Interplay between dietary fibers and gut microbiota for promoting metabolic health

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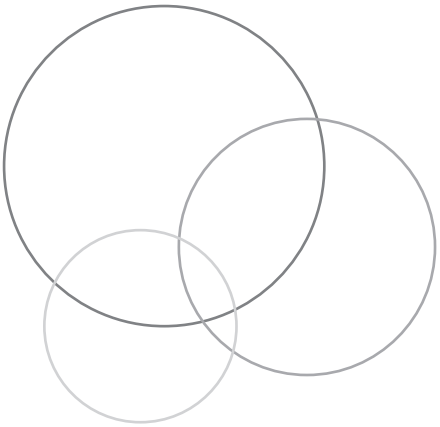

Chapter 4

Reverse cholesterol transport is increased in germ-free mice

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Abstract

The intestinal microbiota is emerging as clinically relevant modulator of atherosclerotic risk. Reverse cholesterol transport (RCT) is an atheroprotective metabolic pathway. How the microbiota impacts RCT has not been investigated. Therefore, the aim of the present study was to characterize (chole)sterol metabolism and RCT in germ-free compared with conventional mice. In chow-fed germ-free mice plasma cholesterol was unchanged, while liver cholesterol content was higher (1.5-fold, $P<0.05$) than in conventional controls. Biliary secretion of cholesterol (2-fold, $P<0.001$) and bile acids (3-fold, $P<0.001$) was substantially increased in the germ-free model, while fecal neutral sterol excretion was unaltered and fecal bile acid excretion was decreased ($P<0.01$). However, fecal bile acid profiles of germ-free mice were dominated by the presence of β-muricholic acid ($P<0.001$), pointing towards a higher contribution of the alternative acidic pathway to total bile acid synthesis in these mice. As expected, secondary bile acids were absent in the germ-free model. *In vivo* macrophage-to-feces RCT was increased more than 2-fold ($P<0.01$) in the absence of intestinal bacteria. These data demonstrate that absence of the intestinal microbiota stimulates RCT more than 2-fold. Thereby, our results support the importance of intestinal bacteria for metabolic regulation and indicate that specific targeting of the microbiota bears therapeutic potential to prevent and treat cardiovascular disease (CVD).

Introduction

Evidence is accumulating that the intestinal microbiota has a substantial impact on the (patho)physiological regulation of metabolism. The human microbiota in general represents not only the first line of contact with the environment, but intestinal bacteria in particular also express approximately 100-fold more genes than present in the human genome.[1] The intestine is one of the key organs in the regulation of cholesterol metabolism with relevance for atherosclerotic cardiovascular disease (CVD). Enterocytes are responsible for cholesterol absorption, can synthesize cholesterol and form HDL particles.[2] As a precedence for the impact of bacteria on CVD it was demonstrated that specific diet-microbe-host interactions can enhance experimental atherosclerosis via the coordinate production of the pro-atherosclerotic metabolite trimethylamine-N-oxide (TMAO).[3] Subsequently, TMAO was also identified as a prospective biomarker for the future development of CVD events in the general population, further stressing the relevance of the microbiota for human disease.[4] In addition, bacteria can modulate bile acid metabolism and thereby impact cholesterol turnover.[5] In conventional mice, the intestine was shown to contribute to reverse cholesterol transport (RCT), a key atheroprotective pathway.[6] However, the impact of the intestinal microbiota *per se* on RCT has not been determined. Therefore, the aim of the present work was to establish the importance of the intestinal microbiota for RCT by comparing conventional with germ-free mice.

Materials and Methods

Experimental animals

Wildtype conventional C57BL/6 mice were obtained from Harlan (Horst, The Netherlands). Wildtype germ-free C57BL/6 mice were generated in our animal facility and housed in sterile flexible gnotobiotic isolators. All animals were housed in controlled rooms with an alternating 12h light-dark cycle. Mice were fed standard chow diet (Ssniff, Germany). All experiments were approved by the Committee of Animal Experimentation at the University of Groningen and performed in accordance with Dutch National Law on Animal Experimentation and international guidelines on animal experimentation.

