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ORIGINAL ARTICLE

In vitro evaluation of the cholesterol-reducing ability of a potential probiotic *Bacillus* spp

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Abstract The cholesterol removal ability of three potential probiotic *Bacillus* spp., namely, *B. flexus* MCC 2458, *B. flexus* MCC 2427 and *B. licheniformis* MCC 2514, has been studied. All the three isolates were deprived of bile salt hydrolase enzyme, but they were still able to remove cholesterol by other mechanisms which were strain-dependent. *B. flexus* MCC 2427 used cholesterol oxidase (2.5 U/mL) for cholesterol removal, whereas *B. flexus* MCC 2458 underwent co-precipitation in the presence of bile. *B. licheniformis* MCC 2514 was involved in cholesterol assimilation, which was also confirmed through an increase in saturated and unsaturated fatty acid contents in the cellular lipid profile. An interesting observation was that the cell-free supernatants of all three isolates exhibited cholesterol-reducing ability and that all were stable at both acidic and alkaline pH. Although the cultures were observed to differ in behavior, their hypocholesterolemic effect satisfies a criterion for their potential application in the food and pharmaceutical industries.

Keywords *Bacillus* spp. · Co-precipitation · Probiotic · Cholesterol oxidase · Fatty acid profile

Introduction

Cholesterol is a basic building block for body tissues mainly involved in the formation of membranes as well as the synthesis of steroid hormones and vitamin D. In addition, one of the major uses of cholesterol in the human body is the synthesis of bile acids that are involved in the emulsification of fats, their ingestion and absorption (Kumar et al. 2012). Elevated blood cholesterol is a well-known major risk factor for coronary heart diseases (Aloglu and Oner 2006) and is estimated to be threefold higher in hypercholesterolemic people than in those with a normal lipid profile. This has led researchers to focus on finding novel approaches to reduce cholesterol levels. It is estimated that a reduction of even 1 % serum cholesterol can reduce the risk of coronary artery disease by 2–3 % (Manson et al. 1992).

Recent modalities for lowering blood cholesterol levels involve dietary management, regular exercise and drug therapy (Dunn-Emke et al. 2001). Several drugs have been introduced onto the market to treat hypercholesterolemia, including 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, such as lovastatin and simvastatin, which inhibit cholesterol biosynthesis (Pazzucconi et al. 1995). However, the undesirable side effects of these compounds have raised concerns regarding their widespread therapeutic usage (Sima and Stancu 2001; Florentin et al. 2008). Although the consumption of low-fat diets is one of the more effective means of reducing cholesterol level, but due to consumer's poor compliance and acceptability of such a dietary regime, attempts have been made to identify alternative strategies to reduce cholesterol levels.

Probiotics are being extensively studied as a modern strategy in the prevention and treatment of hypercholesterolemia. The efficiency of probiotic cultures in reducing cholesterol has been hypothesized based on the results of many experiments which have demonstrated the presence of several mechanisms, including enzymatic deconjugation of bile acids by bile-salt hydrolase (Lambert et al. 2008), assimilation of cholesterol (Pereira and Gibson 2002), co-precipitation of

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cholesterol with deconjugated bile (Liong and Shah 2006), cholesterol binding to cell walls (Liong and Shah 2005), incorporation of cholesterol into the cellular membranes (Lye et al. 2010a), conversion of cholesterol into coprostanol (Lye et al. 2010b) and the production of short-chain fatty acids upon fermentation (De Preter et al. 2007). This has led to several studies being carried out involving lactic acid bacteria (LAB) with the aim to identify novel factors involved in reducing cholesterol levels (Kawase et al. 2000; Haberer et al. 2003). To date, the major cholesterol-lowering mechanism in LAB has been attributed to their bile salt hydrolase activity that catalyzes the hydrolysis of the amide group of glycine- and taurine-conjugated bile salts into amino acid residues and free bile salts (Brashears et al. 1998; Pereira et al. 2003). Gilliland et al. (1985) and Noh et al. (1997) proposed that LAB may incorporate cholesterol from the medium into cellular membranes during growth. Cholesterol thus incorporated into or attached to LAB cells in the intestine cannot be absorbed into the blood (Kimoto et al. 2002). LAB may also stimulate the secretion of bile acids into the gastro-intestinal (GI) tract, triggering a feedback mechanism which regulates hepatic cholesterol synthesis and its subsequent transformation into bile acids, thus reducing the serum cholesterol concentration (Liong and Shah 2005).

Cholesterol degradation, cholesterol oxidase (CHO) activity and bile salt hydrolase activity are well-known cellular processes in various microorganisms in the natural environment. However, knowledge of the mechanism and the factors involved in cholesterol reduction by probiotic *Bacillus* sp. is still limited. Consequently, we initiated an investigation of the cholesterol-lowering ability of *Bacillus* strains with potential probiotic properties (Shobharani and Halami 2014) using various in vitro assays.

