Antioxidative potential of folate producing probiotic *Lactobacillus helveticus* CD6

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Revised: 12 January 2011 / Accepted: 16 January 2011 / Published online: 14 February 2011
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Abstract Folate producing *Lactobacillus* sp. CD6 isolated from fermented milk showed 98% similarity with *Lactobacillus helveticus* based on 16S rRNA gene sequence analysis. It was found to produce a folic acid derivative 5-methyl tetrahydrofolate (5-MeTHF). The intracellular cell-free extract of strain demonstrated antioxidative activity with the inhibition rate of ascorbate autoxidation in the range of $27.5\% \pm 3.7\%$. It showed highest metal ion chelation ability for Fe^{2+} (0.26 ± 0.06 ppm) as compared to Cu^{2+} . The DPPH (α, α -Diphenyl- β -Picrylhydrazyl) radical scavenging activity for intact cells were found to be $24.7\% \pm 10.9\%$ proved its antioxidative potential. Furthermore, it demonstrated 14.89% inhibition of epinephrine autoxidation, 20.9 ± 1.8 μg cysteine equivalent reducing activity and $20.8\% \pm 0.9\%$ hydroxyl radical scavenging effect. The strain was evaluated for probiotic properties as per WHO and FAO guidelines. It showed 90.61% survival at highly acidic condition (pH 2.0), 90.66% viability in presence of synthetic gastric juice and 68% survivability at 0.5% bile concentration for 24 h. It was susceptible to many antibiotics which reduces the prospect to offer resistance determinants to other organisms if administered in the form of probiotic preparations. It showed *in vitro* mucus binding and antimicrobial activity against enteric

pathogens like *Salmonella typhimurium* (NCIM 2501), *Streptococcus pyogenes* (NCIM 2608), and *Staphylococcus aureus* (NCIM 5021) and moreover it showed non-hemolytic activity on sheep blood agar.

Keywords Probiotic · *Lactobacillus helveticus* · Fermented milk · 5-methyl tetrahydrofolate · Ascorbate autoxidation

Introduction

Lactic acid bacteria (LAB) are the most beneficial part of the intestinal microbiota, since early studies of Metchnikoff had proved (Metchnikoff 1908) the favorable effects of soured milk products in humans. LAB comprise wide range of genera including a considerable number of species which are often found in commercial probiotic preparations (Anonymous 1990), but spore-forming bacilli or bifidobacteria may be used as well. A probiotic has been defined by the ILSI (International Life Sciences Institute), Europe working group as “a viable microbial food supplement which beneficially influences the health of the host” (Salminen et al. 1998). This definition depicts the need of safety and efficacy of probiotics to be demonstrated scientifically for each strain and product comprising it while demonstration of health effects includes mechanistic investigations and clinical studies with human subjects. The same ILSI Europe working group also defined probiotic foods as functional, if they have been satisfactorily demonstrated to beneficially affect one or more target functions in the body beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and or reduction in the risk of diseases (Diplock et al. 1999; Vanaja et al. 2011; Ghosh and Chattopadhyay 2010). FAO & WHO (2002) have defined

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probiotics as, ‘Live micro-organisms which when administered in adequate amounts confer a health benefit on the host’. These definitions have set the basis for the assessment of the health promoting potential of probiotics.

Human nutrition is associated with oxidative metabolism, which beside production of energy is involved in a number of vital functions of the host. Under physiological conditions, the reactive species (including peroxy radicals, nitric oxide radical, and superoxide anion) stature a crucial role in primary immune defence of the human body by phagocytic cells against harmful microorganisms (Halliwell and Gutteridge 1999). On the other hand, a prolonged excess of reactive species are highly damaging for the host biomolecules and cells, exerting a variety of pathological effects (e.g. DNA damage, carcinogenesis and cellular degeneration) and inducing many diseases including aging, cancer, atherosclerosis, diabetes and rheumatoid arthritis (Seifried et al. 2007; Valko et al. 2007; Verma and Banerjee 2010). Humans and other organisms possess antioxidative defence and repair systems evolved to protect them against oxidative damage, but these systems are not effective enough to prevent the damage totally (Simic 1988). In order to reduce oxidative damage, various synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene were proposed and used. However, these synthetic antioxidants are considered to be responsible for liver damage and carcinogenesis (Grice 1988). This necessitates searching for natural antioxidants that can protect human body from free radicals and retard the progress of many chronic diseases.

