Indian Journal of Biochemistry & Biophysics
Vol. 56, October 2019, pp. 363-372

Cholesterol lowering and antioxidant potential of probiotic bacteria isolated from locally fermented milk product *kalarei*

Bilqeesa Bhat, Bisma Habib, Neha Bhagat & Bijender Kumar Bajaj*

School of Biotechnology, University of Jammu, Jammu- 180 006, Jammu and Kashmir, India

Received 11 December 2018; *revised 29 March 2019*

Probiotics due to their multifaceted health promoting attributes have gained immense research impetus in recent years. The current study reports the hypocholesterolemic potential of lactic acid bacteria (LAB) isolated from indigenous sources. LAB may use several mechanisms for lowering serum cholesterol level *viz.* cholesterol assimilation, bile salt deconjugation, and cholesterol adsorption on cell surface of live, resting and dead probiotic cells. Cholesterol lowering is generally a strain dependent phenomenon, and different LAB isolates exhibited varying level of hypocholesterolemic effects. Among the LAB isolates, K2 *i.e*. *Enterococcus faecalis* K2 showed the highest *in vitro* cholesterol lowering ability (82.32%), and strong bile salt deconjugation potential, and released about 128.43 µM/mL of cholic acid upon bile salt deconjugation. Furthermore, cholesterol removal by live, resting and dead *E. faecalis* K2 probiotic cells was shown to the extent of 72.46, 44.93, and 45.88%, respectively. Scanning electron microscopy displayed appreciable adherence of cholesterol on to the cellular surfaces of *E. faecalis* K2 cells. The antioxidant potential of the cell free cultural fluid of LAB isolates was quite variable. LAB isolate *E. faecalis* K2 showed appreciable DPPH radical scavenging activity (37.36%), hydroxyl radical scavenging ability (26.35%), and superoxide radical scavenging ability (42.67%). Most of the LAB probiotic isolates were susceptible to conventionally used antibiotics, and lacked biogenic amine producing ability and haemolytic activity. The probiotic isolate *E. faecalis* K2 may have potential for application for management of hypercholesterolemia related coronary heart diseases, however, after thorough *in vivo* investigation.

Keywords: Cholesterol, *Enterococcus faecalis* K2, Hypercholesterolemia, *Kalarei*, Probiotics

Multifaceted health-promoting effects of probiotics have attracted a great deal of research attention in recent years[1]. As the health benefits of probiotics exhibit huge variations, bioprospecting of new/novel strains of efficacious probiotics (lactic acid bacteria, LAB and others) has always been an ongoing practice[2]. Cholesterol lowering potential is considered as one of the most imperative functional attribute of probiotics[3]. Raised serum cholesterol level poses high risk for cardiovascular diseases (CVDs), obesity and other metabolic disorders[4]. Recently CVDs have become one of the major causes of mortality[5]. Considering the adverse effects of therapeutic approaches of cholesterol lowering, application of probiotics for managing raised cholesterol levels, and hence the CVDs has gained immense research impetus[6,7]. Antioxidant potential is another important health promoting feature of probiotics[8]. Safety analysis of probiotics is absolutely essential prior to their commercilization[9]. In the current study the hypocholesterolemic potential of LAB probiotic isolated from several sources including an indigenously milk fermented product *kalarei*[10] was examined by bile salt hydrolysis, bile salt deconjugation, and cholesterol assimilation. Furthermore, probiotic isolates were studied for antioxidant activity and biosafety properties.

*Correspondence:
E-mail: bajajbijenderk@gmail.com

Materials and Methods

Probiotics strains, culture medium, and growth conditions

Five probiotic LAB isolates F11, F17 (from faeces of healthy infants), M16, M17 (human breast milk), and K2 (local fermented milk product, *kalarei*) were procured from culture collection of Fermentation Biotechnology Laboratory, School of Biotechnology, University of Jammu, Jammu[11]. These LAB isolates have previously been characterized for several of the probiotic functional attributes[11]. Each culture was activated from stock cultures maintained in 20% glycerol (−80°C). Bacterial cells were propagated in De Man-Rogosa-Sharpe (MRS) broth by incubating at 37°C for18 h.

The probiotic isolates were examined for cholesterol lowering, and antioxidant potential, and

biosafety aspects. The isolate (K2) which exhibited most of the desired attributes was identified based on 16S rDNA sequence analysis[1]. Universal primer pair used for PCR amplification was lac1-27F 5′-AGAGTTTGATCCTGGCTCAG and lac1-1492R 5′-TACGGYTACCTTGTTACGACT). PCR-amplification was executed and the amplicon was sequenced and analyzed.

