Chapter 5

Absence of intestinal microbiota increases ß-cyclodextrin stimulated reverse cholesterol transport

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Abstract

Non-digestible oligosaccharides are used as prebiotics for perceived health benefits, among these modulating lipid metabolism. However, the mechanisms of action are incompletely understood. The present study characterized the impact of dietary ß-cyclodextrin (ßCD, 10%, w/w), a cyclic oligosaccharide, on sterol metabolism and reverse cholesterol transport (RCT) in conventional and also germ-free mice to establish dependency on metabolism by intestinal bacteria. In conventional ßCD-fed C57BL/6J wild-type mice plasma cholesterol decreased significantly (-40%, $p<0.05$), largely within HDL, while fecal neutral sterol excretion increased (3-fold, $p<0.01$) and fecal bile acid excretion was unchanged. Hepatic cholesterol levels and biliary cholesterol secretion were unaltered. Changes in cholesterol metabolism translated into increased macrophage-to-feces RCT in ßCD-administered mice (1.5-fold, $p<0.05$). In germ-free C57BL/6J mice ßCD similarly lowered plasma cholesterol (-40%, $p<0.05$). However, ßCD increased fecal neutral sterol excretion (7.5-fold, $p<0.01$), bile acid excretion (2-fold, $p<0.05$) and RCT (2.5-fold, $p<0.01$) even more substantially in germ-free mice compared with the effect in conventional mice. In summary, this study demonstrates that ßCD lowers plasma cholesterol levels and increases fecal cholesterol excretion from a RCT-relevant pool. Intestinal bacteria decrease the impact of ßCD on RCT. These data suggest that dietary ßCD might have cardiovascular health benefits.

Introduction

Atherosclerotic cardiovascular disease (CVD) represents the major cause of death worldwide.[1] Drug treatment, primarily with statins, is currently the mainstay of therapy to prevent heart attacks and strokes. However, despite the vast use of statins, the incidence of CVD events has decreased at best between 25-30%.[2,3] Therefore, additional intervention strategies are warranted. In recent years non-digestible oligosaccharides have received increasing interest for their perceived health promoting properties.[4,5] Thereby, nutritional interventions have the advantage of being a complementary and cost effective approach in addition to drug treatment. ß-cyclodextrin (ßCD) is one such non-digestible dietary carbohydrate with proposed cholesterol metabolism modulating properties.[6,7] It is primarily produced from starch through an enzymatic reaction and consists of cyclic oligosaccharides with seven glucopyranosyl units linked in a ring structure. Thereby, ßCD has a high affinity to bind hydrophobic molecules such as cholesterol.[8]

Apart from cholesterol binding, the intestinal microbiota supposedly ferments the non-digestible ßCD for producing bioactive metabolites such as short-chain fatty acids (SCFA).[9,10] However, the extent to which intestinal bacteria are required to mediate the potential lipid modulating effects of ßCD is not known. Furthermore, it has not been investigated if the effects of ßCD on cholesterol metabolism extend to the atheroprotective pathway of reverse cholesterol transport (RCT), i.e. mobilization of cholesterol from macrophage foam cells for final excretion from the body *via* the feces.

Therefore, the aim of the present study was to characterize the impact of dietary administered ßCD on cholesterol metabolism and RCT in the presence or absence of an intestinal microbiota. Our data demonstrate that ßCD reduces plasma cholesterol and effectively increases fecal sterol excretion and RCT in conventional as well as in germ-free mice. Interestingly, the RCT-promoting effect of ßCD was more pronounced in the absence of intestinal bacteria.

Materials and Methods

Experimental animals and diets

Female conventional C57BL/6 mice (N=6) were obtained from Harlan (Horst, The Netherlands). Age-matched wildtype germ-free (GF) C57BL/6 mice (N=4) were generated and maintained in our animal facility. Mice were housed in temperature controlled rooms with alternating 12 h light-dark cycles. GF C57BL/6J mice were maintained in sterile flexible gnotobiotic isolators. GF status was verified regularly by PCR for bacterial 16S rDNA within fecal samples. All animal experiments were approved

by the Committee of Animal Experimentation at the University of Groningen and were performed in accordance with the Dutch Law on Animal Experimentation as well as international guidelines on animal experimentation. Mice were randomly assigned to two different diets for a period of 14 days in a parallel design with a control diet and a 10% ßCD (Roquette, Lastrem, France) supplemented experimental diet (Safe, Augy, France). Diets were sterilized with γ-irradiation (50 kGy). The control diet (given as wt/wt %) contained 60.94% corn starch, 0.06% cholesterol, 20% caseinate, 0.3% L-cystine, 7% carbohydrate mix, 7% soya bean oil, 0.2% choline bitartrate, 3.5% mineral mixture, 1% vitamin mixture. The experimental diet contained 10% (w/w) βCD replacing an equal amount of corn starch with otherwise identical composition characteristics.

