

Original article

Evaluation of the *in vitro* cholesterol-lowering activity of the probiotic strain *Bacillus coagulans* MTCC 5856Muhammed Majeed,^{1,2,3} Shaheen Majeed,^{1,2,3} Kalyanam Nagabhushanam,²  Sivakumar Arumugam,¹
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Summary Emerging scientific evidence suggests that the supplementation of probiotics may help to reduce/manage blood cholesterol levels in humans. We evaluated the *in vitro* cholesterol-lowering activity of the probiotic strain *Bacillus coagulans* MTCC 5856. This probiotic de-conjugated bile salts and liberated deoxycholic acid, confirming its bile salt hydrolase activity. Furthermore, *B. coagulans* MTCC 5856 also significantly ($P < 0.05$) reduced cholesterol levels in culture media under growing (48.42%), resting (live but suspended in buffer, 36.47%) and even heat-killed (dead, 8.5%) conditions. *Bacillus coagulans* MTCC 5856 significantly reduced the cholesterol levels in cholesterol-rich foods, such as egg yolk (39.79%), chicken liver (45.44%) and butter (49.51%), when incubated for 24 h in conditions mimicking the *in vivo* environment. *Bacillus coagulans* MTCC 5856 also produced significant ($P < 0.05$) amounts of propionic acid and butyric acid while fermenting cholesterol-rich foods. The multitudinous ways by which *B. coagulans* MTCC 5856 reduces cholesterol levels endorses its application in functional food formulations and as a dietary ingredient for the management of hypercholesterolemia, potentially reducing the incidence of coronary heart disease and other related disabilities.

Keywords *Bacillus coagulans* MTCC 5856, bile salt hydrolase, hypercholesterolemia, LactoSpore[®], probiotics.

Introduction

Cardiovascular disease (CVD) is one of the foremost causes of death and other disabilities in developing countries and in the western world (Nascimento *et al.*, 2002). The World Health Organization (WHO) estimates that nearly 23 million people will be affected by CVD and that it will become the major cause of death by 2030 (Mendis *et al.*, 2011). Several studies have reported that higher than normal serum cholesterol levels is the leading cause of CVD and other disabilities (Cheung *et al.*, 2000). Furthermore, cholesterol undergoes oxidation, resulting in the formation of arterial plaques, the principal cause of coronary heart disease (Lusis, 2000). In the human body, cholesterol is biosynthesised in the liver or directly absorbed from the diet, mainly from foods containing animal fats and dairy products (Lecerf & De Lorgeril, 2011). Pharmacological agents, such as statins, which inhibit the *de*

novo synthesis of cholesterol, are reported to be effective at reducing blood cholesterol levels. However, such agents are expensive, and adverse side effects have been reported, namely muscle pain/damage, liver damage, liver inflammation, type 2 diabetes and neurological side effects. This has naturally led patients to seek alternative safe options, such as weight reduction, use of dietary supplements, fat-free diets and exercise (Michael *et al.*, 2016). Furthermore, dietary guidelines for Americans in 2010 recommended the consumption of less than 300 mg day⁻¹ of dietary cholesterol (DGA, 2010). However, the Dietary Guidelines Advisory Committee (DGAC) discontinued this recommendation in 2015 due to the lack of scientific evidence on the substantial relationship between the consumption of dietary cholesterol and serum cholesterol, indicating that the change in diet and lifestyle alone cannot reduce the risk of coronary heart disease (DGA, 2015). Regardless of the recommendation on the cholesterol consumption by the DGAC, the benefits of low cholesterol intake cannot be ignored in the management of hypercholesterolemia. Nevertheless, the

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need to manage hypercholesterolemia has provided an opportunity to explore new alternative approaches, including the use of plant sterols, soluble fibres and, most importantly, probiotics, which can reduce blood cholesterol levels and serve as a useful dietary approach. Probiotics are defined as 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). Ample scientific evidence suggests the positive impact of some probiotic organisms on cholesterol metabolism, leading to lower blood cholesterol levels and positive coronary heart disease-related outcomes (Kumar *et al.*, 2012; Kechagia *et al.*, 2013). While the exact mechanisms by which probiotics lower cholesterol are unclear, it is speculated that bile salt hydrolase (BSHs) in the probiotics and/or their inherent ability to scavenge cholesterol could underlie this desired activity. Probiotic organisms, such as the *Lactobacillus*, *Bifidobacterium* and *Bacillus* species, are used for human consumption in various products, such as infant foods, cultured milks and pharmaceutical preparations (tablet, capsules, sachets, etc.). However, a lack of shelf stability of *Lactobacillus* and *Bifidobacterium*, with the consequential loss of viability/activity during storage and transportation/distribution, requires microencapsulation and possibly refrigeration during transportation/storage (Corona-Hernandez *et al.*, 2013). In contrast, due to their spore forming nature, the probiotic strains of *Bacillus* species are shelf-stable and do not require microencapsulation or refrigeration during transportation, storage and market distribution. The nonpathogenic strains of *Bacillus* species are reported to show probiotic potential in humans and animals. However, *Bacillus coagulans* is the most clinically studied organism as a probiotic for human consumption among all *Bacillus* species (Elshahabee *et al.*, 2017). *Bacillus coagulans* is a Gram-positive, rod-shaped, spore-forming bacteria reportedly used for the treatment of gastrointestinal disorders, vaginal infections, lactose intolerance and hepatic comas and as an adjuvant to antibiotic therapy (Majeed & Prakash, 1998; Jurenka, 2012; Majeed *et al.*, 2016a). *Bacillus coagulans* MTCC 5856 is a probiotic strain that has been sold as a commercial probiotic preparation for human and animal consumption under the trade name LactoSpore® by the Sabinsa Corporation globally for over two decades (Majeed & Prakash, 1998). *Bacillus coagulans* MTCC 5856 was observed to inhibit the gastrointestinal (GI) motility of rats in a castor oil-induced diarrhoea model (Majeed *et al.*, 2016b) and found to be effective in improving the clinical symptoms of diarrhoea predominant in irritable bowel syndrome (IBS) patients in a controlled human trial (Majeed *et al.*, 2016c). *Bacillus coagulans* MTCC 5856 at a dose of 2×10^9 spores day⁻¹ was safe and tolerable when taken as a supplement

