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Characterisation and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content

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Abstract

Three out of 297 *Lactobacillus* strains isolated from pig faeces were selected for a feeding trial on account of their high bile-salt hydrolase (BSH) activity, bile-salt resistance, low pH tolerance and the production of antimicrobial substances. Two strains were identified as *Lactobacillus johnsonii* and one as *Lactobacillus reuteri* by DNA–DNA hybridisation. *L. johnsoniii* BFE 1061 produced a bacteriocin active against a range of lactic acid bacteria (LAB) and nonrelated bacteria including *Clostridium perfringens*. Six minipigs were maintained on a high-fat, high-cholesterol ('Western Style') diet for 17 weeks after which the diet was supplemented with the 'probiotic mixture' containing the above mentioned three *Lactobacillus* strains at 2×10^{12} CFU per pig per day for five weeks. The mixture was given as a resuspended lyophilisate. During a two week follow-up period the minipigs received only the 'Western-style' diet without probiotic supplementation. A lowering effect on serum cholesterol levels was indicated after three weeks probiotic feeding, concomitant with an increase in the moisture content of the faeces and *Lactobacillus* cell numbers. Triglycerides, pH and number of lactic acid bacteria in faeces were not significantly influenced by probiotic supplementation.

Keywords: Probiotic; *Lactobacillus*; Bile-salt hydrolysis; Cholesterol; Lactic acid bacteria; Minipigs; Enterohepatic cycle; Intestinal microflora

1. Introduction

Lactic acid bacteria (LAB), especially *Lactobacillus* and *Bifidobacterium* spp., are normal residents of the complex ecosystem of the gastrointestinal tract (GIT) (Mitsuoka, 1992). *Lactobacillus acidophilus* and *Bifidobacterium* spp. are proposed to exert health promoting or 'probiotic' effects in humans and animals. Such effects are considered to include the inhibition of pathogenic microorganisms (Gilliland, 1989; Havenaar and Huis in't Veld, 1992; Salminen

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et al., 1993; Hudault et al., 1997), antimutagenic and anticarcinogenic activity (Fernandes et al., 1987; Fuller, 1989; Gilliland, 1989; Fernandes and Shahani, 1990; Pool-Zobel et al., 1993), increase of the immune response (Fernandes and Shahani, 1990; Perdigon et al., 1990; Mikeš et al., 1995; Tortuero et al., 1997; Malin et al., 1996) and the reduction of cholesterol levels (Mann and Spoerry, 1974; Rao et al., 1981; Grunewald, 1982; Gilliland et al., 1985; Danielson et al., 1989; Lin et al., 1989; Hølund, 1993; Mikeš et al., 1995; Fukushima and Nakano, 1996; Tamai et al., 1996).

One of the major risk factors of coronary heart diseases is hypercholesterolemia. Cholesterol and bile-salt metabolism are closely linked, bile salts being the water-soluble excretory end-products of cholesterol. These bile salts may be transformed by enzyme activities of some intestinal bacteria during the enterohepatic circulation. Bile-salt hydrolase (BSH) (E.C.3.5.1.24) is the enzyme responsible for deconjugation of bile acid, where glycine or taurine are split from the steroid moiety, resulting in free (deconjugated) bile salts. BSH activity is observed in some strains associated with the GIT, representing different species of *Lactobacillus*, *Enterococcus*, *Peptostreptococcus*, *Bifidobacterium*, *Clostridium* and *Bacteroides* (Gilliland and Speck, 1977; Chikai et al., 1987). It was hypothesised that deconjugation of bile salts may contribute to lower cholesterol levels as free bile salts may be excreted more likely from the GIT than conjugated bile salts (Chikai et al., 1987). However, this hypothesis is not undisputed (Marteau et al., 1995) and is incompatible with current knowledge about the passive absorption kinetics of free bile salts in the GIT (Wilson, 1991; Aldini et al., 1996). Faecal loss of bile salts may indeed result in an increased requirement for cholesterol as a precursor for the synthesis of new bile salts and thus may reduce the cholesterol levels (Driessen and de Boer, 1989; De Rodas et al., 1996). Klaver and van der Meer (1993) suggested that the in vitro cholesterol reduction by some *Lactobacillus* spp. results from its coprecipitation with deconjugated bile salts. De Smet et al. (1994) suggested that highly BSH-active *Lactobacillus* spp. may thus reduce serum cholesterol levels, and De Smet et al. (1995) hypothesised that BSH activity may be an important factor for bile tolerance. BSH-active lactobacilli may thus have an advantage to survive and colonise the lower small intestine where the enterohepatic cycle takes place.

