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Engineering the Gut Microbiome for Metabolic, Reproductive, and Cognitive Health

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Erica Maissy

Committee in charge:

Professor Amir Zarrinpar, Chair
Professor Paula Desplats
Professor Rob Knight
Professor Varykina Thackray

2024

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The Dissertation of Erica Maissy is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

DEDICATION

For the mice I sent to Italy – *grazie mille*.

EPIGRAPH

“out pops from her father’s head

a girl with wide brain of her own

yes – do not underestimate daughters

these girls who are born knowing

who are as close to the core of earth’s secrets

as you could ever hope to be

listen – do you promise?

if you can look past her smallness

she;

you

are saying something huge”

- **Hannah Monsour**

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VITA

2018 Bachelor of Science in Biomedical Engineering with Minors in Neuroscience and Dance, University of South Carolina, Honors College

2021 Micro MBA Certificate, UCSD Rady School of Management

2024 Doctor of Philosophy in Biomedical Sciences, University of California San Diego

PUBLICATIONS

Maissy ES, Chiang S, Ryan E, Dorrestein P, Knight R, Thackray V, Zarrinpar A. Luminal Bile Acid Modifications Ameliorate Metabolic Features of Polycystic Ovary Syndrome. *Under review.*

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Russell BJ, Brown SD, Siguenza N, Mai I, Saran AR, Lingaraju A, **Maissy ES**, Dantas Machado AC, Pinto AFM, Sanchez C, Rossitto L, Miyamoto Y, Richter RA, Ho SB, Eckmann L, Hasty J, Gonzalez DJ, Saghatelian A, Knight R, Zarrinpar A. Intestinal transgene delivery with native E. coli chassis allows persistent physiological changes. *Cell.* 2022.

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ABSTRACT OF THE DISSERTATION

Engineering the Gut Microbiome for Metabolic, Reproductive, and Cognitive Health

by

Erica Maissy

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2024

Professor Amir Zarrinpar, Chair

Recent advancements in microbiome research have unveiled promising avenues for therapeutic interventions while underscoring the need for mechanistic studies of microbiome function and temporal dynamics. This dissertation explores the interplay between the gut microbiome, luminal bile acid modifications, and circadian dynamics in the context of women's health and neurodegenerative disease.

Chapter 1 reviews the current research on engineered microbial strains as novel therapeutics for gynecological health, including polycystic ovary syndrome (PCOS). Engineered

probiotics, designed to express specific therapeutic functions such as antiviral peptides and anti-biofilm agents, offer targeted approaches for treating conditions like HIV and Candida albicans infections. The chapter underscores the integration of synthetic biology with clinical medicine, highlighting the therapeutic potential of targeting the microbiome.

Chapter 2 delves into one of these specific microbiome functions that impacts metabolism and the hypothalamic-gonadal-pituitary axis in the context of PCOS. Through murine models, this chapter demonstrates how bacterial bile acid modifications by bile salt hydrolase (BSH) can ameliorate metabolic dysfunction and some reproductive parameters associated with PCOS. This mechanistic insight illustrates the therapeutic promise of microbiome-targeted therapies, and highlights the role of bile acid receptors FXR and TGR5 in glucose homeostasis and hormonal regulation.

Chapter 3 explores the impact of time-restricted feeding (TRF) on the gut microbiome in an Alzheimer's disease (AD) mouse model. The study reveals that TRF modulates gut microbial composition, function, and rhythmicity, influencing metabolites crucial for regulating metabolism, neuroinflammation, and amyloid pathology. The microbiome emerges as a key player contributing to the mechanism of TRF's mitigation of AD-related cognitive decline and neuropathology.

Together, these chapters contribute to a deeper understanding of how characterization and manipulation of the microbiome can elucidate mechanisms of disease and shape therapeutic strategies across diverse health conditions, from reproductive health challenges like PCOS to neurodegenerative diseases such as Alzheimer's. Continued research using synthetic biology and multi-omic analyses to elucidate mechanistic functions of the microbiome will further contribute to improved scientific tools and healthcare outcomes.

Chapter 1 Engineered Microbial Strains for Gynecological Health and PCOS

1.1 Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is a complex endocrine disease affecting roughly 6–10% of individuals with ovaries, primarily during reproductive years (Bozdag *et al.* 2016). It is diagnosed using the Rotterdam criteria, requiring two out of the following three features: clinical and/or biochemical hyperandrogenism, oligo- or anovulation, and polycystic ovarian morphology ('Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome', 2004). PCOS significantly affects physical and emotional well-being, resulting in infertility, menstrual irregularities, acne, excessive male-pattern hair growth, and an increased risk of anxiety and depression. Over 80% of individuals with PCOS also have metabolic dysfunction including insulin resistance (IR) with or without obesity, which can lead to an increased risk of type 2 diabetes, gestational diabetes, cardiovascular disease, and non-alcoholic fatty liver disease (Sanchez-Garrido & Tena-Sempere 2020). While a connection exists between higher body mass index (BMI) and PCOS, it is not a direct cause-and-effect relationship. Elevated BMI, particularly in cases of obesity, can contribute to IR. This, in turn, disrupts hormone levels, potentially worsening PCOS symptoms. Notably, not all women with PCOS exhibit a high BMI, and conversely, not all women with a high BMI develop PCOS (Sam 2007). While the etiology of PCOS is unclear, substantial evidence demonstrating the importance of the gut microbiome in shaping glucose homeostasis and driving metabolic disorders has led to the hypothesis that alterations in the microbiome are also involved in the pathology of PCOS (Giampaolino *et al.* 2021, Rizk & Thackray 2021). While there is inconsistency in the specific gut bacteria reported to be altered in PCOS, a recent meta-analysis of 17 studies showed that there is a consistent decrease in alpha diversity (Sola-Leyva *et al.* 2023), indicating that reduced microbial biodiversity

may be another hallmark of PCOS. Furthermore, recent studies are beginning to consider the effect of hyperandrogenism on the vaginal microbiome, where significant differences are observed in vaginal bacteria between individuals with and without PCOS (Hong *et al.* 2020). However, similar to HPV, PCOS also presents a 'chicken and egg' paradox, with the interplay between host biology and disease shaping one another.

Pre-clinical studies and clinical trials target the vaginal and gut microbiomes for the prevention or treatment of PCOS and other prevalent gynecologic conditions. Treatment options to target the gut microbiome make up a promising field of research, primarily using the methods of transplant, probiotics, prebiotics and diet, and synthetically designed microbial strains (**Fig. 1.1**).

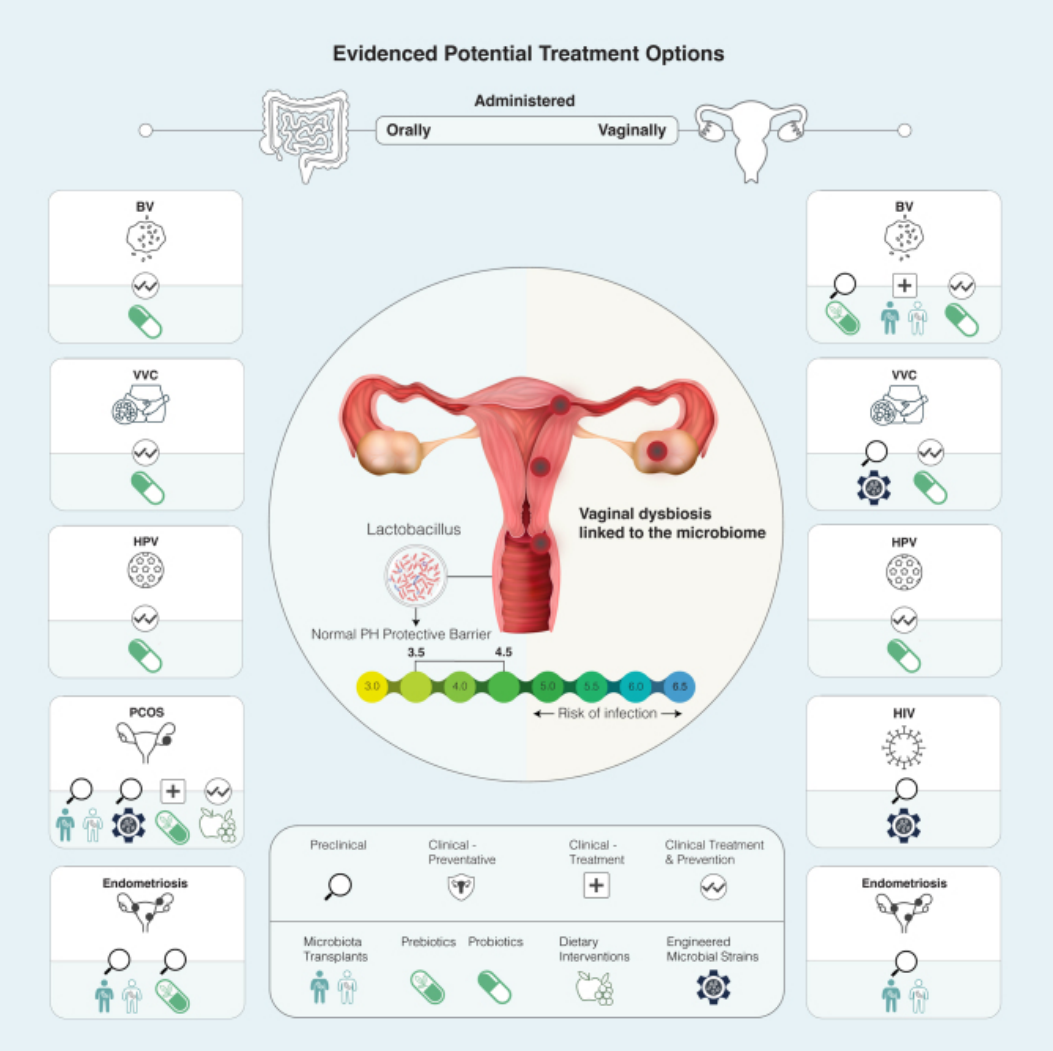


Figure 1.1: Overview of four strategies designed to support the role of the microbiome in gynecological health.

These approaches, as evidenced by clinical trials and preclinical studies, aim to manipulate the microbiome of the gut or vagina to improve gynecologic health and thereby hold the potential for preventing, managing, and treating a range of gynecological issues. 1. Microbiota Transplants: transfer of health-associated bacteria to remediate the vaginal and gut microbiome. 2. Pre and Probiotics: the consumption of beneficial bacteria or specific nutritional components to maintain and promote health. 3. Diet: support of beneficial microbes through dietary components. 4. Engineered Microbial Strains: designing genetically engineered bacteria to achieve health outcomes.

1.2 Engineered Microbial Strains

Engineered probiotics use bacteria that are genetically designed to express a specific function as an alternative to traditional pharmaceutical treatments. Treatment with genetically modified organisms should be considered drug therapy and not, like traditional probiotics, a dietary supplement. The treatment vesicle as a chassis, a bacterial strain that serves as a platform for genetic modification. The choice of chassis is crucial based on manipulability, safety, and scalability, and most studies have selected chassis that are easy to engineer, food-grade, or a predominant species. Recent studies show that there are also some chassis that can be scaled with the use of a prebiotic (Shepherd et al. 2018) or using bacteria derived from the system itself, i.e. native bacteria (Russell et al. 2022), which can implement a more persistent change. The engineered therapeutic functions themselves include but are not limited to, the production of small molecules, metabolic enzymes, nano-bodies, toxins, and immunomodulators. These engineered probiotics have been designed to treat a range of diseases, including GI disease, infection, dysmetabolism, rare genetic disorders, and cancer (Ma et al. 2022, Brevi & Zarrinpar 2023). While studies evaluating individual strains or consortia of bacteria have been somewhat successful in treating vaginal and reproductive conditions, gene-edited probiotics which express deliberate functions are on the horizon for more targeted treatment of these conditions (Vieira-Baptista et al. 2022).

For example, several biotechnology companies and academic groups are developing genetically enhanced probiotics against HIV by inserting potent antiviral genes into bacteria that naturally colonize the vagina. Unlike conventional microbicide delivery systems such as gels and films, once administered, engineered probiotics can serve as a more sustained, self-replicating delivery method for the treatment of HIV. The risk of this more sustained treatment method,

however, is the persistence of any potentially harmful effects for the patient or the environment and is as of now a risk of unknown impact.

Researchers engineered L. jensenii 1153 to produce the potent HIV entry inhibitor cyanovirin-N (CV-N) using chromosomal integration. Pre-clinical testing in macaques showed consistently high levels of colonization with CV-N expressing L. jensenii after vaginal administration, with no significant antibody response, and the strain was easily cleared with topical antibiotic administration. In a repeated low-dose challenge model, HIV acquisition was reduced by 63% in macaques, and this engineered bacterium is being explored as a platform to co-express microbicides against HIV and other STIs (Liu et al. 2006, Lagenaur et al. 2011). More work is needed to examine safety and efficacy in humans.

Furthermore, increased activated genital CD4+ T cells (associated with L. crispatus deficiency) and elevated levels of high-risk bacteria indicate a higher HIV risk, initially discovered in a cohort of South African females (Gosmann et al. 2017). Investigators are leveraging this discovery to develop targeted engineered bacteria to locally modulate CD4+ expression in the vaginal microenvironment (Gosmann et al. 2017). L. acidophilus ATCC 4356 engineered to display human CD4 on its surface can adsorb HIV-1 particles by binding to its envelope protein and successfully reduce infection in vitro and in a murine model (Wei et al. 2019). Lactobacillus has also been modulated to express broadly neutralizing nanobodies (VHH) against HIV, which may be a promising immunization method for females at high risk of HIV-1 transmission (Kalusche et al. 2020).

Engineered strains are also being actively explored as a method to combat C. albicans-driven VVC. Investigators engineered a commercial Saccharomyces boulardii strain to produce medium-chain fatty acids (MCFAs) with anti-biofilm and anti-hyphal effects in vivo. MCFAs also

upregulated the expression of virulence-related genes in the strain of C. albicans, SC5314. The constitutive production and secretions of MCFAs serve as a proof-of-concept for the potential of probiotic yeast as a therapeutic strategy for C. albicans and other opportunistic pathogens in the reproductive tract (Ling et al. 2023).

Finally, the basic science surrounding the role of the gut microbiome in reproductive disease is leading to the development of live bacterial therapeutics. One study showed elevated B. vulgatus in females with PCOS, and in murine models, it was linked to a bile acid – IL22 axis as a potential mediator of PCOS pathology. This suggests that genetically engineering a bile acid-modifying or IL22-expressing engineered bacteria could be effective for the treatment of PCOS (Qi et al. 2019).

The development of live therapeutics is a rapidly growing field, with an increasing number of scientists recognizing the need for the fusion of synthetic biology, clinical medicine, and basic science. As the field grows, it will hopefully closely integrate these three areas to provide optimal and personalized therapies.

1.3 Circadian Dynamics in the Vaginal and Gut Microbiome

There is a gap in knowledge when it comes to understanding temporal dynamics within the female gut and vaginal microbiome. There are clear circadian dynamics within host–microbe relationships; however, much of the research investigating these diurnal rhythms focuses on only male gut microbiota (Frazier & Leone 2022). There is some consensus that vaginal microbiome composition fluctuates with the menstrual cycle phase; however, further evidence is needed regarding the links to gut microbiota with cycle phase, and the role of hormonally based contraception (Song et al. 2020, Krog et al. 2022). Genetically engineered mice that express human

genes related to menstrual cycle regulation and attempt to mimic hormonal patterning have been developed. However, this model lacks physiological menstruation, and therefore cannot be used to study the vaginal environmental shift that occurs during menstruation (Liu et al. 2020). As a result, there is a need for more human studies that analyze the influence of menstrual bleeding on the vaginal microbiome.

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Chapter 1, in full, is a reprint of sections of the material as it appears in Reproduction and Fertility. Caitriona Brennan*, Kristina Chan*, Tanya Kumar*, Erica Maissy, Linda Brubaker, Marisol I Dothard, Jack A Gilbert, Katharine E Gilbert, Amanda L Lewis, Varykina G Thackray, Amir Zarrinpar, Rob Knight. Harnessing the power within: engineering the microbiome for enhanced gynecologic health. 2024. The dissertation author was the primary author on the sections of this paper presented in the dissertation.