Folic acid (FA) plays a key role in the biosynthesis of purines and pyrimidines and regeneration of methionine. *In vivo*, FA is reduced to dihydrofolate (DHF) and subsequently to tetrahydrofolate (THF). THF is enzymatically converted into 5-methyl tetrahydrofolate (5-MeTHF), the actual active form (Stanger 2002). Neurological disorders and cardiovascular diseases are the most severe crisis in human health whose origin lies mainly in FA and vitamin B₁₂ deficiency. FA is key factor in preventing neural tube defects (NTD), neurological disorders, and cancer including cervical, lung, brain, pancreatic, colorectal and breast cancer (Duthie et al. 2002; Wolff et al. 2009).

The present study was intended to isolate a potential folate producing probiotic *Lactobacillus* sp. from fermented milk and study their antioxidative ability in order to enhance antioxidative and nutritional status of food for the benefits of consumers.

Materials and methods

Isolation of bacterial strain Bacterial strain was isolated from conventionally fermented milk from a household by

using microbiological techniques. The samples were collected and serially diluted in sterile phosphate buffer saline pH 7.3 (PBS). A 100 µl of respective dilution was spread on de Man-Rogosa-Sharpe (MRS) medium agar plates (Himedia, Mumbai) and incubated for 24–48 h at 37 °C. Colonies with different morphologies were selected at random and identified further by Gram staining, morphological and biochemical characteristics especially mannitol utilization, casein hydrolysis, phosphoketolase, growth at high temperature and high salt concentration (Axelsson 2004; Holt et al. 1994). Glycerol stocks were prepared and stored at –86 °C. The working cultures were maintained at 4 °C in MRS broth and MRS stabs. Working cultures were sub cultured at every week in MRS broth.

Strain identification A selected bacterial strain was identified by 16S rRNA gene sequence analysis followed by phylogenetic studies. Universal primers 16S27F (5'-CCAGAGTTT GATCMTGGCTCAG-3') and 16S1488R (5'-ACATTTCA CAACAC GAGCTG-3') (Brosius et al. 1978) were used for the amplification of 16S rRNA gene of the isolate.

Screening for folate production

The folate production ability of selected isolates was determined at 37 °C by inoculating single colony in folic acid assay medium (Himedia, Mumbai). The 18 h old culture supernatant was subjected further for disc diffusion assay and HPLC analysis.

Disc diffusion assay The disc diffusion assay was performed as per the method described by Vakil and Shahani (1968) with major modifications. A 100 µl of assay organism *Lactobacillus casei* NCIM 2364 was seeded in 15 ml of FA assay medium containing 0.46% agar. Plates were allowed to solidify and paper discs of uniform size, impregnated with known concentrations of standard FA (Pte Glu) (Fluka) were placed on top of the seed agar plate along with test sample and control. The plates were kept at cool (4 °C) for 20 min. Then all plates were incubated at 37 °C for 18–24 h for zone of exhibition.

Folate analysis by high pressure liquid chromatography (HPLC) The chromatographic analysis was performed using Knauer HPLC (Germany) with isocratic pump using Eurospher C18 reverse phase column (100–5, 250×4.6 mm with pre-column) as stationary phase, equipped with UV-visible detector (280 nm), interrogator (Iris 32, Lite data processor) and acetonitrile: water (95:05, v/v) as mobile phase with a flow rate of 1 ml /min. Individual folate

standards, tetrahydrofolate (THF), 5-methyl tetrahydrofolate (5-MeTHF), and FA (Pte Glu), were purchased from Sigma, Fluka and used without further purification.

Studies on antioxidative potential

Growth conditions and media The culture was grown at 37 °C in fresh modified MRS (mMRS) medium in which magnesium sulfate, manganese sulfate, and dipotassium phosphate were eliminated from the formula (Lin and Yen 1999). Strain was serially transferred at least three times in mMRS prior to use in antioxidative assays.

