Title

Beneficial metabolic effects of PAHSAs depend on the gut microbiota in diet-induced obese mice but not in chow-fed mice.

Journal

Proceedings of the National Academy of Sciences, 121(28)

Authors

Lee, Jennifer

Wellenstein, Kerry

Rahnavard, Ali

et al.

Publication Date

2024-07-09

DOI

10.1073/pnas.2318691121

Peer reviewed

PNAS RESEARCH ARTICLE | PHYSIOLOGY

Beneficial metabolic effects of PAHSAs depend on the gut microbiota in diet-induced obese mice but not in chow-fed mice

Jennifer Lee[a,1,2], Kerry Wellenstein[a,2], Ali Rahnavard[b], Andrew T. Nelson[c,3], Marlena M. Holter[d], Bethany P. Cummings[e,f], Vladimir Yeliseyev[g], Angela Castoldi[h], Clary B. Clish[i], Lynn Bry[g], Dionicio Siegel[c], and Barbara B. Kahn[a,1]

Affiliations are included on p. 11.

Contributed by Barbara B. Kahn; received November 9, 2023; accepted May 31, 2024; reviewed by Fredrik Bäckhed and Sean S. Davies

Dietary lipids play an essential role in regulating the function of the gut microbiota and gastrointestinal tract, and these luminal interactions contribute to mediating host metabolism. Palmitic Acid Hydroxy Stearic Acids (PAHSAs) are a family of lipids with antidiabetic and anti-inflammatory properties, but whether the gut microbiota contributes to their beneficial effects on host metabolism is unknown. Here, we report that treating chow-fed female and male germ-free (GF) mice with PAHSAs improves glucose tolerance, but these effects are lost upon high fat diet (HFD) feeding. However, transfer of feces from PAHSA-treated, but not vehicle-treated, chow-fed conventional mice increases insulin sensitivity in HFD-fed GF mice. Thus, the gut microbiota is necessary for, and can transmit, the insulin-sensitizing effects of PAHSAs in HFD-fed GF male mice. Analyses of the cecal metagenome and lipidome of PAHSA-treated mice identified multiple lipid species that associate with the gut commensal *Bacteroides thetaiotaomicron* (*Bt*) and with insulin sensitivity resulting from PAHSA treatment. Supplementing live, and to some degree, heat-killed *Bt* to HFD-fed female mice prevented weight gain, reduced adiposity, improved glucose tolerance, fortified the colonic mucus barrier and reduced systemic inflammation compared to HFD-fed controls. These effects were not observed in HFD-fed male mice. Furthermore, ovariectomy partially reversed the beneficial *Bt* effects on host metabolism, indicating a role for sex hormones in mediating the *Bt* probiotic effects. Altogether, these studies highlight the fact that PAHSAs can modulate the gut microbiota and that the microbiota is necessary for the beneficial metabolic effects of PAHSAs in HFD-fed mice.

gut microbiota | PAHSAs | diet-induced obesity | *Bacteroides thetaiotaomicron* | glucose metabolism

Significance

Over one billion people worldwide are obese. Safe, affordable methods for sustainable weight loss are needed to reduce the risk of diabetes and comorbidities. The gut microbiota contributes to regulating host metabolism. We report that beneficial Palmitic Acid Hydroxy Stearic Acid (PAHSA) effects on host metabolism in diet-induced obese mice require the gut microbiota. We identify cecal lipid metabolites that correlate with *Bacteroides thetaiotaomicron* (*Bt*) and insulin sensitivity in PAHSA-treated mice. *Bt* supplementation reduced adiposity, improved glucose tolerance, and fortified the gut barrier in female, but not male, diet-induced-obese mice. Thus, PAHSAs have therapeutic potential to modify the gut microbiota. This may be relevant to humans since *Bt* levels are low in humans with obesity and restored with bariatric surgery.

Obesity in adults has nearly tripled worldwide in the last 50 y (1), and this is associated with comorbidities and complications that raise long-term healthcare costs and human disease burden (2, 3). The gastrointestinal tract houses the gut microbiota, and its stratified organization of heterogeneous cell types provides both a physical barrier and immune protection to help regulate host metabolism (4–6). Strong evidence supports an essential role for the gut microbiota in the development of obesity (7–12). Obesity caused by high fat diets (HFD) changes gut microbial composition and increases the production of gram-negative bacteria-derived lipopolysaccharide (LPS), which crosses the gut mucosal barrier that has been rendered leaky from the HFD. This initiates the chronic low-grade inflammation observed in mice and in many humans with obesity (13–16). Thus, major efforts are focused on developing therapeutic gut-based strategies to restore gut microbiota composition and gut epithelial function to treat metabolic disease. However, the gut microbiota is highly variable in taxonomy and function between individuals, and differences in experimental design further complicate findings and interpretation of results across studies. Furthermore, few studies report sex-specific responses to gut microbial interventions, resulting in major knowledge gaps that limit the identification of optimal treatment strategies for women and men.

Palmitic Acid Hydroxy Stearic Acids (PAHSAs) are a family of lipids with antidiabetic and anti-inflammatory properties (17–21). PAHSAs were first identified from lipidomics analysis of white adipose tissue (WAT) from mice overexpressing the glucose transporter, Glut4 (AG4OX) (20). AG4OX mice despite having greater adiposity, have lower fasting glycemia, enhanced glucose tolerance (22, 23), and markedly elevated PAHSA levels in adipose tissue versus controls (20). Circulating and adipose PAHSA levels are low in insulin-resistant people and the levels correlate highly with insulin sensitivity in humans (20). PAHSA treatment regulates multiple components of glucose homeostasis, including

[1]To whom correspondence may be addressed. Email: jen.lee@tufts.edu or bkahn@bidmc.harvard.edu.

[2]Present address: The Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging, Graduate School of Biomedical Sciences, Tufts University, Boston, MA 02111.

[3]Present address: Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75390.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2318691121/-/DCSupplemental.

Published July 5, 2024.

augmenting glucose-stimulated insulin secretion through the G-protein coupled receptor GPR40 in insulin-resistant aged chow-fed mice and in human islets (19), and enhancing insulin action on hepatic glucose production in diet-induced obese mice (21). PAHSAs also have direct effects in the gastrointestinal tract. Daily PAHSA treatment delayed the onset and reduced the severity of dextran sodium sulfate-induced colitis in mice by modulating innate immune responses and attenuating inflammation (18). Evidence that PAHSAs protect the gut from inflammatory injury along with their beneficial effects on glucose metabolism led us to posit that PAHSAs may alter the gut microbiota in a manner that contributes to their beneficial metabolic effects in diet-induced-obese mice.

In this study, we report that the gut microbiota is essential for, and can transmit, some of the beneficial metabolic effects of PAHSA treatment in male HFD-fed mice. Functional profiling of the cecal metagenome and metabolome from PAHSA-treated chow-fed mice revealed that specific bacterial species including *Bacteroides thetaiotaomicron* (*Bt*) and unique signatures of lipid metabolites are altered with PAHSA treatment. Subsequent studies in diet-induced-obese mice demonstrate sex-specific responses to *Bt* supplementation resulting in distinct effects on mucus-producing Goblet cells that line the gut epithelium, intestinal immune phenotypes, and host metabolism. These studies further elucidate the mechanism of action of PAHSAs. They also demonstrate the therapeutic utility of modulating the gut microbiota for the prevention and treatment of obesity and associated metabolic disease and highlight the major role of sex as a biological variable when studying host–microbiome interactions.

Materials and Methods

Animals. Gnotobiotic (GF) C57BL/6J mice from the Massachusetts Host-Microbiome Center (Brigham and Women's Hospital, BWH) were used as indicated. 4- to 6-wk-old GF mice were cohoused 3 to 5 mice/cage in Optimice cages [for fecal microbiota transplantation (FMT) studies] or in gnotobiotic isolators. 4- to 5-wk-old C57BL/6J conventional male mice (Jackson Labs, 000664) were acclimated for 1 wk prior to starting PAHSA treatment. These mice were maintained in a specific pathogen-free barrier facility with a standard 12:12 light:dark cycle at Beth Israel Deaconess Medical Center (BIDMC). In *Bt* supplementation studies, C57BL/6J conventional male and female mice were bred in-house and singly caged (BIDMC). *Bt* supplementation started at 24 wk of age in chow-fed conventional mice and at 36 wk of age in HFD-fed conventional mice. Fecal pellets were collected from GF mice at baseline and serially sampled throughout treatment intervention to ensure gnotobiotic status by microbial plate culture and PCR (*SI Appendix*, Fig. S2 *C* and *D*) in mice. GF, conventional, and ovariectomized (OVX) mice were fed HFD for 12 wk prior to starting treatments. OVX surgeries were performed as described in ref. 24 in mixed genetic background (C57BL/6J and FVB) female conventional mice on the HFD. All experiments were conducted in accordance with and approved by the Institutional Animal Care and Use Committees of BWH and BIDMC.

Chronic Treatments in Experimental Mice.

PAHSAs. 4- to 5-wk-old conventional chow-fed male C57BL/6J mice (Jackson Labs) were singly housed and gavaged with either vehicle (50% PEG400: 0.5% Tween-80: 49.5% water) or 5- and 9- PAHSAs (15 mg/kg BW of each in vehicle; synthesized by the UC San Diego Center for Compound Resources) once daily for 21 d. Animals were terminated on day 21 after 6 h of refeeding following an overnight fast, and tissues were collected. Fecal pellets were collected for FMT studies. Serum and plasma (baseline and terminal time points) and cecal contents were collected for metabolomics and metagenomics analyses. Six- to eight-wk-old GF male and female C57BL/6J mice were cohoused 3 to 5 mice/cage in gnotobiotic isolation chambers at BWH. Mice were gavaged once daily with vehicle or 5- and 9- PAHSAs (chow dose same as above, HFD dose 45 mg/kg BW of each in vehicle). Untreated GF control HFD-fed mice did not receive any treatment.

Bt VPI-5482. Twenty-wk-old conventional C57BL/6J male and female mice fed chow or HFD were treated with *Bt* inoculum. Chow mice were gavaged 3 times/week and HFD-fed mice received daily gavage. Live *Bt* was administered at a dose of 2×10^9CFU/100μL PBS. Heat-killed *Bt* treatment was generated from the same stock of live *Bt* and heat-treated at 121 °C for 15 min. All inoculums were prepared under anaerobic conditions.

Microbiota Data Analysis and Functional Profiling. To ensure read quality, raw reads were assessed using FastQC and MultiQC, and host reads with low quality ends (Phred scores < 28) were filtered using Kneaddata quality control software (25) for automatic adapter detection, trimming low-quality read bases, and removing host (mouse genome) reads prior to downstream analyses. The quality-controlled cleaned reads were then categorized for relative abundance using MetaPhlAn embedded in HUMAnN (26), where they were mapped against microbial marker genes for microbial species and genes for pathway profiling. In Fig. 1*D*, operational taxonomic units (OTUs) were assigned to sequences above 300 bp with 97 to 99% identity after removal of singleton sequences clustering at 1% divergence and taxonomically classified using BLASTn against the NCBI reference genome database. OTUs were compiled by taxonomic level as a relative abundance matrix (genus). Functional profiling of these reads was used for multiomics Spearman correlation analysis with the metabolites from the same cecal sample. For data in Fig. 1*G*, we performed microbial species profiling from metagenomics shotgun reads using MetaPhlAN2 embedded in the HUMAnN2 pipeline (26). DADA2 was used to analyze 16S sequencing reads at the amplicon sequence variant (ASV) level (27).

