Removal of cholesterol by lactobacilli via incorporation and conversion to coprostanol

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ABSTRACT

Fifteen strains of *Lactobacillus* and *Bifidobacterium* were screened based on their ability to adhere to hydrocarbons via the determination of cellular hydrophobicity. *Lactobacillus acidophilus* ATCC 314, *L. acidophilus* FTCC 0291, *Lactobacillus bulgaricus* FTCC 0411, *L. bulgaricus* FTDC 1311, and *L. casei* ATCC 393 showed greater hydrophobicity and, thus, were selected for examination of cholesterol-removal properties. All selected strains showed changes in cellular fatty acid compositions, especially total fatty acids and saturated and unsaturated fatty acids in the presence of cholesterol compared with those grown in the absence of cholesterol. In addition, we found that cells grown in media containing cholesterol were more resistant to sonication and enzymatic lysis compared with those grown without cholesterol. We further evaluated the location of the incorporated cholesterol via the insertion of fluorescence probes into the cellular membrane. In general, enrichment of cholesterol was found in the regions of the phospholipid tails, upper phospholipids, and polar heads of the cellular membrane phospholipid bilayer. Our results also showed that lactobacilli were able to reduce cholesterol via conversion of cholesterol to coprostanol, aided by the ability of strains to produce cholesterol reductase. Our results provided experimental evidence to strengthen the hypothesis that probiotics could remove cholesterol via the incorporation of cholesterol into the cellular membrane and conversion of cholesterol to coprostanol. The strains studied may be potential health adjunct cultures in fermented dairy products with possible in vivo hypocholesterolemic effects.

Key words: *Lactobacillus*, cholesterol, incorporation, coprostanol

INTRODUCTION

Probiotics such as lactobacilli and bifidobacteria represent a “live microbial supplement that beneficially affects the host animal by improving its intestinal microbial balance” (Lye et al., 2009). Probiotics are normal components of the intestinal microflora in both humans and animals and have been associated with various health-promoting properties including hypocholesterolemia (Liu et al., 2007). Therefore, there has been considerable interest in developing dairy products containing these bacteria as dietary adjuncts (Pereira et al., 2003). Yogurt and fermented milk have received much attention as carriers of live probiotic cultures, and other dairy foods such as some cheese varieties, frozen yogurts, and ice cream have also been evaluated as potential carriers (Stanton et al., 2001).

Past studies have found that fermented dairy products with probiotics not only maintained the intestinal microbial balance but also exerted hypocholesterolemic effects in humans and animals (Park et al., 2008). A wide variety of probiotic strains such as *Lactobacillus acidophilus* ATCC 43121 (De Rodas et al., 1996), *Lactobacillus casei* NCDC-19 (Sindhu and Khetarpaul, 2003), *L. acidophilus* 145, and *Bifidobacterium longum* 913 (Keiling et al., 2002) have been clinically proven to positively modulate serum lipid profiles. Probiotic fermented products such as milk (Khedkar et al., 1993), yogurt (El-Gawad et al., 2005), and cheese (Biong et al., 2004) have been reported to significantly reduce serum cholesterol in in vivo models.

It has been reported that probiotics could reduce serum cholesterol via different mechanisms; however, the exact mechanisms involved remain unclear. It has been found that cholesterol is incorporated into the cellular envelope of lactobacilli, and experimental evidence has shown that this altered the cellular membrane of the organism, produced cells that were more robust toward sonication death, cellular lysis, and exhibited changes in the membrane fatty acid profiles (Liong and Shah, 2005). However, to our knowledge, little information is available on the effects of bile salts on the incorporation of cholesterol into the cellular membrane of probiotics.

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Also, the location of the incorporated cholesterol is yet to be determined.

Another plausible mechanism is the conversion of cholesterol to coprostanol by cholesterol reductase producers (Gerard et al., 2004). This hypothesis was raised based on findings that reported increased removal of cholesterol in the form of coprostanol. Coprostanol is absorbed less in the human intestines compared with its parent cholesterol molecule, leading to increased excretion into feces and subsequently a reduced blood cholesterol level. Very little information is available on the ability of probiotics to produce cholesterol reductase. To our knowledge, there has also been no detailed study evaluating the conversion of cholesterol to coprostanol by probiotics in conditions similar to that of the human gastrointestinal tract.

Thus, the aims of this study were to investigate the incorporation of cholesterol into the cellular membrane of probiotics and the location of incorporation in in vitro conditions similar to that of the human gut. In addition, the conversion of cholesterol to coprostanol by probiotics was evaluated.

MATERIALS AND METHODS

Bacteria Cultures

Strains of *Lactobacillus* sp. FTDC 1211, *L. acidophilus* ATCC 4962, *L. acidophilus* ATCC 4356, *L. acidophilus* ATCC 314, *L. acidophilus* FTCC 0291, *L. acidophilus* FTDC 1733, *L. acidophilus* FTDC 2333, *L. acidophilus* FTDC 2131, *Lactobacillus bulgaricus* FTCC 0411, *L. bulgaricus* FTDC 1511, *L. bulgaricus* FTDC 1311, *L. casei* ATCC 393, *L. casei* FTCC 0442, *Lactobacillus fermentum* FTDC 8312, and *Bifidobacterium animalis* BB 12 were obtained from the Culture Collection Center of Universiti Sains Malaysia (Penang, Malaysia). Each stock culture was stored at -20°C in 40% (vol/vol) sterile glycerol. The organisms were successively activated 3 times in sterile de Man, Rogosa, and Sharpe (MRS) broth (Hi-Media, Mumbai, Maharashtra, India) supplemented with 0.15% (wt/vol) L-cysteine hydrochloride (MRS-LC; Hi-Media) before experimental use.

Screening and Selection of Probiotics

The strains of probiotics were screened based on their ability to adhere to hydrocarbons over 24 h via the determination of cellular hydrophobicity (Perez et al., 1998). Briefly, cultures were harvested in the stationary phase by centrifugation at $12,000 \times g$ for 5 min at 4°C (Eppendorf centrifuge 5417R, B. Braun, Melsungen, Hesse, Germany) and washed twice in 50 mM phos-

phate buffer (pH 6.5), followed by resuspension in the same buffer. The cell suspension was adjusted to an optical density of 1.0 with the same buffer at 600 nm. Bacterial suspensions (3 mL) were mixed with 0.6 mL of xylene (Sigma-Aldrich, St. Louis, MO) and vortexed for 2 min. The 2 phases were allowed to separate. The aqueous phase (bottom) was carefully removed, and the optical density was measured at 600 nm (Genesys 10 UV, Thermospectronic, Madison, WI). The cell surface hydrophobicity (H %) was calculated according to the following equation:

$$\text{H \%} = [(A_0 - A)/A_0] \times 100, \quad [1]$$

where A_0 and A are the absorbance before and after extraction with xylene, respectively. Strains with the greatest hydrophobicity were selected for subsequent analyses.

