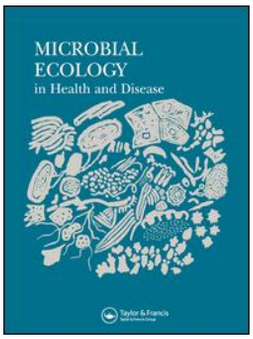

Microbial Ecology in Health and Disease

ISSN: (Print) 1651-2235 (Online) Journal homepage: informahealthcare.com/journals/zmeh20

In Vitro Study of Bile Salt Hydrolase (BSH) Activity of BSH Isogenic *Lactobacillus plantarum* 80 Strains and Estimation of Cholesterol Lowering through Enhanced BSH Activity

I. De Smet, L. Van Hoorde, N. De Saeyer, M. Vande Woestyne & W. Verstraete

To cite this article: I. De Smet, L. Van Hoorde, N. De Saeyer, M. Vande Woestyne & W. Verstraete (1994) *In Vitro* Study of Bile Salt Hydrolase (BSH) Activity of BSH Isogenic *Lactobacillus plantarum* 80 Strains and Estimation of Cholesterol Lowering through Enhanced BSH Activity, Microbial Ecology in Health and Disease, 7:6, 315-329, DOI: 10.3109/08910609409141371

To link to this article: https://doi.org/10.3109/08910609409141371

Published online: 11 Jul 2009.

MICROBIAL ECOLOGY IN HEALTH AND DISEASE VOL. 7: 315–329 (1994)

In Vitro Study of Bile Salt Hydrolase (BSH) Activity of BSH Isogenic *Lactobacillus plantarum* 80 Strains and Estimation of Cholesterol Lowering through Enhanced BSH Activity

I. DE SMET, L. VAN HOORDE, N. DE SAEYER, M. VANDE WOESTYNE and W. VERSTRAETE*

Division of Microbial Ecology, Faculty of Agricultural and Applied Biological Sciences, University of Gent, Coupure Links 653, B-9000 Gent, Belgium

Received 7 July 1994; revised 15 September 1994

Growth and bile salt hydrolase (BSH) activity of the isogenic *Lactobacillus plantarum* 80 (LP80) strains were studied *in vitro*. In pure culture experiments viability and growth performance of the BSH$^-$ LP80 strain was negatively affected by the presence of conjugated bile salts. The LP80 wild type (WT) and BSH overproducing LP80 (pCBH1) strains did not show a die-off upon supplementation of bile salts. The latter strains hydrolysed glyco-conjugated deoxycholate (GDCA) more readily than tauro-conjugated deoxycholate (TDCA), indicating substrate specificity of the enzyme. BSH activities towards TDCA of LP80 WT and LP80 (pCBH1) stationary phase cells were 0·17 and 1·02 μmol/mg CDW.h respectively; activities towards GDCA of the respective strains were 3·52 and 54·80 μmol/mg CDW.h respectively. The study of BSH activity as a function of growth revealed a marked difference in behaviour between LP80 WT and LP80 (pCBH1) with LP80 WT hydrolysing GDCA when reaching the exponential phase, whereas LP80 (pCBH1) immediately started to hydrolyse GDCA. TDCA hydrolysis increased after GDCA hydrolysis was completed. BSH activity of LP80 (pCBH1) in a mixed microbial association, resembling that of the small intestine, was comparable to that determined under pure culture conditions, indicating that BSH activity will probably not be influenced by the presence of the normal intestinal microbiota. Based on the BSH activity of LP80 (pCBH1) and on physiological data on the bile salt-cholesterol metabolism interrelationship, it was calculated that a daily intake of a realistic amount of highly BSH active *Lactobacillus* cells, e.g. in the form of yoghurt, might lead to a significant reduction of cholesterol. Hence, this *in vitro* study indicates that altering BSH activity can be a valid (micro) biological alternative treatment for patients with severe hypercholesterolaemia.

KEY WORDS—Bile salt hydrolysis; Lactobacilli; Cholesterol; Hypercholesterolaemia; Probiotic food products.

INTRODUCTION

During the last few decades, numerous epidemiologic, laboratory and clinical studies have demonstrated the connection between elevated serum cholesterol levels and increased risk for atherosclerosis and coronary heart disease (CHD), the latter being a major cause of death in Western countries.[2,4,42,51] Potential hypocholesterolaemic pharmaceuticals and food products are continuously being developed in order to control serum cholesterol levels in persons with abnormally high levels. These pharmaceuticals are mostly based on interruption of the enterohepatic circulation (EHC) of bile salts.[73]

Bile salt metabolism and cholesterol metabolism are closely linked. Bile salts are the water-soluble excretory end-products of cholesterol, and are essential for emulsification of fats in the digestive tract. They are synthesised in the liver mainly as glyco- or tauro-conjugates. During EHC, the bile salt pool (5 to 10 mmol) is secreted several times a day (six on average) in the duodenum, and passes through the jejunum into the ileum. During intestinal transit, most of the bile salts are absorbed to return to the liver via the portal vein. The daily faecal loss of bile salts that escape reabsorption is about 1 mmol.[30,50,73] Since the body bile salt pool

*Corresponding author

CCC 0891–060X/94/060315–15

is approximately constant, this loss is to be newly synthesised from cholesterol. The liver is the key organ regulating the overall economy of cholesterol and bile salts. It is the major site of cholesterol synthesis. In addition, it controls the fate of cholesterol, i.e., its conversion to bile salts, its secretion into bile, and its input into plasma with lipoproteins.[5,26] Although affected by, for example, heredity, age and nutrition,[60] the whole body cholesterol content remains approximately unchanged throughout life under normal, healthy conditions. It is the net result of all inputs and outputs. The cholesterol inputs are its endogenous synthesis (80 to 90 per cent) and absorption of dietary cholesterol (10 to 20 per cent).[11,50,61,62] The major excretory pathway of cholesterol involves secretion into bile.[70]

Upon surgical, pharmacological or pathological interruption of the EHC of bile salts, bile salt synthesis is increased, leading to an increased demand for cholesterol in the liver.[30,73]

Apart from the therapeutical or surgical attempts to lower serum cholesterol levels through interruption of the EHC, it has been suggested that the ingestion of certain bacterial cells might also influence cholesterol levels through interference with bile salt metabolism. Lactobacilli have been frequently associated with health-promoting effects in the human and animal intestinal tract, and the use of lactobacilli as a probiotic has been a subject of interest for many years.[16] One of the so-called probiotic effects of lactobacilli is the reduction of serum cholesterol levels.[18–20,25,27,67] Although the underlying mechanism is not yet fully understood, it is suggested that the capacity to hydrolyse bile salts might be responsible for lowering the cholesterol level. During intestinal transit, bile salts undergo a number of bacterial transformations,[34] of which one of the most important is bile salt hydrolysis (BSH). The ability to hydrolyse bile salts is encountered in many intestinal *Lactobacillus* spp., but also in numerous other genera including *Enterococcus, Peptostreptococcus, Bifidobacterium, Fusobacterium, Clostridium*, and *Bacteroides*.[21,44,48] Upon bile salt hydrolysis, glycine or taurine is liberated from the steroid moiety of the molecule, resulting in the formation of free (deconjugated) bile salts. Free bile salts are more easily precipitated at low pH or with Ca^{2+}.[71,74] They are less efficiently reabsorbed than their conjugated counterparts. Hence, they are excreted in the faeces. Since the steady state requires that the amount of bile salts extracted from the EHC is matched by *de novo* synthesis of bile salts from cholesterol, elevated BSH activity will lead to an increased demand for cholesterol. Moreover, it was stated by Chikai *et al.*[7] that deconjugated bile salts are adsorbed to a higher extent to bacterial cells and dietary fibre, which enhances their faecal excretion. In addition, deconjugated bile salts might also physicochemically interact with cholesterol. Klaver and Van der Meer[41] showed that bile salts are able to co-precipitate cholesterol at sufficiently low pH (below 5·5). They presented *in vitro* experimental evidence on the role of BSH activity of lactobacilli in relation to cholesterol lowering in culture liquid. This co-precipitation was earlier reported as 'cholesterol assimilation' by Gilliland *et al.*,[20] who suggested that the hypocholesterolaemic effect of *L. acidophilus* is due to the capacity of bile resistant strains to assimilate cholesterol. However, their experiments to prove cholesterol assimilation did not take into account the effects of bacterial BSH activity. Finally, cholesterol absorption from the intestinal content may be impaired. Since cholesterol must be in a soluble state to be absorbed, its absorption is limited by the amounts that can be solubilised into micelles, i.e. by the intraluminal availability of bile salts and phospholipids, which expand mixed micelles.[55,65] In this respect, it is assumed that deconjugated bile salts, due to their greatly reduced solubility, reduce the solubility of cholesterol.[57]

In this work, BSH activity was studied *in vitro* using BSH isogenic *L. plantarum* 80 strains in view of the potential *in vivo* cholesterol lowering through enhanced BSH activity. Therefore, the experimental *in vitro* data were validated by a theoretical evaluation on the level of bile salt and cholesterol balances. This study is to be regarded as an *in vitro* assessment of the potential use of cultured dairy products to lower serum cholesterol.

