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## Effects of 5 $\alpha$ -Dihydrotestosterone (DHT) on Mouse Gut Microbiome– A Study of Sex Differences and Hormonal Effects on Gut Microbiome Composition

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Effects of 5 $\alpha$ -Dihydrotestosterone (DHT) on Mouse Gut Microbiome—  
A Study of Sex Differences and Hormonal Effects on  
Gut Microbiome Composition

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A Thesis  
Presented to  
The Graduate Faculty  
Central Washington University

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In Partial Fulfillment  
of the Requirements for the Degree  
Master of Science  
Biology

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by  
Bikesh Shrestha  
May 2019

CENTRAL WASHINGTON UNIVERSITY

Graduate Studies

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## ABSTRACT

# EFFECTS OF 5 $\alpha$ -DIHYDROTESTOSTERONE (DHT) ON MOUSE GUT MICROBIOME— A STUDY OF SEX DIFFERENCES AND HORMONAL EFFECTS ON GUT MICROBIOME COMPOSITION

by

Bikesh Shrestha

May 2019

Type 1 Diabetes (T1D) is a polygenic and multifactorial disease, traditionally attributed to genetic susceptibility and diet. Over the past decade, novel studies have placed a higher significance on the role of gut microbiome in T1D pathogenesis. Furthermore, diabetic mouse models have shown higher incidence of T1D in females compared to males, attributed to the differences in gut microbial community structure. Interestingly, female mouse models elicit male-like protection from T1D when transplanted with the male gut microbiome. In a previous study, we observed that female Non-obese diabetic (NOD) mice implanted with slow release 5 $\alpha$ -dihydrotestosterone (DHT) for 90 days showed improved glucose tolerance when compared to untreated females. We hypothesized that DHT treatment alters female gut microbial profile to resemble a male-like gut microbiome that induces improved glucose tolerance, a determinant of T1D protection. We compared the gut microbiome composition of DHT-treated female mice with placebo-treated females and age-matched males to identify and characterize changes in the gut microbiome. Extracted bacterial DNA from intestinal samples were subjected to 16S rRNA sequencing. Sequence reads were analyzed using MicrobiomeAnalyst and Piphillin, two web-based programs for phylogenetic and functional analysis. We identified a significant increase in *Bacteroides acidifaciens* in DHT-treated females, which can potentially improve

glucose tolerance and attenuate T1D. Additionally, we noticed strong similarity trends in the proportional composition of the most abundant taxa between DHT-treated females and age-matched males. Our study shows that DHT-treatment alters the female gut microbial profile to resemble a male-like microbiome and possibly induce improved glucose tolerance, a determinant of T1D protection.

**Keywords: Type 1 Diabetes, Dihydrotestosterone, Gut microbiome**

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## CHAPTER I

### INTRODUCTION AND LITERATURE REVIEW

The human body serves as complex ecosystem that supports a vast community of microorganisms. This microbial community is primarily dominated by bacteria colonizing different regions, including skin, urogenital tract, oral cavity, nasal cavity, and the gastrointestinal tract. The Human Microbiome Project funded by the National Institutes of Health (NIH) has revealed the presence of signature microbial communities among different niches in human beings, with some individual variations [1]. Such variations in abundance and diversity are mostly attributed to the founder effect (initial gut colonizers), environment, diet, host genetics and delivery mode during birth [1,2].

Among the different microbiome niches, the gastrointestinal tract is the largest and most functionally prominent. The gut microbiome contains at least ten times more cells and a hundred times more genes than its host [3,4]. The microbial density is the largest at the distal end of the digestive tract, with the colon housing up to  $10^{12}$  microbial cells per gram of fecal content [4]. Firmicutes and Bacteroidetes are the two most abundant bacterial phyla in the gut microbiome [3,4,5]. Bacteria belonging to the Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria and Actinobacteria phyla are also commonly observed in the human gut, but in smaller proportions [3]. Collectively, the human gastrointestinal tract contains up to a hundred trillion bacteria belonging to 500—1000 different species [5].

#### **Gut Microbiome Development**

The human fetus develops in a mostly sterile environment in the uterus and colonization of the gut begins primarily at birth. Based on the delivery mode, a newborn is exposed to a wide array of bacteria, which make up the primary gut colonizers. The gut microbiome in vaginally

born babies are primarily composed of *Prevotella*, *Lactobacillus* or *Sneathia* spp. These bacterial species are seeded orally during passage through the birth canal [6]. For those born through caesarean section, the initial gut colonizers consist of *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp, derived from the skin [7,8]. A newborn's intestine is an aerobic environment that supports oxygen-tolerant facultative anaerobes. Within days, strictly anaerobic bacteria take over as the intestinal lumen becomes anoxic. During the first few weeks of development, bacteria from the mother's skin and vaginal microbiome, belonging to families Enterococcaceae, Streptococcaceae, Clostridiaceae, Lactobacillaceae and Bifidobacteriaceae, colonize the infant's gut [2].

Food is another prominent modulator of the gut microbiome, acting as a source of entry and substrate for new colonizers. *Bifidobacterium* species are more prominent prior to weaning due to their oligosaccharide (sugar) fermenting traits. The gut microbiome undergoes successional changes until the age of 2—3 years and starts stabilizing with the introduction of solid food. Once solid food is introduced, the gut microbiome experiences an increase in *Bacteroides*, *Ruminococcus* and *Clostridium* species, and a decrease in milk oligosaccharide fermenters [2].

Microbial composition in infants prior to weaning starts with relatively low bacterial diversity but large inter-individual variations. As the infant gets older, the gut microbiome increases in diversity and decreases in inter-individual variations, as it is further shaped by food, environmental exposure and exposure to medications, such as antibiotics. A stable and mature adult-like gut microbiome composition is established around age 3. By age 7, the phyla Firmicutes and Bacteroidetes make up 90% of the bacterial composition in the gut [2,8]. A healthy and functionally significant gut microbiome community aids polysaccharide breakdown,

and helps to regulate immune and neural development, as well as hormonal functions and other metabolic activities [9].

### **Healthy Immune System and the Gut Microbiome**

The gut microbiome helps their hosts in synthesis of amino acids and vitamins, and in the processing of indigestible cellulosic compounds from plant polysaccharides [2] A mutualistic relationship exists between the gut microbiome and the host, whereby the microbiome gets a nutrient-rich environment while it regulates metabolic and homeostatic functions. Compositional shifts in the microbiome have been shown to have adverse effects on hosts' health [10].

A healthy immune system requires a healthy gut microbiome. A properly functioning immune system sustains a healthy microbiome while minimizing any risk of infection, controlling the balance between regulatory and inflammatory response. The mucosal immune system regulates anti-inflammatory activities by producing the secretory antibodies; secretory Immunoglobulin A and secretory Immunoglobulin B. Secretory antibodies regulate bacterial colonization in the gut and prevent colonization by harmful agents. [8,11]. Additionally, the gut microbiome regulates host immune cells and mediators. The early life microbiome provides the necessary stimuli for differentiation of cells and tissues in the immune system and plays crucial roles in the development of intestinal and systemic lymphoid tissue [12]. Comparative studies between mice without gut microbiome, termed Germ Free (GF) mice, and conventionally-raised mice have shown a significant decrease in gut mucus thickness in the absence of a gut microbiome. GF mice are axenic, specially raised to be devoid of all microorganisms and therefore lack a gut microbial community. Similarly, GF mice had lower blood vessel density, less stem cell differentiation, reduced antibody production, reduced production of antimicrobial

peptides in the mucus lining, and poorly developed lymphoid tissue [3,13]. A healthy gut microbiome and healthy immune system have a mutualistic relationship.

A healthy gut microbiome is also crucial for proper functioning of T cells. T cells, also called T lymphocytes, are essential part of our immune system with several subsets, each with a distinct function. One of its subsets, the Type 1 regulatory (Tr1) or regulatory (suppressor) T cells, are involved in minimizing T cell mediated immunity and suppressing autoreactive T cells. Tr1 cells suppress inflammation and regulate tolerance to self antigens. Shifts in the microbiome composition may alter this regulatory function and trigger erroneous inflammatory responses. Several autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis and T1D have been attributed to uncontrolled inflammatory responses brought on by an increase in another T cell subset, the T-helper cells (CD4<sup>+</sup> cells) and subsequent activation of killer T cell subset, the cytotoxic T cells (CD8<sup>+</sup> cells) [14,15]. T helper cells help activate cytotoxic T cells while regulatory T cells regulates tolerance to self antigens and autoimmune responses. A balance between T helper cells and regulatory T cells is vital in keeping erroneous activation of cytotoxic T cells in check and preventing autoimmune disease such as T1D [16].

### **Gut Microbiome and Diseases**

Metabolic diseases such as obesity and T1D are polygenic and multifactorial. While a number of gene variants are associated with them, environmental factors such as diet and lifestyle strongly influence disease progression. In addition to genetics, lifestyle and diet, the gut microbiome is another important factor that affects calorie intake and subsequently impacts the severity of metabolic diseases [9]. As we uncover the relationship between clinical parameters and the gut microbiome in disease pathogenesis, understanding microbial communities and their

functional implications for the host can provide better understanding of human health and disease progression.

A healthy gut microbiome requires a healthy diet. Studies have shown that the human population in developed Western nations has undergone shifts in gut microbiome diversity and composition, attributed to diet rich in sugar and processed foods, compared to rural populations that rely on a more traditional diet [17,18]. Scientists have observed gut microbial imbalance, termed dysbiosis, in many cases of inflammatory and immune-mediated diseases prevalent in Western populations including Crohn's disease, rheumatoid arthritis, ankylosing spondylitis, ulcerative colitis, and T1D [19,20,21,22].

### **Type 1 Diabetes**

Type 1 Diabetes (T1D), also known as insulin-dependent diabetes, is an autoimmune disease. It is characterized by the destruction of pancreatic  $\beta$  cells by cytotoxic T cells and other immune cells [4,16,23]. It is a consequence of immune regulation breakdown resulting from expansion of  $CD4^+$  and cytotoxic  $CD8^+$  cells, autoantibody producing B lymphocytes and the activation of the innate immune system, which collectively destroy the pancreatic  $\beta$  cells [16]. When  $\beta$  cells are destroyed, our body cannot produce enough insulin to regulate glucose levels in the blood stream.

Genetic and environmental factors are attributed to its etiology. Population studies and clinical studies in patients have indicated that T1D is associated with genes linked to the Major Histocompatibility complex (MHC), mainly in the Class II region. The gene complex Human Leukocyte Antigen system codes for the MHC proteins. Several MHC class II haplotypes have been associated with T1D susceptibility. Additionally, polymorphisms in the regulatory region of



the insulin gene, cytotoxic T lymphocyte-4 (CTLA-4) gene and other genes are associated with T1D susceptibility [24].

While more than 50 genes have been associated with T1D, the increased prevalence of T1D in recent years suggests that environmental factors, particularly influences from the gut microbiome, may play a larger role [5]. The continuous rise in the prevalence cannot be explained by genetic factors alone. The MHC class II haplotypes found in T1D patients can also be found in normal individuals [24]. The incongruous occurrence of T1D in only one member of monozygotic twins in another study further shows that T1D is heavily regulated by non-genetic factors [26]. Novel studies are examining the gut microbiome as a prominent non-genetic environmental modulator in T1D pathogenesis [5].

### **Non-Obese Diabetic Mouse—a T1D Model**

Non-obese Diabetic (NOD) mice are an animal model for T1D that develop spontaneous insulinitis by 5–6 weeks of age due to cell-mediated immunity. Insulinitis is the inflammation of the islet of Langerhans followed by destruction of  $\beta$  cells. NOD mice share similarities in T1D progression and traits with humans, including presence of pancreas specific autoantibodies, autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> cells and similar genetic linkage. The major genetic contributor to diabetes susceptibility in NOD mice is the MHC class II molecule [24].

T1D onset in NOD mice occurs at about 12–14 weeks of age in females and relatively later in males [46,58]. The disease onset is observed as early as 10 weeks of age in females with a cumulative incidence of 70%–80% by 30 weeks of age. In contrast, T1D in male NOD mice begins around 20 weeks of age with cumulative incidence of around 20% by age 30 weeks [24]. Interestingly, this difference in T1D incidence between the sexes in NOD mice is not observed in GF strains, pointing to the role of gut microbiome in eliciting protection from T1D [25]. Before

puberty, the gut microbial community does not differ significantly between male and female NOD mice. Post puberty, the male gut microbiome profile deviates while the female gut microbiome profile stays similar to that of young mice. Adult male and female NOD mice have different gut microbial composition, and the gut microbiome of castrated males is more similar to females than to non-castrated males [26]. Hormonal changes at puberty likely alter the gut microbiome composition and this change elicits the sex bias in T1D incidence. The transfer of the male gut microbiome from male NOD mice to GF female NOD mice caused elevated testosterone levels in the females [25].

Furthermore, identification and quantification of metabolic products in serum (serum metabolomics analysis) of the GF female NOD mice recipients of the male NOD mice gut microbiome showed lowered concentrations of sphingolipid and glycerophospholipid long-chain fatty acid in the serum, compared to control NOD females, indicating downstream metabolic changes triggered by the male gut microbiome transplantation. Such metabolic changes were not observed upon transfer of the control female NOD mice gut microbiome to GF female NOD mice recipients. This suggested that the metabolic outcome is determined by the sex of the gut microbiome donor. Blocking the androgen receptor (AR) signaling pathway using flutamide attenuated all male gut microbiome specific metabolic changes observed in female recipients. This suggests that elevated testosterone elicited metabolic changes upon male gut microbiome transfer to females.

Additionally, the same study quantified insulin specific autoantibodies (Aab) between different NOD mice treatment groups. Insulin specific Aab is an autoimmune phenotype in pre-diabetic NOD mice and in humans. Aab in female recipients of male gut microbiome was significantly lower than in unmanipulated females. Once again, this difference was attenuated in

female recipients of male gut microbiome treated with flutamide [25]. The study suggests that gut microbiome and androgens regulate each other through a reciprocal feedback mechanism, affecting the metabolome and autoimmune responses.

