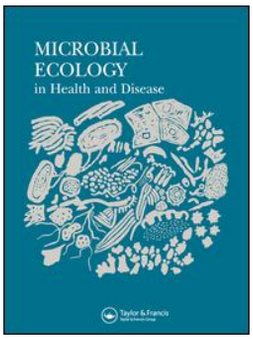

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Probiotics in gnotobiotic mice

Conversion of cholesterol to coprostanol in vitro and in vivo and bile acid deconjugation in vitro

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The intestinal flora may act on cholesterol metabolism either, directly by converting the molecule to coprostanol, or indirectly, by influencing upon intestinal bile acid metabolism, mainly by deconjugation. In the present study, sixteen probiotic strains belonging to the genera: *Bifidobacterium*, *Enterococcus*, *Lactobacillus* and *Streptococcus* were investigated for possible direct action on cholesterol metabolism *in vitro* and *in vivo* in ex-germfree mice monoassociated with each probiotic. Additionally, deconjugation of taurodeoxycholic acid (TDC) and of glycodeoxycholic acid (GDC) was investigated *in vitro* as an indirect effect upon cholesterol metabolism, using the same strains. Gas-liquid chromatography and thin-layer chromatography were used. None of the probiotic strains, was able to transform cholesterol to coprostanol *in vitro* or *in vivo*. Of the total probiotics analyzed, *B. bifidum* B12, *L. acidophilus* La5, *L. acidophilus* ATCC4356, *L. fermentum* ATCC14931, *L. plantarum* 299v, *L. rhamnosus* ATCC7469 and *E. faecium* were able to deconjugate both TDC and GDC *in vitro*. We conclude that the formation of coprostanol does not account for the supposed cholesterol-lowering effect of the probiotics tested. As some of the probiotics were able to deconjugate TDC and/or GDC *in vitro*, this microbial function should be studied more extensively *in vivo*. *Key words*: germfree, monoassociated, probiotics, cholesterol, coprostanol, bile acid, taurodeoxycholic acid, glycodeoxycholic acid.

INTRODUCTION

Cholesterol is an essential component of all cellular membranes in mammals and the precursor of primary bile acids and steroid hormones. The total body cholesterol pool is derived from both endogenous and exogenous sources and this pool is affected by the intestinal microflora, among many other factors.

Cholesterol plays an important role in the development of atherosclerosis and coronary heart disease in humans. Consequently, many efforts have been made to control serum cholesterol levels, not only by using pharmaceutical products, but also with the consumption of cultured dairy products i.e., probiotics, often defined as 'live microbial feed supplements which beneficially affect the host, by improving its intestinal microbial balance' (1).

The most likely mechanisms for microorganisms to act upon the cholesterol metabolism are: i) direct action on the cholesterol molecule, converting it into the non-absorbable coprostanol (2–4), ii) indirect influence upon the cholesterol metabolism, by interfering with the enterohepatic circulation of bile acids (5, 6), and iii) direct 'assimilation' or adsorption of the cholesterol molecule to the microbes *in vivo* (7).

Over the years, many studies have been designed to investigate microbial influence(s) upon cholesterol and bile acid metabolisms *in vitro* as well as *in vivo* (for review, see (4)). In general, microbial conversion of cholesterol to coprostanol is a rare event, and only some few strains have been isolated (8–10). Li et al. fed *E. coprostanoligenes* to rabbits (3) and also to GF mice (11) and found a significant decrease in serum cholesterol levels, with the concomitant increase of coprostanol excretion. Conversely, they did not find any changes in serum cholesterol levels after feeding the same strain to laying hens (12), despite a significant increase in coprostanol excretion.

Microbial transformation of bile acids has been studied in more detail and deconjugation of bile acids has been reported in several species (13–16). Recently, Elkins & Savage (17) working with *Lactobacillus johnsonii* 100-100, hypothesized that deconjugation of bile acid is an important fact for both supplying bacteria with energy as well as for protecting them from bile acid toxicity.

