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RESEARCH PAPER

Gut microbiota remodeling reverses aging-associated inflammation and dysregulation of systemic bile acid homeostasis in mice sex-specifically

Junli Ma[a], Ying Hong[a], Ningning Zheng[a], Guoxiang Xie[b], Yuanzhi Lyu[c], Yu Gu[a], Chuchu Xi[a], Linlin Chen[a], Gaosong Wu[a], Yue Li[d], Xin Tao[a], Jing Zhong[a,e], Zhenzhen Huang[f], Wenbin Wu[f], Lin Yuan[f], Min Lin[f], Xiong Lu[f], Weidong Zhang[a,g], Wei Jia[h,i], Lili Sheng[a], and Houkai Li[a]

[a]Functional Metabolomic and Gut Microbiome Laboratory, Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai, China; [b]Human Metabolomics Institute, Inc, Shenzhen, Guangdong, China; [c]Department of Dermatology, School of Medicine, University of California, Davis, Sacramento, CA, USA; [d]Department of Endocrinology, Shanghai Fifth People's Hospital Affiliated to Fudan University, Shanghai, China; [e]Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, Affiliated Cent Hospital Huzhou University, Huzhou, China; [f]Experiment Center for Science and Technology, Shanghai University of Traditional Chinese Medicine, Shanghai, China; [g]Department of Phytochemistry, College of Pharmacy, Second Military Medical University, Shanghai, China; [h]Shanghai Key Laboratory of Diabetes Mellitus and Center for Translational Medicine, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China; [i]Hong Kong Traditional Chinese Medicine Phenome Research Centre, School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China

ABSTRACT

Aging is usually characterized with inflammation and disordered bile acids (BAs) homeostasis, as well as gut dysbiosis. The pathophysiological changes during aging are also sexual specific. However, it remains unclear about the modulating process among gut microbiota, BA metabolism, and inflammation during aging. In this study, we established a direct link between gut microbiota and BA profile changes in the liver, serum, and four intestinal segments of both sexes during aging and gut microbiota remodeling by co-housing old mice with young ones. We found aging reduced Actinobacteria in male mice but increased Firmicutes in female mice. Among the top 10 altered genera with aging, 4 genera changed oppositely between male and female mice, and most of the changes were reversed by co-housing in both sexes. Gut microbiota remodeling by co-housing partly rescued the systemically dysregulated BA homeostasis induced by aging in a sex- and tissue-specific manner. Aging had greater impacts on hepatic BA profile in females, but intestinal BA profile in males. In addition, aging increased hepatic and colonic deoxycholic acid in male mice, but reduced them in females. Moreover, muricholic acids shifted markedly in the intestine, especially in old male mice, and partially reversed by co-housing. Notably, the ratios of primary to secondary BAs in the liver, serum, and all four intestinal segments were increased in old mice and reduced by co-housing in both sexes. Together, the presented data revealed that sex divergent changes of gut microbiota and BA profile in multiple body compartments during aging and gut microbiota remodeling, highlighting the sex-specific prevention and treatment of aging-related disorders by targeting gut microbiota-regulated BA metabolism should particularly be given more attention.

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Introduction

Aging-related chronic inflammation, also called inflammaging, is a hallmark and a risk factor for the development of age-associated pathologies such as cardiovascular diseases, stroke, insulin resistance, diabetes, Alzheimer's disease, and Parkinson's diseases.[1–4] Although the cause of chronic inflammation remains poorly understood, emerging evidence implicates aging-associated alterations in the composition of bile acids (BAs) and gut microbiota play critical roles in modulating host inflammation and metabolism.[5–11]

BAs are synthesized from cholesterol in the hepatocyte and pass into the small intestine where they promote intestinal absorption of dietary lipids prior to the reabsorption into the liver through enterohepatic circulation or excretion in the feces.[12,13] In addition to the well-established role for dietary lipid absorption and maintaining

CONTACT Houkai Li houkai1976@126.com Functional Metabolomic and Gut Microbiome Laboratory, Institute for Interdisciplinary Medicine Sciences, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China; Lili Sheng fine919@163.com Functional Metabolomic and Gut Microbiome Laboratory, Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China; Wei Jia weijia1@hkbu.edu.hk Chinese Medicine Clinical Study Center, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong SAR, China

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cholesterol homeostasis, BAs also function as signaling molecules regulating glucose, lipid, energy metabolism, and immunity through BA receptors such as nuclear farnesoid X receptor (FXR) and membrane Takeda G protein receptor 5 (TGR5).[13–18] In the liver, hepatic enzymes generate free and conjugated primary BAs. In the intestine, BAs restrict bacterial proliferation, whereas bacterial enzymes bile salt hydrolase (BSH) and 7α-dehydroxylase deconjugate BAs and convert primary BAs into secondary BAs, respectively.[19–21] Given the joint co-metabolism of both host and gut microbiota on BAs homeostasis, and the diversified binding affinities on BA receptors among free and conjugated BAs, eubiosis is essential for maintaining BA homeostasis and health.

Gut microbiota plays a pivotal role in maintaining metabolic homeostasis of host.[5–8,22] However, the composition, diversity, and function of the microbiota are not always constant during the lifetime of the host.[5,6,23] Therefore, the shifted microbial composition and its related dysregulation of host metabolism contribute to the development of various diseases such as metabolic diseases and aging-associated disorders.[5,24,25] Moreover, various microbiota-targeted interventions have shown favorable effects on host health.[26–29] Interestingly, fecal microbiota transplantation is effective in extending mouse lifespan by restoring secondary BA synthesis.[30] Therefore, exploration of host BA pool homeostasis during aging is of great significance for better understanding the physiological process of aging and its related disorders. Meanwhile, there are significant sexual differences in terms of the susceptibility to aging and the occurrence of aging-related disorders such as insulin resistance, energy balance, organ dysfunctions, and even death rate.[31–34] However, the pathophysiological mechanisms underlying the sexual differences in longevity and aging-related diseases are quite complex and poorly understood. Our previous study reveals the sex dissimilarity in metabolism is closely associated with the differences in both BAs and microbiota profiles.[35] Therefore, the systemic investigation on the compositional changes of BA pool in old male and female mice bears much significance for elucidating the sexual differences during aging and susceptibility to aging-related disorders.

In our current study, we performed a systemic investigation on BA pool profiles in liver tissue, serum, and four different segments of intestinal contents (jejunum, ileum, cecum, and colon) in both male and female of young and old mice with targeted BA metabolomics, as well as the composition of gut microbiota. Moreover, we further evaluated the impacts of microbiota remodeling on aging-related disorders and BA pool homeostasis by co-housing young and old mice of both sexes, respectively. Our results showed that aging caused gut dysbiosis and comprehensively dysregulated BA homeostasis with increased systemic inflammation in a sex-specific manner. Gut microbiota remodeling by co-housing partially rescued the above changes in old mice. Therefore, our current study highlights that gut microbiota is a potential target for improving the health of the old population by restoring BA homeostasis, and sex-specific strategy should particularly be given more attention.

Results

Aging causes hepatic inflammation and splenomegaly

Since inflammaging is the common character for most aging-related disorders,[1,36] we first compared the liver histology and the gene expression of pro-inflammatory cytokines between young (3-month-old) and old (26-month-old) mice of both sexes. The results showed that old mice had massive hepatic lymphocyte infiltration in both sexes, but not in young mice (Figure 1(a-b)). The mRNA levels of *Tnf-α* (tumor necrosis factor-alpha) and *Il-1β* (interleukin 1 beta), as well as *Saa1* (serum amyloid A1), which is highly expressed in response to inflammation and tissue injury, were significantly higher in old mice than young mice suggesting the aging-related hepatic inflammation in old mice of both sexes (Figure 1(c-d)). In addition, we also found that old mice had enlarged spleen size and increased spleen to body weight ratio (Figure 1 (e-f)), which is a probable physiological response to bacterial infection.[37] Together, the data indicated that old mice were characterized by higher systemic inflammation than young mice.

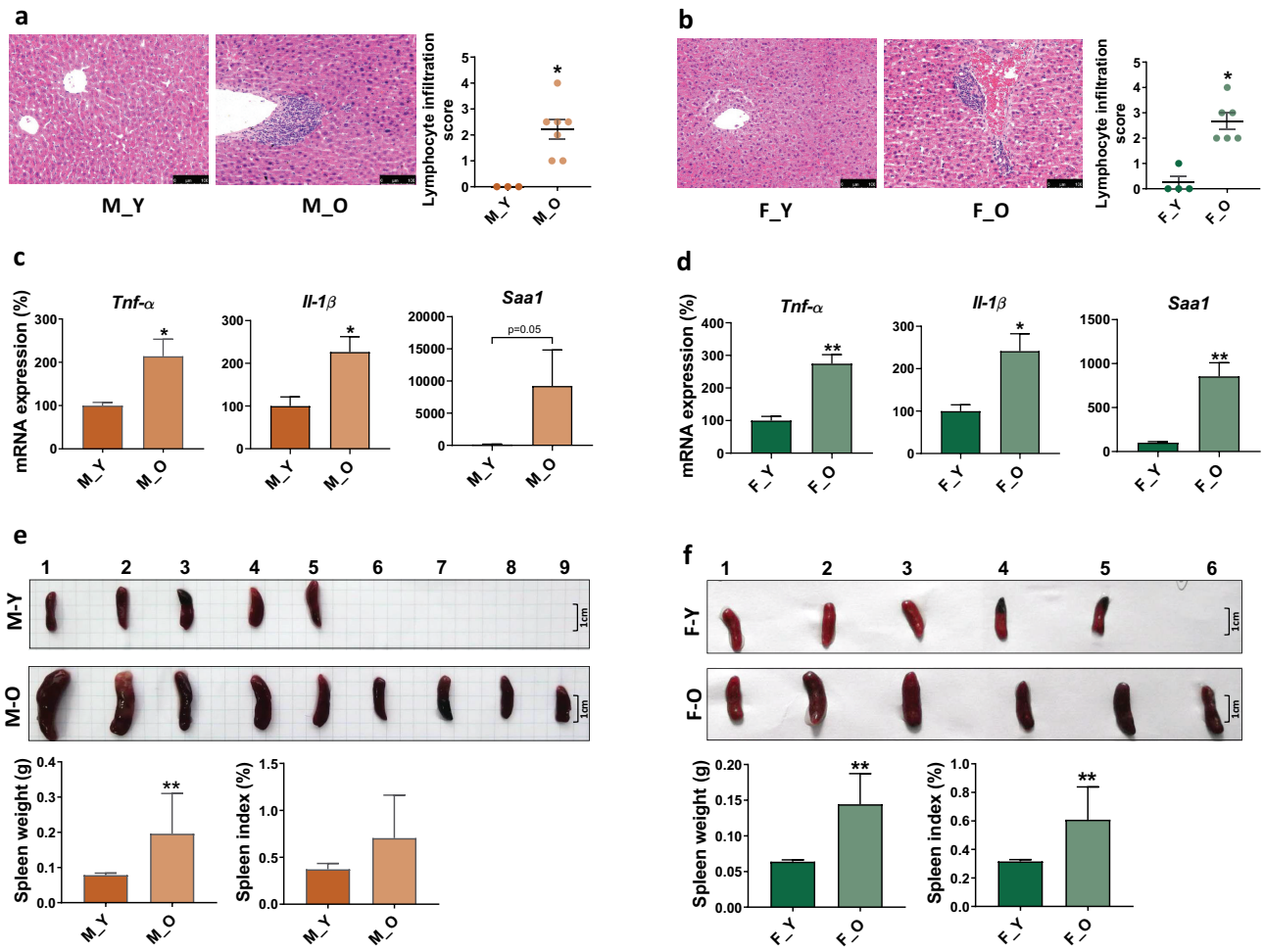

Figure 1. Histological and phenotypic changes in young and old mice of both sexes. M_Y: young male mice; M_O: old male mice; F_Y: young female mice; F_O: old female mice. (a-b) H&E-stained liver sections and lymphocyte infiltration score. Scale bars, 100 μm. (c-d) Hepatic inflammatory gene expression. (e-f) Spleen morphology, spleen weight, and spleen to body weight ratio. *$p < .05$, **$p < .01$.n = 3–8 mice per group.