Cholesterol assimilation ability of the LAB isolates

Cholesterol was dissolved in 50% ethanol (5 mg/mL), filter sterilized and added to MRS-broth supplemented with 0·3% ox-bile at a final concentration of 100 µg/mL. The MRS medium was inoculated with (1%, *v/v*) overnight grown probiotic culture (10^8-10^{10} cfu/mL) and incubated anaerobically at 37°C for 18 h. The culture was centrifuged (10000 × g at 4°C for 10 min) to get the cell free supernatant (CFS). CFS was examined for the remaining cholesterol concentration[12]. For analysis of cholesterol 1.0 mL aliquot of CFS was mixed with 1.0 mL of KOH (33%, *w/v*) and 2.0 mL of absolute ethanol. The contents were well mixed and allowed to stand at 37°C for 15 min. Then 2.0 mL of distilled water and 3.0 mL of hexane were added and mixed well. One mL of top hexane layer was transferred into a glass tube and evaporated under nitrogen atmosphere. The residue was immediately dissolved in 2.0 mL of o-phthalaldehyde reagent and mixed well. Then 0.5 mL of concentrated H_2SO_4 was added, and the content was mixed and incubated at room temperature for 10 min. Finally, the absorbance was read at 550 nm. The amount of cholesterol lowered was determined by subtracting the amount of cholesterol in each sample (µg/mL) from the amount present in the uninoculated control.

Bile salt deconjugation assay for the LAB isolates

Bile salt deconjugation ability was measured[6] by inoculating MRS-broth supplemented with 6 mM sodium glycocholate with overnight grown culture (10^8-10^{10} cfu/mL) at (1% *v/v*) and by incubating anaerobically at 37°C for 18 h. Then 10 mL of the culture was adjusted to pH 7·0 with NaOH (1.0 M) and centrifuged at 10000 × g at 4°C for 10 min, and the pH of the supernatant, thus, obtained was adjusted to 1.0 with HCl (1.0 M). Bile salt deconjugation assay was set up by adding 1.0 mL of the supernatant into 2.0 mL of ethyl acetate followed by vortexing for 1 min. Then 2.0 mL of the top ethyl acetate layer was transferred into a glass tube and evaporated under nitrogen atmosphere at 60°C. The residue was immediately dissolved in 1.0 mL of NaOH (0·01 M). After complete mixing, 1.0 mL of furfuraldehyde (1%) and 1.0 mL of H_2SO_4 (8.0 M) were added, and the mixture was vortexed for 1 min before heating at 65°C in a water-bath for 10 min. After cooling, 2.0 mL of glacial acetic acid was added, and the mixture was mixed well, and read for absorbance at 660 nm. The amount of cholic acid released from deconjugation of bile salts was determined from the cholic acid standard curve.

Cholesterol removing potential of growing, dead and resting cells probiotic cells

Comparative cholesterol lowering potential of growing, dead and resting cells was studied[13]. For growing cell assay, overnight grown cells were inoculated (1%, *v/v*) in MRS broth supplemented with 0.1 g/L of cholesterol and 0.5% (*w/v*) sodium glycocholate and the cultures were incubated anaerobically at 37°C for 18 h. Similarly, for resting cell assay, MRS broth was inoculated with test cultures (1%, *v/v*) and incubated anaerobically at 37°C for 18 h. Then, contents were centrifuged at 5000 × g for 5 min and the cell pellet was washed with sterile saline. The cell pellet was suspended in 0.05 M phosphate buffer (pH 6.8) containing 0.5% oxgall, 0.1 g/L of cholesterol and 0.5% (*w/v*) sodium glycocholate. For dead cell assay, the cells were grown in MRS broth for 18 h and then cells were harvested and re-suspended in phosphate buffer saline (pH 7.0) and heat-killed at 100°C for 15 min followed by centrifugation (5000 × g for 5 min). The heat killed cells were resuspended in 0.05 M phosphate buffer (pH 6.8) containing 0.1 g/L of water soluble cholesterol and 0.5% (*w/v*) sodium glycocholate. The test strains were incubated at 37°C for 24 h and then the mixture was centrifuged (10000 × g for 5 min at 4°C) and the CFS was used for cholesterol assay. In all the three setups, the remaining cholesterol concentration (µg/mL) was determined from CFS using the method described by Rudel and Morris[14].

