Gut microbiota and nuclear receptors in bile acid and lipid metabolism

Out, Carolien

CHAPTER 5

Gut microbiota inhibit ASBT-dependent intestinal bile acid reabsorption via GATA4

Carolien Out, Jay V. Patankar, Marije Boesjes, Trijnie Bos, Sanna de Boer, Rick Havinga, Henk Wolters, Renze Boverhof, Theo H. van Dijk, Anna Smoczek, Andre Bleich, Dagmar Kratky, Folkert Kuipers, Henkjan J. Verkade and Albert K. Groen

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ABSTRACT

Background & aim: Regulation of bile acid homeostasis in mammals is a complex process regulated via extensive cross-talk between liver, intestine and intestinal microbiota. Here we studied the effects of gut microbiota on bile acid homeostasis in mice.

Methods: Bile acid homeostasis was assessed in four mouse models. Germfree mice, conventionally-raised mice, Asbt-KO mice and intestinal-specific Gata4-iKO mice were treated with antibiotics (bacitracin, neomycin and vancomycin; 100mg/kg) for 5 days and subsequently compared with untreated mice.

Results: Attenuation of the bacterial flora by antibiotics strongly reduced fecal excretion and synthesis of bile acids, but increased the expression of the bile acid synthesis enzyme *Cyp7A1*. Similar effects were seen in germfree mice. Intestinal bile acid absorption was increased and accompanied by increases in plasma bile acid levels, biliary bile acid secretion and enterohepatic cycling of bile acids. In the absence of microbiota, the expression of the intestinal bile salt transporter Asbt was strongly increased in the ileum and was also expressed in more proximal parts of the small intestine. Most of the effects of antibiotic treatment on bile acid homeostasis could be prevented by genetic inactivation of either Asbt or the transcription factor Gata4.

Conclusions: Attenuation of gut microbiota alters Gata4-controlled expression of Asbt, increasing absorption and decreasing synthesis of bile acids. Our data support the concept that under physiological conditions microbiota stimulate Gata4, which suppresses Asbt expression, limiting the expression of this transporter to the terminal ileum. Our studies expand current knowledge on the bacterial control of bile acid homeostasis.

INTRODUCTION

The dynamic community of bacteria colonizing the intestinal tract interacts symbiotically with the human host (1). These bacteria – also known as gut microbiota – exert a variety of effects on the host, ranging from shaping the structure and functions of the gut and the immune system, to altering host energy metabolism (1). In return, the bacteria benefit by inhabiting a protected, nutrient-rich environment.

Intestinal bacteria are also known to be involved in the metabolism of bile acids (BAs). BAs are produced in the liver from cholesterol through a complex multi-enzyme pathway. The key enzyme in this pathway is cholesterol 7 alpha-hydroxylase (CYP7A1). Prior to their secretion in bile, BAs are conjugated with the amino acids taurine or glycine. In the intestine, they facilitate the absorption of lipids and lipid-soluble vitamins. Intestinal bacteria can deconjugate BAs and metabolize primary BAs (*i.e.* those synthesized in the liver) through oxidation and dehydroxylation into more hydrophobic, so-called secondary Bas (2-4). Under physiological conditions, BAs are efficiently reabsorbed by ileal enterocytes and transported back to the liver via the portal circulation, in a cycle known as the enterohepatic circulation. Normally, relatively small amounts of BAs reach the colon, where some are absorbed and the remainder is excreted in the feces. To maintain a constant circulating pool, a small amount of BAs is synthesized each cycle, which is equivalent to the loss in feces under steady state conditions.

Active BA absorption is mediated by the apical sodium-dependent BA transporter (Asbt)(5), which is almost exclusively expressed in the terminal part of the ileum, thus ensuring that BAs have sufficient time to aid lipid digestion in the more proximal parts of the small intestine. In Asbt knock-out (Asbt-KO) mice, fecal BA excretion is 10 to 20 times higher than in wildtype mice. Despite increased BA synthesis in these mice, the BA pool size is reduced by 80%, indicating that alternative (absorptive) mechanisms are unable to compensate for loss of Asbt function (6). Asbt is clearly a key regulator of BA recycling and homeostasis.

The expression of Asbt in the small intestine is under direct negative control of the transcription factor Gata4 (7). In the adult mouse, Gata4 expression is repressed in the terminal ileum, thereby allowing expression of Asbt in the enterocytes of this part of the intestine. Consequently, intestinal-specific Gata4 knock-out (Gata4-iKO) induces the expression of Asbt in the duodenum and jejunum (8).

Previous studies in germfree animals have observed that these animals have less fecal BA excretion (9, 10), while the total concentration of BAs in the small intestines is higher (9, 11). Accordingly, germfree animals have an increased half-life of ^{14}C-labeled tauro-cholic acid (12). Similar results have recently been reported in mice treated with antibiotics (13, 14). A possible explanation for these results is that microbial deconjugation of BAs is thought to enhance fecal BA excretion (15). However, when germfree rats are colonized with a bacterial strain capable of deconjugating BAs, this has no effect on the excretion levels of either fecal BAs or of the tracer (12, 16). The fact that conventional animals have higher levels of fecal BA excretion than those in germfree animals is therefore likely to be due to mechanisms other than enhanced microbial deconjugation of BAs.

BAs are made from cholesterol and plasma cholesterol levels are a major risk factor for cardiovascular diseases. If we know how bacteria affect BA homeostasis, this could offer opportunities to modulate cholesterol levels in the future. In this study, we compared germfree and conventional mice and gave mice short-term antibiotic treatment. We demonstrate that germfree and antibiotic-treated mice reabsorb BAs more efficiently than control animals do. Antibiotic treatment in Asbt-KO and Gata4-iKO mice showed that an increase and proximal shift in the expression of Asbt mediated the effects of antibiotic treatment on BA homeostasis and that the induction of Asbt was partly Gata4-dependent. Thus, normally gut microbiota decrease the recirculation of BAs, which results in higher rates of cholesterol catabolism for *de novo* BA synthesis.

