Dissertation

# The gut microbiome in polycystic ovary syndrome: Next-generation sequencing studies of women and mice


submitted by

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# Declaration

*I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".*

*Graz, July 11, 2017*

# Disclosures

Numerous individuals contributed to the data presented in this work.

The human study was conducted at the Medical University of Graz, Division of Endocrinology and Diabetology, headed by Thomas Pieber, under the supervision of Barbara Obermayer-Pietsch. Gregor Gorkiewicz and Vanessa Stadlbauer-Köllner were involved in study planning, Christian Trummer, Roswitha Gumpold, Cornelia Missbrenner, Hannelore Pock, and Sandra Lemesch in recruitment, study visits, and sample collection, Julia Münzker, Verena Zachhuber, Julia Brunner, and the team of Ingeborg Klymiuk, including Martina Hatz, Alexandra Benharkou, and Theresa Maierhofer, in laboratory analyses, and Mina Bashir, Slave Trajanoski, Andrea Groselj-Strele, and Katharina Eberhard in bioinformatic and statistical analyses. Karl Kashofer and Gregor Gorkiewicz at the Institute of Pathology, Christoph Sensen at the Technical University of Graz, Institute for Computational Biotechnology, and the Huttenhower Lab at the Department of Biostatistics, Harvard University, Boston, MA, USA, provided server access for microbiome data analysis. Gut permeability assays were performed in collaboration with the group of Vanessa Stadlbauer-Köllner at the Division of Gastroenterology and Hepatology, including Angela Horvath, Bettina Leber, Monika Tawdrous, and Bianca Schmerböck. Fecal calprotectin measurements were performed by the team of Wolfgang Erwa at the Clinical Institute of Medical and Chemical Laboratory Diagnostics.

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# Abbreviations

| | |
|---|---|
| 17OH-P | 17-hydroxyprogesterone |
| AE-PCOS | Androgen Excess and PCOS Society |
| AFABP | adipocyte-derived fatty acid binding protein |
| AMH | anti-Muellerian hormone |
| ASRM | American Society for Reproductive Medicine |
| AUC | area under the curve |
| BDI | Beck's Depression Inventory |
| BMI | body mass index |
| CD | control diet |
| DAO | diamine oxidase |
| DHEA | dehydroepiandrosterone |
| DHEAS | dehydroepiandrosterone sulfate |
| DHT | dihydrotestosterone |
| E1 | estrone |
| E2 | 17-estradiol |
| EPM | elevated plus maze |
| ESHRE | European Society of Human Reproduction and Embryology |
| F0 | maternal generation (mice) |
| F1 | offspring generation (mice) |
| FAI | free androgen index |
| FDR | false discovery rate (multiple testing correction) |
| FFQ | food frequency questionnaire |
| FG | Ferriman-Gallwey (scoring system for hirsutism) |
| FMT | fecal microbiota transplantation |
| FSH | follicle-stimulating hormone |
| GD | gestational day |
| GF | germ-free |
| GWAS | genome-wide association study |
| HDL | high-density lipoprotein |

| | |
|---|---|
| HF/HS | high-fat, high-sucrose diet |
| HOMA2-IR | homeostasis model assessment for insulin resistance |
| hsCRP | high-sensitivity C-reactive protein |
| IL-6 | interleukin-6 |
| IQR | interquartile range |
| LBP | lipopolysaccharide binding protein |
| LEfSe | linear discriminant analysis effect size |
| LH | luteinizing hormone |
| LPS | lipopolysaccharide (=endotoxin) |
| MDS | multidimensional scaling |
| NIH | National Institutes of Health |
| OF | open field |
| oGTT | oral glucose tolerance test |
| OTU | operational taxonomic unit |
| PBS | phosphate-buffered saline |
| PCoA | principal coordinate analysis |
| PCOS | polycystic ovary syndrome |
| PD | phylogenetic diversity |
| PNA | pre-natal androgenization |
| RSV | ribosomal sequence variant |
| SHBG | sex hormone-binding globulin |
| sCD14 | soluble CD14 (LPS receptor) |
| SPF | specific pathogen-free |
| sRAGE | soluble receptor for advanced glycation end-products |
| TNF-α | tumor necrosis factor-α |
| TSH | thyroid-stimulating hormone |
| WGS | whole-genome shotgun |
| WT | wild-type |

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# Abstract (Deutsch)

Das polyzystische Ovarsyndrom (PCOS) ist ein vielseitiger endokrinologischer Symptomenkomplex, welcher sich durch Androgenüberschuss, gestörte Ovulation und mulifollikuläre Ovarien auszeichnet. PCOS betrifft einen erheblichen Anteil aller Frauen im reproduktionsfähigen Alter und birgt langfristige reproduktive und metabolische Folgen. Die Ursache des PCOS ist nach wie vor ungeklärt und beinhaltet genetische, intrauterine und Lebensstilfaktoren. Wir untersuchten die potenzielle Rolle des Darmmikrobioms in der Pathophysiologie des PCOS, da dieses bekanntlich einen gesundheits- sowie krankheitsfördernden Einfluss im Menschen ausübt.

Wir führten die erste publizierte Studie des Speichel- und Stuhlmikrobioms bei PCOS mittels next-generation sequencing durch. Unsere Hypothese war, dass das Darmmikrobiom zu der Pathophysiologie des PCOS beiträgt, indem bakterielles Endotoxin durch eine gestörte Darmbarriere in den systemischen Kreislauf gelangt und eine systemische Entzündungsreaktion auslöst, welche Insulinresistenz und Hyperandrogenämie begünstigt. Unsere zweite Hypothese war, dass das Darmmikrobiom hormonell aktive Isoflavone pflanzlichen Ursprungs metabolisiert, wodurch sich ein modulierender Effekt auf die Symptome des PCOS ergibt. Das Stuhlmikrobiom von Frauen mit PCOS zeigte eine reduzierte Vielfalt ("richness"), Veränderungen im Gesamtmikrobiomprofil und ein geringeres relatives Vorkommen mehrerer mit Schlankheit assoziierter Bakteriengruppen. Das Speichelmikrobiom war nahezu unverändert. Frauen mit PCOS zeigten beginnende Anzeichen einer Darmbarrierestörung, woraus sich ein möglicher Mechanismus für den Einfluss des Darmmikrobioms auf die Symptomausprägung des PCOS ergibt. Die bakterielle Produktion des Isoflavonmetaboliten Equol unterschied sich nicht zwischen Frauen mit PCOS und Kontrollfrauen.

Als zweiten Ansatz verwendeten wir ein Mausmodell, welches fett- und zuckerreiche (HF/HS) Nahrung mit pränataler Androgenexposition (PNA) durch Dihydrotestosteron (DHT) kombiniert, um die metabolischen, reproduktiven und affektiven Komponenten des PCOS widerzuspiegeln. Unsere Hypothese war, dass sowohl HF/HS Nahrung als auch PNA

bleibende Veränderungen im Darmmikrobiom dieser Mäuse bewirken. Wir fanden geschlechtsspezifische Einflüsse von mütterlicher Ernährung, PNA und der Ernährung der Nachkommen auf die fäkale bakterielle Vielfalt und taxonomische Zusammensetzung.

Wir führten fäkale Mikrobiomtransplantation (FMT) durch, um zu untersuchen, ob sich die Zyklusstörungen und vermehrte Ängstlichkeit der weiblichen PNA Nachkommen auf gesunde Wildtyp Empfänger übertragen ließen. FMT führte nicht zu Zyklusstörungen, beeinflusste jedoch das ängstliche Verhalten in umgekehrter Weise in einer der Empfängergruppen.

Zusammenfassend wurden eine reduzierte Artenvielfalt mit einem veränderten Gesamtmikrobiomprofil bereits in mehreren Human- und Mausstudien des PCOS beobachtet. Eine gestörte Darmbarriere beeinflusst möglicherweise die Symptomausprägung bei gewissen PCOS-Subtypen. Eine Wiederherstellung der Darmbakterienvielfalt, der Stabilität des Ökosystems und der Darmbarriere sollten als ergänzende Therapieansätze bei PCOS in Erwägung gezogen werden.

# Abstract (English)

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder characterized by androgen excess, impaired ovulation, and multifollicular ovarian appearance. PCOS affects a significant percentage of women of reproductive age and is associated with long-term reproductive and metabolic health consequences. The etiology of PCOS is still unclear and comprises genetic, intrauterine, and lifestyle-related factors. We investigated whether the gut microbiome, an important determinant of human health and disease, is involved in the pathophysiology of this disorder.

We conducted the first published study on the stool and saliva microbiome of women with PCOS using next-generation sequencing. We hypothesized that the gut microbiome contributes to PCOS pathophysiology through an impaired gut barrier, which allows leakage of bacterial endotoxin into the systemic circulation, driving the inflammatory processes which promote insulin resistance and hyperandrogenemia. We further hypothesized that gut bacterial metabolism of hormonally active dietary isoflavones can have a modulating effect on PCOS symptom severity. Women with PCOS showed decreased fecal bacterial richness and an altered stool microbiome profile with reductions in the relative abundance of several bacterial genera associated with leanness. The saliva microbiome was largely unchanged. Women with PCOS showed some signs of gut barrier dysfunction, pointing to a potential mechanism by which gut bacteria may drive the disease phenotype. Bacterial production of the isoflavone metabolite equol did not differ between women with PCOS and control women.

In a second approach, we utilized a mouse model combining a high-fat/high-sucrose (HF/HS) diet with dihydrotestosterone (DHT)-induced pre-natal androgenization (PNA) to reproduce the metabolic, reproductive, and behavioral features of human PCOS. We hypothesized that both HF/HS diet and PNA cause lasting changes in the gut microbiome of these mice. We found sex-dependent effects of maternal diet, PNA, and offspring diet on fecal bacterial richness and taxonomic composition.

We performed fecal microbiota transplantation (FMT) to determine whether disturbed estrous cyclicity and increased anxiety-like behavior seen in female PNA offspring could be transferred to healthy wild-type recipients. FMT did not cause reproductive dysfunction, but affected anxiety-like behavior in a donor-discordant manner in one group of recipients.

In conclusion, reduced bacterial richness and shifts in fecal global microbiome composition are recurrent findings in both human and mouse studies of PCOS. An impaired gut barrier might be involved in the pathophysiology of the syndrome in some PCOS phenotypes. The restoration of gut bacterial diversity, community stability, and gut barrier integrity should be considered as an adjuvant treatment approach for PCOS.

# 1. Introduction

## 1.1. Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) affects 6-18 % of reproductive-aged women worldwide, making it one of the most prevalent female endocrine disorders (Diamanti-Kandarakis et al., 1999; Asuncion et al., 2000; Azziz et al., 2004; March et al., 2010). Originally named after sonographic observations in the ovaries of affected women, PCOS is a complex systemic condition involving reproductive, metabolic, and psychological components (Fauser et al., 2012). In addition to reduced fertility, cosmetic issues due to acne and hirsutism, and an increased risk of pregnancy complications, women with PCOS are more likely to develop obesity, insulin resistance, anxiety, and depression (Dunaif, 1997; Fauser et al., 2012; Kollmann et al., 2015; Cooney et al., 2017).

The diagnosis of PCOS has historically been difficult, owing to the large variability in symptom penetrance and severity and the lack of clearly defined diagnostic criteria (Dunaif and Fauser, 2013). The first guideline aiming to standardize the diagnosis of PCOS was issued in 1990 by the National Institutes of Health (NIH) and required the presence of hyperandrogenism and oligo/anovulation, but not polycystic ovarian morphology (Legro et al., 2013). It soon became apparent that a considerable percentage of women with reproductive dysfunction in line with PCOS were not captured by the NIH criteria because they displayed only one of the two required symptoms. This led to a revision of the PCOS diagnostic criteria in 2003 by the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASMR). Informally known as the Rotterdam criteria, this guideline weighs hyperandrogenism, oligo/amenorrhoea, and polycystic ovarian morphology equally, requiring at least two of these symptoms to be present for the diagnosis (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). In 2006, the Androgen Excess and PCOS Society (AE-PCOS) defined a third set of diagnostic criteria, which required the presence of hyperandrogenism and one additional symptom indicative of ovarian dysfunction (oligo/anovulation or polycystic ovarian morphology) (Azziz et al., 2006). The Endocrine Society recommends the use of the Rotterdam criteria for the diagnosis of PCOS and has published detailed guidelines for the clinical and laboratory measures, cut-offs, and assay

types that should be used to assess PCOS symptoms (Legro et al., 2013). These recommendations should lead to a more uniform body of PCOS research in the future. Existing data are confounded by different diagnostic criteria and measurements used, making it difficult to compare and draw conclusions across studies.

The difficulties in identifying diagnosis criteria for PCOS stem in part from the fact that the pathophysiology of this syndrome is complex and its etiology is still unclear after decades of research. PCOS shows familial clustering and a study of more than 3,000 twins estimated the heritability factor to be greater than 70 % (Kahsar-Miller et al., 2001; Vink et al., 2006). The first genome-wide association studies (GWASs) were performed in Han Chinese and identified 11 genetic loci associated with PCOS in or near the genes LHCGR, THADA, DENND1A, FSHR, c9orf3, INSR, YAP1, RAB5B/SUOX, HMGA2, TOX3, ERBB3, and SUMO1P1 (Chen et al., 2011; Shi et al., 2012). Later studies in Caucasian cohorts replicated the loci in c9orf3, THADA, and YAP1 and identified novel loci in GATA4/NEIL2, ERBB4, RAD50, KRR1, and FSHB (Day et al., 2015; Hayes et al., 2015). These candidate genes are implicated in various processes including gonadotropin action, insulin signaling, type 1 and 2 diabetes, cell proliferation, and chromatin remodeling (Welt and Duran, 2014). Each of the identified loci accounted for only a small percentage (<1 % in the study by Hayes et al.) of the total heritability and all had an effect size<2 (Welt and Duran, 2014; Hayes et al., 2015). The genetic basis of PCOS is most likely a combined additive effect of many uncommon variants which have yet to be detected. Epigenetic, environmental, and life-style related factors may have a significant modulating impact on the onset and clinical presentation of the syndrome (Escobar-Morreale, Luque-Ramírez and San Millán, 2005). Non-human primates with high circulating testosterone exhibit reproductive and metabolic abnormalities resembling those in women with PCOS, suggesting that shared, evolutionarily conserved mechanisms may underlie this disorder (Abbott et al., 2017).

A simplified overview of the pathophysiology of PCOS is presented in Figure 1. A key component is a dysregulated hypothalamus-pituitary-adrenal axis. Women with PCOS have elevated circulating levels of luteinizing hormone (LH) and heightened LH pulsatility (Blank, McCartney and Marshall, 2006). As a result of this relative LH-excess compared to follicle-stimulating hormone (FSH) and the impaired LH secretion pattern, recruited follicles in the ovaries fail to fully mature and ovulation is impaired (Blank, McCartney and

Marshall, 2006). The typical ovarian phenotype of PCOS consists of multiple follicles ("cysts") arrested in different stages of development. These immature follicles secrete testosterone, androstenedione, dehydroepiandrosterone (DHEA), and Anti-Muellerian hormone (AMH) (Dumesic, Abbott and Padmanabhan, 2007). The adrenal glands also contribute to circulating androgens by secreting DHEA and DHEA sulfate (DHEAS), androstenedione, and testosterone (Dumesic, Abbott and Padmanabhan, 2007). Increased androgens inhibit the physiological post-ovulatory suppression of LH, driving the vicious cycle of LH-excess, anovulation, and hyperandrogenemia (Rojas et al., 2014).

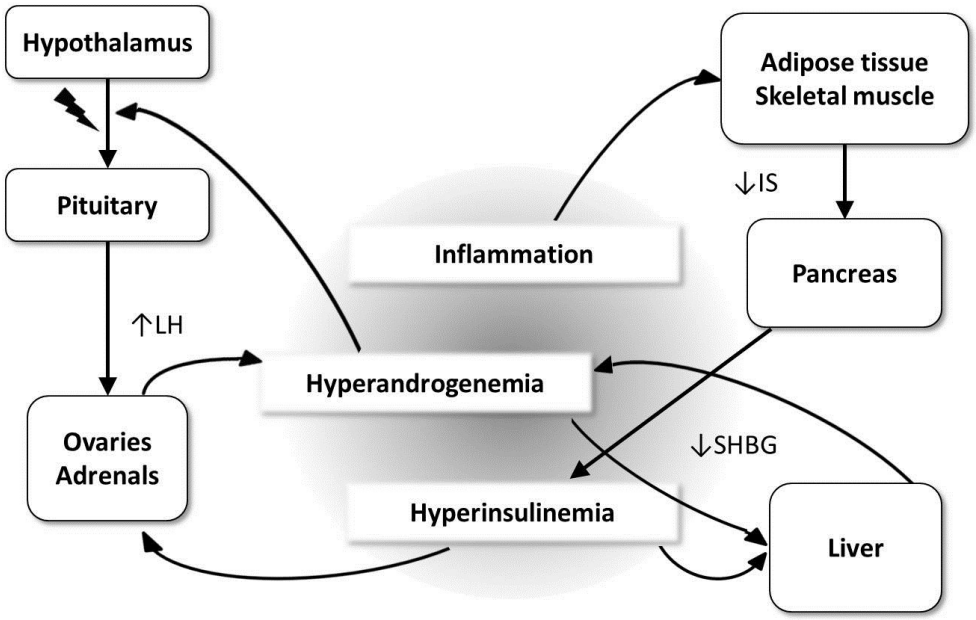


***Figure 1. Simplified depiction of the pathophysiological processes underlying PCOS****. Disturbed hypothalamic signaling to the pituitary gland leads to abnormal LH secretion, which causes follicular arrest and increased androgen production by the ovaries and adrenal glands. Simultaneously, low-grade inflammation causes insulin resistance in adipose tissue and skeletal muscle, leading to compensatory hyperinsulinemia, which further stimulates androgen production. Suppression of hepatic SHBG production by circulating androgens and insulin leads to increased free androgen levels, exacerbating the hyperandrogenic component of the syndrome. LH, luteinizing hormone; SHBG, sex hormone-binding globulin; IS, insulin sensitivity.*

A second key aspect of PCOS pathophysiology is insulin resistance, which is prevalent in a large percentage of both lean and overweight/obese women with PCOS and typically manifests as a heightened insulin response after a meal and can progress to type 2 diabetes (Dunaif, 1997; Moran et al., 2010). While women with PCOS develop insulin resistance in skeletal muscle, adipose tissue, and liver, the ovaries and adrenal glands remain sensitive to insulin and increase androgen production in response to higher insulin levels (Rojas et al., 2014). In the liver, both androgens and insulin suppress sex hormone-binding globulin (SHBG) production, resulting in a greater proportion of free androgens (Rojas et al., 2014). Impaired insulin action and adipose tissue dysfunction in PCOS lead to a chronic inflammatory state with elevated circulating high-sensitivity C-reactive protein (hsCRP), tumor necrosis factor-α (TNF-α), and adipokines such as leptin (Escobar-Morreale, Luque-Ramirez and Gonzalez, 2011; Spritzer et al., 2015). Thus, analogous to gonadotropin dysregulation, hyperinsulinemia feeds the feed-forward cycle of hyperandrogenism in PCOS. Recent research suggests that increased androgen production within the adipose tissue may also contribute to hyperandrogenemia in PCOS (O'Reilly et al., 2017).

A widely acknowledged hypothesis for the development of PCOS is excessive prenatal androgen exposure. In rodents, sheep, and nonhuman primates, a PCOS-like phenotype can be induced by androgen administration to a pregnant animal during a critical developmental window in late gestation (Padmanabhan et al., 2010; Abbott et al., 2013; Maliqueo, Benrick and Stener-Victorin, 2014). Gestational androgen excess is hypothesized to cause a reprogramming of the gonadotropic axis to a masculine pattern, leading to a disturbed LH/FSH ratio and ovulatory dysfunction (Foecking et al., 2005). Gestational androgen excess may also reprogram glucose and lipid utilization in muscle and adipose tissue, possibly via epigenetic mechanisms (Kokosar et al., 2016). Due to ethical considerations, it is difficult to study the direct exposure of the human fetus to androgens during pregnancy. Elevated testosterone has been measured in human maternal blood and amniotic fluid at mid-gestation and in umbilical cord blood at term (Barry et al., 2010; Palomba et al., 2012). Indirect measurements for intrauterine androgen exposure are anogenital distance and the second to fourth digit length ratio, which have been found to be masculinized in women with PCOS (Cattrall, Vollenhoven

and Weston, 2005; Wu et al., 2017). While animal models provide support for the role of prenatal androgen exposure in the development of PCOS, human studies are less clear, with some authors reporting no change in maternal circulating, amniotic fluid, and fetal cord blood testosterone, and the second to fourth digit ratio (Dumesic, Abbott and Padmanabhan, 2007).

As the underlying cause is still unknown, current treatment options for PCOS are symptomatic. They include different formulations of the oral contraceptive pill for hyperandrogenism, the selective estrogen receptor modulator clomiphene and the aromatase inhibitor letrozole to induce ovulation in women desiring pregnancy, and metformin for insulin resistance (Legro et al., 2013). Metformin may also be used off-label in hyperandrogenic women who do not tolerate or wish to use hormonal contraceptives and for whom cosmetic methods provide insufficient symptom improvement (Legro et al., 2013). Other antiandrogenic drugs, such as spironolactone and flutamide, are currently not recommended as a first-line therapy by the Endocrine Society (Legro et al., 2013). Lifestyle interventions focusing on diet and exercise are successful in improving glucose and lipid metabolism in women with PCOS, which has been shown to ameliorate the hyperandrogenic component in some cases (Domecq et al., 2013). The lack of a causal treatment and serious side effects of the available treatment options emphasize the need for continued research in this area to identify new potential causal and modulating factors in PCOS, which can support the development of novel causal or adjuvant therapies. The gut microbiome is one such research target.

## 1.2. Gut microbiome

The microbiome refers to the collective genomes of microorganisms, i.e. bacteria, viruses, fungi, protozoa, and eukaryota, that inhabit a particular habitat such as the human body (Human Microbiome Project Consortium, 2012). It has been termed the "forgotten organ", as it was poorly studied until recent decades despite the fact that bacteria outnumber human cells by an order or magnitude and gut bacteria alone account for approximately 1.5 kg of biomass in an average person (O'Hara and Shanahan, 2006; Karlsson et al., 2013). Currently, most microbiome studies target bacteria, as these

compose the overwhelming majority of the human microbiome, though studies on the human virome and mycobiome are emerging (Wylie, Weinstock and Storch, 2012; Sam, Chang and Chai, 2017). Next-generation sequencing technologies have enabled the study of the global microbiome composition of a habitat in its natural state without the need for prior isolation or cultivation of single microorganisms. This approach, combined with in vivo manipulations of the gut microbiome using germ-free (GF) animal models, fecal microbiota transplantation (FMT), various microbiome-targeted interventions, and sophisticated molecular and microbiological techniques has immensely furthered our understanding of the role of the microbiome, particularly the gut microbiome, in human disease.

Two main next-generation sequencing approaches are used in practice. In whole metagenome shotgun (WGS) sequencing, all available DNA in a sample is sequenced. This allows for inference of the microbial composition of a sample at species-level resolution, as well as the functional capacity of the microbial community (Morgan and Huttenhower, 2012). WGS is high in costs and computationally demanding and can be complicated by high amounts of host DNA in the sample, as is the case for stool (Morgan and Huttenhower, 2012). The second approach is amplicon-based sequencing of a pre-defined variable region of the 16S rRNA gene. This gene is exclusive to bacteria and archaea, eliminating the problem of host contamination (Tringe and Hugenholtz, 2008). The 16S rRNA gene contains conserved regions, ideal for primer placement, and variable regions which allow for identification of the bacteria with the use of a well-curated database (Tringe and Hugenholtz, 2008). 16S rRNA amplicon sequencing has a considerably lower cost than WGS, but does not provide functional information and species-level classification is not always possible (Morgan and Huttenhower, 2012).

There is evidence to suggest that the gut microbiome is involved in PCOS. Obesity, insulin resistance, systemic low-grade inflammation, anxiety, and depression have all been associated with an altered gut microbiome (P J Turnbaugh et al., 2009; Ding et al., 2010; Le Chatelier et al., 2013; Forslund et al., 2015; Zheng et al., 2016). While many studies are limited to providing descriptive and associative data, several landmark studies have uncovered causal effects of the gut microbiome in these conditions.

Obesity is associated with an altered gut microbiome in humans and rodents, and rodent studies have shown that the gut microbiome has the capacity to promote fat storage and weight gain via increased nutrient uptake from food and an up-regulation of hepatic lipogenesis (Bäckhed et al., 2004; Turnbaugh et al., 2006; P J Turnbaugh et al., 2009; Le Chatelier et al., 2013). FMT from an obese mouse to lean GF recipients caused a greater increase in body fat than FMT from a lean donor, despite reduced food intake (Turnbaugh et al., 2006). GF mice are resistant to diet-induced obesity and insulin resistance and FMT can modify insulin sensitivity depending on the donor phenotype (Ding et al., 2010; Ellekilde et al., 2014; Chevalier et al., 2015). In humans, insulin resistance and type 2 diabetes are associated with gut microbiome changes (Qin et al., 2012; Zhang et al., 2013; Forslund et al., 2015). Due to ethical concerns, treatment-resistant *Clostridium difficile* infection is the only current indication for FMT in humans (König et al., 2017). In one notable study, metabolic syndrome patients received either autologous FMT or FMT from a lean donor (Vrieze et al., 2012). Patients who received FMT from a lean donor had improved peripheral insulin sensitivity six weeks after the transfer (Vrieze et al., 2012). An association between gut microbiome changes and anxiety and depression in humans has been reported, and FMT from humans suffering from major depressive disorder induced anxiety- and depressive-like behavior in mice (Zheng et al., 2016).

## 1.3. Role of the gut microbiome in energy metabolism and reproduction

Though commensal microbes are generally regarded as beneficial for health, this effect can be reversed in the context of microbial dysbiosis. In a healthy state, gut microbes are separated from the systemic circulation by tightly linked intestinal epithelial cells and a thick mucus layer (Jakobsson et al., 2015). A high-fat diet can compromise the intestinal barrier and lead to unnatural proximity of bacteria and intestinal epithelial cells, impaired tight junction function, and leakage of bacterial endotoxin into the systemic circulation (Cani et al., 2008; Hamilton et al., 2015; Jakobsson et al., 2015; Müller et al., 2015). This "metabolic endotoxemia" drives a pro-inflammatory response, which is detrimental for insulin receptor function, resulting in insulin resistance and compensatory hyperinsulinemia (Cani et al., 2007). This effect is stronger in conventionally raised compared to specific pathogen-free (SPF) mice, and can be mitigated by antibiotic

treatment, indicating that gut bacteria play a key role in this process (Cani et al., 2008; Müller et al., 2015). Human studies have found associations between gut barrier dysfunction and visceral adiposity in healthy women and between metabolic endotoxemia and type 2 diabetes risk (Gummesson et al., 2011; Pussinen et al., 2011). As obesity and chronic low-grade inflammation are seen in women with PCOS, and as hyperinsulinemia is known to drive hyperandrogenism, it is quite plausible that the gut microbiome may affect the clinical presentation of this condition.

Prior to the start of this project, Tremellen and Pearce postulated the dysbiosis of gut microbiota (DOGMA) hypothesis for PCOS (Tremellen and Pearce, 2012) (Figure 2). It states that "disturbances in bowel bacterial flora (…) brought about by a poor diet creates an increase in gut mucosal permeability, with a resultant increase in the passage of lipopolysaccharide (LPS) from Gram negative colonic bacteria into the systemic circulation. The resultant activation of the immune system interferes with insulin receptor function, driving up serum insulin levels, which in turn increases the ovaries production of androgens and interferes with normal follicle development" (Tremellen and Pearce, 2012).

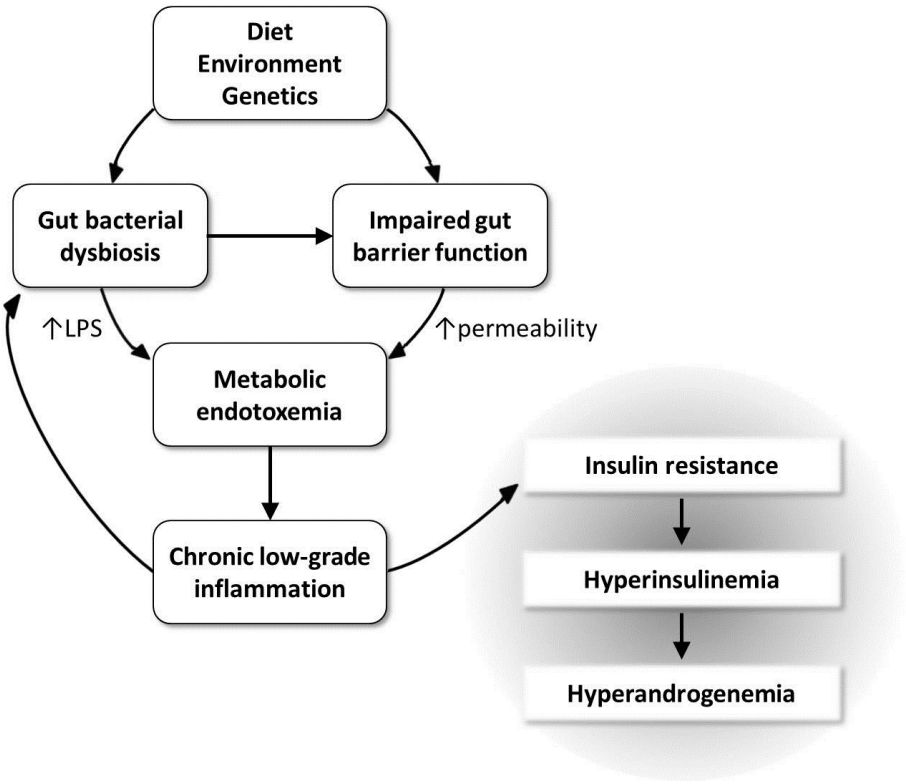


***Figure 2. Modified scheme of the DOGMA hypothesis.*** *Gut bacterial dysbiosis and a disturbed gut barrier lead to leakage of bacterial endotoxin into the circulation. This initiates a pro-inflammatory response which leads to impaired insulin signaling and compensatory hyperinsulinemia, a driver of hyperandrogenemia in PCOS. LPS, lipopolysaccharide (=endotoxin); DOGMA, dysbiosis of gut microbiota hypothesis posed by Tremellen and Pearce* (Tremellen and Pearce, 2012).

Few data are available on the interplay between gut bacteria and sex steroids. In mice, the male and female microbiome profiles differ after puberty and FMT from a post-pubertal male to a GF female causes an increase in circulating testosterone compared to FMT from a female donor (Markle et al., 2013; Yurkovetskiy et al., 2013). Changes in the human gut microbiome have been observed in pregnancy, becoming more pronounced with each trimester (Koren et al., 2012; Gomez-Arango et al., 2016). As pregnancy represents a state of profound hormonal and metabolic adjustments, it is unclear which mechanisms drive these changes. In post-menopausal women and men, levels of urinary estrogens and their metabolites correlated with fecal bacterial richness, but no such correlation was seen in pre-menopausal women (Flores et al., 2012).

The gut microbiome has the capacity to metabolize phytoestrogens, naturally occurring estrogenic compounds, from dietary sources. The best-studied are isoflavones, found predominantly in soy beans, which have been shown to exert estrogen-modulating, anti-inflammatory, and antioxidant activity (Hwang et al., 2006; Wei et al., 2012; Sankar et al., 2015). Daidzein and genistein are two isoflavones present in relevant concentrations in soy beans. Both compounds are weak ligands for the estrogen receptor (Hwang et al., 2006). Daidzein can be metabolized by gut bacteria to the more potent compound equol, which has been found to act as an anti-androgen by binding to free dihydrotestosterone, thereby preventing it from activating the androgen receptor (Lund et al., 2004). Intervention trials suggest that isoflavones might exert a beneficial effect on metabolic and reproductive function in PCOS (Romualdi et al., 2008; Khani et al., 2011). The benefits of consuming soy isoflavones may be greater in or even limited to individuals with a gut microbiota capable of equol production (Setchell, Brown and Lydeking-Olsen, 2002).

Taken together, there are compelling grounds to study the gut microbiome in PCOS. Apart from the article proposing the DOGMA hypothesis, not a single publication on this topic existed at the start of this project.

In this thesis, I present the first published description of the gut microbiome in PCOS in humans. We hypothesized that women with PCOS have gut microbial dysbiosis with concomitant gut barrier dysfunction and endotoxemia, and that these factors are correlated with PCOS symptom severity. In addition to stool, we analyzed the saliva microbiome to evaluate its suitability as a convenient alternative to identify microbial dysbiosis in PCOS. We further hypothesized that women with PCOS have a reduced capacity to metabolize dietary isoflavones to equol, and that the lack of this ability is associated with a worse clinical phenotype.

In a subsequent study in a PCOS mouse model, we performed a detailed investigation of the role of prenatal androgen exposure and a maternal and offspring high-fat/high-sucrose diet on reproductive, behavioral, and metabolic outcomes.

We aimed to gain mechanistic insights into the role of the gut microbiome in these processes by performing an FMT experiment to reproduce the characteristics of the PCOS mouse model in healthy recipient mice.

# 2. Materials and Methods

## 2.1. Part 1: Human Study

### 2.1.1. Study design

The study was undertaken at the Division of Endocrinology and Diabetology, University Clinic Graz, Austria. Women with PCOS and healthy controls were recruited from the endocrinological outpatient clinic and the larger Graz area. The recruitment period lasted from June-December 2014, during which time 25 women each were recruited in the PCOS and control groups. The study protocol was approved by the ethical committee of the Medical University Graz (EK 26-347 ex 13/14). All study participants were at least 18 years old and gave their written informed consent.

The in- and exclusion criteria are presented in Table 1. PCOS was diagnosed according to the Rotterdam criteria when at least two of the following were present: biochemical or clinical hyperandrogenism, oligo- or anovulation, and polycystic ovarian morphology (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Biochemical hyperandrogenism was defined according to guidelines published by the ESHRE and the AE-PCOS using serum total testosterone and dehydroepiandrosterone sulfate (DHEAS) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004; Azziz et al., 2006). Serum total testosterone>2.1 nmol/l and/or DHEAS>7.5 µmol/l were considered abnormal, based on normal ranges previously determined from a representative population sample (Haring et al., 2012). Clinical hyperandrogenism was assessed using the Ferriman-Gallwey (FG) scoring system for hirsutism and a score >8 was considered abnormal (Yildiz et al., 2010). Oligo-ovulation was defined as menstrual cycles with a duration >35 days and anovulation as the absence of menstrual bleeding for three or more consecutive months. Polycystic ovaries or absence thereof were self-reported by both the PCOS and control groups based on previous gynecological ultrasounds. Thyroid disorder, congenital adrenal hyperplasia, Cushing's disease, hyperprolactinemia, and androgen-secreting tumors were excluded by clinical examination and laboratory measurements of thyroid-stimulating hormone (TSH), 17-hydroxyprogesterone (17OH-P), cortisol, and prolactin. Pregnancy was excluded by pregnancy test. Only pre-menopausal women were included in the study. Women in the control group did not meet any of the

Rotterdam Criteria, with the exception of one case of isolated long-standing hirsutism (FG score=10). Exclusion criteria for all women were oral contraceptive, antidiabetic, or antibiotic drug use within the preceding three months, acute or chronic gastrointestinal or periodontal disease, active infections at any body site, a body mass index (BMI)<18, a known allergy to soy, and smoking.

***Table 1. In- and exclusion criteria for the human study.***

| | **PCOS** | **Control** |
|---|---|---|
| **Inclusion Criteria** | - PCOS diagnosed according to Rotterdam criteria<br>- Pre-menopausal<br>- Informed consent | - None of the Rotterdam criteria met (exception: isolated long-standing hirsutism)<br>- Pre-menopausal<br>- Informed consent |
| **Exclusion Criteria** | - Hormonal contraceptive, oral antidiabetic, or antibiotic use in the preceding three months<br>- Acute or chronic gastrointestinal or periodontal disease<br>- Active infections at any body site<br>- Body mass index<18<br>- Soy allergy<br>- Smoking | |

### 2.1.2. Study visits and sampling

A schedule and summary of the study visits is provided in Figure 3.

Study participants came to the endocrinological outpatient clinic in the morning after an overnight fast, having last brushed their teeth the evening before. A saliva sample was taken by collecting saliva in the mouth for several minutes and then voiding it into Sali-Tubes (DRG Diagnostics, Marburg, Germany). Saliva samples were immediately cooled on ice and frozen in liquid nitrogen within ten minutes.