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Chapter 2 : Luminal Bile Acid Modifications Ameliorate Metabolic Features of Polycystic Ovary Syndrome

2.1 Summary

Murine and human studies suggest that gut microbial functions, particularly changes in bacterially-modified bile acids, are key players in polycystic ovary syndrome (PCOS), a major cause of infertility and increases the risk of cardiometabolic conditions. However, the precise role of bacterial bile acid modifications on PCOS-associated metabolic and reproductive dysfunction have yet to be elucidated. Here we show that over-expression of the bacterial enzyme bile salt hydrolase (BSH) in the mouse gut lumen improves glucose homeostasis, body composition, and hormonal dysregulation in a mouse model of PCOS. Furthermore, this amelioration is mediated by the bile acid receptors farnesoid X receptor (FXR) and Takeda G-protein-receptor-5 (TGR5). This highlights the potential for microbiome-targeted PCOS therapies and provides mechanistic insight into the relationship between the gut microbiome, bile acids, and features of PCOS.

2.2 Introduction

PCOS is a lifelong multifactorial disorder, affecting up to 18% of reproductive-aged women.[1, 2] It is characterized by androgen excess, ovulatory dysfunction, and polycystic ovaries, and is the leading cause of anovulatory.[3-6] PCOS independently increases the risks for endometrial hyperplasia and cancer, type II and gestational diabetes, hypertension, non-alcoholic or metabolic dysfunction-associated fatty liver disease, obstructive sleep apnea, anxiety, and depression.[3,7] The current medical management of PCOS is suboptimal since it focuses on symptoms of the syndrome rather than its root causes. Therapeutic goals for women with PCOS include amelioration of hyperandrogenic features, management of metabolic risk factors, prevention of endometrial hyperplasia, contraception in those not pursuing pregnancy, and ovulation induction in those who

are.[8] However, there are no specific treatments for PCOS. Thus, there is a need for better understanding of the pathophysiology of the disease and development of targeted therapies.

Importantly, there is a significant relationship between the gut microbiome and PCOS. Individuals with PCOS have gut microbiota communities that are distinct from healthy controls and compositional diversity correlates with clinical parameters.[9-12] Both human studies and murine models suggest that compositional and functional changes in the microbiome contribute to the underlying mechanisms of PCOS.[12-16] In the letrozole (LET) model, which uses treatment of an aromatase inhibitor to elevate testosterone levels and recapitulate PCOS pathophysiology, alterations in the gut microbiome are correlated with hyperandrogenism.[17-18] However, these relationships are not merely correlational. When LET-treated mice were co-housed with littermate controls, the exposure to a healthy gut microbiome ameliorated both the metabolic and reproductive phenotypes of the PCOS mice.[13] This established a direct link between the gut microbiome and PCOS and suggests that microbiome-targeted therapies could be effective for PCOS.

Bile acids may play a key role in facilitating the connection between the gut microbiome and PCOS. The gut microflora perform a variety of biotransformations to weaken bile acid detergent and antimicrobial properties primarily by deconjugating them using bile salt hydrolase (BSH), and further modifying them into secondary bile acids[19] which act as signaling molecules. Conjugated, deconjugated, and secondary bile acids are versatile, microbially-modified ligands that modulate many physiological processes[20] including metabolism[21-31], neuroinflammation32-36, and circadian rhythms[37,38]. Bile acids and BSH have been implicated in the pathophysiology of PCOS in multiple human and murine studies.[12, 39-41] However, evidence supporting this link is correlational, inconsistent, and lacks a conclusive mechanism. For example, Qi, et al., showed *B.*

vulgatus, a bacterial species identified in the microbiome of patients with PCOS, was sufficient to recapitulate the reproductive and metabolic effects of PCOS in mice.[12] The authors showed that effects of *B. vulgatus* can be reversed with the addition of conjugated bile acids and increased interleukin-22 (IL-22) and thus presumed that its effects were mediated by increased BSH activity. However, the published genomes of *B. vulgatus* strains show that they contain a gene homologous to BSH that can only deconjugate highly specific moieties of bile acids.[42] *B. vulgatus* has extremely limited effects on human bile acids.[43] The same group demonstrated that removing the bile acid modifying genes from *B. vulgatus* still contributed to PCOS through a bile acid-independent mechanism involving the metabolite agmatine.[44] Moreover, women with PCOS have higher levels of conjugated bile acids in systemic circulation suggesting decreased BSH activity.[40] Thus, though BAs likely contribute to the pathophysiology of the PCOS, the role of bacterial functions in general, and BSH in particular, is not clear.

There is a complex interplay between bile acid signaling, insulin resistance, and reproductive features of PCOS. Insulin resistance affects 65-70% of individuals with PCOS, whereas only 40-60% of individuals with PCOS are overweight or obese, suggesting, along with cellular mechanisms, that insulin resistance is independent of obesity.[45-46] Bile acids can mediate glucose homeostasis through farnesoid X receptor (FXR) or the Takeda G-protein-receptor-5 (TGR5).[47] Further, there is a bidirectional "chicken and egg" relationship between insulin resistance and hyperandrogenism in PCOS, each contributing to the maintenance of the other. Insulin sensitizers like metformin can improve serum androgen reduction in conjunction with other therapies, and conversely, antiandrogen therapies can partially reverse peripheral insulin resistance.[48] Increasing luminal BSH activity improves glucose homeostasis[49], therefore microbial BSH could improve reproductive dysfunction in PCOS as a downstream effect of insulin

sensitization. BSH and its impact on the bile acid pool could also modulate PCOS reproductive features through the hypothalamic-pituitary-gonadal (HPG) axis directly. Some androgens such as androsterone can activate FXR receptors and influence bile acid synthesis and composition.[50] Conversely, bile acids can influence plasma testosterone concentrations and alter male fertility in a healthy state and in the context of metabolic syndrome.[51,52] However, whether BAs affect female reproductive hormones and fertility is not clear. Nevertheless, these studies suggest that the gut microbiome can influence fertility and reproduction through bile acid cross-reactivity with sex hormones.

To investigate the specific role of luminal bile acid modifications by BSH on PCOS *in vivo*, we developed a tool using a native *E. coli* (EcAZ-2) as a chassis for functional modification of the mouse microbiome.[49, 53] Using an engineered native bacteria that expresses BSH (EcAZ-2^{BSH+}), we facilitate perpetual engraftment into the conventionally raised mouse gut with a single oral gavage. EcAZ-2^{BSH+} functionally alters the bile acid pool without altering overall microbiome composition, and improves glucose homeostasis in wild-type (WT) male and female mice.[49] Thus, we aimed to assess whether introducing EcAZ-2^{BSH+} into a mouse model of PCOS could ameliorate disease-associated metabolic and reproductive features or exacerbate it as it has been hypothesized by others.[12]

2.3 Results

EcAZ-2^{BSH+} treatment improves glucose homeostasis and body composition in LET-induced PCOS mice.

To explore the impact of luminal bile acid modifications in the context of PCOS, we used the aromatase inhibitor, letrozole (LET) to increase androgen levels and recapitulate both the reproductive and metabolic features of PCOS.[54] Forty conventionally-raised, C57BL/6 female

mice were distributed into four groups based on body weight (n = 10). The groups were colonized with a single gavage of either BSH-containing engineered native *E. coli* (EcAZ-2^{BSH+}) (n = 20) or a control native *E. coli* without BSH (EcAZ-2) (n = 20) at 3.5 weeks old. After confirming colonization at ~10^6 CFU/g (**Fig. 2.S1A**), half of the mice from each bacterial condition received daily gavage of either LET or vehicle control (VEH). Thus, the study design consisted of 4 groups: VEH EcAZ-2, LET EcAZ-2, VEH EcAZ-2^{BSH+}, and LET EcAZ-2^{BSH+} (n = 10/group). Towards the end of the 5 week paradigm, we conducted metabolic and reproductive testing to assess PCOS phenotype (**Fig. 2.1A**).

In the EcAZ-2 control mice, LET mice exhibited higher glucose levels during an insulin tolerance test (ITT) with a 30% increase in AUC compared to VEH EcAZ-2 mice ($p < 0.001$). However, glucose levels were normalized in LET EcAZ-2^{BSH+} mice which demonstrated a 23% reduction in AUC to levels comparable to healthy controls ($p < 0.001$) (**Fig. 2.1B**). LET EcAZ-2 mice also had 16% higher fasting blood glucose levels (FBG) compared to VEH EcAZ-2 mice, recapitulating the hyperglycemia in individuals with PCOS (155.6 mg/dL ± 2.9 in VEH EcAZ-2 and 181.0 mg/dL ± 6.9 in LET EcAZ-2; $p = 0.0030$). EcAZ-2^{BSH+} prevented this LET-induced hyperglycemia with an 11% reduction in FBG (162.9 mg/dL ± 7.2 in LET EcAZ-2^{BSH+}; $p = 0.0296$) (**Fig. 2.1C**). Insulin levels after an overnight fast were elevated in LET EcAZ-2^{BSH+} mice compared to controls, suggesting a compensatory mechanism to restore glucose homeostasis (**Fig. 2.S1B**). Overall, these results demonstrate that the microbial bile acid modifications could help correct the glucose dysregulation in the letrozole-induced PCOS model.

We next assessed the effects of our letrozole model on body weight and composition. LET EcAZ-2 mice had 10% increased body weight compared to VEH EcAZ-2 (19.0 g ± 0.4 in VEH EcAZ-2 and 17.3 g ± 0.3 in LET EcAZ-2; $p = 0.0205$). However, LET mice treated with EcAZ-

2^{BSH+} did not gain weight (17.5 g ± 0.7 in LET EcAZ-2^{BSH+}) compared to LET EcAZ-2 (**Fig. 2.1D**). Interestingly, body compositional analysis revealed that the elevated body weight observed in LET EcAZ-2 mice was not due to an increase in adiposity (**Fig. 2.1E**), corroborated by dissection and measurement of parametrial fat pads after sacrifice (**Fig 2.S1C**). Rather, the increase in body weight was due to a 13% increase in lean body mass in the LET EcAZ-2 condition compared to VEH EcAZ-2 (14.2 g ± 0.3 in VEH EcAZ-2 and 16.0 g ± 0.4 in LET EcAZ-2; p = 0.0042), likely an effect of androgen-mediated muscle hypertrophy. The increase in lean mass was ameliorated in LET EcAZ-2^{BSH+} mice, with a 10% reduction to lean mass and overall body composition similar to controls (14.5 g ± 0.6 in EcAZ-2^{BSH+} LET; p = 0.0173) (**Fig. 2.1F**). This body weight difference between LET EcAZ-2 and LET EcAZ-2^{BSH+} occurred in the setting of no decrease in food consumption or caloric intake, but rather an increase in LET EcAZ-2^{BSH+} mice (**Fig. 2.S1D**). These results suggest that hyperandrogenic signaling in LET mice may be attenuated with EcAZ-2^{BSH+} colonization. Taken together, these results demonstrate that luminal bile acid deconjugation by BSH ameliorates metabolic features of PCOS in a LET mouse model.

EcAZ-2^{BSH+} treatment improves glucose homeostasis in DHT-induced PCOS mice.

To confirm that this effect wasn't model specific, we replicated our findings in another mouse model of PCOS using a subcutaneous pellet of the non-aromatizable androgen dihydrotestosterone (DHT). The 90-day DHT model has a stronger dysmetabolic phenotype than the LET model[55], and it maintains the core hallmarks of PCOS, including hyperandrogenism, anovulation, and impaired glucose homeostasis. The paradigm for this experiment lasted ninety days to establish both metabolic and reproductive features of PCOS, however, study design and

assessments of PCOS phenotype were unchanged (**Fig. 2.1G**). After confirming steady colonization with EcAZ-2 and EcAZ-2^{BSH+} (**Fig. 2.S1E**), we observed that DHT EcAZ-2 mice had significant impairment in glucose handling compared to VEH EcAZ-2 mice, with a 35% increase in ITT AUC ($p < 0.0001$). As in the LET model, the DHT EcAZ-2^{BSH+} mice had improved glucose handling, with a 13% reduction in ITT AUC compared to DHT controls ($p < 0.05$) (**Fig. 2.1H**). We also performed an oral glucose tolerance test (OGTT) which similarly demonstrated a prevention of the impaired glucose homeostasis of DHT mice with EcAZ-2^{BSH+} colonization (**Fig. 2.1I**). OGTT was conducted only in the DHT cohort due to the limited time scale of the LET paradigm. In the DHT model of PCOS, we found no significant differences in fasted blood glucose (**Fig. 2.1F**), fasted insulin (**Fig. 2.S1G**), body weight (**Fig. 2.S1H**), food intake (**Fig. 2.S1I**), adiposity (**Fig. 2.S1J, 2.S1K**), or lean mass (**Fig. 2.S1L**) in DHT EcAZ-2^{BSH+} mice compared to DHT EcAZ-2 controls. These results confirm that the beneficial effect of BSH on hyperandrogenic dysmetabolism is observed in multiple models of PCOS, suggesting that bile acids may provide a promising therapeutic target for individuals with PCOS.

