Diet-induced alterations of host cholesterol metabolism are likely to affect gut microbiota composition in hamsters

INÉS MARTÍNEZ¹, DIAHANN J. PERDICARO¹, ANDREW W. BROWN², SUSAN HAMMONS¹, TREVOR J. CARDEN², TIMOTHY P. CARR², KENT M. ESKRIDGE³, AND JENS WALTER¹*

Department of Food Science and Technology¹, University of Nebraska, Lincoln, NE 68583-0919, USA, and Department of Nutrition and Health Sciences², University of Nebraska, Lincoln, NE 68583-0806, USA, and Department of Statistics³, University of Nebraska, Lincoln, NE 68583-0963, USA.

*Corresponding author. Mailing address: Jens Walter, Department of Food Science and Technology, University of Nebraska, 333 Food Industry Complex, Lincoln, NE 68583-0919. Phone: (402) 472-2615; Fax.: (402) 472-1693.

E-mail: jwalter2@unl.edu
Abstract

The gastrointestinal microbiota impacts the metabolism of the mammalian host with consequences for health. However, the complexity of gut microbial communities and host metabolic pathways make functional connections difficult to unravel, especially in terms of causation. In this study, we have characterized the fecal microbiota of hamsters whose cholesterol metabolism was extensively modulated by the dietary addition of plant sterol esters (PSE). PSE intake induced dramatic shifts in the fecal microbiota, reducing several bacterial taxa within the families Coriobacteriacea and Erysipelotrichaceae. The abundance of these taxa displayed remarkably high correlations with host cholesterol metabolites. Most importantly, the associations between several bacterial taxa with fecal and biliary cholesterol excretion showed an almost perfect fit to a sigmoidal inhibitory nonlinear model describing bacterial inhibition, suggesting that host cholesterol excretion can shape microbiota structure through the antibacterial action of cholesterol. In vitro experiments suggested a modest antibacterial effect of cholesterol, and especially of cholesteryl-linoleate, but not plant sterols when included into model bile micelles. The findings obtained in this study are relevant to our understanding of gut microbiota-host lipid metabolism interactions, as they provide first evidence for a role of cholesterol excreted with the bile as a relevant host factor that modulates the gut microbiota. The findings further suggest that the connections between Coriobacteriacea and Erysipelotrichaceae and host lipid metabolism, which have been observed in several studies, might be caused by a metabolic phenotype of the host (cholesterol excretion) affecting the gut microbiota.
Introduction

The mammalian gastrointestinal tract is colonized by trillions of microbial community (the gut microbiota), a large fraction of which are bacteria. This microbial community has an extensive impact on host metabolism with important implications for health (1, 12, 43). The contribution of the gut microbiota to energy harvest from the diet and fat storage constitutes a key beneficial trait that underlies host-microbiota symbiosis in mammals (4). However, this contribution has likely become detrimental to modern humans living in societies with excess food resources, as it increases susceptibility to metabolic disorders, such as obesity, type 2 diabetes, and coronary heart disease. Accordingly, the gut microbiota is increasingly being accepted as an important factor that contributes to pathological conditions associated with obesity (9), and in humans, metabolic pathologies are often associated with alterations in the gut microbiota (which is referred to as dysbiosis) (23, 24, 39, 50). Unfortunately, there is still little consensus on the bacterial groups that are associated with obesity-associated diseases and metabolic phenotypes (43). In addition, although comparisons between germ-free and conventional mice and rats have clearly established a role of the microbiota in modulating host lipid metabolism (3, 12, 13, 46), it is still unclear whether dysbioses contribute to metabolic pathologies. However, such basic information is essential for our understanding of the diet-microbiota-host metabolism interplay, especially for the development of dietary strategies to prevent metabolic disorders through a modulation of the gut microbiome (14, 43, 48).

Novel molecular technologies based on massive parallel sequencing have enabled the identification of associations between host lipid metabolism and gut microbial community structure in both humans and animals. Two bacterial families, the Erysipelotrichaceae and Coriobacteriaceae, have been repeatedly linked to the host lipid metabolism and associated with the dyslipidemic phenotypes. Spencer and coworkers (39) showed that levels of Erysipelotrichaceae were positively associated with changes in liver fat in humans, and higher
proportions of this bacterial group have been also identified in morbidly obese individuals (50).

Erysipelotrichi have been also linked to lipidemic imbalances in mice and in a hamster model of hypercholesterolemia (28, 49). For Coriobacteriaceae, strong positive links have been determined with plasma non-HDL in hamsters (28). Moreover, Claus and colleagues (12) showed an association between Coriobacteriaceae, in particular the genus *Eggerthella*, with host metabolism and especially hepatic triglyceride levels in mice. The recurrent identification of associations between Coriobacteriaceae and Erysipelotrichaceae, and specific taxa within these families, with host lipid and cholesterol phenotypes in different host species (humans, mice, and hamsters), suggests a genuine link between these bacterial groups and the host lipid metabolism (12, 28, 48).