Since the early studies in humans by Mann & Spoerry (18), showing a surprising decrease in serum cholesterol levels after ingestion of large amounts of fermented milk, other investigators have claimed hypocholesterolaemic

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properties of different milk products in humans (19, 20), as well as in other mammals (5, 21). However, others have failed to demonstrate any cholesterol-lowering capacity of dairy products, following similar trails (22, 23).

The three likely mechanisms of microbial influence on cholesterol metabolism are all included in the Germfree Animal Characteristic/Microflora Associated Characteristic (GAC/MAC) concept (24).

The aim of this study was to investigate two of those mechanisms in some probiotic bacteria: i) conversion of cholesterol to coprostanol *in vitro* and *in vivo* and ii) deconjugation of TDC and of GDC *in vitro*. For this purpose, sixteen bacterial strains from the lactic acid bacteria group and from the *Bifidobacterium* genus, were selected among the most commonly ones used in the manufacture of dairy products.

MATERIAL AND METHODS

Animals

A total of ninety male and female NMRI-KI mice, about 3 months old, were allotted to eighteen groups. Sixteen of them, 4–5 GF mice per group were monoassociated with a probiotic strain. The other two groups were the GF and CV controls, 14 and 10 mice, respectively. The GF mice were reared under GF conditions in light-weight stainless steel isolators (25) and the CV mice in an ordinary animal room with artificial light between 6 a.m. to 6 p.m., temperature $24 \pm 2°C$ and humidity $55 \pm 10\%$. All the animals were fed an autoclaved rodent diet, R36 (Lactamin, Sweden) and had free access to water. The study was approved by the Ethical Committee for Animal Research, Stockholm Nord, Sweden.

Bacterial strains

The probiotic strains used as monoassociates are listed in Table I. They were purchased from international collections or received as donations, and their labels are presented as given by the donors. All the strains were tested for conversion of cholesterol to coprostanol *in vitro* and *in vivo*. All but three strains were tested for TDC deconjugation and all but two strains were tested for GDC *in vitro*. Each strain was cultured in de Man, Rogosa & Sharpe (MRS) or Todd Hewitt (TH) broth according to requirement, and incubated anaerobically at 37°C for 72 h. Thereafter, aliquots of 0.5 ml were inoculated into 10 ml of the *in vitro* test media and aliquots of 10 ml were transferred into ampoules that were sealed and sterilized on the outside with chromsulfuric acid and taken to the GF isolators, to infect each animal group.

Cholesterol conversion

- Test media and procedure *in vitro*. Calf brain-peptone yeast (CB-PY) medium was used to test cholesterol conversion and it was prepared as follows. Aliquots of 100 ml of peptone yeast extract, as described in the

Table I

Deconjugation of taurodeoxycholic (TDC) and of glycodeoxycholic acid (GDC) by some probiotic strains

Bacterial strain	Label*	Source	TDC & GDC† deconjugation	
Bifidobacterium				
B bifidum B11	0014405	Tine, Norway	−	+
B bifidum B12	5001151	Ch. Hansen, Denmark	+	+
Lactobacillus				
L acidophilus La5	0014410	Ch. Hansen, Denmark	+	+
L acidophilus	ATCC4356	Arla, Sweden	+	+
L casei	strain Shirota	Yakult, Japan	NT	NT
L delbrückii subsp *bulgaricus*	DSM20081	Ch. Hansen, Denmark	NT	−
L fermentum	ATCC14931		+	+
L plantarum 271	strain 26	ProViva, Sweden	−	+
L plantarum 299	strain LP1	ProViva, Sweden	−	+
L plantarum 299v	strain LP2	ProViva, Sweden	+	+
L reuteri		BioGaia, USA	NT	NT
L rhamnosus	ATCC7469		+	+
L rhamnosus GG	ATCC53103	Valio, Finland	+	−
Streptococcus and *Enterococcus*				
S thermophilus	ATCC19258	Ch. Hansen, Denmark	−	−
S thermophilus B16	1344506-1	Ch. Hansen, Denmark	−	+
E faecium		Gaio, Denmark	+	+

* type strain by the culture collection or labeled by the donor, † deconjugation of TDC & GDC performed by thin layer chromatography; NT not tested.