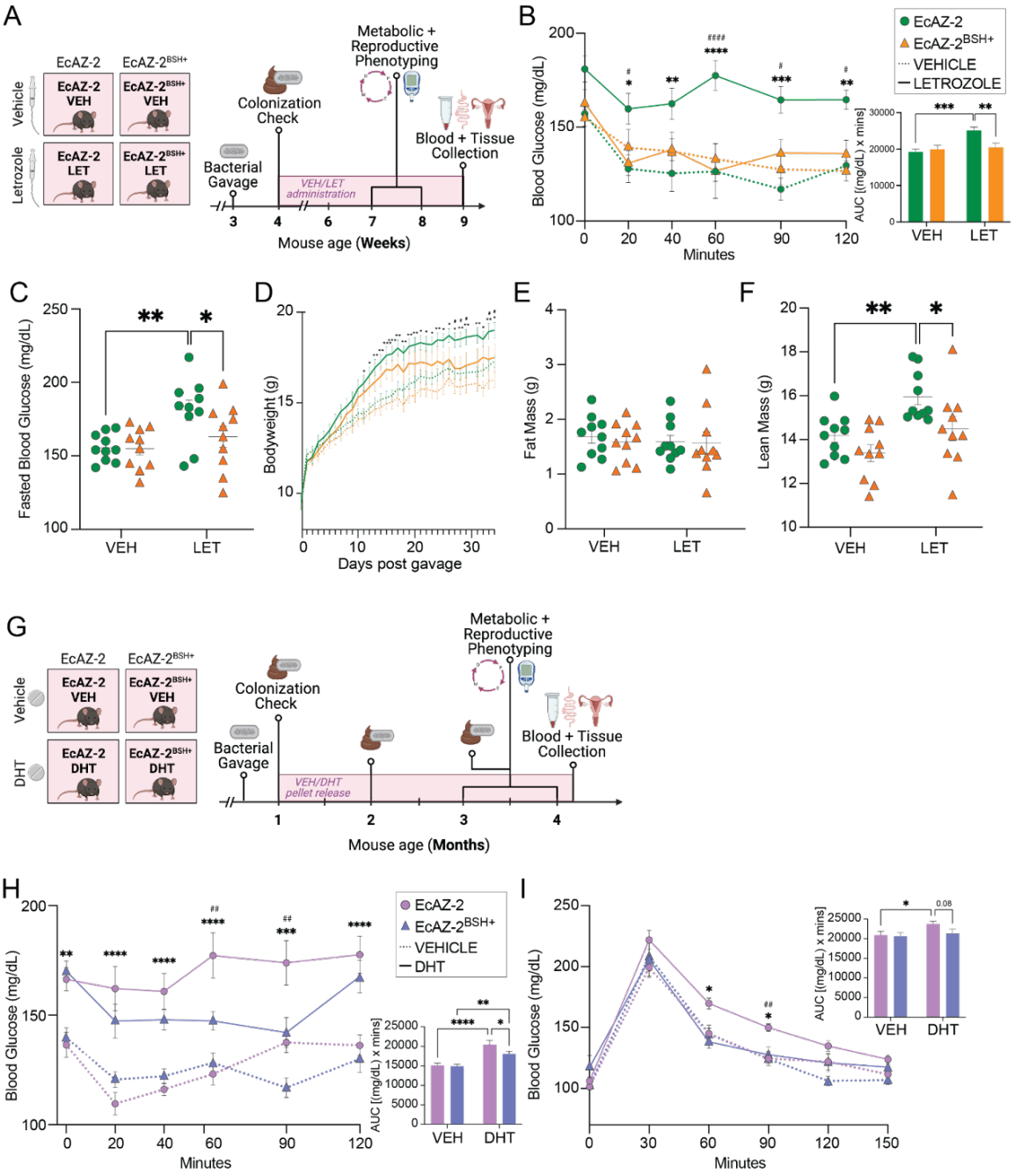
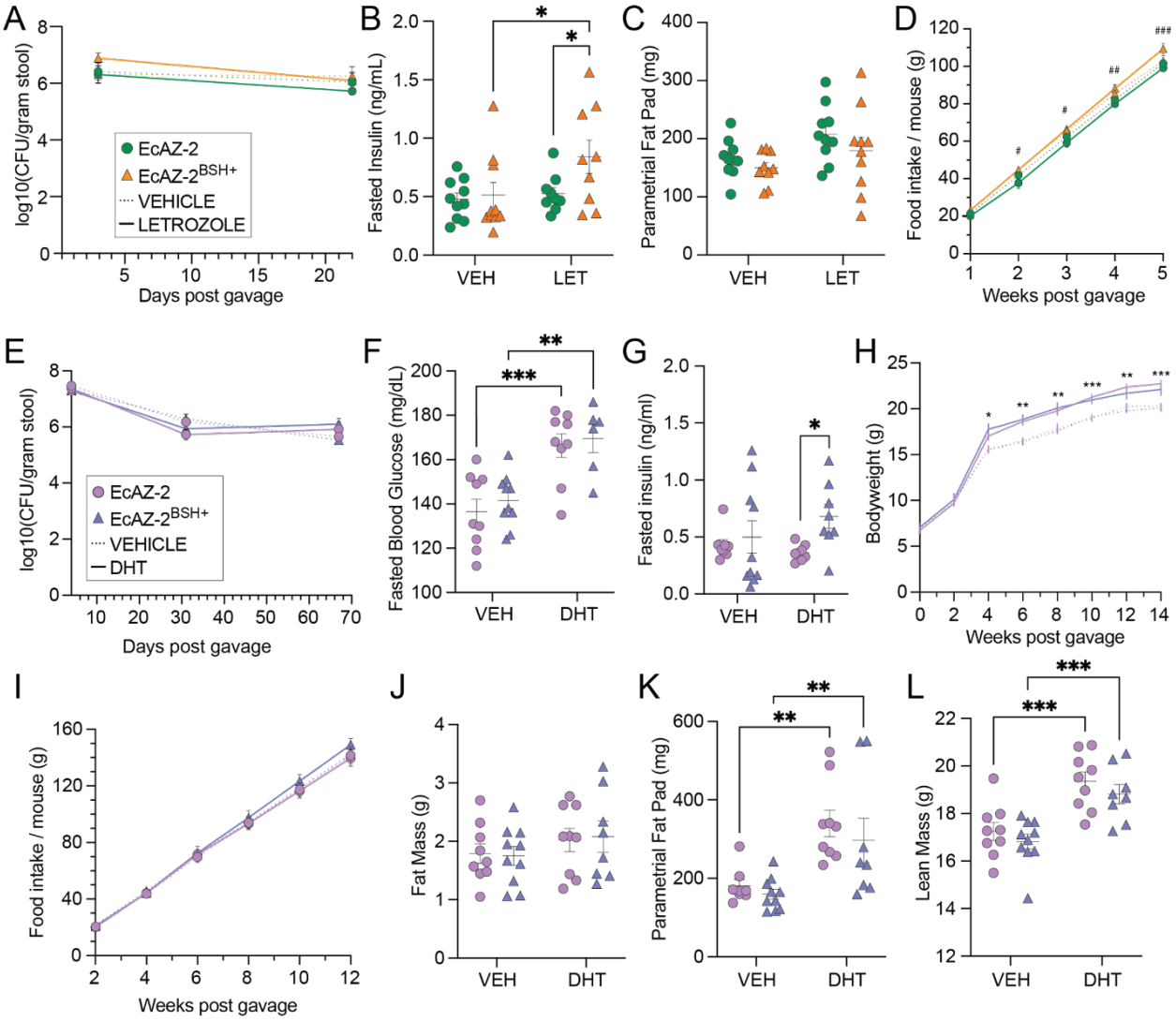


Figure 2.1: EcAZ-2^{BSH+} treatment ameliorates metabolic dysfunction in PCOS mice.

a, LET Study design and experiment timeline. b, ITT after IP injection of insulin. Inset shows area under the curve (AUC). c, Fasted blood glucose levels from ITT timepoint, t = 0. d, Daily body weight measurements. e-f, Fat (e) and lean (f) body mass. g, DHT study design and experiment timeline. h, ITT after IP injection of insulin. Inset shows AUC. i, OGTT after oral gavage of glucose. (b-f, h-i) n = 9-10 mice/group. Plots show individual points (c,e,f) and mean ± SEM. Significance was determined by 2-way ANOVA and is represented by *p < 0.05, **p < 0.01, ***p < 0.001, ****P < 0.0001.



Supplemental Figure 2.1: 3

a, EcAZ-2 and EcAZ-2^{BSH+} bacterial colonization levels at 3 and 22 days post gavage. b, Fasted insulin levels at time of sacrifice. c, Parametrial fat pad weight at time of sacrifice. d, Cumulative food intake per mouse measured weekly (n = 5 cages). (a-d) LET experiment, n = 9-10 mice/group. e, EcAZ-2 and EcAZ-2^{BSH+} bacterial colonization levels at 4, 30, and 68 days post gavage. f, Fasted blood glucose levels from ITT timepoint, t = 0. g, Fasted insulin levels at time of sacrifice. h, Daily body weight measurements. i, Cumulative food intake per mouse measured bi-weekly (n = 5 cages). j-k, Fat mass (j) and parametrial fat pad weight (k) at time of sacrifice. l, Lean mass. (b-c, f-g, j-l) n = 9-10 mice/group. Plots show individual points (b-c, f-g, j-l) and mean ± SEM. Significance was determined by 2-way ANOVA and is represented by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

EcAZ-2^{BSH+} treatment affects some reproductive features of PCOS models.

Next, we investigated whether EcAZ-2^{BSH+} colonization also impacts reproductive features of PCOS. The LET EcAZ-2 mice recapitulated the hyperandrogenism associated with PCOS, with a ⅔-fold increase in testosterone level compared to VEH EcAZ-2 controls (23.5 ng/dL ± 1.6 in VEH EcAZ-2 and 39.3 ng/dL ± 5.9 in LET EcAZ-2; $p = 0.0122$) (**Fig. 2.2A**). However, EcAz-2^{BSH+} did not prevent hyperandrogenism; serum testosterone levels were similar to those in controls (39.9 ng/dL ± 5.1 in LET EcAz-2^{BSH+}). Interestingly, the LET model also elevated luteinizing hormone (LH) levels (0.29 ng/mL ± 0.02 in VEH EcAZ-2 and 1.14 ng/mL ± 0.14 in LET EcAZ-2; $p < 0.0001$), which is also elevated in human patients with PCOS. This was partially ameliorated by EcAz-2^{BSH+} treatment (0.83 ng/mL ± 0.10 in LET EcAz-2^{BSH+}, $p = 0.0284$) (**Fig. 2.2B**). Thus, factors other than LH might be maintaining the elevated testosterone levels. Follicle-stimulating hormone (FSH) was elevated in the LET model, but not affected by EcAZ-2^{BSH+} (**Fig. 2.2C**). In the DHT model of PCOS, we confirmed that levels of DHT were elevated compared to VEH controls (1303 pg/mL ± 82 in DHT EcAZ-2, 1361 pg/mL ± 133 in DHT EcAz-2^{BSH+}, undetectable in VEH controls, $p < 0.0001$) (**Fig. 2.S2A**). In both models hyperandrogenism in PCOS is driven by exogenous addition of aromatase inhibitor (i.e., LET) or androgen (i.e., DHT). Hence, the primary cause of hyperandrogenism in both cases cannot be easily reversed by the addition of EcAz-2^{BSH+} treatment. However, the decrease of LH despite high androgen levels demonstrates that EcAz-2^{BSH+} impacts one of the key drivers of androgen production that contributes to PCOS symptoms. We next wanted to determine whether EcAz-2^{BSH+} treatment affects anovulation and ovarian cysts.

EcAZ-2^{BSH+} treatment doesn't prevent anovulation in PCOS.

To determine if EcAZ-2^{BSH+} improved anovulation, we conducted vaginal cytology to determine the estrous cycle of the mice over seven consecutive days. LET mice were stuck in diestrus, indicating anovulation, and cycling was not restored by EcAZ-2^{BSH+} (**Fig. 2.2D-E)**. Similarly, DHT mice were anovulatory and EcAZ-2^{BSH+} colonized mice remained in diestrus. (**Fig. 2.S2B-C**). Ovary weight was increased by 59% in LET EcAZ-2 mice compared to VEH controls, which is associated with hyperandrogenism and polycystic ovarian morphology (PCOM) (2.2mg ± 0.2 in VEH EcAZ-2 and 3.5mg ± 0.2 in LET EcAZ-2; $p < 0.0001$). This increase in ovary weight was ameliorated by 17% by EcAZ-2^{BSH+} (2.9mg ± 0.3 in LET EcAZ-2^{BSH+}; $p = 0.0447$) (**Fig. 2.2F**). To investigate if this reflected PCOM, we conducted H&E staining of ovaries to enumerate the number of corpora lutea (CL), transient structures that emerge following rupture of the developed follicle, indicating ovulation. Histological results confirmed that LET-treated mice were indeed anovulatory, and demonstrated that EcAZ-2^{BSH+} did not rescue PCOM (**Fig. 2.2G-H**). DHT EcAZ-2^{BSH+} mice had no change in ovary weight compared to DHT EcAZ-2 mice, and were anovulatory as detected by ovarian histology (**Fig. 2.2SD-F**). Taken together, although we observed some subtle reproductive changes, EcAZ-2^{BSH+} does not prevent acyclicity and anovulation in LET and DHT models of PCOS.

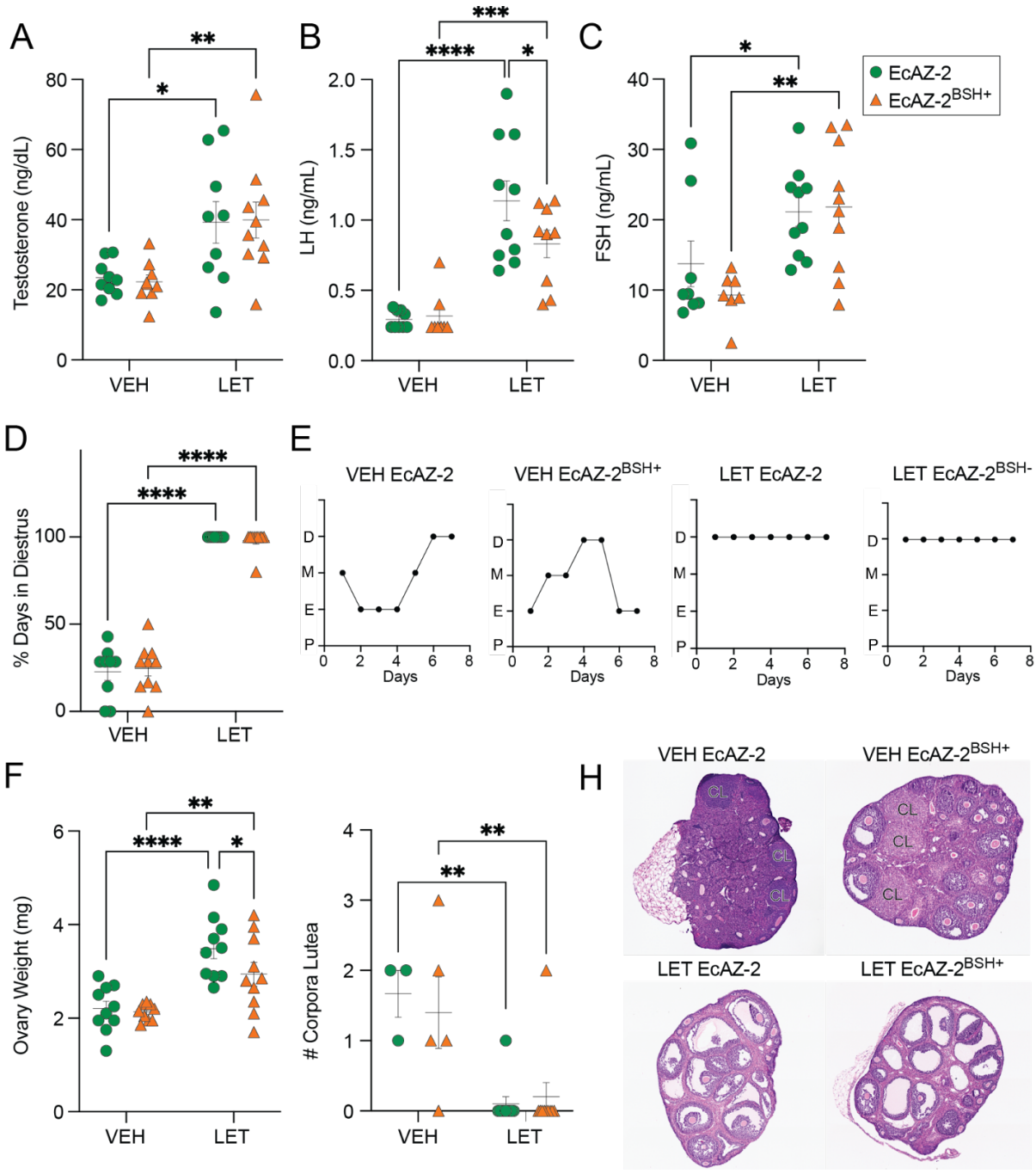
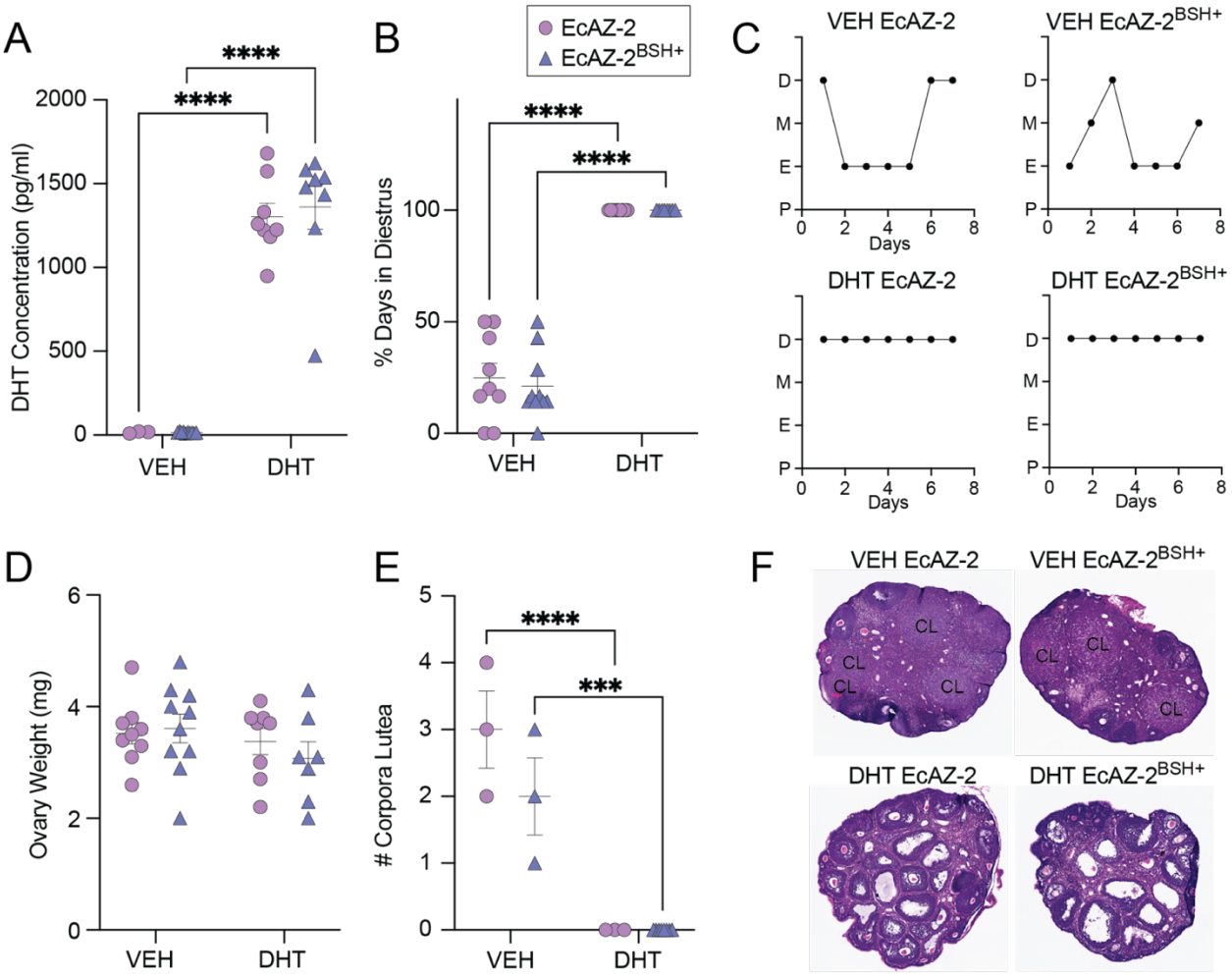