The important similarities between hamsters and humans in terms of lipid profiles, enzymatic pathways in lipoprotein and bile metabolism, and susceptibility to diet-induced atherosclerosis pose advantages in using these animals to investigate functional interactions between cholesterol metabolism and the gut microbiota (29). In a previous study, we have used the hamster model of hypercholesterolemia and investigated the interplay between grain sorghum lipid extract in the diet, gut microbial ecology, and cholesterol metabolism. This study showed that specific bacterial groups in the fecal samples were tightly linked to dietary-induced improvements in host cholesterol metabolism (28). In particular, Coriobacteriaceae and unclassified members of the Erysipelotrichaceae showed negative correlations with non-HDL cholesterol and cholesterol absorption, while bifidobacteria showed positive correlations with HDL cholesterol. Some of these correlations were highly significant, but the directionality of these interactions was not established. Unfortunately, hamsters cannot be reared germ-free (2), which precludes the study of causation between specific bacterial taxa and host cholesterol metabolism employing gnotobiotic approaches in this animal model. However, it is possible to specifically modulate the hamster’s cholesterol metabolism and study the effects on the gut.
microbiota. For example, plant sterols and their esters offer an opportunity to modulate host cholesterol metabolism in hamsters (34). These compounds reduce cholesterol absorption in the intestine by a displacement of cholesterol by the plant sterol in intestinal micelles, co-crystallization between plant sterols and cholesterol leading to the formation of insoluble crystals, and impediment of cholesterol hydrolysis by lipases and cholesterol esterases (6, 7, 19, 32, 33, 35, 42). The chemical processes by which plant sterols work have been extensively studied in vitro and do not require the participation of intestinal bacteria.

In this study, we have characterized the fecal microbiota of hamsters whose cholesterol metabolism was extensively modulated by dietary addition of plant sterol esters (34). In these hamsters, PSE intake reduced cholesterol absorption and increased cholesterol excretion, and consequently decreased plasma non-HDL cholesterol and liver esterified cholesterol levels. Pyrosequencing of 16S rRNA tags revealed that PSE also induced dramatic shifts in the fecal microbiota with remarkably high correlations with host cholesterol metabolites. Most importantly, the associations between several bacterial taxa with fecal and biliary cholesterol excretion showed an excellent fit to a nonlinear sigmoidal inhibitory model used to describe dose-response relations between bacteria and inhibitory compounds (25, 30), suggesting that host cholesterol excretion can shape microbial community structure through the antimicrobial action of cholesterol excreted in the gut.

**Materials and Methods**

*Animal experiments and diets*

The fecal samples analyzed were obtained in a previous study that determined the effect of dietary PSE on hamsters’ lipid metabolism (34). The handling of animals, feed composition, plant sterol composition of the diets, sample collection, and metabolic analysis has been described in this report. Briefly, Bio-F1B male Syrian hamsters (Bio Breeders, Watertown, MA)
were individually caged and randomly assigned to four dietary treatments throughout a 4-week period: a modified AIN-93M diet containing no PSE (C), or containing 5% (w/w) plant sterols esterified with fatty acids from beef tallow (BT), or stearic acid (SA). The final energy distribution of each diet was 36% fat, 35% carbohydrate and 29% protein. The animals were housed in a facility with controlled atmosphere (25°C) under 12-hour light:dark cycles, and had access to food and water *ad libitum*. The animals were euthanized by CO₂ asphyxiation after 4 weeks of dietary intervention. Blood was collected by cardiac puncture, and plasma was obtained by centrifugation (2,000 × g for 30 min at 4°C). Total and HDL cholesterol were enzymatically quantified in the plasma samples. Livers were excised and immediately frozen in liquid nitrogen. Total cholesterol, triglycerides, free cholesterol, esterified cholesterol and phospholipids were measured in the livers. Cholesterol absorption was quantified in fecal samples collected at week 3 with radiolabeled sterols, as previously described (Schneider et al., 2000). The complete fecal output was collected during week 4 and stored frozen (−80°C). Fecal concentration of neutral sterols, bile acids, cholesterol, dihydrocholesterol, coprostan-3-one and coprostan-3-ol was determined as previously described (34).

**Characterization of the fecal microbiota**

The gut microbial composition was determined in fecal samples of hamsters fed a control diet (C) (n=7) or plant sterols esterified with stearic acid (SA) (n=9) and beef tallow (BT) (n=6). DNA was extracted using a standard method that combined enzymatic and mechanical cell lysis with phenol-chloroform extractions (28). The fecal microbial community was characterized by massive parallel sequencing of the V3 region of the 16S rRNA. PCRs were performed with the forward primer (A-338F) 5’-gcctcctctgcccgctcagACTCCTACGGGAGGCAGCAG-3’ and the reverse primer (B-518R) 5’-gccttgccagcccgctcagNNNNNNATTACCGCGGCTGCTGG-3’ (with the A and B adaptors indicated in lower case, and an eight nucleotide barcode shown as
Ns), and products were sequenced using the Roche Genome Sequencer GS-FLX (454 Life Sciences) as described previously (28). The sequence data set is available upon request.

