

Dissertation

**Energy Sensing and Eating Behavior
in Patients with Anorexia Nervosa, Athletes, Obese, Overweight
and Normal Weight Controls: The Gut Microbiome**

submitted by
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2017

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Disclosures

Part of this thesis has been published in Mörkl S, Lackner S, Müller W, Gorkiewicz G, Kashofer K, Oberarscher A, et al. *Gut microbiota and body composition in anorexia nervosa inpatients in comparison to athletes, overweight, obese and normal weight controls*. International Journal of Eating Disorders (1).

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Foreword

„Ich kann mir kein seligeres Wissen denken, als dieses Eine: dass man ein Beginner werden muss. Einer, der das erste Wort schreibt hinter einem jahrhundertelangen Gedankenstrich.“ (Rainer Maria Rilke)

The gut microbiome was long forgotten in modern western medicine. Two thousand years ago, it was already known in Traditional Chinese Medicine that the gut is closely connected to emotions and immune-regulation. In our western world, the concept of the gut is widely integrated in our daily language (“gut feelings”, “gut instinct”, “it takes guts”). However, this knowledge just recently come back into focus of modern western medicine as scientists have begun to explore the influence of the gut on modulating appetite, the immune system and brain functions.

In this dissertation the role of the gut microbiome in a life threatening psychiatric disorder, anorexia nervosa, is investigated. Anorexia nervosa is a severe psychiatric illness with a wide range of comorbidities and a narrow field of treatment possibilities. At the beginning of this project in 2013 it was not known whether the gut microbiome is altered in anorexia nervosa compared to healthy controls. In the meantime, there have been a small number of studies which have indicated that gut microbial composition of anorectic patients is in fact severely different compared to normal weight controls. However, has been no study thus far which has focused on assessing microbial features between different body mass index groups including anorexia nervosa patients and a group of athletes. Along with gut microbial composition, we investigated many possible influence factors on the gut microbiome, such as physical activity, anthropometric measurements, subcutaneous adipose tissue thickness and dietary patterns. This basic scientific research is essential for furthering the understanding of the pathophysiology of the disease, indicating the urgent need for more longitudinal interventional studies and providing the scientific background which is needed in order to expand the treatment possibilities for anorexia nervosa and other forms of over- and undernutrition.

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Language is a very important tool to transmit research and without proper communication knowledge would never be able to spread and reach fields where it is needed. Therefore, I want to express my gratitude to **Prof. Peter Holzer** for proof-reading our research articles, to **Trent Haigh** for teaching me the subtleties of the English language and to **Prof. Wolfram Müller** for introducing me to the philosophy of science.

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Abbreviations and Definitions

5-HT	5-hydroxytryptophan, serotonin
ACC	Anterior cingulate cortex
AN	Anorexia nervosa
AT	Athletes
B.	Bifidobacterium
BBB	Blood brain barrier
BCM	Body cell mass
BDD	Body dysmorphic disorder
BDI	Beck Depression Inventory
BDNF	Brain derived neurotrophic factor
BIA	Bioimpedance analysis
BMI	Body mass index
bp	Base pairs
CCK	Cholecystokinin
CNS	Central nervous system
CRP	C-reactive protein
CVs	Coefficients of variation
D _{INCL}	Sum-Score of subcutaneous adipose tissue measurements
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical Manual of Diseases
ECM	Extracellular water
EEC	Enteroendocrinic cells
ELISA	Enzyme-linked immunoassay
ENS	Enteral nervous system
FB-Ratio	Firmicutes/Bacteroidetes Ratio
FDR	False discovery rate
FFA	Free fatty acids
FFM	Fat free Mass
FM	Fat Mass
FTND	Fagerström Test for Nicotine Dependence
GABA	Gamma-aminobutyric acid
GBA	Gut-brain-axis
GI	gastrointestinal
GLP-1	Glucagon-like-peptide 1
GM	Gut microbiota
HAMD	Hamilton Inventory for Depression
HDL	High density lipoprotein
HMP	Human Microbiome Project
HPA	Hypothalamic pituitary adrenal axis
IASMS	International Association of Sciences in Medicine and Sports
ICD-10	International classification of diseases
IgA	Immunglobulin A
IL	Interleukin

IPAQ	International physical activity questionnaire
ISAK	International Society for the Advancement of Kinanthropometry
KEGG	Kyoto Encyclopedia of Genes and Genomes
L.	Lactobacillus
LBM	Lean Body Mass
LCFA	Long Chain fatty acids
LDA	Linear discriminant analysis
LDL	Low Density Lipoprotein
LEfSe	Linear discriminant analysis effect size
LOQ	Limit of quantification
LPS	Lipopolysaccharides
LPS	Microbiota derived lipopolysaccharide
m	Body mass
M.	Methanobacter
MET	Metabolic Equivalent of Task
NCBI	National Center for Biotechnology Information
nt	Number of nucleotides
NW	Normal weight
OB	Obese
OTU	Operational Taxonomic Unit
OW	Overweight
PCoA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PICRUS _t	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PYY	Peptide YY
Q	Phred Quality Score
QIIME	Quantitative Insights into Microbial Ecology
R	Resistance
RNA	Ribonucleic acid
S	Svedberg-Unit
SAT	Subcutaneous Adipose Tissue
SCFA	Short chain fatty acid
Sp.	Species
SSRI	Selective serotonin reuptake inhibitor
TAG	Triacylglycerine
TBF	Total Body Fat
TBW	Total Body Water
TCA	Tricyclic antidepressant
TLR4	Toll-like receptor 4
WHO	World Health Organization
WHR	Waist-hip-ratio
WHtR	Waist-height-ratio
X _c	Sum of membrane capacities

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Zusammenfassung

Einleitung: Anorexia nervosa (AN) ist eine heterogene Essstörung mit weitreichender klinischer Relevanz und ausgeprägten Einflüssen auf die Körperzusammensetzung. Das Darmmikrobiom ist essentiell für die Energiegewinnung aus der Nahrung und beeinflusst die Verdauung, das Sättigungsgefühl und das Körpergewicht. In diesem Zusammenhang kann eine bakterielle Fehlbesiedelung im Darm zur Pathogenese von Erkrankungen wie AN beitragen. Das Ziel dieser Arbeit ist es Korrelationen des Darmmikrobioms, der Körperzusammensetzung, körperlicher Aktivität, Ernährung, Depressionsskalen und Laborparametern in einer großen Gruppe von Patientinnen mit AN sowie Frauen mehrerer body mass index (BMI)-Gruppen und Athletinnen darzustellen.

Methoden: In dieser Querschnittsstudie wurde das Darmmikrobiom bei 106 Personen, darunter 18 AN-Patientinnen, 20 Athletinnen, 26 normalgewichtigen, 22 übergewichtigen und 20 adipösen Teilnehmerinnen erhoben. Bakterielle DNA wurde aus Stuhlproben isoliert und die bakterielle Komposition wurde durch 16SrRNA Sequenzierung bestimmt. QIIME, eine bioinformatische Pipeline, wurde verwendet um die Ergebnisse zu analysieren. Die Teilnehmerinnen füllten Fragebögen zu Diät, körperlicher Aktivität und Depressionssymptomen aus. Darüber hinaus erfolgten anthropometrische Messungen, Messungen der Körperzusammensetzung, des subkutanen Fettgewebes und die Erhebung von Laborwerten (Serumlipide, Entzündungsmarker und Marker der Darmbarriere (Zonulin)).

Ergebnisse: AN Patientinnen und adipöse Teilnehmerinnen hatten im Vergleich zu den anderen Gruppen eine signifikant verminderte Alpha-Diversität, während Athletinnen die höchste bakterielle Artenvielfalt aufwiesen. Bakterielle Gemeinschaftsstrukturen wiesen signifikante Assoziationen zu Körperfettparametern, Serumlipiden, C-reaktivem Protein (CRP), Depressionsskalen und Rauchen auf. Die Familie der Coriobacteriaceae war im Vergleich zu den anderen Gruppen deutlich erhöht bei AN-Patientinnen.

Diskussion: Diese Studie unterstreicht Veränderungen des Darmmikrobioms bei AN, zeigt Unterschiede zwischen BMI Gruppen auf und beschreibt Korrelationen des Darmmikrobioms, anthropometrischen Messungen, der Körperzusammensetzung, der

Körperfettverteilung, Entzündung und Depression. Diese Erkenntnisse stellen die Grundlage dar um die Standardtherapie bei AN durch Beeinflussung des Darmmikrobioms zu erweitern und zu verbessern.

Schlüsselwörter: body mass index, Darmmikrobiom, anorexia nervosa, Essstörungen, Darm-Gehirn-Achse, Ultraschall, Depression.

Abstract

Background: Anorexia nervosa (AN) is a heterogeneous eating disorder with growing clinical relevance and a distinct impact on body composition. The gut microbiota is essential for the energy harvest from the diet and might therefore have an influence on digestion, satiety and body weight. In this context, gut dysbiosis might contribute to the pathogenesis of AN. The aim of this work is to investigate correlations between the gut microbiota, body composition, physical activity, diet, depression measures and laboratory parameters in a large female cohort divided into different body mass index (BMI) groups, AN patients and normal weight athletes (AT).

Methods: This cross-sectional study investigated the gut microbiome of 106 female participants (AN patients (n=18), AT (n=20), normal weight (n=26), overweight (n=22) and obese women (n=20)). Genomic DNA was isolated from stool samples and bacterial composition was characterized by sequencing of the 16S rRNA gene. QIIME, a bioinformatic pipeline, was used to analyze data. Participants completed questionnaires about diet, physical activity and depression. Furthermore, anthropometric measurements, assessments of body composition and subcutaneous adipose tissue and laboratory parameters (serum lipids, markers of inflammation and the gut barrier marker zonulin) have been performed.

Results: AN patients and obese participants had significantly lower alpha diversity compared to the other groups, while AT showed highest species richness. Bacterial community structures were significantly associated with body fat parameters, serum lipids, CRP, depression scales and smoking. The bacterial family of Coriobacteriaceae was identified as a significantly enriched phylotype in AN compared to the other groups.

Conclusion: This study demonstrates evidence of microbial alterations in AN, points out differences in the gut microbiome among BMI groups and shows correlations between the gut microbiota profile, anthropometric measurements, body composition, body fat distribution, inflammation and depression. These insights may provide new opportunities to improve the standard therapy of AN by addressing the gut microbiota.

Keywords: body mass index, gut microbiota, anorexia nervosa, eating disorders, gut-brain-axis, ultrasound, depression.

1. Introduction

Anorexia nervosa (AN) is a serious eating disorder with high rates of comorbidities and complications. It is characterized by excessive weight loss and distorted body self-perception (4). The gut microbiota is essential for the energy harvesting of food and might therefore have an influence on digestion, satiety and body weight. Up to now, the gut microbiota in AN patients and the underlying processes which have an impact on weight gain and satiety, such as gut barrier function, have not been investigated in detail and compared to profiles of other BMI-groups and athletes (AT).

In this dissertation it is attempted to answer the question whether and to what extent gut microbiota play a role in the pathogenesis of anorexia nervosa and obesity. Our results show associations of gut microbial composition, bacterial community structures, anthropometric measurements, body fat distribution, physical activity, depression scores and laboratory parameters. Furthermore, possible future directions regarding the treatment of AN are pointed out; for example, expanding current therapies with “psychobiotics”(5). Finally, a narrative review of key evidence-based studies about gut microbiota and the gut-brain-axis is presented.

1.1 Anorexia nervosa

1.1.1 Diagnostic criteria

AN can be diagnosed either by the diagnostic criteria of DSM-IV (6) or ICD-10 (7).

According to DSM IV, the diagnosis of AN relies on objective and measurable criteria (6):

1. Refusal to maintain body weight at or above a minimally normal weight for age and height (e.g. weight loss leading to maintenance of body weight at less than 85% of that expected or failure to make expected weight gain during period of growth, leading to body weight less than 85% of that expected).
2. Intense fear of gaining weight or becoming fat, even though underweight.
3. Disturbance in the way in which one's body weight or shape is experienced, undue influence of body weight or shape on self-evaluation, or denial of the seriousness of the current low body weight.
4. In post-menarcheal females, amenorrhoea, which is the absence of at least three consecutive menstrual cycles.

ICD-10 specifies the following criteria (7):

- A. Weight loss, or in children a lack of weight gain, leading to a body weight of at least 15% below the normal or expected weight for age and height.
- B. The weight loss is self-induced by avoidance of "fattening foods".
- C. A self-perception of being too fat, with an intrusive dread of fatness, which leads to a self-imposed low weight threshold.
- D. A widespread endocrine disorder involving the hypothalamic-pituitary-gonadal axis, manifest in the female as amenorrhea, and in the male as a loss of sexual interest and potency (an apparent exception is the persistence of vaginal bleeds in anorexic women who are on replacement hormonal therapy, most commonly taken as a contraceptive pill).
- E. Does not meet criteria A and B of Bulimia nervosa (F50.2).

Anorexia nervosa can be divided into two different types:

Restricting type: There is no binge-eating or purging behavior like self-induced vomiting or laxative-abuse.

Binge-eating/purging type: The patient has regularly engaged in purging or binge-eating behavior (7).

1.1.2 History

The term *Anorexia nervosa* derives from the Greek word ἀνορεξία (Greek for loss of appetite) and was first used by the French physiologist Fleury Imbert (8).

The disease was then described in two case-reports by William Gull, an English physician in 1868 (9). Other sources claim that it was first described by Morton in 1689 (10) and the French physician Lasegue in 1873 who wrote the most detailed description of AN in a case-paper entitled *De l'Anorexie hystérique* (11).

In 1971, AN was first defined as a disease identity on its own. Formerly, it had been believed to be a special form of schizophrenia, obsessive-compulsive disorder or depression (12). First studies about long-term treatment of AN were published between 1980 and 1985 (13, 14).

1.1.3 Epidemiology

For Austria, there are no specific data regarding the incidence of AN. Numbers for prevalence and incidence differ according to the country of origin, age, sex and population, and are widely heterogeneous. Overall, AN occurs in 0.9-4.3% of women and 0.2-0.3% of men in the western countries (15). Between 90-97% percent of affected patients are women (16). Women of high socioeconomic status in industrialized societies are more likely to develop AN in comparison to less industrialized countries. There are also some high-risk occupations for the development of AN like fashion modeling and dancing (17). AN mostly has its onset in teen years and young adulthood. While the overall incidence rate has remained stable over the past decades, there has been an increase in the high-risk-group of 15-19-year-old girls (15). Nevertheless, it remains unclear whether this is due to an increase of frequency or better diagnosis (18). Approximately 50% of AN patients develop a chronic and relapsing illness which is indicated by severe biopsychosocial impairment (19-21).

1.1.4 Comorbidities

AN is associated with a number of comorbidities such as personality disorders (for instance obsessive-compulsive personality disorder, borderline disorder and dependent personality disorders) (22), anxiety disorders (23), alcoholism (24), attention deficit hyperactivity disorder (25), body dysmorphic disorder (BDD) (26) and mood disorders (27). Depression and anxiety are the most common comorbid conditions. For example, anxiety disorders are present in up to 65% of AN patients (28) and the lifetime prevalence of major depression in AN patients is up to 75% (29, 30).

Mood disorders may increase vulnerability in developing an eating disorder, while in some cases comorbidities follow after the development of an eating disorder (27). Also, autism spectrum disorders occur frequently among patients with AN (31).

1.1.5 Genetics and AN

The underlying genetic mechanisms of AN are not well understood. Genetic studies show that there might be a heritability of approximately 50 to 80% (32). The function of neurotransmitters and their receptors seems to be impacted by genetic variants which alter their metabolism and function (33). Furthermore, there are a few specific genome-wide association studies in AN but unfortunately, they lack significant results due to low power (34).

1.1.6 Causes and consequences of AN

The function of a healthy brain relies on a steady nutrient source from all food groups. In AN, brain function is negatively influenced by insufficient food intake. Since AN patients avoid carbohydrates and simple sugars, neurons lack glucose, which is their sole energy source (35). Furthermore, myelin, a high-density fat which isolates neurons in the brain and permits fast neurotransmission, cannot be generated without proper fat intake. Fat and carbohydrate consumption are both restricted in AN (36). Insufficient myelination in adolescence could lead to problems with abstract thinking, executive functions and decision-making, all of which are common in AN (37). For example, Wierenga et al. pointed out that AN patients have difficulties in reward learning compared to healthy controls (38). Decreased food ingestion activates the hypothalamic area and releases orexins and melanin-concentrating hormone in the tegmental area. This leads to a

dopamine increase in the nucleus accumbens and thus the reward system is stimulated. Therefore, AN patients are rewarded following food aversion, which reinforces their decreased food intake (39). In addition, patients with AN tend to have a reduced awareness of satiety or hunger, as well as an alteration of gustatory processes (40, 41).

Proteins are an important factor for generating neurotransmitters and their receptors. AN is highly associated with protein malnutrition. In some cases, Kwashiorkor occurs: this condition is characterized by peripheral edema, hypoalbuminemia, fatty liver, skin and hair lesions, apathy, immune-depression and increased risk for infections (42). Further, serotonin and dopamine are synthesized from essential amino acids like tryptophan and phenylalanine. With a low intake of amino acids, the availability of these neurotransmitters can be altered; this affects mood and motivation (43).

Furthermore, AN patients show a number of brain abnormalities regarding size and function compared to healthy controls. Reduced thickness of the hippocampi and the anterior cingulate has been shown in patients with AN. However, these regions recover in size after weight gain (44). In a study from Connan et al. hippocampal volume was found to be reduced in AN patients, however this was not associated with functional aspects such as cognitive function (45). In another study, it was shown that the right dorsal anterior cingulate cortex (ACC) was significantly reduced in AN patients compared to healthy controls, and correlated with lower IQ-performance (46). Moreover, there may be a correlation between cortical thickness and body mass index (BMI). A study from Lavagnino et al. found reduced cortical thickness in AN, especially in the left superior parietal/occipital cortex and left post central cortex (47). Whether this is a cause or a consequence of AN still needs to be established.

1.1.7 Therapy and Treatment

Presently, there are very few effective treatments for AN available; this leads to high rates of chronicity and mortality (48, 49). The core symptoms of AN remain difficult to treat using current behavioral or psychiatric approaches (19, 50-53). Moreover, AN patients often fail to acknowledge their illness or assess it correctly and are therefore not motivated to start a therapy. So far, several psychosocial, behavioral and pharmacologic interventions have been investigated for AN, but the treatment effects found are usually small (51, 54, 55).

In Austria, a multifactorial therapy consisting of medical supervision, behavioral therapy and group therapy is used in inpatient treatment and in specialized clinics. Most AN patients require a long-term combination treatment of outpatient- and inpatient therapy (54).

Important factors of the disorder-specific therapy are the stabilization of eating behavior as well as psychotherapeutic interventions. The degree of underweight is commonly described by the BMI, which is derived from the following formula: weight, in kilograms divided by height squared, in meters (weight/height²). The normal BMI range in adults is 18.5-25 (56, 57).

If the patient reaches a critical, life-threatening level of underweight, which is defined by a BMI lower than 12, an inpatient treatment with parenteral nourishment is indicated (58). Often tube feeding devices or intravenous nourishment are used to prevent the patient from dying due to malnourishment and electrolyte imbalances.

1.1.7.1 Pharmacological therapy

Fifty percent of AN patients are treated with psychopharmacological interventions (59). Selective serotonin reuptake inhibitors (SSRI) (60), tricyclic antidepressants (TCA) (61, 62) and antipsychotics (62) are currently used in the treatment of AN, although controlled medication trials have not found significant treatment effects and further, there is a lack of supporting efficacy data. According to a study by Garner et al. antidepressants are the medication most commonly prescribed and there is a greater use of medication among adults compared to adolescents (63). Treatment recommendations suggest that pharmacotherapy should not be the first treatment for AN since empirical evidence has shown pharmacotherapy to be widely ineffective (64).

1.1.7.2 Psychotherapy

In terms of psychotherapy, cognitive behavioral interventions are recommended. Some controlled trials have indicated that it shows at least some efficacy in assisting with weight gain in AN (65, 66). A large study which compared treatment as usual, behavioral therapy and psychodynamic therapy, found psychodynamic therapy to be advantageous in terms of

12-month recovery, and cognitive behavior therapy to be more effective regarding weight gain; however, overall there were no significant differences from treatment as usual (67). Attrition rates of psychotherapy are high in patients with AN; the majority of patients remains underweight even after psychotherapeutic and pharmacological treatment (32).

In comparison to other psychiatric diseases, the advances in treatment effectiveness in AN are low and there have been few randomized controlled trials. The lack of response to available treatment regimens emphasizes the need of new treatment models and improving understanding of the underlying factors of AN in order to target its symptomatology more specifically.

1.3 Microbiota and microbiome

“*We are not individuals, we are colonies of creatures.*”

(Dr. Bruce Birren, Genome sequencing and analysis program, MIT, Boston)

In recent years, the composition of human germs, which includes diverse microbial communities living in and on the body, their genetic material and their interactions with the environment have become a flourishing area of study.

Macroscopic hosts exist alongside and symbiotic with commensal microorganisms. Human symbionts outnumber our body cells by a factor of 10 and express 10 fold more genes than the genome of their host (68). Microbial communities living in and on our body include bacteria, viruses, fungi and other eukaryotic species.

1.3.1 Definitions

A microbial community which occupies a specific habitat is called *microbiota*. Microbiota resides on different body parts such as the skin, the oral cavity, the urogenital tract and the gastrointestinal tract. Every microbe contains genetic material. The collective of genes and genomes found in the microbiota are therefore referred to as the *microbiome* (69).

The genetic potential (referring to the total genomic DNA or RNA transcripts of all organisms within a community) of the human microbiome is called the *metagenome*. *Metagenomics* is the study of structures, functions and dynamic operations of microbial communities. It uses DNA sequencing methods (70).

The composition of the microbiota can vary with age, sex, environmental factors (such as mode of delivery, breastfeeding, medication, hygiene, exposure to toxins), geography, diet and disease (71-74). The human gut microbiome is shared among family members.

Depending on the anatomical region, there are differences in the bacterial communities. In humans, bacterial concentration increases distally, in the large intestine. The small intestine has very low bacterial cell counts (10^3 - 10^8 cells/g) compared to the large intestine (10^{11} /g feces) (75). This microbiota gradient is due to luminal flow, different pH levels and the different secretion of antibacterial substances such as bile (76). The microbiota of the large

intestine is the most widely studied via fecal samples. Each person's gut microbiome varies; however, there is a wide array of shared microbial genes, which can be identified as "core microbiome" (69).

1.3.2 Classification of Bacteria

Bacteria are prokaryotes since their DNA is stored in their cytoplasm in nucleoids instead of being kept in a nucleus (77). Over the course of our lives, we are colonized by a diversity of commensal microbes which inhabit the skin, the oral cavity and the gut (78). These microbes are normally commensal or mutualists which help to digest food and maintain the immune system and also play a role in health and disease (69, 79).

In 1977, Woese and Fox (2) published a paper in which they divided the phylogenetic tree of domains (which were then termed as kingdoms). These divisions of Woese and Fox, which are still used today, specify the three domains of Bacteria, the Archaea and the Eucarya. Each of these three domains can be divided into kingdoms, phyla, classes, orders, families, genera, species and subspecies (Figure 1). Different species and subspecies can be further divided according to serotypes and strain levels (80). To name and classify bacteria, the Linnean system is widely used in biology (81). It consists of a hierarchy of groupings and naming conventions. The lowest is the species level, and similar species are then grouped into a genus. The naming convention, called binomial nomenclature, gives every species a two-word name. The first word is the name of the genus, the second word is the name of the species (e.g. *Lactobacillus acidophilus*) (82).