by healthy participants for 30 days (Majeed *et al.*, 2016d), as demonstrated in a double-blind, placebo-controlled clinical trial. A recent randomised, double-blind, placebo-controlled, multicentre trial showed improvements in the clinical symptoms of patients with both major depression and IBS after *B. coagulans* MTCC 5856 supplementation at a dose of 2×10^9 spores day⁻¹ for 90 days (Majeed *et al.*, 2018a). Further, *B. coagulans* MTCC 5856 exhibited high resistance to the processing temperatures of various functional food (baked food) preparations and was stable for up to 36 months of storage at the ambient temperature (Majeed *et al.*, 2016a,e).

Although various probiotic strains have been reported for their cholesterol-lowering potential via bile salt hydrolysis and cholesterol assimilation (Pereira *et al.*, 2003; Guijie, 2012), the probiotic strain *B. coagulans* MTCC 5856 has not yet been explored for its cholesterol-lowering activity. Furthermore, it is well-known that the biological effects of probiotics are strain specific and cannot be generalised among genera, species and strains (Papadimitriou *et al.*, 2015), prompting the investigation of the *in vitro* cholesterol-lowering potential of the probiotic strain *B. coagulans* MTCC 5856 in this study.

Material

Oxyrase was procured from Oxyrase, Inc. (Mansfield, OH, USA). Glucose yeast extract agar (GYEA), De Man, Rogosa, and Sharpe (MRS) broth and trypticase soy agar were purchased from HiMedia (Mumbai, India). Lysozyme, pepsin, pancreatin from porcine, bile salt, taurodeoxycholate sodium salt, deoxycholic acid, cholesterol and SCFA standards (propionic and butyric acids) were procured from Sigma-Aldrich (St. Louis, MO, USA). TLC Silica Gel 60 F254 was procured from Merck KGaA (Darmstadt, Germany). Probiotic bacteria *B. coagulans* MTCC 5856 is a proprietary strain of Sabinsa Corporation/Sami Labs Limited deposited into the Microbial Type Culture Collection and Gene Bank (MTCC) (Chandigarh, India).

Methods

Bile salt hydrolase activity by the plate method

A plate assay method for BSH activity was performed according to the method described by Dashkevich & Feighner (1989) and Ahn *et al.* (2003) with minor modifications. Soft MRS agar was prepared by adding agar (1%, w/v) to MRS broth and then supplementing with bile salts (0.3% w/v; ox bile) and CaCO₃ (0.3%, w/v). This mixture was autoclaved at 121 °C for 15 min, and plates were prepared and dried at room

temperature. *Bacillus coagulans* MTCC 5856 ($10 \mu\text{L}$, corresponding to $2 \times 10^8 \text{ cfu mL}^{-1}$) that had been cultivated for 18 h was inoculated on MRS agar by puncturing the soft agar. Subsequently, plates were incubated at 37°C for 72 h. Clearly, visible halos around the punctures indicated the positive BSH activity of the *B. coagulans* MTCC 5856. *Bacillus coagulans* MTCC 5856 grown on MRS agar without bile salts was used as the negative control, and measurements were repeated three times.

