Exposure to a Healthy Gut Microbiome Protects Against Reproductive and Metabolic Dysregulation in a PCOS Mouse Model

Pedro J. Torres,[1] Bryan S. Ho,[2] Pablo Arroyo,[2] Lillian Sau,[2] Annie Chen,[2] Scott T. Kelley,[1] and Varykina G. Thackray[2]

[1]Department of Biology, San Diego State University, San Diego, California 92182; and [2]Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Diego, La Jolla, California 92093

ORCiD numbers: 0000-0002-6672-6117 (V. G. Thackray).

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting ~10% to 15% of reproductive-aged women worldwide. Diagnosis requires two of the following: hyperandrogenism, oligo-ovulation or anovulation, and polycystic ovaries. In addition to reproductive dysfunction, many women with PCOS display metabolic abnormalities associated with hyperandrogenism. Recent studies have reported that the gut microbiome is altered in women with PCOS and rodent models of the disorder. However, it is unknown whether the gut microbiome plays a causal role in the development and pathology of PCOS. Given its potential role, we hypothesized that exposure to a healthy gut microbiome would protect against development of PCOS. A cohousing study was performed using a letrozole-induced PCOS mouse model that recapitulates many reproductive and metabolic characteristics of PCOS. Because mice are coprophagic, cohousing results in repeated, noninvasive inoculation of gut microbes in cohoused mice via the fecal-oral route. In contrast to letrozole-treated mice housed together, letrozole mice cohoused with placebo mice showed significant improvement in both reproductive and metabolic PCOS phenotypes. Using 16S rRNA gene sequencing, we also observed that the overall composition of the gut microbiome and the relative abundance of *Coprobacillus* and *Lactobacillus* differed in letrozole-treated mice cohoused with placebo mice compared with letrozole mice housed together. These results suggest that dysbiosis of the gut microbiome may play a causal role in PCOS and that modulation of the gut microbiome may be a potential treatment option for PCOS. **(*Endocrinology* 160: 1193–1204, 2019)**

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting ~10% to 15% of women worldwide (1). Diagnosis of PCOS, based on the Rotterdam Consensus criteria (2003), requires two of the following: hyperandrogenism, oligomenorrhea or amenorrhea, and polycystic ovaries. PCOS is the leading cause of anovulatory infertility in women, and women with PCOS also have an elevated likelihood of miscarriage and pregnancy complications (1–3). Although it is often perceived as a reproductive disorder, PCOS is also a metabolic disorder. Women with PCOS have an elevated risk of developing obesity, type 2 diabetes, hypertension, and nonalcoholic fatty liver disease (2, 4, 5). PCOS-related metabolic dysfunction is associated with hyperandrogenism and occurs irrespective of body mass index (6, 7). Although studies indicate that androgen excess is an important contributor to metabolic dysregulation in women with PCOS, the mechanisms that lead to obesity and insulin resistance in PCOS are not well understood. Although genetic and environmental factors undoubtedly influence the development and pathology of PCOS (6, 8–10), it is worth exploring whether gut microbes contribute to this disorder.

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Abbreviations: CAP, canonical analysis of principal coordinates; FBG, fasting blood glucose; LET, letrozole mice housed together; LET^{ch}, letrozole mice cohoused with placebo mice; P, placebo mice housed together; P^{ch}, placebo mice cohoused with letrozole mice; PCoA, principal coordinate analysis; PCOS, polycystic ovary syndrome; PD, phylogenetic diversity; PERMANOVA, permutational multivariate analysis of variance; SV, sequence variant.

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Studies over the past decade have shown that the gastrointestinal tract harbors a complex microbial ecosystem (the gut microbiome) that is important for human health and disease (11, 12). Gut microbes offer many benefits to the host, including protection against pathogens and regulation of host immunity and the integrity of the intestinal barrier (13–15). The gut microbiome is also involved in the production of short-chain fatty acids via fermentation of dietary fibers, production of essential vitamins such as folic acid and B12, and modification of bile acids, neurotransmitters, and hormones (16, 17). Studies have also shown that changes in the gut microbiome are associated with metabolic disorders such as obesity and type 2 diabetes (18, 19). Moreover, studies have reported that transplantation of stool from obese donors into germ-free mice results in an obese phenotype (20), suggesting that the gut microbiome may play a causative role in metabolic dysregulation. These transplantation studies were complemented with cohousing studies that took advantage of the fact that, because mice are coprophagic, cohousing provides a means for repeated, noninvasive microbial inoculation. Cohousing germ-free mice transplanted with stool from obese donors with germ-free mice transplanted with stool from lean donors was shown to protect the mice transplanted with obese donor stool from developing obesity (20–22). Altogether, these studies suggest that modulation of the gut microbiome may be a potential treatment option for metabolic disorders.

With regard to PCOS, several recent studies reported that changes in the gut microbiome are associated with PCOS (23–26). These studies detected lower α-diversity and differences in the relative abundances of specific Bacteroidetes and Firmicutes in women with PCOS compared with controls (23–25). In particular, changes in the relative abundance of bacterial genera from the Bacteroidaceae, Clostridaceae, Erysipelotrichidae, Lachnospiraceae, Lactobacillaceae, Porphyromondaceae, Ruminococcaceae, and S24-7 families were observed in several studies. In addition, changes in the gut microbiome correlated with hyperandrogenism (23–25), suggesting that testosterone may influence the composition of the gut microbiome in women. In addition to studies in humans, several studies reported a significant association between the gut microbiome and PCOS in rodent models (27–30). Because the rodent models are diet independent, these studies suggest that the mechanisms that result in an altered gut microbiome in PCOS are distinct from diet-induced effects on the gut microbiome observed in high-fat diet–induced obesity models. Overall, these studies indicate that the gut microbiome of women with PCOS differs significantly from that of healthy women and suggest that a microbial imbalance or "dysbiosis" in the gut may contribute to the pathology of PCOS.

We previously developed a PCOS mouse model that uses treatment with letrozole, a nonsteroidal aromatase inhibitor, to increase testosterone levels and decrease estrogen levels by inhibiting the conversion of testosterone to estrogen (31). Letrozole treatment of pubertal female mice results in reproductive hallmarks of PCOS including hyperandrogenism, acyclicity, polycystic ovaries, and elevated LH levels (31). This model also exhibits metabolic dysregulation similar to the phenotype in women with PCOS, including weight gain, abdominal adiposity, elevated fasting blood glucose (FBG) and insulin levels, impaired glucose-stimulated insulin secretion, insulin resistance, and dyslipidemia (32). Our studies also showed that letrozole treatment did not alter food intake or energy expenditure, even though locomotion was decreased (32), suggesting that other mechanisms contribute to the metabolic dysregulation in this model. Although letrozole treatment results in estrogen levels that are lower than estrogen levels in women with PCOS, we used this model to study the role of the gut microbiome in PCOS because it recapitulates both reproductive and metabolic aspects of PCOS. As in women with PCOS, 16S rRNA gene sequencing showed that letrozole treatment was associated with lower gut microbial richness, a shift in the overall gut microbial composition, and changes in specific Bacteroidetes and Firmicutes (27). A recent study examining the effects of nonantibiotic drugs on the gut microbiome found that letrozole did not alter growth of ~40 representative gut bacteria (33), which suggests that differences in the gut microbial composition found in the PCOS mouse model are not a direct effect of letrozole.