Determination of plasma and liver lipid analysis

At the time of termination, liver and blood were collected by heart puncture. Plasma total cholesterol and triglycerides were measured using commercially available reagents

(Roche Diagnostic, Basel, Switzerland). Pooled plasma of each group was subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Health, Uppsala, Sweden) essentially as described previously.[1] Livers were homogenized and lipids extracted following the general procedure described by Bligh & Dyer.[2] Lipids were redissolved in water containing 2% Triton X-100. Total cholesterol was measured as detailed above.

Bile measurements

Continuous bile cannulation was performed under anesthesia (hypnorm 1 ml/kg body weight; diazepam 10 mg/kg body weight) for 20 minutes. Respective biliary bile acid and cholesterol concentrations were determined and secretion rates calculated as described previously.[1]

Fecal sterol and bile acid analysis

Fecal samples from individually housed mice collected over 24 hours were dried, weighed and ground. Neutral sterols and bile acids were extracted and measured using gas-liquid chromatography as published.[1]

Macrophage-to-feces RCT studies

C57BL/6 donor mice were used to harvest primary peritoneal thioglycollate-elicited macrophages.[3] Macrophages were loaded *in vitro* with 5μg/ml acetylated LDL and 3μCi/ml [^{3}H] cholesterol (Perkin Elmer, Boston, MA) for 24 hours to become foam cells.[3] Then macrophages were injected into individually housed recipient mice. At indicated time points plasma, liver and fecal recovery of labeled cholesterol was analyzed using liquid scintillation counting (Packard 1600CA Tri-carb, Packard, Meriden, CT). For this purpose a piece of liver was solubilized in Solvable (Packard).[4] Fecal neutral sterol and bile acid fractions were extracted from feces as described above.[2] The counts were expressed relative to the injected dose.

Hepatic gene expression analysis

Hepatic mRNA was extracted using TriReagent (Sigma) and quantified with a Nanodrop ND-100UV-vis spectrometer (NanoDrop Technologies Wilmington DE). cDNA was made from 1μg of RNA using reagents from Invitrogen (Carlsbad CA). Primers were synthesized by Eurogentec (Seraing, Belgium). Real-time PCR

was performed using an ABI Prism 7700 machine (Applied Biosystems, Damstadt Germany). mRNA expression levels of individual genes were calculated relative to the housekeeping gene *36B4* and normalized to the relative mean expression level of the respective control group.

Statistics

Statistical analysis was performed using GraphPad Prism software (San Diego, CA). All data are presented as mean ± SEM. Differences between groups were determined using the Mann-Whitney U-test. P-values below 0.05 were considered statistically significant.

Results

First, we characterized cholesterol metabolism in germ-free mice compared with conventional controls. Both groups had similar body weights 21.3±1.1 vs 21.4±1.5 g. While plasma total cholesterol (Fig. 1A) and triglyceride levels (0.26±0.02 vs 0.28±0.02 mmol/l) were comparable between groups, FPLC profiles showed a discrete shift towards higher LDL-C and lower HDL-C in germ-free animals (Supplementary Fig. IA). HDL cholesterol efflux capacity did not differ between groups (Supplementary Fig. IB). Hepatic cholesterol content was higher in germ-free mice by 1.5-fold (5.90±1.25 vs. 4.23±0.73 nmol/mg, $P<0.05$,) and in agreement with these data, expression of the sterol regulatory element-binding protein (*Srebp2)* target genes low density lipoprotein receptor (*Ldlr)* (Table 1, $P<0.001$) and HMG-CoA reductase (*Hmcg-Coar)* (Table 1, $P<0.05$) was decreased.