The sequences obtained were quality controlled using the QIIME pipeline (10). Sequences <150 bp or >350 bp in length were removed, as well as sequences containing one or more ambiguous nucleotides or mismatches to the primer or barcode, an average quality score below 25, and homopolymer runs longer than 6 bp. Chimera removal was performed using the Blast Fragments algorithm in QIIME. An average of at least 1,700 sequences per sample was obtained after quality control. Taxonomical characterization of the sequences was done with the Classifier tool from the Ribosomal Database Project (RDP) (45), which classified the sequences from the phylum to the genus level. Additionally, Operational Taxonomic Units (OTU) were determined using a 97% sequence similarity cutoff to characterize the microbiota at a lower taxonomic level roughly equivalent to bacterial species. OTUs were generated by aligning the quality-controlled sequences with the Infernal Alignment algorithm of RDP, followed by clustering with the Complete Linkage Clustering tool of RDP. OTUs determined to be significantly affected by plant sterol consumption or associated with host physiological parameters were confirmed using BLASTn as described previously (27). Briefly, 5 representative sequences of the selected OTUs were taxonomically assigned and aligned by ClustalW within their respective phylum. Phylogenetic trees were constructed for each phylum with the Neighbor-joining algorithm and distance matrices were generated (MEGA 4.0) (41). Sequences that clustered in the tree with >97% similarity were combined into one single OTU, and consensus sequences were generated within these clusters. The consensus sequences were used to assign all sequences in the entire sequence set to respective OTUs by aligning them in BioEdit (18) with the BLASTn algorithm (>97% similarity and at least 95% overlap) against a local database composed of all the quality-controlled sequences. UniFrac analysis
was performed using the QIIME pipeline to investigate the beta-diversity of microbial
communities (10).

**In-vitro inhibition bacterial assays**

Representative bacterial strains originating from the mammalian gastrointestinal tract were
selected to test for antibacterial activity of cholesterol, cholesteryl-linoleate, and the plant sterols
\(\beta\)-sitosteryl-stearate and stigmastanyl-stearate when incorporated into micelles containing bile-
salts and lecithin. The bacteria and growth media used were: *Bifidobacterium longum* subsp.
*infantis* ATCC15697\(^T\) (in MRS medium supplemented with 0.5 mg/L L-cysteine), *Lactobacillus
reuteri* Lpuph-1 (in MRS medium), *Eggerthella lenta* ATCC 25559\(^T\) (in MRS medium), *Slackia
heliofrinireducens* ATCC 29202\(^T\) (in MRS medium), *Collinsella intestinalis* ATCC 13228\(^T\) (in
Peptone Yeast Glucose medium), two human isolates of *Collinsella aerofaciens* KD-D8-5 and
IM-D3-18 (in PYG medium), *Clostridium histolyticum* ATCC 19401\(^T\) (in PYG medium). All media
were pre-reduced for 24 h, and bacterial inoculums for inhibition tests were prepared as follows
under anaerobic conditions at 37°C (Bactron IV Anaerobic Chamber, Shel Lab, USA): Bacterial
cultures started from one single colony were grown for 24 h, transferred to fresh media (1%
inoculum), and then grown for another 16 h.

Micelles containing cholesterol (>98% purity, Sigma), cholesteryl-linoleate (>99% purity Sigma),
or plant sterols (stigmastanol and \(\beta\)-sitosterol, synthesized as described in (7)), were prepared
as described by Brown et al. (7), with minor modifications. Briefly, 48 \(\mu\)l of a solution containing
either cholesterol, cholesteryl-linoleate, stigmastanol, or \(\beta\)-sitosterol (161 mg/ml in chloroform)
was mixed with 41 \(\mu\)l of a lecithin solution (221.3 mg/ml in chloroform), and chloroform was
removed under a stream of nitrogen. The resulting mixture of lecithin and cholesterol/sterols
was dissolved in a solution containing 5.4 mg/ml of the bile salt sodium taurocholate (>97%
purity, Sigma) in distilled water. The solutions were sonicated for 3-6 min with 30% amplitude
using a Branson 450 Sonifier (Danbury, CT) and afterwards filter sterilized with 0.45 μm pore filters (Fisherbrand, Fisher Scientific). To test for the antibacterial capacity of cholesterol, cholesteryl-linoleate, or the plant sterols, 5 μl of a 1:10 dilution of the bacterial inoculums were transferred into a solution made of 500 μl of pre-reduced micelle suspensions and 500 μl of the appropriate pre-reduced double-concentrated (2X) media. Given the antibacterial activity of bile acids, micelle suspensions containing bile acids but no sterols were inoculated and used as control. Cultures were incubated anaerobically at 37°C for 12 h (mid log phase), and spectrophotometric optical density (OD) quantifications were performed at 600 nm of wavelength (BioMate3, Thermo Scientific). Three biological replicates were conducted for the experiments. The concentration of cholesterol in these experiments (386 μg/ml media) is approximately double the concentration of the average cholesterol levels measured in the fecal samples of hamsters consuming the control diet (170 ± 111 μg/g), but still around 20 times lower than the fecal cholesterol levels present in SA treated hamsters (4303 ± 1930 μg/g).

Unfortunately, generation of micelles containing higher amounts of cholesterol, which would have better reflected the in vivo conditions, was not possible due to the formation of a precipitate.