BSH activity by the TLC method

The BSH activity of *B. coagulans* MTCC 5856 was confirmed using the TLC (thin layer chromatography) plate method as described previously by Guo *et al.* (2011). *Bacillus coagulans* MTCC 5856 was grown in MRS broth at 37°C for 18 h and then centrifuged at $8000 \times g$ for 10 min at 4°C . The cell pellets were then washed with 2 mL of sterile buffer (0.1 M PBS, pH 6.5). After washing, 5 mL of buffer solution was added to the cell pellets of *B. coagulans* MTCC 5856. A bacterial suspension (1 mL) was mixed with the reaction mixture (1 mL). The reaction mixture comprised MRS broth and taurodeoxycholate sodium salt (TDC, 0.3% w/v) in phosphate buffer (0.1 mol L^{-1}) at a final pH of 6.5. The strain was then cultivated at 37°C for 8 h. After cultivation, the samples were evaporated under vacuum, and the residue was subsequently dissolved in methanol (1 mL) and centrifuged at $8000 \times g$ for 5 min. The supernatants as well as standard solutions, namely taurodeoxycholate and deoxycholic acid (each of them 5 mM in methanol), were spotted onto the baselines of silica TLC plates ($10 \times 10 \text{ cm}$, TLC Silica Gel 60 F254; Merck). The mobile phase contained isoamyl acetate, propionic acid, n-propanol and water at a ratio of 40: 30: 20: 10. The TLC plates were developed for 30 min. The plates were then dried and sprayed with a 10% w/v solution of phosphomolybdic acid in ethanol, followed by drying at 80°C for 5 min using a hot air oven. Deoxycholic acid was shown to be liberated from bile salts by BSH-positive *B. coagulans* MTCC 5856. The results of TLC were evaluated by a comparison with standards.

Bacterial growth and cholesterol removal

The growth of *B. coagulans* MTCC 5856 in the presence of cholesterol was assessed at different time intervals for up to 24 h. *Bacillus coagulans* MTCC 5856 was inoculated in sterilised MRS broth media supplemented with 0.30% ox bile and $100 \mu\text{g mL}^{-1}$ water-soluble cholesterol (Polyoxethylene cholesterol sebacate). This mixture was then incubated at 37°C with gentle shaking (100 r.p.m.) for 24 h. Samples

were taken at different time intervals (0, 6, 12, 18 and 24 h), and the bacterial growth was measured at 600 nm using a UV-VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Cholesterol removal was determined according to the method described by Rudel & Morris (1973) with some modifications proposed by Liong & Shah (2005). After incubation, the cells were centrifuged ($8000 \times g$ at 4°C for 10 min), and the remaining cholesterol concentration in the supernatant was determined using a spectrophotometer (Shimadzu). One millilitre of the aliquot was added to 1 mL of KOH (33% w/v) and 2 mL of absolute ethanol; the mixture was vortexed for 1 min and heated at 37°C for 15 min. After cooling, 2 mL of distilled water and 3 mL of hexane were added, and the mixture was vortexed for 1 min. One millilitre of the hexane layer was transferred into a glass tube and evaporated. The residue was immediately dissolved in 2 mL of o-phthalaldehyde reagent. After complete mixing, 0.5 mL of concentrated sulphuric acid was added, and the mixture was vortexed for 1 min. The absorbance was read at 550 nm after 10 min as described by Rudel & Morris (1973). All experiments were performed in triplicate and repeated twice. Similarly, *Lactobacillus casei* ATCC 393 served as a positive control in this study. The cholesterol concentration was determined from a standard curve prepared using a cholesterol stock solution. The ability of *B. coagulans* MTCC 5856 to remove cholesterol was expressed as the percentage of cholesterol removed at each incubation interval as follows:

$$\text{Cholesterol removal (\%)} = 100 - \left[\frac{\text{residual cholesterol after fermentation}}{\text{initial cholesterol added}} \times 100 \right]$$

Cholesterol removal by growing, resting and heat-killed (dead) *B. coagulans* MTCC 5856 cells

The ability of nongrowing *B. coagulans* MTCC 5856 cells, such as resting cells and heat-killed (dead) cells, to remove cholesterol was determined and compared with the ability of growing cells to remove cholesterol. Resting cells were prepared by growing *B. coagulans* MTCC 5856 in MRS media overnight followed by centrifugation ($8000 \times g$ at 4°C for 10 min) to obtain a cell mass. This cell mass was further resuspended in sterile phosphate buffer (0.05 M, pH 6.5) containing 0.3% (w/v) ox bile and $100 \mu\text{g mL}^{-1}$ water-soluble cholesterol and then incubated for 24 h at 37°C . Heat-killed (dead) cells were prepared by growing *B. coagulans* MTCC 5856 in MRS media overnight followed by autoclaving at 121°C for 15 min. After autoclaving, the cells were collected by centrifugation ($8000 \times g$ at 4°C for 10 min), resuspended aseptically in MRS broth supplemented with 0.3% (w/v) ox bile

acid and $100 \mu\text{g mL}^{-1}$ water-soluble cholesterol and then incubated for 24 h at 37°C . Growing cells were prepared by growing *B. coagulans* MTCC 5856 in MRS media overnight and then adding 1 mL of this mixture to MRS broth supplemented with 0.3% (w/v) ox bile acid and $100 \mu\text{g mL}^{-1}$ water-soluble cholesterol and incubating for 24 h at 37°C . After incubation, all the samples were centrifuged ($8000 \times g$ at 4°C for 20 min), and the cholesterol concentrations of the supernatants were measured according to the colorimetric method using a spectrophotometer as described previously (Rudel & Morris, 1973; Liong & Shah, 2005). All experiments were performed in triplicate and assayed twice.