Anthropometric and medical history data were collected (Appendix 7.1 and 7.2) and a baseline blood sample was drawn. Next, a 75 g oral glucose tolerance test (oGTT; Glucoral 75 Citron, Germania Pharmazeutika, Vienna, Austria) was performed, with blood sampling after 30, 60, and 120 minutes. A spot urine sample was provided. Blood was centrifuged within half an hour and serum/plasma separated. Serum, plasma, and urine

were either assayed immediately in the routine endocrinological and central laboratories of the University Clinic Graz or stored at -20°C until later use.

Study participants completed a food frequency questionnaire (FFQ), developed by dieticians of the Clinical Medical Nutrition Therapy Unit, University Clinic Graz, and modified to include soy products, to assess the intake of major food groups (Appendix 7.3) and a Beck's Depression Inventory (BDI, Appendix 7.4) to screen for the presence of depressive symptoms.

Following the first study visit, a functional test to evaluate dietary isoflavone metabolism by the gut microbiome was administered according to a published protocol (Setchell and Cole, 2006). Study participants consumed a soy drink (Joya© Soja Schoko Drink 200 ml, Mona Naturprodukte GmbH, Vienna, Austria) twice per day (morning and evening) on three consecutive days. Stool samples were self-collected before the first soy drink using empty stool collection tubes with a built-in spatula (Praxisdienst GmbH, Longuich, Germany) and provided instructions (Appendix 7.5). Stool was immediately frozen at -16°C and returned to the outpatient clinic on cool packs on the morning following the last soy drink. At this time, a post-soy urine sample was collected. The interval between the first and second study visits was 3-7 days.

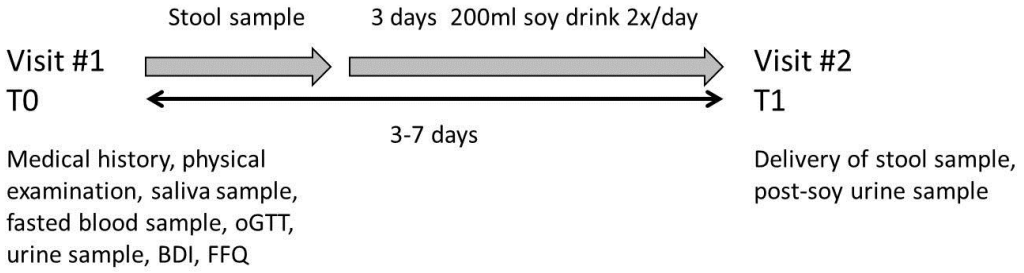


***Figure 3. Study design for the human study.*** *oGTT, oral glucose tolerance test; BDI, Beck's depression inventory; FFQ, food frequency questionnaire. N=24 PCOS and 19 controls.*

### 2.1.3. Biochemical measurements

Serum estrone (E1), 17-estradiol (E2), total testosterone, androstenedione, dehydroepiandrosterone (DHEA), DHEAS, dihydrotestosterone (DHT), and saliva total testosterone and androstenedione were measured by liquid chromatography-tandem mass spectrometry in collaboration with the team of Brian Keevil at the Department of Clinical Chemistry, University Hospital of South Manchester, Manchester, UK (Chadwick, Owen and Keevil, 2005; Owen, Wu and Keevil, 2014; Owen et al., 2015).

Serum and urine genistein, daidzein, and equol were measured using liquid chromatography-tandem mass spectrometry at LGC (Cambridgeshire, United Kingdom) according to a published method (Grace et al., 2007).

Serum cortisol, TSH, prolactin, insulin, anti-Muellerian hormone (AMH), and sex hormone-binding globulin (SHBG) were measured by chemiluminescence immunoassay. Serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), 17OH-P, high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), the soluble receptor for advanced glycation end-products (sRAGE), adipocyte-derived fatty acid-binding protein (AFABP), leptin, adiponectin, visfatin, resistin, zonulin, diamine oxidase (DAO), lipopolysaccharide binding protein (LBP), soluble CD14 (sCD14), stool calprotectin, and stool zonulin were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers´ instructions. Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, glucose, and urine creatinine were measured by enzymatic colorimetric assay. Complete and differential blood counts were performed. The assays, detection limits, and reliability indices are summarized in Table 2.

Serum endotoxin (=lipopolysaccharide, LPS) was measured in collaboration with Vanessa Stadlbauer-Köllner of the Division of Gastroenterology and Hepatology, University Clinic Graz, using the HEK-Blue LPS Detection Kit (Invivogen, San Diego, USA). Cells were cultured in 24-well plates ($5x10^4$ cells/well). After 24 hours, medium was discarded and replaced with samples/endotoxin standards and detection medium. Cells were incubated for 21 hours at 37°C and color intensity was measured at a wavelength of 650 nm.

***Table 2. Technical specifications of used assays.***

| Analyte | Assay type | Detection limit | Intra-assay CV (%) | Inter-assay CV (%) |
|---|---|---|---|---|
| E1 | LC-MS-MS | 3.9 pmol/l | 4.7 | 2.1-3.4 |
| E2 | LC-MS-MS | 8.0 pmol/l | 6.7 | 3.4-5.0 |
| Total testosterone | LC-MS-MS | 0.3 nmol/l | <10 | <6 |
| Androstenedione | LC-MS-MS | 0.3 nmol/l | <10 | <7 |
| DHEA | LC-MS-MS | 1.0 nmol/l | <10 | <10 |
| DHEAS | LC-MS-MS | 1.0 µmol/l | 2.9-11.3 | 3.7-7.3 |
| DHT | LC-MS-MS | 0.3 nmol/l | <10 | <10 |
| Saliva total testosterone | LC-MS-MS | 5.0 pmol/l | <7.5 | <4 |
| Saliva androstenedione | LC-MS-MS | 6.3 pmol/l | <3 | <7.5 |
| Cortisol | Chemiluminescence immunoassay, ADVIA Centaur XP (Roche) | 3.9 nmol/l | 2.9-4.2 | 4.4-6.0 |
| TSH | Chemiluminescence immunoassay, ADVIA Centaur XP (Roche) | 0.01 mU/l | 2.4-2.5 | 2.1-5.3 |
| Prolactin | Chemiluminescence immunoassay, ADVIA Centaur XP (Roche) | 6.4 mU/l | 1.9-4.4 | 2.0-5.3 |
| Insulin | Chemiluminescence immunoassay, ADVIA Centaur XP (Roche) | 0.5 mU/l | 3.2-4.6 | 2.6-5.9 |
| AMH | Chemiluminescence immunoassay, Access2 (Beckman Coulter) | 0.1 pmol/l | 0-1.7 | 0-3.1 |
| SHBG | Chemiluminescence immunoassay, Cobas e411 (Roche) | 0.4 nmol/l | 2.1-2.7 | 2.7-5.6 |
| LH | ELISA (DiaSource) | 1.3 IU/l | 4.5-7.6 | 3.2-11.0 |
| FSH | ELISA (DiaSource) | 0.9 IU/l | 4.2-7.9 | 5.2-7.2 |
| 17OH-P | ELISA (IBL International) | 0.1 nmol/l | 2.8-4.9 | 5.8-9-2 |
| hs-CRP | ELISA (BioVendor) | 0.02 mg/l | 4.1-6.9 | 5.8-6.3 |
| IL-6 | ELISA (BioVendor) | 0.9 pg/ml | 0.2-7.8 | 0-17.8 |
| TNF-α | ELISA (BioVendor) | 2.3 pg/ml | 1.9-10.4 | 4.5-10.3 |
| Zonulin (serum) | ELISA (Immundiagnostik) | 0.2 ng/ml | 3.4-6.0 | 13.3-13.6 |
| Zonulin (stool) | ELISA (Immundiagnostik) | 0.2 ng/ml | 3.2-6.2 | 12.7-13.9 |
| DAO | ELISA (Immundiagnostik) | 0.12 U/m | 1.4-1.7 | 7.9-10.7 |
| LBP | ELISA (Hycult Biotech) | 4.4 ng/ml | 0.05-8.5 | § |
| sCD14 | ELISA (R&D Systems) | 125 pg/ml | 4.8-6.4 | 4.8-7.4 |
| Calprotectin | ELISA (Bühlmann Laboratories) | 10 µg/g | 2.7-8.1 | 6.6-14.5 |
| sRAGE | ELISA (BioVendor) | 19.2 pg/ml | 2.6-5.3 | 5.5-8.8 |
| AFABP | ELISA (BioVendor) | 0.05 ng/ml | 2.3-2.7 | 2.3-5.5 |
| Leptin | ELISA (BioVendor) | 0.2 ng/ml | 4.2-7.6 | 4.4-6.7 |
| Adiponectin | ELISA (BioVendor) | 26 ng/ml | 3.9-5.9 | 6.3-7.0 |
| Visfatin | ELISA (BioVendor) | 30 pg/ml | 2.3-9.1 | 4.7-7.2 |
| Resistin | ELISA (BioVendor) | 0.01 ng/ml | 5.2-6.6 | 7.0-8.1 |
| Total cholesterol | Enzymatic colorimetric assay, Cobas c (Roche) | 0.1 mmol/l | 0.6 | 1.4-1.6 |
| HDL cholesterol | Enzymatic colorimetric assay, Cobas c (Roche) | 0.1 mmol/l | 0.5-0.7 | 0.9 |
| Triglycerides | Enzymatic colorimetric assay, Cobas c (Roche) | 0.1 mmol/l | 0.7 | 1.9 |

| | | | | |
|---|---|---|---|---|
| Glucose | Enzymatic colorimetric assay, Cobas c (Roche) | 0.1 mmol/l | 0.5-0.7 | 1.1-1.2 |
| Urine creatinine | Enzymatic colorimetric assay, Cobas c (Roche) | 0.03 mmol/l | 0.9-2.2 | 1.5-2.5 |
| LPS | HEK-Blue LPS detection kit (Invivogen) | 0.01 EU/ml | § | § |
| Serum genistein | LC-MS-MS | 0.5 ng/ml | <20 | <20 |
| Serum daidzein | LC-MS-MS | 0.5 ng/ml | <20 | <20 |
| Serum equol | LC-MS-MS | 0.5 ng/ml | <20 | <20 |
| Urine genistein | LC-MS-MS | 0.5 ng/ml | <20 | <20 |
| Urine daidzein | LC-MS-MS | 0.5 ng/ml | <20 | <20 |
| Urine equol | LC-MS-MS | 0.5 ng/ml | <20 | <20 |

*CV, coefficient of variation. § not provided by manufacturer.*

### 2.1.4. Calculation of indices and definition of cut-offs

BMI was calculated as $\frac{Weight\ (kg)}{Height\ (m)^2}$. The homeostasis model assessment for insulin resistance (HOMA2-IR) index was calculated using the HOMA calculator V2.2.3 provided by the Diabetes Trial Unit, University of Oxford, UK (www.dtu.ox.ac.uk/homacalculator/, last accessed Dec 17, 2015).

The area under the curve (AUC) for glucose and insulin was calculated from the oGTT using the trapezoidal method. The free androgen index (FAI) was calculated according to the formula $100 \times \frac{Total\ testosterone\ (nmol/l)}{SHBG\ (nmol/l)}$. Free testosterone and free DHT were calculated from total testosterone/DHT and SHBG according to Mazer, assuming a blood albumin concentration of 6.2 µmol/l (Mazer, 2009). Measured urine isoflavone concentrations were normalized to urine creatinine using the formula $100 \times \frac{[C]analyte}{[C]creatinine}$.

Overweight was defined as a BMI≥25 (World Health Organization, http://www.who.int). Insulin resistance was defined as a HOMA2-IR≥2 (Gayoso-Diz et al., 2013). Bacterial metabolism of daidzein to equol was assessed using the equol:daidzein quotient (E:D). An equol producer was defined as having a logE:D>-1.5 (Setchell and Cole, 2006). Two diet types were defined from the FFQ; a high-carbohydrate type, consuming mainly foods from the "Grains" and "Fruit/Vegetables" categories, and a high-animal protein type, consuming a greater proportion of foods from the "Meat/Fish" and "Dairy" categories.

### 2.1.5. Next-generation sequencing of stool and saliva samples

DNA was extracted from stool and saliva samples using the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) on the MagNA Pure Instrument (Roche). For stool samples, the sample was thawed partially and an amount approximately the size of a maize kernel was homogenized in 500 µl 1x phosphate-buffered saline (PBS). 250 µl of the diluted sample were added to 250 µl bacteria lysis buffer in a sample tube containing MagNA Lyser Green Beads (1.4 mm diameter ceramic beads, Roche). For saliva samples, the sample was thawed fully, vortexed, and 250 µl sample were added directly to 250 µl lysis buffer in a sample tube containing MagNA Lyser Green Beads. The subsequent DNA extraction procedure was identical for stool and saliva samples.

Samples were homogenized in a MagNA Lyser Instrument (2 x 6000 rpm for 30 s, separated by 1 min cooling) and treated with 25 µl lysozyme (Roth, Karlsruhe, Germany) at 37 $^{o}$C for 30 minutes, then with 43.3 µl proteinase K (Roche) at 60 $^{o}$C for 1 hour. Lysates were incubated at 95 $^{o}$C for 10 minutes, cooled on ice for 5 minutes, and centrifuged for 5 minutes at full speed. DNA was isolated from the lysate supernatant by the MagNA Pure Instrument using the manufacturer's software. The volume of supernatant used was 100 µl for stool and 200 µl for saliva samples. Both sample types were eluted in 100 µl elution buffer. A PCR reaction was performed to amplify the V1-2 region of the bacterial 16S rRNA gene using the primers F27 (AGAGTTTGATCCTGGCTCAG) and R357 (CTGCTGCCTYCCGTA) (Eurofins Genomics, Ebersberg, Germany) and the FastStart High Fidelity PCR System, dNTPack (Roche) with initial denaturation at 95°C for three minutes followed by 28 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for one minute, one final extension cycle at 72°C for seven minutes, and a final cooling step to 10°C. Triplicates were pooled, visualized on a 1.5 % agarose gel, and 15 µl of pooled PCR product were normalized according to manufacturer's instructions on a SequalPrep Normalization Plate (Life Technologies, Vienna, Austria). 15 µl of the normalized PCR product were used as a template for indexing PCR in a 50 µl single reaction to introduce barcode sequences to each sample according to Kozich *et al.* (Kozich et al., 2013). Cycling conditions were initial denaturation at 95°C for three minutes followed by eight cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for one minute,

one final extension cycle at 72°C for seven minutes, and a final cooling step to 4°C. After indexing, 5 µl of each sample were pooled and 50 µl of the unpurified library were loaded on a 1 % agarose gel and purified from the gel with the Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The pooled DNA was quantified using QuantiFluor ONE dsDNA dye on a Quantus Fluorometer (Promega, Mannheim, Germany) according to the manufacturer's instructions and visualized for size validation on a 2100 Bioanalyzer Instrument (Agilent Technologies, Santa Clara, USA) using a high-sensitivity DNA assay according to the manufacturer's instructions. The final 6 pM library containing all pooled samples was run with 20 % PhiX and version v3, 600 cycles chemistry according to the manufacturer's instructions on a MiSeq desktop sequencer (Illumina, Eindhoven, Netherlands). Samples were sequenced in two subsequent runs, with samples from PCOS patients and controls distributed equally between the runs. One negative control consisting of 250 µl sterile PBS was included in each DNA extraction run (8 negative controls in total) and underwent the same procedure as the samples. A mock community containing genomic DNA from twenty bacterial species was included beginning at the 16S rRNA PCR step to estimate bias due to PCR and sequencing errors (HM-782D, BEI Resources, Manassas, USA, Appendix 7.6).

### 2.1.6. Processing of sequencing data

Raw reads were processed using the open-source software mothur V1.35.0 according to a published protocol (accessed April 2015) (Kozich et al., 2013). Forward and reverse reads were assembled into contigs and reads with ambiguous base calls or homopolymer runs>8 bases were discarded. Sequences were aligned to the Silva 119 database (www.arb-silva.de), pre-clustered to allow one mismatch per 100 bases, and non-bacterial sequences were removed. Chimeric sequences were removed using UCHIME (Edgar et al., 2011). The remaining sequences were degapped, deuniqued, split into individual samples, and formatted for open reference operational taxonomic unit (OTU) picking in QIIME 1.8.0, which was performed using UCLUST against the greengenes 13.8 database (DeSantis et al., 2006; Caporaso et al., 2010; Edgar, 2010). The sequence similarity threshold for OTUs was set at 97 %. Alpha rarefaction analyses were based on the number of observed OTUs and Faith's phylogenetic diversity (Faith's PD) index and performed in QIIME. Principal coordinate analyses (PCoA) were based on unweighted and

weighted UniFrac distances calculated in QIIME (Lozupone and Knight, 2005). Taxa summaries were performed in QIIME. Three OTU abundance cut-offs were tested to assess the impact of high- and low-abundance taxa and OTUs inflation due to sequencing errors. The OUT cut-offs were "no singletons", "relative abundance <0.01 %", and "relative abundance <0.1 %". All samples were normalized to the sample with the lowest read count for alpha and beta diversity comparisons. For taxa comparisons, relative abundances calculated from all reads in each sample were used.

### 2.1.7. Real-time qPCR

We confirmed differences in relative abundance of bacteria from the Tenericutes phylum by real-time quantitative PCR using the primer set Ten662F and Ten862R, normalized to total 16S rRNA gene content using the primer set 926F and 1062R, as described by Yang *et al.* (Yang et al., 2015). 20 ng DNA template were used in a 10 µl reaction on a LightCycler 480 Instrument (Roche) according to the manufacturer's instructions.

### 2.1.8. Statistical analysis

Statistical analysis was performed in IBM SPSS Statistics Version 22 or 23 unless otherwise stated. All continuous data were screened for normality and equality of variance prior to statistical testing. Normally distributed data were compared using unpaired student's t-tests. Non-normally distributed data were either log-transformed, followed by parametric testing, or compared using Mann-Whitney U tests. Categorical data were compared using Fisher's Exact tests. Correlations were tested using Spearman's correlations. In the case of missing values, patients were excluded from the analysis for that variable. All data are expressed as median and interquartile range (IQR) unless otherwise stated.

Statistical tests on microbiome data were performed in QIIME. Non-parametric student's t-tests using Monte Carlo permutations were used for alpha diversity comparisons. Mann-Whitney U tests followed by Benjamini-Hochberg false discovery rate (FDR) correction were used for taxa comparisons. Adonis was used to compare UniFrac distance matrices. Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify taxa that best explain variation in the PCOS and control microbiome profiles (Segata et al., 2011).

A p-value<0.05 was considered statistically significant. The following symbols are used to denote p-values: *p<0.05; **p<0.01; ***p<0.001.

## 2.2. Part 2: Mouse study

### 2.2.1. PNA model

The study design for the prenatal androgenization (PNA) model is summarized in Figure 4. Fifty 12-week old virgin female C57/Bl6j mice (F0 generation) were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Mice were housed five to a cage under a 12 h light/dark cycle at a temperature of 21-22°C and 55-65 % humidity. After one week of acclimatization, mice were randomly assigned to either a control diet (CD) (#98052602, Research Diets Inc., New Brunswick, NJ, USA) containing 10 kcal% fat, 73 kcal% carbohydrates, and 17 kcal% protein, or a high-fat, high-sucrose diet (HF/HS) consisting of i) high-fat diet (# D12079B, Research Diets Inc., Appendix 7.7) containing 40 kcal% fat, 43 kcal% carbohydrates, and 17 kcal% protein and ii) 20 % sucrose solution (S9378, Sigma-Aldrich) supplemented with a vitamin mix (V10001, Research Diets Inc., 10 g/4000 kcal) and a mineral mix (S10001 Research Diets Inc., 35 g/4000 kcal). All animals were provided with tap water ad libitum.

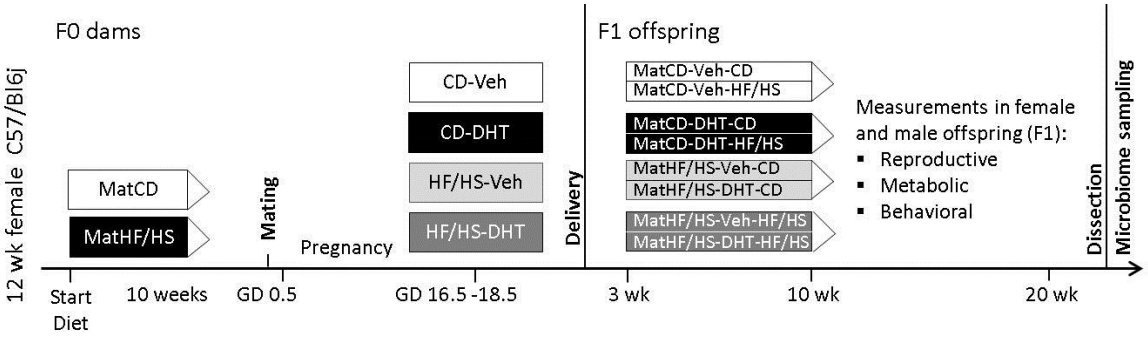


***Figure 4. Study design for the PNA and HF/HS-diet mouse model.*** *CD, control diet; HF/HS, high-fat, high-sucrose diet; Veh, vehicle, DHT, dihydrotestosterone; GD, gestational day; wk, week. N=50 dams at start of experiment and 9-12 offspring/group. An additional group of dams was sacrificed at GD 18.5 and fecal material collected for gut microbiome analysis (N=4-6/group).*

After 10 weeks, females in estrus phase, determined by vaginal smear cytology, were mated overnight with a CD-fed male. The presence of a post-copulation plug was confirmed the following morning, which was defined as gestational day (GD) 0.5. Plug-

positive animals were placed in single cages and continued on their respective diets. At GD 16.5 the HF/HS and CD groups were sub-divided into four groups: CD-Veh, CD-DHT, HF/HS-Veh, and HF/HS-DHT, testing two factors: diet and injection. All mice received a 100 µl subcutaneous injection in the interscapular area of either 250 µg/kg dihydrotestosterone (A8380, Sigma-Aldrich) dissolved in a mixture of 5 µl benzyl benzoate (B6630, Sigma-Aldrich, St. Louis, MO, USA) and 95 µl sesame oil (S3547, Sigma-Aldrich) or vehicle alone for three days (until GD 18.5). All injections were given between 9 and 10 AM. An additional group of twenty mice underwent the same procedures as F0 mice, but was sacrificed at GD 18.5 and colonic fecal material was collected, frozen immediately in liquid nitrogen, and stored at -80°C until later analysis.

All offspring (F1 generation) were weaned at 21 days, separated by sex, and randomly assigned to continue on the diet of the mother or switch to the opposite diet. Mice from the F1 generation were divided into 8 groups: MatCD-Veh-CD, MatCD-DHT-CD, MatHF/HS-Veh-CD, MatHF/HS-DHT-CD, MatCD-Veh-HF/HS, MatCD-DHT-HF/HS, MatHF/HS-Veh-HF/HS, and MatHF/HS-DHT-HF/HS, testing three factors: maternal diet, maternal injection, and offspring diet.

F1 mice were housed 2-6 to a cage. Body weight and food intake were measured weekly. At four months of age, F1 mice underwent behavioral testing to assess anxiety-like behavior in the elevated plus maze (EPM) and 4-5 days later the open field (OF). Animals were tracked for ten minutes in each arena using the EthoVision XT system (Noldus Information Technology, Wageningen, Netherlands) and the percentage of time spent in pre-defined areas of the arena was recorded (periphery and center for OF, open and closed arms for EPM). The behavior tests were performed in a soundproof room with no daylight. Prior to the start of the experiment, mice were acclimatized to the behavior room for 20 minutes. The arena was cleaned with 70 % ethanol before each mouse to remove bias due to olfactory cues. Vaginal smear cytology was performed on female mice immediately after behavior testing, to confirm that results were based on female mice in all cycle stages. At 20-21 weeks of age, vaginal smear cytology was performed on female mice to assess estrous cyclicity over ten consecutive days. Vaginal smears were collected by flushing the vagina with 15 µl sterile 0.9 % saline solution, followed by microscopic inspection of the fluid on a glass slide and identification of the cycle phase according to

published protocols (Caligioni, 2009; Byers et al., 2012). At 22 weeks of age, F1 mice were sacrificed and colonic fecal material was collected and frozen immediately in liquid nitrogen. Cecal material was collected from a subset of mice for use in FMT as described below.

All animal procedures were approved by the Animal Ethics Committee Karolinska Institute (121-16) in accordance with the legal requirements of the European Community (Decree 86/609/EEC).

### 2.2.2. Preparation of cecal inoculate for FMT experiment

The donors for the FMT experiment were female offspring which remained on the same diet as their mothers (MatCD-Veh-CD, MatCD-DHT-CD, MatHF/HS-Veh-HF/HS, and MatHF/HS-DHT-HF/HS). Three cages per group were selected (n=1-4 mice/cage, 6-8 mice/group) and the cecal contents were pooled and homogenized in sterile PBS containing 25 % glycerol (4 ml per cage). The stool suspension was briefly centrifuged to remove particulate matter and the supernatant was collected and pooled for each group. The absorption of the pooled supernatants was measured on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 620 nm and the dilutions adjusted to the levels of the least concentrated sample. The inoculates were aliquoted and fresh aliquots were thawed on each day of FMT. Immediately prior to gavage, FMT aliquots were diluted 1:5. This dilution approach enabled us to use the same FMT inoculate stocks for all mice in each group for the whole duration of the experiment.

### 2.2.3. FMT experiment

An overview of the study design for the FMT experiment is given in Figure 5. Four-week old female C57/Bl6j mice (Janvier) were conventionally housed six to a cage and fed standard irradiated chow. Weight gain, food, and water intake were monitored throughout the experiment. Mice were randomly assigned to one of five treatment groups, four receiving FMT and one control group (n=12/group). Beginning at five weeks of age, mice in the FMT groups received a sterile-filtered antibiotic mix consisting of 10 mg/kg vancomycin, 20 mg/kg neomycin, 20 mg/kg metronidazole, 20 mg/kg ampicillin, 10 mg/kg gentamicin, and 1 mg/kg amphotericin B (all Sigma-Aldrich), prepared in sterile water, via once daily oral gavage for two weeks. The control group received sterile water

without antibiotics. Beginning on the day following the last antibiotic gavage, mice in the FMT groups received 150 µl FMT inoculate via oral gavage. FMT was given once daily for seven days, every second day for the next seven days, and then once weekly until the end of the experiment. Mice in the control group received sterile PBS with 25 % glycerol following the same schedule. Estrous cyclicity was assessed three weeks after the first FMT as described earlier. Anxiety-like behavior was assessed in the OF and EPM 5-6 weeks after the first FMT as described earlier. Following behavior testing, mice were sacrificed and cecal contents were collected and frozen immediately in liquid nitrogen.

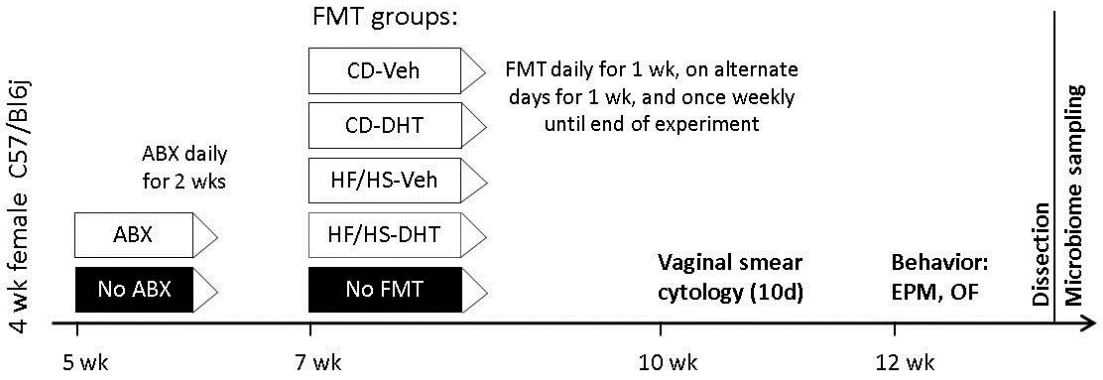


***Figure 5. Study design for the FMT experiment.*** *FMT, fecal microbiota transplantation; ABX, antibiotic; CD, donor control diet; HF/HS, donor high-fat, high-sucrose diet; Veh, donor maternal vehicle injection; DHT, donor maternal dihydrotestosterone injection; wk, week. N=12/FMT group.*

### 2.2.4. Next-generation sequencing of fecal samples and FMT inoculates

DNA was extracted from fecal/cecal samples and FMT inoculates using the Maxwell 16 Tissue DNA Purification Kit (Promega Corporation, Madison, WI, USA) on the Maxwell 16 Instrument according to the manufacturer's instructions. Up to 50 mg fecal/cecal material per sample and 300 µl FMT inoculate were used. DNA was eluted in 300 µl Elution Buffer. The eluted DNA was centrifuged at full speed for two minutes and the supernatant collected. The DNA concentration was measured on a NanoDrop 2000 and DNA was diluted to 5 ng/µl with DEPC-treated water. One negative control was included in each DNA extraction run and negative controls were pooled prior to PCR. 50 ng DNA were used in a PCR reaction to amplify the V4 region of the bacterial 16S rRNA gene using the

primers 515fB (GTGYCAGCMGCCGCGGTAA) and 806rB (GGACTACNVGGGTWTCTAAT) (Eurofins Genomics, Ebersberg, Germany) and the Phusion Hot Start II High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA). The PCR conditions were initial denaturation at 95°C for three minutes followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds, one final extension cycle at 72°C for ten minutes, and a final cooling step to 10°C. A sample of DEPC-treated water, used to dilute the samples, was included as a PCR negative control. PCR products were visualized on a 1 % agarose gel and purified using Agencourt AMPure XP beads (Beckman Coulter, Brea. CA. USA) with a ratio of 1.2x beads to DNA. The purified PCR amplicons were indexed at the Science for Life Laboratory (Stockholm, Sweden) using the Nextera DNA Library Prep Kit (Illumina) and sequenced with v3, 600 cycles chemistry on a MiSeq Instrument (Illumina). F0 and F1 samples were sequenced in one run, FMT samples in a second run. An additional negative control from the indexing PCR was included. A microbial community standard consisting of eight bacterial species underwent the same DNA isolation, PCR, and sequencing protocol as samples (D6300, Zymo Research, Irvine, CA, USA, Appendix 7.8).

### 2.2.5. Processing of sequencing data

Primers were trimmed from raw reads using cutadapt (Martin, 2011). Trimmed raw reads were processed in R 3.4.0 using the DADA2 1.4.0 package (Callahan et al., 2016). Forward and reverse reads were filtered based on their quality score profiles and trimmed at the point where read quality began to drop (F=230 and R=180 bases for PNA samples, F=260 and R=180 bases for FMT samples). Error rates of forward and reverse reads were calculated using the DADA2 algorithm and error-corrected reads were merged. Chimeric sequences were identified and removed. Taxonomic assignments were made for each ribosomal sequence variant (RSV) using the Silva v123 database (www.arb-silva.de). Subsequent analyses were performed in R using the phyloseq package (McMurdie and Holmes, 2013). Alpha rarefaction analyses were based on the number of observed RSVs and the Shannon diversity index. Beta diversity analyses were based on Bray-Curtis distances and visualized using multidimensional scaling (MDS).

### 2.2.6. Statistical analysis

Statistical analyses were performed in IBM SPSS Statistics Version 22 or 23 unless otherwise stated. All continuous data were screened for normality and equality of variance prior to statistical testing. Non-normally distributed data were log-transformed. Data were analyzed using 2-way or 3-way ANOVA depending on the number of factors tested (diet and injection in F0 and FMT mice, maternal diet, maternal injection, and offspring diet in F1 mice). ANOVA was followed by post-hoc tests with Bonferroni correction. Male and female offspring were analyzed separately.

For the microbiome data, alpha diversity was compared in SPSS using 2- or 3-way ANOVA with post-hoc testing and p-value correction as described above. Bray-Curtis distance matrices were compared using Adonis as part of the phyloseq package. Phylum and genus comparisons were made using DESeq2 (Love, Huber and Anders, 2014).

A p-value<0.05 was considered statistically significant. The following symbols are used to denote p-values: *p<0.05; **p<0.01; ***p<0.001.

# 3. Results

## 3.1. Part 1: Human study

### 3.1.1. Patient characteristics

Clinical and laboratory characteristics of the PCOS and control groups are shown in Table 3 and Figure 6. Six participants were excluded from the analysis due to a BMI<18 (n=1 PCOS), previously undetected hyperandrogenemia (n= 3 control), and smoking during the study period (n= 2 control). Women with PCOS were significantly younger than controls (p=0.003). BMI and WHR were not significantly different between the groups. In the assessment of glucose and lipid metabolism, women with PCOS showed a less favorable phenotype, with higher fasting insulin (p=0.022), HOMA2-IR (p=0.027), and AUC insulin in the oGTT (p=0.009). Glucose tolerance was not impaired. Women with PCOS had higher triglyceride (p=0.010) and lower HDL-cholesterol levels (p=0.006) than control women.

***Table 3. Anthropometric, metabolic, and steroid hormone parameters in women with PCOS and control women.***

| | **Reference range** | **Control (n=20)** | | **PCOS (n=24)** | | |
|---|---|---|---|---|---|---|
| | | **median** | **IQR** | **median** | **IQR** | **p-value** |
| Age | | 32 | 12.0 | 27 | 5.9 | 0.003** |
| **Anthropometric parameters** | | | | | | |
| BMI | 18.5-25.0# | 22.3 | 4.10 | 24.9 | 11.75 | 0.147 |
| Waist to hip ratio | < 0.85# | 0.80 | 0.063 | 0.82 | 0.077 | 0.439 |
| **Metabolic parameters** | | | | | | |
| Fasting glucose (mmol/l) | < 7.0† | 4.5 | 0.50 | 4.7 | 0.59 | 0.209 |
| 2h glucose (mmol/l) | < 11.1† | 4.3 | 1.09 | 4.8 | 1.15 | 0.296 |
| AUC glucose (mmolh/l) | § | 10.2 | 4.52 | 10.9 | 3.61 | 0.273 |
| Fasting insulin (pmol/l) | 20.9-173.8 | 41.4 | 51.08 | 84.4 | 55.25 | 0.022* |
| 2h insulin (pmol/l) | § | 129 | 140.0 | 188 | 336.7 | 0.371 |
| AUC insulin (mmolh/l) | § | 353 | 427.3 | 691 | 562.0 | 0.009** |
| HOMA2-IR | < 2.0 | 0.8 | 1.05 | 1.7 | 1.20 | 0.027* |
| Total cholesterol (mmol/l) | < 5.2 | 4.6 | 0.64 | 4.5 | 1.13 | 0.699 |
| HDL-cholesterol (mmol/l) | > 1.0 | 2.0 | 0.42 | 1.7 | 0.49 | 0.006** |
| Triglycerides (mmol/l) | < 1.65 | 0.59 | 0.248 | 0.74 | 0.242 | 0.010* |
| **Serum sex hormones** | | | | | | |
| FSH (IU/l) | 0.5-61.2‡ | 9.2 | 8.11 | 7.5 | 2.73 | 0.178 |
| LH (IU/l) | 2.0-22.0‡ | 5.8 | 9.34 | 9.3 | 8.60 | 0.042* |
| LH:FSH ratio | § | 1.2 | 1.19 | 1.5 | 1.06 | 0.035* |

| | Reference range | Control (n=20) | | PCOS (n=24) | | |
|---|---|---|---|---|---|---|
| | | median | IQR | median | IQR | p-value |
| AMH (pmol/l) | 1.4-65.2 | 26.8 | 22.42 | 61.1 | 52.59 | 0.0002*** |
| Total testosterone (nmol/l) | 0.37-2.1 | 1.1 | 0.56 | 1.3 | 0.77 | 0.002** |
| DHT (nmol/l) | § | 0.34 | 0.241 | 0.46 | 0.528 | 0.096 |
| Androstenedione (nmol/l) | 0.89-7.5 | 2.6 | 1.61 | 4.2 | 2.69 | 0.0003*** |
| DHEA (nmol/l) | § | 13.7 | 11.37 | 21.4 | 12.40 | 0.015* |
| DHEAS (µmol/l) | § | 3.3 | 3.74 | 4.9 | 2.35 | 0.073 |
| E1 (pmol/l) | § | 274 | 184.8 | 195 | 118.9 | 0.138 |
| E2 (pmol/l) | § | 436 | 285.8 | 163 | 181.1 | 0.0005*** |
| FAI | § | 1.3 | 0.68 | 3.1 | 2.75 | <0.0001*** |
| Free testosterone (pmol/l) | § | 10.6 | 5.86 | 20.9 | 13.00 | <0.0001*** |
| Free DHT (pmol/l) | § | 1.3 | 1.03 | 3.0 | 2.19 | <0.0001*** |
| **Saliva sex hormones** | | | | | | |
| Saliva testosterone (pmol/l) | § | 15.3 | 10.50 | 25.9 | 17.50 | 0.002** |
| Saliva androstenedione (pmol/l) | § | 240.3 | 149.67 | 415.3 | 164.10 | 0.0003*** |
| **Psychological assessment** | | | | | | |
| BDI (score) | | 1.5 | 5.75 | 4.5 | 9.75 | 0.027* |
| **Dietary assessment** | | | | | | |
| Total points on FFQ | | 69 | 27.0 | 68 | 31.8 | 0.318 |
| Grains (% of total points) | | 19 | 9.4 | 17 | 8.9 | 0.025* |
| Dairy (% of total points) | | 13 | 8.6 | 13 | 10.2 | 0.502 |
| Meat/Fish (% of total points) | | 6 | 6.4 | 7 | 6.8 | 0.649 |
| Fruits/Vegetables (% of total points) | | 17 | 11.4 | 19 | 12.6 | 0.856 |
| Fats (% of total points) | | 10 | 6.0 | 12 | 5.2 | 0.258 |
| Soy (% of total points) | | 1 | 1.9 | 1 | 2.5 | 0.658 |

*IQR, interquartile range; BMI, body mass index; AUC, area under the curve; HOMA2-IR, homeostasis model assessment for insulin resistance; HDL, high-density lipoprotein; FSH, follicle-stimulating hormone; LH, luteinizing hormone; AMH, anti-Muellerian hormone; DHT, dihydrotestosterone; DHEA, dehydroepiandrosterone; DHEAS, DHEA sulfate; E1, estrone; E2, 17-estradiol; FAI, free androgen index; BDI, Beck's Depression Inventory; FFQ, food frequency questionnaire. #as defined by the World Health Organization; †as defined by the American Diabetes Association; ‡depending on menstrual cycle phase; §reference range not defined. Adapted from* (Lindheim et al., 2016).