The aim of this study was to characterise highly BSH-active lactobacilli isolated from minipigs and to assess the effect of three BSH-positive isolates on minipigs maintained on a high cholesterol diet. These strains were host specific and were selected also for other probiotic characteristics such as bile tolerance, acid resistance and the production of antimicrobial substances. The parameters monitored during the feeding trial were the cell counts for total LAB and for *Lactobacillus* spp., serum cholesterol levels, serum triglycerides, faeces pH and faeces moisture contents.

2. Materials and methods

2.1. Isolation of bacterial strains

Serial dilutions of freshly collected pig faeces were made in quarter-strength Ringer's solution, and 100 μl of each dilution was spread-plated onto MRS (Merck) and Rogosa (Merck) agar. Plates were incubated anaerobically at 37°C for 48 h in a MK3 Anaerobic Workstation (dw Scientific, Shipley, West Yorkshire, UK). One to three colonies were randomly selected from each of duplicate MRS and Rogosa plates of the highest dilution showing growth. Colonies were subcultured in MRS broth, and restreaked onto MRS agar to ensure purity. Purified cultures were maintained at −80°C in 20% (v/v) glycerol. Authentic reference strains and their origin are listed in Tables 1 and 2. They were cultured as described in the respective culture collection catalogues.

2.2. Identification of isolates

Isolates were initially selected on the basis of Gram reaction, morphology and catalase activity. All Gram-positive and catalase-negative rods were selected for further studies to determine if they were BSH positive. Isolates were screened for BSH activity by impregnating a sterile filter disk in an overnight culture, and placing the filter on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA) (Sigma) and 0.37 g/l $CaCl_2$ (Dashkevicz and Feighner, 1989). Plates were

Table 1
DNA-homology values recorded between the three lactobacilli with high BSH activity, isolated from pig faeces, and reference strains

Strains	% DNA homology of isolates		
	L. reuteri BFE 1058	*L. johnsonii* BFE 1059	*L.johnsonii* BFE 1061
L. acidophilus DSM 20079^T		12	
L. brevis DSM 20054^T	53		
L. crispatus[a] CHN-1-4		48	41
L. fermentum DSM 20052^T	19		
L. gallinarum[a] T 50		35	0
L. gasseri DSM 20077		54	
L. johnsonii DSM 20553		76	
L. johnsonii[a]Omniflora		78	91
L. reuteri DSM 20016^T	100		

[a] Received from E. Lauer.
DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

Table 2
Inhibitory spectrum of the bacteriocin produced by *L. johnsonii* BFE 1061 according to the method of Uhlman et al. (1992)

Indicator strains	Inhibitory activity[a]
L. acidophilus ATCC 43121	+
L. amylophilus DSM 20533	−
L. amylovorus DSM 20531	+
L. animalis NCFB 2425	−
L. crispatus CHN-1-4[b]	+
L. delbrueckii subsp. *lactis* DSM 20072	−
L. gasseri DSM 20077	+
L. jensenii DSM 20577	+
L. johnsonii Omniflora[b]	+
L. reuteri DSM 20016	−
L. sake DSM 20017	+
Carnobacterium divergens L66[c]	+
C. gallinarum DSM 4847	+
Lactococcus lactis subsp. *lactis* DSM 20481	−
Enterococcus faecalis DSM 20478	−
E. faecium DSM 20477	+
Bacillus cereus DSM 345	−
Clostridium perfringens DSM 7576	+
C. butyricum DSM 552	+
Listeria monocytogenes DSM 20600	−
Staphylococcus aureus ATCC 14458	−
Staph. aureus ATTC 6538	+
Streptococcus mutans DSM 6178	+

[a] + = Clear inhibition zone of 1 mm or more, − = no inhibition zone.
[b] Received from E. Lauer.
[c] Strain from the culture collection of the Institute of Hygiene and Toxicology, Karlsruhe, Germany.
ATCC = American Type Culture Collection, USA; DSM = Deutsche Sammlung von Mikro-organismen, Braunschweig; Germany; NCFB = National Collection of Food Bacteria, UK.

incubated anaerobically at 37°C for 72 h, after which the diameter of the precipitation zones was measured. The three strains which displayed the largest precipitation zones were further identified. Fermentation of carbohydrates was determined by using the API 50 CH system (BioMérieux S.A., La Balme les Grottes, France) according to the manufacturer's instructions. The ability to grow at 15°C and 45°C was assessed in MRS broth after 5 and 3 days of incubation, respectively. Tests for the hydrolysis of arginine, gas production from glucose and presence of meso-diaminopimelic acid (m-DAP) in cell walls were performed using the methods of Schillinger and Lücke (1987). The configuration of lactic acid produced was determined using the spectrophotometric method of Boehringer-Mannheim (1989). The phenotypic characteristics of these strains (Table 3) were compared with the typical characteristics given for each *Lactobacillus* sp. according to Kandler and Weiss (1986); Hammes et al. (1991).