MATERIALS AND METHODS

Animal experiments

All experiments were performed on male mice. C57Bl/6J mice (Charles River, France), Asbt-KO mice (kindly provided by Prof. P.A. Dawson, Wake Forest University School of Medicine, Winston-Salem, NC, USA), Gata4-iKO mice (8) and their control littermates were housed individually in a temperature and light-controlled facility with a 12-hour light-dark cycle. All mice were fed commercially available laboratory chow (RMH-B; Hope Farms, Woerden, the Netherlands) *ad libitum*, which was supplemented with antibiotics (bacitracin, neomycin and vancomycin; 100mg/kg) as required. Germfree

NMRI mice and their conventional counterparts were housed in groups of five and fed an irradiated diet (Ssniff® M-Z, Ssniff Spezialdiäten GmbH, Soest, Germany).

After 5 days of antibiotic or control treatment, gallbladder cannulation was performed to collect hepatic bile. Bile was collected during 20 minutes under Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg) anesthesia using a humidified incubator to maintain body temperature. Blood was obtained via heart puncture and, after sacrificing the mice, the liver and ileum were excised and snap-frozen in liquid nitrogen for gene expression and protein analysis. All experiments were approved by the Ethics Committee for Animal Experiments of the University of Groningen.

Measurements

Analysis of BAs, cholesterol and phospholipids, as well as cDNA measurements and western blotting were all performed as described previously. For detailed information please refer to supplemental material and methods.

RESULTS

Bile acid homeostasis in germfree mice

When we compared BA homeostasis between germfree and conventional mice, we found the BA concentration in the feces of germfree mice to be less than one third of that in the feces of their conventional counterparts (Figure 1A). Only primary BAs were present in the feces of germfree mice and virtually all fecal BAs were conjugated, mainly with taurine, while in the feces of conventional mice also secondary BAs were present and only 19% of BAs were conjugated (Supplemental figure 1A). The main BAs detected in plasma were taurocholic acid (tCA) and tauro-beta-muricholic acid (tβ-MCA), levels that were much higher in germfree mice than in conventional controls (Supplemental figure 1B).

Bile flow was higher in germfree mice than in conventional mice (Figure 1B). Secretion of biliary BAs was also significantly higher in these mice (Figure 1C), and consisted mainly of CA and β-MCA (Supplemental figure 1C). When we compared the levels of cholesterol and phospholipids secreted in bile between the 2 groups of mice, we found germfree mice had 4.2-fold higher amounts of biliary cholesterol (3.3 ± 1.3 vs. 0.8 ± 0.2 nmol/min/100g; p=0.04) and 2.4-fold higher amounts of phospholipids (26.3 ± 7.2 vs. 10.8 ± 2.5 nmol/min/100g; p=0.04).

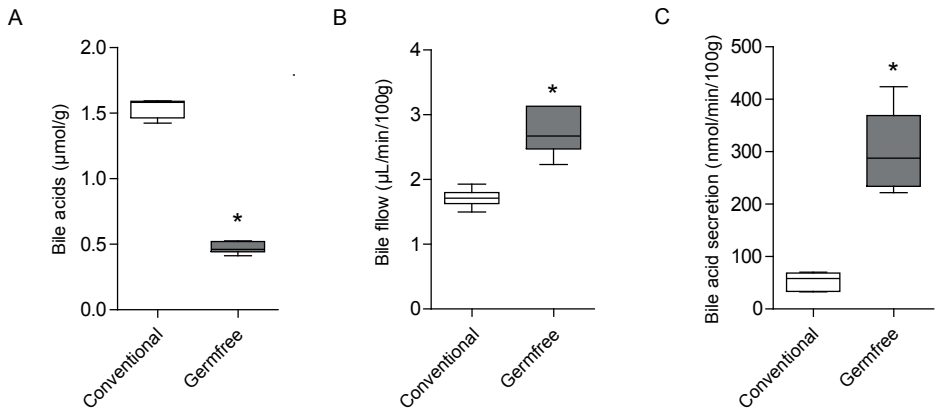


Figure 1. Bile acid homeostasis in germfree mice. (A) Total BA concentration in feces (µmol/g feces) of germfree and conventional mice. (B) Bile flow (µL/min/100g bodyweight) measured by bile cannulation and (C) secretion of biliary BAs (nmol/min/100g bodyweight) in germfree and conventional mice. Median ± range; n = 6-9/group; * $p < 0.05$.

Bile acid homeostasis in conventional mice treated with antibiotics

BA homeostasis in germfree mice is clearly different to that in their conventional counterparts. However, since the differences in the colonization of the gut are present from birth, metabolic compensation may have occurred. Thus, to identify the short-term effects of a decrease in intestinal bacteria on BA metabolism, conventional mice were treated with cocktail of three non-absorbable antibiotics for 5 days, which significantly reduced fecal bacterial DNA concentrations (Supplemental figure 2A). Antibiotic treatment markedly reduced total fecal BA excretion (Figure 2A), indicating an inhibition of BA synthesis. To validate this, we injected mice intravenously with ^{13}C-cholate and subsequently determined the turnover and pool size of cholate. The cholate pool size was larger in antibiotic-treated mice than in control mice (34 ± 5 vs. 31 ± 8 µmol/100g), as apparent from the lower ^{13}C-cholate enrichment seen in the plasma of these mice (Supplemental figure 2B). In line with the lower fecal BA excretion, cholate turnover rate (Figure 2B) and cholate synthesis were also lower in treated mice than in control mice (3.1 ± 2.5 vs. 6.7 ± 2.5 µmol/24h/100g).