SEM analysis for adsorption of cholesterol to probiotic cell surface

Cholesterol adsorption by the cell surface of growing, and dead probiotic cells was studied using scanning electron microscopy (SEM). Growing and dead cells were prepared[13]. In the assay three sets of cells were studied *i.e.* (1) cells grown in MRS broth supplemented with cholesterol only, (2) cell grown in

MRS broth supplemented with cholesterol and bile salt, and (3) heat killed cells suspended in 0.05 M phosphate buffer (pH 6.8) containing cholesterol. The cells grown in MRS broth without any supplementation were used as a control. Cholesterol attached to the cell surfaces (three conditions and control) was observed using scanning electron microscopy (Electron Microscope, JEOL JEM - 100CXII, CSIR-IIIM, Jammu). Probiotic cells were fixed in 2.5% (*v/v*) glutaraldehyde (pH 7.4) for 2 h at 4°C. The bacterial cells were washed with 0.1 M of phosphate buffer saline and centrifuged at 5000 rpm for 10 min. Cell pellet was fixed for 1.0 h in 1.0% osmium tetraoxide prepared in phosphate buffer. The sample was washed two times with distilled water for 10 min and centrifuged. After proper processing, the probiotic cells were coated with gold and viewed under scanning electron microscope and images were taken at 5000 and 10000x.

Antioxidant potential of LAB isolates

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity of CFS of each probiotic culture (10^8-10^{10} cfu/mL) was determined[15]. Briefly, 1.0 mL of CFS was added to 2.0 mL ethanolic DPPH radical solution (0.05 mM) and the mixture was mixed vigorously and incubated at room temperature in the dark for 30 min. The blank contained only ethanol and the cells. The control included deionized water and DPPH solution. Ascorbic acid was taken as positive control. Absorbance of the resulting solution was measured at 517 nm. The scavenging ability was expressed as:

$$\text{Scavenging activity } (\%) = 1 - \frac{\text{A sample} - \text{A blank}}{\text{A control}} \times 100$$

Hydroxyl radical scavenging assay was conducted by a Fenton reaction method[15]. Briefly, the reaction mixture containing 2.0 mL of $FeSO_4$ (0.5 mM), 1.0 mL of brilliant green (0.435 mM), 1.5 mL of H_2O_2 (3.0%, *w/v*), and 1.0 mL of CFS was incubated at room temperature for 20 min, and then the absorbance was measured at 624 nm. The absorbance change of the reaction mixture indicated the scavenging ability of the probiotic strains for hydroxyl radicals. Hydroxyl radical scavenging activity is expressed as:

$$\text{Scavenging activity } (\%) = 1 - \frac{(\text{As} - \text{A0})}{(\text{A} - \text{A0})} \times 100$$

Where, as the absorbance in the presence of the sample, A0 is the absorbance of the control in the absence of the sample, and A is the absorbance without the sample and Fenton reaction system.

The superoxide radical scavenging activity of CFS of probiotic cells was determined[16]. The superoxide radical was generated in 3.0 mL of 0.1 M sodium phosphate buffer (pH 7.4) containing 156 µM β-nicotinamide adenine dinucleotide (NADH), 52 µM nitroblue tetrazolium (NBT) and 20 µM phenazine methosulfate (PMS). After addition of 1.0 mL CFS, the reaction mixture was incubated at 25°C for 5 min. The scavenging activity of superoxide radical was expressed as:

$$\text{Scavenging activity } (\%) = 1 - \frac{\text{A sample}}{\text{A blank}} \times 100$$

Deionized water and ascorbic acid (Vc) were used as the blank and positive control, respectively.

***In vitro* biosafety evaluation LAB probiotic isolates**

Blood hemolysis test for probiotics LAB was done. Fresh bacterial cultures were streaked on agar plates containing 5% (*v/v*) calf blood. The plates were incubated at 37°C for 48 h under anaerobic condition and the plates examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies)[17].

Gelatin hydrolyzing activity of probiotics LAB was done by analyzing their gelatinase producing ability on TND-gelatin agar (1.7% tryptone, 0.3% neo-peptone, 0.25% dextrose, 0.5% NaCl, 0.25% K_2HPO_4 and 1.5% agar, 0.4% gelatin). LAB cultures were spotted on TND-gelatin agar plates and incubated at 37°C for 3 days. Then the plates were flooded with saturated ammonium sulfate solution. Appearance of clear zones around the inoculated culture spots against the opaque background indicates gelatinase producing ability.

Biogenic amines production by LAB probiotics was examined on the decarboxylation medium[18]. The LAB isolates were grown in MRS broth containing 0.1% (*w/v*) amino acids (tyrosine and histidine) and 0.005% pyridoxal phosphate to promote the enzyme induction, for 4 days at 37°C. Visual observation of the cultures was made after end of incubation. Change in the coloration of culture from yellow to violet is an indicative of decarboxylase positive reaction.

Antibiotic susceptibility of five probiotic cultures was assessed by employing disk diffusion method[19]. Ten conventionally prescribed antibiotics used were Penicillin-G (P), Gentamycin (GEN), Vancomycin (VA), Erythromycin (E), Ampicillin (AMP), Chloramphenicol (C), Fusidic acid (FC), Clindamycin (CD) and Kanamycin (K), Tetracycline (TE). Details of respective concentration, group and mode of action of antibiotics is given in (Table 1).