Materials and methods

Media chemicals and reagents All chemicals used to prepare the culture media were obtained from Hi Media Pvt Ltd (Mumbai, India). Water-soluble cholesterol (polyoxyethanylcholesteryl sebacate), sodium glycocholate, sodium taurocholate, horseradish peroxide and fatty acid methyl ester standards were procured from Sigma-Aldrich (St. Louis, MO, USA). Ox-bile, phenol, *O*-dianisidine dihydrochloride and *O*-phthaldehyde were purchased from Hi Media, Pvt Ltd, and 4-amino antipyrine was purchased from Rolex Chemical Industries (Mumbai, India).

Bacterial culture and growth conditions Three *Bacillus* cultures which had been previously characterized as potential probiotic (Shobharani and Halami 2014), namely, *B. flexus* MCC 2458, *B. flexus* MCC 2427 and *B. licheniformis* MCC 2514, were investigated for their cholesterol removal ability. All cultures were grown in Luria Bertani (LB) medium at 37 °C under shaking conditions (120 rpm).

Bile salt hydrolase activity of *Bacillus* spp. The bile salt hydrolase (BSH) activity of the *Bacillus* cultures was determined by growing the isolates on LB agar supplemented with 0.5 % sodium taurocholic acid or sodium deoxycholic acid (Du Toit et al. 1998). The appearance of a white precipitate around the colony and clearing of the media were indicative of BSH activity.

CHO oxidase activity of *Bacillus* spp. Initially, the CHO-producing ability of the *Bacillus* cultures was examined by a colony-staining assay using filter papers dipped in a solution containing 0.5 % cholesterol, 1.7 % 4-aminoantipyrin, 6 % phenol and 3000 U/L horseradish peroxidase in 100 mM potassium buffer phosphate (pH 7.0). A filter paper soaked in this solution was placed onto the *Bacillus* colonies grown on LB agar medium and incubated at 37 °C for 24–48 h. CHO activity was confirmed by the development of a red color due to the formation of quinoneimine dye. The CHO-producing colonies were then grown on LB agar media supplemented with 0.1 % cholesterol, 0.1 % Triton X-100, 0.01 % *O*-dianisidine and 1000 U/L peroxidase and incubated at 37 °C for 24–48 h. In the presence of active CHO, H_2O_2 is released from cholesterol which then reacts with *O*-dianisidine to form an azo compound that turns the color of medium into an intense brown. CHO activity was also quantified by a spectrophotometric method as described by Richmond (1973). Briefly, the crude enzyme solution [cell-free supernatant (CFS); 0.05 mL] was incubated in the reaction mixture containing 0.05 % triton X-100 in 3 mL of 0.1 M sodium phosphate buffer (pH 7.0) and 6 mM cholesterol in isopropanol (0.05 mL). The reaction was carried out at 30 °C for 1 min, and the increase in absorbance resulting from the oxidation of cholesterol was measured at 240 nm (MultiGo spectrophotometer; Thermo Fischer Scientific Inc, Milford, MA, USA). The enzyme unit of CHO was defined as the amount of enzyme oxidizing 1 μM cholesterol per minute at 30 °C.

Estimation of cholesterol in media Cholesterol content in the spent broth was determined by the *O*-phthaldehyde method described by Rudel and Morris (1973). Briefly, the *Bacillus* cultures, grown in LB broth supplemented with water-soluble cholesterol (100 mg/L), were centrifuged (8000*g*, 4 °C, 15 min), and the CFS was analyzed for the residual cholesterol. Briefly, 0.5 mL of CFS was added to 2 mL of KOH (50 % wt/vol) and 3 mL of absolute ethanol, vortexed for 1 min and then heated at 60 °C for 15 min. After cooling, 3 mL of distilled water and 5 mL of hexane were added and the mixture vortexed for 1 min, following which 2.5 mL of the hexane layer was transferred into a glass tube and evaporated under nitrogen. The residue was immediately dissolved in 4 mL *O*-

phthalaldehyde reagent (0.5 mg/mL in acetic acid) and left at room temperature for 10 min. After complete mixing, 2 mL concentrated sulfuric acid was added slowly and the mixture vortexed for 1 min; after standing for 10 min at room temperature, absorbance was read at 550 nm (MultiGo spectrophotometer; Thermo Fisher Scientific). The percentage reduction in cholesterol level was determined by the difference between cholesterol level in the control (un-inoculated LB broth) and test samples (CFS of culture).