In mice given antibiotics, we also observed higher levels of plasma BAs, bile flow and biliary BA secretion (Figure 2C-E), suggesting that antibiotic treatment enhanced intestinal BA absorption and enterohepatic cycling. Biliary secretion of CA and β-MCA was far higher in treated mice than in controls, and also the secretion of several secondary BAs

was higher (Supplemental figure 2C). As biliary BA secretion is the driving force behind the biliary secretion of cholesterol and phospholipids, it was not unexpected to see an increase in the biliary secretion of these lipids (Supplemental figure 2D-E). The fecal excretion of neutral sterols was lower in antibiotic-treated mice (Supplemental figure 2F). Treatment with a different antibacterial drug (ampicillin 100mg/kg) showed similar results, and treatment of germfree mice with antibiotics had no effect on any of the parameters related to BA and cholesterol metabolism studied above (data not shown).

Effects of antibiotic therapy on gene expression and bile acid reabsorption

In order to study the mechanism by which gut microbiota influence BA reabsorption, we measured the gene expression of key enzymes and transporters within the enterohepatic circulation. While antibiotic treatment increased the hepatic expression of *Cyp7A1*, there were no significant differences between antibiotic-treated mice and controls with regard to the hepatic expression of the nuclear receptors *Fxr* and *Lrh-1* or their target gene *Shp*, which are known to be involved in the regulation of *Cyp7a1* expression (Figure 2F).

Antibiotic treatment strongly increased *Asbt* expression in all segments of the small intestine (Figure 2G), absolute amounts were highest in the terminal ileum. Western blot analysis showed that Asbt protein levels in the terminal ileum were higher than those in controls (Figure 2H). Despite increased Asbt expression, ileal expression of the BA-responsive genes *Shp* and *Fgf15* was much lower than in controls (Figure 2I), suggesting decreased BA activation of Fxr in this part of the small intestine.

Increased Asbt expression in the proximal small intestine may induce BA absorption in this part of the intestine. We analyzed luminal BA concentrations in several segments of the small intestine, luminal BA concentrations were lower in the jejunum and ileum of mice treated with antibiotics than in control mice (data not shown), indicating that enhanced absorption may have occurred in these parts. In line with proximal Asbt expression, antibiotic treatment induced the expression levels of the BA-responsive genes *Shp* and *Fgf15* in the proximal small intestine (Figure 2J). These findings are compatible with enhanced import of BAs into proximal enterocytes. These data suggest that when there is a reduction of bacterial flora, BAs are also absorbed in the proximal small intestine, which may result in a reduction of BA absorption and of subsequent Fxr activation in distal parts.

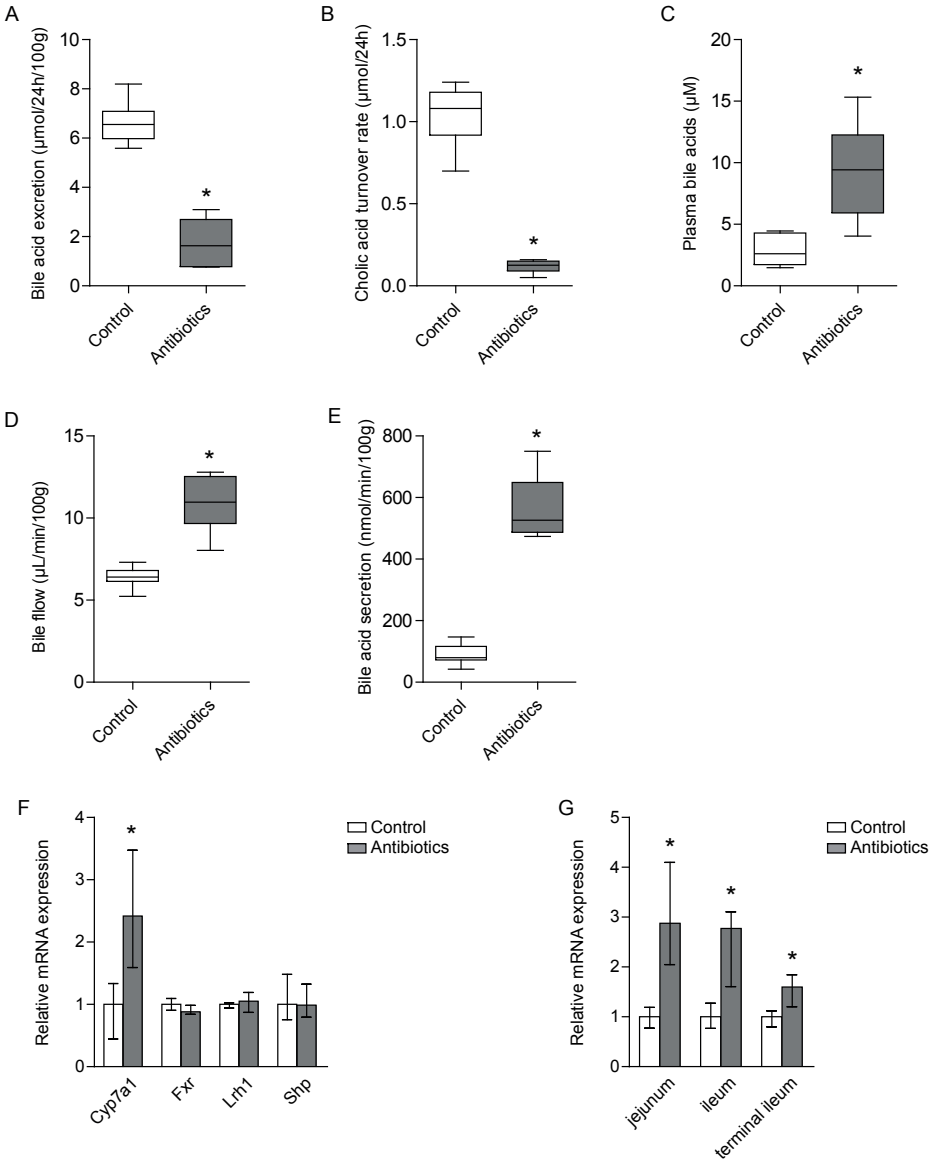
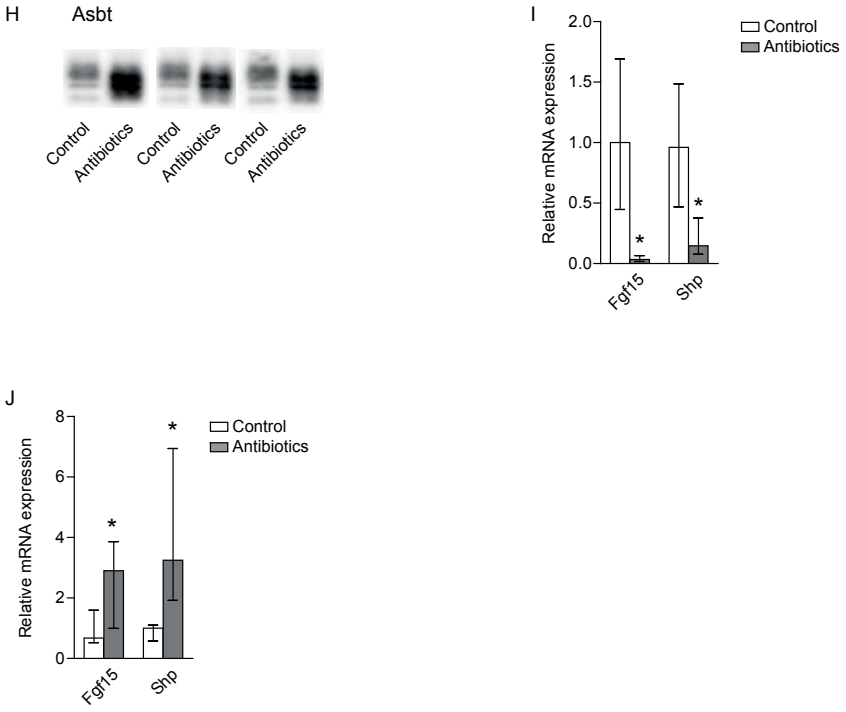