MATERIALS AND METHODS

Media and chemicals

Sodium salts of taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), glycocholic acid (GCA) and deoxycholic acid (DCA) were obtained from Sigma. De Man-Rogosa-Sharpe (MRS) broth was obtained from Difco. Other selective and non-selective media used were purchased from Oxoid.

Bacterial strains and growth conditions

The strains used in this study are three bile salt hydrolytic (BSH) isogenic *Lactobacillus plantarum* 80 (LP80) strains, i.e. the BSH wild type strain (LP80 WT), the BSH negative mutant (LP80 BSH^-), and the BSH overproducing LP80 (pCBH1) strain (Table 1). The LP80 WT strain[38] carries the chromosomal *bsh* gene. The LP80 BSH^- strain was constructed by Leer *et al.*[43] by insertional inactivation of the chromosomal *bsh* gene with a chloramphenicol resistance gene. Overproduction of the BSH enzyme in LP80 was obtained as described by Christiaens *et al.*[8] The BSH overproducing LP80 strain harbours the multicopy plasmid pCBH1 carrying the LP80 chromosomal *bsh* gene and an erythromycin resistance gene.

The *Lactobacillus* strains were grown in MRS broth[10] at 37°C in an anaerobic cabinet (Forma Scientific Anaerobic System model 1024) which contained a mixture of 80 per cent N_2, 10 per cent CO_2 and 10 per cent H_2. The BSH overproducing LP80 strain was grown in MRS broth supplemented with 100 µg/ml erythromycin. Viable counts were performed on Rogosa agar plates.[1] LP80 (pCBH1) was enumerated on Rogosa plates which contained 100 µg/ml erythromycin.

Simulation of the Human Intestinal Microbial Ecosystem—SHIME

This five-stage reactor to simulate the human gastrointestinal microbial ecosystem was developed by Molly *et al.*[53,54] This multi-stage reactor was used to study the bile salt hydrolytic activity of the isogenic LP80 strains in a complex mixed microbial association that is representative for the *in vivo* intestinal microbiota. This *in vitro* gastrointestinal microbial ecosystem is subjected to relevant physicochemical stresses that play an important role *in vivo* in the control of the population levels and the activity of the human gut bacteria, i.e. gastric and pancreatic juice, and bile salt stress.

Bile salt hydrolase assay

A modification of the HPLC-procedure described by Scalia[59] was used to determine BSH activity. A reversed phase column PLRP-S (300 Å, 8 µm; 150 × 4·6 mm; Alltech) and an appropriate pre-column were used. The HPLC was equipped with a DAD 440 detector (Kontron), an autosampler MSI T-660 (Kontron), a pump 325 system (Kontron) and DEGASYS DG 1210 (Kontron). Software DS 450 (version 1) was used. The solvents used were HPLC-grade methanol (solvent A), and solvent B, which consisted of a mixture of 65 per cent methanol in 0·03 M sodium acetate adjusted to pH 4·3 by means of phosphoric acid. An isocratic elution of 35 per cent solvent A and 65 per cent solvent B was used at a flow rate of 1 ml/min. An injection loop of 10 µl was used, and UV-detection occurred at 202 nm within 25 min after injection of the bile salt extract.

One ml samples to be analysed were acidified by the addition of 10 µl 6 N HCl to stop BSH activity. GCA was added as an internal standard. From the 1 ml sample, bile salts were then extracted using isopropanol (1:4; v:v).[6] The samples were mixed vigorously for 10 min and centrifuged at 1000 ***g*** for 10 min. Four ml of the isopropanol supernatant was evaporated under an N_2-flow at elevated temperature (37°C) to accelerate the evaporation process. After complete isopropanol removal, the bile salt extract was redissolved in 800 µl methanol, and filtered through a 0·45 µm HPLC-filter (Gelman Sciences, Ann Arbor, MI, USA). Prior to injection in the HPLC, samples were stored at − 20°C.

BSH activity of resting cells

Cells of the *L. plantarum* 80 isogenic strains were grown in MRS broth of pH 6·5. When the cultures had reached the stationary phase, 5·0 mM GDCA or 5·0 mM TDCA were added. During 3·5 h of anaerobic incubation, samples were taken at regular time intervals to analyse for conjugated bile salt content by HPLC. At the same time, viable counts were performed on Rogosa agar plates after 10-fold dilution in physiological saline (8·5 g/l NaCl). The cell dry weight (CDW) of the LP80 isogenic strains was (0·75 ± 0·04) g CDW/Log_{10} 12 CFU. BSH activity was expressed as µmol DCA formed/mg CDW.h.

BSH activity of growing cells of LP80 WT and LP80 (pCBH1)

A 1 per cent inoculum of an overnight grown culture of the LP80 WT and overproducing LP80 (pCBH1) strains was added to fresh MRS broth (pH 6·0) to which 5·0 mM GDCA and 5·0 mM TDCA were added. During the incubation, pH was not controlled. The initial cell concentration was Log_{10} 7·35 ± 0·15 CFU/ml. Samples were taken at regular time intervals during 9 h incubation to determine bile salt content and viable cell concentration. The experiment was performed in duplicate.

Table 1. *Lactobacillus plantarum* 80 (LP80) strains used in this study

L. plantarum 80 strain	Genetic markers or description	Reference
L. plantarum 80		
Wild type (LP80 WT)	Grass silage starter; chromosomal *bsh* gene	Josson *et al.*[38]
BSH$^-$ mutant (LP80 BSH$^-$)	Inactivated chromosomal *bsh* gene through insertion of chloramphenicol resistance gene	Leer *et al.*[43]
BSH overproducer (LP80 (pCBH1))	Multicopy plasmid pCBH1 with *bsh* gene and erythromycin resistance gene	Christiaens *et al.*[8]

BSH activity of LP80 (pCBH1) in the gastrointestinal simulator (SHIME)

To investigate the BSH activity of the BSH overproducing LP80 (pCBH1) strain in a mixed intestinal microbial association, batch experiments in SHIME-reactor content of the small intestine (reactor vessel 1) were performed. One hundred ml of reactor medium was supplemented with 7·5 mM GDCA and 2·5 mM TDCA, thus simulating the average bile secretion into the proximal small intestine after a meal.[30] Three batch experiments were set up, i.e. a control batch (i) in which no LP80 (pCBH1) cells were inoculated to determine the background BSH activity present in the SHIME reactor. In the second batch (ii), content of the SHIME reactor corresponding with the small intestine was supplemented with LP80 (pCBH1) to a final concentration of Log_{10} 8·70 ± 0·10 CFU/ml. In the third batch experiment (iii), filter-sterilised SHIME content (0·22 μm; Millipore) was supplemented with about Log_{10} 8·70 ± 0·10 LP80 (pCBH1) CFU/ml. This experimental set-up in batch was not intrinsically different from the normal SHIME performance since this system simulates the small intestine as a fill-and-draw system.[53] At regular time intervals, the different incubation mixtures were sampled, and bile salts were extracted and analysed by HPLC. Estimation of a number of representative gastrointestinal bacterial groups were performed at the start and after 6·5 h incubation respectively. The following selective and non-selective growth media were used: Violet Red Bile Glucose agar (Oxoid) for Enterobacteriaceae,[37] Slanetz and Bartley medium (Oxoid) for faecal streptococci,[69] MRS agar (Difco) for lactic acid bacteria,[10] Rogosa agar (Oxoid) for *Lactobacillus* spp.,[1] and Nutrient Agar (Oxoid) for total aerobes.[1] The experiments were performed in duplicate.