Removal of cholesterol from different food sources by *B. coagulans* MTCC 5856 under gastric stress

The effect of cholesterol removal by *B. coagulans* MTCC 5856 from cholesterol-rich food sources was investigated by a method described previously (de Palencia *et al.*, 2008; Ritter *et al.*, 2009) with some modifications. Chicken liver was washed with water and soaked in 70% ethanol for 30 min, rinsed with sterile distilled water and then homogenised in phosphate buffer (0.2 M, pH 7.0). Similarly, egg yolk was isolated from egg, added to phosphate buffer (0.2 M, pH 7.0) and then homogenised. Butter was procured from a local market and homogenised in phosphate buffer (0.2 M, pH 7.0). To mimic the *in vivo* conditions, all samples were added to 10 mL of a sterile electrolyte solution (6.2 g L^{-1} NaCl, 2.2 g L^{-1} KCl, 0.22 g L^{-1} CaCl_2 and 1.2 g L^{-1} NaHCO_3), lysozyme (0.01%, Sigma-Aldrich) and pepsin (0.3%, Sigma-Aldrich). The pH of the samples was decreased to 1.5 by adding 1 M HCl. *Bacillus coagulans* MTCC 5856 was added to each group and incubated at 37°C with gentle shaking (100 r.p.m.) for 3 h. After the incubation, the pH of each sample was adjusted aseptically to 7.0 using a sterile sodium bicarbonate saturated solution (8%, w/v). The oxygen-reducing enzyme oxyrase (Oxyrase Inc.) was also added to each flask to induce an anaerobic environment. Sterile bile salt (0.3%, w/v; Sigma-Aldrich) and pancreatin from porcine (0.1%, w/v; Sigma-Aldrich) were also added to samples from each group. All the samples were incubated at 37°C with gentle shaking (100 r.p.m.) for 24 h. After incubation, the samples were centrifuged ($8000 \times g$ at 4°C for 10 min), and the cholesterol content was estimated in the supernatant by a method described previously (Rudel & Morris, 1973; Liong & Shah, 2005). The production of short-chain fatty acids (propionic and butyric acids) by *B. coagulans* MTCC 5856 while fermenting egg yolk, butter and chicken liver was carried out using the method described in our previous study (Majeed *et al.*, 2018b). After 24 h

of incubation, 5 mL of each sample was collected and diluted with 5 mL of distilled water, and the pH was then adjusted to 1.5 using 3 M H_2SO_4 . Chilled (-20°C) diethyl ether (10 mL) was added to each sample, and the samples were mixed in a vortex mixer for 1 min. Sodium chloride was added, and the mixture was then centrifuged at $3000 \times g$ for 10 min. After centrifugation, the organic layer was separated and transferred to a fresh vial. SCFA was quantified using gas chromatography (GC) (Agilent Technologies 6890N gas chromatograph) by comparisons with SCFA standards as described previously (Majeed *et al.*, 2018b).

Statistical analysis

All data are presented as the mean \pm standard deviation (SD) of the assigned number of independent experiments. For single comparisons, values of *P* were determined using Student's *t* test. For multiple comparisons, *P* values were determined by one-way analysis of variance (ANOVA) using GraphPad prism software version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). Significance was defined at 5% level.

Results

Bile salt hydrolase activity of *B. coagulans* MTCC 5856

Bacillus coagulans MTCC 5856 growth was observed on an agar plate containing ox bile and calcium carbonate, which indicates its tolerance against ox bile and the presence of BSH activity. There was an 18 ± 1.8 mm clear zone in the soft agar plate as shown in Fig. 1, which indicated the presence of BSH activity. Furthermore, BSH activity was confirmed by the TLC method. The hydrolysis of sodium taurodeoxycholate and the formation of deoxycholic acid by *B. coagulans* MTCC 5856 clearly suggested the production of BSH enzyme. The R_f value for standard deoxycholate was 0.89, matching the R_f values of the deoxycholate liberated from the sodium taurodeoxycholate when incubated with *B. coagulans* MTCC 5856.

Removal of cholesterol by growing *B. coagulans* MTCC 5856 cells

As shown in Fig. 2, growing *B. coagulans* MTCC 5856 cells removed cholesterol in a time-dependent manner. At 24 h of incubation, the highest removal of cholesterol and the maximum growth was observed, indicating that the growth/colonisation of *B. coagulans* MTCC 5856 was responsible for the removal of cholesterol. *Lactobacillus casei* ATCC 393 was used as a control to compare the cholesterol removal activity of the *B. coagulans* MTCC 5856 strain. No significant