Cholesterol assimilation and cellular fatty acid profiling The *Bacillus* cultures were grown in LB medium supplemented with different concentrations of water-soluble cholesterol (50, 100 and 150 mg/L). After 24 h of incubation at 37 °C, the reduction in cholesterol level was analyzed in spent broth by the *O*-phthalaldehyde method as described in the previous section. The assimilation of cholesterol into the cellular membrane was determined by fatty acid profiling using gas chromatography (GC; Shimadzu Corp., Kyoto, Japan). Briefly, the *Bacillus* cultures grown at various concentrations of cholesterol were centrifuged and the cellular lipids was extracted from the cell pellet using chloroform and methanol as described by Bligh and Dyer (1959). The lipids were then methylated with 2 N KOH in methanol for 1 h, and the resulting fatty acid methyl esters were analyzed by GC using an RTX-1 capillary column (100 % dimethyl polysiloxane; 30 m × 0.32 mm ID × 0.25 μm). The injector and detector temperatures of the GC system were 230 and 250 °C, respectively, and the nitrogen flow rate was 1.5 mL/min. Fatty acid methyl esters were identified by comparison with authentic standards (Sigma-Aldrich) and confirmed by comparing GC-mass spectrometry spectra (Turbomass Gold; PerkinElmer Inc., Waltham, MA, USA).

Co-precipitation of cholesterol with deconjugated bile Co-precipitation of cholesterol with bile was analyzed by growing the culture in LB broth adjusted to acidic pH (5.0) with HCl and supplemented with filter-sterilized water-soluble cholesterol (polyoxyethanyl cholesteryl sebacate) and 6 mM sodium glycocholate or 6 mM sodium taurocholate. After an incubation period (48 h), the cells were centrifuged (8,000*g*, 4 °C, 10 min) and the cholesterol content in the broth determined by the *O*-phthaldehyde method as already described.

Reduction of cholesterol by the CFS The LB broth in which each of the three *Bacillus* cultures was grown was centrifuged, and the respective CFS obtained was filter sterilized and used to investigate cholesterol-reducing ability. Each CFS was diluted with fresh LB broth to various concentrations (25, 50, 75 and 100 %) and supplemented with cholesterol at a final concentration of 100 mg/L and 0.2 % ox-bile. These preparations were then incubated at 37 °C. At regular intervals (1, 2, 3, 4 days) the residual cholesterol was estimated by the *O*-phthaldehyde method. The stability of each CFS at various temperatures and pH was determined by exposing the CFS to a range of temperatures (30–80 °C) and pH (2–10) for 30 min, followed by incubation with cholesterol media. Post-incubation period residual cholesterol was analyzed as described elsewhere in the Materials and methods.

Statistical analysis Data analysis was carried out using STATISTICA version 6 software (StatSoft Inc., Tulsa, OK, USA). Each experiment was carried out in duplicate trials in three separate runs, and significant differences between samples were analyzed by analysis of variance (ANOVA). Mean separation was determined using by Duncan's Multiple range test (StatSoft Inc. 1999).

Results and discussion

The three *Bacillus* cultures (MCC 2458, MCC 2427, and MCC 2514) used in our study were previously isolated from raw milk samples and found to have potential probiotic properties (Shobharani and Halami 2014). In preliminary studies, *Bacillus* cultures were evaluated for their probiotic properties, including GI surveillance, non-virulent property, antimicrobial activity and antibiotic susceptibility, as well as cholesterol-reducing ability. Among the cultures tested, *B. flexus* MCC 2458, *B. flexus* MCC 2427 and *B. licheniformis* MCC 2514 exhibited cholesterol-reducing ability in addition to other probiotic properties. Interestingly, none of the preferred cultures exhibited BSH activity. Although the presence of BSH in several bacterial species of the GI tract, such as *Lactobacillus* sp., *Bifidobacterium longum*, *Clostridium perfringens* and *Bacteroides fragilis* ssp. *fragilis* (Corzo and Gilliland 1999), was considered to be the main factor determining cholesterol reduction, its absence raised our interest to analyze other factors or mechanisms involved in the selected cultures that are responsible for reducing cholesterol from the media.

Effect of growth phase on cholesterol removal

The three cultures tested showed the characteristic coloration with filter paper assay and the CHO indicator plate (Fig. 1), demonstrating their ability to produce CHO. CHO activity and cholesterol level in media were monitored in all of the selected cultures during the 4-day growth period. The cell viability of cultures grown in media supplemented with cholesterol (100 mg/L) was 2–3 log lower than that in cultures grown in media with out cholesterol. Cells grown on the cholesterol-supplemented media showed a steady and significant ($p<0.05$) increase in cell viability during the first 24 h, with the highest increase in *B. licheniformis* MCC 2514 (log increase 8.06±0.23) followed by *B. flexus* MCC 2427 (log