Multiomics Correlation Analysis. Correlations between microbial species abundance and metabolite intensities were assessed using the *"btest"* Python package (28) (*SI Appendix, Supplemental Methods*). Within *btest*, the *P*-value and correlation are determined using the Spearman correlation test. Adjustments for multiple testing were made using the Benjamini–Hochberg false discovery rate and differences considered significant at $P \leq 0.05$ unless otherwise noted.

Results

PAHSA Treatment in Mice Increases Insulin Sensitivity and Alters the Gut Microbiota and Plasma and Cecal Metabolomes. To determine the effects of PAHSA treatment on gut microbiota function and community composition, we measured the cecal and plasma metabolomes and cecal microbiome in conventional chow-fed mice (Fig. 1*A*). Consistent with previous reports, PAHSA treatment had no effect on body weight (19, 21) (Fig. 1*B*) but improved insulin sensitivity as early as 13 d of treatment (Fig. 1*C*). Microbiota analyses of cecal contents showed comparable abundance levels for most genera between vehicle and PAHSA-treated mice. An exception is *Bacteroides,* the most abundant genera, which increased with PAHSA treatment (Fig. 1*D*). PAHSAs had major effects on plasma lipid metabolite levels (Fig. 1*E* and *SI Appendix*, Fig. S1*A*) including increasing circulating levels of 8 cholesterol ester (CE) species and lowering levels of 85 plasma lipid metabolites compared to controls (*SI Appendix*, Fig. S1*A*). Sixty-nine of these species were triacylglycerols (TAGs), 10 DAGs (diacylglycerides), 3 PEs (phosphatidylethanolamine), and 2 PCs (phosphatidylcholine) (*SI Appendix*, Fig. S1*A*). In the cecum, 113 lipid species increased with PAHSA treatment versus control mice (*SI Appendix*, Fig. S1*B*) including 17 PCs, 8 PEs, 41 TAGs, 30 NH4-TAGs, and 9 plasmalogens (*SI Appendix*, Fig. S1*B*).

Thirty-five of these lipids were changed in both plasma and cecum, with levels in plasma being lower and levels in the cecum being higher in PAHSA-treated mice compared to controls (Fig. 1 *E* and *F*). These included C34:0 PC, two PEs, C38:4 DAG, nineteen TAGs, and twelve NH4-TAGS. Among them, five species highlighted in red boxes are differentially regulated by PAHSAs in both plasma and cecal contents and correlated with abundance levels of *Bt, Parasutterella excrementihominis (Pe),* and *Akkermansia muciniphila* (*Am,* Fig. 1*G*). Also, three plasma TAGs

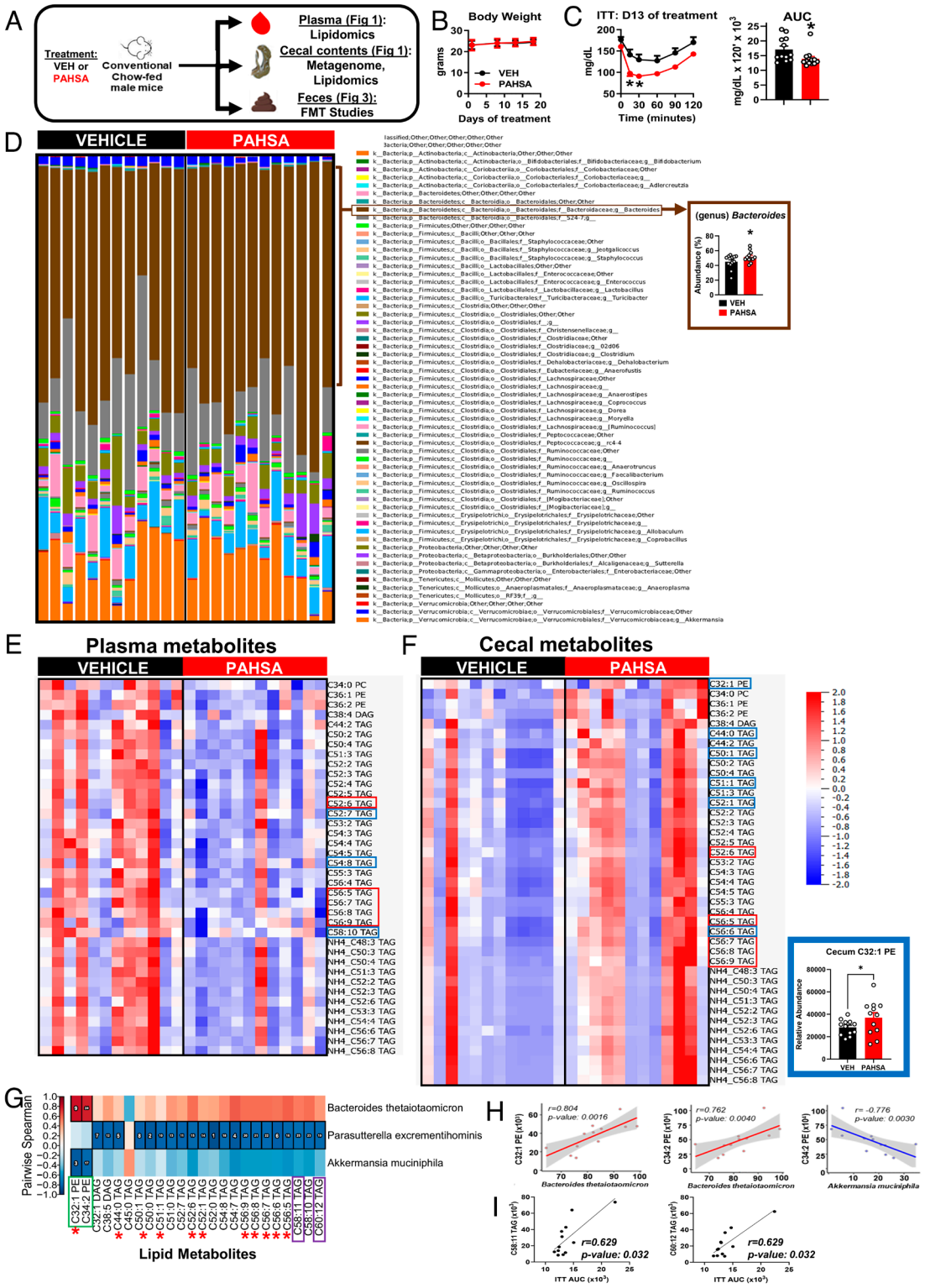

Fig. 1. PAHSA treatment in mice increases insulin sensitivity and alters the gut microbiota and plasma and cecal metabolomes. (*A*) Experimental design in which conventional chow-fed male mice were treated once daily with oral vehicle or PAHSAs. Effects of treatment on (*B*) body weight, (*C*) insulin sensitivity after 13 d of treatment, with area under the curve (AUC) (*$P < 0.05$ PAHSA versus VEH). (*D*) Stacked bar graph of gut microbiota abundance at the genus level in vehicle and PAHSA-treated mice, and bar graph of abundance levels of *Bacteroides* (genus), (*$P < 0.05$ PAHSA versus VEH). Heatmaps of abundance levels of (*E*) plasma metabolites and (*F*) cecal metabolites from vehicle and PAHSA-treated mice. Each column represents one mouse, *$P < 0.05$ PAHSA versus VEH. (*G*) Heatmap showing that *Bt* strongly associates with a subset of cecal lipid metabolites from PAHSA-treated mice. (*H*) Correlations of *Bt* with C34:2 PE and C32:1 PE, and *Am* with C34:2 PE. (*I*) Correlations of ITT AUC with C58:11 TAG and C60:12 TAG. n = 12/group. Statistical analyses: (*C* and *F*) Repeated-measures two-way ANOVA followed by Tukey's post hoc test and Student's *t* test. (*H*) btest using data in (*E* and *F*). (*I*) Spearman correlation using data in (*C*) and (*E*). (*H* and *I*) Spearman correlation to calculate r- and *P*-values (*B–D*), and the bar graphs in (*D*) and (*F*) show means ± SEM. In (*E* and *F*), red boxes indicate PAHSA-regulated metabolites in both plasma and cecal contents, although sometimes in the opposite direction, from the same experimental mice, and are also found in the lipidomics signature in (*G*). Blue boxes in (*E* and *F*) indicate PAHSA-regulated metabolites which are changed in the same experimental mice in either plasma or cecal contents but not in both; these metabolites are in the lipidomics signature in (*G*). In (*G*), red asterisks indicate the 11 cecal metabolites from (*F*) that are increased with PAHSA treatment, green boxes indicate metabolites that positively correlate with *Bt* abundance levels in (*H*), and purple boxes indicate metabolites that positively correlate with ITT AUC of PAHSA-treated mice in (*I*).

(Fig. 1*E*) and six cecal lipids (C32:1 PE and five TAGs) (Fig. 1*F*) highlighted in the blue boxes were differentially regulated by PAHSAs and were also associated with the same gut microbial species (*Bt, Pe, Am*) (Fig. 1*G*).

To understand how PAHSAs affect gut microbiota function, we tested for correlations between gut microbiota abundance and cecal metabolite levels in PAHSA-treated mice (26). Co-occurrence between microbiota abundance levels and metabolite levels was measured by the Spearman correlation test to validate microbe-metabolite associations. These analyses identified 24 lipid metabolites (two PEs, two DAGs, and 20 TAGs) with significant positive correlations with three microbial species in PAHSA-treated mice: *Bt* (positive), *Pe* (negative), and *Am* (negative) (Fig. 1*G*). Among the 24 cecal metabolites, 23 correlated significantly with *Bt.* For example, C32:1PE was one of 11 cecal lipid species that was increased with PAHSA treatment (Fig. 1*F*) and positively correlated with *Bt* abundance (Fig. 1*H*). Similarly, C34:2PE correlated positively with *Bt* and negatively with *Am* levels (Fig. 1*H*). Furthermore, within the subset of 24 cecal lipid metabolites (Fig. 1*G*), two (C58:11 TAG, C60:12 TAG) positively correlated with the ITT AUC of chow-fed mice. These data indicate that some lipids that are associated with *Bt* (Fig. 1*G*) may contribute to the enhanced insulin sensitivity from PAHSA treatment (Fig. 1*I*). Overall, PAHSAs have specific effects on the gut microbiota and lipid metabolites, and these gut microbe-metabolite signatures may contribute to the improved insulin sensitivity in PAHSA-treated mice.