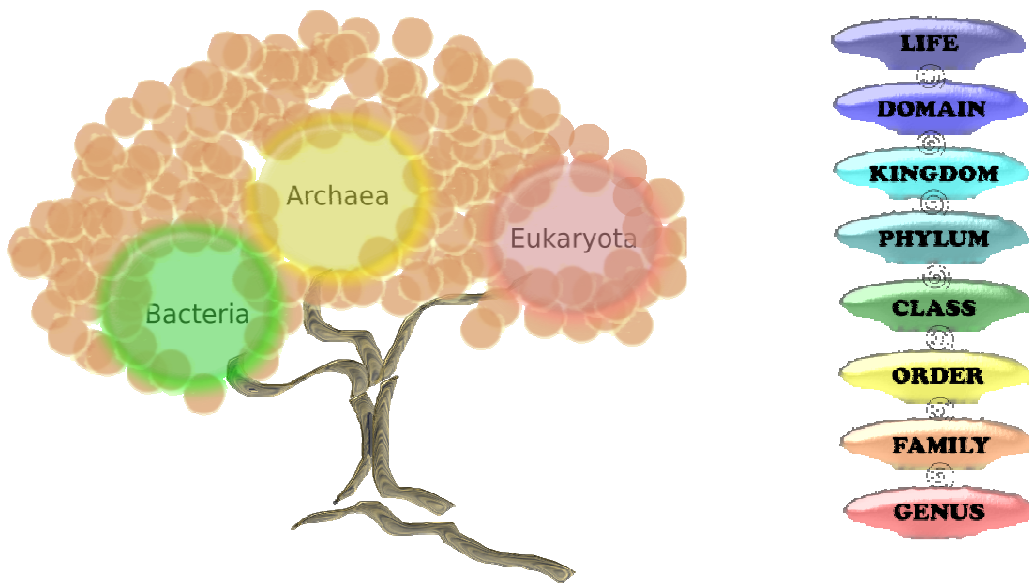


Figure 1: Phylogenetic tree of life and classification of bacteria according to Woese and Fox (2).

1.3.3 Development of the human microbiome

In each generation, human microbiota is newly acquired. In the prenatal period, the developing organism is first exposed to maternal gut-derived metabolites and intrauterine microbes (83) and then is further exposed to commensals during the passage through the birth canal. According to study results from Dominguez-Bello et al. (2010), babies born vaginally acquire a different microbiota than babies born via cesarean section (84). For this reason, caesarean section has been associated with increased risk for immune and metabolic disorders. However, the microbiota of cesarean born infants can be partially restored via vaginal microbial transfer (85). The microbiota of the infant is further shaped through maternal milk, which contains live microbes, metabolites, immunoglobulin and cytokines (86). In turn, the acquired microbiota induce the building of lymphoid structures (87) and fortify the intestinal barrier by inducing the maturation of epithelial cells and angiogenesis (88, 89). The microbial succession during the first two years of life varies in connection with a more diverse diet (90). From the second year of life onwards, the gut microbiome composition stabilizes and an adult microbiome is developed (91, 92). Stability of the gut microbiome is reached at the age of two to three years (90). However, over the lifetime, the gut microbiome does change again. Elderly people have a different microbiome than middle-aged adults (93). A recent study found that the microbiota of

elderly patients with frailty correlates with an overall decrease in microbial diversity, including increases in *Bacteroides* and decreases in *Firmicutes* (94).

1.3.4 16 S rRNA marker gene

To differentiate bacteria, it is important to determine evolutionary relationships. Therefore, a molecule of broad distribution is needed. Ribosomal RNA (rRNA) is highly appropriate because it is crucial for protein synthesis. Mutations of rRNA have a very high probability of being fatal to the bacterium. As a result, rRNA mutations are rare and their sequence changes very slowly over time, which permits the detection of relatedness among distant species. 16 S rRNA is a part of the 30S subunit of bacterial ribosomes (2) and contains highly conserved regions which link different species of bacteria (95). These highly conserved regions are interspersed with hypervariable regions which reflect different evolutionary species. Consequently, 16S rRNA sequencing is a method for phylogenetic classification of bacteria and can be used to calculate trees of life. Most genetic relationships calculated in this way can be verified with other methods which focus on morphological and physiological features. Therefore, 16S rRNA sequencing is now a standard for the identification and classification of microbes. 16S rRNA can be amplified with polymerase chain reaction (PCR) using universal primers which span variable and conserved regions. The mixture of RNA fragments is then separated by a chemical denaturing gradient or a temperature gradient gel electrophoresis. The variations of the migration-distances reflect the diversity of 16S species in the sample (96). For most bacteria, strains of 16S rRNA gene sequences are available in public databases such as the database of the National Center for Biotechnology Information (NCBI) (97).

1.3.5 Operational Taxonomic Units (OTUs)

First, total DNA is extracted from the sample and the composition of the community is determined by amplifying and sequencing the 16S rRNA gene. Bacteria that share a high similarity level in DNA-DNA hybridization, also share a high degree of their 16S rRNA gene (98). Highly similar sequences are then grouped in operational taxonomic units (OTUs) (99). An OTU represents strains or groups with similar phenotypic properties. Based on the 16 S rRNA gene, a similarity level of 97% is used to delineate sequences at the species level (100). If two sequences present a similarity of at least 97% over the entire

length of both sequences, then they belong to the same OTU (80). These sequences can be analyzed to identify how many of which species are present (abundance, OTU counts) and in terms of phylogenetic diversity (80).

The first common method of classification is the reference method: comparing OTUs with databases of recognized organisms, and then classify them on the family or genus level. But there are limitations: Not all sampled sequences are covered in databases and species-level assignments are hard to find. The second method is called “de novo clustering”. 16S rRNA sequences are clustered based on sequence similarity. In our study, we used de novo clustering to ensure a more exact display of community composition, and chose an open reference method which is better in terms of speed and computational power.

Summing up, while both reference based and de novo OTU classification methods are used to analyze OTU data, specifically, reference-based OTU classification methods are commonly used to analyze the genus-level and de novo classification is used to analyze the species level (101).

1.3.6 Alpha diversity

Alpha diversity is a term which was first used by Robert Whittaker in 1960 (102).

If a specific area like the gut is a good habitat for lots of different microbes, the alpha diversity is high. It is also referred to as within-sample diversity. The abundances of species are not important for alpha diversity; alpha diversity results from competition between different bacterial species, during the time that each species becomes adapted to its niche. Quantification of alpha diversity emphasizes both the richness of species, which is the total number of organisms in the sample, and evenness, which refers to the even distribution of species. Specifically, evenness measures the relative abundance of the different species which make up the richness of a habitat. Therefore, species diversity combines species richness with evenness: thus, taking not only how many species are present into account, but also how evenly they are distributed (99).

Alpha diversity can be calculated from a random sample. It can only be compared between different samples if the size of samples is nearly identical, since diversity increases with an increase of sample size (103). Differences in alpha diversity can be depicted using rarefaction plots, which indicate if further sequences would affect the number of observed

OTUs. For example, if the rarefaction curve is flat, no further OTUs are likely to be observed. Rarefaction is used to downsample to the lowest count of sequences and to discard sequences which do not match quality criteria (low quality, short sequences, chimeric sequences, sequences with homopolymers, contamination). Representative sequences are then chosen and compared. The same rarefaction is performed many times and sequences are removed at random. Then, the average is calculated. Species richness is either calculated by the number of observed species, where the different kinds of species in a sample (including singletons, doubletons and rare OTUs) are counted, or it is estimated with richness estimators, like the Chao-1-estimator (104). The Chao-1 is based on the number of observed species and is corrected for singletons and doubletons, since this leads to a more accurate account of the observed number of species. There are also other diversity estimators like the Shannon-Index or the Simpson-Index (105). Every index has its benefits and drawbacks and currently, there are no specific guidelines indicating which matrices are recommended. The difference between diversity measures can be considerable; thus, influencing the significance of the results. For example, Shannon's Index is more sensitive to species richness while Simpson's Index is more sensitive to species evenness (105). In this thesis, the species richness is calculated using the number of observed species, the Chao-1-estimator and Shannon's Index.

1.3.7 Beta diversity

Beta diversity refers to the degree of species variation between defined communities. In other words, it is the amount of organismal diversity shared between two or more communities (99). The less conformity there is between the species of two samples, the bigger the beta diversity. If there is high conformity between two samples, there is minimal beta diversity. The maximum amount of beta diversity is reached when two communities have no identical species.

Beta diversity is used to divide between local and regional effect. If a local habitat has many different species (high alpha diversity), but is ubiquitous in all regions, there is low beta diversity. In contrast, if there is low alpha diversity, which means there are not many different species in one sample, but if this sample is different to samples of other regions, there is high beta diversity. Thus, high alpha- and high beta diversity don't necessarily need to be linearly associated. Beta diversity is calculated by dividing the number of

species by the number of measurements (106). Many statistical methods have been established to quantify beta diversity. A widely used distance metric for describing differences in beta diversity is UniFrac. UniFrac includes information on the relationship of members within a community. While weighted (or quantitative) UniFrac includes the abundance of observed organisms (weighted sum of branch lengths in a phylogenetic tree), unweighted UniFrac only considers the presence or absence of species (107).

1.3.8 Population and diversity of the human gut

According to human microbiome project (HMP) data, the gut and the oral cavity have the most diverse microbiome among all sampled body habitats (108). The gut microbiome has high inter-individual variability and is highly diverse. The phyla of *Bacteroidetes* (gram-negative) and *Firmicutes* (gram-positive) dominate, but their abundance in different subjects ranges from >90% of *Bacteroidetes* to >90% of *Firmicutes* (109). Further, only a few taxa are present in >95% of the subjects sampled (110).

It has been hypothesized that the gut microbiome forms “enterotypes” which are stable core sets or clusters of microbiome configurations (111). In a study of individuals from six nations, three gut microbiome types were observed, which were dominated by either *Prevotella*, *Ruminococcus* or *Bacteroides* (111). Some species, like *Escherichia coli*, have a low abundance. But even low-abundance community members could have an influence on health (112). Under specific circumstances, they can overgrow and like *Clostridium difficile*, be the reason for life-threatening infections. However, the concept of enterotypes is not universally accepted due to inter-individual variation of clusters (113).

Diverse species of bacteria have been found to encode similar core functions, despite their heterogeneity. Therefore, while individuals have a unique microbe collection, we share genes that encode for common functions (114).

1.4 Gut-brain-axis

1.4.1 Definition

Changes of human behavior are not only connected with the external environment, but also with the internal environment.

Over the past years, lots of effort has been directed towards the characterizing the bidirectional interactions between the central nervous system (CNS), the enteric nervous system (ENS) and the gastrointestinal tract (GI). The brain and the gut are interrelated through a bidirectional communication network which is referred to as the gut-brain-axis (115), illustrated in Figure 2. The gut-brain-axis has a regulatory effect on neuroinflammation (116), neuroendocrine stress response (117) and neural development (118). It remains to be determined if gut microbiome alterations are a causal or causative factor of health conditions such as obesity (119), depression (120) or AN (121), but regardless of the sequence of events, alterations of the microbial community are likely to affect the communication between the gut and the brain.

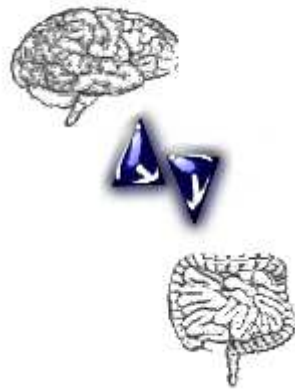


Figure 2: Gut-brain-axis.

1.4.2 Communication pathways between the gut microbiota and the brain

The gut microbiome has direct and indirect ways to send signals to the brain in order to influence processes regarding neuroinflammation, neurotransmission and neurogenesis. Moreover, it may have an influence on the digestive tract and eating behavior (signaling to the brain), and therefore ensure an optimal supply of the nutrients it needs (122, 123).

The gut is the largest endocrine organ. Enteroendocrine cells (EEC) have apical processes and are in direct contact with the gut lumen. They sense luminal contents and elevated substance levels which have been absorbed in the epithelial layer. EEC respond to changes in the lumen through interaction with a number of gut peptides which act locally, peripherally and centrally. Through vagal fibres, which extend into the lamina propria of the intestinal villi in the mucosal layer, the vagal nerve is activated through microbiota and gut peptides such as glucagon-like peptide-1 (GLP-1), peptide YY (PYY), cholecystokinin (CCK), leptin and others (124); these act as nutrient sensors. The vagal nerve is the main afferent pathway of the gut. Afferent nerve fibres lead to the nucleus solitaries of the hindbrain. Information from the gut microbiota is then processed in brain regions involved in neuroendocrine and behavioral processes. For example, *Lactobacilli* have been shown to obtain physiological effects through activation of the vagus nerve (125). The vagus also regulates gastrointestinal inflammatory response; study results have shown a link between vagal afferent neurons and the local release of intestinal inflammatory mediators in response to gut bacteria (126).

Microbiota can signal from the periphery via the vagus nerve or directly through the blood brain barrier (BBB). According to study results from Braniste et al., the gut microbiome regulates the permeability of the BBB (127) through the expression of tight junction proteins (like occludin and claudin), and therefore influences the way immunological and microbial metabolites are capable of infiltrating into the brain.

These microbial metabolites and immune mediators (cytokines) are important signaling mechanisms from the gut microbiota to the brain (115, 128). Inflammatory mediators such as chemokines and cytokines are produced in connection with gut microbiota. They affect the barrier of the gut epithelium (129), cause high intestinal permeability and infiltrate into the systemic circulation where they induce an immune response. This leads subsequently to a leaking of bacteria into the systemic circulation where inflammatory reactions follow (130). Thus, the gut microbiota has modulatory effects on this intestinal barrier (131, 132). For example, increased gut permeability is associated with low counts of *Bifidobacteria*, which are known to improve gut barrier function (133). A measurement of gut permeability is the protein zonulin (haptoglobin 2 precursor), which was measured in this

study. High zonulin serum concentrations indicate a “leaky gut”(134) through increasing the permeability of tight junctions between enterocytes(134).

A number of signaling molecules have been identified as possibly being the communication tools between the gut microbiota and their host. Catecholamines, serotonin, dynorphin, gamma-aminobutyric acid (GABA) and cytokines are released in the gut lumen by neurons, immune cells and enterochromaffin cells (135). A high proportion of identified metabolites originates from the gut, which provides the theoretical basis for a microbiota to brain-signaling structure (136).

1.5 The influence of the gut microbiota on health parameters

“The states of health or disease are the expressions of the success or failure experienced by the organism in its efforts to respond adaptively to environmental challenges.”

Rene Dubos (1965)

The idea that microbes might contribute to human health is not new: In 1892, A. Döderlein found out about the importance of Lactobacilli as gatekeepers of the vaginal ecosystem (137); a few years later, in 1908 Metchnikoff associated prolonged life with the consumption of fermented dairy products (138).

1.5.1 Gut microbiota and body weight

Gut microbiota composition correlates with diet and health and is essential for different body functions; furthermore, it is responsible for digestion and energy harvest of enteral food (94, 139-141). Research focusing on the role of the gut microbiota in regard to body weight will be discussed the following, firstly in rodent and then in human models. Table 1 gives an overview on certain bacterial genera which were found to be connected with high or low body weight.

<i>Model</i>	<i>Increased Weight, Obesity</i>	<i>Decreased Weight/ lean individuals</i>
Mice/Rats	High <i>Firmicutes</i> (142-144) High <i>Methanobrevibacter smithii</i> (145) High <i>Bacteroides thetaiotaomicron</i> (145) High <i>Enterobacteriaceae</i> (144, 146) High <i>Mollicutes</i> (144, 147)	High <i>Bacteroidetes</i> (148)
Humans	Reduced bacterial diversity (69) Low <i>Bacteroidetes</i> (68, 149) High <i>Prevotella</i> (150) High methanogenic <i>Archaea</i> (150) High <i>Lactobacillus</i> (149, 151, 152) High <i>Tropheryma whipplei</i> (153) High <i>Escherichia coli</i> (154) High <i>Bacteroides, Clostridium, and Staphylococcus</i> (155) High <i>Bacteroidetes</i> (156)	High <i>Bacteroidetes</i> (68) High <i>Bifidobacterium</i> genus (149, 157) High <i>Methanobacter smithii</i> (149, 158) High <i>Akkermansia</i> (154)

Table 1: Overview of gut microbiota strains and body weight in animal models and humans

Gut microbiota plays a role in weight gain and weight loss (68, 79, 143, 149, 159). This is supported by reports involving mouse models.

For example, adult germ-free mice that had been colonized with gut microbiota from conventionally raised mice, produced a significant increase in body fat within 10-14 days,

despite having decreased food consumption (160). When germ-free mice were colonized with gut microbiota from mice with obesity, they gained more weight compared to those, who have been colonized by gut microbiota from lean mice (142). These results suggest that microbiota affects the energy balance of their hosts in different ways (142).

Turnbaugh and colleagues conducted 16S rRNA studies to identify the gut microbiome of two different mouse models: ob/ob mice (which are genetically deficient in leptin) and diet-induced obese mice (with a high fat and sugar diet). Ob/ob mice had more *Firmicutes* than *Bacteroidetes* in their gut microbiome compared lean mice (143, 144). In a study from Santacruz et al. obese mice were found to have OTUs which lean mice lacked. These belonged to the *Firmicutes* phylum and were related to the *Clostridium* cluster. *Clostridia* are butyrate producing gut bacteria, which could be important for weight gain; weight loss has been associated with reductions of *Clostridia* (147). Another study colonized mice with human gut microbiota. Swift and substantial changes in gut microbiome composition and stable changes in taxa abundance and gene expression could be shown in response to changes in diet (161).

In humans, the gut microbiota was shown to differ between obese and lean individuals (68, 159). But study results regarding specific taxa have been variable. For example, the phylum of *Bacteroidetes* has been reported to either decrease, increase or stay the same during weight reduction (142). Therefore, the *Firmicutes/Bacteroidetes* (FB-Ratio) is often used to depict phylum level changes of gut microbiota. For example, Ley and colleagues demonstrated, using a weight loss program, that a decrease in the FB-ratio in obese individuals correlated with weight loss; they concluded that a modulation of the microbiota might be beneficial for the treatment of obesity (68). In a study from Arumugam (111) a reduction of *Bacteroidetes* was found in obese patients compared to normal-weight subjects; this is quite in contrast to mouse models, where the *Bacteroidetes* member *Bacteroides thetaiotaomicron* has been associated with host adiposity (145).

Taking into account that *Firmicutes* produce more short chain fatty acids (SCFA; such as butyrate) (160), it has been suggested that the gut microbiome in obese subjects affects energy harvest and promotes fat disposition. However, since both *Firmicutes* and *Bacteroidetes* are known to produce SCFA, which are a core factor for energy harvest, their roles in obesity are not completely clear. Furthermore, other studies have not found increased *Firmicutes/Bacteroidetes* ratios in obesity (150, 162).

The *Bifidobacterium* genus (*Bifidobacterium animalis*) and *Methanobacter smithii* have been associated with lower BMI in humans, while other studies have shown that *Lactobacillus* (*Lactobacillus reuteri*, *Lactobacillus sakei*) are associated with obese status (149, 158). *Lactobacillus* species concentration was higher in obese patients than in lean controls and AN patients (149). Interestingly, in farm animals, probiotics such as the *Lactobacillus* species are used as growth promoters to induce weight gain (163). Further, obesity was also shown to be associated with reduced bacterial diversity (69).

Interestingly there are even changes in gut microbiota in surgically induced weight loss which reflect the changes in diet induced weight loss, that is increased levels of *Bacteroidetes* and decreased levels of *Firmicutes* (164).

1.5.2 Gut microbiota, nutrition and diet

After a meal, a large amount of undigested food containing lipids, polysaccharides and peptides passes through the small intestine and enters the large intestine, where these substances provide a perfect medium for rich microbiota growth. We are in a symbiotic relationship with our gut microbiota. While we provide them with nutrition, they are in part responsible for health and disease.

A number of metabolic pathways are influenced by gut microbes (114). It stands to reason that the microbiota plays an important role in energy harvest and the extraction of nutrients from the diet (143). For example, gut microbiota contributes to fermentation, reduction of nitrate and sulfate, esterification, aromatic fission, hydrolysis and deconjugation (glycosides in plant food, steroid hormones and other endogenous compounds excreted in bile). Furthermore, microbiota supplies the host with folates, vitamin K, biotin, riboflavin, cobalamin and other B-vitamins (165).

Additionally, the GI provides feedback after eating a meal via the gut-brain-axis. Enteroendocrine cells sense and respond to specific nutrients, release gut peptides and regulate energy output.

Recent evidence has suggested that the gut microbiota controls energy balance via intestinal nutrient-sensing mechanisms (166). This extraction and storage of calories from food are impacted by the gut microbiome; this could be defined as a diet-microbiome interaction. Microbiota also seems to have an impact on taste preferences (123).

In studies of nutrition in mouse models, germ-free mice were colonized with a human gut microbiome. When mice were fed a Western diet (with high fat and sugar) versus conventional mouse food, rapid effects of diet on the gut microbiome have been observed. Even a single day on a high-fat diet had a significant effect on the microbiome; this suggests that the gut microbiome is dynamic and responds quickly to dietary changes (161). Furthermore, certain food components such as artificial sweeteners have been found to change the composition and function of the gut microbiome and could lead to glucose intolerance (167).

Germ-free mice, which have not had exposure to any microbiota, have less adiposity than normal mice and are resistant to diet-induced obesity (168). In humans, it has also been confirmed that the microbiota seems to be influenced by dietary patterns (169-171). Obese subjects suffering from metabolic syndrome and diabetes showed an increase in *Firmicutes* and low bacterial diversity, which is generally associated with poor health status (144).

In a controlled-feeding study the *Prevotella* enterotype was associated with a high-carbohydrate diet and *Bacteroides* with an animal fat and protein diet (169). Short-term dietary shifts from high-fat to high protein to high carbohydrate (and vice versa) can modify the abundances of bacteria (169, 172) but rarely change the presence or absence of specific gut microbes (169). The gut microbiome might also adapt to certain stages of life. For example, the microbiomes of infants have higher levels of folate-producing enzymes than those of adults. The biosynthetic capacity for the vitamins cobalamin, thiamine and biotin increases with age (90).

1.5.3 Gut microbiota, body fat and nutrient sensing

Most human studies use the BMI to measure obesity; however, BMI is a rather imprecise measure of adiposity and only accounts for overall body mass without distinguishing between lean mass and fat mass (173). Adipose tissue is distributed in different anatomical depots (174). The fat store size is highly variable within individuals, with ranges from five to 60% of total body weight. Over 80% of body fat is stored in subcutaneous adipose tissue (SAT). Subcutaneous fat layers interfere with metabolic functions and thus contribute to the complications of obesity (175). Interestingly, men and women have a different fat distribution; for example, women accumulate more lower body fat compared to men (176).

Central obesity is known to lead to an increased risk of metabolic complications (177). Body fat mass has also been shown to be regulated by the gut microbiota (178). Specifically, it was shown in studies with germ free mice that the gut microbiota can regulate circuitries in the hypothalamus and the brainstem and decrease the fat suppressing peptides brain derived neurotrophic factor (BDNF) and GLP-1. Subsequently, gut microbiota dysbiosis impacts on body fat mass (178).

Microbiota in the GI tract have an important function in nutrient sensing and signaling to the brain in order to ensure optimal resources of nutrients (122, 123). As mentioned above, gut-brain signals through afferent vagal fibres sense and control food intake and satiety. In vivo and in vitro studies have shown that carbohydrates, proteins and fat induce the secretion of PYY and GLP-1 (179). In EEC there are similar taste receptors to those in oral taste signaling (such as the sweet taste receptor) (180). Furthermore, receptors for SCFAs and long chain fatty acids (LCFA) mediate the release of GLP-1, PYY and CCK (181). Nutrients generate local intestinal signals which evoke different pathways for regulating the absorption of nutrients (182). Lipids might trigger a feedback system via the vagal nerve (183). Celiac vagal afferents are activated by lipid infusion and reduce food intake via gut sensory nerves (184), which are activated through CCK (185).