Bile collection and analysis of bile acid, cholesterol and phospholipid secretion

The gallbladder was cannulated under anesthesia (hypnorm 1 ml/kg body weight (bw); diazepam 10 mg/kg bw). Bile was collected for 20 minutes and the rate of secretion was determined gravimetrically. Biliary bile salt, cholesterol and phospholipid concentrations were analyzed and the respective rates of biliary secretion determined as described previously.[11]

Plasma lipid and lipoprotein analysis

Blood was collected by heart puncture at the time of termination and immediately placed on ice. After isolation of plasma, aliquots were stored at -80°C until further analysis. To measure plasma total cholesterol and triglycerides commercially available reagents were used (Roche Diagnostic, Basel, Switzerland). Lipoprotein fractions were obtained from pooled plasma samples subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Health, Uppsala, Sweden) as described.[12]

Mass fecal sterol and bile acids excretion

Mice were housed individually and feces were collected over a 24 h period. Fecal samples were dried, weighed and ground. Neutral sterols and bile acids were extracted from 50 mg of feces and measured using gas-liquid chromatography as published.[12] Bile acids were methylated with a mixture of methanol and acetyl chloride and trimethylsilytated with pyridine, N, O-Bis (trimethylysilyl) trifluoroacetamide and trimethylcholorosilane.

Analysis of hepatic lipid composition

Liver was excised at the time of termination and homogenized. Lipids were extracted from the homogenates using the Bligh and Dyer procedure and redissolved in water containing 2% Triton X-100. Commercially available kits were employed to measure total cholesterol and triglyceride content (Roche Diagnostics).

Macrophage-to-feces RCT studies

Primary peritoneal macrophages elicited by thioglycollate were harvested from C57BL/6J donor mice followed by loading *in vitro* with 50 µg/ml acetylated LDL and 3 µCi/ml [^{3}H] cholesterol (Perkin Elmer, Boston, MA) for 24 h to generate foam cells.[11] Then macrophages were injected into individually housed recipient mice fed the different diets. Counts were measured in plasma obtained at 24 and 48 h after macrophage injection using liquid scintillation counting (Packard 1600CA Tri-carb, Packard, Meriden, CT). Liver was collected at the time of termination (48 h), solubilized with Solvable (Packard) followed by liquid scintillation counting. Feces were collected at 24 and 48 h, dried, weighed and ground. Fecal neutral sterol and bile acid extraction was performed[13] and counts were determined in each fraction using liquid scintillation counting. Counts were expressed relative to the administered dose.

LCAT assay

Plasma LCAT activity was measured following the general procedure of Glomset and Wright with the following minor modifications.[14] Plasma samples were incubated with labelled [^{3}H] cholesterol substrate for 6 hours at 37 ˚C. Cold ethanol was added at the end to stop the reaction. Free and esterified cholesterol were separated using silica columns and hexane was used to elute [^{3}H] cholesteryl esters from the column. The samples were dried under a stream of nitrogen and counts were measured using liquid scintillation counting (Packard 1600CA Tri-carb, Packard, Meriden, CT). LCAT activity was expressed in arbitrary units (AU) in which 100 AU represents the equivalent of 87 nmol cholesterol esterified per ml plasma per hour.

HDL efflux assay

Primary peritoneal macrophages were harvested from control and ßCD fed C57BL/6J mice followed by loading *in vitro* with 50 µg/ml acetylated LDL and 1 µCi/ml [^{3}H] cholesterol (Perkin Elmer, Boston, MA) for 24 h to generate foam cells.[11] Overnight equilibration was performed by incubation with RPMI 1640 Glutamax medium

(Gibco, Carlsbad, CA) containing 2% BSA. Thereafter the cells were washed with PBS and incubated with 2% apoB-depleted plasma samples added in RPMI 1640 Glutamax medium containing penicillin/streptomycin.[15] After 5 h of efflux, the medium was collected and centrifuged for 5 min at 10,000 rpm; 0.1M NaOH was added to the cells and incubated for at least 30 min. The radioactivity in both medium and cells was determined using liquid scintillation counting (Packard 1600CA Tri-carb, Packard, Meriden, CT) and efflux calculated from as: efflux(%) = [counts in medium /(counts in cells + counts in medium)]*100.[15] Values of negative control wells without the addition of HDL were subtracted from all experimental data.

Cholesterol absorption studies and calculation of TICE

Fractional cholesterol absorption was measured in conventional control and βCD fed C57BL/6J mice with the plasma dual isotope ratio method using blood samples obtained after intravenous (D7) and oral (D5) administration of stable isotopically labelled cholesterol as described.[16] Trans-intestinal cholesterol efflux (TICE) was calculated according to the following formula: TICE = fecal cholesterol excretion – [% intestinal cholesterol absorption x (biliary cholesterol secretion + dietary cholesterol intake)].

Quantitative real-time PCR gene expression analysis

Total mRNA was extracted from the liver using TriReagent (Sigma) and quantified with a Nanodrop ND-100UV-vis spectrometer (NanoDrop Technologies Wilmington DE). One μg of mRNA was used for synthesizing cDNA with reagents from Invitrogen (Carlsbad CA). Real-time PCR was performed on an ABI Prism 7700 machine (Applied Biosystems, Damstadt Germany). The primers were synthesized by Eurogentec (Seraing, Belgium). The mRNA expression of each gene was calculated relative to the housekeeping gene cyclophilin and further normalized to the relative expression levels of the respective control groups.