2.3. Characterisation of three BSH-active isolates

2.3.1. Determination of DNA base composition and DNA–DNA homology

The DNA was isolated according to the method described by Marmur (1961). The mol% guanine plus cytosine was calculated from the thermal melting point (T_m) of the DNA as described by Marmur and Doty (1962) using a Gilford Response spectrophotometer (Gilford, Ciba-Corning, Giessen, Germany) with the corresponding software. The DNA

Table 3
Phenotypic characteristics of three strains of *Lactobacillus* sp. isolated from pig faeces[a]

Characteristics	*L. reuteri* BFE 1058	*L. johnsonii* BFE 1059	*L. johnsonii* BFE 1061
Gas from glucose	+	−	−
Ammonia from arginine	+	−	−
Mol% G + C	40.4	33.4	33.4
Fermentation of:			
Arabinose	+	−	−
Cellobiose	−	+	+
Esculin	−	+	+
Fructose	−	+	+
Lactose	+	−	+
Mannose	−	+	−
Melibiose	+	−	+
Ribose	+	−	−
Salicin	−	−	−
Trehalose	−	+	+

[a] Isolates were selected on the basis that they displayed the largest precipitation zones in the BSH plate assay. All strains fermented galactose, glucose, maltose, raffinose and xylose, grew at 45°C, but not at 15°C and produced DL-lactic acid. Amygdaline, gluconate, mannitol, melezitose, rhamnose and sorbitol were not fermented by any of the strains. None of the strains contained m-DAP in their cell walls.

homology was determined spectrophotometrically using the procedure described by De Ley et al. (1970).

2.3.2. Bile-salt hydrolytic activity

Quantitative BSH activity of stationary phase cultures was kindly determined by I. De Smet at the University of Ghent, Belgium, using HPLC analysis (De Smet et al., 1994). The effect of 2.0 mM TDCA and 2.0 mM sodium salt of glycodeoxycholic acid (GDCA Sigma) on the growth of cultures was determined at the start and after two hours of incubation at 37°C by determining the viable cell counts on MRS agar. The BSH activity was expressed as nmol DCA formed per 10^{10} colony forming units (CFU) initially present per min.

2.3.3. Bile-salt tolerance

Isolates were compared for their ability to grow in the presence of bile. MRS broth was supplemented with 0.3% (w/v) oxgall (Difco) and 0.3% (v/v) pasteurized native porcine bile, respectively. Native porcine bile was collected from healthy pig carcasses directly after slaughtering at the communal slaughterhouse in Karlsruhe. It was pooled, filtered at 0.2 μm and stored in 100-ml portions at − 18°C. A microplate with 180 μl of medium was inoculated with 20 μl of an overnight culture. The plates were incubated at 37°C and the absorbance at 580 nm was monitored at hourly intervals using a microplate reader (Thermomax microplate reader, Molecular Devices, USA).

2.3.4. Low pH tolerance

MRS broth was adjusted to pH 2, 3, 4, 5, 6 and 8, respectively, using 1 N HCl or 1 N NaOH. The media (180 μl) were inoculated with 20 μl of an overnight culture and incubated at 37°C. The absorbance at 580 nm was measured at two-hourly intervals using a microplate reader, and growth curves were plotted.

2.3.5. Antagonism

Isolates were screened for antagonistic activity by the deferred inhibition test described by Schillinger and Lücke (1989), using bacteriocin screening medium (BSM) (Tichaczek et al., 1993) without catalase. Indicator strains used were *L. acidophilus* ATCC 43121, *Lactobacillus crispatus* CHN-1-4 (received from Dr. E. Lauer Novartis Consumer Health GmbH, Berlin), *Lactobacillus gasseri* DSM 20077, *L. johnsonii* Omniflora (received from Dr. E. Lauer), *Lactobacillus reuteri* DSM 20016, *Lactobacillus sake* DSM 20017, *Enterococcus faecalis* DSM 20478, *Enterococcus faecium* DSM 20477 and *Listeria monocytogenes* DSM 20600.

2.3.6. Spectrum of bacteriocin activity

The antibacterial activity of the isolates was determined using cell-free neutralised supernatants (CFNS). The CFNS were obtained from cultures grown in MRS broth for 18 h at 37°C. Cultures were centrifuged and the pH of the supernatant adjusted to 6.5–7.0 with 1 N NaOH. The supernatant was then heated for 5 min at 100°C and stored at − 20°C. The neutralised supernatants were tested against a wide variety of LAB and pathogens (see Table 2) using the agar spot test method of Uhlman et al. (1992).