Results and Discussion

The current study was conducted to investigate the *in vitro* cholesterol lowering potential of probiotic lactic acid bacteria isolated[20] from local fermented milk products *kalarei* (isolate K2), infant feces (F11, F17) and human breast milk (M16, M17). These LAB isolates have previously been characterized for various probiotic functional attributes like excellent adhesion, coaggregation and autoaggregation, antimicrobial potential, and exopolysaccharide producing ability[11,20]. Exopolysaccharides were isolated and characterized from probiotic bacterium *Enterococcus faecium* K1 isolated from fermented indigenous milk product *kalarei*[20]. In the current study, cholesterol lowering potential of these probiotic LAB was evaluated based on bile salt hydrolase (BSH) activity, bile salt deconjugation ability and the cholesterol assimilation potential. The cell surface change of probiotic bacteria in presence of cholesterol and bile salts was also studied using scanning electron microscopy (SEM). Furthermore, the probiotic isolates were also studied for their antioxidant potential, and bio-safety analysis.

Cholesterol assimilation ability of the LAB isolates

The LAB isolates were able to remove cholesterol from MRS-broth supplemented with ox-bile (0.3%) and cholesterol (100 µg/mL) during growth. The LAB isolate K2 showed the highest *in vitro* cholesterol lowering ability of 82.32% followed by isolate M17, F11, and M16 which displayed cholesterol lowering ability of 53.41, 41.09, and 31.05%, respectively, after 24 h of incubation (Fig. 1A). The isolate F18, however, exhibited meagre cholesterol lowering potential (13.65%). Similar to current study, a probiotic isolate *Lactobacillus reuteri* LR6 showed

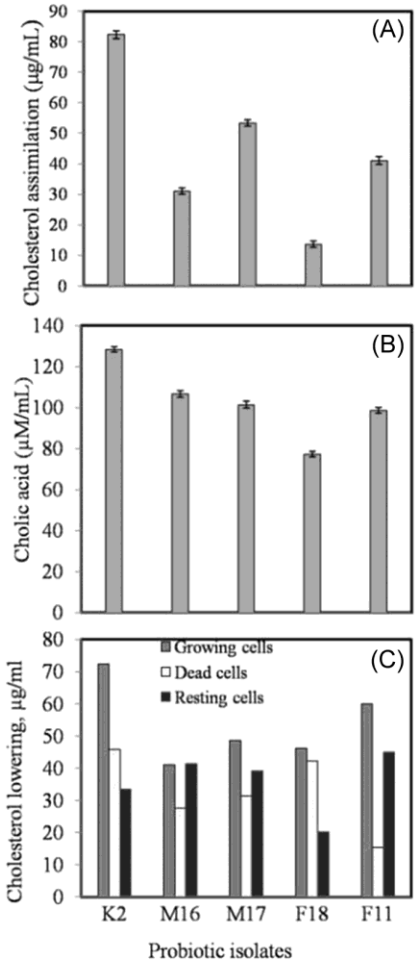

Fig. 1 — Cholesterol lowering by probiotic isolates based upon cholesterol assimilation (A), Bile salt deconjugation (B); Cholesterol removal by live; and (C) dead and resting cells

Table 1 — Antibiotics used for Antibiotic susceptibility assay of LAB probiotic isolates

Antibiotic	Amount in the disc (µg)	Group of antibiotic	Mode of action
Penicillin-G (P)	10 µg	β-Lactams	Cell wall synthesis inhibitors
Gentamycin (GEN)	10 µg	Aminoglycosides	Protein synthesis inhibitors
Vancomycin (VA)	30 µg	Glycopeptides	Cell wall synthesis inhibitors
Erythromycin (E)	15 µg	Macrolides	Cell wall synthesis inhibitors
Ampicillin (AMP)	10 µg	β-Lactams	Cell wall synthesis inhibitors
Chloramphenicol (C)	30 µg	Others	Protein synthesis inhibitors
Fusidic acid (FC)	10 µg	Fusidane	Protein synthesis inhibitors
Clindamycin (CD)	2 µg	Lincosamide	Protein synthesis inhibitors
Kanamycin (K)	10 µg	Aminoglycoside	Protein synthesis inhibitors
Tetracycline (TE)	30 µg	Tetracyclines	Protein synthesis inhibitors

cholesterol lowering[21] by 68.19±0.86%, whereas *Lactobacillus pentosus* KF923750 did remove cholesterol by 62.4% after 24 h of incubation[22]. Cholesterol assimilation by probiotic bacteria in the gastrointestinal tract allows the reduced absorption of cholesterol by enterocytes, and more excretion of the cholesterol from the host, thus, lowering the serum cholesterol level, and potentially resulting in decreased risk of cardiovascular diseases[23]. Thus, cholesterol reduction by probiotic bacteria *via* assimilation, enzymatic degradation and/or other mechanisms may help managing hypercholesterolemia related CVDs. In this study probiotic isolate K2 exhibited significant cholesterol lowering activity *in vitro* indicating its potential for pharmacological applications.