1.5.4 Microbiota, immune regulation, intestinal barrier and inflammation

In recent research, it has been pointed out that the microbiota contributes fundamentally to the induction, proper function and training of the host's immune system. In this symbiotic relationship, the immune system and the microbiota cooperate in inducing protective responses to pathogens, and in playing a major role in the function, education, calibration and promotion of immune response. Immune cells, such as dendritic cells, sample antigens or bacteria from the intestinal lumen and transport them to the mesenteric lymph nodes. Subsequently, they induce a protective immunoglobulin A (IgA) secretion which coats bacteria and allows controlled uptake in order to train the immune system. Similarly, micro-RNAs are released by gut epithelial cells which regulate gene expression and growth of bacteria (186).

Nowadays, the overuse of antibiotic agents and changes in dietary patterns and lifestyle have led to a reduced resilience and diversity of microbiota, and therefore to a less balanced immune system. It has been suspected that this could partly account for the rise in autoimmune and inflammatory disorders in high-income, western countries (187).

Interactions between the microbiota and the immune-system have to be balanced, as failures in this complex interactional system could lead to misdirected immune responses such as allergies, autoimmune and inflammatory disorders. According to certain aspects (nutrition, co-infection, genetics), the same microbe could either act as a mutualist or parasite. To minimize inflammation and to maintain a homeostatic microbiota-host relationship, the epithelial cell surfaces are protected with a “mucosal firewall” containing mucus secreted by goblet cells, IgA and antimicrobial peptides (188). Furthermore, antimicrobial proteins secreted by the mucosa protect the host from microbial invasion. Behind the epithelial layer, immune cells in the lamina propria prevent microbial penetration (189). Studies have shown that certain bacterial strains influence the gut barrier (such as *Faecalibacterium prausnitzii* or *Akkermansia muciniphila*) (190).

In this context, zonulin has been found to work as a peripheral serum marker of intestinal permeability (134). When gut permeability is increased, the immune system is confronted with antigens from food and an immune response is induced. A dysbiosis can cause an altered immune response and induce inflammation. For example, increased zonulin has been found in inflammatory bowel disease (191), obesity with insulin resistance (192) and coronary heart disease (193). Results from animal studies have shown that gut permeability can be affected by food restriction and physical activity. For example, in an animal model of AN, mice with activity-based AN showed higher intestinal permeability and histological changes of the intestinal mucosa (194, 195).

In further breakthroughs in research, a link has been shown between members of the microbiota and the induction of regulatory T-cells which cut down mucosal inflammation (196, 197). Additionally, SCFA control various immune responses and regulate the T-cell network (198, 199). For example, Burelin and Cani identified a microbiota-derived lipopolysaccharide (LPS) which might have an impact on the beginning of low-grade inflammation processes. LPS is a proinflammatory molecule in the cell wall of gram-negative bacteria and binds to the toll-like receptor 4 (TLR4). Mice which had been on

high-fat diet showed a significant increase of circulating LPS (200). Increased LPS is associated with inflammation in the liver, muscle and adipose tissue (133, 200).

1.5.5 Gut microbiota and exercise

Exercise has shown to have an impact on immunological, metabolic and neural pathways (201) and offers a variety of proven health benefits in patients with coronary heart disease (202), diabetes (203), irritable bowel syndrome (204), colon cancer (205) and depression (206). Early studies have shown an interaction between physical activity and gastrointestinal function (207). Physical exercise shows effects on transit time, digestion and absorption rates of food (208). It modulates the vagal tone, interrelates with the immune system, and may consequently influence the brain-gut-microbiome-axis (209). Regular physical activity mediates interactions between the skeletal muscle and other organs such as the brain, the adipose tissue and the gut (201, 210).

Studies on the relationship of exercise and gut microbiota in mice have produced inconsistent results, due to of differences in genetic strains, as well as deviations in techniques and procedures. This is further explained in the following.

A study by Mika et al. demonstrated that increasing activity levels in mice also increased the abundance of beneficial microbiota. They performed a six-week long training program (wheel running) and compared the microbiota of trained mice to mice without an exercise program. The microbial communities in mice with exercise were less even and less diverse, which reflects malleability. Exercise changed phyla, increased *Bacteroidetes* and decreased *Firmicutes*, which may be associated with slimness. In juveniles, exercise led to changes of the phyla which were associated with adaptive metabolic consequences (148).

Similar results were found by Evans and colleagues in their study, observing an increased percentage of *Bacteroidetes* and a decreased percentage of *Firmicutes* after a 12-week exercise program (running wheel) in mice. Contrary to these findings, Lambert et al. actually found a greater abundance of select *Firmicutes* species and lower *Bacteroides* species in normal and diabetic exercised mice compared to non-exercised mice (211). A rodent study from Kang et al. found that a high-fat diet altered the gut microbial

community, and exercise caused massive shifts in the gut microbiome, which were nearly of the same magnitude as the high-fat diet; they concluded that the gut microbiome is impacted by both diet and exercise (212).

In humans, few investigations regarding gut microbiota and activity have been performed. Some studies have shown that exercise-level may also modify the composition of the human gut. For example, in the elderly, lack of exercise and frailty is linked with low microbial diversity (94). Clarke et al. compared elite-level international rugby players during a pre-World Cup training with matched inactive controls (213). As most rugby players were classified as overweight or obese according to BMI alone, two control groups, one with low BMI and one with high BMI had been established. Rugby players displayed a greater alpha diversity than the control groups: while rugby players had 22 phyla of bacteria, the low BMI controls had 11 and the high BMI controls 9 phyla. *Firmicutes* were significantly more abundant, while *Bacteroidetes* were significantly less abundant. They also found a greater abundance of *Lactobacillaceae* amongst rugby players. Furthermore, higher counts of *Akkermansiaceae* and *Akkermansia* have been found among AT compared with high BMI controls (213). *Akkermansia muciniphila* is an organism which degrades mucin and occupies a niche at the mucinous layer of the gut. In mice, it appears to be important for improving barrier function and metabolic status (214). *Akkermansia* are SCFA producing bacteria. Their abundance is decreased in obesity (214).

In an intervention study, Santacruz et al. investigated the effects of an obesity treatment intervention program on the gut microbiota, including an energy restricted diet and 10 weeks of moderate exercise. The *Firmicutes* decreased after the combined intervention, while there was an increase of *Bacteroidetes*. The *Lactobacillus* group was also found to have been increased after the intervention (146).

1.6 Microbiota and anorexia nervosa

*“All disease begins in the gut.”
(Hippocrates)*

A dysregulation or dysbiosis has been identified in a number of psychiatric diseases such as AN (121), autism spectrum disorders (215, 216), Parkinson’s disease (217), and disorders of mood and affect (120, 215). Interestingly, some case studies have described the development of psychotic symptoms after broad-spectrum antibiotic intake (218, 219). The exact reason for psychiatric adverse reactions after antibiotic treatment is not known, but besides direct toxic effects on the brain, gut microbiota alterations could be responsible. Figure 3 depicts the relationship between psychiatric diseases such as AN, microbiota, and nutrition.

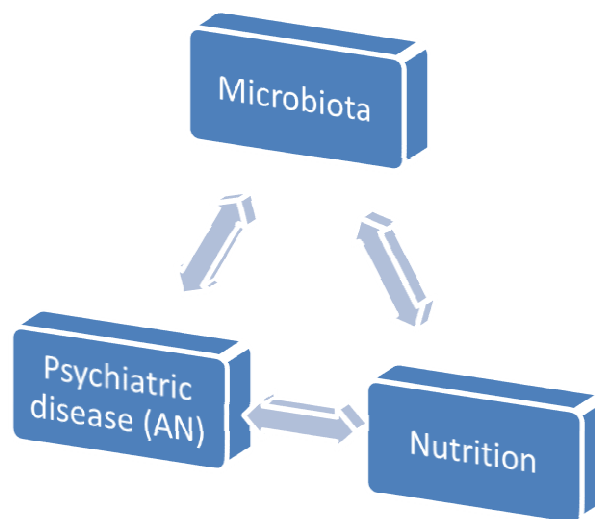


Figure 3: Relationship between microbiota and psychiatric disease.

So far, six studies have explored gut microbial composition in AN (121, 149, 158, 220-222); they found profound perturbations. Table 2 gives an overview of the different gut microbiota alterations in AN.

<i>Gut microbiota alterations in AN</i>	<p>Lower alpha diversity (220)</p> <p>Lower amounts of total bacteria (121)</p> <p>Increased levels of <i>Methanobrevibacter smithii</i> (149)</p> <p>Higher abundance of <i>Ruminococcaceae</i> (220)</p> <p>Low levels of <i>Lactobacillus reuteri</i>, <i>Lactobacillus sakei</i> (158)</p> <p>High levels of <i>Bifidobacterium animalis</i>, <i>Methanobrevibacter smithii</i> and <i>Escherichia coli</i> (158)</p> <p>Increased levels of <i>Clostridium coccooides</i>, <i>Clostridium leptum</i> subgroup, <i>Bacteroides fragilis</i> (121)</p> <p>Low <i>Streptococcus</i> (121)</p> <p>Low SCFA levels (121)</p> <p>Higher levels of mucin degraders and <i>Clostridium</i> clusters I, XI and XVIII (222)</p> <p>Reduced levels of <i>Roseburia</i> (222)</p>
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Table 2: Overview of the different gut microbiota alterations in AN.

Regarding bacterial diversity, studies have confirmed lowered alpha diversity levels in AN which correlated with depression levels (220).

A culture based study identified 11 completely new bacterial species in a single AN stool sample (221). Further, in a molecular based study, Armougom et al. analyzed the microbiota of nine AN patients in comparison to 20 obese participants and 20 lean subjects, and discovered increased levels of the archaeon *Methanobrevibacter smithii*. This archaeon comprises ten percent of all anaerobes living in a healthy colon.

Methanobrevibacter smithii and other methanogens recycle hydrogen gas from the gut and have a role in microbial fermentation (145). This is important for energy extraction and may, therefore, be an adaptive response for optimizing energy intake from a low-calorie diet in AN. However, another explanation of this is that AN patients often suffer from constipation and this could be a cause for the increase of *Methanobrevibacter smithii* in their gut (223). In a re-nourishment study, Kleiman et al. (220) found changes in the composition of intestinal microbiota in AN subjects, specifically, a higher abundance of *Ruminococcaceae*. This bacterial family is associated with inflammation and irritable bowel syndrome (224). Million et al. (158) investigated the gut microbiome of 15 AN patients, as well as 134 obese, 38 overweight and 76 lean controls. They found a positive correlation between BMI and *Lactobacillus reuteri* and *Lactobacillus sakei*, while *Bifidobacterium animalis*, *Methanobrevibacter smithii* and *Escherichia coli* were negatively correlated with BMI.

Morita et al. (121), demonstrated that AN patients showed reduced amounts of total bacteria and obligate anaerobes including those from the *Clostridium coccooides* group, *Clostridium leptum* subgroup, and *Bacteroides fragilis* group compared to age-matched healthy controls. Lower numbers of *Streptococcus* were also found in the AN group, compared to the control group. Furthermore, lower SCFA levels (acetate and propionate) were identified in the AN group. The gut microbiota before and after weight gain in AN patients was also investigated by Mack et al. (222). They found higher levels of mucin-degraders and members of *Clostridium clusters I, XI and XVIII* and reduced levels of the butyrate-producing *Roseburia spp.*

AN is a disease with comorbid anxiety and depression. Up to 80% of AN patients experience depression at some point in their lifetime (225). Interestingly, alterations of the gut microbiome have been discovered in depression (226, 227). For example, Jiang and colleagues observed an increase in bacterial alpha diversity, with a decrease of *Firmicutes* and an increase in *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* in those with depression (227).

Altogether, the findings from the above-mentioned studies are not consistent. This underlines the need for further research considering possible impact factors on the gut microbiome in over- and undernutrition as well as in psychiatric disease.

1.7 Aims and hypothesis

The major aims of this study were (1):

- (1) To identify to what extent the gut microbiota in AN differs in comparison to other BMI groups and AT.
- (2) To investigate associations between gut microbiota community structures, parameters of body composition (anthropometric measurements, total body fat and fat distribution), depression scales, smoking status, physical activity, nutrition and laboratory parameters (serum lipids, gut barrier function and inflammation).

We hypothesized that:

- (1) Gut microbiota would be significantly different between groups, in terms of alpha- and beta diversity.
- (2) Gut community structure would be associated with the parameters investigated (such as body composition, depression scales, smoking status, physical activity, nutrition and laboratory parameters).
- (3) Study groups would demonstrate significant differences in the abundance of taxa on phylogenetic levels.
- (4) Alpha diversity would be correlated with the assessed parameters.
- (5) Zonulin concentrations in AN would be significantly higher compared to other groups, indicating impaired gut barrier function in AN.

2. Materials and methods

2.1 Participants

2.1.1 Recruitment and group characteristics

The data of this doctoral thesis was collected as a part of the “Energy sensing in anorexia nervosa”- study. This study had a prospective, exploratory design and was conducted at the Medical University of Graz. Results from this study were recently published (1).

The recruitment of AN patients took place in three psychiatric inpatient wards in Graz, Austria (Department of Psychiatry and Psychotherapeutic Medicine, Medical University of Graz; Landeskrankenhaus Graz Südwest, Standort Süd; Krankenhaus der Barmherzigen Brüder, Graz Eggenberg). Female competitive AT were enlisted from local volleyball and water polo sports teams. Normal weight (NW), overweight (OW) and obese (OB) women were recruited from the Campus of the Karl Franzens University of Graz and the Medical University of Graz(1). In accordance with the BMI classification from the World Health Organization (WHO) (56) the recruited participants were divided into the groups “normal weight”, “overweight”, and “obese”. All participants were of Caucasian ethnicity. Informed written consent was obtained from all participants before study participation. This study was performed according to the Declaration of Helsinki. It was approved by the Ethics Committee of the Medical University of Graz (Ethics committee number: MUG-26-383ex13/14)(1).

2.1.2 Inclusion and exclusion criteria

All women who fitted the following criteria were included in our study: 1) age between 18 and 40 years, 2) AN patients who met the ICD-10 criteria in a structured diagnostic interview, and were diagnosed by an experienced consultant psychiatrist 3) AT with a regular training agenda (at least 7 hours per week and regular participation in local competitions). We excluded women who, in the preceding two months, had taken antibiotics or antifungals, prebiotics or probiotics. Furthermore, we excluded women with acute or chronic infections (also including upper respiratory tract infections, fever, chronic inflammatory and autoimmune-disorders, drug- or alcohol addiction, cognitive deficits,

inflammatory syndromes of the digestive tract (such as inflammatory bowel disease or irritable bowel syndrome), history of GI-surgery (other than appendectomy) and women during pregnancy or while breastfeeding(1).

2.2 Body composition assessments

2.2.1 Anthropometric assessments

Anthropometric measurements including weight, height, waist and hip circumference, upper arm circumference, triceps skinfold thickness and waist-hip-ratio (WHR) were performed (1) (228). Weight and height were measured with a calibrated digital stadiometer and platform scale (Secca 764). Anthropometric circumferences were all measured according to WHO guidelines (229). Triceps skinfold thickness was assessed using a calibrated skinfold caliper in accordance with the International Society for the Advancement of Kinanthropometry (ISAK) recommendations (230). BMI was calculated using the following formula: $\text{weight}[\text{kg}]/\text{height}[\text{m}^2]$ (231).

2.2.2 Subcutaneous adipose tissue (SAT) measurements

In all participants, body fat distribution was assessed using an exact ultrasound method for the detection of subcutaneous adipose tissue (SAT) as described by Müller et al. (232-234). This novel ultrasound method is capable of measuring SAT layers on eight specific body sites (upper abdomen, lower abdomen, erector spinae, distal triceps, brachioradialis, lateral thigh, front thigh and medial calf) without compression, thus enabling a precise SAT determination that is only limited by furrowed borders and the plasticity of the tissues. This method is also suitable for very slim people such as AN patients, who often demonstrate SAT thicknesses below one millimeter (233).

It should be noted that external oblique was used instead of the lateral thigh measurement point, since the lateral thigh point was not listed on the original set of measurement points at the time our measurements were performed. The marking of the specific sites was done relative to the person's body height (233), in order to ensure the comparability of sites. All ultrasound measurements were performed by a trained researcher according to the guidelines of the International Association of Sciences in Medicine and Sports (IASMS) (233). Ultra sound pictures of specific body sites were evaluated with a semi- automatic

image evaluation approach (FAT-Software, Rotosport). Recent studies have confirmed the reliability of this method (233, 234).

2.2.3 Bioimpedance analysis (BIA)

Bioelectrical impedance spectroscopy (BIA) is an easy-to-use, non-invasive and relatively inexpensive technique to evaluate changes in body water distribution of different populations (235-237). It uses an electrical current as well as the different resistances of water and fat tissue, in order to measure the resistance (R) of cellular tissue and the sum of all membrane-capacities (Xc). We performed BIA in the pre-defined measurement position: The participant had to lie flat on her back with arms and legs slightly bent. Four adhesive electrodes were placed on the dorsal sides of both hands and feet. The impedance was measured from the wrist to the contralateral ankle. A small current (1-10 μ A) was passed between the electrodes while the voltage was measured. As hydration is a crucial factor known to alter BIA results by increasing the electrical resistance of the body, all participants needed to have been fasted overnight. Fat-free body mass consists of approximately 74% water. Since fat is a poor conductor of electricity, there is a direct relationship between resistance and highly conductive body areas. Intact cell membranes have a high capacitive resistance (Xc).

High Xc-values could, therefore, be used as an indicator of a healthy nutritional state. The higher the Xc is in relation to total resistance, then the higher the phase angle. The phase angle is reduced by malnourishment, while properly nourished, healthy and athletic participants show an increased phase angle (235, 236). We used single frequency BIA (BIA 101 – Body Impedance Analyzer Akern) as described in the manual (238), and analyzed the BIA output with the Body Composition software (BodyComposition – Professional v9.0.14325), which uses the equations from Sun et al. (239) for calculating fat-free mass (FFM) and total body water (TBW), and the equations from Sergie et al. (240) for calculating the extracellular water (ECW). Total body fat (TBF) was calculated by subtracting FFM from body mass (m): $TBF=m-FFM$.

2.3 Questionnaires

2.3.1 Clinical characteristics

2.3.2 Depression scales

All groups were screened for psychopathology in a short psychiatric assessment, and then they also completed the Beck Depression Inventory (BDI (241)) and the Hamilton Depression Inventory (HAMD (242)) , both of which will be described in the following section.

2.3.2.1 Beck Depression Inventory (BDI)

The BDI is a self- rating-questionnaire developed by Aaron T. Beck to measure depressive symptoms (241). It is the most widely used depression inventory. The person filling it out needs to answer items relating to symptoms of depression during the previous two weeks. The BDI consists of 21 categories, each with four response options. In its current version, the BDI categories consist of the following themes: sadness, pessimism, past failure, self-dislike, self-criticism, suicidal thoughts or wishes, crying, agitation, loss of interest, indecisiveness, worthlessness, loss of energy, changes in sleeping patterns, irritability, changes in appetite, difficulty concentrating, tiredness or fatigue, and loss of interest in sex. The test takes five to ten minutes to complete. To evaluate the results, the values of each category are added and compared to cut-off values (Table 3) (241).

<i>Score</i>	<i>Interpretation</i>
0-13	Minimal Depression
14-19	Mild Depression
20-28	Moderate Depression
29-63	Severe Depression

Table 3: BDI cut-off-scores (241).

2.3.2.2 Hamilton Depression Inventory (HAMD)

The Hamilton inventory (242) is a widely-used third party assessment tool for the evaluation of depressive symptoms. The original scale consisted of 17 items. In the current study, the revised scale containing 21 items (depressed mood, feelings of guilt, suicidality, difficulties initiating and maintaining sleep, sleep disturbances in the early hours of the morning, work and hobbies, depressive inhibition, anxiety, inner tension, somatic concerns, gastrointestinal symptoms, general somatic symptoms, genital symptoms, hypochondria, weight-loss, insight and acknowledgment of the illness, fluctuations of symptoms, depersonalization and derealization, paranoid symptoms and compulsions) was used. Each item is rated by the examiner based on a 30-minute patient interview. For each item, there is a scale with a brief description of symptoms. The term of reference of the symptoms is the week before the interview. The higher the score reached, the more severe the depression. 66 is the highest score, while 0 is the lowest score. There is no standardized cut-off for the HAMD, but there is a commonly used evaluation guideline for clinical practice (Table 4) (242, 243).

<i>Score</i>	<i>Interpretation</i>
0-9	No depression
10-20	Mild depression
21-30	Moderate depression
>30	Severe depression

Table 4: Hamilton cut-off scores (242).

2.3.3 Fagerström test for nicotine dependence (FTND)

Smoking status was assessed using the Fagerström test for nicotine dependence (FTND) (244). The FTND is a psychometric test allowing the classification of dependent and non-dependent cigarette consumption. It contains six questions covering different dimensions of dependence. The FTND has a high validity and reliability (245) and has been shown to correlate with biochemical parameters (exhaled carbon monoxide, cotinine-level) (246). It includes the following aspects: smoking in the early hours of the morning, smoking more than ten cigarettes per day and failed abstinence.

All smokers completed the FTND and were classified as either low, moderate, strong or severe smokers (Table 5) (244).

<i>Score</i>	<i>Interpretation</i>
0-2	Low dependence
3-5	Moderate dependence
6-7	Strong dependence
8-10	Severe dependence

Table 5: FTND cut-off scores (244).

2.3.4 International Physical Activity Questionnaire (IPAQ)

The international physical activity questionnaire (IPAQ) was used to quantify and compare amounts of physical activity between BMI groups, AN patients and AT (1). The IPAQ was developed by an International Consensus Group in 1998–1999 to establish a standardized and culturally adaptable measurement tool which is valid across various populations in the world. It is intended to evaluate the levels of habitual physical activity for young to middle-aged adults (i.e. 15–69 years old). The IPAQ is a self-report questionnaire commonly used in mental health care settings to assess physical activity (247) and has been previously used in patients with mental disorders such as schizophrenia (248) or AN (249).

The long version of the IPAQ used in our study consists of 27 questions and is divided into five parts: (1) job-related physical activity, (2) transportation physical activity, (3) housework, house maintenance and caring for family, (4) recreation, sport and leisure-time physical activity, (5) time spent sitting. Each activity is further divided into vigorous activity (defined by hard physical effort and significantly increased breath frequency) and moderate activity (defined by moderate physical effort and moderately increased breath frequency). The participants of our study had to report exactly (in total hours and minutes) how much time they had spent being physically active in the last seven days. There are no generally validated cut-offs for the IPAQ. Further, the scores reported in the guidelines depict comparisons of mean values of different populations. The scores are divided into three different activity levels: low, medium and high. A high activity level is reached with

12.500 steps per day or at least one hour of moderate physical activity, half an hour of vigorous physical activity or at least half an hour more moderate activity than the medium level. Medium activity includes a minimum of half an hour moderate activity per day. Low activity is defined by not fulfilling the criteria of moderate or high physical activity. The results of the IPAQ are expressed in metabolic equivalent of task (MET)-minutes per week (250). The activity levels described are based on the results of different studies of physical activity (247, 251, 252).

2.4 Nutritional data collection

A nutritionist interviewed every participant and completed a detailed food interview, including a follow up after one to four weeks, according to a standardized protocol (1) (253). A photographic food atlas was used to quantify foods and beverages.