2.3.7. Effect of enzymes, pH and heat on bacteriocin activity

The effect of heat, different enzymes and pH on the bacteriocin activity was determined according to

the methods described by Van Laack et al. (1992). *L. sake* DSM 20017 was used as indicator strain.

2.4. Feeding trial

2.4.1. Preparation of the three Lactobacillus isolates for the minipig feeding trial

The pure cultures of *L. johnsonii* BFE 1059 and BFE 1061 and *L. reuteri* BFE 1058 were cultivated in MRS broth in a 1000-l online fermenter by the Gesellschaft für Biotechnologische Forschung mbH (GBF, D-38124 Braunschweig, Germany). An inoculum of 10 l in MRS was transferred online to 1022-kg sterilised fermentation medium (glucose 20 kg, tryptic digested casein peptone 10 kg, meat extract 5 kg, sodium acetate 5 kg, dipotassium hydrogen phosphate · $3H_2O$ 2 kg, disodium hydrogen citrate 2 kg, Tween 80 1 kg, magnesium sulphate · $7H_2O$ 0.2 kg, Tegosipon (anti-foam) 0.1 kg and Aqua demin. ad 1094 l). After centrifugation, the resulting 7.37 kg of cell biomass were lyophilised in 10% (w/v) skim milk containing 5% (w/v) glycerol. The resulting 3.74 kg lyophilisate were subsequently packed and sealed in a glove box under nitrogen and water free (2.5% humidity) conditions in order to obtain 450 portions of 8 g each. Viability was checked weekly during storage at 4°C. For preparation of the final probiotic mixture, packages were chosen at random. Each package was transferred to a sterile jug containing 50 ml sterile quarter-strength Ringer solution. The mixture was stirred for 5 min using a sterile electric stainless steel mixer (Krups, Germany). In order to check viability, two solutions were prepared and used to determine CFU by serial dilution and plating on MRS agar. Viability checks were performed on a weekly basis using these methods, which showed viability reduction of $<2\%$ during the whole study period.

2.4.2. Animals and diet

Six male Göttingen minipigs between 3 and 6 years were used. Mean bodyweight was 55.31 kg before and 66.66 kg after finishing the study. They received a 'Western-style' diet for a baseline period of 17 weeks to develop hypercholesterolemia, followed by 5 weeks on a 'Western-style' diet with the probiotic supplement and three weeks follow up feeding with the 'Western-style' diet. This diet was composed of (g/100 g diet): wheat starch 50.0, casein 20.0, mineral/vitamin mixture 8.0, lard 7.5, margarine 7.5, cellulose 6.0 and cholesterol 1.0. The pigs received 308 g of the diet twice a day. The doses of viable bacteria amounted to 2×10^{12} CFU per pig per day. During the probiotic feeding period, 8 g of the freeze-dried probiotic mixture was suspended in 50 ml of quarter-strength Ringer solution and mixed with 308 g of the diet administered both in the morning and afternoon. The minipigs had water available ad libitum, except one hour before feeding to ensure that the probiotic mixture was completely consumed.

2.4.3. Determination of viable bacterial counts in faeces

Serial dilutions of faeces were made in quarter-strength Ringer and plated out on MRS-agar (Merck) and on Rogosa agar (Merck). The plates were incubated anaerobically in a MK3 Anaerobic workstation (dw Scientific) (3% H_2, 10% CO_2, 87% N_2) for 48 h at 37°C. The colony forming units per ml (CFU/ml) for LAB and lactobacilli were determined on the above-mentioned medium respectively. Typical colonies were isolated from highest dilutions both on MRS and Rogosa agar plates and their phenotypic features determined.

2.4.4. Determination of serum cholesterol and serum lipoproteins

Blood samples were collected from the brachial–jugular region after 12 h of fasting. Total serum cholesterol was determined in the medical laboratory of Wulff and Wöhrmann (D-24306 Plön) on a fully automatized Hitachi 705 analyser (Hitachi, Kyoto, Japan) using the enzymatic CHOD-PAP method from Boehringer-Mannheim according to Richmond (1973). Serum triglycerides were measured enzymatically using the GPO-PAP method according to Fossati and Prencipe (1982).

2.4.5. Determination of faeces pH and faeces moisture contents

The faeces was diluted 1:10 with quarter-strength Ringer solution and mixed in a stomacher 400 (Colworth, UAC House, London, UK) until homogeneous, upon which the pH was determined using a calomel electrode and a WTW pH-meter (WTW, Weilheim, Germany). The moisture content was determined after freeze drying the faeces.

