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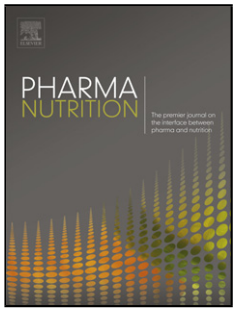

Prebiotics may alter bile salt hydrolase activity: Possible implications for cholesterol metabolism

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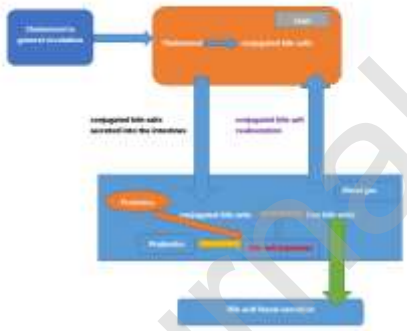
Graphical abstract



Highlights

- Bile salt hydrolase levels and deconjugation rates vary with different probiotics.
- Bile salt deconjugation rates will vary in the presence of prebiotics.

- Prebiotics could alter bile salt deconjugation rates and lead to changes in serum cholesterol.

Abstract

Probiotics secrete bile salt hydrolase (BSH) which catalyses the deconjugation and excretion of bile salts in the GI tract altering cholesterol metabolism in the liver. Many probiotic preparations include prebiotics to promote probiotic growth but little is understood about how prebiotics affect BSH activity. In this study the ability of probiotic Lactobacilli species to deconjugate bile salts in the presence of various prebiotics was determined by measuring cholic acid release. The kinetic properties of BSH was assessed to determine the impact the prebiotics on bile salt deconjugation. When *L. acidophilus* NCTC 1723 was incubated with inulin (1%) there was a significant ($p<0.01$) increase in cholic acid release by 0.16 nmol/min. Lactulose and lactobionic acid at 1% decreased cholic acid release to 0.2 nmol/min and 0.06 nmol/min respectively. In the presence of the pure BSH, inulin and lactulose (0-6%) altered K_m and V_{max} of the enzyme with a K_i of 12% and 10.5% respectively. By contrast, lactobionic acid (2%) increased BSH activity two-fold ($p<0.01$).

These results confirm that prebiotics are capable of altering BSH activity *in vitro*. Similar changes *in vivo* could potentially affect the claimed health benefits of synbiotics particularly where the desired outcome is lowering of serum cholesterol.

Keywords: prebiotics, probiotics, bile acids, Bile salt hydrolase, metabolism, cholesterol.

1. Background

Probiotics were first demonstrated to exhibit hypocholesterolemic effects in humans as early as 1963 [1-2]. The mechanism is thought to involve the metabolism of bile salts by intestinal bacteria reducing the amount of bile salts reabsorbed by enterohepatic circulation [3]. To compensate for the loss of bile salts there is an increase in bile salt biosynthesis from cholesterol in the liver, leading to a reduction in serum cholesterol levels. This shift in cholesterol metabolism by probiotics has been suggested to decrease the risk of cardiovascular disease and is thought by many to be one of the putative benefits of probiotics [4-7]. The metabolism of bile salts in the GI tract is dependent on the chemical form, for example, whether it is a primary or secondary bile salt, the level of conjugation and hydroxylation [8-10]. In terms of cholesterol metabolism, perhaps the most important bile salt modification takes place in the colon with the deconjugation of primary bile salts. Deconjugation is catalysed by bile salt hydrolase (BSH) enzymes secreted by gut bacteria including members of the genera, *Bifidobacterium and Lactobacillus* which account for the majority of bacterial species in probiotic preparations [11-14]. Deconjugation generally reduces the chances of bile salt reabsorption from the terminal ileum and increases faecal excretion [15-18]. The process of deconjugation involves hydrolysis of the C-24 N-acyl amide bond linking bile acids to their amino acid conjugates [19] thus liberating unconjugated bile salts [11, 20].