Statistics

Statistical analysis was performed using GraphPad Prism software (San Diego, CA). Data are presented as median (interquartile range). Statistical differences between groups were assessed using the Mann-Whitney U-test. Statistical significance for all comparisons was assigned at $P<0.05$.

Results

ßCD lowers plasma cholesterol and increases fecal cholesterol excretion.

Feeding mice for 2 weeks with ßCD supplemented diet significantly reduced plasma cholesterol levels (P<0.05, Fig. 1A). FPLC profiles indicated a cholesterol decrease mainly in the HDL fraction with a slight shift towards smaller HDL particles (Fig. 1B). Correspondingly, plasma LCAT activity (Fig. 1C) and also hepatic *Lcat* mRNA expression (Table 1) were somewhat lower in the ßCD group, however, not significantly.

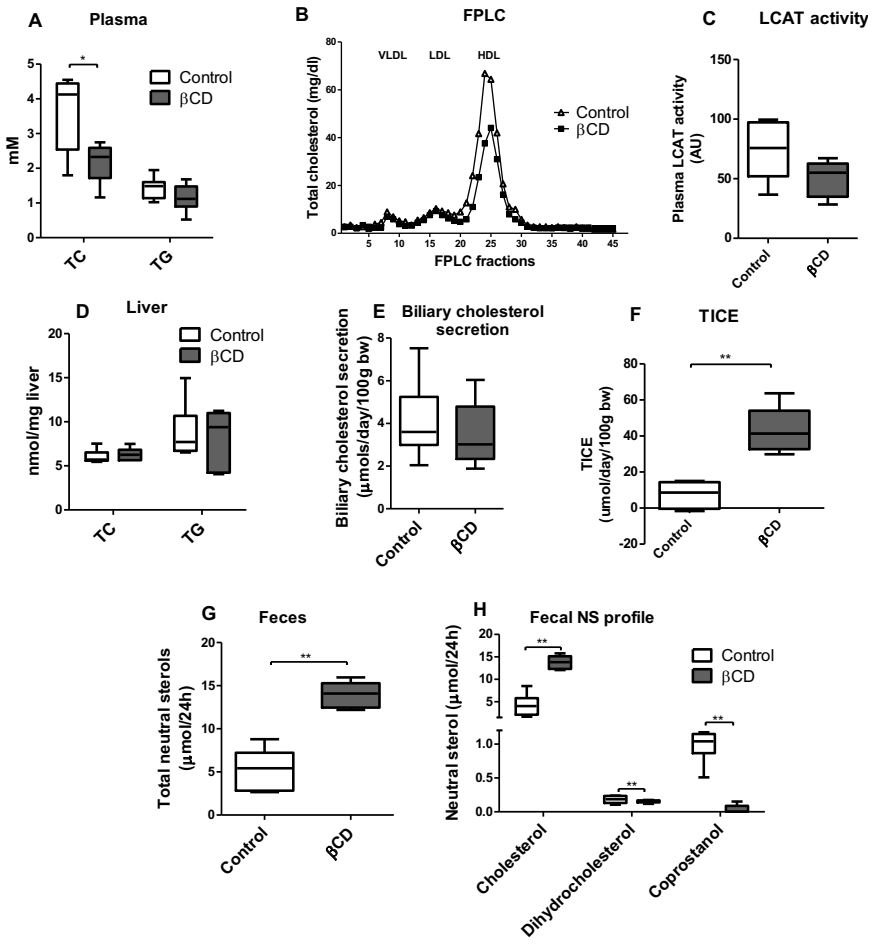

Figure 1: βCD reduces plasma cholesterol and increases fecal neutral sterol excretion. (A) plasma total cholesterol (TC) and triglycerides (TG); (B) FPLC profile; (C) LCAT activity (D) hepatic TC and TG; (E) biliary cholesterol secretion; (F) trans-intestinal cholesterol excretion (TICE); (G) fecal total neutral sterol (NS) excretion; (H) fecal NS profile. Data are presented as box plots showing median (interquartile range) and min/max; at least N=6 for each group. Statistically significant differences are indicated as *P<0.05; **P<0.01.

Hepatic cholesterol and triglyceride levels remained unchanged (Fig. 1D), as were biliary cholesterol secretion rates (Fig. 1E). ßCD fed mice exhibited unchanged hepatic mRNA expression of *Hmgcoar, Hmgcs1* expression was lower, but not significantly (Table 1) and plasma lathosterol:cholesterol ratios were higher in ßCD receiving mice compared to controls (0.0045 (0.0042-0.0054) *vs* 0.0100 (0.0095-0.0143), $p<0.05$) indicating that overall ßCD might increase endogenous cholesterol synthesis to a certain extent.