Manufacturer's weights on packaging and household measures were also used to quantify foods. Habitual dietary intake was then quantified and analyzed by a nation-specific software. All participants were screened for the use of pro- and prebiotics intake or vegetarian habits(1). The nutrient analysis was calculated by the software nut.s® (www.nutritional-software.at/, Vienna, Austria) using an adaptive Austrian food database.

2.5 Laboratory parameters

2.5.1 Serum lipids

Serum lipids (total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol) were compared in all patients and controls following an overnight fasting. Cholesterol, HDL-cholesterol and triglycerides were measured by enzymatic photometric methods (Roche Diagnostics, Mannheim, Germany). The limit of quantification (LOQ) for total cholesterol, HDL-cholesterol and triglycerides was 0.1 mmol/L. LDL-cholesterol concentrations were determined by Friedewald's formula (1, 254).

2.5.2 Markers of inflammation

Additionally, laboratory markers of inflammation such as high sensitive C-reactive protein (CRP) and Interleukin (IL)-6 were analyzed at the clinical institute for laboratory diagnostics in accordance with the standard procedures. A particle enhanced turbidimetric assay on a Cobas 6000 chemical routine analyzer (Roche Diagnostics, Mannheim, Germany) was used. The LOQ for high sensitivity CRP was 0.25mg/L. The intra-assay and inter-assay coefficients of variation (CV) of all routine assays were below 5% (1).

2.5.3 Zonulin

Zonulin concentrations in serum were measured with a commercially available enzyme-linked immunoassay (ELISA) test kit (K5600, Immundiagnostik AG, Bensheim, Germany). The ELISA kit used for zonulin measurement detects the active (uncleaved) form of zonulin. Within-day CVs at different concentrations were below 8 %. The between-day CVs were below 12 %.

2.6 Microbiome analysis

2.6.1 Sample collection, processing and storage

The PSP spin stool DNA collection kit (Stratec, Birkenfeld, Germany) was used to collect stool samples from all study participants. A pea-sized amount of stool (approximately 1g) was suspended in the buffer solution of the PSP-Spin-Stool-DNA-Plus-Kit. All samples were immediately frozen at -20°C and remained frozen until DNA isolation and sequencing analysis (1).

2.6.2 DNA isolation

The PowerLyzerPower Soil DNA Isolation Kit (MO BIO Laboratories Inc, CA, USA) was used in accordance with the instructions of the manufacturer to extract bacterial DNA from the acquired stool samples. Picogreen fluorescence (Thermo Fisher Scientific, MA, USA) was used to quantify DNA concentration (1).

2.6.3 Polymerase chain reaction (PCR)

With PCR, the variable V1-V2 region of the 16SrRNA bacterial gene was multiplied. PCR is a method used in molecular biology to amplify DNA and generate copies of DNA sequences. In the first step, called DNA melting, the DNA double helix is separated at a high temperature. Then, in the annealing phase, the temperature is lowered. 50ng of DNA were used as templates for the DNA polymerase to amplify the target DNA (1).

The following oligonucleotide primers (complementary to the targeted DNA region) were used: (1)16s_515_S3_fwd:GATTGCCAGCAGCCGCGGTAA and (2) 16s_806_S2_rev:GGACTACCAGGGTATCTAAT. As mentioned above, these 16S rDNA regions showed strong taxonomic classification and can be used for community clustering. Mastermix 16S Complete PCR Kit (Molzym, Bremen, Germany) was used for the amplification of bacterial 16SrRNA. Using the product of the first PCR a second round of PCR was performed with primers combining the 16S primer sequence to the A and P adapters which are needed for Ion Torrent Sequencing. Moreover, a molecular barcode sequence was included to allow multiplexing of up to 86 samples at once (1).

2.6.4 Electrophoresis

Next, agarose gel electrophoresis was used with the PCR products. QiaQuick (Qiagen, Hilden, Germany) gel extraction system was used to excise and purify the band of expected length (330 nucleotides (nt)) from the agarose gel. The final PCR product was again measured by Picogreen fluorescence (Thermo Fisher Scientific, MA, USA) (1).

2.6.5 Sequencing

The amplicons from up to 60 samples were pooled equimolarly. Then, an emulsion PCR using the Ion Torrent One Touch 2.0. kit was performed according to the manufacturer's instructions. Further, the beads were purified on the Ion ES station and sequenced on the Ion Torrent 318 chips. Sequencing reactions were carried out on the Ion Torrent PGM using the Ion 400 base-pairs (bp) Sequencing Kit running for 1082 flows (all reagents were from Thermo Fisher Scientific, MA, USA). Subsequently, the sequences were split by

barcode and transferred to the Torrent suite server. As bioinformatical input, unmapped .bam-files were used (1).

2.6.6 Bioinformatics

The sequences used for bioinformatics were subjected to trimming by a sliding window quality filter with a width of 20nt and a cutoff at the Phred Quality Score (Q) 20. Deconseq (255) was used to remove reads mapping the human genome and reads shorter than 100 nucleotides. The Acacia tool (256) was used for error correction, which led to a correction of 10-20% of reads. Chimeras were removed with the de-novo and reference based usearch algorithm (257). Sequence files were analyzed by Quantitative Insights Into Microbial Ecology (QIIME) 1.8 workflow scripts (258) and then clustered into operational taxonomic units (OTUs) based on a 97% similarity level. OTU search was performed using the parallel_pick_open_reference_otus workflow script and the greengenes 13_8 reference database. Species richness was determined by the number of observed species in each sample, the Chao-1 estimator (104) and the Shannon Index (259) (1).

2.6.7 Phylogenetic Analysis

Initially, to avoid the inflation of OTUs due to sequencing errors, the data set was denoised using the method described by Quince et al.(260, 261). All sequences shorter than 150 base pairs containing any ambiguous characters or not matching to the forward primer were discarded (109). Chimeric sequences were identified with Uchime (262) and removed together with contaminant human sequences. The remaining sequences were assigned to their respective samples by using the sample specific 6 base pair barcode , which preceded the primer. In order to perform sample wide comparisons, OTUs were generated with an extended ribosomal database project (RDP)-pyrosequencing approach (263), which was integrated into the phylotyping pipeline. All sequences were pooled and aligned with Infernal (V1.0I using a 16S r RNA secondary structure based model for accurate position alignment of sequences (264)). The aligned sequences were clustered by complete linkage to form OTUs with sequence distances ranging from 0% to 5%. For each OTU, a representative sequence was extracted and a taxonomic classification was assigned to it using the RDP Bayesian classifier 2.0.1 (265). Finally, the pooled sequences were again separated according to their sample affiliation (1).

2.7 Statistical analysis and visualization

Descriptive statistics are stated with the average of the parameter, the standard deviation is given in brackets. When data are not normally distributed, the median (Mdn) is shown. P-values lower than 0.05 were regarded as statistically significant.

Categorical and continuous variables were compared by the χ^2 -test and t-test, respectively. The analyses were performed using SPSS V23.0 (IBM, Paris, France) and R version 2.14.0 (R Foundation, Vienna, Austria). The Shapiro-Wilk test and the Kolmogorov Smirnov test were used to test for normal distribution. Heterogeneity of samples was analyzed with Levene's test. ANOVAs were used to investigate group differences between AN patients, other BMI groups and AT. Covariates (such as age, BMI, smoking) were introduced to correct for possible confounding effects. When data were not normally distributed, Kruskal-Wallis and Mann-Whitney U-Tests were used to test for significant differences. Sequence abundance in each sample was normalized to the sample with the maximum number of sequences. Additionally, abundance data were log transformed after uniformly adding a value, in order to down weight OTUs with high abundance, and to resemble the normal Gaussian distribution more closely. Principal component analysis (PCoA) was performed on the normalized, log-transformed data in QIIME (1).

To account for multiple comparisons, p-values were adjusted using the method proposed by Benjamini and Hochberg, false discovery rate (FDR) (266). Adjusted p-values less than 0.05 were considered statistically significant. Linear discriminant effect size (LEfSe) (267) analysis was performed, in order to detect statistically relevant strains which were present in several of the study groupings. LEfSe is a biomarker discovery and explanation tool for high-dimensional data; it determines the taxa most likely to explain differences between classes by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance. We performed LEfSe on the galaxy-server and used the default settings (Alpha value for the factorial Kruskal-Wallis-Test=0.05, threshold on the logarithmic LDA score for discriminative features= 2.0, all-against-all multiclass analysis) (1). LEfSe first applies a Kruskal-Wallis Test without p-value adjustment, then performs a Wilcoxon Test on subclasses and determines an LDA score, which is a measurement of effect size.

Metagenomic modeling of taxonomic information (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States- PICRUSt) was used to evaluate differences in the metagenome (268).

OTUs were visualized as PCoA plots using the QIIME core microbiome script.

Additionally, associations between gut microbiome community structures and study parameters were tested for statistical significance using the QIIME implementation of the Adonis test, and significance of individual bacterial strains was determined by the Kruskal-Wallis test. Adonis, a nonparametric statistical method, uses a QIIME distance matrix file (weighted or unweighted UniFrac distance matrix), a mapping file and a category in the mapping file to determine the sample grouping. Then, an R²-value (effect size) is calculated which displays the percentage of the variation explained, as well as the p-value to determine the statistical significance. Before performing the Kruskal-Wallis-Test, data were tested for Homogeneity of Variance. Data visualization was performed using GraphPad-Prism v5 and the Calypso software (3), with permission of Prof. L. Krause, University of Queensland, Australia (cgenome.net/calypso) (1).

3. Results

3.1 Descriptive statistics and tests for group differences

3.1.1 Characterization of groups

18 AN patients, 26 NW, 22 OW, 20 OB women and 20 AT took part in our study.

Demographical and clinical characteristics are depicted in Table 6. AN patients had a mean BMI of 15.29 ± 1.28 kg/m². BMI ranges of study groups are depicted in Table 7.

The mean age of the participants was 24.5 ± 4.6 years. Obese participants (M=26.9, SD=6.1) were significantly older than AT (M=22.15, SD=3.86, p=0.006) and AN patients (M=22.44, SD=3.20; p=0.015). All other groups did not show significant differences regarding age. All AT were normal weight. Six AN patients had the purging type and 12 patients had the non-purging type of AN. Mean age at onset of AN was 21.79 (3.62) years and mean duration of illness was 3.14 (3.51) years. Five patients had received high caloric nutritional supplements for up until seven to 19 days before stool-sampling. Stool samples of AN patients were acquired 10.61 (13.01) days after admission to the hospital (1).

<i>Population</i>	<i>AN</i>	<i>NW</i>	<i>OW</i>	<i>OB</i>	<i>AT</i>	<i>p-</i>
<i>Characteristics</i>	<i>(n=18)</i>	<i>(n=26)</i>	<i>(n=22)</i>	<i>(n=20)</i>	<i>(n=20)</i>	<i>Value</i>
Age	22.44	24.93	25.32	26.9	22.15	0.002
Mean (SD)	(3.20)	(3.75)	(3.98)	(6.09)	(3.86)	
[years]						
BMI	15.29	21.89	26.99	34.55	22.14	<0.001
Mean (SD)	(1.28)	(1.73)	(1.13)	(4.43)	(1.76)	
[kg/m ²]						
BMI range	13.24-	19.02-	25.12-	25.12-	19.01-	
	17.19	24.89	29.34	29.34	25.88	
Education	13.86	17.21	18.39	16.28	15.42	<0.001

Mean (SD)	(2.58)	(3.17)	(3.57)	(4.01)	(2.84)
[years]					

Table 6: Characteristics of the study population. This table was modified and reproduced from Mörkl et al., 2017 (1) with permission from the International Journal of Eating disorders (Copyright Transfer Agreement Section B, paragraph 4b).

3.1.2 Body composition assessments

3.1.2.1 Results of anthropometric assessments

We measured weight, height, height when sitting, waist and hip circumference and triceps skinfold thickness and calculated waist-hip-ratio, waist-height-ratio and triceps skinfold thickness. Results are depicted in Table 7.

<i>Anthropometric assessments</i>	<i>AN</i> <i>(n=18)</i>	<i>NW</i> <i>(n=26)</i>	<i>OW</i> <i>(n=22)</i>	<i>OB</i> <i>(n=20)</i>	<i>AT</i> <i>(n=20)</i>	<i>p-Value</i>
Weight	42.3	62.24	75.38	97.00	64.39	<0.001
Mean (SD)	(4.84)	(6.64)	(5.03)	(13.57)	(5.58)	
[kg]						
Height	1.67	1.68	1.66	1.68	1.68	0.211
Mean (SD)	(0.06)	(0.06)	(0.06)	(0.06)	(0.05)	
[m]						
Height when sitting	85.95	89.43	88.95	89.20	90.90	0.001
Mean (SD)	(3.60)	(3.61)	(3.18)	(3.76)	(2.88)	
[cm]						
Waist circumference	59.97	71.09	79.58	94.70	70.90	<0.001
Mean (SD)	(3.95)	(4.95)	(4.49)	(10.69)	(4.09)	
[cm]						

Hip circumference	79.31	93.46	106.59	121.84	97.94	<0.001
Mean (SD)	(4.15)	(6.54)	(6.57)	(7.99)	(5.79)	
[cm]						
Waist-hip-ratio	0.75	0.76	0.75	0.78	0.73	0.03
Mean (SD)	(0.04)	(0.04)	(0.06)	(0.07)	(0.04)	
Waist-height-ratio	0.36	0.42	0.48	0.57	0.42	<0.001
Mean (SD)	(0.03)	(0.03)	(0.03)	(0.07)	(0.03)	
Triceps Skinfold	7.36	19.85	26.41	35.13	14.53)	<0.001
Thickness	(3.40)	(4.35)	(5.32)	(8.31)	(4.30)	
Mean (SD)						
[mm]						

Table 7: Results of anthropometric assessments. This table was modified and reproduced from Mörkl et al., 2017 (1) with permission from the International Journal of Eating disorders (Copyright Transfer Agreement Section B, paragraph 4b).

3.1.2.2 Results of SAT patterning

SAT was measured on eight specific body sites with a novel ultrasound technique as described in section 2.2.2 of materials and methods (233). Table 8 shows the results of the measurements in our groups.

<i>Specific body sites</i>	<i>AN</i> <i>(n=18)</i>	<i>NW</i> <i>(n=26)</i>	<i>OW</i> <i>(n=22)</i>	<i>OB</i> <i>(n=20)</i>	<i>AT</i> <i>(n=20)</i>	<i>p-Value</i>
Brachioradialis	1.49	4.26	6.43	7.90	3.27	<0.001
Mean (SD)	(1.30)	(1.37)	(1.77)	(1.31)	(1.19)	
[mm]						
Upper Abdomen	3.89	13.58	27.48	46.27	9.82	<0.001
Mean (SD)	(2.52)	(8.65)	(9.61)	(14.87)	(7.53)	

[mm]						
Lower Abdomen	6.27	19.90	33.90	51.41	16.60	<0.001
Mean (SD)	(4.51)	(9.64)	(9.09)	(11.64)	(8.84)	
[mm]						
External	1.96	7.45	15.32	25.48	5.75	<0.001
Oblique	(1.88)	(3.75)	(5.15)	(10.60)	(4.17)	
Mean (SD)						
[mm]						
Front Thigh	4.99	12.63	19.09	24.04	10.33	<0.001
Mean (SD)	(3.35)	(3.12)	(4.00)	(7.88)	(3.31)	
[mm]						
Medial Calf	3.38	9.05	12.73	15.41	6.26	<0.001
Mean (SD)	(2.27)	(3.34)	(3.38)	(3.43)	(3.04)	
[mm]						
Erector spinae	3.22	7.54	13.36	23.29	7.05	<0.001
Mean (SD)	(2.07)	(2.70)	(4.11)	(7.24)	(4.25)	
[mm]						
Sum-score	29.12	85.05	144.31	205.63	66.87	<0.001
Mean (SD)	(18.69)	(29.46)	(29.69)	(28.87)	(30.77)	
(D _{INCL})						

Table 8: Results of subcutaneous adipose tissue patterning

3.1.2.3 Results of bioimpedance analysis (BIA)

Total body fat and basal metabolic rate were assessed by using BIA (235, 236). The results of the BIA are depicted in Table 9.

<i>Bioimpedance analysis</i>	<i>AN</i> (<i>n=18</i>)	<i>NW</i> (<i>n=26</i>)	<i>OW</i> (<i>n=22</i>)	<i>OB</i> (<i>n=20</i>)	<i>AT</i> (<i>n=20</i>)	<i>p-Value</i>
BIA- Total Body fat Mean (SD) [kg]	4.27 (2.46)	18.18 (4.21)	28.52 (2.92)	43.01 (8.68)	16.68 (3.70)	<0.001
BIA- Total Body fat Mean (SD) [%]	9.69 (5.05)	28.91 (4.21)	37.79 (2.25)	44.09 (3.24)	25.74 (4.17)	<0.001
BIA- Basal metabolic rate Mean (SD) [kcal]	1217.83 (43.37)	1319.46 (43.14)	1363.23 (41.85)	1466.70 (97.10)	1404.70 (66.76)	<0.001

Table 9: Results of Bioimpedance analysis. This table was modified and reproduced from Mörkl et al., 2017 (1) with permission from the International Journal of Eating disorders (Copyright Transfer Agreement Section B, paragraph 4b).

3.1.3 Results of questionnaires

3.1.3.1 Depression scales

AN patients (n=18) had mean BDI and HAMD scores of 21.72 and 18.22 respectively, reflecting moderate depression levels. All other groups did not show signs of depression on BDI and HAMD. AT showed lowest BDI ($M= 3.1$, $SD= 2.72$) and HAMD-scores ($M= 2.9$, $SD= 2.9$) compared to the other groups (1).

3.1.3.2 Fagerström Score

Out of all study participants, 25.5% reported being smokers. The average Fagerström score of all participants who reported to be smokers was 2.48. An ANOVA indicated significant differences in the Fagerström score ($F(4,101)=6.93$, $p<0.001$). All AT were non-smokers. The Fagerström score from AN patients was highest ($M=2.22$, $SD=3.84$) as shown by Tukey's post-hoc test, and differed significantly from the score of all other groups (NW: $M=0.15$, $SD=0.61$; OW: $M=0.32$, $SD=0.72$; OB: $M=0.80$, $SD=1.61$; AT: $M=0.00$, $SD=0.00$) (1).

3.1.3.3 International physical activity questionnaire (IPAQ)

The mean IPAQ score was calculated with total physical activity (MET) minutes per week. The mean score reached for all groups was 5820.09 (SD 8146.58). Because the IPAQ scores were not normally distributed, the Median (Mdn) and interquartile ranges (IQR) are given. AT reported highest MET-minutes (Mdn= 5888.50, IQR 4138.5) compared to other groups while AN patients reported the lowest scores (Mdn= 2199.75, IQR 5007.63) (1).

3.1.4 Results of nutritional data collection

Using the results of a dietary recalls, micro- and macronutrients were calculated. OB participants reported highest total energy intake, while NW subjects reported lowest total intakes. However, reported energy intakes between groups did not differ significantly ($p=0.33$). Groups differed regarding fibre intake and vitamin D intake. OW subjects reported lowest fibre intake while AT had highest fibre intake of our study groups. Patients

with AN reported highest vitamin D intake (mainly from food supplements) while normal weight subjects reported the lowest intake (1). Dietary supplements of AN patients were included in the analysis. The results of the nutrient intake in our study groups are presented in Table 10.

<i>Nutrients</i>	<i>AN</i> (<i>n=18</i>)	<i>NW</i> (<i>n=26</i>)	<i>OW</i> (<i>n=22</i>)	<i>OB</i> (<i>n=20</i>)	<i>AT</i> (<i>n=20</i>)	<i>p</i>
Energy	1950.39	1926.39	1700.79	2076.45	2021.35	0.33
Mean (SD)	(934.02)	(611.30)	(350.97)	(548.37)	(537.64)	
[kcal]						
Carbohydrates	238920.92	217542.51	182888.04	236804.11	230464.26	0.19
Mean (SD)	(126350.8)	(83857.52)	(52982.50)	(80835.24)	(73131.17)	
[mg]						
Fat	71513.26	83649.42	72384.51	88207.19	81385.81	0.25
Mean (SD)	(39105.88)	(29863.54)	(16530.96)	(26497.10)	(25917.06)	
[mg]						
Protein	78254.62	61383.35	64090.97	76845.933	75958.05	0.08
Mean (SD)	(36498.48)	(20438.39)	(19273.45)	(23740.49)	(27950.46)	
[mg]						
Fibre	21262.26	21459.98	17023.52	19433.18	26651.99	0.03
Mean (SD)	(12459.87)	(6717.67)	(6240.05)	(8017.22)	(13847.30)	
[mg]						
Magnesium	367.69	352.62	303.85	325.68	412.52	0.13
Mean (SD)	(173.78)	(117.91)	(89.74)	(107.66)	(192.35)	
[mg]						

Vitamin D	7.53	1.76	3.26	2.52	3.13	<0.00
Mean (SD)	(7.58)	(1.04)	(3.78)	(1.86)	(2.63)	1
[mg]						

Table 10: Nutrient intake of study participants

3.1.5 Results of laboratory parameters

Table 11 shows laboratory parameters of study groups. We detected significant differences of HDL ($p=0.013$), Cholesterol-HDL-ratio ($p=0.001$) and CRP ($p<0.001$) (1). AN patients had highest means for zonulin, while AT had the lowest values. Unexpectedly, we could not find significant differences of peripheral zonulin concentrations measured in blood serum ($H(4)=5.911$, $p=0.404$). Zonulin was significantly positively correlated with CRP ($r=0.244$, $p=0.012$), IL-6 ($r=0.269$, $p=0.005$), BMI ($r=0.192$, $p=0.049$), waist-circumference ($r=0.216$, $p=0.026$), upper arm circumference ($r=0.224$, $p=0.021$) and total SAT-mass ($r=0.203$, $p=0.042$). Further, we found associations of zonulin with nutritional intake. There was a positive correlation between zonulin and total energy intake ($r=0.225$, $p=0.020$), carbohydrate intake ($r=0.221$, $P=0.023$), protein intake ($r=0.210$, $p=0.031$) and fat intake ($r=0.207$, $p=0.034$).

<i>Laboratory parameters</i>	<i>AN</i> <i>(n=18)</i>	<i>NW</i> <i>(n=26)</i>	<i>OW</i> <i>(n=22)</i>	<i>OB</i> <i>(n=20)</i>	<i>AT</i> <i>(n=20)</i>	<i>p-Value</i>
Cholesterol	176.55	179.11	178.86	191.40	178.40	0.701
Mean (SD)	(42.22)	(34.47)	(26.45)	(38.44)	(38.66)	
[mg/dl]						
HDL	75.66	79.11	74.54	63.65	81.10	0.013
Mean (SD)	(14.42)	(16.88)	(16.45)	(22.20)	(11.89)	
[mg/dl]						
LDL	83.11	85.31	85.72	103.65	81.10	0.109

Mean (SD)	(33.86)	(28.32)	(23.51)	(30.80)	(29.18)	
[mg/dl]						
Cholesterol-	2.36	2.34	2.49	3.23	2.21	0.001
HDL-Ratio	(0.56)	(0.64)	(0.57)	(1.03)	(0.37)	
Mean (SD)						
Triglycerides	88.67	73.85	93.09	120.75	80.55	0.008
Mean (SD)	(38.38)	(36.42)	(38.87)	(65.38)	(31.76)	
[mg/dl]						
C-reactive	0.87	1.81	3.00	6.96	1.44	<0.001
protein	(1.23)	(1.77)	(3.91)	(5.04)	(2.08)	
Mean (SD)						
[mg/l]						
Interleukine-6	4.73	2.03	2.40	3.99	1.90	0.342
Mean (SD)	(12.81)	(0.93)	(1.21)	(1.68)	(0.62)	
[pg/ml]						
Zonulin	120.27	47.34	56.81	64.13	57.95	0.404
Mean (SD)	(306.30)	(18.84)	(23.20)	(30.12)	(29.67)	
[ng/ml]						

Table 11: Laboratory parameters. This table was modified and reproduced from Mörkl et al., 2017 (1) with permission from the International Journal of Eating disorders (Copyright Transfer Agreement Section B, paragraph 4b).