Women with PCOS scored significantly higher than control women on the BDI (p=0.027), indicating a greater tendency towards depressive disorder. Women with PCOS reported a lower percentage consumption of grains than women in the control group (p=0.025). There was no statistically significant difference in any other assessed food group.

Women with PCOS showed the expected abnormalities in serum levels of steroid hormones and other reproductive parameters. LH (p=0.042), AMH (p=0.0002), total testosterone (p=0.002), androstenedione (p=0.0003), and DHEA (p=0.015) were significantly higher and E2 was significantly lower (p=0.0005) in the PCOS group than in controls. The hyperandrogenemia was reflected in increased saliva testosterone (p=0.002) and androstenedione (p=0.0003) levels in the PCOS group.

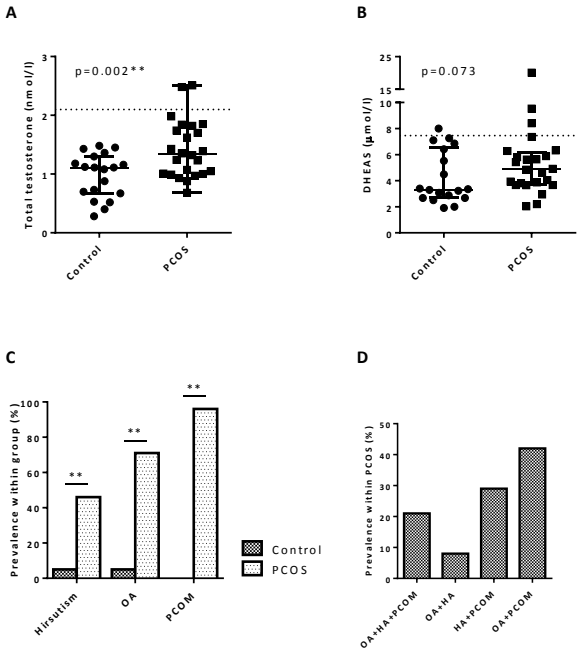


***Figure 6. PCOS diagnostic criteria and phenotypes in the studied cohort.*** *Serum total testosterone (A) and DHEAS (B) levels in women with PCOS and control women. C. Prevalence of hirsutism, OA, and PCOM in women with PCOS patients and control women. D. Prevalence of PCOS phenotypes according to the Rotterdam Criteria. PCOM, polycystic ovarian morphology; OA, oligo-/amenorrhoea; HA, hyperandrogenism (clinical and/or biochemical).* (Lindheim et al., 2017).

Median free testosterone was twice as high and median free DHT and the FAI were two- to threefold higher in women with PCOS than in control women (p<0.0001 for all). Women with PCOS reported hirsutism, oligo-/amenorrhoea, and polycystic ovarian morphology in 46 %, 71 %, and 96 % of cases, respectively (Figure 6C). When categorizing women with PCOS into the four possible phenotypes under the Rotterdam diagnostic criteria, the majority presented as type D (OA+PCOM, 42 %), while the remaining phenotypes were represented with a prevalence of 29 % for type C (HA+PCOM), 21 % for type A (OA+HA+PCOM, "classic" PCOS), and 8 % for type B (OA+HA) (Figure 6D).

### 3.1.2. Assessment of sequencing bias using a mock community

A mock bacterial community was examined to estimate OTU taxonomic assignment bias due to PCR and sequencing errors (Table 4). When comparing expected and observed relative abundance data for the mock community, most genera were within ±50 % of the expected percentage. Bacteria from the genera *Bacteroides* and *Helicobacter* were overestimated by 2-2.5-fold, while *Acinetobacter*, *Enterobacteriaceae*, and *Pseudomonas* were underestimated by 2-3-fold. Of these, only *Bacteroides* was found in samples with a relative abundance>1 %.

***Table 4. Assessment of taxonomy assignment bias due to PCR and sequencing in the human study.***

| **Genus** | **Expected RA** | **Observed RA** | | **Fold Δ** | |
|---|---|---|---|---|---|
| | | **OTU RA >0.1%** | **OTU RA >0.01%** | **OTU RA >0.1%** | **OTU RA >0.01%** |
| #Acinetobacter | 0.05 | 0.02 | 0.02 | -2.4 | -2.4 |
| †Actinomyces | 0.05 | 0.03 | 0.03 | -1.6 | -1.5 |
| ‡Bacillus | 0.05 | 0.04 | 0.04 | -1.2 | -1.2 |
| §Bacteroides | 0.05 | 0.12 | 0.12 | 2.4 | 2.4 |
| ‡Clostridiaceae unclass. | 0.05 | 0.06 | 0.06 | 1.2 | 1.2 |
| \|\|Deinococcus | 0.05 | 0.04 | 0.04 | -1.2 | -1.2 |
| ‡Enterococcaceae unclass. | 0.05 | 0.04 | 0.04 | -1.4 | -1.4 |
| #Enterobacteriaceae unclass. | 0.05 | 0.02 | 0.02 | -2.2 | -2.2 |
| #Helicobacter | 0.05 | 0.11 | 0.11 | 2.2 | 2.2 |
| ‡Lactobacillus | 0.05 | 0.06 | 0.06 | 1.3 | 1.3 |
| ‡Listeria | 0.05 | 0.05 | 0.05 | 1.0 | 1.0 |
| #Neisseria | 0.05 | 0.06 | 0.06 | 1.3 | 1.3 |
| †Proprionibacterium | 0.05 | 0.06 | 0.06 | 1.1 | 1.1 |
| #Pseudomonas | 0.05 | 0.02 | 0.02 | -2.7 | -2.7 |
| #Rhodobacter | 0.05 | 0.04 | 0.04 | -1.2 | -1.2 |
| ‡Staphylococcus (2 spp.) | 0.10 | 0.09 | 0.09 | -1.2 | -1.1 |
| ‡Streptococcus (3 spp.) | 0.15 | 0.13 | 0.13 | -1.1 | -1.1 |
| #Comamonadaceae unclass. | 0.00 | <0.0001 | <0.0001 | | |

*Expected and observed relative abundances of bacterial genera in a mock community at OTU relative abundance cut-offs of 0.1 % and 0.01 %. RA, relative abundance; unclass., unclassified (the lowest classified taxonomic level is shown); spp., species. Square brackets indicate a greengenes suggested taxonomic assignment. #Proteobacteria; †Actinobacteria; ‡Firmicutes; §Bacteroidetes; ||[Thermi]. Adapted from* (Lindheim et al., 2016).

The mock community contained DNA in equimolar amounts from 20 bacterial species from 17 genera. All 17 genera were classified correctly at relative abundance cut-offs of 0.01 % and 0.1 %. One contaminating genus from the Comamonadaceae family was detected at a very low relative abundance (Table 4). When only singleton OTUs were removed from the dataset, the number of genera was overestimated, with a total of 29 identified genera (Table 5). This classification bias became more obvious on the OTU level, with 188 OTUs identified in the singleton-filtered data and 53 and 31 OTUs at the 0.01 % and 0.1 % cut-offs, respectively. A cut-off of 1 % resulted in the removal of the majority of reads and OTUs across all samples and was therefore deemed too conservative. From the mock community data alone, a relative abundance cut-off of 0.1 % seems appropriate. However, this cut-off would also result in the removal of many true, low-abundance OTUs. Therefore, we performed all analyses using both a 0.1 % and a 0.01 % OTU relative abundance cut-off for both stool and saliva samples.

***Table 5. Effect of relative abundance cut-offs on sequencing data of a bacterial mock community and samples in the human study.***

| | **Singleton-filtered** | **OTU RA >0.01 %** | **OTU RA >0.1 %** | **OTU RA >1 %** | **Expected** |
|---|---|---|---|---|---|
| **Mock community** | | | | | |
| Genera observed | 29 | 19 | 19 | 0 | 17 |
| OTUs observed | 188 | 53 | 31 | 0 | 20 |
| Reads analyzed (x1,000) | 106.4 | 105.8 | 105.1 | 0 | |
| **Stool samples** | | | | | |
| Genera observed | 81 ± 7.9 | 49 ± 4.6 | 25 ± 2.4 | 4 ± 1.1 | |
| OTUs observed | 2,626 ± 422.6 | 666 ± 124.1 | 109 ± 18.3 | 12 ± 0.0 | |
| Reads analyzed (x1,000) | 65.4 ± 13.48 | 61.1 ± 12.96 | 47.7 ± 11.81 | 16.5 ± 9.29 | |
| **Saliva samples** | | | | | |
| Genera observed | 72 ± 5.1 | 52 ± 2.8 | 32 ± 1.4 | 12 ± 0.0 | |
| OTUs observed | 773 ± 114.8 | 489 ± 48.1 | 116 ± 8.6 | 24 ± 0.0 | |
| Reads analyzed (x1,000) | 82.2 ± 15.17 | 80.4 ± 15.09 | 74.8 ± 14.84 | 46.9 ± 12.19 | |

*RA, relative abundance. Data are presented as mean ± standard deviation. Adapted from* (Lindheim et al., 2016).

### 3.1.3. Stool and saliva microbiome composition in PCOS

All included study participants provided a saliva sample. All women in the PCOS group and 19/20 women in the control group provided a stool sample. The most abundant phyla and genera in PCOS and control stool and saliva samples are summarized in Tables 6-9, using OTU relative abundance cut-offs of 0.1 % and 0.01 %. The stool microbiome was dominated by the phyla Bacteroidetes and Firmicutes, with *Bacteroides* as the dominant genus. Saliva samples were dominated by the phylum Bacteroidetes, followed by Firmicutes and Proteobacteria. The dominant genus in saliva was *Prevotella*, followed by *Streptococcus*. There were no significant differences between PCOS and control samples in stool phyla and genera with a relative abundance>1 % or in the Firmicutes:Bacteroidetes ratio. In saliva, the phylum Actinobacteria was less abundant in the PCOS group at the 0.1 %-filter ($p=0.024$), but not at the 0.01 %-filter ($p=0.075$).

***Table 6. Most abundant bacterial genera and phyla in stool samples from women with PCOS and control women using an OTU relative abundance cut-off of 0.01 %.***

| **OTU RA>0.01 %** | **% of total bacteria in Control (n=19)** | | **% of total bacteria in PCOS (n=24)** | | |
|---|---|---|---|---|---|
| **Top genera in stool** | **median** | **IQR** | **median** | **IQR** | **FDR p-value** |
| †Bacteroides | 30.1 | 16.87 | 38.6 | 14.78 | 0.701 |
| #Ruminococcaceae unclass. 1 | 6.9 | 3.76 | 6.6 | 4.45 | 0.879 |
| #Lachnospiraceae unclass. 1 | 5.6 | 3.45 | 6.4 | 4.48 | 0.669 |
| #Clostridiales unclass. 1 | 4.6 | 2.71 | 3.7 | 2.43 | 0.669 |
| #Lachnospiraceae unclass. 2 | 2.8 | 2.77 | 3.8 | 2.57 | 0.693 |
| #Clostridiales unclass. 2 | 3.6 | 3.81 | 2.7 | 2.57 | 0.087 |
| †Rikenellaceae unclass. | 2.5 | 5.25 | 3.1 | 2.55 | 0.970 |
| #Faecalibacterium | 2.3 | 2.19 | 3.5 | 3.12 | 0.651 |
| #Dialister | 1.5 | 4.27 | 1.6 | 4.08 | 0.693 |
| †Parabacteroides | 1.9 | 2.38 | 1.2 | 1.45 | 0.916 |
| #Ruminococcus | 2.6 | 2.18 | 0.4 | 1.48 | 0.087 |
| †[Barnesiellaceae] unclass. | 1.0 | 2.26 | 1.1 | 1.57 | 0.942 |
| #Ruminococcaceae unclass. 2 | 1.4 | 1.66 | 0.9 | 3.07 | 0.697 |
| #Erysipelotrichaceae unclass. | 0.9 | 0.64 | 1.2 | 0.69 | 0.151 |
| §Sutterella | 1.6 | 1.58 | 0.6 | 1.20 | 0.347 |
| **Top phyla in stool** | **median** | **IQR** | **median** | **IQR** | **FDR p-value** |
| †Bacteroidetes | 48.0 | 9.49 | 50.8 | 13.64 | 0.980 |
| #Firmicutes | 48.1 | 12.13 | 47.6 | 11.76 | 0.867 |
| §Proteobacteria | 2.5 | 3.15 | 1.5 | 1.94 | 0.229 |
| F:B ratio | 1.0 | 0.34 | 0.9 | 0.49 | 0.922 |

*RA, relative abundance; unclass., unclassified (the lowest classified taxonomic level is shown); FDR, Benjamini Hochberg false discovery rate correction. Square brackets indicate a greengenes suggested taxonomic assignment. #Firmicutes; †Bacteroidetes; §Proteobacteria. Adapted from* (Lindheim et al., 2017)

***Table 7. Most abundant bacterial genera and phyla in stool samples from women with PCOS and control women using an OTU relative abundance cut-off of 0.1 %.***

| **OTU RA>0.1%** | **% of total bacteria in Control (n=19)** | | **% of total bacteria in PCOS (n=24)** | | |
|---|---|---|---|---|---|
| **Top genera in stool** | **median** | **IQR** | **median** | **IQR** | **FDR p-value** |
| †Bacteroides | 37.5 | 18.21 | 44.6 | 15.53 | 0.693 |
| #Lachnospiraceae unclass. 1 | 4.4 | 4.25 | 5.9 | 3.94 | 0.431 |
| #Ruminococcaceae unclass. 1 | 4.0 | 2.59 | 4.6 | 4.60 | 0.825 |
| #Clostridiales unclass. 1 | 3.8 | 2.61 | 2.9 | 2.68 | 0.545 |
| †Rikenellaceae unclass. | 2.9 | 6.52 | 3.5 | 2.75 | 0.903 |
| #Faecalibacterium | 2.7 | 2.16 | 2.5 | 3.27 | 0.735 |
| #Lachnospiraceae unclass. 2 | 2.1 | 2.73 | 2.9 | 2.86 | 0.545 |
| †Parabacteroides | 2.0 | 2.82 | 1.5 | 1.68 | 0.903 |
| #Ruminococcaceae unclass. 2 | 1.6 | 1.81 | 0.6 | 2.78 | 0.545 |
| #Clostridiales unclass. 2 | 2.4 | 2.08 | 1.1 | 2.09 | 0.545 |
| †[Barnesiellaceae] unclass. | 1.2 | 2.67 | 1.3 | 2.05 | 0.903 |
| #Dialister | 0.1 | 5.69 | 1.6 | 5.28 | 0.615 |
| #Erysipelotrichaceae unclass. | 1.1 | 1.03 | 1.2 | 0.80 | 0.361 |
| §Sutterella | 2.2 | 1.95 | 0.7 | 1.51 | 0.333 |
| #Coprococcus | 0.4 | 1.50 | 0.7 | 1.12 | 0.903 |
| #Ruminococcus | 2.2 | 3.35 | 0.1 | 1.18 | 0.058 |
| **Top phyla in stool** | **median** | **IQR** | **median** | **IQR** | **FDR p-value** |
| #Firmicutes | 57.6 | 16.09 | 60.9 | 13.25 | 0.788 |
| †Bacteroidetes | 40.0 | 15.81 | 38.2 | 13.13 | 0.788 |
| §Proteobacteria | 2.4 | 2.94 | 1.5 | 2.65 | 0.610 |
| F:B ratio | 0.7 | 0.40 | 0.6 | 0.38 | 0.922 |

*RA, relative abundance; unclass., unclassified (the lowest classified taxonomic level is show); FDR, Benjamini Hochberg false discovery rate correction. Square brackets indicate a greengenes suggested taxonomic assignment. #Firmicutes; †Bacteroidetes; §Proteobacteria.*

***Table 8. Most abundant bacterial genera and phyla in saliva samples from women with PCOS and control women using an OTU relative abundance cut-off of 0.01 %.***

| **OTU RA>0.01 %** | **% of total bacteria in Control (n=20)** | | **% of total bacteria in PCOS (n=24)** | | |
|---|---|---|---|---|---|
| **Top genera in saliva** | **median** | **IQR** | **median** | **IQR** | **FDR p-value** |
| #Prevotella | 31.3 | 8.41 | 29.9 | 4.62 | 0.991 |
| †Streptococcus | 10.9 | 2.56 | 11.9 | 8.39 | 0.913 |
| †Veillonella | 8.2 | 3.07 | 8.1 | 5.47 | 0.991 |
| #[Prevotella] | 6.8 | 7.16 | 6.2 | 5.57 | 0.871 |
| §Neisseria | 6.5 | 5.27 | 5.2 | 6.95 | 0.913 |
| ‡Fusobacterium | 3.9 | 2.99 | 5.5 | 3.91 | 0.871 |
| #Porphyromonas | 3.3 | 5.01 | 5.7 | 4.49 | 0.913 |
| ¶Rothia | 4.3 | 2.67 | 2.5 | 2.02 | 0.871 |
| ¶Actinomyces | 2.4 | 0.96 | 2.5 | 1.00 | 0.991 |
| §Haemophilus | 2.9 | 2.38 | 2.2 | 1.77 | 0.940 |
| ‡Leptotrichia | 2.8 | 3.72 | 1.8 | 2.16 | 0.913 |
| †Granulicatella | 1.7 | 0.92 | 1.6 | 0.87 | 0.945 |
| **Top phyla in saliva** | **median** | **IQR** | **median** | **IQR** | **FDR p-value** |
| †Bacteroidetes | 42.9 | 6.92 | 45.0 | 6.68 | 0.953 |
| #Firmicutes | 25.7 | 6.18 | 27.7 | 5.19 | 0.953 |
| §Proteobacteria | 10.0 | 7.74 | 10.6 | 7.65 | 0.953 |
| ‡Fusobacteria | 7.6 | 4.17 | 7.9 | 4.38 | 0.953 |
| ¶Actinobacteria | 8.1 | 1.84 | 6.1 | 2.76 | 0.075 |
| TM7 | 1.3 | 1.12 | 1.1 | 1.00 | 0.619 |

*RA, relative abundance; unclass., unclassified (the lowest classified taxonomic level is shown); FDR, Benjamini Hochberg false discovery rate correction. Square brackets indicate a greengenes suggested taxonomic assignment. #Firmicutes; †Bacteroidetes; §Proteobacteria; ‡Fusobacteria; ¶Actinobacteria.*

***Table 9. Most abundant bacterial genera and phyla in saliva samples from women with PCOS and control women using an OTU relative abundance cut-off of 0.1 %.***

| **OTU RA>0.1 %** | **% of total bacteria in Control (n=20)** | | **% of total bacteria in PCOS (n=24)** | | |
|---|---|---|---|---|---|
| **Top genera in saliva** | **median** | **IQR** | **median** | **IQR** | **FDR p-value** |
| #Prevotella | 32.5 | 8.63 | 30.8 | 4.17 | 0.981 |
| †Streptococcus | 10.3 | 2.69 | 12.2 | 8.29 | 0.740 |
| †Veillonella | 8.4 | 3.65 | 8.2 | 5.42 | 0.847 |
| #[Prevotella] | 7.1 | 7.67 | 6.5 | 5.75 | 0.740 |
| §Neisseria | 6.9 | 5.74 | 5.5 | 7.63 | 0.740 |
| ‡Fusobacterium | 3.8 | 3.44 | 5.4 | 4.11 | 0.749 |
| #Porphyromonas | 3.0 | 5.63 | 5.5 | 4.60 | 0.749 |
| ¶Rothia | 4.5 | 3.31 | 2.7 | 2.33 | 0.726 |
| §Haemophilus | 3.0 | 2.06 | 2.3 | 1.81 | 0.910 |
| ‡Leptotrichia | 2.1 | 4.00 | 1.5 | 2.25 | 0.740 |
| ¶Actinomyces | 2.4 | 0.93 | 2.3 | 1.30 | 0.749 |
| †Granulicatella | 1.5 | 1.04 | 1.4 | 0.83 | 0.740 |
| ¶Atopobium | 1.0 | 1.11 | 0.7 | 0.61 | 0.740 |
| †Gemellaceae unclass. | 0.9 | 0.76 | 0.7 | 0.69 | 0.941 |
| §Campylobacter | 0.8 | 0.50 | 0.7 | 0.53 | 0.749 |
| \|\|TM7-3 unclass. | 0.8 | 0.78 | 0.6 | 0.75 | 0.740 |
| **Top phyla in saliva** | **median** | **IQR** | **median** | **IQR** | **FDR p-value** |
| †Bacteroidetes | 43.9 | 6.70 | 46.4 | 6.88 | 0.706 |
| #Firmicutes | 24.8 | 6.21 | 27.1 | 7.98 | 0.706 |
| §Proteobacteria | 9.5 | 8.94 | 10.1 | 8.21 | 0.706 |
| ‡Fusobacteria | 7.2 | 4.58 | 7.3 | 5.37 | 0.706 |
| ¶Actinobacteria | 8.2 | 2.19 | 6.1 | 2.82 | 0.024* |
| \|\|TM7 | 1.3 | 1.38 | 1.1 | 1.11 | 0.492 |

*RA, relative abundance; unclass., unclassified (the lowest classified taxonomic level is shown); FDR, Benjamini Hochberg false discovery rate correction. Square brackets indicate a greengenes suggested taxonomic assignment. #Firmicutes; †Bacteroidetes; §Proteobacteria; ‡Fusobacteria; ¶Actinobacteria; ||TM7. Adapted from* (Lindheim et al., 2016).

### 3.1.4. Differentially abundant taxa

When examining rare taxa with a relative abundance<1 %, stool samples from women with PCOS showed a lower relative abundance of bacteria from the phylum Tenericutes ($p<0.0001$), the Bacteroidales family S24-7 ($p=0.026$), and the Tenericutes order ML61528-J ($p=0.039$) (Figure 7A, C, D). The lower Tenericutes gene count was confirmed with real-time qPCR ($p=0.022$) (Figure 7B).

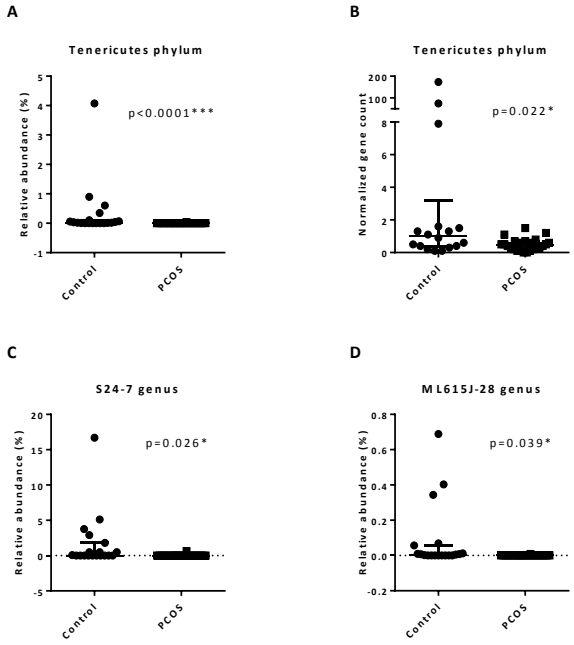


***Figure 7. Differentially abundant stool bacterial genera and phyla in women with PCOS and control women.*** *A, C, D. Groups were compared using Mann-Whitney U tests followed by Benjamini-Hochberg false discovery rate p-value correction. An OTU relative abundance cut-off of 0.01 % was used. B. Tenericutes differential gene counts assessed by real-time qPCR. Bars represent median and IQR. Adapted from* (Lindheim et al., 2017).

In addition to taxa comparisons, we used linear discriminant analysis effect size (LEfSe) to identify discriminant taxa for PCOS and control samples (Figure 8). In stool, six genera were enriched in PCOS samples at the 0.01 % OTU relative abundance cut-off and ten genera and two phyla were enriched in control samples (Figure 8B). Four of these genera were also significant at the 0.1 % OTU relative abundance cut-off (Figure 8A). Among the taxa enriched in control samples were the phylum Tenericutes, the family S24-7, and the order ML615J-28, all of which were significant in the taxa comparisons. In saliva, bacteria from the phylum Actinobacteria and the family Lachnospiraceae were enriched in control samples at both OTU relative abundance cut-offs, in line with the results of the taxa comparisons (Figure 9). No discriminant taxa for PCOS were found in saliva samples.

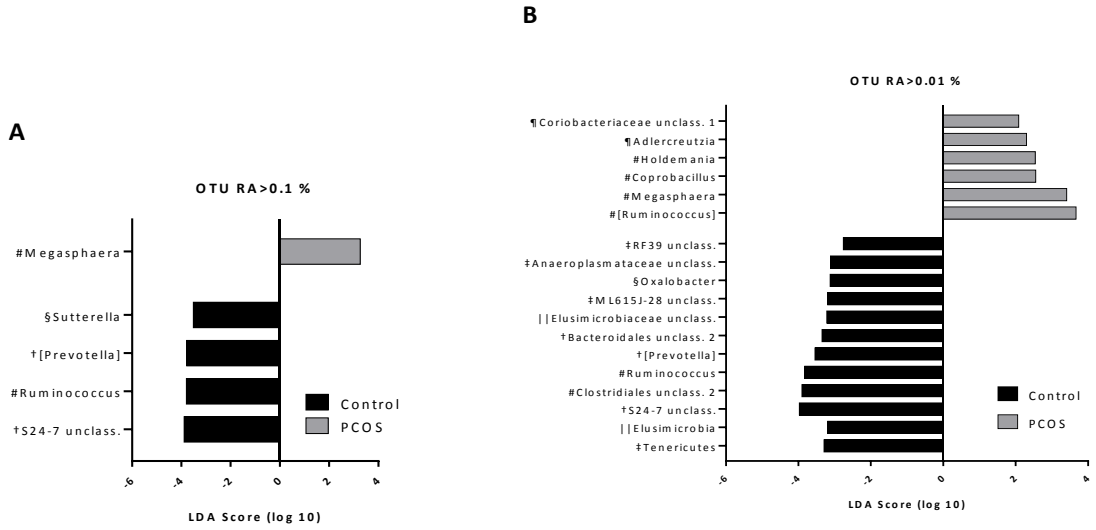


***Figure 8. Discriminant genera and phyla in stool samples from women with PCOS and control women.*** *Linear discriminant analysis effect size (LEfSe) plots using OTU relative abundance cut-offs of 0.1 % (A) and 0.01 % (B). RA, relative abundance. §Proteobacteria; †Bacteroidetes; #Firmicutes; ¶Actinobacteria; ‡Tenericutes; ||Elusimicrobia.*

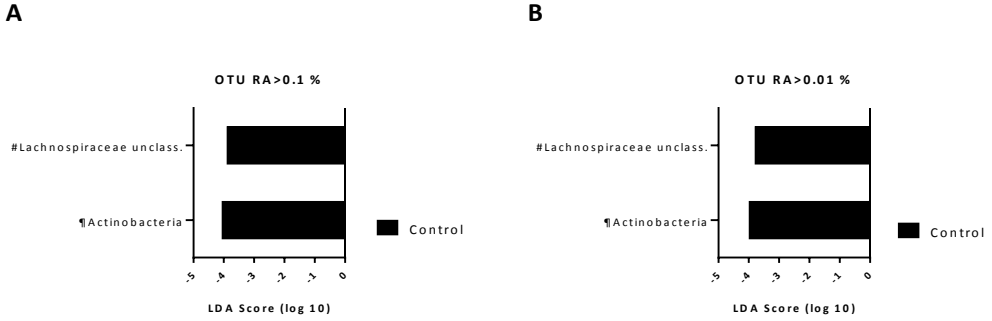


***Figure 9. Discriminant genera and phyla in saliva samples from women with PCOS and control women identified by LEfSe.*** *Linear discriminant analysis effect size (LEfSe) plots at OTU relative abundance cut-offs of 0.1 % (A) and 0.01 % (B). RA, relative abundance. #Firmicutes; ¶Actinobacteria.*

### 3.1.5. Alpha diversity

Women with PCOS had lower bacterial richness and diversity than controls, seen in the total number of observed OTUs (p=0.030) and Faith's phylogenetic diversity (p=0.027), at the 0.01 % OTU relative abundance cut-off (Figure 10C, D). This difference was not seen at the 0.1 % cut-off, indicating that rare species caused this result (Figure 10A, B). In saliva samples, there was no difference in bacterial richness or diversity between women with PCOS and control women (Figure 11).

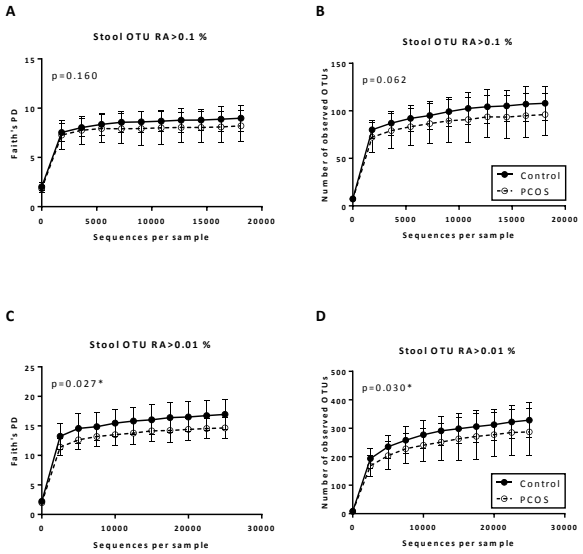


***Figure 10. Alpha diversity of stool samples from women with PCOS and control women.*** *Faith's phylogenetic diversity (A, C) and the number of observed OTUs (B, D) in stool samples using OTU relative abundance cut-offs of 0.1 % (A, B) and 0.01 % (C, D). Samples were rarefied to the smallest observed number of reads (18,100 for 0.1 %-filtered data and 24,990 for 0.01 %-filtered data). Median and IQR are plotted. RA, relative abundance. Adapted from* (Lindheim et al., 2017).

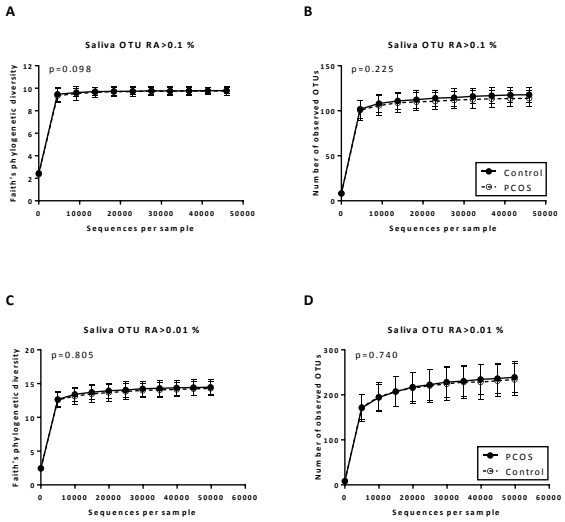


***Figure 11. Alpha diversity of saliva samples from women with PCOS and control women.*** *Faith's phylogenetic diversity (A, C) and the number of observed OTUs (B, D) in saliva samples using OTU relative abundance cut-offs of 0.1 % (A, B) and 0.01 % (C, D). Samples were rarefied to the smallest observed number of reads (45,940 for 0.1 %-filtered data and 49,810 for 0.01 %-filtered data). Median and IQR are plotted. RA, relative abundance. Adapted from* (Lindheim et al., 2016).

### 3.1.6. Beta diversity

Samples from women with PCOS and controls formed distinct clusters in unweighted, but not weighted UniFrac PCoA plots at both OTU relative abundance cut-offs (p=0.018 and 0.004 for the 0.1 % and 0.01 % cut-off, respectively) (Figure 12). Unweighted UniFrac analysis takes only presence and absence of OTUs into account, while weighted UniFrac analysis also utilizes abundance. In saliva, no difference in microbiome profiles was found between women with PCOS and controls (Figure 13).

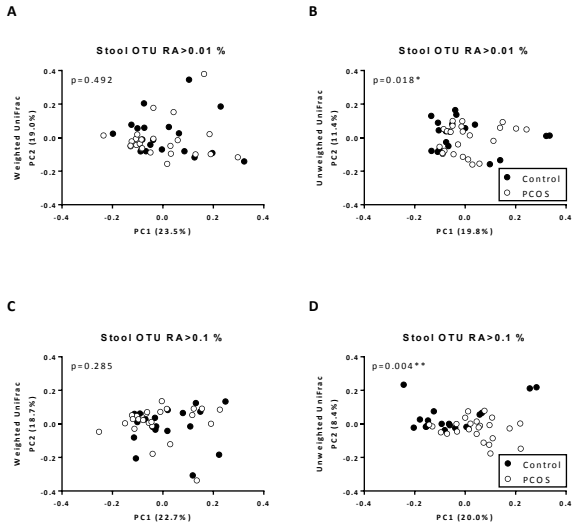


***Figure 12. Beta diversity of stool samples from women with PCOS and control women.*** *PCoA plots of weighted (A, C) and unweighted (B, D) UniFrac distances of stool samples at OTU relative abundance cut-offs of 0.1 % (A, B) and 0.01 % (C, D). Each dot represents the total bacterial community composition of one patient sample. The amount of variation explained by each principal coordinate is indicated in brackets. RA, relative abundance. Adapted from* (Lindheim et al., 2017).

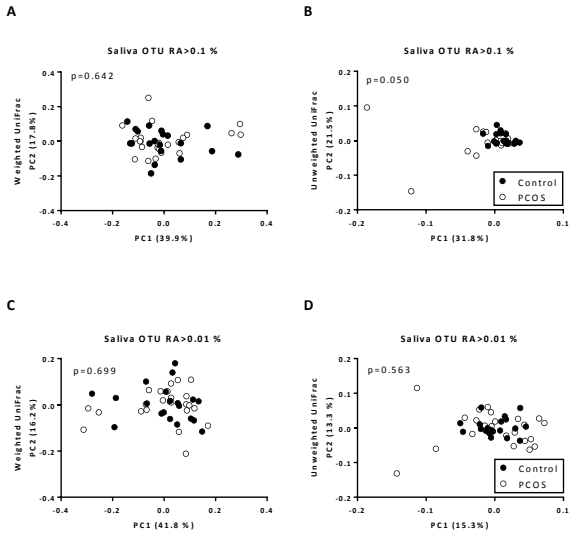


***Figure 13. Beta diversity of saliva samples from women with PCOS and control women.*** *PCoA plots of weighted (A, C) and unweighted (B, D) UniFrac distances of saliva samples at OTU relative abundance cut-offs of 0.1 % (A, B) and 0.01 % (C, D). Each dot represents the total bacterial community composition of one patient sample. The amount of variation explained by each principal coordinate is indicated in brackets. RA, relative abundance. Adapted from* (Lindheim et al., 2016).