RESULTS

BSH activity of LP80 resting cells

Cells of the BSH isogenic LP80 strains were harvested at stationary phase and subjected to 5·0 mM GDCA and 5·0 mM TDCA respectively. Conjugated bile salt analyses of these mixtures at regular time intervals showed a linear decrease in the concentration of GDCA and TDCA in the respective LP80 WT and LP80 (pCBH1) cultures. The BSH$^-$ mutant strain did not hydrolyse either of the bile salt conjugates. BSH activities of the BSH WT and overproducing LP80 strains towards GDCA and TDCA respectively were determined and are given in Table 2. These data indicate that the BSH overproducing LP80 (pCBH1) strain showed a 15-fold higher BSH activity towards GDCA than the LP80 WT strain. Both the BSH WT and overproducing LP80 strains show a much lower activity towards TDCA. Furthermore, the glyco-conjugated bile salts exerted an acute bactericidal effect on LP80 BSH$^-$ in that the concentration of viable cells decreased by about 2

Table 2. Bile salt hydrolytic activity (μmol DCA formed/mg CDW.h) of the *L. plantarum* 80 BSH wild type (LP80 WT) strain, the BSH overproducing *L. plantarum* 80 (pCBH1) strain, and the BSH negative *L. plantarum* 80 mutant strain (LP80 BSH$^-$) in stationary phase

	BSH activity (μmol DCA/mg CDW.h) towards	
Strain	GDCA	TDCA
LP80 WT	3·52	0·17
LP80 (pCBH1)	54·80	1·02
LP80 BSH$^-$	0	0

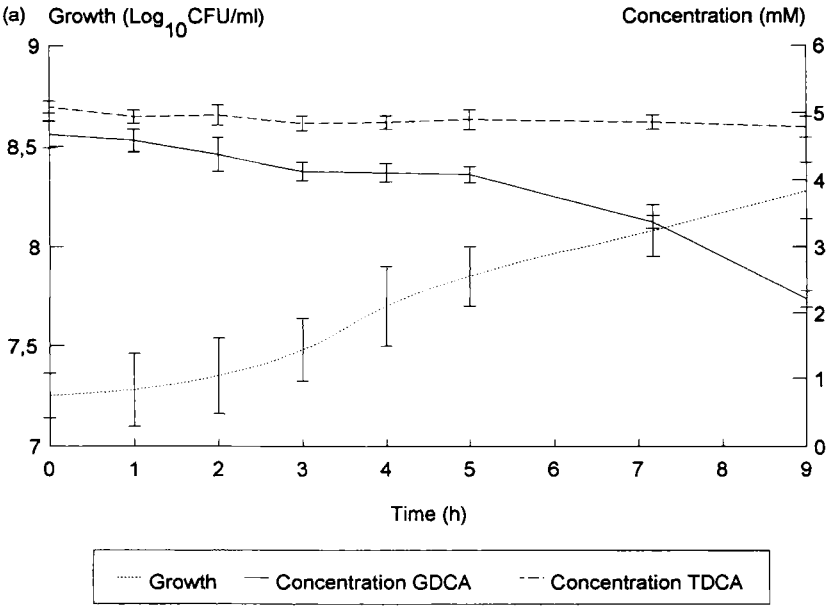

Figure 1a. Growth (Log_{10} CFU/ml) and BSH activity (mM) as a function of time of *L. plantarum* 80 wild type in MRS (pH 6·0) supplemented with 5·0 mM GDCA and 5·0 mM TDCA. The experiment was performed in duplicate; error bars indicate standard deviations

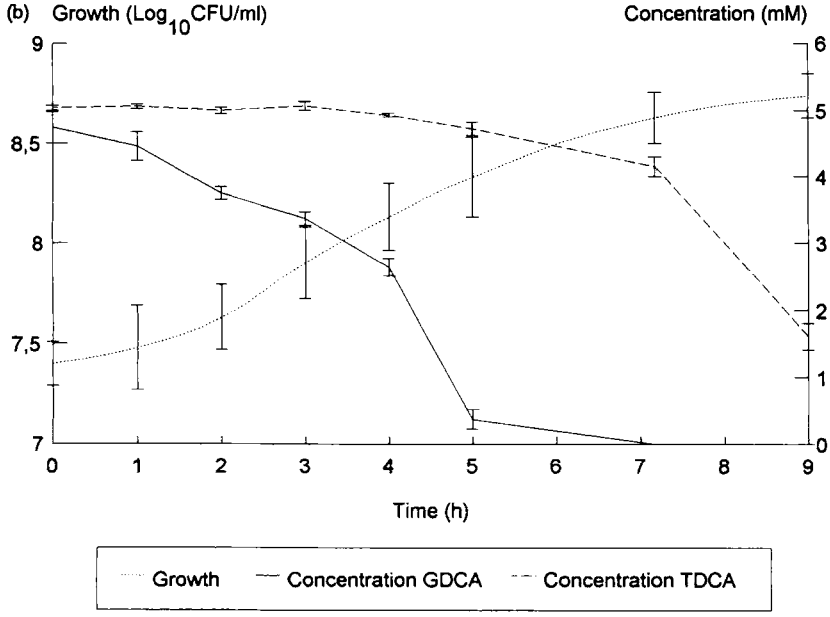

Figure 1b. Growth (Log_{10} CFU/ml) and BSH activity (mM) as a function of time of *L. plantarum* 80 (pCBH1) in MRS (pH 6·0) supplemented with 5·0 mM GDCA and 5·0 mM TDCA. The experiment was performed in duplicate; error bars indicate standard deviations

Log_{10}-units within 30 min after the start of the incubation. Tauro-conjugates did not cause such a reduction (data not shown). Growth of LP80 WT and LP80 (pCBH1) was not negatively affected by the presence of glyco- and tauro-conjugates (Figures 1a and 1b).

Table 3. BSH activity of *L. plantarum* 80 (pCBH1) in a mixed microbial association. pH and viable counts (Log_{10} CFU/ml small intestinal SHIME content) of a number of representative gastrointestinal bacterial groups in simulated gastrointestinal fluid at the onset of the experiment (t=0 h) and after 6·5 h incubation respectively. The experiment was performed in duplicate. Data are the means of two values; standard deviations of pH measurements were $\leq$0·08 pH-units; standard deviations of viable counts were $\leq Log_{10}$ 0·45 CFU/ml

	Control SHIME fluid (i)		SHIME fluid+LP80(pCBH1) (ii)		Sterile SHIME fluid+LP80(pCBH1) (iii)	
	t=0 h	t=6·5 h	t=0 h	t=6·5 h	t=0 h	t=6·5 h
pH	6·25	5·94	6·22	5·81	6·19	6·08
Total aerobes	5·89	8·96	5·78	8·59	<1*	<1*
Enterobacteriaceae	2·78	8·64	2·86	5·60	<1*	<1*
Faecal streptococci	3·37	7·21	3·45	4·06	<1*	<1*
Lactic acid bacteria	3·89	7·54	8·78	8·84	8·74	8·92
Lactobacillus spp.	<1*	2·08	8·65	8·73	8·72	8·79
LP80 (pCBH1)	<1*	<1*	8·61	8·65	8·68	8·76

*<1: value lower than experimental detection limit (Log_{10} 1 CFU/ml).

BSH activity of growing cells of LP80 WT and LP80 (pCBH1)

To investigate whether BSH activity is growth-related, growth of a fresh 1 per cent inoculum of the BSH WT and overproducing LP80 strains in MRS broth (pH 6·0) supplemented with 5·0 mM GDCA and 5·0 mM TDCA was monitored over 9 h and BSH activity was examined at regular intervals. Figures 1a and 1b show the results obtained from the experiments with the LP80 WT strain and the BSH overproducing LP80 (pCBH1) strain respectively. It is clear from Figure 1a that BSH activity of the WT strain towards GDCA only started when cells had reached the exponential growth phase (after about 5 h of incubation). TDCA was not hydrolysed at all during the 9 h incubation period (Figure 1a). The BSH overproducing LP80 (pCBH1) strain started to hydrolyse GDCA immediately, and after about 5 h of incubation, all of the glyco-conjugated bile salts were hydrolysed (Figure 1b). Hydrolysis of TDCA only started after 5 h incubation, i.e. when all of the GDCA had been hydrolysed (Figure 1b).