Incorporation of Cholesterol into the Cellular Membrane

Cellular Fatty Acids Composition. Sterile MRS-LC broth containing 0.3% (wt/vol) individual bile acid, 0.1% (wt/vol) pancreatin (Sigma-Aldrich), and cholesterol (100 $\mu\text{g/mL}$; Hi-Media) was inoculated with 10% (vol/vol) of individual probiotic. Bile salts used were oxgall, taurocholic, and cholic acids (Sigma-Aldrich). The resulting mixture was adjusted to pH 8.0 and incubated at 37°C for 20 h. Sterile MRS-LC broth without cholesterol was used as a control. Upon incubation, the mixture was centrifuged (Eppendorf centrifuge 5417R, B. Braun) at $12,000 \times g$ at 4°C for 15 min, and the cell pellet was harvested for the determination of cellular lipids. The cellular lipids were extracted using the method of Murga et al. (2000). The lipids were converted into fatty acid methyl esters and quantified using gas chromatography (GC-17A, Shimadzu, Kyoto, Japan). The GC was equipped with a capillary column (50 m $\times$ 0.53 mm inside diameter $\times$ 0.70 mm outside diameter; Varian CP-Sil 5CB, Walnut Creek, CA), and oven temperature was held at 100°C after sample injection and increased to 225°C at a rate of 5°C/min . The injector and detector temperatures were 260°C and 280°C , respectively. Helium was used as the carrier gas (3 mL/min), split ratio was 1:100, and the injection volume was 1 μL . The concentration of fatty acid methyl esters in samples was determined using a standard of fatty acid methyl esters (C:4-C:24; Supelco, Bellefonte, PA).

Tolerance Toward Sonication and Enzymatic Treatment. Sterile MRS-LC broth containing 0.3% (wt/vol) individual bile acid, 0.1% (wt/vol) pancre-

atin (Sigma-Aldrich), and cholesterol (100 µg/mL; Hi-Media) was inoculated with 10% (vol/vol) of individual probiotic. The resulting mixture was adjusted to pH 8.0 and incubated at 37°C for 20 h. The cells were harvested by centrifugation (Eppendorf centrifuge 5417R, B. Braun) at $12,000 \times g$ at 4°C for 15 min. The cells were lysed enzymatically using the method of Andrighetto et al. (2000) and via sonication (45 kHz, 15 min; ELMA, Singen, Hohentwiel, Germany) using the method of Psomas et al. (2003). Tolerance toward sonication and enzymatic lysis was determined as growth in colony-forming units per milliliter using the pour plate method. Briefly, MRS-LC agar (Hi-Media) was used for plating, and plates were incubated at 37°C for 48 h.

Fluorescence Anisotropy. Sterile MRS-LC broth containing 0.3% (wt/vol) individual bile acid, 0.1% (wt/vol) pancreatin (Sigma-Aldrich), and cholesterol (100 µg/mL; Hi-Media) was inoculated with 10% (vol/vol) of individual probiotic. The resulting mixture was adjusted to pH 8.0 and incubated at 37°C for 20 h. The cells were collected upon incubation by centrifugation at $14,000 \times g$ at 4°C for 15 min (Eppendorf centrifuge 5417R, B. Braun). The cell pellet was then washed twice with phosphate buffer (50 mM, pH 6.5) and then resuspended in the same buffer. Glass beads (2 g; 0.43–0.60 mm; Sigma-Aldrich) were added to the mixture, vortexed for 2 min, and placed in an ice bath for 1 min. This step was repeated 5 times to yield crude cellular extract. Cellular membrane was separated from the crude cellular extract via centrifugation ($14,000 \times g$, 4°C, 15 min), and the membrane lipid order was determined via fluorescence anisotropy (**FAn**). Fluorescence probes were inserted into the cellular membrane, namely 1,6-diphenyl-1,3,5-hexatriene (**DPH**; Sigma-Aldrich), 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene (**TMA-DPH**; Sigma-Aldrich), and 8-anilino-1-naphthalenesulfonic acid (**ANS**; Sigma-Aldrich). All fluorescence probes were prepared according to Cooper et al. (1980). Cellular membranes with an optical density of 0.3 were suspended in probe solutions at a ratio of 3:1 and incubated at 37°C. Fluorescence anisotropy was measured using a fluorescence spectrophotometer (Cary Eclipse, Varian, Palo Alto, CA). Excitation wavelength was 365 nm for DPH and TMA-DPH and 390 nm for ANS. Emission was determined at 445 nm for DPH and TMA-DPH and 490 nm for ANS (Barshtein et al., 1997). Unlabeled cellular membrane was used as a blank.

Conversion of Cholesterol to Coprostanol

Fluorometric Assay for Cholesterol Reductase Activity. Sterile MRS-LC broth containing 0.3% (wt/

vol) individual bile acid and 0.1% (wt/vol) pancreatin (Sigma-Aldrich) was inoculated with 10% (vol/vol) of individual probiotic. The resulting mixture was adjusted to pH 8.0 and incubated at 37°C for 20 h. Both intracellular and extracellular cholesterol reductase activities were determined in this experiment. Upon incubation, bacterial cells were harvested by centrifugation at $3,500 \times g$ at 4°C for 15 min (Eppendorf centrifuge 5417R, B. Braun). The supernatant was collected and used as crude enzyme extract for the determination of extracellular enzyme activity. The cell pellet obtained was lysed to yield crude cellular extract as described earlier.

Intracellular and extracellular cholesterol reductase activity was determined according to the fluorescence method of Yang and Beitz (1992). Cholesterol reductase activity was expressed as the oxidation of 1 µmol of reduced NAD per minute. The protein concentration of the crude enzyme extracts was estimated by using the Bradford assay (Bradford, 1976). Specific activity was expressed as units of activity per milligram of protein.

Determination of Cholesterol and Coprostanol Content. Sterile MRS-LC broth containing 0.3% (wt/vol) individual bile acid, 0.1% (wt/vol) pancreatin (Sigma-Aldrich), and cholesterol (100 µg/mL; Hi-Media) was inoculated with 10% (vol/vol) of individual probiotic. The resulting mixture was adjusted to pH 8.0 and incubated at 37°C for 20 h. Upon incubation, the mixture was centrifuged (Eppendorf centrifuge 5417R, B. Braun) at $3,500 \times g$ at 4°C for 15 min. The supernatant was filtered through a 0.45-µm membrane and then analyzed using HPLC as described by Skrede et al. (1985). A Luna C18(2) column (150 × 4.6 mm, 5-µm particle size; Phenomenex, Torrance, CA) was used with methanol:water (95:5, vol:vol) as the mobile phase and a flow rate of 1 mL/min. The chromatograph was equipped with a constant flow pump (LC 20AD, Shimadzu) and a differential refractometer (RID 10A, Shimadzu). The concentrations of cholesterol and coprostanol were determined using known concentrations of cholesterol (Hi-Media) and coprostanol (Sigma-Aldrich).

Statistical Analysis

Data analysis was performed using SPSS Inc. software (version 15.0; Chicago, IL). A repeated-measure ANOVA was used for time-based analyses. A one-way ANOVA was used to evaluate the significant differences between sample means, with the significance level at $\alpha = 0.05$. Mean comparisons were assessed by Tukey's test. All data presented are mean values of duplicates obtained from 2 separate runs ($n = 4$), unless stated otherwise.