To begin to address whether the gut microbiome contributes to the pathophysiology of PCOS and whether manipulation of the gut microbiome can be used to treat PCOS, we used a cohousing paradigm to determine whether exposure to a healthy gut microbiome protected against development of PCOS metabolic and reproductive phenotypes. Because mice are coprophagic, gut microbes can be readily transferred from one mouse to another through the fecal-oral route. In this study, pubertal female mice were treated with placebo or letrozole and housed two per cage in three different housing arrangements. The study groups consisted of placebo mice housed together (P), letrozole mice housed together (LET), placebo mice cohoused with letrozole mice (P^{ch}), and letrozole mice cohoused with placebo mice (LET^{ch}). Overall, cohousing letrozole with placebo mice resulted in substantial improvement in both PCOS metabolic and reproductive phenotypes compared with letrozole mice housed together. Furthermore, 16S rRNA gene sequence analysis demonstrated that cohousing letrozole with placebo mice resulted in changes in the

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β-diversity of the gut microbiome and highlighted bacteria that may be candidates for probiotic therapy. Our findings support the idea that there may be a causal link between the gut microbiome and PCOS and that modulation of the gut microbiome may be a potential treatment option for PCOS.

Materials and Methods

PCOS mouse model

C57BL/6NHsd female mice from Envigo were housed in a vivarium under specific pathogen-free conditions with an automatic 12 hour:12 hour light/dark cycle (light period: 06:00 to 18:00) and *ad libitum* access to water and food (Teklad S-2335 Mouse Breeder Irradiated Diet, Envigo). To establish the pubertal PCOS model, 4-week-old female mice were implanted subcutaneously with a placebo or 3-mg letrozole pellet (3 mm diameter; 50 μg/d; Innovative Research of America) for 5 weeks. The 50 μg/d dose was based on the original letrozole mouse model study (31). For the cohousing paradigm, mice were housed two per cage in three different cage arrangements: two placebo mice, two letrozole mice, or one placebo and one letrozole mouse. The cohousing experimental design resulted in four groups of mice (n = 8 per group): P, LET, P^{ch}, and LET^{ch}. All animal procedures in this study were approved by the University of California, San Diego Institutional Animal Care and Use Committee (Protocol Number S14011).

Analysis of reproductive phenotype

Mice were weighed weekly. Estrous cycle stage was determined from the predominant cell type in vaginal epithelial smears obtained during weeks 4 to 5 of treatment as previously described (31). At the end of the experiment, ovaries were dissected, weighed, fixed in 4% paraformaldehyde, paraffin-embedded, sectioned at 10 μm, and stained with hematoxylin and eosin (Zyagen). Serum testosterone and LH levels were measured using a mouse ELISA (34) (range 10 to 800 ng/dL) and a radioimmunoassay (35, 36) (range 0.04 to 75 ng/mL), by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Facility.

Analysis of metabolic phenotype

After 5 weeks of treatment, mice were fasted for 5 hours and blood from the tail vein was collected to measure fasting insulin levels. Blood glucose was measured with a handheld glucometer (One Touch UltraMini, LifeScan, Inc), and an intraperitoneal insulin tolerance test was performed. Tail vein blood glucose was measured just before (time 0) an intraperitoneal injection of insulin (0.75 U/kg in sterile saline; Humulin R U-100, Eli Lilly) was given and at 15, 30, 45, 60, 90, and 120 minutes after injection. At the end of the experiment, the mice were anesthetized with isoflurane, blood was collected from the posterior vena cava, and parametrial fat pads were dissected and weighed. Serum insulin was measured with a mouse ELISA (37) by the University of California, Davis Mouse Metabolic Phenotyping Center.

Quantitative real-time PCR of ovarian genes

Total RNA was isolated from ovaries with an RNeasy Mini Plus kit (Qiagen), which also removes genomic DNA. cDNA was made by reverse transcription of total RNA with an iScript cDNA synthesis kit (Bio-Rad Laboratories). cDNA products were detected with SYBR Green Supermix (Bio-Rad Laboratories) on a Bio-Rad CFX Connect quantitative real-time PCR system (Bio-Rad Laboratories) with previously described primers (31). Data were analyzed by the $2^{-\Delta\Delta CT}$ method (38) by normalizing the gene of interest to glyceraldehyde 3-phosphate dehydrogenase. Data were represented as mean fold change compared with placebo ± the SEM.

Statistical analysis of reproductive and metabolic phenotypes

The statistical package JMP 13 (SAS) was used to analyze differences between groups by one-way ANOVA followed by *post hoc* comparisons with the Tukey-Kramer honestly significant difference test or two-way repeated-measures ANOVA followed by *post hoc* comparisons of individual time points. Different letters or an asterisk were used to indicate significant differences ($P < 0.05$).

Fecal sample collection, DNA isolation, and 16S rRNA gene sequencing

Fecal samples were collected from 8 mice per group (32 mice total) before treatment and once per week for 5 weeks. Fecal samples were frozen immediately after collection and stored at −80°C. Bacterial DNA was extracted from the samples with the DNeasy PowerSoil Kit (Qiagen) and stored at −80°C. The V4 hypervariable region of the 16S rRNA gene was PCR amplified with primers 515F and 806R (39). The reverse primers contained unique 12-bp Golay barcodes that were incorporated into the PCR amplicons (39). Amplicon sequence libraries were prepared at the Scripps Research Institute Next Generation Sequencing Core Facility, where the libraries were sequenced on an Illumina MiSeq as previously described (27).

16S rRNA gene sequence analysis

Raw sequences were imported into QIIME 2 (version 2018.4) with the q2-tools-import script, and sequences were demultiplexed with the q2-demux emp-single script. This procedure resulted in 7.3 million sequences, with an average of 36,000 sequences per sample. The 16S rRNA sequences generated in this study were deposited into the European Nucleotide Archive (Study Accession Number PRJEB29583). DADA2 software was used to obtain a set of observed sequence variants (SVs) (40). Based on the quality scores, the forward reads were truncated at position 240 with the q2-dada2-denoise script. Taxonomy was assigned with a pretrained naive Bayes classifier (Greengenes 13_8 99% operational taxonomic units) and the q2-feature-classifier plugin (41). Out of 192 samples, 5 were removed because of insufficient sequence coverage (one placebo at week 4, two P^{ch} at week 5, and two LET^{ch} at week 5), resulting in 187 samples. In total, 318 SVs were identified from 186 fecal samples. The resulting SVs were then aligned in MAFFT (42), and a phylogenetic tree was built in FastTree (43). Taxonomic distributions of the samples were calculated with the q2-taxa-barplot script. α- and β-diversity metrics were computed with the q2-diversity core-metrics script at a rarefied sampling depth of 1250. The α-diversity metric, Faith phylogenetic diversity (PD), was used to measure phylogenetic biodiversity by calculating the total branch lengths on a phylogenetic tree of all members in a community (44). UniFrac

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was used to compare the similarity (β-diversity) between the microbial communities by calculating the shared PD between pairs of microbial communities (45, 46).