### 3.1.7. Role of contamination

The main contaminant found in the negative controls was from the family Comamonadaceae, followed by *Pseudomonas* and an unclassified genus from the family Enterobacteriaceae (Table 10). Bacteria found in negative controls were generally absent or at a very low read count in samples. Some genera showed a high read count in samples and few reads in the negative controls, indicating cross-contamination from samples to negative controls. Genera showing this pattern were *Bacteroides, Streptococcus, Neisseria, Prevotella, Rothia, Haemophilus, Leptotrichia, Granulicatella, Fusobacterium, Campylobacter, Veillonella*, and bacteria from the Clostridiales family. This cross-contamination appeared to be on a very small scale and any contamination of samples due to bacterial DNA in the reagents was most likely removed during the OTU relative abundance filtering steps. No genera which were found to discriminate between women with PCOS and control women were found in negative controls with two exceptions, an unclassified genus from the Bacteroidales and one from the Clostridiales family.

***Table 10. Read counts (RC) of bacterial genera which were detected with at least two reads in negative controls (NCs) and their read counts across all unfiltered samples in the human study.***

| | **Read count in NCs (n=8)** | | **RC in samples (n=87)** | |
|---|---|---|---|---|
| **Genus** | **median** | **IQR** | **median** | **IQR** |
| §Comamonadaceae unclass. | 1,326 | 738.5 | 1 | 4.0 |
| §Pseudomonas | 256 | 235.0 | 0 | 0.0 |
| §Enterobacteriaceae unclass. | 154 | 130.5 | 1 | 18.5.0 |
| †Bacteroides | 123 | 685.5 | 35 | 2,1442.5 |
| #Alkalibacterium | 92 | 137.5 | 0 | 0.0 |
| §Delftia | 59 | 79.5 | 0 | 0.0 |
| §Xanthomonadaceae unclass. 1 | 38 | 67.5 | 0 | 0.0 |
| §Stenotrophomonas | 25 | 58.5 | 0 | 0.0 |
| §Caulobacteraceae unclass. | 20 | 35.0 | 0 | 0.0 |
| ¶Microbacterium | 14 | 31.0 | 0 | 0.0 |
| §Neisseriaceae unclass. | 11 | 33.5 | 2 | 38.5 |
| #Streptococcus | 11 | 29.0 | 4,215 | 9,296.0 |
| §Oceanospirillales unclass. | 5 | 11.5 | 0 | 0.0 |
| ‖Fimbriimonas | 5 | 24.5 | 0 | 0.0 |
| §Aeromonadaceae unclass. | 4 | 13.5 | 0 | 0.0 |
| §Acinetobacter | 4 | 13.0 | 0 | 0.0 |

| | | | | |
|---|---|---|---|---|
| §Neisseria | 3 | 21.5 | 144 | 4,237.0 |
| ¶Proprionibacterium | 3 | 37.5 | 0 | 1.0 |
| †Pedobacter | 2 | 24.5 | 0 | 0.0 |
| §Ochrobactrum | 1 | 17.5 | 0 | 0.0 |
| §Gluconacetobacter | 1 | 8.0 | 0 | 0.0 |
| †Prevotella | 1 | 3.0 | 15,496 | 23,423.5 |
| †Bacteroidales unclass. | 0 | 43.0 | 1 | 3.0 |
| ¶Corynebacterium | 0 | 11.5 | 3 | 36.0 |
| ¶Rothia | 0 | 0.5 | 435 | 2,464.0 |
| §Bradyrhizobium | 0 | 18.0 | 0 | 0.0 |
| §Alteromonadales unclass. | 0 | 11.5 | 0 | 0.0 |
| #Staphylococcus | 0 | 0.5 | 0 | 1.0 |
| #Acidaminococcus | 0 | 9.5 | 0 | 0.0 |
| §Haemophilus | 0 | 7.0 | 303 | 1,966.5 |
| ‡Leptotrichia | 0 | 0.5 | 240 | 1,873.0 |
| #Clostridium | 0 | 12.0 | 2 | 105.5 |
| §Sulfurospirillum | 0 | 12.0 | 0 | 0.0 |
| #Granulicatella | 0 | 9.0 | 495 | 1,185.5 |
| †Chryseobacterium | 0 | 4.0 | 0 | 0.0 |
| ‡Fusobacterium | 0 | 0.5 | 667 | 3,898.5 |
| ¶Actinomycetales unclass. | 0 | 3.0 | 0 | 0.0 |
| †Barnesiellaceae unclass. | 0 | 1.0 | 0 | 603.0 |
| §Xanthomonadaceae unclass. 2 | 0 | 4.0 | 0 | 0.0 |
| §Shewanella | 0 | 4.5 | 0 | 0.0 |
| §Sphingomonadaceae unclass. | 0 | 3.0 | 0 | 0.0 |
| §Oxalobacteraceae unclass. | 0 | 4.0 | 0 | 0.0 |
| #Lactobacillus | 0 | 0.5 | 1 | 5.0 |
| #Listeria | 0 | 1.0 | 0 | 0.0 |
| §Campylobacter | 0 | 0.5 | 148 | 694.5 |
| §Enhydrobacter | 0 | 2.0 | 0 | 0.0 |
| #Veillonella | 0 | 1.0 | 1,929 | 6,795.5 |
| **F16 unclass. | 0 | 0.5 | 3 | 189.5 |
| #Enterococcaceae unclass. | 0 | 0.5 | 0 | 0.0 |
| §Vibrio | 0 | 1.0 | 0 | 0.0 |
| #Clostridiales unclass. | 0 | 0.5 | 692 | 2,210.5 |

*unclass., unclassified (the lowest classified taxonomic level is shown). §Proteobacteria; †Bacteroidetes; #Firmicutes; ¶Actinobacteria; ||Armatimonadetes; ‡Fusobacteria; **TM7.* (Lindheim et al., 2016).

### 3.1.8. Gut barrier permeability and inflammation

To investigate the hypothesis that a disrupted gut barrier leads to translocation of endotoxin into the bloodstream and a subsequent pro-inflammatory response, we assessed surrogate markers for gut permeability and inflammation in serum and stool (Figure 14). Women with PCOS showed higher serum levels of the tight-junction modulator zonulin (p=0.006), while stool levels were not different to those of controls (p=0.063). Serum DAO, which is released from intestinal epithelial cells in response to inflammatory stimuli, was higher in women with PCOS (p=0.044). Stool calprotectin, a marker for intestinal inflammation, did not differ between the groups. There was no significant difference in serum endotoxin, the soluble LPS receptor sCD14, or LBP.

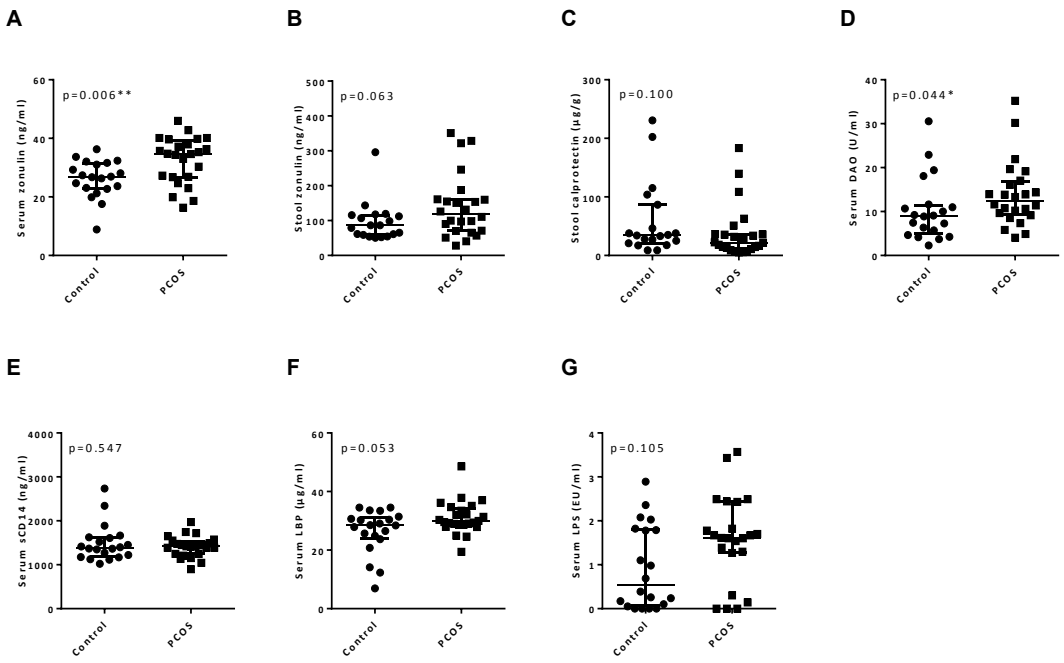


***Figure 14. Gut permeability and endotoxemia in women with PCOS and control women.*** *Serum and stool markers of gut permeability (A, B), gut inflammation (C, D), and endotoxemia (E-G). DAO, diamine oxidase; sCD14, soluble CD14 (endotoxin co-receptor); LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide (=endotoxin). Dots represent individual study subjects. Bars represent median and IQR. Adapted from* (Lindheim et al., 2017).

Of the studied inflammatory parameters, blood leukocytes were higher in women with PCOS than in healthy controls (p=0.042) due to a greater lymphocyte fraction (p=0.001) (Figure 15B, C). Serum hsCRP and sRAGE were not significantly different between the two groups. IL-6 and TNF-α were undetectable in almost all samples (data not shown). We investigated several adipocyte-derived markers to determine whether adipose tissue dysfunction might contribute to low-grade inflammation and insulin resistance in our PCOS cohort (Figure 15E-I). There was no significant difference in serum levels of leptin, adiponectin, resistin, or visfatin. Serum AFABP, an adipokine associated with insulin resistance, was significantly higher in women with PCOS than in controls (p=0.004).

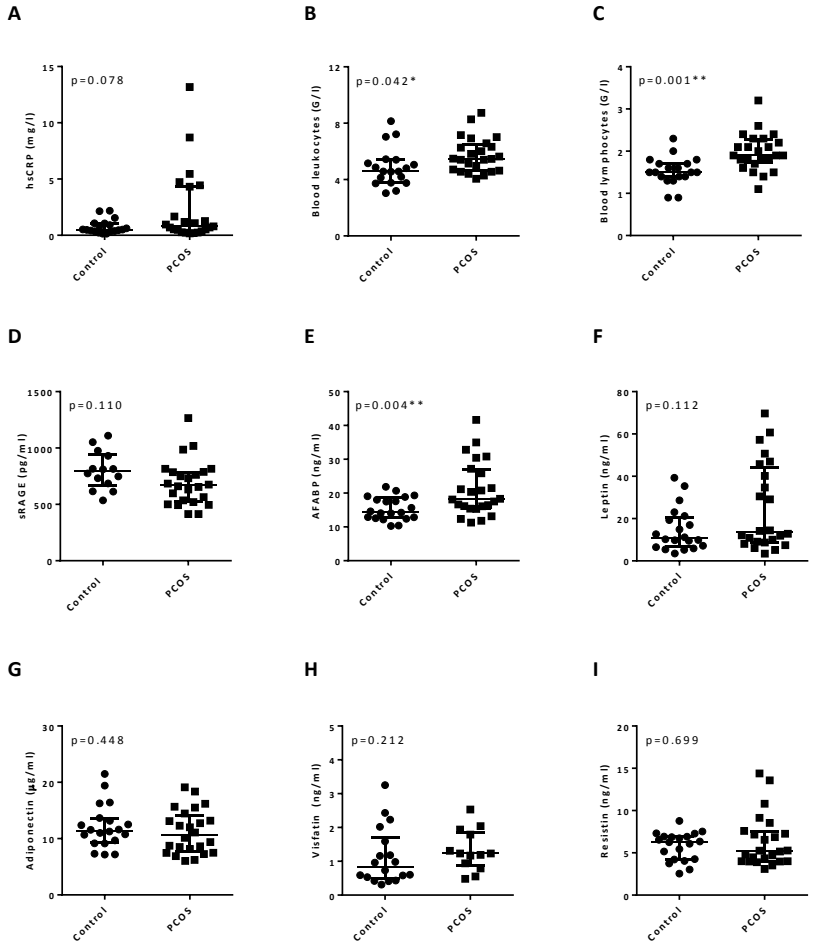


***Figure 15. Inflammation and adipose tissue dysfunction in women with PCOS and control women.*** *hsCRP, high-sensitivity C-reactive protein; sRAGE, soluble receptor for advanced glycation end-products; AFABP, adipocyte-derived fatty acid binding protein. Dots represent individual study subjects. Bars represent median and IQR. Adapted from* (Lindheim et al., 2017).

### 3.1.9. Isoflavone metabolism

We measured urine levels of daidzein, genistein, and equol before and after a soy challenge to detect differences in equol production capacity between women with PCOS and controls (Figure 16). At baseline, levels of all three compounds were low, reflecting the generally low consumption of soy in the study population (Figure 16A). Daidzein levels were significantly higher in control women than in women with PCOS at baseline (p=0.036). After three days of regular consumption of a moderate amount of soy protein (13.2 g/day), urine levels of all three compounds increased significantly in the whole cohort (p<0.0001 for daidzein and genistein and p=0.003 for equol). We distinguished between equol producers and non-producers according to Setchell *et al*., using a log10(E:D) ratio of -1.5 as a threshold (Setchell and Cole, 2006) (Figure 16B). The overall prevalence of equol producers was 30 % in the whole cohort (Figure 16C). 42 % of control women were classified as equol producers, compared with 21 % of women with PCOS. This difference was not statistically significant (p=0.120).

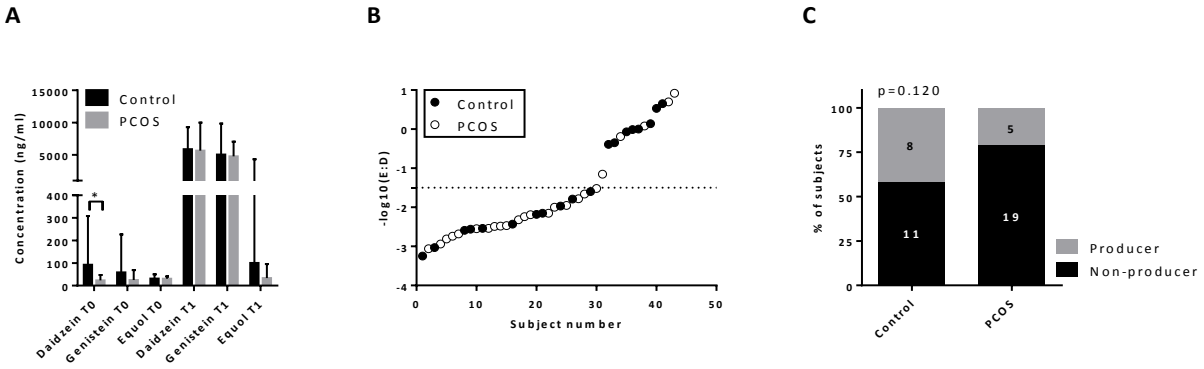


***Figure 16. Bacterial isoflavone metabolism in women with PCOS and control women.*** *A. Daidzein, genistein, and equol levels in urine before (T0) and after (T1) a 3-day soy challenge. Data are presented as median and IQR. B. Determination of equol producers and non-producers using log10(equol:daidzein). An equol producer was defined as having a quotient>-1.5. C. Comparison of equol producer prevalence in PCOS and control groups. The number of subjects in each category is indicated on segments.*

### 3.1.10. Exploring associations between the gut microbiome and the studied parameters

To gain insight into the interactions between the gut microbiome and the large number of parameters which are known to be relevant to PCOS, as well as novel parameters assessed in the context of this study, we performed an exploratory analysis of correlations between these various biomarkers.

First, we explored the hypothesis that a disturbed gut microbiome leads to gut barrier dysfunction (seen in elevated serum and stool zonulin and serum DAO), endotoxemia (seen in elevated LPS and LBP), and inflammation (seen in elevated hsCRP), thereby aggravating the PCOS phenotype (assessed by serum total and free testosterone, AMH, LH:FSH, FG-score, HOMA2-IR, and BDI) (Figure 17). In the global analysis, which included the whole study cohort, we saw inverse correlations between fecal bacterial diversity and serum and stool zonulin, LPS, total testosterone, AMH, and the LH:FSH ratio (Figure 17A). In the PCOS group, we found significant positive correlations between stool zonulin and serum total testosterone and AMH and a correlation between serum LBP and DAO and clustering in unweighted UniFrac analysis (Figure 17C). This effect was completely absent in the control group (Figure 17B). We found some indications for an influence of total testosterone, AMH, and LH:FSH on clustering in unweighted and weighted UniFrac analysis. BDI score was inversely correlated with bacterial diversity, serum DAO, and LBP in control women, but not in women with PCOS.

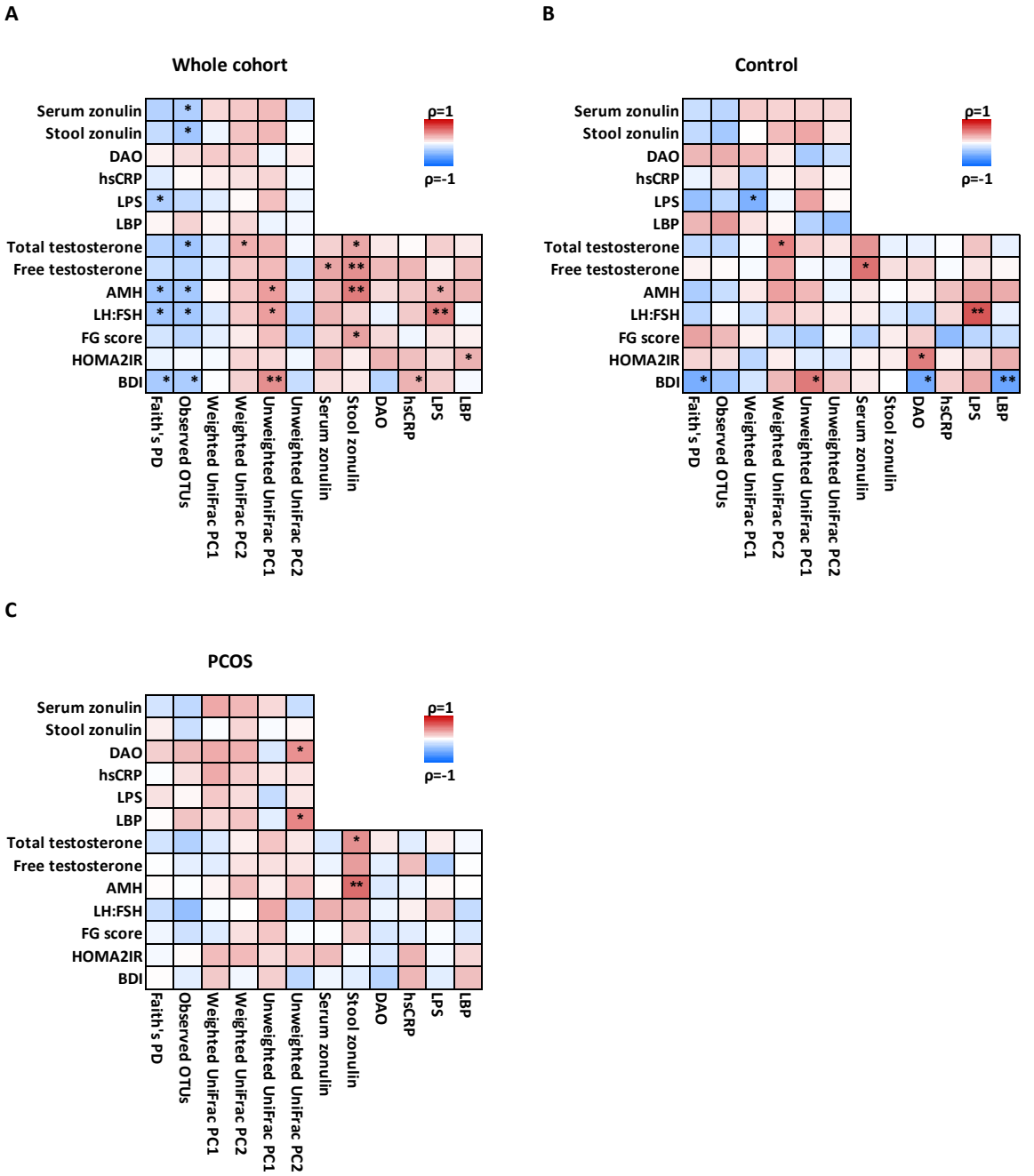


***Figure 17. Exploratory analysis of correlations between parameters related to stool microbiome, gut permeability, inflammation, and PCOS.*** *A. The whole study cohort. B. Control women only. C. Women with PCOS only. Stool microbiome parameters were derived from the 0.01 %-filtered dataset. PD, phylogenetic diversity; OTU, operational taxonomic unit; PC, principal coordinate; DAO, diamine oxidase; hsCRP, high-sensitivity C-reactive protein; LPS, lipopolysaccharide (=endotoxin); LBP, LPS-binding protein; AMH, anti-Muellerian hormone; LH:FSH, ratio of luteinizing to follicle-stimulating hormone; FG, Ferriman-Gallwey; HOMA2IR, homeostasis model assessment for insulin resistance; BDI, Beck's Depression Inventory. Cells are colored according to Spearman's ρ. Significant correlations are marked as follows: *p<0.05; **p<0.01; ***p<0.001.*

We next investigated whether age, BMI, or diet type could have confounded our obtained results (Figure 18). We found significant negative correlations between age and total and free testosterone, AMH, and the LH:FSH ratio. As these parameters are known to decline with age, this finding was not surprising. Upon analyzing controls and women with PCOS separately, this physiological influence of age disappeared in the PCOS group, with the exception of AMH (Figure 18C). In the whole cohort, age was positively correlated with bacterial diversity, and showed an effect on clustering in the unweighted UniFrac analysis (Figure 18A). We can therefore not exclude the possibility that the significant results we obtained were due to the higher age of the control group rather than the PCOS diagnosis.

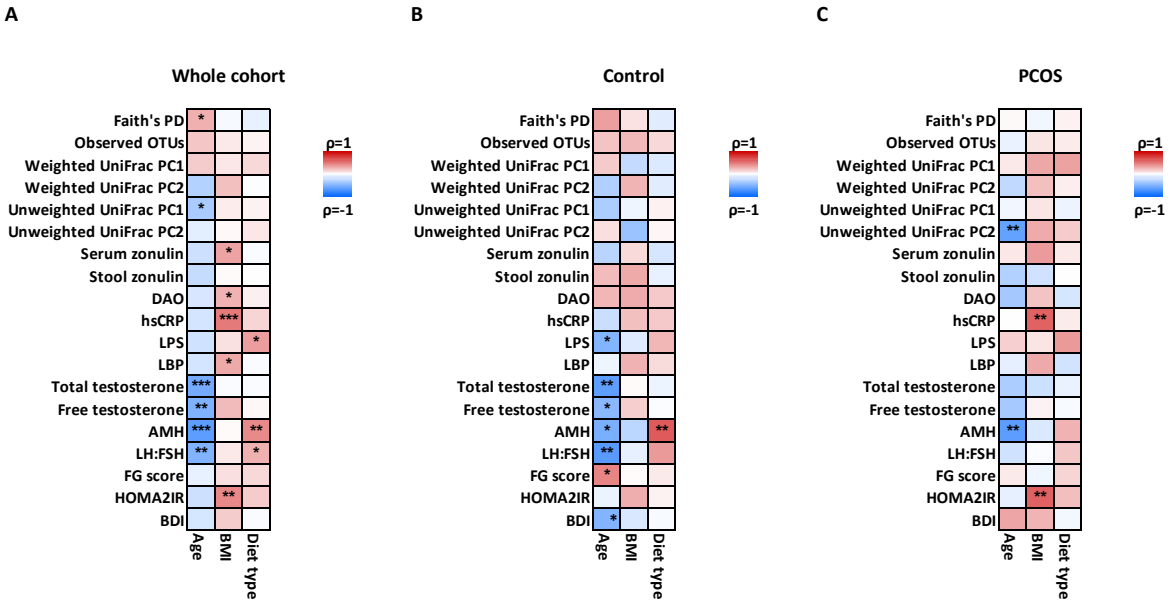


***Figure 18. Exploratory analysis of correlations between the suspected confounders age, BMI, and diet type and parameters related to the stool microbiome, gut permeability, inflammation, and PCOS.*** *A. The whole study cohort. B. Control women only. C. Women with PCOS only. Stool microbiome parameters were derived from the 0.01 %-filtered dataset. PD, phylogenetic diversity; OTU, operational taxonomic unit; PC, principal coordinate; DAO, diamine oxidase; hsCRP, high-sensitivity C-reactive protein; LPS, lipopolysaccharide (=endotoxin); LBP, LPS-binding protein; AMH, anti-Muellerian hormone; LH:FSH, ratio of luteinizing to follicle-stimulating hormone; FG, Ferriman-Gallwey; HOMA2IR, homeostasis model assessment for insulin resistance; BDI, Beck's Depression Inventory; BMI, body mass index. Cells are colored according to Spearman's ρ. Significant correlations are marked as follows: *p<0.05; **p<0.01; ***p<0.001.*

BMI correlated with parameters of gut permeability and inflammation (Figure 18A). It has long been known that obesity is a pro-inflammatory state. In the PCOS group, BMI correlated strongly and significantly with hsCRP and HOMA2IR, indicating more severe metabolic inflammation in these women (Figure 18C). Diet was associated with serum LPS, AMH, and LH:FSH in the whole cohort, with the animal protein type showing higher levels of these parameters (Figure 18A).

There were almost no significant correlations between saliva microbiome parameters and parameters related to PCOS in control women (Figure 19B). In women with PCOS, hsCRP, salivary and serum androgen levels, and the FG score correlated significantly with clustering in weighted UniFrac analysis (Figure 19C). Of the suspected confounders, only BMI appeared to be associated with clustering in the weighted UniFrac analysis, while age and diet type had no effect. In control women, BDI and HOMA2-IR correlated significantly with clustering in weighted UniFrac analysis (Figure 19B).

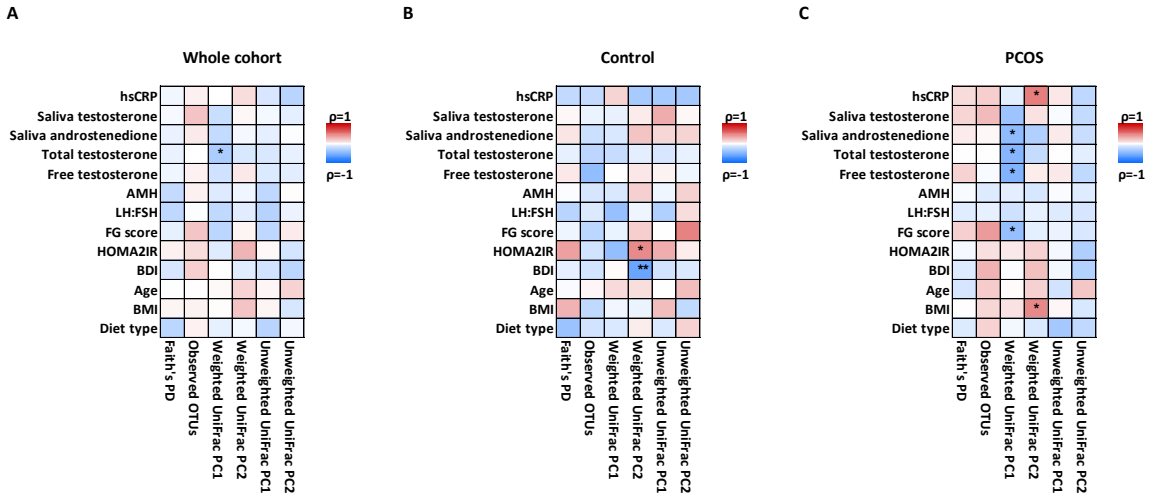


***Figure 19. Exploratory analysis of correlations between parameters related to saliva microbiome, inflammation, and PCOS as well as the suspected confounders age, BMI, and diet type.*** *A. The whole study cohort. B. Control women only. C. Women with PCOS only. Saliva microbiome parameters were derived from the 0.01 %-filtered dataset. PD, phylogenetic diversity; OTU, operational taxonomic unit; PC, principal coordinate; hsCRP, high-sensitivity C-reactive protein; AMH, anti-Muellerian hormone; LH:FSH, ratio of luteinizing to follicle-stimulating hormone; FG, Ferriman-Gallwey; HOMA2IR, homeostasis model assessment for insulin resistance; BDI, Beck's Depression Inventory; BMI, body mass index. Cells are colored according to Spearman's ρ. Significant correlations are marked as follows: *p<0.05; **p<0.01; ***p<0.001.*

Finally, we investigated whether bacterial isoflavone metabolism was associated with changes in the gut microbiome, gut barrier parameters, inflammation, and parameters related to PCOS. In the control group, all investigated variables related to isoflavone metabolism were associated with clustering in the weighted UniFrac analysis Figure 20B). In the PCOS group, few significant correlations were seen (Figure 20C). Overall, a greater increase in urine equol levels after soy consumption was associated with lower serum androgens, zonulin, and AMH, indicating that equol producing capacity may have a beneficial effect on PCOS pathophysiology (Figure 20A).

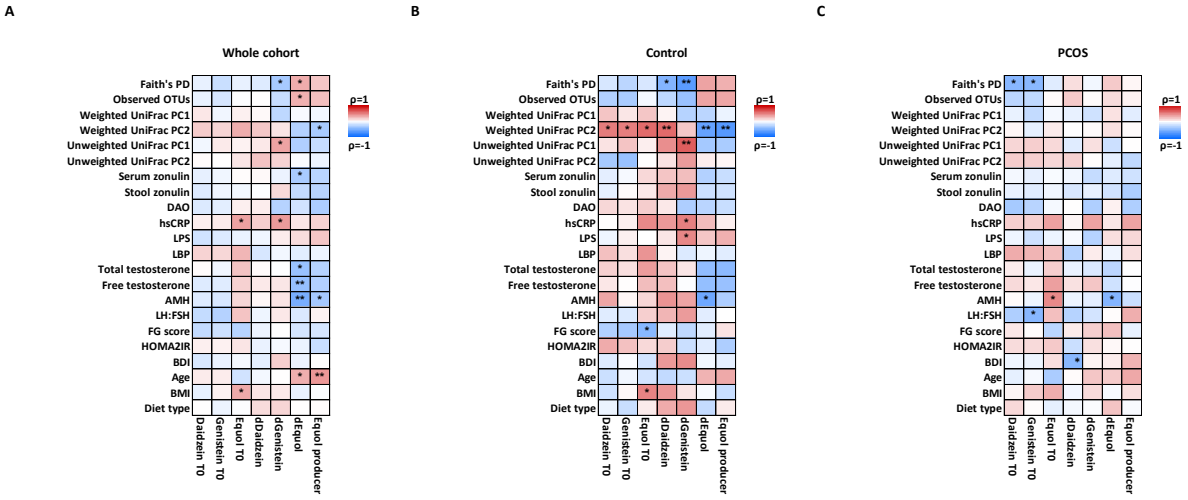


***Figure 20. Exploratory analysis of correlations between parameters related to isoflavone metabolism, stool microbiome, gut permeability, inflammation, and PCOS as well as the suspected confounders age, BMI, and diet type.*** *A. The whole study cohort. B. Control women only. C. Women with PCOS only. Stool microbiome parameters were derived from the 0.01 %-filtered dataset. PD, phylogenetic diversity; OTU, operational taxonomic unit; PC, principal coordinate; DAO, diamine oxidase; hsCRP, high-sensitivity C-reactive protein; LPS, lipopolysaccharide (=endotoxin); LBP, LPS-binding protein; AMH, anti-Muellerian hormone; LH:FSH, ratio of luteinizing to follicle-stimulating hormone; FG, Ferriman-Gallwey; HOMA2IR, homeostasis model assessment for insulin resistance; BMI, body mass index; T0, baseline value; d, change from baseline after soy challenge. Cells are colored according to Spearman's ρ. Significant correlations are marked as follows: *p<0.05; **p<0.01; ***p<0.001.*

## 3.2. Part 2: Mouse study

The following experiments were performed at the Reproductive Endocrinology and Metabolism group, Department of Physiology and Pharmacology, Karolinska Institutet in Stockholm, Sweden. The experiments were supervised by Associate Professor Elisabet Stener-Victorin and were carried out in collaboration with PhD students Maria Manti and Romina Fornes. The data related to F0 mice are part of the PhD thesis of Romina Fornes and the data related to F1 mice are part of the PhD thesis of Maria Manti. My original contribution to these projects are the fecal microbiome analysis in the two sets of mice and the FMT experiment. To provide the context for these results, the phenotype of the PNA mouse model will be briefly summarized with Elisabet and Maria's permission. Next-generation sequencing and the FMT experiment were performed in collaboration with the group of Eduardo Villablanca at the Department of Medicine, Karolinska Institutet.

The interaction between diet-induced obesity and the gut microbiome is already well-described. Therefore, it was decided to focus this project on the reproductive and behavioral phenotype of the combined PNA-HFD mouse model. The metabolic measurements discussed in this thesis will be limited to body weight and food intake.

### 3.2.1. Metabolic and reproductive phenotype of male and female PNA offspring

Body weight and food intake of F1 mice were followed until 19 weeks of age starting at weaning (Figure 21). As expected, mice fed a HF/HS diet consumed more calories than mice on a CD (main effect of offspring diet, $p<0.05$). In male offspring, weight gain was determined by an interaction of maternal injection and offspring diet (maternal injection*offspring diet, $p<0.05$). A HF/HS-diet led to weight gain in all groups, but male offspring exposed to DHT displayed an attenuated weight gain compared to vehicle-exposed mice. This difference was not explained by a lower calorie intake. In female offspring, weight gain was determined by an interaction of maternal and offspring diet (maternal diet*offspring diet, $p<0.05$), with no significant effect of DHT exposure. Female offspring showed greater weight gain on a HF/HS-diet when additionally exposed to a maternal HF/HS-diet. This difference was not explained by a higher calorie intake.

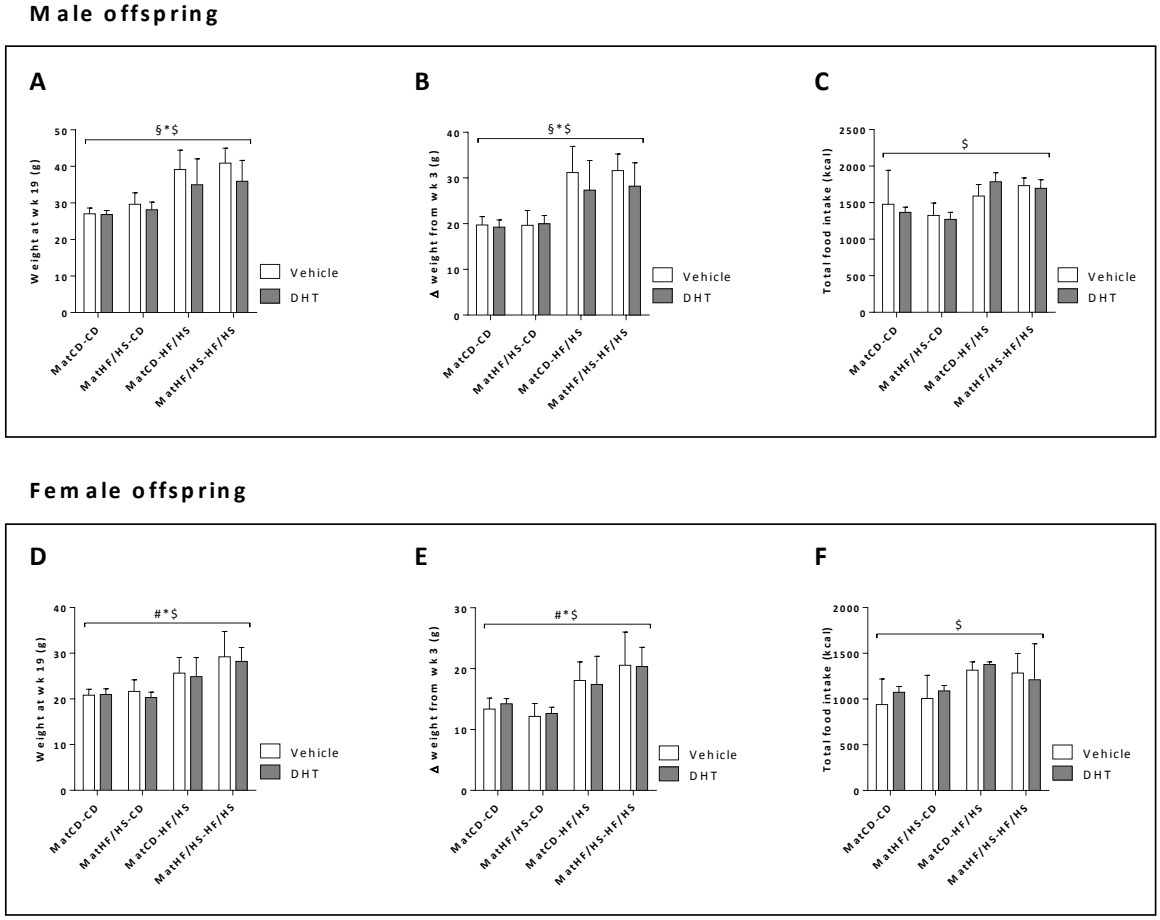


***Figure 21. Body weight development and food intake in PNA offspring.*** *Body weight at week 19 (A, D), weight gain from week 3 to week 19 (B, E), and total food intake during the experiment (C, F) in male (A-C) and female (D-F) offspring. Food intake was normalized to the number of mice per cage. #p<0.05 main effect of maternal diet; §p<0.05 main effect of maternal injection; $p<0.05 main effect of offspring diet. * denotes interactions between factors. Mean and SD are shown. Groups were compared using 3-way ANOVA. N=9-12 mice per group for body weight and weight gain and N=3-8 cages per group for food intake.*

Female DHT-exposed offspring had disrupted estrous cycles, with more days spent in diestrus and fewer days spent in proestrus (main effect of maternal injection, $p<0.05$) (Figure 22). In addition, there was an interaction between maternal and offspring diet in the proportion of days spent in proestrus (maternal diet*offspring diet, $p<0.05$). This may be due to the rather large within-group variation. As the effect size for the interaction was small (partial Eta-squared=0.062), the effect on the overall result is negligible.