Table 1. The binding ability of lactobacilli to hydrocarbon over 24 h¹

Strain ²	Fermentation time (h)						
	0	4	8	12	16	20	24
<i>L. acidophilus</i> FTDC 1733	29.41 ± 0.31	34.82 ± 0.22	40.22 ± 0.22	44.56 ± 0.21	71.77 ± 0.34	50.83 ± 0.43	27.09 ± 0.14
<i>L. acidophilus</i> ATCC 4962	10.04 ± 0.24	17.25 ± 0.15	33.55 ± 0.37	53.43 ± 0.72	49.07 ± 0.14	40.38 ± 0.37	27.12 ± 0.45
<i>L. bulgaricus</i> FTDC 1511	19.39 ± 0.29	26.72 ± 0.23	30.47 ± 0.37	34.57 ± 0.48	46.95 ± 0.18	52.12 ± 0.74	60.03 ± 0.30
<i>L. acidophilus</i> FTDC 2333	12.45 ± 0.25	27.00 ± 0.38	39.44 ± 0.40	48.91 ± 0.59	52.18 ± 0.26	63.87 ± 0.14	20.19 ± 0.31
<i>L. acidophilus</i> ATCC 4356	10.15 ± 0.35	19.79 ± 0.35	29.47 ± 0.19	35.50 ± 0.62	40.20 ± 0.75	26.96 ± 0.27	18.21 ± 0.33
<i>L. acidophilus</i> FTDC 2131	10.55 ± 0.33	28.76 ± 0.43	40.09 ± 0.46	48.16 ± 0.07	58.36 ± 0.27	37.93 ± 0.16	27.47 ± 0.89
<i>L. acidophilus</i> ATCC 314*	15.24 ± 0.88	34.76 ± 0.23	40.14 ± 0.53	46.22 ± 0.86	68.64 ± 0.56	61.10 ± 0.17	56.05 ± 0.49
<i>L. bulgaricus</i> FTDC 1311*	19.24 ± 0.40	26.39 ± 0.35	54.28 ± 0.43	68.50 ± 0.30	72.55 ± 0.37	55.73 ± 0.64	30.90 ± 0.49
<i>L. casei</i> ATCC 393*	12.12 ± 0.71	27.65 ± 0.71	45.78 ± 0.27	47.57 ± 0.32	53.87 ± 0.23	67.98 ± 0.35	73.61 ± 0.36
<i>L. fermentum</i> FTDC 8312	10.62 ± 0.83	26.09 ± 0.48	44.59 ± 0.55	67.24 ± 0.35	60.18 ± 0.63	44.99 ± 0.45	22.50 ± 0.91
<i>L. acidophilus</i> FTCC 0291*	19.23 ± 0.69	28.08 ± 0.55	51.20 ± 0.28	58.24 ± 0.90	80.62 ± 0.22	43.86 ± 0.61	27.07 ± 0.91
<i>L. casei</i> FTCC 0442	19.90 ± 0.69	33.46 ± 0.74	41.62 ± 0.86	48.74 ± 1.18	58.67 ± 0.27	49.56 ± 0.33	21.90 ± 0.68
<i>L. bulgaricus</i> FTCC 0411*	26.18 ± 0.61	37.27 ± 0.86	45.24 ± 0.70	52.40 ± 0.23	70.20 ± 0.55	49.25 ± 0.46	36.66 ± 0.71
<i>B. animalis</i> BB 12	11.15 ± 0.45	25.77 ± 0.52	40.50 ± 0.64	61.18 ± 0.46	56.07 ± 0.73	41.08 ± 0.66	23.61 ± 0.69
<i>Lactobacillus</i> sp. FTDC 1211	8.20 ± 0.87	15.67 ± 0.89	24.83 ± 0.91	44.71 ± 0.32	52.97 ± 0.50	67.43 ± 0.35	46.89 ± 0.33

¹Results express hydrophobicity (%) as means ± SD; value are means of duplicates from 2 separate runs (n = 4). Effect of strains, $P < 0.0001$; effect of fermentation time, $P < 0.0001$; interaction of strains and fermentation time, $P < 0.0001$.

²*L.* = *Lactobacillus*; *B.* = *Bifidobacterium*.

*Significantly the greatest hydrophobicity ($P < 0.05$) from repeated-measure design.

RESULTS AND DISCUSSION

Screening and Selection of Probiotics

Fifteen strains of *Lactobacillus* and *Bifidobacterium* were screened based on their ability to adhere to hydrocarbons via the determination of cellular hydrophobicity over a period of 24 h (Table 1). Binding abilities of these strains to hydrocarbon were time and strain dependent. Our results showed that all strains studied exhibited hydrophobicity toward hydrocarbon. This adhesion ability might be due to the hydrophobic nature of the outermost surface of the microorganisms, which enables their attachment to host tissue (Vinderola and Reinheimer, 2003). Adhesion is an important characteristic for probiotics to survive within the gastrointestinal tract and persist at high levels and at closer contact with the epithelial cells to exert health benefits. The hydrophobicity values of strains varied and ranged from 8.20 to 80.62%. There was a general increase ($P < 0.05$) in the hydrophobicity of probiotics during the first 12 to 20 h of fermentation, and a decrease was shown during the later stage of fermentation. The greatest hydrophobicity ($P < 0.05$) was observed from strains of *L. acidophilus* ATCC 314, *L. acidophilus* FTCC 0291, *L. bulgaricus* FTCC 0411, *L. bulgaricus* FTDC 1311, and *L. casei* ATCC 393. Thus, these strains were selected for subsequent analyses.

Incorporation of Cholesterol into the Cellular Membrane

Cellular Fatty Acids Composition. The fatty acid composition of probiotics grown in the presence

and absence of cholesterol is shown in Table 2. In general, greater concentrations of saturated, unsaturated, and total fatty acids ($P < 0.05$) were observed for cells grown in media supplemented with cholesterol compared with the control (without cholesterol). Considering that the lipids of gram-positive bacteria are normally found in the membrane, and fatty acids in bacteria are mainly the precursors of cellular phospholipids, we postulate that the alteration of fatty acid profiles of cells grown in the presence of cholesterol might be the result of the incorporation of cholesterol into the cell membrane of probiotic cells. It has also been previously suggested that an increase in fatty acids via the incorporation of cholesterol could occur as part of a homeostatic mechanism to maintain the membrane integrity and fluidity (Berlin et al., 1998). Among all of the bile salts studied, cells grown in media supplemented with oxgall showed greater concentrations of saturated, unsaturated, and total fatty acids ($P < 0.05$) compared with media containing taurocholic and cholic acids. The bacterial membrane bilayers normally contain saturated and monounsaturated fatty acids but lack polyunsaturated fatty acids (Hulbert, 2003). In the presence of cholic acid, all strains with the exception of *L. acidophilus* ATCC 314 contained eicosapentaenoic acid (C20:5n-3); however, this fatty acid was absent in cells grown in media containing oxgall and taurocholic acid. Lactobacilli have been reported to possess primary physiological responses to bile salt, leading to the incorporation of low-melting-point unsaturated fatty acids into the cellular membrane to strengthen membrane stability (Taranto et al., 2006). Thus, we postulate that the