BSH activity of LP80 (pCBH1) in the gastrointestinal simulator

BSH activity of the BSH overproducing LP80 (pCBH1) was examined in the reactor content of vessel 1 of the simulation of the human intestinal microbial ecosystem. The initial bile salt concentration was set at 7.5 mM GDCA and 2·5 mM TDCA. Three parallel experiments were performed (Table 3), i.e. (i) a control to which no LP80 (pCBH1) cells were administered to the simulated gastrointestinal fluid, (ii) SHIME fluid to which Log_{10} 8·70 ± 0·10 LP80 (pCBH1) CFU/ml were added, and (iii) filter-sterilised SHIME fluid containing Log_{10} 8·70 ± 0·10 LP80 (pCBH1) CFU/ml.

Table 3 shows the pH and viable cell concentrations of a number of representative gastrointestinal bacterial groups at the onset of the experiment and after 6·5 h incubation respectively. The pH was not controlled during the 6·5 h incubation period. The pH decreased about 0·30 units in the control fluid (i) and about 0·45 units in (ii) [SHIME+LP80 (pCBH1)]. The (iii) batch [sterile SHIME+LP80 (pCBH1)] showed a decrease in pH of about 0·2 units (Table 3). The LP80 (pCBH1) concentrations in experiments (ii) and (iii) remained at Log_{10} 8·70 ± 0·10 CFU/ml throughout the 6·5 h incubation period (Table 3). Levels of all other bacterial groups were higher after 6·5 h incubation (Table 3). However, comparison between the viable counts of batches (i) and (ii) shows a marked difference in number of Enterobacteriaceae and faecal streptococci. The presence of about Log_{10} 8·70 LP80 (pCBH1) CFU/ml in batch (ii) caused the number of Enterobacteriaceae and faecal streptococci to be decreased by about 3 Log_{10}-units compared to the control batch (i) (Table 3). The BSH activities against 7·5 mM GDCA and 2·5 mM TDCA in these batch

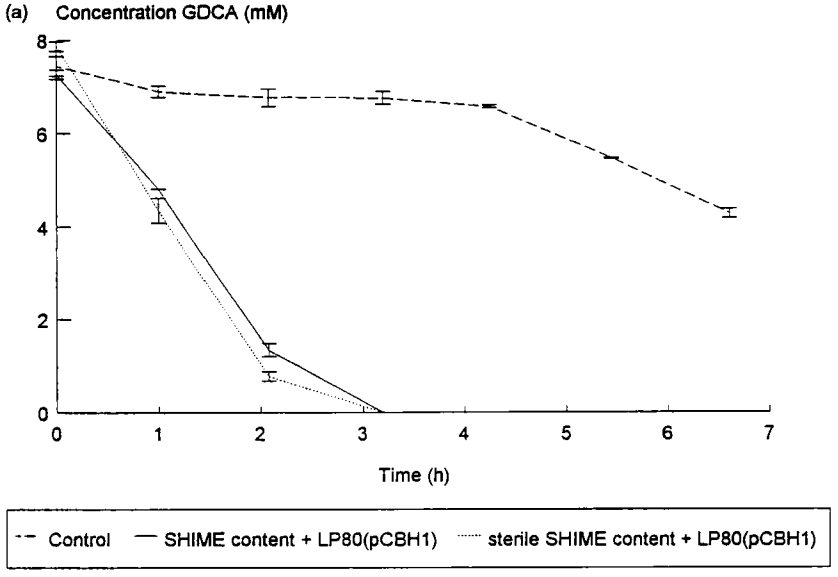

Figure 2a. BSH activity of *L. plantarum* 80 (pCBH1) in mixed microbial population. Hydrolysis of GDCA (mM) as a function of incubation time. The control treatment shows the background BSH activity in SHIME small intestinal content without LP80 (pCBH1) supplementation. The experiment was performed in duplicate; error bars indicate standard deviations

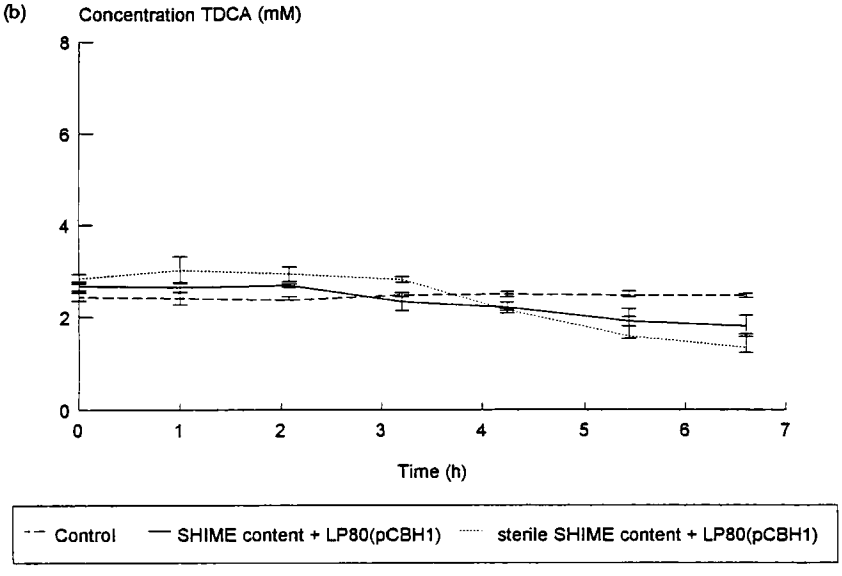

Figure 2b. BSH activity of *L. plantarum* 80 (pCBH1) in mixed microbial population. Hydrolysis of TDCA (mM) as a function of incubation time. The control treatment shows the background BSH activity in SHIME small intestinal content without LP80 (pCBH1) supplementation. The experiment was performed in duplicate; error bars indicate standard deviations

experiments are given in Figures 2a and 2b respectively. The hydrolysis of GDCA as a function of incubation time is shown in Figure 2a. It is clear from this figure that background BSH activity of the intestinal microbiota was of very limited importance, whereas in both experiments with

LP80 (pCBH1), GDCA was readily hydrolysed. Comparison between the non-sterile inoculated small intestinal content (ii) and the filter-sterilised inoculated small intestinal content (iii) showed no significant difference in BSH activity (Figure 2a). In both experiments, 7·5 mM GDCA was completely hydrolysed within about 3·5 h by the addition of LP80 (pCBH1). Figure 2b shows that the level of TDCA hydrolysis was significantly lower than that of GDCA hydrolysis in each of the three batches. In addition, hydrolysis of TDCA in both the filter-sterile (iii) and non-sterile LP80 (pCBH1) inoculated (ii) experiments only started after about 3·5 h incubation, which coincided with complete GDCA hydrolysis.

DISCUSSION

Research on mutant bacterial strains that differ in a single characteristic from their wild type strain may assist in answering many basic questions relating to the microecology of the gastrointestinal tract.[66] In this study, bile salt hydrolytic (BSH) isogenic strains of *L. plantarum* 80 were compared for their BSH activity and growth in the presence of bile salt conjugates *in vitro*. Although *L. plantarum* 80 is a silage isolate, earlier experiments had clearly shown that the *bsh* gene of LP80 shows homology with the DNA of intestinal lactobacilli isolates displaying BSH activity.[8] The presence of the BSH enzyme in a *Lactobacillus* silage isolate may be explained by the physical linkage between the silage ecosystem and the gastrointestinal ecosystem.[68,72]

From the BSH activity testing with resting cells towards either GDCA or TDCA, it was demonstrated that both LP80 WT and LP80 (pCBH1) showed a higher affinity for glyco-conjugates than for tauro-conjugates (Table 2). BSH activity of the overproducing strain towards GDCA corresponds to 54·80 μmol DCA formed/mg CDW.h, whereas that of the WT strain was about 15 times lower (3·52 μmol DCA formed/mg CDW.h) (Table 2). BSH activities towards TDCA of the LP80 (pCBH1) and LP80 WT strains were 1·02 and 0·17 μmol DCA formed/mg CDW.h respectively. This specificity was confirmed when BSH activity was examined in mixtures containing both GDCA and TDCA (Figures 1a and 1b; Figures 2a and 2b), indicating that glyco-conjugated bile salts were being hydrolysed preferentially. Indeed, the BSH enzyme has substrate specificity, depending on the glycine or taurine amino acid moiety.[8,22,40,47,63]