Aging alters hepatic BA profile sex-specifically

As BAs are synthesized in the liver and play important roles in regulating host metabolism and inflammation, we first analyzed BA composition in liver tissue in the young and old mice of both sexes. The quantity of total BAs in liver remained relatively constant with aging in male mice but reduced in female mice (Figure 2(a-b)). The old male mice showed increased ratios of conjugated to unconjugated and primary to secondary BAs, while the total hepatic BA, and ratios of conjugated to unconjugated and primary to secondary BAs were much higher in females than males of either young or old mice (Figure 2(a-b)). Regarding the individual BAs, many unconjugated hepatic BAs showed decreasing trend with aging in male mice; however, only DCA level was significantly higher in old male mice at 15-fold than young ones. In contrast, there were 16 BAs that were markedly lower in old female mice than young mice, and most of them were unconjugated primary and secondary BAs. Interestingly, the DCA levels in old male and female were oppositely altered in the context of similar levels of CA between old and young mice regardless of their sexes (Figure 2(a-b)), suggesting the differential expression of bacterial 7α-dehydroxylase in these mice. Additionally, the proportions of BAs such as TβMCA and TαMCA were also differently changed with aging in male or female mice, respectively (Figure S1 A). These results suggested that aging divergently altered hepatic BA composition in a sex-specific manner with a more dramatic variation in female mice than male mice.

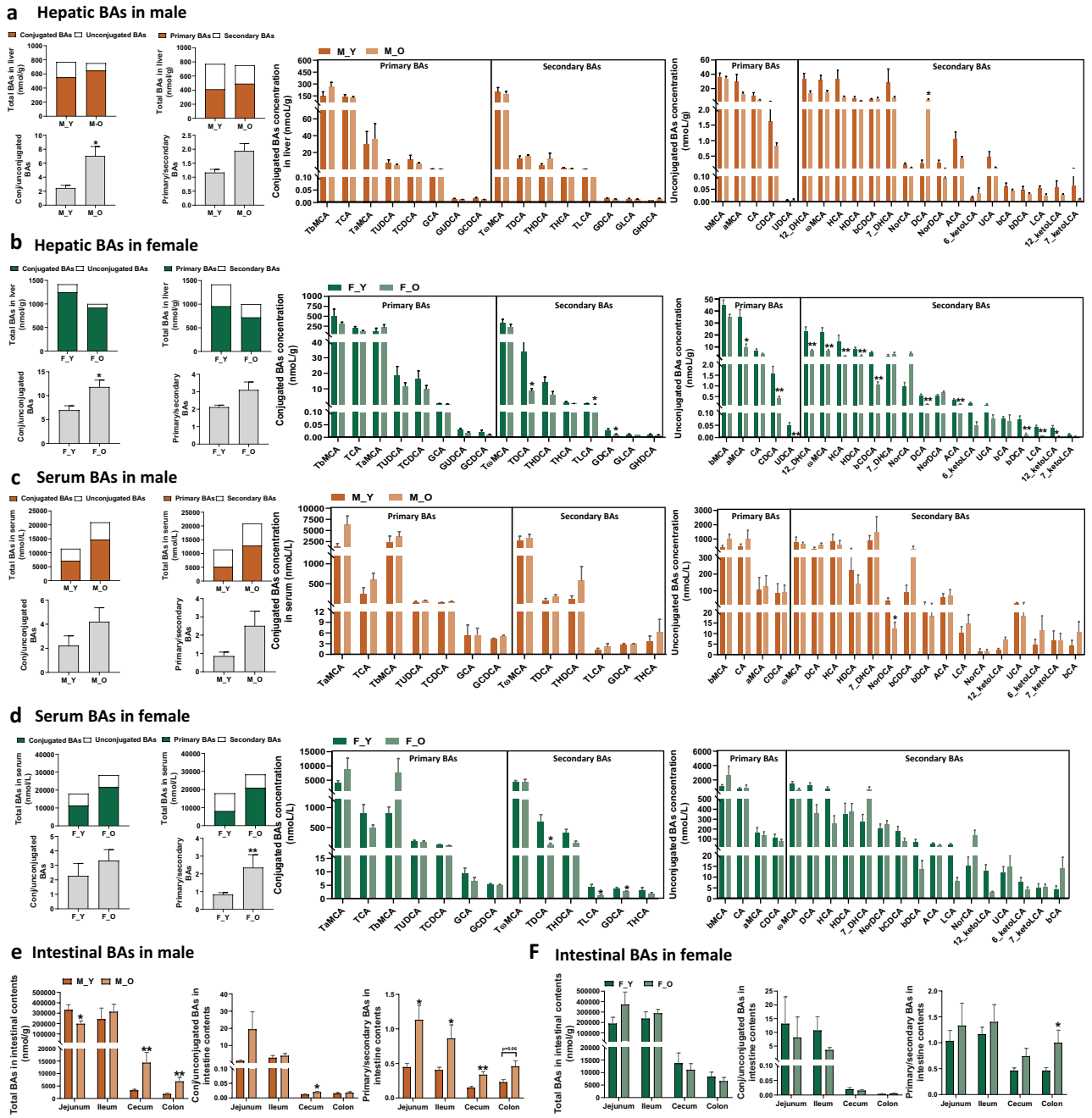

Figure 2. BA profile in the liver, serum, and intestine of young and old mice. Total bile acids, ratios of conjugated to unconjugated, primary to secondary bile acids and concentrations of individual BAs in liver (a-b), serum (c-d), and intestine (e-f) of young and old mice of both sexes. *$p < .05$, **$p < .01$. $n = 5–9$ mice per group.

Aging alters BA profile in serum sex-specifically

To further assess the aging-related dysregulation on BA homeostasis, we quantified the BA composition in serum of these mice. We found that both old male and female mice had 1.8-fold and 1.6-fold increase in total BAs in serum compared with young mice of same sex, respectively (Figure 2(c-d)). The increase in total BAs in serum of old mice was mainly due to an increase in the conjugated primary BAs, such as TαMCA in male mice and Tα+βMCA in female mice. Moreover, increased ratios of conjugated to unconjugated, and primary to secondary BAs were observed in serum of old mice of both sexes. Aging reduced NorDCA in male mice, and conjugated

secondary BAs, such as TDCA, TLCA, and GDCA in female mice (Figure 2(c-d)). The proportions of TαMCA, TβMCA, and TωMCA were differentially changed in the two sexes during aging (Figure S1 B). The results suggested that aging resulted in sex-specific alterations in serum BA profiles.

Aging alters BA profiles in different intestinal segments sex-specifically

Since intestinal tract is the main site for BA functions for either facilitating dietary lipids absorption or regulation on host metabolism through intestinal BA receptors,[38–40] we then quantified the BA profiles in the contents of four intestinal segments, including jejunum, ileum, cecum, and colon. BAs are mainly reabsorbed into the portal vein at ileum, and approximately 5% excreted from feces, so the total BAs concentration was reduced dramatically in cecum and colon contents in all mice (Figure 2 (e-f)). In male mice, the total BA concentrations were lower in jejunum, but higher in cecum and colon contents than young mice. Moreover, the ratios of primary to secondary BAs were significantly higher in old mice than those of young mice (Figure 2(e)). In contrast, the old and young female mice showed comparable levels of either total BAs or the ratios of conjugated to unconjugated, and primary to secondary BAs in the four intestinal segments, except for the significant higher ratio of primary to secondary BAs in colon contents (figure 2(f)), suggesting the abnormally sex-specific BA synthesis, transport, or metabolism during aging.

The concentrations of individual BAs were altered with aging in a segment- or sex-dependent manner. For example, 9 out of 19 detected unconjugated BAs were reduced in the jejunum and 7 unconjugated BAs were increased in the cecum of old male mice, whereas most unconjugated BAs were relatively constant in other two intestinal segments of male mice (Figure S2). In female mice, unconjugated BAs were relatively constant in the intestinal segments with the exception of ileum (Figure S3). In old male mice, βMCA tended to be consistently increased in the ileum, cecum, and colon, while secondary BAs DCA and LCA were consistently increased in cecum and/or colon (Figure S2). In contrast, βMCA was only elevated in the ileum in old female mice (Figure S3). Moreover, Tα+βMCA, which are FXR antagonists, as well as TωMCA and TDCA had increased trend in the cecum and colon in old male mice but not in old female mice (Figure S2 and S3). In general, more BAs were changed in the intestine of male mice than female mice, especially in the jejunum, cecum, and colon. Importantly, MCAs were significantly shifted in the intestine of male mice but not female mice (Figure S2 and S3). These findings indicated divergent changes in intestinal BA composition sex-specifically during aging, which might lead to different metabolic effects.