Table 2. Fatty acid composition of lactobacilli grown in different bile media¹

Fatty acid ²	Strain ³	MRS ⁴ broth (control)	MRS broth + cholesterol	MRS broth + cholesterol + 0.3% oxgall	MRS broth + cholesterol + 0.3% taurocholic acid	MRS broth + cholesterol + 0.3% cholic acid
C8:0	<i>L. acidophilus</i> ATCC 314	0.29 ± 0.02	0.43 ± 0.03	0.38 ± 0.07	0.35 ± 0.12	0.31 ± 0.05
	<i>L. acidophilus</i> FTCC 0291	0.20 ± 0.02	0.92 ± 0.06	0.85 ± 0.11	0.75 ± 0.18	0.69 ± 0.17
	<i>L. bulgaricus</i> FTCC 0411	0.20 ± 0.01	0.83 ± 0.07	0.74 ± 0.18	0.20 ± 0.01	0.52 ± 0.07
	<i>L. bulgaricus</i> FTDC 1311	0.25 ± 0.01	0.77 ± 0.11	0.63 ± 0.10	0.31 ± 0.06	0.61 ± 0.08
	<i>L. casei</i> ATCC 393	0.21 ± 0.03	0.80 ± 0.06	0.70 ± 0.06	0.60 ± 0.12	0.46 ± 0.05
C16:0	<i>L. acidophilus</i> ATCC 314	0.16 ± 0.02	0.62 ± 0.09	0.41 ± 0.09	0.13 ± 0.03	0.30 ± 0.04
	<i>L. acidophilus</i> FTCC 0291	0.14 ± 0.02	0.49 ± 0.07	0.26 ± 0.02	0.17 ± 0.01	0.27 ± 0.03
	<i>L. bulgaricus</i> FTCC 0411	0.19 ± 0.02	0.73 ± 0.11	0.49 ± 0.04	0.47 ± 0.06	0.37 ± 0.04
	<i>L. bulgaricus</i> FTDC 1311	0.13 ± 0.02	0.90 ± 0.16	0.60 ± 0.07	0.18 ± 0.10	0.19 ± 0.04
	<i>L. casei</i> ATCC 393	0.14 ± 0.02	0.63 ± 0.05	0.51 ± 0.08	0.50 ± 0.04	0.60 ± 0.10
C18:0	<i>L. acidophilus</i> ATCC 314	0.26 ± 0.02	0.39 ± 0.12	0.14 ± 0.04	0.23 ± 0.12	0.10 ± 0.01
	<i>L. acidophilus</i> FTCC 0291	0.29 ± 0.02	0.45 ± 0.35	0.41 ± 0.03	0.38 ± 0.15	0.29 ± 0.06
	<i>L. bulgaricus</i> FTCC 0411	0.27 ± 0.01	0.43 ± 0.06	0.25 ± 0.03	0.35 ± 0.13	0.17 ± 0.05
	<i>L. bulgaricus</i> FTDC 1311	0.23 ± 0.03	0.74 ± 0.05	0.50 ± 0.06	0.36 ± 0.27	0.68 ± 0.12
	<i>L. casei</i> ATCC 393	0.21 ± 0.03	0.85 ± 0.14	0.36 ± 0.06	0.57 ± 0.04	0.25 ± 0.05
C18:1n-9c	<i>L. acidophilus</i> ATCC 314	0.21 ± 0.01	0.72 ± 0.12	0.28 ± 0.05	0.63 ± 0.04	0.45 ± 0.01
	<i>L. acidophilus</i> FTCC 0291	0.20 ± 0.02	0.57 ± 0.05	0.47 ± 0.10	0.35 ± 0.04	0.25 ± 0.02
	<i>L. bulgaricus</i> FTCC 0411	0.20 ± 0.11	0.39 ± 0.08	0.14 ± 0.02	0.18 ± 0.08	0.31 ± 0.04
	<i>L. bulgaricus</i> FTDC 1311	0.18 ± 0.02	0.64 ± 0.19	0.58 ± 0.09	0.32 ± 0.06	0.34 ± 0.13
	<i>L. casei</i> ATCC 393	0.20 ± 0.02	0.96 ± 0.19	0.71 ± 0.15	0.56 ± 0.07	0.51 ± 0.09
C18:2n-6t	<i>L. acidophilus</i> ATCC 314	0.37 ± 0.04	0.89 ± 0.09	0.73 ± 0.06	0.30 ± 0.06	0.19 ± 0.01
	<i>L. acidophilus</i> FTCC 0291	0.37 ± 0.03	0.39 ± 0.04	0.28 ± 0.10	0.21 ± 0.12	0.13 ± 0.03
	<i>L. bulgaricus</i> FTCC 0411	0.33 ± 0.04	0.53 ± 0.06	0.50 ± 0.07	0.36 ± 0.09	0.29 ± 0.07
	<i>L. bulgaricus</i> FTDC 1311	0.35 ± 0.05	0.61 ± 0.02	0.54 ± 0.07	0.44 ± 0.12	0.72 ± 0.09
	<i>L. casei</i> ATCC 393	0.37 ± 0.07	0.87 ± 0.12	0.64 ± 0.07	0.39 ± 0.07	0.30 ± 0.04
C20:5n-3	<i>L. acidophilus</i> ATCC 314	ND ^b	ND	ND	ND	ND
	<i>L. acidophilus</i> FTCC 0291	0.18 ± 0.04	0.38 ± 0.06	1.60 ± 0.35	0.50 ± 0.03	1.04 ± 0.14
	<i>L. bulgaricus</i> FTCC 0411	ND	ND	ND	ND	0.94 ± 0.32
	<i>L. bulgaricus</i> FTDC 1311	ND	ND	ND	ND	0.62 ± 0.05
	<i>L. casei</i> ATCC 393	ND	ND	1.20 ± 0.18	0.62 ± 0.08	1.03 ± 0.08
C24:0	<i>L. acidophilus</i> ATCC 314	0.19 ± 0.01	0.98 ± 0.08	0.69 ± 0.11	0.77 ± 0.09	0.65 ± 0.07
	<i>L. acidophilus</i> FTCC 0291	0.17 ± 0.01	0.79 ± 0.06	0.77 ± 0.34	0.44 ± 0.13	0.74 ± 0.37
	<i>L. bulgaricus</i> FTCC 0411	0.21 ± 0.03	0.58 ± 0.05	0.23 ± 0.04	0.21 ± 0.10	0.47 ± 0.06
	<i>L. bulgaricus</i> FTDC 1311	0.17 ± 0.01	0.78 ± 0.13	0.68 ± 0.11	0.52 ± 0.16	0.32 ± 0.12
	<i>L. casei</i> ATCC 393	0.16 ± 0.01	0.77 ± 0.14	0.31 ± 0.07	0.41 ± 0.10	0.39 ± 0.09
SFA	<i>L. acidophilus</i> ATCC 314	0.90 ± 0.02 ^{c,A}	2.42 ± 0.13 ^{a,C}	1.62 ± 0.22 ^{b,C}	1.48 ± 0.21 ^{b,B}	1.36 ± 0.08 ^{b,B}
	<i>L. acidophilus</i> FTCC 0291	0.79 ± 0.03 ^{c,BC}	2.65 ± 0.32 ^{a,BC}	2.29 ± 0.29 ^{ab,AB}	1.74 ± 0.37 ^{b,AB}	1.99 ± 0.52 ^{ab,A}
	<i>L. bulgaricus</i> FTCC 0411	0.86 ± 0.03 ^{d,AB}	2.57 ± 0.10 ^{a,BC}	1.71 ± 0.26 ^{bc}	1.22 ± 0.08 ^{c,B}	1.53 ± 0.07 ^{b,AB}
	<i>L. bulgaricus</i> FTDC 1311	0.79 ± 0.03 ^{d,CD}	3.19 ± 0.28 ^{a,A}	2.41 ± 0.22 ^{b,A}	1.37 ± 0.29 ^{c,B}	1.80 ± 0.24 ^{c,AB}
	<i>L. casei</i> ATCC 393	0.72 ± 0.03 ^{d,D}	3.05 ± 0.20 ^{a,AB}	1.88 ± 0.12 ^{bc,BC}	2.13 ± 0.23 ^{b,A}	1.68 ± 0.09 ^{c,AB}
UFA	<i>L. acidophilus</i> ATCC 314	0.58 ± 0.04 ^{c,B}	1.61 ± 0.15 ^{a,AB}	1.02 ± 0.10 ^{b,B}	0.93 ± 0.06 ^{b,BC}	0.58 ± 0.02 ^{c,C}
	<i>L. acidophilus</i> FTCC 0291	0.75 ± 0.06 ^{d,AB}	1.34 ± 0.02 ^{b,BC}	2.35 ± 0.19 ^{a,A}	1.05 ± 0.14 ^{c,B}	1.42 ± 0.13 ^{b,B}
	<i>L. bulgaricus</i> FTCC 0411	0.53 ± 0.10 ^{c,B}	0.92 ± 0.12 ^{b,D}	0.65 ± 0.08 ^{bc,C}	0.54 ± 0.01 ^{c,D}	1.54 ± 0.32 ^{a,AB}
	<i>L. bulgaricus</i> FTDC 1311	0.53 ± 0.05 ^{c,B}	1.25 ± 0.20 ^{b,C}	1.11 ± 0.12 ^{b,B}	0.76 ± 0.18 ^{c,CD}	1.69 ± 0.14 ^{a,AB}
	<i>L. casei</i> ATCC 393	0.57 ± 0.08 ^{d,B}	1.84 ± 0.18 ^{bc,A}	2.55 ± 0.14 ^{a,A}	1.57 ± 0.09 ^{c,A}	1.84 ± 0.09 ^{b,A}
Total FA	<i>L. acidophilus</i> ATCC 314	1.48 ± 0.05 ^{d,AB}	4.01 ± 0.25 ^{a,BC}	2.63 ± 0.29 ^{b,C}	2.40 ± 0.19 ^{b,BC}	1.94 ± 0.08 ^{c,B}
	<i>L. acidophilus</i> FTCC 0291	1.55 ± 0.04 ^{d,A}	3.99 ± 0.32 ^{ab,BC}	4.65 ± 0.30 ^{a,A}	2.80 ± 0.51 ^{c,B}	3.41 ± 0.40 ^{bc,A}
	<i>L. bulgaricus</i> FTCC 0411	1.40 ± 0.09 ^{c,BC}	3.49 ± 0.18 ^{a,C}	2.36 ± 0.33 ^{b,C}	1.76 ± 0.06 ^{c,D}	3.07 ± 0.28 ^{a,A}
	<i>L. bulgaricus</i> FTDC 1311	1.32 ± 0.06 ^{d,C}	4.44 ± 0.25 ^{a,AB}	3.53 ± 0.20 ^{b,B}	2.13 ± 0.22 ^{c,CD}	3.49 ± 0.26 ^{b,A}
	<i>L. casei</i> ATCC 393	1.29 ± 0.08 ^{c,C}	4.89 ± 0.36 ^{a,A}	4.44 ± 0.23 ^{a,A}	3.70 ± 0.16 ^{b,A}	3.53 ± 0.13 ^{b,A}