### **Gut Microbiome and T1D Pathogenesis**

Studies on both rodent models and human subjects have revealed correlation between gut dysbiosis and T1D progression (Table 1). In a T1D human study, bacteria from phylum Bacteroidetes increased proportionally in abundance in diabetic children while members of phylum Firmicutes increased over time in healthy infants. Also, the gut microbiomes in diabetic children were less diverse and differed more between patients, while healthy children had similar gut microbiome composition with higher diversity [27]. A similar cross-sectional study identified phylum level decreases in Actinobacteria and Firmicutes, and the corresponding Firmicutes-to-Bacteroidetes ratio in diabetic children compared to healthy children [28]. Similar compositional changes were also observed at the genus level with significantly reduced proportions of lactic acid-producing bacteria, mucin-degrading bacteria and butyrate-producing bacteria, all known to be essential to the maintenance of gut integrity [28]. Poor health corresponds with decreased diversity and reduced stability in the gut microbiome [27,34]. The current challenge is to identify gut microbiome markers associated with these metabolic diseases and define their causative roles.

Despite advancements in recent studies, we know very little about the role of the gut microbiome in T1D pathogenesis. While a number of correlations have been identified, we have yet to make progress in understanding the causal relationship. Current studies suggest that gut microbiome alters T1D pathogenesis through its effects on gut permeability as well as molecular mimicry of self antigens by bacterial proteins and metabolites. Additionally, gut microbiota

alters autoimmunity by modulating our immune system (Fig. 1). Various pathogen-associated molecular patterns such as lipopolysaccharides found in the gut microbiome can activate Toll-like receptors that can induce pro diabetogenic or anti-diabetogenic signals. Additionally, the gut microbiome can modulate the immune system by regulating T cells and their subsets [5].

Table 1

Alterations in the gut microbiome and its possible effects as listed in published studies

<b>Gut microbiome change</b>	<b>Model</b>	<b>Possible Effects</b>	<b>Reference</b>
Increase in <i>Dialister invisus</i> , <i>Gemella sanguinis</i> and <i>Bifidobacterium longum</i>	Human	Increased permeability	24
Decrease in <i>Bifidobacterium adolescentis</i> and <i>Bifidobacterium pseudocatenulatum</i> Increases in genus <i>Bacteroides</i>	Human	Increased permeability Decrease in lactate and butyrate producing species	30
Decrease in <i>Akkermandia</i> , <i>Prevotella</i> (mucin degraders) Decrease in <i>Faecalibacterium</i> , <i>Subdoligranulum</i> (butyrate producers) Increase in <i>Alistipes</i> , <i>Bacteroides</i> , <i>Veillonella</i> (producers of other Short-Chain-Fatty-Acids)	Human	Decreased intestinal integrity and increased gut permeability	15
Decreased abundance of <i>Staphylococci</i> and <i>Lactobacilli</i> in patients	Human	Limited ability to regulate proinflammatory response –	31
Increase in segmented filamentous bacteria in female	NOD mice	Stimulation of Th17 cells and inhibition of Th1 effector cells	32
Increase in <i>Fusobacteria</i>	NOD mice	Stimulation of CD8+ T cells	33
Decreased <i>Lactobacillus</i> , <i>Bryantella</i> , <i>Bifidobacterium</i> , and <i>Turibacter</i> and increased <i>Bacteroides</i> , <i>Eubacterium</i> and <i>Ruminococcus</i>	Rat	Possible role of gut flora in antigenic load in the intestine and subsequent effect on immune system.	34
Decrease in Firmicutes and increased Bacteroidetes	Human	Decreased diversity and reduced stability	28

In a human case-control study between 10 children at risk for T1D and 10 controls, a significant increase in *Dialister invisus*, *Gemella sanguinis* and *Bifidobacterium longum* were

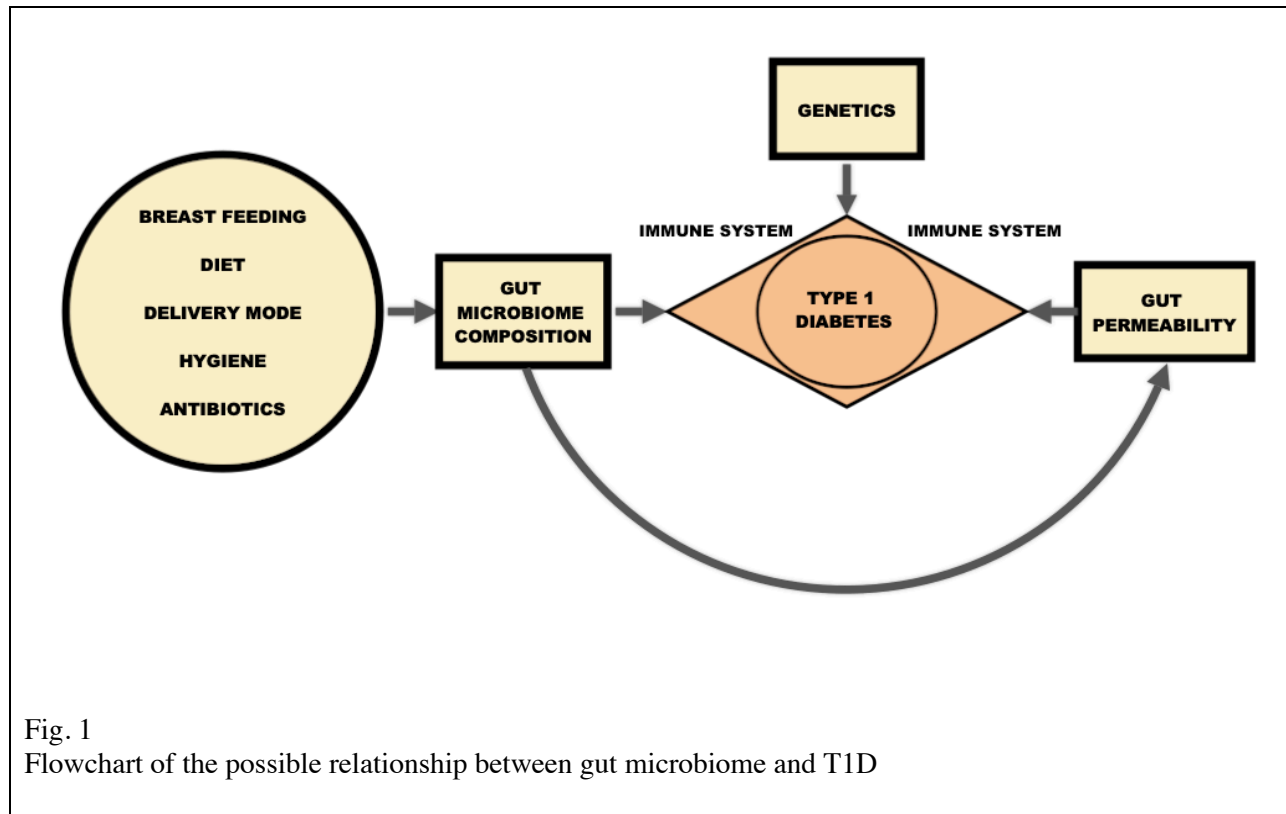


Fig. 1  
Flowchart of the possible relationship between gut microbiome and T1D

observed in T1D cases. This corresponded with significant increase in gut permeability detected through the lactulose/mannitol test [23]. While the relationship between the microbiome and gut permeability was not determined, this study suggests that T1D is associated with change in microbiome composition and increased gut permeability. A similar human study that compared the gut microbiome between children with at least two diabetes-associated autoantibodies and age and sex-matched autoantibody negative controls revealed that pancreatic  $\beta$ -cell autoimmunity is associated with lower abundance of lactate and butyrate-producing bacterial species [30]. Children with  $\beta$ -cell autoimmunity had increased abundance of *Bacteroides* bacteria and decreased *Bifidobacterium adolescentis* and *B. pseudocatenulatum*. *Bifidobacterium* species are known to produce butyrate [29].

The gut microbiome of healthy humans is composed of butyrate-producing and

mucin-degrading bacteria, while that of human diabetic patients is composed of producers of other short-chain-fatty-acids (SCFAs) such as succinate, acetate and propionate [13]. Butyrate is known to contribute to mucin synthesis, regulate tight junctions and maintain gut permeability, possibly helping in T1D regulation. Lactate can be further metabolized into butyrate. Mucin is a glycoprotein produced by mammals to maintain gut integrity. The presence of mucin degraders is an indicator of abundant mucin and a healthy gut [14].

While human studies are limited, studies on mouse models have fueled further exploration of relationships between the gut microbiome and T1D. Infecting the gut of NOD mice with the wild-type enteric pathogen *Citrobacter rodentium*, disrupted the intestinal epithelial barrier and accelerated insulinitis [35]. A modified strain of *C. rodentium* lacking the *Escherichia coli* secreted protein F, which is associated with virulence in *E. coli* strains, was incapable of disrupting the gut epithelial barrier. Infecting the mice with this modified strain did not disrupt gut integrity or affect insulinitis [35]. We know from recent studies that an altered gut microbiome is associated with T cell-mediated destruction of pancreatic  $\beta$  cells in T1D patients [32]. An increasingly permeable gut fails to prevent translocation of unwanted toxins, antigens and other infection factors into the intestinal mucosal components, possibly triggering an autoimmune reaction [36]. When gut integrity is compromised, CD8<sup>+</sup> T cells, in pancreatic lymphatic system become activated and proliferate, triggering insulinitis [5]. These Cytotoxic T cells (CD8<sup>+</sup>) are subsets of T cells that recognize and destroy infected cells and tumor cells in our body. They identify their target by binding to antigens presented by the infected cells. CD8<sup>+</sup> T cells are predominantly involved in pancreatic islet infiltration and subsequent  $\beta$  cell destruction in T1D in both human and NOD mice [37]. Recent studies have attempted to bridge the gap in understanding the role of gut microbiome in regulation of CD8<sup>+</sup> T cell-mediated autoimmunity.

Certain transporter protein peptides expressed by *Leptotrichia goodfellowii*, a member of the phylum Fusobacteria, directly mimic CD8<sup>+</sup> T cell antigens and stimulate the immune cells and thus accelerate insulinitis in NOD mice [32]. Since T cell antigen recognition is nonspecific, the gut microbiome can present a lot of potential antigens that can mimic CD8<sup>+</sup> T cell antigens and accelerate diabetes [32]. Molecular mimicry of T cell antigens is one of the proposed mechanisms of diabetes progression induced by the gut microbiome.

In addition to CD8<sup>+</sup> T cells, certain gut bacteria have been known to regulate other T cell subsets and their functions. One such subset is CD4<sup>+</sup> T helper cells, which helps suppress and regulate different immune responses. The crosstalk between CD4<sup>+</sup> T helper cells and intestinal microbiota helps regulate immune response during homeostasis and inflammation. *Listeria monocytogenes* can induce T helper type 1 (Th1) response and segmented filamentous bacteria regulate T helper type 17 (Th17) response [5,38]. Th1 cells help promote macrophage activation and CD8<sup>+</sup> T cell proliferation in response to microbial pathogens. Similarly, Th17 cells are pro-inflammatory and play similar defensive role against extracellular pathogens by recruiting macrophages to infected tissue [39]. Aberrant regulation of CD4<sup>+</sup> T helper cells are associated with several autoimmune disorders [40]. A healthy crosstalk between CD4<sup>+</sup> T helper cells and intestinal microbiota is essential for immune homeostasis.

Tr1 cells minimize T cell mediated immunity and suppress autoreactive T cells. Changes in the gut microbiome can increase the number of Tr1 cells in the intestine. Tr1 cells can inhibit activation of effector T cells, decreasing incidence of diabetes [5,12]. Although the exact mechanism for the gut microbiome to regulate proliferation and activation of certain T cell subsets is yet to be understood, SCFAs secreted by the microbes seem to exert important roles. Comparative studies between Specific Pathogen Free (SPF) and Germ Free (GF) mice show that

SPF mice have significantly higher SCFAs such as butyrate and propionate produced as metabolites in the gut. These SCFAs induce increased T regulatory cell generation and accumulation in the colon and decreased pro-inflammatory Th17 cell production [5,15]. It is evident that the gut microbiome and their metabolites cross talks with the immune system to maintain homeostasis and any dysbiosis may possibly result in inflammation and autoimmune disorders.

### **Variation in Gut Microbiome Along the Gastrointestinal Tract**

Many recent studies and observations from human and rodent models indicate possible relationships between gut dysbiosis and T1D. In particular, scientists have looked into the abundance of two phyla, Firmicutes and Bacteroidetes, and the Firmicutes/Bacteroidetes ratio as a possible indicator of diabetes. T1D is reportedly associated with a decreased proportion of Firmicutes and Actinobacteria and increased proportion of Bacteroidetes in fecal bacteria [27,28]. Most studies on T1D and gut microbiome rely on gut microbial data obtained from fecal samples. The bacterial composition in fecal samples may not fully represent the gut microbiome composition. The gut microbiome along the gastrointestinal (GI) tract in rodent models have regionally distinct profiles along the tract and distinct alterations between control and T1D patients [41] A study on GI tract of C57BL/6 mice showed that *Lactobacillaceae* are more abundant in proximal gut while the distal gut is primarily inhabited by bacteria belonging to family *Ruminococcaceae*, *Lachnospiraceae*, *Rikenellaceae*, *Prevotellaceae* and *Bacteroidaceae* [42]. This clearly suggests variations in gut microbial composition along the GI tract. The core gut microbiome differs along the GI tract due to differences in selective pressures within their physiochemical conditions. Cecum, colon and fecal samples share more similarity and differ from stomach and small intestine gut microbiome composition [42]. Since gut microbiome



composition varies along the GI tract, we should pay special attention to what samples we use for microbiome related study.