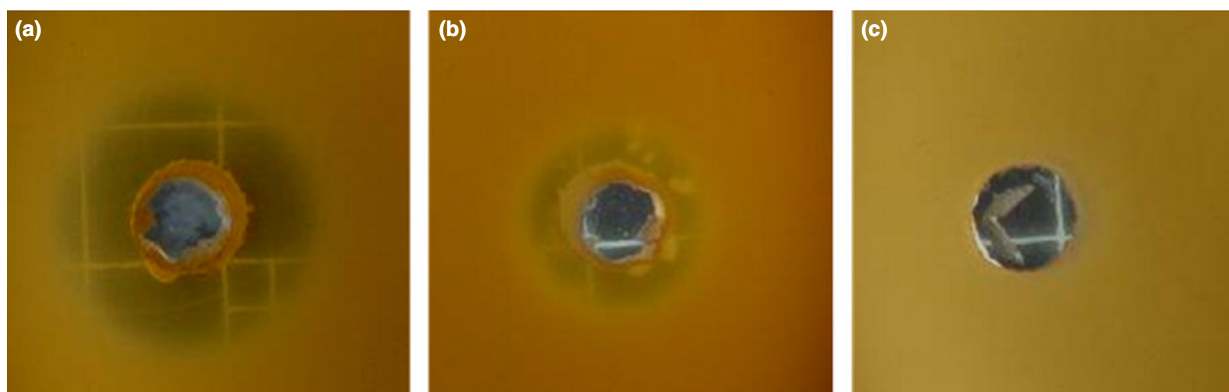


Figure 1 BSH activity of *Bacillus coagulans* MTCC 5856. (a) Determination using soft MRS agar supplemented with ox bile (0.3%, w/v) and CaCO_3 (0.3%, w/v). (b) *Lactobacillus casei* ATCC 393 was used as a positive control, and MRS agar without bile salts served as the negative control (c). Hollow zones suggest the BSH activity of *B. coagulans* MTCC 5856. Measurements were repeated three times ($n = 3$).

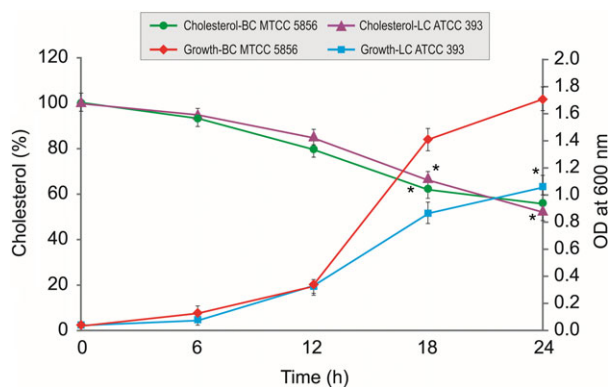


Figure 2 Cholesterol removal (%) by *Bacillus coagulans* MTCC 5856 or *Lactobacillus casei* ATCC 393 incubated in MRS supplemented with $100 \mu\text{g mL}^{-1}$ water-soluble cholesterol and 0.3% ox bile for 24 h at 37°C . There was a significant cholesterol reduction after 18 h of growth, suggesting that *B. coagulans* MTCC 5856 was more effective in the growth phase ($P < 0.05$). *Lactobacillus casei* ATCC 393 was used as a control in this study to compare with *B. coagulans* MTCC 5856. No significant difference in the abilities of the *B. coagulans* MTCC 5856 and *L. casei* ATCC 393 groups to remove cholesterol from media was observed. Each value represents the mean \pm SD ($n = 3$). * $P < 0.05$ (Student's *t* test). LC ATCC 393, *L. casei* ATCC 393; BC MTCC 5856, *B. coagulans* MTCC 5856.

($P > 0.05$) difference was observed between *L. casei* ATCC 393 and the *B. coagulans* MTCC 5856 group after 24 h of incubation at 37°C (Fig. 2). Furthermore, different concentrations of cholesterol were added to the media and incubated with *B. coagulans* MTCC 5856 for 24 h in the presence of ox bile (bile salt). A time-dependent removal of cholesterol was observed (Table 1). However, regardless of the minimum concentration ($25 \mu\text{g mL}^{-1}$) or maximum

concentration ($200 \mu\text{g mL}^{-1}$) of cholesterol, the level of removal was nearly similar to the maximum level of cholesterol removal observed at 24 h of incubation (Table 1).

Removal of cholesterol by growing, resting and heat-killed *B. coagulans* MTCC 5856 cells

The ability of *B. coagulans* MTCC 5856, either growing or nongrowing (resting or heat-killed dead cells), to remove cholesterol was investigated. The removal of cholesterol varied remarkably between growing cells (48.42%), resting cells (36.47%) and dead cells (8.5%) at 24 h (Fig. 3). However, resting and heat-killed dead cells were also able to remove cholesterol, demonstrating the ability of *B. coagulans* MTCC 5856 to remove cholesterol in both its growing and nongrowing forms.

Removal of cholesterol and production of SCFAs by *B. coagulans* MTCC 5856 in cholesterol-rich foods

The ability of *B. coagulans* MTCC 5856 to remove cholesterol from cholesterol-rich food sources, such as egg yolk, chicken liver and butter, under gastric stress (acid and bile acids) was investigated, with an aim to understand the *in vivo* efficacy of *B. coagulans* MTCC 5856. A significant ($P < 0.05$) reduction in cholesterol levels was observed in all cholesterol-rich food sources after 24 h of *B. coagulans* MTCC 5856 fermentation (Fig. 4). The reductions of cholesterol levels in egg yolk, chicken liver and butter were 39.79%, 45.14% and 49.51%, respectively, after 24 h of *B. coagulans* MTCC 5856 fermentation. The study results clearly demonstrate that *B. coagulans* MTCC 5856 has the ability to remove cholesterol from various cholesterol-rich foods under gastric stress mimicking *in vivo* conditions. The production of