Figure 2. Antibiotic treatment alters bile acid composition, recirculation and gene expression in the enterohepatic circulation. (A) Fecal excretion of BAs in antibiotic-treated and control mice (µmol/24h/100g bodyweight). (B) Cholic acid turnover rate (µmol/24h), (C) total plasma BA levels (µM), (D) bile flow (µL/min/100g bodyweight) and (E) secretion of biliary BAs (nmol/min/100g bodyweight) in antibiotic-treated and control mice. (F) Hepatic mRNA expression in antibiotic-treated and control mice. (G) *Asbt* mRNA expression in different segments of the small intestine.



(H) Representative western blot of ileal Asbt from 3 control and 3 antibiotic-treated mice. (I) Ileal mRNA expression of BA-responsive genes. (J) mRNA expression of BA-responsive genes in the jejunum. Median ± range; n = 8/group; * $p < 0.05$.

Effects of antibiotic therapy in Asbt-KO mice

The results described above suggested that the bacterial flora may influence Asbt expression and thereby BA absorption. Recently, Annaba *et al.* showed that certain intestinal *E. coli* species can indeed inhibit Asbt expression (17). To identify the role of Asbt in the relationship between gut microbiota and BA absorption, we treated Asbt-KO mice with antibiotics.

In untreated animals, fecal BA excretion was higer in Asbt-KO mice than in wildtype (WT) mice (Figure 3A), which coincided with lower biliary BA secretion, despite no changes in either bile flow rate or plasma BA concentration (Figure 3B-D). Secondary BAs made up 51% of the plasma BA pool in Asbt-KO mice, compared with only 12% in WT mice, suggesting that in Asbt-KO mice colonic absorption of secondary BAs com-