**Statistical analysis**

Results were expressed as mean ± SD unless otherwise stated. The impact of dietary treatments on the abundance of individual bacterial taxa was analyzed by one-way ANOVAs and Tukey’s post-hoc tests. Correlations between bacterial groups and host physiological measurements were assessed with Pearson’s correlations and non-linear regressions. A non-linear four-parameter sigmoidal inhibitory model that describes dose-response relations between bacteria and inhibitory compounds was used to fit the data (25, 30). The model is represented by equation (1), where X represents the metabolic parameter, Y the abundance of the bacterial taxon in fecal sample, Yo and Ymax are the minimum and maximum effects,
respectively EC50 is the concentration where 50% of the maximum effect is measured, and the slope is the sigmoidicity coefficient.

\[ Y = Y_0 + \frac{Y_{max} - Y_0}{EC50 + X^{slope}} \]

The statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, USA). \( P < 0.05 \) in the ANOVAs, and correlation coefficients \( r > 0.60 \) (in absolute value) were considered significant. In addition to the analyses above, partial correlations were performed to evaluate the associations between metabolic markers and abundance of bacterial populations when excluding the effect of diet using linear regressions, using SAS/STAT® software. To decrease the number of variables in this analysis and increase the power of the test, only a few bacterial taxa (Coriobacteriaceae, Erysipelotrichaceae, unclassified Erysipelotrichaceae, OTU1), and metabolic markers (whole body cholesterol synthesis, fecal cholesterol, fecal neutral sterols, fecal bile acid) that were determined pertinent based on the previous analyses were selected to conduct the partial correlations statistics.

Results

Dietary supplementation with 5% plant sterols induced substantial alterations of the fecal microbiota of hamsters

454 pyrosequencing was used to characterize the fecal microbiota of hamsters fed a control diet, or a diet supplemented with 5% plant sterols esterified with stearic acid (SA) or beef tallow (BT). The inclusion of PSE in the diet, in particular SA, had extensive effects on the fecal microbiota composition. UniFrac analysis revealed that the fecal microbial communities of hamsters fed the SA diet clustered separate from those of the control group (Figure 1A). Analysis of Shannon diversity coefficients revealed that dietary addition of SA reduced the diversity of the fecal microbiota when compared to control \((P < 0.05)\) (Figure 1B).
Dietary PSE significantly altered the gut microbiota at all taxonomic levels (Table 1). PSE had a dramatic effect on the abundance of the phylum Actinobacteria, and especially on the family Coriobacteriaceae, which declined tenfold and fourfold with SA or BT intake, respectively. Three OTUs belonging to this family were significantly reduced through PSE (Table 1, Supplementary Figure 1). SA induced a significant increase in the phylum Firmicutes which was to a large degree due to a bloom of a single OTU (OTU16) within the family Eubacteriaceae (Table 1). It is possible that this increase could be due to the ability of this organism to utilize the additional cholesterol that became available as a growth substrate. However, the presence of OTU16 is strictly linked to the absence of the OTUs whose abundance was reduced by PSE (data not shown), suggesting that the bloom was caused by OTU16 expanding into niches that became vacant. Although total Firmicutes showed an increase with SA, five OTUs belonging to the family Erysipelotrichaceae did not follow this general trend and instead showed a dramatic reduction (Table 1, Supplementary Figure 2).

Correlation analysis showed an extensive link between the microbiota and host lipid metabolism. The feeding of PSE induced extensive changes in the lipid and cholesterol metabolism of hamsters (34), and many of the individual metabolites were highly interrelated (Figure 2A). One of the goals of the present study was to investigate whether the lipidemic effects were associated with shifts in the gut microbiota. The correlation analysis revealed that host cholesterol metabolism was extensively interlinked with the bacterial community (Figure 2). The tightest associations were observed within the phylum Actinobacteria, and especially the family Coriobacteriaceae, which showed remarkable associations with cholesterol absorption ($r = 0.75$, $P < 0.0001$), whole body cholesterol synthesis ($r = -0.75$, $P < 0.0001$), fecal biliary cholesterol excretion ($r = -0.75$, $P < 0.0001$), liver free cholesterol ($r = 0.73$, $P < 0.0001$), plasma non-HDL cholesterol ($r = 0.68$, $P = 0.0005$), and liver weight ($r = 0.82$, $P < 0.0001$), among others. Similar high correlations were observed for the OTUs belonging to the Coriobacteriacea and
Erysipelotrichaceae families that were affected by PSE administration. Total Firmicutes and unclassified Erysipelotrichaceae also displayed significant associations with host lipidemic markers, although they were less significant than associations detected for Coriobacteriaceae and the individual OTUs (Figure 2).

Improvements in the host lipid metabolism appear to be independent from metabolic modifications of intestinal cholesterol by the gut microbiota

One mechanism through which gut bacteria could lower host cholesterol levels is the transformation of cholesterol to coprostan-3-one, coprostan-3-ol and dihydrocholesterol, which are to a large degree excreted (38). However, fecal concentrations of these cholesterol derivatives were not affected by PSE intake, and their fecal concentrations did not correlate with any of the host lipid metabolism markers tested (Figure 2A). In addition, the correlation analysis did not provide evidence for an association between the coprostan-3-one, coprostan-3-ol, and dihydrocholesterol and specific bacterial members of the gut microbiota (Figure 2). These findings suggest that PSE-induced shifts of the gut microbiota did not impact bacterial cholesterol metabolites in the gastrointestinal lumen, nor was the bacterial action on the cholesterol pool responsible for the improvements observed in the host lipid metabolism.