Anaerobe Laboratory Manual (26), were mixed with 2.5 g freeze-dried calf brain homogenized in 100 ml of phosphate citrate buffer. Thereafter, 0.8 ml of resazurin solution (11 mg resazurin in 44 ml distilled water) were added. This medium was dispensed in 10 ml test tubes, autoclaved at 115°C for 10 min, inoculated with the bacteria (see above) and incubated anaerobically for 72 h at 37°C.

- Positive control. For the conversion of cholesterol to coprostanol in the *in vitro* study, the so-called 'long line' was used. This consists of a microbial mixture originating from a caecum of a CV AGUS rat, subcultured in the CB-PY medium around 2300 times, starting almost 30 years ago. The cholesterol-converting property has routinely been checked at intervals.
- Cholesterol conversion *in vivo*. Each group of mice was transferred into a small stainless steel rearing isolator (SRI) together with a fresh bacterial suspension of the respective probiotic strain (see above), which was spread on the bedding material and fur of the mice. The animals remained within the SRI for 10–15 days. Thereafter, they were taken out, anesthetized and killed by cervical dislocation. To verify bacterial establishment, two inocula from caecum of 1 ωl each, were cultured in MRS or TH broth and agar. From the inoculated broth, additional aliquots of 10 ωl and 1 ωl were plated onto agar. All the media were incubated anaerobically at 37°C for 72 h. To investigate cholesterol conversion, cecal and large intestinal contents from each animal were sampled and stored frozen at -20°C until analysis.
- Pretreatment of samples prior to gas liquid chromatography (GLC) analysis. The caecum and colon content samples were thawed and aliquots of 0.5–1.0 g diluted with 2 ml of saline and homogenized. These samples as well as the contents of the *in vitro* culture tubes, were further hydrolyzed with 2 ml of a solution of 95% ethanol and 10 M sodium hydroxide (2:1) in a water bath at 60°C for 45 min. The hydrolysate was extracted twice with n-hexane; the combined hexane phases were extracted with 70% ethanol until pH was neutral and evaporated to dryness.
- GLC analysis of cholesterol conversion. The dried samples were dissolved in acetone and assayed in a gas liquid chromatograph equipped with a glass column packed with 3% OV-17 maintained at 290°C and with a flame ionization detector. The results were expressed as the percentage of coprostanol out of the total cholesterol plus coprostanol present. By using this method, peak areas less than 5% were regarded as impurities. GF animals do not excrete coprostanol (GAC) in feces, whereas CV animals do (MAC).

Bile acid deconjugation

For TDC and GDC deconjugation analyses, each inoculated MRS or TH broth (see above) was supplemented either, with 2.5 mM TDC or 2.5 mM GDC and incubated anaerobically at 37°C for 72 h. Thereafter, the free bile acids were extracted by reflux boiling twice for 2 hours: i) with 96% ethanol and ii) with chloroform: methanol (1:1), followed by extraction with 70% ethanol/hexane. Then, aliquots were assayed by thin-layer chromatography (TLC) which was performed on pre-coated plates with silica gel 60 (27), using n-butanol: acetic acid: water (110:8:12) as a mobile phase for 8 h run and the glass plates were developed with pure iodine. The results were expressed as positive or negative with regard to deconjugation capacity.

RESULTS

All the animals remained healthy throughout the study. All strains except *L. delbrückii* subsp *bulgaricus* were established in the large intestine of the mice in numbers higher than 10^7. Lower numbers of microbes (10^3) were found in those animals inoculated with *L. delbrückii* subsp *bulgaricus*.

Cholesterol conversion in vitro

Coprostanol was not detected in any of the probiotic cultures (data not shown). The 'long line' control showed 90% conversion of cholesterol to coprostanol (Fig. 1A).

Cholesterol conversion in vivo

No coprostanol was found in any of the mice inoculated with the probiotic strains tested and this value corresponds to a GAC (Fig. 1B). The chromatograms of the CV mice tested, showed a mean value of 21% cholesterol conversion; a result from one conventional mouse is presented in Fig. 1C. A similar conversion has been found when the 'long line' is established in ex-germfree mice (data not shown).