Table 1: Hepatic gene expression in conventional mice fed diet containing βCD

mRNA expression	**Control**	**βCD**
Hepatic gene expression		
Hmgcoar	1.00 (0.44-1.12)	1.08 (0.55-1.11)
Ldlr	1.00 (0.77-1.03)	1.62 (1.04-1.35)*
Fxr	1.00 (0.88-1.13)	1.28 (1.14-1.26)
Abcg5	1.00 (0.47-0.62)	1.12 (0.51-0.60)
Abcg8	1.00 (0.42-0.54)	1.05 (0.44-0.53)
Bsep	1.00 (0.70-0.82)	0.93 (0.60-0.69)
Srb1	1.00 (0.58-0.63)	1.00 (0.59-0.63)
Cyp7a1	1.00 (0.42-1.04)	1.44 (0.47-1.11)
Cyp8b1	1.00 (0.82-1.21)	0.55 (0.33-0.67)*
Cyp27a	1.00 (0.67-0.87)	0.88 (0.65-0.96)
Hmgcs1	1.00 (0.50-1.26)	0.48 (0.49-0.74)
Lcat	1.00 (0.52-1.07)	0.69 (0.57-0.65)
ApoE	1.00 (0.56-0.75)	0.91 (0.53-0.68)
Abca1	1.00 (0.55-0.67)	1.12 (0.53-0.72)
Abcg1	1.00 (0.50-0.75)	1.08 (0.56-0.83)
Scd1	1.00 (0.46-0.70)	1.40 (0.69-0.90)
*Pgc-1*β	1.00 (0.28-0.33)	1.77 (0.46-0.71)
Fasn	1.00 (0.70-1.56)	1.08 (0.89-2.36)
Gpat	1.00 (0.43-0.79)	0.84 (0.48-0.51)
Srebf-1c	1.00 (0.43-0.56)	1.56 (0.27-0.88)
Proximal small intestine		
Npc1l1	1.00 (2.44-3.91)	1.07 (2.42-3.97)
Abca1	1.00 (0.84-2.58)	0.32 (0.41-0.76)*

*Data are presented as median (interquartile range), at least N=6 for each group. Statistically significant differences are indicated as *P<0.05.*

ßCD increased total fecal neutral sterol excretion by approximately two-fold (P<0.01, Fig. 1G), mainly due to an increased excretion of cholesterol, whereas dihydrocholesterol and coprostanol were significantly reduced (Fig. 1H). To investigate the metabolic basis for the increased fecal neutral sterol excretion we first explored cholesterol absorption. *Npc1l1* mRNA expression in the small intestine was unchanged (Table 1) and also plasma campesterol:cholesterol (0.010 (0.010-0.012) *vs* 0.010 (0.009-0.010), respectively, n.s.) and ß-sitosterol:cholesterol (0.025 (0.023-0.026) *vs* 0.028 (0.026-0.031), respectively, n.s.) ratios were not different between the control and the ßCD group. However, fractional cholesterol absorption measured with the plasma dual isotope ratio method indicated a slight but not significant decrease in ßCD fed mice (48.3 (43.3-57.5) *vs* 40.9 (21.7-49.4) %) indicating that decreased absorption might contribute to increased fecal neutral sterol output. Next, we explored TICE, a pathway by which enterocytes can increase fecal neutral sterol excretion independent of the biliary pathway.[17] Our results indicated that TICE was significantly elevated in mice fed the ßCD containing diet by about seven-fold (P<0.01, Fig. 1F). Since TICE is stimulated by LXR agonists [16] and ßCD was suggested to stimulate LXR activity [18], we further explored the expression of relevant model transcriptional targets of LXR in addition to *Npc1l1*. Hepatic expression of *Abcg5, Abcg8 Abca1, Abcg1 and ApoE* remained unaffected by the ßCD diet, while *Abca1* expression in proximal small intestine was even downregulated in ßCD fed mice (P<0.05). Furthermore, macrophage mRNA expression of *Abca1* and *Abcg1* were not different between the groups (data not shown) and consistent with these data there was also no discernible difference in cholesterol efflux capacity between peritoneal macrophages isolated from control and ßCD fed conventional mice (10.3 (8.4-12.8) *vs* 13.5 (11.8-14.4) %, respectively).

ßCD does not affect total bile acid excretion but changes bile acid composition.

Total bile acids in plasma remained unaffected (Fig. 2A). However, the plasma bile acid profile revealed significant shifts in individual bile acid species. ßCD increased the percentage of circulating cholic, taurocholic, chenodeoxycholic, tauro-α-muricholic and ω-muricholic acid (Fig. 2B). On the other hand, plasma β-muricholic acid concentrations were significantly decreased. Biliary secretion of bile acids tended to be higher, while overall fecal bile acid excretion tended to be lower in the ßCD group (Fig. 2C and 2D). Particularly fecal deoxycholic and ω-muricholic acid excretion was significantly decreased (P<0.01, Fig. 1E). Liver mRNA gene expression analysis of bile acid biosynthesis-related genes showed a downregulation of sterol 12-alpha-hydroxylase (*Cyp8b1*) in ßCD receiving mice. However, other bile acid synthesis-related genes

such as cholesterol 7α-hydroxylase (*Cyp7A1*), also a LXR target in mice, and sterol 27-hydroxylase (*Cyp27A*) remained unchanged (Table 1).