^{a-d}Means in the same row with different superscripts are significantly different ($P < 0.05$).^{A-D}Means in the same column with different superscripts are significantly different ($P < 0.05$).¹Results express concentration of fatty acid (g/g of dry weight of cells) as means ± SD; values are means of duplicates from 2 separate runs (n = 4).²c = cis; t = trans; SFA = total saturated fatty acids; UFA = total unsaturated fatty acids; Total FA = total saturated and unsaturated fatty acids.³L. = *Lactobacillus*.⁴MRS = de Man, Rogosa, and Sharpe.⁵ND = not determined.

Table 3. Resistance of lactobacilli strains toward sonication and enzymatic lysis in the presence and absence of cholesterol and bile salts¹

Strain ²	MRS ³ broth (control)	MRS broth + cholesterol	MRS broth + cholesterol + 0.3% oxgall	MRS broth + cholesterol + 0.3% taurocholic acid	MRS broth + cholesterol + 0.3% cholic acid
Sonication					
<i>L. acidophilus</i> ATCC 314	7.55 ± 0.44 ^{d,AB}	9.56 ± 0.04 ^{a,C}	8.69 ± 0.04 ^{b,A}	8.46 ± 0.46 ^{bc,A}	7.97 ± 0.01 ^{cd,AB}
<i>L. acidophilus</i> FTCC 0291	7.34 ± 0.12 ^{d,AB}	10.13 ± 0.09 ^{a,B}	8.91 ± 0.03 ^{b,A}	7.67 ± 0.04 ^{c,AB}	7.96 ± 0.03 ^{c,AB}
<i>L. bulgaricus</i> FTCC 0411	7.96 ± 0.03 ^{b,A}	10.62 ± 0.35 ^{a,A}	8.56 ± 0.32 ^{b,A}	8.16 ± 0.40 ^{b,AB}	8.06 ± 0.22 ^{b,A}
<i>L. bulgaricus</i> FTDC 1311	6.62 ± 0.07 ^{d,C}	8.86 ± 0.01 ^{a,D}	7.89 ± 0.49 ^{b,B}	7.34 ± 0.50 ^{bc,C}	6.96 ± 0.04 ^{cd,C}
<i>L. casei</i> ATCC 393	7.24 ± 0.52 ^{c,BC}	9.62 ± 0.09 ^{a,C}	8.75 ± 0.03 ^{b,A}	8.41 ± 0.44 ^{b,A}	7.50 ± 0.45 ^{c,B}
Enzymatic lysis					
<i>L. acidophilus</i> ATCC 314	7.40 ± 0.14 ^{b,AB}	8.82 ± 0.01 ^{a,BC}	8.18 ± 0.47 ^{ab,A}	7.95 ± 0.61 ^{ab,A}	5.96 ± 0.45 ^{c,B}
<i>L. acidophilus</i> FTCC 0291	6.93 ± 0.43 ^{d,ABC}	9.58 ± 0.11 ^{a,A}	8.55 ± 0.04 ^{b,A}	7.34 ± 0.03 ^{cd,AB}	7.56 ± 0.08 ^{c,A}
<i>L. bulgaricus</i> FTCC 0411	7.58 ± 0.05 ^{b,A}	9.27 ± 0.06 ^{a,AB}	6.55 ± 0.03 ^{c,B}	6.39 ± 0.24 ^{c,B}	5.78 ± 0.48 ^{d,B}
<i>L. bulgaricus</i> FTDC 1311	6.30 ± 0.45 ^{bc,C}	8.46 ± 0.47 ^{a,C}	7.15 ± 0.12 ^{b,B}	6.87 ± 0.58 ^{b,B}	5.68 ± 0.05 ^{c,B}
<i>L. casei</i> ATCC 393	6.72 ± 0.35 ^{c,BC}	9.10 ± 0.13 ^{a,AB}	5.14 ± 0.54 ^{d,C}	8.16 ± 0.44 ^{b,A}	4.63 ± 0.28 ^{d,C}

^{a-d}Means in the same row with different superscripts are significantly different ($P < 0.05$).

^{A-D}Means in the same column with different superscripts are significantly different ($P < 0.05$).

¹Results express cell count (log cfu/mL) as means ± SD; values are means of duplicates from 2 separate runs (n = 4).

²*L.* = *Lactobacillus*.

³MRS = de Man, Rogosa, and Sharpe.

presence of cholic acid increased the incorporation of cholesterol into the cell membrane, which subsequently increased the concentration of low-melting-point eicosapentaenoic acid (C20:5n-3; -114.1°C) in the cell membrane to exhibit a quick adaptive response to bile. In most cases, the least concentrations of saturated, unsaturated, and total fatty acids ($P < 0.05$) were observed from cells grown in media containing taurocholic acid. Our results also showed that most cells grown in the presence of cholesterol alone had greater

concentrations of saturated fatty acids and total fatty acids compared with cells grown in the presence of both cholesterol and bile salts. Acids and bile salts in the human gastrointestinal tract are the main hurdles for probiotic organisms before their influx and proliferation in the lower gut. Thus, we postulate that the growth of probiotics was inhibited in the presence of bile salt, leading to reduced ability for cholesterol incorporation and subsequently lesser cellular concentrations of saturated fatty acids and total fatty acids.