Cholesterol excreted by the host via bile appears to inhibit specific bacterial taxa

Upon visual inspection of the correlation plots, the associations of bacterial taxa with the host cholesterol synthesis and excretion appeared to follow an exponential relationship. In addition, these associations appeared to resemble dose-response curves observed in microbial antibiotic assays. We therefore tested how bacterial proportions and cholesterol synthesis/excretion fitted a four-parameter sigmoidal model for bacterial inhibition used to describe dose-response relations between bacteria and inhibitory compounds (25, 30). This analysis revealed that the relationships between cholesterol synthesis, fecal biliary cholesterol excretion, and fecal
cholesterol with several bacterial taxa showed an almost perfect fit to the inhibitory model (Figure 3). For example, the associations between Coriobacteriaceae and host cholesterol synthesis, fecal biliary cholesterol excretion, and fecal cholesterol fitted the model with regression coefficients of -0.93, -0.87, and -0.91, respectively. The model was an even better fit for the data obtained for OTU1, which is related to *Eggerthella lenta*. Interestingly, the fecal proportions of OTU13, which was classified as a member of the Erysipelotrichaceae family, also showed a tight association with cholesterol synthesis and excretion with an excellent fit to the model. In total, four OTUs, two belonging to the Coriobacteriaceae (OTUs 1 and 4), and two belonging to the Erysipelotrichaceae (OTUs 13 and 15) displayed a significant fit to the four-parameter sigmoidal model (R = -0.94, R = -0.71, R = -0.73 and R = -0.68, respectively). The fact that the abundance of taxonomically distinct bacterial taxa displayed associations to fecal cholesterol concentrations that fit an inhibition model suggests an antibacterial role of the excreted cholesterol. In contrast, bile acid concentrations showed no negative associations with the fecal proportions of Coriobacteriaceae or Erysipelotrichaceae related phylotypes that were affected by the feeding of PSE (Supplementary Figure 3).

Partial correlation analysis revealed that the negative associations between the abundance of Erysipelotrichaceae with whole body cholesterol synthesis and concentrations of fecal neutral sterols (*P* = 0.033 and *P* = 0.034, respectively) were independent of dietary PSE. This analysis also suggested that the links between fecal proportions of Erysipelotrichaceae and Coriobacteriaceae with fecal cholesterol might also be independent of PSE intake (*P* = 0.076, *P* = 0.069). Importantly, the abundance of the family Coriobacteriaceae (*P* = 0.0726, *r* = -0.7122) and the OTUs 1 (*P* = 0.0244, *r* = -0.82) and 13 (*P* = 0.0032, *r* = -0.92) also showed highly significant negative associations with fecal cholesterol excretion in hamsters on the control diet alone (Supplementary figure 4). These findings suggest that the associations detected between
host cholesterol metabolism and the microbiota are not solely caused by PSE, but instead by a
direct association of the host phenotype with the bacterial taxa.

Cholesteryl-linoleate but not plant sterols inhibit growth of gut bacteria in vitro

To gain insight into the functional interactions between bacteria and cholesterol metabolism, we
determined the capability of cholesterol, cholesteryl-linoleate, and the plant sterols stigmastanol
and β-sitosterol to inhibit bacterial growth when included in model bile mixed micelles containing
a bile salt and lecithin (which are natural components of micelles in the gut) (31). We used plant
sterols for these experiments as PSE get hydrolyzed in the gut, and the unesterified sterol is the
compound that gets incorporated into intestinal micelles (6). We tested for the inhibition of
seven strains of gut bacteria including strains belonging to the Coriobacteriaceae, as members
of this bacterial family were most affected in our animal experiments. No statistically significant
levels of inhibition were detected for any of the treatments. However, out of the seven strains
tested, Lactobacillus reuteri, Clostridium histolyticum, and Collinsella aerofaciens showed a
modest degree of inhibition. All three showed reduced growth in the presence of micelles
containing cholesteryl-linoleate when compared to control micelles containing only lecithin and
bile acid (Figure 4). Growth of Lactobacillus reuteri also appeared to be inhibited by micelles
containing cholesterol.

Discussion

In this study, we have characterized the interplay between the gut microbiota and cholesterol
metabolism in hamsters treated with PSE. The study revealed that PSE-induced alterations of
cholesterol metabolism were tightly associated with specific compositional shifts of the gut
microbiota. The strongest associations were identified between the families Coriobacteriaceae
and Erysipelotrichaceae, and several OTUs within these families, and host cholesterol
concentrations in plasma, the liver, and fecal samples. Although it is difficult to determine cause-
effect relationships among these associations because of the impossibility to raise hamsters germ-free, this study provided several lines of evidence that indicate that the bacterial shifts induced by PSE are a consequence of changes in host cholesterol metabolism.