In the same cohort of mice, PAHSA treatment increased ileal expression of *gcg* and the Goblet cell marker *clca1* (*SI Appendix*, Fig. S1*C*). In another cohort of HFD-fed mice, PAHSA treatment reduced expression of proinflammatory cytokines and increased *occludin*, a tight junction marker in the ileum (*SI Appendix*, Fig. S1*D*). These data indicate that PAHSAs have direct beneficial effects on the gut epithelium. PAHSA treatment did not alter cecal levels of gut microbe-derived short-chain fatty acids (SCFA) (data not shown). However, in another cohort of chow-fed mice that were refed for 4 h, PAHSA treatment for 28 d raised fecal propionate levels (data not shown). These differences in SCFA levels may be due to sampling location within the gastrointestinal tract and the fed versus fasted/refed state of the animals.

The Gut Microbiota Is Necessary for PAHSA Effects to Improve Glucose Homeostasis in HFD-Fed Mice but Not in Chow-Fed Mice. To determine whether the gut microbiota contributes to the antidiabetic effects of PAHSAs, we treated germ-free (GF) chow-fed female mice with oral PAHSAs or vehicle (Fig. 2*A*). PAHSA treatment had no effect on body weight (Fig. 2*B*), but it improved glucose tolerance (Fig. 2 *C* and *D*) versus vehicle-treated and untreated controls. Similarly, in chow-fed GF male mice (Fig. 2*E*), PAHSA treatment had no effect on body weight (Fig. 2*F*), but improved glucose tolerance (Fig. 2 *G* and *H*) versus vehicle-treated controls. The effect of PAHSAs to increase ileal *gcg* and *clca1* expression in chow-fed conventional mice (*SI Appendix*, Fig. S1*C*) was attenuated in chow-GF mice (*SI Appendix*, Fig. S2 *A* and *B*), suggesting that the gut microbiota may mediate the expression of gut enteroendocrine cell and Goblet cell genes. These data indicate that the PAHSA effects to improve glucose tolerance are independent of the gut microbiota in both sexes of chow-fed mice.

To determine the role of the gut microbiota in mediating PAHSA effects in HFD-fed mice, we treated GF-HFD-fed mice with oral PAHSAs or vehicle (Fig. 2 *I–P*). Consistent with previous studies in conventional diet-induced obese mice (19, 21), there was no effect of PAHSA treatment on body weight (Fig. 2 *J* and *N*). Unlike in GF-chow mice, PAHSA treatment did not improve glucose tolerance (Fig. 2 *K* and *O*) or insulin sensitivity (Fig. 2 *L* and *P*) in GF-HFD female or male mice. Thus, the PAHSA effects to mediate glucose metabolism depend on the gut microbiota in HFD-fed female and male mice.

The Beneficial PAHSA Effects on Glucose Homeostasis Are Transmissible by FMT. To determine whether the PAHSA effects to improve host metabolism can be transmitted by FMT, we transplanted donor feces from conventional male mice treated with vehicle or PAHSAs (Fig. 1*A*) into recipient GF-HFD male mice (Fig. 3*A*). There was no effect of PAHSA-FMT on body weight in recipient mice versus vehicle-FMT controls (Fig. 3 *B* and *C*), consistent with PAHSA treatment having no effect on body weight in donor mice. However, PAHSA-FMT recipient male mice were more glucose tolerant and insulin sensitive versus vehicle-FMT recipient mice (Fig. 3 *D–F*). Interestingly, treatment with vehicle-FMT appeared to worsen glucose tolerance versus no FMT. Since PAHSAs are dissolved in the same vehicle, vehicle-FMT is the appropriate control for PAHSA-FMT. By contrast, there was no effect of PAHSA-FMT on any of these metabolic outcomes in GF-HFD female mice (*SI Appendix*, Fig. S3 *A–F*). This indicates sex-specific responses in HFD-fed GF mice to FMT from PAHSA-treated mice. These data demonstrate that FMT from PAHSA-treated chow-fed mice can transmit PAHSA-mediated improvements in glucose metabolism to male GF-HFD mice.

The Gut Microbiome Has Sex-Specific Responses to *Bt* Supplementation in Mice. Functional profiling of the gut microbiota from PAHSA-treated chow-fed mice identified *Bt* to associate with a subset of cecal lipid metabolites (Fig. 1*G*). Since a subset of these lipids correlated with insulin sensitivity, we aimed to determine the effects of heat-killed (HKBT) and live (LBT) *Bt* supplementation on the gut microbiota in HFD-fed conventional mice. Principal Components Analysis of gut metagenome sequencing data showed that HFD feeding had the greatest effect on gut microbial community composition in both female and male conventional mice compared to effects of treatment with either heat-killed or live *Bt* (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S4 *A* and *B*). Within HFD-fed female mice, microbial composition was slightly more variable in LBT-supplemented HFD-female mice compared to HFD-PBS and HFD-HKBT-treated controls (Fig. 4*A*). *Bt* supplementation had minimal effect on gut microbiota composition in HFD-fed male mice (Fig. 4*B*).

Supervised classification and hierarchical clustering of gut microbial taxa demonstrated that female (Fig. 4*C* and *SI Appendix*, Fig. S4*A*) and male (Fig. 4*D* and *SI Appendix*, Fig. S4*B*) mice have distinct sex-specific responses to HFD feeding and *Bt* supplementation. Female chow-fed mice had elevated genera levels of *Turicibacter, Muribaculum,* and *Lachnospiraceae* (*UCG-008* and *UCG-001*) versus all HFD-fed mice (Fig. 4*C*). HFD-PBS female mice had increased genera levels of *Lactobacillus* and *Odoribacter* compared to all other groups (Fig. 4*C*). Although LBT and HKBT had minimal effects on microbial composition, there were some sex-specific effects at the genera level. HFD-HKBT female mice had higher genera levels of *Bifidobacterium* and *Coriobacteriaceae* versus chow and HFD-PBS mice, while LBT treatment restored levels of *Blautia* (genera), *Rikenellaceae* (family), and *Clostridia* (class) toward chow levels (Fig. 4*C*). Interestingly, *Blautia* (genera) abundance changed with *Bt* treatment, with levels increasing with HKBT and reaching highest levels with LBT supplementation versus chow and HFD-PBS controls (Fig. 4*C*).

In contrast, male chow-fed mice had elevated genera levels of *Bacteroides, Parasutterella, Muribaculum,* and *Lachnospiraceae*

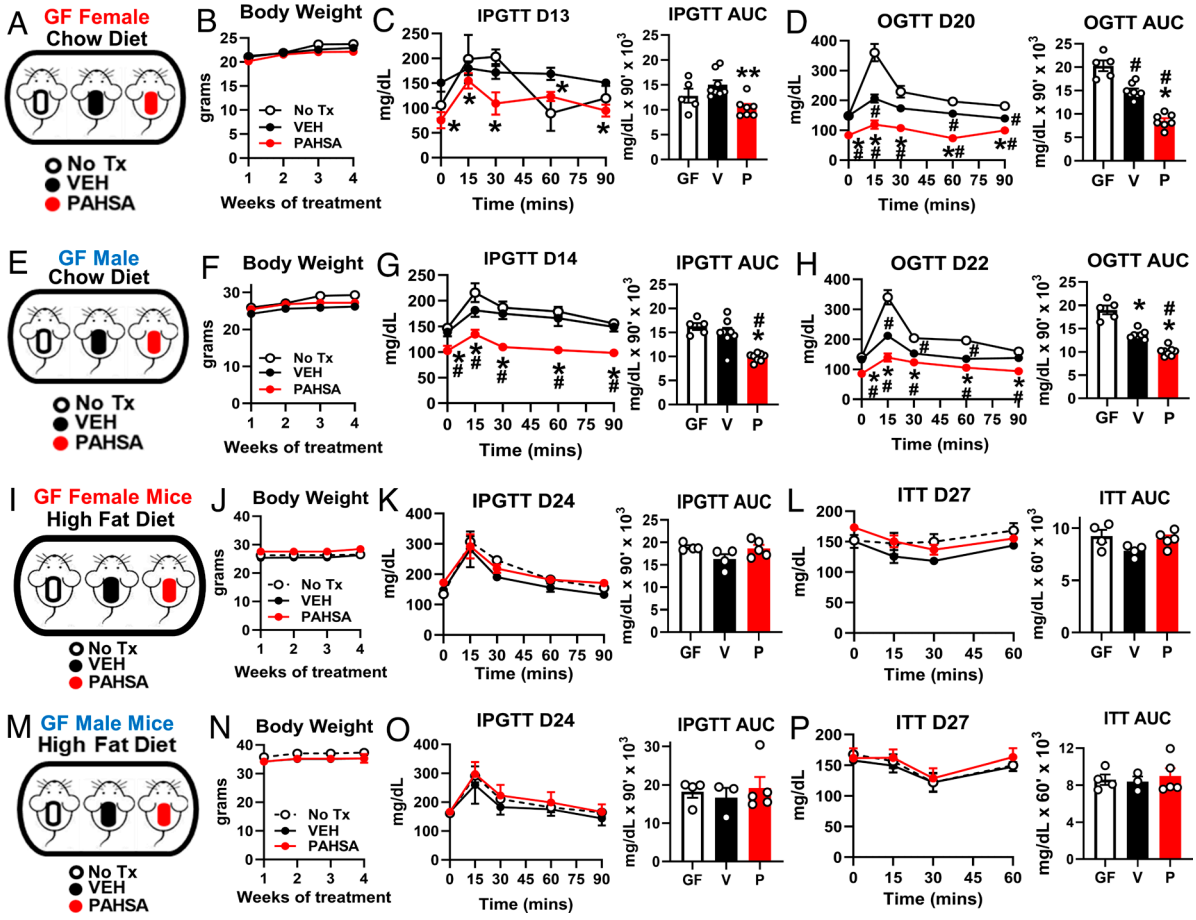

Fig. 2. The gut microbiota is necessary for PAHSAs to improve glucose homeostasis in HFD-fed mice but not in chow-fed mice. (*A*) Experimental design for chow-fed GF female mice receiving no treatment (No Tx) or treated with oral vehicle (VEH) or PAHSAs. Effects of once daily oral vehicle or PAHSA treatment in GF-chow female mice on (*B*) body weight, (*C*) intraperitoneal glucose tolerance after 13 d of treatment with corresponding AUC graph, and (*D*) oral glucose tolerance after 20 d of treatment with corresponding AUC graph. (*E*) Experimental design for chow-fed GF male mice receiving no treatment (No Tx) or treated with oral vehicle (VEH) or PAHSAs. Daily oral vehicle or PAHSA treatment effects in GF-chow male mice on (*F*) body weight, (*G*) intraperitoneal glucose tolerance after 14 d of treatment with AUC graph, and (*H*) oral glucose tolerance after 22 d of treatment with AUC graph. (*I*) Experimental design for HFD-fed GF female mice receiving no treatment (No Tx) or treated with oral vehicle (VEH) or PAHSAs. Daily oral vehicle or PAHSA treatment effects in GF-HFD female mice on (*J*) body weight, (*K*) intraperitoneal glucose tolerance after 24 d of treatment with AUC graph, and (*L*) insulin sensitivity after 27 d of treatment with AUC graph. (*M*) Experimental design for GF-HFD-fed male mice receiving no treatment (No tx) or treated once daily with oral vehicle (VEH) or PAHSAs. Daily oral vehicle or PAHSA treatment effects in male GF-HFD mice on (*N*) body weight, (*O*) intraperitoneal glucose tolerance after 24 d of treatment with AUC graph, and (*P*) insulin sensitivity after 27 d of treatment with AUC graph. n = 4 to 5/group in female mice and n = 3 to 5/group in male mice. Data are means ± SEM.

versus HFD-fed mice (Fig. 4*D*). Within HFD-fed male mice, HKBT treatment, and LBT to a lesser degree, increased genera levels of *Blautia, Lactobacillus,* and *Oscillobacter* compared to PBS controls. HFD-LBT male mice had increased levels of genera that included *Ileibacterium, Coriobacteriaceae* (*UCG-002*), and *Odoribacter* versus all other groups. These data show that the gut microbiota has sex-specific responses to *Bt* oral bacteriotherapy in HFD-fed mice.