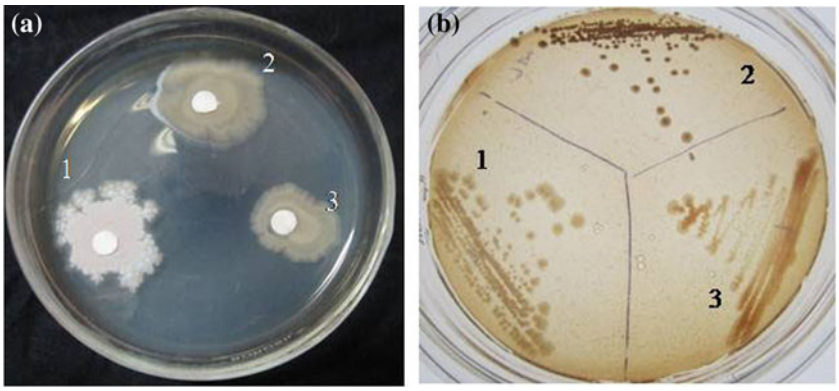

Fig. 1 Selection of cholesterol oxidase (CHO) producers. **a** Colony staining method using a filter disc containing 4-aminoantipyrin, phenol and horseradish peroxidase, **b** plate assay using *O*-dianisidine and horseradish peroxidase. *1* Control (*Bacillus cereus* MCC 2015), *2 B. flexus* MCC 2458, *3 B. flexus* MCC 2427

increase 7.18±0.76) and *B. flexus* MCC 2458 (log increase 6.18±0.76). With further incubation, an extensive and significant ($p<0.05$) variation was observed in the growth pattern in each culture (Fig. 2a). *B. flexus* MCC 2458 showed an increase in cell viability on the second day of growth, followed by a reduction on the third and fourth days, whereas the opposite was observed for *B. licheniformis* MCC 2514, with a reduction in cell viability on day 2 and an increase on days 3 and 4. *B. flexus* MCC 2427 showed a steady reduction in cell viability during the 4-day incubation period. Remagni et al. (2013) also observed variations in cell growth in the presence of cholesterol. In their study, *Lactobacillus acidophilus* and *Enterococcus italicus* 989 had the highest cell count after 16 h of incubation, whereas the number of viable cells of *Lactobacillus plantarum* 885 and *L. delbrueckii* ssp. *bulgaricus* V15 increased after 24 h. On further incubation, viable cells decreased until the end of the 48-h of incubation. Variation in cultivation time is observed in a large number of microbial species; for example, *Arthrobacter simplex* (Liu et al. 1983), *Brevibacterium* sp. (Yang and Zhang 2012), *Rhodococcus equi* (Sojo et al. 1997) and *Streptomyces* sp. (Tomioka et al. 1976) have been reported to produce their maximal level of CHO after 96, 36, 100 and 40 h of cultivation, respectively.

The highest level of CHO was observed in *B. flexus* MCC 2427 (2.5 U/mL) after 24 h of incubation, following which there was a gradual reduction in CHO level with increasing incubation time (correlation coefficient >0.8 with cell viability) (Fig. 2b). No significant ($p>0.05$) difference in CHO activity was observed between *B. flexus* MCC 2458 and *B. licheniformis* MCC 2514. Kim et al. (2002) reported a positive correlation between growth and CHO production in *B. subtilis* SFF34, with the maximal level of CHO (3.14 U/mL) reached after 24 h of incubation. *Bacillus* sp. isolated from fermented flatfish has been found to produce CHO with a specific activity of 1.4 U/mg, which upon purification by ion-exchange chromatography was found to consist of two fractions with an activity of 7.6 and 8.0 U/mg, respectively (Rhee et al. 2002). CHO, a bi-functional flavin adenine dinucleotide-containing microbial enzyme belonging to the family of oxidoreductases, is considered to be the first enzyme in the cholesterol degradation pathway that catalyzes the oxidation of cholesterol into 4-cholesten-3-one, a molecule which is more susceptible to degradation by bacteria (MacLachlan et al. 2000). Therefore, culture MCC 2427, with its relatively high CHO activity (2.5 U/mL), may be a potent microbial source to degrade cholesterol.

The percentage residual cholesterol in the culture broth and cholesterol assimilation in the cell pellet during growth are presented in Fig. 2c. During growth of the cultures, we observed a constant reduction in cholesterol content in the broth with a concomitant increase in the concentration of cholesterol in the cell pellet. After 4 days of incubation, the maximum reduction of cholesterol in the culture broth (42.4 %) was observed in the culture broth of *B. licheniformis* MCC 2514, with concurrent assimilation of 68.51 μg/mg of cholesterol in the cell pellet.