The study of BSH activity of the WT and overproducing LP80 strains as a function of growth phase revealed a marked difference in behaviour between the LP80 WT and LP80 (pCBH1) strains (Figures 1a and 1b respectively). It was shown that BSH activity of the LP80 WT strain was growth dependent. The LP80 WT culture started to hydrolyse glyco-conjugates when it had reached the exponential growth phase, i.e. after a lag-phase of about five hours. Tauro-conjugates were not hydrolysed during the nine hour incubation period observed (Figure 1a). This growth dependency was also observed for *Lactobacillus* sp. strain 100-100 by Lundeen and Savage.[47] However, the BSH overproducing LP80 (pCBH1) strain readily started to hydrolyse GDCA from the start of the incubation, and after about five hours, complete GDCA hydrolysis was observed (Figure 1b). At that stage, TDCA hydrolysis started. In the latter experiments, BSH activity was not induced by the addition of bile salts to the growth medium prior to the BSH activity assay. This indicates that BSH activity is expressed constitutively, which is in agreement with several other reports.[21,35,49]

These experiments also investigated if BSH activity of the overproducing LP80 (pCBH1) strain in a mixed microbial community matched the activity shown in pure culture experiments. For that purpose, content of the small intestine as produced by the SHIME,[53,54] containing a microbiota representative of the *in vivo* human gastrointestinal microbiota, was used. Three parallel batch experiments were performed, each of which initially contained about 10 mM total bile salt concentration (Figures 2a and 2b), corresponding to the average bile salt secretion in the upper small intestine after a meal.[30] The control experiment revealed that background BSH activity in the SHIME was very low, whereas supplementation of the highly active LP80 (pCBH1) strain in both normal and filter-sterilised SHIME content markedly increased the level of BSH activity (Figures 2a and 2b). In addition, no significant differences were observed between the normal and sterile batch, indicating that the presence of the normal gastrointestinal microbiota was not suppressing the BSH active LP80 (pCBH1) strain. Under the experimental conditions used, it took LP80 (pCBH1) less than four hours (i.e. the average *in vivo* residence time in the small intestine) to completely hydrolyse the 7·5 mM glyco-conjugated bile salts initially present. The activity

towards tauro-conjugates was shown to be much lower. However, when extrapolating these data to *in vivo* conditions, where about 7·5 mM glyco- and 2·5 mM tauro-conjugates are excreted in the small intestine after a meal,[30] it can be concluded that the majority of these bile salt conjugates can be hydrolysed by the BSH overproducing *L. plantarum* 80 strain. In addition, this experiment demonstrated the effect of high levels of *L. plantarum* 80 (pCBH1) cells on a number of representative bacterial groups of the gastrointestinal tract (Table 3) in that certain bacterial groups were being inhibited by the lactobacilli. Although the levels of all bacterial groups tested increased during the experimental six and a half hour incubation period, LP80 (pCBH1) had remained at the same level as at the time of inoculation. However, a marked difference in the numbers of enterobacteriaceae and faecal streptococci was observed between the experiments with or without LP80 (pCBH1) addition. Due to the presence of high numbers of LP80 (pCBH1) cells in batch (ii) the numbers of Enterobacteriaceae and faecal streptococci were significantly decreased (Table 3). This confirmed *in vivo* antagonistic effects of lactobacilli observed by several investigators.[36,37,39,45] Indeed, lactobacilli have often been used to control the gastrointestinal microbiota so that potential enteropathogens are eliminated, whereas desirable organisms are maintained. For example *L. acidophilus* was shown to be effective in the treatment of different types of diarrhoea in humans colonised with pathogenic *Escherichia coli*.[3,9]

Based on the physiological interrelationship between bile salt and cholesterol metabolism and the experimental data described above, the potential positive net effect on the overall cholesterol level was calculated. A daily intake of 250 ml yoghurt, containing about Log_{10} 9·50 CFU/ml of the BSH overproducing LP80 (pCBH1) strain (0·75 g CDW/Log_{10} 12 CFU), was assumed. This dose corresponds to about 500 mg CDW. Based on *in vivo* data, it was further assumed that 1 per cent[46,58] of this *Lactobacillus* inoculum (i.e. 5 mg CDW) survives gastric transit and enters the small intestine. In addition, these cells were assumed to reside transiently before being carried along with the small intestinal content without actually colonising the small intestine. Therefore, taking into account the BSH activity of strain LP80 (pCBH1), i.e. about 55 µmol DCA formed/mg CDW.h, and an average residence time of four hours in the small intestine, one can calculate that about 1 mmol (0·5 g) of deconjugated bile salts can be formed through BSH activity by this lactobacillus inoculum. This estimate has to be evaluated against an average hepatic bile salt secretion of 2 mmol/4 h.[50] Hence, the assumed amount of BSH active lactobacilli can deconjugate about half of the bile salt conjugates that are excreted in the small intestine during their residence. In order to evaluate to what extent this altered bile salt metabolism may affect the cholesterol net balance, a number of *in vivo* data on the physiology and physicochemistry of bile salts and cholesterol were considered. In healthy humans about 75 per cent of the bile salt pool is reabsorbed in the conjugated form in an active sodium-dependent way, the remaining 25 per cent being hydrolysed during intestinal transit (Figure 3). Maximally about 15 per cent of the bile salt pool is reabsorbed daily in the unconjugated form via passive diffusion. Thus, about 60 per cent of the deconjugated bile salts are passively reabsorbed. Accordingly, 40 per cent of the deconjugated bile salts are being excreted in the faeces. One mmol deconjugated bile salts formed through increased BSH activity thus yields a supplementary faecal excretion of 0·4 mmol bile salts (40 per cent of 1 mmol). This amount then requires to be newly synthesised from cholesterol to maintain bile salt homeostasis. With an average daily faecal loss of bile salts in the order of 1 mmol,[30,73] this extra loss would be significant and might potentially lead to a decrease of about 30 per cent of the half life of bile, thus increasing the demand for cholesterol as a precursor for bile salt synthesis. In this way, about 30 per cent of the average amount of cholesterol daily passing into the intestine (1·4 mmol[50]) is needed for *de novo* bile salt synthesis.

The alteration in bile salt metabolism through enhanced BSH activity might also affect cholesterol in a more direct way, i.e. by influencing its solubility and intestinal absorption. Mixed micelles, being the main form of bile salts encountered in the small intestine, can maximally dissolve 4 per cent (wt/wt) cholesterol.[33] The intestinal solubility of cholesterol depends on the integration of cholesterol in such micelles. Since the critical micellar concentration values of the conjugated bile salts and their deconjugated counterparts are not significantly different,[32] the 4 per cent (wt cholesterol/wt conjugated bile salts in micelles) ratio can also be used for deconjugated bile salts. Deconjugated bile salts are less resistant to precipitation with Ca^{2+} or to precipitation at pH lower