Next, we determined the effect of HFD feeding and *Bt* supplementation on the biomass of detectable *Bt species* in vivo. Metagenome sequencing detected levels of both heat-killed and live *Bt.* HFD-feeding reduced *Bt* levels in female (Fig. 4*E*) and tended to decrease *Bt* levels in male (Fig. 4*F*) mice compared to chow control levels, and both HKBT and LBT supplementation tended to raise *Bt* levels toward those of chow-fed mice. We found no effect of HKBT or LBT supplementation on gut microbe-derived SCFA concentrations in the cecum of HFD-fed female (Fig. 4*G*) or male (Fig. 4*H*) mice.

***Bt* Supplementation Improves Host Metabolism in Female Diet-Induced Obese Mice.** We next tested the effects of *Bt* supplementation on host metabolism in conventional female and male mice. LBT supplementation decreased weight gain and adiposity in HFD-fed female mice versus HFD-PBS controls (Fig. 5 *A*–*C*) without affecting lean mass (Fig. 5*D*). The decreased adiposity could not be explained by a change in food intake (Fig. 5*E*). HFD-fed mice receiving either heat-killed (HKBT) or LBT supplementation tended to have lower ambient glycemia versus HFD-PBS controls throughout the course of intervention (Fig. 5*F*). Glucose tolerance (Fig. 5 *G* and *H*) was improved in HFD-HKBT and HFD-LBT female mice, and this was independent of insulin secretion (Fig. 5*H*). Female LBT-treated mice had increased GLP-1 levels in response to oral lipids versus HFD-PBS controls (Fig. 5*I*). However, *Bt* supplementation had no effect on insulin sensitivity measured by the insulin tolerance test (Fig. 5*J*). The reduced weight gain and adiposity in HFD-LBT mice was independent of brown adipose tissue (BAT) *ucp1* expression (Fig. 5*K*). Adipocyte number in perigonadal (PG) and subcutaneous (SQ) WATs was variable and adipocyte size did not differ (Fig. 5*L*). Fecal energy content was increased in HKBT-treated mice but unchanged in LBT-treated mice versus controls (Fig. 5*M*). In HFD-fed males, there was no effect of *Bt* administration on any of these metabolic outcomes (*SI Appendix*, Fig. S5 *U–AE*). In chow-fed females (*SI Appendix*, Fig. S5 *A–J*),

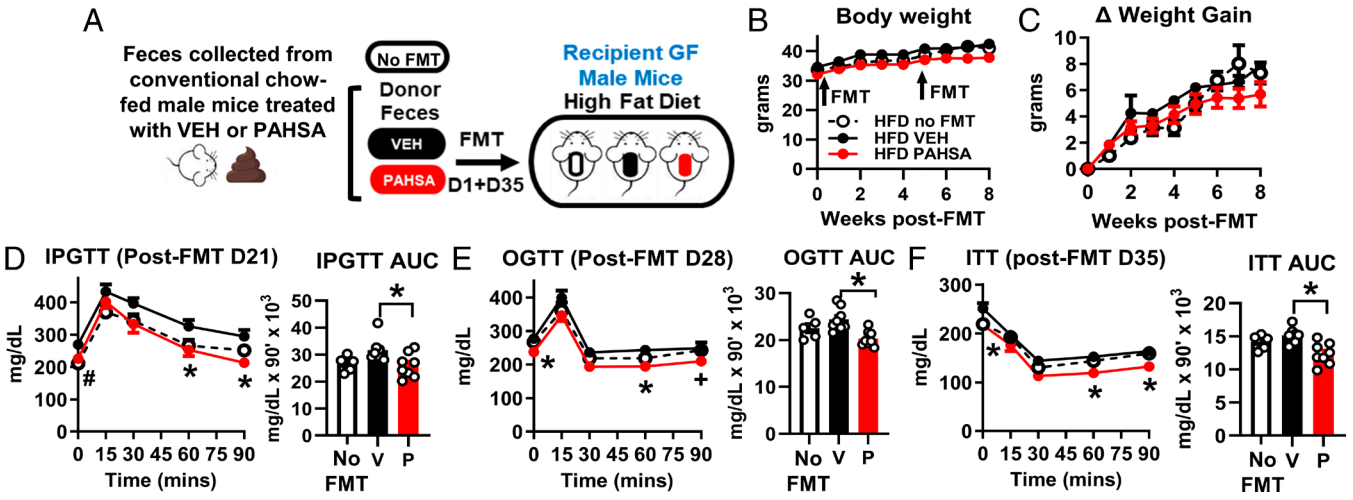

Fig. 3. The beneficial PAHSA effects on glucose homeostasis are transmissible by FMT. (*A*) Experimental design for FMT in GF mice which received fecal contents from donor chow-fed male mice treated with vehicle (V) or PAHSAs (P). FMT effects in recipient GF-HFD male mice on (*B*) body weight, (*C*) weight gain, (*D*) intraperitoneal glucose tolerance 21 d post-FMT, (*E*) oral glucose tolerance 28 d post-FMT, and (*F*) insulin sensitivity 35 d post-FMT. $^{*}P < 0.05$ GF-HFD mice receiving FMT from VEH-treated chow-fed mice versus GF-HFD mice receiving FMT from PAHSA-treated chow-fed mice, $^{\#}P < 0.05$ GF-HFD mice receiving FMT from VEH-treated chow-fed mice versus all groups. $^{+}P < 0.05$ GF-HFD mice receiving FMT from PAHSA-treated mice versus GF-HFD mice receiving no FMT. n = 5 GF-HFD receiving no FMT, n = 8 GF-HFD receiving FMT from VEH-treated mice, n = 8 GF-HFD mice receiving FMT from PAHSA-treated mice. Data are means ± SEM. Statistical analysis using repeated measures one- or two-way ANOVA followed by Tukey's post hoc test.

only the effects of *Bt* on lowering adiposity versus PBS controls were observed. In chow-fed males, *Bt* supplementation had no effect on body weight, body composition, food intake, or insulin sensitivity, but lowered serial glycemia, improved glucose tolerance, and increased glucose-stimulated insulin secretion (*SI Appendix*, Fig. S5 *K–T*).

Considering the sex-specific *Bt* supplementation effects on host metabolism, we asked whether sex hormones were involved (*SI Appendix*, Fig. S5*AF*). In another cohort of mixed-strain background female HFD-fed mice, LBT supplementation reduced weight gain (*SI Appendix*, Fig. S5*AG*), which is consistent with LBT effects on body weight in C57BL6 female mice (Fig. 5*B*). As expected, ovariectomized (OVX) mice gained more weight and tended to have increased adiposity and worsened glucose tolerance (*SI Appendix*, Fig. S5 *AG–AJ*) versus sham controls. HKBT- and LBT-treated HFD-OVX female mice tended to gain less weight and adiposity and were more glucose-tolerant compared to HFD-OVX controls (*SI Appendix*, Fig. S5 *AG–AJ*), indicating that estrogen only partially mediates the *Bt* effects on host metabolism in diet-induced obese female mice. These data demonstrate that live and/or heat-killed *Bt* supplementation has beneficial sex-specific effects on host metabolism in conventional diet-induced obese mice.

***Bt* Supplementation Affects the Gut Mucosa and Intestinal Innate Immune Cells in Diet-Induced Obese Mice.** Intestinal barrier function plays a critical role in regulating glucose metabolism and this is mediated in part by the gut mucosa and gut inflammatory status. HFD feeding decreased colonic mucus thickness in female mice compared to chow-fed controls (Fig. 6*A*). LBT supplementation increased colonic mucus thickness toward those of chow-fed controls (Fig. 6*A*). Similarly, colonic MUC2 levels, the predominant intestinal mucin protein, tended to decrease with HFD feeding, and were ~70% lower versus levels in LBT-supplemented mice (Fig. 6*B*). MUC2 is synthesized and secreted by Goblet cells that line the intestinal epithelium. Thus, we measured the number of Goblet cells in the ileum from the same mice. Goblet cell frequency was not different between chow and HFD-PBS or HFD-HKBT-treated mice (Fig. 6*C*). However, LBT supplementation increased the number of ileal Goblet cells versus HFD-PBS controls (Fig. 6*C*). By contrast, there was no effect of *Bt* supplementation on the intestinal mucosa in HFD-fed male mice (*SI Appendix*, Fig. S6 *A* and *B*), which may explain the lack of metabolic improvement (*SI Appendix*, Fig. S5 *U–AD*).

Plasma lipopolysaccharide (LPS) binding protein (LBP), a proxy for LPS levels, and interleukin-6 (IL-6) can indicate whether the increased gut mucosa architecture with LBT treatment prevents translocation of proinflammatory molecules into systemic circulation. Plasma LBP levels were reduced in both HKBT- and LBT-treated HFD-fed female mice (Fig. 6*D*). Similarly, LBT lowered and HKBT tended to reduce circulating IL-6 levels in diet-induced obese female mice (Fig. 6*E*). Despite no change in mucosal thickness or Goblet cell frequency, *Bt* supplementation tended to lower levels of circulating LBP and IL-6 in HFD-fed male mice (*SI Appendix*, Fig. S6 *C* and *D*). These data indicate that LBT has sex-specific effects to fortify the intestinal mucosa, and this associates with a reduction in proinflammatory molecules that are known to translocate into systemic circulation in diet-induced obese mice.