Statistical analysis of 16S rRNA sequences

Statistical calculations were performed in the R statistical package (version 3.5.1) with the phyloseq (version 1.26.1) (47) and vegan package (version 2.5.3). α-Diversity data were tested for normality via the Shapiro-Wilk test. Variables that were not normally distributed were ranked. Changes in α-diversity over time were analyzed via simple linear regression and Pearson rank correlation on ranked diversity measures. Linear mixed effects analysis of the relationship between α-diversity and time was done with the lme4 R package (version 1.1.19). *P* values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. Principal coordinate analysis (PCoA) and canonical analysis of principal coordinates (CAP) plots (48) were constructed in the phyloseq R package. PCoA plots were used to represent the similarity of posttreatment (weeks 1 to 5) fecal microbiome samples based on multiple variables in the data set, and CAP was used to visualize the relationship of the fecal microbiome with specific parameters. Permutational multivariate analysis of variance (PERMANOVA) used posttreatment weighted UniFrac distance measures to assess bacterial community compositional differences and its relationship to cohousing treatment group (999 permutations "vegan" package). DESeq2 (49) (version 1.14.1) in the microbiomeSeq package (version 0.1, http://www.github.com/umerijaz/microbiomeSeq) was used to identify bacterial genera that were differentially abundant between placebo and LET mice and between LET^{ch} and LET.

Results

Cohousing letrozole mice with placebo mice resulted in less weight gain and abdominal adiposity

To investigate whether exposure to a healthy gut microbiome can protect against the development of a PCOS metabolic and reproductive phenotype, we performed a cohousing study. Female mice were implanted with a placebo or letrozole pellet at 4 weeks of age and housed two mice per cage in three different housing arrangements. This study design resulted in four groups of mice (n = 8 per group): P, LET, P^{ch}, and LET^{ch} (Fig. 1A). As shown in Fig. 1B, weight was measured each week during the 5 weeks of treatment. Similarly to previously published studies (27, 32), 2 weeks of letrozole treatment resulted in increased weight compared with placebo treatment that was maintained for the rest of the study (Fig. 1B). Five weeks of letrozole treatment also resulted in greater abdominal adiposity compared with placebo treatment (Fig. 1C). Interestingly, P^{ch} mice had similar weight gain and abdominal adiposity compared with placebo mice. In contrast, LET^{ch} mice gained less weight and exhibited a trend toward less abdominal adiposity compared with LET mice (Fig. 1B and 1C). Notably, the protective effect of cohousing letrozole mice with placebo mice on weight gain manifested only after several weeks of treatment.

Cohousing letrozole mice with placebo mice resulted in reduced FBG and insulin levels and insulin resistance

As reported in previous studies (27, 31, 32), 5 weeks of letrozole treatment resulted in increased FBG and insulin levels and insulin resistance (Fig. 1D–1F). P^{ch} mice had similar serum glucose and insulin levels and insulin sensitivity to that of placebo mice, whereas LET^{ch} mice had reduced FBG and insulin levels as well as less insulin resistance compared with LET mice (Fig. 1D–1F).

Cohousing letrozole mice with placebo mice resulted in estrous cyclicity

In addition to characterizing the effect of cohousing on the PCOS metabolic phenotype, we also assessed the effect on the reproductive axis. As previously published (27, 31), letrozole treatment resulted in hallmarks of PCOS, including elevated testosterone and LH levels and acyclicity in LET mice (Fig. 2A–2C). P^{ch} mice did not have changes in testosterone, LH, or estrous cyclicity compared with placebo mice (Fig. 2A–2C). On the other hand, LET^{ch} mice had decreased testosterone and LH levels compared with LET mice (Fig. 2A and 2B). In addition, LET^{ch} mice displayed changes in the morphology of vaginal epithelial cells representative of different stages of the estrous cycle compared with the constant diestrus exhibited by LET mice (Fig. 2C).

Cohousing letrozole mice with placebo mice protected ovarian function

Consistent with previous reports (27, 31), the ovaries of LET mice lacked corpora lutea and displayed cystic follicles and hemorrhagic cysts, whereas the ovaries of P^{ch} mice had a similar morphology to that of placebo mice (Fig. 3A). Interestingly, the ovaries of LET^{ch} mice lacked cystic follicles and hemorrhagic cysts and contained corpora lutea (Fig. 3A). As in previous reports (31), LET mice showed a significant increase in both ovarian weight and ovarian mRNA expression levels of follicle-stimulating hormone receptor (*Fshr*), cytochrome P450 17A1 (*Cyp17*), and aromatase (*Cyp19*) compared with placebo mice (Fig. 3B–3E). The ovarian weight and mRNA expression levels in P^{ch} mice mirrored those of placebo mice (Fig. 3B–3E). Compared with LET mice, LET^{ch} mice showed a significant decrease in ovarian weight and mRNA expression levels of *Cyp17,* whereas *Fshr* and *Cyp19* mRNA expression levels were comparable to those of LET mice (Fig. 3B–3E).