3.2 Results of microbiota analysis

3.2.1 Intestinal microbiota composition

In total, we acquired 106 stool samples and attained 6.180.771 sequences with on average 58.309 sequences per sample (range 7.012- 143.584) (1).

After removing chimeric reads and quality filtering, 5.475.612 sequences (range 25.083- 77.296) remained corresponding to 51,189 sequences per sample (range 25,083-77,296) on average. OTUs were clustered using a 97% similarity threshold (1).

Samples showed heterogenous prevalence of bacterial genera. On the phylum level, *Firmicutes* and *Bacteroidetes* were most dominantly found in all samples. *Leuconostoc* was the rarest genus while *Bacteroides* was the most abundant genus. Figure 4 depicts a hierarchical tree of all identified taxa in our samples (1).

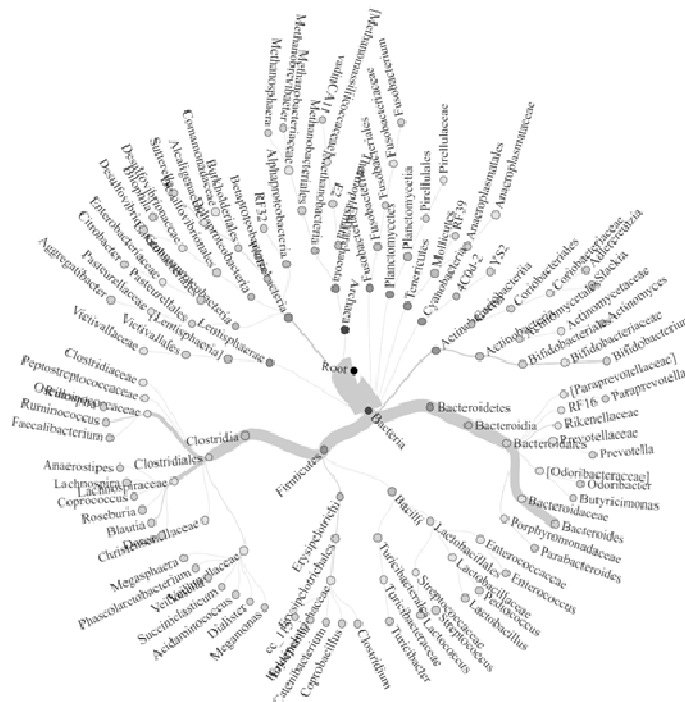


Figure 4: Hierarchical tree of identified taxa, starting from phylum-level (root) to genus level (branch). This figure was created using the Calypso-software (3) and is reproduced from Mörkl et al. 2017 (1) with permission from the International Journal of Eating disorders (Copyright Transfer Agreement Section B, paragraph 4b).

3.2.2 Alpha diversity

Figure 5 depicts the color-coded rarefaction curves of alpha diversity, with the Chao-1-estimator on the left and number of observed species on the right. This rarefaction analysis assesses how well the sequence data represent the diversity of microbial community studies. Microbial sequences are randomly drawn from each sample. The number of observed species is counted and plotted as a function of sequences sampled. The slope of the curve indicates if the underlying microbial community is well represented by the sequence data. A steep slope indicates that a large fraction of the diversity remains undiscovered, and if the curve becomes flatter to the right, a reasonable number of sequence reads has been obtained. Our obtained rarefaction curves showed adequate saturation at the selected rarefaction level (Figure 5).

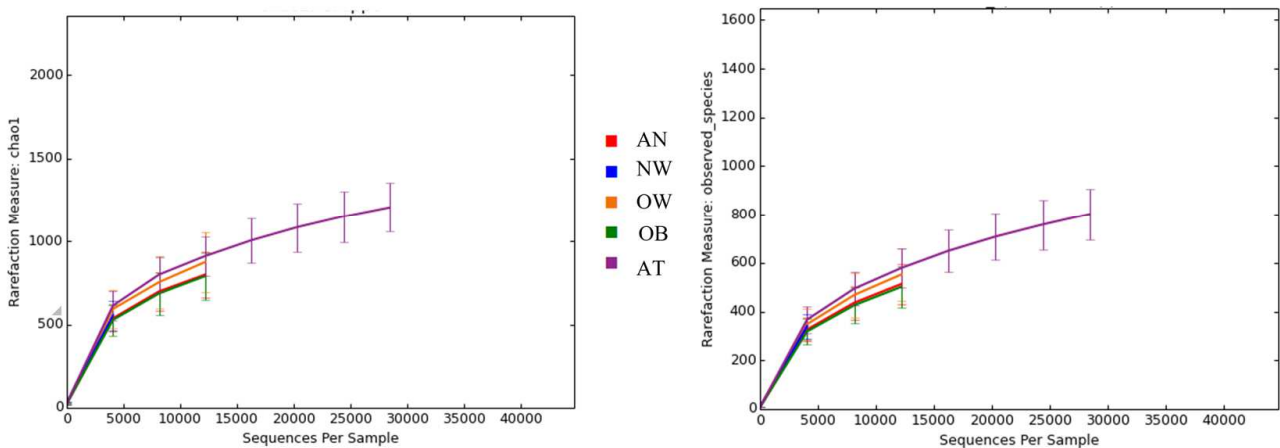


Figure 5: Rarefaction curves of groups.

Chao-1-diversity ($H(4)=10.26$, $p=0.04$) and the number of observed species ($H(4)=10.13$, $p=0.04$) differed significantly between the groups. AT displayed the highest Chao-1-diversity compared to OB ($p=0.011$), NW ($p=0.037$) and AN patients ($p=0.019$). AT showed a higher number of observed species in comparison to AN ($p=0.038$) and OB ($p=0.012$), while OB participants presented a significantly lower diversity than OW participants ($p=0.047$) (1). Figure 6A illustrates the number of observed species and Figure 6B the Chao-1-Diversity.

Diversity and evenness measure how equally the taxa within a microbial community are distributed. The Shannon-Index, which is a measurement of how many species are in a

dataset, simultaneously takes into account how evenly individuals within a community are distributed (269). We found a significant difference of the Shannon Index between groups ($F(98,4)=2.73$, $p=0.034$), even after correction for age ($F(4,97)=2.63$, $p=0.039$) (Figure 6C) Post hoc testing identified that OW ($M=6.56$, $SD=0.47$) and OB ($M=6.07$, $SD=0.55$) participants ($p=0.037$) differed significantly in their Shannon-Index (1).

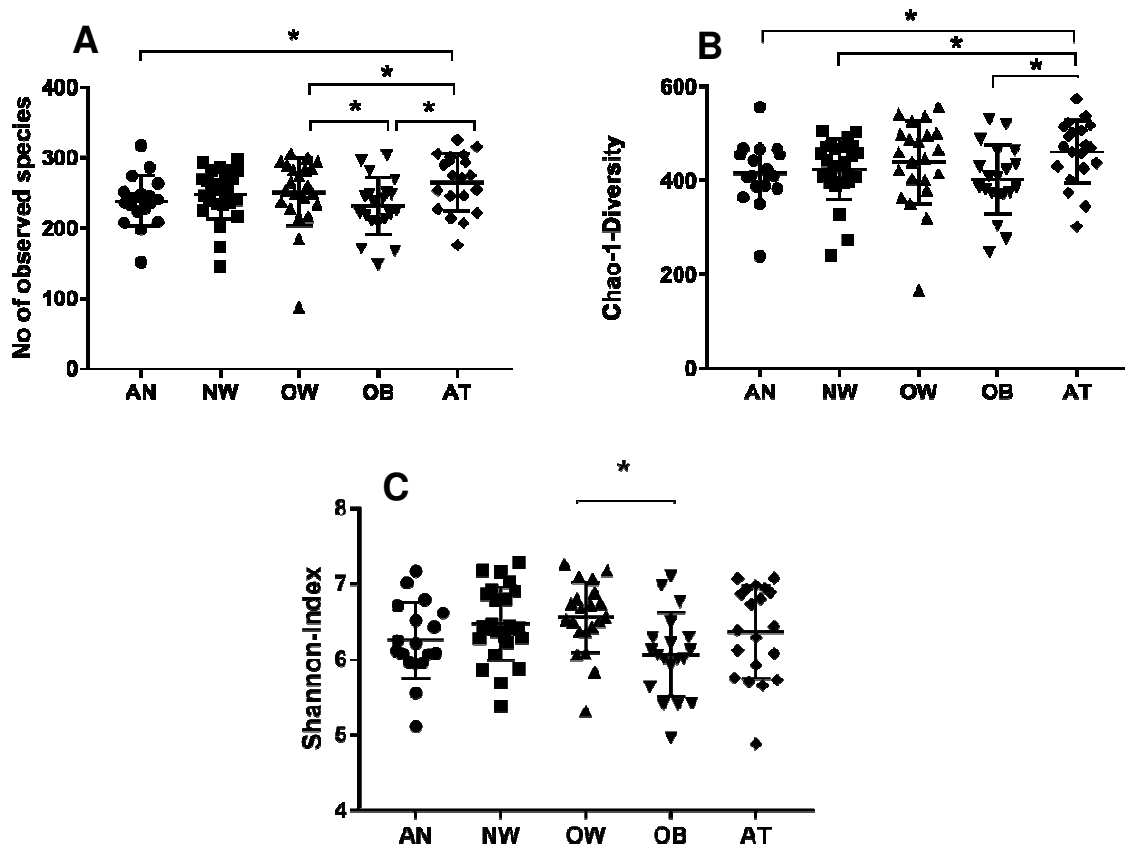


Figure 6: Evaluation of alpha diversity in groups. Richness was characterized by the number of observed species in each sample (A), the Chao-1-estimator of diversity (B) and the Shannon-Index (C). An asterisk indicates $p < 0.05$. This figure is reproduced from Mörkl et al., 2017 (1) with permission from the International Journal of Eating disorders (Copyright Transfer Agreement Section B, paragraph 4b).

3.2.3 Beta-Diversity

As a measure for beta-diversity, which compares samples based on bacterial community structures, weighted and unweighted UniFrac distances were used. Figure 7 (unweighted UniFrac Distance) and Figure 8 (weighted UniFrac Distance) show the color-coded principal component analysis plot (PCoA) of the groups. Using ANOSIM, a non-parametric method used for detecting whether groups of samples differ from each other, we identified significant differences of unweighted UniFrac distance matrix between all groups ($R=0.0602$, $p=0.002$) and weighted UniFrac distance matrix between all groups ($R=0.044$, $p=0.002$) (1).

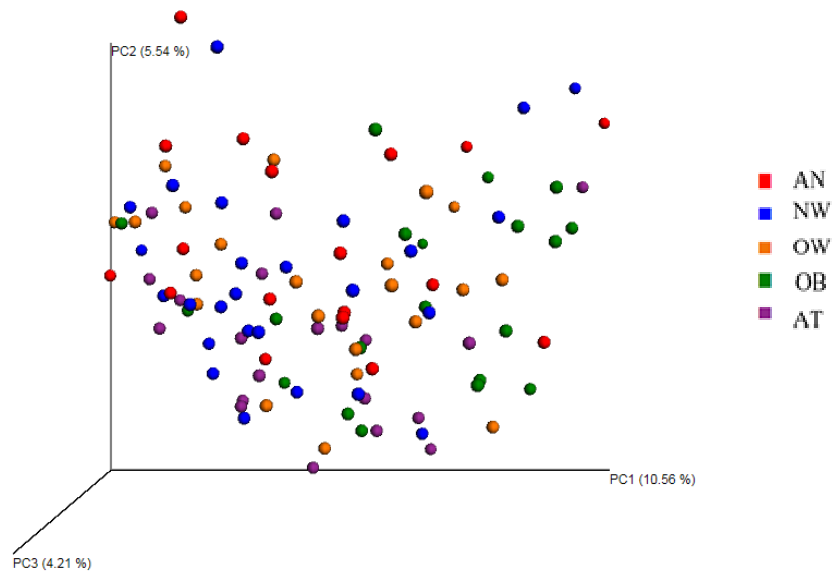


Figure 7: Principal component analysis (PCoA) of groups (unweighted UniFrac Distance). Each dot symbolizes the bacterial community composition of one individual stool sample. Axis titles indicate the percentage of the explained variation. This figure was modified and is reproduced from Mörkl et al., 2017 (1) with permission from the International Journal of Eating disorders (Copyright Transfer Agreement Section B, paragraph 4b).

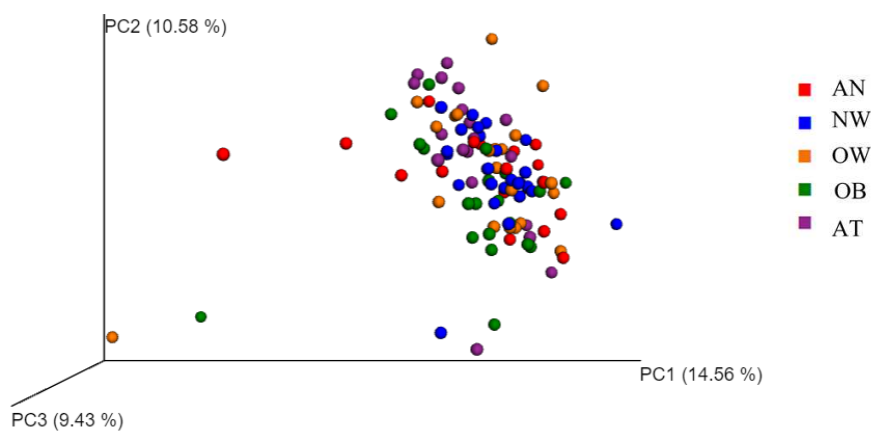


Figure 8: Principal component analysis (PCoA) of groups (Weighted UniFrac distance). Each dot symbolizes the bacterial community composition of one individual stool sample. Axis titles indicate the percentage of the explained variation. This figure was modified and is reproduced from Mörkl et al., 2017 (1) with permission from the International Journal of Eating disorders (Copyright Transfer Agreement Section B, paragraph 4b).

3.2.4 Adonis-Analysis

Adonis-analysis describes the strength and significance that a categorical or continuous variable (grouping) has in determining the variation of distances between bacterial species in a phylogenetic tree.

3.2.4.1 Adonis analysis of unweighted UniFrac

The results of an unweighted Adonis analysis showed significant associations of beta diversity with BMI, anthropometric measurements (upper arm circumference, hip circumference), the results of BIA (phase angle, fat mass, body cell mass, basal metabolic rate), SAT measurements (measurement locations: erector spinae, lower abdomen, medial calf) and smoking status (FTND) (1) (Table 12).

<i>Parameters</i>	<i>Sum of squares</i>	<i>Mean squares</i>	<i>F-Model</i>	<i>R2</i>	<i>Pr(>F)</i>
BMI	0.117	0.11701	17356	0.0169	0.036
Upper arm circumference	0.1299	0.12994	19.311	0.0188	0.029
Hip circumference	0.1128	0.11283	16.726	0.0163	0.045
BIA- body cell mass	0.119	0.11898	17654	0.0172	0.042
BIA- body fat mass	0.1396	0.1396	20776	0.0202	0.014
BIA- basal metabolic rate	0.1169	0.11687	17335	0.0169	0.043
BIA- phase angle	0.1541	0.15411	22985	0.0223	0.008
US- Erector Spinae	0.1409	0.14094	21002	0.0208	0.01
US- Lower Abdomen	0.1488	0.14876	2203	0.0216	0.011
US- Medial Calf	0.1192	0.11916	17615	0.0173	0.041
FTND Score	0.1926	0.19256	28883	0.0278	0.005

Table 12: Results of unweighted Adonis analysis.

3.2.4.2 Adonis analysis of weighted UniFrac

A weighted Adonis analysis, which takes abundances of bacteria into account, identified significant associations of gut microbiota with BMI group, BIA phase angle, ultrasound measurements (measurement locations erector spinae, lower abdomen), results of clinical chemistry (CRP, Cholesterol-HDL-ratio), smoking status (FTND score) and depression (HAMD) (1) (Table 13).

<i>Category</i>	<i>Sum of squares</i>	<i>Mean squares</i>	<i>F-Model</i>	<i>R2</i>	<i>Pr(>F)</i>
BMI group	0.3778	0.37781	15862	0.0155	0.001
BIA- phase angle	0.3199	0.31986	13397	0.0131	0.008
US- Erector Spinae	0.3265	0.3265	13718	0.0137	0.005
US- Lower Abdomen	0.2869	0.28686	1199	0.0119	0.04
CRP	0.2947	0.29471	12331	0.0121	0.043
Cholesterol-HDL-ratio	0.2992	0.29918	1252	0.0122	0.024
FTND Score	0.3698	0.36982	15522	0.0151	0.002
HAMD Score	0.2852	0.28517	11919	0.0118	0.047

Table 13: Results of weighted Adonis analysis.

3.2.5 Differences of taxa abundance

Differences in taxa abundance have been calculated with Calypso (3) after log₁₀-transformation of data. P-values were adjusted for multiple testing by FDR (266). Top 300 most abundant taxa have been included in the analysis.

3.2.5.1 Phylum level

At the phylum level, we identified significant differences in the taxa of *Firmicutes* and *Bacteroidetes*. AT and OB participants showed highest counts of *Firmicutes*, while AN patients, NW and OW participants showed comparatively low counts. *Bacteroidetes* was found with the highest abundances in AT and the lowest in NW participants. Figure 9 and Table 14 depict these results.

<i>Taxa</i>	<i>P</i>	<i>FDR</i> ▼	<i>AN</i> <i>mean</i>	<i>NW</i> <i>mean</i>	<i>OW</i> <i>mean</i>	<i>OB</i> <i>mean</i>	<i>AT</i> <i>mean</i>
Firmicutes	0.000037	0.00035	4.26	4.13	4.24	4.28	4.51
Bacteroidetes	0.000058	0.00035	4.16	4.14	4.24	4.30	4.46

Table 14: Significantly different abundances of taxa at phylum level between groups.

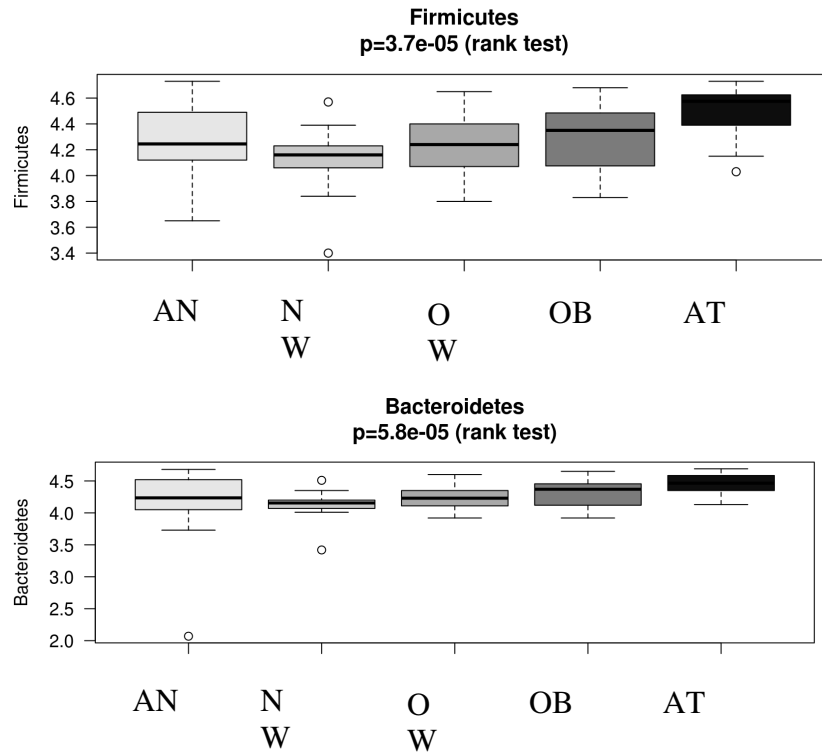


Figure 9: Boxplots of *Bacteroidetes* and *Firmicutes* abundance in groups. This figure was created using the Calypso-software (3).

3.2.5.2 Class level

At the class level, significant differences have been identified in the classes of *Clostridia* and *Bacteroidia*. AT showed the highest counts of Clostridia and Bacteroidia while NW participants showed the lowest counts. Results are depicted in Table 15.

<i>Taxa</i>	<i>P</i>	<i>P</i> <i>Bonferroni</i>	<i>FDR</i> ▼	<i>AN</i> <i>mean</i>	<i>NW</i> <i>mean</i>	<i>OW</i> <i>mean</i>	<i>OB</i> <i>mean</i>	<i>AT</i> <i>mean</i>
Clostridia	0.000057	0.0011	0.00058	4.22	4.1	4.17	4.23	4.46
Bacteroidia	0.000058	0.0012	0.00058	4.16	4.14	4.24	4.3	4.46

Table 15: Significantly different abundances of taxa at class level between groups.

3.2.5.3 Order level

At the order level, AT showed the highest means for *Clostridiales* and *Bacteroidales* compared to the other groups (Table 16).

<i>Taxa</i>	<i>P</i>	<i>P</i> <i>Bonferroni</i>	<i>FDR</i> ▼	<i>AN</i> <i>mean</i>	<i>NW</i> <i>mean</i>	<i>OW</i> <i>mean</i>	<i>OB</i> <i>mean</i>	<i>AT</i> <i>mean</i>
Clostridiales	0.000057	0.0014	0.0007	4.22	4.1	4.17	4.23	4.46
Bacteroidales	0.000058	0.0014	0.0007	4.16	4.14	4.24	4.3	4.46

Table 16: Significantly different abundances of taxa at order level between groups.

3.2.5.4 Family level

At the family level, significantly different abundances of *Ruminococcaceae*, *Bacteroidaceae*, *Lachnospiraceae*, *Clostridiaceae*, *Odoribacteraceae* and unclassified bacteria were observed (Table 17).

<i>Taxa</i>	<i>P</i>	<i>P</i> <i>Bonferroni</i>	<i>FDR</i> ▼	<i>AN</i> <i>mean</i>	<i>NW</i> <i>mean</i>	<i>OW</i> <i>mean</i>	<i>OB</i> <i>mean</i>	<i>AT</i> <i>mean</i>
Ruminococcaceae	0.000031	0.0012	0.0012	3.84	3.75	3.85	3.84	4.13
Bacteroidaceae	0.00017	0.0066	0.0033	4.03	3.99	4.12	4.17	4.32
Lachnospiraceae	0.00085	0.033	0.011	3.77	3.65	3.68	3.81	3.97
Clostridiaceae	0.0031	0.12	0.03	2.01	1.8	2.18	2.17	2.55
Unclassified	0.0046	0.18	0.036	3.42	3.2	3.29	3.13	3.55
Odoribacteraceae	0.0079	0.31	0.051	1.53	1.65	2.83	0.1	2.01

Table 17: Significantly different abundances of taxa at family level between groups.

3.2.5.5 Genus level

Table 18 shows significant differences at genus level identified by Kruskal-Wallis-Test.