Figure 2.2: EcAZ-2^{BSH+} treatment doesn't restore reproductive function in PCOS mice. 4

a, Serum testosterone, b, LH, c, and FSH levels at time of sacrifice. d, Quantitative analysis of % days in diestrus. e, Representative estrous cycles from one mouse per condition. The stages of the estrous cycle are designated as diestrus (D), metestrus (M), estrus (E), and proestrus (P). f, Ovary weight at time of sacrifice. g, Quantitative analysis of corpora lutea (n = 3-10/group) h, Representative histology of ovaries stained with hematoxylin and eosin (H&E) from one mouse per condition. Corpora lutea labeled as (CL). (a-d,f) n = 9-10 mice/group. (a-d,f-g) Plots show individual points and mean ± SEM. Significance was determined by 2-way ANOVA and is represented by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$.



Supplemental Figure 2.2: 5

a, DHT concentration from serum at time of sacrifice. b, Quantitative analysis of time in diestrus. c, Representative estrous cycles from one mouse per condition. The stages of the estrous cycle are designated as diestrus (D), metestrus (M), estrus (E), and proestrus (P). d, Ovary weight at time of sacrifice. e, Quantitative analysis of corpora lutea (n = 3-10). f, Representative H&E histology of ovaries from one mouse per condition. (a-b,d) n = 9-10 mice/group. (a-b,d-e) Plots show individual points and mean ± SEM. Significance was determined by 2-way ANOVA and is represented by *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.

LET model and EcAZ-2^{BSH+} treatment affect fecal bile acid composition.

We have reported that introduction of EcAZ-2^{BSH+} bacteria to conventionally raised male mice increases the level of fecal total BAs and secondary BAs compared to controls, without changing overall gut microbiome composition in healthy male mice.[49] We now investigate the effects of luminal BSH overexpression on fecal bile acids and microbiome composition not only in female mice, but also in the context of a PCOS model. Targeted fecal metabolomics revealed that LET decreased total fecal BAs (177.5 ± 28.4 in VEH EcAZ-2 and 42.9 ± 3.6 in LET EcAZ-2; p = 0.0013) compared to VEH, largely driven by a decrease in primary BAs (121.0 ± 28.4 in VEH EcAZ-2 and 17.1 ± 4.2 in LET EcAZ-2; p = 0.0052; **Fig. 2.3A**). EcAZ-2^{BSH+} did not impact total or primary bile acid levels in either LET or VEH mice **(Fig. 2.3B)**. EcAZ-2^{BSH+} did increase levels of secondary BAs in the VEH condition (56.5 ± 8.2 in VEH EcAZ-2 and 92.2 ± 20.2 in VEH EcAZ-2^{BSH+}; p = 0.0328) as we had reported in male mice[49], however, it did not have the same effect in the LET condition, perhaps due to the overall reduction in bile acid levels (**Fig. 2.3C**). Cholic acid (CA) levels, which were lower in the LET model (7.2 ± 1.4 in VEH EcAZ-2 and 2.6 ± 0.5 in LET EcAZ-2; p = 0.0851)**,** were greater in LET EcAZ-2^{BSH+} mice, at levels similar to healthy controls (8.6 ± 1.7 in LET EcAZ-2BSH+; p = 0.0258) (**Fig. 2.3D**). Chenodeoxycholic acid (CDCA) was also significantly increased by EcAZ-2^{BSH+} in LET mice (4.9 ± 0.7 in LET EcAZ-2 and 7.2 ± 0.7 in LET EcAZ-2 BSH+; p = 0.0192) but not affected by LET compared to VEH (**Fig. 2.S3A**). No other changes were observed in the relative abundance of specific BAs as a percentage of total BAs due to EcAZ-2^{BSH+}. These results indicate that while the LET PCOS model significantly changes the overall bile acid pool, EcAZ-2^{BSH+} more specifically increases the deconjugated primary BAs CA and CDCA in LET mice.

LET model and EcAZ-2^{BSH+} treatment modulate microbiome composition and function.

To further understand the role of EcAZ-2^{BSH+} on the microbiome, we performed metagenomic sequencing on fecal samples from each condition. Shannon diversity index, which reflects both richness and evenness, revealed a 34% increase in α-diversity in LET mice compared to VEH, and no effect of EcAZ-2^{BSH+}on α-diversity (**Fig. 2.3E**). Principle coordinate analysis using Bray-Curtis dissimilarity (β-diversity) (**Fig. 2.3F**) recapitulated other studies.[13, 17, 18, 56] in distinguishing microbiome composition between LET and VEH mice (p = 0.018) (**Fig. 2.3G)**, and as we previously reported in male mice[49], EcAZ-2^{BSH+} did not cause overall compositional changes in VEH mice (p = 0.244) (**Fig. 2.3SB)** or in LET mice (p = 0.809) (**Fig. 2.3H**). However, LET EcAZ-2^{BSH+} mice were also indistinguishable from VEH EcAZ-2 (**Fig. 2.S3C**, PERMANOVA p = 0.176), suggesting a subtle shift to a microbiome composition between that of LET EcAZ-2 mice and VEH EcAZ-2.

To determine what compositional changes underlie these differences, we performed differential abundance analysis using ALDEx2 (Fernandes et al., 2014). At the species level, we observed a reduction in the relative abundance of several bacteria within the order Bacteroidales, including the genera *Prevotella, Bacteroides, Parabacteroides*, and an increase in the relative of abundance of several species within the order Clostridiales, including those from the genus *Butyrivibrio,* in LET compared to VEH. EcAZ-2^{BSH+} induced unique differences in the relative abundance of species, including a depletion of several bacteria in the genus *Lactobacillus*. However, the abundance of one species is affected by both LET administration and EcAZ-2^{BSH+} colonization, the archaeon *Halobiforma nitratareducens,* which is depleted in LET mice compared to VEH but higher in abundance in LET EcAZ-2^{BSH+} compared to LET EcAZ-2 (**Fig. 2.3I**). To further investigate functional changes, ALDEx2 at the KEGG orthologue (KO) level illustrates a

number of functions that follow a similar pattern, more abundant in LET EcAZ-2 compared to VEH controls and less abundant in LET EcAZ-2^{BSH+} compared to EcAZ-2 controls, or vice versa, revealing potential mechanisms through which EcAZ-2^{BSH+} prevents LET-associated dysbiosis (**Fig. 2.3J).** These KOs relate to amino acid metabolism, carbohydrate metabolism, cell growth, and notably, nitrate reduction, an alternative form of respiration that is a capability of *H. nitratireducens.*[57] Taken together, these results indicate that EcAZ-2^{BSH+} shifts microbiome function in LET mice to a state more similar to healthy controls.

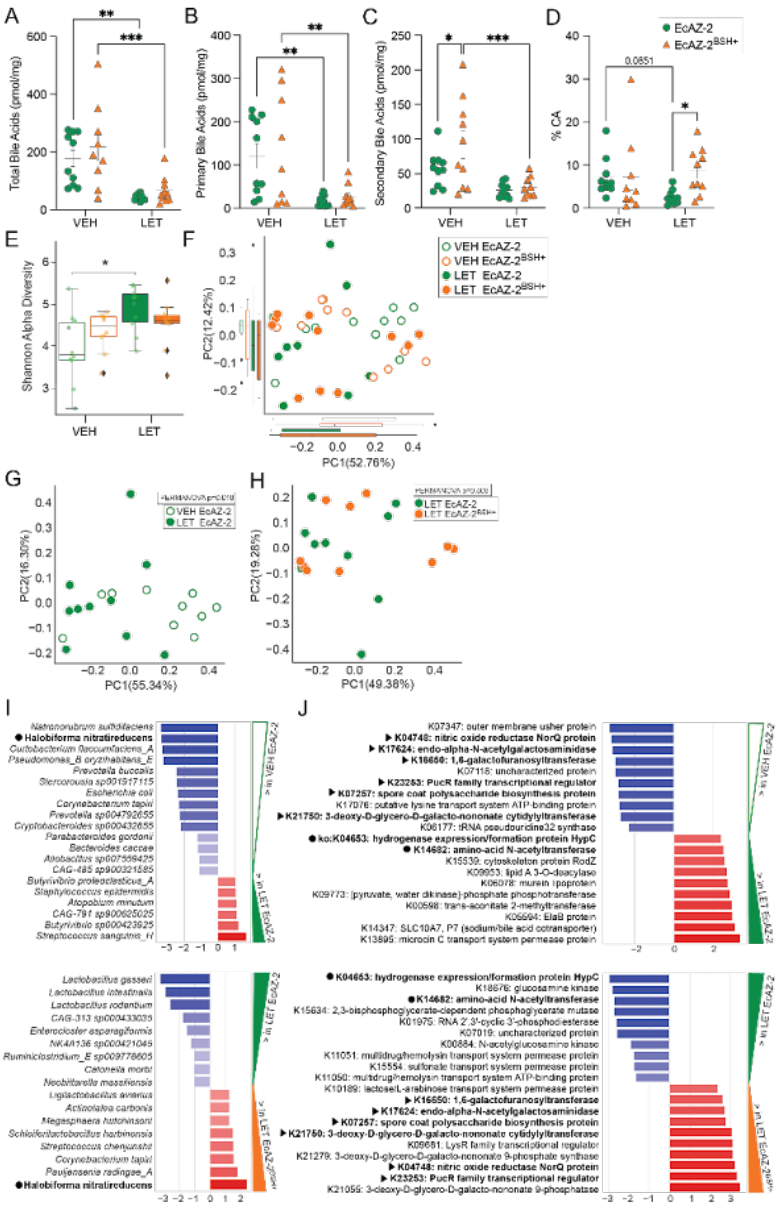
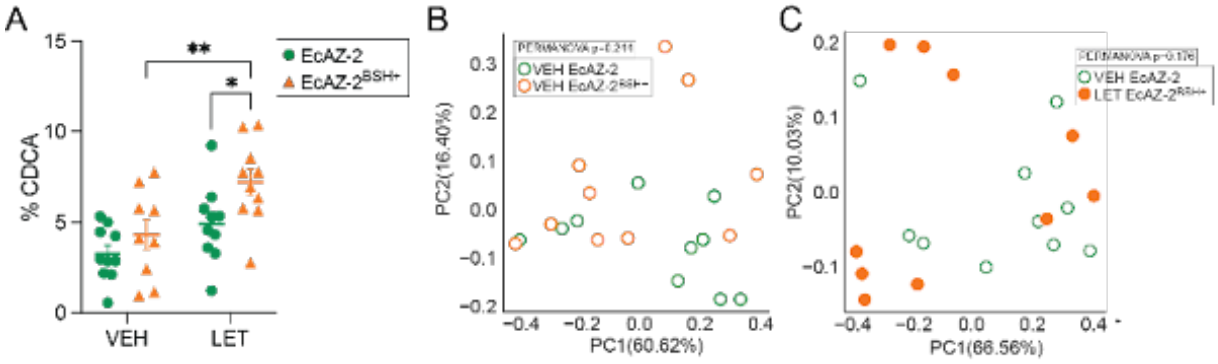


Figure 2.3: Bile acid composition and microbiome composition and function are altered by LET model and EcAZ-2^{BSH+} colonization. 6

a-d, Total bile acids (a), primary bile acids (b), secondary bile acids (c), and % cholic acid (d) in fecal samples collected from mice prior to sacrifice. Significance was determined by 2-way ANOVA and is represented by *p < 0.05, **p < 0.01, ***p < 0.001. Plots show individual points and mean ± SEM. e, Shannon diversity index (a measure of α-diversity) from metagenomic sequencing of fecal samples collected from mice prior to sacrifice. f-h, Principal coordinate analysis based on Bray-Curtis dissimilarity (a measure of beta diversity) in all four conditions (f), LET compared to VEH (g), and EcAZ-2^{BSH+} compared to EcAZ-2 in the context of the LET model (h). Significance was determined by PERMANOVA. i,j, ALDEx2 differential abundance analysis at species level (i) and KEGG orthologue (KO) level. Effect size >= 0.5. (j). (e-j) Metagenomic sequencing of fecal samples collected from mice prior to sacrifice. (a-j) n = 9-10 mice/group.



Supplemental Figure 2.3: 7

a % chenodeoxycholic acid in fecal samples collected from mice prior to sacrifice**.** Significance was determined by 2-way ANOVA and is represented by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Plots show individual points and mean ± SEM. **b,c,** Principal coordinate analysis based on Bray-Curtis dissimilarity (a measure of beta diversity) in EcAZ-2^{BSH+} compared to EcAZ-2 in VEH mice (b) and LET EcAZ-2^{BSH+} compared to VEH EcAZ-2 (c). Significance was determined by PERMANOVA. (a-c) n = 9-10 mice/group.

Effects of EcAZ-2^{BSH+} treatment on PCOS metabolic and reproductive features are mediated by FXR.

Even with subtle effects on bile acid composition, we postulated that bile acid receptors mediate the physiological effects of increased luminal BSH activity on PCOS mice. Bile acids are ligands for the nuclear hormone receptor farnesoid X receptor (FXR) which is a major metabolic regulator[58-60] and may have a direct role in reproductive function.[61] To investigate the extent to which FXR mediates the effect of EcAZ-2^{BSH+}, we repeated the paradigm outlined in **Fig. 2.1A** using *Nr1h4*$^{-/-}$ mice (FXR KO).

In the absence of FXR, the engineered native bacteria maintained stable colonization (**Fig. 2.S4A**). LET EcAZ-2 mice still demonstrated significant impairment in glucose handling, with a 38% greater ITT AUC compared to VEH EcAZ-2 mice. However, LET EcAZ-2^{BSH+} no longer lowered glucose during ITT or increased fasted insulin levels compared to LET EcAZ-2 in the context of the FXR KO (**Fig. 2.4B, 2.S4B**). Similarly, LET treatment in the FXR KO EcAZ-2 mice resulted in a 24% increase in FBG levels (139.43 mg/dL ± 4.87 in VEH EcAZ-2 and 173.0 mg/dL ± 7.4 in LET EcAZ-2; $p = 0.0007$), which was not prevented by EcAZ-2^{BSH+} in FXR KO mice (**Fig. 2.4B**). LET EcAZ-2 mice had 18% greater body weight than VEH EcAZ-2 mice (16.6 ± 0.5 in VEH EcAZ-2 and 18.7 ± 0.2 in LET EcAZ-2; $p = 0.0020$), which unlike in the WT cohort, was not significantly different between LET mice colonized with EcAZ-2^{BSH+} compared to LET EcAZ-2 controls. (**Fig. 2.4C**). There were no significant differences in lean body mass between the four groups (**Fig. 2.4D**). As in the WT LET cohort, EcAZ-2^{BSH+} did not affect adiposity (**Fig. 2.S4C,D**) in the absence of FXR. Taken together, these results suggest that global knockout of *Nr1h4* didn't interfere with the LET model's recapitulation of PCOS dysmetabolism. However, FXR appears to mediate the effect of EcAZ-2^{BSH+} in ameliorating metabolic features of PCOS, suggesting that

FXR is necessary for the beneficial effect of luminal bile acid deconjugation on PCOS pathophysiology.