Intestinal innate immune cell types play a critical role in regulating gut homeostasis and contribute to host metabolic processes (5). Here, HFD feeding increased the percentage of colonic intraepithelial CD8+ T-cells versus chow controls (*SI Appendix*, Fig. S6*E*), and within this population, *Bt* supplementation tended to lower the percentage and MFI of $CD8^{+}IFN\gamma^{+}$ T-cells versus HFD-PBS and chow controls (Fig. 6*F* and *SI Appendix*, Fig. S6*E*). Increasing evidence supports a role for $TCR\gamma\delta^{+}$ cells in maintaining gut barrier function and regulating gut and host metabolism. HFD feeding tended to lower the percentage of colonic intraepithelial $TCR\gamma\delta^{+}$ T-cells versus chow (Fig. 6*G*), with LBT treatment reducing $TCR\gamma\delta^{+}$ levels, although the subset of $TCR\gamma\delta^{+}IFN\gamma^{+}$ T-cells was not altered (*SI Appendix*, Fig. S6*F*). Similarly, *Bt* supplementation reduced the expression of T-cell and myeloid cell markers (*tbx21* and *arg1),* and related proinflammatory cytokines (*il-17, il-10, tnf-α)* versus HFD-PBS controls (*SI Appendix*, Fig. S6*H*). These data indicate that *Bt* supplementation reduces colonic intraepithelial proinflammatory immune cell profiles and function.

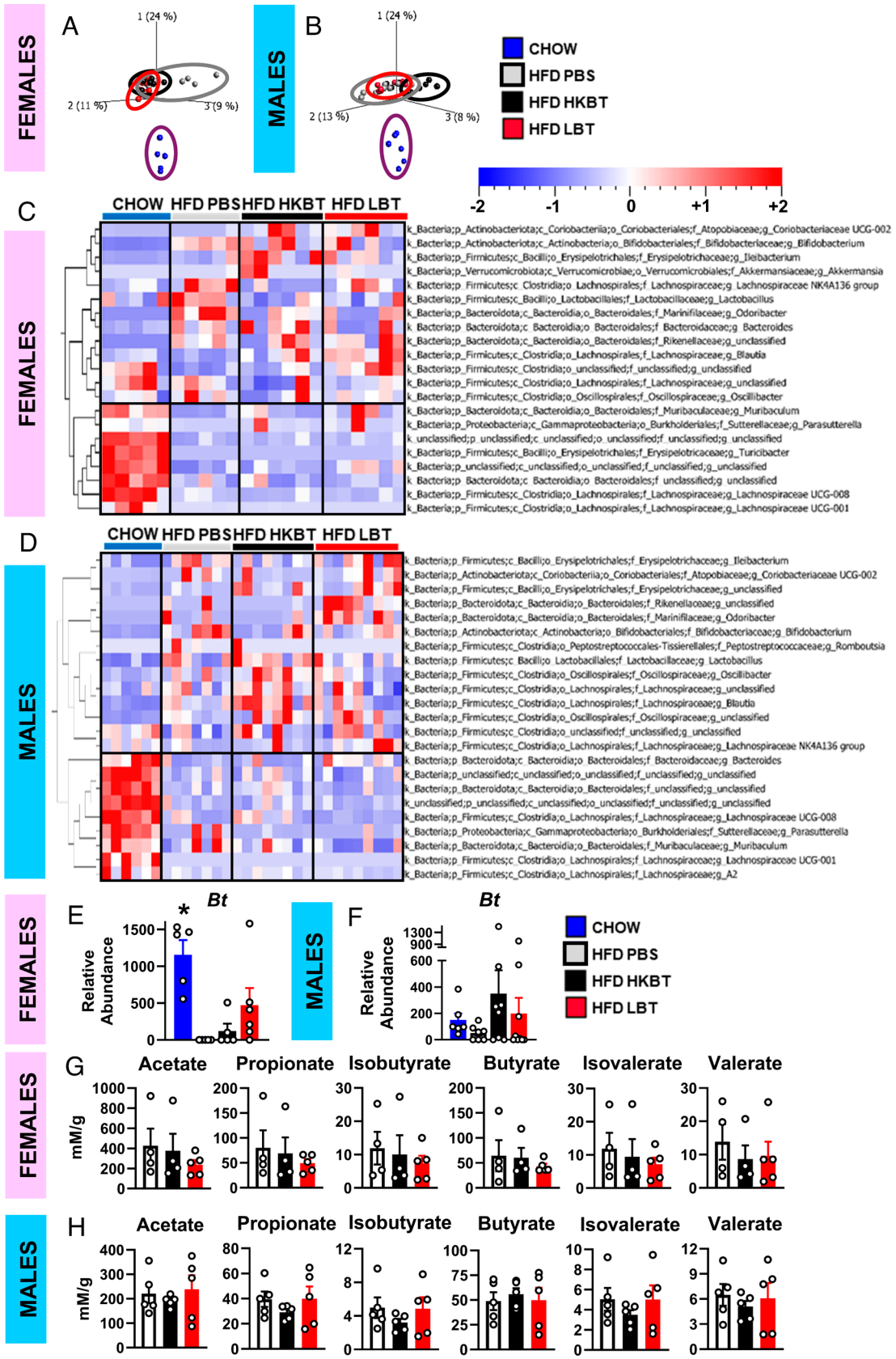

Fig. 4. The gut microbiome has sex-specific responses to *Bt* supplementation in diet-induced obese mice. Conventional C57BL6/J mice were fed chow or HFD supplemented with PBS, heat-killed *Bt* (HKBT), or live *Bt* (LBT), and cecal contents were collected for gut metagenome sequencing. Principal components analyses illustrating similarities in the way HFD alters gut microbial composition in (*A*) female and (*B*) male mice with lesser effects of HKBT or LBT. (*C* and *D*) Heatmap with hierarchical clustering of gut microbial species in (*C*) female and (*D*) male mice. Relative abundance levels of *Bt* in (*E*) female and (*F*) male mice calculated as the frequency of ASVs normalized to gram of fecal DNA, and SCFA levels in the same cecal contents in (*G*) female and (*H*) male mice. For (*A*, *C*, and *E*) female mice, n = 5 to 6/group. For (*B*, *D*, and *F*) male mice, n = 6 to 9/group. For (*G* and *H*) n = 4 to 5/group. *$P < 0.05$ chow versus all groups. Data are means ± SEM. Statistical analysis conducted using one-way ANOVA followed by Tukey's post hoc test.

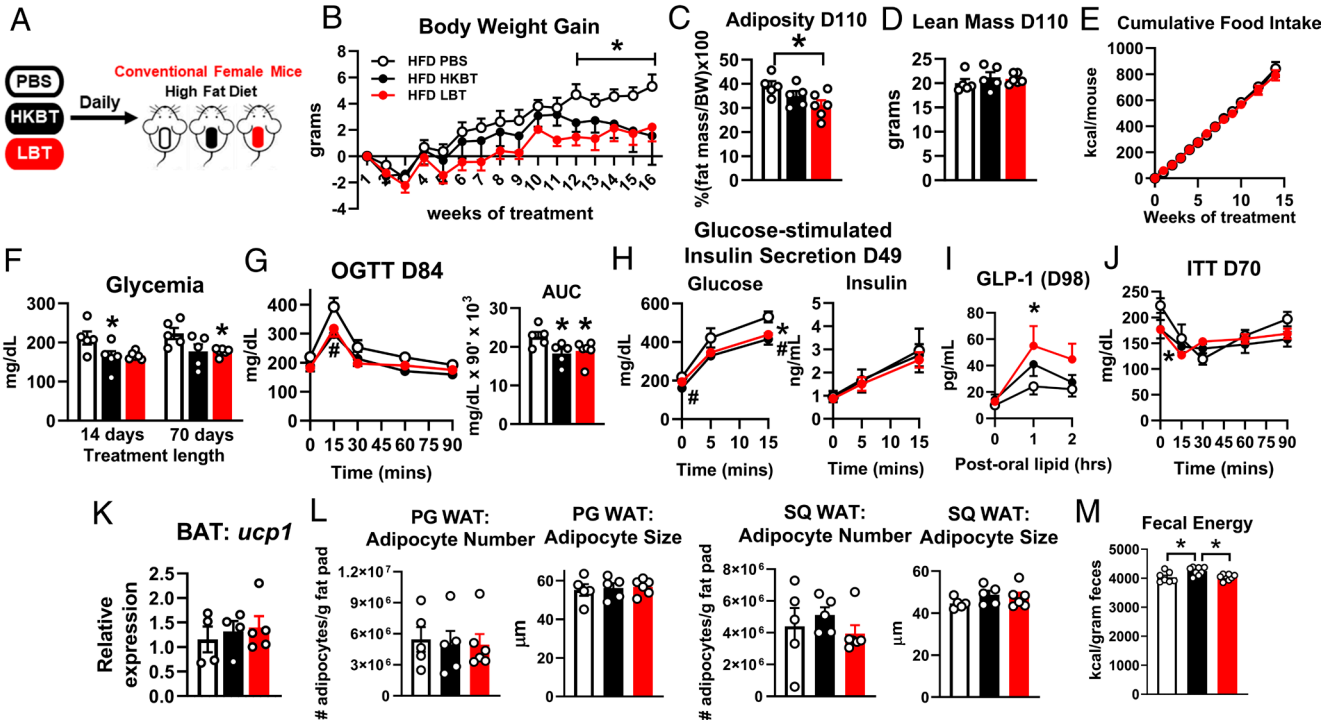

Fig. 5. *Bt* supplementation improves host metabolism in conventional diet-induced obese mice. (*A*) Experimental design in which conventional HFD-fed female mice were orally supplemented once daily with PBS, heat-killed *Bt* (HKBT), or live *Bt* (LBT). Effects of *Bt* supplementation on (*B*) weight gain, (*C*) adiposity (110 d of treatment), (*D*) lean mass (110 d of treatment), (*E*) cumulative food intake, (*F*) ambient glycemia following a 5-h food removal (14 and 70 d of treatment), (*G*) oral glucose tolerance test with AUC graph (84 d of treatment), (*H*) circulating glucose and insulin in response to oral glucose (49 d of treatment), (*I*) plasma GLP-1 levels in response to oral lipid (98 d of treatment), (*J*) insulin sensitivity (70 d of treatment), (*K*) *ucp1* expression in BAT, (*L*) adipocyte number and size in PG and SC WATs, and (*M*) fecal energy (77 d of treatment). n = 5 to 7 per group. **P* < 0.05 HFD LBT versus HFD PBS; #*P* < 0.05 HFD HKBT versus HFD PBS. Data are means ± SEM. Statistical analysis conducted using one-way ANOVA or repeated-measures one-way ANOVA followed by Tukey's post hoc test.

Discussion

The gut microbiota offers therapeutic opportunities to improve host metabolism. Here, we demonstrate how antidiabetic effects of PAHSAs do not depend on the gut microbiota in chow-fed mice, but they do in HFD-fed mice. In multiomic analyses of cecal microbial communities and lipid metabolites in mice with increased insulin sensitivity resulting from PAHSA treatment, we identified *Bt* as a target species associated with alterations in multiple lipids. Two of these lipids correlated with the insulin-sensitizing effects of PAHSAs. We then treated diet-induced obese mice with *Bt* and found distinct sex-specific effects on the gut microbiome, intestinal barrier function, adiposity, and glucose metabolism. The studies highlight the therapeutic value of targeting the gut microbiota and demonstrate that beneficial effects of the gut microbiota on host metabolism can be diet and sex specific.