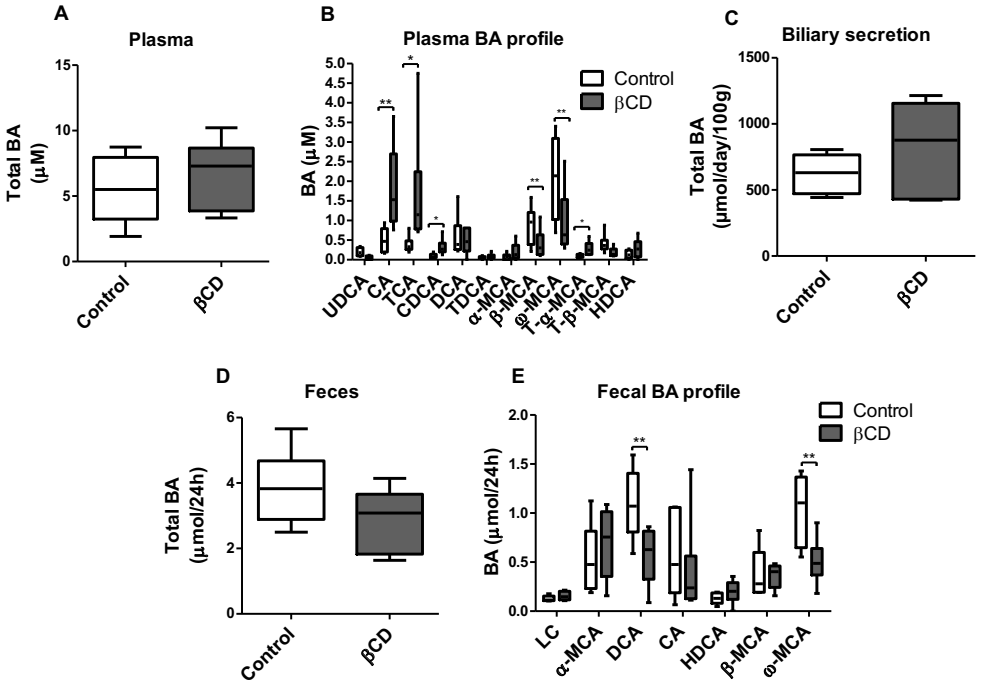

Figure 2: ßCD induces shifts in bile acid profiles. (A) total plasma bile acids; (B) plasma bile acids profile; (C) Biliary bile acid (BA) secretion; (D) Fecal total BA excretion; (E) Fecal BA profile. LC, lithocholic acid; α-MCA, α-muricholic; DCA, deoxycholic acid; CA, cholic acid; HDCA, hyodeoxycholic acid; β-MCA, β-muricholic acid; ω-MCA, ω-muricholic acid; UDCA, ursodeoxycholic acid; CDCA, chenodeoxycholic acid; TCA, tauro-cholic acid; TDCA, tauro-deoxycholic acid; T-α-MCA, tauro-α-muricholic; T-β-MCA, tauro-β-muricholic acid T-ω-MCA, tauro-ω-muricholic acid. Data are presented as box plots showing median (interquartile range) and min/max; at least N=6 for each group. Statistically significant differences are indicated as *P<0.05; **P<0.01.

ßCD increases fecal neutral sterol excretion to a greater extent in germ-free mice lacking intestinal microbiota.

In order to determine the extent to which the intestinal microbiota mediates the cholesterol lowering effect of ßCD, a dietary intervention with ßCD was performed in germ-free mice. Comparable to the conventional mice, plasma cholesterol was significantly reduced mainly in the HDL fraction (P<0.05, Table 2, Supplemental figure I).

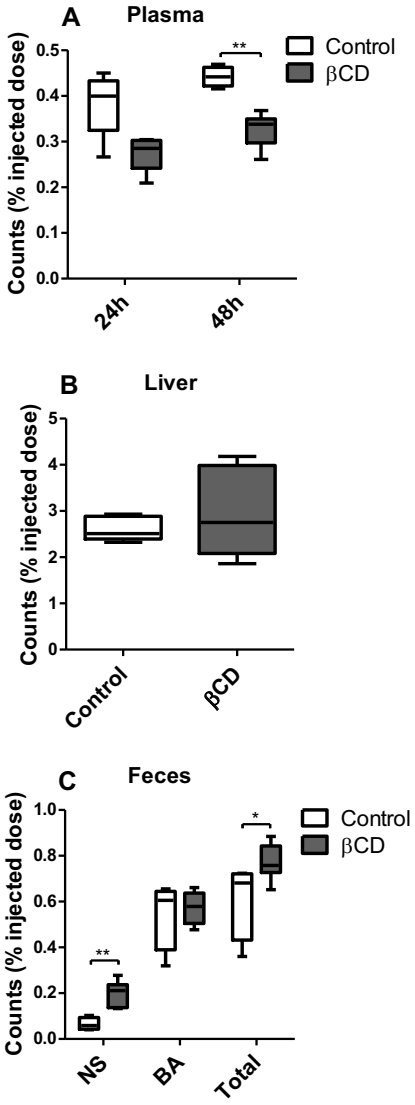

Figure 3: Effect of ßCD containing diet on macrophage-to-feces reverse cholesterol transport in conventional mice. Macrophage-derived ^{3}H-cholesterol tracer recovered in (A) plasma; (B) liver; (C) fecal neutral sterols (NS) and bile acids (BA). Data are presented as box plots showing median (interquartile range) and min/max; at least N=6 for each group. Statistically significant differences are indicated as *P<0.05; **P<0.01.