First, the capability of PSE to decrease cholesterol absorption and ultimately modify the host cholesterol metabolism is based on physico-chemical interactions independent of bacterial action. Second, levels of cholesterol derivatives (coprostan-3-one, coprostan-3-ol and dihydrocholesterol) that are considered to contribute to the cholesterol lowering activity of the gut microbiota were not affected by PSE and showed no association with host cholesterol phenotypes. Third, PSE have not been described to be antibacterial, and are therefore unlikely to directly cause the dramatic shifts in the microbiota when added to the diet. In contrast, cholesterol-derivatives, and especially cholesterol-linoleate, have been shown to be antibacterial (16, 26), and associations between several bacterial taxa affected by dietary PSE with fecal and biliary cholesterol excretion showed an excellent fit to a sigmoidal inhibitory nonlinear model of dose-response relations between bacteria and inhibitory compounds (Figure 3). Fourth, fecal cholesterol excretion was negatively associated with bacterial taxa when only hamsters in the control treatment were considered.

The data obtained in this study therefore suggests that changes in host cholesterol metabolism induced through dietary PSE were the main drivers of the modulation in gut microbiota composition. A schematic summary illustrating the physiological processes that are likely to have caused the PSE-induced associations between host cholesterol metabolism and the gut microbiota is shown in Figure 5. Intake of PSE decreased plasma and liver cholesterol levels through an inhibition of both dietary and biliary cholesterol absorption in the small intestine, with a consequent increase in fecal cholesterol excretion (Figure 5A). In order to maintain cholesterol homeostasis, the host compensated for the decrease in the total cholesterol pool by increasing
cholesterol synthesis (Figure 5B), which resulted in a further increase of bile-excreted cholesterol. The combination of higher biliary cholesterol excretion and decreased cholesterol absorption resulted in higher concentrations of free and esterified cholesterol in the gastrointestinal tract. These cholesterol derivatives exert an antibacterial effect on specific members of the gastrointestinal microbiota, causing alterations in the microbial community. Since cholesterol excretion strongly correlated with plasma cholesterol levels, the antimicrobial effect of cholesterol is ultimately causing detectable correlations between specific bacterial taxa and host plasma and liver cholesterol levels (Figure 5B).

The findings obtained in this study are relevant to our understanding of the gut microbiota-host lipid metabolism interplay. The linkages between the gut microbiota and cholesterol metabolism found in PSE-treated hamsters recapitulated previous findings in hamsters fed grain sorghum lipid extracts (GSL) (28). In addition, negative correlations between Coriobacteriaceae and Erysipelotrichaceae with fecal cholesterol excretion were also detected by our group in an independent experiment with hamsters that were fed whole sorghum kernels (Martínez et al., unpublished observations). Bacterial taxa within the families Coriobacteriaceae and Erysipelotrichaceae have been recurrently associated with host dyslipidemic phenotypes in mice and humans, in the context of obesity, metabolic syndrome, and hypercholesterolemia (39, 49, 50). If microbiome alterations would contribute to lipidemic aberrancies, they could constitute potential pharmaceutical targets to improve host metabolic functions. In fact, we have previously suggested Coriobacteriaceae as potential therapeutic targets to improve host cholesterol metabolism (28). However, the data obtained with PSE treated hamsters suggest that the strong associations between Coriobacteriaceae and Erysipelotrichaceae and host cholesterol metabolism are caused by the host phenotype affecting the bacteria. Analogous interactions could exist in mice and humans, especially in relation to metabolic disorders that are associated with an altered cholesterol metabolism, such as obesity and the metabolic
syndrome. The findings also indicate that diet can modulate gut microbiota composition through an effect on host metabolism, which has also been demonstrated for dietary-fat-induced changes in host bile-acid composition (15).

The findings obtained in PSE-treated hamsters provided the first evidence for a role of cholesterol as a relevant host factor that modulates the gut microbiota. The in vitro assays performed in this study confirmed the antibacterial effect of cholesteryl-linoleate on selected strains of gut bacteria. Although the antibacterial effect detected in the in vitro experiments was small, even small levels of inhibition could be relevant under the competitive conditions in the gastrointestinal tract where even a minor reduction in growth rate could translate into a significant ecological disadvantage. It is important to point out that the approximate concentrations of cholesterol in the in vitro experiments, due to experimental limitations, were around 20 times lower than those present in the gut of hamsters during PSE treatment. Interestingly, cholesterol and its derivatives have antibacterial activity in the nose and eye epithelial linings (16, 26). In addition, the findings of Do and co-workers suggest that the antibacterial effect of cholesterol-esters in nasal fluid acts in synergism with that of the α-defensin HNP-2. Enteric defensins play a significant role in regulating the gut microbiota (37), and the strong inhibitory effects of cholesterol detected in hamsters might therefore be caused by a synergistic effect of the two compounds. Clearly, the in vitro experiments on the antibacterial effects of cholesterol included in this study are only preliminary, and further research should be targeted towards the role of cholesterol as a host factor that modulates the gut microbiota.