FMT can transmit host phenotype (11, 29), making FMT an attractive strategy to treat and/or prevent certain human diseases. For example, FMT using healthy donor stool can improve the clinical response of some patients with recurrent *Clostridiodes difficile* infections and irritable bowel disease (IBD) (30–32), although effects are variable with rare cases of complications (33, 34). Despite the risks, interest remains in applying FMT for treatment and/or prevention of obesity and associated metabolic diseases (35–40). We find the beneficial PAHSA effects to improve insulin sensitivity in mice are transmissible by FMT. Donor feces from male mice resulted in increased insulin sensitivity in male, but not female, recipient mice, highlighting the importance of sex in mediating gut microbiome effects on host metabolism (41, 42). Overall, our studies demonstrate that the gut microbiota plays a key role in mediating PAHSA effects to improve glucose metabolism in HFD-fed male mice and support the broader notion that the gut microbiome is a therapeutic target that may be leveraged to improve host metabolism in obesity.

Phenotype transmission by FMT led us to ask whether a specific microbiota contributed to the beneficial PAHSA effects on glucose metabolism. We found that PAHSAs did not alter *Bt* abundance, but did alter a signature of cecal lipids, some of which positively associated with *Bt* abundance levels. While Bt levels were unchanged, PAHSAs may alter *Bt* metabolic activity since one study comparing the gut microbial proteomes of obese and lean humans demonstrated that *Bacteroidetes* were more metabolically active in obese microbiomes despite their lower abundance levels (43). Thus, the abundance and activity of *Bacteroidetes* can be dissociated. While we cannot conclude that the elevation of the cecal lipids shown in Fig. 1*G* is specific to *Bt,* these results suggest that PAHSAs can affect *Bt* and/or other gut microbial genomes and regulate levels of multiple cecal lipids. This is consistent with a report demonstrating that diet can regulate enzyme activity and metabolite biosynthesis in *Bt* (44). Further studies are needed to determine whether any of these lipids mediate the beneficial effects of PAHSAs.

Next, we tested whether *Bt* supplementation has beneficial effects to improve host metabolism in diet-induced obese mice. This could be relevant to human obesity since *Bt* levels are low in humans with obesity and restored with bariatric surgery (45). We found that *Bt* supplementation reduced weight gain and adiposity, and improved glucose tolerance in HFD-fed female, but not male mice. This sex-specific response to *Bt* supplementation may be due, in part, to inherent differences in the gut microbiotas of male and female C57BL6 mice (46). One study in 15-wk-old HFD-fed male mice showed that 7 wk of *Bt* supplementation reduced weight gain, adiposity, and adipocyte size (45). In another study,

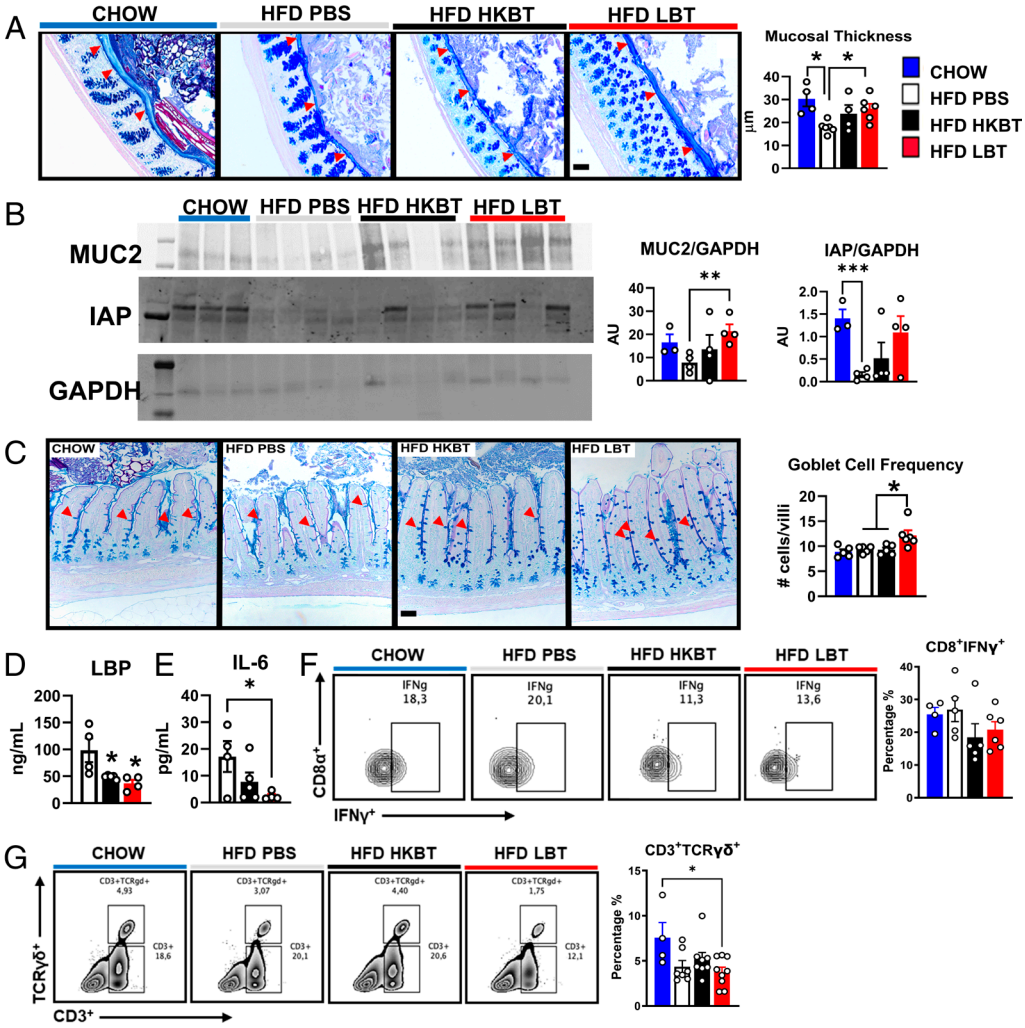

Fig. 6. Effects of *Bt* supplementation on the gut mucosa and intestinal innate immune cells in diet-induced obese mice. Effects of *Bt* supplementation in chow or HFD-fed conventional female mice supplemented with PBS, HKBT, or LBT on (*A*) colon mucus thickness, indicated by red arrows (n = 4 to 6/group), (*B*) Western blots and corresponding densitometric quantification for MUC2 (mucin2), IAP, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) in the mouse ileum (n = 3 to 4/group), (*C*) Goblet cell frequency in the mouse ileum, indicated by red arrows, and quantification graph (n = 5/group), (*D*) circulating LPS-binding protein ([LBP]; n = 4 to 5/group), (*E*) circulating interleukin-6 (IL-6; n = 4 to 5/group). Intraepithelial lymphocytes (IELs) were isolated from the colon, and the percentages of (*F*) $CD8^+IFN\gamma^+$ cells and (*G*) $CD3^+TCR\gamma\delta^+$ cells were measured by flow cytometry (n = 4 to 9/group). All images taken at 20× magnification by light microscopy. Scale bar indicated by the black line = 50 μm. *$P < 0.05$ HFD HKBT or HFD LBT versus HFD PBS, or as indicated. Data are means ± SEM. Statistical analysis using one- or two-way ANOVA followed by Tukey's post hoc test.

3 wk of *Bt* supplementation to 6-wk-old C57BL6 HFD-fed male mice improved insulin sensitivity (47). There are also reports that *Bt* supplementation adversely affects host metabolism. In one study, 6-wk-old HFD-fed male mice that were pretreated with antibiotics prior to starting *Bt* supplementation three times a week for 6 wk, gained weight (48). The divergent outcomes across *Bt* studies may be due to differences in mouse strain and age, type of HFD, mouse vendor sources, and housing conditions, all of which can impact gut microbiome-host outcomes (49, 50). Also, we administered *Bt* daily, whereas *Bt* was administered three times a week in other studies (45, 47). Overall, our findings suggest that *Bt* supplementation in female mice with diet-induced obesity may improve several metabolic parameters.

Sex hormones regulate the function of metabolic tissues (51) and contribute to differences in gut microbiome composition and host function (41, 52). However, the role of sex hormones in mediating probiotic supplementation effects on host metabolism is less known. To address this and because *Bt* supplementation improved several metabolic parameters only in HFD-fed female mice, we ovariectomized diet-induced obese mice. Ovariectomy partially reversed some of the *Bt* effects on host metabolism. It is possible that longer *Bt* supplementation may have greater benefits to host metabolism in ovariectomized mice. Many commercial probiotics are marketed for specific demographics (infant, women, all adults) and specific health outcomes (immune health, bloating, diarrhea). A growing market of next-generation probiotics targets metabolism and weight management, but their effects in both sexes are less clear (53). Our *Bt* supplementation and ovariectomy studies are among the few to date determining the role of estrogen and sex as a biological variable in probiotic effects on host metabolism. Our work highlights the need for more studies to determine sex-specific responses to probiotic supplementation.

Both heat-killed and live *Bt* had comparable effects to reduce weight gain, improve glucose tolerance, and lower circulating LPS and IL-6 in HFD-fed female mice. Both live probiotics and heat-killed postbiotics have therapeutic efficacy to improve multiple metabolic parameters in mice and humans (54, 55). For example, live and heat-killed *Am* supplementation reduced weight gain and improved insulin sensitivity in mice and in human studies (53–55). In one study, heat-killed *Am* retained its ability to bind to and activate TLR2, and this improved host metabolism in mice (55). Gut microbiota-derived lipids could explain the

effects of heat-killed Bt in our studies. Sphingolipids are a major component of *Bacteroides* membranes and can induce signal transduction in the host (56). The generation of sphingoid backbones during sphingolipid biosynthesis can form other complex lipid structures, including phosphatidylethanolamine dihydroceramides, which comprise up to 30% of the total lipid content in *Bacteroidetes* cells (57). *Bt,* a prominent member of *Bacteroides* (genus), can generate ceramides, a type of sphingolipid, and upon *Bt* supplementation to HFD-fed mice, these lipids can regulate gut inflammation and hepatic ceramide levels (58). The fact that sphingolipids are resistant to heat shock may explain the conserved effects of heat-killed *Bt* on improving glucose metabolism in HFD-fed mice observed here.