Table 4. Fluorescence anisotropy of cellular membrane of cells grown in the presence and absence of cholesterol and bile salts¹

Fluorescence anisotropy ²	Strain ³	MRS ⁴ broth (control)	MRS broth + cholesterol	MRS broth + cholesterol + 0.3% oxgall	MRS broth + cholesterol + 0.3% taurocholic acid	MRS broth + cholesterol + 0.3% cholic acid
DPH	<i>L. acidophilus</i> ATCC 314	0.24 ± 0.01 ^{bc,B}	0.22 ± 0.01 ^{c,C}	0.27 ± 0.01 ^{b,C}	0.31 ± 0.03 ^{a,AB}	0.25 ± 0.01 ^{bc,B}
	<i>L. acidophilus</i> FTCC 0291	0.24 ± 0.01 ^{bc,B}	0.27 ± 0.01 ^{b,B}	0.33 ± 0.01 ^{a,B}	0.34 ± 0.02 ^{a,A}	0.23 ± 0.03 ^{c,B}
	<i>L. bulgaricus</i> FTCC 0411	0.28 ± 0.01 ^{d,A}	0.31 ± 0.01 ^{c,B}	0.38 ± 0.01 ^{a,A}	0.29 ± 0.01 ^{cd,B}	0.35 ± 0.01 ^{b,A}
	<i>L. bulgaricus</i> FTDC 1311	0.15 ± 0.01 ^{c,C}	0.36 ± 0.03 ^{a,A}	0.18 ± 0.01 ^{c,D}	0.35 ± 0.02 ^{a,A}	0.25 ± 0.01 ^{b,B}
	<i>L. casei</i> ATCC 393	0.15 ± 0.01 ^{d,C}	0.16 ± 0.01 ^{d,D}	0.18 ± 0.01 ^{c,D}	0.28 ± 0.01 ^{a,B}	0.23 ± 0.01 ^{b,B}
ANS	<i>L. acidophilus</i> ATCC 314	0.23 ± 0.01 ^{bc,A}	0.25 ± 0.03 ^{ab,A}	0.21 ± 0.01 ^{c,B}	0.25 ± 0.01 ^{ab,B}	0.28 ± 0.02 ^{a,A}
	<i>L. acidophilus</i> FTCC 0291	0.20 ± 0.02 ^{ab,AB}	0.19 ± 0.01 ^{c,BC}	0.21 ± 0.01 ^{ab,B}	0.22 ± 0.02 ^{a,C}	0.18 ± 0.03 ^{b,B}
	<i>L. bulgaricus</i> FTCC 0411	0.21 ± 0.01 ^{bc,A}	0.20 ± 0.01 ^{c,BC}	0.22 ± 0.02 ^{abc,B}	0.24 ± 0.01 ^{a,BC}	0.23 ± 0.03 ^{ab,A}
	<i>L. bulgaricus</i> FTDC 1311	0.14 ± 0.01 ^{d,C}	0.18 ± 0.02 ^{c,C}	0.25 ± 0.01 ^{b,A}	0.31 ± 0.01 ^{a,A}	0.26 ± 0.01 ^{b,A}
	<i>L. casei</i> ATCC 393	0.17 ± 0.02 ^{c,BC}	0.23 ± 0.02 ^{b,AB}	0.27 ± 0.01 ^{a,A}	0.19 ± 0.01 ^{c,D}	0.17 ± 0.02 ^{c,B}
TMA-DPH	<i>L. acidophilus</i> ATCC 314	0.13 ± 0.01 ^{d,C}	0.22 ± 0.01 ^{c,B}	0.31 ± 0.02 ^{a,B}	0.29 ± 0.01 ^{b,BC}	0.25 ± 0.01 ^{c,B}
	<i>L. acidophilus</i> FTCC 0291	0.23 ± 0.01 ^{c,B}	0.30 ± 0.01 ^{b,A}	0.36 ± 0.01 ^{a,A}	0.32 ± 0.03 ^{b,A}	0.24 ± 0.01 ^{c,B}
	<i>L. bulgaricus</i> FTCC 0411	0.14 ± 0.01 ^{b,C}	0.15 ± 0.01 ^{b,C}	0.16 ± 0.01 ^{b,D}	0.19 ± 0.01 ^{a,D}	0.17 ± 0.01 ^{ab,C}
	<i>L. bulgaricus</i> FTDC 1311	0.28 ± 0.01 ^{b,A}	0.24 ± 0.02 ^{c,B}	0.29 ± 0.02 ^{b,B}	0.31 ± 0.02 ^{b,AB}	0.49 ± 0.01 ^{a,A}
	<i>L. casei</i> ATCC 393	0.13 ± 0.02 ^{c,C}	0.15 ± 0.02 ^{c,C}	0.21 ± 0.01 ^{b,C}	0.26 ± 0.01 ^{a,C}	0.20 ± 0.02 ^{b,C}

^{a-d}Means in the same row with different superscripts are significantly different ($P < 0.05$).

^{A-D}Means in the same column with different superscripts are significantly different ($P < 0.05$).

¹Results are expressed as means ± SD; value are means of duplicates from 2 separate runs (n = 4).

²DPH = 1,6-diphenyl-1,3,5-hexatriene; ANS = 8-anilino-1-naphthalenesulfonic acid; TMA-DPH = 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene.

³*L.* = *Lactobacillus*.

⁴MRS = de Man, Rogosa, and Sharpe.

Table 5. Total intracellular cholesterol reductase activity, protein content, and specific cholesterol reductase activity of probiotics in the presence of different bile salts¹

Strain	Bile salt			
	Control	Oxgall	Taurocholic acid	Cholic acid
Total activity (U/mL)				
<i>L. acidophilus</i> ATCC 314	0.09 ± 0.006 ^{ab,B}	0.11 ± 0.028 ^{a,A}	0.02 ± 0.009 ^{c,BC}	0.06 ± 0.022 ^{b,B}
<i>L. acidophilus</i> FTCC 0291	0.03 ± 0.004 ^{c,C}	0.04 ± 0.003 ^{b,B}	0.15 ± 0.002 ^{a,A}	0.02 ± 0.002 ^{d,C}
<i>L. bulgaricus</i> FTCC 0411	0.15 ± 0.031 ^{a,A}	0.02 ± 0.009 ^{b,B}	0.01 ± 0.004 ^{b,C}	0.04 ± 0.015 ^{b,C}
<i>L. bulgaricus</i> FTDC 1311	0.05 ± 0.012 ^{a,C}	0.05 ± 0.009 ^{a,B}	0.06 ± 0.035 ^{a,B}	0.03 ± 0.004 ^{a,C}
<i>L. casei</i> ATCC 393	0.14 ± 0.007 ^{b,A}	0.04 ± 0.007 ^{d,B}	0.16 ± 0.007 ^{a,A}	0.12 ± 0.003 ^{c,A}
Total protein (mg/mL)				
<i>L. acidophilus</i> ATCC 314	0.12 ± 0.010 ^{a,B}	0.03 ± 0.002 ^{bc,A}	0.02 ± 0.002 ^{c,A}	0.05 ± 0.005 ^{b,AB}
<i>L. acidophilus</i> FTCC 0291	0.06 ± 0.005 ^{a,C}	0.01 ± 0.003 ^{c,D}	0.02 ± 0.004 ^{bc,A}	0.04 ± 0.009 ^{ab,AB}
<i>L. bulgaricus</i> FTCC 0411	0.04 ± 0.003 ^{a,CD}	0.03 ± 0.003 ^{b,AB}	0.02 ± 0.001 ^{b,A}	0.03 ± 0.004 ^{b,B}
<i>L. bulgaricus</i> FTDC 1311	0.02 ± 0.002 ^{b,D}	0.02 ± 0.003 ^{b,C}	0.01 ± 0.001 ^{b,B}	0.04 ± 0.003 ^{a,AB}
<i>L. casei</i> ATCC 393	0.19 ± 0.010 ^{a,A}	0.03 ± 0.004 ^{c,BC}	0.03 ± 0.001 ^{c,A}	0.07 ± 0.017 ^{b,A}
Specific activity (U/mg)				
<i>L. acidophilus</i> ATCC 314	0.80 ± 0.105 ^{b,C}	3.24 ± 0.971 ^{a,B}	0.98 ± 0.297 ^{b,C}	1.26 ± 0.331 ^{b,B}
<i>L. acidophilus</i> FTCC 0291	0.57 ± 0.039 ^{b,C}	7.42 ± 1.403 ^{a,A}	6.82 ± 0.932 ^{a,A}	0.38 ± 0.082 ^{b,C}
<i>L. bulgaricus</i> FTCC 0411	3.89 ± 1.034 ^{a,A}	1.31 ± 0.101 ^{b,C}	0.51 ± 0.186 ^{b,C}	1.21 ± 0.143 ^{b,B}
<i>L. bulgaricus</i> FTDC 1311	2.26 ± 0.385 ^{ab,B}	2.91 ± 0.864 ^{ab,BC}	4.01 ± 2.378 ^{a,B}	0.67 ± 0.119 ^{b,C}
<i>L. casei</i> ATCC 393	0.77 ± 0.006 ^{c,C}	2.03 ± 0.186 ^{b,BC}	6.45 ± 0.178 ^{a,AB}	1.76 ± 0.318 ^{b,A}

^{a-d}Means in the same row with different superscripts are significantly different ($P < 0.05$).