Microbial bile acid deconjugation in vitro

The results of the TDC and GDC deconjugation are presented in Table I. When tested, all strains except two were capable of splitting bile acid conjugates. Five of them were able to split one of either conjugates.

DISCUSSION

Conversion of cholesterol to coprostanol is a microbial function carried out by anaerobic microorganisms present in the intestine of mammals. By definition, GF animals lack that conversion (GAC). As early as 1959, Danielsson & Gustafsson (28) found significantly higher serum cholesterol levels in GF than in CV animals fed the same diet.

The results of this study show that none of the strains tested was able to convert cholesterol to coprostanol i.e.,

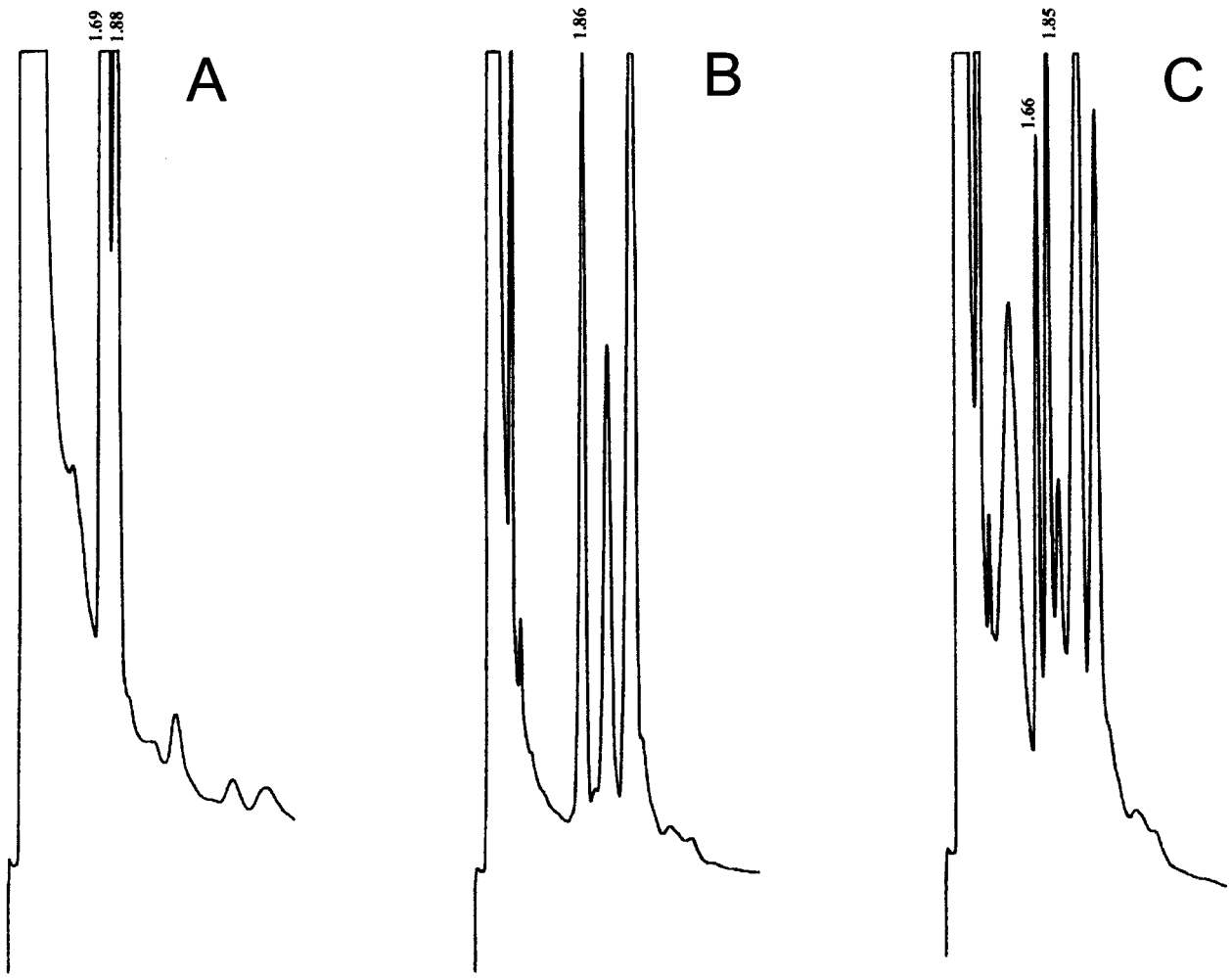

Fig. 1. Gas-liquid chromatograms from: A. calf brain-peptone yeast (CB-PY) medium inoculated with the positive control 'long line'; peaks 1.69 and 1.88 represent retention time for coprostanol (90%) and cholesterol, respectively, B. large intestinal sample from a NMRI mouse inoculated with the probiotic *L plantarum* 299v; peak 1.86 represents retention time for cholesterol; no coprostanol (0%) peak is observed, C. large intestinal sample from a NMRI conventional mouse; peaks 1.66 and 1.85 represent retention time for coprostanol (40%) and cholesterol, respectively.

to act directly upon the cholesterol molecule. To the best of our knowledge, no probiotic strain has shown the ability to perform that conversion. It might be reasonable to assume that this capability does not account for the supposed cholesterol-lowering effect of probiotics, claimed by some investigators (19, 20, 29, 30).

The other two mechanisms of lowering cholesterol levels—increase in deconjugation of bile acids and cholesterol 'assimilation'—, are indirect effects on the molecule, and they are mainly based on the capacity of the strains to interact with bile acid metabolism.

Cholic and chenodeoxycholic acids are the most common primary bile acids in several mammalian species, including man and are yield as the main end products from cholesterol metabolism in the liver. They are conjugated within the liver mainly with glycine or taurine, followed by secretion into the bile. In their conjugated form, they perform emulsification of dietary lipids. When reaching the intestine, they undergo several microbial transformations, including deconjugation (4). Thus, bile acids in CV animals are excreted in feces mainly in their free form, while in GF animals, as conjugates. The bile salt pool turns over 6–10 cycles per day. Most of the bile acids are reabsorbed to return to the liver via the portal vein; a small amount is excreted in the stools per day, which balances hepatic synthesis.