2.4.6. Statistical analyses

The data were analysed with the Friedman rank test, followed by the Wilcoxon/Wilcox multiple rank-sum test because of the nonnormal distribution of the measurements (Ramm and Hofmann, 1982). It was taken into account that, without a control group and with only six subjects, the power of the tests and the significance of the results might be influenced.

3. Results and discussion

3.1. Isolation and characterisation of strains

The Gram-positive, catalase-negative rods isolated on MRS and Rogosa agar from pig faeces were preliminarily identified as lactobacilli. Out of 297 strains of *Lactobacillus* spp. screened for BSH activity on plates, 191 were positive, with precipitation zones varying in size (Fig. 1). The three strains (BFE 1058, 1059 and 1061) which displayed the largest precipitation zones were selected for further studies. Strains BFE 1059 and 1061 were identified as members of the *L. acidophilus* group and BFE 1058 as *L. reuteri* on the basis of their physiological characteristics, sugar fermentation patterns and mol% G + C content (Table 3). Preliminary identification of strain BFE 1058 as *L. reuteri* was confirmed by DNA–DNA homology studies (Table 1). According to DNA–DNA hybridisations, strains BFE 1059 and 1061 are both members of the species *L. johnsonii*.

Bile-salt tolerance of the three strains was quantified according to Chateau et al. (1994). The results are shown in Table 4. *Lactobacillus reuteri* BFE 1058 and *L. johnsonii* BFE 1061 are bile-salt resistant, as indicated by a growth delay < 10 min recorded in the presence of 0.3% (w/v) oxgall. *L. johnsonii* BFE 1059 was considered bile-salt tolerant with a 'growth delay' value of 25 min (Table 4). Native porcine bile exhibited a stronger bactericidal effect when compared to the results obtained for oxgall (Table 4). Bile-salt tolerance is important for strains to grow and survive in the upper small

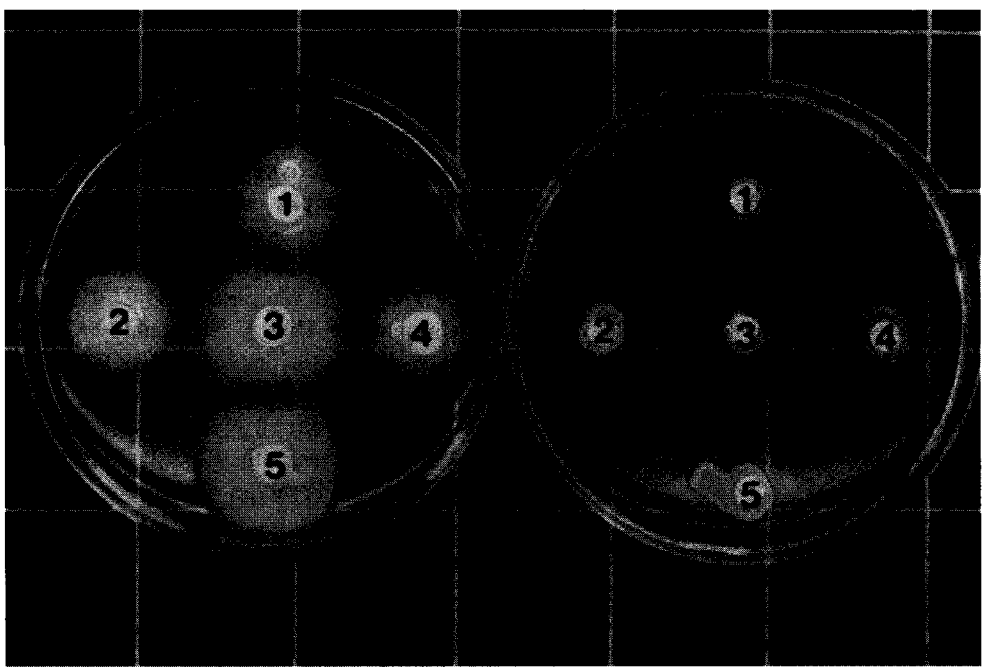

Fig. 1. Bile-salt hydrolytic acitivity as detected by the plate-assay method on A: MRS supplemented with 0.5% TDCA (sodium salt of taurodeoxycholic acid) and 0.37 g/l $CaCl_2$ and B: MRS as control. (1) *L. reuteri* BFE 1058, (2) *Lactobacillus* sp. BFE 1062, (3) *L. acidophilus* BFE 1059, (4) *L. reuteri* BFE 1060 and (5) *L. johnsonii* BFE 1061.