A previous study on rats reported that bacterial species from human duodenum instilled in the pancreatic ductal system induced rapid cellular infiltration and subsequent B cell destruction [43]. With shared blood supply and close functional relationship with the pancreas, analysis of small intestine samples offers a better probe into relationship between gut microbiome and T1D. Hence, it only seems logical that we look into the small intestine microbiome in more depth than merely find correlation between fecal data and T1D.

### **Androgens and T1D in NOD Mice**

Castration increases while oophorectomy decreases T1D in NOD mice [44,45]. Previous studies have confirmed significant reduction in T1D occurrences in female NOD mice treated with sub-cutaneous DHT implants (15mg) over a period of 60 days. DHT is a reduced form of testosterone and a more potent agonist of AR, that cannot be converted to estrogen. When spleen cells from non-treated female NOD mice were transferred to DHT-treated females, the protective effects of DHT was diminished with increased incidence of diabetes at an earlier age [46].

The androgen-dependent attenuation diabetes in NOD mice is due to the alteration of gut microbiome [25]. Androgens support expansion of certain microbial composition, forming a positive feedback mechanism that contributes to sexual dimorphism in T1D incidence. There is a clear relationship between alteration in gut microbiome during puberty and T1D incidence in NOD mice, with suppressed autoimmunity in males. Sex specific microbiome difference due to androgens in males induces metabolite changes and changes in serum androgen level that opposes T1D pathogenesis [25]. T1D in human is not sex biased since the peak onset of T1D precedes puberty.

## Approaches to Investigating the Microbiome

There are two common approaches to studying the gut microbiome, diversity analysis and functional analysis (metagenomics) (Fig. 2). Diversity analysis compares the different taxonomic groups identified through 16S rRNA sequencing. Metagenomics is a more expensive technique that sequences every gene from a sample to reveal the biological functions of the entire community. Alternatively, we can use 16S rRNA data to infer metagenomic content by utilizing available genome databases. Recent studies have shown high accuracies in predicting metagenomic content by matching OTU sequences to its nearest-neighbor genome [47,48].

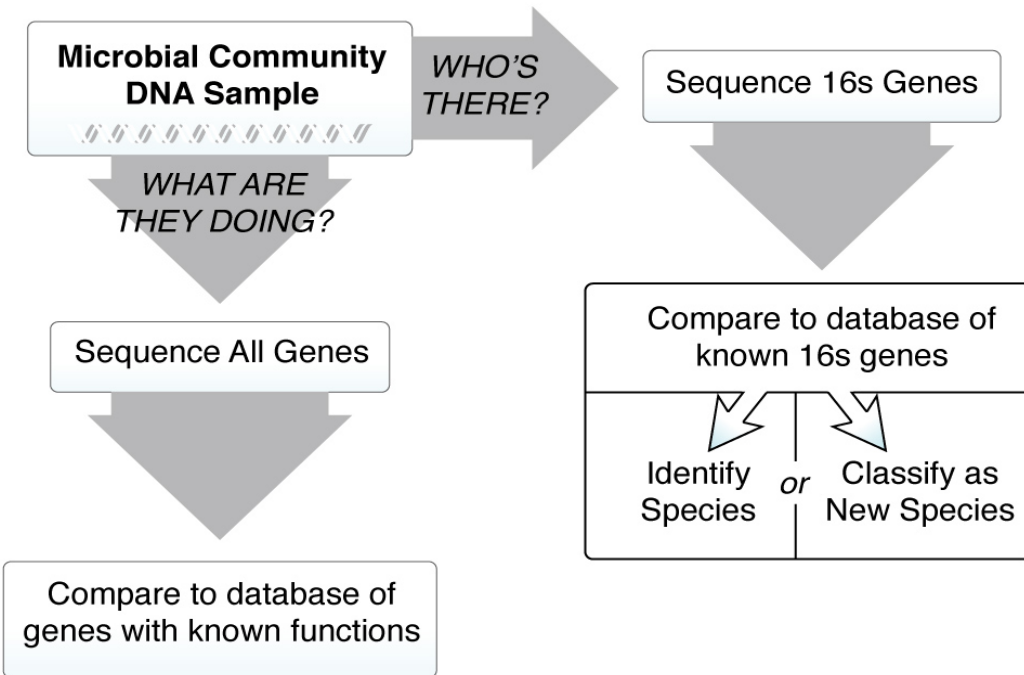


Fig. 2  
Microbiome study flowchart [49]

## 16S Ribosomal RNA (rRNA) Sequencing

The use of the 16S rRNA gene as a marker gene for taxonomic assignments and phylogenetic analysis in a microbial community has become a common practice among the scientific community. The 16S rRNA gene is the preferred marker gene to study phylogeny for three main reasons: it is present in almost all bacteria; its function is mostly conserved, any random sequence change is an accurate measure of evolution; and it is large enough for informatics analysis (1500 bp) [50]. The 16S sequence consists of nine hypervariable regions that are separated by nine conserved regions [51,52]. For phylogenetic analysis, most studies use partial sequences of individual variable regions instead of sequencing the entire gene [53,54,55]. The 16S rRNA fourth variable (V4) region (Fig. 3) provide a reliable measure to represent full length 16S rRNA sequence in phylogenetic analysis of bacteria [56].

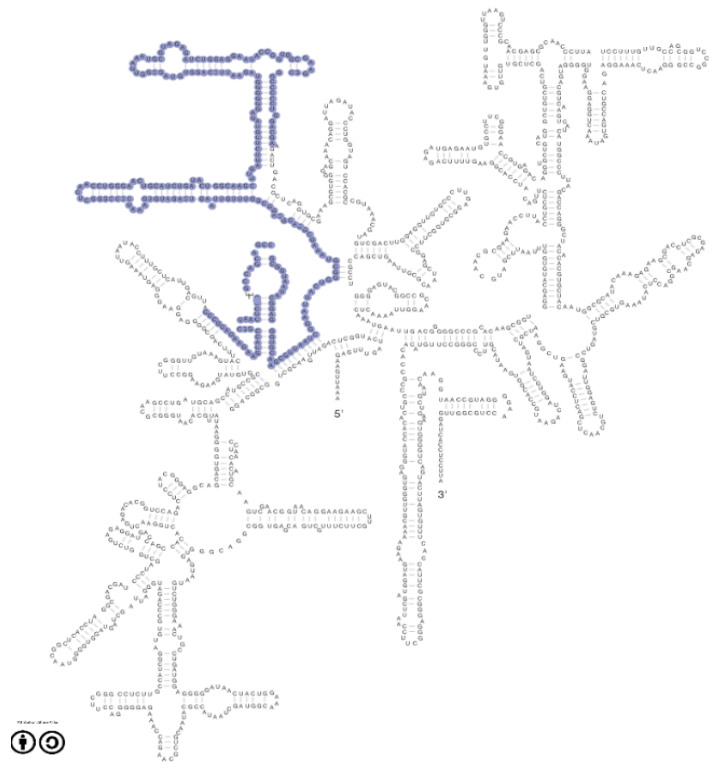


Fig. 3  
16S reference sequence from *Escherichia coli* with V4 region (highlighted in blue) [57]

## **Metagenomic Inference with Piphillin**

16S rRNA data can provide information on microbial community structure. However, functional analysis is necessary to understand the biological implications of the microbial community. Although shotgun metagenomic sequencing allows comprehensive quantification of functional genes and possibly interference of their roles in the community, this usually tend to be rather expensive and technologically challenging. Novel computational algorithms are now being utilized to employ 16S rRNA sequence data to predict metagenomic content with a high level of accuracy. This is achieved by matching identified OTUs with the nearest sequenced genome from available database, such as KEGG (Kyoto Encyclopedia of Genes and Genomes) [48,49].

Piphillin is one such web-based algorithm that can predict metagenome by using nearest-neighbor matching between 16S rRNA sequences and genome reference database. It searches OTU sequences against the genome references to generate a genome abundance table. The inferred genome contents are summed to generate the total metagenomic content of the sample. This metagenomic contents are expressed in terms of KEGG Orthology (KO) [49]. Each KO entry obtained, identified by the K number, represents computational prediction of cellular processes and functions of the community, based on genomic information [56].

## **Data Analysis with MicrobiomeAnalyst**

MicrobiomeAnalyst is a user-friendly web-based tool that offers comprehensive statistical analysis, visual exploration and integration of microbiome data. Using 16S rRNA gene data it offers compositional profiling through common statistical methods for different alpha and beta diversity analyses at different taxonomic levels. Alpha diversity analysis allows us to investigate species abundance and richness within a particular treatment group. Beta diversity analysis allows us to compare variation in species distribution among different treatment groups.

The program also supports functional profiling by predicting metabolic potential. It supports data filtering and normalization technique coupled with differential analysis methods for comparative analysis. This is a powerful tool that supports many common data formats and offers many statistical and visual analysis tools for diversity and functional analysis of the microbial community [59].

### **Significance and Potential Therapeutic Applications**

Millions of people around the world live with T1D and obesity. While there are several management options for these metabolic diseases, there are still no cures. Recent studies have shown that the interaction between hormones and gut microbiome modifies the T1D progression whereby males are more protected. The regulatory relationship between the gut microbiome and hormones in disease progression cannot be simply discarded. Identifying the relationship between gut microbiome, hormones and metabolic diseases has important health implications in disease diagnosis and treatments.

Once we develop a clearer understanding, we can look into probiotics as potential tools to improve gut integrity in T1D. Furthermore, we can explore antigen specific therapies that target beta cell reactive T cells, without affecting our immune system. The use of probiotics to improve gut integrity and using engineered bacterial strain to transport auto-antigen to induce tolerance are some possible methods to help attenuate T1D.

By understanding the relationship between gut microbiome, hormones and their metabolic effects, we can learn how they play a role in regulating health and disease. Furthermore, understanding the sex-difference in microbiome composition will help us understand gender-associated differences in diseases and identify high risk populations. Understanding the underlying microbial reason behind such dimorphisms and disease prognosis

can open the possibilities of non-invasive microbial therapy techniques, such as the use of genetically modified probiotics and effective vectors of auto-antigens, in disease treatments. Once we understand the regulatory mechanism, we can potentially alter the gut microbiome make up to elicit disease protection and support better health. What we know so far is just the tip of the iceberg. With new evidence emerging, we now know that microbiome alterations are causal factors in disease progression and not merely a consequence of disease.

## CHAPTER II

### JOURNAL ARTICLE

#### **Sub-cutaneous dihydrotestosterone (DHT) implants in female non-obese diabetic (NOD) mice alters the gut microbiome**

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#### **Abstract**

**Background:** Non-obese Diabetic (NOD) mice are a polygenic model for Type 1 Diabetes (T1D) research that spontaneously develop the disease. T1D is polygenic and multifactorial, traditionally attributed to genetic susceptibility and diet. Novel studies have alluded to the role of gut microbiome in T1D pathogenesis. Interestingly, NOD mice have shown higher incidence of T1D in females compared to males, attributed to the sex specific gut microbial composition. In a previous study, female NOD mice implanted with slow release 5 $\alpha$ -dihydrotestosterone (DHT) pellets for 90 days showed improved glucose tolerance compared to placebo-treated females. In order to investigate whether a difference in gut microbiome existed in these mice, we compared the gut microbiome composition of DHT-treated females with placebo-treated females and age-matched males. Samples from the small intestine were used for DNA analysis, utilizing the 16S rRNA sequences to determine species richness and diversity, and infer metabolic potential of the respective microbial communities.

**Results:** We identified significant increases in *Bacteroides acidifaciens* in DHT-treated females. Additionally, males and DHT-treated females showed strong similarity trends in the proportional composition of the most abundant taxon.

**Conclusion:** Our study shows that DHT treatment alters the female gut microbial profile to resemble a male-like microbiome which may induce improved glucose tolerance, a determinant

of T1D protection.

## **Keywords**

DHT, Gut microbiome, NOD mice, Type 1 Diabetes

## **Background**

The human body serves as an ecosystem to a diverse microbiome, which colonizes different regions such as the skin, urogenital tract, oral cavity, nasal cavity and the gastrointestinal tract. Among the different microbiome niches, the gastrointestinal tract is the largest and most functionally prominent. The gut microbiome contains at least ten times more cells and a hundred times more genes than its host [3,4]. This microbial density is the largest at the distal end of the digestive tract, with the colon housing up to  $10^{12}$  microbial cells per gram of fecal content, primarily dominated by two bacterial phyla: Bacteroidetes and Firmicutes [3,5]. Collectively, the human gastrointestinal tract contains up to a hundred trillion bacteria belonging to 500–1000 different species [5].

The gut microbiome helps their hosts in the synthesis of amino acids and vitamins, and processing indigestible cellulosic compounds from plant polysaccharides [2]. They share a mutualistic relationship whereby the microbiome gets a nutrient rich environment while it regulates certain metabolic and homeostatic functions. Compositional shifts in the microbiome adversely affects the host's health [10]. Gut microbial imbalance (dysbiosis) has been associated with several inflammatory and immune-mediated diseases prevalent in Western populations, including Type 1 diabetes (T1D) [19,21,22].

T1D, also known as juvenile diabetes or insulin-dependent diabetes, is an autoimmune disease characterized by the destruction of pancreatic  $\beta$  cells by cytotoxic T cells and other immune cells [6,17]. It is a consequence of immune regulation breakdown resulting from



expansion of CD4<sup>+</sup> and cytotoxic CD8<sup>+</sup> cells, autoantibody producing B lymphocytes and the activation of the innate immune system, which collectively destroy the pancreatic  $\beta$  cells [16]. When  $\beta$  cells are destroyed, the body cannot produce enough insulin to regulate glucose levels in the blood stream.