Table 1 Effect of *Bacillus coagulans* MTCC 5856 on the reduction of cholesterol in MRS media supplemented with water soluble cholesterol and 0.3% ox bile

Time (h)	Cholesterol $\mu\text{g mL}^{-1}$ (%)					
0	25 \pm 1.2 (100) ^a	50 \pm 1.4 (100) ^a	75 \pm 1.5 (100) ^a	100 \pm 1.8 (100) ^a	150 \pm 2.1 (100) ^a	200 \pm 2.5 (100) ^a
6	21.25 \pm 1.1 (85.0) ^b	45.21 \pm 1.2 (90.4) ^b	67.21 \pm 1.6 (89.6) ^b	93.91 \pm 1.9 (93.9) ^b	144.92 \pm 1.9 (96.6) ^b	187.05 \pm 1.9 (93.5) ^b
12	16.4 \pm 0.8 (53.6) ^c	37.12 \pm 1.3 (74.2) ^c	48.15 \pm 1.4 (64.2) ^c	79.92 \pm 1.2 (79.9) ^c	119.15 \pm 1.8 (79.43) ^c	156.11 \pm 2.5 (78.0) ^c
18	14.14 \pm 0.7 (48.5) ^d	30.25 \pm 1.1 (60.5) ^d	36.15 \pm 1.1 (48.2) ^d	62.1 \pm 1.4 (62.1) ^d	93.1 \pm 1.4 (62.0) ^d	95.1 \pm 2.6 (47.5) ^d
24	11.42 \pm 0.8 (45.6) ^e	24.16 \pm 0.7 (48.32) ^e	30.92 \pm 1.3 (41.2) ^e	55.58 \pm 1.1 (55.5) ^e	81.64 \pm 1.8 (54.4) ^e	91.04 \pm 1.8 (45.5) ^e

Values are average mean of triplicate performed at two different occasions. Different letters in the same column indicate significant differences ($P < 0.05$). Percentage values of cholesterol in a given row which are followed by the same letter are not statistically different ($P > 0.05$).

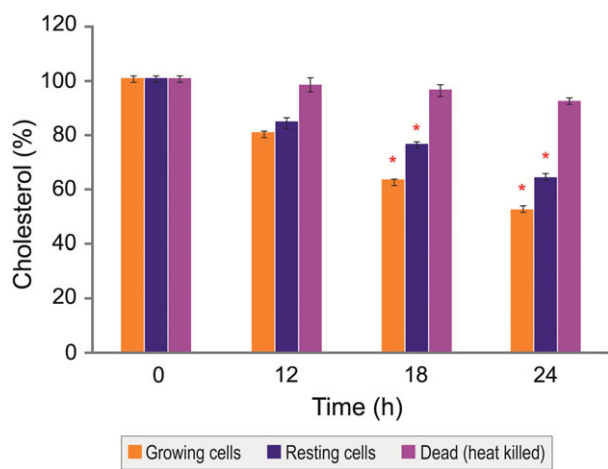


Figure 3 Effects of growing, resting and heat-killed (dead) *Bacillus coagulans* MTCC 5856 cells on the removal of cholesterol (%) in the presence of ox bile (0.3%, w/v). Resting and dead cells were also effective in removing cholesterol, but growing cells significantly reduced the cholesterol content after 24 h of incubation at 37 °C ($P < 0.5$). Each value represents the mean \pm SD ($n = 3$). * $P < 0.05$ (Student's t test).

SCFAs (propionic and butyric acids) by *B. coagulans* MTCC 5856 while fermenting egg yolk, chicken liver and butter in a GIT hostile environment was also evaluated. At 24 h, the maximum production of butyric acid was observed in egg yolk ($4.16 \pm 0.05 \text{ mg g}^{-1}$), followed by butter ($3.46 \pm 0.06 \text{ mg g}^{-1}$) and chicken liver ($3.16 \pm 0.05 \text{ mg g}^{-1}$) (Fig. 5). However, the production of propionic acid was highest in the chicken liver group ($1.68 \pm 0.01 \text{ mg g}^{-1}$), followed by butter ($1.36 \pm 0.01 \text{ mg g}^{-1}$) and egg yolk ($2.14 \pm 0.01 \text{ mg g}^{-1}$) (Fig. 5). Furthermore, the production of butyric acid by *B. coagulans* MTCC 5856 was significantly ($P < 0.05$) higher than that of propionic acid while fermenting egg yolk, chicken liver and butter.