Bile salt deconjugation potential of LAB isolates

Bile salt deconjugation mechanism is based on the ability of certain probiotics to enzymatically deconjugate bile acids, and there upon increasing their rates of excretion *via* feces[24]. Since, cholesterol being a precursor of bile acids, deconjugation enhances the conversion of cholesterol to bile acids replacing those lost during excretion, and thus resulting in lowering of the serum cholesterol level[24]. All the LAB probiotic isolates caused substantial reduction of bile salt and exhibited strong deconjugation activity when grown in MRS broth supplemented with bile salt sodium glycocholate. The probiotic isolate K2 deconjugated sodium glycocholate maximally and released an amount of 128.43 µM/mL of cholic acid after 24 h. Probiotic isolates M16, M17, F11 and F18 also exhibited considerable bile salt deconjugation activity and released 106.69, 101.47, 98.65 and 77.34 µM/mL of cholic acid, respectively (Fig. 1B). *Lactobacillus fermentum* CAT19 and *L. fermentum* CABA16 were reported to show bile salt deconjugation ability of sodium glycocholate, and released free cholic acid in the range of 0.12 to 0.40 µM/mL[24]. In general, bile salt deconjugation is species and strain dependent phenomenon which may explicate the variations in deconjugation ability of various strains.

Cholesterol lowering by live, dead and resting probiotic cells

The living, dead, and resting probiotic cells were examined for their cholesterol lowering potential. Among living cells, isolate K2 exhibited highest hypocholesterolemic ability of 72.46%, followed by isolate F11 (60.05%) after incubation of 24 h. Among dead cells, isolate K2, and F18 showed almost similar cholesterol lowering ability of 45.88 and 42.24%, respectively (Fig 1C). Cholesterol removal by dead cells might be attributed to cell surface adhesion of cholesterol by probiotic cells. Among resting cells (live but non-dividing), isolate F11 showed highest cholesterol lowering ability of 44.93, followed by isolate M16 (41.43%). Choi and Chang[13] found that living cells of *Lactobacillus plantarum* EM showed cholesterol removing ability of 88.12%. Miremadi *et al.*[25] observed that growing, resting and dead cells removed cholesterol in range of 34-65%, 29-56% and 9-37%, respectively. The cholesterol removal by resting and dead probiotic cells confirmed that their cellular membrane still had the ability to adhere to the cholesterol. Results indicated that cholesterol lowering phenomenon is not only displayed by living cells involving assimilation mechanism, but also by an adhesion mechanism (non-metabolic process) to cell surfaces of dead and resting cells[13,25]. Current study shows that maximum cholesterol lowering was exhibited by live and growing cells of probiotic LAB isolate K2, however, the resting and dead counterparts also displayed significant cholesterol lowering which suggests that the non-viable cells of the probiotics may also explored as potential cholesterol lowering agents.

SEM analysis for cell surface adsorption of cholesterol by probiotics

SEM analysis was performed to examine the adherence of cholesterol to probiotic cell surface, which is assumed to be a possible mechanism for cholesterol lowering. SEM analysis was executed for LAB isolate K2 grown in MRS broth without cholesterol Control (Fig. 2A), with cholesterol only (Fig. 2B), and with cholesterol and bile salt (Fig. 2C). Also SEM analysis was carried out for heat killed cells which were suspended in MRS broth containing cholesterol to determine the surface adsorption of cholesterol by heat killed cells (Fig. 2D). The results of SEM analysis of live and dead cells would give insights about the differences (if any) between the adhesion mechanisms employed by live and dead probiotic cells. The surface adhesion properties of probiotics represent important mechanism for cholesterol removal[13].