Genetic and environmental factors are attributed to its etiology. Population studies and clinical studies in patients have indicated that T1D is associated with genes linked to the Major Histocompatibility complex (MHC), mainly in the Class II region. The gene complex, Human Leukocyte Antigen system codes for the MHC proteins. Several MHC class II haplotypes have been associated with T1D susceptibility [24]. Additionally, polymorphisms in the regulatory region of insulin gene, cytotoxic T lymphocyte-4 (CTLA-4) gene and other genes are associated with T1D susceptibility [24]

The continuous rise in the T1D prevalence in recent years cannot be explained by genetic factors alone. While more than 50 associated genes have been identified, such increased prevalence suggests that environmental factors may play a bigger role [5]. The MHC class II haplotypes found in T1D patients can also be found in normal individuals [24]. Novel studies in the Non-Obese Diabetic (NOD) mouse are looking into gut microbiome as a prominent non-genetic environmental modulator in T1D progression [25,26].

NOD mice are animal model for T1D that develop spontaneous insulinitis by 5–6 weeks of age, due to cell mediated immunity. Insulinitis is the inflammation of the islet of Langerhans followed by destruction of insulin producing  $\beta$  cells, characterized as T1D. NOD mice share similarities in T1D progression and traits with humans, including presence of pancreas-specific autoantibodies, autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells and similar genetic linkage [61]. The major genetic contributor to diabetes susceptibility in NOD mice is the MHC class II molecule [24].

T1D onset in NOD mice occurs at about 12–14 weeks of age in females and relatively later in males [46,60]. The disease onset is observed as early as 10 weeks of age in females with a cumulative incidence of 70%–80% by 30 weeks of age. In contrast, T1D in male NOD mice begins around 20 weeks of age with cumulative incidence of around 20% by age 30 weeks [24]. Interestingly, this difference in T1D incidence between the sexes in NOD mice is not observed in Germ Free (GF) strains (axenic mice with no microorganisms in the gut), pointing to the role of gut microbiome in eliciting protection from T1D [25].

Before puberty, the gut microbial community does not differ significantly between male and female NOD mice. Post puberty, the male gut microbiome profile deviates while the female gut microbiome profile stays similar to that of young mice. Adult male and female NOD mice have different gut microbial composition, but the gut microbiome of castrated males is more similar to females than to non-castrated males [26]. Hormonal changes at puberty likely alter the gut microbiome composition and this change elicits the sex bias in T1D incidence. The transfer of the male gut microbiome from male NOD mice to GF female NOD mice caused elevated testosterone levels in the females. This increase in testosterone correlated with increase in T1D protection in the GF female NOD mice [25].

Furthermore, identification and quantification of metabolic products in serum (serum metabolomics analysis) of the GF female NOD mice recipients of the male NOD mice gut microbiome showed lowered concentrations of sphingolipid and glycerophospholipid long-chain fatty acid compared to control NOD females, indicating downstream metabolic changes triggered by the male gut microbiome transplantation. Such metabolic changes were not observed upon transfer of the control female NOD mice gut microbiome to GF female NOD mice recipients. This suggested that the metabolic outcome is determined by the sex of the gut microbiome

donor. Blocking the AR signaling pathway using flutamide attenuated all male gut microbiome specific metabolic changes observed in female recipients. This suggests that elevated testosterone elicited metabolic changes upon male gut microbiome transfer to females.

Additionally, the same study also quantified insulin specific autoantibodies (Aab) between different NOD mice treatment groups. Insulin-specific Aab is an autoimmune phenotype in pre-diabetic NOD mice and in humans. Aab in female recipients of male gut microbiome was significantly lower than in unmanipulated females. Once again, this difference was attenuated in female recipients of male gut microbiome treated with flutamide [25]. The study suggests that gut microbiome and androgens regulate each other through a reciprocal feedback mechanism, affecting the metabolome and autoimmune responses. Previous studies have confirmed a significant reduction in T1D occurrences in female NOD mice treated with sub-cutaneous DHT implants (15mg) over a period of 60 days [46]. It is hypothesized that androgen-dependent attenuation diabetes in NOD mice is due to the alteration of gut microbiome [25].

NOD mice share many immunological and genetic traits with the human form of the disease and spontaneously develop diabetes. In a previous study, it was observed female NOD mice, implanted with slow release DHT pellets for 90 days at 19–20 days old, showed improved glucose tolerance, a determinant of improved T1D [58]. We used 16S rRNA sequence data of the gut microbial community using intestine samples preserved from that study to assign operational taxonomic unit (OTUs) and categorize them into taxonomic units. We then compared the gut microbiome composition and diversity between DHT-treated female NOD mice, placebo-treated female NOD mice and age-matched male NOD mice to identify the effect of increased androgen concentration on the gut microbiome composition. Furthermore, we looked

at the differences in potential metabolic pathways between the three treatment groups through functional analysis. We hypothesized that the DHT-treated female gut microbiome would be more similar to the male gut microbiome than to the placebo-treated female gut microbiome, both in terms of diversity and composition, and in functional profile.

## **Results**

### **Phylum Level Analysis:**

The four most abundant phyla in our samples were *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*. Although the proportion of different phyla varied among individuals, we noticed a clear trend in proportional distribution of the two most abundant phyla between the treatment groups (Fig. 4). The mean percent distribution of *Firmicutes* in DHT-treated females and untreated males was 43.5 % and 54.5 % respectively while placebo-treated females had a higher percentage of *Firmicutes* at 62.73 %. Conversely, the mean percent distribution of *Bacteroidetes* in males and DHT-treated females was 45.6 % and 42.2 % respectively while placebo treated females had a lower *Bacteroidetes* proportion of 28.8 % (Table 2). Although we could not identify any statistical significance ( $p \leq 0.05$ ), we noticed a trend of higher similarity between males and DHT-treated females than between DHT-treated females and placebo-treated females. We also noticed a slight (but non-significant) increase in *Proteobacteria* in DHT-treated females.

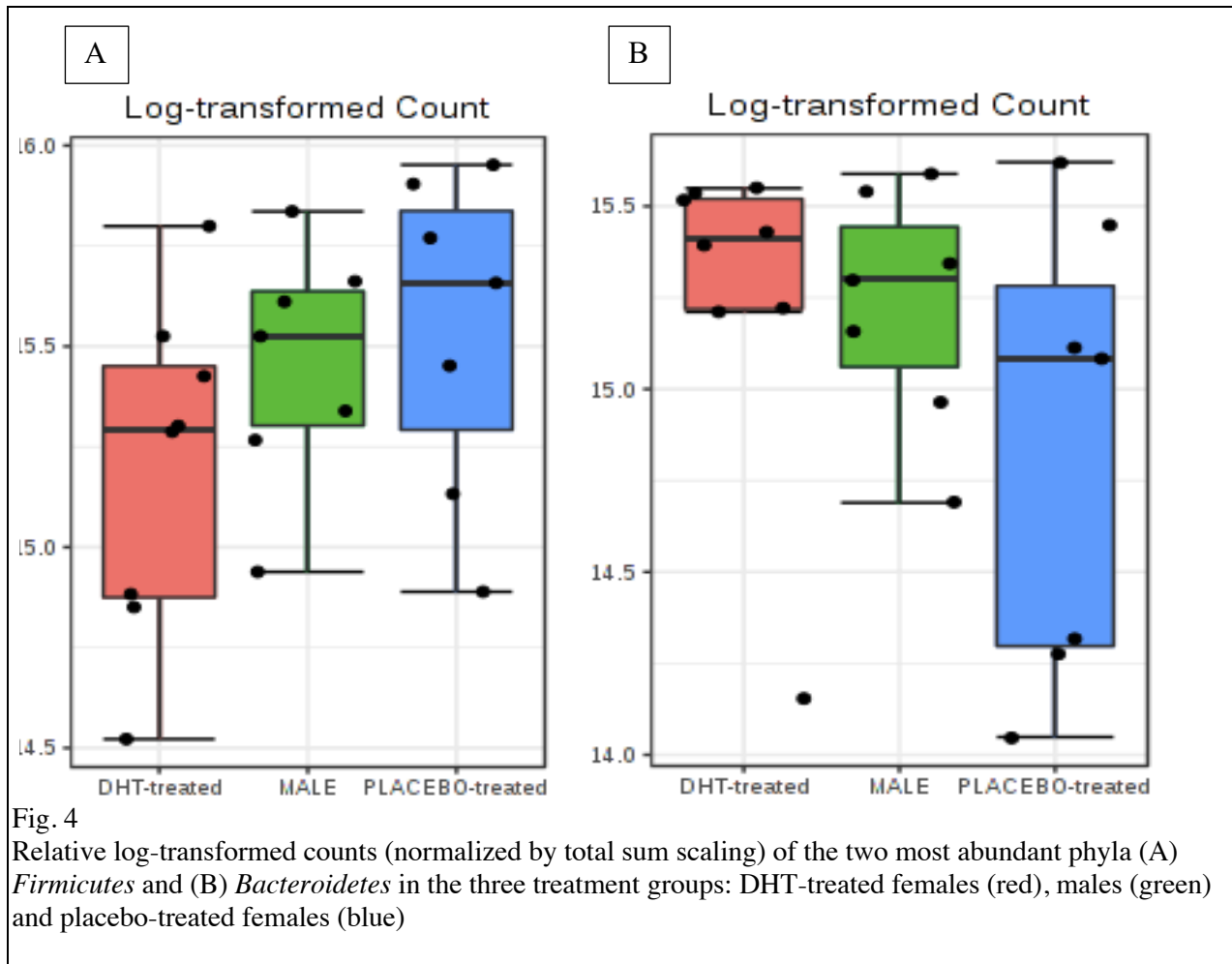


Fig. 4 Relative log-transformed counts (normalized by total sum scaling) of the two most abundant phyla (A) *Firmicutes* and (B) *Bacteroidetes* in the three treatment groups: DHT-treated females (red), males (green) and placebo-treated females (blue)

Table 2 Relative mean proportional abundance (in percent) of all phyla identified in DHT-treated females, males and placebo-treated females.

S No.	PHYLUM	DHT-treated	MALE	PLACEBO-treated	p-value
1	<i>Firmicutes</i>	43.25	54.50	62.73	0.21
2	<i>Bacteroidetes</i>	45.60	42.22	28.92	0.28
3	<i>Proteobacteria</i>	9.63	2.09	5.36	0.13
4	<i>Actinobacteria</i>	1.48	0.99	2.96	0.35
5	<i>Tenericutes</i>	0.005	0.16	0.008	0.08
6	<i>Verrucomicrobia</i>	0.024	0.018	0.006	0.49
7	<i>Cyanobacteria</i>	0.007	0.0033	0.013	0.58
8	<i>Thermotogae</i>	0.0023	0.00097	0.0013	0.62

## Class Level Analysis:

We identified 15 classes in our samples. The two most abundant classes were *Bacilli* and *Bacteroidia* (Fig. 5). Class *Bacilli* was the most abundant class in placebo-treated females (non-significant) while *Bacteroidia* was the most abundant class in males (non-significant) and DHT-treated females. Class *Erysipelotrichia* was significantly more abundant in males than in placebo-treated and DHT-treated females (Table 3).

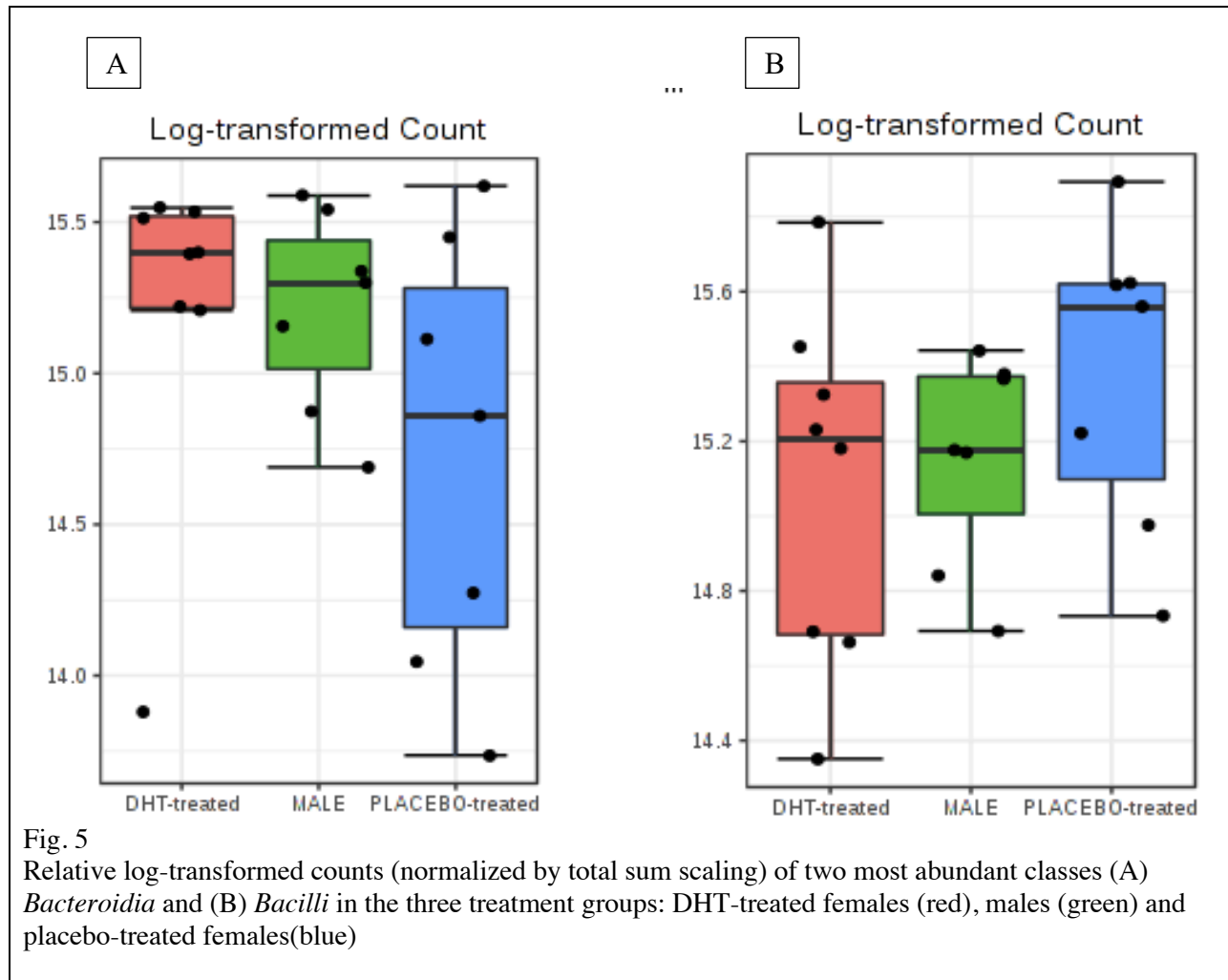


Table 3

Relative mean proportional abundance (in percent) of all classes identified in DHT treated-females, males and placebo-treated females. \* represents significance ( $p \leq 0.05$ ; two-way ANOVA). Mean values not sharing a letter within each row are significantly different from each other ( $p \leq 0.05$ ; Tukey's test). Mean values sharing letters or without letters are non-significant within the row.