Discussion

It is now evident that probiotic organisms exert various health benefits, one of them being the

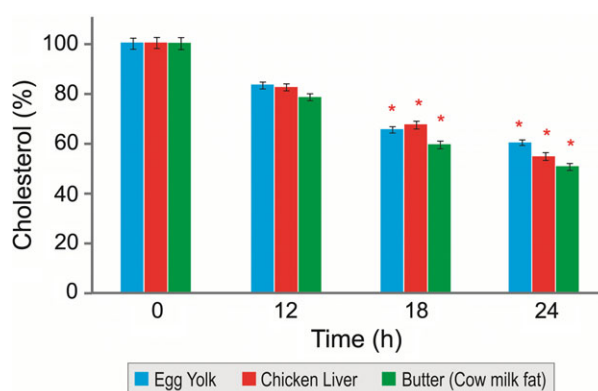


Figure 4 Effects of *Bacillus coagulans* MTCC 5856 on the removal of cholesterol from different food sources (egg yolk, chicken liver and butter). A time-dependent reduction in cholesterol from egg yolk, chicken liver and butter was observed, and the highest reduction was observed at 24 h. Each value represents the mean \pm SD ($n = 3$). * $P < 0.05$ (Student's t test).

cholesterol-lowering potential in humans via bile salt hydrolysis and cholesterol assimilation (Pereira *et al.*, 2003; Guijie, 2012). The presence of BSH activity in a probiotic strain has always been linked to cholesterol-lowering potential and becomes the main criteria and biomarker for the selection of probiotic strain adjuncts to manage hypercholesterolemia. Nevertheless, FAO/WHO guidelines also suggest that the BSH activity of a probiotic strain is one of the main activities required for characterisation as a probiotic strain along with other properties (FAO/WHO, 2002). We unequivocally demonstrated the BSH activity of *B. coagulans* MTCC 5856 by the plate method, which was further proven by the TLC method of sodium taurodeoxycholate deconjugation upon incubation with this strain. The probiotic strains of *Lactobacillus* and *Bifidobacterium* have been exclusively studied and reported to have BSH activity that interferes with cholesterol metabolism; these strains thereby have cholesterol-lowering potential in humans and animal models. Furthermore,

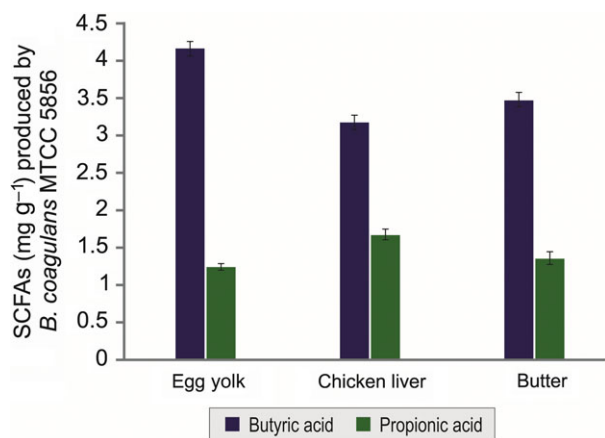


Figure 5 Production of short-chain fatty acids (propionic and butyric acid) by *Bacillus coagulans* MTCC 5856 while grown using egg yolk, butter and chicken liver as nutritional sources. Short-chain fatty acids were quantified after 24 h of incubation. The production of butyric acid was better than that of propionic acid after 24 h of incubation. Each value represents the mean \pm SD ($n = 3$).

BSH activity has been reported for the probiotic strains of *Lactobacillus* and *Bifidobacterium*, but the probiotic strains of *B. coagulans* have not been investigated more exhaustively. To the best of our knowledge, this is the first study to report the BSH activity of the probiotic strain *B. coagulans* MTCC 5856. Conjugated and un-conjugated bile acids are reportedly absorbed by the entire gut, but de-conjugated bile salts are less efficiently reabsorbed than conjugated bile acids, leading to the excretion of significant amounts of free bile acids in human faeces (Carey & Duane, 1994; Begley *et al.*, 2006). It was also reported that probiotic BSH activity plays a pivotal role in increasing the *de novo* synthesis of bile salts in the liver to replace the excreted free bile salts that help to reduce the serum cholesterol. It was further suggested that the BSH activity may also indirectly prevent cholesterol absorption by reducing its solubility, as cholesterol is less soluble in bile acids than bile salts. Thus, the probiotic strain *B. coagulans* MTCC 5856 could be quite useful in managing the cholesterol level in humans via bile salt hydrolysis due to its BSH activity.

Furthermore, another important property of a probiotic is its ability to metabolise cholesterol by assimilating and removing the cholesterol in the gut, thus preventing the excess absorption of cholesterol. We herein report cholesterol removal during the growth of *B. coagulans* MTCC 5856 in the presence of 0.3% ox bile, as the cholesterol level was reduced by 54.4% in 24 h. Furthermore, a time-dependent cholesterol reduction was observed, suggesting that the removal of cholesterol is correlated with the growth of *B. coagulans*