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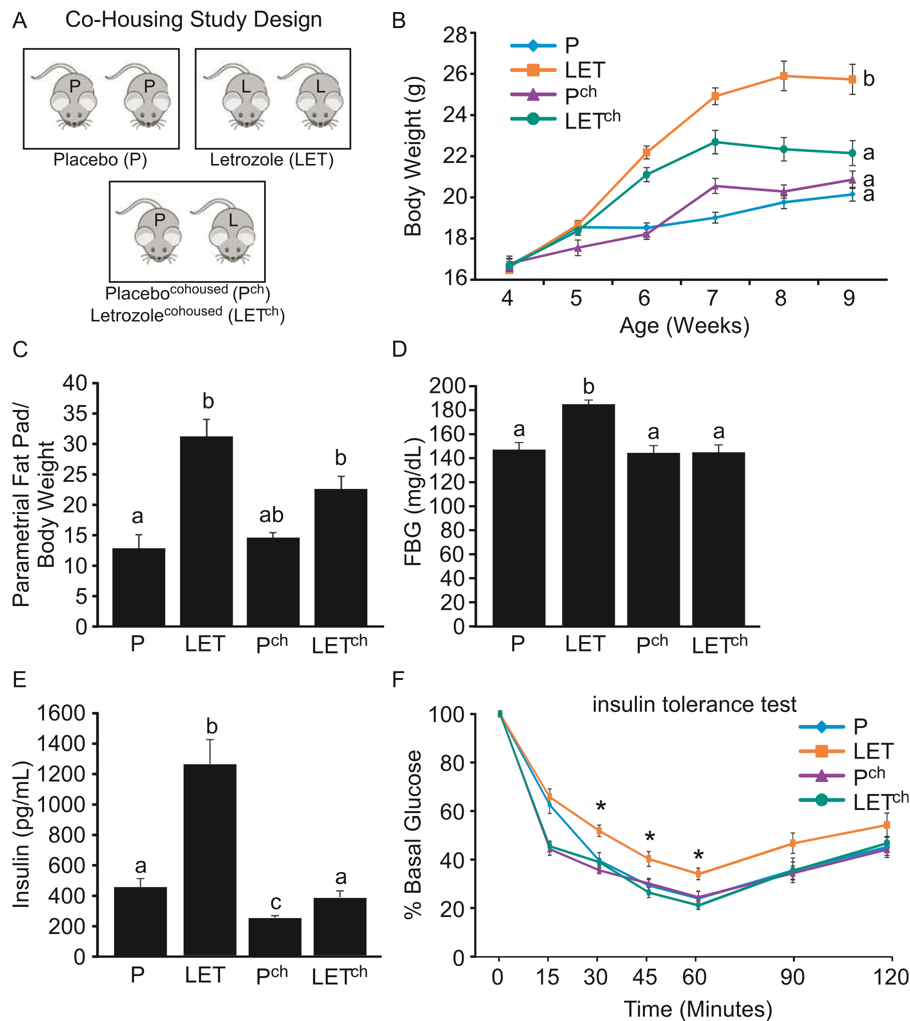

Figure 1. Cohousing letrozole mice with placebo mice protected against development of the PCOS metabolic phenotype. Design of cohousing study with pubertal female mice housed two per cage in three different housing arrangements that resulted in four groups of mice (n = 8 per group): P, LET, P^{ch}, and LET^{ch} (A). Letrozole treatment resulted in metabolic dysfunction compared with placebo including (B–F) increased weight, abdominal adiposity, FBG, and insulin levels and insulin resistance. (B–F) Compared with LET mice, LET^{ch} mice showed a decrease in body weight, a decrease in abdominal adiposity, a decrease in FBG and insulin levels, and restored insulin sensitivity. Graph error bars represent SEM. Different letters or an asterisk symbol were used to indicate significant differences in a one-way ANOVA or repeated-measures two-way ANOVA followed by *post hoc* comparisons with the Tukey-Kramer honestly significant difference test ($P < 0.05$).

Gut microbial richness did not correlate with an improved PCOS phenotype

The overall composition of the gut microbiome from samples collected before placebo and letrozole treatment (time 0) was compared between the four groups. No significant difference in α- or β-diversity was observed between the groups, indicating that the gut microbiomes were similar before treatment (Fig. 4). Linear regression was used to examine the relationship between α-diversity of the gut microbiome (Faith PD) and time. There was a strong positive relationship between α-diversity and time in placebo mice ($r = 0.23$) but not LET mice ($r = 0.05$) (Fig. 5A and 5B). To account for the repeated measures in this longitudinal study, we also used a linear mixed-effect model to examine the association between microbial diversity and time. This analysis confirmed that there was a significant effect of time on α-diversity in placebo mice ($P = 0.003$) but not LET mice ($P = 0.2$) (Fig. 5A and 5B). We then investigated whether changes in α-diversity correlated with improved metabolic and reproductive phenotypes in the cohoused mice. In contrast to placebo mice, we did not observe a significant effect of time on α-diversity on linear regression or the linear mixed-effect model in P^{ch} mice ($r = 0.009$; $P = 1$) or LET^{ch} mice ($r = 0.08$; $P = 0.71$) (Fig. 5C and 5D).

Composition of the gut microbiome was altered by cohousing

In addition to investigating changes in α-diversity, we used weighted UniFrac distances to compare the similarity of gut microbial composition (β-diversity) between the different groups. Although visualization of the UniFrac distances via PCoA did not result in distinct clustering, a

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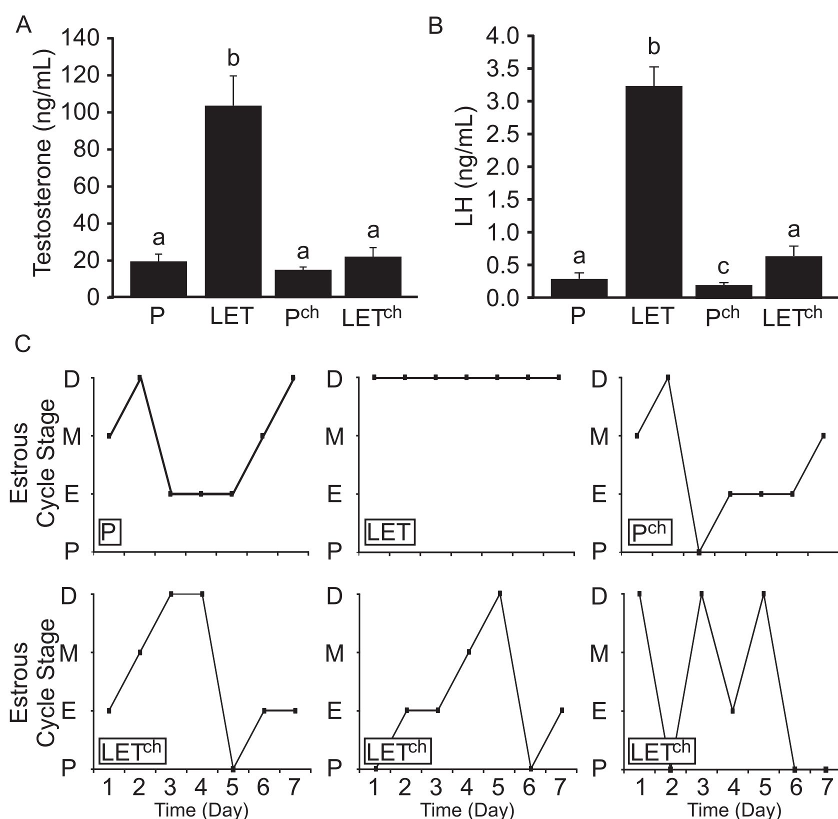

Figure 2. Letrozole mice cohoused with placebo mice did not become hyperandrogenemic or acyclic. The cohousing study included four groups of mice (n = 8 per group): P, LET, P^{ch}, and LET^{ch}. Letrozole treatment resulted in increased (A) testosterone and (B) LH levels compared with placebo. LET^{ch} mice displayed a decrease in (A) testosterone and (B) LH and (C) a restoration of estrous cyclicity compared LET mice stuck in diestrus. Stages of the estrous cycle are indicated as diestrus (D), metestrus (M), estrus (E), and proestrus (P). Graphs illustrating the estrous cycle stages of representative mice from the four groups are shown. Graph error bars represent SEM. Different letters were used to indicate significant differences in a one-way ANOVA followed by *post hoc* comparisons with the Tukey-Kramer honestly significant difference test ($P < 0.05$).