S No.	CLASS	DHT-treated	MALE	PLACEBO-treated	p-value
1	<i>Bacilli</i>	39.58	41.78	56.93	0.31
2	<i>Bacteroidia</i>	44.95	41.49	26.86	0.24
3	<i>Clostridia</i>	3.36	11.58	5.56	0.09
4	<i>Deltaproteobacteria</i>	8.51	1.713	4.01	0.18
5	<i>Actinobacteria</i>	1.48	0.999	2.96	0.35
6	<i>Flavobacteriia</i>	0.60	0.72	2.04	0.32
7	<i>Gammaproteobacteria</i>	0.98	0.32	1.26	0.12
8	<i>Erysipelotrichia</i>	0.29 <sup>a</sup>	0.96 <sup>b</sup>	0.16 <sup>a</sup>	<b>0.031*</b>
9	<i>Alphaproteobacteria</i>	0.05	0.20	0.097	0.57
10	<i>Betaproteobacteria</i>	0.11	0.028	0.076	0.02
11	<i>Mollicutes</i>	0.004	0.16	0.008	0.083
12	<i>Cytophagia</i>	0.045	0.013	0.022	0.23
13	<i>Verrucomicrobiae</i>	0.024	0.018	0.006	0.49
14	<i>Cyanobacteria</i>	0.007	0.003	0.013	0.58
15	<i>Thermotogae</i>	0.0023	0.0009	0.0012	0.62

### Order Level Analysis:

We identified 22 different orders with *Lactobacillales*, *Bacteroidales* and *Clostridiales* as the most abundant groups. *Lactobacillales* was more abundant (non-significant) in placebo-treated females while *Bacteroidales* was more abundant (non-significant) in males and DHT-treated females (Fig. 6). *Clostridiales* was more abundant (non-significant) in males (Table 4). Although in small proportion, *Erysipelotrichales* was significantly more abundant in males than in placebo-treated females and DHT-treated females.

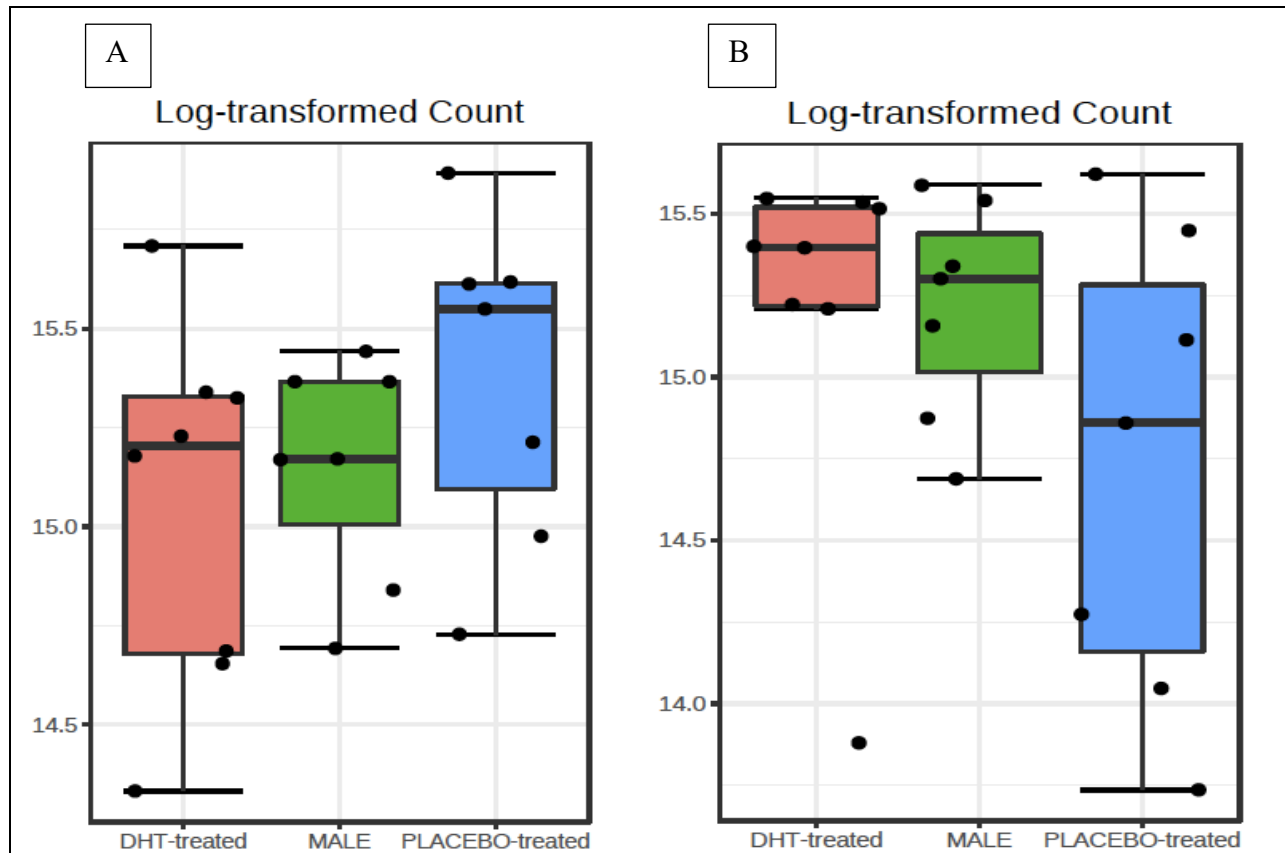


Fig. 6  
Relative log-transformed counts (normalized by total sum scaling) of two most abundant orders (A) *Lactobacillales* and (B) *Bacteroidales* in the three treatment groups: DHT-treated females (red), males (green) and placebo-treated females (blue)

Table 4  
Relative mean proportional abundance (in percent) of the five most abundant orders (1–5) and significantly different order (6) identified in DHT-treated females, male and placebo-treated females. \* represents significance ( $p \leq 0.05$ ; two-way ANOVA). Mean values not sharing a letter within each row are significantly different from each other ( $p \leq 0.05$ ; Tukey's test). Mean values sharing letters or without letters are non-significant within the row.

S No.	ORDER	DHT-treated	PLACEBO-treated	MALE	Significance
1	<i>Lactobacillales</i>	38.58	56.74	41.58	0.25
2	<i>Bacteroidales</i>	44.95	26.86	41.49	0.24
3	<i>Clostridiales</i>	3.36	5.56	11.58	0.09
4	<i>Desulfovibrionales</i>	8.51	4.01	1.71	0.18
5	<i>Actinomycetales</i>	1.05	2.73	0.46	0.29
6	<i>Erysipelotrichales</i>	0.29 <sup>a</sup>	0.16 <sup>a</sup>	0.96 <sup>b</sup>	<b>0.03*</b>



### Family Level Analysis:

We identified 42 families in our analysis. *Lactobacillaceae*, *Porphyromonadaceae* and *Bacteroidaceae* were the most abundant families. *Lactobacillaceae* was the most abundant family in placebo-treated females (non-significant) while *Porphyromonadaceae* and *Bacteroidaceae* were more abundant in males and DHT-treated females (non-significant) (Fig. 7). Bacteria belonging to family *Ruminococcaceae* were significantly higher in males compared to DHT-treated and placebo-treated females. *Erysipelotrichaceae* was significantly higher in males compared to placebo treated females. *Clostridiales* was significantly higher in males compared to DHT-treated females (Table 5).

Table 5

Relative mean proportional abundance (in percent) of the five most abundant family (1—5) and significantly different families (6—8) identified in DHT-treated females, males and placebo-treated females. \* represents significance ( $p \leq 0.05$ ; two-way ANOVA). Mean values not sharing a letter within each row are significantly different from each other ( $p \leq 0.05$ ; Tukey's test). Mean values sharing letters or without letters are non-significant within the row.

S No.	FAMILY	DHT-treated	MALE	PLACEBO-treated	Significance
1	<i>Lactobacillaceae</i>	35.04	28.73	53.12	0.10
2	<i>Porphyromonadaceae</i>	21.22	23.47	16.06	0.43
3	<i>Bacteroidaceae</i>	23.72	17.94	10.61	0.28
4	<i>Streptococcaceae</i>	2.28	12.55	3.01	0.11
5	<i>Desulfovibrionaceae</i>	8.51	1.71	4.01	0.18
6	<i>Ruminococcaceae</i>	0.25 <sup>a</sup>	1.06 <sup>b</sup>	0.16 <sup>a</sup>	0.004*
7	<i>Erysipelotrichaceae</i>	0.29 <sup>a</sup>	0.96 <sup>b</sup>	0.16 <sup>a</sup>	0.03*
8	<i>Clostridiales</i>	0.0008 <sup>a</sup>	0.07 <sup>b</sup>	0.01	0.02*

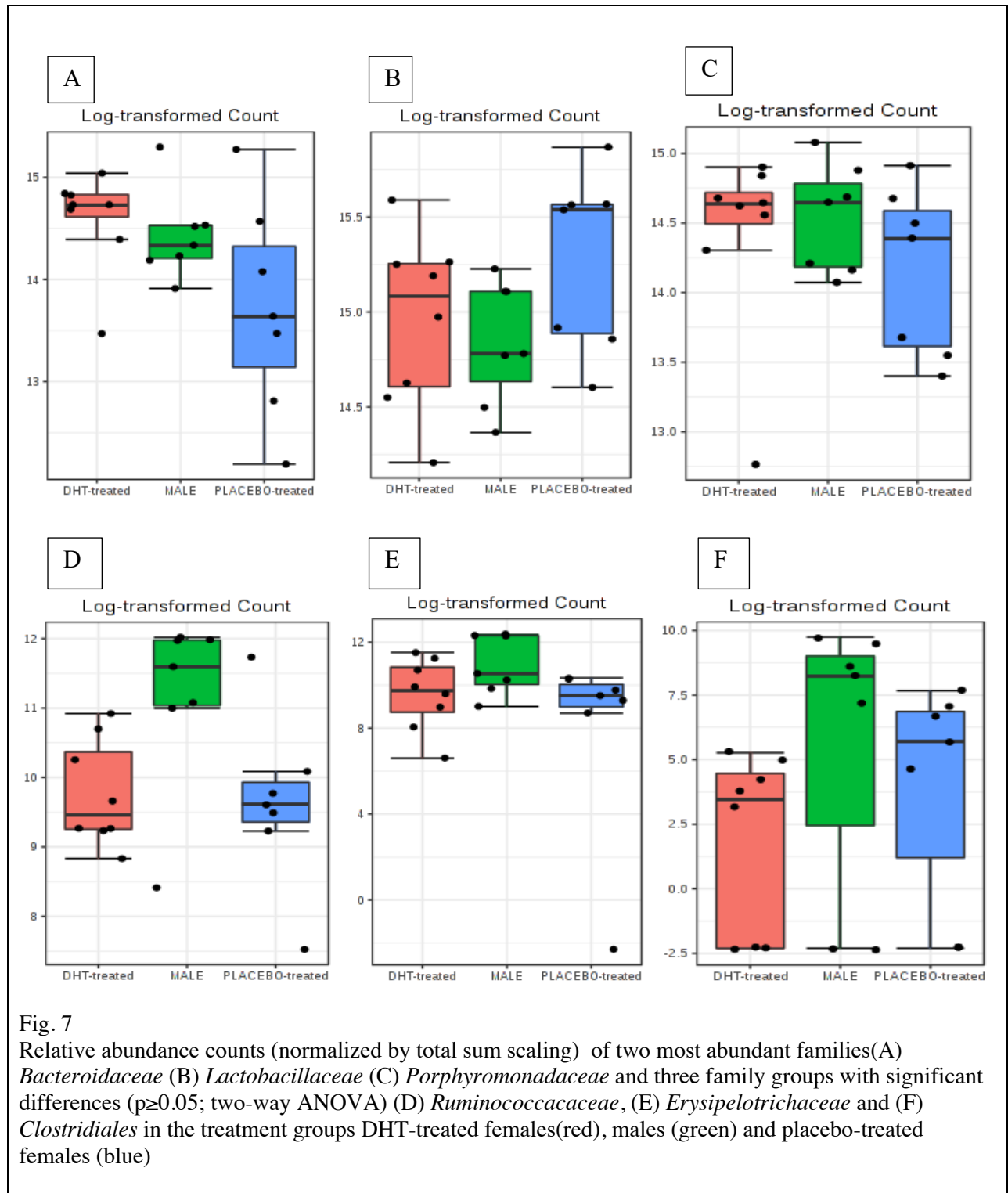


Fig. 7  
 Relative abundance counts (normalized by total sum scaling) of two most abundant families(A) *Bacteroidaceae* (B) *Lactobacillaceae* (C) *Porphyromonadaceae* and three family groups with significant differences ( $p \geq 0.05$ ; two-way ANOVA) (D) *Ruminococcaceae*, (E) *Erysipelotrichaceae* and (F) *Clostridiales* in the treatment groups DHT-treated females (red), males (green) and placebo-treated females (blue)

## Genus Level Analysis:

We identified 93 genera among our samples. *Lactobacillus*, *Bacteroides* and *Barnesiella* were the three most abundant genera, *Lactobacillus* with the highest proportional abundance in placebo-treated females, and the latter two were more abundant in males and DHT-treated females respectively (non-significant) (Fig. 8). *Ruminococcus* was significantly more abundant in males than both DHT-treated females and placebo-treated group. *Intestinimonas*, *Pseudoflavonifractor* and *Porphyromonas* were significantly more abundant in males than DHT-treated females. *Coprobacter* was significantly more abundant in DHT-treated females compared to both males and placebo-treated females (Table 6).