Bile salt deconjugation is now listed as one of the key selection criteria for probiotics [21]. Other proposed mechanisms by which probiotics could reduce serum cholesterol include binding of cholesterol to the probiotic cell membrane [22] and direct metabolism of cholesterol by probiotic cultures reducing the amount available to the host [23-24]. Products containing probiotics alone or in “synbiotic” combination with prebiotics (indigestible carbohydrates) are now increasingly proposed to maintain

cardiovascular health [25-30]. The indigestible nature of prebiotics allow them to be able to reach the lower intestine and serve as substrates for probiotic growth *in vivo* [31]. Presently little is understood about how the prebiotics in "synbiotic" preparations affect bile salt and cholesterol metabolism. Prebiotics have also been shown to possess other properties which are independent of probiotic growth including inhibition of apoptosis of colon cells and reducing the level of genotoxicity [32].

Although a limited number of studies have been carried out on the synbiotic applications of prebiotics and probiotics very little is understood about how prebiotics impact on BSH activity, particularly on bile salt deconjugation [33-35]. In an attempt to determine how prebiotics could alter bile salt deconjugation and ultimately affect cholesterol metabolism, the BSH activity from five probiotic Lactobacilli species was assessed in the presence of various prebiotics. In addition, the basic enzyme kinetic properties of a typical BSH enzyme known as Choloylglycine hydrolase (CGH) was also assessed in the presence of three widely used prebiotics. The results confirm a complex interaction between prebiotics and BSH activity and the potential to affect cholesterol metabolism.

2. Materials and Methods

2.1. Materials

Inulin, lactulose, lactobionic acid, CGH and all chemicals including bile acids/ salts used in this study were of analytical grade and were obtained from Sigma-Aldrich, Dorset, UK. *L. acidophilus* NCTC 1723, and *L. delbrueckii ss bulgaricus* NCTC 12712, were purchased from National Collection of Type Cultures (NCTC), *L. brevis* NCIMB 11973 was purchased from National Collection of Industrial, Marine and Food Bacteria

(NCIMB), *L. acidophilus* NCFM was a gift from Danisco, UK and *L. reuteri* NCIMB 11951 was from UEL culture collection. Anaerobic incubation was achieved using CO_2 gaspaks in anaerobic jars (Sigma-Aldrich, Dorset, UK).

2.2. Microorganisms, media and culture conditions

The strains were maintained at -80^{0}C in 30% glycerol and initially grown anaerobically on MRS agar plates at 37^{0}C for 48 h. Growth of *Lactobacillus* cultures were achieved anaerobically in MRS broth overnight, 1% of the overnight culture was then introduced in the MRS medium supplemented with sodium salts of different bile salts (glycocholic, taurocholic, glycodeoxycholic, taurodeoxycholic, glycochenodeoxycholic and taurochenodeoxycholic acid) at concentrations ranging from 1-6 mM to represent the concentration in the intestine [36-38]. Cultures were incubated 37˚C for 15h until the cells achieved a late exponential growth phase. The broth cultures (10 ml) were adjusted to pH 7 with 1M NaOH and centrifuged at 10,000 x g for 15 min at 5˚C. The cell pellet was discarded, and the supernatant broth adjusted to pH 1 with 10M HCL and deconjugation determined by thin layer chromatography.

2.3. The detection of bile salt deconjugation by thin layer chromatography

Deconjugated bile acids were determined by spotting 3µl of the supernatant from the broth cultures along with appropriate bile salt standards onto a 5cm x 20cm silica coated (0.25mm) TLC plate (Sigma-Aldrich, Dorset, UK), previously activated at 110˚C for 10 min. Chromatograms were developed in glass tanks lined with filter paper saturated with the solvent. The solvent system contained: Cyclohexane-chloroform-methanol-

acetic acid (15:65:10:1) [39]. After development and drying, the plates were sprayed with vanillin-sulfuric acid reagent and heated at 120˚C as described by Stahl, 1965. Results were expressed as R_f values calculated using the formula x/y, where x is the distance moved from the point of application and y the solvent front.