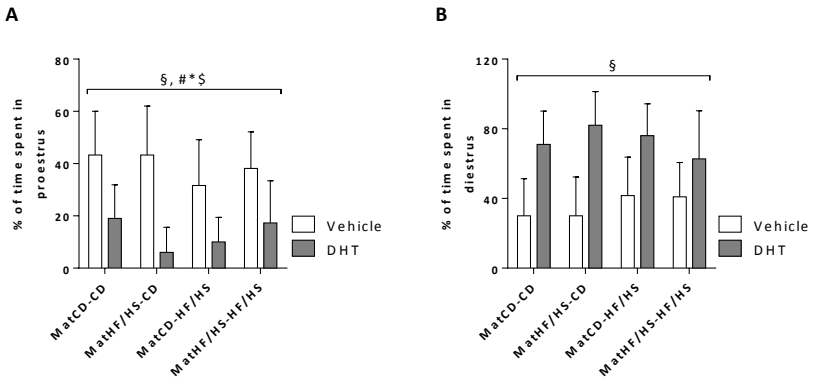


***Figure 22. Estrous cyclicity in female PNA offspring.*** *Percentage of time spent in proestrus (A) and diestrus (B) over ten consecutive days. #p<0.05 main effect of maternal diet; §p<0.05 main effect of maternal injection; $p<0.05 main effect of offspring diet. * denotes interactions between factors. Mean and SD are shown. Groups were compared using 3-way ANOVA. N=9-12 mice per group.*

In previous work by Elisabet Stener-Victorin's group, PNA exposure induced anxiety-like behavior in female rats in an androgen and estrogen receptor-dependent manner (Hu et al., 2015). We therefore performed behavior testing on male and female PNA offspring in the EPM and OF. In line with previous data, we found a reduction in the time spent in the open areas of the EPM and OF in female PNA offspring, indicating increased anxiety-like behavior (main effect of maternal injection, $p<0.05$) (Figure 23E-H). The maternal DHT injection effect was completely absent in male offspring (Figure 23A-D). Maternal diet significantly affected behavior in male offspring, with males exposed to a maternal HF/HS diet displaying greater anxiety-like behavior in the OF irrespective of DHT exposure (main effect of maternal diet, $p<0.05$).

The total distance traveled in the OF was not different in female offspring, while in male offspring there was a three-way interaction (maternal diet*maternal injection*offspring diet, $p<0.05$) (Figure 23D, H). The effect size of this interaction was not large enough to affect the result (partial Eta-squared=0.051). The total distance traveled in the EPM was influenced by an interaction of maternal and offspring diet in both male and female offspring (maternal diet*offspring diet, $p<0.05$) (Figure 23B, F). These interactions had negligible effect sizes (partial Eta-squared=0.090 for female and 0.046 for male offspring).

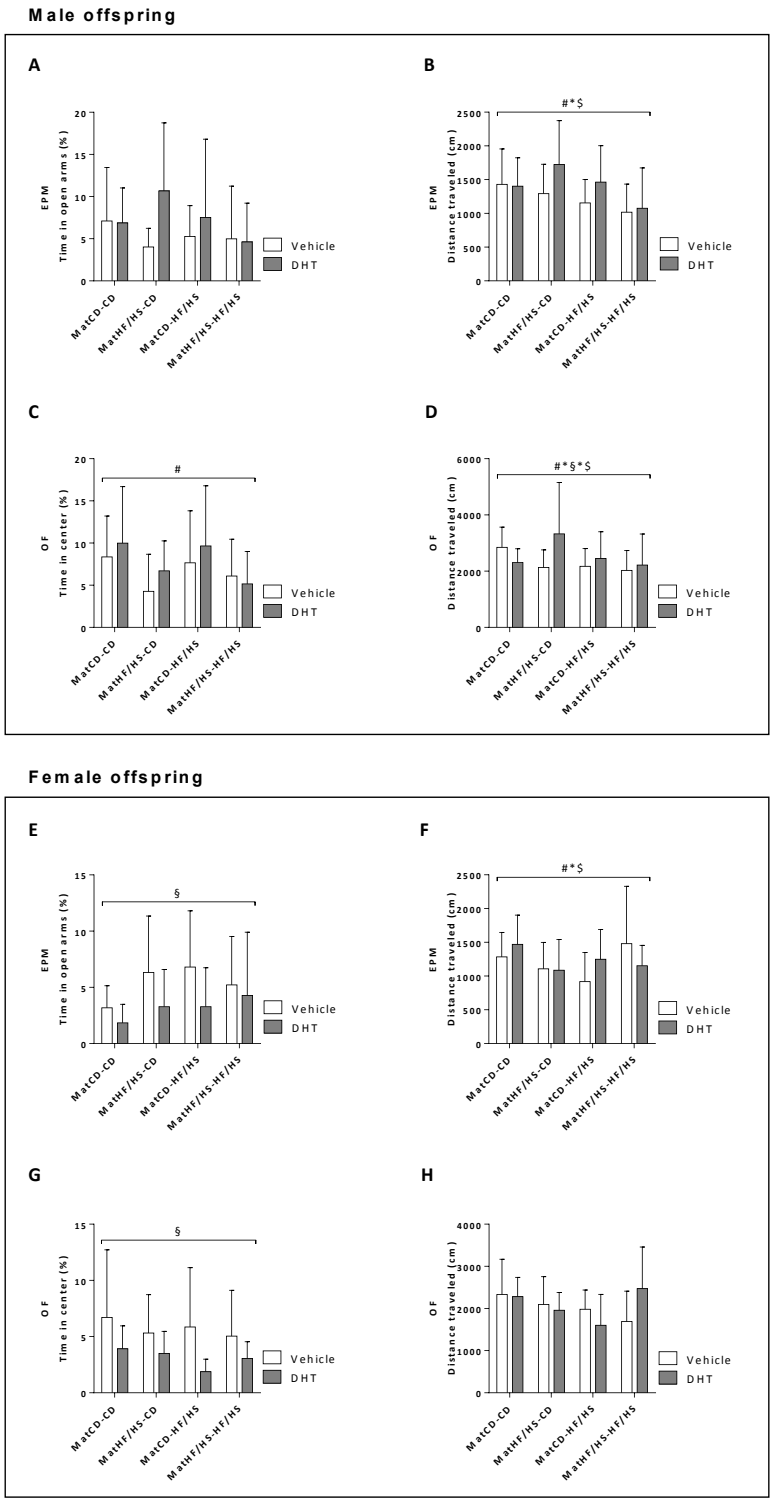


***Figure 23. Anxiety-like behavior in PNA offspring.*** *Time spent in the open arms of the elevated plus maze (A, E) and open field (C, G), as well as total distance traveled (B, D, F, H) for male (A-D) and female (E-H) offspring. #p<0.05 main effect of maternal diet; §p<0.05 main effect of maternal injection; $p<0.05 main effect of offspring diet. * denotes interactions between factors. Mean and SD are shown. Groups were compared using 3-way ANOVA. N=9-12 mice per group.*

### 3.2.2. Fecal microbiome composition in PNA offspring and dams

Fecal samples were collected from male and female PNA mice at 23 weeks of age and from an unrelated group of dams which underwent the same experimental procedures as the mothers of the PNA mice, but were sacrificed at gestational day (GD) 18.5.

The predominant phyla and genera in F0 dams, F1 male offspring, and F1 female offspring are presented in Table 11. Fecal samples were dominated by bacteria from the phylum Bacteroidetes, followed by comparable proportions of Firmicutes and Proteobacteria. F0 samples showed an unusually high percentage of Verrucomicrobia, which was not seen in F1 samples. This finding was reflected on the genus level, where *Akkermansia* was the dominant genus in F0 samples. The dominant genera in F1 mice were *Desulfovibrio*, members of the Bacteroidales family S24-7, and the Rikenellaceae group *RC9*.

***Table 11. Relative abundances of the top (>1 %) bacterial genera and phyla in fecal samples from PNA offspring and dams.***

| | % of total bacteria in F0 dams (n=20) | | % of total bacteria in male offspring (n=76) | | % of total bacteria in female offspring (n=74) | |
|---|---|---|---|---|---|---|
| **Top genera** | **median** | **IQR** | **median** | **IQR** | **median** | **IQR** |
| ‡Akkermansia | 24.6 | 35.12 | 5.8 | 24.24 | 6.4 | 38.67 |
| §Desulfovibrio | 13.9 | 17.26 | 15.3 | 28.06 | 13.2 | 37.03 |
| †S24-7 unclass. | 13.0 | 11.62 | 16.1 | 10.26 | 13.9 | 11.66 |
| †Rikenellaceae RC9 | 15.3 | 12.47 | 10.6 | 12.67 | 6.9 | 10.77 |
| #Clostridiales vadinBB60 | 0.0 | 0.46 | 7.5 | 7.59 | 4.1 | 7.64 |
| †Bacteroides | 3.0 | 2.87 | 1.3 | 3.42 | 2.5 | 9.73 |
| #Lachnospiraceae NK4A136 | 0.7 | 1.23 | 4.7 | 7.79 | 2.0 | 4.25 |
| †Alistipes | 2.8 | 2.88 | 3.5 | 3.17 | 3.3 | 2.26 |
| #Lachnospiraceae unclass. | 2.1 | 2.54 | 3.0 | 3.63 | 2.8 | 4.04 |
| #Allobaculum | 2.5 | 5.09 | 0.9 | 1.67 | 0.7 | 1.29 |
| †Alloprevotella | 3.5 | 5.04 | 0.0 | 0.44 | 0.0 | 0.00 |
| §Parasutterella | 0.1 | 0.37 | 0.5 | 0.89 | 0.5 | 1.06 |
| #Ruminococcaceae unclass. | 0.4 | 0.83 | 0.7 | 0.74 | 0.6 | 1.07 |
| **Top phyla** | **median** | **IQR** | **median** | **IQR** | **median** | **IQR** |
| †Bacteroidetes | 43.7 | 24.38 | 39.8 | 25.46 | 36.8 | 26.36 |
| #Firmicutes | 15.4 | 11.46 | 24.5 | 16.77 | 19.5 | 19.91 |
| §Proteobacteria | 14.5 | 17.65 | 15.8 | 24.60 | 14.5 | 34.40 |
| ‡Verrucomicrobia | 24.6 | 35.12 | 5.8 | 24.24 | 6.4 | 38.67 |

*Median values across all treatment groups. RA, relative abundance; unclass., unclassified (the lowest classified taxonomic level is shown). #Firmicutes; †Bacteroidetes; §Proteobacteria; ‡Verrucomicrobia.*

### 3.2.3. Differentially abundant taxa

We investigated whether the factors maternal diet, maternal injection, and offspring diet, caused significant changes in genus relative abundances (Figure 24). In F0 dams, three genera were significantly more abundant and two genera were significantly less abundant due to a HF/HS diet, with fold changes ranging from -2 to 4 (Figure 24A). DHT injection did not cause any significant genus relative abundance changes in F0 dams.

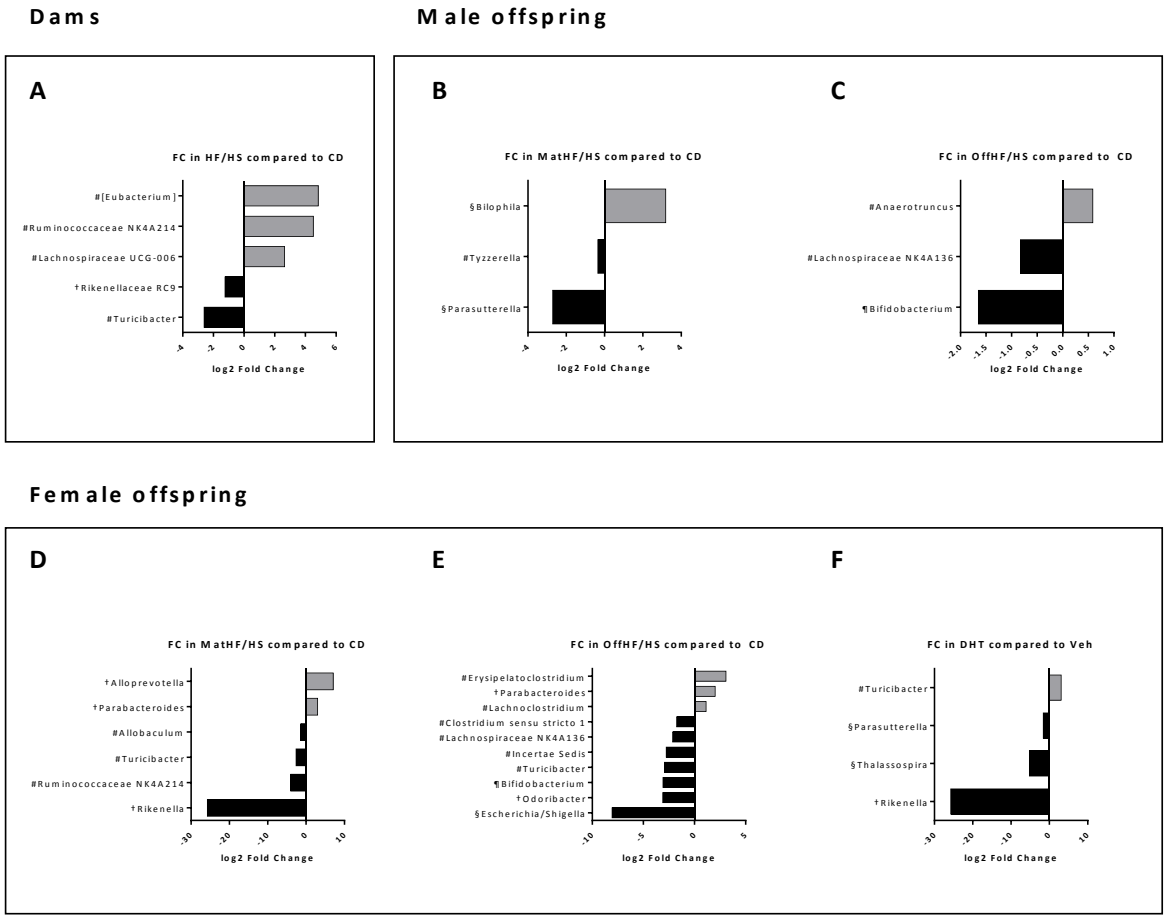


***Figure 24. Differentially abundant genera in fecal samples from PNA offspring and dams identified by DESeq2.*** *Log2 fold change (FC) of relative abundance for genera which were significantly changed in dams (A), male (B, C) and female (D-F) PNA offspring due to the factors maternal diet (A, B, D), offspring diet (C, E), and maternal injection (F). No genera were significantly changed due to maternal injection in mothers or male offspring. Square brackets indicate a Silva suggested taxonomic assignment. P<0.05 for all after Benjamini-Hochberg false discovery rate correction. #Firmicutes; †Bacteroidetes; §Proteobacteria; ¶Actinobacteria.*

In male offspring, both maternal and offspring HF/HS diet led to changes in genus relative abundances (Figure 24B, C). These changes ranged from -2 to 3-fold, and did not overlap with the significant genera found in dams. No genera were significantly changed in male offspring due to maternal injection.

In female offspring, we saw the largest effects of all three studied factors, with relative abundance changes ranging from -25 to 10-fold (Figure 24D-F). Two genera were found to be regulated by offspring diet in a concordant way in both male and female offspring. These were Lachnospiraceae group *NK4A136* and *Bifidobacterium*, which both had a lower relative abundance in response to a HF/HS diet (Figure 24 C, E). There was no clear pattern of specific phyla being consistently more or less abundant due to any of the studied factors.

### 3.2.4. Alpha diversity in PNA offspring

Alpha diversity of fecal samples was assessed using the number of observed ribosomal sequence variants (RSVs) and the Shannon index (Figure 25). In F0 dams, there was an interaction effect of diet and injection on the number of observed RSVs, with an increase in diversity both in the CD-DHT and the HF/HS-Veh groups, but no change in the HF/HS-DHT group (diet*injection, $p<0.05$) (Figure 25A). There was no significant difference in the Shannon index in F0 dams.

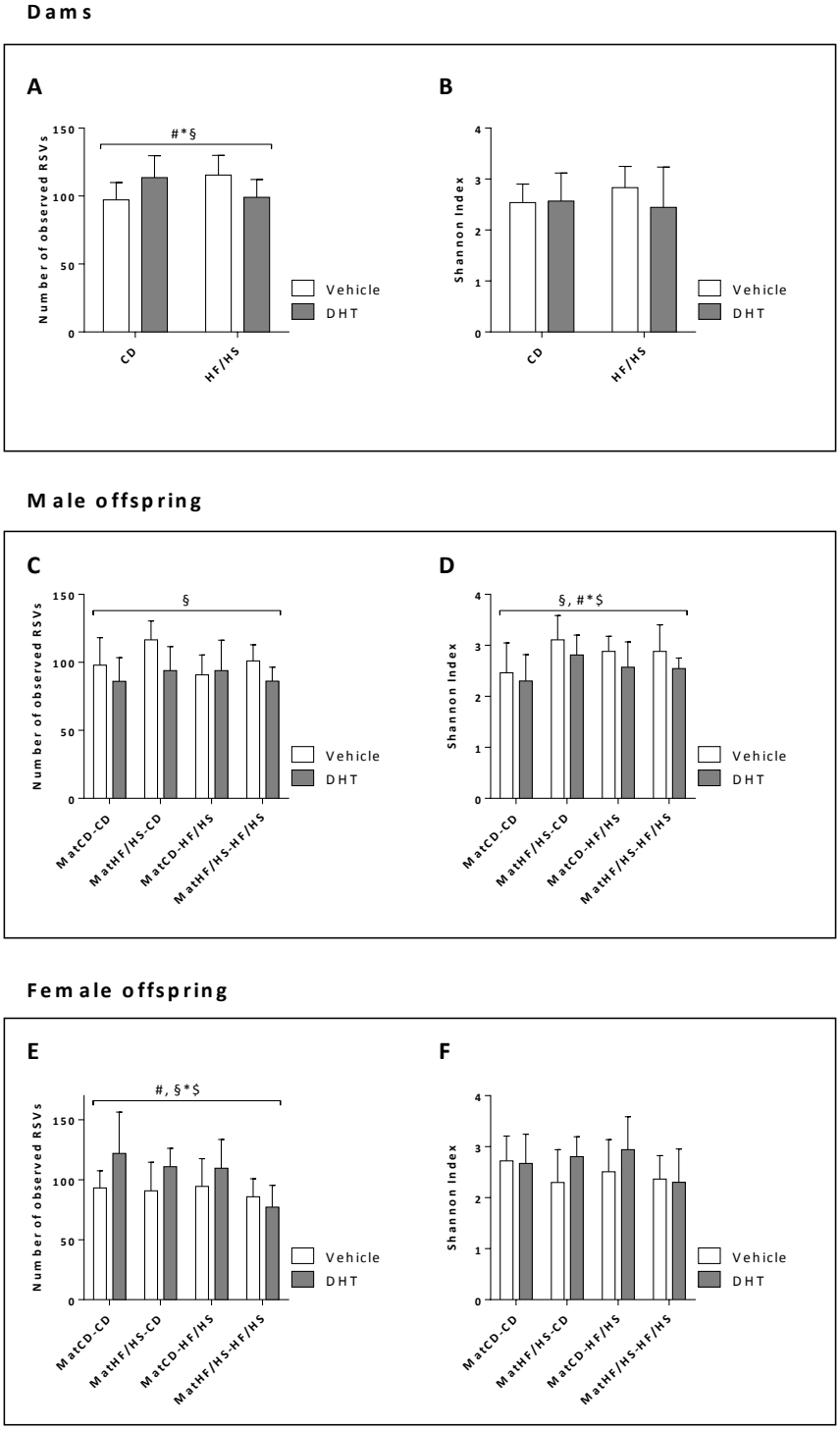


***Figure 25. Alpha diversity of fecal samples from dams (A, B), male (C, D), and female (E, F) PNA offspring.*** *The number of observed RSVs (A, C, E) and Shannon Index (B, D, F) are presented. Mean and SD are plotted. RSV: ribosomal sequence variant. #p<0.05 main effect of maternal diet; §p<0.05 main effect of maternal injection; $p<0.05 main effect of offspring diet. * denotes interactions between factors. Mean and SD are shown. Groups were compared using 2- or 3-way ANOVA. N= 4-6 animals per group for dams and 7-10 animals per group for offspring.*

In male offspring, DHT-exposed offspring exhibited lower diversity in both diversity measures (main effect of maternal injection, $p<0.05$) (Figure 25C, D). For the Shannon index, there was an additional interaction between maternal and offspring diet, with offspring from mothers fed a HF/HS diet showing a higher diversity on a CD than offspring from mothers fed a CD (maternal diet*offspring diet $p<0.05$).

In female offspring, a maternal HF/HS diet led to a reduced richness, seen in the number of observed RSVs (main effect of maternal diet, $p<0.05$) (Figure 25E). Additionally, there was an interaction between maternal injection and offspring diet, where DHT exposure caused an increase in richness in CD-fed offspring, but not in HF/HS diet-fed offspring (maternal injection*offspring diet, $p<0.05$). There was no significant difference in the Shannon index in female offspring.

### 3.2.5. Beta diversity in PNA offspring

Bray-Curtis distances were calculated and plotted using multidimensional scaling (MDS) to compare whole bacterial communities across treatment groups. In F0 dams, neither diet nor injection significantly affected clustering of samples in the MDS plot (Figure 26).

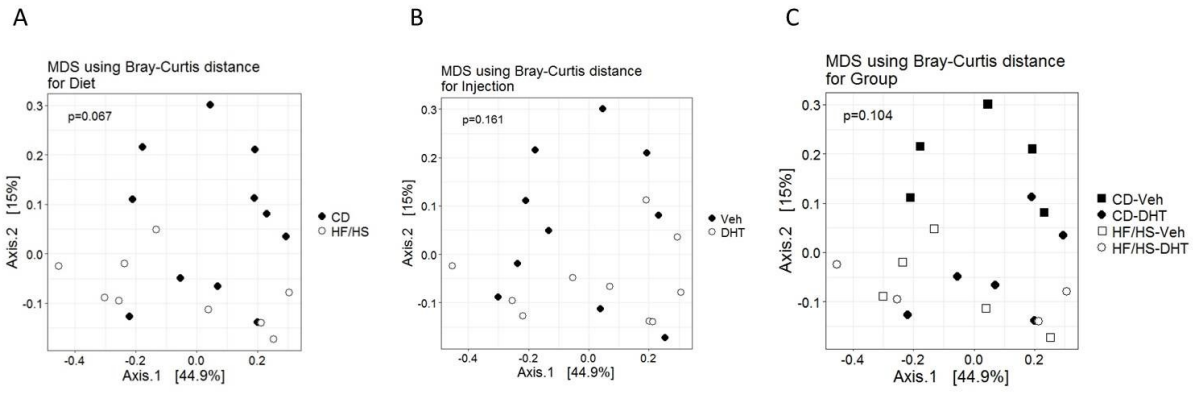


***Figure 26. Beta diversity of fecal samples from dams.*** *Multi-dimensional scaling (MDS) plots of Bray-Curtis distances for the factors diet (A), injection (B), and group (C). Each dot represents the total bacterial community composition of one sample. The amount of variation explained by each MDS coordinate is indicated in square brackets. Groups were compared using Adonis.*

In male offspring, both maternal and offspring diet caused a significant clustering in the MDS plot (p=0.001 for both factors), while DHT exposure had no significant effect (Figure 27). In female offspring, only offspring diet led to a significant clustering, with no significant effect of maternal diet or injection (Figure 28).

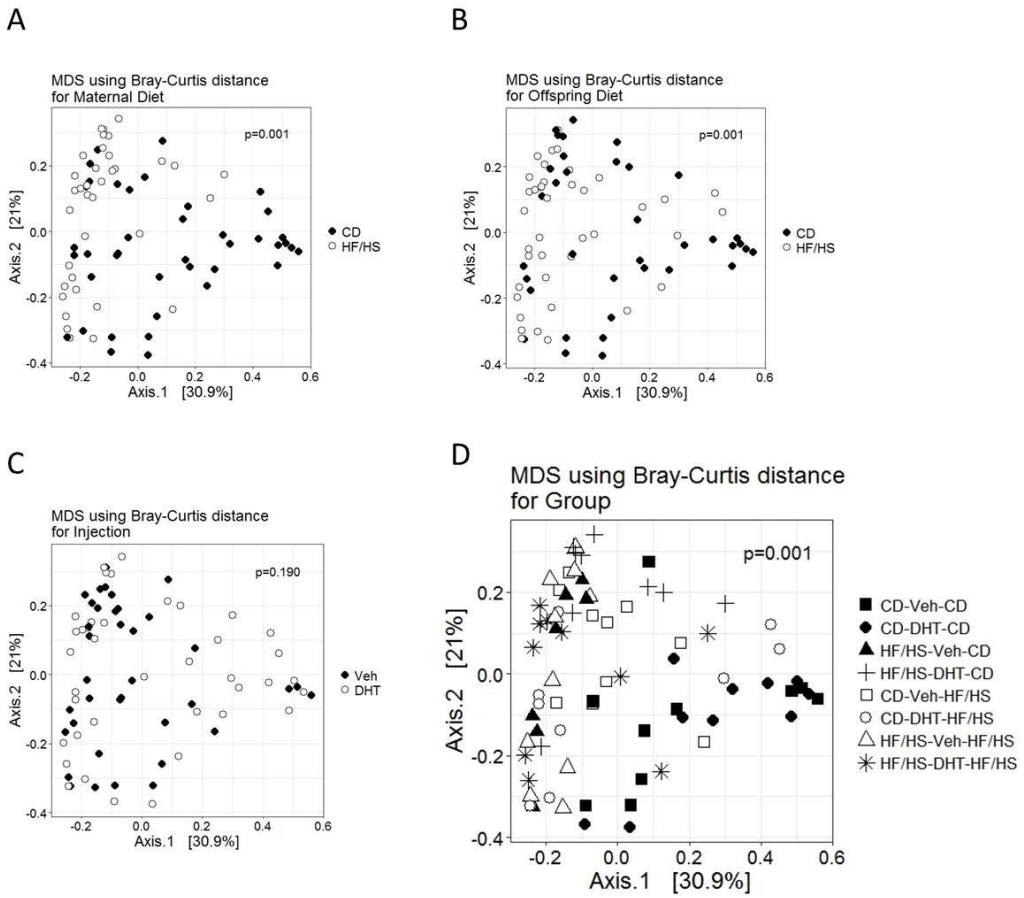


***Figure 27. Beta diversity of fecal samples from male PNA offspring.*** *Multi-dimensional scaling (MDS) plots of Bray-Curtis distances for the factors maternal diet (A), offspring diet (B), maternal injection (C), and group (D). Each dot represents the total bacterial community composition of one sample. The amount of variation explained by each MDS coordinate is indicated in brackets. Groups were compared using Adonis.*

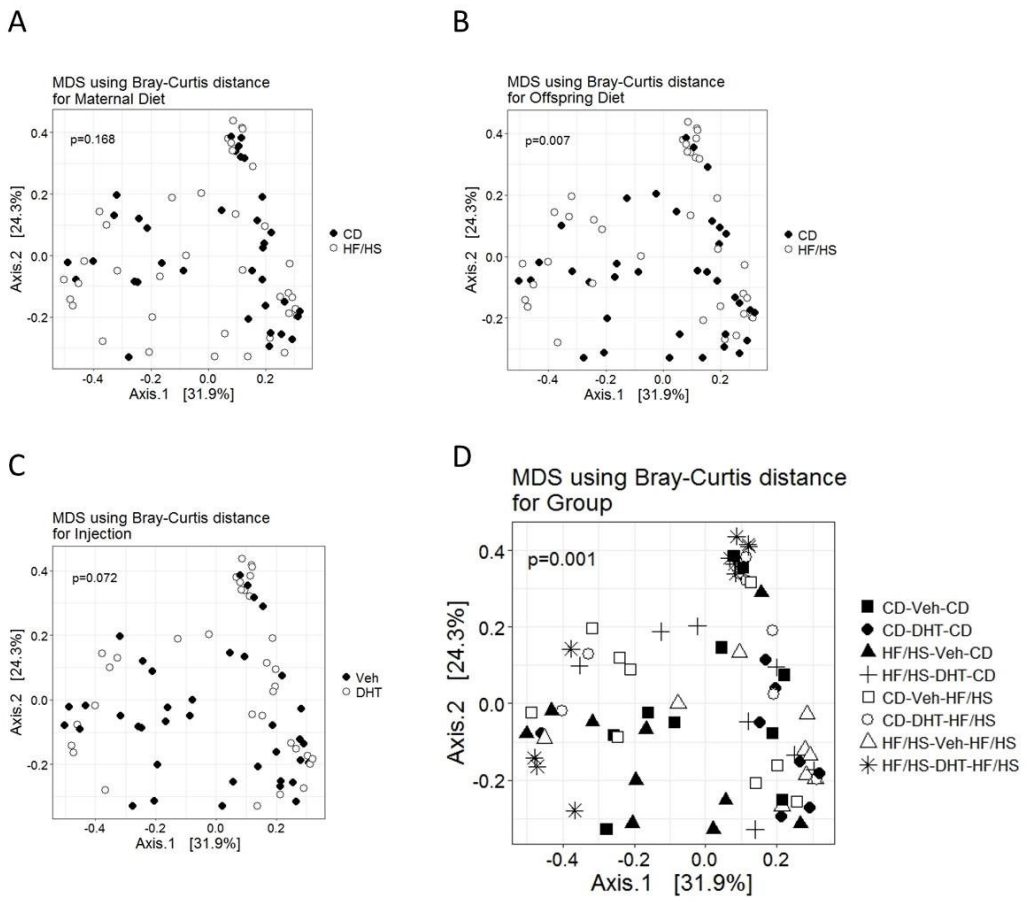


***Figure 28. Beta diversity of fecal samples from female PNA offspring.*** *Multi-dimensional scaling (MDS) plots of Bray-Curtis distances for the factors maternal diet (A), offspring diet (B), maternal injection (C), and group (D). Each dot represents the total bacterial community composition of one sample. The amount of variation explained by each MDs coordinate is indicated in brackets. Groups were compared using Adonis.*

### 3.2.6. Role of sequencing bias and contamination in PNA samples

We included a mock community composed of eight bacterial species to assess classification bias due to PCR and sequencing limitations. Bacteria from the mock community underwent the same DNA extraction and library preparation procedures as samples. We found a strong distortion in the representation of most bacteria from the mock community (Table 12). The genus *Lactobacillus* was overrepresented by almost 5-fold, while *Bacillus*, *Listeria*, *Enterococcus*, and *Staphylococcus* were underrepresented by 8 to 12-fold. The mock community only contained bacteria from the phyla Proteobacteria

and Firmicutes. None of the genera from the mock community were present in samples with a relative abundance greater than 1 %. *Salmonella*, *Listeria*, and *Staphylococcus* were not found in any samples.

***Table 12. Assessment of taxonomic assignment bias due to PCR and sequencing in the PNA mouse study.***

| Genus | Expected RA | Observed RA | FC |
|---|---|---|---|
| #Lactobacillus | 0.10 | 0.47 | 4.9 |
| §Salmonella | 0.12 | 0.20 | 1.7 |
| §Escherichia | 0.13 | 0.21 | 1.6 |
| §Pseudomonadaceae unclass. | 0.15 | 0.04 | -3.3 |
| #Bacillus | 0.12 | 0.01 | -8.5 |
| #Listeria | 0.13 | 0.01 | -9.2 |
| #Enterococcus | 0.13 | 0.01 | -9.7 |
| #Staphylococcus | 0.10 | 0.01 | -11.9 |

*Expected and observed relative abundances of bacterial genera in a mock community. RA, relative abundance; FC, fold change; unclass., unclassified (the lowest classified taxonomic level is shown); spp., species. §Proteobacteria; #Firmicutes.*

The included negative controls showed some evidence of reagent and sample cross-contamination (Table 13). Most genera that were found in NCs were not present in samples, or were present with a much higher read count, suggesting that NCs were likely contaminated by samples and not the contrary. NCs also contained some genera which were found by DESeq2 to be significantly changed due to the studied factors. These genera were *Bilophila, Alloprevotella, Erysipelatoclostridium*, and *Odoribacter*. These results should therefore be interpreted with caution.

***Table 13. Read counts (RC) of bacterial genera which were detected with at least two reads in negative controls (NCs) and their read counts across all samples in the PNA mouse study.***

| | **Total RC in NCs (n=3)** | **RC in samples (n=170)** | |
|---|---|---|---|
| **Genus** | | **median** | **IQR** |
| §Pseudomonadaceae unclass. | 10347 | 0 | 0.0 |
| §Escherichia/Shigella | 2066 | 0 | 2.0 |
| §Undibacterium | 534 | 0 | 0.0 |
| §Massilia | 473 | 0 | 0.0 |
| †Rikenellaceae RC9 | 388 | 3549 | 3561.0 |
| ¶Cellulomonas | 278 | 0 | 0.0 |
| §Acinetobacter | 199 | 0 | 0.0 |
| §Ralstonia | 153 | 0 | 0.0 |
| ‡Akkermansia | 133 | 2735 | 11177.0 |
| ¶Propionibacterium | 105 | 0 | 0.0 |
| †Cloacibacterium | 89 | 0 | 0.0 |
| §Desulfovibrio | 73 | 4938 | 9512.3 |
| #Staphylococcus | 72 | 0 | 0.0 |
| †S24-7 unclass. | 68 | 4989 | 3283.5 |
| †Prevotella 2 | 62 | 0 | 0.0 |
| §Comamonadaceae unclass. | 49 | 0 | 0.0 |
| §Azomonas | 44 | 0 | 0.0 |
| §Comamonas | 28 | 0 | 0.0 |
| #Anaerotruncus | 26 | 147 | 188.0 |
| §Brevundimonas | 19 | 0 | 0.0 |
| §Sphingobium | 19 | 0 | 0.0 |
| §Pseudomonas | 17 | 0 | 0.0 |
| #Allobaculum | 16 | 304 | 654.5 |
| §Bilophila | 16 | 8 | 26.8 |
| ¶Microbacteriaceae unclass | 15 | 0 | 0.0 |
| †Alloprevotella | 15 | 0 | 283.5 |
| †Parabacteroides | 11 | 147 | 361.5 |
| §Methylobacterium | 10 | 0 | 0.0 |
| #Lachnospiraceae unclass. | 9 | 914 | 1346.3 |
| §Defluviimonas | 9 | 0 | 0.0 |
| #Cohnella | 8 | 0 | 0.0 |
| †Alistipes | 7 | 1080 | 950.8 |
| #Erysipelatoclostridium | 6 | 14 | 39.0 |
| \|\|Acidobacteria unclass. | 5 | 0 | 0.0 |
| ¶Pseudonocardia | 5 | 0 | 0.0 |
| †Odoribacter | 2 | 3 | 526.5 |
| §Sphingomonas | 2 | 0 | 0.0 |
| §Gammaproteobacteria unclass. | 2 | 0 | 0.0 |

*unclass., unclassified (the lowest classified taxonomic level is shown). §Proteobacteria; †Bacteroidetes; #Firmicutes; ¶Actinobacteria; ‡Verrucomicrobia ||Acidobacteria.*

### 3.2.7. FMT experiment

To investigate whether the changes we observed in F1 female mice due to DHT and HF/HS-diet exposure were mediated by the gut microbiome, healthy wild-type (WT) mice were depleted of their gut microbiome via an orally administered antibiotic mix. Following two weeks of daily antibiotic treatment, mice received FMT via oral gavage of feces from female donors belonging to four of the F1 treatment groups: CD-Veh-CD, CD-DHT-CD, HF/HS-Veh-HF/HS, HF/HS-DHT-HF/HS. To simplify, since maternal and offspring diet in the donors were the same, these groups will be referred to as CD-Veh, CD-DHT, HF/HS-Veh, and HF/HS-DHT. One control group, named No FMT, was included. This group did not receive antibiotics or FMT, but instead was gavaged with sterile water/PBS.