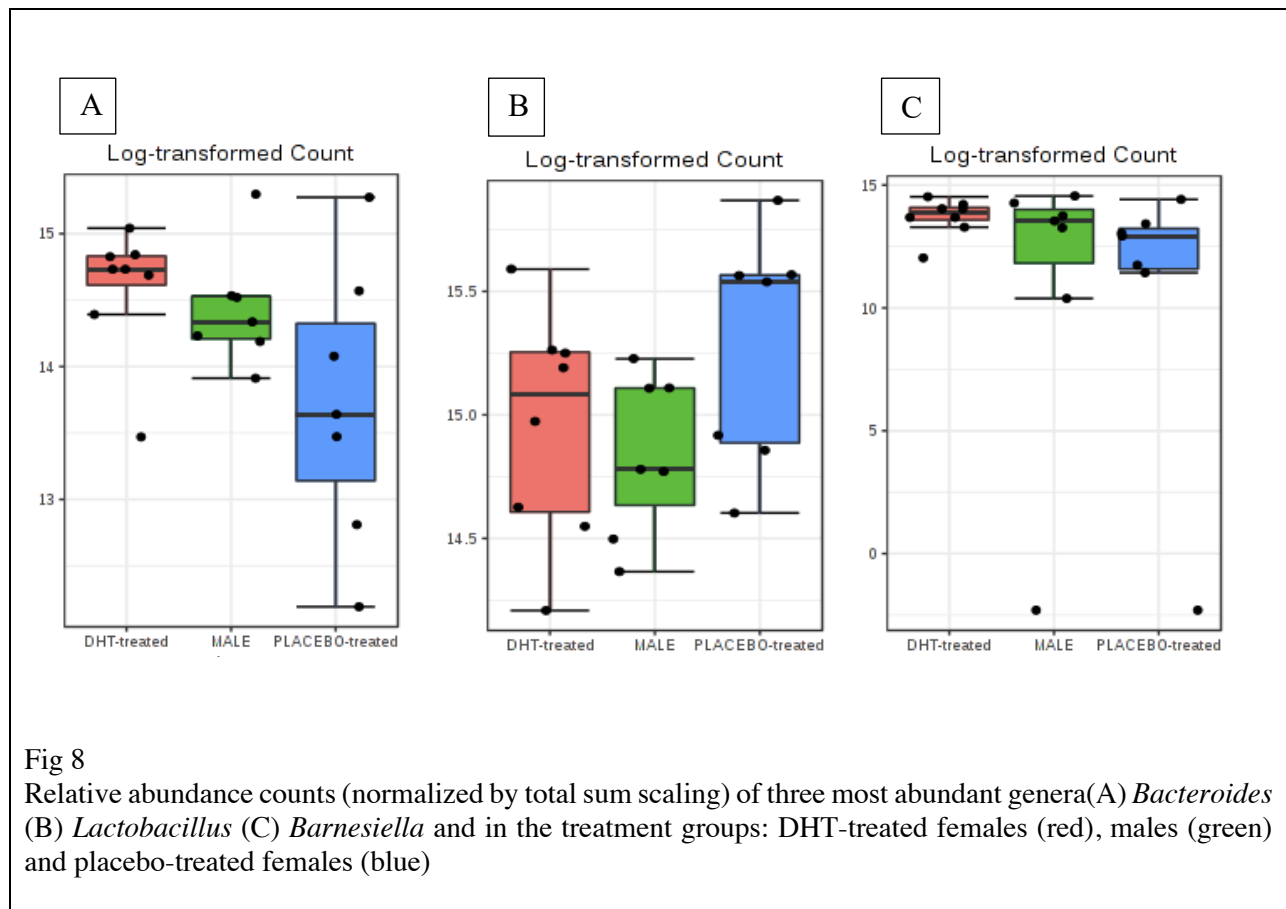


Table 6

Relative mean proportional abundance (in percent) of the five most abundant genera (1—5) and significantly different genera (6—9) identified in DHT-treated females, males and placebo-treated females. \* represents significance ( $p \leq 0.05$ ; two-way ANOVA). Mean values not sharing a letter within each row are significantly different from each other ( $p \leq 0.05$ ; Tukey's test). Mean values sharing letters or without letters are non-significant within the row.

S No.	GENUS	DHT-treated	MALE	PLACEBO-treated	Significance
1	<i>Lactobacillus</i>	35.04	28.72	53.12	0.10
2	<i>Bacteroides</i>	23.72	17.94	10.61	0.28
3	<i>Barnesiella</i>	10.84	10.25	6.12	0.29
4	<i>Lactococcus</i>	2.27	12.55	2.99	0.11
5	<i>Porphyromonas</i>	2.78 <sup>a</sup>	8.27 <sup>b</sup>	5.84	<b>0.05*</b>
6	<i>Coprobacter</i>	0.79 <sup>a</sup>	0.16 <sup>b</sup>	0.25 <sup>b</sup>	<b>0.01*</b>
7	<i>Ruminococcus</i>	0.07 <sup>a</sup>	0.56 <sup>b</sup>	0.07 <sup>a</sup>	<b>0.03*</b>
8	<i>Intestinimonas</i>	0.00059 <sup>a</sup>	0.040 <sup>b</sup>	0.003	<b>0.035*</b>
9	<i>Pseudoflavonifractor</i>	0.0002 <sup>a</sup>	0.026 <sup>b</sup>	0.002	<b>0.040*</b>

### Species Level Analysis:

After low count filtering, we retained 176 species among all our samples. *Lactobacillus johnsonii* and *Bacteroides acidifaciens* collectively made the majority of the bacterial composition in our samples. Placebo-treated females showed a trend of increased *L. johnsonii* (Fig. 9). *B. acidifaciens* was significantly more abundant in both males and DHT-treated females than in placebo-treated females. There were no significant differences in the proportion of *B. acidifaciens* between males and DHT-treated females. *Clostridium avalense*, *Lactobacillus reuteri*, *Ruminococcus spp.*, *Intestinimonas butyriproducens*, *Bacteroides capillosus*, *Clostridium indolis*, *Porphyromonas spp* and *Anaerostipes sp* were all significantly more abundant in males. *Coprobacter fastidious* and *Desulfovibrio desulfuricans* were significantly more abundant in DHT-treated females (Table 7).

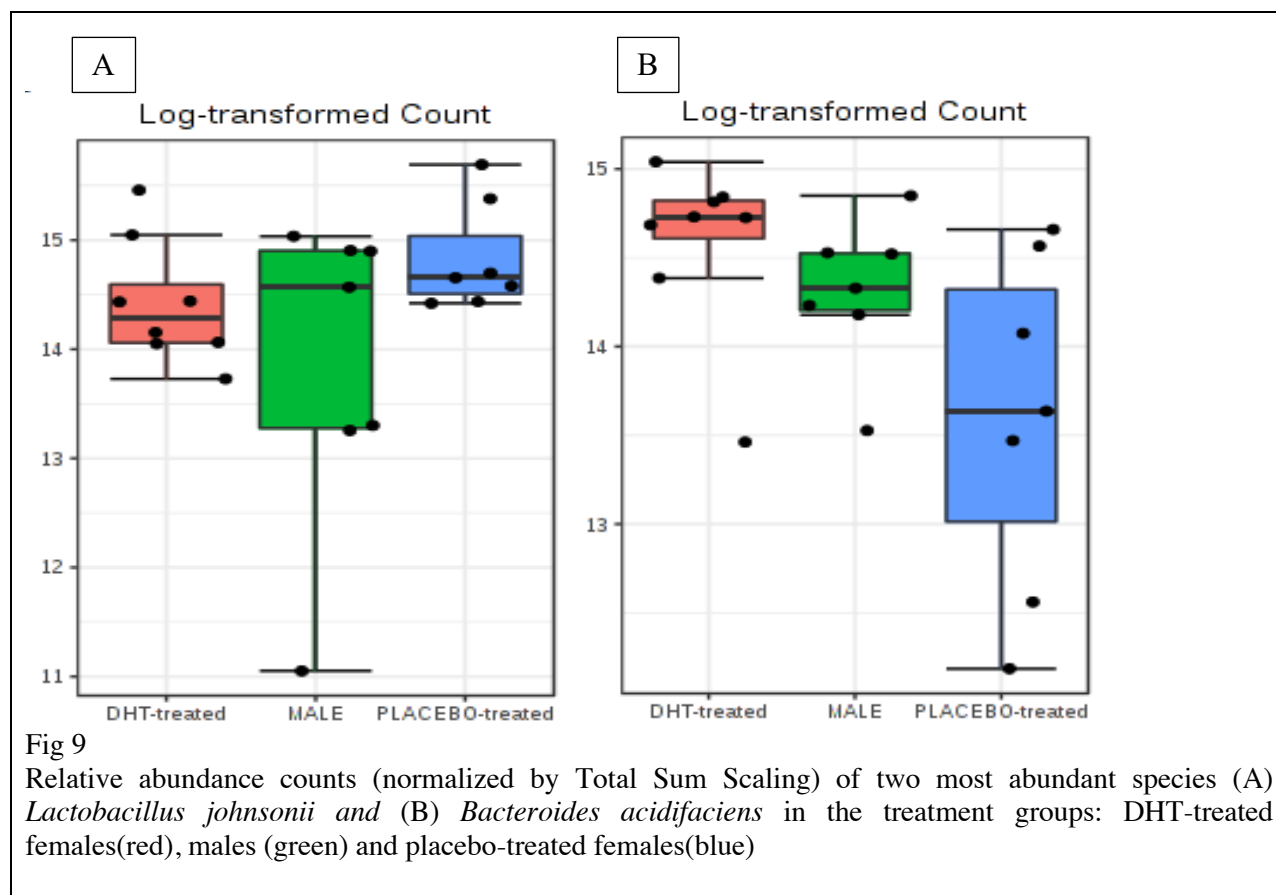


Fig 9 Relative abundance counts (normalized by Total Sum Scaling) of two most abundant species (A) *Lactobacillus johnsonii* and (B) *Bacteroides acidifaciens* in the treatment groups: DHT-treated females (red), males (green) and placebo-treated females (blue)

Table 7

Relative mean proportional abundance (in percent) of the two most abundant species (1–2) and significantly different species (2–12) identified in DHT-treated females, males and placebo-treated females. \* represents significance ( $p \leq 0.05$ ; two-way ANOVA). Mean values not sharing a letter within each row are significantly different from each other ( $p \leq 0.05$ ; Tukey's test). Mean values sharing letters or without letters are non-significant within the row.

S No.	SPECIES	DHT-treated	MALE	PLACEBO-treated	Significance
1	<i>Lactobacillus johnsonii</i>	23.15	16.21	39.03	0.28
2	<i>Bacteroides acidifaciens</i>	23.60 <sup>a</sup>	17.24 <sup>a</sup>	10.05 <sup>b</sup>	<b>0.02*</b>
3	<i>Clostridium lavalense</i>	0.045 <sup>a</sup>	0.557 <sup>b</sup>	0.090 <sup>a</sup>	<b>0.003*</b>
4	<i>Lactobacillus reuteri</i>	0.001 <sup>a</sup>	0.018 <sup>b</sup>	0.0003 <sup>a</sup>	<b>0.004*</b>
5	<i>Coprobacter fastidiosus</i>	0.79 <sup>a</sup>	0.16 <sup>b</sup>	0.25 <sup>b</sup>	<b>0.01*</b>
6	<i>Ruminococcus spp.</i>	0.0004 <sup>a</sup>	0.37 <sup>b</sup>	0.0007	<b>0.02*</b>
7	<i>Intestinimonas butyriciproducens</i>	0.0005 <sup>a</sup>	0.04 <sup>b</sup>	0.003	<b>0.03*</b>
8	<i>Bacteroides capillosus</i>	0.02 <sup>a</sup>	0.03 <sup>b</sup>	0.002	<b>0.04*</b>
9	<i>Clostridium indolis</i>	0.07 <sup>a</sup>	0.50 <sup>b</sup>	0.21	<b>0.05*</b>
10	<i>Porphyromonas spp</i>	2.78 <sup>a</sup>	8.27 <sup>b</sup>	5.84	<b>0.05*</b>
11	<i>Desulfovibrio desulfuricans</i>	1.26 <sup>a</sup>	0.18 <sup>b</sup>	0.48	<b>0.05*</b>
12	<i>Anaerostipes sp</i>	0.004 <sup>a</sup>	0.113 <sup>b</sup>	0.007	<b>0.05*</b>

## Alpha Diversity Analysis:

We used the Shannon index to measure species diversity, taking into account the species richness and abundance from our data. Although the male gut microbiome seemed to have a higher alpha diversity based on increased average index value (Fig. 10), we did not find significant difference among treatment groups ( $p = 0.28$ ).