^{A-D}Means in the same column with different superscripts are significantly different ($P < 0.05$).

¹Results are expressed as means ± SD; value are means of duplicates from 2 separate runs (n = 4).

²*L.* = *Lactobacillus*.

Tolerance Toward Sonication and Enzymatic Treatment. Resistance of probiotics toward sonication and enzymatic lysis in the presence of different bile salts is shown in Table 3. All strains were more resistant ($P < 0.05$) to lysis in media containing cholesterol compared with the control (without cholesterol) after sonication and enzymatic treatment. We postulate that the incorporation of cholesterol into the cellular membrane increased the concentration of saturated and unsaturated fatty acids that subsequently increased membrane strength. Dambekodi and Gilliland (1998) showed that the incorporation of cholesterol increased tensile strength of the membranes leading to increased cellular resistance toward lysis. A greater resistance ($P < 0.05$) toward sonication was found in most cells grown in the presence of oxgall compared with those grown in the presence of taurocholic and cholic acids. This was most prevalent for *L. casei* ATCC 393, for which growth was one log cycle greater than that of those grown in media containing cholic acid. However, all strains showed a growth value exceeding 10^6 cfu/mL upon sonication. Similarly, strains grown in media supplemented with oxgall and taurocholic acids showed a greater ability to withstand enzymatic lysis compared with those grown in the presence of cholic acid. Cells grown in the presence of cholic acid showed growth below 10^7 cfu/mL upon enzyme lysis, and this was most prevalent for *L. bulgaricus* FTCC 0411.

FAn. The effect of supplementation of cholesterol and different bile salts on membrane fluidity was assessed

via FAn (Table 4). The fluorescence probe DPH is an apolar fluorophore that has a high affinity for different hydrophobic regions within the membrane, whereas ANS is a fluorophore that has a high affinity toward the interface between the apolar tail and polar head of phospholipids, and TMA-DPH is a cationic probe with the fluorophore TMA incorporated in the head group and DPH incorporated in the acyl chain (Liong et al., 2007). In general, the FAn of DPH, ANS, and TMA-DPH were greater ($P < 0.05$) for most strains grown in the media containing cholesterol compared with the control (without cholesterol). This indicates greater saturation of cholesterol in the hydrophobic regions of the phospholipid tails, upper phospholipids region, and polar heads of the membrane bilayer in cells grown in the presence of cholesterol. This enrichment of cholesterol in various regions may further increase the packing order and decrease membrane permeability and fluidity. In the presence of bile salts, greater FAn of DPH ($P < 0.05$) was observed for cells grown in media supplemented with taurocholic acid compared with those grown in media containing oxgall and cholic acid. Similarly, the FAn of ANS for strains grown in media containing taurocholic acid was greater ($P < 0.05$) compared with those grown in media supplemented with oxgall and cholic acid. This was most prevalent for *L. bulgaricus* FTDC 1311. On the other hand, the FAn of TMA-DPH of cells grown in media supplemented with oxgall was the greatest, followed by taurocholic acid and the least in the presence of cholic acid ($P < 0.05$).

Table 6. Total extracellular cholesterol reductase activity, protein content, and specific cholesterol reductase activity of probiotics in the presence of different bile salts¹

Strain ²	Bile salt			
	Control	Oxgall	Taurocholic acid	Cholic acid
Total activity (U/mL)				
<i>L. acidophilus</i> ATCC 314	0.003 ± 0.001 ^{ab,B}	0.090 ± 0.003 ^{a,A}	0.006 ± 0.001 ^{c,BC}	0.018 ± 0.003 ^{b,B}
<i>L. acidophilus</i> FTCC 0291	0.002 ± 0.001 ^{c,C}	0.005 ± 0.001 ^{b,B}	0.033 ± 0.007 ^{a,A}	0.017 ± 0.004 ^{d,C}
<i>L. bulgaricus</i> FTCC 0411	0.027 ± 0.003 ^{a,A}	0.061 ± 0.004 ^{b,B}	0.025 ± 0.011 ^{b,C}	0.053 ± 0.017 ^{b,C}
<i>L. bulgaricus</i> FTDC 1311	0.010 ± 0.004 ^{a,C}	0.037 ± 0.012 ^{a,B}	0.026 ± 0.004 ^{a,B}	0.019 ± 0.006 ^{a,C}
<i>L. casei</i> ATCC 393	0.004 ± 0.001 ^{b,A}	0.005 ± 0.001 ^{d,B}	0.007 ± 0.002 ^{a,A}	0.014 ± 0.004 ^{c,A}
Total protein (mg/mL)				
<i>L. acidophilus</i> ATCC 314	0.137 ± 0.012 ^{a,B}	0.143 ± 0.005 ^{bc,A}	0.185 ± 0.004 ^{c,A}	0.120 ± 0.001 ^{b,AB}
<i>L. acidophilus</i> FTCC 0291	0.125 ± 0.004 ^{a,C}	0.111 ± 0.057 ^{c,D}	0.142 ± 0.018 ^{bc,A}	0.110 ± 0.015 ^{ab,AB}
<i>L. bulgaricus</i> FTCC 0411	0.108 ± 0.005 ^{a,CD}	0.198 ± 0.075 ^{b,AB}	0.109 ± 0.003 ^{b,A}	0.095 ± 0.005 ^{b,B}
<i>L. bulgaricus</i> FTDC 1311	0.147 ± 0.010 ^{b,D}	0.02 ± 0.003 ^{b,C}	0.159 ± 0.015 ^{b,B}	0.120 ± 0.030 ^{a,AB}
<i>L. casei</i> ATCC 393	0.101 ± 0.010 ^{a,A}	0.139 ± 0.008 ^{bc,BC}	0.101 ± 0.010 ^{c,A}	0.103 ± 0.003 ^{b,A}
Specific activity (U/mg)				
<i>L. acidophilus</i> ATCC 314	0.022 ± 0.005 ^{b,C}	0.627 ± 0.008 ^{a,B}	0.030 ± 0.004 ^{b,C}	0.151 ± 0.028 ^{b,B}
<i>L. acidophilus</i> FTCC 0291	0.015 ± 0.001 ^{b,C}	0.044 ± 0.011 ^{a,A}	0.236 ± 0.067 ^{a,A}	0.150 ± 0.026 ^{b,C}
<i>L. bulgaricus</i> FTCC 0411	0.246 ± 0.024 ^{a,A}	0.329 ± 0.088 ^{b,C}	0.230 ± 0.097 ^{b,C}	0.557 ± 0.160 ^{b,B}
<i>L. bulgaricus</i> FTDC 1311	0.069 ± 0.031 ^{ab,B}	0.327 ± 0.160 ^{ab,BC}	0.167 ± 0.040 ^{a,B}	0.174 ± 0.036 ^{b,C}
<i>L. casei</i> ATCC 393	0.043 ± 0.008 ^{c,C}	0.037 ± 0.007 ^{b,BC}	0.067 ± 0.024 ^{a,AB}	0.137 ± 0.034 ^{b,A}

^{a-d}Means in the same row with different superscripts are significantly different ($P < 0.05$).