Bile acids, which are synthesized from cholesterol, also have antimicrobial activity (17, 40). Bile acids have been demonstrated to modulate gut microbiota composition (15, 20), and since concentration and composition of bile excreted are influenced by dietary fat, bile acids have
been suggested to be one potential cause for the dysbiosis that is associated with obesity-related pathologies (48). However, bile acids did not appear to be a contributing factor in the population shifts observed in PSE treated hamsters, as fecal bile acid excretion was reduced by PSE and showed significant positive correlations with the bacteria of the Coriobacteriacea and Erysipelotrichaceae family that were affected by PSE. Although the reduction of bile excretion in PSE treated hamsters was likely caused by the lower cholesterol pool, only fecal cholesterol showed highly significant correlations with both the improvements in host lipid metabolism and the abundance of bacterial taxa.

Although this study revealed an example by which a host metabolic factor influences the gut microbiota composition, research with germ-free animals has clearly shown that gut microbes impact host metabolism (including cholesterol metabolism), and it is likely that some alterations of the gut microbiome associated with host metabolism have functional consequences for the host (5, 8, 44, 46). Specific bacterial taxa have been determined to improve lipid markers in the host. Bifidobacteria, which have shown positive associations with plasma HDL cholesterol in our previous study (28), have been identified to alleviate dyslipidemia and high-fat-induced insulin resistance when administered as probiotics (11, 21, 22, 36, 47). In addition, some changes in gut microbiota composition induced through host factors might still have pathologic consequences, as shown recently for a fat-induced pathobiont expansion caused by changes in the bile acid pool (15). Given that the gut microbiota reduces liver and plasma cholesterol levels (13, 46), we cannot exclude that the dramatic changes in Coriobacteriacea and Erysipelotrichaceae contribute to the cholesterol lowering effects of PSEs. However, the findings obtained in this study provide evidence that interactions between the gut microbiota and host metabolism are bi-directional, and some patterns of dysbiosis associated with metabolic dysfunctions might be a consequence rather than a cause of the host phenotype.
Acknowledgements

Susan Hammons was supported by the UCARE Program of the University of Nebraska. We thank Dr. John H. Rupnow for organizing and supporting D. P.’s internship at the University of Nebraska.

References


Figure legends

Figure 1. Alpha- and beta- diversity measurements of the fecal bacterial communities. Principal coordinate analysis based on Unifrac distances, segregated fecal microbial communities of hamsters fed plant sterols esterified with stearic acid (dark grey closed circles) and beef tallow (open circles) from animals fed a control diet (light grey closed circles) (A). Shannon’s diversity index (B). * P < 0.05.

Figure 2. Associations among host markers of the lipid metabolism, and with fecal bacterial populations. Heatmap displaying correlation coefficients among markers of the host lipid metabolism (A), and between host lipid profile and abundance of fecal bacterial populations (B).

Figure 3. Inhibitory associations between fecal bacterial populations and markers of host cholesterol metabolism. Four-parameter sigmoidal inhibitory regressions between fecal abundance of Coriobacteriaceae (A, B, C), OTU1 (D, E, F), and OUT13 (G, H, I) with whole body cholesterol synthesis (A, D, G), fecal biliary cholesterol excretion (B, E, H), and fecal cholesterol excretion (C, F, I).