2.4. Determination of bile salt hydrolase activity

The effect of varying concentrations of prebiotic on Lactobacilli BSH activity was measured as the rate of enzymatic hydrolysis of the C-24 N-acyl amide bond linking bile salts to release the free bile acid and the amino acid conjugate. In this study the release of free cholic acid from taurocholic was quantified as described by Liong and Shah (2005). Briefly, *Lactobacillus acidophilus* NCTC 1723 chosen for its ability to deconjugate taurocholic acid. The overnight culture (1%) was introduced into MRS broth (10mL) and incubated at 37˚C for 15 h. The medium also contained either inulin, lactulose or lactobionic acid 0.5 – 2% and sodium taurocholic acid 6 mM as the most suitable bile acid substrate. At the end of the incubation the pH of the medium was adjusted to pH 7 with NaOH (1N) and centrifuged at 10,000 x g for 15min at 5˚C. The cell pellet was discarded, and the supernatant broth adjusted to pH 1 with HCl (10N). 2 mL ethyl acetate was added 1mL of the supernatant and the mixture was vortexed. The ethyl acetate layer was collected in a glass tube evaporated under a stream of nitrogen gas at 65˚C. The residue then dissolved in 1 ml 0.01N NaOH, once completely mixed 6 ml of H_2SO_4 (16N) and 1ml furfuraldehyde (1%) was added and the mixture boiled at 65˚C for 10 mins. On cooling, 5 mL glacial acetic acid was introduced and vortexed for 1 min prior to taking the absorbance reading at 660nm to determine the level of cholic acid released. To quantify pure cholic acid standards was dissolved in 0.01N

NaOH and for recovery cholic acid was dissolved in media and then treated as above. All experiments were repeated three times.

2.5. The kinetic properties of Choloylglycine hydrolase in the presence of prebiotics

K_m, V_{max} and K_i were determined by incubating CGH (50 units) for 30 minutes at 37˚C with varying concentrations of taurocholic acid (0-6mM) in sodium phosphate buffer (pH 6.4). In a parallel series of studies 2, 4 and 6% inulin, lactulose or lactobionic acid 0.5, 1 and 2% were introduced and incubated under the same conditions. The activity of CGH were determined by measuring the free cholic acid released as described section 2.3.

2.6. Statistical analysis

Statistical analysis was performed using Graphpad prism version 4.0. One-way analysis of variance (ANOVA) at 95% confidence interval and a post tests Dunnett multiple comparison tests were used to determine significant differences on all data (according to the experimental design and runs). The differences were considered significant when $P<0.05$.

3. Results

3.1. Growth of probiotics in the presence of bile salts

Following incubation in MRS broth supplemented with bile salts (1.5mM), growth was observed for cultures of *L. acidophilus* NCTC 1723, *L. delbrueckii ss bulgaricus* NCTC 12712, *L. brevis* NCIMB 11973, *L. acidophilus* NCFM and *L. reuteri* NCIMB 11951 in glycocholic, taurocholic, taurodeoxycholic and taurochenodeoxycholic acid. No growth was observed in the presence of glycodeoxycholic acid and glycochenodeoxycholic acid for cultures of *L. acidophilus* NCTC 1723 and *L. delbrueckii ss bulgaricus* NCTC 12712 (Table 1). There was also no growth for *L. reuteri* NCIMB 11951 in the presence of glycochenodeoxycholic acid (Table 1).

3.2. Deconjugation of bile salts by L. acidophilus NCTC 1723

Deconjugation of the bile salts was assessed using TLC and confirmed by comparing the R_f values to the pure conjugated bile salts and the appearance of the free bile acids in the medium. *L. acidophilus* NCTC 1723 deconjugated the sodium salts of glycocholic, taurocholic, taurodeoxycholic and taurochenodeoxycholic acid (Table 2). However incomplete deconjugation was observed for glycocholic and taurodeoxycholic acid (Table 2) with both free and conjugated bile acids appearing on the TLC plate. Deconjugation was not observed for cultures of *L. delbrueckii ss bulgaricus* NCTC 12712, *L. brevis* NCIMB 11973, *L. acidophilus* NCFM and *L. reuteri* NCIMB 11951 despite growth in media containing the bile salts (Table 2).