Table 4
Bile tolerance, bile-salt hydrolytic activity and bactericidal effect of bile salts on three strains of *Lactobacillus* isolated from pig faeces

	L. reuteri BFE 1058	*L. johnsonii* BFE 1059	*L. johnsonii* BFE 1061
Bile tolerance[a]			
0.3% (w/v) Oxgall	9 min	25 min	5 min
0.3% (v/v) native porcine bile	45 min	58 min	13 min
Bile salt hydrolytic activity[b]			
TDCA	618	1204	1079
GDCA	4458	4462	2231
Bactericidal effect of conjugated bile salts[c]			
0 h	5.62×10^9	1.50×10^9	6.92×10^8
2 h	2.95×10^9	3.89×10^5	1.23×10^7

[a] Delay of growth (min) from the optical density (measured at 580 nm) to increase by 0.3 units between MRS and MRS containing either 0.3% (w/v) Oxgall or 0.3% (v/v) native porcine bile.
[b] BSH activity is expressed as nmol deoxycholic acid (DCA) formed per 10^{10} CFU initially present per min.
[c] Viable counts (CFU/ml) were determined in the presence of 2.0 mM TDCA and 2.0 mM GDCA at the beginning of the assay and after 2 h.

intestine, where BSH activity of such lactobacilli may play a role in the enterohepatic cycle.

Lactobacillus reuteri BFE 1058 and *L. johnsonii* BFE 1061 were able to grow at pH 3 and 4, and may thus be regarded as acid tolerant (results not shown). Acid-tolerant strains have an advantage in surviving the low pH conditions in the stomach (pH 2.0 in extreme cases), where hydrochloric and gastric acids are secreted. *L. johnsonii* BFE 1059 did not grow at pH 3.0; speculatively this strain might still survive passage of the stomach, although in lower numbers than the other two strains.

The quantitative BSH activity of resting cells is shown in Table 4. The data indicated a higher affinity for GDCA than for TDCA. These pig isolates displayed BSH activities that were in the same range as the BSH activities of the two hamster strains identified as *L. animalis* and that of *L. plantarum* LP80, a BSH overproducing strain studied by De Smet et al. (1995). Furthermore, concentrations of 2.0 mM TDCA and 2.0 mM GDCA had a bactericidal effect on the *L johnsonii* strains BFE 1059 and BFE 1061, but not on *L. reuteri* BFE 1058 (Table 4). This indicates that BFE 1058 may be better adapted to tolerate the intestinal bile conditions. De Smet et al. (1994) suggested that lactobacilli with high BSH activity may contribute to a reduction of cholesterol levels, as the free bile salts (deconjugated) are not as easily reabsorbed in the intestines as conjugated bile salts. Free bile salts may then be excreted in the faeces as poly-bile acid-polymers (Benson et al., 1993). De Smet et al. (1995) also hypothesised that BSH activity may be important for bile-salt resistance, and may therefore be considered an important colonisation factor.

Antagonistic activity of the viable cells of all three isolates was observed against different strains of *Lactobacillus* spp., *Enterococcus* spp. and *Listeria monocytogenes* (data not shown). Strains that exert antagonism against undesired intestinal bacteria may interact to stabilise or control the autochthonous intestinal microflora. The nonspecific antagonism of *L. reuteri* BFE 1058 was probably due to lactic and acetic acid since the neutralised supernatant exerted no inhibitory effect. The CFNS of *L. johnsonii* BFE 1059 was inactivated by catalase and proteases, which indicates that the inhibition was probably due to both a protein and hydrogen peroxide (Table 5). The CFNS of *L. johnsonii* BFE 1061 active against a variety of LAB and pathogens (Table 2) could be inactivated by proteinase K, trypsin, α-chymotrypsin and pepsin, but not by lysozyme and catalase, which indicated a proteinaceous nature of the antagonistic compound (Table 5). The assumed bacteriocin was heat-stable and was not inactivated at 121°C for 15 min. It remained stable at pH 2–10, suggesting that it would be active at the pH of the GIT.

The inhibitory spectrum of the bacteriocin from *L. johnsonii* BFE 1061 is different from that of most known *L. acidophilus* bacteriocins which are generally active only against other lactic acid bacteria (Barefoot and Klaenhammer, 1983; Muriana and Klaenhammer, 1987; Toba et al., 1991; Tahara et al., 1996). Further studies on primary protein structure, however, are necessary to determine whether the bacteriocin produced by *L. johnsonii* BFE 1061 is different from other bacteriocins described for *L. acidophilus* or *L. johnsonii*.