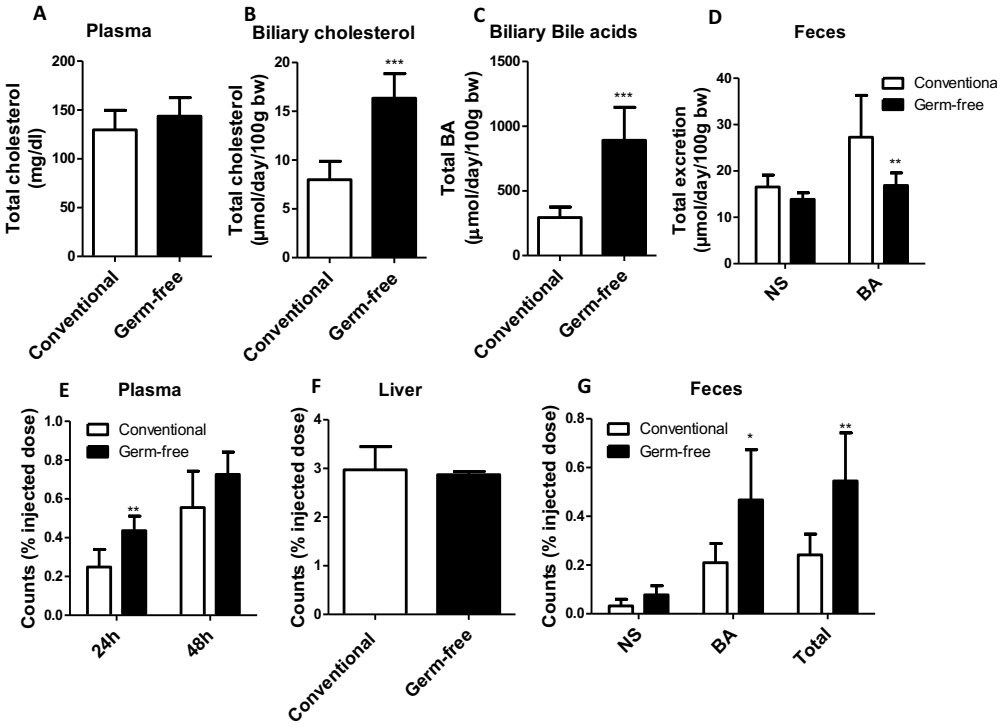

Figure 1: Absence of intestinal microbiota stimulates macrophage-to-feces reverse cholesterol transport. Mass measurements (A) Plasma total cholesterol; (B) biliary cholesterol secretion; (C) total biliary bile acid (BA) secretion; (D) fecal mass neutral sterol (NS) and bile acid excretion; RCT experiment, macrophage-derived cholesterol tracer recovered in (E) plasma; (F) liver; (G) fecal neutral sterols and bile acids. Data are presented as means ± SD, N=7 for each group. Statistically significant differences are indicated as *P<0.05; **P<0.01, ***P<0.001.

Biliary cholesterol and bile acid secretion was about 2- and 3-fold increased, respectively, in mice lacking microbiota (P<0.001, Fig. 1B and 1C). Fecal output was significantly increased in germ-free mice (213±26 vs 162±12 mg/day, P<0.05). However, fecal excretion of neutral sterols remained unchanged, while bile acid excretion was reduced by 1.5-fold (Fig. 1D, P<0.01). Increased mRNA expression of Nieman-Pick C1-like1 (*Npc1l1)* in the proximal small intestine (Table 1, p<0.05) indicated increased cholesterol absorption in germ-free mice.

Next, a RCT experiment was performed. Plasma ^{3}H-cholesterol tracer recovery was significantly increased by about 2-fold in germ-free mice after 24h (P<0.01, Fig. 1E) and tended to be higher at 48h. Tracer recovery in the liver at 48h remained unchanged (Fig. 1F). Overall RCT, determined as fecal recovery of macrophage-derived ^{3}H-cholesterol, was 2-fold higher in germ-free mice (Fig. 1G, P<0.01,). This increase

was largely due to significantly more tracer recovered in fecal bile acids (P<0.01). Stress could potentially explain these findings[7], but plasma corticosterone levels were comparable between conventional and germ-free mice making this possibility less likely (1466±219 vs 1496±159 nmol/l, respectively).