3.3. Effect of prebiotics on L. acidophilus NCTC 1723 growth

The lowest growth of *L. acidophilus* NCTC 1723 was in the absence of both glucose and the prebiotics at 5.95×10^4 CFU/ml. In the presence of glucose without prebiotics

the *L. acidophilus* NCTC 1723 cell number increased to 7.83 x 10^8 CFU/ml (Table 3). The addition of inulin at 1% or 2% to the media resulted in a slight increase in the number of *L. acidophilus* NCTC 1723 cells to 8.21 x 10^8 CFU/ml ($p<0.05$) and 8.32 x 10^8 CFU/ml ($p<0.05$) respectively. By contrast, the presence of lactobionic acid the number was 7.0 x 10^8 CFU/ml ($p<0.001$) and 5.18 x 10^7 CFU/ml ($p<0.001$) for 0.5% and 1% respectively. At 2% lactobionic acid no growth of *L. acidophilus* NCTC 1723 was observed. With lactulose (0.5-2%) as the prebiotic, there was no significant change in cell growth when compared to glucose.

3.4. Effect of prebiotics on L. acidophilus NCTC 1723 deconjugation activity

The effect of prebiotics on *L. acidophilus* NCTC 1723 BSH activity was determined quantitatively by assaying for the corresponding release of cholic acid from taurocholic acid (6mM) (Figure 1). Cholic acid released following growth with glucose as the carbon source was 0.25 nmol/min, whereas with inulin at 1% there was significant increases in deconjugation with 0.42 nmol/min cholic acid ($P<0.01$) released. At 2% inulin deconjugation fell back to 0.28 x 10^{-2} nmol/min cholic acid released ($P<0.05$). By contrast both lactulose and lactobionic acid resulted in a decrease in BSH deconjugation activity with the greatest inhibition caused by 1% lactobionic acid with only 0.009 nmol/min ($P<0.01$) cholic acid released. At 2% lactulose cholic acid release was 0.066 nmol/min ($P<0.01$).

3.5. Effects of prebiotics on the enzyme kinetic properties of bile salt hydrolase.

Bile salt hydrolase activity was assessed using a pure sample of the bile salt hydrolysing enzyme CGH [38]. Kinetics studies with the pure CGH and the prebiotics (inulin,

lactulose, lactobionic acid) revealed contrasting results with either a concentration dependent increase or decrease in enzymatic activity (Table 4). There was no significant difference in CGH kinetic activity in the presence of inulin at 2%. At higher concentrations (4 and 6% w/v) enzyme activity was inhibited by 12 and 27% respectively. Lineweaver burk plots showed uncompetitive inhibition at 4% inulin and mixed inhibition at 6% with a K_i of 12% (Table 4). The V_{max} for the release of free bile acids decreased with increasing inulin concentrations from 25±3.7 to 17.24±4.3 nmol/min, and K_m values also decreased from 2.2±0.3, to 1.72±0.9 mM (Table 4). Similarly with lactulose (2, 4 and 6%) CGH enzyme activity was inhibited by 25, 32 and 36% respectively was observed (Table 4). Lineweaver burk plots showed an uncompetitive inhibition at 2 and 4 % and mixed inhibition at 6%, the K_i was 10.5%. V_{max} values decreased in increasing lactulose concentrations from 16.39±2.3 nmol/min, K_m values decreased to 1.72±0.7 mM (Table 4). With lactobionic acid at concentrations up to 2%, CGH activity increased dose dependently with V_{max} increasing to 33.3±4.2 nmol/min and 50±6.9 nmol/min at 1% and 2% respectively. Similarly K_m values increased to 4.3±0.5mM (Table 4).

4. Discussion

Although bile salt deconjugation is an accepted property of BSH enzymes, little is understood about how dietary ingredients and in particular prebiotics affect the kinetic properties of these essential enzymes. For individuals on certain diets particularly those with a high fibre content, high levels of prebiotics could reach the colon and impact on BSH activity. Given the importance of BSH in bile salt and cholesterol metabolism