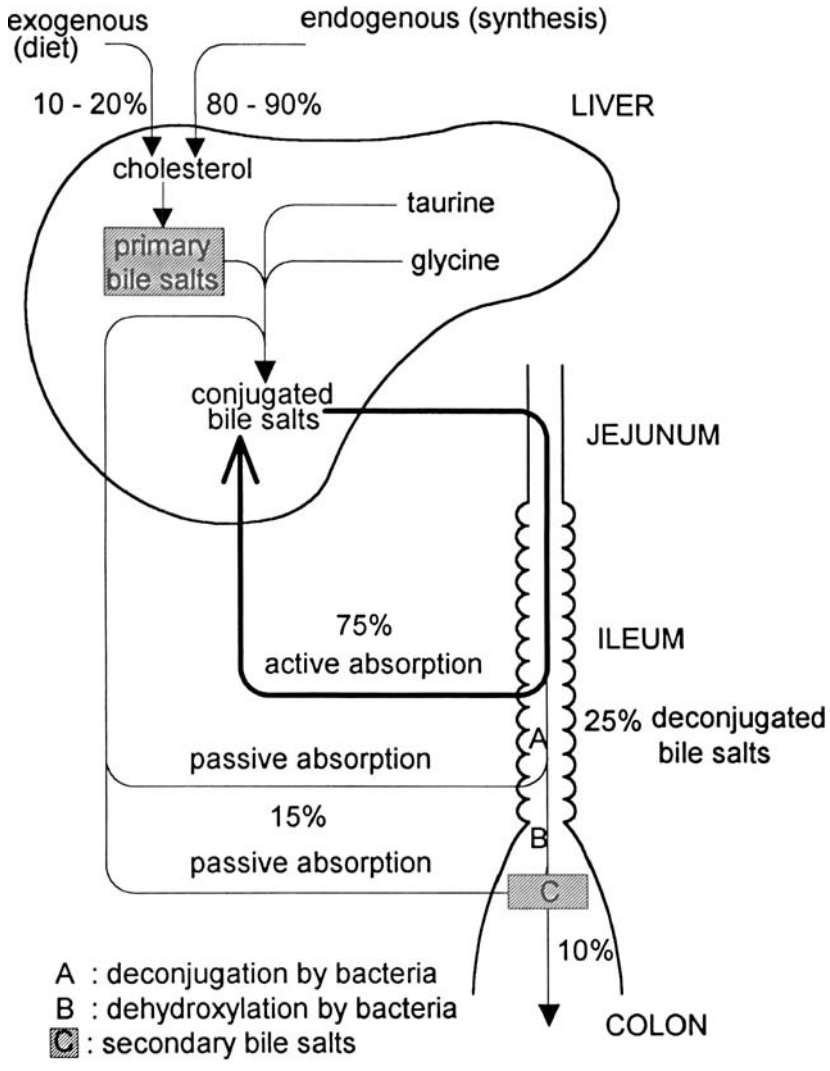

Figure 3. Enterohepatic circulation of bile salts. Daily bile salt fluxes are given as percentages of the bile salt pool.[30,50,73]

than 6·0[71,74] and in this way decrease cholesterol solubility in the intestine.[41] *In vitro* co-precipitation of cholesterol with deconjugated bile salts has been observed when BSH active lactobacilli were cultured in the presence of conjugated bile salts under acidifying conditions.[41] From control experiments without BSH active lactobacilli but with unconjugated bile salts, it appeared that at a pH lower than 5·5, even in the absence of bacterial cells, cholesterol was removed from the medium by precipitation with the unconjugated bile salts. It was therefore assumed that the amount of cholesterol that might be removed from suspension and prevented from absorption, is proportional to the amount of deconjugated bile salts that is formed. Based on the increased BSH activity yielding about 1 mmol of free bile salts, and the 4 per cent solubility-ratio,[33] one can calculate that about 0·05 mmol cholesterol can be solubilised in mixed micelles formed out of the 1 mmol deconjugated bile salts. Thus, 4 per cent of the total amount of cholesterol that passes the small intestine daily (1·4 mmol[50]) can potentially be precipitated. However, due to the pH-dependency, this phenomenon is probably only of limited importance *in vivo* since the pH is neutral to alkaline in

the major part of the small intestine. Indeed, when studying the physicochemical interaction between deconjugated bile salts and cholesterol, Klaver and Van der Meer[41] found that when the pH was maintained at 6·0, no co-precipitation of cholesterol with deconjugated bile salts was observed. Normally, about 0·4 mmol cholesterol (about 30 per cent of the cholesterol passing the small intestine) is absorbed from the intestine daily.[30,50] As cholesterol needs to be in a soluble state before it can be absorbed, its absorption is limited by the amounts that can be solubilised into micelles. Consequently, any change in intestinal bile salt availability might influence cholesterol absorption. When bile salts are diverted from the intestine, or absent as in total biliary obstruction, it was demonstrated that no cholesterol is absorbed.[30] However, due to the complexity of the bile salt—cholesterol interactions, a quantitative estimation of the absorption phenomenon is difficult to make.

Based on the above calculation, it may be concluded that the overall effect of increased intestinal BSH activity on cholesterol would be expected to be due to increased amounts of bile salts excreted in the faeces, and to influence intestinal cholesterol absorption to a lesser extent. This is no more than an amplification of the steady state *in vivo* conditions: only about one third of the intestinal cholesterol is normally reabsorbed in the small intestine, whereas the majority is excreted in the faeces.[30] Thus, enhanced intestinal BSH activity might be compared to a microbial cholestyramine effect, increasing hepatic bile salt synthesis, thus lowering cholesterol levels. However, a question of crucial importance remains, i.e. how adaptive is the bile salt synthesis pathway from cholesterol to make up for the increased faecal bile salt losses. It has been shown by Dowling *et al.*[12] that the increase in bile salt synthesis is only proportional to the degree of interruption of the EHC until the maximal rate of synthesis is achieved. The latter authors were the first to show that in Rhesus monkeys the capacity of bile salt synthesis in response to an interruption of the EHC was limited and reached a maximum at 20 per cent interruption of the EHC. Higher levels of interruption led to bile salt depletion. Grundy *et al.*[24] demonstrated that the normal human bile salt pool is maintained despite a 2- to 3-fold increase in faecal bile salt excretion. To meet the cholesterol requirement for supplementary bile salt synthesis, more cholesterol is gathered in the liver through the up-regulation of LDL receptors and through increased cholesterol synthesis. Grundy[23] demonstrated that cholestyramine treatment of persons without hypercholesterolaemia reduced serum cholesterol levels by 15 to 30 per cent. Patients suffering from hypercholesterolaemia showed average reductions between 20 and 30 per cent, except for those with homozygous familial hypercholesterolaemia, who showed more variable results. Surgical removal of a part of the ileum resulted in decreases of more than 30 per cent.[23]

The net positive effect due to enhanced BSH activity, calculated under very favourable conditions, would therefore be in the same order of magnitude compared with literature data obtained from therapeutic or surgical alterations of the EHC. Based on the one-to-two rule,[15,28] which states that a 1 per cent reduction of the serum cholesterol level causes a 2 per cent lowering of the risk for coronary heart disease (CHD), a significant positive effect for patients suffering from elevated cholesterol might be obtained by ingesting a realistic amount of BSH active lactobacilli. Side-effects of altered bile salt metabolism have been reported. It is known that bile salt malabsorption, resulting from ileal resection, ileal disease or other disruption of ileal physiology, can reduce water and electrolyte absorption from the colon, leading to watery diarrhoea.[29,31,56] Digestion of dietary fat and fat-soluble compounds is relatively unimpaired except for patients with large ileal resections.[30] Based on these pathophysiological data, increased small intestinal BSH activity might hypothetically lead to watery diarrhoea. However, in patients with severe hypercholesterolaemia, this inconvenience has to be evaluated against the positive effect on the risk for atherosclerosis and coronary heart disease.

In conclusion, it needs to be stated that this *in vitro* study is only of indicative value in the research on the hypocholesterolaemic effect of fermented dairy products, a field which has been gaining much attention recently. *In vivo* testing in suitable animal models is necessary. Partial evidence for the impact of bacterial BSH activity on the animal host has already been given by Feighner and Dashkevicz[13,14] and Fuller *et al.*[17] These investigators observed an inverse relationship between growth performance of poultry and cholyltaurine hydrolase activity. Growth depression in chickens could be alleviated by reducing the population of BSH active microorganisms through the administration of sub-therapeutic concentrations of feed additive antibiotics. This

indicates that the established BSH active gastrointestinal microbiota directly influences diet utilisation and energy conversion of its host. However, when extrapolating *in vivo* data from animal experiments to humans, one should take into account that bile salt and cholesterol metabolism in a particular test animal may differ from that of humans. Cholesterol metabolism in the hamster has been widely studied because the male hamster was recognized as a good model for man in this respect.[61] Other investigators stated that man is even more sensitive to bile salt removal through cholestyramine in the extent to which this is reflected in lowered serum cholesterol levels when compared to small laboratory animal models.[52,64]

The BSH isogenic *L. plantarum* 80 strains used in this study are not of human or animal origin. Research is now being focused on the isolation of highly BSH active gastrointestinal lactobacilli for two prevalent reasons. First, the lactic acid bacteria, mainly lactobacilli and/or streptococci, used in probiotic products are mostly intestinal isolates, and the use in food and feed production of microorganisms normally inhabiting other biotopes is not generally approved. Furthermore, the *L. plantarum* 80 strains are genetically modified microorganisms. Although biotechnology has already had a major effect on the animal feed trade with products contributing to better feed utilisation, improved health and welfare of livestock, there remains ambiguity about what is possible and what is acceptable in food production systems. As for the current situation with regard to the use of genetically engineered microoganisms in foods, public awareness requires extensive validation in terms of proof of safety and efficiency.