3.2. Feeding trial

In the second part of the study the three strains were used for feeding trials with a group of minipigs. Feeding the 'Western style' diet supplemented with

Table 5
Effect of different enzymes and heat treatments on the inhibitory activity of the cell-free neutralised supernatant (CFNS) of *L. johnsonii* strains BFE 1061 and BFE 1059

Treatment	Activity	
	L. johnsonii BFE 1061	*L. johnsonii* BFE 1059
α-Chymotrypsin (Serva, 17160)	−	−
Lysozyme (Serva, 28262)	+	+
Trypsin (Sigma, T-8253)	−	−
Catalase (Sigma, C-10)	+	−
Pepsin (Merck, 7189)	−	−
Proteinase K (Sigma, P-0390)	−	−
10 min at 100°C	+	+
20 min at 100°C	+	+
15 min 121°C	+	−

+ = no inhibition of activity.
− = complete inhibition of activity.

1% crystalline cholesterol caused an increase in serum cholesterol levels of the minipigs with 'steady-state' reached after 17 weeks. This phenomenon (i.e. the increase of serum cholesterol levels of pigs through dietary cholesterol intake) has been reported by other workers (Gilliland et al., 1985; De Rodas et al., 1996). Hypercholesterolemia is considered the most common risk factor associated with coronary heart diseases in westernized countries, and it appears therefore important to find ways of reducing serum cholesterol levels.

Pigs were selected as experimental animals as their digestive and circulation systems are comparable to those of humans (Ratcliffe and Luginbühl, 1971). Gilliland et al. (1984) showed that bile resistant *Lactobacillus* strains survived in greater numbers in the upper small intestine. Production of bacteriocins by lactobacilli may be advantageous to compete and survive in the presence of other lactic acid bacteria (Gilliland and Walker, 1990). According to literature intestinal strains of lactic acid bacteria exhibit host specificity. The three selected *Lactobacillus* strains used in the probiotic mixture were isolated from pigs and may therefore be regarded host specific. Furthermore, they possessed the above-mentioned characteristics and were, therefore, regarded to be better adapted to survive and colonise the intestinal tract of the minipigs used in the in vivo experiment.

A slight but nonsignificant overall increase in faecal *Lactobacillus* cell counts was observed on Rogosa-agar during the feeding period (Table 2). Two weeks after commencement of probiotic feeding the *Lactobacillus* population increased by about one log unit, the highest level reached during the feeding trial. No changes were, however, observed for numbers of the LAB population (consisting mainly of lactobacilli and enterococci; data not shown) on MRS agar during the feeding period, which remained constant at a level between 10^8 and 10^9 CFU/ml (Table 6). The slight increase observed in the *Lactobacillus* numbers on Rogosa agar is probably the direct result of feeding the probiotic mixture.

In our study BSH-active lactobacilli were administered with the aim of testing the BSH hypothesis for cholesterol reduction. Probiotic feeding showed a

Table 6
Effect of the probiotic mixture (existing of two strains of *L. johnsonii* and one of *L. reuteri*) (see Table 3) administered at 10^{12} CFU per pig per day on the *Lactobacillus* and total LAB populations of minipigs maintained on a high-fat and high-cholesterol diet

Bacterial type	Before probiotic feeding	After three weeks probiotic feeding	Two weeks after terminating probiotic feeding
Lactic acid bacteria	8.5±0.37[a]	8.7±0.43	8.5±0.26
Lactobacilli	7.8±0.55	8.4±0.51	8.1±0.18

[a] Mean values in log CFU/g wet faeces±SD.

Table 7
Effect of the probiotic mixture (see Table 1) on total serum cholesterol and triglycerides of minipigs fed on a 'Western style' diet

Parameter	Before probiotic feeding	After three weeks probiotic feeding	Two weeks after terminating probiotic feeding
Total cholesterol	3.25±0.59[a]	2.74±0.39	3.39±1.36
Triglycerides	0.59±0.19	0.75±0.22	0.58±0.26

[a] Mean values in mmol/l±SD.

lowering effect on serum cholesterol in this study (Table 7). The serum cholesterol levels seemed to reach the baseline level again two weeks after probiotic treatment was terminated. The results indicate that BSH-active lactobacilli probably reduce cholesterol levels; however, a feeding trial with more experimental animals including a control group would be required to confirm the results observed in this preliminary study. The real effect on serum cholesterol levels might have been overshadowed as the minipigs unfortunately gained ca. 10–15 kg in body weight over the experimental period (see also under 2.4.2 for more details on the experimental animals). The triglycerides increased during the feeding of the probiotic mixture, but only nonsignificantly. This undesired effect was in contrast with the results obtained by Danielson et al. (1989), but could have been due to the extremely long period of feeding the high-cholesterol, high-fat diet to reach a definite steady state of elevated cholesterol levels. The diet led to excessive hypertriglyceridemia in one of the minipigs (no. 3). This animal had to be withdrawn from the feeding trial at week 24, shortly before termination of the study, as nutritive toxic liver damage could have been caused.