PERMANOVA test (ADONIS) detected a significant effect of cohousing treatment on the microbial community structure ($P = 0.001$) (Fig. 6A). This trend was also observed in unweighted UniFrac (data not shown). CAP was then used to analyze the microbial composition in response to an *a priori* defined experimental variable (cohousing treatment). PERMANOVA demonstrated a strong relationship between cohousing treatment and the overall composition of the gut microbiome ($P = 0.001$) (Fig. 6B), suggesting that cohousing resulted in a distinct gut microbial community in LET^{ch} mice compared with LET mice. To understand when the gut microbiome diverged, we then compared the fecal samples from the four groups at each time point (Fig. 6C–6G). We observed a significant separation of the bacterial communities in the treatment groups after 2 weeks (ADONIS, $P = 0.004$) (Fig. 6D). Separation of the bacterial communities also occurred in weeks 3 and 4 but not in week 5, possibly because of convergence of P^{ch} and LET^{ch} with a placebo-like gut microbiome phenotype.

Differentially abundant genera are associated with cohoused letrozole mice

Differential abundance of gut bacteria between placebo- and letrozole-treated mice was determined with DESeq2. This approach used a negative binomial regression for modeling count variables and is commonly used for overdispersed data, which is typical of microbiome data (33). DESeq2 identified five bacterial genera that were of higher relative abundance and four bacterial genera that were of lower relative abundance in placebo compared with LET mice (Fig. 7A). The gram-positive bacteria included *Coprobacillus, Candidatus Arthromitus, Roseburia, Dorea, Lactobacillus,* and *Adlercreutzia,* and the gram-negative bacteria included *Akkermansia, Christensenella,* and *Turicibacter*. DESeq2 also identified three bacterial genera that had an altered relative abundance in LET^{ch} compared with LET mice: *Coprobacillus, Christensenella,* and *Lactobacillus* (Fig. 7B).

Discussion

This study demonstrated that exposure to a healthy gut microbiome resulted in protection from developing a metabolic phenotype in a PCOS mouse model. In particular, cohousing letrozole-treated mice with placebo mice resulted in LET^{ch} mice with body weight, FBG and insulin levels, and insulin resistance similar to those of placebo mice (Fig. 1). Although our results demonstrated that cohousing letrozole with placebo mice resulted in protection from metabolic dysregulation by the end of the study, future studies will be needed to ascertain how much time cohousing takes to exert a protective effect on these metabolic factors. The improved PCOS metabolic phenotype obtained via cohousing is consistent with previous cohousing studies with obese mice, in which exposure to a healthy gut microbiome provided protection from weight gain to germ-free mice inoculated with feces from obese donors (22). It is worth noting that the letrozole-induced metabolic phenotype does not involve changes in diet, food intake, or total energy expenditure (32), suggesting that other mechanisms are responsible for the development of metabolic dysregulation in PCOS. For instance, because studies have

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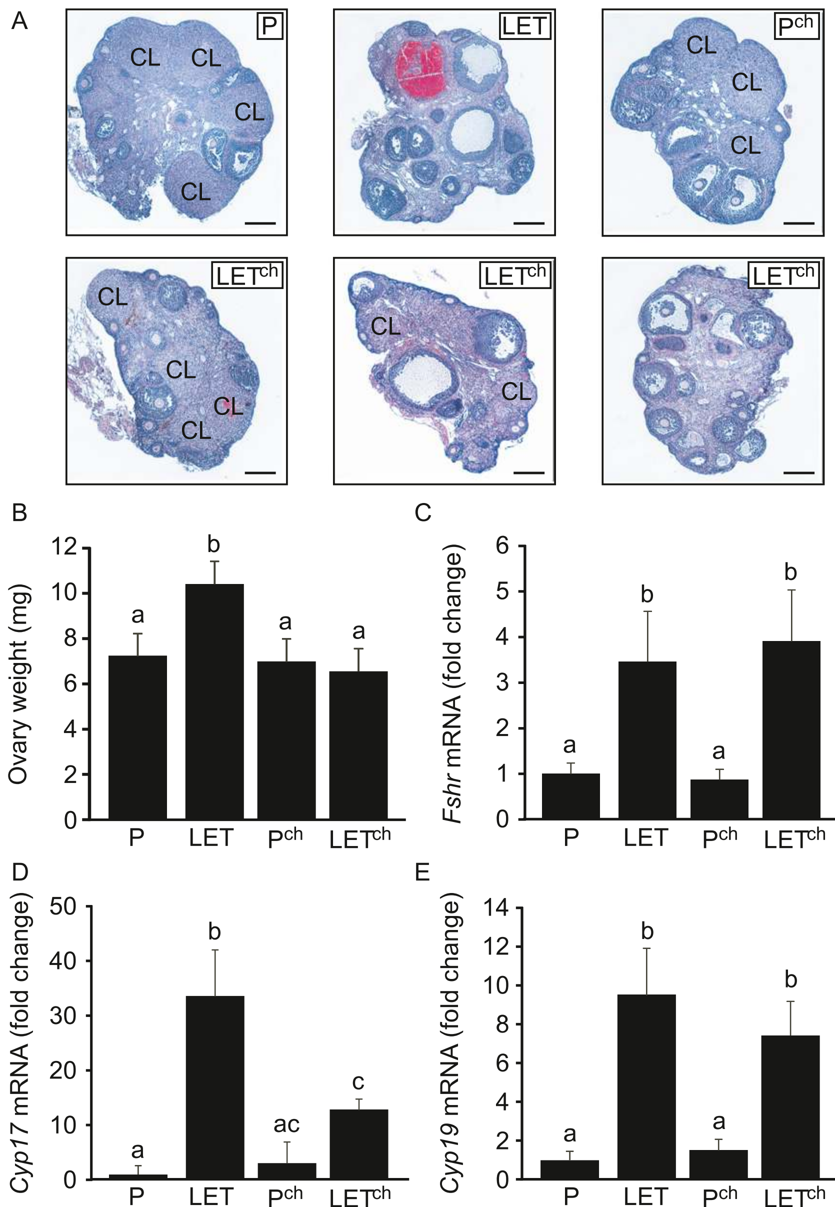