ACKNOWLEDGEMENTS

This work was financially supported by the European Community research programme AIR1-CT92-0256.

REFERENCES

1. Allison C, McFarlan C, MacFarlane GT. (1989). Studies on mixed population of human intestinal bacteria grown in single-stage and multi-stage continuous culture systems. *Applied and Environmental Microbiology* **55,** 672–678.
2. Barr DP, Russ EM, Eder HA. (1951). Protein-lipid relationship in human plasma, II. In atherosclerosis and related conditions. *American Journal of Medicine* **11,** 480–493.
3. Beck C, Necheles H. (1961). Beneficial effects of administration of *Lactobacillus acidophilus* in diarrheal and other intestinal disorders. *American Journal of Gastroenterology* **35,** 522–533.
4. Brown MS, Goldstein JL. (1984). How LDL receptors influence cholesterol and atherosclerosis. *Scientific American* **251,** 58–66.
5. Brown MS, Goldstein JL. (1986). A receptor-mediated pathway for cholesterol homeostasis. *Science* **232,** 34–47.
6. Cantafora A, Di Biase A, Alvaro D, Angelico M. (1987). High-performance liquid chromatography with tauro-7α, 12α-dihydroxy-5β-cholanic acid as internal standard. *Journal of Chromatography* **386,** 367–370.
7. Chikai T, Naka H, Ushida K. (1987). Deconjugation of bile acids by human intestinal bacteria implanted in germ-free rats. *Lipids* **22,** 669–671.
8. Christiaens H, Leer RJ, Pouwels PH, Verstraete W. (1992). Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* using a direct plate assay. *Applied and Environmental Microbiology* **58,** 3792–3798.
9. Clements ML, Levine MM, Black RE, Robins-Browne RM, Cisneros LA, Drusano GL, Lanata CF, Saah AJ. (1981). *Lactobacillus* prophylaxis for diarrhea due to enterotoxigenic *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **20,** 104–108.
10. De Man JC, Rogosa M, Sharpe ME. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology* **23,** 130–135.
11. Dietschy JM, Siperstein MD. (1967). Effect of cholesterol feeding and fasting on sterol synthesis in seventeen tissues of the rat. *Journal of Lipid Research* **8,** 97–104.
12. Dowling RH, Mack E, Small DM. (1970). Effects of controlled interruption of bile salts by biliary diversion and by ileal resection on bile salt secretion, synthesis, and pool size in the rhesus monkey. *Journal of Clinical Investigation* **49,** 232–242.
13. Feighner SD, Dashkevicz MP. (1987). Subtherapeutic levels of antibiotics in poultry feeds and their effect on weight gain, feed efficiency and bacterial cholyltaurine hydrolase activity. *Applied and Environmental Microbiology* **53,** 331–336.
14. Feighner SD, Dashkevicz MP. (1988). Effect of dietary carbohydrates on bacterial cholyltaurine hydrolase in poultry intestinal homogenates. *Applied and Environmental Microbiology* **54,** 337–342.
15. Frick M, Elo O, Haapa K. (1987). Helsinki heart study: Primary prevention trial with gemfibrozil in middle-aged men with dyslipidemia. *New England Journal of Medicine* **317,** 1237–1245.
16. Fuller R. (1989). A review: Probiotics in man and animals. *Journal of Applied Bacteriology* **66,** 365–378.

17. Fuller R, Cole CB, Coates ME. (1984). The role of *Streptococcus faecium* in antibiotic-relieved growth depression in chickens. In: Woodbine M. (ed) *Antimicrobials and Agriculture*. London, Butterworths, pp. 395–403.
18. Gilliland SE. (1989). Hypocholesteremic action of lactobacilli. In *Proceedings of the NIZO-Workshop Fermented Milks and Health*, NIZO, Ede, The Netherlands, pp. 77–88.
19. Gilliland SE. (1990). Health and nutritional benefits from lactic acid bacteria. *FEMS Microbiology Reviews* **87,** 175–188.
20. Gilliland SE, Nelson CR, Maxwell C. (1985). Assimilation of cholesterol by *Lactobacillus acidophilus*. *Applied and Environmental Microbiology* **49,** 377–381.
21. Gilliland SE, Speck ML. (1977). Deconjugation of bile acids by intestinal lactobacilli. *Applied and Environmental Microbiology* **33,** 15–18.
22. Gopal-Srivastava R, Hylemon PB. (1988). Purification and characterization of bile salt hydrolase from *Clostridium perfringens*. *Journal of Lipid Research* **29,** 1079–1085.
23. Grundy SM. (1972). Treatment of hypercholesterolemia by interference with bile acid metabolism. *Archives of Internal Medicine* **130,** 638–648.
24. Grundy SM, Ahrens EH Jr, Salen G. (1971). Interruption of the enterohepatic circulation of bile acids in man: Comparative effect of cholestyramine and ileal exclusion on cholesterol metabolism. *Journal of Laboratory and Clinical Medicine* **78,** 94–121.
25. Grunewald KK. (1982). Serum cholesterol levels in rats fed skim milk fermented by *Lactobacillus acidophilus*. *Journal of Food Sciences* **47,** 2078–2079.
26. Havel RJ. (1986). Functional activities of hepatic lipoprotein receptors. *Annual Review of Physiology* **48,** 119–134.
27. Hepner G, Fried R, St Jeor S, Fusetti L, Morin R. (1979). Hypocholesterolemic effect of yogurt and milk. *American Journal of Clinical Nutrition* **32,** 19–24.
28. Hjermann I, Byre K, Holme I. (1981). Effect of diet and smoking intervention on the incidence of coronary heart disease. *Lancet* **2,** 1303.
29. Hofmann AF. (1967). The syndrome of ileal disease and the broken enterohepatic circulation: choleretic enteropathy. *Gastroenterology* **52,** 752–757.
30. Hofmann AF. (1989). The enterohepatic circulation of bile acids in health and disease. In: Sleisinger MH, Fordtran JS (eds) *Gastro-intestinal diseases. Pathophysiology, Diagnosis, Management*. W.B. Saunders Company, New York, pp. 144–161.
31. Hofmann AF, Poley JR. (1972). Role of bile acid malabsorption in pathogenesis of diarrhea and steatorrhea in patients with ileal resection. Response to cholestyramine or replacement of dietary long chain triglyceride by medium chain triglyceride. *Gastroenterology* **62,** 918–934.
32. Hofmann AF, Roda A. (1984). Physicochemical properties of bile acids and their relationship to biological properties: an overview of the problem. *Journal of Lipid Research* **25,** 1477–1489.
33. Hofmann AF, Small DM. (1967). Detergent properties of bile salts. Correlation with physiological function. *Annual Review of Medicine* **18,** 333–376.
34. Hylemon PB. (1985). Metabolism of bile acids in intestinal microflora. In: Danielson H, Sjövall J (eds) *Steroids and bile acids: new comprehensive biochemistry*. vol. 12. Elsevier Publishing Inc., Amsterdam, pp. 331–334.
35. Hylemon PB, Glass TL. (1983). Biotransformation of bile acids and cholesterol by the intestinal microflora. In: Hentges DJ (ed) *Human intestinal microflora in health and disease*. Academic Press Inc., New York, pp. 189–213.
36. Isolauri E, Juntunen M, Rautanen T, Sillanaukee P, Koivula T. (1991). A human *Lactobacillus* strain (*Lactobacillus casei* sp. strain GG) promotes recovery from acute diarrhea in children. *Pediatrics* **88,** 90–97.
37. Johansson M-L, Molin G, Jeppsson B, Nobaek S, Ahrené S, Bengmark S. (1993). Administration of different *Lactobacillus* strains in fermented oatmeal soup: *in vivo* colonization of human intestinal mucosa and effect on the indigenous flora. *Applied and Environmental Microbiology* **59,** 15–20.
38. Josson K, Scheirlinck T, Michiels F, Platteeuw C, Stansens P, Joos H, Dhaese P, Zabeau M, Mahillon J. (1989). Characterization of a gram-positive broad host range plasmid isolated from *Lactobacillus hilgardii*. *Plasmid* **11,** 9–20.
39. Juven BJ, Meinersmann RJ, Stern NJ. (1991). Antagonistic effects of lactobacilli and pediococci to control intestinal colonization by human enteropathogens in live poultry. *Journal of Applied Bacteriology* **70,** 95–103.
40. Kawamoto K, Horibe I, Urchida K. (1989). Purification and characterization of a new hydrolase for conjugated bile acids, chenodeoxycholyltaurine hydrolase, from *Bacteroides vulgatus*. *Journal of Biochemistry* **106,** 1049–1053.
41. Klaver FAM, Van der Meer R. (1993). The assumed assimilation of cholesterol by lactobacilli and *Bifidobacterium bifidum* is due to their bile salt deconjugating activity. *Applied and Environmental Microbiology* **59,** 1120–1124.
42. LaRosa JC, Hunninghake D, Bush D, Criqui MH, Getz GS, Gotto AM Jr *et al.* (1990). The cholesterol facts. A summary of the evidence relating dietary fats, serum cholesterol, and coronary heart disease. A joint statement by the American Heart Association and the National Heart, Lung, and