Preparation of intracellular cell-free extract (ICFE) It was obtained as per the method of Lin and Yen (1999) with minor modifications. Bacterial cells were harvested by centrifugation at 4,000×g for 10 min after 18 h of incubation in mMRS broth at 37 °C. Cell pellets were then quickly washed twice with deionized water and resuspended in same for ultrasonic disruption. Sonication was performed at 130 W with 30% amplitude for 5 min in 50 s on/10 s off cycle by keeping it in ice bath using sonicator (VCX 130, Sonics Vibra cell, USA). Cell debris were removed by centrifugation at 6,000×g for 10 min and resulting supernatant was used as ICFE for antioxidative studies. The colony forming units (CFU) were determined prior to preparation of ICFE.

Inhibition of ascorbate autoxidation The antioxidative activity of isolate was assayed by the method of inhibition of ascorbate autoxidation (Mishra and Kovachich 1984). A 0.1 ml of ICFE or buffer which served as control was mixed with an ascorbate solution (0.1 ml, 5.0 mM) and phosphate buffer (9.8 ml, 0.2 M, pH 7.0). After 10 min incubation at 37 °C, A_{265} was measured by an UV spectrophotometer (UV-VIS 1601, Shimadzu, Japan). The ascorbate autoxidation inhibition was then calculated as, % Inhibition effect = $[1 - A/A'] \times 100$, where A : test and A' : control.

Reducing activity assay The method developed by Oyaizu (1986) was used to evaluate the reducing activity of isolate. A 0.5 ml of potassium ferricyanide (1%) was mixed with the same volume of phosphate buffer (0.02 M, pH 7.2) and ICFE. The reaction mixture was incubated at 50 °C for 20 min, and then cooled rapidly, and 0.5 ml of trichloroacetic acid (10%) was added. A 0.5 ml of upper phase was obtained by centrifugation at 300×g for 5 min, and 0.2 ml of FeCl_3 (0.1%) was added and A_{700} was recorded. Cysteine was used as the standard for expression of reducing activity.

Metal ion chelating assay The chelating ability of ICFE of isolate for ferrous and cupric ions was determined. The ferrous ion chelating ability of ICFE of isolate was determined by using method of Yamauchi et al. (1984) with some modification. A 0.5 ml of ICFE was mixed with 0.1 ml of ascorbate (1%), 0.1 ml of FeSO_4 (0.4%), and 1 ml of NaOH (0.2 M). The mixture was incubated at 37 °C in water bath; 0.2 ml of trichloroacetic acid (TCA; 10%) was added following incubation for 20 min. Supernatant was obtained by centrifugation at 300×g for 10 min, and 0.5 ml of *o*-phenanthroline (0.1%) was added. After 10 min of reaction, A_{510} was measured.

The cupric ion chelating ability of ICFE of isolate was determined as per the method described by Looyenga and Boltz (1971) with certain modifications. A 0.1 ml of ICFE (ultra pure water as control) was mixed with 0.1 ml of CuSO_4 (100 μM), 0.2 ml of HCl (0.02 M), and 0.1 ml of HOCl (0.5%). This mix was incubated at 37 °C for 20 min; 0.2 ml of pyrrolidinedithiocarbamate ammonium salt (APDTC; 0.1 %) was added and allowed to stand for 5 min. The extract was obtained by adding 2 ml of chloroform with vigorous shaking for 1 min. The content of Cu in extract was determined by using S2-Atomic absorption spectrophotometer (Thermo Electron Corporation, USA).

Hydroxyl radical scavenging assay The hydroxyl radical scavenging activity was measured by the method of Jin et al. (1996). The hydroxyl radical was generated in the mixture containing 1 ml of 0.75 mM 1, 10-phenanthroline, 1.5 ml of 0.15 M sodium phosphate buffer (pH 7.4), 1 ml of 0.75 mM FeSO_4 and 1 ml of H_2O_2 (0.01%, v/v). After addition of 1 ml of ICFE (test sample), the mixture was incubated at 37 °C for 30 min and A_{536} was measured. The % hydroxyl radical scavenging activity = $(A_{\text{test}} - A_{\text{blank}})/(A' - A_{\text{blank}}) \times 100$, where A' was the absorbance of the deionised water instead of H_2O_2 and test in the assay system. Deionised water and ascorbic acid were used as the blank and control, respectively.