<i>Taxa</i>	<i>P</i>	<i>P</i> <i>Bonferroni</i>	<i>FDR</i> ▼	<i>AN</i> <i>mean</i>	<i>NW</i> <i>mean</i>	<i>OW</i> <i>mean</i>	<i>OB</i> <i>mean</i>	<i>AT</i> <i>mean</i>
Bacteroides	0.00017	0.0088	0.0033	4.03	3.99	4.12	4.17	4.32
Roseburia	0.00019	0.0099	0.0033	1.87	1.24	2.25	2.64	2.65
Unclassified	0.00013	0.0068	0.0033	4.09	3.9	4.08	3.98	4.29
Coprococcus	0.0027	0.14	0.035	2.57	2.58	2.53	2.61	2.97
Dorea	0.004	0.21	0.042	2.27	2.03	2.17	2.5	2.4

Table 18: Significantly different abundances of taxa at genus level between groups.

3.2.6 Linear discriminant effect size (LEfSe)

Taxa associated with different biological conditions were identified with LEfSe (267). LEfSe is less likely to identify discriminative features with a high group number; this is due to the fact that LDA effect size is done in a multiclass analysis in an all against all approach. Therefore, we chose to perform a stepwise LEfSe-approach in groups.

First, LEfSe revealed only one significantly enriched phylotype in AN compared to other entities (LDA score >3.5): the family of *Coriobacteriaceae* (1). Figure 10 depicts the cladogram of the three groups.

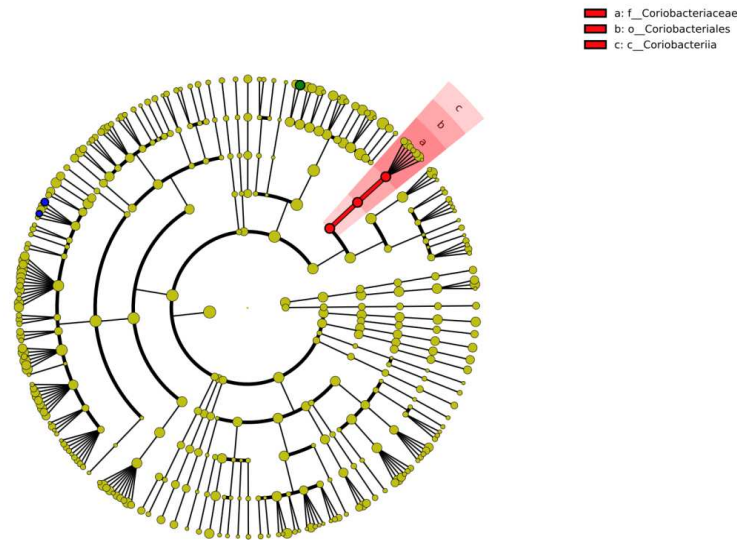


Figure 10: Cladogram representing the LefSe output. Only one highly significant association with entities (LDA score of >3.5) was found, namely Coriobacteriaceae. The diameter of each circle is proportional to the taxon's abundance. This figure is reproduced from Mörkl et al., 2017 (1) with permission from the International Journal of Eating disorders (Copyright Transfer Agreement Section B, paragraph 4b).

Secondly, we performed LefSe on the gut microbiome data of AN patients in comparison to NW participants. We could identify significantly discriminant features which are depicted in Figure 11.

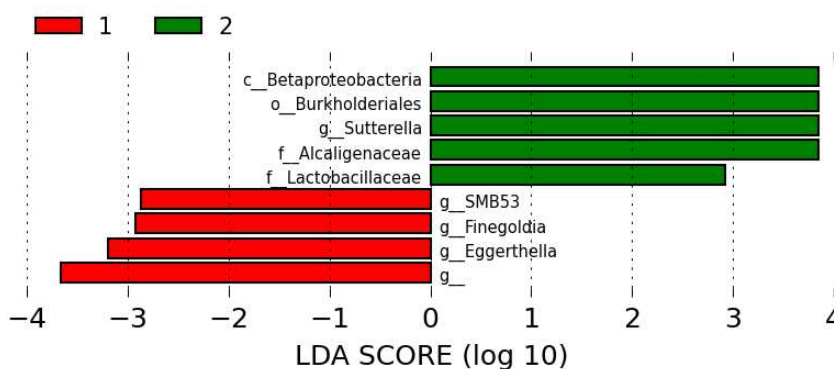


Figure 11: Comparison of discriminant features in LDA score between AN (1) patients and NW (2) controls.

Third, we compared AN patients to OW participants. LefSe identified 14 discriminant features between the two groups which are depicted in Figure 12 below.

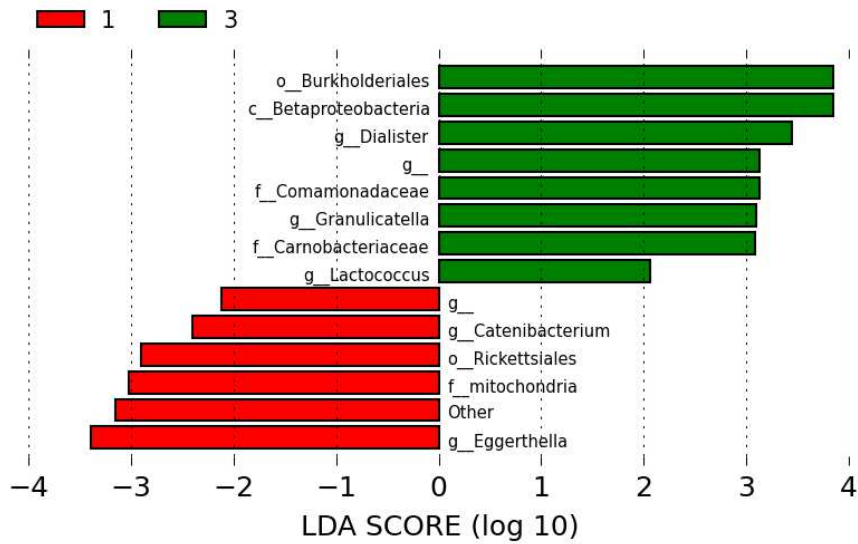


Figure 12: Comparison of discriminant features in LDA score between AN (1) patients and OW (3) controls.

After this, AN patients were compared to OB participants. The results are depicted in Figure 13. Overall, we could identify 34 discriminative features with LDA-score above 2.0.

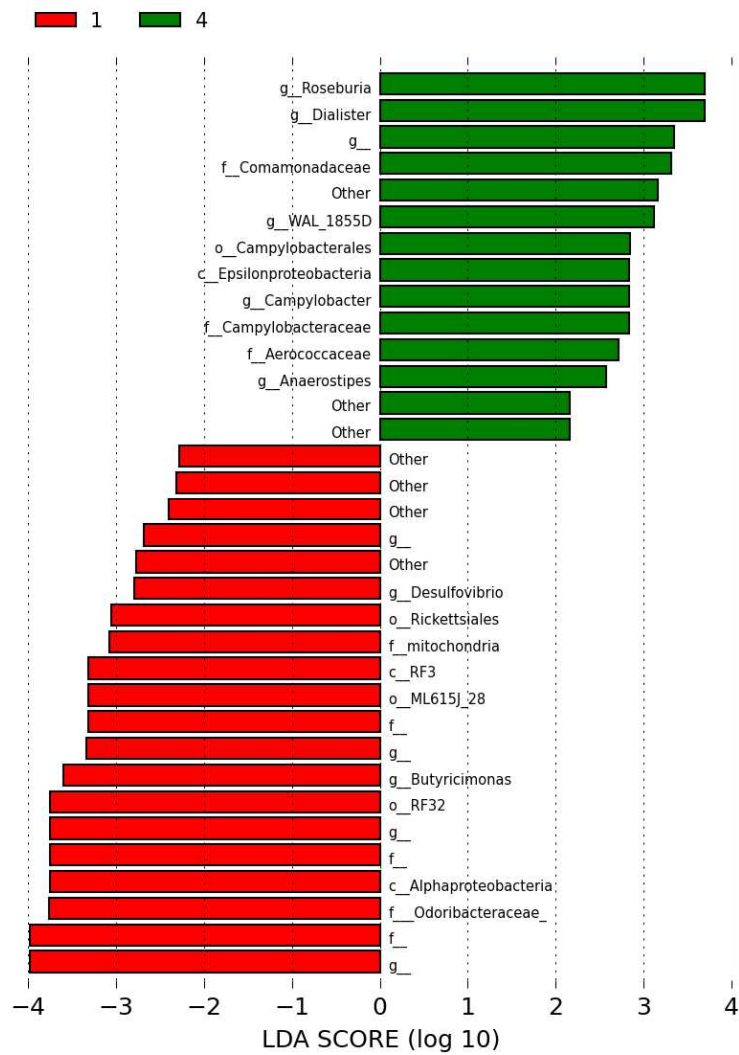


Figure 13: Comparison of discriminant features in LDA score between AN (1) patients and OB (4) controls.

Following this, we compared AN to AT using LEfSe. We found 21 discriminant features, which are depicted in Figure 14.

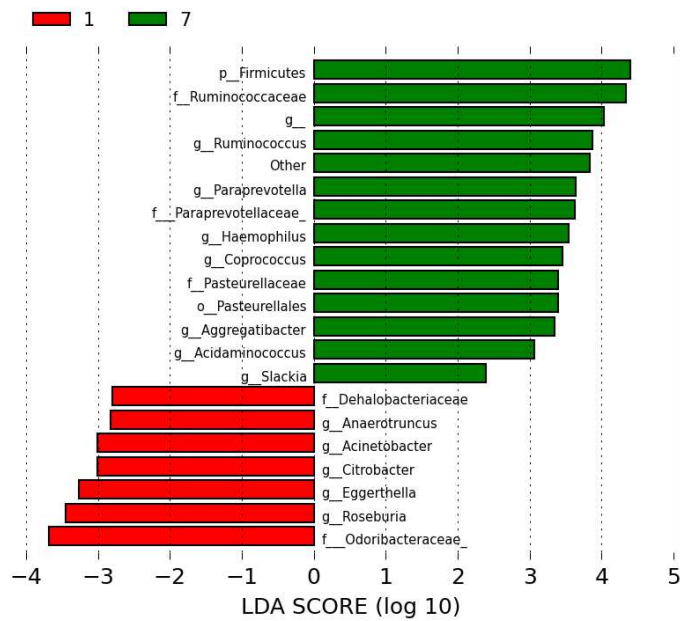


Figure 14: Comparison of discriminant features in LDA score between AN (1) patients and AT (7).

3.2.7 Metagenomic modeling of taxonomic information (PICRUSt)

PICRUSt was used to evaluate differences in the metagenome (1). Only stilbenoid-biosynthesis could be found as a differential pathway, as it was more active in AN compared to the other groups (Figure 15); however, the effect was relatively small.

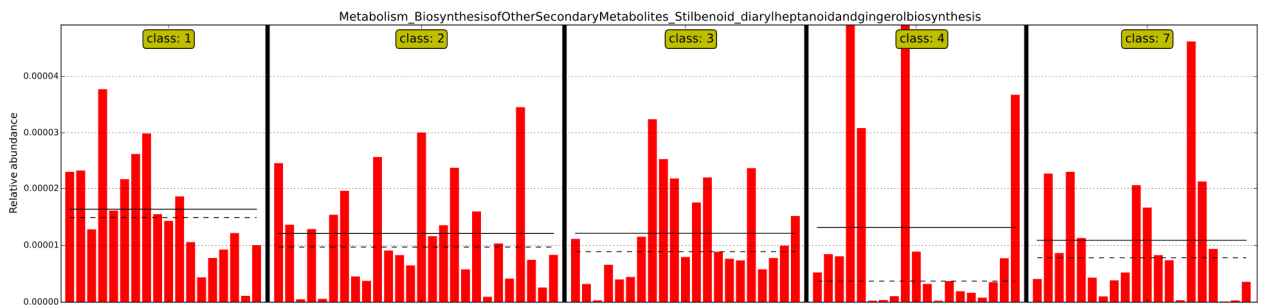


Figure 15: Results of PICRUSt analysis.

3.3 Analysis of correlations

3.3.1 Correlations with alpha diversity

First, we examined correlations between alpha diversity and dietary compounds (carbohydrates, fat, protein, fibre, vitamin D and magnesium). Positive correlations were revealed between Chao-1-index, fibre intake ($r=0.273$, $p=0.005$) and vitamin D ($r=0.263$, $p=0.006$), as well as between the number of observed species, fibre intake ($r=0.302$, $p=0.002$), vitamin D ($r=0.246$, $p=0.006$) and magnesium ($r=0.237$, $p=0.014$) (1). There were no significant correlations of alpha diversity and laboratory parameters. Alpha diversity was correlated with BDI-scores, demonstrating that greater levels of depression correlated with a lower number of observed species ($r= -0.256$, $p= 0.032$) and Chao-1-diversity ($r=-0.212$, $p=0.010$), but only when all groups were included in the analysis (1). This correlation could not be shown in the group of AN patients. Furthermore, there were no significant correlations of alpha diversity and SAT, or alpha diversity and BIA parameters, and also no significant correlations of alpha diversity parameters and the IPAQ score (1).

3.3.2 Correlations of taxa with BMI

We performed Spearman's correlations in Calypso (3) to identify taxa correlating with BMI. Table 19 shows the results.

<i>Taxa</i>	<i>P</i>	<i>R</i>	<i>Mean Abundance</i>	<i>Positive Samples</i>
Roseburia	0.00037	0.343	2.079	101
Dialister	0.00400	0.2802	0.235	79
Clostridium	0.03500	0.207	0.686	92
Anaerostipes	0.04000	0.2017	0.585	94

Table 19: Correlations on taxonomic level with BMI.

4. Discussion

4.1 Summary of the findings

This study demonstrates for the first time alterations in gut microbiota in a large female cohort divided into different BMI groups, AN patients and normal weight AT.

Further, associations of gut microbiota community structures, parameters of body composition, lifestyle and nutrition, along with gut barrier integrity and inflammation parameters, have been identified (1).

We report alterations of gut microbial diversity indices, microbial community structures, and the relationship of community structures with anthropometric measurements, SAT thicknesses, depression scores, BIA and laboratory values such as inflammation markers and zonulin. Moreover, as far as we are aware, this is the first that time serum levels of zonulin in AN patients compared to other BMI groups and AT have been described.

The results of this study can be summarized as follows: Species depletion of the gut microbiome is prominent in AN and OB participants, while AT show highest species counts compared to the other groups. Groups differ significantly concerning gut community structure, and further, gut community structures are related to disease parameters. Furthermore, significant clade differences on every taxonomic level are evident between the investigated groups. *Coriobacteriaceae* were identified as a significantly altered clade in the AN group in comparison to OB and AT (1). In addition, we confirmed the results of a study by Mack et al. (222) showing a depletion of *Roseburia* in AN, and point out possible microbial candidates for future interventional studies.

This work therefore contributes to the development of future recommendations regarding the treatment of AN. Observations from this field of research may help in the identification of novel therapies and targeted interventions for eating disorders and other forms of over- and undernutrition.

4.2 Answers to research questions, results of the study

The major aims of this study were:

- (1) To identify to what extent gut microbiota in AN differ in comparison to other BMI groups and AT.
- (2) To investigate associations of gut microbiota community structures, parameters of body composition (anthropometric measurements, total body fat and fat distribution), depression measures, smoking status, physical activity, nutrition and laboratory parameters (serum lipids, markers of gut barrier and inflammation).

We could verify our hypotheses 1-4:

- (1) Gut microbiota are significantly different between groups in terms of alpha- and beta diversity (1).
- (2) Gut community structure is associated with the investigated parameters (body composition, depression scales, smoking status, physical activity, nutrition and laboratory parameters) (1).
- (3) There are significant differences between study groups in the abundance of taxa on phylogenetic levels (1).
- (4) Alpha diversity is correlated with fibre intake, vitamin D, magnesium and BDI-scores (1).

However, we had to reject hypothesis 5:

- (5) Zonulin concentrations in AN were not significantly higher compared to other groups.

4.3 Differences in alpha and beta diversity of groups

As expected, alpha diversity, measured by the number of observed species and Chao-1, was significantly different between the investigated groups (1). Our analyses of the gut microbiota of AN patients, AT and the other BMI groups fit in part with previous studies on AN patients. We found that AT displayed the most diverse gut microbiota, while both OB and AN individuals presented with a microbial community of reduced diversity, relative to AT. While our data do not demonstrate that alpha diversity is lower in OB

individuals compared to NW individuals as reported previously (270, 271), they do confirm that AT display a higher diversity of the gut microbiota than NW participants (213).

A diverse gut microbiome is essential for the energy harvest from food (166, 272). In a study from Mack et al. it was shown that alpha diversity was decreased in AN, and that it increased after weight gain in AN patients (222); this is in line with our study results showing that AN patients showed a comparably low alpha diversity (1).

Alpha diversity might be important in the pathogenesis of diseases; it has been shown that lower bacterial diversity is associated with inflammatory bowel disease (273), lower resistance to pathogenic bacteria (74) and with greater depression and anxiety in AN (220), indicating that a more diverse gut is healthier. This was also confirmed by our study: higher BDI-scores were correlated with lower species-richness but only when all study groups were included in the analysis (1). An increasing amount of evidence suggests that alpha diversity is particularly lower in disease states compared to healthy controls, indicating that low diversity and species richness might be a general feature associated with several human disease conditions. Low alpha diversity is a common feature of industrialized, western countries. Among others, candidate risks for decreasing alpha diversity are lifestyle factors such as eating behaviors (western diet), increases in antibiotic consumption and disruptions of circadian rhythms (274). Therefore, low alpha diversity might also be a key regulator influencing the course and the progress of eating disorders such as AN. On the contrary, an increase in diversity levels (which could be accomplished through prebiotics, probiotics, a nutrient dense diet and physical activity) could lead to improved weight restoration and influence mood states through the gut-brain-axis in a positive way through normalizing brain function.

Additionally, alpha diversity is known to be influenced by physical activity (213). It has been publicized that physical training leads to an increase of alpha diversity (213, 275). In the current study, AT reported highest median IPAQ-scores (MET-minutes) compared to other groups, while AN patients reported the lowest scores, which could have influenced the alpha diversity levels measured (1). However, since the IPAQ is a self-rating questionnaire, the results could have been affected by over- or underreporting. As the

IPAQ only assesses the physical activity from the previous seven days, general long-term physical activity may not be well reflected by our results. Additionally, the data are solely based on the subjective rating of the participants and further, might have lower reliability in study participants with very low (252) or high BMI (279).

Furthermore, there exist various methods for calculating alpha diversity, besides Chao-1 index, Shannon diversity and the number of observed species, which are used in most studies. Up to now, there has been no strict consensus about alpha diversity measures, however, a consistent terminology related to species diversity exists. Diversity itself is not to be confused with a diversity index, as an index serves as a surrogate for diversity itself. Certain indices are sensitive to the presence of “rare” sequences which often leads to an overestimation of diversity. However, it remains difficult to distinguish between sequencing artefacts which could be mistaken for rare OTUs and the “real” rare sequences (276).

Moreover, it has been suggested that diversity alone might not be enough to account for a “healthy state” of the gut microbiome. For example, in autism, Finegold et al. reported higher microbial diversity in children suffering from autism compared to controls; this was largely attributed to diverse species of *Clostridia* (277), which were suspected to contribute to a pathogenic state. Therefore, alpha diversity must be seen as a context-dependent parameter and not as a stand-alone indicator for a healthy gut microbiome.

We used ANOSIM to detect whether BMI groups and AT differed from each other, in terms of community structures. Indeed, we identified significantly different community structures, using weighted and unweighted UniFrac distance matrices. Our results are consistent with a study from Dominianni et al., who found significant differences in the gut microbiome composition according to BMI, in normal weight and obese participants (278). However, the variances explained by BMI which contributed to the differences found in our study were, overall, small; only 6% and 4% of the variance were explained by the BMI groups (1).

4.4 Differences in the abundance of taxa on phylogenetic levels

Overall, we could identify differences on every phylogenetic level between the included BMI groups, AN patients and athletes. The differences from phylum to genus level arise primarily from bacteria which are known to be responsible for energy harvesting from diet.

The “energy harvest hypothesis”(143) suggests that a high intake of dietary fibre (which is the main source of SCFA produced by gut bacteria), leads to weight gain. Nonetheless, in western countries, the daily intake of fibre is low and it is unknown whether it is predominantly relevant for the development of obesity (279). Therefore, energy harvesting properties may also be strain specific or dependent on gut bacterial communities (different bacteria sharing an environment). In the following, differences in the abundance of main taxa will be discussed with regard to the related literature.

The two dominating phyla in the gut are *Bacteroidetes* and *Firmicutes*, both of them are responsible for energy harvesting from diet (143). AT and OB participants showed the highest counts of *Firmicutes*, while AN patients, NW and OW showed comparatively low counts. *Bacteroidetes* were most prominent in AT, while NW participants showed the lowest abundances.

Firmicutes and *Bacteroidetes* have been connected to body weight and BMI in rodent and human studies. For example, it was shown that ob/ob mice have more *Firmicutes* than *Bacteroidetes* in their gut microbiome compared to genetically lean mice (143, 144); this is also in line with the results of our study: OB participants had higher mean abundances of *Firmicutes* than AN and NW participants. In a study from Turnbaugh et al. it was demonstrated, that obese participants suffering from metabolic syndrome and diabetes showed an increase in *Firmicutes* along with low bacterial diversity, which was associated with poor health status (144).

However, we did not find a reduction of *Bacteroidetes* in OB participants such as that found by Arumugam (111), in comparison to NW subjects. This could be due to the fact, that *Bacteroidetes* are not only the most abundant, but also the most variable genus in the human gut (111). The phyla of *Bacteroidetes* and *Firmicutes* are also highly variable in

terms of abundance between subjects; moreover, the phylum of *Bacteroidetes* has been reported to either decrease, increase or stay the same during weight reduction (142). However, the findings from a murine study from Samuel et al. are in line with our results: *Bacteroidetes* were more abundant in obese hosts (145).

Interestingly, in our study, AT showed the highest levels of *Firmicutes* and *Bacteroidetes*. Since *Firmicutes* are known to produce SCFAs such as butyrate, and fulfil the function of energy harvesting (143), it might be the case that the gut microbiome of AT adapts itself to higher energy needs in order to generate an appropriate energy supply for athletic performance, through increasing the total abundance of *Firmicutes* and *Bacteroidetes*. This is in line with the study results from Mika et al. and Evans et al., which showed that activity increased the overall abundance of beneficial microbiota (148, 280). Exercise was shown to change phyla, in particular, leading to an increase of *Bacteroidetes* and decrease of *Firmicutes* in mice (148). On the contrary, the study results from Lambert et al. showed a greater abundance of select *Firmicutes* species and lower abundance of *Bacteroides* species in normal and diabetic exercised mice compared to non-exercised mice (211). Interestingly, it was shown that this could be dependent on the duration of regular exercise: short term exercise in rats was associated with higher *Bacteroidetes* and lower *Firmicutes*, but long term (six-week exercise activity) led to a greater abundance of *Firmicutes*.

In a study from Clarke et al. who investigated the gut microbiome of AT (40 elite rugby players) it was found that *Bacteroidetes* was the only taxon which was significantly less abundant in AT. Moreover, AT had greater amounts of *Firmicutes* than controls (213). In line with this, it was concluded in the American gut project, that increasing moderate exercise drives diversity in the *Firmicutes* phylum which contributes to a healthy gut environment (281). This underlines the results of our study, namely, that AT showed even more *Firmicutes* than OB participants.

In our study, AT showed the highest counts of *Clostridia* and *Bacteroidia* while NW showed the lowest counts. *Clostridia* are a part of the *Firmicutes* phylum and can further be divided into the clusters *Ruminococcaceae* and *Lachnospiraceae* (282). The presence of *Clostridia* is thought to be influenced by high physical activity (213). *Clostridium* species are important for supplying colonocytes with butyrate produced by the fermentation of

otherwise indigestible fibre (283). The SCFA butyrate provides energy gained from diet and is the preferred source of energy for colonocytes (284). The relatively high number of *Clostridia* in the AT group studied here could be due to exercise and to stress during the phase of competition. This inference is supported by the observation that mice exposed to stress present with significant increases of caecal *Clostridium* levels (285).