Probiotic isolate K2 clearly showed strong cholesterol binding capability to its cell surface when compared with control (Fig. 2B), thus, indicating its potential effectiveness for reducing the serum cholesterol level[13]. Similar cholesterol binding affinity

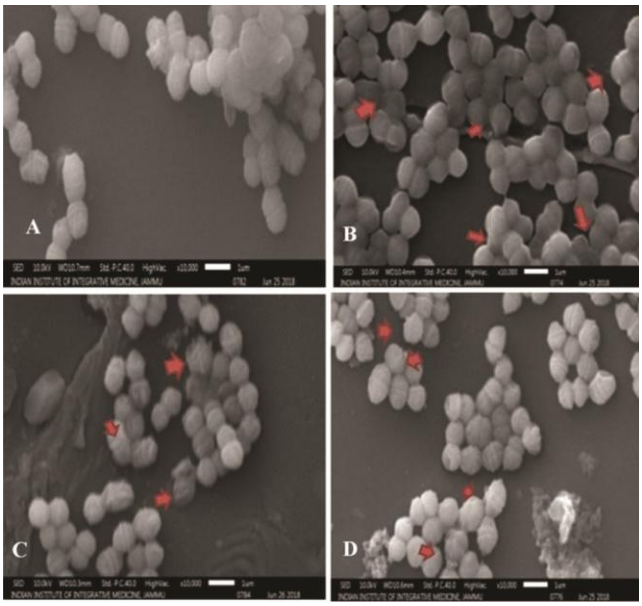

Fig. 2 — SEM analysis of cell surface adsorption based cholesterol removal by *Enterococcus faecalis* K2 (10000×); (A) Cells grown in MRS broth; (B) Control, cells grown in MRS supplemented with cholesterol; (C) Cells grown in MRS supplemented with cholesterol and bile salt; and (D) Heat killed cells suspended in phosphate buffer

was shown by probiotic strain K2 in MRS medium supplemented with bile salt (Fig. 2C). The cholesterol binding abilities can be attributed to chemical and structural properties of cell wall peptidoglycans containing amino acids that might have capability of binding to the cholesterol[26]. SEM micrograph of heat killed probiotic K2 cells also showed strong cholesterol binding affinity to cell surfaces (Fig. 2D). Similar results were shown by *Lactobacillus plantarum* EM and *L. acidophilus* ATCC 43121 where both probiotic strains showed cholesterol adhesion to cell membrane in dead state[13]. Cholesterol adherence was found to the surface of lactobacilli and the attachment of cholesterol was hypothesised to be dependent on growth[26]. Adherence of cholesterol to bacterial cell surface is a physical phenomenon that might be affected by cell wall polymers containing protein moieties which are able to bind cholesterol. This feature indicates that probiotic microorganisms both in their live, dead and attenuated forms may have potential application for lowering down the cholesterol level.

Antioxidant activity potential of LAB isolates

In the current study, antioxidant activity of LAB probiotic isolates was determined by DPPH radical, hydroxyl radical and superoxide radical scavenging potential. The isolate F18 showed the highest DPPH

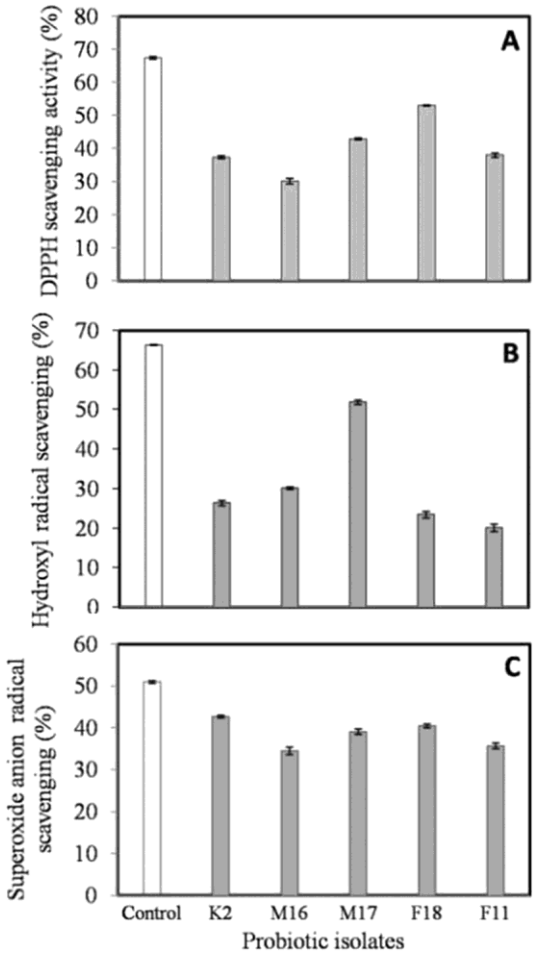

Fig. 3 — (A) Antioxidant activity potential of probiotic LAB isolates based upon DPPH free radical scavenging activity; (B) Hydroxyl radical scavenging activity; and (C) Superoxide radical scavenging activity