With regards to reproductive phenotype in the FXR KO mice, LET EcAZ-2 mice maintained six-fold higher LH levels compared to VEH EcAZ-2 mice (1.48 ng/mL ± 0.3 in VEH EcAZ-2 and 9.4 ng/mL ± 1.4 in LET EcAZ-2; $p < 0.0001$) (**Fig. 2.4E**) and had 34% increased ovary weight (2.06 g ± 0.17 in VEH EcAZ-2 and 2.77 g ± 0.30 in LET EcAZ-2; $p = 0.0150$) (**Fig. 2.4F**). EcAZ-2^{BSH+} mice did not, in the absence of *FXR*, ameliorate LH levels or ovary weight in LET mice. As in the WT LET cohort, EcAZ-2^{BSH+} did not affect serum levels of testosterone (**Fig. 2.S4E**) and FSH (**Fig. 2.S4F**), or prevent anovulation (**Fig. 2.S4G**) in FXR-/- mice. This suggests that FXR is also not required for the reproductive phenotype of the LET model, but is necessary for the effects of EcAZ-2^{BSH+} on LH and ovary weight, revealing that FXR mediates the effect of BSH on both metabolic and reproductive parameters of PCOS.

Effects of EcAZ-2^{BSH+} treatment on PCOS metabolic and reproductive features are partially mediated by TGR5.

Bile acids are also ligands for the G protein–coupled receptor Takeda G-protein-receptor-5 (TGR5), which mediates metabolism[62-65] and reproduction[51, 66-68]. To test the role of TGR5 in mediating the effect of EcAZ-2^{BSH+} colonization, we repeated the same paradigm (**Fig. 2.1A**) again in *Gpbar1*$^{-/-}$ (TGR5 KO) mice.

In a global knockout of TGR5, as in the WT LET cohort, EcAZ-2^{BSH+} did not affect colonization levels of the engineered native bacteria (**Fig. 2.S4G**). LET mice still demonstrated significantly elevated glucose levels in the ITT compared to VEH controls, with a 38% greater ITT AUC, however LET EcAZ-2^{BSH+} mice did not have improved glucose handling compared to

LET EcAZ-2 mice (**Fig. 2.4G**). LET EcAZ-2 mice also maintained a significantly higher FBG level compared to VEH EcAZ-2 mice (175.13 mg/dL ± 3.2 in VEH EcAZ-2 and 191 mg/dL ± 6.7 in LET EcAZ-2; $p = 0.0204$). In contrast to the FXR knockout mice, however, LET EcAZ-2^{BSH+} still demonstrated significantly lower FBG levels compared to LET controls (172.75 mg/dL ± 3.1 in LET EcAZ-2^{BSH+}; $p = 0.0086$) (**Fig. 2.4H**), whereas fasted insulin levels remained unchanged (**Fig. 2.S4H**). In the TGR5 KO mice, LET EcAZ-2 mice had significantly greater body weight than VEH EcAZ-2 mice, not prevented by EcAZ-2^{BSH+} (**Fig. 2.4I**). Similar to the WT LET cohort, in the absence of TGR5, LET EcAZ-2 had an 11% increase in lean mass compared to VEH controls (14.2 ± 0.5 in VEH EcAZ-2 and 15.8 ± 0.4 in LET EcAZ-2; $p = 0.0272$), which was prevented EcAZ-2^{BSH+}colonization, with a 10% decrease to lean mass similar to VEH controls (14.4 mg/dL ± 0.4 in LET EcAZ-2^{BSH+}; $p = 0.0583$) (**Fig. 2.4J**). As in the WT LET cohort, EcAZ-2^{BSH+} did not affect adiposity (**Fig. 2.S4I-J**) in the TGR5 KO mice. In summary, LET effectively recapitulates the PCOS metabolic phenotype in the absence of TGR5, and TGR5 is necessary for the effect of EcAZ-2^{BSH+}on LET-induced insulin response and bodyweight, suggesting the effect of bile acid signaling in mediating those features of PCOS are regulated by both FXR and TGR5. TGR5 does not, however, mediate the effect of EcAZ-2^{BSH+} on reducing LET-induced hyperglycemia or lean mass hypertrophy, suggesting that these PCOS features are regulated by FXR alone.

With regards to reproductive phenotype, LET EcAZ-2 mice had four-fold higher LH levels compared to VEH EcAZ-2 mice in the TGR5 KO (3.6 ng/mL ± 0.8 in VEH EcAZ-2 and 15.3 ng/mL ± 1.4 in LET EcAZ-2; $p < 0.0001$). Elevated LH levels were ameliorated with a 54% reduction in LET EcAZ-2^{BSH+} mice compared to LET controls (10.0 ng/mL ± 1.4 in LET EcAZ-2^{BSH+}; $p = 0.0021$) (**Fig. 4K**). In TGR5 KO mice, serum testosterone levels were nearly doubled in LET EcAZ-2^{BSH+} compared to LET controls ($p = 0.0103$), so negative feedback from high T

levels could explain the decrease in LH level (**Fig. 2.S4K**). Similar to WT, LET EcAZ-2 mice had 52.9% greater ovary weight compared to VEH EcAZ-2 mice in the TGR5 KO (2.1 g ± 0.2 in VEH EcAZ-2 and 3.2 g ± 0.2 in LET EcAZ-2; $p = 0.0007$), however, the increase in ovary weight was not prevented by EcAZ-2^{BSH+} in LET mice (**Fig. 2.4L**). As in the WT LET cohort, EcAZ-2^{BSH+} did not impact serum levels of FSH (**Fig. 2.S4L**), or prevent anovulation (**Fig. 2.S4M**) in the absence of TGR5. In addition to the metabolic phenotype, LET effectively recapitulates the PCOS reproductive phenotype in the TGR5 KO, and TGR5 is necessary for the effect of EcAZ-2^{BSH+}on LET-induced increase in ovary weight, in addition to FXR. TGR5 is not, however, necessary for the effect of EcAZ-2^{BSH+} in reducing LH levels, suggesting that the effect of bile acid deconjugation on LH levels is mediated more so by FXR. In summary, both FXR and TGR5 play a role in mediating the effect of EcAZ-2^{BSH+} in ameliorating features of PCOS (**Fig. 2.4M**).

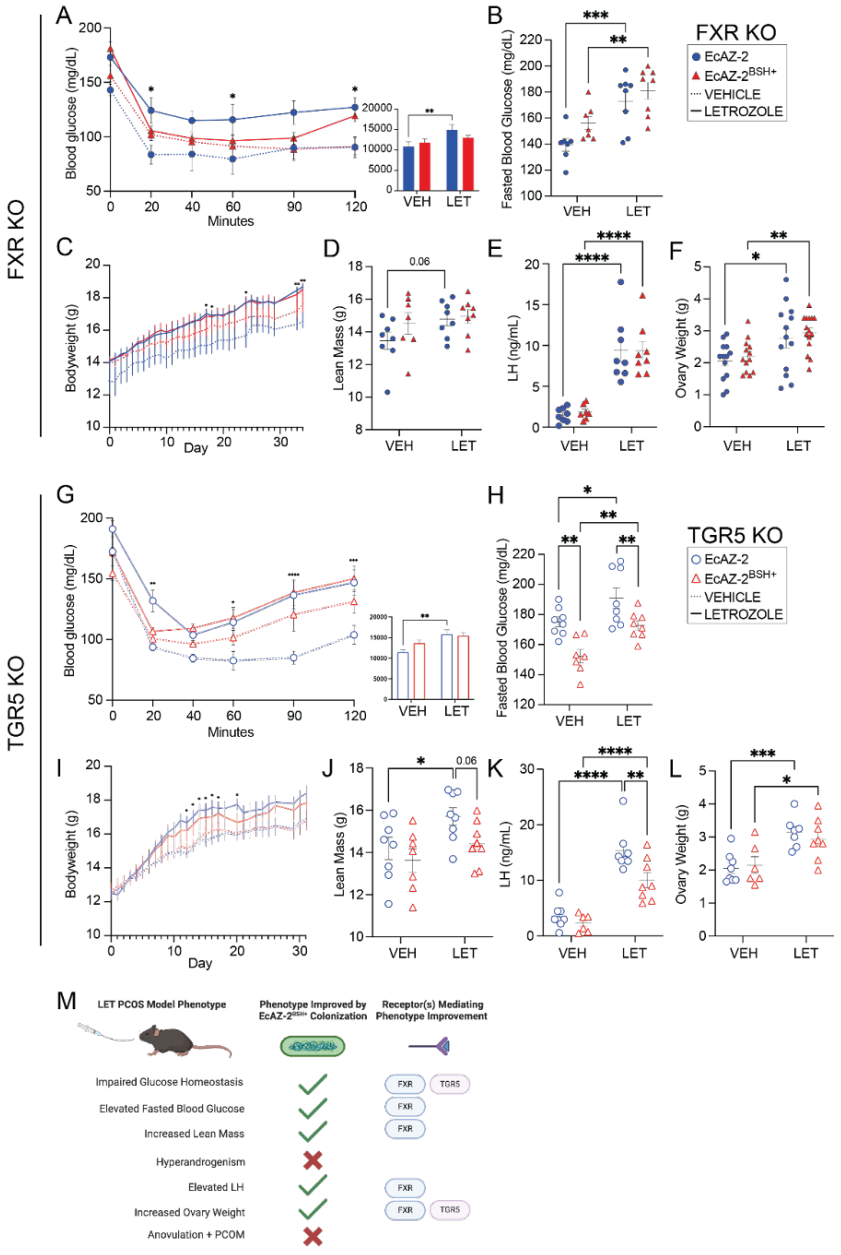
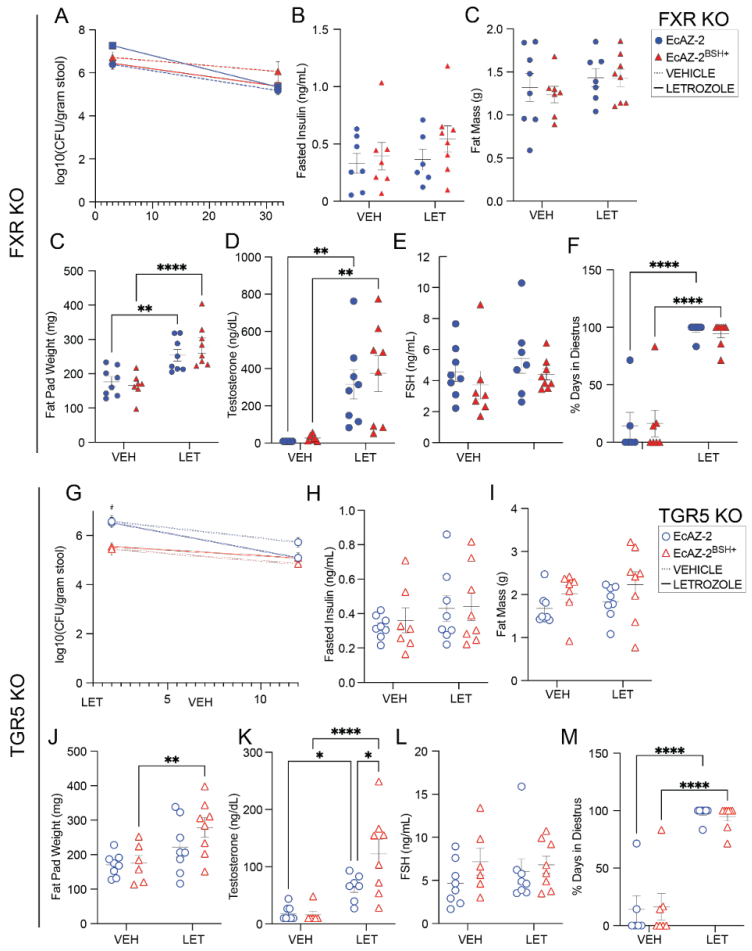


Figure 2.4: EcAZ-2^{BSH+} effects on PCOS-related metabolic and reproductive phenotype are mediated by FXR and TGR5. 8

LET study in FXR KO (a-f) and TGR5 KO (g-l) mouse cohorts follows the same study design and timeline as the WT LET cohort. n=7-8 per group for all phenotyping. a,g Insulin tolerance test showing blood glucose levels 0-120 minutes after IP injection of insulin. Inset shows AUC. b,h Fasted blood glucose levels from ITT timepoint, t=0. c,i Daily body weight measurements. d,j Lean body mass measured from EchoMRI. e-f,k-l Serum LH levels (e,k) and ovary weight (f,l) at time of sacrifice. (b, d-f, h, j-l) Plots show individual points and mean ± SEM. m, Summary figure of the role of EcAZ-2^{BSH+} and bile acid receptors in PCOS phenotype. (a-l) n = 7-8 mice/group. Significance was determined by 2-way ANOVA and is represented by *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.



Supplemental Figure 2.4: 9

LET study in FXR KO (a-f) and TGR5 KO (g-m) mouse cohorts. a,g Fasted insulin levels at time of sacrifice. b,h EcAZ-2 and EcAZ-2^{BSH+} bacterial colonization levels at two timepoints. c,i Fat body mass measured from EchoMRI. d,j Parametrial fat pad weight at time of sacrifice. e-f,k-l Serum testosterone (d) and FSH (e) levels at time of sacrifice. g,m Quantified percent days in diestrus based on vaginal cytology. (b-f, h-m) Plots show individual points and mean ± SEM. (a-m) n = 7-8 mice/group. Significance was determined by 2-way ANOVA and is represented by *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.

2.4 Discussion

The role of the gut microbiome in PCOS is complex and multidimensional, and the specific role of bile acids on metabolic and reproductive dysfunction has yet to be fully elucidated. In this study, we demonstrate that hyperandrogenism, dysregulation of glucose homeostasis, anovulation, and polycystic ovarian morphology are recapitulated by LET and DHT models of PCOS, as seen in previous studies.[54,69] Engineered native bacteria overexpressing BSH in conventional mice demonstrate that bacterial bile acid biotransformations protect against glucoregulatory dysfunction in PCOS. Importantly, overexpression of BSH did not alter normal female metabolic and reproductive phenotypes in WT mice. Moreover, BSH over-expression improves glucose handling in two models of PCOS, which both have elevated glucose levels in an ITT but with varying levels of adiposity, providing evidence for modulation of BSH as a promising therapeutic strategy for both lean and obese individuals with PCOS. LET-treated mice had a shift in the fecal bile acid pool, which has been reported in PCOS patients[12, 70], where untargeted bile acid metabolomics significantly improves random forest classification accuracy between LET and placebo-treated mice compared to 16S alone.[71] We replicated a change in the relative abundance of bacteria between LET and control mice that were similar to previous reports, specifically a decrease in species belonging to Bacteroidales and an increase in species belonging to Clostridiales.[18] Notably, our results did not replicate the higher abundance of *B. vulgatus* and other Bacteroides species that discriminated PCOS subjects from control subjects in the Qi et. al. study.[12] Analysis of metagenomic sequencing allowed us to further characterize the gut microbiome of LET mice at the level of gene functions, for which we also report differences in the LET model. EcAZ-2^{BSH+} colonization shifts composition and function of the gut microbiome of PCOS model mice, without altering overall microbial diversity or bile acid levels, demonstrating its efficacy as a precise means to functionally manipulate the gut microbiome.