Similar to conventional mice, ßCD increased fecal cholesterol excretion also in germ-free animals (7.5-fold, P<0.05), however, to a substantially greater extent. In the germ-free group, the campesterol:cholesterol (0.012 (0.011-0.012) *vs* 0.018 (0.016-0.022), respectively, P<0.05) and ß-sitosterol:cholesterol (0.028 (0.026-0.029) *vs* 0.055 (0.045-0.066), respectively, P<0.05) ratios increased upon ßCD-feeding.

Table 2: Lipid and sterol profiles in plasma, liver and feces of germ-free mice fed control and βCD diet

	Control	βCD
Plasma		
Total cholesterol (mM)	1.83 (1.69-1.89)	0.94 (0.82-1.11)*
Triglycerides (mM)	0.10 (0.06-0.15)	0.10 (0.06-0.15)
Liver		
Total cholesterol (nmol/mg of liver)	10.26 (9.95-10.61)	6.48 (6.24-6.70)*
Triglycerides (nmol/mg of liver)	21.66 (20.19-23.86)	7.75 (6.48-10.48)*
Fecal neutral sterol profiles		
Cholesterol (µmol/24h)	1.33 (1.20-1.62)	11.36 (9.54-13.01)*
Dihydrocholesterol (µmol/24h)	0.12 (0.11-0.13)	0.12 (0.09-0.14)
Coprostanol (µmol/24h)	n.d.	n.d.
Fecal bile acids profiles		
α-Muricholic acid (µmol/24h)	0.12 (0.11-0.18)	1.02 (0.95-1.05)*
β-Muricholic acid (µmol/24h)	0.60 (0.45-0.70)	0.15 (0.13-0.17)*
ω-Muricholic acid (µmol/24h)	n.d.	n.d.
Deoxycholic acid (µmol/24h)	n.d.	n.d.
Cholic acid (µmol/24h)	0.41 (0.37-0.44)	0.35 (0.34-0.37)
Ursodeoxycholic acid (µmol/24h)	n.d.	n.d.
Chenodeoxycholic acid (µmol/24h)	0.10 (0.07-0.11)	0.68 (0.60-0.75)*

*Data are presented as median (interquartile range), at least N=4 for each group. N.d. Not detected. Statistically significant differences are indicated as *P<0.05.*

In the liver, a reduction in cholesterol and triglyceride levels was observed (P<0.05, Table 2). Liver mRNA expression of *Hmgcoar* was upregulated in ßCD receiving germ-free mice compared to controls (P<0.05, Table 3) and also the plasma lathosterol:cholesterol ratio was significantly increased (0.0025 (0.0023-0.0026) *vs* 0.0208 (0.0197-0.0230), respectively, P<0.05) by ßCD administration in germ-free mice. On the other hand, hepatic mRNA expression of genes associated with fatty acid metabolism such as steroyl-CoA desaturase (*Scd1*), fatty acid synthase (*Fasn*) and sterol regulatory element-binding protein 1c (*Srebp-1c*) were significantly lower in germ-free mice receiving ßCD compared to the control diet group (all P<0.05, Table 3). In terms of fecal bile acid excretion, α-muricholic andchenodeoxycholic acid were increased compared to the control group (P<0.05, Table 2), whereas secondary BA such as ω-muricholic and deoxycholic acid, which were lowered in conventional mice upon ßCD feeding, were not detected in both groups under germ-free conditions.

Table 3: Hepatic gene expression in germ-free mice fed control and βCD diet

mRNA expression	Control	βCD
Hmgcoar	1.00 (0.50-0.58)	2.71 (1.20-1.58)*
Ldlr	1.00 (0.94-1.05)	1.27 (1.01-1.88)
Fxr	1.00 (1.56-2.04)	1.00 (1.34-1.92)
Abcg5	1.00 (0.65-0.74)	0.75 (0.43-0.57)
Abcg8	1.00 (0.64-0.80)	0.68 (0.30-0.66)
Bsep	1,00 (0.69-1.02)	0.68 (0.42-0.71)
Srb1	1.00 (0.59-0.74)	0.79 (0.43-0.60)
Cyp7a1	1.00 (0.32-0.44)	1.22 (0.29-0.76)
Cyp8b1	1.00 (0.48-0.90)	2.17 (0.83-1.99)
Cyp27a	1.00 (0.57-0.72)	1.08 (0.64-0.81)
Scd1	1.00 (1.10-1.95)	0.11 (0.16-0.17)*
Fasn	1.00 (0.66-0.83)	0.81 (0.48-0.69)*
Srebf-1c	1.00 (1.12-1.18)	0.13 (0.13-0.19)*

*Data are presented as median (interquartile range), at least N=4 for each group. Statistically significant differences are indicated as *P<0.05.*

ßCD increases macrophage-to-feces RCT and this effect is more pronounced in germ-free mice.