^{A-D}Means in the same column with different superscripts are significantly different ($P < 0.05$).

¹Results are expressed as means ± SD; value are means of duplicates from 2 separate runs (n = 4).

²*L.* = *Lactobacillus*.

This was most prevalent for *L. acidophilus* ATCC 314 and *L. acidophilus* FTCC 0291.

In general, the FAn of DPH and ANS were greater for most strains grown in the media containing cholesterol compared with the FAn of TMA-DPH. In the presence of oxgall, greater FAn of TMA-DPH was detected for most strains compared with the FAn of DPH and ANS. The FAn of DPH of cells grown in media supplemented with taurocholic acid was the greatest, followed by the FAn of TMA-DPH, and the least in the FAn of ANS.

Greater FAn of DPH and TMA-DPH were detected for most strains grown in the media supplemented with cholic acid compared with the FAn of ANS.

Conversion of Cholesterol to Coprostanol

Fluorometric Assay for Cholesterol Reductase Activity. Both intracellular and extracellular cholesterol reductase activities of probiotics varied among strains (Tables 5 and 6), as illustrated by the varying

Table 7. Conversion of cholesterol to coprostanol by lactobacilli in media containing cholesterol and bile salts¹

Strain ²	MRS broth + cholesterol	MRS broth + cholesterol + 0.3% oxgall	MRS broth + cholesterol + 0.3% taurocholic acid	MRS broth + cholesterol + 0.3% cholic acid
Concentration of cholesterol (μg/mL)				
<i>L. acidophilus</i> ATCC 314	69.79 ± 3.96 ^{b,A}	74.91 ± 8.11 ^{ab,A}	82.18 ± 3.12 ^{a,A}	39.48 ± 0.81 ^{c,C}
<i>L. acidophilus</i> FTCC 0291	49.63 ± 1.30 ^{b,C}	57.45 ± 2.91 ^{ab,B}	61.32 ± 7.45 ^{a,B}	65.16 ± 1.89 ^{a,A}
<i>L. bulgaricus</i> FTCC 0411	60.70 ± 2.68 ^{a,B}	49.92 ± 1.31 ^{c,B}	54.59 ± 1.39 ^{bc,B}	57.98 ± 3.30 ^{b,B}
<i>L. bulgaricus</i> FTDC 1311	40.03 ± 4.20 ^{a,D}	25.03 ± 0.85 ^{c,C}	30.01 ± 0.98 ^{b,C}	35.61 ± 1.14 ^{a,C}
<i>L. casei</i> ATCC 393	45.87 ± 2.72 ^{b,CD}	32.10 ± 0.27 ^{c,C}	36.47 ± 1.98 ^{c,C}	68.43 ± 3.30 ^{a,A}
Concentration of coprostanol (μg/mL)				
<i>L. acidophilus</i> ATCC 314	24.76 ± 1.23 ^{a,A}	21.05 ± 0.57 ^{b,B}	8.00 ± 0.48 ^{d,C}	17.81 ± 0.71 ^{c,A}
<i>L. acidophilus</i> FTCC 0291	22.98 ± 0.71 ^{b,A}	32.49 ± 2.40 ^{a,A}	19.98 ± 0.64 ^{c,A}	7.85 ± 0.60 ^{d,B}
<i>L. bulgaricus</i> FTCC 0411	11.17 ± 0.61 ^{a,C}	8.97 ± 0.68 ^{b,D}	8.19 ± 0.21 ^{b,C}	4.35 ± 0.52 ^{c,D}
<i>L. bulgaricus</i> FTDC 1311	7.11 ± 0.19 ^{b,D}	13.51 ± 0.98 ^{a,C}	8.34 ± 0.88 ^{b,C}	8.19 ± 0.39 ^{b,B}
<i>L. casei</i> ATCC 393	18.27 ± 1.07 ^{a,B}	12.39 ± 0.73 ^{b,C}	16.47 ± 1.10 ^{a,B}	6.26 ± 0.62 ^{c,C}

^{a-d}Means in the same row with different superscripts are significantly different ($P < 0.05$).

^{A-D}Means in the same column with different superscripts are significantly different ($P < 0.05$).

¹Results are expressed as means ± SD; value are means of duplicates from 2 separate runs (n = 4).

²*L.* = *Lactobacillus*.

amounts of reduced NAD oxidized in broth containing oxgall, taurocholic, and cholic acids. Our results showed that all strains studied were able to produce cholesterol reductase intracellularly. Extracellular cholesterol reductase was also detected, indicating that the bioconversion of cholesterol to coprostanol could also occur outside of the cell. This could benefit the host by reducing blood cholesterol levels and might also find potential application in the development of dairy functional foods. It has been found that dairy components such as lactose and incomplete digestion of carbohydrates inhibited the conversion of cholesterol to coprostanol by intestinal microflora (Macdonald et al., 1983). Therefore, the incorporation of probiotics into dairy products such as milk and yogurt might alleviate this setback; probiotics could degrade lactose and other dairy carbohydrates, allowing conversion of cholesterol to coprostanol by gut microorganisms or by cholesterol reductase-producing probiotics. Our results also showed that greater specific intracellular and extracellular cholesterol reductase activity was observed in media containing oxgall and taurocholic acid ($P < 0.05$) compared with the control. This was most prevalent for *L. acidophilus* FTCC 0291. Among all the bile salts, strains grown in media supplemented with cholic acid showed the least specific activity ($P < 0.05$) in both intracellular and extracellular cholesterol reductase activity.

Concentration of Cholesterol and Coprostanol.

The conversion of cholesterol to coprostanol in the presence of different bile salts was measured by determining the concentrations of cholesterol and coprostanol in the media (Table 7). The concentration of cholesterol in different bile media upon fermentation varied and ranged from 25.03 to 82.18 $\mu\text{g/mL}$, whereas the concentration of coprostanol ranged from 4.35 to 32.49 $\mu\text{g/mL}$. In general, the concentration of cholesterol upon fermentation was lesser ($P < 0.05$) in media supplemented with oxgall and taurocholic acid compared with the control. Among all the bile salts studied, the greatest concentration of cholesterol upon fermentation was observed in media supplemented with cholic acid, and this was most prevalent for *L. bulgaricus* FTDC 1311 and *L. casei* ATCC 393 ($P < 0.05$). The concentration of cholesterol detected in media upon fermentation was least in the presence of oxgall ($P < 0.05$) in most strains studied. In the presence of bile salts, the greatest concentration of coprostanol was observed in media containing oxgall, and this was most prevalent for *L. acidophilus* FTCC 0291 and *L. bulgaricus* FTDC 1311 ($P < 0.05$), whereas the least concentration of coprostanol was often observed in media containing cholic acid compared with other bile salts studied ($P < 0.05$).

CONCLUSIONS

All strains studied were able to remove or reduce the concentration of cholesterol in vitro through incorporation of cholesterol into the cellular membrane and conversion of cholesterol to coprostanol. Most cholesterol was incorporated in the regions of the phospholipid tails, upper phospholipids, and polar heads of the membrane bilayer. Our results indicated that the strains studied may be promising candidates for use as adjunct cultures in fermented dairy products with possible hypocholesterolemic potentials upon consumption.

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