We hypothesized that improvement of glucose handling would have downstream effects on reproductive function in the context of PCOS. Insulin stimulates androgen synthesis in ovarian theca cells independently from LH-induced androgen synthesis[72-73], suggesting that insulin resistance further perpetuates PCOS-associated reproductive dysfunction. Furthermore, improving insulin sensitivity through dietary and pharmacological agents in individuals with PCOS improves reproductive features.[74] Despite the marked improvement in the metabolic symptoms of PCOS as well as a significant reduction in LH levels and ovary weight, EcAZ-2^{BSH+} did not prevent PCOS-associated hyperandrogenism, anovulation, or polycystic ovarian morphology. Further research is needed to determine whether this effect on LH and ovary weight is biologically relevant, including investigation of LH pulsatility to improve temporal resolution of LH frequency and amplitude. We suggest EcAZ-2^{BSH+} was not able to overcome the high bolus and frequency of exogenous aromatase inhibitor or androgens, respectively, in the letrozole and DHT PCOS mouse models. However, we propose that a future study using a continuous LET or DHT administration model to recapitulate PCOS, or even a study in PCOS patients whose hyperandrogenism is less severe than most mouse models, may reveal that increasing BSH activity is sufficient to reduce hyperandrogenism and restore ovulation.

EcAZ-2^{BSH+} colonization significantly reduced PCOS-associated elevated LH serum levels and increased ovary weight, demonstrating that luminal BSH over-expression indeed impacts indicators of reproductive function in PCOS. In addition to downstream effects of metabolic improvements, there is evidence that bile acid signaling directly modulates the HPG axis. FXR is present in ovarian follicles[75] within granulosa cells[61], which also contain enzymes for bile acid synthesis[76], and bile acids are present in high concentration within bovine and human follicular fluid.[77,78] TGR5 has been identified in ovarian granulosa cells[79], and is expressed in the

hypothalamus and pituitary of mice[80], suggesting potential for direct bile acid receptor activation in the HPG axis. Although it has not yet been investigated in females, FXR and TGR5 activation in testicular Leydig cells regulate testosterone levels and reproductive function in male mice.[51, 66, 67, 68] Additionally, due to structural similarity between androgens and bile acids[50, 81-83], crosstalk between these molecules and their receptors are likely to affect reproductive endocrine signaling and PCOS physiology. Neither the FXR or TGR5 KO impaired the estrous cycle of female control mice, suggesting that they are not independently necessary for reproductive function. Future studies using WT littermate controls can address the specific effects of these KOs on normal female reproduction. Our results suggest that FXR mediates the decrease of LH levels by BSH, and both FXR and TGR5 are necessary for the effect of BSH on ovary weight. Further research, such as tissue specific FXR and TGR5 KOs, can elucidate whether these effects are dependent on metabolic improvements or HPG-axis specific.

PCOS is a heterogeneous condition whose phenotype varies between individuals based on age, genotype, environment, and lifestyle factors.[84] There is a growing appreciation for the role of the microbiome in gynecological health[85] and the reproductive endocrine system.[86,87] The gut microbiome is increasingly becoming a target for PCOS treatment, with clinical trials demonstrating that pre- and probiotic supplementation reduces testosterone levels and BMI in PCOS patients.[88] Compositional studies of the microbiomes of PCOS individuals can be biased by dietary and environmental factors, and methodological variability between laboratories further contributes to difficulties with replicability and reproducibility.[89] This underscores the value of mechanistically investigating microbial functions, such as with engineered native bacteria, to identify conserved mechanisms across populations. Since bacterial and bile acid composition was measured in feces, this reflects the microbiome in the large intestine lumen, but not the small

intestine, where the majority of bile acid biotransformations occur. This caveat could be addressed by a future study characterizing microbial and bile acid composition in ileal contents in response to LET administration and EcAZ-2^{BSH+}. Targeted approaches to unravel metabolic and reproductive features will support tailoring therapeutic approaches to the individual, depending on goals of treatment. Future directions will elucidate the precise pathways through which microbial bile acid deconjugation influences metabolic and reproductive health, in both healthy states and in the context of PCOS. Investigating the interplay between the gut microbiome, insulin signaling, and reproductive endocrine regulation will provide deeper insights into disease mechanisms and lead to potential therapeutics for individuals with PCOS.

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Zyagen. Microscopy was performed at the UCSD Microscopy core. Testosterone, LH, and FSH assays were performed at the UVA Ligand Laboratory.

Author Contributions

Conceptualization: ESM, SC, VT, AZ; Methodology, Investigation, and Validation: EM, SC, ER, RAR, AFMP, LS, JDB, SMR, RK, VGT, AZ; Software: RAR; Formal Analysis: EM, SC, ER, RAR, SMR, AZ; Resources: EM, SC, ER, AFMP, THB, MA, JDB, AS, RK, VGT, AZ; Data Curation: EM, SC, RAR; Writing – Original Draft Preparation: EM, SC, ER, AZ; Writing – Reviewing and Editing: EM, SC, ER, SMR, RK, VGT, AZ; Visualization: EM, SC, ER, RAR; Supervision: AZ; Project administration: AZ; Funding Acquisition: EM, AZ.

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Chapter 3: Gut microbiome composition, function, and rhythmicity are modulated by time-restricted feeding in Alzheimer's disease mice accompanying rescue of brain pathology and memory.

3.1 Introduction

Alzheimer's disease (AD), the most prevalent form of dementia, is a multifactorial neurodegenerative disorder with an increasingly overwhelming disease burden, currently impacting an estimated 6.7 million Americans.[1] Its complex etiology presents a critical need to identify pathological mechanisms beyond the amyloid cascade to better understand the molecular underpinnings of AD and identify new biomarkers and therapeutics.

Circadian rhythmicity disruption is an early clinical marker for more than 80% of patients with AD, and emerging evidence suggests that these alterations directly contribute to advancing neuropathology.[2,3] Modulation of the circadian clock is being investigated as a strategy to improve health outcomes in AD and its associated risk factors such as dysmetabolism and inflammation.[4,5] Specifically, our group demonstrated that entraining circadian rhythms through time-restricted feeding (TRF; 6 h feed, 18 h fast cycles) without caloric restriction rescues neuropathology and improves sleep and memory in mouse models of AD.[6] Preliminary evidence suggests that the analogous human intervention, time restricted eating, may produce similar neuroprotective effects for patients with mild cognitive impairment (MCI) and AD.[7] While transcriptional regulation by TRF points to several potential targets, the precise mechanisms by which TRF modulates brain transcription, cognition, and neuropathology remains to be elucidated and are likely pleiotropic.

Growing evidence supports a role for the gut-brain axis in neurodegenerative, metabolic, and inflammatory disease[8,9] and emerging data show that the microbiome and its secondary metabolites influence health and behavior.[10-13] Studies using AD models and human subjects suggest that gut microbiome-based biomarkers are linked to amyloid pathology and

neuroinflammation, thus supporting their role in neurodegeneration.[14-17] Still, studies defining the mechanistic contribution of the microbiome to AD pathology is needed.

A key potential mediator of the gut microbiome and TRF in AD are bile acids (BA), a family of metabolites modulated by the gut microbiome which serve critical roles in cholesterol, lipid, and glucose homeostasis, blood–brain barrier permeability, neuroinflammation, circadian rhythms, and neurodegeneration.[18-20] Importantly, BAs are dysregulated in AD.[21] Recent studies using large cohorts from the Alzheimer's Disease Neuroimaging Initiative (ADNI) showed that serum BA associate with AD biomarkers and alterations in BA correlate with cognitive impairment, with AD patients showing lower serum concentrations of cholic acid (CA) and increased levels of the bacterially produced, secondary BA, deoxycholic acid (DCA), and its glycine and taurine conjugated forms. These studies demonstrate that genes involved in BA metabolism are expressed in the brain and are dysregulated in AD postmortem samples, particularly those associated with taurine transport, BA synthesis, and cholesterol metabolism.[22,23]

BAs effects are mediated through binding and activation of nuclear receptors such as Farnesoid X receptor (FXR)[24] or membrane receptors such as Takeda G-protein-coupled bile acid receptor 5 (TGR5).[25] The presence of BAs and their receptors and transporters in the brain, as well as peripheral crosstalk between the gut, liver, and brain, points to a role of BAs in the CNS and neurological disease. TUDCA has been shown to reduce cognitive impairment and neuroinflammation in AD models, and in combination with sodium phenylbutyrate (AMX0035) is being evaluated in a phase II clinical trial for MCI and early dementia due to AD.[12,26]

Cholesterol also emerges as a potential mediator linking the gut microbiome, TRF, and AD. TRF decreases serum cholesterol levels, the production of which is diurnally regulated by clock genes and modulated by microbially modified metabolites.[27,28] Genome-wide association

studies have identified some cholesterol metabolism genes as major susceptibility loci for AD.[29] Alterations in cholesterol metabolism and transport are hallmarks of AD, exacerbated by the high-risk ApoE4 allele, and resulting in glial cell activation, increased inflammatory signaling, and impaired phagocytic activity, all contributing to AD pathology.[30] A failure to clear excess lipids leads to lipid aggregation and the formation of micelles, cholesterol crystals, and lipid droplets that can damage cells. [30] Importantly, neural cholesterol clearance involves the conversion of cholesterol into BA for removal, while liver cholesterol surplus is metabolized through the classical pathway via the synthesis of BA, that are later modified in the gut. ApoE is a major determinant of cholesterol and lipid homeostasis[31], thus the crucial roles of ApoE isoforms in AD underscores the impact of cholesterol and associated BA on pathology.

Overall, this study aims to demonstrate that composition, function, and rhythmicity of the gut microbiome, metabolome, and transcriptome identify unique signatures of AD-linked pathology mediating the gut-brain axis. Further, we narrow in on mechanisms by which TRF modulates this signature to ameliorate neuroinflammation, circadian dysregulation, and AD pathology in mouse models.

3.2 Results

Microbiome composition is altered by TRF in the APP23 mouse model of AD .

We previously established that time-restricted feeding (TRF) modulates brain transcription and improves amyloid beta clearance and deposition, sleep and activity rhythms, and cognition in the APP23 mouse model of AD.[6] To explore the impact of the microbiome on TRF-altered AD pathophysiology, we collected fecal and liver samples from the same mice at 10-12 months of age. Transgenic (TG) and non-transgenic (NTG) littermates were randomly assigned to either *ad libitum* feeding (ALF) or time-restricted feeding (TRF), with a 6 h feeding/18 h fasting regimen

and the feeding window aligned to the middle of the active period (ZT15–ZT21). Fecal samples were collected every 4 h during a 24-h period (ZT 0, 4, 6, 8, 12, 16, 20) for metagenomic and metabolomic analysis, and liver was collected during sacrifice at either ZT 0 or ZT 12 for transcriptome analysis (3-4 mice per time point per genotype per feeding condition) (**Fig. 3.1A**).

The composition of the stool microbiome, as determined by metagenomic sequencing of the host fecal sample, was largely shared between conditions (2971 species in NTG ALF, TG ALF, and TG TRF). However, about one third of species were unique to 1 or 2 conditions, demonstrating that composition was shaped by both genotype and feeding pattern (**Fig. 3.1B**). TRF increased fecal microbiome α-diversity, as determined by Chao1 diversity index ($p \leq 0.05$ compared to ALF in TG and NTG, **Fig. 3.1C**). α-diversity (i.e., Chao1) was not an indicator of improved pathology since the TG ALF condition was unchanged compared to NTG ALF.

To assess similarity of the microbiome between NTG and TG mice, without the factor of diet pattern, we conducted a principal coordinates analysis (PCoA) of Bray Curtis β-diversity distances using the Woltka per-genome quantitation against the Refseq database. Our results demonstrate that TG fecal microbiomes were distinct from NTG (TG ALF versus NTG ALF permutational multivariate analysis of variance [PERMANOVA] $p = 0.001$) (**Fig. 3.1D**), in agreement with previous reports.[32] Because significant compositional differences between male and female mice were not detected, samples were combined by sex within each condition (**Fig. 3.S1A).** Furthermore, stool metagenomics revealed taxonomic alterations in these TG mice using the Wolkta taxonomic classifier and ALDEx2 compositional differential abundance analysis (**Fig. 3.1E**). Notably, we detected significant differences in numerous populations in TG mice, including reductions in Turicibacteraceae, which are associated with the modification of bile acids and host lipids.[33] *Turicibacter* is depleted in AD patients, and this deficiency inversely correlates with YKL-

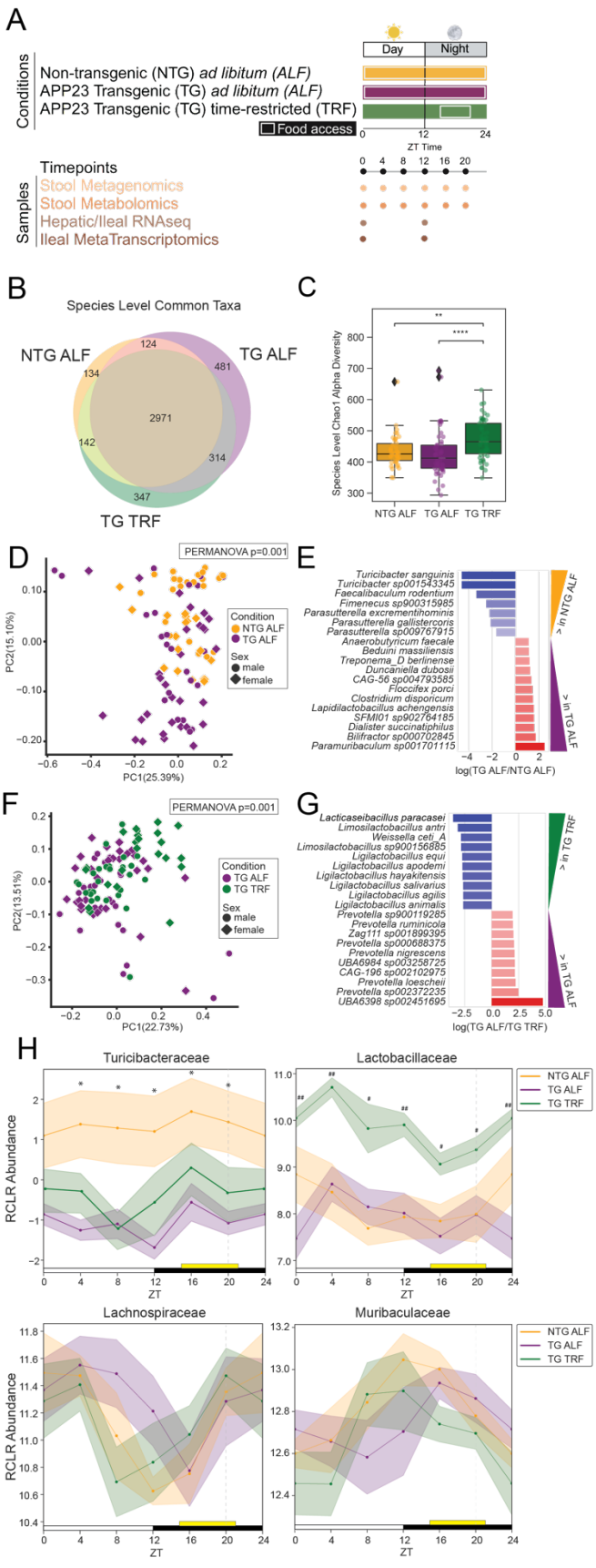
40 levels in CSF, a marker of astroglial and microglial activation.[34] TG mice also showed reductions in species belonging to Lactobacillaceae, Lachnospiracaea, and Muribaculaceae.

Similarly, we wanted to assess the effect of TRF in the context of the TG mouse model, and found that TG TRF microbiomes were distinct from TG ALF controls (TG ALF versus TG TRF [PERMANOVA] p = 0.001) (**Fig. 3.1F**). Species level differential abundance analysis with ALDEx2 revealed taxonomic changes due to feeding pattern (**Fig. 3.1G**). Species belonging to Lactobacillaceae, a family which produces short-chain fatty acids and has been shown to improve cognitive dysfunction and suppress inflammation and Aβ deposition in another AD mouse model[35], are more abundant in TG TRF compared to TG ALF. The genus Prevotella, which is reportedly more abundant in one population of patients with AD[36]**,** was less abundant in the TG TRF compared to TG ALF.