DPPH radical scavenging activity The scavenging of α, α -Diphenyl- β -Picrylhydrazyl (DPPH) radical by ICFE and intact cells of isolate was analyzed by modifying the method of Shimada et al. (1992). A 0.8 ml of test and 1 ml of freshly prepared DPPH solution (0.2 mM in methanol) were mixed and allowed to react for 30 min in dark. Blank samples contained pure methanol or PBS (pH 7.3). The scavenged DPPH was then monitored by measuring the decrease in A_{517} . The % scavenging ability was determined as follows;

$$[A_{\text{blank}} - A_{\text{test}}/A_{\text{blank}}] \times 100.$$

Superoxide dismutase (SOD) activity and its induction It was performed by the method of Sun and Zigman (1978) with certain modifications. ICFE was prepared from the culture and SOD activity was analyzed. In brief, 0.3 ml of ICFE or ultra pure water (used as a control), 2 ml of 50 mM sodium carbonate buffer (pH 10.5), 0.3 ml (0.3 mM) epinephrine were mixed together. The mixture was transferred to cuvette and A_{320} was measured for 3 min.

$$\% \text{ Inhibition effect} = [A_{\text{control}} - A_{\text{test}} / A_{\text{control}}] \times 100.$$

The SOD induction studies were done by growing culture in modified MRS containing 0.01% metal ion such as Mn^{2+} , Fe^{2+} , Cu^{2+} , and Zn^{2+} at 37 °C for 18 h (Lin and Yen 1999).

SOD detection by native gel electrophoresis The presence of SOD in ICFE of isolate was determined by using 10 % non-denaturing polyacrylamide gel electrophoresis. Electrophoresis was performed at 50 V for 3–4 h in tris-glycine buffer (pH 8.3). After electrophoresis, a modified photochemical method of Beauchamp and Fridovich (1971) was used to visualize SOD activities. The gel was first soaked in 1.225 mM nitroblue tetrazolium solution for 15 min, briefly washed, then soaked in 100 mM potassium phosphate buffer, pH 7.0, containing 0.028 mM riboflavin and 28 mM TEMED for another 15 min. After the gel was briefly washed, it was illuminated on a light box with intensity of $30 \text{ uE s}^{-1} \text{ m}^{-2}$ for 15 min to initiate the photochemical reaction. Duplicate gel was subjected for coomassie brilliant blue staining for protein pattern of ICFE of isolate.

Evaluation of probiotic properties

Acid tolerance Culture was grown overnight in MRS broth at 37 °C followed by centrifugation at $8,000 \times g$ for 5 min. Cell pellet was harvested to wash twice in sterile phosphate buffered saline (PBS) pH 7.3 and resuspended in 1 ml of PBS. The culture suspension was diluted 1:100 in PBS at pH 1, 2, and 3. Mixture was incubated at 37 °C and viable bacterial cells were determined at 0, 1, 2, and 3 h time interval by plating on MRS agar plates. Growth of bacteria was expressed in colony forming units per milliliter ($\log_{10}$ CFU/ml) and the percentage survival of strain at different pH values was calculated.

Gastric juice tolerance Gastric juice tolerance was determined as per method of Pedersen et al. (2004) with some modifications. Overnight grown culture of isolate was cell pelleted to wash with PBS (pH 7.3) and suspended in same. The cell suspension was diluted 1:10 in synthetic gastric juice and incubated at 37 °C. Samples were taken at 0, 30, and 180 min. intervals and survival rate was measured by

spreading 100 μl sample on MRS agar plates, which were incubated at 37 °C for 24 h. Growth of bacteria was expressed in colony forming units per milliliter ($\log_{10}$ CFU/ml).

Bile salt tolerance It was determined by inoculating 100 μl overnight grown culture in 900 μl growth medium supplemented with 0.2%, 0.3%, 0.5%, 1.0%, 1.5%, and 2.0% bile salt and was incubated at 37 °C for 24 h. The viable bacteria were enumerated by plating 100 μl of culture on to the MRS agar plate incubated at 37 °C for 24 h. Growth of bacteria was expressed in colony forming units per milliliter ($\log_{10}$ CFU/ml) and the percent survival of strain was then calculated.