Blood Institute. The task force on cholesterol issues, American Heart Association. *Circulation* **8,** 1721–1733.
43. Leer RJ, Christiaens H, Peters L, Posno M, Verstraete W, Pouwels PH. (1993). Nucleotide sequence and gene disruption of the conjugated bile acid hydrolase gene of *Lactobacillus plantarum* strain 80. *Molecular and General Genetics* **239,** 269–272.
44. Lewis R, Gorbach S. (1972). Modification of bile acids by intestinal bacteria. *Archives of Internal Medicine* **130,** 545–549.
45. Lidbeck A, Geltner Allinger U, Arrhage KM, Ottoya J, Brismar B, Gustafsson J-A, Rafter JJ, Nord CE. (1991). Impact of *Lactobacillus acidophilus* supplements on the faecal microflora and soluble faecal bile acids in colon cancer patients. *Microbial Ecology in Health and Disease* **4,** 81–88.
46. Lin SY, Ayres JW, Winkler W Jr, Sandine WE. (1989). *Lactobacillus* effects on cholesterol: *in vitro* and *in vivo* results. *Journal of Dairy Sciences* **72,** 2885–2899.
47. Lundeen SG, Savage DC. (1990). Characterization and purification of bile salt hydrolase from *Lactobacillus* sp. strain 100-100. *Journal of Bacteriology* **172,** 4171–4177.
48. Macdonald IA, Bokkenheuser VD, Winter J, Mclernon AM, Mosbach EH. (1983). Degradation of steroids in the human gut. *Journal of Lipid Research* **24,** 675–700.
49. Masuda N. (1981). Deconjugation of bile salts by *Bacteroides* and *Clostridium*. *Microbiology and Immunology* **25,** 1–11.
50. McGilvery RW. (1983). Turnover of fats and lipoproteins: the cholesterol connection. In *Biochemistry. A functional approach*. W.B. Saunders, Company, Tokio, pp. 555–571.
51. Mellies MJ, DeVault AR, Kassler-Toub K, McGovern ME, Pan HY. (1993). Provastatin experience in elderly and non-elderly patients. *Atherosclerosis* **101,** 97–110.
52. Miettinen TA, Lempinen M. (1977). Cholestyramine and ileal by-pass in the treatment of familial hypercholesterolaemia. *European Journal of Clinical Investigation* **7,** 509–514.
53. Molly K, Vande Woestyne M, Verstraete W. (1993). Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. *Applied Microbiology and Biotechnology* **39,** 254–258.
54. Molly K, De Smet I, Vande Woestyne M, Verstraete W. (1994). Validation of the Simulation of the Human Intestinal Microbial Ecosystem (SHIME)-reactor using microorganism-associated activities. *Microbial Ecology in Health and Disease* **7,** 191–200.
55. Pihl A. (1955). The effect of dietary fat on the intestinal cholesterol and on the cholesterol metabolism in the liver of rats. *Acta Physiologica Scandinavica* **34,** 183–196.
56. Popovic OS, Kostic KM, Milovic VB, Milutinovic-Djuric S, Miletic VD, Sesic L *et al.* (1987). Primary bile acid malabsorption. Histologic and immunologic study in three patients. *Gastroenterology* **92,** 1851–1858.
57. Reynier MO, Montet JC, Gerolami A, Marteau C, Crotte C, Montet AM, Mathieu S. (1981). Comparative effects of cholic, chenodeoxycholic, and ursodeoxycholic acids on micellar solubilization and intestinal absorption of cholesterol. *Journal of Lipid Research* **22,** 467–473.
58. Saxelin M, Elo S, Salminen S, Vapaatola H. (1991). Dose response colonisation of faeces after oral administration of *Lactobacillus casei* strain GG. *Microbial Ecology in Health and Disease* **4,** 209–211.
59. Scalia S. (1988). Simultaneous determination of free and conjugated bile acids in human gastric juice by high performance liquid chromatography. *Journal of Chromatography* **431,** 259–269.
60. Schaefer EJ, Camon-Fava S, Cohn SD, Schaefer MM, Ordovas JM, Castelli WP, Wilson PWF. (1994). Effects of age, gender, and menopausal status on plasma low density lipoprotein cholesterol and apolipoprotein B levels in the Framingham Offspring study. *Journal of Lipid Research* **35,** 779–792.
61. Spady DK, Dietschy JM. (1983). Sterol synthesis *in vivo* in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster, and rat. *Journal of Lipid Research* **24,** 303–315.
62. Spady DK, Woollett LA, Dietschy JM. (1993). Regulation of plasma LDL-cholesterol levels by dietary cholesterol and fatty acids. *Annual Review of Nutrition* **13,** 355–381.
63. Stellwag EJ, Hylemon PB. (1976). Purification and characterization of bile salt hydrolase from *Bacillus fragilis* subsp. *fragilis*. *Biochimica et Biophysica Acta* **452,** 165–176.
64. Suckling KE, Benson GM, Bond B, Gee A, Glen A, Haynes C, Jackson B. (1991). Cholesterol lowering and bile acid excretion in the hamster with cholestyramine treatment. *Atherosclerosis* **89,** 183–190.
65. Swell L, Flick DF, Field H Jr, Treadwell CR. (1955). Role of fat and fatty acid absorption of dietary cholesterol. *American Journal of Physiology* **180,** 124–128.
66. Tannock GW. (1988a). Mini Review: Molecular Genetics: A new tool for investigating the microbial ecology of the gastro-intestinal tract. *Microbial Ecology* **15,** 239–256.
67. Tannock GW. (1988b). The normal microflora: New concepts in health promotion. *Microbiological Sciences* **5,** 4–8.

68. Tannock GW. (1990). The microecology of lactobacilli inhabiting the gastrointestinal tract. *Advances in Microbial Ecology* **11,** 147–171.
69. Taylor EW, Burman NP. (1964). The application of membrane filtration techniques to the bacteriological examination of water. *Journal of Applied Bacteriology* **27,** 297–303.
70. Turley SD, Dietschy JM. (1988). The metabolism and excretion of cholesterol by the liver. In: Arias IM, Jakoby WB, Popper H, Schachter D, Shafzitz DA (eds) *The liver: biology and pathobiology*, 2nd edn. Raven Press Ltd, New York, pp. 617–641.
71. Van der Meer R, De Vries HT. (1985). Differential binding of glycine- and taurine-conjugated bile acids to insoluble calcium phosphate. *Biochemical Journal* **229,** 265–267.
72. Van Renterghem B, Huysman F, Rygole R, Verstraete W. (1991). Detection and prevalence of *Listeria monocytogenes* in the agricultural ecosystem. *Journal of Applied Bacteriology* **71,** 211–217.
73. Vlahcevic ZR, Heuman DM, Hylemon PB. (1990). Physiology and pathophysiology of enterohepatic circulation of bile acids. In: Zakim D, Boyer TD (eds) *Hepatology. A textbook of liver disease*, vol 1. W.B. Saunders Company, Philadelphia, pp. 341–377.
74. Williamson BWA, Percy-Robb IW. (1979). The interaction of calcium ions with glycocholate micelles in aqueous solution. *Biochemical Journal* **181,** 61–66.