Body weight and food intake of FMT recipient and control mice are shown in Figure 29. Antibiotic treatment was tolerated well and did not cause a drop in body weight or food intake. Body weight, weight gain, and food intake remained constant between all groups with one exception. Donor DHT exposure caused increased body weight gain in the HF/HS groups at day 53 (main effect of donor maternal injection, $p<0.05$) (Figure 29B). The small effect size (partial Eta-squared=0.09) indicates that this was most likely a spurious result.

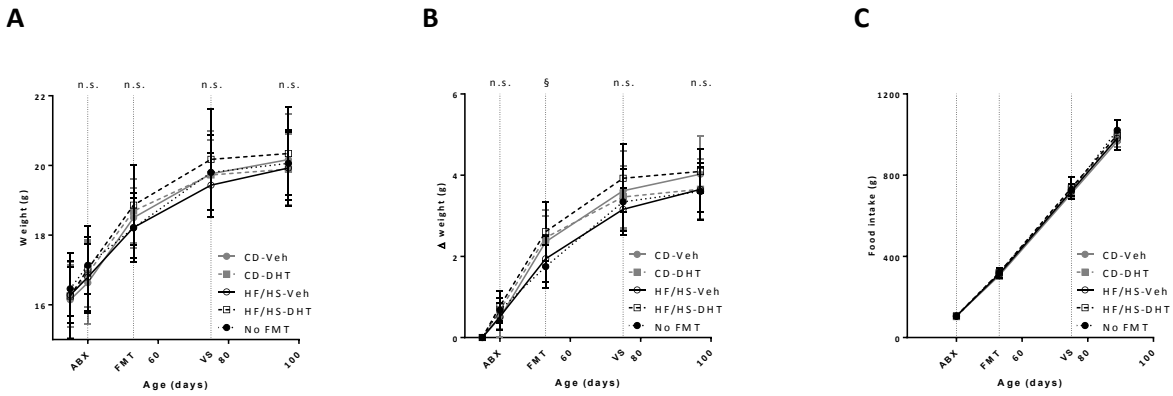


***Figure 29. Body weight development and food intake in FMT recipients.*** *Body weight (A), body weight change from baseline (B), and cumulative food intake (C). Mean and standard deviation (SD) are plotted. ABX, start of antibiotic treatment; FMT, start of fecal microbiome transplantation; VS, start of vaginal smears. Control animals received sterile water/PBS instead of ABX/FMT. §p<0.05 main effect for donor maternal injection. Groups were compared using 2-way ANOVA. N=12 mice per group for A and N=2 cages per group for C.*

We investigated whether FMT could transfer the reproductive dysfunction and anxiety-like behavior observed in female PNA offspring. There was no significant difference in the percentage of time spent in proestrus or diestrus or in the number of proestrus to estrus transitions in FMT recipients (Figure 30).

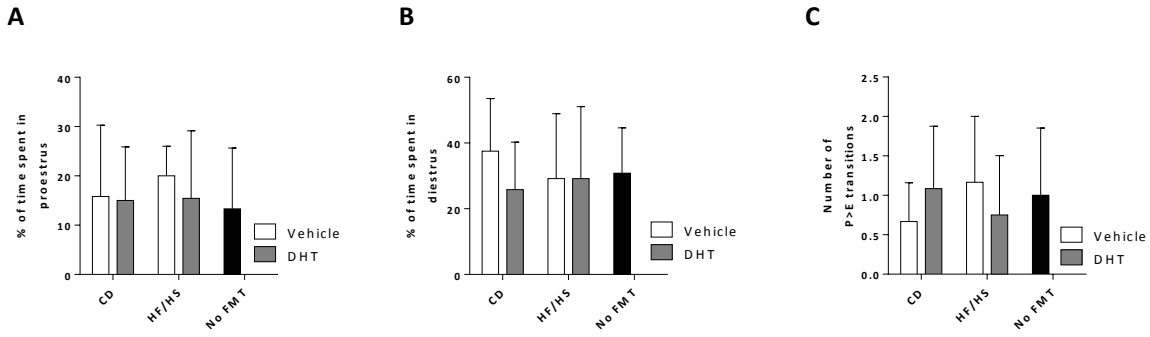


***Figure 30. Estrous cyclicity in FMT recipients.*** *The percent of time spent in proestrus (A), diestrus (B), and the number of proestrus to diestrus transitions (C) over ten consecutive days. Mean and standard deviation (SD) are plotted. Groups were compared using 2-way ANOVA. N=12 mice per group.*

There was no significant difference between the groups in the time spent in the open arms or the total distance traveled in the EPM (Figure 31). In the OF, mice that received FMT from HFD-DHT donors spent significantly more time in the center than the other three groups of FMT recipients (main effect of donor maternal injection, $p<0.05$), indicating reduced anxiety-like behavior in these mice (Figure 31C). There was no difference in the total distance traveled in the OF.

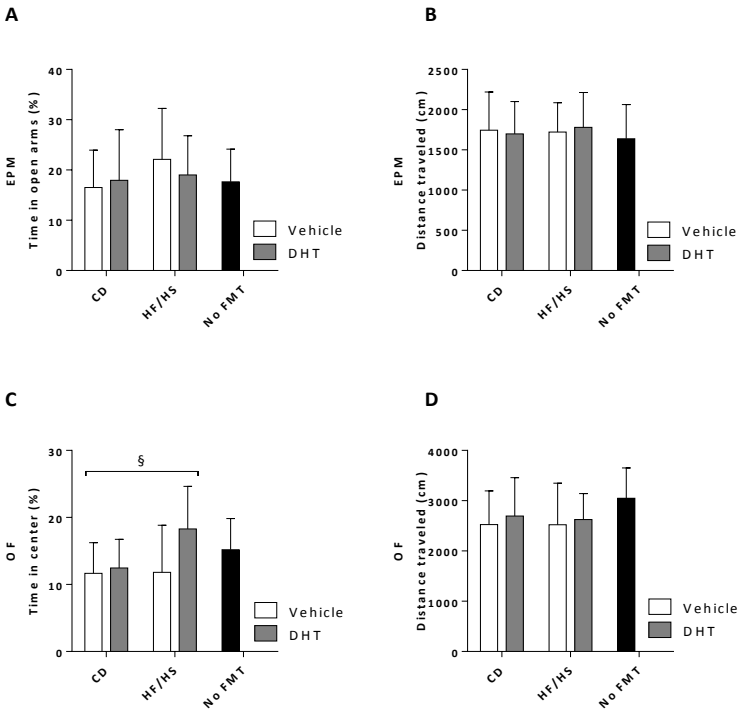


***Figure 31. Anxiety-like behavior in FMT recipients.*** *Time spent in the open arms of the elevated plus maze (A) and open field (C) and the total distance traveled (B, D). Mean and SD are shown. §p<0.05 main effect of donor maternal injection. Groups were compared using 2-way ANOVA. N=12 mice per group.*

### 3.2.8. Fecal microbiome analysis of FMT recipients

The most prevalent phyla and genera in fecal material from FMT recipients and in FMT inoculates are summarized in Tables 14 and 15. The most abundant phylum in FMT recipient samples was Bacteroidetes, followed by Firmicutes. The most abundant genus was from the Bacteroidales S24-7 group, followed by Lachnospiraceae *NK4A136* and *Desulfovibrio*. The dominant phyla and genera were the same as those found in the PNA dams and offspring, but there were some differences in their relative abundance across samples.

***Table 14. Relative abundances of the top (>1 %) bacterial genera and phyla in cecal samples from FMT recipients.***

| | % of total bacteria by group (n=12 animals per group) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **CD-Veh** | | **CD-DHT** | | **HF/HS-Veh** | | **HF/HS-DHT** | | **No FMT** | |
| **Top genera FMT recipients** | **med.** | **IQR** | **med.** | **IQR** | **med.** | **IQR** | **med.** | **IQR** | **med.** | **IQR** |
| †S24-7 unclass. | 59.2 | 12.48 | 60.2 | 6.92 | 36.6 | 27.10 | 53.6 | 5.55 | 34.3 | 11.43 |
| #Lachnospiraceae NK4A136 | 13.8 | 9.38 | 9.7 | 6.37 | 15.2 | 5.45 | 11.7 | 9.19 | 9.7 | 8.93 |
| §Desulfovibrio | 4.1 | 1.37 | 8.4 | 1.77 | 7.9 | 3.43 | 11.0 | 3.30 | 8.6 | 3.29 |
| †Bacteroides | 3.8 | 1.64 | 4.1 | 3.56 | 6.5 | 10.47 | 5.1 | 4.73 | 10.3 | 6.45 |
| #Lachnospiraceae unclass. | 2.4 | 2.10 | 2.1 | 0.87 | 5.5 | 7.18 | 2.8 | 1.30 | 8.0 | 8.20 |
| ‡Ackermanns | 3.8 | 5.59 | 3.8 | 6.19 | 0.9 | 2.82 | 1.1 | 1.94 | 0.0 | 0.00 |
| #Clostridiales vadinBB60 | 2.4 | 1.25 | 0.5 | 0.34 | 1.1 | 1.73 | 1.4 | 0.23 | 1.9 | 0.85 |
| †Rikenellaceae RC9 | 1.4 | 2.75 | 0.3 | 0.59 | 1.2 | 2.02 | 0.4 | 0.44 | 2.5 | 2.09 |
| §Parasutterella | 0.9 | 0.51 | 0.6 | 0.77 | 0.8 | 1.76 | 1.8 | 0.93 | 1.5 | 1.53 |
| #Ruminococcaceae unclass. | 0.9 | 0.55 | 0.7 | 0.71 | 1.4 | 1.23 | 1.0 | 0.36 | 2.1 | 1.83 |
| †Alistipes | 0.9 | 0.56 | 0.5 | 0.28 | 0.5 | 0.17 | 0.7 | 0.38 | 3.1 | 1.11 |
| †Alloprevotella | 0.8 | 0.32 | 0.5 | 0.25 | 0.5 | 0.59 | 1.0 | 0.56 | 1.3 | 3.20 |
| **Top phyla FMT recipients** | **med.** | **IQR** | **med.** | **IQR** | **med.** | **IQR** | **med.** | **IQR** | **med.** | **IQR** |
| †Bacteroidetes | 66.4 | 7.58 | 67.9 | 8.20 | 54.0 | 21.19 | 61.7 | 6.22 | 55.4 | 7.07 |
| #Firmicutes | 23.5 | 5.59 | 16.0 | 4.26 | 26.3 | 18.72 | 19.7 | 7.93 | 32.5 | 11.98 |
| §Proteobacteria | 5.4 | 1.54 | 9.2 | 2.09 | 10.7 | 5.34 | 13.3 | 3.67 | 12.7 | 3.44 |
| ‡Verrucomicrobia | 3.8 | 5.59 | 3.8 | 6.19 | 0.9 | 2.82 | 1.1 | 1.94 | 0.0 | 0.00 |

*Unclass., unclassified (the lowest classified taxonomic level is shown). #Firmicutes; †Bacteroidetes; §Proteobacteria; ‡Verrucomicrobia.*

FMT recipient samples did not contain the *Akkermansia* overgrowth that was found in the F0 dams. FMT recipient samples contained a markedly lower proportion of Rikenellaceae *RC9* and a higher proportion of Lachnospiraceae *NK4A136* than PNA samples.

FMT inoculates contained a greater proportion of bacteria from the phylum Proteobacteria and a lower proportion of Bacteroidetes than FMT recipient and PNA samples (Table 15). The dominant genus in FMT inoculates was *Desulfovibrio*, followed by *Akkermansia*. Though the proportion of Akkermansia was not as high as in F0 dams, there may have been some carry-over of this bacterial bloom from the PNA experiment. The sterile PBS/glycerol solution used to inoculate no-FMT control mice was largely devoid of bacteria and contained almost only bacteria from the phylum Proteobacteria, which we assume to be reagent contamination.

***Table 15. Relative abundances of the top (>1 %) bacterial genera and phyla in FMT inoculates.***

| **% of total bacteria by group (n=12 animals per group)** | | | | | |
|---|---|---|---|---|---|
| | **CD-Veh** | **CD-DHT** | **HF/HS-Veh** | **HF/HS-DHT** | **No FMT** |
| **Top genera FMT inoculate** | | | | | |
| §Desulfovibrio | 41.8 | 40.9 | 36.5 | 14.0 | 0.0 |
| ‡Akkermansia | 11.0 | 8.8 | 18.9 | 22.9 | 0.0 |
| †S24-7 unclass. | 9.0 | 10.6 | 12.3 | 9.7 | 0.0 |
| #Lachnospiraceae unclass. | 8.1 | 6.7 | 6.7 | 9.9 | 0.0 |
| †Rikenellaceae RC9 | 3.9 | 5.1 | 2.6 | 8.6 | 0.0 |
| #Lachnospiraceae NK4A136 | 4.4 | 6.8 | 3.2 | 5.2 | 0.0 |
| #Ruminococcaceae unclass. | 3.5 | 3.1 | 3.8 | 2.8 | 0.0 |
| †Alistipes | 3.0 | 3.2 | 3.7 | 1.9 | 0.0 |
| †Bacteroides | 1.9 | 1.0 | 1.5 | 6.1 | 0.0 |
| #Clostridiales vadinBB60 | 3.2 | 2.5 | 1.4 | 3.0 | 0.0 |
| #Ruminiclostridium | 1.6 | 1.5 | 2.0 | 1.6 | 0.0 |
| #Anaerotruncus | 1.7 | 1.1 | 1.2 | 1.8 | 0.0 |
| †Parabacteroides | 0.4 | 0.3 | 1.6 | 2.7 | 0.0 |
| #Lachnoclostridium | 1.0 | 1.1 | 0.8 | 1.7 | 0.0 |
| **Top phyla FMT inoculate** | | | | | |
| §Proteobacteria | 42.8 | 41.5 | 36.9 | 16.2 | 95.0 |
| #Firmicutes | 27.3 | 27.5 | 22.2 | 30.0 | 0.0 |
| †Bacteroidetes | 18.7 | 22.0 | 22.1 | 31.0 | 0.0 |
| ‡Verrucomicrobia | 11.0 | 8.8 | 18.9 | 22.9 | 0.0 |

*Unclass., unclassified (the lowest classified taxonomic level is shown). #Firmicutes; †Bacteroidetes; §Proteobacteria; ‡Verrucomicrobia.*

DESeq2 analysis identified several differentially abundant bacterial genera in response to donor diet and DHT exposure (Figure 32). Donor HF/HS diet led to a lower relative abundance of bacteria from the families Ruminococcaceae and Lachnospiraceae and the genus *Rikenella* (Figure 32A). These same groups were also found to be less abundant in female PNA offspring fed a HF/HS diet (Figure 24E). Donor DHT exposure led to a lower relative abundance of the genera *Thalassospira* and *Rikenella*, which were also less abundant in the corresponding PNA mice (Figures 32B and 24F). That we could replicate these results in FMT recipients speaks for the effectiveness of the FMT procedure and strengthens the hypothesis that these genera may in fact be regulated by a HF/HS diet and DHT exposure.

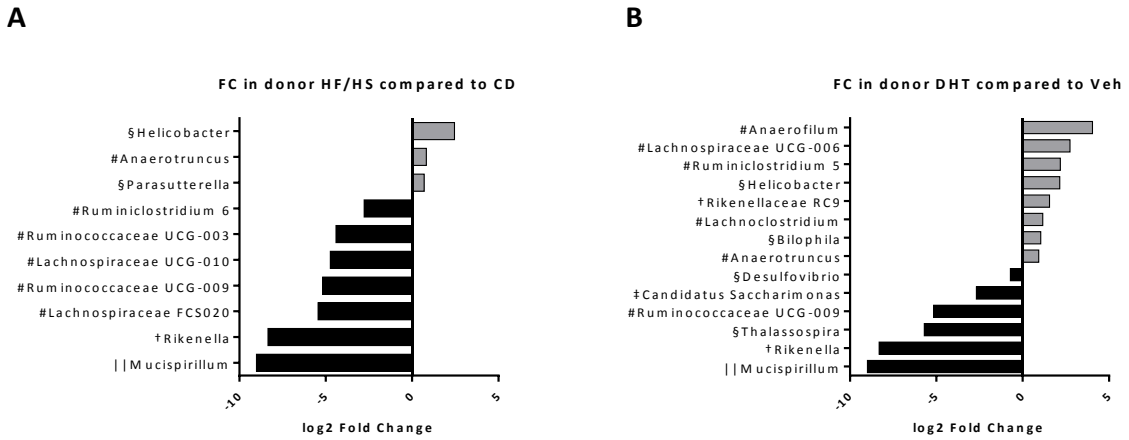


***Figure 32. Differentially abundant genera in FMT recipient samples identified by DESeq2.*** *Log2 fold change (FC) of relative abundance for genera which were significantly changed due to donor diet (A) and DHT exposure (B). P<0.05 for all after Benjamini-Hochberg false discovery rate correction. #Firmicutes; †Bacteroidetes; §Proteobacteria; ||Deferribacteres; ‡Saccharibacteria.*

### 3.2.9. Alpha diversity in FMT recipients

The number of observed RSVs in FMT recipients was determined by an interaction between donor diet and DHT exposure (donor diet*donor maternal injection, $p<0.05$), with the highest diversity observed in the HF/HS-Veh group (Figure 33A). The Shannon index was influenced independently by both donor diet and DHT exposure (main effect of donor diet and donor maternal injection, $p<0.05$) (Figure 33B). Donor DHT exposure led to a decreased Shannon index, while donor HF/HS diet led to an increased Shannon index.

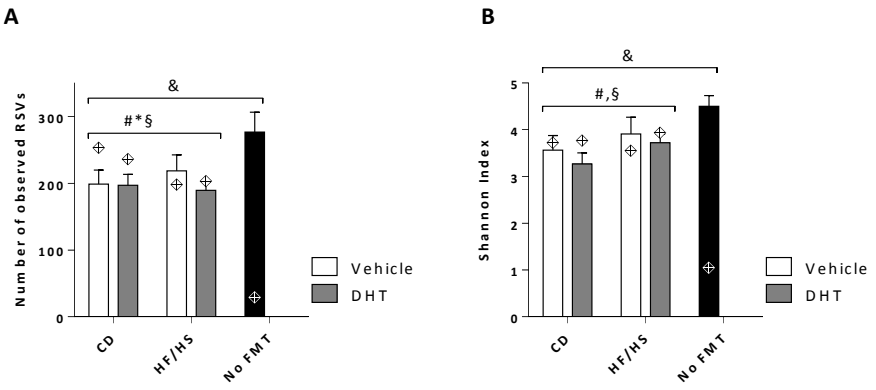


***Figure 33. Alpha diversity of cecal samples from FMT recipients and inoculates.*** *The number of observed RSVs (A) and Shannon index (B). Diamonds show the diversity of the respective FMT inoculate. RSV, ribosomal sequence variant. #p<0.05 main effect of donor diet; §p<0.05 main effect of donor maternal injection; &p<0.05 for all groups compared to No FMT. * denotes interactions between factors. Mean and SD are shown. N=12 animals per group.*

Both the number of observed RSVs and the Shannon index were significantly reduced in all FMT recipients. This may be a result of the antibiotic treatment, but might also be due to the fact that the FMT inoculate, which had been diluted to prepare it for oral gavage, was less diverse than the undiluted cecal sample of the no-FMT group. FMT recipients showed a very similar diversity as their respective inoculates, indicating that the FMT was successful in transferring the alpha diversity attribute.

### 3.2.10. Beta diversity in FMT recipients

In beta diversity analyses, both donor diet and donor DHT exposure significantly affected clustering in the MDS plot based on Bray-Curtis distance matrices (Figure 34). All mouse fecal samples clustered separately from all FMT inoculate samples.

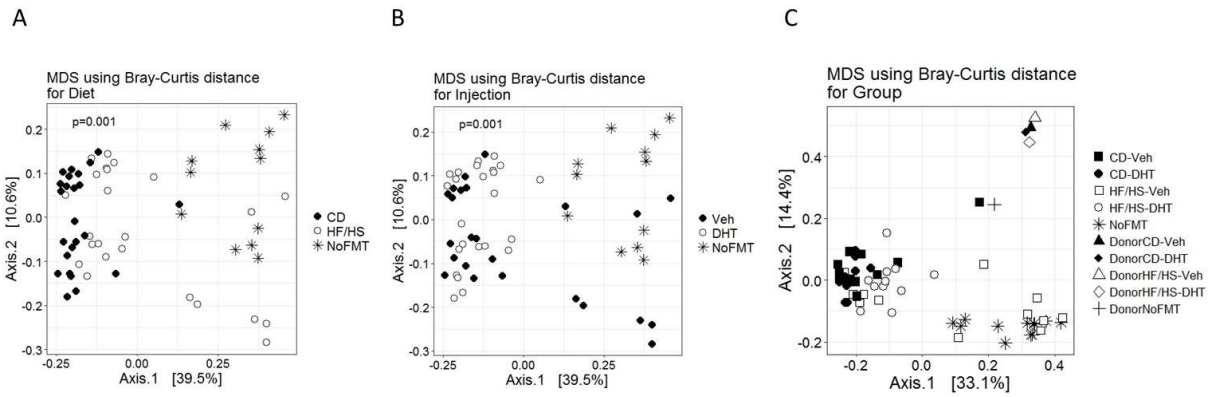


***Figure 34. Beta diversity of cecal samples from FMT recipients and inoculates.*** *Multi-dimensional scaling (MDS) plots of Bray-Curtis distances for the factors donor diet (A), donor maternal injection (B), and group (C). Each dot represents the total bacterial community composition of one sample. The amount of variation explained by each MDS coordinate is indicated in brackets. Groups were compared using Adonis.*

### 3.2.11. Efficiency of the FMT protocol

We assessed the efficiency of our FMT protocol based on the proportion of shared genera (Figure 35) and pairwise Bray-Curtis dissimilarity estimates (Figure 36). We found that approximately 90 % of genera in the inoculate were transferred to at least one recipient, with only few genera that were present in the inoculate, but not in any recipient (Figure 35A). When including only genera that were shared between the inoculate and all recipients, we found a lower percentage of transferred genera (44-60 %) (Figure 35B). However, these genera made up 70-90 % of genera that were found in all recipients, with only few genera present in all recipients that did not come from the inoculate. To summarize, while only about half of the genera in the inoculate were transferred to all recipients, these genera made up over 70 % of "core" genera that were present in all

recipients. Of the remaining "non-core" genera, mice collectively adopted about 90 % of the original inoculate, but also acquired individual-specific bacteria, which were not derived from the inoculate and not found in all animals from the same group.

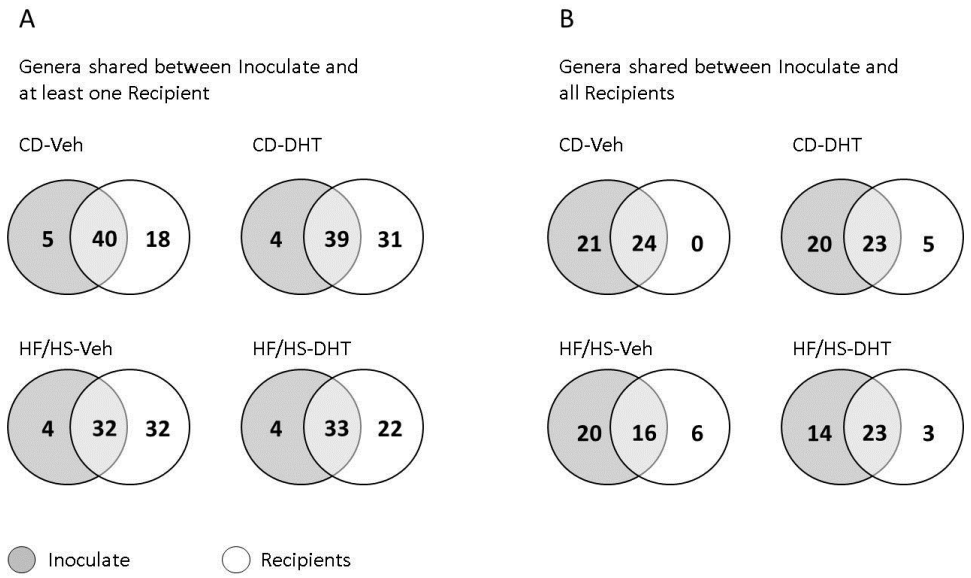


***Figure 35. Efficiency of the FMT based on the number of genera shared between FMT inoculate and recipient samples.*** *A. Genera which were shared between FMT inoculate and at least one recipient sample. B. Genera which were shared between FMT inoculate and all recipient samples.*

When comparing Bray-Curtis dissimilarity measures, mice within the same treatment group were more similar to each other than to all other mice (Figure 36A). Mice that received FMT were more different from no-FMT control mice than the overall between-group difference, indicating that either the antibiotic pre-treatment or the composition of the inoculate significantly impacted microbial communities (Figure 36A). Mice receiving FMT from DHT-exposed donors were more similar to each other than mice receiving FMT from vehicle-exposed donors (Figure 36B). When comparing the four FMT recipient groups, mice receiving FMT from HF/HS-DHT exposed donors were significantly more similar to each other than the other three groups, indicating that this treatment, combining both exposures, might lead to a more uniform microbiome, which is preserved to a greater degree after FMT (Figure 36C).

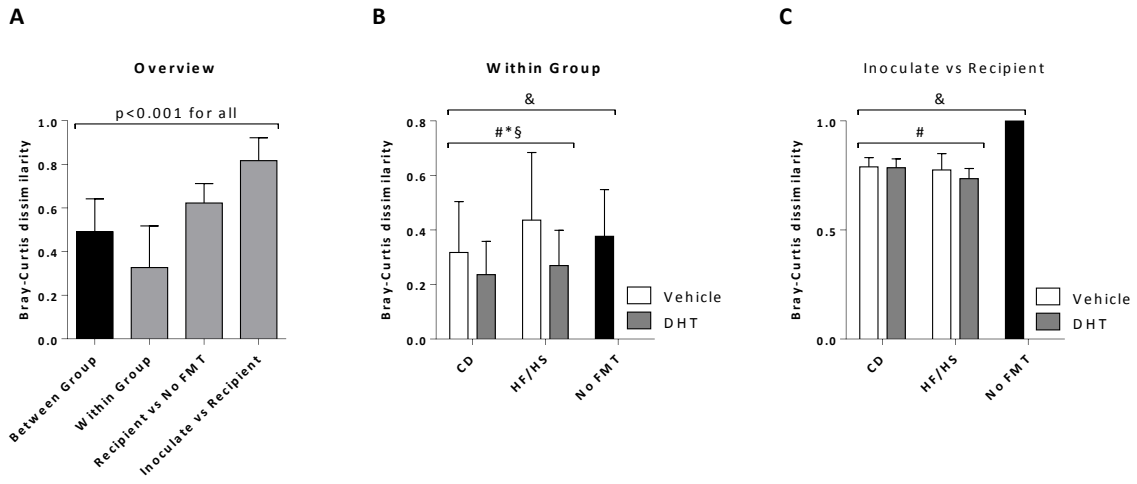


***Figure 36. Bray-Curtis dissimilarities of samples from FMT recipients, FMT inoculate, and no-FMT controls.*** *A. Samples from all groups were pooled together to evaluate dissimilarities of microbiome profiles between groups, within groups, between FMT recipients and No FMT controls, and between FMT recipients and the inoculate. B. Detailed analysis of within-group dissimilarities in FMT recipients compared to No FMT controls. C. Detailed analysis of dissimilarities between FMT groups and the inoculate. #p<0.05 main effect of donor diet; §p<0.05 main effect of donor maternal injection; &p<0.05 for all groups compared to No FMT. * denotes interactions between factors. Mean and SD are shown. N=12 animals per group.*

### 3.2.12. Role of contamination in FMT samples

In the included negative controls (NCs), we found evidence for reagent contamination as well as some cross-contamination from samples (Table 16). Bacteria detected in the NCs were almost totally absent from samples and did not overlap with the significant genera identified by DESeq2. We therefore conclude that contamination was not a relevant influence in this experiment.

***Table 16. Read counts (RC) of bacterial genera which were detected with at least two reads in negative controls (NCs) and their read counts across all samples in the FMT experiment.***

| | **Total RC in NCs (n=2)** | **RC in samples (n=60)** | |
|---|---|---|---|
| **Genus** | | **median** | **IQR** |
| §Pseudomonadaceae unclass. | 83959 | 5 | 7.0 |
| §Escherichia/Shigella | 15812 | 0 | 0.0 |
| ¶Cellulomonas | 4311 | 0 | 0.0 |
| §Undibacterium | 1445 | 0 | 0.0 |
| §Massilia | 1109 | 0 | 0.0 |
| §Sphingobacteriales env.OPS 17 | 892 | 0 | 0.0 |
| ¶Propionibacterium | 604 | 0 | 0.0 |
| §Ralstonia | 334 | 0 | 0.0 |
| §Sphingobium | 292 | 0 | 0.0 |
| §Azomonas | 141 | 0 | 0.0 |
| \|\|Planctomycetaceae unclass. | 137 | 0 | 0.0 |
| §Acinetobacter | 60 | 0 | 0.0 |
| §Phyllobacterium | 41 | 0 | 0.0 |
| †S24-7 unclass. | 33 | 23201 | 8123.3 |
| #Cohnella | 18 | 0 | 0.0 |
| ‡Saccharibacteria unclass. | 17 | 0 | 0.0 |
| ¶Actinobacteria unclass. | 16 | 0 | 0.0 |
| §Pelomonas | 12 | 0 | 0.0 |
| §Methylophilaceae unclass. | 10 | 0 | 0.0 |
| †Cloacibacterium | 9 | 0 | 0.0 |
| ¶Microbacteriaceae unclass | 7 | 0 | 0.0 |
| §Methylobacterium | 7 | 0 | 0.0 |
| §Phyllobacteriaceae unclass. | 7 | 0 | 0.0 |
| §Bradyrhizobiaceae unclass. | 6 | 0 | 0.0 |
| *SHA-109 unclass. | 5 | 0 | 0.0 |
| ¶Williamsia | 4 | 0 | 0.0 |

*unclass., unclassified (the lowest classified taxonomic level is shown). §Proteobacteria; †Bacteroidetes; #Firmicutes; ¶Actinobacteria; ||Planctomycetes; ‡Saccharibacteria; *SHA-109.*

# 4. Discussion

## 4.1. Part 1: Human study

The pilot study presented in this work is the first published description of the stool and saliva microbiome in women with PCOS (Lindheim et al., 2016, 2017). At the start of our investigations, the DOGMA hypothesis had been published and served as the basis for our human pilot study (Tremellen and Pearce, 2012). Other reports of bacterial involvement in PCOS at the time were limited to saliva in the context of gingivitis and did not use next-generation sequencing (Akcali et al., 2014). During the course of our studies, two groups published data on the gut microbiome in rodent models of PCOS (Guo et al., 2016; Kelley et al., 2016). Shortly after the publication of our human study, Liu *et al.* published the only other descriptive study of the gut microbiome in women with PCOS (Liu et al., 2017).

Our main finding was a reduction in bacterial richness and diversity in stool samples from women with PCOS compared to control women, which was accompanied by a significant clustering of samples from the PCOS and control groups in unweighted UniFrac analysis. The diversity loss was approximately 15 % and was significant at an OTU relative abundance cut-off of 0.01 %, but not 0.1 %, indicating that rare species contributed strongly to this result. Indeed, we found differences in relative abundance in taxa contributing <1 % of total sequences. The phylum Tenericutes, the Tenericutes order ML615J-28, and the Bacteroidales family S24-7 had a significantly lower relative abundance in the PCOS group than in controls. LEfSe analysis found these taxa to be discriminant for control samples, while several genera from the Firmicutes and Actinobacteria phyla were discriminant for PCOS.

Our results agree with the only other published study on the gut microbiome in women with PCOS. This study was conducted in a Chinese cohort of 33 women with PCOS and 15 control women, sub-divided into obese and lean women using a BMI cut-off of 25 (Liu et al., 2017). The authors found a reduction in fecal bacterial richness in women with PCOS, which was exacerbated by obesity. The authors also found a separation of samples due to both BMI and PCOS in the beta diversity plot, as well as changes in several bacterial taxa. Most of these taxa did not overlap with our findings; however, both Liu *et al.* and we report a reduction in relative abundance of bacteria from the families Clostridiales and

Ruminococcaceae in women with PCOS compared to controls. Contrary to Liu *et al.*, we found no correlation of BMI with gut microbiome richness or taxonomic profiles in either the PCOS or control group. The sample size of our study may have been too small to detect a BMI effect. On the other hand, the Chinese study's result might have arisen due to another PCOS-associated component which was more prevalent in the obese PCOS group. Since both studies were limited by a small sample size, they are susceptible to spurious results due to factors other than those investigated. The overlap of our findings related to bacterial richness is nonetheless encouraging, particularly in light of a recent study reporting a similar reduction in richness in a letrozole-induced mouse model of PCOS (Kelley et al., 2016). In this study, letrozole-treated animals showed a decrease in species from the Bacteroidales family S24-7, which we also found to be less abundant in our cohort of women with PCOS.

Previous studies have reported a loss of bacterial richness in individuals with obesity and an inverse association between richness and adiposity, insulin resistance, hyperlipidemia, and circulating inflammatory markers (Turnbaugh et al., 2006; Le Chatelier et al., 2013). In type 2 diabetes, studies have reported both a decrease and no change in fecal bacterial richness (Qin et al., 2012; Forslund et al., 2015; Org et al., 2017).

While initial studies reported a diversity reduction of up to 30 % and an increase in the Firmicutes to Bacteroidetes ratio in obese individuals, more recent meta-analyses concluded that microbiome differences are more subtle than previously thought (Ley et al., 2005; P J Turnbaugh et al., 2009; Walters, Xu and Knight, 2014; Sze and Schloss, 2016). According to these meta-analyses, the richness decrease in obesity averages at around 7 %. Evaluating our results in the context of these findings, the 15 % loss of richness we found in the PCOS group is substantial, consistent with other PCOS studies, and likely not the sole result of a higher BMI of some of the women in the PCOS group.

Changes in the F:B ratio and the majority of differentially abundant taxa due to obesity and/or diabetes have been inconsistent across studies (Qin et al., 2012; Walters, Xu and Knight, 2014; Forslund et al., 2015; Sze and Schloss, 2016; Org et al., 2017). Meta-analyses concluded that the small effect of obesity on the gut microbiome is dwarfed by the variability between individuals and studies (Walters, Xu and Knight, 2014; Sze and Schloss,

2016). Only three bacterial taxa were persistently associated with obesity after multiple testing correction, one of which, the genus *Megasphaera*, was a discriminant genus for PCOS in our study, as identified by LEfSe analysis (Walters, Xu and Knight, 2014).

Of the taxa that showed a significant difference in relative abundance in our study, the phylum Tenericutes and the order ML615J-28 have been associated with a healthy metabolic phenotype (Goodrich, Waters, et al., 2014; Lim et al., 2016). The Bacteroidales family S24-7 decreased in relative abundance in diet-induced obese mice, an effect which was reversed by exercise (Evans et al., 2014). At least two studies have identified genera from the phylum Tenericutes, including those identified in our study, to have a heritable component (Goodrich, Waters, et al., 2014; Lim et al., 2016). As PCOS is known to have a heritable component, we can speculate that there may be a link between the genetic factors predisposing to PCOS and to an unfavorable gut microbiome, initiating a feed-forward cycle that drives the clinical phenotype (Vink et al., 2006).

Richness and other diversity measures are useful tools to investigate the gut microbiome as they are quantitative, informative about the overall health of the microbial community, and much less variable due to study center or sample collection, preparation, and sequencing procedures (Goodrich, Di Rienzi, et al., 2014). On the other hand, identifying specific species or groups of bacteria associated with disease is a challenge. To overcome methodological and biological confounders, large changes need to consistently occur in a great proportion of the affected individuals, as in inflammatory bowel disease (Walters, Xu and Knight, 2014). The task is additionally complicated by the fact that little is known about many genera, species, or even phyla identified by next-generation sequencing, as most have not been cultured and studied in detail (Morgan and Huttenhower, 2012).