Ruminococcaceae are a member of the *Firmicutes* phylum. They are one of the three major groups of dominant gut bacteria (*Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3)) regardless of nationality, and are therefore seen as common gut microbes (111). *Ruminococcaceae* are also responsible for the breakdown of complex carbohydrates and are predominantly found in subjects with high-carb diets consisting of unprocessed whole grains and legumes (111, 286). In fact, AT in our study reported the highest fibre intake and third highest total carbohydrate intake of the investigated groups (1), which could account for the high amounts of *Ruminococcaceae* found in the AT group.

Importantly, we identified *Roseburia* to be less abundant in AN patients and NW subjects, while in AT, *Roseburia* was found to be relatively highly abundant. *Roseburia* is a member of the *Lachnospiraceae* family (287). Mack et al. found that *Roseburia* was the only genus that correlated with butyrate in AN patients (222). *Roseburia* is known as a carbohydrate degrader (288) and is also a key SCFA (butyrate) producer (289, 290). Mack et al. reasoned that low abundances of *Roseburia* might explain decreased butyrate proportions in AN before weight gain. Furthermore, a depletion of *Roseburia* has been linked to inflammatory bowel disease (291) and associated with intestinal barrier dysfunction (292). We also confirm the results of former studies, which have identified that *Roseburia* is more abundant in obese subjects (293, 294); this was the case also in the current study, and it emphasizes the important role of *Roseburia* as a butyrate producer for energy harvest. Therefore, *Roseburia* could be a candidate for interventional probiotic studies in AN patients by increasing gut barrier function and energy harvest as its main mechanism of action.

In general, AT showed higher total abundances of genera and higher diversity, which might be important factors of a healthy gut microbiome. Furthermore, besides the abundance of certain bacterial genera, the function of the microbiota and interactions

between microbiota in this group, might be central for health. In the next step, we used LEfSe analysis to identify differentially abundant features which could be biomarkers of the AN group in comparison to other groups.

4.5 Identification of differentially abundant taxa in AN compared to other study groups, using LEfSe analysis

Using LEfSe, there was revealed to be only one significantly enriched family in AN compared to other entities (LDA score >3.5); this was the family of *Coriobacteriaceae* (1).

Coriobacteriaceae, which belong to the phylum *Actinobacteria* within the order of *Coriobacteriales*, might therefore play a role in the disease mechanism of AN, since they are known to influence a range of metabolic functions such as bile salt and steroid conversion, the activation of dietary polyphenols, and lipid metabolism (295-297). They have also been described as acting as pathobionts (bacteria which are pathogens under certain conditions), and causing bacteremia, periodontitis and vaginosis (295, 298). Animal studies have shown correlations of *Coriobacteriaceae* and non-HDL-plasma cholesterol levels and triglycerides (299, 300). High serum lipids are common in AN patients; *Coriobacteriaceae* may therefore be a contributing factor to high serum lipids in AN (301). Interestingly, *Coriobacteriaceae* have been associated with animals not getting obese while receiving a high fat diet (302, 303). The relation between *Coriobacteriaceae* and bile acid metabolism could be an important factor here, since it has been shown that bile acids are also important for gut barrier function (304). An overgrowth of *Coriobacteriaceae* might be involved in the gut dysbiosis of AN patients, however, further studies are needed to gain insight in the role of *Coriobacteriaceae* in eating disorders such as AN.

In a second step, we again performed LEfSe, this time including the AN group and the other BMI groups in separate steps to identify differentially abundant genera. In the following, the main differentially abundant bacterial genera are discussed in detail.

While *Eggerthella*, *SMB53* and *Finegoldia* were significantly more abundant in AN patients compared with NW, NW participants showed higher abundances of *Betaproteobacteria*, *Burkholderiales*, *Sutterella*, *Alcaligenaceae*, and *Lactobacillaceae*.

Interestingly, *Eggerthella* were identified as being more abundant in NW controls in comparison to OW participants and AT, while not being as abundant in NW in comparison to OB participants.

Eggerthella is a genus of the family of *Coriobacteriaceae*. *Coriobacteriaceae* have already been identified as a differentially abundant family in AN in the first LEfSe analysis which included three groups, mentioned above. In addition to the functions of the *Coriobacteriaceae* family described above, *Eggerthella* have been specifically connected to inflammatory processes, for example in ulcerative colitis and the formation of abscesses in internal organs (305).

Both *SMB 53* and *Finegoldia* were found to be more abundant in AN patients, and they belong to the family of *Clostridiaceae*. An increased abundance of *Clostridia* has been connected to activation of the HPA axis in mice (285). Since the HPA axis is also activated in AN (306), *Clostridiaceae* like *SMB53* and *Finegoldia* could be a contributing factor in this. *Clostridia* are butyrate producing gut bacteria, responsible for energy extraction from diet (147). It might be the case that the gut microbiota in AN patients adapts to extract even more energy from restricted dietary intakes. However, although statistically significant differences in abundances were found, the total abundances of *SMB 53* and *Finegoldia* were below promille range in the groups. Therefore, the clinical importance of this finding may be low.

In comparison to AN, NW participants showed significantly higher abundances of the classes *Betaproteobacteria*, *Burkholderiales*, *Sutterella*, *Alcaligenaceae* and *Lactobacillaceae*. When AN patients were compared to overweight participants, again the *Betaproteobacteria* and the order of *Burkholderiales*, as well as the family of *Comamonadaceae* were found to be differentially abundant features of overweight participants compared to AN.

Burkholderiales (order), *Alcaligenaceae* (family) and *Sutterella* (genus) form subclasses in the class of *Betaproteobacteria*. *Betaproteobacteria* are one of six bacterial classes which are most commonly found in western countries and are considered as a normal part of the

gut microbiome (307). However, an overgrowth of certain genera, like the *Sutterella* genus, has been observed in autism spectrum disorders (308).

The family of *Lactobacillaceae* and the family of *Carnobacteriaceae* both belong to the order of *Lactobacillales* of the *Firmicutes* phylum. Members of the *Lactobacillales* order have been found to be higher in obese patients than lean controls (149, 151, 158).

Granulicatella and *Lactococcus*, which in the current study were more abundant in OB participants than in AN, are specific genera in the order of *Lactobacillales*. *Granulicatella* are fastidious microorganisms, which means that they require a complex nutritional state and can only grow in a specific diet of certain nutrients. The *Granulicatella* genus is known to be in the normal flora of the upper respiratory, gastrointestinal and urogenital tracts of humans (309). The *Lactococcus* are so called ‘homofermentors’ and produce lactic acid as a single product of glucose fermentation. This fermentation is dependent on certain environmental factors such as pH, glucose concentration and nutrition (310).

Lactobacillales and especially the genus *Lactobacillus* are used as growth-promoters in farm animals to improve weight gain. However, the weight promoting effects seem to be strain specific (311, 312), since various strains produce different amounts of SCFA, leading to weight gain (313). Nevertheless, our study results are in line with previous studies (312), regarding our findings that members of the order of *Lactobacillales* were more abundant in participants with higher BMI.

Dialister, which in the current study was found predominantly in overweight and obese subjects, also belongs to the *Firmicutes* phylum and the family of *Veillonellaceae*. In a study from Nakayama et al., *Dialister* abundance has been found to correlate with carbohydrate intake (314). Carbohydrates are metabolized to SCFA, and contribute to weight gain as described above (313). Further, *Dialister* might play a role in inflammatory processes, as they have been found to correlate with disease activity in spondyloarthritis (315).

Catenibacterium belongs to the family of *Erysipelotrichidae* and has only one known species, which is *Catenibacterium mitsuokai*. In a study from Brahe et al., it was connected to an unhealthy fasting serum lipid profile (119). Since AN patients often show

pathological serum lipids (301), an overgrowths of *Catenibacterium mitsuokai* could be a co-responsible factor in this pathology.

Overall, the most differentially abundant clades were identified between AN patients and OB participants. Again, *Roseburia* were identified as more abundant in OB compared to AN, and less abundant in AN compared to AT. Therefore, our study results confirm the results from Mack et al, finding that AN patients showed reduced levels of *Roseburia*. Mack et al. suggested using *Roseburia* in interventional studies, due to their effect on SCFA production (222).

4.6 PICRUSt analysis

PICRUSt identified Stilbenoid-biosynthesis as a differential pathway which was significantly more pronounced in AN patients compared to the other groups; however, the general effect was relatively small. Stilbenoids are plant sources of phytoalexins and have also been connected to natural anti-inflammatory and anti-cancer functions (316). One example is the polyphenol resveratrol, which is found in grapes (309). Resveratrol seems to have some anti-inflammatory properties, however, study results have been inconsistent (317).

Up until now, there have been no human studies which have found high stilbenoids in AN patients. Human trials concerning resveratrol in relation to weight gain or loss, have also shown conflicting results. Christenson et al. investigated the effects of resveratrol on weight reduction, however, no significant anti-obesity effect could be found (317). Quite to the contrary, in animal models high stilbenoid synthesis has been found in high weight rabbits (318). In agreement with our results, an upregulated stilbenoid syntheses was found in lean mice in a study from Garcia-Mazcorro, however, the sample size (n=10) was small (319).

It is not clear why stilbenoid synthesis was upregulated in AN patients in our study. Since the PICRUSt method is dependent on a secondary model based on the so-called Kyoto Encyclopedia of Genes and Genomes (KEGG)-pathways it is likely that these were meta-effects without significance (1).

4.7 Associations with investigated parameters

4.7.1 Microbiota and BMI

Using Spearman's correlations, certain taxa showing correlations with BMI were identified in our study, some of which have already been identified in taxa abundance comparisons and LEfSe analysis. Remarkably, all genera correlating with BMI in our study were *Firmicutes* and known SCFA producers. SCFA (such as acetate, propionate and butyrate) are produced by fermenting carbohydrates, and serve as substrates for de novo lipogenesis in the liver resulting in high energy harvest from food (143).

Roseburia, *Dialister* and *Clostridium* have already been demonstrated as differentially abundant in BMI groups, while the genus *Anaerostipes* showed no significant differences of abundance between groups; however, it did show a positive correlation with BMI.

Roseburia showed the highest correlation coefficient with BMI. As discussed above, the *Lachnospiraceae* (287) member *Roseburia* contributes to energy harvest through its carbohydrate degrading properties and its role as a key SCFA (butyrate) producer (289). *Dialister*, which was found predominantly in OW and OB participants in our study, is a member of the family of *Veillonellaceae*. It is connected to the intake of carbohydrates, which are further metabolized to SCFA (314, 315). *Clostridia* are the main butyrate producers and supply colonocytes with energy through the fermentation of fibre and therefore might contribute to weight gain and high BMI (147, 283, 284).

Anaerostipes have not been identified as a genus correlating with BMI before. However, their role as an SCFA producer is evident. As common gut inhabitants, *Anaerostipes* are efficient lactate converters and produce butyrate from lactate formed by *Bifidobacteria* (320, 321).

Butyrate has been linked with a healthy state and is typically decreased in several pathologies (322); on the contrary, an increase of *Clostridiales* has been shown to cause an increased production of butyrate and has been associated with visceral hypersensitivity and inflammatory bowel syndrome (323). Further, high concentrations of butyrate have been shown to correlate with metabolic syndrome risk factors, while weight loss in obese adults through reduced carbohydrate-intake was shown to decrease butyrate concentration by reducing butyrate producing bacteria in the gut (324).

However, stool SCFA content including butyrate was not investigated in our study, so our assumptions can only be made based on the abundance of SCFA producing bacteria.

Nevertheless, effects of interactions between bacteria have to be taken into account, since some genera such as *Lactobacillus paracasei* have been found to react to butyrate concentrations by reducing it when its concentrations are high and increasing it when its concentrations are low (325).

4.7.2 Microbiota and body composition

We found associations of gut community structures with anthropometric measurements such as upper arm circumference and hip circumference as well as SAT (1). Moreover, we confirmed the study results from Goffredo et al. who found associations of fat patterns and the gut microbiome in adolescents (272). In that study, SAT tissue was measured by fast magnetic resonance imaging. However, to the best of our knowledge, our study was the first study to investigate SAT patterns using a more reliable ultrasound technique (234) together with examining features of the gut microbiome (1). The measurement points erector spinae, lower abdomen, and medial calf showed associations with community structure(1). In contrast to men, woman store fat subcutaneously due to sex hormone differences (326). Most of the non-visceral SAT is found in the hypodermis and is not related to the obesity related health conditions such as heart disease and stroke. As a matter of fact, abdominal SAT has even been found to be a protective factor (327). However, according to the “adipose tissue expandability hypothesis” from Danforth (328), there is a limited capacity for the expansion of SAT tissue for lipid storage. When this capacity is surpassed, the body forms ectopic fat depots including visceral adipose tissue, which is associated with insulin resistance and metabolic dysfunction (329). SAT is strongly involved in the endocrine system and secretes hormones which are active in a range of processes such as nutritional intake (leptin, angiotensin), inflammation (resistin, visfatin, adiponectin, IL-6) and control of insulin-sensitivity (330), processes which have also been found to be connected to the gut microbiome (143, 331). However, further research is needed in order to verify possible associations of microbiota diversity and SAT patterns on specific body sites.

Results of the BIA, in particular the phase angle, total fat mass, body cell mass and basal metabolic rate, showed significant associations with overall gut community structures. The phase angle in particular was associated with gut community structures, using weighted and unweighted UniFrac distances (1). BIA predictions rely on population-specific equations, but the phase angle is estimated directly without conversion to specific body

compartments (332). The phase angle is strongly associated with several biological factors such as the cell quantity, cell membrane integrity and permeability, and the quantity of intra- and extracellular fluid (332). It is associated with the Xc-value which is achieved by intact cell membranes and indicates the nutritional state (235, 236). Malnutrition and inflammation are known to influence the phase angle; low values are normally related to more severe illnesses and worse prognoses (333). Up to now, there have been only a few studies which have investigated parameters of BIA together with the gut microbiome in women with and without metabolic disorder. For example, Munukka et al. found that *Eubacterium rectale* positively correlated with fat mass estimated by BIA (334). Remely et al. used BIA to investigate body fat along with microbiota changes during a weight loss program. Along with changes in the ratio of *Firmicutes/Bacteroidetes* they found an increase in *Lactobacilli* and *Clostridium Cluster IV* with more total body fat, as recorded by BIA (335). So far, there has been no interventional study which has investigated the effects found with BIA, so further studies are urgently warranted.

4.7.3 Microbiota and Smoking

Additionally, gut microbial composition was associated with smoking status (1). This is in line with study results from a Danish study showing associations of beta-diversity (Bray-Curtis distance) with four smoking categories: current smoking status, smoking history, parental smoking and maternal smoking during pregnancy (336). Similar to our study, they could only show very modest effects, while no significant associations for individual species or pathways could be detected (336).

4.7.4 Microbiota and depression

We found associations of community structure and the HAMD. This finding underlines the results of Kleiman et. al. who found correlations of alpha diversity with BDI in 15 AN patients before weight gain (220).

A study from Jiang et al. compared gut microbiota from patients with major depression with healthy controls. However, they could not find associations of gut community structures with depression-scales, due to significant interindividual variations (227). It remains unclear, whether the gut microbiota changed due to high depression levels or because of AN itself, since all AN patients in our study as well as in the study from

Kleiman et al. scored high in depression scales, and our other BMI groups did not. Further, correlations with the BDI and alpha-diversity could only be found in our study when all study groups were included in the analysis. We did not find correlations of alpha-diversity and BDI within the group of AN patients, which has to be considered when interpreting our results (1).

4.7.5 Microbiota and physical activity

Our study confirmed other studies showing that gut microbial diversity is higher in participants with high physical activity levels (AT-group) (213, 275). Physical activity seems to be beneficial for the gut microbiome. However, it is difficult to measure physical activity. In our study, we used a self-rating questionnaire (1). It is a known problem in self-rating questionnaires that over- or underreporting can occur and that participants tend to misjudge their activity level (337). This could be the reason for the current study not finding significant correlations of alpha diversity levels and the IPAQ score, and for not finding correlations between the IPAQ score, and gut community structures of the gut microbiome (1).

The results of our LEfSe analysis show, that AT demonstrated a higher abundance in *Firmicutes* than the low-activity AN patients. Especially the *Ruminococcaceae* of the *Firmicutes* phylum, which are responsible for carbohydrate breakdown, had a higher abundance in AT. *Ruminococcaceae* have been found to be increased in subjects with high carb-diets (111, 286). In fact, AT reported the highest fibre intake and third highest total carbohydrate intake of the investigated groups (1).

In light of these results, an important confounding factor is that people performing regular exercise usually have other diet patterns than inactive subjects which makes it difficult to separate the impact of dietary changes and the impact of exercise and fitness; especially AT usually have discriminant dietary intakes (213, 338). Thus, diet and exercise cannot be easily separated. In this context it is interesting that in weight reduction, exercise alone is not as effective as when it is combined with dietary changes (339).

The type of exercise performed might also have an influence on microbiota. Our AT study participants all played ball sports and took part in competitions on a regular basis (1). While

intensive exercise has been shown to produce multiple metabolites as well as inflammatory mediators (340), habitual, moderate exercise has been shown to lead to the suppression of proinflammatory cytokines (213). Further, regular exercise leads to anti-inflammatory effects and improved immunological profile in many diseases such as diabetes (341) and obesity (342). Excessive, prolonged exercise causes intestinal hypo-perfusion, impairs mucosal homeostasis and causes injury to enterocytes (343), which leads to cramps and diarrhea (344) followed by increased intestinal permeability because of phosphorylation of tight junction proteins (345). Regular, low intensity exercise has been shown to increase intestinal barrier function and the preservation of mucous thickness as well as lower rates of bacterial translocation (346). These factors might contribute to a dose-dependent effect of exercise on gut microbiota and health.

4.7.6 Correlations of microbiota and dietary components

In our study we identified small positive correlations of alpha diversity and fibre intake (1). OW subjects reported the lowest fibre intake while AT had the highest fibre intake out of all our study groups. Indeed, fibre is the major source for SCFA production by gut microbes (156). SCFAs are known to interact with multiple immune and metabolic pathways, can improve intestinal mucosal immunity, provide a source of energy for the liver, and influence brain function (347).

We confirmed the results of other studies which showed that western diets, which are low in fibre, result in the loss of important gut microbes and in a reduction of diversity (347, 348). This has for example been shown in a murine study, where a western diet over a few generations induced irreversible changes to the gut microbiota, which could not be restored by dietary changes (349).

In a modern western diet it is difficult to reach the recommended daily intake of fibre; this is known as the “fibre gap”, and can trigger inflammatory processes (347). Microbiota species depletion has a potential negative effect on various health aspects, therefore, more fibre intake may be beneficial; in fact, a fibre rich diet attenuating SCFA production was recommended in mental disorders such as schizophrenia (350) and could also be beneficial for patients suffering from AN.

Secondly, we found small correlations of alpha diversity and vitamin D (1). Vitamin D is classically known for its effects on calcium absorption and bone health, but it also has extra-skeletal immune effects, as it stimulates innate immune response (351, 352). Vitamin D favors tolerogenic rather than inflammogenic T-cell differentiation and therefore has an important role in microbiota sensing and enhancing the intestinal barrier (353). Consequently, vitamin D could be an important factor for gut health and diversity.

Third, magnesium levels correlated with the number of observed species. For example, in a murine study it was shown that magnesium deficiency led to depressive-like behavior (354). Magnesium is a co-factor responsible for over 300 enzyme-reactions (355); it is critical in neurotransmission (356) and modulates N-Methyl-D-aspartate receptor function (357), and therefore might play a role in psychiatric disorders like depression (358). In fact, it has been shown to have anti-depressive properties (359). Magnesium deficiency has an impact on inflammatory processes which are connected with increased gut permeability and changes of the gut microbiota in mice (360). Since low magnesium levels might contribute to lower alpha diversity, and further, since magnesium has been shown to have anti-depressive effects, a magnesium rich diet might be worth recommending to patients with AN and depression. However, one point to consider is that excessively high magnesium intake could have an impact on colonic transit time, and subsequently actually decrease alpha diversity (361).

4.7.7 Microbiota and laboratory parameters

While AN patients and AT showed lowest CRP, OB participants had highest CRP values (1). However, we did not find correlations between microbial diversity and parameters of clinical chemistry (1). It is known that obesity is characterized by chronic low-grade systemic inflammation, since pro-inflammatory mediators are released from adipose tissue (362, 363); this was also shown in our study results (1). Adipocytes are the source of proinflammatory cytokines such as TNF-alpha and IL-6 (364). Gut dysbiosis, followed by a reduced integrity of the intestinal wall, could be an additional factor driving systemic microinflammation in the two extremes of body weight: AN and OB.

However, serum zonulin, a marker for intestinal permeability, was not significantly different between groups. Even so, participants in these extreme groups (AN and OB) did

have the highest mean levels of serum zonulin. The concept of a “leaky gut” induced by stressors (i.e. reduced intestinal permeability) may play a role in a range of inflammatory diseases, obesity and psychiatric diseases (134, 192, 365). In line with that research, another study showed that high zonulin was connected to low alpha diversity in overweight pregnant women (366). Also, gut barrier function has been shown to be significantly altered in an activity based anorexia model in mice (194, 195). However, zonulin concentrations were not significantly different between our groups. It is important to note that as far as we are aware, this is the first study to investigate gut permeability with zonulin and gut microbiota, using a large female group and AT.

Some possible reasons for the lack of difference in zonulin levels in this study are discussed in the following. As this was a pilot study, we did not calculate the number of cases beforehand. Although we included 106 female participants in total, the number of participants in each BMI group could have been too low in order to detect significant differences in zonulin levels. Another possible reason for this lack of differences is that the anorexia mouse model mentioned above is an activity based model. It could be possible that zonulin levels were higher in that study because of physical activity. In animal models of high physical activity, a higher intestinal permeability together with an ingress of endotoxins in the bloodstream was discovered (367, 368).

Another factor could be diet. Zonulin is a mediator known to regulate intestinal permeability by modulating tight junctions, and is increased by gliadin, a part of gluten (369). Because of this, gluten-free diets have been recommended in order to prevent an increase of intestinal permeability. Based on a study published in the Scandinavian Journal of Gastroenterology in 2006, it was concluded that gliadin activates zonulin signaling, irrespective of the genetic preposition of celiac disease, and leads to intestinal permeability and immune activation (370). Dietary interventions involving gluten free diets have therefore been suggested for patients with autoimmune disorders and have also been tested in psychiatric disorders such as autism (371), however, no significant effects on symptom severity could be determined. On the contrary, a gluten free diet in healthy persons who had not received a diagnosis of celiac disease or gluten sensitivity led to reduction of beneficial bacteria such as *Lactobacilli* and *Bifidobacteria* and induced an immune suppressive effect by reducing tumor necrosis factor- α and interferon- γ (372).

In the current study we could demonstrate small, positive correlations of serum zonulin with total energy intake, carbohydrate intake, protein intake and fat intake, however,

zonulin was not significantly correlated with fibre intake. These findings are partly in line with the study results from Zak-Golab et al., who investigated serum zonulin in 50 obese and 30 normal weight subjects (373). They found correlations with total energy intake but an inverse correlation with dietary protein percentage. We also partly confirmed the results of a study from Moreno-Navarrete et al., who found correlations of serum zonulin with BMI and IL-6, however, in our study, we did not observe correlations with serum lipids such as HDL, triglycerides and cholesterol levels (192).