radical scavenging ability of 53.02% followed by M17 (42.93%), F11 (37.99%) and K2 (37.36%) (Fig. 3A). Similar to current study, *Lactobacillus rhamnosus* R4 displayed 53.78% DPPH scavenging ability[27]. All tested probiotic strains exhibited appreciable scavenging activities against cellular toxic substance like DPPH radicals to various degrees, indicating their detoxifying effects. Probiotics possess antioxidant enzymatic systems and one of the best known of these enzymes is superoxide dismutase (SOD) which catalyzes the breakdown of superoxide into hydrogen peroxide and water and is therefore, a central regulator of reactive oxygen species (ROS) levels. Additionally, probiotics produce other metabolites with antioxidant activity, such as glutathione (GSH), butyrate, and folate which help in neutralization of ROS[8]. Strong DPPH radical scavenging activity of probiotic bacteria indicates

their high antioxidant potential. Though oxidation is important for energy generation in the living organisms, but oxidative stress can lead to severe damage to the biomolecules of vital importance for various physiological and metabolic functions. Antioxidative properties of probiotics thus, may play an important role in combating the drastic effects of ROS.

Antioxidant potential based upon hydroxyl radical scavenging activity was also studied for LAB isolates. The reactive free radicals induce oxidative damage to biomolecules, and results in manifestation of cancer and several other diseases[15]. In the current investigation, LAB isolate M17 showed the highest hydroxyl radical scavenging ability of 51.88% (Fig. 3B) followed by M16 (30.12%), K2 (26.35%) and F18 (23.43%). The isolate F11 showed lowest scavenging ability of 20.08% Similar results were observed for *Lactobacillus rhamnosus* R4 which displayed hydroxyl scavenging ability[27]of 45.79%. Similarly, intracellular cell-free extracts of the 11 *L. plantarum* strains demonstrated hydroxyl radical scavenging activity in a dose-dependent manner within the cell concentration range of 10^8-10^{10} cfu/mL[15]. Hydrogen peroxide is a weak oxidant, but it is extremely diffusible and has longer half life. These two properties of hydrogen peroxide lead to the oxidative damage either directly or due to formation of hydroxyl radicals on reaction with transition metal ions. Significant hydroxyl scavenging activity of probiotic isolates shows their adequate antioxidative potential.

Antioxidant potential of the LAB isolates was also examined by superoxide radical scavenging activity. The LAB isolate K2 showed the highest superoxide radical scavenging ability of 42.67% (Fig 3C) followed by F18 (40.46%), M17 (39.09%), and F11 (35.73%). Similar to current study, *Lactobacillus plantarum* MA2 displayed superoxide radical scavenging ability[28] of 40.22 ± 0.03%. Reactive oxygen species such as superoxide anion is produced during cellular metabolism and plays an important role in cell signalling, apoptosis, gene expression and ion transportation. However, when such ROS molecules are generated in excess or cellular defences are deficient, biomolecules including protein, lipid, and nucleic acids get damaged due to oxidative stress. Such damage may lead to a variety of age-related degenerative diseases, such as cancer, Alzheimer's disease, and Parkinson's disease[15]. Superoxide dismutase scavenges superoxide anions which are precursors of hydrogen peroxide, hydroxyl radical, and singlet oxygen which might induce oxidative damage to lipids proteins and DNA.

In vitro biosafety evaluation of LAB probiotic isolates.

Safety evaluation of probiotics is absolutely essential to ensure their usage for various health benefits for human beings. All the five LAB probiotic isolates were evaluated for their safety aspects *viz.*, blood hemolysis, gelatin hydrolysis and biogenic amines production.

Hemolytic test analysis of the LAB isolates *i.e*. K2, M16, M17, F11 and F18 on blood (bovine) agar plates, showed that none of the probiotic isolates showed α- or β- hemolytic activity (Table 2). For safety concern, it is desired that probiotics should not exhibit haemolysis. Similarly, *Lactobacillus fermentum* strains did not show β-haemolytic activity, however, *L. fermentum* 0-25A and *L. fermentum* 12-18A strains showed α-haemolytic activity[17]. In view of the fact that none of the tested strains showed blood hemolysis so these may be considered biologically safe, and may be further explored for other functional aspects and application potential.

Gelatin hydrolysis ability of the probiotic isolates (K2, M16, M17, F11, and F18) is determined by their ability to produce gelatinase (proteolytic enzyme) that liquefies the gelatin. Two of the probiotic isolates *i.e*. M17 and F18 hydrolyzed the gelatin as indicated by appearance of hydrolytic zones on gelatin agar plates. However, isolate K2, M16 and F11 did not show any gelatin hydrolysis on gelatin agar plates.