Cholesterol assimilation and fatty acid composition

To investigate the ability of *Bacillus* spp. to remove cholesterol from media by incorporating it into their cells, we analyzed the cellular lipid profile. Following supplementation of various concentrations (50, 100 and 150 mg/L) of cholesterol to the LB culture broth, we noted an overall increase in total fatty acids in all the three cultures relative to that grown in the absence of cholesterol (Table 1). In all cases, the maximum increase was observed in C18.0. Overall, total saturated and unsaturated fatty acids increased in the three test cultures with an increasing concentration of cholesterol. Similarly, Kimoto et al. (2002) reported that *Lactococcus lactis* KF147 grown in the presence of cholesterol showed an increased content of saturated and unsaturated fatty acids. Among the three cultures of *Bacillus* sp. tested in our study, MCC 2514 showed the highest increase in total fatty acids (50.35 μg/mg), unsaturated fatty acids (11.63 μg/mg) and saturated fatty acids (38.72 μg/mg), followed by MCC 2458 with 42.3, 7.68 and 34.62 μg/mg of total fatty acids, unsaturated fatty acids and saturated fatty acids, respectively. These results are in agreement with the cholesterol reduction data (Fig. 2c), wherein the maximum reduction in cholesterol (42.41 %) was observed in *B. licheniformis* MCC 2514. Further, as suggested by Lye

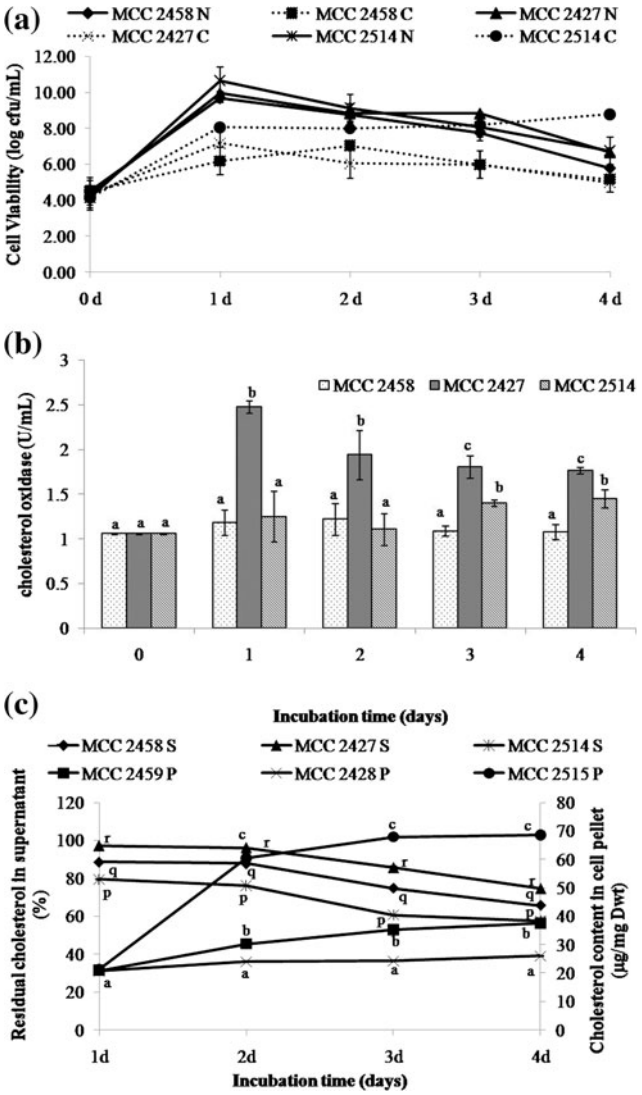

Fig. 2 Time course of cell growth (**a**), CHO activity (**b**) and cholesterol assimilation (**c**) during cultivation of *Bacillus* spp. in normal Luria Bertani (LB) broth (*N*) and LB broth supplemented with 100 mg/L cholesterol (*C*). *P* Cell pellet, *S* cell-free supernatant. CHO activity was defined as the amount of enzyme oxidizing 1 μM cholesterol per minute at 30 °C. Residual cholesterol is the percentage of cholesterol remaining in the culture supernatant during growth as compared to the initial (0 day, *0 d*) concentration. Values are given as the mean±standard deviation (SD) of duplicate trials of three separate experiments. *Different lowercase letters within the group of cultures at any one time point* indicate significant differences ($p<0.05$) in the mean values of that culture over time (**b**, **c**)

et al. (2010a), incorporation of cholesterol into the cellular membrane leads to increased membrane strength and, consequently, to a higher resistance to cell lysis. This may explain, at least partially, the elevated cell viability of *B. licheniformis* MCC 2514 (Fig. 2a) in the presence of cholesterol relative to the other *Bacillus* strains tested. Based on these results, we suggest that *B. licheniformis* MCC 2514 cells incorporated the maximum amount of cholesterol into their cellular membrane, thus facilitating cholesterol removal from the culture media.