there are strong arguments to have a better understanding of how prebiotics could affect BSH kinetic activity, and potentially affect bile salt and serum cholesterol levels. Members of the genus *Bifidobacterium* and *Lactobacillus*, which are widely utilised as probiotics are a major source of BSH enzymes. A number of studies have suggested that the activity of BSH produced by probiotics may play a role in regulating serum cholesterol in animal models and humans [33, 41-42]. The putative hypocholesterolaemic properties is one of the key arguments for the health benefits of probiotics. In this study five *Lactobacillus* cultures (*L. acidophilus* NCTC 1723, *L. delbrueckii ss bulgaricus* NCTC 12712*, L. brevis* NCIMB 11973*, L. acidophilus* NCFM and *L. reuteri* NCIMB 11951) were shown to grow in high concentrations (1.5 - 6mM) of bile salts. The bacteria were also assessed for the ability to deconjugate bile salts presumably through the secretion of BSH. Of the five Lactobacilli cultures only *L. acidophilus* NCTC 1723 deconjugated the five bile salts used. This is not a totally surprising observation given the mutable nature of bacteria and confirms the need for constant testing to confirm any claimed properties of probiotics. No deconjugation was observed for *L. acidophilus* NCFM, *L. brevis* NCIMB 11973, *L. delbrueckii ss bulgaricus* NCTC 12712 and *L. reuteri* NCIMB 11951 although all are also widely used as probiotics. Variations in the ability of the different Lactobacilli species/strains to deconjugate bile salts has been known for some time. For example Brashears *et al*. 1998 [43] showed that *L. acidophilus* 43121, *Lactobacillus acidophilus* L1, *Lactobacillus casei* N19 and *Lactobacillus casei* E5 cultures were capable of deconjugating taurocholic acid whilst others reported the inability of Lactobacilli species to deconjugate bile salts [44-45]. It is important to note that some of the variations in deconjugation reported in the literature might have been due to variations in study design such as low substrate (bile salt) levels, variations in growth conditions, the type

of conjugated bile acids used and differing assay conditions. However, the observations in this study confirm the need for caution when attributing general characteristics and specifically the ability to deconjugate bile salts to putative probiotics.

Another key objective of this study was to assess if prebiotics could affect BSH deconjugation in general for which there is little information. In recent years prebiotics are often added alongside probiotic in what is termed synbiotic application. In this study the impact of prebiotics on bile salt deconjugation by *L. acidophilus* NCTC 1723 was assessed using taurocholic acid with either inulin, lactobionic acid or lactulose as the prebiotics. All three prebiotics have been shown to possess differing ability to support the growth and functionality of probiotics [30,32]. Both inulin and lactulose slightly increased growth of *L. acidophilus* NCTC 1723 whereas lactobionic acid significantly decreased growth. The decreased growth caused by lactobionic acid can be attributed to the lowering of pH below pH 6 (data not shown) in the medium at concentrations above 0.5%, a factor that might be of importance should this compound be used in high concentrations as a prebiotic. The increased growth with inulin was not accompanied by increased deconjugation. For all three prebiotics used in this study deconjugation was reduced as the concentration increased. These results are in line with a study by Sridevi et al. 2009 [46] where the BSH activity varied with carbon source, pH and other and culture conditions. These observations indicate that although prebiotics may promote probiotic growth under certain conditions, they could potentially have other negative effects in the colon such as reduced bile salt deconjugation dependent on the local conditions.

Although these novel results demonstrate that prebiotics can interfere with the ability of probiotics to deconjugate bile salts, other factors must be considered, for example the impact on overall probiotic growth or the change in pH. In an attempt to determine if prebiotics directly alter BSH activity, kinetic studies were undertaken using CGH a commonly used commercially available bile salt hydrolysing enzyme [38]. Using taurocholic acid as the substrate, the rate of deconjugation appeared to follow simple Michaelis-Menten kinetics. Both inulin and lactulose demonstrated uncompetitive inhibition at 4% and mixed inhibition at 6%. The prebiotics also decreased apparent V_{max} and K_m with apparent K_i of about 12%. By contrast, lactobionic acid increased the ability of CGH to deconjugate taurocholic acid, doubling both V_{max} and K_m. The increased activity of lactobionic acid is probably due to the lower pH with increasing lactobionic acid concentrations. By contrast lower pH by the same prebiotic had a detrimental effect on cell viability. Previously a pH of 4 was reported as optimal for CGH activity, so these observations are in line with the activity of the enzyme [47]. BSH are generally released by probiotic bacteria into the lumen of the GI tract where they exert their effects. There is now evidence that changes in the gut microbiota will alter both BSH functionality and bile acid metabolism in vivo [48-49] These results confirm that high level of prebiotics (up to 6%) will not only affect probiotic growth but could affect BSH activity in ways that are not always predictable. In considering the implications for the results it may possible that the effects observed for the taurine conjugated bile salts may be different, if only slightly with glycine conjugated bile salts as a substrate.