Antimicrobial activity Agar spot test was used to detect antimicrobial activities of test organism against potent enteric pathogens. Overnight grown culture of test organism was spotted onto the surface of MRS medium plate and culture was allowed to grow under micro-aerophilic condition at 37 °C for 24 h. Enteric pathogens like *Salmonella typhimurium* NCIM 2501, *Streptococcus pyogenes* NCIM 2608 and *Staphylococcus aureus* NCIM 5021 were inoculated at a concentration of 10^6 cells/ml in 7 ml of soft nutrient medium containing agar agar (0.7%) overlaying the test organism CD6 spots. These plates were incubated at 37 °C for 24 h and growth inhibition of pathogens was observed.

Antibiotic susceptibility test Antibiotic drug susceptibility was determined by seeding 0.1 ml (10^6 cells/ml) of overnight grown culture in 100 ml molten semisoft MRS agar. Standard antibiotic discs (Himedia, India) were placed on the surface of the seeded agar medium. Plates were observed for zones of inhibition after 24 h of incubation at 37 °C.

Hemolytic activity Blood hemolysis was examined on MRS agar plates supplemented with 5% sheep blood, which were incubated at 37 °C for 24 h.

Mucin binding assay The bacteria were grown at 37 °C in MRS broth medium supplemented with 0.1% mucin Type-II from porcine stomach (Sigma) for 24 h to induce binding. Wells of microtitre plate were coated with mucin (150 μl per well) $100 \mu\text{g l}^{-1}$ in 50 mM Na_2CO_3 buffer, pH 9.7, incubated overnight at 4 °C with slow rotation (Pedersen et al. 2004). Wells were blocked with PBS with 1% Tween 20 for 1 h and washed with PBST (PBS supplemented with 0.05% Tween 20, pH 7.3). The bacterial strains were grown as described above, washed once in PBST and diluted to $A_{595} = 0.5 \pm 0.02$ in the same buffer. The Bacterial suspension (100 μl) was added to each well and incubated for 1 h at room temperature. The wells were washed with

PBST and binding was examined with an inverted microscope TCM-400 (Labomed, USA). The buffer was poured off and, after wells had dried, the A_{450} was measured by using micro plate reader (Model 680, BioRad, Japan). All measurements were obtained in triplicate.

Bacterial adhesion to solvent Bacterial adhesion to solvent was measured according to Rosenberg et al. (1980) with some modifications. Isolate was grown overnight at 37 °C. The cells were pelleted at 8,000×g for 5 min and washed twice with PBS pH 7.3, resuspended in 0.1 M KNO₃ (pH 6.2) and A_{600} was measured as A_0 . The 1 ml solvent was added to 3 ml of cell suspension. After 10 min pre-incubation at room temperature, two phases were mixed by gentle vortexing for 2 min. The aqueous phase was removed after 20 min of incubation at room temperature and A_{600} was measured as A_1 . The percentage of bacterial adhesion to solvent was calculated as $(A_0 - A_1/A_0) \times 100$.

Autoaggregation assay Autoaggregation assay was performed according to Del Re et al. (2000) with certain modifications. Isolate was grown over night at 37 °C in MRS broth. The cells were pelleted and washed twice with PBS (pH 7.3) and resuspended in PBS to get A_{600} to 0.5 of which 4 ml was mixed by gentle vortexing for 10 s and incubated at 37 °C for 1 h. After incubation, absorbance of upper suspension was measured. Autoaggregation percentage was expressed as: $A_0 - (A_t/A_0) \times 100$, where A_t represents the absorbance at time $t=1$ h and A_0 the absorbance at $t=0$.

Statistical analysis

The data was subjected to one way analysis of variance (ANOVA) using the Graph Pad InStat® (Version 3.01, GraphPad Software Inc.). The differences in the mean values in each treatment were compared and significance level was calculated at $P<0.01$ and $P<0.001$ level.

Results and discussion

Isolation and identification of folate producing bacteria

Lactobacillus and bifidobacteria create a healthy and beneficial environment and protect from potentially harmful microorganisms in the gut ecosystem when they are present in sufficient numbers. The fermented milk is known as a good source of such organisms since long. Evaluation of the health related beneficial functions of such strains from fermented foods are important and hence its incorporation