Co-precipitation of cholesterol with bile

Co-precipitation of cholesterol with bile is a method used by certain bacteria to remove cholesterol from their environment. The mechanism involves the conversion of cholesterol to replace lost bile acids so as to maintain the normal bile concentration in the body. In the present study, co-precipitation of cholesterol was analyzed under acidic pH (5.0) in the presence of ox-bile and conjugated bile acids (sodium glycocholic and taurocholic acid). The percentage reduction in cholesterol was then compared with the cultures grown in the absence of bile. On the whole, our results showed a significant ($p<0.05$) reduction in cholesterol content in the presence of bile (Table 2): there was a 18.48–19.68 % reduction in cholesterol in the absence of bile, which increased to 27.57–31.22 % with supplementation of bile. Although there was no significant difference ($p>0.05$) in the reduction in cholesterol content in the presence of bile among the tested *Bacillus* spp., *B. flexus* MCC 2458 showed a slightly higher reduction compared to *B. flexus* MCC 2427 and *B. licheniformis* MCC2514. With respect to the conjugated bile acids, all three cultures preferred sodium glycocholic acid to sodium taurocholic acid (Table 2). As sodium glycocholate and sodium taurocholate are present in the human system in a 3:1 ratio, deconjugation or co-precipitation with the predominant sodium glycocholate may be beneficial in lowering cholesterol (Brashears et al. 1998). Similarly, a high deconjugation activity by *Lactobacillus acidophilus* ATCC 33200, 4357, 4962 and *Lactobacillus casei* ASCC 1521 toward sodium glycocholate and sodium taurocholate has been reported (Liong and Shah 2005). Klaver and Van der Meer (1993) have suggested that the removal of cholesterol from the culture medium by *L. acidophilus* strains can be attributed to the co-precipitation of cholesterol with the deconjugated bile salts under acidic conditions.

Reduction of cholesterol by the CFS

The CFS of the *Bacillus* spp. were evaluated for their ability to reduce cholesterol in the presence of 0.2 % bile. The results showed that the percentage of cholesterol reduced in all three cultures tested increased significantly ($p<0.05$) with increasing concentration of CFS. In samples of 100 % CFS, the reduction in cholesterol content was highest with *B. flexus* MCC 2458 (48.4 %) on the first day of growth, which increased to 79.54 % on the fourth culture day (Fig. 3a). As suggested by Kim et al. (2008), some of the components in the CFS may contribute to the reduction in cholesterol content. The stability of the CFS at various pH varied among each of the cultures of *Bacillus* sp. tested. Interestingly, the cultures showed a potential to reduce cholesterol content at both acidic

Table 1 Effect of cholesterol[a] on the cellular fatty acid profile of *Bacillus* spp.