Aging alters gut microbiota composition sex-specifically

Given the intricate role of gut microbiota in aging-related disorders and maintaining BAs pool homeostasis, we further profiled the compositional changes of gut microbiota during aging in these mice. First of all, the Unweighted UniFrac PCoA showed that old mice of both sexes had dramatically different patterns of gut microbiota with their young counterparts (Figure 3(a)), which is consistent with previous reports.[41] Surprisingly, aging had opposite effects on shifting Shannon index in the two sexes (Figure 3(b)), suggesting the sex-specific impacts of aging on bacterial α diversity. At the phylum level, the most significant change caused by aging in male mice was the reduction of Actinobacteria (Figure 3(c)). However, in female mice, the abundance of Actinobacteria as well as Firmicutes was increased, while Proteobacteria was reduced during aging (Figure 3(d)). At the genus level, the top 10 significantly altered genera with aging in both sexes were summarized (Figure 3 (e-f)). Specifically, there were five increased genera including *Lachnospiraceae_NK4A136_group, norank_f_Lachnospiraceae, unclassified_f_Ruminococcaceae, Oscillibacter*, and *Blautia*, and five decreased genera including *norank_f_Erysipelotrichaceae, Faecalibaculum, Enterohabdus, Bifidobacterium, and [Eubacterium]_fissicatena_group* in old male mice (Figure 3(e)). Interestingly, the majority of bacteria among the top 10 significantly changed genera were decreased in old female mice including *Desulfovibrio, norank_f_Lachnospiraceae, norank_f_Bacteroidales_S24-7_group,*

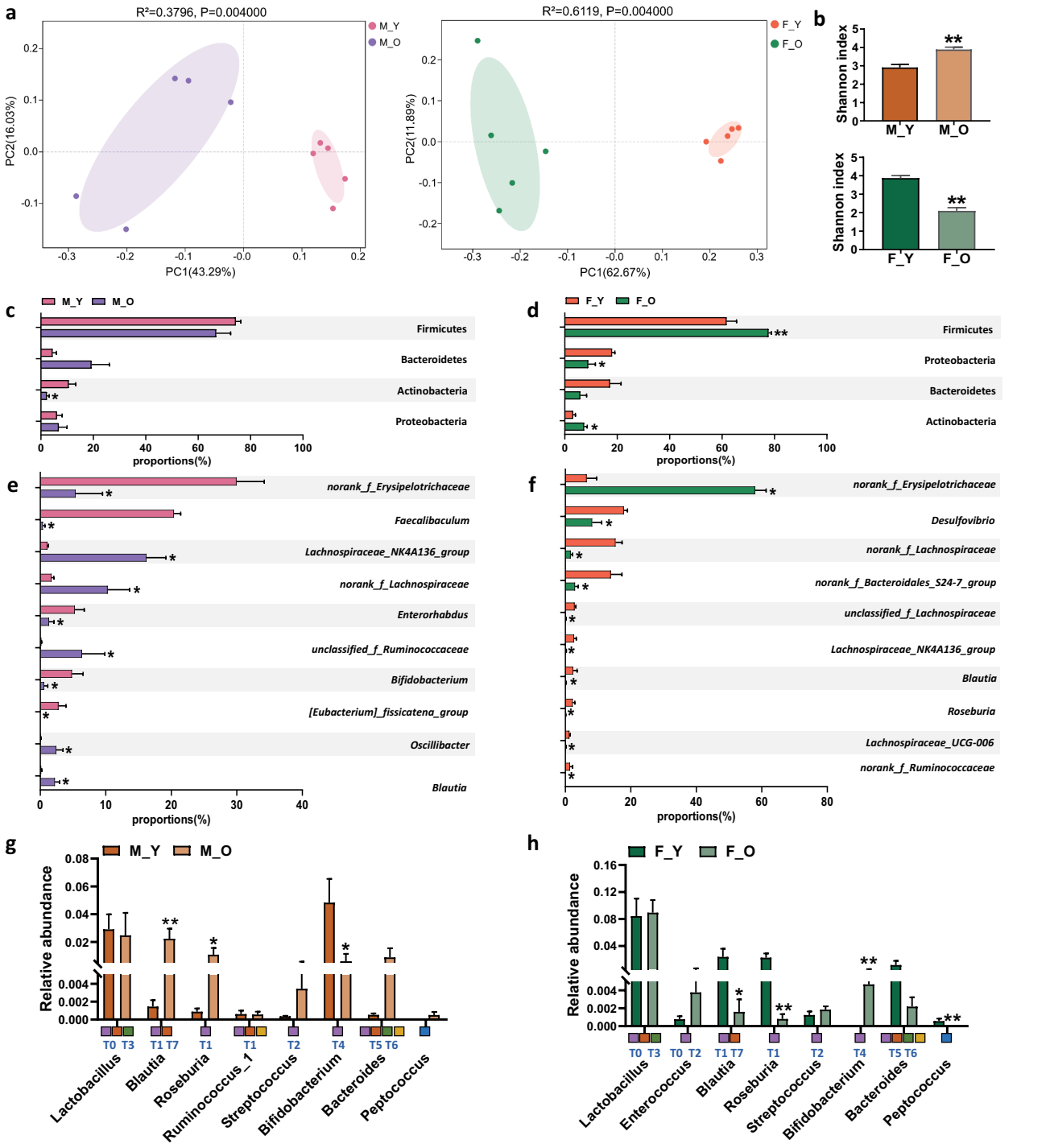

Figure 3. Aging induces gut microbiota disbalance in both sexes. (a) Principal co-ordinates analysis plots of cecal microbiota at OUT level based on unweighted_unifrac. (b) Shannon index. (c-d) The relative abundance of the dominant phyla in young and old mice of both sexes. (e-f) Top 10 differential genera with the highest relative abundance between young and old mice in both sexes. (g-h) Relative abundance of cecal microbiota involved in BAs metabolism at genus level. T0 to T7 that marked below the bar plots are the eight major phylotypes of BSH. Meanwhile, the squares filled with purple, orange, green, yellow, and blue represent bacteria that associated with BSH deconjugation, 7α dehydroxylation, esterify, C-6 epimerization, and desulfation functions, respectively. *$p < .05$, **$p < .01$. $n = 5$–6 mice per group.

unclassified_f_Lachnospiraceae, *Lachnospiraceae_NK4A136_group*, *Blautia*, *Roseburia*, *Lachnospiraceae_UCG-006*, and *norank_f_Ruminococcaceae*, except for *norank_f_Erysipelotrichaceae* (figure 3 (f)). Four genera were oppositely changed with aging between male and female mice, such as

norank_f_Erysipelotrichaceae, Lachnospiraceae_NK4A136_group, norank_f_Lachnospiraceae, and *Blautia*.

Since many bacterial species are involved in BAs metabolism, such as BA deconjugation,[20,40,42] epimerization,[43] 7α-dehydroxylation,[44–46] and desulfation,[47] bacterial genera that contained the main species with BA metabolizing functions were further studied. Based on previous phylogenetic analysis with human gut microbiota, the eight phylotypes of BSH (T0-T7) were also labeled under each genus in Figure 3(g-h) to define their deconjugation activity.[21] Old male mice had increased *Blautia* and *Roseburia* and reduced *Bifidobacterium* when compared with young male mice (Figure 3(g)). Some species under *Blautia* and *Roseburia* contain BSH-T1, which had the highest abundance of BSHs in the gut microbiota of human.[21] It is worth noting that old female mice had reduced *Blautia* and *Roseburia* and increased *Bifidobacterium*, which were totally opposite to the changes in males (Figure 3(h)). These data indicated that the alternation of gut microbiota composition during aging was sex-specific which might account for, at least partially, the disparity of aging-related disorders including the imbalanced BAs metabolism between males and females.

Co-housing with young mice reduces hepatic inflammation and splenomegaly in old mice

To test whether the compositional alteration of gut microbiota is causative for aging-related disorders, we remodeled the gut microbiota by co-housing old mice with their young partners of the same sex periodically for 10 weeks to minimize the impact of lifestyle between old and young mice (See experiment design in Figure 4(a)). First, co-housing experiment did not impact the body weight of old mice in both sexes (Figure 4(b-c)). However, co-housing reduced the hepatic lymphocyte infiltration score and mRNA expression level of *Tnf-α, Il-1β*, and *Saa1*, especially in old male mice (Figure 4(d-g)). Moreover, co-housing also reduced the spleen weight and index, especially in old female mice (Figure 4(h-i)). These results indicated co-housing old mice with young mice exerted beneficial effects to different extent in male and female old mice implying the sex-specific impacts of gut microbiota on aging.

Co-housing with young mice remodels gut microbiota composition of old mice sex-specifically

To determine the role of gut microbiota in improving aging-related disorders by co-housing, we profiled the composition of gut microbiota based on 16 S rRNA gene sequencing. First, Unweighted UniFrac PCoA showed distinct clustering of intestinal microbe communities of each group of both sexes (Figure 5(a-b)). In specific, we observed that young mice were separated away from old mice alongside PC1 at 30.1% and 46.1% interpretation power for male and female mice, respectively. The co-housed old male mice were clustered between young and old mice, but separated with old mice by PC2, while the co-housed old female mice were closer to young female mice, indicating that the microbiota profile in old mice were shifted significantly after co-housing (Figure 5(a)). Meanwhile, we observed that co-housing caused significant restoration in bacterial α diversity in old female mice, but not in male ones compared to their old partners (Figure 5(b)). Then, we further analyzed the relative abundance of bacteria at different levels. At the phylum level, co-housing reversed the decreased abundance of Actinobacteria in old male mice, but showed no significant impacts in old female mice (Figure 5(c-d)). At genus level, more significant impacts of co-housing on gut microbiota were observed with sex-specific alterations. For example, co-housing significantly reversed the relative abundance of *Faecalibaculum, Bifidobacterium, unclassified_f_Ruminococcaceae, Blautia, and Ruminiclostridium* in old male mice (Figure 5(e)), as well as *norank_f_Erysipelotrichaceae, norank_f_Lachospiraceae, unclassified_f_Lachospiraceae, Lachospiraceae_NK4A136_group, Lachnospiraceae_UCG-006, Roseburia*, and *Lachnoclostridium* in old female mice (figure 5(f)). Notably, co-housing led to a dramatic reduction in the abundance of *norank_f_Erysipelotrichaceae* from 58% to 12%, which was comparable to the level of young female mice (figure 5(f)).

In addition, we further compared the impacts of co-housing on BA metabolism-related genera. Co-housing significantly reversed the aging-associated changes of *Blautia* (BSH-T1 and T7) and *Bifidobacterium* (BSH-T4) in male mice. Moreover, *Lactobacillus* (BSH-T0 and T3) was increased in co-housed old male mice (Figure 5(g)). In females, co-