In conclusion, if bile salt deconjugation is a key requirement for probiotic classification then greater caution is needed when selecting species/strain [21]. There are at least seven different BSH genes producing a range of proteins with different

activity. The BSH kinetic observations in this study were based on experiments with purified BSH from Clostridium perfringens and may not necessarily be the same as if the BSH had been isolated from certain probiotics.

These studies confirm that although certain bacteria species/strain are widely used as probiotics they may not always have the ability to support bile salt deconjugation. Similarly, prebiotics such as inulin may have benefits in terms of probiotic growth, however high levels may reduce BSH activity which could be affect bile salt and cholesterol metabolism. Where bile salt deconjugation is the primary objective with a view to lower serum cholesterol, a prebiotic such as lactobionic acid would be more effective probably through the lowering of colonic pH. However where probiotic growth is the main objective, other prebiotics such as inulin should be considered.

Declarations section

Ethical Approval and Consent to participate: Not applicable

Consent for publication: Not applicable

Availability of supporting data: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: WM, OA, OC participated in the overall planning of experiments, research design, data analysis & interpretation, and manuscript preparation. OA performed experimental studies of probiotics, enzyme kinetics. All authors read and approved the final manuscript.

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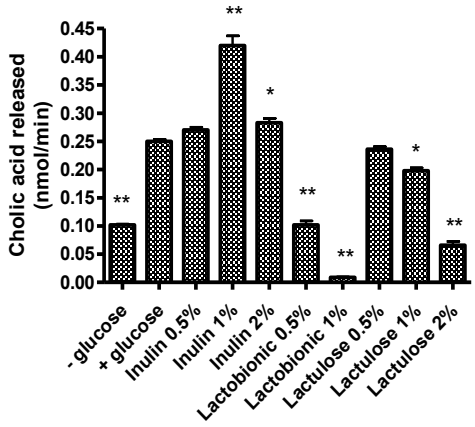
Fig.1. Effect of varying prebiotic concentrations on *L. acidophilus* NCTC 1723 BSH activity. Bacteria cultured in media supplemented with 6mM taurocholic acid, 1% glucose and/or 0.5, 1 and 2% prebiotic. Each point represents the mean and SD of at least three determinations. Post test – Dunnetts multiple comparison tests (all data were compared against the control data glucose with **p≤0.01 and * p<0.05).



TABLE/FIGURE LEGENDS

Table 1. Growth of *L. acidophilus* NCTC 1723, *L. delbrueckii ss bulgaricus* NCTC 12712, *L. reuteri* NCIMB 11951, *L. brevis* NCIMB 11973 *and L. acidophilus* NCFM in bile salts. Following 15 hr growth in glycocholic, taurocholic, glycodeoxycholic, taurodeoxycholic, glycochenodeoxycholic and taurochenodeoxycholic acid all at 1.5mM and the following growth patterns were observed –

Bile acids (1.5mM)	Growth				
	A	B	C	D	E
Glycocholic	+++	+++	+++	+++	+++
Taurocholic	+++	+++	+++	+++	+++
Glycodeoxycholic	NEG	NEG	+	+++	+++
Taurodeoxycholic	+++	++	+++	+++	+++
Glycochenodeoxycholic	NEG	NEG	NEG	++	++
Taurochenodeoxycholic	+++	++	+++	+++	+++

NEG – No growth
+++ - Profuse growth
++ - Minimal growth

A - *L. acidophilus* NCTC 1723,
B - *L. delbrueckii ss bulgaricus* NCTC 12712
C - *L. reuteri* NCIMB 11951

D – *L. brevis* NCIMB 11973
E – *L. acidophilus* NCFM LYO 10

Table 2. R_f values of free and conjugated bile acids. Following 15 hr growth in media supplemented with glycocholic, taurocholic, glycodeoxycholic, taurodeoxycholic or taurochenodeoxycholic acid the ability of *L. acidophilus* NCTC 1723, *L. delbrueckii ss bulgaricus* NCTC 12712, *L. reuteri* NCIMB 11951, *L. brevis* NCIMB 11973 and *L. acidophilus* NCFM to deconjugate bile salts was assayed for on TLC plates.