To compare differentially bacteria from the ALDeX2 analyses between all three conditions, and to evaluate time-dependent changes, we calculated relative centered log ratio (RCLR) abundance levels in key families (**Fig. 3.1H**). Turicibacteraceae*,* which was markedly reduced in TG ALF compared to NTG ALF, is significantly improved by TRF. Similarly, *Lactobacillaceae*, which is similar in abundance between NTG and TG conditions, but less abundant at ZT 0 in TG mice, was significantly increased by TRF. Abundance over time was also altered between conditions for the families Lachnospiraceae and Muribaculaceae, which display a rightward phase shifted in TG ALF compared to NTG ALF, which is corrected by TRF. Taken together, these results suggest that microbiome composition is altered by both genotype and fasting, and may underlie functional changes that contribute to the amelioration of AD pathology.

Figure 3.1: TRF creates a unique compositional signature in the microbiome of APP23 AD model mice. 10

a, Schematic of design and sample collection. b, Venn diagram of shared taxonomy at species rank, using Woltka annotation. c, Alpha diversity using Chao1 at species rank. Significance was determined by Mann Whitney and and is represented by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. d, PCoA of Bray Curtis Beta Diversity comparing genomic composition of bacteria in stool of NTG ALF and TG ALF mice [Permanova $p = 0.001$]. e, Differential abundances using ALDeX2 comparing log fold change (LFC) of bacterial species in stool of NTG ALF and TG ALF mice [effect size ≥ 0.5]. f, PCoA of Bray Curtis Beta Diversity comparing genomic composition of bacteria in stool of TG ALF and TG TRF mice [Permanova $p = 0.001$]. g, Differential abundances using ALDeX2 comparing log fold change (LFC) of bacterial species in stool of TG ALF and TG TRF mice [effect size ≥ 0.5]. h, RCLR abundance of key bacterial families that are differentially abundant based on genotype and/or feeding condition. (b-h) n=3-4/sex/genotype sampled at 6 timepoints across 24 hours.


A
Conditions
Non-transgenic (NTG) ad libitum (ALF)
APP23 Transgenic (TG) ad libitum (ALF)
APP23 Transgenic (TG) time-restricted (TRF)
Food access
Day
Night
0
12
24
ZT Time
Timepoints
0 4 8 12 16 20
Samples
Stool Metagenomics
Stool Metabolomics
Hepatic/Ileal RNAseq
Ileal MetaTranscriptomics
B
Species Level Common Taxa
NTG ALF
TG ALF
TG TRF
124
481
134
2971
142
314
347
C
Species Level Chao1 Alpha Diversity
NTG ALF
TG ALF
TG TRF
D
PERMANOVA p=0.001
PC2(15.10%)
PC1(25.39%)
Condition
NTG ALF
TG ALF
Sex
male
female
E
Turicibacter sanguinis
Turicibacter sp001543345
Faecalibaculum rodentium
Fimenecus sp900315985
Parasutterella excrementihominis
Parasutterella gallistercoris
Parasutterella sp009767915
Anaerobutyricum faecale
Beduini massiliensis
Treponema_D berlinense
Duncaniella dubosii
CAG-56 sp004793585
Floccifex porci
Clostridium disporicum
Lapidilactobacillus achengensis
SFMI01 sp902764185
Dialister succinatiphilus
Bilifractor sp000702845
Paramuribaculum sp001701115
> in NTG ALF
> in TG ALF
log(TG ALF/NTG ALF)
F
PERMANOVA p=0.001
PC2(13.51%)
PC1(22.73%)
Condition
TG ALF
TG TRF
Sex
male
female
G
Lacticaseibacillus paracasei
Limosilactobacillus antri
Weissella ceti_A
Limosilactobacillus sp900156885
Ligilactobacillus equi
Ligilactobacillus apodemi
Ligilactobacillus hayakitensis
Ligilactobacillus salivarius
Ligilactobacillus agilis
Ligilactobacillus animalis
Prevotella sp900119285
Prevotella ruminicola
Zag111 sp001899395
Prevotella sp000688375
Prevotella nigrescens
UBA6984 sp003258725
CAG-196 sp002102975
Prevotella loescheii
Prevotella sp002372235
UBA6398 sp002451695
> in TG TRF
> in TG ALF
log(TG ALF/TG TRF)
H
Turicibacteraceae
Lactobacillaceae
Lachnospiraceae
Muribaculaceae
RCLR Abundance
ZT
NTG ALF
TG ALF
TG TRF

Stool microbiome composition is altered by TRF in the APP23 mouse model of AD .

Entrainment or maintenance of circadian rhythmicity in the microbiome is linked to the benefits of TRF (Saran et al., 2020; Zarrinpar et al., 2016) and microbiome data is highly temporally dynamic (Allaband et al., 2024). Therefore, we investigated metagenomic cyclical patterns using the meta2d algorithm (PMID: 27378304) in MetaCycle (Wu et al., 2016). The proportion (%) of taxa that meet the criteria for rhythmicity (BH.q <0.05) at each level of taxonomic classification is 5 to 20-fold greater in the TG TRF condition compared to the NTG ALF and TG ALF conditions (**Fig 3.2A**). At the species level, 8 species are cycling in all conditions and 83 species (85% of those cycling in TG TRF) are cycling uniquely in the TG TRF condition (**Fig 3.2B**).

To compare differentially abundant bacteria from the ALDeX2 analyses between all three conditions, and to evaluate time-dependent changes, we calculated relative centered log ratio (RCLR) abundance levels in key families (**Fig. 3.2C**). Turicibacteraceae, which was markedly reduced in TG ALF compared to NTG ALF, is significantly improved by TRF. Similarly, *Lactobacillaceae*, which is similar in abundance between NTG and TG conditions, but less abundant at ZT 0 in TG mice, was significantly increased by TRF. Abundance over time was also altered between conditions for the families Lachnospiraceae and Muribaculaceae, which display a rightward phase shifted in TG ALF compared to NTG ALF, which is corrected by TRF. This illustrates that not only does TRF entrain rhythmicity in TG mice, composition of key microbes in all conditions are temporally dynamic.

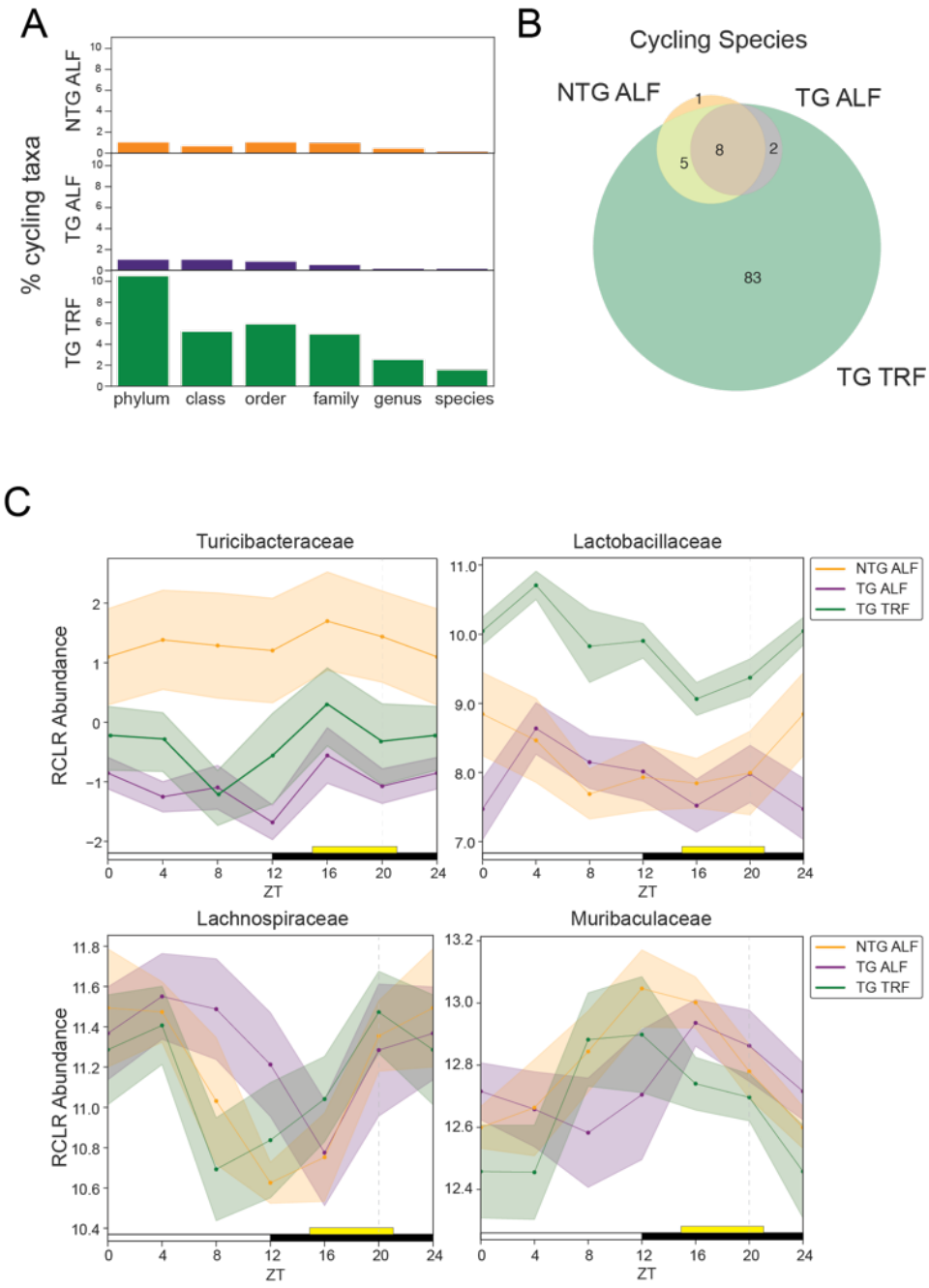


Figure 3.2: TRF entrains microbiome rhythmicity in APP23 AD model mice. 11

a, Cycling (meta2d algorithm, Metacycle, BH.q < 0.05) at phylum, class, order, family, genus, and species level. b, Venn diagram of species level cycling. c, RCLR abundance of key bacterial families that are differentially abundant based on genotype and/or feeding condition. Statistical significance was determined by MannWhitney. *p < 0.05 between NTG ALF and TG TRF; #p < 0.05 between TG ALF and TG TRF; ##p < 0.01 between TG ALF and TG TRF (a-c) n=3-4/sex/genotype sampled at 6 timepoints across 24 hours.

TRF alters stool metabolome composition in the APP23 mouse model of AD .

To elucidate functional changes in the microbiomes between conditions, we analyzed metabolites from the same fecal specimens using untargeted LC-MS. Using untargeted liquid chromatography–mass spectrometry. Using a linear mixed effects model, we observed a small set of stool metabolites that were differentially abundant between TG mice fed according to the TRF paradigm versus ALF (**Fig. 3.3A**). Using CANOPUS predictive annotation, a majority of these metabolites were classified as amino acids, peptides and analogues, and some classified as bile acids, alcohols, and derivatives or fatty acids and conjugates. The increase or decrease in abundance of these metabolites have a reversed pattern in the TG TRF condition. The metabolites presented fall within a p-value < 0.05, however, when adjusting for multiple comparisons, these differences are not statistically significant. Focusing only on the metabolites annotated by GNPS, BIRDMAn differential abundance analysis revealed several metabolites that are differentially abundant, including isovalerylcarnitine, which is involved in fatty acid metabolism, indoxyl sulfate, a neurotoxic intermediate of tryptophan metabolism, and trigonellinamide, an antioxidant plant-derived alkaloid derivative (n=3-4 sex/genotype across 6 timepoints; 95% confidence interval) **(Fig. 3.3B).**

Notably, TRF compared to ALF caused a more significant differentiation in fecal metabolite pool than that caused by genotype. The linear mixed effect model, organized hierarchically by metabolite classification, highlights a large number of amino acids, peptides, and analogues, as well as bile acids and fatty acids, that are differentially abundant in the TG TRF condition compared to TG ALF; unadjusted p-value <0.05 (**Fig. 3.3C**). For these metabolites, NTG ALF mice display a similar abundance pattern to TG ALF mice, underscoring the power of the TRF feeding paradigm to alter the metabolomic pool. BIRDMAn differential abundance analysis

highlights annotated metabolites that are differentially abundant due to TRF, including di and tri-peptides, long chain fatty acids, and metabolites of tryptophan metabolism such as kynurenic acid and L-kynurenine (n=3-4 sex/genotype across 6 timepoints; 95% confidence interval; 0.2-fold log cutoff) **(Fig 3.3D).**

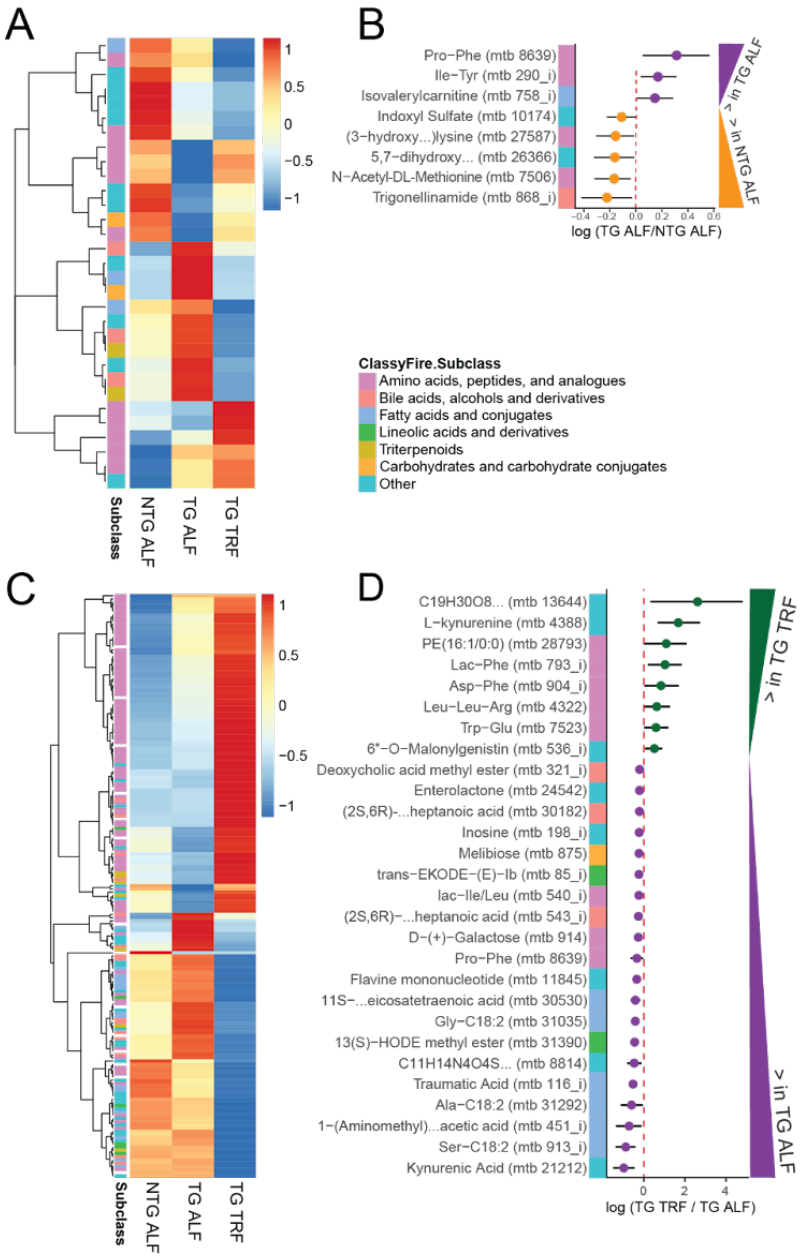


Figure 3.3: TRF modulates key metabolomic alterations in APP23 TG mice.

a, Differential abundance of NTg ALF and Tg ALF stool metabolites using LME, hierarchical categorization by subclass (unadjusted p-value <0.05). b, Differential abundance of NTg ALF and Tg ALF stool metabolites using Birdman, log input (no cutoff). c, Differential abundance of Tg ALF and Tg TRF stool metabolites using LME, hierarchical categorization by subclass (adjusted p-value <0.05). d, Differential abundance of Tg ALF and Tg TRF stool metabolites using Birdman, log input (0.2fold-cutoff). (a-d) n=3-4/sex/genotype sampled at 6 timepoints across 24 hours. Metabolite annotations derived from GNPS and subclassification annotations derived from CANOPUS.