Low grade chronic inflammation may actually be the link between high zonulin concentrations and extreme forms of over- or undernutrition, due to the fact that bacteria colonizing the gut are the source of proinflammatory lipopolysaccharides (LPS), which stimulate the secretion of tumor necrosis factor- α and IL-6 (374). Microbial changes due to very high or very low energy consumption might therefore increase the intestinal permeability in OB and AN and may be the result of long-lasting inappropriate nutritional habits.

4.8 Discussion of Limitations

The following limitations have to be considered when interpreting our study results.

4.8.1 Critical reflection on the methods

For the assessment of body composition we used two different approaches: SAT-US and BIA (1). US detection of SAT is one of the most reliable methods available currently (232-234) and its reliability and accuracy have been proven in various BMI groups due to the fact that it is a compression free method with an accuracy only limited by furrowed borders and plasticity of the tissues (234). The reliability and accuracy of BIA is controversially discussed in the literature. Since many different equations for BIA calibration are used, study results cannot be easily compared among different studies. BIA has nonetheless been shown to produce reasonable estimates of body composition in healthy, euvoletic adults if specific predictive equations are used which take age, gender, level of physical activity, body fat and ethnicity into account (375). Further, a detailed implementation of the method is obligatory in achieving reliable results; this includes a good contact of electrodes,

overnight fasting of the participants, no physical activity before measurement and ambient temperature (238, 375). Indeed, these conditions were met before BIA was carried out in the current study. Nevertheless, certain studies have reported severe shortcomings of BIA, such as problems with accuracy (376), validity, measurement errors (377) and reproducibility (378).

The method used to assess gut microbiome data was 16s Ion torrent sequencing. Along with Illumina sequencing, Ion torrent sequencing is an advanced high-throughput sequencing technology providing a powerful approach for the exploration of microbial diversity (379). However, it is influenced by a range of methodological variables; for example, the method of sampling, sample-storage, DNA extraction, primer pairs, chemistry version, read length, and analysis pipelines (380).

For instance, Clooney et al. pointed out that the variance in microbiota composition is highly dependent on the chosen methodology (380), however, a study from Salipante et al. found that the microbiological characterization of specimens was generally in good agreement for both sequencing platforms, Illumina and Ion torrent (379).

Physical activity was estimated with a self-evaluation questionnaire (IPAQ) (1, 247). Over- and underreporting can be problematic in self-evaluation questionnaires, and could have led to different types of over- or underestimation in our investigated groups (337).

4.8.2 Critical reflection on the results

First, differences between the findings reported here and in other studies, regarding gut microbial parameters, could be attributed to the heterogeneity of AN patients. Some patients included in our study had been inpatients for a longer period of time, while others had just started their inpatient treatment (1). We did not conduct a follow-up investigation of the investigated parameters, since this was a cross-sectional study with a single-time assessment.

Secondly, all of our study participants were female. Given that AN is predominant in females and only about 10% of the individuals with AN are male (381), it was not possible to recruit a sufficient number of male AN patients, in order to have tested for sex differences between groups (1). Several studies have identified sex differentials in the gut

microbiome composition and abundance of specific taxa. For example, lower abundances of *Bacteroidetes* were found in women compared to men (382). In mouse studies, sex differences in the gut microbiome appeared after puberty, which may be because of the hormonal effects shaping the gut microbiome (383). Therefore, the results of this study might not be generalizable to males. We also did not assess the menstrual cycle of our female participants (1). Recently, the “microgenderome” was shown to influence the gut-brain-axis via estrogen (384, 385). Interestingly, the re-absorption of sex hormones such as estrogen relies on the presence of gut microbiota (386). In postmenopausal females, estrogen concentrations have been correlated with microbiota richness, however, in premenopausal females richness was found to be unrelated to the gut microbiome (387). Nevertheless, we cannot rule out potential influences of the menstrual cycle on the gut microbiome of our participants (1).

Thirdly, the influence of factors such as age and diet have to be considered. None of our participants received any standardized diet. It is known that the gut microbiota can be influenced by short and long term dietary changes (94, 171, 288, 349). Further, AN patients were investigated during an inpatient stay. While AN patients had a standardized diet according to meal plans, the other groups had their diet as usual. Nevertheless, food recalls have been performed to estimate dietary intake. All participants consumed a specific European or Austrian diet, and therefore, geographic dietary effects, which have been shown to influence the gut microbiome (90), can be ruled out (1).

Since microbiota do adapt to dietary extremes such as obesity or anorexia, and energy harvest from diet is strongly connected to the SCFA levels produced by gut bacteria (68, 143), the total SCFA content of groups would have been of interest; however, this was not within the initial scope of our study. Therefore, the relationship between obesity and energy harvest related to bacterial SCFA production should be investigated further.

Additionally, AN patients received psychopharmacological medication (mainly antidepressants). Studies have shown that psychotropic drugs, such as SSRI, have antibiotic properties (388, 389) which could influence microbial diversity and composition in both short and long term (390). All other BMI groups did not receive any long-term medication, as our inclusion criteria required the participants to be free from any antibiotic or antifungal treatment two months before their participation in the study. Moreover, all

women with acute or chronic infections or diseases were excluded (1). It is possible that medication effects in the AN group could have influenced laboratory parameters such as zonulin serum concentrations, however, up to now there are no studies which have investigated effects of psychopharmacological treatment on serum zonulin.

Colon transit time is a factor which substantially influences alpha diversity (391). In our study, we did not take colon transit time into account. AN patients often suffer from constipation; this could have led to a falsely elevated species richness (391).

It is important to note however, that all factors mentioned, including reduced dietary intake in AN, reduced physical activity, psychotropic medication, and slow colon transit time, reflect the current clinical reality of AN (1).

Additional factors influencing the gut microbiome include age, geography and pet ownership. The gut microbiome changes over the lifetime and adopts a stable anaerobic pattern around age three (90). It also changes in old age (>65 years) (93). Nevertheless, overall gut microbiome structures have been considered to be stable in adults (392). In our investigated groups, OB participants were significantly older than AN patients and AT, but none of the participants were over 40 (1). Since, as mentioned, the gut microbiome can be considered to be relatively stable throughout adulthood (393), and the age differences between the OB group, AN patients and AT were relatively small, this may not have had any significant impact on the differences found in gut microbial composition. Geographical discrepancies which could have affected the gut microbiome (90) were ruled out by only recruiting participants in Graz, and that all participants lived in Styria. Another interesting factor is that the gut microbiome could even be influenced by sharing the household with a pet; this was shown in a recent study from Tun et. al on infants (394). It is not currently known whether pet ownership is a factor of influence on the adult gut microbiome as well. Since this study was designed in 2013 (before publication of the study results from Tun and colleagues), we did not ask our study participants about pet-ownership, a factor which may have influenced the gut microbiota.

Another aspect is that genetic factors have been shown to influence the abundance of gut bacteria. For example, Goodrich et al. investigated the gut microbiota in monozygotic and dizygotic twin pairs and found out that relative abundances of gut bacteria were more highly correlated in monozygotic twins than in dizygotic twins (271). The family of

Christensenellaceae, gram-negative members from the class of *Clostridia*, were shown to be the most heritable gut microbial family and were found to be associated with lean BMI in twin pairs (271). However, in our groups, *Christensenellaceae* were not found as a differentially abundant family. While some taxa seem to be heritable, others are mostly influenced by environmental factors. For example, environmental factors such as diet have been shown to primarily shape the *Bacteroidetes* (395). In the current study, genetic factors, which might shape the gut microbiota along with environmental factors, were not investigated.

Finally, gut microbiota has a large inter-individual, and short-term, variability. Although microbial composition is considered to be stable over the long term, it could vary on short term scales (396, 397). Since the gut microbiome has even been reported to display circadian behavior following a 24-h cycle (398, 399), the time point for the collection of stool samples might be important. Thaïss et al. found that up to 20% of species in mice and humans undergo diurnal fluctuations in their relative abundance and this causes rhythmic changes of the entire bacterial community. For example, *Lactobacillus* abundance increased during a resting phase in mice and declined in an active phase (400). Therefore, the dimension of time of day should be considered in microbiota studies. The stool samples of our participants were mostly collected in the morning, but we did not make a clear distinction according to certain time points, which might have had an impact on the gut microbial composition.

4.9 Implications for future research

In summary, based on our study results, the following hypotheses can be proposed for future studies:

- 1) Pre-existing changes in the gut microbiota may induce changes in BMI and body composition, and partly influence extreme forms of over- and undernutrition through complex mechanisms of immunity, inflammation, and satiety regulation.
- 2) The gut microbiota influences the nutritional state and alterations of the gut microbiota lead to changes in energy harvest. Manipulation of the gut microbiota through probiotics and prebiotics accompanied with changes of environmental,

dietary and lifestyle factors could therefore have a positive effect on the treatment of AN and obesity.

- 3) According to our results, we would suggest members of the phylum *Firmicutes* and in particular the families of *Coriobacteriaceae* and *Roseburia* as candidates for interventional studies.

In the following, gut microbiome regulation and interventional studies with antibiotics, probiotics, prebiotics and psychobiotics shall be briefly discussed.

4.10 Microbiome regulation: antibiotics, probiotics, prebiotics, psychobiotics

Antibiotics have an impact on bacteria and on their host (401). For instance, the intake of antibiotics at a very young age has been associated with higher weight gain (402) and low-dose antibiotic exposure has been shown to lead to obesity in mice (402). Fecal transplantation in mice revealed that antibiotic-treated stool was capable of increasing total body fat in the host (403). In a randomized controlled clinical trial in 2767 Malawian malnourished children, a one-week antibiotic treatment enhanced the nutritional status and led to a decrease of mortality (404). Based on these studies, interventional studies using antibiotics in states of undernutrition like AN might support energy harvest from diet and could be a first step intervention, followed by a systematic propagation of the gut flora.

In order to change gut microbiota, prebiotics and probiotics are used.

Prebiotics consist of non-digestible food parts which are capable of stimulating the gut microbiota (405). Most prebiotics are carbohydrates, fructans and glucans (galactooligosacharrides) and are capable of influencing the gut microbiome and behavior (406). Probiotics are defined as microorganisms used in food-additives and were first described in 1907 by Elie Metchnikoff (138). The probiotics used most often in human studies are *Lactobacillus* and *Bifidobacteria* (407, 408).

From a psychiatric point of view, the impact of gut microbiota on neurotransmitter metabolism, and especially serotonin metabolism, is of interest for conditions such as AN.

In preclinical research in rodents, it was demonstrated that probiotics have an antidepressant and anxiolytic effect (409). For instance, *Lactobacillus plantarum* showed an antidepressant effect in mice (410, 411) by decreasing 5-HT turnover in the prefrontal cortex (412). In rats, *Lactobacillus helveticus* led to a reduction of 5-HT levels (413). Related to this, psychological distress and anxiety were decreased in healthy adults after *Lactobacillus* and *Bifidobacterium* probiotic intake (414).

Up to now, there is only a minor number of interventional studies in AN using probiotics and one case report where probiotics and prebiotics were used in an AN patient with disseminated intravascular coagulation (415-417). For example, Solis et al. investigated the effects of yoghurt and milk in the diet on interferon- γ production and found that fermented milk and yoghurt did have an effect on interferon gamma production in AN patients (415). Although there were no significant differences found in BMI or clinical parameters (such as length of recovery and hospitalization period), and an increase in BMI was only seen in the first six weeks of the study (as can be expected from patients receiving refeeding therapy), the authors concluded that interferon- γ could be considered as a biological marker for detecting the effect of probiotics on the immune response in nutritional deficiency. Nova et al. aimed to assess the effects of yoghurt on certain immunological parameters in 16 AN patients, compared to 14 AN patients who did not receive yoghurt during the period of refeeding. They suggested that the inclusion of yogurt in refeeding therapy for AN shows positive immunological effects (such as the CD4+/CD8+ ratio and the production of IFN- γ by lymphocytes) (416).

There have been some studies investigating the effects of pre- and probiotics in obesity. For instance, the prebiotic inulin has been shown to induce satiety and prompt the growth of *Bifidobacteria* and *Lactobacilli* (418). Dewulf et al., who performed an interventional study in obese women, pointed out that prebiotics alleviated host obesity (419). Adding to this, SCFA were found to be lowered in the prebiotic treated group, in comparison to controls. This was also affirmed by another study, showing that administration of prebiotics in obese women leads to an increase of *Bifidobacteria* and decreased SCFA production (420).

Only few studies have evaluated the impact of probiotics on gut microbiota in the context of obesity. Larsen et al. studied the impact of *Lactobacillus salivarius* supplementation in obese adolescents. No change in the production of SCFAs was observed (421). Lee et al. found that probiotics led to a reduction of LPS production through altered gut microbiota and found a major reduction in body weight and waist circumference in obese patients after probiotic supplementation (422). In a subsequent study, a probiotic, and a mixture of pre- and probiotic supplementation, was used to investigate effects on biomarkers of obesity in comparison to healthy volunteers. After treatment, positive alterations in serum lipid profile were observed in the probiotic and the symbiotic group along with a decrease in inflammatory cytokines. A more pronounced effect was found in the symbiotic group (423). A study from Brahe et al. investigated the effects of *Lactobacillus paracasei* F19, or flaxseed mucilage, on the gut microbiota and metabolic risk markers in obesity. Comparison of gut microbiota composition at baseline and after six weeks of intervention with flaxseed mucilage showed alterations in the abundance of thirty-three metagenomic species ($P < 0.01$), including decreased relative abundance of eight *Faecalibacterium* species (119). Thus, this intervention was shown to have a positive effect on gut microbiota in obesity.

Probiotics supplementation in AT has shown only modest clinical benefits so far (424). In a study from Haywood et al. elite rugby players were treated with probiotics (containing *Lactobacillus gasseri*, *Bifidobacterium bifidum* and *Bifidobacterium longum*) in order to prevent infections, compared to a placebo-group (425). The authors concluded that those probiotics might have beneficial effects in highly trained rugby players. Similar effects could also be shown in a study by West et al., who investigated gastrointestinal and upper respiratory-tract symptoms in cyclists with *Lactobacillus fermentum* supplementation, compared to a placebo group (426). Thus, the authors indicated that this supplementation may be beneficial for this group. AT often experience problems regarding GI permeability due to forced exercise. Probiotic supplementation might therefore have an effect on the leaky gut in AT (427).

These clinical trials point out that gut microbiota is altered by pre- and probiotic interventions, but none of these trials could clearly show that these alterations were solely responsible for a reduction or gain of body weight. It can be suggested that the use of pre- and probiotics might be beneficial for patients suffering from AN and obesity, and that

they might have effects on inflammation processes. However, up to now this has not been able to be conclusively proven.

Further, the effects of probiotics might not only rely on the specific types of ingested strains and factors such as application time and diet (428), but also on their mode of application. As the bacteria in probiotics may not be able to reach the intestine in a sufficient number to treat diseases other applications like fecal microbiota transplantation, which is now mainly used for the treatment of therapy resistant gastrointestinal diseases like *Clostridium difficile* infections and inflammatory bowel syndrome (429, 430), could be used for the treatment of AN in the future. However, yet there have been no studies or case reports describing the use of fecal microbiota transplantation in the treatment of AN. In patients with metabolic syndrome, fecal microbiota transplantation was conducted in a randomized controlled trial, whereas the lean donor feces group displayed increased insulin sensitivity (431), suggesting fecal microbiota transplantation as a future treatment strategy for severe metabolic syndrome.

Future studies will be required to find suitable probiotics which together with prebiotics and nutritional interventions could be used as “psychobiotics”(living organisms that, when ingested in adequate amounts, produce a health benefit in patients suffering from psychiatric illness)(5) to treat AN and various forms of over- or undernutrition through the regulation of satiety mechanisms (432).

4.11 Implications for clinical practice

We derived the following clinical implications from our study results:

- Since physical activity is a crucial factor for gut diversity and a more diverse gut has health benefits (213, 275), inpatient therapy of AN patients should include physical exercises along with sufficient substitution of micro- and macronutrients. Hyperactivity in AN patients during inpatient therapy has been connected with difficulties in weight gain, a worse clinical outcome and longer hospitalization periods (433). In the past, bed rest was medically prescribed to AN patients to counteract further weight reduction. However, recent study results have shown that basic physical activity should be maintained, as this has a positive impact on body composition, bone structure and affective symptoms (433). In this context, physical

activity could therefore lead to an increase in alpha diversity followed by a more efficient energy absorption (166) (1).

- A diet rich in fibre, vitamin D and magnesium for AN patients might be advisable for maximizing alpha diversity and energy harvest from diet (1).
- Since CRP and smoking status were significantly correlated with gut community structures (1), patients should be advised to stop smoking in order to prevent inflammatory response.
- Further research should be conducted to test the utility of pre- and probiotics in the management of AN and to find bacterial genera or combinations of genera for healthy weight regulation. Since pre- and probiotics are dietary supplements shown to have only minor side effects (434), they could be used as a complementary therapeutic option in in- and outpatient treatment of AN. Also, further studies on stool transplantation for the treatment of severe forms of AN should be conducted.

5. Conclusions and outlook

The results of this thesis highlight diverse alterations in the gut microbiome in different BMI groups and AT. Our study results are important for further understanding the pathomechanisms of dietary extremes and showing trademarks of a “healthy” gut microbiome in normal weight AT. These results form the basis for further interventional studies; studies which will be crucial for investigating implications of future treatments involving new therapeutic strategies.

Importantly, the composition of the gut microbiota is influenced by several genetic and environmental factors. Several lines of evidence indicate that gut microbiota and environmental interactions play a significant pathophysiological role in over- and undernutrition. Gut dysbiosis and a leaky gut may influence numerous pathways implicated in the biology of AN and obesity, including immune activation and neuroplasticity cascades.

Nevertheless, it remains difficult to establish a causality between gut dysbiosis and disease, and to transduce whether gut dysbiosis is the cause or vice versa. The impact of specific taxa on behavior and weight should be more closely identified, in order to distinguish markers of nourishment and inflammation. This could provide an important background for improving the efficiency of therapeutic weight restoration in AN and may influence the future management of the disease via probiotic, prebiotic, symbiotic, and antibiotic treatment.

Furthermore, since gut microbiota has an impact on energy harvest from food, the definition of a calorie is called into discussion: the energy output from food appears to differ according to the individual microbiota composition. Since the gut microbial composition of every individual is highly unique, specific microbial treatments should be personally adapted to every patient. This would again emphasize the need for a personalized therapeutic approach in psychiatric treatment (435). Following on from this, personalized nutrition could be used in the context of the gut microbiome, in order to develop personalized diets and enhance microbiome-targeted interventions.

Further research is necessary to replicate these findings and to investigate the clinical implications of our research.

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7. Appendix

7.1 Original Reports originated from this thesis

Mörkl, S., Lackner, S., Müller, W., Gorkiewicz, G., Kashofer, K., Oberascher, A., ... & Mangge, H. (2017). Gut microbiota and body composition in anorexia nervosa inpatients in comparison to athletes, overweight, obese, and normal weight controls. *International Journal of Eating Disorders*.

Mörkl S., Lackner S., Meinitzer A., Gorkiewicz G., Kashofer K., Painold A., Holasek S.J.- Pilotstudie: Mikrobiom und Darmbarriere bei Anorexia nervosa- Fortschritte der Neurologie und Psychiatrie- Article accepted on 25th, Oct, 2017.

Störchle, P., Müller, W., Sengeis, M., Ahammer, H., Fürhapter-Rieger, A., Bachl, N., **Mörkl, S.,** Lackner, S., Holasek, S. (2017). Standardized Ultrasound Measurement of Subcutaneous Fat Patterning: High Reliability and Accuracy in Groups Ranging from Lean to Obese. *Ultrasound in Medicine & Biology*, 43(2), 427-438.

Lackner, S., **Mörkl, S.,** Müller, W., Fürhapter-Rieger, A., Oberascher, A., Lehhofer, M., Bieberger, C., Wonisch, W., Amouzadeh, O., Moser, M., Mangge, H., Holasek S.J. - Improved assessment of relative body weight and body fat for diagnosis and treatment of anorexia nervosa- under review.

Mörkl, S., Lackner, S., Meinitzer, A., Mangge, H., Holasek, S.J. Gut microbiota and intestinal permeability measured by serum zonulin in women- in preparation.

7.2 Public presentations originated from this thesis

- 2017 Poster Prize- ÖGPP congress 2017
- 2016 3rd Poster Prize- Theodor Escherich Symposium on Medical Microbiome Research
- 2016 2nd poster prize - ÖGE annual meeting "Hot Spots in der Ernährung"

Mörkl, S; Lackner, S; Gorkiewicz, G; Kashofer, K; Blesl, C; Tmava, A; Oberascher, A; Holasek, SJ; Interplay of Gut Microbiota, Body Mass Index and Depression Scores in Anorexia nervosa-preliminary Data
European Psychiatry 2017; 41: -EPA 2017; APR 1-4, 2017; Florence, Italy. [Oral Communication]

Mörkl, S; Lackner, S; Meinitzer, A; Blesl, C; Painold, A; Kashofer, K; Gorkiewicz, G; Oberascher, A; Holasek, S; Der Tryptophan-Kynureninstoffwechsel bei Anorexia nervosa- gibt es einen Zusammenhang mit der Alpha-Diversität des Mikrobioms?

17. Jahrestagung der Österreichischen Gesellschaft für Psychiatrie, Psychotherapie und Psychosomatik; APR 26-29, 2017; Gmunden. 2017. [Poster]

Mörkl, S; Lackner, S; Gorkiewicz, G; Kashofer, K; Oberascher, A; Amouzadeh-Ghadikolai, O; Holasek, SJ. Dietary effects on gut microbiota in different BMI-groups. Ernährung aktuell. 2016; 4/2016: 30--ÖGE Jahrestagung 2016 - Hot Spots in der Ernährung; NOV 24-25, 2016; Vienna, AUSTRIA. [Poster]

Mörkl, S; Lackner, S; Gorkiewicz, G; Kashofer, K; Oberascher, A; Amouzadeh-Ghadikolai, O; Holasek, SJ. Anthropometric measurements in different BMI groups and AT http://www.medunigraz.at/fileadmin/studieren/phd/Abstractbook_DocDay_2016.pdf. 2016; -Doctoral Day 2016; DEZ 14, 2016; Graz, Austria. [Poster]

Mörkl, S; Lackner, S; Gorkiewicz, G; Kashofer, K; Oberascher, A; Wallner-Liebmann, SJ; Dissecting the female gut microbiota- 106 participants, 115 measurements and millions of bugs. Phenotypes and prevention- the interplay of genes, life-style and gut environment- NUGO Week 2016. 2016; -13th Nugo Week- Phenotypes and prevention- the interplay of genes, lifestyle factors and gut environment; SEP 5-8,2016

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Lackner, S; **Mörkl, S;** Müller, W; Oberascher, A; Holasek, SJ. Identification of Body Composition Types in Patients with Anorexia nervosa. Ernährung aktuell. 2016; 4/2016: 29-30.-ÖGE Jahrestagung 2016 - Hot Spots in der Ernährung; NOV 24-25, 2016; Vienna, AUSTRIA. [Poster]

Lackner, S; **Mörkl, S;** Müller, W; Oberascher, A; Holasek, SJ. Body Composition Types in Patients with Anorexia nervosa: Do we need to reconsider dietary treatment? Doctoral Day 2016 -Ventures into new realms - Abstract Book. 2016; -Doctoral Day 2016; DEZ 14, 2016; Graz, AUSTRIA. [Poster]

Lackner, S; **Mörkl, S;** Müller, W; Oberascher, A; Holasek, SJ. Subcutaneous adipose tissue in patients with eating disorders: Application of a standardized ultrasound measurement technique in extremely lean and obese patients. 24th European Congress on Obesity (ECO2017), Porto, Portugal, May 17-20, 2017: Abstracts. Obes Facts 2017;10(suppl 1):1-274. 2017; -European Congress on Obesity; MAY 17-20, 2017; Porto, PORTUGAL. [Poster]

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