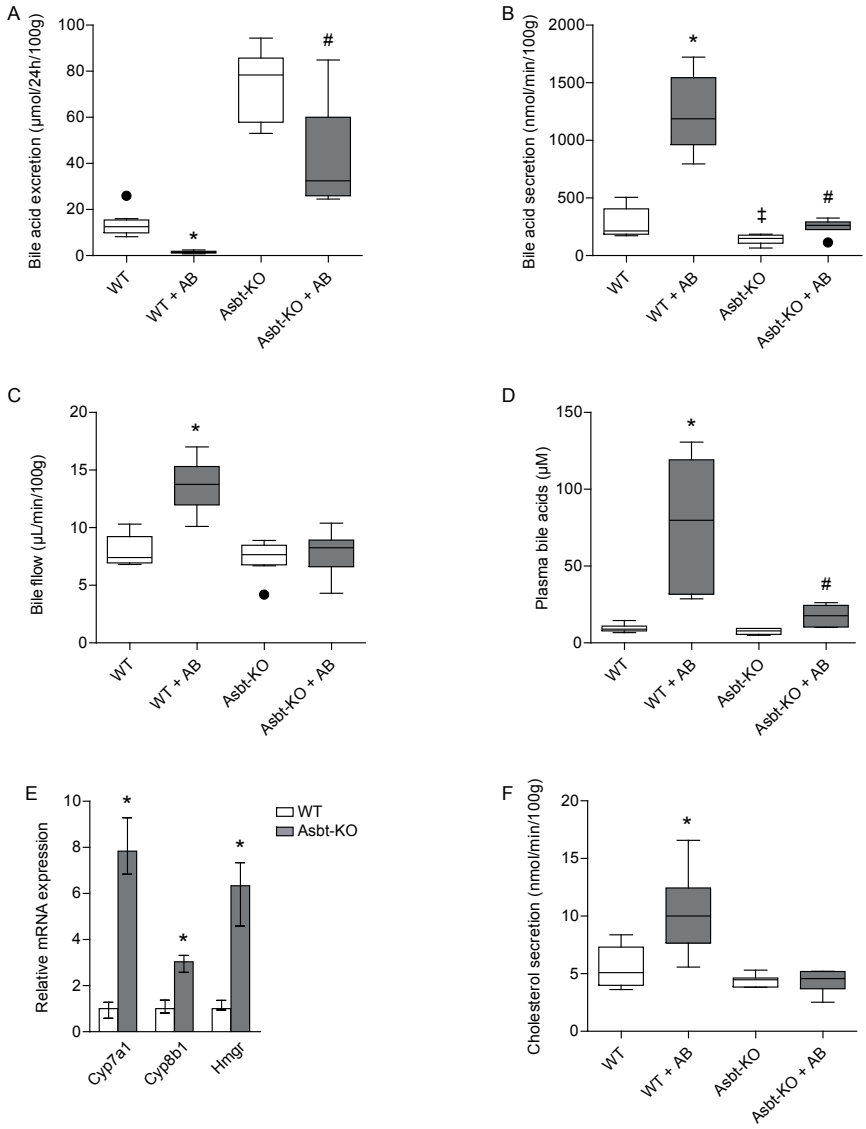
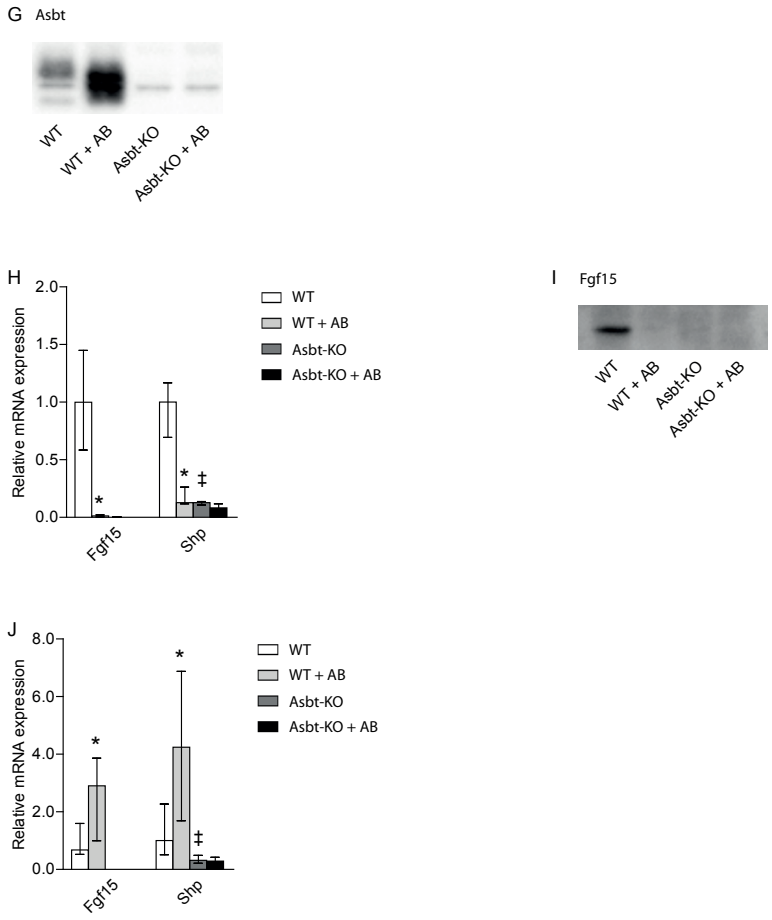


Figure 3. Bile acid homeostasis in Asbt-KO mice treated with antibiotics. (A) Fecal BA excretion (µmol/24h/100g bodyweight) in wildtype and Asbt-KO mice upon antibiotic treatment. (B) Secretion of biliary BAs (nmol/min/100g bodyweight). (C) Bile flow (µL/min/100g bodyweight). (D) Total plasma BAs (µM) in antibiotic-treated and control wildtype and Asbt-KO mice. (E) Hepatic mRNA expression of *Cyp7a1*, *Cyp8b1* and *Hmgr*. (F) Biliary cholesterol secretion (nmol/min/100g bodyweight) in Asbt-KO and wildtype mice.



(G) Ileal Asbt protein expression. (H) Ileal mRNA expression of BA-responsive genes. (I) Ileal FGF15 protein levels. (J) mRNA expression of *Fgf15* and *Shp* in jejunum of wildtype and Asbt-KO mice. Median ± range; n = 8/group; * $p < 0.05$ WT vs. WT + AB, ‡ $p < 0.05$ WT vs. Asbt-KO, # $p < 0.05$ Asbt-KO vs. Asbt-KO + AB.

pensates for enhanced fecal BA loss, as shown before (6). Hepatic mRNA expression of *Cyp7a1*, *Cyp8b1* and *Hmgr* was higher in Asbt-KO mice than in WT mice (Figure 3E), findings compatible with compensation for the increased fecal BA loss.

Antibiotic treatment reduced fecal BA excretion 9.1-fold in WT mice, but only 2.4-fold in Asbt-KO mice (Figure 3A). While in antibiotic-treated WT mice we observed a dramatic 8.9-fold increase in plasma BAs, this increase was only 2.2-fold in antibiotic-treated

Asbt-KO mice (Figure 3D). Bile flow in Asbt-KO mice was not affected by antibiotic treatment (Figure 3C). Biliary BA secretion did increase in Asbt-KO mice upon antibiotic treatment, although not as much as in WT mice (1.8-fold versus 5.5-fold)(Figure 3B). Biliary cholesterol secretion was induced in WT mice upon antibiotic treatment, but unchanged in treated Asbt-KO mice (Figure 3F).