MTCC 5856. The results were in agreement with other studies reporting the cholesterol removal properties of *Lactobacillus* and *Bifidobacteria* species (Pereira & Gibson, 2002; Wang *et al.*, 2012). Previous studies by other researchers revealed that the probiotic strains *Lactobacillus acidophilus* NCDC195, *L. johnsonii* NCDO1693, *L. casei* (N19 and E5), *L. acidophilus* L1 and ATCC 43121 assimilate 50 to 60 $\mu\text{g mL}^{-1}$ of cholesterol in MRS media. Similarly, the probiotic strains *L. plantarum* Lp91 and Lp21 were also reported to assimilate 50.44–69.30 $\mu\text{g mL}^{-1}$ of cholesterol in MRS media (Brashears *et al.*, 1998; Rajesh *et al.*, 2012). Likewise, we observed in this study that the probiotic strain *B. coagulans* MTCC 5856 was able to assimilate 44.5 $\mu\text{g mL}^{-1}$ of cholesterol (44.5%) in MRS media after 24 h of fermentation at 37 °C, comparable with the positive control *L. casei* ATCC 393 (Fig. 2). Moreover, some previous studies also showed that cholesterol removal is highly dependent on the strain growth conditions (Pereira & Gibson, 2002). The enhanced growth of *B. coagulans* 5856 in the presence of cholesterol and ox bile indicated the tolerance of the strain during the growth phase. The levels of cholesterol removal by resting (live but suspended in phosphate buffer) and dead (heat-killed) *B. coagulans* MTCC 5856 cells were herein reported to be 36.46% and 8.5%, respectively, leading us to speculate that the cell membrane has the ability to bind and scavenge cholesterol (Tok & Aslim, 2010). Liong & Shah (2006) reported a similar observation for the resting and dead cells of *Lactobacillus* species. This result indicated that *B. coagulans* MTCC 5856 has the ability to not only assimilate cholesterol during the growth phase but also to remove it via direct binding.

The properties of the probiotic strain *B. coagulans* MTCC 5856 to assimilate and remove cholesterol could be useful in reducing cholesterol and exploited to formulate novel probiotic foods or supplements that can play a role in CVD prevention. Furthermore, being a spore, *B. coagulans* MTCC 5856 is highly resistant to gastric stress and shows resistance to the temperatures and processing conditions of functional foods (Majeed *et al.*, 2016e), unlike other probiotic strains (*Lactobacillus* and *Bifidobacterium*) (Corona-Hernandez *et al.*, 2013). Thus, *B. coagulans* MTCC 5856 has a practical advantage for incorporation into functional foods targeting the management of cholesterol levels.

The major sources of cholesterol in the human body are internal biosynthesis and the dietary amounts consumed mainly from dairy, animal sources and animal fats (Lecerf & De Lorgeril, 2011). Therefore, it is important for a probiotic strain to possess the ability to assimilate and remove the cholesterol from a cholesterol-rich food source. In the present study, we investigated the ability of *B. coagulans* MTCC 5856 to remove the cholesterol from cholesterol-rich food sources, that is, egg yolk, chicken liver and butter

(from cow milk), under gastric stress (acid and bile acids), mimicking the *in vivo* conditions. The cholesterol levels in butter, chicken liver and egg yolk were reduced by *B. coagulans* MTCC 5856 by 49.51%, 45.14% and 39.79%, respectively, after 24 h (Fig. 4). This is the first report to demonstrate that the probiotic *B. coagulans* MTCC 5856 has the ability to reduce cholesterol in food stuffs, mimicking *in vivo* conditions. This study provides the necessary insight that the probiotic *B. coagulans* MTCC 5856 can be incorporated into various foods, especially when cholesterol is present.

Probiotic strains have been reported to produce short-chain fatty acids, namely propionic and butyric acids, which are reported to inhibit the synthesis of liver cholesterol (He & Shi, 2017). The butyrate produced by probiotics inhibits the biosynthesis of cholesterol in the liver, and propionate inhibits the synthesis of fatty acids, which ultimately reduces the rate of cholesterol biosynthesis, thereby lowering the cholesterol levels in the blood (Den Besten *et al.*, 2013). In our study, we observed the production of butyric acid and propionic acid by the probiotic *B. coagulans* MTCC 5856 while grown in cholesterol-rich foods (egg yolk, liver and butter), mimicking the *in vivo* conditions (Fig. 5). Furthermore, *B. coagulans* MTCC 5856 has been reported to produce short-chain fatty acids (acetic, propionic and butyric acids) while fermenting prebiotic fibres, such as cranberry seed fibre and galactomannan from fenugreek (Majeed *et al.*, 2018b,c), thus further demonstrating a role for *B. coagulans* 5856 in the inhibition of liver cholesterol synthesis.

Conclusion

The present study suggests that the probiotic bacteria *B. coagulans* MTCC 5856 exhibits *in vitro* cholesterol-lowering activity via different mechanisms, including bile salt de-conjugation, cholesterol removal, cholesterol binding to resting and dead cells and the production of short-chain fatty acids. This study also reported that the *B. coagulans* MTCC 5856 helped remove cholesterol from cholesterol-rich foods (egg yolk, liver and butter) under a hostile GI environment, thereby suggesting its ability to function *in vivo*. Although further *in vivo* studies may be needed, the findings reported in this study provide clear evidence of the effectiveness of the probiotic strain *B. coagulans* MTCC 5856 in the nutritional treatment of diet-induced hypercholesterolemia.

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Competing interests

All authors approved the final version of the manuscript. Authors are employees of Sabinsa Corporation/Sami labs Limited, manufacturer and marketer of LactoSpore®.

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