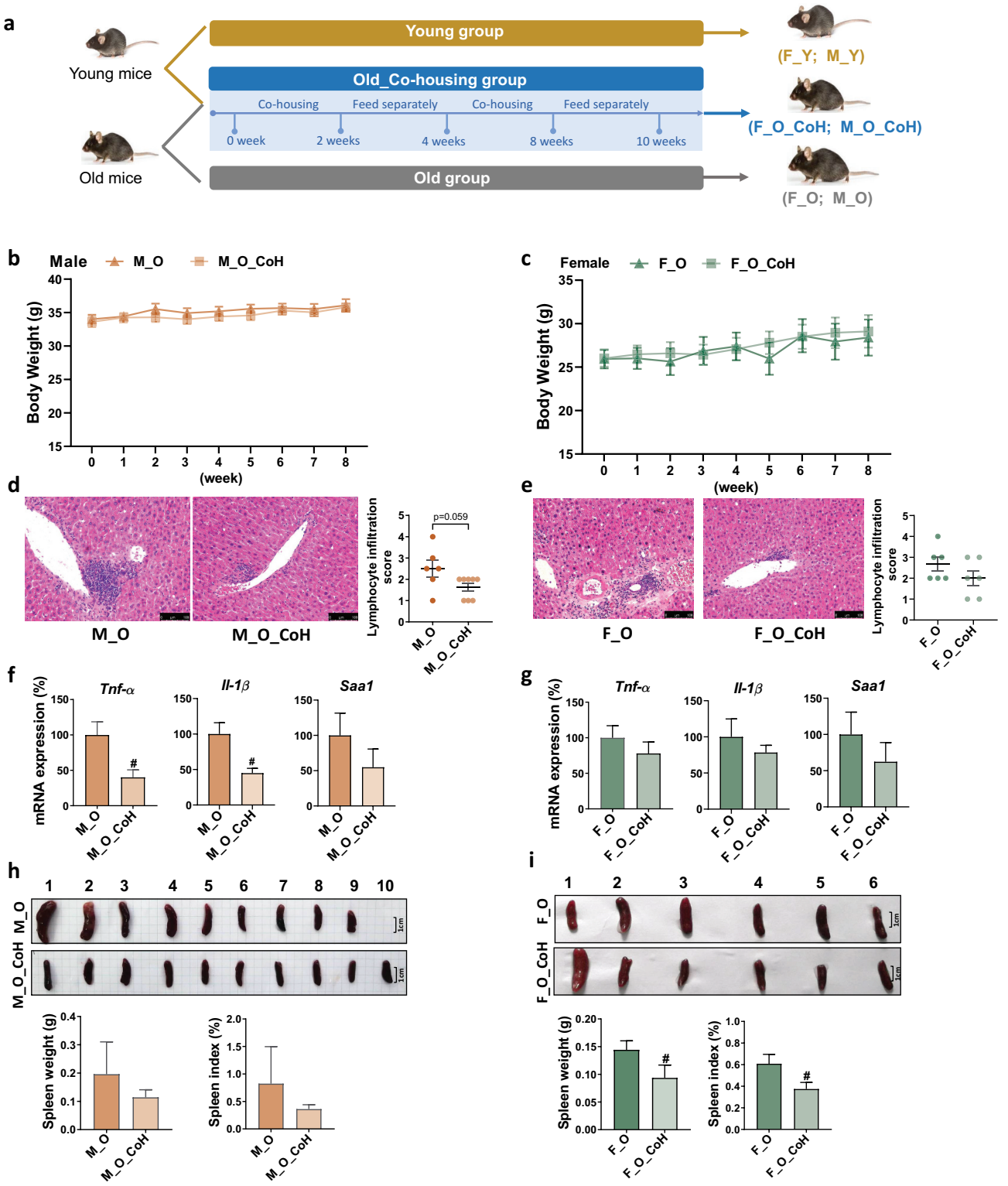

Figure 4. Histological and phenotypic changes in old mice after co-housing. (a) Co-housing experimental design. (b-c) Body weight in old mice (M_O and F_O) and co-housed old mice (M_O_CoH and F_O_CoH) of both sexes. (d-e) H&E-stained liver sections and lymphocyte infiltration score. Scale bars, 100 μm. (F-G) Hepatic inflammation-related gene expression. (h-i) Spleen morphology, spleen weight, and percentage of spleen to body weight ratio. $^{\#}p < .05$. $n = 6$–10 mice per group.

housing normalized the abundance of *Roseburia* (BSH-T1), *Bifidobacterium* (BSH-T4), and *Peptococcus* (Figure 5(h)). In addition to the gut microbiota remodeling, the sex-specific difference in gut microbiota composition was also observed in young mice including *norank_f_Erysipelotrichaceae,*

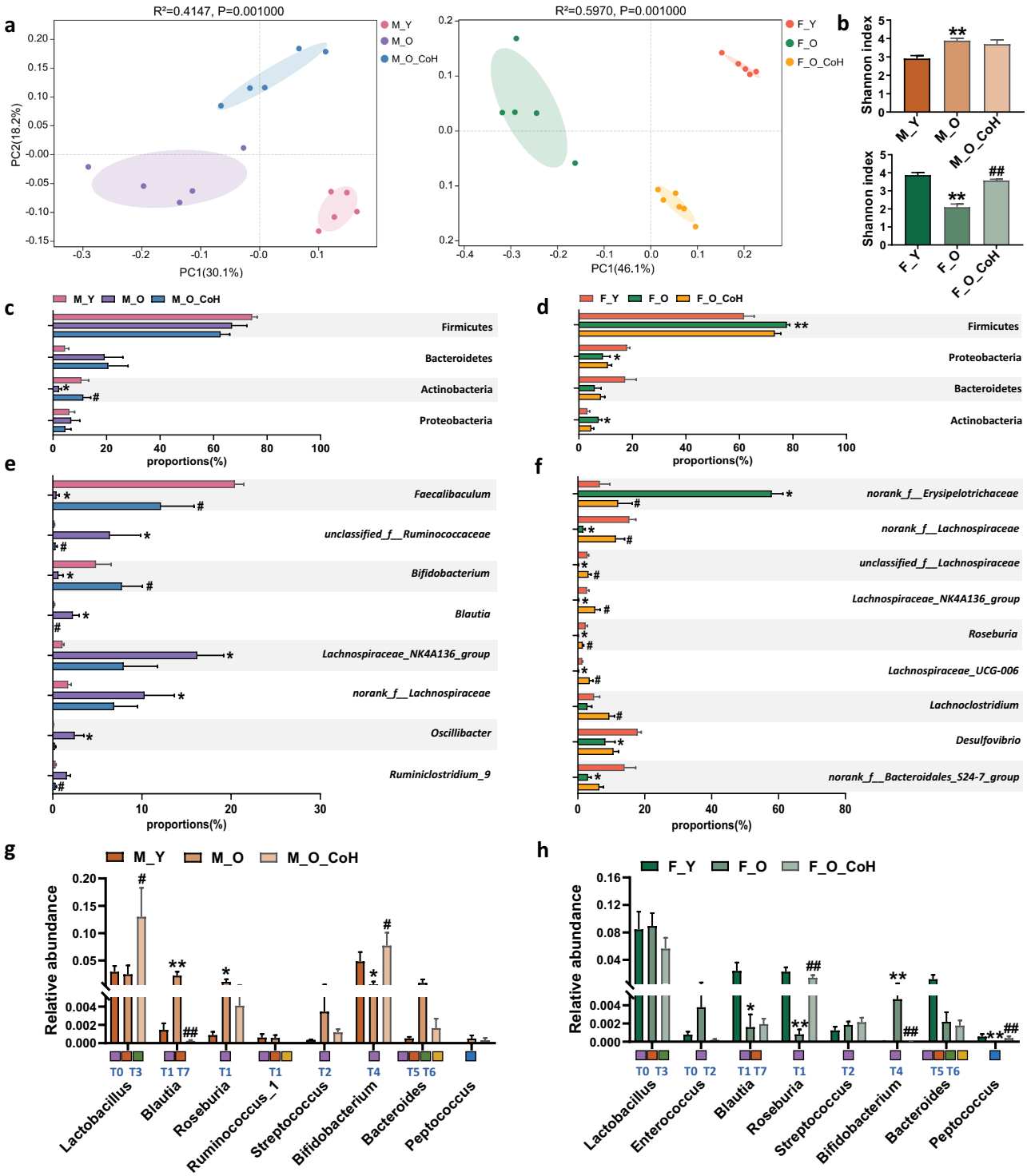

Figure 5. Co-housing improves gut microbiota disbalance induced by aging in both sexes. (a) Principal co-ordinates analysis plots of cecal microbiota at OUT level based on unweighted_unifrac. (b) Shannon index. (c-d) The relative abundance of the dominant phyla after co-housing in both sexes. (e-f) The differential genera that were significantly changed during aging and were reversed by co-housing in male and female mice, respectively. (g-h) Relative abundance of cecal microbiota involved in BAs metabolism at genus level. T0 to T7 that marked below the bar plots are the eight major phylotypes of BSH. Meanwhile, the squares filled with purple, orange, green, yellow, and blue represent bacteria that associated with BSH deconjugation, 7α-dehydroxylation, esterify, C-6 epimerization, and desulfation functions, respectively. *$p < .05$, **$p < .01$, compared with young group. $^{\#}p < .05$, $^{\#\#}p < .01$, compared with old group. $n = 5–6$ mice per group.

Faecalibaculum, Bifidobacterium, Lachospiraceae_NK4A136_group, norank_f_Lachospiraceae, Desulfovibrio, nonrank_f_Bacteroidales_S24-7_group, and *Roseburia*. However, these sex-related differences were abolished or even reversed with aging, while co-housing reverted some of them (Figure S4). Taken together, these data indicated that co-housing old mice with young ones remodeled their gut microbiota composition sex-specifically.

Gut microbiota remodeling alters BA profile in the liver, serum, and intestine

Given the important crosstalk between gut microbiota and BA metabolism, we further tested whether gut microbiota remodeling with co-housing alters BA pool profiles in sex-specific manner. The BA profiles in liver, serum, and different segments of intestine were quantified in both old male and female mice with or without co-housing. In general, co-housing increased total hepatic BAs levels but did not significantly alter the ratios of conjugated to unconjugated, or primary to secondary BAs in both sexes (Figure 6(a-b)). Co-housing resulted in different extent of alterations in hepatic BA signatures between male and female mice. Specifically, co-housing significantly reversed the hepatic concentrations of βDCA and 12-ketoLCA in old male mice (Figure 6(a)), and TDCA, TLCA, and βDCA in old female mice (Figure 6(b)). Notably, co-housing mainly changed the concentrations of secondary BAs, instead of primary BAs in both sexes, which is consistent with the role of gut microbiota in BAs metabolism. Additionally, the proportions of most individual BAs remained relatively constant in response to gut microbiota remodeling (Figure S5).

In serum, co-housing reversed the increased total BA levels in old mice of both sexes (Figure 6(c-d)), and in particular the ratios of conjugated to unconjugated, primary to secondary BAs in old female mice (Figure 6(d)). Although not statistically significant, co-housing resulted in reversed changes of some unconjugated BAs such as 7-DHCA, NorDCA, βCDCA, 6-ketoLCA, and βCA in males and HCA, 7-DHCA, βDCA, LCA, and βCA in females in concentrations (Figure 6(c-d)). In contrast to the concentration, co-housing also led to compositional changes of either conjugated or unconjugated BAs sex-specifically. For example, co-housed male mice showed decreased TαMCA, and increased TβMCA and TωMCA in composition, but increased TωMCA, and decreased TαMCA and TβMCA were observed in co-housed females (Figure S5B). In addition, co-housing caused decreased composition in 7-DHCA, and increased DCA, ωMCA in males, whereas decreased βMCA, and increased ωMCA, NorDCA in females (Figure S5B).

Intestinal tract is the main site for BAs functions where the gut microbiota modulates BA homeostasis through a series of bacterial enzymes and the majority of BAs are then reabsorbed at ileum,[48] so the total BA concentrations were depleted in the cecal and colon contents in all mice (Figure 6(e-f)). In male mice, co-housing reduced the ratio of primary to secondary BAs in all four intestinal segments, which rescued the increased levels caused by aging (Figure 6(e)). In female mice, similar changes were observed, but only the ratios of conjugated to unconjugated in cecal, and primary to secondary BAs in colon were significantly reversed by co-housing (figure 6(f)). Moreover, there were a number of individual BAs that were altered to different extent in concentration upon co-housing sex-specifically (Figure S6). However, there were only two unconjugated secondary BAs that were significantly reversed by co-housing, i.e. 6-ketoLCA and 12-ketoLCA in jejunum of male mice (Figure S6), whereas more significantly altered BAs were observed such as βMCA, βCA, and 7-DHCA in ileum and 12-ketoLCA, 7-DHCA, and NorCA in colon of co-housed old female mice (Figure S7). Since the eight BSH phylotypes have different deconjugation activity on various substrates,[21] we further studied the effects of age and gut microbiota remodeling on the ratio of free to conjugated BAs. The results indicated that aging significantly decreased the ratios of DCA/GDCA and DCA/TDCA, but increased CDCA/GCDCA and CDCA/TCDCA, and in which only CDCA/GCDCA and CDCA/TCDCA were significantly reversed by co-housing in male mice (Figure S8). No significant differences were observed in the ratios of these free to conjugated BAs in female mice (Figure S8).