Bile acids/salts	R_f values of free bile acids/ salts					
	Standards	A	B	C	D	E
Cholic	0.35	0.35	-	-	-	-
Chenodeoxycholic	0.40	0.40	-	-	-	-
Deoxycholic	0.40	0.40	-	-	-	-
Glycocholic	0.20	0.20	0.20	0.20	0.20	0.20
Taurocholic	0.13	-	0.13	0.13	0.13	0.13
Glycodeoxycholic	0.28	NG	NG	0.28	0.28	0.28
Taurodeoxycholic	0.10	0.10	0.10	0.10	0.10	0.10
Taurochenodeoxycholic	0.15	-	0.15	0.15	0.15	0.15

A *L. acidophilus* NCTC 1723

B *L. delbrueckii ss bulgaricus* NCTC 12712

C *L. reuteri* NCIMB 11951

D *L. brevis* NCIMB 11973

E *L. acidophilus* NCFM LYO 10

NG No growth

\- Not detected on TLC plate

Table 3. Varying prebiotic concentration on growth of *L. acidophilus* NCTC 1723. Bacteria was cultured in media supplemented with 6mM taurocholic acid, 1% glucose and/or 0.5, 1 or 2% prebiotic.

Table 3

Glucose and/ or varying prebiotic	Conc. (%)	CFU/ml
Glucose	1.0	7.83×10^{8}
Inulin	0.5	8.07×10^{8}
Inulin	1.0	$8.21 \times 10^{8*}$
Inulin	2.0	$8.32 \times 10^{8*}$
Lactulose	0.5	7.85×10^{8}
Lactulose	1.0	8.08×10^{8}
Lactulose	2.0	8.07×10^{8}
Lactobionic acid	0.5	$7.0 \times 10^{8*}$
Lactobionic acid	1.0	$5.18 \times 10^{7***}$
Lactobionic acid	2.0	-
No glucose or prebiotic	0.0	$5.95 \times 10^{4***}$

Post test - Dunnetts multiple comparison tests (all data were compared against the control data without prebiotics **$p \leq 0.01$, ***$p \leq 0.001$ and * $p < 0.05$)

Table 4. The effect of varying prebiotic concentrations on CGH activity. Enzyme activity was determined by the extent of cholic acid released. The enzymatic activity of (CGH) was carried out in sodium hydrogen phosphate buffer containing taurocholic acid 1-5mM and 2, 4 and 6% prebiotic (inulin), (lactulose) or 0.5, 1 C 2%▲ lactobionic acid. Km and Vmax values were as determined by Lineweaver-Burk plots.

	Prebiotic (%)	% inhibition	Km (mM)	Vmax (nmol/min)	Ki (%)
Inulin	0	-	2.2±0.3,	25.0±3.7,	12.0
	2	7.65	2.5±0.4,	25.0±3.2,	
	4	12.02*	1.7±0.1,	20.0±2.2,	
	6	27.32**	1.7±0.9	17.2±4.3	
Lactulose	0	-	2.2±0.3,	25.0±3.7,	10.5
	2	25.16*	1.4±0.2,	20.0±2.8,	
	4	32.43*	1.5±0.4,	16.7±1.9,	
	6	36.34**	1.7±0.7	16.4±2.3	
Lactobionic acid	0	-	2.2±0.3,	25.0±3.7,	-
	0.5	-17.32	2.0±0.1	26.3±1.7,	
	1	-27.45*	2.9±0.3	33.3±4.2,	
	2	-37.00**	4.3±0.5	50.0±6.9	

Post test - Dunnetts multiple comparison test (all data were compared against the control data without prebiotics with **$p \leq 0.01$ and * $p < 0.05$)