PAHSA treatment increased cecal levels of C32:1PE and several TAG species, which correlated with *Bt* abundance levels in PAHSA-treated mice. We cannot conclude that altered levels of these lipids derive specifically from *Bt.* However, *Bt*-derived lipids, including other PEs and sphingolipids, can regulate sphingolipid bioavailability in the host gut and gut mucosal immunity in mice (59). The PEs that increase with PAHSA treatment and were associated with *Bt* may have similar effects. A number of bacterially produced lipids exert effects on the host. *Am*-derived diacyl phosphatidylethanolamine with two branched chains modulates homeostatic immune responses, *Neisseria meningitidis*-derived *N*-acyl amides regulate immune cell activation, and *Bacteroides* (including *Bt*)-derived sphingolipids affect host lipid metabolism systemically (58, 60, 61). Together, these studies highlight the therapeutic utility of leveraging the gut microbiota lipidome to prevent metabolic disease. The mechanisms by which *Bt*-derived lipids act, either directly or indirectly by way of downstream metabolites, warrant further study. It is advantageous that *Bt* has efficacy as both a probiotic and postbiotic, as this increases its therapeutic application.

Fortifying the intestinal barrier has therapeutic potential to prevent and/or treat metabolic disease (6, 13, 62). HFD-induced obesity increases gut permeability and systemic inflammation, which worsens host metabolism in mice (63). These effects are also observed in humans (16). We report that despite HFD feeding in female mice, live *Bt* supplementation preserves colon mucus thickness, and this is associated with increased mucin protein levels and Goblet cell frequency to levels of chow-fed control mice. This result is consistent with studies in which *Am* supplementation in diet-induced obese mice (55), or *Bt* supplementation in GF rats (64) increased Goblet cell density and elevated expression of mucus-related genes. We also report that *Bt* supplementation increases protein levels of intestinal alkaline phosphatase (IAP), the primary LPS-detoxifying enzyme in the intestinal brush border membrane (65, 66). The reduced weight gain and adiposity in HFD-fed mice supplemented with live *Bt* is associated with elevated colon IAP levels, which may reflect a proportional increase in mucus thickness. This result is consistent with studies in HFD-fed mice in which IAP inhibition raised circulating levels of LBP and TNF-α (46), and in human studies in which people with Type 2 Diabetes have lower stool IAP levels versus people without diabetes (67). We show that *Bt* supplementation fortifies the gut mucus barrier, which may prevent the translocation of proinflammatory gut microbe-derived LPS from entering host circulation.

We also find that colonic intraepithelial T-cells are unlikely to play a role in mediating *Bt* effects on host metabolism in HFD-fed female mice since *Bt* treatment did not change the levels of these cells. This may be due to the lack of change in levels of fecal SCFAs, which are critical regulators of T-cell function (68, 69). Mucosal T-cells play an important role in regulating intestinal epithelial cell turnover and repair to maintain gut barrier function (70, 71). Previous studies demonstrate that different T-cell subtypes (Th1, Th17, Tregs) in the colonic lamina propria in HFD-fed male mice are critical to promote colonic health in mice (5, 68), and therapeutic targeting of specific T-cell subtypes delays HFD-induced senescence in mice (72). We measured intraepithelial T-cell subtypes in female mice, which may explain why our results differ. Overall, our data indicate that *Bt* supplementation has some beneficial effects on the intestinal mucosa which are independent of mucosal T-cells in HFD-fed female mice.

In summary, we report the effects of PAHSA treatment on plasma and cecal lipid profiles in mice. PAHSAs differentially regulated multiple lipid species and some PE levels correlated with *Bt*. This could have therapeutic implications, since levels of PE, the predominant phospholipid in the body, are associated with insulin sensitivity, BMI, and waist-to-hip ratio in humans with obesity (73, 74). The PAHSA-induced increase in some PE species may contribute to the enhanced insulin sensitivity in PAHSA-treated mice, although our studies did not test causality directly. Overall, our results identify lipid species and signatures in mice with increased insulin sensitivity resulting from PAHSA treatment. Future studies are needed to determine the therapeutic efficacy of these lipids in diet-induced obesity.

These studies highlight the therapeutic potential of targeting the gut microbiome to beneficially modulate host metabolism and prevent obesity. They also highlight the critical role of sex dimorphism in regulating host metabolism. Identifying novel gut microbiota and optimizing next-generation microbe-based interventions have major potential to complement existing therapies and/or strategies to support long-term weight and glucose management in people with obesity. Increasing our knowledge of how gut microbiota functions in response to lipids, including PAHSAs, may unlock new therapeutic targets and strategies leveraging the gut microbiome to improve host health.

Limitations of Study

We report the effects of PAHSAs on glucose homeostasis in conventional male but not female mice. However, we do report that PAHSA treatment improves glucose tolerance in both sexes of GF mice on chow diet but not on a HFD, indicating that the requirement of the gut microbiota to mediate PAHSA effects on glucose homeostasis is dependent on diet but not on sex. We previously reported beneficial anti-inflammatory effects of PAHSAs in conventional female chow-fed NOD mice (75, 76). Additional studies are needed to determine whether PAHSAs have beneficial effects on glucose homeostasis in conventional female C57BL/6J mice as we see in conventional C57BL6 male mice. In the current studies, we did not measure circulating GLP-1 levels or the frequency of intestinal Goblet cells. Future studies will include these outcomes. In our FMT studies, donor feces from PAHSA-treated male mice transmitted some of the beneficial effects of PAHSAs on glucose metabolism to recipient GF male, but not female, mice. Whether donor feces from female PAHSA-treated mice can transmit metabolic effects to recipient GF female mice requires further investigation. However, we can still conclude that some of the beneficial effects of PAHSAs on glucose metabolism can be transmitted by FMT. We also determined the effects of *Bt* supplementation on the gut barrier in diet-induced obese mice. We measured colon mucus thickness in AB/PAS-stained Carnoy's-fixed tissues. In future studies, we will employ confocal microscopy to determine mucus thickness as this approach robustly quantifies multilayer mucus properties and reduces artifacts derived from fixation and tissue processing (6, 77). *Bt*-produced lipids may contribute to the *Bt* effects in HFD-female mice. Future studies defining the lipidome

from GF mice monocolonized with *Bt* would provide insight into which *Bt*-derived lipids mediate host metabolism. Last, future studies will determine the effects of supplementation with PAHSA-associated and/or *Bt*-associated lipid metabolites on improving host metabolism in the setting of diet-induced obesity.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information.

ACKNOWLEDGMENTS. The Harvard Digestive Disease Center and NIH grant P30 DK034854 supported the Histology and Imaging Core. P30 DK034854 and a capital grant from the Massachusetts Life Sciences Center supported gnotobiotic mouse studies and microbial preparation of *Bt* by the Massachusetts Host Microbiome Center. We thank Dr. Yana Stackpole for providing bioinformatics support, and Mary Louise Delaney, MA, for microbiology support. 1K01DK114162-01A1 (J.L.), BADERC 5P30DK057521-19 (J.L. and B.B.K.), HDDC P30 DK034854 (J.L.), JPB (B.B.K.), and R01 DK106210 (B.B.K.).

Author affiliations: [a]Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215; [b]Department of Biostatistics and Bioinformatics, Milken Institute School of Public Health, George Washington University, Washington, DC 20052; [c]Division of Pharmaceutical Chemistry, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA 92093; [d]Department of Biomedical Sciences, Cornell University College of Veterinary Medicine, Cornell University, Ithaca, NY 14850; [e]Department of Surgery, School of Medicine, University of California, Davis, Sacramento, CA 95817; [f]Department of Molecular Biosciences, School of Veterinary Medicine, University of California Davis School of Veterinary Medicine, Davis, CA 95616; [g]Massachusetts Host-Microbiome Center, Department of Pathology, Brigham & Women's Hospital and Harvard Medical School, Boston, MA 02115; [h]Laboratory of Immunopathology Keizo Asami, Federal University of Pernambuco, Recife 50670-901, Brazil; and [i]Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA 02142

Author contributions: J.L., A.C., L.B., and B.B.K. designed research; J.L., K.W., M.M.H., B.P.C., V.Y., and C.B.C. performed research; B.B.K. supervised the research and data analysis; A.T.N. and D.S. contributed new reagents/analytic tools; J.L., A.R., M.M.H., B.P.C., A.C., C.B.C., and L.B. analyzed data; and J.L. and B.B.K. wrote the paper.

Reviewers: F.B., Goteborgs universitet; and S.S.D., Vanderbilt University.

Competing interest statement: B.B.K. is an inventor on the following patents: "Lipids That Increase Insulin Sensitivity and Methods of Using the Same." Patent No. 10,604,473 and "Fatty Acid Esters of Hydroxy Fatty Acids (FAHFAs) for use in the treatment of Type 1 Diabetes." Patent No. 11,013,711.