Altogether, these results indicated that systemic BA profiles were greatly influenced by varying

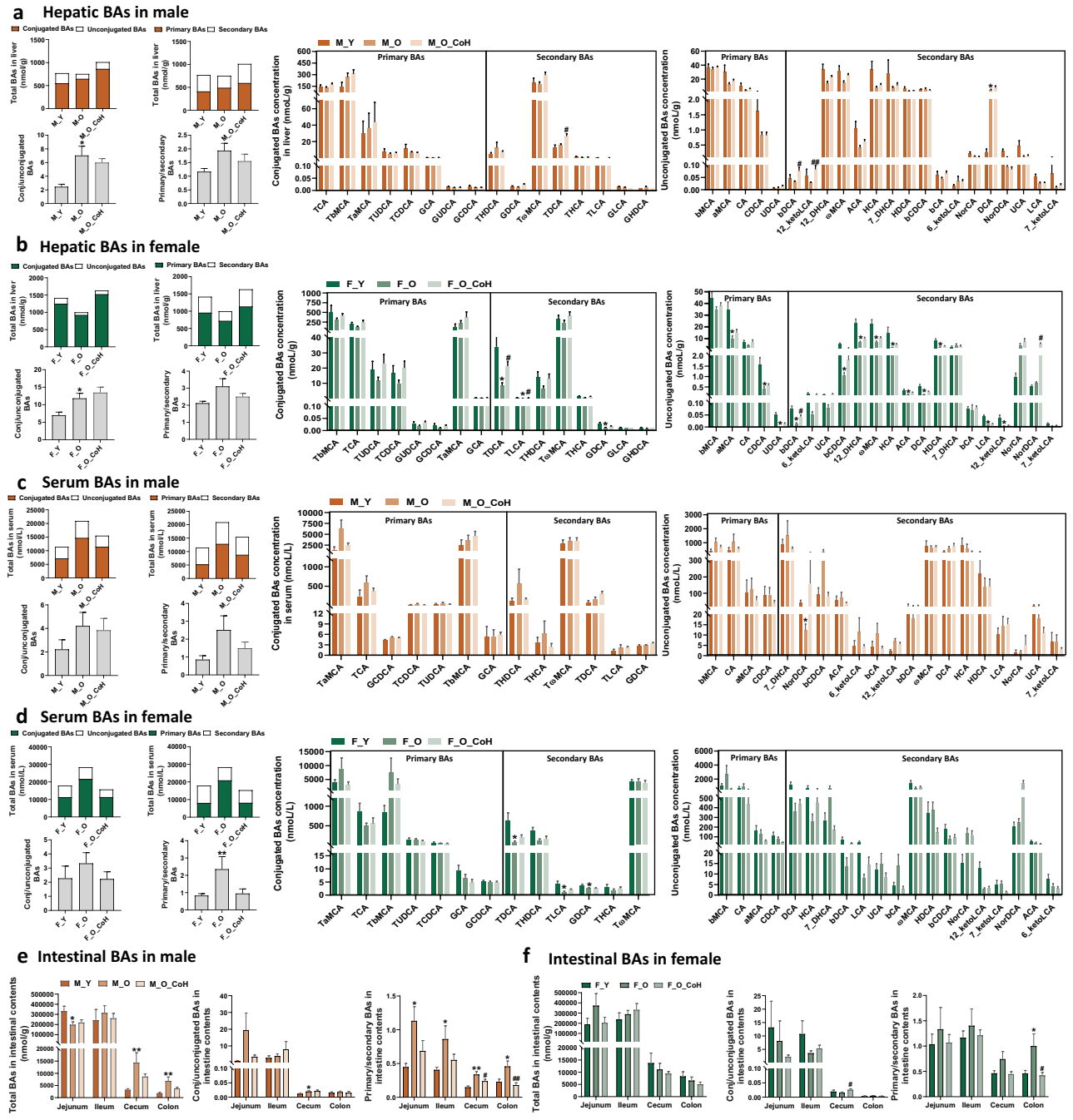

Figure 6. BA profile in the liver, serum, and intestine of male and female mice after co-housing. Total bile acids, ratios of conjugated to unconjugated, primary to secondary bile acids and concentrations of individual BAs in liver (a-b), serum (c-d), and intestine (e-f) of old and co-housing mice of both sexes. *$p < .05$, **$p < .01$, compared with young group. #$p < .05$, # #$p < .01$, compared with old group. n = 5–10 mice per group.

degrees by the gut microbiota remodeling through co-housing in a sex-specific manner, which suggests that the inoculation of bacteria from young partners holds benefits for attenuating aging-related disorders through re-balancing BA homeostasis.

Gut microbiota remodeling alters BA-related gene expression

To test whether gut microbiota remodeling could exert comprehensive impact on hepatic gene expression, liver transcriptome was performed in

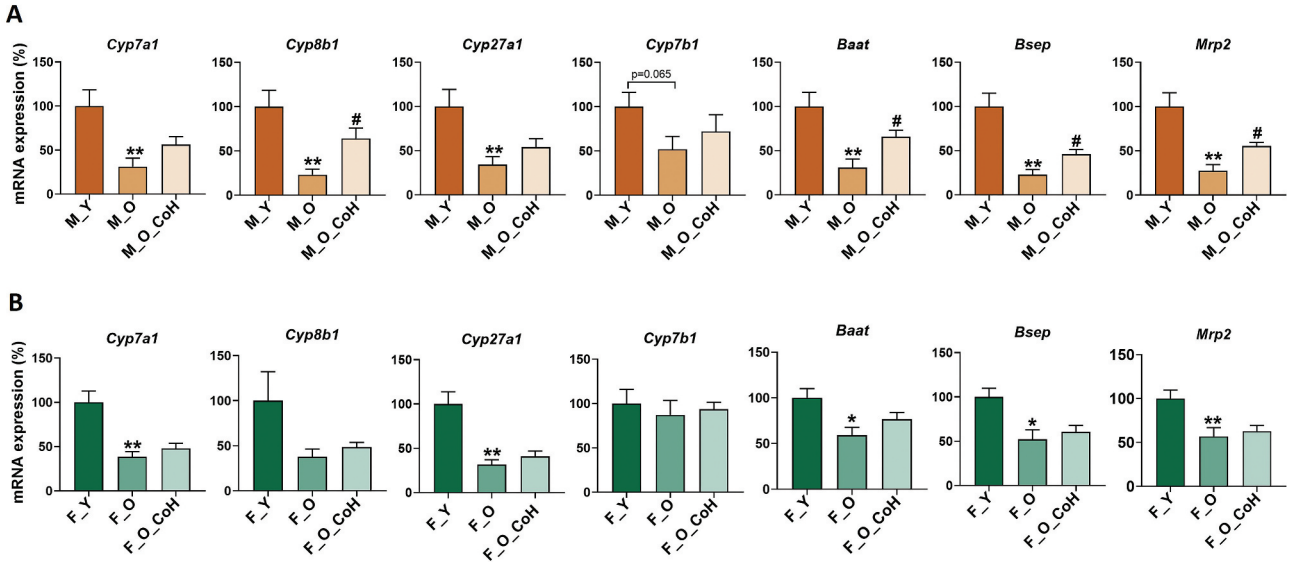

Figure 7. Hepatic BAs metabolism-related gene expression in male (a) and female (b) mice. *p< .05 and **p < .01 compared with young group; # p < .05 compared with old group. n = 5–8 mice per group.

the old male mice with or without co-housing. In total, eighty-one genes were upregulated and 793 genes were downregulated in the co-housed old male mice compared to those of old mice with Q value < 0.05 and fold change ≥ 2 or ≤0.5 used as the cutoff criteria (Figure S9A). Using the Database for Annotation, Visualization and Integrated Discovery (DAVID), we further found more than 10 out of 30 altered pathways were related to infection-induced diseases, including *Staphylococcus aureus* infection, leishmaniasis, malaria, and tuberculosis (Figure S9B). Additionally, seven downregulated pathways were associated with immune response and inflammation, such as NOD-like receptor signaling pathway, cytokine-cytokine receptor interaction, TNF signaling pathway, and NF-kappa B signaling pathway.

To further explore the co-housing-affected genes, Gene Set Enrichment Analysis (GSEA)[49] was employed to identify significantly enriched biological pathways on the basis of normalized enrichment score (NES) ranking. Compared with separated housed old male mice, co-housed old mice significantly activated the expression of "Bile acid and bile salt metabolism" and "TCA cycle and respiratory electron transport" gene sets and inhibited the expression of "Interferon gamma signaling" and "Innate immune system" gene sets (Figure S9 C-D). These data suggest that gut microbiota remodeling by co-housing significantly affect the expression of BA metabolism and inflammation-related genes by the gut-liver axis, which is consistent with the observations on the inflammatory phenotypes and BA profiles of these mice.

Next, BAs hemostasis-related genes were analyzed by qPCR in the liver and ileum during aging and after co-housing in both sexes. In male mice, the mRNA levels of hepatic *Cyp7a1, Cyp8b1, Cyp27a1, Cyp7b1, Baat, Bsep*, and *Mrp2* were reduced in old mice, while co-housing reversed *Cyp8b1, Baat, Bsep*, and *Mrp2* levels (Figure 7(a)). In female mice, aging reduced most of the above genes. However, co-housing had limited effect on reversing those changes (Figure 7 (b)). It should be noted that the mRNA level of *Cyp7b1*, which is involved in alternative BA synthesis, was not changed during aging or co-housing in female mice. Additionally, the expression of ileal BA transporters had reduced trend in old male mice but co-housing did not reverse thus changes. On the contrary, in female mice, aging had no effect on altering these gene expression, but co-housing had the trend to reduce them (Figure S10A-B).