Table 1: Gene expression analysis in conventional and germ-fee mice

Genes	**Conventional**	**Germ-free**
Liver		
Hmgcoar	1.00±0.08	0.83±0.05*
Cyp7a1	1.00±0.42	0.26±0.14***
Cyp8b1	1.00±0.29	0.36±0.15**
Cyp27a1	1.00±0.15	0.84±0.13
Abcg5	1.00±0.19	1.06±0.08
Abcg8	1.00±0.27	1.18±0.06
Bsep	1.00±0.25	0.75±0.24*
Srb1	1.00±0.11	0.95±0.15
Fxr	1.00±0.26	0.89±0.19
Ldlr	1.00±0.12	0.68±0.07***
Proximal intestine		
Npc1l1	1.00±0.09	1.75±0.08*
Distal intestine		
Fgf15	1.00±0.15	2.61±0.30*

*Data are presented as means ± SD, N=7 for each group. Statistically significant differences are indicated as *P<0.05, **P<0.01, ***P<0.001.*

Therefore, we also characterized bile acid metabolism under germ-free conditions more in detail. Bile flow was significantly increased compared to conventional mice (2-fold, P<0.001, Supplementary Fig. IIA). Although fecal mass excretion of bile acids was lowered, there were striking shifts in the fecal bile acid profile in germ-free mice. As expected, secondary bile acids such as deoxycholic acid (DCA) and ω-muricholic acid (ω-MCA) were absent. On the other hand, the percentage of fecal cholic acid (CA) was higher (P<0.05) and the contribution of β-muricholic acid (β-MCA) was increased by 4.5-fold in mice lacking microbiota (P<0.001, Supplementary Figure IIA & B). Quantification of hepatic bile acid synthesis gene expression (Table 1) revealed decreased *Cyp8b1* (P<0.01) and *Cyp7a1* (P<0.001) mRNA levels in germ-free mice, whereas *Cyp27a1*, which initiates the alternative acidic pathway with β-MCA as end

product, remained unchanged. mRNA expression of *fibroblast growth factor 15 (Fgf15)* in the terminal ileum was 2.6-fold increased in the germ-free model (Table 1, $P<0.05$).

Discussion

The results of the present study demonstrate that complete absence of the microbiota (i) does not influence plasma cholesterol levels or mass fecal neutral sterol excretion, (ii) decreases fecal BA excretion and (iii) significantly increases RCT, mainly within the fecal BA fraction. Previous work indicated that differences in cholesterol metabolism between germ-free and conventional mouse models are variable; the observed phenotypes conceivably depend on diet, genetic background and the respective composition of the microbiota in the conventional control groups.[8-10] Thus far, decreased plasma cholesterol, both increased as well as decreased liver cholesterol and higher fecal neutral sterol output were observed in germ-free mice fed Western-type or high-fat diets.[10,11] Interestingly though, the most significant differences between conventional and germ-free mice in our experimental system were seen in bile acids.

The changes in BA metabolism occurring in the germ-free mice are, however, rather complex; on the one hand, there is increased biliary BA secretion, on the other decreased fecal excretion. This difference can be explained by increased BA reabsorption in the terminal ileum of germ-free mice, a notion in general consistent with the increased expression of the farnesoid X receptor (FXR) target gene *Fgf15* that we observed in our study. However, the 2.6-fold increase in *Fgf15* expression in the germ-free model also indicates another relevant change in BA metabolism in these mice. Mice lacking intestinal bacteria have two principal BA species, the more hydrophobic taurocholic acid (TCA), which is a FXR agonist[12], and the hydrophilic tauro-beta-muricholic acid (T-β-MCA), which has been characterized as a FXR antagonist[13,14]. Increased expression of the FXR target gene *Fgf15* indicates thus that relatively more TCA, the FXR agonist, is taken up over T-β-MCA, the FXR antagonist. Indeed, previous work demonstrated a substantially higher affinity of ASBT, the transporter responsible for intestinal BA reuptake, for TCA compared with T-β-MCA[15]. Since due to the absence of bacteria no secondary BA are formed in germ-free mice there is no means of taking up conversion products of T-β-MCA in the colon, thus resulting in substantial amounts of T-β-MCA being excreted into the feces. In agreement, hepatic gene expression analysis indicated that the expression of Cyp27a1 is unchanged, pointing towards a higher relative contribution of the alternative acidic bile acid synthesis pathway with its end product T-β-MCA in the germ-free mice. It is thus to be expected that in the absence of intestinal bacteria

relatively more macrophage-derived cholesterol is converted into T-β-MCA, which is then preferentially excreted into the feces. These mechanisms can in our view explain how increased RCT mainly in the BA fraction occurs in germ-free mice with decreased mass fecal BA excretion. However, more experimentation seems required to fully substantiate this proposed model.