TRF entrains metabolite rhythmicity in the APP23 mouse model of AD.

To determine whether metagenomic cycling was reflected functionally in the metabolic temporal pattern, we used the meta2d algorithm (PMID 27378304) in MetaCycle (Wu et al., 2016) to assess cycling in the LC-MS data. Nearly half of metabolites in the stool of TG TRF mice met the criteria for significant cycling (BH.q < 0.5) compared to 11% and 17% in the NTG ALF and TG ALF conditions, respectively (**Fig. 3.4A**). Nearly 300 metabolites were cycling in all conditions, whereas over 1000 metabolites were cycling uniquely in the TG TRF condition (**Fig. 3.4B**). We conducted a principal coordinates analysis (PCoA) of Bray Curtis β-diversity distances based on ZT time of collection, demonstrating that time plays a strong role in distinguishing metabolomic composition between samples, which clustered into two groups: ZT 8/12/16, during which mice are primarily fasted, and ZT 20/0/4, during which mice are primarily fed (**Fig. 3.4C**).

To further illustrate the temporal dynamics of the metabolome, we compared the abundance of metabolites annotated to CANOPUS subclasses between ZT 12, representing a time point in which mice are primarily not fed, regardless of paradigm, and ZT 0, in which mice are primarily fed (**Fig. 3.4D**). Metabolites classified as amino acids, peptides, and analogues were differentially abundant between TG ALF mice and NTG ALF controls at ZT12, whereas TG TRF mice had significantly greater abundance than the ALF conditions at ZT 0. For fatty acids and conjugates, ZT 12 was the time point with the greatest point of change, whereas for bile acids and triterpenoids, ZT 0 was the time point with the greatest point of change. Taken together, these results demonstrate that TRF modulates key metabolites in the APP23 mouse model of AD in a time-dependent manner.

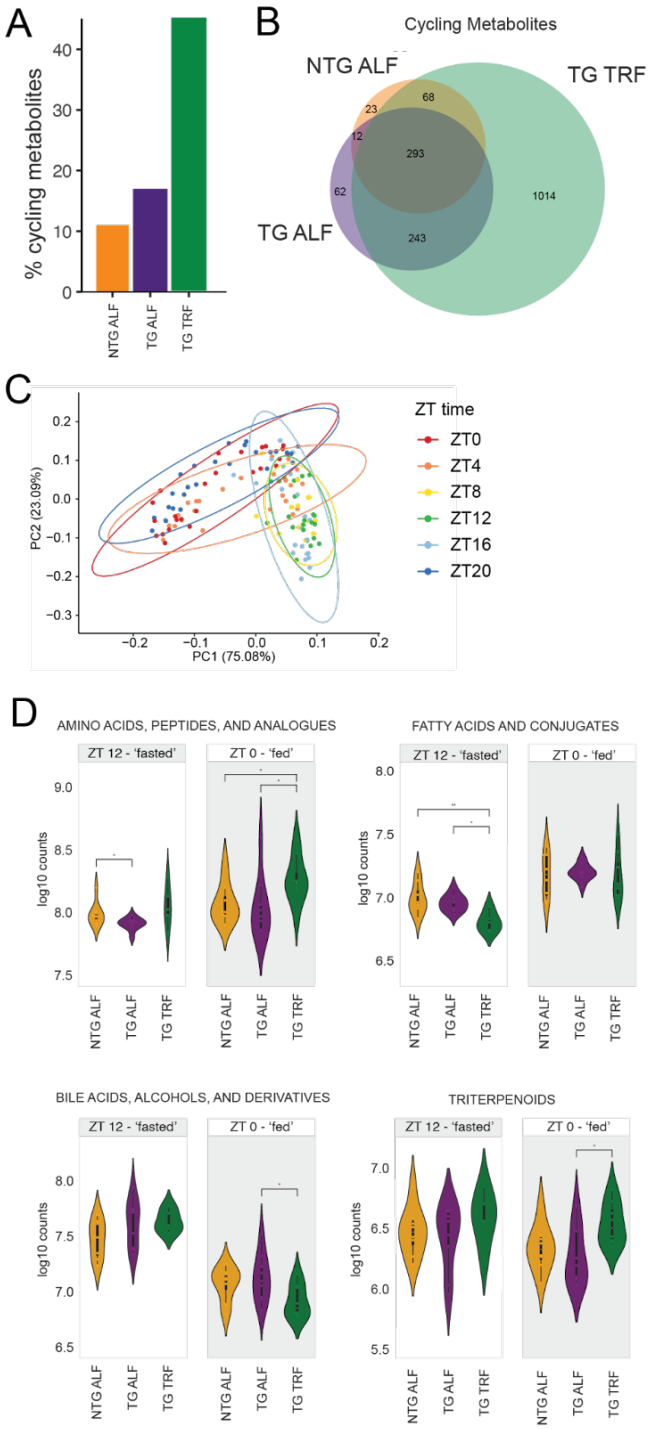


Figure 3.4: TRF entrains metabolomic rhythmicity in APP23 AD model mice.

a, Cycling (meta2d algorithm, Metacycle, BH.q < 0.05) of stool metabolites. b, Venn diagram of cycling metabolites by condition. c, PCoA of Bray Curtis Beta Diversity comparing metabolomic composition by ZT time of collection. d, Log10 abundance of stool metabolites by condition measured at ZT 0 and ZT 12, categorized by CANOPUS subclassification. Statistical significance was determined by MannWhitney. *p < 0.05; **p < 0.01 (a-d) n=3-4/sex/genotype sampled at 6 timepoints across 24 hours.

TRF entrains metabolite rhythmicity in the APP23 mouse model of AD.

In order to understand how these differences in metabolism and signaling molecules were reflected in the host, we conducted RNAseq in ileal and hepatic tissue. Diurnal fluctuation of the microbiome can affect that of the hepatic and ileal transcriptome (Leone et al., 2015; Manella et al., 2021; Weger et al., 2019, Dantas-Machado et al., 2022), so we hypothesized that time-dependent microbiome and metabolomic changes induced by the TG genotype and by TRF would impact the host transcriptome.

Pathway analysis using metascape revealed enriched GO terms of differentially expressed genes in the ileum between NTG ALF and TG ALF mice, and between TG TRF mice and TG ALF controls (**Fig. 3.5A**) We identified shifts relating to lipid metabolism, notably long chain fatty acids and PPAR signaling, monocarboxylic acid metabolic process, which may reflect alterations in tryptophan metabolism, and changes in nuclear receptor signaling, which may reflect changes in bile acids and other derivatives of cholesterol. Notably, TRF induced more transcriptional shifts compared to the TG genotype and some GO terms, such as carbohydrate transport and regulation secretion, were enriched at one time point but not another, illustrating the diurnal nature of ileal gene expression.

Similarly, in the liver, we observed GO term enrichment driven by genes involved in long chain fatty acid metabolism, insulin response, carboxylic acid metabolism, derivatives of cholesterol, and chromatin reorganization (**Fig. 3.5B**). Changes in transcription were primarily driven by TRF and not by the TG genotype, and ZT 12 was the time point with enrichment of the most genes, especially with relation to chromatin reorganization, DNA damage response, and MAPK signaling, which could indicate changes in inflammatory regulation related to AD pathology. In summary, microbiome and metabolomic alterations by TRF are reflected in ileal and

hepatic gene expression, highlighting fatty acid metabolism and signaling, among other metabolic pathways, as a potential mechanism and therapeutic target for AD.

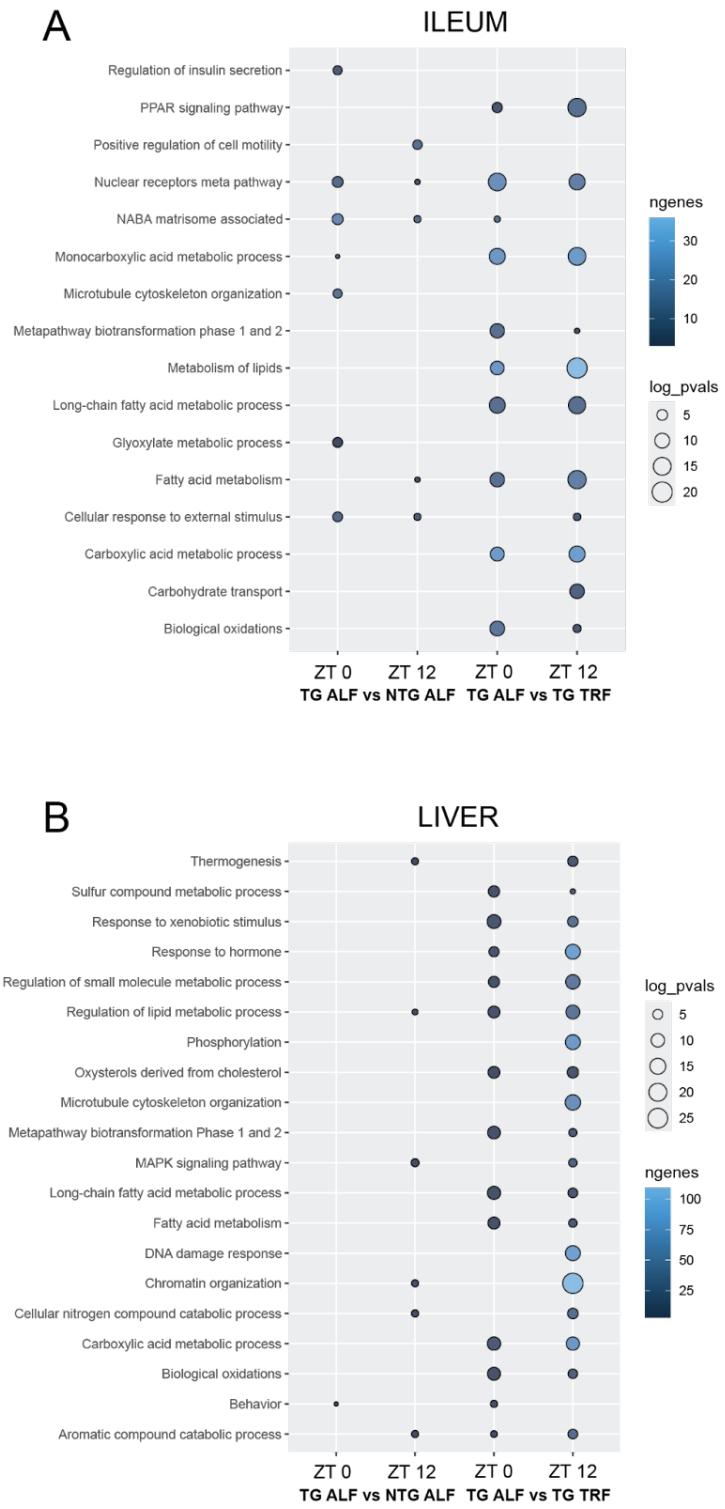


Figure 3.5: TRF alters ileal and hepatic gene expression of key metabolic pathways.

a, Enriched GO annotations obtained from differentially expressed genes in the ileum between conditions, by time point. b, Enriched GO annotations obtained from differentially expressed genes in the liver between conditions, by time point. (a-b) n=3-4/sex/genotype sampled at ZT 0 and ZT 12. Sex and time point were used as covariates in Deseq2 analysis.

3.3 Discussion

This study demonstrates that time-restricted feeding (TRF) profoundly alters the gut-liver-brain axis in the APP23 mouse model of Alzheimer's disease (AD), creating a unique compositional and functional signature characterized by enhanced microbial and metabolic rhythmicity. TRF increased fecal microbiome α-diversity and induced distinct compositional changes, notably enhancing the abundance of beneficial bacteria such as Lactobacillaceae, which has been shown to improve cognitive dysfunction and suppress inflammation in other AD models[35].

These microbiome alterations were accompanied by significant shifts in the metabolome, including changes in amino acids, peptides, fatty acids, and tryptophan metabolites. The metabolomic analysis revealed modulation of key compounds implicated in AD pathology, such as kynurenine pathway metabolites[37-39] and long-chain fatty acids[40-43] . Importantly, TRF dramatically increased the proportion of cycling metabolites, suggesting improved metabolic rhythmicity. These changes were reflected in the host transcriptome, with significant alterations in gene expression in both the ileum and liver, particularly in pathways related to lipid metabolism and nuclear receptor signaling.

Our findings suggest that TRF may exert its beneficial effects on AD pathology through multiple interconnected mechanisms. The modulation of PPARγ signaling, which plays a crucial role in regulating lipid metabolism and insulin sensitivity, emerges as a potential key mediator of AD.[44,45] PPARγ agonists have shown promise in phase II clinical trials for AD, improving cognition and memory[44]. Furthermore, the observed changes in tryptophan metabolism and lipid homeostasis may contribute to reduced neuroinflammation and improved cognitive function, highlighting the kynurenine pathway as a potential therapeutic target. The alterations in cholesterol metabolism and transport pathways are particularly intriguing, given the crucial role of ApoE in

AD risk and the emerging potential of LXR agonists in reducing AD-like damage[46,47]. Our results suggest that TRF may modulate these pathways, potentially offering a non-pharmacological approach to targeting cholesterol-related mechanisms in AD. The observed changes in bile acid metabolism and signaling further support this hypothesis, as bile acids are known to play a role in cholesterol homeostasis and have been implicated in AD pathology[29].

The dramatic increase in cycling metabolites induced by TRF suggests that enhancing metabolic rhythmicity could be a promising therapeutic strategy. This approach might be complemented by pharmacological interventions that mimic or enhance the effects of TRF, such as SGLT2 inhibitors or ketone body supplements, which have shown promise in preclinical AD models[48,49]. Transcriptomic analysis revealed significant changes in gene expression related to chromatin reorganization and DNA damage response, particularly at ZT12. This suggests that TRF may influence epigenetic regulation and cellular stress responses, potentially offering new avenues for therapeutic intervention in AD. Further investigation into these pathways could yield novel targets for drug development.

Although this manuscript does not explore sex differences within the microbiome, metabolome, and transcriptome in the context of TRF in AD, this presents a promising avenue for future investigation. Given the well-documented sex differences in AD prevalence and risk factors[50], as well as emerging evidence of sex-dependent microbiome effects on neuroinflammation and cognitive function in AD[51], larger-scale studies are poised to uncover crucial insights. Exploring sex-specific responses to TRF could lead to more mechanistic insights, potentially addressing the higher prevalence of AD in women and the differential impact of ApoE4 on AD risk. Investigating sex-specific responses to TRF with regards to its impact on microglial

activation, whose modulation by the microbiome is sex-specific[52], also provide crucial insights into the gender disparity observed in AD prevalence and risk.

Future research should focus on validating these findings in human studies and exploring the specific mechanisms by which TRF-induced changes in the microbiome and metabolome contribute to improved cognitive function and reduced AD pathology. Long-term studies examining the effects of TRF on AD progression and the potential for combinatorial therapies targeting the gut-liver-brain axis could yield valuable insights for AD treatment strategies.

In conclusion, our multi-omics approach provides compelling evidence for the therapeutic potential of TRF in AD, highlighting the gut-liver axis as a promising target for intervention. These findings not only advance our understanding of the complex interplay between circadian rhythms, metabolism, and neurodegeneration but also open new avenues for the development of chronotherapeutic approaches in the treatment of Alzheimer's disease. By modulating key pathways involved in lipid metabolism, inflammation, and metabolic rhythmicity, TRF may offer a novel, non-invasive strategy to complement existing therapeutic approaches for AD.

3.4 Acknowledgments

Chapter 3, in part, is currently being prepared for submission for publication of the material. Erica Maissy*; Daniel Whittaker*; Stephany Flores-Ramos; Daniel Carlin; R. Alexander Richter; Pieter Dorrestein; Rob Knight; Paula Desplats P†, Amir Zarrinpar†. Gut microbiome composition, function, and rhythmicity are modulated by time-restricted feeding in Alzheimer's disease mice accompanying rescue of brain pathology and memory. The dissertation author was the primary researcher and author of this paper.

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