Sex-related BA profiles at different age stages

In addition to the shifted BA profiles during aging and after co-housing in two sexes, the concentrations of individual BA were also significantly different between male and female mice (Figure 8). In

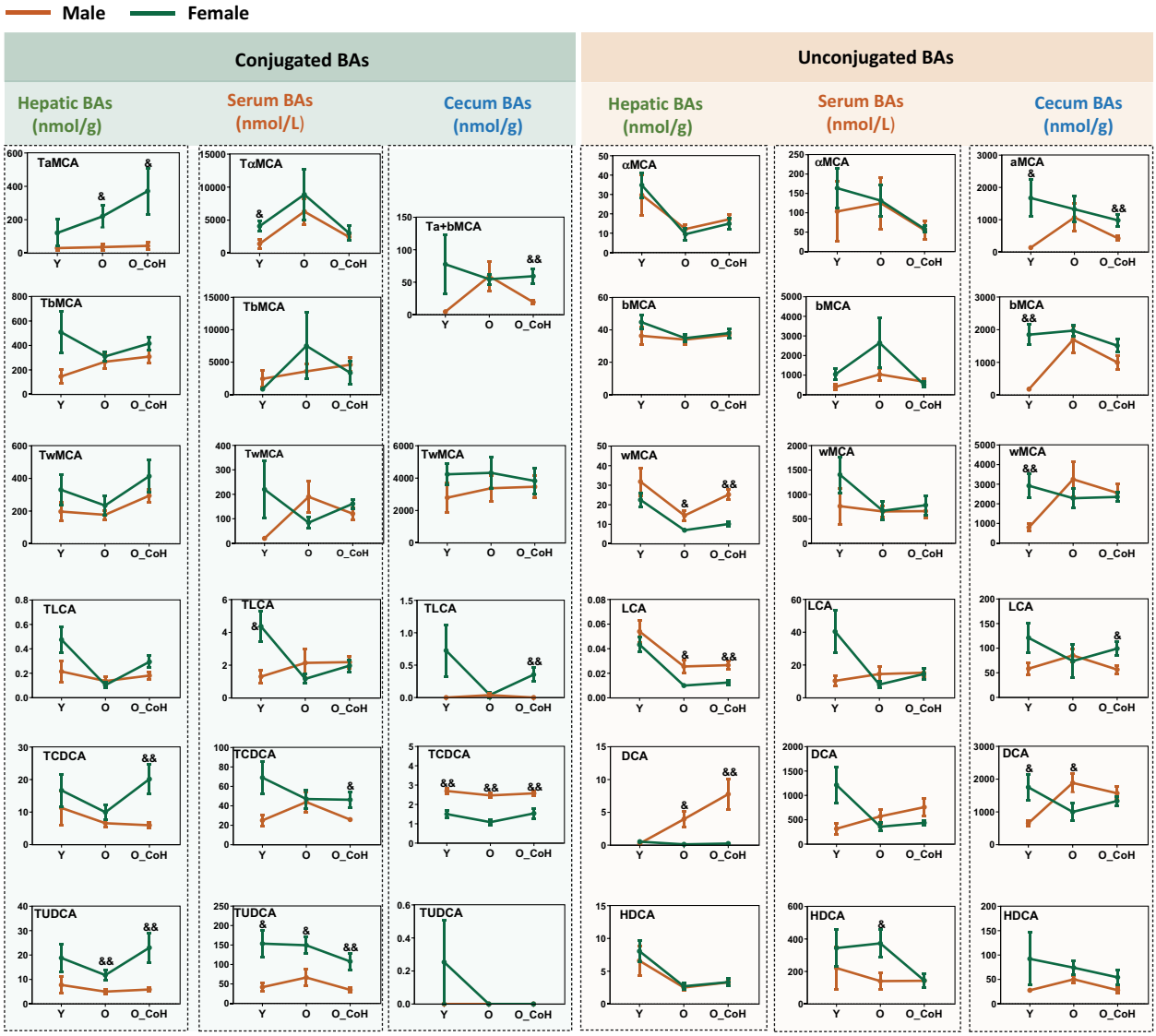

Figure 8. Sex difference in the concentration of certain BAs in the liver, serum, and cecal contents. &$p < .05$, &&$p < .01$ compared with female group. n = 5–10 mice per group.

general, compared with the male mice, female mice had higher level of hepatic conjugated BAs including all conjugated MCA, TLCA, TCDCA, and TUDCA. On the contrary, male mice had higher levels of hepatic free BAs, such as ωMCA, LCA, and DCA. Additionally, aging increased the sex difference of TαMCA, TUDCA, and DCA but narrowed the sex difference of TβMCA and TLCA in liver. Moreover, aging narrowed the sex difference of TLCA, LCA, and DCA in the serum, free, and conjugated αMCA and βMCA in the cecum. Compared with the male mice, female mice had higher concentrations of TCDCA and TUDCA in the liver and serum at young, old, and/or co-housed conditions. Hepatic DCA levels were increased with age and co-housing in male mice, while it remained very low and relatively constant with age in females (Figure 8). These data suggested BA levels were sex different and there were divergent changes of BAs during aging and co-housing in two sexes.

Sex-specific correlation between BAs and gut microbiota

The correlation analysis between the top 20 families of the gut microbiome and individual BA in the cecum was performed in each sex (Figure S11). In male mice, *Erysipelotrichaceae* and *Staphyloc occaceae*, which are under Firmicutes phylum,

were negatively correlated with 6-ketoLCA, GCA, 12-ketoLCA, TωMCA, ωMCA, GDCA, 7-HDCA, CA, and βMCA (Figure S9A). However, most of the above BAs were positively correlated with *Lachnospiraceae* and one unknown family under Saccharibacteria. Probiotic families *Lactobacillaceae* and *Bifidobacteriaceae* were clustered together with *Lactobacillaceae* positively correlated with THCA (Figure S11A). In female mice, *Lactobacillaceae* clustered separately with *Bifidobacteriaceae* and correlated with six BAs but not THCA. In addition, sex-specific correlation of *Erysipelotrichaceae* and *Lachnospiraceae* with BAs was observed (Figure S11B). These results revealed a sex-specific correlationship between the BAs and gut microbiota that was significantly altered by co-housing, which is indicative of the crosstalk between BAs and gut microbiota.

Discussion

The aging-related inflammation and disordered metabolism are sex-specific and presumably associated with the dysregulation in gut microbiota and BA homeostasis.[35,50-53] The presented data establish a direct link between gut microbiota and aging-associated BA profile changes in multiple tissues of both sexes. We demonstrate that aging has a greater impact on changing hepatic BA profiles in females, but gut BA profiles in males. As summarized in Figure 9, co-housing old mice with young mice strikingly changed gut microbiota composition in old mice and shifted BA profiles in multiple tissues to rescue aging-induced imbalanced BA homeostasis in a sex-specific manner. These findings suggest prevention and treatment of aging-associated disorders by targeting gut microbiota-regulated BA signaling should take sex into account.

Gut microbiota plays essential roles in regulating host metabolism and inflammation, while its composition changes throughout the life span and is known to be associated with host sex.[51,52] In the present study, among the top 10 differential genera during aging, four genera had opposite changes in two sexes. *Norank_f_Erysipelotrichaceae* was found to be either reduced or increased in male or female mice during aging. It is consistent with previous report of increased abundance of *Erysipelotrichaceae* in female mouse model of Alzheimer's disease.[55] Meanwhile, *Erysipelotrichaceae* is also associated with the hyperlipidemia.[56,57] Given the fact of higher rate of Alzheimer's disease in females than males,[58] and the association of Alzheimer's disease with dysregulated lipid metabolism,[59,60] the increased abundance of *norank_f_Erysipelotrichaceae* in old female mice is a probable risk factor for occurrence of aging-related disorders in females. The bacteria belonging to *Bifidobacterium* genus have been widely used as probiotics.[61,62] Our data showed the relative abundance of *Bifidobacterium* genus was reduced in male but increased in female mice during aging, which may account for the physiological disparity between males and females. Consistent evidence of reduced *Bifidobacterium* genus has been reported in old male mice and humans.[5,41,63,64] In addition, *Roseburia* was increased or reduced in male and female mice during aging, respectively. Many species of *Roseburia* contain BSHs and can produce health-promoting butyrate. The alteration of *Roseburia* with age in male mice is consistent with previous report in mice[41] but was contrary to the findings in humans, which showed reduced relative abundance in centenarians.[30] It should be taken into account that the changes of the composition of gut microbiota vary in different studies during aging between mice and humans, which might be associated with the differences in anatomical location, diet, lifestyle, sex, and age. The divergent changes of microbiota profile during aging might lead to the disparity in the metabolism and immune response between the two sexes.

The biological functions of BAs are diversified which are associated with their concentrations and organ distributions.[48] Our data showed that total BA concentrations in liver reduced in female mice, whereas they remained relatively constant with age in male mice. In addition, total BA concentrations in serum increased in both female and male mice. However, Fu *et al* found total BA concentrations in liver of both sexes and in serum of male mice remained relatively constant from 3 to 27 months, only total BA in serum of female mice significantly increased with age.[10] This inconsistent finding might be due to the types of BAs detected in different studies. We quantified 39 BAs in our current study, while only 20 of them were measured in previous report, and several BAs with high proportions, such

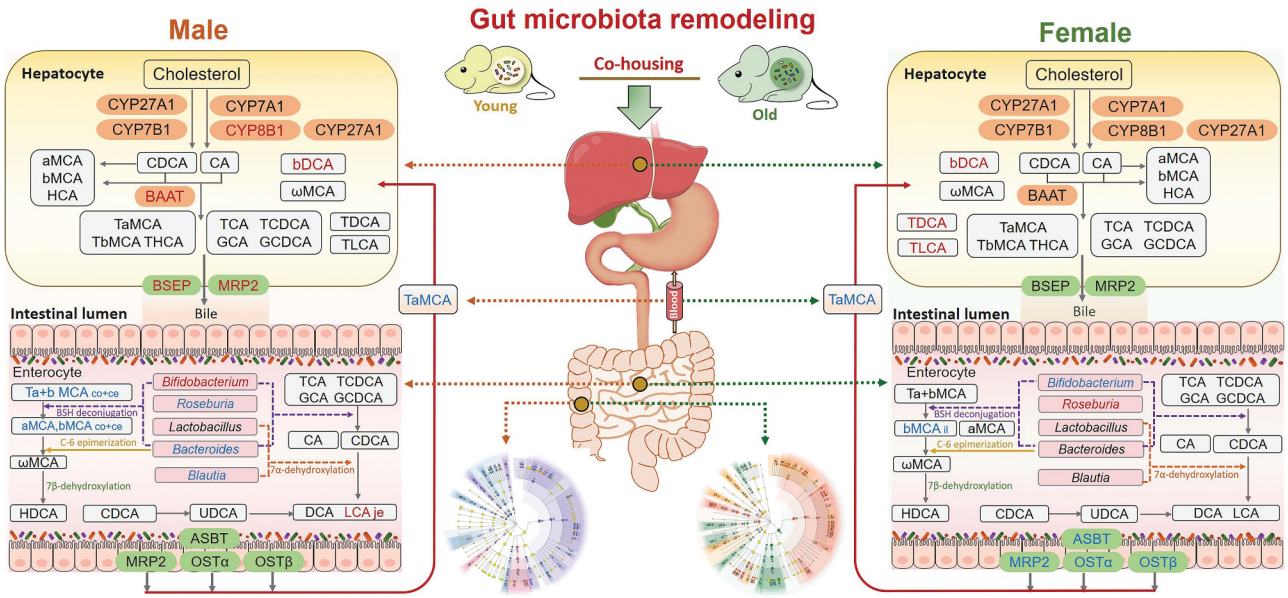

Figure 9. Gut microbiota remodeling by co-housing reverses the dysregulation of systemic BA homeostasis induced by aging. Old mice of both sexes had dramatic changes in gut microbiota composition and BA profiles compared with their young counterparts. To investigate the impact of gut microbiota composition on aging-associated disorders and bile acids metabolism, we performed a co-housing experiment within the same sex. After co-housing, the host phenotypes were reversed, and the microbiota profile in co-housed old mice was shifted. Next, we investigated the expression of genes involved in BA biosynthesis, transport, and metabolism.[12,13,45,54] In the liver, co-housing increased *Cyp8b1, Baat, Bsep*, and *Mrp2* levels in old male mice, while there was no significant change in old female mice. The concentrations of hepatic βDCA were increased after co-housing in both male and female mice, while TLCA and TDCA were only increased after co-housing in female mice. In the ileum, decreased trends of *Asbt, Mrp2, Osta*, and *Ostβ* were observed in co-housed old female mice, while expression of these genes did not show marked changes after co-housing in male mice. Meanwhile, *Bifidobacterium* was increased and reduced in co-housed old male and female mice, respectively. In addition, *Roseburia, Blautia*, and *Bacteroides* were reduced in co-housed old male mice, while these genera did not show consistent changes after co-housing in female mice. Moreover, the intestinal concentrations of individual BAs were divergently altered upon co-housing in the jejunum (je), ileum (il), cecum (ce), and colon (co) in a sex-dependent manner. Furthermore, in the serum, TαMCA was reduced in old male and female mice upon co-housing. In summary, co-housing reversed aging-associated dysregulation of systemic bile acid homeostasis in mice in a sex-dependent manner. Red and blue represent increased and decreased gene expression and BAs levels in co-housed old mice when compared with old mice, respectively.