1. WHO, Obesity and overweight. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight. Accessed 6 July 2023.
2. L. M. Jaacks *et al.*, The obesity transition: Stages of the global epidemic. *Lancet Diabetes Endocrinol.* **7**, 231–240 (2019).
3. A. Afshin *et al.*, Health effects of overweight and obesity in 195 Countries over 25 years. *N. Engl. J. Med.* **377**, 13–27 (2017).
4. D. A. Winer, H. Luck, S. Tsai, S. Winer, The intestinal immune system in obesity and insulin resistance. *Cell Metab.* **23**, 413–426 (2016).
5. H. Luck *et al.*, Regulation of obesity-related insulin resistance with gut anti-inflammatory agents. *Cell Metab.* **21**, 527–542 (2015).
6. B. O. Schroeder *et al.*, Bifidobacteria or Fiber protects against diet-induced microbiota-mediated colonic mucus deterioration. *Cell Host Microbe* **23**, 27–40.e27 (2018).
7. M. Nieuwdorp, P. W. Gilijamse, N. Pai, L. M. Kaplan, Role of the microbiome in energy regulation and metabolism. *Gastroenterology* **146**, 1525–1533 (2014).
8. A. J. Cox, N. P. West, A. W. Cripps, Obesity, inflammation, and the gut microbiota. *Lancet Diabetes Endocrinol.* **3**, 207–215 (2015).
9. R. E. Ley, P. J. Turnbaugh, S. Klein, J. I. Gordon, Microbial ecology: Human gut microbes associated with obesity. *Nature* **444**, 1022–1023 (2006).
10. P. J. Turnbaugh *et al.*, An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027–1031 (2006).
11. V. K. Ridaura *et al.*, Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* **341**, 1241214 (2013).
12. P. J. Turnbaugh, F. Bäckhed, L. Fulton, J. I. Gordon, Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe* **3**, 213–223 (2008).
13. P. D. Cani *et al.*, Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut* **58**, 1091–1103 (2009).
14. C. B. de La Serre *et al.*, Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation. *Am. J. Physiol. Gastrointest. Liver Physiol.* **299**, G440–G448 (2010).
15. M. S. Desai *et al.*, A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. *Cell* **167**, 1339–1353.e21 (2016).
16. B. Seethaler *et al.*, Biomarkers for assessment of intestinal permeability in clinical practice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **321**, G11–g17 (2021).
17. P. Aryal *et al.*, Distinct biological activities of isomers from several families of branched fatty acid esters of hydroxy fatty acids (FAHFAs). *J. Lipid Res.* **62**, 100108 (2021).
18. J. Lee *et al.*, Branched fatty acid esters of hydroxy fatty acids (FAHFAs) protect against colitis by regulating gut innate and adaptive immune responses. *J. Biol. Chem.* **291**, 22207–22217 (2016).
19. I. Syed *et al.*, Palmitic acid hydroxystearic acids activate GPR40, which is involved in their beneficial effects on glucose homeostasis. *Cell Metab.* **27**, 419–427.e4 (2018).
20. M. M. Yore *et al.*, Discovery of a class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. *Cell* **159**, 318–332 (2014).
21. P. Zhou *et al.*, PAHSAs enhance hepatic and systemic insulin sensitivity through direct and indirect mechanisms. *J. Clin. Invest.* **129**, 4138–4150 (2019).
22. E. Carvalho, K. Kotani, O. D. Peroni, B. B. Kahn, Adipose-specific overexpression of GLUT4 reverses insulin resistance and diabetes in mice lacking GLUT4 selectively in muscle. *Am. J. Physiol. Endocrinol. Metab.* **289**, E551–E561 (2005).
23. P. R. Shepherd *et al.*, Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. *J. Biol. Chem.* **268**, 22243–22246 (1993).
24. V. R. Souza *et al.*, Description of ovariectomy protocol in mice. *Methods Mol. Biol.* **1916**, 303–309 (2019).
25. The Huttenhower Lab, KneadData. Github. https://github.com/biobakery/kneaddata. Accessed 16 May 2021.
26. E. A. Franzosa *et al.*, Species-level functional profiling of metagenomes and metatranscriptomes. *Nat. Methods* **15**, 962–968 (2018).
27. B. J. Callahan *et al.*, DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583 (2016).
28. B. Sayoldin, M. Baghbanzadeh, K. A. Crandall, A. Rahnavard, btest: Link, rank, and visualize associations among omics features across multi-omics datasets. Github. https://github.com/omicsEye/btest. Accessed 2 October 2023.
29. P. J. Turnbaugh *et al.*, The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* **1**, 6ra14 (2009).
30. M. El-Salhy, J. G. Hatlebakk, O. H. Gilja, A. Bråthen Kristoffersen, T. Hausken, Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* **69**, 859–867 (2020).
31. M. Hamazaki *et al.*, Fecal microbiota transplantation in the treatment of irritable bowel syndrome: A single-center prospective study in Japan. *BMC Gastroenterol.* **22**, 342 (2022).
32. T. Holvoet *et al.*, Fecal microbiota transplantation reduces symptoms in some patients with irritable bowel syndrome with predominant abdominal bloating: Short- and long-term results from a placebo-controlled randomized trial. *Gastroenterology* **160**, 145–157.e8 (2021).
33. M. J. Blaser, Fecal microbiota transplantation for dysbiosis–Predictable risks. *N. Engl. J. Med.* **381**, 2064–2066 (2019).
34. Z. DeFilipp *et al.*, Drug-resistant E. coli bacteremia transmitted by fecal microbiota transplant. *N. Engl. J. Med.* **381**, 2043–2050 (2019).
35. J. R. Allegretti *et al.*, Effects of fecal microbiota transplantation with oral capsules in obese patients. *Clin. Gastroenterol. Hepatol.* **18**, 855–863.e2 (2020).
36. J. R. Allegretti *et al.*, Impact of fecal microbiota transplantation with capsules on the prevention of metabolic syndrome among patients with obesity. *Hormones (Athens)* **20**, 209–211 (2021).
37. V. Mocanu *et al.*, Fecal microbial transplantation and fiber supplementation in patients with severe obesity and metabolic syndrome: A randomized double-blind, placebo-controlled phase 2 trial. *Nat. Med.* **27**, 1272–1279 (2021).
38. P. de Groot *et al.*, Donor metabolic characteristics drive effects of faecal microbiota transplantation on recipient insulin sensitivity, energy expenditure and intestinal transit time. *Gut* **69**, 502–512 (2020).
39. E. W. Yu *et al.*, Fecal microbiota transplantation for the improvement of metabolism in obesity: The FMT-TRIM double-blind placebo-controlled pilot trial. *PLoS Med.* **17**, e1003051 (2020).
40. A. Vrieze *et al.*, Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology* **143**, 913–916.e7 (2012).
41. A. Gao *et al.*, Sexual dimorphism in glucose metabolism is shaped by androgen-driven gut microbiome. *Nat. Commun.* **12**, 7080 (2021).
42. K. D. Acharya, X. Gao, E. P. Bless, J. Chen, M. J. Tetel, Estradiol and high fat diet associate with changes in gut microbiota in female ob/ob mice. *Sci. Rep.* **9**, 20192 (2019).
43. C. A. Kolmeder *et al.*, Colonic metaproteomic signatures of active bacteria and the host in obesity. *Proteomics* **15**, 3544–3552 (2015).
44. H. Liu *et al.*, Functional genetics of human gut commensal Bacteroides thetaiotaomicron reveals metabolic requirements for growth across environments. *Cell Rep.* **34**, 108789 (2021).
45. R. Liu *et al.*, Gut microbiome and serum metabolome alterations in obesity and after weight-loss intervention. *Nat. Med.* **23**, 859–868 (2017).
46. K. Kaliannan *et al.*, Estrogen-mediated gut microbiome alterations influence sexual dimorphism in metabolic syndrome in mice. *Microbiome* **6**, 205 (2018).
47. T. Takeuchi *et al.*, Gut microbial carbohydrate metabolism contributes to insulin resistance. *Nature* **621**, 389–395 (2023).
48. S. H. Cho, Y. J. Cho, J. H. Park, The human symbiont Bacteroides thetaiotaomicron promotes diet-induced obesity by regulating host lipid metabolism. *J. Microbiol.* **60**, 118–127 (2022).
49. J. A. Barnett, D. L. Gibson, H(2)Oh No! The importance of reporting your water source in your in vivo microbiome studies. *Gut Microbes* **10**, 261–269 (2019).
50. K. D. Parker, S. E. Albeke, J. P. Gigley, A. M. Goldstein, N. L. Ward, Microbiome composition in both wild-type and disease model mice is heavily influenced by mouse facility. *Front. Microbiol.* **9**, 1598 (2018).
51. K. Karastergiou, S. R. Smith, A. S. Greenberg, S. K. Fried, Sex differences in human adipose tissues–The biology of pear shape. *Biol. Sex Differ.* **3**, 13 (2012).
52. J. G. Markle *et al.*, Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* **339**, 1084–1088 (2013).
53. F. Perraudeau *et al.*, Improvements to postprandial glucose control in subjects with type 2 diabetes: A multicenter, double blind, randomized placebo-controlled trial of a novel probiotic formulation. *BMJ Open Diabetes Res. Care* **8**, e001319 (2020).
54. C. Depommier *et al.*, Supplementation with Akkermansia muciniphila in overweight and obese human volunteers: A proof-of-concept exploratory study. *Nat. Med.* **25**, 1096–1103 (2019).
55. H. Plovier *et al.*, A purified membrane protein from Akkermansia muciniphila or the pasteurized bacterium improves metabolism in obese and diabetic mice. *Nat. Med.* **23**, 107–113 (2017).

56. D. An, C. Na, J. Bielawski, Y. A. Hannun, D. L. Kasper, Membrane sphingolipids as essential molecular signals for Bacteroides survival in the intestine. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 4666–4671 (2011).
57. C. F. Frankfater, M. G. Sartorio, E. Valguarnera, M. F. Feldman, F. F. Hsu, Lipidome of the Bacteroides genus containing new peptidolipid and sphingolipid families revealed by multiple-stage mass spectrometry. *Biochemistry* **62**, 1160–1180 (2023).
58. E. L. Johnson *et al.*, Sphingolipids produced by gut bacteria enter host metabolic pathways impacting ceramide levels. *Nat. Commun.* **11**, 2471 (2020).
59. E. M. Brown *et al.*, Bacteroides-derived sphingolipids are critical for maintaining intestinal homeostasis and symbiosis. *Cell Host Microbe* **25**, 668–680.e7 (2019).
60. M. Bae *et al.*, Akkermansia muciniphila phospholipid induces homeostatic immune responses. *Nature* **608**, 168–173 (2022).
61. W. Cho *et al.*, N-Acyl amides from Neisseria meningitidis and their role in sphingosine receptor signaling. *Chembiochem* **23**, e202200490 (2022).
62. P. Paone *et al.*, Prebiotic oligofructose protects against high-fat diet-induced obesity by changing the gut microbiota, intestinal mucus production, glycosylation and secretion. *Gut Microbes* **14**, 2152307 (2022).
63. X. Wei *et al.*, Fatty acid synthase modulates intestinal barrier function through palmitoylation of mucin 2. *Cell Host Microbe* **11**, 140–152 (2012).
64. L. Wrzosek *et al.*, Bacteroides thetaiotaomicron and Faecalibacterium prausnitzii influence the production of mucus glycans and the development of goblet cells in the colonic epithelium of a gnotobiotic model rodent. *BMC Biol.* **11**, 61 (2013).
65. F. Kühn *et al.*, Intestinal alkaline phosphatase targets the gut barrier to prevent aging. *JCI Insight* **5**, e134049 (2020).
66. J. M. Bates, J. Akerlund, E. Mittge, K. Guillemin, Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host Microbe* **2**, 371–382 (2007).
67. M. S. Malo, A high level of intestinal alkaline phosphatase is protective against type 2 diabetes mellitus irrespective of obesity. *EBioMedicine* **2**, 2016–2023 (2015).
68. P. M. Smith *et al.*, The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* **341**, 569–573 (2013).
69. N. Arpaia *et al.*, Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* **504**, 451–455 (2013).
70. H. Komano *et al.*, Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6147–6151 (1995).
71. R. Boismenu, W. L. Havran, Modulation of epithelial cell growth by intraepithelial gamma delta T cells. *Science* **266**, 1253–1255 (1994).
72. S. Arora *et al.*, Invariant natural killer T cells coordinate removal of senescent cells. *Med* **2**, 938–950 (2021).
73. A. Mousa *et al.*, Lipidomic profiling reveals early-stage metabolic dysfunction in overweight or obese humans. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **1864**, 335–343 (2019).
74. A. E. Mendham *et al.*, Exercise training improves mitochondrial respiration and is associated with an altered intramuscular phospholipid signature in women with obesity. *Diabetologia* **64**, 1642–1659 (2021).
75. M. F. Rubin de Celis *et al.*, PAHSAs reduce cellular senescence and protect pancreatic beta cells from metabolic stress through regulation of Mdm2/p53. *Proc. Natl. Acad. Sci. U.S.A.* **119**, e2206923119 (2022).
76. I. Syed *et al.*, PAHSAs attenuate immune responses and promote β cell survival in autoimmune diabetic mice. *J. Clin. Invest.* **129**, 3717–3731 (2019).
77. M. E. Johansson *et al.*, Normalization of host intestinal mucus layers requires long-term microbial colonization. *Cell Host Microbe* **18**, 582–592 (2015).