When we looked at the expression levels of BA-responsive genes in the intestine, we saw that antibiotic treatment induced ileal Asbt protein expression in WT mice, whereas it was absent in Asbt-KO mice (Figure 3G). Ileal expression of *Fgf15* was also virtually absent in Asbt-KO mice, as was the expression of *Shp*; neither changed upon treatment with antibiotics (Figure 3H). Fgf15 protein levels were also undetectable in both Asbt-KO and WT mice treated with antibiotics (Figure 3I). In the proximal intestine, antibiotic treatment increased the expression of *Fgf15* and *Shp* in WT mice, whereas their expression remained unaffected in Asbt-KO mice (Figure 3J). These results demonstrate that the bacterial flora mediate transcriptional changes in enterocyte Asbt, which in turn govern BA reabsorption.

Effects of antibiotic therapy in Gata4-iKO mice

It is not known how intestinal bacteria might influence the expression of Asbt. The major regulator of intestinal Asbt expression is the transcription factor Gata4, which is expressed throughout the small intestine apart from in the terminal ileum (18). Gata4 inhibits the expression of Asbt, and therefore restricts Asbt expression to the terminal ileum (19, 20).

Recently, it has been shown that intestinal bacteria influence the expression of genes regulated by Gata4 (21). We therefore used intestinal-specific Gata4 knock-out (Gata4-iKO) mice to determine the role of Gata4 in BA reabsorption in response to changes in the intestinal microbiota. Compared with WT mice, untreated Gata4-iKO mice had higher expression levels of *Asbt* in proximal parts of the intestine (Figure 4A), in line with previous studies (7, 8, 18, 22). These higher *Asbt* expression levels were accompanied by lower fecal BA excretion; absolute values were similar to the levels of fecal BA excretion seen in antibiotic-treated WT mice (Figure 4B). Bile flow and biliary BA secretion were higher in Gata4-iKO mice than in WT mice (Figure 4C/D), in accordance with the enhanced BA reabsorption observed in Gata4-iKO mice.

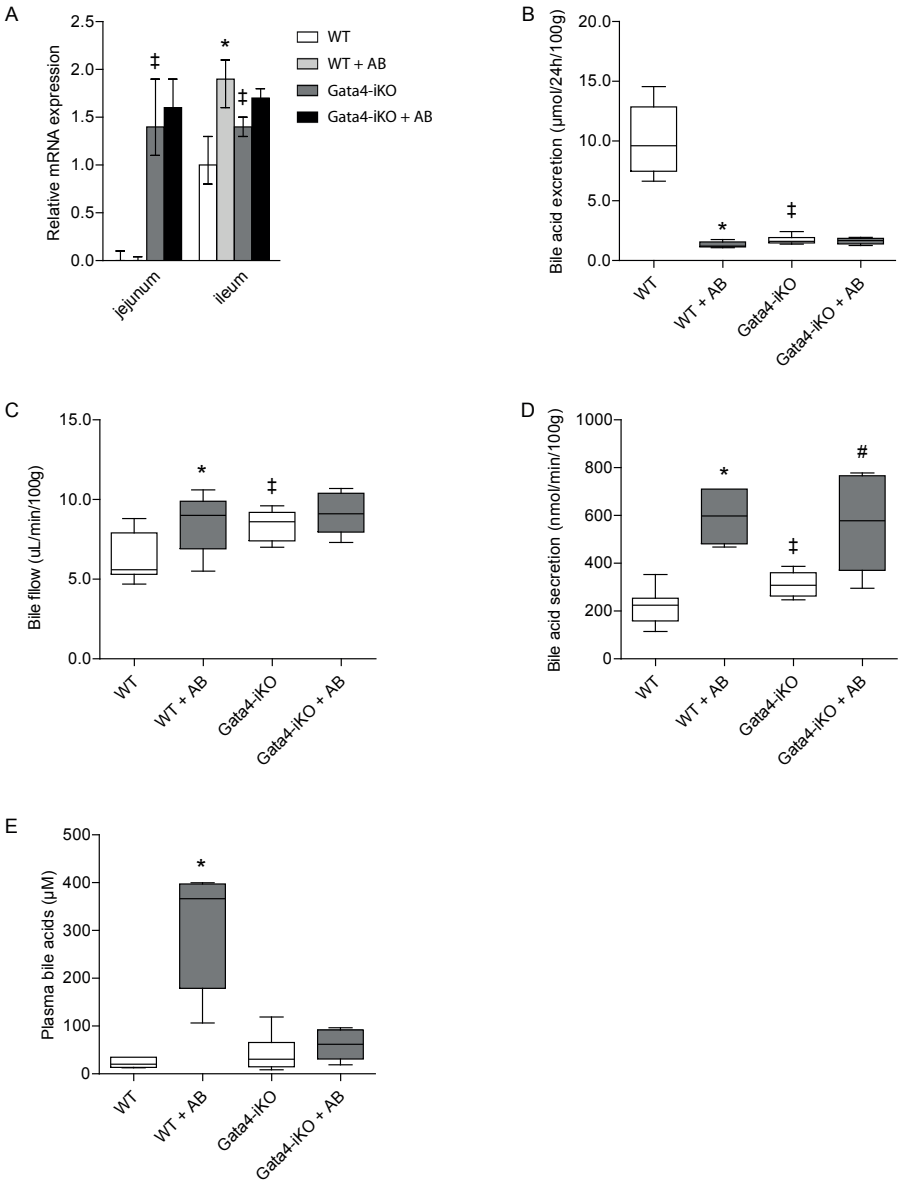


Figure 4. Bile acid homeostasis in Gata4-iKO mice upon antibiotic treatment. (A) Relative mRNA expression of *Asbt* in different segments of the small intestine from wildtype and Gata4-iKO mice. (B) Fecal excretion of BAs (µmol/24h/100g bodyweight). (C) Bile flow (µL/min/100g bodyweight). (D) Biliary BA secretion (nmol/min/100g bodyweight) in wildtype and Gata4-iKO mice. (E) Total plasma BA levels (µM). Median ± range; n = 6-7/group; * $p < 0.05$ WT vs. WT + AB; ‡ $p < 0.05$ WT vs. Gata4-iKO; # $p < 0.05$ Gata4-iKO vs. Gata4-iKO + AB.