as HCA and 7-DHCA, were not included.[10] Secondary BAs, DCA and LCA, which are generated by gut microbiota, tended to be reduced in the serum and liver of old female mice compared with young female mice. Differing with the changes in females, old male mice had 15-fold induction of hepatic DCA level. These two BAs are not only agonists for FXR but also can activate TGR5 signaling to improve metabolism.[65–67] However, excessive DCA produces reactive oxygen species that cause DNA damage and senescence-associated secretory phenotype, which facilitate high fat diet-induced hepatocellular carcinoma growth.[68] Therefore, reduced levels of DCA and LCA in old female mice might lead to less activation of FXR and TGR5 signaling which affect metabolism. On the other hand, elevated hepatic DCA in the liver of male mice may induce inflammatory signaling that are associated with the development of cancer, leading to the sex difference in the incidence of liver diseases.[68–71] These results suggest the potential difference of the activation of BA receptors and DCA induced toxicity between the two sexes which could lead to different immune response and metabolism during aging. In addition, it is well known that the secondary BA concentration in feces is related to the incidence of colonic cancer.[72] The concentration of the major secondary fecal BAs, DCA and LCA, increased with aging and was significantly higher in elderly subjects compared to young adults.[73] In line with the fact of age-related incidence rates of colon cancer are higher for men than women,[74] our results also showed that aging increased DCA (4.6-fold

increase) and LCA (2.7-fold increase) in the colon of male mice but not female mice.

A very intriguing finding in the present study was that aging increased the ratio of primary to secondary BAs while co-housing reduced it in the liver, serum, and all four intestinal segments of both sexes. Gut microbiota has essential effects on secondary BAs production. *Blautia* and *Bacteroides* genera, which include many species that convert primary BAs to secondary BAs by 7α-dehydroxylation,[13,44,46] were increased in old male mice and reduced after co-housing. Though there are many genera that can regulate secondary BAs production, *Blautia* and *Bacteroides* were markedly influenced by age and gut microbiota remodeling in a sex-dependent manner, suggesting their divergent roles in regulating host health in two sexes which need further study.

The beneficial or detrimental effects of BAs are also associated with the extent of hydrophobicity or hydrophilicity. Previous study reports that total BAs in serum and liver became more hydrophilic with aging in both male and female mice.[10] In the present study, we also found increased ratios of conjugated to unconjugated BAs in old mice, leading to increased hydrophilicity of BAs in the liver and serum, which was consistent with the previous finding. Increased hydrophobicity of BAs in tissues can cause cytotoxicity and produce reactive oxygen species, resulting in DNA damage, apoptosis, and necrosis.[75] Since the mRNA level of hepatic *Baat*, which involved in BAs conjugation, was reduced in old mice, it is possible that gut microbiota alters the BA composition during aging in order to increase the hydrophilicity of BAs, which might indicate an initiated protective mechanism to alter BA composition and compensate for disease susceptibility during aging. In the present study, the ratios of CDCA/TCDCA and CDCA/GCDCA were significantly increased in cecum contents of old mice and were decreased after co-housing. BSHs are a set of bacteria-derived enzymes for hydrolysis of conjugated BAs with different substrates and activities in bacteria.[21] Previous study indicated that BSH-T3 showed the highest enzyme activity when the substrates were TCDCA and GCDCA, followed by BSH-T1 and BSH-T4 based on human gut microbiome.[21] Although BSH-T3 showed the highest enzyme activity, it was only found in *Lactobacillus*, which contained 0.15% of the total relative abundance of BSHs. BSH-T1, with the relative abundance of 38.03%, was found in *Blautia, Roseburia*, and *Ruminococcus_1*. In addition, BSH-T4 was found in *Bifidobacterium* with 2.74% of the total relative abundance of BSHs.[21] Interestingly, age and co-housing induced abundance changes of *Blautia* and *Roseburia* in male mice as well as *Bifidobacterium* in female mice were consistent with the ratio changes of CDCA/TCDCA and/or CDCA/GCDCA, indicating these genera may play important roles in aging-associated BA alteration sex-specifically. However, BSHs containing bacteria that have high deconjugation activity toward MCA should be identified in future.

Interesting findings from the present study show both aging and co-housing significantly affect the concentration of MCAs in a tissue- and sex-dependent manner. The unconjugated and conjugated MCAs are unique BAs that only exist in rodents and not in humans. They play an important role as the signal molecules in regulating FXR signaling pathway which in turn affects host metabolism.[76,77] In the liver, aging tended to reduce the concentration of αMCA and ωMCA in both sexes, while co-housing tended to increase ωMCA level in male mice. In the serum, old mice had increased trend of TαMCA, which is a more potent FXR antagonist than TβMCA, while co-housing reduced TαMCA levels. This change happened in both male and female mice suggesting the potent deactivation of FXR signaling in peripheral organs. However, previous study showed increased TαMCA with age in the serum was only found in female mice, not in male mice, which is not consistent with our findings.[10] It is worth noting that MCAs shifted more markedly in the intestine, especially in male mice. In the cecum, Tα+βMCA (13-fold increase), TωMCA (9-fold increase), αMCA (8-fold increase), βMCA (9-fold increase), and ωMCA (4-fold increase) were increased in old male mice. Similarly, in the colon, Tα+βMCA (11-fold increase), αMCA (5-fold increase), and βMCA (8-fold increase) were increased in old male mice while co-housing normalized all of them. Compared with the multiply changes of MCAs in male mice, it is interesting to note that only ileal

βMCA was shifted by aging in female mice. These findings clearly suggest the divergent effects of age and gut microbiota remodeling on regulating BA profiles in two sexes. Because about 95% BAs are efficiently taken up in the ileal segment and active uptake of conjugated BAs is mediated by ileal ASBT,[78,79] the elevated Tα+βMCA in the cecum and colon of old male mice might due to reduced ileal uptake that occurred during aging. Since Tα+βMCA are FXR antagonists, the increased level may have led to reduced intestinal FXR activity. Deactivation of hepatic FXR can cause liver cancer,[27,80,81] but deactivation of intestinal FXR may have some benefits. It has been shown that intestinal FXR deficient mice are resistant to diet-induced steatohepatitis, obesity, and insulin resistance.[15,82] Therefore, old mice may have elevated intestinal Tα+βMCA levels to present beneficial effects while co-housed mice do not need to maintain high Tα+βMCA levels.

The sex differences in the concentrations of individual BAs shown in the present study provide important evidence that BAs may function as markers for longevity. Females have a longer life expectancy than males in many species, including humans.[32,83,84] In our study, both hepatic and serum concentrations of TCDCA, TαMCA, TUDCA were higher in female mice. The long-lived lit/lit mice were shown to have increased CDCA and UDCA in serum.[85] Therefore, the higher hepatic and serum concentrations of TCDCA and TUDCA might correlate with the tendency of increased longevity in female mice.

Although co-housing is increasingly adopted for investigating the role of gut microbiota,[86,87] co-housing of mice for over 6 weeks can result in some extents of chronic stress and behavior dysfunction, but not present after 2 weeks of co-housing.[88] In our current study, to avoid unintended chronic stress or behavior change induced by co-housing, we adopted an intermittent co-housing strategy for gut microbiota remodeling. According to previous report, chronic stress can reduce body weight and disturb gut microbiota, with increased levels of inflammation promoting operational taxonomic units related to *Helicobacter, Peptostreptococcaceae, Streptococcus*, and *Enterococcus faecalis*.[89] Our data showed the body weight gain of co-housed old mice was comparable with their counterparts in either male or female mice during 10 weeks intermittent co-housing experiment. In addition, none of the stress-related bacteria was increased in the co-housed old mice, suggesting the intermittent co-housing did not induce obviously chronic stress in our current study. Even though, it should be cautious to interpret the results obtained from co-housing experiment, and more considerations should be taken in further study to clearly differentiate the impacts contributed by gut microbiota itself, or jointly with stress.

The current study provided a comprehensive description of the age-related changes of gut microbiota and BA profiles in male and female C57BL/6 mice. In addition, we found co-housing partially changed gut microbiota composition in a sex-specific manner. This might be due to the differential colonization and competitive ability of different gut bacteria, which leads to differential alteration of the relative abundance of each strain. In terms of the complicated crosstalks between gut microbiota and BAs, as well as the complex gut-liver axis in regulation of BA metabolism, our current results demonstrated that co-housing induced diversified changes of BAs either in absolute concentrations or relative composition sex-specifically.

In conclusion, aging is characterized by a series of disorders and susceptibility to various diseases with sexual disparity. Our current report reveals the interplay between gut microbiota and BA metabolism during aging, and more importantly, we demonstrate that gut microbiota remodeling can attenuate aging-related disorders, at least partially, through reverses the imbalanced BA homeostasis in a sex-specific manner. Our current findings highlight the potential of sex-specific strategy to prevent or treat aging-related disorders by targeting gut microbiota-regulated BA metabolism axis. Further investigations are warranted to elucidate the exact roles of altered BAs during aging in different sexes.

Methods and materials

Mice

Male and female C57BL/6 J mice of 24-month-old were provided by Laboratory Animal Center of Xiamen University (Xiamen, China). Four-week-

old C57BL/6 J mice of both sexes were provided by Shanghai Laboratory Animal Center (Shanghai, China). All mice were housed in a 12-hour light (7 AM to 7 PM) and 12-hour dark (7 PM to 7 AM) cycle, with free access to water and chow diet. The experiments were conducted under the Guidelines for Animal Experiment of Shanghai University of Traditional Chinese Medicine and the protocol was approved by the institutional Animal Ethics Committee.

Co-housing experiments

An intermittent co-housing experiment was performed by consolidating 24-month-old old mice with 1-month-old young mice of the same sex for 10 weeks according to the reference with some modification.[88] In detail, the first round of co-housing lasted for 2 weeks and followed by 2 weeks interval of separation. Then, the second round of co-housing lasted for 4 weeks and followed by 2 weeks of separation. The experimental design is shown in Figure 4(a). In total, there were six groups including M_Y (young male), M_O (old male), M_O_CoH (co-housed old male), F_Y (young female), F_O (old female), and F_O_CoH (co-housed old female). At the end of the experiment, overnight fasted mice were sacrificed after anesthesia with 1% pentobarbital sodium solution by intraperitoneal injection. Samples were collected and immediately frozen at −80°C for further analysis.