Fatty acids	*Bacillus flexus* MCC 2458				*Bacillus flexus* MCC 2427				*Bacillus licheniformis* MCC 2514			
	Control	50 mg/L	100 mg/L	150 mg/L	Control	50 mg/L	100 mg/L	150 mg/L	Control	50 mg/L	100 mg/L	150 mg/L
C8	0.88±0.01	1.81±0.34	4.13±1.09	3.41±0.14	0.45±1.31	4.05±0.03	2.92±0.01	1.91±0.57	1.04±0.04	4.06±0.07	6.05±0.01	6.66±0.11
C10	0.26±0.21	0.72±0.06	1.22±0.06	2.05±0.08	0.51±0.27	0.99±0.02	1.21±0.18	1.09±0.05	0.36±0.08	1.70±0.04	1.43±0.21	1.12±0.34
C12	0.27±0.28	1.03±0.01	1.73±0.26	4.16±0.08	0.33±1.70	2.00±0.88	2.56±0.04	1.52±0.73	0.36±0.08	7.73±0.02	4.46±0.28	3.89±1.31
C13	0.18±0.08	0.90±0.02	1.85±0.34	4.58±0.11	0.55±0.57	0.45±0.58	1.69±0.01	1.61±0.67	0.23±0.14	2.40±0.01	1.92±0.08	1.87±0.27
C14	0.01±0.14	0.13±1.09	0.60±0.14	0.52±0.34	0.05±0.05	0.48±0.73	0.23±0.08	0.24±0.07	0.02±0.12	0.57±0.01	0.63±0.14	0.45±1.70
C15	0.04±0.07	0.19±0.34	0.13±0.28	0.60±0.06	0.29±0.22	0.31±0.67	0.46±0.14	0.29±0.08	0.07±0.11	0.63±0.01	0.69±0.07	0.67±0.57
C16	0.07±0.08	0.80±1.56	2.84±0.21	3.97±0.01	0.27±0.91	1.50±0.05	1.79±0.07	1.43±0.08	0.14±0.08	3.06±0.08	2.76±0.08	2.98±0.05
C16.1	0.02±0.04	0.15±0.01	0.25±0.14	0.47±0.08	0.05±1.94	0.06±0.01	0.24±0.08	0.23±0.08	0.54±0.08	4.22±0.08	4.27±0.04	4.57±0.22
C17	0.01±0.09	0.13±0.18	0.21±0.08	0.40±0.08	0.05±0.78	0.07±0.30	0.29±0.00	0.15±0.11	0.34±0.09	1.22±0.11	1.41±0.18	2.88±0.91
C18	0.20±0.34	1.92±0.04	7.06±0.08	11.83±0.14	0.56±0.11	4.64±0.03	5.13±0.91	5.17±0.34	0.38±0.14	7.97±0.34	15.43±0.04	16.51±1.94
C18.1	0.06±0.56	0.30±1.99	1.99±0.07	1.98±0.12	0.12±0.00	1.02±0.91	1.09±0.00	0.82±0.06	0.06±0.04	2.14±0.57	2.92±0.01	5.67±1.09
C18.3	0.06±0.01	1.22±0.21	2.57±0.04	2.81±0.14	0.42±0.91	0.41±0.94	0.41±0.12	1.52±0.19	0.11±0.07	0.26±0.05	0.25±0.21	0.74±0.06
C20	ND	0.71±0.08	ND	1.47±0.07	ND	0.08±0.78	0.42±0.03	1.21±0.21	0.01±0.06	1.22±0.22	0.80±0.08	0.30±0.26
C21	0.04±0.03	0.39±0.08	ND	1.37±0.08	0.02±0.14	0.29±0.11	0.38±0.01	1.50±0.01	0.04±0.01	1.08±0.91	1.09±0.08	0.51±0.34
C22	0.01±0.45	0.06±0.14	0.09±0.01	0.27±0.01	0.06±0.07	0.08±0.01	0.15±0.01	0.13±0.21	0.05±0.01	0.60±1.94	0.92±0.14	0.90±0.14
C22.1	0.07±0.62	0.35±0.07	1.67±0.01	2.42±0.01	ND	0.31±0.01	0.70±0.01	1.85±0.04	0.09±0.01	1.42±0.00	1.20±0.07	0.65±0.28
TFA	2.18	10.79	26.35	42.30	3.73	16.73	19.69	20.66	3.83	40.26	46.24	50.35
TUSFA	0.20	2.02	6.48	7.68	0.59	1.80	2.44	4.42	0.80	8.03	8.64	11.63
TSFA	1.98	8.77	19.87	34.62	3.14	14.93	17.24	16.24	3.04	32.23	37.60	38.72

Values are presented as the mean± standard deviation (SD)of duplicate trials and expressed as micrograms per milligram dry weight

TFA, Total fatty acids; TUSFA, total unsaturated fatty acids (16.1, 18.1, 18.3, 22.1); TSFA, total saturated fatty acids; ND, not detected

[a] The three *Bacillus* spp. were cultivated in normal Luria Bertani (LB) broth (control; no cholesterol) or in LB broth supplemented with 50, 100 or 150 mg/L cholesterol

Table 2 Co-precipitation of cholesterol by *Bacillus* spp.

Culture	Percentage reduction of cholesterol			
	Control (only cholesterol)	Ox-bile	Sodium taurocholate	Sodium glycocholate
MCC 2458	19.68±0.01 b	31.23±0.01 b	38.60±0.02 c	39.67±0.05 b
MCC 2427	18.49±0.02 a	27.57±0.04 a	30.90±0.05 a	34.48±0.01 a
MCC 2514	19.47±0.01 b	31.02±0.01 b	37.48±0.03 b	39.53±0.00 b

Values are presented as the mean±SD of duplicate trials from three separate runs

Values in a column followed by the same lowercase letters do not differ significantly ($p > 0.05$)