In PCOS and in obesity without PCOS, inter-individual variation and environmental influences such as diet and geography appear to be much greater than disease-associated factors in shaping the gut bacterial community, drowning out small changes that might be observed in the affected individuals (Costello et al., 2009; Caporaso et al., 2011; Chen et al., 2016). This is assuming that such a signature exists, which at present has not been convincingly shown. The involvement of the gut microbiome in PCOS might occur in the context of general dysbiosis and loss of diversity, with no specific reproducible bacterial

signature. The significant taxa identified in our study represented a very small percentage of total bacteria, with a mean relative abundance of 0.3 % for Tenericutes and 1.7 % for S24-7 bacteria. While single low-abundance species can have a profound impact on the host, such as segmented filamentous bacteria in early development of the immune system, we must interpret our results with caution until they have been replicated in larger patient cohorts (Yin et al., 2013; Farkas et al., 2015).

Despite difficulties in detecting and qualifying microbiome changes in metabolic disease, fecal transplantation studies have shown that the gut microbiome is fundamentally altered in these conditions and is able to transfer disease features to previously healthy organisms (commonly germ-free mice) (Turnbaugh et al., 2006). A recent study in a letrozole-induced rat model of PCOS could improve hyperandrogenemia, estrous cyclicity, and even ovarian morphology through fecal transplantation from healthy rats and through the administration of a single *Lactobacillus* strain (Guo et al., 2016). The failure of past studies to report consistent results may be due to limitations of the methodological approach and the tools used. As next-generation sequencing methods continue to become more sophisticated and affordable, many of these obstacles will be eliminated.

We hypothesized that the gut microbiome might exert its effect in PCOS through a modulation of the gut barrier, allowing bacterial endotoxin to enter the circulation and drive inflammation, insulin resistance, hyperinsulinemia, and androgen hypersecretion. The gold-standard method to measure gut permeability in humans is an oral lactulose-mannitol test (Sapone et al., 2006). It is a shortcoming of our study that we did not perform functional testing, but rather measured surrogate markers. For a first assessment in the context of this pilot study, this was a legitimate approach, as serum zonulin, has been described to correlate well with the lactulose:mannitol ratio (Sapone et al., 2006). We did not rely on zonulin alone, but measured a panel of markers related to gut permeability both in serum and stool.

We detected an increase in serum zonulin in women with PCOS, indicating increased intestinal permeability. Increased serum zonulin levels have been reported in women with PCOS and have been shown to correlate with BMI and HOMA-IR (Zak-Golab et al., 2013; Zhang et al., 2015). Of the remaining gut barrier parameters, only DAO was

significantly higher in women with PCOS than in controls. DAO is produced in intestinal epithelial cells and serum levels rise upon gut barrier damage (Wang et al., 2015; Yu et al., 2016). Our findings suggest that the gut barrier may be impaired in PCOS, but not to the degree that we could detect increased circulating endotoxin. Gut barrier dysfunction and endotoxemia may be present in some PCOS phenotypes, such as those with obesity and insulin resistance, but absent in others. Since our small sample size did not allow us to stratify for these criteria we were unable to further test this hypothesis. In a study of 15 obese and 13 lean subjects, obese individuals showed an altered gut microbiome profile with an increased relative abundance of Proteobacteria and increased fecal calprotectin levels, but gut permeability was unchanged compared to controls (Verdam et al., 2013). We hypothesize that gut dysbiosis and inflammation may precede gut barrier dysfunction in parallel with the spectrum of PCOS severity.

Our investigation of inflammatory markers showed higher blood lymphocyte counts in women with PCOS, but no significant difference in circulating hsCRP. Increased lymphocytes have been observed previously in PCOS, but in the absence of increased hsCRP cannot be interpreted as a sign of inflammation (Phelan et al., 2013; Papalou et al., 2015). Chronic low-grade inflammation and adipose tissue dysfunction are more prevalent in women with PCOS than in the general population, although whether this is due primarily to adiposity or a PCOS-specific factor is still unclear (Escobar-Morreale, Luque-Ramirez and Gonzalez, 2011; Spritzer et al., 2015). We found a significant increase in AFABP, but not in other common adipokines, in the PCOS group. While adipose tissue dysfunction does not seem to be severe in our study cohort, we cannot exclude that the gut barrier abnormalities were the result of pro-inflammatory signaling due to secreted adipokines.

The modest changes in gut microbiome, gut permeability, and systemic inflammation in our cohort may be attributable to the fact that women in the PCOS group on average displayed a mild clinical phenotype. Circulating androgens, basal and secreted insulin in the oGTT, and serum lipids were significantly elevated in women with PCOS compared to control women despite a comparable BMI, confirming that these women have demonstrable reproductive and metabolic impairments. However, the median values of all studied parameters in women with PCOS were within the normal range. This may be

partly because empirically determined reference ranges are often derived from populations including women with undiagnosed PCOS, a fact that most laboratories are oblivious to. In the calculated free androgen values in our study cohort, differences between the groups became much more pronounced, with two- to threefold higher levels in women with PCOS. When categorizing women in the PCOS group into the four possible phenotypes dictated by the Rotterdam criteria, we found that most women fell into phenotype D (OA+PCOM). This phenotype, which does not display hyperandrogenism, has been described as the phenotype with the mildest symptoms and lowest cardiometabolic risk (Moghetti et al., 2013; Lizneva et al., 2016). As we were not able to preselect our consecutively recruited study participants, it may be coincidence that this phenotype was so highly represented in our cohort. According to previous reports, phenotype A ("classic" PCOS with all three diagnostic criteria fulfilled) is the most common in the general population, with phenotypes B, C, and D displaying an approximately equal lower prevalence (Lizneva et al., 2016). Therefore, our PCOS cohort did not represent what to our knowledge is the "typical" manifestation of PCOS in the population, but rather a milder form of the syndrome. Knowing this, it is perhaps more striking that we found bacterial diversity loss and signs of gut barrier dysfunction and inflammation in these women. If our cohort had included more women with the phenotype A we might have seen much more pronounced changes in these parameters.

Our study suffers from certain weaknesses implicit in its design as a pilot study. Our patient cohort, though adequate in number for parametric and nonparametric statistical comparisons, was small and heterogeneous. We did not match our groups according to age or BMI or stratify according to Rotterdam phenotypes, obesity, or insulin resistance. Doing so would have required much more resources, time, and a much larger number of participants, which was judged not feasible for a first investigation of this kind. The aim of this study was to provide a first description of the investigated entities, providing a valuable knowledge base for future studies. As BMI was not statistically different between groups, our cohort can be considered homogenous in this regard despite the relatively small sample size.

Gut microbiome profiles are notoriously variable both within and between subjects (Costello et al., 2009; Caporaso et al., 2011). It has been estimated that with 20 samples per group, differences with effect sizes of 0.008 in unweighted and 0.04 in weighted pairwise distances can be detected with 90 % power (Kelly et al., 2015). Our study was underpowered to detect small effects in weighted UniFrac analysis and likely also in taxa comparisons. The study was undertaken with the understanding that only large and consistent changes would be detectable with the statistical methods used and that the obtained results would be liable to confounding by known or unknown factors. We attempted to address these limitations by adhering strictly to the assumptions of the statistical tests used and adjusting for multiple hypothesis testing wherever necessary, using multiple approaches to detect differences between the groups (such as LEfSe in addition to taxonomic comparisons), performing additional exploratory analyses without a stringent multiple testing correction to detect associations that might otherwise have been missed, and accounting for suspected confounders by defining them as an exclusion criterion for the study or by including them in the analyses.

In the less stringent exploratory analyses, we found multiple associations in support of our original hypothesis. Fecal bacterial diversity was inversely correlated with serum and stool zonulin, LPS, serum total testosterone, AMH, and the LH:FSH ratio in the whole study cohort. In the PCOS group, there was an inverse correlation between stool zonulin and serum total testosterone and AMH, which was not observed in the control group. These results, while descriptive in nature, are encouraging incentives to continue testing this hypothesis in larger patient cohorts.

Regarding suspected confounders, we found that the diet of the PCOS and control groups differed slightly, with control women consuming significantly more grains. However, when categorizing study participants into a high-carbohydrate or a high-animal protein diet, there was no significant difference between the groups. Diet type and BMI did not appear to affect microbiome parameters. Recent studies suggest that the influence of these and other suspected confounding factors is much lower than previously assumed, possibly accounting for <1 % of variability in microbiome profiles (Stahringer et al., 2012; Belstrom et al., 2014; Chen et al., 2016). In our analysis, age correlated positively with richness and with clustering in the unweighted PCoA plot. We acknowledge that the

divergent mean age of the PCOS and control groups might have confounded the obtained result. Having said this, we propose an alternative explanation. It has been reported that PCOS symptoms are often more severe in young patients and ameliorate with aging (Brown et al., 2011; Carmina, Campagna and Lobo, 2012; Pinola et al., 2015). The age effect we observed may therefore result from a change in the PCOS phenotype occurring with age. Older women displaying an ameliorated PCOS phenotype may resemble control women more closely in their steroid, inflammatory, and metabolic profile than younger, more symptomatic women with PCOS. This phenotypic shift could be reflected in the microbiome profile, giving the impression that age was responsible for the observed difference. Indeed, we observed a significant association of age on clustering in the unweighted PCoA analysis only in the PCOS, and not in the control group.

Both BMI and diet were associated with parameters of gut permeability and inflammation. This finding is not surprising, as obesity is known to promote inflammation and a leaky gut and vice versa (Cani et al., 2007; Zak-Golab et al., 2013; Hamilton et al., 2015). BMI was not significantly different between the two groups and there were no visible outliers in the measured parameters, making it unlikely that a subgroup of obese women in the PCOS group were responsible for the obtained results.

Although not classical features of PCOS, depression and anxiety are more prevalent among this group than in the general population (Cooney et al., 2017). In our exploratory analyses we found inverse correlations between BDI score and stool bacterial diversity, DAO, and LBP in the control group, while these associations were not seen in PCOS. Gut microbiome changes in depression have been reported previously (Zheng et al., 2016). With a BDI score >8 indicating mild depression, both groups scored in the normal range, though women with PCOS scored significantly higher than control women. It is unclear how the association of BDI and the stool microbiome in control women is to be interpreted. This result may be an artifact due to another factor that was not considered.

The saliva microbiome was largely unchanged in PCOS. We found a significant reduction of the phylum Actinobacteria at the 0.1 % OTU relative abundance cut-off and the same phylum was found to be discriminant for control samples in the LEfSe analysis. A reduction of Actinobacteria has been reported in periodontal disease, which is more

prevalent among women with PCOS than in the general population (Liu et al., 2012; Wang et al., 2013; Porwal et al., 2014; Rahiminejad et al., 2015). We specifically recruited women without signs of gingivitis or periodontitis to avoid this confounding factor. In our exploratory analyses, we found an association of hsCRP and saliva steroids with clustering in weighted UniFrac analysis. We hypothesize that women with PCOS have a saliva microbiome which is more permissive for oral pathogens, raising the risk to develop periodontal disease in these women. Based on our results, the saliva microbiome seems to be a less promising biomarker for PCOS than the stool microbiome.

We have previously shown that women with PCOS have increased salivary androgen levels and that a higher saliva testosterone to androstenedione ratio is associated with an adverse metabolic phenotype (Münzker et al., 2017). Although in the current study saliva androgens were markedly elevated in women with PCOS, we did not find significant differences in saliva bacterial richness or clustering in beta diversity analyses. Our exploratory analyses showed a correlation between saliva and serum androgens and clustering in weighted UniFrac analysis. Oral bacteria may react to altered androgen levels and our failure to detect this change may have been due to lack of statistical power.

Lastly, the role of isoflavone metabolism and equol production in PCOS will be addressed. The overall equol producer prevalence in our study cohort was around 30 %, as previously reported for Western populations (Song et al., 2006; Atkinson, Newton, Bowles, et al., 2008; Degen et al., 2011). Though not statistically significant, women with PCOS were only half as likely to be equol producers as control women. Baseline isoflavone levels and the responses on the FFQ revealed that overall soy consumption was very low in our cohort. This is one of the central limitations when investigating the role of soy isoflavones and their metabolites in Western populations. The bulk of studies on the role of isoflavones in disease stems from Asian populations, where soy is regularly consumed in large amounts and up to 80 % of the population has the gut bacteria needed for equol production (Fujimoto et al., 2008). If the precursor compounds are not ingested, one cannot expect to observe a direct effect on hormonal or metabolic parameters. However, we cannot dismiss entirely the possibility that isoflavones and equol producing bacteria might have a beneficial effect in PCOS.

It is critical to disentangle the effects of ingested isoflavones (genistein and daidzein) from their metabolites and the functional capacity of the gut microbiome to produce them. In this study, we did not perform an intervention with isoflavones. Rather, we tested for the capacity of the gut microbiome of our study participants to produce equol. This was of relevance for two reasons. Firstly, equol produced by the gut microbiome may have direct beneficial effects, for example by binding to DHT and preventing it from activating the androgen receptor or by decreasing steroidogenic enzyme expression, as has been shown in vitro and in a rat model of PCOS (Lund et al., 2004; Rajan, M. and Balaji, 2017). Secondly, the capacity to produce equol may be a biomarker of sorts, indicating the presence of gut bacteria which exert a beneficial influence on metabolic and reproductive processes via a still unknown mechanism.

Some evidence exists that the capacity to produce equol alone is associated with a more favorable hormonal and metabolic profile in Western populations (Ingram et al., 1997; Duncan et al., 2000; Reverri et al., 2016). Other studies have found no difference in circulating androgen levels between female equol producers and non-producers (Frankenfeld et al., 2004; Atkinson, Newton, Stanczyk, et al., 2008). We observed an association of the rise in urinary equol after a soy challenge with lower circulating androgens, zonulin, and AMH. All variables related to isoflavone metabolism were associated with clustering in weighted UniFrac analysis, suggesting that equol production capacity may be associated with a gut microbiome profile that exerts beneficial effects on pathophysiological processes in PCOS.

Three studies in PCOS showed improvements in plasma lipid and androgen profiles after an isoflavone intervention (Romualdi et al., 2008; Khani et al., 2011; Jamilian and Asemi, 2016). A meta-analysis found that isoflavone consumption reduced circulating levels of LH and FSH (Hooper et al., 2009). Women with PCOS may therefore benefit from consuming more soy regardless of their equol producing capacity.

## 4.2. Part 2: Mouse study

In the first part of this study, we aimed to describe the gut microbiome of a mouse model of PCOS, induced by maternal HF/HS diet and PNA, and to describe the effect of the individual factors maternal diet, maternal injection, and offspring diet on gut microbiome parameters. Next, we performed FMT to test the hypothesis that the gut microbiome contributes to reproductive and behavioral dysfunction in this mouse model.

Various rodent models of PCOS exist. Commonly, pre-pubertal or adult animals are made hyperandrogenic by direct application of androgens, such as testosterone or DHT, or the aromatase inhibitor letrozole, to induce a PCOS-like phenotype (Maliqueo, Benrick and Stener-Victorin, 2014). For the PNA model, mice are indirectly exposed to high androgen levels in utero, programming them to develop reproductive dysfunction and polycystic ovarian morphology with or without hyperandrogenemia (Roland et al., 2010; Witham et al., 2012; Caldwell et al., 2014; Maliqueo, Benrick and Stener-Victorin, 2014). Depending on the species, time of drug administration, and dosage used, PCOS rodent models can display impaired or normal glucose and lipid metabolism (Roland et al., 2010; Caldwell et al., 2014; Maliqueo, Benrick and Stener-Victorin, 2014; Kauffman et al., 2015; Noroozzadeh et al., 2015). The DHT-induced PNA model in mice typically displays long-term reproductive, but not metabolic features of PCOS (Witham et al., 2012). Despite the apparent mild phenotype of the DHT-PNA model, we consider it preferable to other models as it mimics one of the suspected pathophysiological mechanism of PCOS, this being indirect in-utero exposure to high maternal androgen levels (Dumesic, Abbott and Padmanabhan, 2007; Abbott and Bacha, 2013). As is evident from our human study, hyperandrogenemia is not required for diagnosis and may be absent in a substantial proportion of women with PCOS. By combining the DHT-induced PNA model with a HF/HS diet, we successfully recapitulated both the reproductive and metabolic components of PCOS. By using DHT rather than testosterone, we eliminated potential secondary effects due to metabolism of testosterone to estradiol and other steroid hormone receptor ligands. Of the available experimental animals, only a mouse model allowed us to conduct an experiment of this size and duration. Therefore, we believe that we selected the best possible model to address our research question.

We found sex-dependent effects of the studied factors on the gut microbiome of PNA offspring. In males, we found a DHT-dependent reduction in bacterial richness and diversity, measured by the Shannon index. This result agrees with the findings of our human pilot study and with a recent study that found decreased fecal bacterial diversity in a letrozole-induced mouse model of PCOS (Kelley et al., 2016). Maternal and offspring diet had an interaction effect on the Shannon index in male offspring, with lower diversity observed in CD-CD groups. In beta diversity analyses and taxa comparisons, there was a significant effect of both maternal and offspring diet, but no effect of maternal injection.

In female offspring, there was an interaction between maternal injection and offspring diet and a main effect of maternal diet on the number of observed RSVs. DHT exposure tended to increase richness in all diet groups, with the exception of the HF/HS-HF/HS groups, where richness was decreased compared to the other diet groups and lowest with additional DHT exposure. In beta diversity analyses, only offspring diet had a significant effect on clustering. All three studied factors caused significant changes in genus relative abundances. This pattern resembles that found in F0 dams, where fecal bacterial richness was increased in the CD-DHT and HF/HS-Veh groups.

Compared to male offspring, female offspring were more susceptible to alpha diversity changes due to maternal diet, while the maternal effect on community composition was less pronounced. Female mice appear to be more susceptible to changes in offspring diet than males, with a larger number of differentially abundant genera identified by DESeq2. The majority of genera identified by DESeq2 were unique for males and females; however, Lachnospiraceae *NK4A136* and *Bifidobacterium* showed a concordant decrease in relative abundance due to offspring HF/HS diet in both male and female offspring.

Sex-dependent responses of the gut microbiome to diet in mice have been reported previously (Org et al., 2016). In this study, gonadectomy affected the microbiome profiles of both male and female mice. The gonadectomy effect was more prevalent in males on a control diet, while females showed greater changes on a high-fat diet. These results fit well with our observations, where a DHT-induced diversity reduction was stronger in males on a CD and females on a HF/HS-diet. Female mice also showed more genus

relative abundance changes due to a HF/HS diet than males. In F0 dams, only diet, but not DHT injection, led to significant changes in genus relative abundances.

Regarding the effect of maternal factors, maternal high-fat diet and stress have been reported to cause lasting sex-dependent changes in offspring gut microbiota in humans and rodents (Chu et al., 2016; Paul et al., 2016; Jašarević et al., 2017). This effect is most likely mediated through a direct transfer of microbes from mother to offspring immediately after birth, but may occur already in-utero through transmission of maternal bacteria in the amniotic fluid (Chu et al., 2016). There was no concordance in altered genera due to any studied factor between dams and offspring. This is not surprising considering that the sampled dams were not the biological mothers of the offspring. Furthermore, pregnancy itself causes profound changes in gut microbiome profiles in humans and mice and pregnant and non-pregnant animals cannot readily be compared (Koren et al., 2012; Paul et al., 2016; Jašarević et al., 2017).

Contradictory data exist on the effect of diet-induced obesity on alpha diversity in mice, with different studies reporting a decrease (Turnbaugh et al., 2008), an increase (Evans et al., 2014; Shang et al., 2017; Xiao et al., 2017), or no change (Kim et al., 2012). As in humans, different bacterial taxa were found to be altered in mice fed a high-fat diet, with variable results across studies. Lachnospiraceae and *Bifidobacterium*, the two taxa that showed consistent changes in male and female offspring in our study, were previously found to both increase and decrease in high-fat diet-fed mice (Kim et al., 2012; Parks et al., 2013; Evans et al., 2014; Shang et al., 2017). High-fat diet-induced gut microbiome changes may depend on diet composition rather than diet-induced obesity (Evans et al., 2014; Xiao et al., 2017). Isocaloric diets containing different fat sources were shown to induce specific gut microbiome changes in mice (Huang et al., 2013). Differences not only in the percentage of macronutrients, but also the macronutrient composition of high-fat diets used may explain the lack of congruency across studies regarding changes in specific bacteria. The gut microbiome is complex and varies depending on genetic, environmental, and individual factors. Individual and time have been described as two of the strongest contributors to variation in the mouse gut microbiome (Hildebrand et al., 2013; Hoy et al., 2015). Our experiment involved both a large number of individual mice and a long duration, so diet- or DHT-induced changes may have been obscured by these effects.

The overall bacterial composition in PNA male and female samples agreed with previously published data from the same mouse strain (Gu et al., 2013). Samples from F0 dams showed an unexpected bloom of *Akkermansia*, which was not observed in offspring. Such blooms of single species can occur due to a loss of stability in the intestinal ecosystem as a result of stress, disease, or antibiotic treatment, among other factors (Stecher, Maier and Hardt, 2013). Pregnancy may represent a stressful situation in itself and blooms of single taxa, including *Akkermansia*, have been reported (Gohir et al., 2015; Jašarević et al., 2017). Turnbaugh *et al.* have described the blooming of a single genus in response to a high-fat diet (Turnbaugh et al., 2008). The *Akkermansia* bloom in F0 dams may have been the result of the pregnant state combined with other stressors acting together. Alternatively, environmental factors during breeding, handling, and shipping of these animals may have allowed this genus to establish itself as a dominant member of the gut microbiome prior to the start of the experiment. The proportions of the remaining dominant genera were comparable between F1 and F0 samples, indicating that *Akkermansia* crowded out low- rather than high-abundance bacteria in F0 mice. As dams in this study were not the biological mothers of the offspring, we cannot say whether the real mothers showed the same microbiome profile and whether these microbes could have been transferred between generations.

We aimed to transfer the reproductive and behavioral phenotype observed in female PNA offspring to healthy female WT mice via FMT. As the role of the gut microbiome in energy harvest and obesity is already well-described, we chose to omit the metabolic aspect from our investigations. DHT-exposed FMT donors showed disrupted estrous cyclicity, with a larger percentage of days spent in diestrus at the expense of proestrus. In the OF and EPM, DHT-exposed FMT donors spent less time in the open areas of the arenas, indicating increased anxiety-like behavior as a direct result of maternal DHT injection. Increased anxiety-like behavior has been reported previously in rat models of PCOS induced by post-natal DHT administration and PNA using testosterone (Feng et al., 2011; Hu et al., 2015). Women with PCOS have an increased prevalence of anxiety and depression, a finding which was underscored in our human study, where the PCOS group scored higher on the BDI than controls (Jedel et al., 2010; Cooney et al., 2017). A maternal HF/HS diet had no effect on anxiety-like behavior in female PNA offspring in our study,

but caused increased anxiety-like behavior in the OF in male PNA offspring. These results agree with a recently published study, where a maternal high-fat diet increased anxiety-like behavior in male, but not female offspring (Bruce-Keller et al., 2017).

WT FMT recipients in our study did not show changes in estrous cyclicity. Few data exist on the effect of the gut microbiome on reproductive function. It has been reported that male germ-free (GF) mice have lower testosterone levels than specific pathogen-free (SPF) mice, and transfer of male-type microbiota to female GF mice resulted in increased testosterone levels (Markle et al., 2013; Yurkovetskiy et al., 2013). In a rat model of PCOS, FMT from healthy donors was able to reverse hyperandrogenemia, disrupted estrous cyclicity, and polycystic ovarian morphology after just two weeks (Guo et al., 2016). While there may be some potential of the gut microbiome to affect reproductive function, no such effect was discernible in our study. One reason for this may be that the FMT donors in our study developed changes in the gut microbiome in response to DHT exposure, not the other way around. Therefore, the microbiome that was transferred may have been reactive to DHT without exerting a reciprocal effect on gonadotropin and androgen production and action. Prenatal DHT exposure has been described to impair the maturation of the hypothalamic-pituitary-gonadotropic axis in female rats, preventing the pre-ovulatory gonadotropin surge and leading to oligo- or anovulation (Foecking et al., 2005). We suspect that the reproductive changes observed in our PCOS model were the result of early developmental mechanisms such as this, which was not dependent on the gut microbiome.

We could not replicate the behavioral phenotype of female PNA offspring in FMT recipients. Instead of the expected increase in anxiety-like behavior in mice receiving FMT from DHT-exposed donors, we found reduced anxiety in the HF/HS-DHT recipients in the OF, while the remaining three groups showed no significant differences. No differences due to FMT were found in the EPM. Changes in anxiety-like behavior have been reported in GF mice and in response to gut microbiome manipulations such as antibiotic or pre-biotic treatment (Luczynski et al., 2016; Burokas et al., 2017; Leclercq et al., 2017). FMT from humans suffering from depression led to anxiety-and depressive-like behaviors in mice (Zheng et al., 2016). Several studies have used FMT to transfer an anxiety-like phenotype between mice (Collins, Kassam and Bercik, 2013; Bruce-Keller et al., 2015;

Gacias et al., 2016). Though FMT led to changes in anxiety-like behavior in our study, these changes did not mirror the donor phenotype and we cannot conclude that the gut microbiome was involved in mediating the anxiety effects of DHT exposure.

While both OF and EPM are suitable for testing anxiety-like behavior in mice, they are not identical, with mice displaying more exploratory behavior in the OF and a greater stress response in the EPM (Carola et al., 2002; Polissidis et al., 2017). We cannot currently explain the reduced anxiety-like behavior in the HF/HS-DHT recipient group. We can speculate that the greater stress of a dual exposure to HF/HS-diet and DHT caused the gut microbiome of PNA offspring to develop protective anxiety-reducing mechanisms, which caused an overshooting response in healthy WT mice. It must be noted that PNA offspring were overall more anxious than FMT recipients, spending only around 5 % of time in the open areas of the OF and EPM, compared to 10-20 % in the FMT recipients. The long duration and repeated testing procedures may have provoked an anxiety-like response in PNA offspring, which was exacerbated by HF/HS-diet and DHT exposure. In the shorter and less intensive FMT experiment, mice may have been more resilient to any anxiety-inducing influence of the donor gut microbiome.

As this was the first attempt to transfer a PCOS phenotype via FMT, we opted to use conventionally raised, antibiotic-treated FMT recipients. GF mice are considered the gold-standard for investigating microbiome effects on host phenotype, particularly when the disease in question is not yet known to be microbiota-dependent (Lundberg et al., 2016). GF mice have been used to show a causal role of the gut microbiome in obesity, insulin resistance, and anxiety- and depressive-like behavior (Bäckhed et al., 2004; Bercik et al., 2011; Ridaura et al., 2013; Chevalier et al., 2015; Zheng et al., 2016). However, GF mice also have disadvantages as they differ from conventionally raised mice in a number of immunological, anatomical, reproductive, and metabolic characteristics and may not accurately represent a healthy state (Nicklas, Keubler and Bleich, 2015; Lundberg et al., 2016). Changes in anxiety-like behavior and a prolonged diestrus period have been described in female GF mice, which could have confounded the results of our experiment (Shimizu et al., 1998; Luczynski et al., 2016). GF mice are cost- and labor-intensive and require special housing facilities and handling procedures. For these reasons, we did not use GF mice in this study, but rather the best-suited alternative.

Numerous studies have achieved a successful metabolic and behavior phenotype transfer in mice using conventional housing, frozen FMT material, and antibiotic pre-treatment (Bercik et al., 2011; Ellekilde et al., 2014; Bruce-Keller et al., 2015; He et al., 2015; Gacias et al., 2016). We chose a cocktail of five antibiotics and one antifungal drug based on previously published protocols for microbiome depletion in mice (Hill et al., 2010; Bruce-Keller et al., 2015; Gacias et al., 2016). The route of antibiotic administration, preparation of the FMT inoculate from frozen cecal material, and the time between FMT administration and testing were based on a thorough literature search focused on studies with positive FMT outcomes.

Antibiotic pre-treatment may not be necessary for successful colonization. In the earlier-mentioned letrozole-induced rat model of PCOS, FMT was used in conventional animals without antibiotic pre-treatment to successfully rescue the PCOS phenotype (Guo et al., 2016). Manichanh *et al.* reported a stronger resemblance to the donor in non-antibiotic-treated FMT recipients, while antibiotic pre-treatment led to a reshaping of the community, which then represented a combination of the antibiotic and FMT effects (Manichanh et al., 2010). Despite these caveats, we found it necessary to deplete the gut bacteria in our recipient animals to provide optimal conditions for the donor microbiome to establish itself in the host in a conventional, open-housed setting. We acknowledge that no antibiotic regimen can completely sterilize the gastrointestinal tract of a mammal, that we might have induced an overgrowth of opportunistic species prior to FMT, and that mouse-or cage-dependent effects might have affected the results of the experiment.

The overall community composition between inoculate and recipient samples differed substantially, as seen in beta diversity plots and comparisons of Bray-Curtis dissimilarity. This was not unexpected, as inoculates had been diluted prior to FMT and mice were exposed to bacteria from themselves, their cagemates, handlers, and the environment. As 5-6 weeks passed between the first FMT and cecal sampling, the microbiome composition was expected to change due to time alone. We did not expect to achieve perfect colonization efficiency with our protocol, but rather aimed to seed the recipient gut with dominant microbes from the donors, allowing the bacterial community to organize and stabilize around these species. Booster FMT doses were given once weekly until the end of the experiment to reinforce the donor microbiome profile, but these were likely much

less effective than the initial inoculation. We found comparable colonization efficiency, measured in the number of shared genera between inoculate and recipient, in all FMT groups. A large percentage of the core microbial community, found in all FMT recipients, was derived from the FMT inoculate. The FMT groups clustered in beta diversity analyses both due to donor diet and donor DHT exposure. Several genera that showed altered relative abundances in FMT donors showed concordant changes in FMT recipients, including a down-regulation of bacteria from the families Lachnospiraceae and Ruminococcaceae in response to a HF/HS diet and a down-regulation of *Thalassospira* and *Rikenella* due to DHT exposure. Alpha diversity measures were comparable between inoculate and recipient samples. We conclude that our FMT protocol was successful in transferring various aspects of the donor microbiome, including dominant taxa, discriminant taxa for the donor exposures, and alpha diversity characteristics. Meanwhile, overall community structure was not preserved.

Antibiotic treatment has been reported to cause long-lasting shifts in the gut microbiome and affect anxiety-like behavior in mice, even if non-absorbable antibiotics are used (Manichanh et al., 2010; Bercik et al., 2011; Cox et al., 2014; Nobel et al., 2015; Tochitani et al., 2016). In our study, antibiotic treatment had no detectable negative effects on body weight or food intake. Antibiotic-treated animals did not perform differently to no-FMT control mice in the EPM or OF. We conclude that our antibiotic regimen did not significantly affect the results of the behavior testing. However, we observed a non-significant decrease in three of the four FMT groups in the time spent in the center of the OF compared to no FMT controls, indicating that the antibiotic administration may have the potential to promote anxiety-like behavior. All mice receiving FMT had significantly lower alpha diversity than non-FMT controls, confirming that there was a reduction in intestinal bacterial load due to antibiotic treatment, which was incompletely restored by FMT. As the inoculates had been diluted in preparation for oral gavage and to enable the use of the same inoculate stocks throughout the whole experiment, the lower alpha diversity observed in FMT recipients may not be due to a lasting antibiotic effect, but rather because the inoculates themselves were not more diverse. Had undiluted material been used, diversity levels after FMT may have risen to those of non-FMT controls.

It is difficult to determine the degree to which the results from our human and mouse studies are comparable. A naturally occurring PCOS-like phenotype has been described in female rhesus monkeys, but a similar phenotype in mice is lacking (Abbott et al., 2017). As discussed earlier, the PNA model is one of the best-suited available tools to study PCOS, but its pathogenesis is still considerably different from that of human PCOS. Regarding the gut microbiome, the gastrointestinal tracts of humans and mice differ morphologically and functionally (Nguyen et al., 2015). While bacterial composition is comparable between the two species on the phylum level, greater differences appear at lower taxonomic levels (Ley et al., 2005). However, the functional capacity of human and mouse gut microbiomes is highly similar, which allows for the creation of humanized mice to study the role of the microbiome in disease (P. J. Turnbaugh et al., 2009; Xiao et al., 2015). While we hesitate to compare bacterial profiles or single taxa from human and mouse gut samples for these reasons, we consider richness and diversity to be suitable measures for cross-species comparison. We observed a reduced diversity in women with PCOS, as well as male and a subgroup of female PNA offspring, a finding which is consistent with other studies (Kelley et al., 2016; Liu et al., 2017). While a reduction in diversity may be a general indicator of poor gut health rather than a PCOS-specific phenomenon, it nevertheless provides an interesting target for future studies and possible new treatments in PCOS.

As the mouse study was conducted after the human study, the microbiome analysis workflow was adjusted based on location and newly published bioinformatic tools. The DNA extraction methods between the two studies differed due to the instruments available on-site. For the mouse study, the V4 segment was chosen for sequencing rather than V1-2 as the shorter length of this gene region permitted a longer overlap of forward and reverse reads, leading to more accurate sequence inference (Kozich et al., 2013). The clustering-based, open-reference OTU-picking approach that was used in the human study was replaced by the DADA2 algorithm in the mouse study. DADA2 circumvents the need for clustering according to a predefined similarity threshold, eliminating the earlier-discussed problem of artificial OTUs caused by sequencing errors (Callahan et al., 2016). Instead, DADA2 calculates sequencing error rates and infers sequences ad hoc. DADA2 has been shown to outperform other sequencing methods on various mock community

and biological datasets (Callahan et al., 2016). While these changes in protocol make it harder to compare our two studies, we feel they added overall value by giving a more accurate representation of the sequenced samples. As mentioned earlier, even if identical methods are used, microbiome data from humans and mice cannot be compared directly.

The 16S rRNA gene-based approach of our two studies did not provide functional information about the bacteria in our sample. Whole-genome shotgun (WGS) sequencing is a preferable approach since it provides functional and taxonomic information with species-level resolution (Morgan and Huttenhower, 2012). However, WGS is more expensive and computationally challenging than amplicon sequencing, which is why we opted for amplicon sequencing for these early studies. Ideally, microbiome studies should be based on WGS data and include large sample sizes, which is not feasible in many cases. If financial means are limited, amplicon sequencing from a larger number of samples may be preferable. However, in a well-selected sample designed to minimize confounding effects and inter-individual variation, WGS will provide a wider range of valuable information, even when a smaller number of samples is used.

# 5. Conclusion

In this thesis, I present the first published description of the gut microbiome in women with PCOS and a large-scale, first-of-its-kind rodent study investigating whether there is a gut microbiome-dependent mechanism in the influence of pre-and post-natal high-fat/high-sucrose diet and prenatal androgen exposure on PCOS-typical reproductive and behavioral abnormalities in mice. At the time of writing, the total available body of literature on this subject comprised our publications, two rodent studies, and one other human study. Clearly, we are still in the beginning stages of uncovering the role of the gut microbiome in PCOS and related conditions. While it will take a considerable number of years, studies, and most of all human samples to reach any definitive conclusions, our studies provide a valuable reference point and will serve to inform and guide future research.

PCOS is a very common condition among women and though not directly life-threatening, it causes considerable physical and emotional distress and long-term health consequences that are anything but trivial. The available treatment options are scarce, symptomatic, and not always well-accepted or tolerated. Our goal with this project was to broach a new research field in PCOS, with the ultimate intent of identifying novel treatment approaches capable of providing additional relief to the affected women, be it causal or symptomatic. The gut microbiome is an enormously promising and attractive treatment avenue, as it provides a large natural interface to the systemic circulation and can be manipulated in a simple, rapid, and non-invasive way. A dietary, probiotic, and perhaps even a targeted antibiotic approach to manipulate the gut microbiome for greater systemic health would be cost-effective and virtually free of harmful side effects. Such an intervention would empower women with PCOS, enabling them to manage their symptoms in a holistic manner, while simultaneously reducing their risk for long-term health complications. We therefore consider this work to be of the highest importance, both for its novelty and for its potential to promote future research towards improving treatment options in PCOS.