It is well established that deconjugated bile acids have a greatly reduced solubility, which in turn affects the solubility of cholesterol (31). It has been shown that some factors such as pH and presence of a keto group in position C7, influences upon adsorption of the bile acids to intestinal microorganisms (32). Klaver & van Der Meer showed that 'assimilation' of cholesterol to lactobacilli and *Bifidobacterium bifidum* is due to their bile acid-deconjugating activity (16).

All three hypothesis of a serum cholesterol-lowering effect of probiotics have in common that the interactions have to take place in the small intestine i.e., the area in which cholesterol and bile acids are absorbed.

As it is evident from Table I, the results show that several probiotic strains can deconjugate bile acids. If this were the case *in vivo* and if the strain could reside at least temporarily in the lower part of the small intestine, we would expect interference with the bile acid enterohepatic circulation and thus indirectly affecting the total cholesterol pool. Additionally, deconjugated bile acids may have a reduced absorption rate and their higher excretion would influence the cholesterol metabolism through an increased *de novo* synthesis of bile acids.

Interestingly, the claimed cholesterol-lowering capacity of probiotics are all related to species that are able to deconjugate bile acids *in vitro* (21, 33–35). However, whether and to what extent bile acids hydrolases deriving from probiotic strains, exert splitting of the conjugates in the small intestine, have as far as we are aware of, not been investigated in man.

The adsorption or 'assimilation' hypothesis is based upon some *in vitro* models (7, 36, 37). As in the case of the deconjugation hypothesis, adsorption has never been shown to take place in the small intestine. Thus, further research is needed to approach and clarify the complex mechanisms by which a specific probiotic may act on blood cholesterol levels.

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