It was shown by Gilliland et al. (1985); De Rodas et al. (1996); Danielson et al. (1989) that a selected *L. acidophilus* strain (ATCC 43121) had a lowering effect on serum cholesterol levels in pigs maintained on a high cholesterol diet. Gilliland et al. (1985) attributed this effect to the in vitro cholesterol assimilation of this *L. acidophilus* strain, whereas Danielson et al. (1989) did not present a hypothesis on the possible mechanism to explain their observation. De Rodas et al. (1996) claimed that the assimilation of cholesterol and the deconjugation of bile salts had a lowering effect on serum cholesterol levels through interference with the enterohepatic cycle. According to Klaver and van der Meer (1993) it is not likely that real bacterial uptake of cholesterol occurs in *Lactobacillus* species, but they proved that deconjugated bile salts, which is the effect of BSH-active strains, do coprecipitate cholesterol under acidifying conditions. Although this conclusion appears convincing, it has to be taken into account that values below pH 6.0 are not typical of the region in the small intestine where the cholesterol and bile absorption take place. Therefore, this hypothesis seems not applicable as the only mechanism responsible for cholesterol lowering. We would agree more with the conclusions of Marshall and Taylor (1995), who reported that for some strains, removal of cholesterol may occur both in the presence and in the absence of bile and that coprecipitation at low-pH values does not account for all cholesterol removed in vitro. They therefore assumed that an unknown mode of physical association of cholesterol with the bacterial surface may be present, which can be further increased if the bacteria are able to deconjugate bile acids. This is in agreement with our recent observations in studies on the 'cholesterol assimilating' strain *L. salivarius* ssp. *salicinius* NCFB 1555^T using radio-HPLC. No metabolites of the radiolabelled cholesterol were found and all added cholesterol could be recovered after incubation (Haberer et al., 1997), i.e assimilation can be excluded. Results of coprecipitation/solubilisation studies in the presence and absence of living cells at different pH values and at different bile acid compositions and concentrations indicate that even in the absence of bile acids, the bacterial surface of this strain was able to bind 10% of the added cholesterol. This ability could be further increased by up to 80% of the added cholesterol using higher concentrations of deconjugated bile acids. The cholesterol is suggested to be bound in a more specific association to the bacterial cell surface, as it was resistant to resolubilisation at pH 9 for more than 24 h.

The administration of the probiotic mixture had no effect on the pH values of the faeces (Table 8). The

Table 8
Effect of probiotic mixture (see Table 1) on the faeces moisture content and faeces pH of minipigs maintained on a high-fat, high-cholesterol diet

Parameter	Before probiotic feeding	Three weeks after probiotic feeding	Two weeks after terminating probiotic feeding
Faecal moisture content	41.9±4.1[a]	46.6±2.6	39.7±1.6
Faecal pH	7.5±0.23[b]	7.5±0.26	7.8±0.18

[a] Faecal water content measured in % wet weight of faeces (mean±SD.).
[b] Faecal pH (mean±SD).

pH values remained constant between 7.0 and 8.0 for the different minipigs. These values are relatively high and typical for a 'Western-style' diet. A gradual, but definitive increase of the moisture contents of the faeces was observed during probiotic feeding. After termination of probiotic feeding a significant reduction ($P < 0.05$) of the moisture content was measured (Table 8). Compared to pigs on a normal diet, the faeces of the minipigs on a 'Western-style' diet are drier and may cause mild constipation. No diarrhoea, however, was observed from feeding the high doses of lactobacilli.

4. Conclusions

The data are strongly indicative of a meaningful change in serum cholesterol levels of hypercholesterolaemic subjects through high levels of BSH-active lactobacilli. The future involvement of a larger number of experimental animals is suggested as this may serve to confirm the trend observed in cholesterol reduction during the probiotic feeding period. Measurement of primary and secondary bile-salt composition in faeces and faecal water, determination of excreted cholesterol and its metabolites, and monitoring of different physiological bile acid pools could elucidate the mechanism behind cholesterol reduction and should ensure that there is no increase in faecal cytotoxicity or comutagenicity caused by the observed changes in cholesterol and bile-salt metabolism.

Considering that several BSH positive 'probiotic' LAB strains are consumed via commercial novel type yoghurt products (own, unpublished results), the need for further studies is apparent, also with respect to possible implications for the human enterohepatic cycle.

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