Due to the exploratory nature of both our human and mouse studies, as well as the pilot design of the human study, our data are primarily association-based and lack statistical

power for many of the attempted comparisons. Gut microbiome studies require large sample sizes as inter-individual variation and the danger of confounding due to environmental factors are high. The heterogeneous nature of PCOS does not lend itself to small sample sizes. Ideally, studies such as the one carried out here should have a large sample size, control for as many potential confounders as possible through well-defined in- and exclusion criteria and/or case-control matching, and stratify according to discriminant features of PCOS including the four Rotterdam phenotypes, body mass index, and insulin resistance measures. It was neither practicable nor reasonable to expend such a large amount of resources on a first investigation of this kind. We opted instead to perform a pilot study on a small, well-characterized, carefully selected (through in- and exclusion criteria) cohort of women with PCOS and controls and later supplemented this data with data from a large mouse study with controlled exposures in a laboratory setting. We consider it a major strength of this work that we combined observational data in the target group of women with PCOS with a mouse study that allowed for specific manipulation of the factors of interest. By performing FMT, we gained mechanistic insights into the role of the gut microbiome in this pathology, elevating our study beyond the level of mere associative results.

We found that women with PCOS have an altered gut microbiome with reduced bacterial richness and some signs of abnormal gut barrier function and endotoxemia. While we cannot conclude from our results that the DOGMA (dysbiosis of gut microbiota, see Tremellen *et al*.) hypothesis holds true, we expect these results to become clearer in larger, well-stratified cohorts. It is very likely that gut dysbiosis and metabolic endotoxemia are absent or less pronounced in certain subgroups of women with PCOS, such as lean, insulin-sensitive, and normoandrogenic women, and present to a larger degree in obese, insulin resistant, and hyperandrogenic women. The results from our rodent studies demonstrate that both prenatal androgen exposure and pre- and postnatal diet affect the gut microbiome, though the direction and magnitude of these effects in male and female offspring remain to be confirmed. We could not induce the observed reproductive and behavioral phenotype in healthy recipients via FMT, discrediting, but not disproving, the hypothesis that the gut microbiome drives these processes in PCOS. Although the pathological microbiome did not induce PCOS-like

changes in healthy mice, this does not exclude the possibility that a restitution of the dysbiotic microbiome could improve PCOS symptoms. While there may or may not be a PCOS-specific microbiome, the observed bacterial dysbiosis in our human study is sufficient to justify further research into the gut microbiome as a treatment target in this condition. The expected benefits of a microbiome-targeted intervention far outweigh the risks, and there is ample evidence that a healthy gut promotes whole body health.

For future research, both animal and human studies are indispensable and should be designed intelligently to advance the current state of knowledge in a synergistic manner. In humans, the focus should be on defining the true state of the gut microbiome and gut barrier function in PCOS. This will be achieved through large-scale studies with a focus on minimizing the confounding effects of internal and external factors, as described earlier. In parallel, intervention studies aimed at improving gut bacterial diversity and community stability should be tested for their potential to manage symptom severity and improve quality of life in the affected women. Augmenting this research, animal studies will provide valuable knowledge on the mechanisms underlying the influence of the gut microbiome on whole body reproductive and metabolic homeostasis. These studies should avail themselves of germ-free and conventional models and explore the effects of gut microbiome manipulations through fecal microbial transplantation, humanization, and administration of various antibiotics, mono- and multistrain probiotics, candidate bacteria, PCOS-relevant pharmaceuticals, and nutraceuticals. Further exploration of the pathophysiological mechanisms underlying the development of PCOS is critical to refine animal models to better reflect the human situation. As next-generation sequencing methods continue to develop, it will be possible to generate large amounts of data in a short time and at relatively low cost. While this resource should be used to the fullest, thorough and mindful planning of study designs as well as statistical analyses is essential to produce high-quality data which exceed simple descriptions of associations and advance towards a true understanding of causality.

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# 7. Appendix

## 7.1. Case report form for human study

Version 1.0 vom 07.05.2014

**Isoflavonstoffwechsel bei PCOS - Pilotstudie** Seite 1 von 2

**Anamnesebogen** | Pat. ID: _____

Einwilligungserklärung unterschrieben? ❍ ja ❍ nein

**Soziodemographische Angaben:** | **Untersuchungsdatum** _ _ / _ _ / 2 0 _ _ (T T M M J J J J)

Geschlecht ❍ männlich ❍ weiblich

Geburtsdatum _ _ / _ _ / _ _ _ _ (T T M M J J J J)

Ethnische Zugehörigkeit: ❍ kaukasisch ("Weiße") ❍ türkisch ❍ afrikanisch ❍ asiatisch ❍ andere

**PCOS Anamnese:**

Wurde bereits ein polyzystisches Ovarsyndrom diagnostiziert? ❍ ja ❍ nein

Wurden im gynäkologischen Ultraschall jemals polyzystische Ovarien (= kleine Bläschen in den Eierstöcken) entdeckt? ❍ ja ❍ nein

Datum der letzten gynäkologischen Untersuchung (Monat/Jahr) _____

Ist eine Erhöhung der Androgene (männliche Hormone) im Blut bekannt? ❍ ja ❍ nein

Neigen Sie zu Akne, unreiner oder fettiger Haut? ❍ ja ❍ nein

Haben Sie verstärkten Haarausfall (Kopf) bemerkt? ❍ ja ❍ nein

*(vom Studienpersonal auszufüllen)* FG-Score _____

**Zyklusanamnese:**

Alter zum Zeitpunkt der ersten Menstruation _____

Beginn des aktuellen Zyklus (1. Tag der Blutung) _____

Durchschnittliche Zykluslänge (ausgenommen Zeiten, in denen Sie hormonell verhütet haben)

❍ <21 Tage ❍ 21-25 Tage ❍ 26-30 Tage ❍ 31-35 Tage ❍ >35 Tage ❍ ganz unregelmäßig

Anzahl Schwangerschaften _____

Anzahl Geburten _____

Verwenden Sie derzeit ein hormonelles Verhütungsmittel (Pille, Hormonspirale, NuvaRing, Stäbchen, Pflaster, 3-Monats-Spritze)? ❍ ja: _____ ❍ nein

Haben Sie in der Vergangenheit ein hormonelles Verhütungsmittel verwendet? ❍ ja ❍ nein

Wenn ja, welches zuletzt? _____

Dauer der Einnahme/Verwendung _____

Wann abgesetzt _____

# Isoflavonstoffwechsel bei PCOS - Pilotstudie



## Anamnesebogen

Pat. ID: _____

**Allgemeine Anamnese:**

Sind Vorerkrankungen bekannt? ❍ ja: _____ ❍ nein

Wurden Sie schon einmal operiert? ❍ ja: _____ ❍ nein

*Bitte kreuzen Sie alle Punkte an, die **innerhalb der letzten 3 Monate** auf Sie zugetroffen haben:*

- Entzündungen des Mund- Rachenraums ❍ ja ❍ nein
- Karies ❍ ja ❍ nein
- Zahnfleischbluten ❍ ja ❍ nein
- Magen- Darm Erkrankungen ❍ ja: _____ ❍ nein
- Sonstige Darmbeschwerden ❍ ja: _____ ❍ nein
- Sonstige Infektionen oder Erkrankungen ❍ ja: _____ ❍ nein
- Einnahme von Antibiotika ❍ ja ❍ nein
- Einnahme von Medikamenten ❍ ja: _____ ❍ nein
- Gewichtsveränderung (mehr als 3kg) ❍ ja: _____ ❍ nein

Ist eine Sojaallergie bekannt? ❍ ja ❍ nein

Sonstige Allergien ❍ ja: _____ ❍ nein

Rauchen Sie? ❍ ja ❍ nein

Wieviel körperliche Bewegung (bei mäßiger Anstrengung) betreiben Sie in der Woche?

❍ <30 min ❍ 30-60min ❍ 60-90 min ❍ 90-120 min ❍ >120 min

**Körperliche Untersuchung:** (– Bitte messen, nicht erfragen!) BMI ⌴⌴⌴ kg/m²

Körpergröße ⌴⌴⌴ cm

Gewicht ⌴⌴⌴ kg

Blutdruck (sitzend, nach 10 min Ruhe) ⌴⌴⌴ / ⌴⌴⌴ mm Hg systolisch / diastolisch

Herzfrequenz (sitzend, nach 10 min Ruhe) ⌴⌴⌴ / min

Umfang Taille ⌴⌴⌴ cm

Umfang Hüfte ⌴⌴⌴ cm

Körperliches Befinden:

❍ **1** (schlecht) ❍ **2** (eher schlecht) ❍ **3** (mittelmässig) ❍ **4** (gut) ❍ **5** (sehr gut)

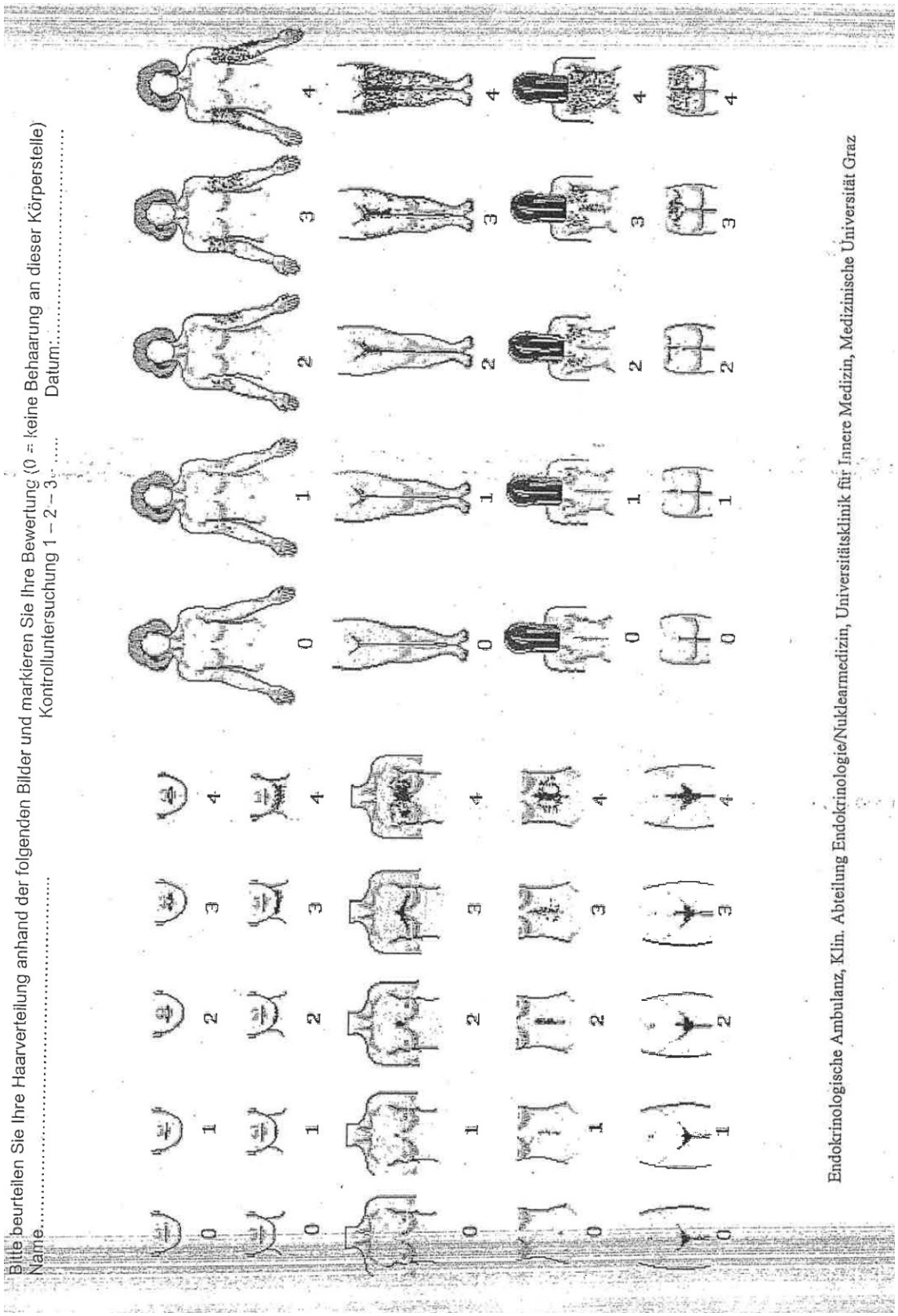
Psychisches Befinden/Lebensqualität:

❍ **1** (schlecht) ❍ **2** (eher schlecht) ❍ **3** (mittelmässig) ❍ **4** (gut) ❍ **5** (sehr gut)

Kommentar:

Untersucher/in: _____

## 7.2. Ferriman-Gallwey scoring sheet



Bitte beurteilen Sie Ihre Haarverteilung anhand der folgenden Bilder und markieren Sie Ihre Bewertung (0 = keine Behaarung an dieser Körperstelle)
Name:.....
Kontrolluntersuchung 1 – 2 – 3
Datum:.....

Endokrinologische Ambulanz, Klin. Abteilung Endokrinologie/Nuklearmedizin, Universitätsklinik für Innere Medizin, Medizinische Universität Graz

## 7.3. Food frequency questionnaire

**Landeskrankenhaus - Universitätsklinikum Graz**
**Ernährungsmedizinischer Dienst**
**Ltd. Diaetologin: Eisenberger Anna Maria**
A-8036 Graz, Auenbruggerplatz 21, Telefon: +43 (316) 385-82958, Fax: +43 (316) 385-4745
*Steiermärkische Krankenanstaltengesellschaft m.b.H.* *Medizinische Universität Graz*

# Ernährungsfragebogen

StudienID

PatientenID

**Datum:**

| Wie häufig verzehren Sie folgende Lebensmittel und Getränke? | | | | | | |
|---|---|---|---|---|---|---|
| **Getreideprodukte** | mehrmals täglich | täglich | mehrmals pro Woche | etwa 1 x pro Woche | mehrmals pro Monat | seltener/ nie |
| Brot | | | | | | |
| Müsli | | | | | | |
| Vollkornprodukte | | | | | | |
| Kartoffeln, Reis, Nudeln | | | | | | |
| **Milch und Milchprodukte** | mehrmals täglich | täglich | mehrmals pro Woche | etwa 1 x pro Woche | mehrmals pro Monat | seltener/ nie |
| Käsesorten mind. 45% Fett i. Tr. | | | | | | |
| fettarme Käsesorten mit weniger als 45% Fett i. Tr. | | | | | | |
| Milch, Topfen, Joghurt o.ä. mit 3,5% Fett | | | | | | |
| Milch, Topfen, Joghurt o.ä. mit 1,5%Fett | | | | | | |
| Sauerrahm, Obers, Creme fraîche | | | | | | |
| **Fleisch- und Fischprodukte** | mehrmals täglich | täglich | mehrmals pro Woche | etwa 1 x pro Woche | mehrmals pro Monat | seltener/ nie |
| Fleisch | | | | | | |
| Fettreiche Wurstsorten (z.B. Leberstreichwurst, Salami, Bergsteiger, Mortadella, …) | | | | | | |
| Fettarme Wurstsorten (z.B. Putenwurst, Schinken, Krakauer,…) | | | | | | |
| Fisch | | | | | | |
| Fischkonserven | | | | | | |
| **Obst und Gemüse** | mehrmals täglich | täglich | mehrmals pro Woche | etwa 1 x pro Woche | mehrmals pro Monat | seltener/ nie |
| Gemüse und Salat | | | | | | |
| Obst | | | | | | |

| **Fette** | mehrmals täglich | täglich | mehrmals pro Woche | etwa 1 x pro Woche | mehrmals pro Monat | seltener/ nie |
|---|---|---|---|---|---|---|
| Butter, Margarine | | | | | | |
| Halbfettbutter oder -margarine | | | | | | |
| Öle | | | | | | |
| Schmalz | | | | | | |
| **Extras** | mehrmals täglich | täglich | mehrmals pro Woche | etwa 1 x pro Woche | mehrmals pro Monat | seltener/ nie |
| Süßigkeiten, Schokolade | | | | | | |
| Kuchen, Kekse, Torten | | | | | | |
| Eis, Pudding und süße Speisen | | | | | | |
| In Fett gebackene Speisen (Wiener Schnitzel, Pommes frites,….) | | | | | | |
| Chips, Erdnüsse, Knabbereien | | | | | | |
| **Getränke** | mehrmals täglich | täglich | mehrmals pro Woche | etwa 1 x pro Woche | mehrmals pro Monat | seltener/ nie |
| Mineralwasser, verdünnte Obstsäfte, Wasser, Tee | | | | | | |
| Limonaden, Cola | | | | | | |
| Limonaden oder Cola mit Süßstoff | | | | | | |
| Obst- und Gemüsesäfte unverdünnt | | | | | | |
| Kakao, Milch, Milchshakes | | | | | | |
| Kaffee | | | | | | |
| Bier, Wein, sonstige alkoholische Getränke | | | | | | |
| Energiedrinks | | | | | | |
| **Sojaprodukte** | mehrmals täglich | täglich | mehrmals pro Woche | etwa 1 x pro Woche | mehrmals pro Monat | seltener/ nie |
| Sojamehl, Sojabohnen | | | | | | |
| Tofu, Tempeh, Miso, Natto, Sojasprossen | | | | | | |
| Fleischersatzprodukte mit Soja (Sojaburger, Sojawürstchen etc.) | | | | | | |
| Milchersatzprodukte mit Soja (Sojamilch, Drinks, Joghurt, Dessert, Käse, Eis etc.) | | | | | | |
| Nahrungsergänzungsmittel mit Soja oder Isoflavonen | | | | | | |
| Andere: | | | | | | |

## 7.4. Beck's Depression Inventory

### BDI

Name: _____ Ausfülldatum: _____

Dieser Fragebogen enthält 21 Gruppen von Aussagen. Bitte lesen Sie jede Gruppe sorgfältig durch. Suchen Sie dann die eine Aussage in jeder Gruppe heraus, die am besten beschreibt, wie Sie sich in dieser Woche einschließlich heute gefühlt haben und kreuzen Sie die dazugehörige Ziffer (1, 2, 3 oder 4) an. Falls mehrere Aussagen einer Gruppe gleichermaßen zutreffen, können Sie auch mehrere Ziffern markieren. Lesen Sie auf jeden Fall alle Aussagen in jeder Gruppe, bevor Sie Ihre Wahl treffen.

01. A
- [1] Ich bin nicht traurig.
- [2] Ich bin traurig.
- [3] Ich bin die ganze Zeit traurig und komme nicht davon los.
- [4] Ich bin so traurig oder unglücklich, dass ich es kaum noch ertrage.

02. B
- [1] Ich sehe nicht besonders mutlos in die Zukunft.
- [2] Ich sehe mutlos in die Zukunft.
- [3] Ich habe nichts, worauf ich mich freuen kann.
- [4] Ich habe das Gefühl, dass die Zukunft hoffnungslos ist, und dass die Situation nicht besser werden kann.

03. C
- [1] Ich fühle mich nicht als Versager.
- [2] Ich habe das Gefühl, öfter versagt zu haben als der Durchschnitt.
- [3] Wenn ich auf mein Leben zurückblicke, sehe ich bloß eine Menge Fehlschläge.
- [4] Ich habe das Gefühl, als Mensch ein völliger Versager zu sein.

04. D
- [1] Ich kann die Dinge genauso genießen wie früher.
- [2] Ich kann die Dinge nicht mehr so genießen wie früher.
- [3] Ich kann aus nichts mehr eine echte Befriedigung ziehen.
- [4] Ich bin zu allem unzufrieden oder gelangweilt.

05. E
- [1] Ich habe keine Schuldgefühle.
- [2] Ich habe häufig Schuldgefühle.
- [3] Ich habe fast immer Schuldgefühle.
- [4] Ich habe immer Schuldgefühle.

06. F
- [1] Ich habe nicht das Gefühl, gestraft zu sein.
- [2] Ich habe das Gefühl, vielleicht gestraft zu werden.
- [3] Ich erwarte bestraft zu werden.
- [4] Ich habe das Gefühl, bestraft zu sein.

07. G
- [1] Ich bin nicht von mir enttäuscht.
- [2] Ich bin von mir enttäuscht.
- [3] Ich finde mich fürchterlich.
- [4] Ich hasse mich.

08. H
- [1] Ich habe nicht das Gefühl, schlechter zu sein als alle anderen.
- [2] Ich kritisiere mich wegen meiner Fehler und Schwächen.
- [3] Ich mache mir die ganze Zeit Vorwürfe wegen meiner Mängel.
- [4] Ich gebe mir für alles die Schuld, was schief geht.

09. I
- [1] Ich denke nicht daran, mir etwas anzutun.
- [2] Ich denke manchmal an Selbstmord, aber ich würde es nicht tun.
- [3] Ich möchte mich am liebsten umbringen.
- [4] Ich würde mich umbringen, wenn ich die Gelegenheit hätte.

10. J
- [1] Ich weine nicht öfter als früher.
- [2] Ich weine jetzt mehr als früher.
- [3] Ich weine jetzt die ganze Zeit.
- [4] Früher konnte ich weinen, aber jetzt kann ich es nicht mehr, obwohl ich es möchte.

11. K

[1] Ich bin nicht reizbarer als sonst.
[2] Ich bin jetzt leichter verärgert oder gereizt als früher.
[3] Ich fühle mich dauernd gereizt.
[4] Die Dinge, die mich früher geärgert haben, berühren mich nicht mehr

12. L

[1] Ich habe nicht das Interesse an Menschen verloren.
[2] Ich interessiere mich jetzt weniger für Menschen als früher.
[3] Ich habe mein Interesse an anderen Menschen zum größten Teil verloren.
[4] Ich habe mein ganzes Interesse an anderen Menschen verloren.

13. M

[1] Ich bin so entschlussfreudig wie immer.
[2] Ich schiebe Entscheidungen jetzt öfter als früher auf.
[3] Es fällt mir jetzt schwerer als früher, Entscheidungen zu treffen.
[4] Ich kann überhaupt keine Entscheidungen mehr treffen.

14. N

[1] Ich habe nicht das Gefühl, schlechter auszusehen als früher.
[2] Ich mache mir Sorgen, dass ich alt oder unattraktiv aussehe.
[3] Ich habe das Gefühl, dass Veränderungen in meinem Aussehen eintreten, die mich hässlich machen.
[4] Ich finde mich hässlich.

15. O

[1] Ich kann so gut arbeiten wie früher.
[2] Ich muss mir einen Ruck geben, bevor ich eine Tätigkeit in Angriff nehme.
[3] Ich muss mich zu jeder Tätigkeit zwingen.
[4] Ich bin unfähig zu arbeiten.

16. P

[1] Ich schlafe so gut wie sonst.
[2] Ich schlafe nicht mehr so gut wie früher.
[3] Ich wache 1 bis 2 Stunden früher auf als sonst, und es fällt mir schwer, wieder einzuschlafen.
[4] Ich wache mehrere Stunden früher auf als sonst und kann nicht mehr einschlafen.

17. Q

[1] Ich ermüde nicht stärker als sonst.
[2] Ich ermüde schneller als früher.
[3] Fast alles ermüdet mich.
[4] Ich bin zu müde, um etwas zu tun.

18. R

[1] Mein Appetit ist nicht schlechter als sonst.
[2] Mein Appetit ist nicht mehr so gut wie früher.
[3] Mein Appetit hat sehr stark nachgelassen.
[4] Ich habe überhaupt keinen Appetit mehr.

19. S

[1] Ich habe in letzter Zeit kaum abgenommen.
[2] Ich habe mehr als 2 Kilo abgenommen.
[3] Ich habe mehr als 5 Kilo abgenommen.
[4] Ich habe mehr als 8 Kilo abgenommen.

Ich esse absichtlich weniger, um abzunehmen.
☐ Ja ☐ Nein

20. T

[1] Ich mache mir keine größeren Sorgen um meine Gesundheit als sonst.
[2] Ich mache mir Sorgen über körperliche Probleme, wie Schmerzen, Magenbeschwerden oder Verstopfung.
[3] Ich mache mir große Sorgen über gesundheitliche Probleme, dass es mir schwerfällt, an etwas anderes zu denken.
[4] Ich mache mir so große Sorgen über gesundheitliche Probleme, dass ich an nichts anderes mehr denken kann.

21. U

[1] Ich habe in letzter Zeit keine Veränderung meines Interesses an Sex bemerkt.
[2] Ich interessiere mich weniger für Sex als früher.
[3] Ich interessiere mich jetzt viel weniger für Sex.
[4] Ich habe das Interesse an Sex völlig verloren.

Danke!

## 7.5. Stool collection instructions for human study

Version 2 vom 06.08.2014

Einfluss des bakteriellen Isoflavonstoffwechsels auf metabolische und endokrine Parameter bei Frauen mit polyzystischem Ovarsyndrom – Pilotstudie

**Information für Probandinnen**

Hinweise zum Sojakonsum:

Bitte nehmen Sie während der Studienzeit keine anderen Sojaprodukte außer denen, die Sie von uns bekommen, zu sich.

Bitte verzichten Sie während der Studienzeit auf Alkohol, da dieser die Messungen verfälschen kann.

Bitte trinken Sie an drei aufeinanderfolgenden Tagen jeweils 2 der Sojadrinks, die sie von uns bekommen haben. Trinken Sie jeweils ein Sojagetränk am Morgen/Vormittag und eines am Abend und notieren sie die Uhrzeit der Einnahme hier:

| | | |
|---|---|---|
| Tag 1 | morgens_____ | abends_____ |
| Tag 2 | morgens_____ | abends_____ |
| Tag 3 | morgens_____ | abends_____ |
| Tag 4 | 2. Studientermin | |

Sollten während der Studienzeit unerwünschte Nebenwirkungen auftreten, notieren Sie diese bitte hier:

| Datum, Uhrzeit | Nebenwirkung |
|---|---|
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |
| _____ | _____ |

Version 2 vom 06.08.2014

Hinweise zur Stuhlprobensammlung:

Bitte sammeln Sie eine Stuhlprobe zu einem Zeitpunkt vor dem ersten Sojagetränk (Probe 1) und eine zweite am Tag des letzten Sojagetränks oder am Morgen der 2. Studienvisite (Probe 2).

1. Die Stuhlproben sollten so bald wie möglich nach der Entnahme gekühlt werden. Vergewissern Sie sich daher vor der Probensammlung, dass genug Platz für alle 4 Sammelgefäße in Ihrem Tiefkühler vorhanden ist.
2. Der Stuhl darf **nicht mit Harn oder dem Toilettenwasser in Berührung kommen**. Leeren Sie daher vor dem Stuhlgang Ihre Blase und bringen Sie erst danach den Stuhlfänger auf dem Toilettensitz an (Anleitung ist am Stuhlfänger aufgedruckt).
3. Entnehmen Sie die Stuhlprobe mit dem zur Verfügung gestellten Stuhlsammelbehälter. Verwenden Sie den Löffel im Deckel des Behälters um eine kleine Menge Stuhl von drei unterschiedlichen Stellen zu entnehmen. Bitte füllen Sie den Behälter maximal bis zur Hälfte! Schrauben Sie im Anschluss den Sammelbehälter in den Transportbehälter.
4. Wiederholen Sie Schritt 3 mit dem zweiten Stuhlsammelbehälter. Nach erfolgreicher Probenentnahme kann der Stuhl samt Stuhlfänger hinuntergespült werden.
5. Frieren Sie die Stuhlproben sofort im Gefrierschrank ein und lagern sie bis zur 2. Visite dort.
6. Bringen Sie alle vier Stuhlbehälter gekühlt in der von uns zur Verfügung gestellten Verpackung zur 2. Studienvisite mit.

Notieren Sie hier Datum und Uhrzeit der Stuhlprobenentnahme:

Probe 1 _____

Probe 2 _____

Bei Fragen und Problemen können Sie sich jederzeit an uns wenden.

Dr.in Lisa Lindheim, Studienkoordinatorin: 0681-814-835-44

Univ.Prof.in Dr.in Barbara Obermayer-Pietsch, Studienleiterin: 0316-385-80253

Wir bedanken uns herzlich für Ihre Mitarbeit!

# 7.6. Mock community data sheet, BEI Resources

b|e|i RESOURCES
SUPPORTING INFECTIOUS DISEASE RESEARCH

## Product Information Sheet for HM-782D

### Genomic DNA from Microbial Mock Community B (Even, Low Concentration), v5.1L, for 16S rRNA Gene Sequencing

### Catalog No. HM-782D

### For research use only. Not for human use.

**Contributor and Manufacturer:**
Sarah K. Highlander, Associate Professor, Department of Molecular Virology and Microbiology; Baylor College of Medicine, Houston, Texas

**Product Description:**
HM-782D contains genomic DNA from 20 bacterial strains containing equimolar (Even) ribosomal RNA operon counts (100,000 copies per organism per µL). This mock community is recommended for 16S rRNA gene sequencing by Sanger or amplicon sequencing methods. The recommended amount to use per experiment is 1 µL.[1] The bacterial strains that DNA was extracted from are listed in Table 1.

Note: The label for HM-782D is incorrect. HM-782D contains genomic DNA from microbial mock community B and not microbial mock community A.

**Table 1. Microbial Mock Community B**

| Organism | NCBI Reference Sequence |
|---|---|
| *Acinetobacter baumannii*, strain 5377 | NC_009085 |
| *Actinomyces odontolyticus*, strain 1A.21 | NZ_AAYI02000000 |
| *Bacillus cereus*, strain NRS 248 | NC_003909 |
| *Bacteroides vulgatus*, strain ATCC® 8482™ | NC_009614 |
| *Clostridium beijerinckii*, strain NCIMB 8052 | NC_009617 |
| *Deinococcus radiodurans*, strain R1 (smooth) | NC_001263, NC_001264 |
| *Enterococcus faecalis*, strain OG1RF | NC_17316 |
| *Escherichia coli*, strain K12, substrain MG1655 | NC_000913 |
| *Helicobacter pylori*, strain 26695 | NC_000915 |
| *Lactobacillus gasseri*, strain 63 AM | NC_008530 |
| *Listeria monocytogenes*, strain EGDe | NC_003210 |
| *Neisseria meningitidis*, strain MC58 | NC_003112 |
| *Propionibacterium acnes*, strain KPA171202 | NC_006085 |
| *Pseudomonas aeruginosa*, strain PAO1-LAC | NC_002516 |
| *Rhodobacter sphaeroides*, strain ATH 2.4.1 | NC_007493, NC_007494 |
| *Staphylococcus aureus*, strain TCH1516 | NC_010079 |
| *Staphylococcus epidermidis*, FDA strain PCI 1200 | NC_004461 |
| *Streptococcus agalactiae*, strain 2603 V/R | NC_004116 |
| *Streptococcus mutans*, strain UA159 | NC_004350 |
| *Streptococcus pneumoniae*, strain TIGR4 | NC_003028 |

HM-782D has been qualified for PCR applications by amplification of approximately 1500 base pairs of the 16S ribosomal RNA gene.

**Material Provided:**
Each vial contains approximately 20 µL of the bacterial genomic DNA mixture suspended in Tris-HCl, pH ~ 7.5. The concentration is shown on the Certificate of Analysis. The vial should be centrifuged prior to opening.

**Packaging/Storage:**
HM-782D was packaged aseptically in screw-capped plastic cryovials. The product is provided frozen on ice and should be stored at -20°C or colder immediately upon arrival. Freeze-thaw cycles should be minimized.

**Citation:**
Acknowledgment for publications should read "The following reagent was obtained through BEI Resources, NIAID, NIH as part of the Human Microbiome Project: Genomic DNA from Microbial Mock Community B (Even, Low Concentration), v5.1L, for 16S RNA Gene Sequencing, HM-782D."

**Biosafety Level: 1**
Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, and National Institutes of Health. Biosafety in Microbiological and Biomedical Laboratories. 5th ed. Washington, DC: U.S. Government Printing Office, 2009; see www.cdc.gov/biosafety/publications/bmbl5/index.htm.

**Disclaimers:**
You are authorized to use this product for research use only. It is not intended for human use.

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**References:**

1. S. K. Highlander, Personal Communication.

ATCC® is a trademark of the American Type Culture Collection.

Support Provided by NIAID

**BEI Resources**
www.beiresources.org

E-mail: contact@beiresources.org
Tel: 800-359-7370
Fax: 703-365-2898

## 7.7. High-fat diet formulation used for PNA model

OpenSource DIETS®

**D12079B, 98121701 and 98052602**
**RD Western Diets**
**and Low Fat RD Western Diets With or Without Cholesterol**

Formulated by:
Research Diets, Inc.

| Product # | D12079B | | 98121701 | | 98052602 | |
|---|---|---|---|---|---|---|
| % | gm | *kcal* | gm | *kcal* | gm | *kcal* |
| Protein | 20 | *17* | 17 | *17* | 17 | *17* |
| Carbohydrate | 50 | *43* | 71 | *73* | 71 | *73* |
| Fat | 21 | *40* | 4 | *10* | 4 | *10* |
| Total | | *100* | | *100* | | *100* |
| kcal/gm | 4.68 | | 3.91 | | 3.91 | |
| | | | | | | |
| Ingredient | gm | *kcal* | gm | *kcal* | gm | *kcal* |
| | | | | | | |
| Casein, 80 Mesh | 195 | *780* | 195 | *780* | 195 | *780* |
| DL-Methionine | 3 | *12* | 3 | *12* | 3 | *12* |
| | | | | | | |
| Corn Starch | 50 | *200* | 404.4 | *1617.6* | 404.4 | *1617.6* |
| Maltodextrin 10 | 100 | *400* | 100 | *400* | 100 | *400* |
| Sucrose | 341 | *1364* | 341 | *1364* | 341 | *1364* |
| | | | | | | |
| Cellulose, BW200 | 50 | *0* | 50 | *0* | 50 | *0* |
| | | | | | | |
| Milk Fat, Anhydrous | 200 | *1800* | 0 | *0* | 0 | *0* |
| Corn Oil | 10 | *90* | 52.5 | *472.5* | 52.5 | *472.5* |
| Ethoxyquin | 0.04 | *0* | 0.04 | *0* | 0.04 | *0* |
| | | | | | | |
| Mineral Mix S10001 | 35 | *0* | 35 | *0* | 35 | *0* |
| Calcium Carbonate | 4 | *0* | 4 | *0* | 4 | *0* |
| | | | | | | |
| Vitamin Mix V10001 | 10 | *40* | 10 | *40* | 10 | *40* |
| Choline Bitartrate | 2 | *0* | 2 | *0* | 2 | *0* |
| | | | | | | |
| Cholesterol | 1.5 | *0* | 0 | *0* | 1.5 | *0* |
| | | | | | | |
| FD&C Yellow Dye #5 | 0 | *0* | 0.05 | *0* | 0 | *0* |
| FD&C Blue Dye #1 | 0 | *0* | 0.05 | *0* | 0 | *0* |
| FD&C Red Dye #40 | 0 | *0* | 0 | *0* | 0.1 | *0* |
| | | | | | | |
| **Total** | **1001.54** | ***4686*** | **1197.04** | ***4686.1*** | **1198.54** | ***4686.1*** |

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New Brunswick, NJ 08901 USA
info@researchdiets.com

RESEARCH DIETS INC.
www.ResearchDiets.com

## 7.8. Mock community data sheet, Zymo Research

ZYMO RESEARCH
*The Beauty of Science is to Make Things Simple*

# Genome and Composition

## ZymoBIOMICS™ Microbial Community Standard

Catalog Nos. D6300
Lot No.: ZRC183430

**Composition:** Table 1 shows the microbial composition, containing both the theoretical values and values measured by next generation sequencing techniques.

**Table 1: Microbial Composition**

| Species | Theoretical Composition (%) | Measured Composition[1] (%) |
|---|---|---|
| | Genomic DNA | Genomic DNA[2] |
| *Pseudomonas aeruginosa* | 12.0 | 14.7 |
| *Escherichia coli* | 12.0 | 12.6 |
| *Salmonella enterica* | 12.0 | 12.1 |
| *Lactobacillus fermentum* | 12.0 | 9.5 |
| *Enterococcus faecalis* | 12.0 | 12.6 |
| *Staphylococcus aureus* | 12.0 | 10.2 |
| *Listeria monocytogenes* | 12.0 | 12.6 |
| *Bacillus subtilis* | 12.0 | 12.3 |
| *Saccharomyces cerevisiae* | 2.0 | 1.9 |
| *Cryptococcus neoformans* | 2.0 | 1.5 |

[1] Prior to sequencing the DNA was extracted from the microbial standard using ZR Fungal/Bacterial DNA MiniPrep™ (Cat. No. D6005), after 5-minute bead bashing with FastPrep®-24.

[2] Shotgun sequencing was performed using the Illumina® MiSeq™ (2x150bp) and with sequencing library prepared with Kapa HyperPlus. The composition in terms of genomic DNA abundance was calculated by summarizing the raw sequencing reads mapped to the genome of each strain.

**Genome Information:** The 16S/18S rRNA sequences (fasta format) and genomes (fasta format) of these strains are available at: https://s3.amazonaws.com/zymo-files/BioPool/ZymoBIOMICS.STD.genomes.ZR160406.zip.

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