Figure 3. Cohousing letrozole mice with placebo mice improved the ovarian phenotype. The cohousing study included four groups of mice (n = 8 per group): P, LET, P^{ch}, and LET^{ch}. (A) Letrozole treatment resulted in a lack of corpora lutea, cystlike follicles, and hemorrhagic cysts in the ovaries compared with placebo mice. (A) Unlike LET mice, LET^{ch} mice lacked polycystic ovaries, and their ovaries contained corpora lutea (CL) which is evidence of ovulation. Scale bars represent 250 μm. (B–E) Letrozole treatment also resulted in increased ovarian weight and increased mRNA expression of several ovarian genes important in ovarian follicular development and steroidogenesis. (B) Ovarian weight was lower in LET^{ch} mice compared with LET mice. *Fshr* and *Cyp19* mRNA levels were similar between LET and LET^{ch} mice, whereas *Cyp17* was lower in LET^{ch} mice compared with LET mice. Graph error bars represent standard error of the mean. Different letters were used to indicate significant differences in a one-way ANOVA followed by *post hoc* comparisons with the Tukey-Kramer honestly significant difference test ($P < 0.05$).

indicated that obesity may be influenced by an increased capacity of the gut microbiome to harvest energy from dietary fiber (21), it would be informative to test whether this occurs in letrozole-treated mice.

In addition to an effect of cohousing on the PCOS metabolic phenotype, this study reports an effect of cohousing on a reproductive phenotype. Specifically, cohousing letrozole mice with placebo mice resulted in LET^{ch} mice with normalized testosterone and LH levels as well as estrous cycling and ovarian morphology similar to those of placebo mice (Figs. 2 and 3). The presence of estrous cycles and corpora lutea in many of the LET^{ch} mice suggests that the mice were able to ovulate. Future superovulation studies could be informative in determining whether exposure to a healthy microbiome restores ovulation in letrozole-treated mice cohoused with placebo mice. Because ovarian *Cyp17* gene expression is induced by both androgens and insulin (50, 51), it is unsurprising that *Cyp17* levels were normalized in LET^{ch} mice (Fig. 3) that had reduced circulating levels of testosterone and insulin. On the other hand, it is not clear why *Fshr* mRNA levels were increased in both LET and LET^{ch} mice. With regard to aromatase (*Cyp19*) expression, one possible explanation why *Cyp19* mRNA levels did not resolve in LET^{ch} mice, despite normalization of testosterone levels, is that LET^{ch} mice were still exposed to letrozole. These results support the idea that suppression of the aromatase enzyme with letrozole treatment results in a compensatory increase in Cyp19 mRNA. This study also indicates that normalization of *Cyp19* mRNA was not necessary for an improved PCOS phenotype and suggests that the protective effect of cohousing did not occur because of decreased letrozole activity in LET^{ch} mice.

To further characterize the effects of cohousing on the letrozole-induced PCOS mouse model, we examined the effects of cohousing on the gut microbiome. As published previously (27), we observed lower α-diversity in letrozole-treated female mice compared with placebo mice. These results are consistent with studies that reported lower α-diversity of the gut microbiome in women with PCOS compared with controls (23–25). However, because we did not observe higher α-diversity in LET^{ch} mice compared with LET mice (Fig. 5), these results

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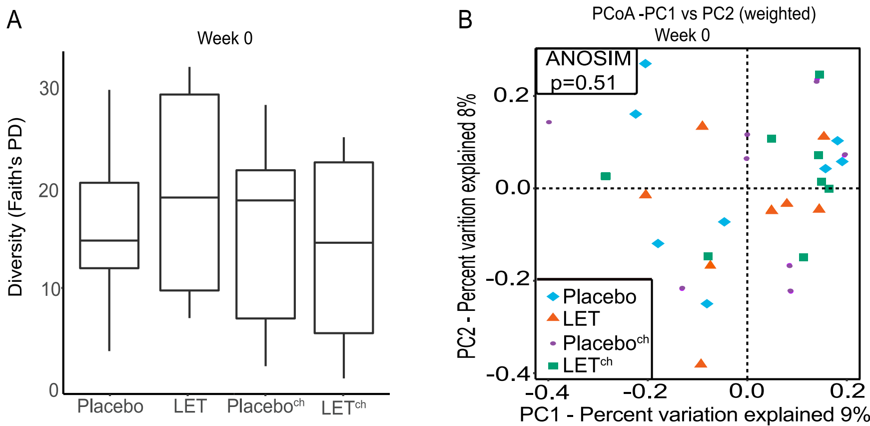

Figure 4. Gut microbiome was similar in all cohousing treatment groups before treatment. The cohousing study included four groups of mice: P, LET, P^{ch}, and LET^{ch} (n = 8 per group with the exception of n = 7 for P time 4 and n = 6 for P^{ch} and LET^{ch} time 5). No significant differences in (A) gut microbial richness (α-diversity, Faith PD) or (B) community composition (β-diversity, weighted UniFrac) were observed among cohousing treatment groups before treatment (week 0; n = 8 per group). One-way ANOVA was used to compare α-diversity among the groups, and analysis of similarity (ANOSIM) test was used to compare β-diversity among the groups.

indicate that the physiological differences between LET and LET^{ch} mice are probably not due to changes in α-diversity *per se* but may reflect changes in specific gut microbes. Supporting this idea, we observed changes in the overall gut bacterial composition at the same time (Fig. 6; week 2) that we observed a protective effect of cohousing on weight gain in LET^{ch} mice (Fig. 1B). Additional support for this idea comes from the identification of specific gut bacteria such as *Coprobacillus* and *Lactobacillus* that had a relative abundance altered by letrozole treatment and restored by cohousing letrozole with placebo mice. Interestingly, these bacteria have been linked with host metabolism. *Coprobacillus* was reported to be enriched in healthy subjects compared with obese subjects and was proposed as a novel probiotic because of its association with a healthy gut microbiome (52, 53). Although *Lactobacillus* is commonly used as a probiotic, increased abundance of some *Lactobacillus* species has been reported in obese humans (54, 55), suggesting that the effect of *Lactobacillus* on metabolism may be species and strain specific (56). Our results highlight the need for bacterial species- and strain-level identification in future studies focused on the role of the gut microbiome in PCOS.

