der NutriAging Studie ist es, Faktoren zu finden, welche nicht nur die Lebens-, sondern vor allem die Gesundheitserwartung beeinflussen, während sich diese Arbeit dabei auf den Effekt einer erhöhten Protein-Aufnahme und Krafttraining bei älteren Personen auf die Zusammensetzung und Diversität des Darm-Mikrobioms fokussiert.

Material und Methoden: Grundlage der Arbeit war eine verblindete, randomisierte, kontrollierte Studie mit zwei Interventions- und einer Kontrollgruppe mit insgesamt 117 Personen im Alter von 73 ± 5 Jahren (MW ± SD) über eine Dauer von 15 Wochen. Die Intervention beinhaltete eine erhöhte Proteinaufnahme von 1.0 (Protein Low) oder 1.6 (Protein High) g/kg KG/d und zusätzlich angeleitetes Krafttraining zweimal wöchentlich, während die Kontrollgruppe lediglich beobachtet wurde und ihre Lebens- bzw. Ernährungsgewohnheiten nicht veränderte. Zu drei Zeitpunkten wurden Stuhlproben gesammelt, welche verwendet wurden, um die bakterielle DNA mit Next Generation Sequencing Methoden zu identifizieren. Hierfür wurde Illumina Sequencing und PCR-Analyse verwendet. Nach bioinformatischer Datenaufbereitung konnten durch die Programmiersprache R statistische und grafische Auswertungen erfolgen.

Ergebnisse: Die Diversität der Proben wurde durch Kennzahlen aus dem Bereich der mikrobiellen Ökologie berechnet. Weder die beobachtete (Shannon Index, Simpson Index) noch die geschätzte (Chao1 Index, ACE Estimator) Diversität wurden durch die Intervention signifikant beeinflusst. Eine PCoA führte, bei 4% erklärter Varianz, zu dem Schluss, dass eventuell auftretende Veränderungen des Mikrobioms auf zahlreiche Einflussfaktoren zurückzuführen sind. Der Faktor Interventionsgruppe hatte einen signifikanten Einfluss auf die β-Diversität, jedoch war dieser Effekt nicht zeitabhängig, was die Intervention als Ursache ausschließt. Ein Trend konnte hinsichtlich des *Firmicutes/Bacteroidetes*-Verhältnisses beobachtet werden, welches in den Interventionsgruppen höher war, als in der Kontrollgruppe. Dieser Umstand veränderte sich im Laufe der Studie nicht, und sollte demnach mit weiteren Variablen verglichen werden, wie BMI oder der Gesamt-Diversität, um einen Effekt der Intervention zu

bestätigen. Die Zusammensetzung der Darmbakterien blieb im Laufe der Studie bei allen Teilnehmern stabil, was auch durch eine Cluster Analyse bestätigt wurde.

Conclusio: Die Zusammensetzung und Diversität des Darmmikrobioms haben signifikante Auswirkungen für den Menschen und beeinflussen Entzündungsprozesse, Metabolismus, Alterung und Wohlbefinden. Die Signifikanz des Mikrobioms bei diesen Prozessen wird erst seit Kurzem erkannt, und die Auswirkungen einer hohen Proteinaufnahme ist noch nicht hinreichend geklärt. Weitere Forschung auf diesem Gebiet ist essenziell für die Formulierung von Empfehlungen, nicht nur für die ältere, sondern für die gesamte erwachsene Bevölkerung. Die Ergebnisse dieser Arbeit deuten auf keinen Effekt der Menge an Protein (0.8-2.0g/kg KG/d) auf die Diversität des Darm-Mikrobioms hin. Weder positive noch negative Auswirkungen konnten gefunden werden, was auch für die Erhöhung der Protein-Empfehlungen für ältere Personen spricht.

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