Table 2 — Biosafety analysis of LAB probiotic isolates based upon hemolysis, gelatin hydrolysis and biogenic amines production

Probiotic LAB isolates	Hemolysis pattern			Gelatin hydrolysis	Biogenic amines
	Growth	Zone	Hemolysis		
K2	-	-	-	-	-
M16	-	-	-	+	-
M17	-	-	-	+	-
F11	-	-	-	-	-
F18	-	-	-	+	-

Table 3 — Antibiotic susceptibility analysis* of probiotic LAB isolates

Probiotic LAB isolates	Antibiotics*									
	P	GEN	VA	E	AMP	C	FC	CD	K	TE
K2	S	S	R	S	S	S	S	S	S	S
M16	S	S	R	S	S	S	S	S	S	S
M17	S	S	S	S	S	S	S	S	S	S
F11	S	S	R	S	S	S	S	S	S	S
F18	S	S	S	R	S	S	S	S	S	S

*S- susceptible; R-resistant;

Antibiotics* P -Penicillin-G , GEN- Gentamycin, VA- vancomycin, E- Erythromycin, AMP-Ampicillin, C- Chloramphenicol, FC- Fusidic acid, CD- Clindamycin, K- Kanamycin, TE- Tetracycline

Ramesh *et al.*[29] observed that KADR5 and KADR6 strains of *Bacillus* sp. isolated from the gut of *Labeo rohita* showed considerable gelanotilytic activity.

Biogenic amines production assay was done for LAB isolates. Several toxic conditions and food poisoning cases among human are associated with histamine and tyramine amines. Secondary amines may undergo nitrosation and form nitrosamines[30]. Therefore, the probiotic isolates were studied for their ability to produce biogenic amines using decarboxylation medium containing tyrosine and histidine. The results indicated that no biogenic amine like tyramine and histamine was produced by any of the probiotic LAB isolates. Similar to current study, none of seven strains tested for histidine, lysine, ornithine, and tyrosine, showed any of the amine production[9]. However, *Lactobacillus casei* (TISTR 389) and *Lactobacillus delbrueckii* subsp. bulgaricus (TISTR 895) produced biogenic amines histamine and tyramine[31]. Therefore, careful screening for biogenic amines production is recommended before selecting probiotics for appropriate application in food, dairy and pharmaceutical industry.

All the LAB isolates were examined for their antibiotic susceptibility. Antibiotic susceptibility is one of the essential functional characteristic of potential probiotics, and probiotics intended for human/animal application must not possess any antibiotic resistance against commonly used antibiotics[32]. Most of the LAB probiotic isolates showed susceptibility towards ten conventionally prescribed antibiotics (Table 3). However, strain K2, M16, and F11 showed moderate resistance towards vancomycin which might be due to interference of vancomycin[33] in cell wall peptidoglycan synthesis. Similarly, *Lactobacillus* strains isolated from pharmaceutical and dairy products were highly resistant to vancomycin and streptomycin[34].

Molecular identification of LAB isolate equipped with the most desired characteristics

The LAB probiotic isolate K2 from *kalarei* exhibited ample hypocholesterolemic potential, antioxidant activity and safety attributes, thus, was selected for its identification by 16S rDNA sequence analysis. The 16S rDNA sequence of LAB isolate K2 showed close resemblance (97%) with that of several of *Enterococcus faecalis* strains available in GenBank database (National Centre for Biotechnology Information, NCBI). Therefore, the strain was designated as *Enterococcus faecalis* K2. The sequence was deposited in GenBank under the accession number MG754449.

Conclusion

The current study was carried out to study the cholesterol lowering potential of LAB probiotic isolates from indigenous sources. Several of the isolates exhibited hypocholesterolemic and antioxidant potential, and qualified for the bio-safety aspects. The probiotic strain *Enterococcus faecalis* K2 was found to be the most potential cholesterol lowering strain that also possessed other functional attributes. The cholesterol lowering by probiotic *E. faecalis* K2 was mediated by several mechanisms like assimilation, bile salt deconjugation, and cells surface adhesion. Even the dead cells showed sufficient cholesterol removing ability. However, for harnessing full biotechnological potential of *E. faecalis* K2 in food/feed and pharmaceutical industry, further *in vivo* studies must be executed for cholesterol lowering activity and other functional attributes.

Acknowledgement

Dr. Bijender Kumar Bajaj gratefully acknowledges VLIR-UOS (Government of Belgium) for 'Short Research Stay Scholarship' ('SRS' Scholarship) at the

Department of Bioscience Engineering, University of Antwerp, Antwerpen, Belgium, Department of Science and Technology (Government of India), for granting Research Project (Ref. SR/SO/BB-66/2007), and Department of Biotechnology (DBT), Government of India, for financial support. Ms Bilqeesa Bhat gratefully acknowledges the Council of Scientific and Industrial Research (CSIR) for CSIR-Senior Research Fellowship for Doctoral Research.

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