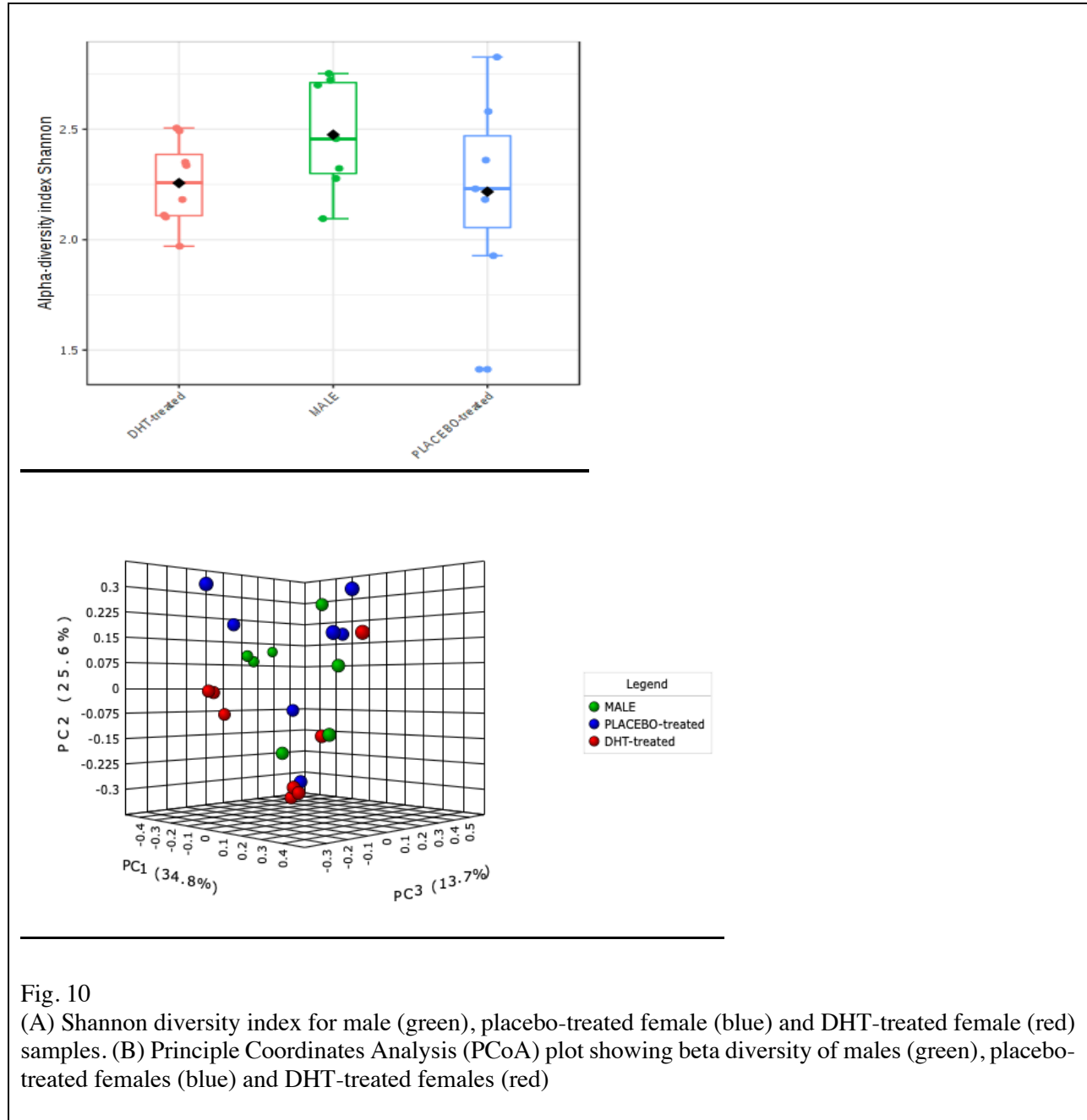


Fig. 10

(A) Shannon diversity index for male (green), placebo-treated female (blue) and DHT-treated female (red) samples. (B) Principle Coordinates Analysis (PCoA) plot showing beta diversity of males (green), placebo-treated females (blue) and DHT-treated females (red)

We used Principal Coordinate Analysis (PCoA) to visualize differences in species composition among treatment groups using the Bray-Curtis Index as a measure of Beta diversity and a permutational MANOVA (PERMANOVA) for statistical significance. Fig. 10 shows the PCoA plot with individual samples from DHT-treated females, males and placebo-treated females ( $p < 0.054$ ).

**Functional Analysis:**

We looked into KEGG pathways using KEGG ortholog abundance data obtained from Piphillin, which identified 138 pathways. While none showed significant differences, a few trends were noted (Table 8). Compared to males, placebo-treated females had increased potential (non-significant) for Lipopolysaccharide (LPS) synthesis. This was even higher in DHT-treated

Table 8  
Relative abundance (per million reads) of KEGG pathways in DHT-treated females, males and placebo-treated females.

PATHWAY	DHT-treated	Placebo-treated	Male	p-value
Lipopolysaccharide biosynthesis	630.51	450.90	218.42	0.13
Biosynthesis of unsaturated fatty acids	1185.52	1711.43	531.65	0.17
Fatty acid biosynthesis	2782.34	3617.05	1344.99	0.26
Fatty acid elongation	38.915	44.32	4.91	0.33
Fatty acid degradation	1340.96	1707.77	513.47	0.15
Inositol phosphate metabolism	593.04	896.057	272.94	0.17
alpha-Linolenic acid metabolism	167.90	298.15	60.39	0.38
Linoleic acid metabolism	58.07	50.19	16.46	0.13
Limonene and pinene degradation	593.31	412.94	138.49	0.40
Fluorobenzoate degradation	200.77	178.73	65.69	0.35
Steroid degradation	132.52	63.59	12.70	0.30
Metabolism of xenobiotics by cytochrome P450	361.88	405.10	105.97	0.40
Drug metabolism - cytochrome P450	517.40	567.07	155.08	0.41
Geraniol degradation	600.43	626.41	167.50	0.41
Lipoic acid metabolism	1679.67	2593.13	675.59	0.43

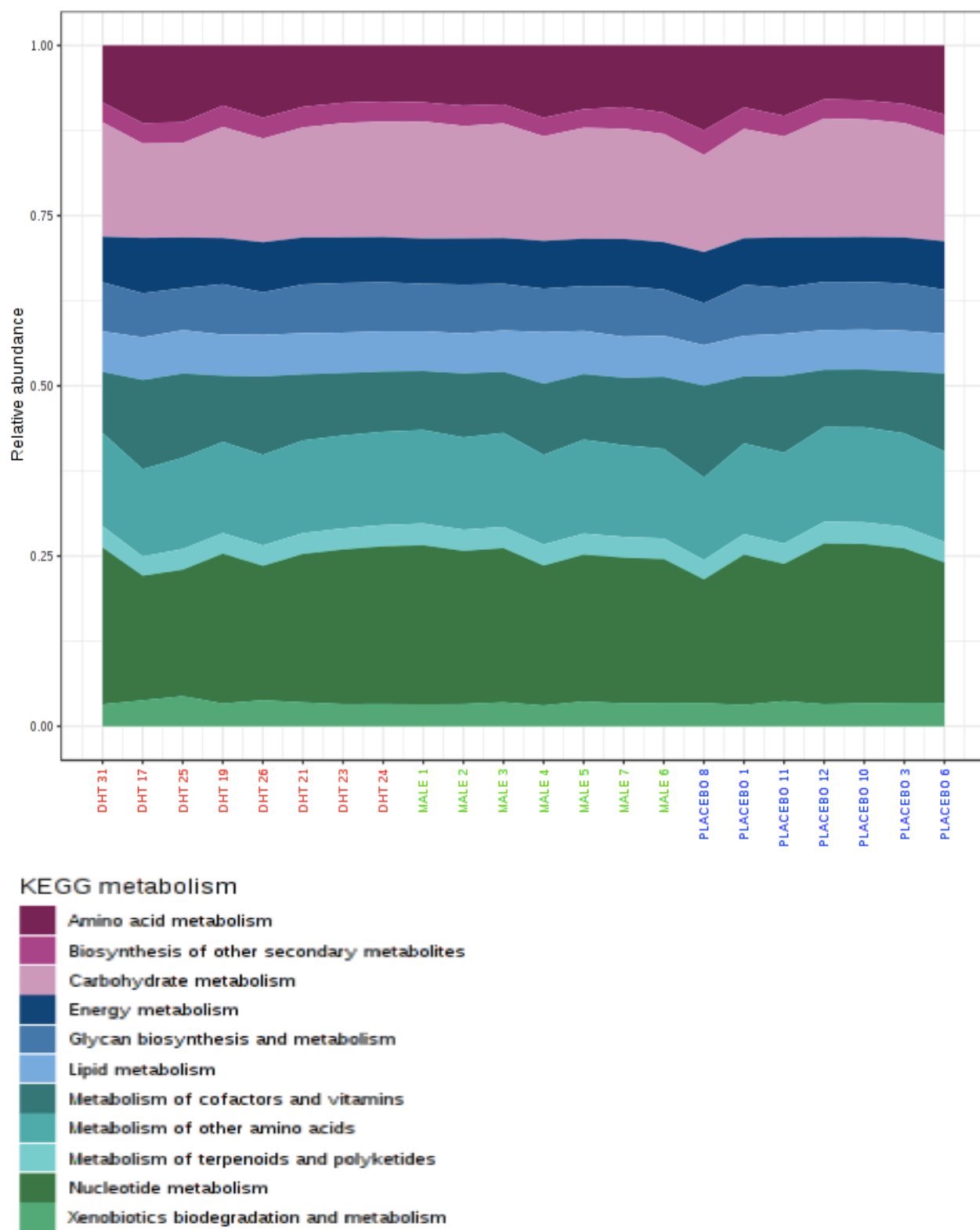


Fig. 11  
Area plot of metabolisms across different sample



females. Fatty acid metabolism potential (biosynthesis, elongation and degradation) was relatively higher (non-significant) among placebo-treated females. Both DHT-treated and placebo-treated females showed increase potential (non-significant) for xenobiotic and drug metabolism. Additionally, we identified 11 metabolic processes from different samples to create their functional profile for comparative analysis (Fig. 11). None of the treatment groups showed any significance differences with regards to metabolic processes.

## **Discussion**

We examined the diversity and functional profiles of the small intestine bacterial composition in NOD mice between DHT-treated females, untreated males and placebo-treated females. Our data were analyzed at different taxonomic levels to identify microbiome compositional traits that differed between the treatment groups and identify any possible similarity between males and DHT-treated females.

Although NOD mice develop spontaneous T1D, there is a strong sex bias with higher incidence of disease progression in females. The attenuation of T1D in male NOD mice is due to the altered gut microbiome induced by higher androgen concentration [25]. A previous study had shown improved glucose tolerance in DHT-treated female mice and male mice compared to placebo-treated females [60]. We hypothesized that DHT altered gut microbiome in female NOD mice to resemble a composition similar to male gut microbiome and this altered microbiome is responsible for improved glucose tolerance. The goal of this study was to understand the effects of androgens on gut microbiome and infer possible protective effects against T1D.

Four major phyla: *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria* comprised the gut microbiome in the small intestine of NOD mice in our study. We noticed a trend of reduced *Bacteroidetes* and increased *Firmicutes* in placebo-treated females, mainly

explained by increase in bacteria in the class *Bacilli*. Males and DHT-treated females showed similar abundance profile trends with reduced *Firmicutes* and increased *Bacteroidetes*. These do not support findings from previous studies on human stool samples which associate T1D with increased *Bacteroidetes* and reduced *Firmicutes* [27]. However, these are consistent with similar study conducted on human duodenal mucosa samples, which showed an increase in phylum *Firmicutes* in human T1D subjects and a subsequent decrease in phylum *Bacteroidetes* [60]. Increase in phylum *Firmicutes* was also observed in the duodenal gut microbiome in rats with Streptozotocin-induced T1D [41]. Although our differences were not statistically significant, the observed trend of increase in phylum *Bacteroidetes* and reduced *Firmicutes* in DHT-treated female NOD mice, corresponds with duodenal gut microbiome composition in healthy subjects in T1D studies.

Our data support studies conducted on small intestines and show a clear trend in similarity in phyla abundance profiles of the microbiomes in males and DHT-treated females. We also observed similar trends at both class and order levels. Males and DHT-treated females had higher proportions of *Bacteroidia* and lowered proportions of *Bacilli* compared to placebo-treated females (non-significant). Similarly, placebo-treated females had a higher proportion of *Lactobacillales* and lower *Bacteroidales* compared to both males and DHT-treated females (non-significant). *Erysipelotrichales* were present in significantly higher proportion in male samples only. This suggests that DHT shifts certain gut microbial populations in females to more closely resemble male-like profiles but does not have any effect on other female microbial profile features, which remain unchanged. Based on this observation, we can infer that DHT-treated females and placebo-treated females still share some similarity, as DHT alone cannot completely shift the female gut microbiome composition into male-like composition.

We observed similar trends at the family level, with increased proportions of *Lactobacillaceae* and decreased proportions of *Porphyromonadaceae* and *Bacteroidaceae* in placebo-treated females compared to both males and DHT-treated females (non-significant). Decreased *Porphyromonadaceae* is consistent with a previous observation on stool samples from diabetic children, which showed lowered *Porphyromonadaceae* in patients than in healthy controls [27]. Three families, *Ruminococcaceae*, *Erysipelotrichaceae* and *Clostridiales*, occurred in significantly high proportions in males compared to both DHT-treated and placebo-treated females. We do not know what roles these play in the small intestines with regards to T1D pathogenesis.

Similar trends were also observed at the genus level, with the most abundant genus showing clear similarity between males and DHT-treated females. Genus *Bacteroides* showed a trend of higher proportion in males and DHT-treated females than in placebo-treated females (non-significant). Low level of *Bacteroides* is considered an indicator of high blood glucose level in the elderly population [63]. Similarly, the genus *Barnesiella* showed a trend of increased proportional abundance in males and DHT-treated females. Although these traits were not statistically significant, increased *Barnesiella* has been associated with reduced T1D incidence, whereby gluten-containing diets increased *Barnesiella* sp. among others and attenuated T1D in NOD mice [64].

At the species level, *Lactobacillus johnsonii* was most abundant and showed a trend to higher proportion in placebo-treated females compared to both males and DHT-treated females. Additionally, *Lactobacillus reuteri* has a significantly higher proportion in males and DHT-treated females compared to placebo-treated females. Although they made a small portion of the microbiome, *L. reuteri* is believed to improve incretin and insulin secretion [65].