Histological evaluation on the degree of hepatic lymphocyte infiltration

Liver tissues were fixed with 10% neutral formalin for 24 hours, embedded in paraffin, stained with hematoxylin-eosin staining (H&E) and sections were observed for the degree of hepatic lymphocyte infiltration under the light microscope. The degree of hepatic lymphocyte infiltration was evaluated according to a previous publication in a blinded way.[29] The criteria for scoring including 0 (absent), 1 (rare), 2 (mild), 3 (moderate), and 4 (severe).

Quantitative RT-PCR

Total RNA from ileum tissue was isolated using a RNeasy mini kit (#74104, QIAGEN, Germany) and total RNA of liver tissue was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized by the high Capacity cDNA Reverse Transcription Kit (#K1682, Thermo Fisher Scientific, USA). QRT-PCR was performed using SYBR Green (A25777, Thermo Fisher Scientific, USA). Gene expression was normalized to 18 s. The primers used are shown in supplementary table 1.

16 S rDNA sequencing

DNA samples were extracted from 50 to 100 mg cecal contents using E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA.). Qualified DNA samples were applied to amplification of 16 S rDNA V3-V4 region using the universal primers 338 F (ACTCCTACGGGAGGCAGCAG) and 806 R (GGACTACHVGGGTWTCTAAT). The sequencing was performed by the Illumina MiSeq PE300 system (Illumina, San Diego, USA) according to the standard protocols. Raw data files were demultiplexed, quality-filtered, and sequences whose overlap longer than 10 bp were merged using FLASH. The reads were clustered to OTUs with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) and chimeras were removed using UCHIME. The taxonomy of each sequence was analyzed by Ribosomal Database Project Classifier algorithm (http://rdp.cme.msu.edu/) against the 16 S rDNA database Silva (SSU123) using confidence threshold of 70%. The principal coordinates (PCoA) analysis based on unweighted_unifrac was conducted to reflect community similarity and overall difference of gut microbiota in each group, the difference between groups was analyzed by Adonis test.

BA extraction and quantification

Chemicals

All of the 39 bile acids standards including taurohyocholic acid (THCA), hyocholic acid (HCA), ω-muricholic acid (ωMCA), tauro-ω-muricholic acid (TωMCA), tauro-α muricholic acid (TαMCA), tauro-β-muricholic acid (TβMCA), tauroursodeoxycholic acid (TUDCA), glycoursodeoxycholic acid (GUDCA), glycohyodeoxycholic acid (GHDCA), taurohyodeoxycholi acid (THDCA), taurocholic

acid (TCA), glycoursodeoxycholic acid (GCA), 12-dehydrocholic acid (12-DHCA), β-muricholic acid (βMCA), α-muricholic acid (αMCA), 7-dehydrocholic acid (7-DHCA), 3-dehydrocholic acid (3-DHCA), taurochenodeoxycholic acid (TCDCA), 3β-cholic acid (βCA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDCA), glycol deoxycholic acid (GDCA), ursodeoxycholic acid (UDCA), hyodeoxycholic acid (HDCA), cholic acid (CA), ursocholic acid (UCA), 23-nordeoxycholic acid (NorDCA), norcholic acid (NorCA), allocholic acid (ACA), 3β-chenodeoxycholic acid (βCDCA), taurolithocholicacid (TLCA), 3β deoxycholic acid (βDCA), glycolithocholic acid (GLCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), 6-ketolithocholic acid (6-ketoLCA), 7-ketolithocholic acid (7-ketoLCA), 12-ketolithocholic acid (12-ketoLCA), lithocholic acid (LCA) were purchased from Steraloids Inc. (Newport, RI) and TRC Chemicals (Toronto, ON, Canada), and 9 stable isotope-labeled standards were obtained from C/D/N Isotopes Inc. (Quebec, Canada) and Steraloids Inc. (Newport, RI). The standards and stable isotope-labeled standards were accurately weighed and prepared in methanol at a concentration of 5.0 mM (stock solution). Further dilution was performed to obtain a series of calibration concentration of 2000, 400, 160, 32, 12.8, 2.5, or 1 nM with methanol/water (50/50, v/v). Internal Standard (IS) concentrations were kept constant at all the calibration points at 100 nM for GCA-d4, TCA-d4, TCDCA-d9, UDCA-d4, CA-d4, GCDCA-d4, GDCA-d4, DCA-d4, and 200 nM for LCA-d4.

Methanol (Optima LC-MS), acetonitrile (Optima LC-MS), and formic acid (Optima LC-MS) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ). Ultrapure water was produced by a Mill-Q Reference system equipped with an LC-MS Pak filter (Millipore, Billerica, MA).

Sample preparation

Quantitative analysis of BAs was conducted according to the previous publications.[90,91] Briefly, each 20 μL of serum or standard solution was spiked with 180 μL of acetonitrile:methanol = 80:20 containing 9 internal standards (100 μl IS to acetonitrile:methanol = 80:20) and the extraction of bile acids was conducted at a laboratory shaker at 10°C and 1,500 rpm for 15 min. After centrifugation, the supernatant (170 μl) was transferred to a microcentrifuge tube for lyophilization using a freeze dryer system (Labconco, Kansas City, MO). The residue was reconstituted with 1:1 (v/v) mobile phase B (30 μl, acetonitrile/methanol = 80:20, v/v) and mobile phase A (30 μl, water), and centrifuged at 13,500 g and 4°C for 20 min. The supernatant was transferred to a 96-well plate for LC-MS analysis.

Each 100 mg liver tissue sample was homogenized with 100 μL of 50% methanol using a Bullet Blender Tissue Homogenizer (Next Advance, Inc., Averill Park, NY). An aliquot of 150 μL of acetonitrile containing 9 internal standards was added and the second step extraction was performed using the homogenizer. After centrifugation, the supernatant was divided into two aliquots (200 μL and 10 μL) and transferred to a microcentrifuge tube for lyophilization. The residue was reconstituted in 1:1 (v/v) mobile phase B (acetonitrile/methanol = 95:5, v/v) and mobile phase A (water with formic acid, pH = 3.25), and centrifuged at 13,500 g and 4°C for 20 min. The supernatant was transferred to a 96-well plate for LC-MS analysis.

Each 100 mg Intestinal contents (jejunum, ileum, cecum, colon) sample were homogenized with 500 μL of ice-cold water. The mixture was vortexed for 4 min and then centrifuged at 13,200 rpm for 10 min at 4°C. A 300 μL aliquot of supernatant was transferred to a 2-ml tube, and the pellets were further extracted with ice-cold methanol using the same protocol. Another 300 μL aliquot of supernatant was added to the same tube as the initial aliquot, and 10 μL of IS (*p*-chlorophenylalanine in water, 5 g/ml) was added. The extraction was vortexed for 30 s and centrifuged at 13,000 rpm for 20 min. The supernatant was transferred to a 96-well plate for LC-MS analysis.

Instrumentation

An ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA) was used to quantitate 39 bile acids in the mouse liver, serum, and intestine samples. All chromatographic separations were performed with ACQUITY UPLC BEH C18 1.7 μM VanGuard pre-column (2.1 × 5 mm) and ACQUITY UPLC BEH C18 1.7 μM analytical

column (2.1 × 100 mm). The mobile phase consisted of 10 mM ammonium acetate adjusted to pH 3.25 using formic acid (mobile phase A) and acetonitrile/methanol (mobile phase B). The flow rate was 0.45 mL/min with the following mobile phase gradient: 0–1 min (5% B), 1–5 min (5–25% B), 5–15.5 min (25–40% B), 15.5–17.5 min (40–95% B), 17.5–19 min (95% B), 19–19.5 min (95–5% B), and 19.6–21 min (5% B). The column was maintained at 45°C and the injection volume of all samples was 5 μL. The mass spectrometer was operated with source and desolvation temperatures set at 150°C and 550°C. Bile acids were detected in the negative mode. The mass spectrometer was operated in negative ion mode with a 1.2kV capillary voltage. The source and desolvation gas temperature were 150°C and 550°C, respectively. The data were collected with multiple reaction monitor (MRM), and the cone and collision energy for each BA used the optimized settings from QuanOptimize application manager (Waters), according to the previous publications.

Data analysis

The raw data were processed using the TargetLynx application manager (Waters Corp., Milford, MA) to obtain calibration equations and the measured concentration of each bile acid in the samples.

RNA sequencing analysis

Hepatic total RNA was extracted using RNeasy mini kit (Qiagen, Germany), paired-end libraries were synthesized by using the TruSeq™ RNA Sample Preparation Kit (Illumina, USA) following TruSeq™ RNA Sample Preparation Guide. Purified libraries were quantified by Qubit® 2.0 Fluorometer (Life Technologies, USA) and validated by Agilent 2100 bioanalyzer (Agilent Technologies, USA) to confirm the insert size and calculate the mole concentration. Cluster was generated by cBot with the library diluted to 10 pM and then were sequenced on the Illumina HiSeq (Illumina, USA). The raw reads were filtered by Seqtk before mapping to genome using Tophat (version: 2.0.9).[92] The fragments of genes were counted using HTSeq followed by TMM (trimmed mean of M values) normalization.[93] Significant differential expressed genes were identified as those with a False Discovery Rate value above the threshold ($Q < 0.05$) and fold-change >2 using edgeR software.[94] RNAseq data can be accessed in NCBI and the accession number is PRJNA611506.

Correlation analysis between bacterial taxonomy and BAs

Correlation between the top 20 families and bile acids was estimated by Spearman's correlation coefficient analysis, which was visualized in heatmap including positive (red) or negative (blue) relationship. The significant correction was performed with the criteria of $^{*}p < .05$, $^{**}p < .01$.

Statistical analysis

Data are shown as means ± SEM unless otherwise noted. All the bar plots in this study were generated with Prism 8.0 (GraphPad, La Jolla, CA, USA). Differences between groups were calculated by Mann–Whitney U test or Kruskal–Wallis H test using SPSS 24.0 (IBM, SPSS, USA). In addition, the p value was adjusted using Benjamini-Hochberg to control the multiple testing false discovery rate in the analysis of bile acids and the differential bacteria. $p < .05$ was considered statistically significant.

Author contributions

Junli Ma took the responsibility for the animal experiments, data analysis, and manuscript writing; Ying Hong helped animal experiments and data analysis; Ningning Zheng contributed to analysis of microbiome data; Guoxiang Xie was responsible for performing targeted metabolomics on bile acids; Yuanzhi Lyu analyzed the RNAseq data; Zhenzhen Huang and Wenbin Wu contributed to the animal experiment and sample collection; Gaosong Wu and Yu Gu contributed to data analysis of bile acid profile; Chuchu Xi and Linlin Chen helped the animal experiments and sample processing; Yue Li, Xin Tao, and Jing Zhong helped the data analysis and manuscript writing; Lin Yuan, Min Lin, and Xiong Lu were responsible for the HE staining and histological analysis of liver tissue; Weidong zhang helped in project design; Wei Jia participated in the design of this study and bile acids data analysis; Lili Sheng was responsible for the data

analysis and manuscript writing; Houkai Li supervised the project and revised the manuscript.

Disclosure statement

The authors declare no competing financial interests.

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ORCID

Yue Li http://orcid.org/0000-0001-5603-104X

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