Sex differences in the gut microbiome probably arise after puberty through the action of sex steroids (57). However, the mechanisms by which sex steroids influence the gut microbiome remain unclear. In the case of PCOS, previous studies in humans and mouse models of the disorder (23–25, 27) suggest that elevated testosterone levels select for different gut microbes via unknown mechanisms. Future studies investigating the role of androgen and estrogen receptors in immune cells, intestine, liver, or other relevant tissues will be important in determining whether steroid receptor signaling in the host is necessary for steroid-dependent changes in the gut microbiome. Additional studies will also be needed to determine whether androgens can directly regulate gut bacteria by acting as substrates for bacterial enzymes, such as β-glucuronidases,

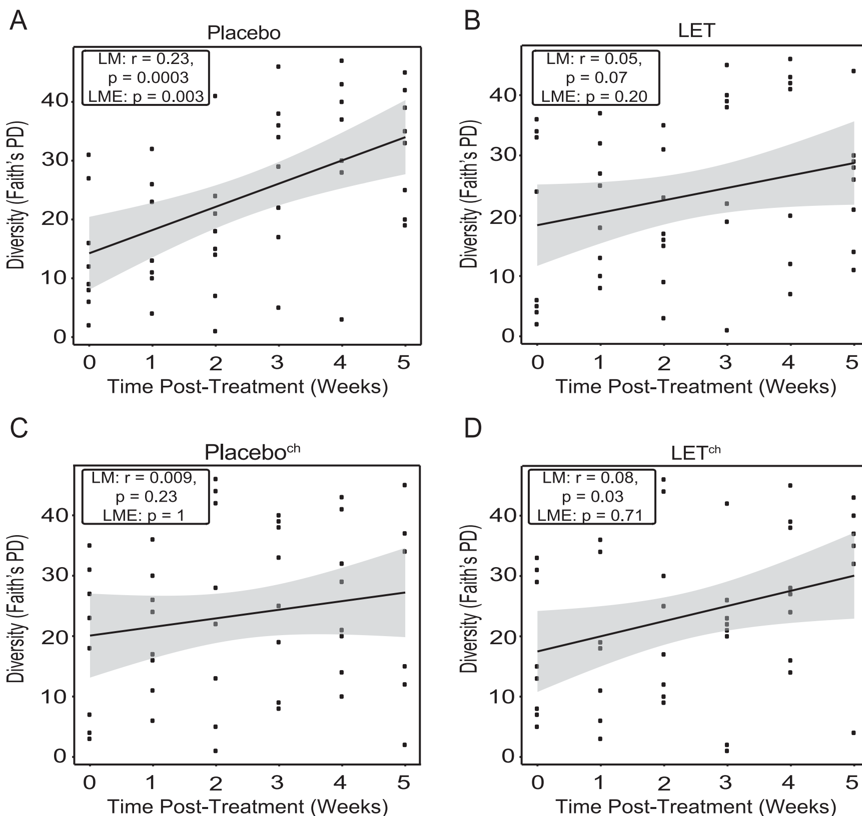

Figure 5. Cohousing letrozole mice with placebo mice did not restore α-diversity of the gut microbiome. The cohousing study included four groups of mice: (A) P, (B) LET, (C) P^{ch}, and (D) LET^{ch} (n = 8 per group with the exception of n = 7 for P time 4 and n = 6 for P^{ch} and LET^{ch} time 5). α-Diversity as approximated by Faith PD ranked estimate was graphed over time for the four groups. Results of linear regression model (LM) and *P* value are in the box insets, and the gray shaded area indicates the 95% CI for the line of best fit. *P* values for the linear mixed effects model (LME) were obtained by the likelihood ratio test of the full model, with the effect in question (time) against the model without the effect in question, and are in the box insets.

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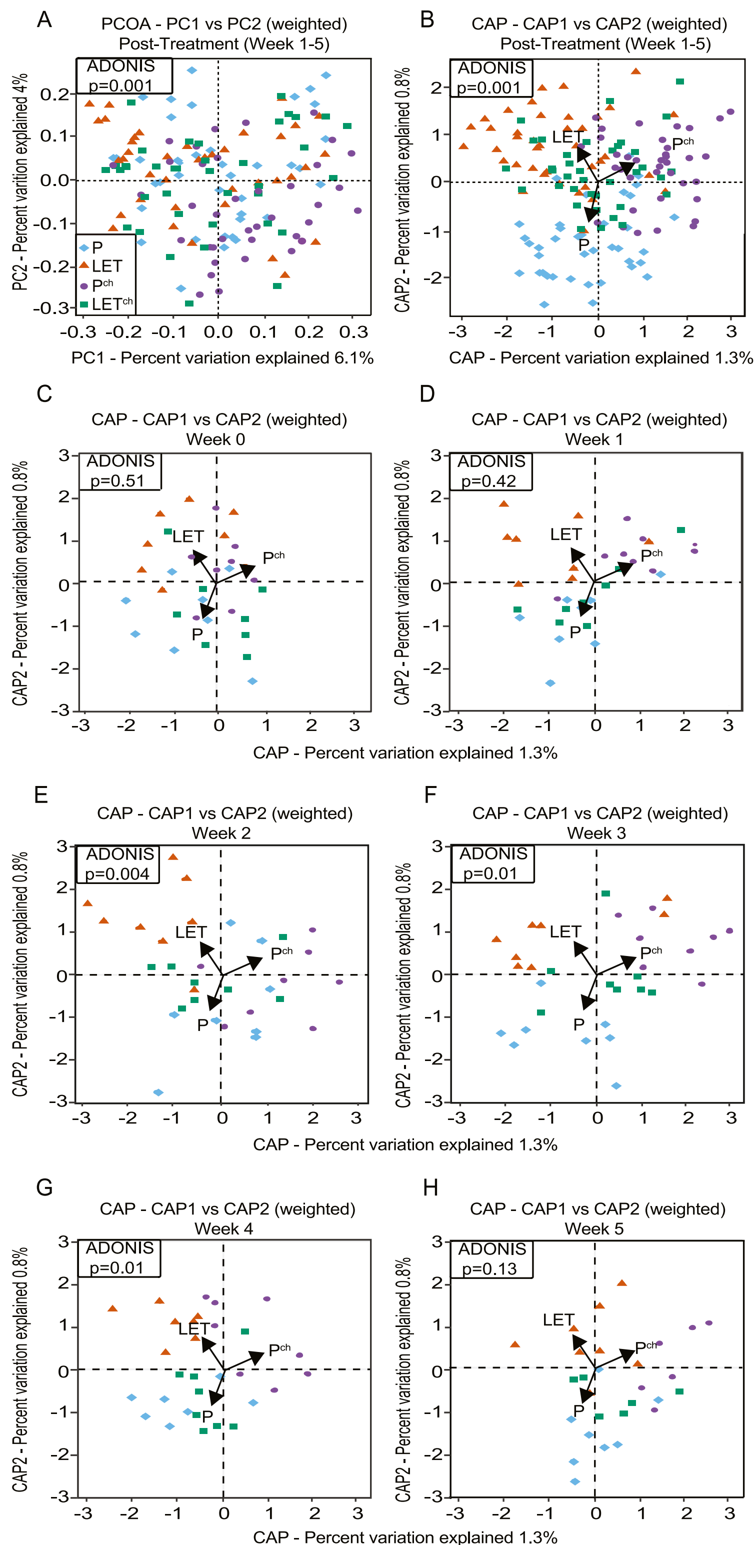

Figure 6. Cohousing letrozole mice with placebo mice influenced the overall composition of the gut bacterial community over time. The cohousing study included four groups of mice: P, LET, P^{ch}, and LET^{ch} (n = 8 per group with the exception of n = 7 for P time 4 and n = 6 for P^{ch} and LET^{ch} time 5). (A) Unconstrained PCoA of weighted UniFrac distances demonstrated changes in the microbial composition (β-diversity) among samples collected after treatment. Permutational ANOVA of the weighted UniFrac distances indicated that cohousing had a strong influence on the gut microbial community (P = 0.001). (B) Constrained CAP of weighted UniFrac distances further illustrated the relationship between β-diversity and posttreatment with a significant effect of constraining the data based on the cohousing treatment group (P = 0.001). (C–H) Samples from the different groups were then compared at each time point. Permutational ANOVA of the weighted UniFrac distances was done for each time point.

important for producing carbon and energy as described for estrogens (58).