Figure 4. In vitro inhibition of fecal bacterial isolates by sterols. Optical density difference between micelles containing cholesterol, cholesteryl-linoleate, β-sitosterol, and stigmasterol compared to control micelles without sterols for the gut bacterial isolates Lactobacillus reuteri (A), Collinsella aerofaciens (B), and Clostridium histolyticum (C).
Figure 5. Model depicting the impact of plant sterol esters on the host lipid metabolism and on the gut microbiota. The inclusion of dietary plant sterol esters (PSE) precludes the incorporation of cholesterol into the micelles in the small intestine, lowering cholesterol absorption and increasing cholesterol excretion. The removal of cholesterol results in reductions in plasma non-HDL, liver weight and hepatic liver esterified cholesterol (A). The decreased cholesterol pools are compensated by increased hepatic cholesterol synthesis. The higher concentrations of fecal cholesterol coupled with the inhibitory capacity of cholesterol result in the decreased abundance of bacterial populations of Coriobacteriaceae and Erysipelotrichaceae in the gastrointestinal tract (B).
Table 1. Abundance of fecal bacterial taxa of hamsters fed a control diet, or diets enriched in plant sterol esters. The taxa presented were significantly affected by plant sterols esterified with stearic acid (SA) or beef tallow (BT), or were determined to be associated with host metabolic markers of the lipid metabolism.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Control</th>
<th>SA</th>
<th>BT</th>
<th>ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria</td>
<td>16.55 ± 7.27</td>
<td>3.24 ± 1.90***</td>
<td>8.37 ± 5.04*</td>
<td>0.0002</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>72.88 ± 5.50</td>
<td>85.07 ± 7.25***</td>
<td>76.44 ± 4.90</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>Control</th>
<th>SA</th>
<th>BT</th>
<th>ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coriobacteriaceae</td>
<td>11.63 ± 7.30</td>
<td>0.88 ± 0.49***</td>
<td>2.71 ± 2.32**</td>
<td>0.0002</td>
</tr>
<tr>
<td>Eubacteriaceae</td>
<td>0.02 ± 0.05</td>
<td>10.85 ± 11.58***</td>
<td>0.61 ± 1.32</td>
<td>0.0156</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genus</th>
<th>Control</th>
<th>SA</th>
<th>BT</th>
<th>ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unc. Coriobacteriaceae</td>
<td>11.57 ± 7.30</td>
<td>0.87 ± 0.49***</td>
<td>2.66 ± 2.27**</td>
<td>0.0002</td>
</tr>
<tr>
<td>Unc. Erysipelotrichaceae</td>
<td>1.08 ± 0.30*</td>
<td>0.66 ± 0.24</td>
<td>0.76 ± 0.40</td>
<td>0.0409</td>
</tr>
<tr>
<td>Unc. Eubacteriaceae</td>
<td>0.02 ± 0.05</td>
<td>10.85 ± 11.58***</td>
<td>0.61 ± 1.32</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OTUs (family, genus, species)</th>
<th>Control</th>
<th>SA</th>
<th>BT</th>
<th>ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU1 (Coriobacteriaceae, <em>Eggerthella lenta</em> 97%)</td>
<td>6.98 ± 7.46</td>
<td>0.24 ± 0.31*</td>
<td>0.04 ± 0.07*</td>
<td>0.0078</td>
</tr>
<tr>
<td>OTU2 (Coriobacteriaceae, <em>Gordonibacter pamelaeae</em> 93%)</td>
<td>2.05 ± 0.76</td>
<td>0.12 ± 0.10***</td>
<td>0.81 ± 1.19*</td>
<td>0.0002</td>
</tr>
<tr>
<td>OTU4 (Coriobacteriaceae, <em>Slackia heliotrinireducens</em> 97%)</td>
<td>1.22 ± 0.93</td>
<td>0.03 ± 0.10**</td>
<td>0.24 ± 0.30*</td>
<td>0.0011</td>
</tr>
<tr>
<td>OTU8 (Erysipelotrichaceae, <em>Allobaculum stercoricanis</em> 91%)</td>
<td>6.56 ± 4.80</td>
<td>1.48 ± 2.24*</td>
<td>2.71 ± 2.51</td>
<td>0.0205</td>
</tr>
<tr>
<td>OTU9 (Erysipelotrichaceae, <em>Allobaculum stercoricanis</em> 86%)</td>
<td>3.30 ± 3.19</td>
<td>0.15 ± 0.14**</td>
<td>0.31 ± 0.28*</td>
<td>0.0051</td>
</tr>
<tr>
<td>OTU10 (Erysipelotrichaceae, <em>Eubacterium cylindroides</em> 87%)</td>
<td>1.03 ± 1.00</td>
<td>0.08 ± 0.07*</td>
<td>0.28 ± 0.23</td>
<td>0.0125</td>
</tr>
<tr>
<td>OTU12 (Erysipelotrichaceae, <em>Allobaculum stercoricanis</em> 94%)</td>
<td>3.14 ± 1.55</td>
<td>0.71 ± 0.86**</td>
<td>1.43 ± 1.08*</td>
<td>0.0021</td>
</tr>
<tr>
<td>OTU13 (Erysipelotrichaceae, <em>Eubacterium biforme</em> 87%)</td>
<td>0.52 ± 0.25</td>
<td>0.09 ± 0.09**</td>
<td>0.22 ± 0.40</td>
<td>0.0100</td>
</tr>
<tr>
<td>OTU15 (Eubacteriaceae, <em>Clostridium sulfavum</em> 90%)</td>
<td>0.91 ± 0.67</td>
<td>0.20 ± 0.29*</td>
<td>0.29 ± 0.21*</td>
<td>0.0105</td>
</tr>
<tr>
<td>OTU16 (Eubacteriaceae, <em>Eubacterium limosum</em> 93%)</td>
<td>0.04 ± 0.06</td>
<td>8.55 ± 12.71</td>
<td>0.62 ± 1.49</td>
<td>0.0972</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01, *** P < 0.001 compared to Control
§ P < 0.05, §§ P < 0.01, §§§ P < 0.001 compared to BT
A

PC2 (20%)
PC1 (29%)
PC3 (15%)

B

Shannon's diversity index

Control  SA  BT
Coriobacteriaceae abundance (%)  

OTU1 abundance (%)  

OTU13 abundance (%)  

Whole body cholesterol synthesis (mmol/day*100g BW)  

Fecal biliary cholesterol excretion (mmol/day*100g BW)  

Fecal cholesterol excretion (mmol/day*100g BW)  

A  R = -0.93  

B  R = -0.87  

C  R = -0.91  

D  R = -0.98  

E  R = -0.89  

F  R = -0.94  

G  R = -0.75  

H  R = -0.75  

I  R = -0.73
Liver Plant Sterol Esters in Diet
Gallbladder Chylomicron Plasma
Fecal cholesterol excretion
Liver weight Cholesterol absorption
Host cholesterol synthesis
Exclusion of cholesterol from micelles
Liver esterified cholesterol
Cholesterol circulation
Plasma non-HDL and total cholesterol
Inhibition of susceptible bacterial taxa through cholesterol
Coriobacteriaceae Erysipelotrichaceae OTUs within these families
Fecal cholesterol excretion
Biliary cholesterol excretion
Host cholesterol synthesis