Next, Gata4-iKO mice were treated with antibiotics. Antibiotic treatment influenced neither *Asbt* expression nor fecal BA excretion in Gata4-iKO mice (Figure 4A/B). Bile flow and plasma BA levels were also unaffected by antibiotic treatment in Gata4-iKO mice (Figure 4C/E). However, biliary BA secretion was induced by antibiotic treatment in Gata4-iKO mice (Figure 4D). When we looked at individual BAs, we found that this increase was mainly due to increased biliary secretion of β-MCA (Supplemental figure 3). The induction of β-MCA secretion upon antibiotic treatment was 4.5-fold in WT mice and 1.6-fold in Gata4-iKO mice; some of the β-MCA detected in Gata4-iKO mice was present in the form of Δ22-β-MCA. Antibiotic treatment of WT mice greatly increased the biliary secretion of CA, which was unchanged in antibiotic-treated Gata4-iKO mice.

Biliary cholesterol and phospholipid secretion were higher in Gata4-iKO mice than in wildtype animals, but were not induced upon antibiotic treatment (data not shown). Taken together, most of the effects seen in WT mice treated with antibiotics were absent in Gata4-iKO mice.

DISCUSSION

The regulation of BA homeostasis in mammals is a complex process regulated via extensive cross-talk between liver, intestine and intestinal microbiota. The role of bacterial metabolism was noted back in the early 1980s, when several studies reported that BA kinetics change considerably in germfree rats (9-12, 16, 23). After a long standstill, interest in the underlying regulatory mechanisms has seen a revival, especially over the past three years.

In this study we have shown that under physiological conditions gut microbiota inhibit enterohepatic recycling of BAs. One hypothesis regarding the effect of gut microbiota on BA kinetics involves interference with the activation of the BA receptor Fxr. Sayin *et al.* recently argued that the shift in BA composition to β-muricholate antagonizes intestinal Fxr, leading to a decrease in ileal *Fgf15* expression and an increase in BA synthesis (24). Hu *et al.* have also recently postulated that α and β-muricholate antagonize Fxr (25). However, when Miyata *et al.* treated *Fxr* knock-out mice with ampicillin, they reported increased *Asbt* expression, decreased fecal BA excretion, increased levels

of portal BAs and decreased *Fgf15* expression (13, 14), supporting an Fxr-independent pathway for the changes in BA metabolism seen upon antibiotic treatment.

In the present study we investigated the effects of gut microbiota on BA physiology in more detail. Our data confirm that fecal BA excretion is decreased considerably in germfree animals and antibiotic-treated mice. We speculate that induction of Asbt expression throughout the ileum and expression in more proximal parts of the intestine, contribute to more efficient absorption of BAs from the intestinal lumen, resulting in elevated plasma BA levels, increased biliary BA secretion and decreased fecal BA excretion. Absorption of BAs in proximal enterocytes induced BA-responsive genes proximally, whereas distal enterocytes may encounter fewer BA molecules than usual, leading to decreased activation of Fxr and lower expression of BA-responsive genes in the distal small intestine.

The fact that we observed increased hepatic expression of *Cyp7a1* following antibiotic treatment suggested an increase in BA synthesis. However, the data presented in our study, as well as data presented by Miyata *et al.* and Sayin *et al.,* show decreased fecal BA excretion in antibiotic-treated or germfree mice, indicating a decrease in BA synthesis. Both studies also speculated that intestinal BA reabsorption may be enhanced, contributing to the enhanced total BA pool size (14, 24). Other studies in germfree animals have also reported increases in the uptake of tCA in the ileal epithelium (26) and in the half-life of ^{14}C-labeled tauro-cholic acid (12), indicating enhanced BA reabsorption and decreased turnover. The "classic view" on BA homeostasis implies that the BA pool size is maintained by BA synthesis, which, under steady-state conditions, compensates for fecal BA loss. Therefore, despite increased hepatic *Cyp7a1* expression, the strongly decreased fecal BA excretion rate in the face of increased intestinal Asbt expression, biliary BA secretion and circulating pool, command the conclusion that antibiotic treatment induces more effective intestinal BA conservation, instead of inducing BA synthesis (Supplemental figure 4). When we performed cholate kinetic studies in antibiotic-treated mice to validate the direct relationship between fecal excretion and BA synthesis once more, we saw a decrease in cholate synthesis, confirming that antibiotic treatment inhibited BA synthesis. One might question whether animals treated with antibiotics are in a steady state, however germfree mice certainly are.

The homeostasis of cholesterol, the substrate for BA synthesis, has also been studied. In germfree animals hepatic cholesterol synthesis from labeled acetate is only 13% of

that of conventional animals (27). When germfree rats are injected with cholesterol-26-^{14}C they expire 50% less ^{14}C as $^{14}C0_2$ than do conventional rats, with higher specific activities in plasma and liver (28). Also, 7α-hydroxylation of [4-^{14}C]-cholesterol and 5α-reduction and 12α-hydroxylation of the BA precursor 7α-hydroxy-4-cholesten-3-one are lower in germfree rats (29, 30). These findings are in accordance with germfree animals having slower cholesterol turnover than conventional animals. Although at the time no mechanism was known for the differences observed between germfree and conventional animals, such valuable data should not be forgotten.