Finally, macrophage-to-feces RCT experiments were performed in conventional mice to determine whether the observed alterations in sterol metabolism upon ßCD feeding would also translate into changes in RCT, a pathway with prime relevance for the protection against atherosclerotic CVD. Plasma ^{3}H-cholesterol tracer recovery was significantly lower in ßCD-receiving mice compared to the control group 48 h after macrophage injection (P<0.01, Fig. 3A), while liver tracer recovery remained unchanged (Fig. 3B). Overall fecal tracer excretion was increased (P<0.01, Fig. 3C). This increase was mainly due to a significantly higher tracer recovery in the fecal neutral sterol fraction (P<0.01) whereas tracer recovery in the bile acid fraction was comparable in both groups.

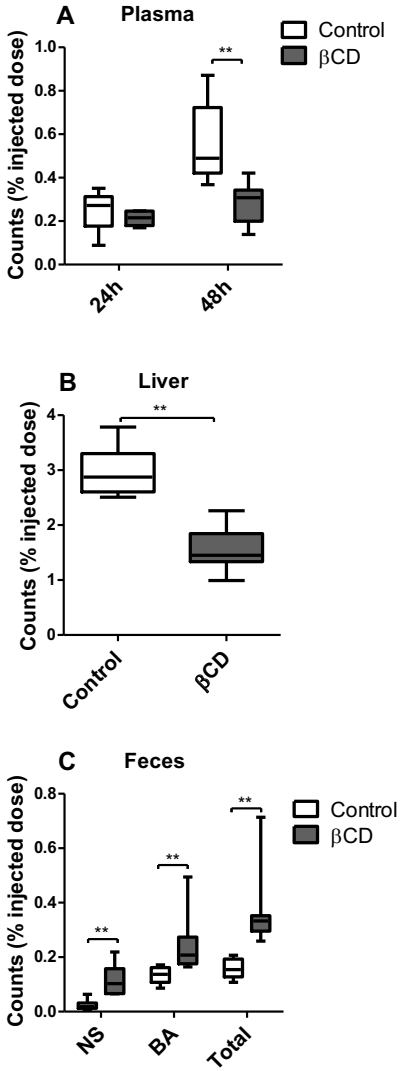

Figure 4: Effect of ßCD containing diet on macrophage-to-feces reverse cholesterol transport in germ-free mice. Macrophage-derived cholesterol tracer recovered in (A) plasma; (B) liver; (C) fecal neutral sterols (NS) and bile acids (BA). Data are presented as box plots showing median (interquartile range) and min/max; at least N=6 for each group. Statistically significant differences are indicated as **P<0.01.

Next, the RCT study was performed in germ-free mice. Here, ^{3}H-cholesterol tracer recovery was two-fold lower in plasma 48 h after macrophage injection (P<0.01, Fig.4A). Under germ-free conditions ßCD reduced liver tracer recovery compared to the control group (P<0.01, Fig 4B). Overall fecal excretion of the ^{3}H-cholesterol tracer and thereby functional RCT increased more than two-fold in ßCD-fed GF mice (P<0.01, Fig. 4C). Interestingly, both the neutral sterol as well as the bile acid fractions significantly contributed (P<0.01) to the increase in total fecal tracer elimination. Combined, these data indicate that the metabolic effects of ßCD on sterol homeostasis translate into a significant increase in RCT, which is even more pronounced in the absence of intestinal bacteria.

Discussion

The results of this study demonstrate that (i) the non-digestible cyclic carbohydrate ßCD increases fecal neutral sterol excretion from a RCT-relevant pool in mice, and (ii) ßCD even more substantially enhances fecal neutral sterol and bile acid excretion in germ-free mice, translating into an overall 2.5-fold increase in RCT. Importantly, our data thereby establish that the beneficial effects of ßCD do not primarily depend on bacterial fermentation products. In contrast, it appears that the effectiveness of ßCD to increase RCT is even higher in the absence of microbiota in germ-free mice.

Non-digestible oligosaccharides such as ßCD can exert metabolic health effects either directly through binding with lipid molecules such as cholesterol or indirectly via microbiota-dependent generation of bioactive metabolites. The present study is unique in the sense that it addresses both direct as well as indirect effects of ßCD. In conventional as well as germ-free mice ßCD reduced plasma total cholesterol levels. This observation in conventional mice is consistent with previous studies in rats, hamsters and pigs.[7,19,20] The reduction occurred mainly in HDL cholesterol, which has been shown in human population studies to be inversely correlated with risk of atherosclerotic cardiovascular disease.[21] However, a prediction of how treatment with ßCD would impact atherosclerosis is difficult, since in humans increasing HDL-C by pharmacological interventions with e.g. CETP inhibitors or niacin failed to show benefit in terms of cardiovascular disease outcomes.[21] In addition, there are data in mice that steady-state HDL-C levels are less reliable predictors for atherosclerosis than the overall flux through the RCT pathway. Hepatic SR-BI overexpression lowers plasma HDL-C while increasing RCT and decreasing atherosclerosis [22], whereas in SR-BI knockout mice high HDL-C levels fail to increase RCT or protect against atherosclerotic lesion formation.[23]