Although our results suggest that bacterial exchange may protect against the PCOS phenotype, it is possible that exposure to other fecal microbes (*e.g.*, archaea), microbial metabolites, or even steroids in feces and urine could play a protective role. Fecal microbiome transplant studies will be critical in ruling out the influence of pheromones, behavioral interactions, or urine in the protective effect of cohousing. Future reconstitution experiments will also be important to determine whether specific bacteria or metabolites are necessary for a protective effect. If bacteria prove to be an important component of the protective effect of cohousing, it is worth considering how modulating gut bacterial composition could improve reproductive function. Because studies have shown that weight loss in women with PCOS results in decreased androgen levels and improved menstrual cycling and fertility (59), it is possible that gut microbes may indirectly regulate the reproductive axis through effects on metabolism. Alternatively, gut bacteria may have a direct effect on reproduction by controlling the amount of steroid hormones excreted or reabsorbed into enterohepatic circulation through deconjugation of steroids conjugated in the liver (60). Although there are some similarities in the types of bacteria that are altered in PCOS and obesity, it remains unclear whether microbial dysbiosis and metabolic dysregulation in these two disorders result from similar mechanisms. Studies demonstrating that changes in the gut microbiome and metabolism are associated with hyperandrogenism and that PCOS metabolic dysregulation occurs in a body mass index–independent manner indicate that some of the mechanisms driving PCOS metabolic disturbances are distinct from those driving metabolic dysregulation in obesity (6, 23–25, 7).

In summary, our study demonstrated that exposure to a healthy gut

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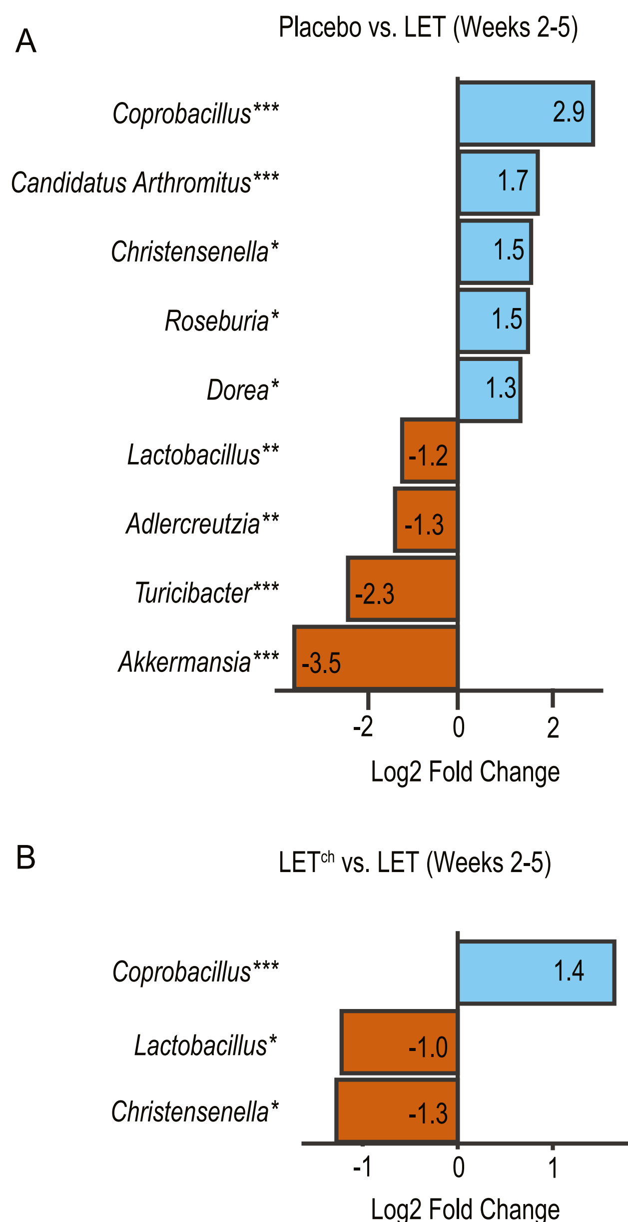

Figure 7. Specific bacterial genera were associated with improvement of the PCOS phenotype during cohousing. The cohousing study included four groups of mice: P, LET, P^{ch}, and LET^{ch} (n = 8 per group with the exception of n = 7 for P time 4 and n = 6 for P^{ch} and LET^{ch} time 5). Results from the DESeq2 differential abundance analysis were expressed as log2 fold change for (A) the comparison of P and LET mice and (B) the comparison of LET^{ch} and LET mice. Positive log2 fold changes represent bacterial genera increased in (A) P mice relative to LET mice or (B) LET^{ch} relative to LET mice, whereas negative changes represent bacterial general increased in (A) LET relative to P mice or (B) LET relative to LET^{ch} mice. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

microbiome via a cohousing paradigm resulted in protection from developing metabolic and reproductive phenotypes in a letrozole-induced PCOS mouse model. The physiological phenotypes were associated with changes in the composition of the gut microbiome, suggesting that modulation of gut microbes toward a dysbiotic or healthy state may influence the degree of pathology. It is notable that cohousing also resulted in changes in the gut bacterial community of placebo mice cohoused with letrozole mice compared with placebo mice housed together. However, because these changes were not sufficient to alter the metabolic and reproductive phenotypes of the host, these results suggest that the healthy gut microbiome was resistant to any pathological influence from the feces of the letrozole mice. To elucidate how exposure to a healthy gut microbiome protected mice from developing PCOS, future studies are needed to characterize the effects of cohousing on the composition and function of the gut microbiome in the letrozole-induced PCOS mouse model by using metagenomics and metabolomics. In addition, studies are needed to investigate the mechanisms by which changes in the gut microbiome influence metabolism and reproduction. Moreover, because these results imply that modulating the composition of the gut microbiome may be a potential treatment option for women with PCOS, future studies should also investigate whether supplementation with prebiotics or novel probiotics such as *Coprobacillus* can protect against the development and pathology of PCOS.

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Correspondence: Varykina G. Thackray, PhD, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Diego, La Jolla, California 92093. E-mail: vthackray@ucsd.edu.

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