in commercial dairy products and foods is highly desired. In this context, we selectively isolated bacterial strain from fermented milk. Three different colonies on MRS agar CD5, CD6, and CD7 were selected and identified further by Gram staining, morphology and biochemical characterization. Owing to better growth in folic acid deficient medium indicative of folate production and based on the morphological and biochemical characteristics, isolate CD6 was selected and employed throughout these studies. Colony morphology of CD6 appeared small (0.2 to 2 mm), grayish brown with entire margins, convex, rough, and opaque on MRS agar. Microscopically, it appeared straight rod shaped Gram positive bacilli. It showed no bubbling when hydrogen peroxide was added, hence considered as catalase negative. Utilization of glucose was detected without production of gas in Durham's tube and failed to show pentose fermentation. It was able to produce acid on utilization of mannitol. Casein hydrolysis and phosphoketolase activity was negative. It showed growth at high temperature (45 °C) and high salt concentration such as 6.5% NaCl. CD6 was identified with 16S rRNA gene sequence analysis where it exhibited 98% similarity with *Lactobacillus helveticus*.

This culture produced folic acid which was further detected by disc diffusion assay and confirmed by HPLC analysis. The cell-free extract of isolate showed distinguishable growth exhibition of auxotrophic mutant *Lactobacillus casei* NCIM 2364 and peak at 6.5 min of retention time in HPLC chromatogram, which was indicative of production of 5-MeTHF (data not shown). Considering this fact strain CD6 was screened for antioxidative potential and probiotic properties.

Studies on antioxidative potential

Free radicals are produced in the human body in the course of regular metabolism, but when exposed to xenobiotic agents from foods and environment, the risk of radical production significantly increases. The oxidative damage is related to many diseases and generated a risk of atherosclerosis, and other civilization diseases (Agerholm-Larsen et al. 2000). In order to evaluate antioxidative potential of LAB from conventionally fermented milk; a variety of different antioxidant assay systems were implicated in this study. The approx $7.5 \pm 0.2 \text{ Log}_{10}$ CFU/ ml were used for antioxidant studies.

The modified MRS medium containing no added metals was executed in ascorbate system to reduce the catalytic metal dependant ascorbate autoxidation (Buettner 1990). The values obtained by ascorbate autoxidation inhibition were higher than that of strains of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Bifidobacterium longum* (Lin and Yen 1999) (Table 1).

Table 1 Antioxidative potential of *Lactobacillus helveticus* CD6

Antioxidative potential	Intracellular cell-free extract	Intact cells
Inhibition of ascorbate autoxidation (%)	27.5±3.7	n/d
Reducing activity (equivalent cysteine, µg)	20.9±1.8	n/d
DPPH radical scavenging activity (%)	6.8±2.0	24.7±10.9
Hydroxyl radical scavenging effect (%) (equivalent ascorbate, µg)	20.8±0.9	n/d
Metal ion chelating assay (ppm)		
Ferrous ions	0.26±0.06	n/d
Cupric ions	0.03±0.0	

Mean (± standard deviation) of results from three separate experiments ($n=3$); n/d: not determined

DPPH (α,α -diphenyl- β -picrylhydrazyl) radical

The deficiencies and overload of transition metals especially iron and copper leads to generate oxidative stress (Evans and Halliwell 2001). It has been proposed that copper ion is required for caeruloplasmin, which loads iron on to transferrin, and CuZnSOD. On the other hand these metals are responsible for skin damage, coronary heart diseases, and Alzheimer's (Sarri 1989; Smith et al. 1997). Therefore, the chelating ability of LAB towards iron and copper ions was evaluated. The ICFE of isolate showed higher Fe^{2+} ion chelation than that of Cu^{2+} ion. These results are in agreement with studies of metal ion utilization by *Lactobacillus sakei* (Duhutrel et al. 2010). The factors responsible for metal ion chelation were not well understood in LAB (Duhutrel et al. 2010; Lin and Yen 1999). Although, iron chelators of probiotic cultures viz. *Escherichia coli*, *Bacillus* spp. are thought to be accountable for iron nutrition (Ahire et al. 2010, 2011; Patel et al. 2010).

Hydroxyl radicals are the most reactive moieties among the reactive oxygen species and are known for lipid peroxidation and DNA damages (Halliwell and Chirico 1993). Iron plays central role in the hydroxyl radical formation via Fenton reaction. The ICFE of isolate showed hydroxyl radical scavenging activity equivalent with ~ 8 µg of ascorbic acid *in vitro*. The % hydroxyl radical scavenging activity was found to be comparable with *Lactobacillus fermentum* (Wang et al. 2009). In addition, the iron chelation states inhibition of hydroxyl radical formation via Fenton reaction.