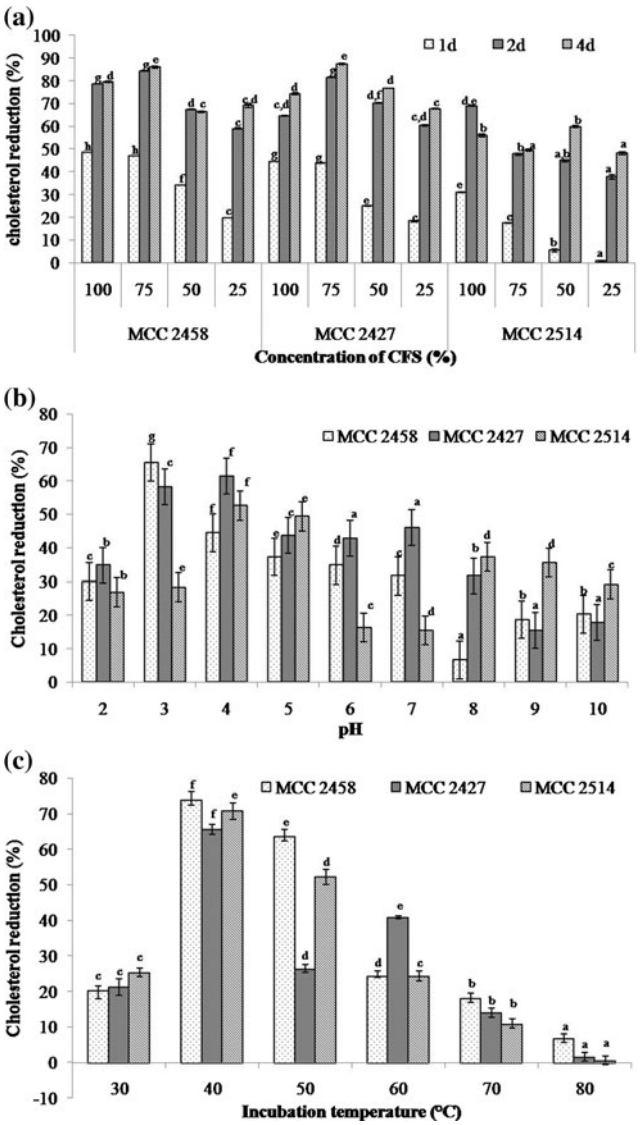

Fig. 3 **a** Cholesterol lowering effect of the cell-free supernatant (*CFS*) at various concentrations (**a**). Effect of pH (**b**) and temperature (**c**) on cholesterol-lowering ability of CFS. Values are given as the mean±SD of three separate experiments. *Different lowercase letters indicate* significant differences in cholesterol reduction for that culture at that concentration and time point (**a**) and significant differences between cultures at the variable tested (*X-axis*) (**b**, **c**)

and alkaline pH. In terms of the association between cholesterol reduction and pH, *B. flexus* MCC 2458 achieved its highest efficiency for cholesterol reduction (65.48 %) at acidic pH (pH 3.0); this efficiency decreased at neutral pH and gradually increased under an increasingly alkaline condition, reaching 20.3 % at pH 10.0. In comparison, both *B. flexus* MCC 2427 and *B. licheniformis* MCC 2514 also displayed maximum cholesterol reduction at an acidic pH (pH 4.0), with a reduction of 61.45 and 52.57 %, respectively; under the alkaline condition, maximum cholesterol reduction was achieved at pH 9 and pH 8 by MCC 2514 (35.63 %) and MCC 2427 (31.60 %), respectively (Fig. 3b). The stability study of CFS at various temperatures revealed that 40 °C was optimum in terms of stability for all three cultures. *B. flexus* MCC 2458 displayed maximum (73.81 %) reduction at 40 °C, followed by *B. licheniformis* MCC 2514 (70.72 %) and *B. flexus* MCC 2427 (65.57 %) (Fig. 3c). Kim et al. (2008) reported similar results with *Lactobacillus acidophilus* ATCC 43121 CFS, which was able to reduce cholesterol in broth in a dose-dependent manner. Rhee et al. (2002) detected two extracellular CHO (CO1 and CO2) in *Bacillus* sp. SFF34 with optimum pH of 6.25 and 6.0 and with a temperature optimum of 60 °C and 40 °C, respectively.

Conclusions

The results of this study reveal that the possible mechanism(s) or factor(s) involved in removing cholesterol varied among the individual *Bacillus* cultures tested. In contrast to previous studies, where BSH was considered to be the main mechanism for cholesterol removal, the cultures tested in our study were able to reduce the cholesterol level even in the absence of BSH. The cholesterol removal mechanism associated with CHO, cholesterol assimilation and cholesterol co-precipitation with bile was found to be culture-dependent, with *B. flexus* MCC 2427 involving CHO production and *B. licheniformis* MCC 2514 and *B. flexus* MCC 2458 exhibiting cholesterol reduction through the assimilation and co-precipitation mechanism, respectively. Given the

importance of cholesterol in cardiovascular disease and other related illness in humans, these three cultures with cholesterol removing ability may prove to be potential candidates for food and pharmaceutical applications. Our study can only be considered to be preliminary in terms of elucidating the mechanism exhibited by *Bacillus* spp. to eliminate cholesterol. More properly planned in vivo studies will have to be undertaken in the future to precisely understand the mechanism involved for a safer and effective probiotic culture with anti-hypercholesterolemic effects.

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Conflict of interest The authors declare no conflicts of interest.

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