Further, it has to be noted that converting chenodexycholic acid (CDCA) into β-MCA occurs in mice but not in humans.[16] Therefore, further studies are needed to investigate, if the results of our current study can be translated to a clinical setting in humans. In addition, also regarding bile acid metabolism in germ-free mice variable data have been generated, likely dependent on genetic background and diet; e.g. unchanged as well as decreased fecal bile acid excretion were reported.[8,14] Clearly, also with respect to this issue, more studies are needed to better characterize the response of different germ-free mouse lines to varying experimental conditions. In summary, our present work supports the importance of the intestinal microbiota for metabolic regulation and extends previous observations to RCT, a pathway with a high relevance for atherosclerosis protection. Specific targeting of the intestinal microbiota with the aim to modulate bile acid metabolism bears therapeutic potential with the goal to prevent and treat CVD.

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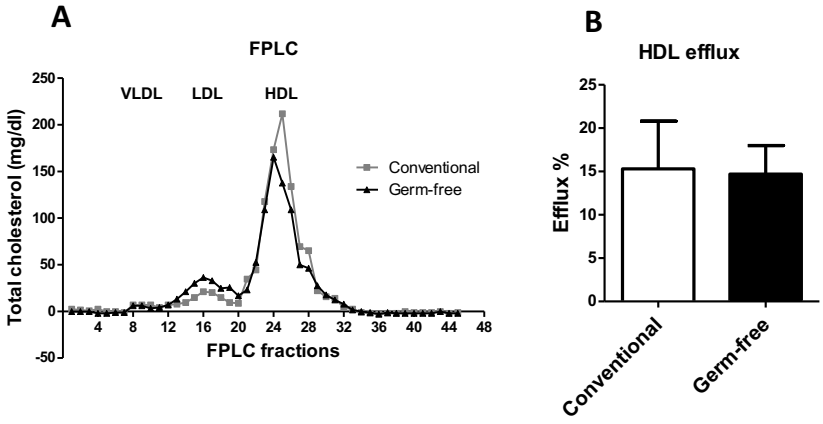
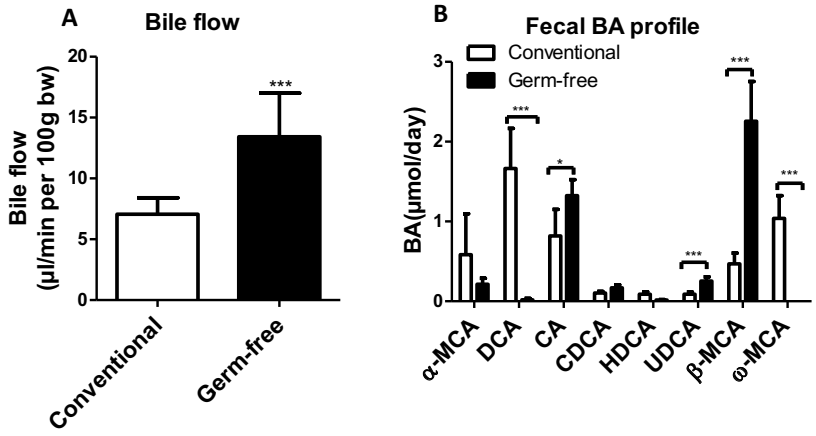
Supplemental Figures



Supplementary Figure I: FPLC profiles (A) and HDL cholesterol efflux (B) in conventional and germ-free mice. Data are presented as means ± SD, N=7 for each group.



Supplementary Figure II: Bile flow (A) and fecal bile acid profiles (B) in germ-free and conventional mice. α-MCA, α-muricholic acid; DCA, deoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; HDCA, hyodeoxycholic acid; UDCA, ursodeoxycholic acid; β-MCA, βmuricholic acid; ω-MCA, ω-muricholic acid. Data are presented as means ± SD. N=7 for each group. Statistically significant differences are indicated as *P<0.05; **P<0.01, ***P<0.001.