Furthermore, ßCD receiving mice had a two-fold higher fecal neutral sterol excretion. This observation can partly be attributed to the ability of ßCD to form inclusion complexes with hydrophobic molecules such as cholesterol [7], and partly to the stimulating effect that ßCD has on TICE. The molecular regulation of TICE is at present still unclear [17] making it difficult to find a mechanistic explanation for our result. The reasoning that ßCD directly binds cholesterol on the other hand is further substantiated by the significant reduction in fecal coprostanol excretion we observed in ßCD-fed conventional mice compared to the control group. Coprostanol is a product of a reaction between cholesterol and intestinal bacteria, in which bacteria-derived enzymes reduce the double-bonds between carbons 5 and 6 of the cholesterol molecule to form coprostanol.[24–26] Since cholesterol is shielded by ßCD, access by intestinal bacterial is partly or completely prevented reducing the conversion reaction and

thereby the overall excretion of coprostanol. Similarly, inclusion of certain bile acids into the hydrophobic ßCD cavity, as indicated by previous work [27–30], could lead to non-availability of these for the formation of the secondary bile acids DCA and ω-MCA by the microbiota thereby explaining the reduction in secondary bile acids in plasma as well as feces observed in our present study in conventional mice.

The role of the intestinal microbiota in mediating metabolic effects of ßCD has not been analyzed before to the best of our knowledge. It is known that SCFA which are derived from microbial fermentation can impact hepatic lipid metabolism, particularly, by acting as precursors for cholesterol synthesis.[31–34] In rats, supplementation of ßCD in the diet increased the production of acetate and propionate by two-fold.[9] However, in the present study we demonstrate that intestinal bacteria are dispensable for the impact of ßCD on sterol metabolism. On the contrary, fecal excretion of neutral sterols and bile acids as well as RCT were even substantially more increased upon ßCD administration in the germ-free animals. How can intestinal bacteria modulate cholesterol metabolism? Firstly, lactic acid bacterial strains such as *Lactobacillus acidophilus* have been suggested to impact cholesterol absorption in the intestine by downregulating Niemann-Pick C1-Like 1 (NPC1L1) expression [35,36], which could decrease cholesterol absorption and thereby increase RCT comparable to the use of the chemical cholesterol absorption inhibitor ezetimibe.[37–39] This possibility is unlikely to explain our findings, since we observed the opposite, namely increased fecal sterol excretion in the absence of intestinal bacteria. Secondly, intestinal bacteria play a crucial role in the conversion of primary into secondary bile acids. CA and CDCA are two primary bile acids synthesized in humans and mice, which are converted by the intestinal bacteria into DCA and LCA, respectively. Furthermore, in mice CDCA is converted into MCA. CDCA, CA or DCA are known agonists for FXR activation in the liver, resulting in a suppression of bile acid synthesis.[40–42] On the other hand, MCA has been implied as an antagonist of FXR activation, and is thus expected to increase hepatic bile acid synthesis[43]. This could partially explain the considerable 2-fold increase in bile acid excretion and increased recovery of RCT-relevant cholesterol in the fecal bile acid fraction (2-fold) of germ-free mice. Thirdly, bacteria have been shown to sequester or assimilate cholesterol in their membranes.[44–46] In conventional mice this scenario would be expected to enhance cholesterol excretion somewhat in competition to ßCD and we believe that such a concept could serve as a likely explanation for the results of our work. In addition, it can also not be excluded that ßCD fermentation might decrease the amount of ßCD available for cholesterol and bile acid binding.

Since in our hands, dietary administered ßCD acts mostly in the intestinal compartment to increase fecal sterol excretion and RCT we expect that ßCD could

reduce the incidence of CVD events, also when given on top of statin therapy, similar to the drug ezetimibe.[47] In addition, ßCD has no known toxic effects even up to a very high intake of 20% [19,20], thus presenting in our view a viable dietary health supplement for primary or secondary prevention indications. However, further studies in at least animal models of atherosclerosis are first required to substantiate these expectations.

Previous studies that explored potential anti-atherogenic effects of ßCD have indicated that ßCDs such as hydroxypropryl-ßCD (HP-ßCD) and methyl-ßCD increase the removal of cholesterol from macrophage foam cells *in vitro.*[48,49] More recently, it was shown that *ex vivo* incubation of human atherosclerotic plaques with HP-ßCD led to cholesterol efflux from the plaque tissue into the supernatant.[18] Further, repeated subcutaneous administration of HP-ßCD to mice was demonstrated to have the potential to reduce atherosclerotic lesion formation. The authors of this study suggested that the beneficial effects of HP-ßCD would occur by stimulating LXR activation. In our study, using a dietary route of ßCD administration, however, we could not detect any indication for an increased expression of bona fide LXR target genes.

In summary, our study demonstrates that ßCD as a dietary fiber can effectively increase fecal sterol excretion and RCT. These beneficial metabolic effects of ßCD are further enhanced in the absence of intestinal bacteria and thus do not require metabolic conversion of ßCD by the gut microbiota. Based on our results we would propose to further explore the potential clinical benefits of using dietary ßCD for the prevention and treatment of atherosclerotic cardiovascular disease.

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