Clearly, in the antibiotic-treated and germfree animals there is a discrepancy between *Cyp7a1* mRNA expression and actual *in vivo* BA synthesis. If synthesis was in fact increased in these mice, along with enhanced intestinal BA reabsorption and decreased fecal excretion, then the BA pool would also be permanently increased. Previous studies on BA synthesis under different conditions have shown that altered synthesis of BAs is not always correlated with changes in *Cyp7a1* mRNA expression (31-33). In a recent paper on *Apobec-1* – an enzyme involved in the production of apolipoprotein B48 – *Cyp7a1* mRNA expression was decreased by approximately 75% in *Apobec-1*$^{-/-}$ mice, while no difference in fecal BA excretion was found (34). The authors suggested that post-transcriptional regulation of *Cyp7a1* expression and alteration in *Cyp7a1* mRNA stability accounted for the changes observed in *Apobec-1*$^{-/-}$ mice. These findings underscore the importance of physiological measurements – along with data on gene expression – to properly study the enterohepatic circulation of BAs *in vivo*.

Thus, increased intestinal BA reabsorption due to enhanced Asbt expression leads to a compensatory decrease in BA synthesis. The question then arises how the microbiota might regulate Asbt expression. Previously, Annaba *et al.* suggested a link between certain intestinal bacterial species and regulation of Asbt (17). Post-transcriptional regulation of Asbt has also been suggested (35). Our data confirm the importance of Asbt for the microbiota-induced alterations in BA homeostasis. The fact that a small effect remained in Asbt-KO mice treated with antibiotics could be related to the massive flow of BAs into the colon in the absence of Asbt, and hence increased uptake of these BAs across the colonocytes. This Asbt-independent BA absorption was also seen in untreated Asbt-KO mice, reflected in the higher amounts of circulating secondary BAs in these mice, DCA in particular. However, we cannot exclude the fact that DCA may also be taken up by the small intestine after coprophagic recycling of the excreted

BAs. Taken together, Asbt is important in the interaction between the bacterial flora and BA reabsorption.

Asbt expression is thought to be primarily controlled by the transcription factor Gata4, which plays an important role in maintaining jejunal-ileal differences in absorptive enterocyte gene expression and restricts expression of Asbt to the terminal ileum (7, 18, 20, 22). Studies in mice have shown that intestinal *Gata4* deletion induces *Asbt* expression and BA absorption in the proximal small intestine, leading to tauro-β-muricholate enrichment of the BA pool (7). This phenotype shows a striking resemblance to that seen in our experiments with antibiotic treatment and it should also be noted that others have previously linked enterocyte Gata4 to the commensal flora (21). We now show that antibiotic treatment in Gata4-iKO mice failed to elicit the changes on *Asbt* expression, fecal BA excretion, bile flow rate and plasma BA concentrations that were observed in wildtype mice. Therefore, we postulate that under physiological conditions gut microbiota stimulate Gata4-dependent Asbt repression throughout the intestine expect in the terminal ileum – since Gata4 is not expressed here – thereby restricting BA reabsorption to this part of the small intestine.

The exact mechanism by which intestinal bacteria regulate Gata4 remains to be elucidated. Shulzhenko *et al.* recently proposed a molecular switch between Gata4-dependent metabolic and interferon-dependent immune processes within epithelial cells, influenced by gut microbiota (21). Munakata *et al.* have found that the absence of gut mirobiota induces the expression of several interferon regulatory factors, as well as IFN-α-inducible genes and IFN receptors. The authors suggested that the regulation of this small subset of genes reflects an adaptive response of the immune system to prevent excessive inflammation during continuous exposure to commensal bacteria (36). In line with these studies, the lack of bacteria-derived signals in germfree animals or following antibiotic treatment may change anti-inflammatory responses in these animals. This may shift the balance of enterocyte metabolism at the expense of Gata4-dependent metabolic functions, ultimately leading to diminished inhibition of Asbt expression and increased BA reabsorption.

Since BA and cholesterol metabolism are tightly coupled, more efficient recirculation of BAs could result in slower rates of cholesterol catabolism for *de novo* BA synthesis. In fact, germfree rats are known to accumulate more cholesterol than their conventional counterparts (10). Long-term antibiotic treatment may therefore have negative effects

on plasma cholesterol levels, which are a major risk factor for cardiovascular diseases. Conversely, manipulation of gut microbiota by administration of probiotics is known to significantly reduce cholesterol levels in mice and man, and prevents hypercholesterolemia in mice fed a fat-enriched diet (37-40). It is therefore tempting to speculate that specific probiotics might reduce cholesterol levels via eliciting benign stimuli that downregulate immune responses. Such an immune downregulation may increase Gata4-dependent metabolic functions, which inhibits Asbt expression and promotes BA excretion, thereby increasing cholesterol catabolism for *de novo* BA synthesis. A further possible implication of our findings is that it may be possible to induce Asbt expression in the proximal small intestine by interventions that promote specific intestinal bacteria. Such interventions may be useful in the development of therapies for patients with BA malabsorption and associated maldigestion of fat due to ileal disease or resection.

Taken together, we demonstrate that antibiotic treatment induced a strong increase in the expression of Asbt in the ileum and a shift in expression in the direction of the jejunum. We saw an increase in enterohepatic cycling of BAs, resulting in decreased fecal BA excretion, higher levels of β-muricholate and cholate in the BA pool, and increased biliary BA secretion. As a consequence, hepatic BA synthesis is reduced. Regulation of Asbt expression by Gata4 is crucial in mediating the effects that intestinal microbiota exert on host BA reabsorption.

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