*Bacteroides acidifaciens*, the second most abundant species had a significantly higher proportion in DHT-treated females and males, compared to placebo treated females.

Feeding mice with *B. acidifaciens* increases their insulin level and lowers blood glucose concentrations, compared to control mice. *B. acidifaciens* also enhances production of glucagon-like-peptide (GLP-1) and decreases the expression of dipeptidyl peptidase-4 (DPP-4), an enzyme that degrades GLP-1 in the small intestine [64]. GLP-1 is an incretin that can decrease blood sugar level by enhancing insulin secretion. This suggests that *B. acidifaciens* or their metabolites improves glucose tolerance and insulin sensitivity. Additionally, we know that *B. acidifaciens* also plays a major role in inducing production of immunoglobulin A (IgA) in both small and large intestines [67,68]. IgA are antibodies secreted in the mucus membrane in both large and small intestine. Type 1 diabetes has been long associated with selective IgA deficiency [69].

*Desulfovibrio desulfuricans* was significantly higher in DHT-treated females.

*Desulfovibrio* species are known to oxidize butyrate in the presence of sulphate as an electron acceptor in anaerobic environment [71]. Abundance of *Desulfovibrio* species signifies production of butyrate in the small intestine. Butyrate is known to be anti-diabetogenic and contributes to mucin synthesis, regulates tight junctions and maintains gut integrity [14].

The Shannon diversity index for alpha diversity and the Principal Coordinate Analysis (PCoA) for beta diversity using the Bray-Curtis Index showed differences in diversity between individual samples. We could not demonstrate similarity between males and DHT-treated females, or between the two female treatment groups. We observed variations in the diversity between individual samples. Interestingly, this variability is also observed in the development of T1D. Although female NOD mice develop T1D at an earlier age than males, the onset could begin anytime between 10—14 weeks with a cumulative incidence of about 70% to 80% by 30 weeks

of age. Similarly, males usually develop T1D after 20 weeks of age with a cumulative incidence of only 20%—30% age. Although females have a higher T1D incidence, age of T1D development is a variable in NOD mice. The variation in diversity between individual samples could be a result of age-based variation in the development of T1D.

Functional analysis allowed us to compare different possible metabolic pathways in the microbial communities. We noticed a higher average occurrence of LPS biosynthesis in DHT-treated females, followed by placebo-treated females and males. LPS production is attributed to Gram-negative bacteria [71]. The increase in LPS production potential in DHT-treated females coincides with an increase in Bacteroidetes (which are Gram-negative). Elevated LPS concentrations in plasma has been associated with several metabolic disorders and is known to induce inflammation. Studies suggest that LPS from the gut can translocate into plasma and induce metabolic endotoxemia, triggering inflammatory disorders [72]. T1D prognosis in NOD mice is associated with TLR4 and the Myeloid differentiation primary response 88 pathway and these can be directly affected by LPS levels [73,74]. In general, a high amount of LPS coupled with increased gut permeability is seen as a biomarker for inflammation and metabolic disorders, including insulin resistance. Normally, the lipid A domain of LPS binds and activates TLR-4, which further triggers downstream activation of NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway. However, not all subtypes of LPS may stimulate the immune system and some have even shown inhibitory effects [74]. One study highlighted this difference in immunogenicity through intraperitoneal injection of LPS derived from *Escherichia coli* and *Bacteroides dorei* whereby, LPS from *E. coli* led to delayed onset and reduced incidence of T1D. LPS from *B. dorei* did not show such protective effects [74].

Another related study showed that LPS produced from Bacteroidetes can trigger protective effects in Inflammatory Bowel Disease in mouse models. LPS from Proteobacteria did not confer such protections. The study raises the possibility of immunoinhibitory effects of certain LPS and cautions against directly connecting LPS levels with immunogenicity [75]. Hence, we cannot draw any definite conclusion based on higher metabolic potential of LPS biosynthesis in DHT treated females.

It is also worth noting that placebo-treated females had a higher proportion of fatty acid metabolism. Although, we did not observe any significant differences the observed trend coincides with the previous observations of distinct differences in male and female NOD mice in serum metabolite levels of a subset of glycerophospholipid and sphingolipid metabolites [25]. Additionally, all experimental groups with implants (DHT or placebo) exhibited higher abundance of xenobiotic and drug metabolism pathways. This could be due to the presence of exogenous sub-cutaneous pellet implants.

## **Methods**

### **Tissue Collection:**

We used three NOD mouse treatment groups for this study: DHT-treated females, untreated males and placebo-treated females. All mice used in the study were housed in 12:12 light-dark cycle and fed NIH-31 chow and water *ad libitum*. Female mice were implanted with either a placebo pellet or a DHT 90-day slow release pellet (2.5 mg) (Innovative Research of America, Sarasota FL) at 19-20 days. Pellets were implanted before the onset of insulinitis, which normally occurs at 5-6 weeks of age. Age-matched males did not get any treatments. At the completion of the study (90 days after pellet insertion), all mice were euthanized via CO<sub>2</sub>. Following euthanasia, the small intestines were collected and stored individually at -80 degrees,

until thawed for DNA extraction [1]. Animals were cared for and handled in accordance with the National Institute of Environmental Health Sciences Institutional Animal Care and Use Committee approval (Protocol # 01-30) at Reproductive & Developmental Biology Laboratory, NIEHS, NIH, Research Triangle Park, NC. Intestine samples from age-matched untreated males (n = 7), DHT-treated-females (n = 8) and placebo-treated females (n = 7) were obtained for this study.

#### **DNA Extraction:**

Frozen intestine tissues were equilibrated to room temperature before extraction. Upon thawing, the intestines were stretched out in a standard sterile dissecting tray. All instruments were treated with 95% ethanol and heated over a Bunsen burner to eliminate contaminants. The first 1.3 cm from each end of the intestinal sample was then removed to minimize contamination. Then, 25 mg tissue was collected from the proximal and distal ends. Genomic DNA was extracted from each sample using DNeasy™ Blood and Tissue Kits (Qiagen Inc, Germantown, MD) according to the manufacturer's instructions, with the exception that the final DNA elution step was repeated to increase DNA yield. UV absorbance ratio at 260nm was used to quantify DNA and the purity was determined using 260/280 ratio.

#### **16S rRNA Sequencing and OTU Assignment:**

Isolated genomic DNA was sent to MR DNA Lab (Shallowater, TX) a commercial sequencing service in order to identify the bacterial members present in each sample using 16S rRNA (Ribosomal Ribonucleic Acid) amplicon sequencing. 16S rRNA gene sequencing is a well-established and a reliable method to identify bacterial taxa and compare bacterial populations from complex microbiomes. 16S rRNA genes are highly conserved and used by scientists to identify and assign phylogeny to all bacterial species in a sample [76]

The 16S rRNA gene V4 variable region Polymerase Chain Reaction (PCR) primers 515/806 were used for sequencing. The PCR followed a single-step 30 cycle using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute. This was followed by the final elongation step at 72°C for 5 minutes. Sequencing was performed using an Ion Torrent PGM sequencer and operational taxonomic units (OTUs) were subsequently generated for each sequence. OTUs were defined by clustering at 1% divergence (99% similarity). The final OTUs were categorized taxonomically using the BLASTn option for searching the database derived from NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and from RDPII (<http://rdp.cme.msu.edu>). The OTUs were then compiled into most relevant taxonomic level based on percent identity to the reference sequence as defined in Table 9.

Table 9  
Percent identity associated by Taxonomic designation

<b>Identity to reference sequence</b>	<b>Identity Designation</b>
> 97%	Species
Between 97% and 95%	Genus assignment
Between 95% and 90%	Family assignment
Between 90% and 85%	Order assignment
Between 85% and 80%	Class assignment
Between 80% and 77%	Phylum assignment
< 77%	(unknown)

### **Diversity Analysis:**

We used MicrobiomeAnalyst, a web based comprehensive statistical and analytical tool for microbiome data for diversity and functional analysis [60]. OTU abundance data were used for the diversity analysis. All samples with their respective sequence read counts (Fig 12).



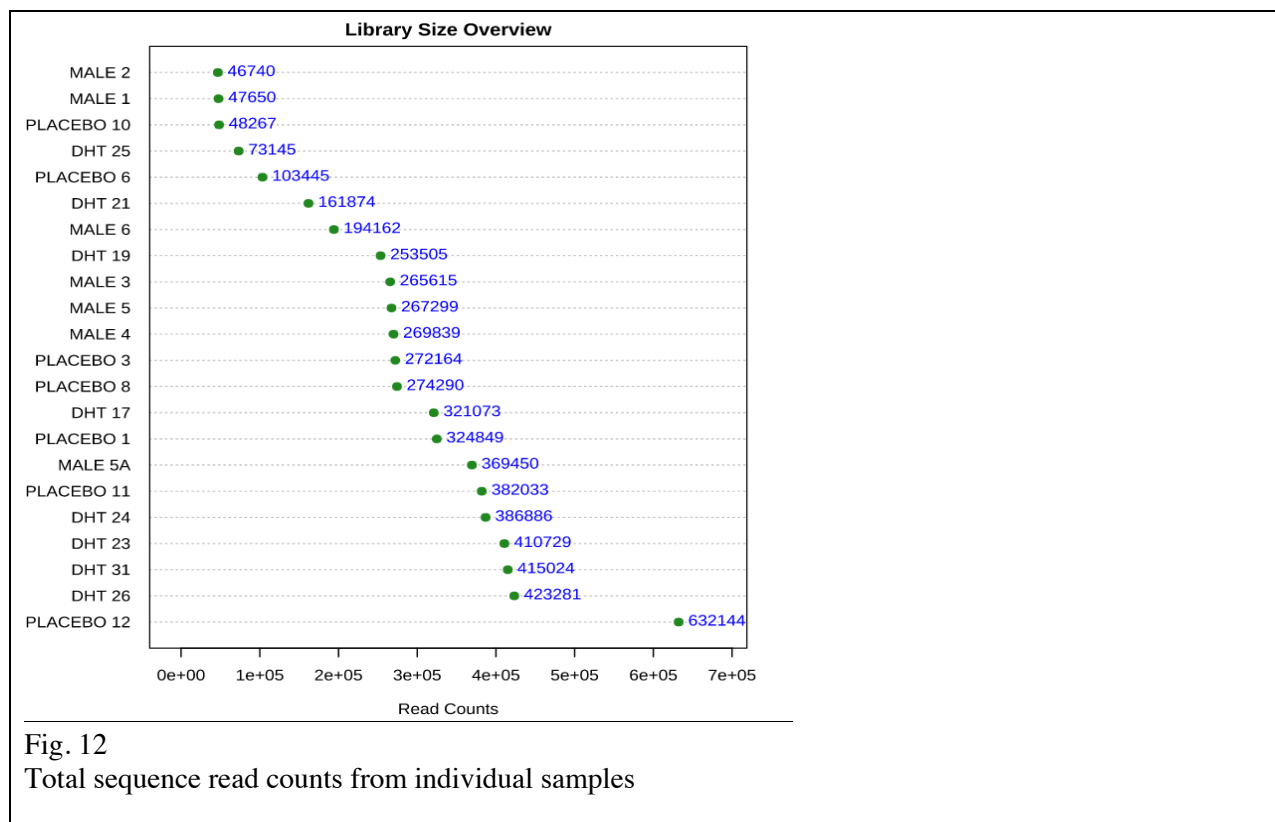


Table 10  
Sample characteristics of three treatment groups: males, DHT-treated females and placebo-treated females

Reads	Number
Total number of samples	22
Total Read counts	5943464
Average Counts per sample	270175
Maximum counts per sample	632144
Minimum counts per sample	46740
Total species identified	805
Total species $\geq 2$ counts	462

Marker Data Profiling (MDP) feature in MicrobiomeAnalyst was used to analyze microbial composition and diversity in our samples. The OTU abundance table and metadata file was uploaded in the program for differential abundance testing. We used the SILVA format for taxonomic assignments for our analysis. Our data contained 462 total species with two or more read counts. To account for any sequencing errors, we used the default low count filter with

minimum count of 4 and 20% prevalence; only those features with at least 20% of its values containing at least 4 counts were retained for analysis. This resulted in 286 low abundance species being removed from the analysis. 176 species remained for comparative analysis. We used total sum scaling to normalize data and account for sampling depth. Relative abundance was compared at different taxonomic levels. Shannon index was calculated at species level to determine alpha diversity within each test groups. One-way ANOVA with post hoc Tukey HSD test were used to test for significant differences ( $p \leq 0.05$ ) among groups. Principal Coordinate Analysis (PCoA) was used to analyze beta diversity using the Bray-Curtis Index to analyze dissimilarity in communities and permutational MANOVA (PERMANOVA) was employed to determine statistical significance.

### **Functional Analysis**

Piphillin was used to generate KEGG Orthologs (KO) from the OTU abundance table and representative sequence file [51]. KEGG orthologs are functional orthologs derived from KEGG Orthology database that represent a computational prediction of cellular processes and functions of the community, based on genomic information. The KEGG ortholog abundance table was entered into MicrobiomeAnalyst for functional analysis. MicrobiomeAnalyst uses this input format to generate KEGG pathway abundance table and a metabolic profile ([https://www.genome.jp/kegg-bin/get\\_htext#B2](https://www.genome.jp/kegg-bin/get_htext#B2)). We compared metabolic features among experimental groups and used a one-way ANOVA to identify any significant differences.

### **Conclusions**

Our study suggests that exogenous androgen treatment in female NOD mice using DHT implants can shift female gut microbiota to resemble the composition of gut microbiota in males. Although we observed only a few significant differences, we noticed strong similarity trends in

the proportional composition of the most abundant taxon between DHT-treated females and age-matched males. We identified significant increases in *Bacteroides acidifaciens* in DHT-treated females, a bacterial species known to enhance glucose tolerance and attenuate T1D. Taken together, our findings suggest that gut microbiome modulations play causative roles in T1D progression.

## **Declarations**

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### ***Ethics approval and consent to participate***

Not applicable

### ***Consent for publication***

Not applicable

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