Both intact cells and ICFE of isolate showed good antioxidant effects on DPPH radical scavenging *in vitro*. Although the conditions in gastrointestinal tract are very complicated, the results of Kaizu et al. (1993) showed that the intracellular extract is antioxidative *in vivo* too. The reducing ability of strain claims its potential to inhibit oxidation. It is

very well known that those substances exhibited reducing activity having potential to decompose hydroperoxides to hydroxyoctadecadienoic acids (Gardner 1975).

SOD is an important physiological defence mechanism in neutralization of superoxides among aerobic organisms. The studies of Sanders et al. (1995) demonstrated the presence of such a key enzyme in *Lactococcus* sp. preventing damage caused by toxic superoxides. In this study, ICFE of isolate exhibited 14.89% inhibition of epinephrine autoxidation. The effect of metal ions on SOD activity was analyzed by using one way analysis of variance (ANOVA) in which mMRS used as control. After addition of Fe^{2+} , Cu^{2+} the SOD activity was not affected significantly, however; on addition of Mn^{2+} and Zn^{2+} , the activity was significantly reduced. The P value for Mn^{2+} was found to be extremely significant ($***P<0.001$) while Zn^{2+} was very significant ($**P<0.01$) (Fig. 1). Further, the effect of metal ions on survival of bacteria was analyzed. Addition of Fe^{2+} , Mn^{2+} , and Zn^{2+} ions was not significant ($ns; P>0.05$) in the context of survival (%) of bacteria when compared to control (mMRS) while on addition of Cu^{2+} , survival was significantly decreased. The P value in case of Cu^{2+} was found to be extremely significant ($***P<0.001$) (Fig. 2). On the basis of analysis, SOD activity of ICFE of isolate was not dependent on metal ions as well as number of viable cells, except Mn^{2+} which drastically decreased the activity to negative value which was undesired. Furthermore, the absence of SOD in activity staining revealed that some other substances also may be responsible for scavenging of superoxides (Lin and Yen 1999) (Fig. 3).

Studies on probiotic properties

Culture CD6 showed $\sim 90\%$ viability in highly acidic condition (Table 2) and synthetic gastric juice (pH 2.5) containing pepsin, lysozyme. This survival was quite high than earlier reported *Lactobacillus acidophilus* A12 (Na-

Fig. 1 Effect of different metal ions on SOD induction. Data are mean ± SD. Error bars represent SD. ns no significance; $**P<0.01$, $***P<0.001$; $n=3$ per group

Fig. 2 Effect of different metal ions on survival of bacteria. Data are mean $\pm$ SD. Error bars represent SD. ns no significance; *** $P < 0.001$; $n = 3$ per group

kyoung et al. 2008) which ensured the arrival of suitable concentration of viable cells in to the intestine, and exerts their probiotic effect. The strain showed 100%, 98%, and 68% survival at varying concentrations of bile salt such as 0.2, 0.3, and 0.5 g % for 24 h, whereas, it failed to survive in 1.0, 1.5, 2.0 g % concentrations of bile salt. This behavior allowed to predict the potential of this strain as a probiotic, since it survived (0.5%) bile concentration which was higher than physiological concentration in the duodenum (Brashears et al. 2003) as well as the concentration (0.3%) which has previously been applied by other investigators (Kuhle et al. 2005). Colonization in intestinal wall is an important desirable property for probiotic

Table 2 Acid tolerance of *Lactobacillus helveticus* CD6

pH	Viable bacterial count (log ₁₀ CFU/ml) incubation period (h)				% Survival (after 3 h)
	0	1	2	3	
1	9.48±0.0	0*	0*	0*	—
2	9.48±0.0	9.32±0.02	9.10±0.03	8.59±0.04	90.61
3	9.48±0.0	9.36±0.03	9.16±0.03	8.65±0.02	91.25

Each value is the mean of three separate experiments ($n = 3$), $\pm$ std. dev; *not determined

bacteria. This colonization efficiency of the strain was determined by evaluating *in vitro* mucin binding ability, surface properties like autoaggregation and microbial adhesion to solvents. In autoaggregation study, cell sedimentation rate was found 19.33% $\pm$ 3.05% after 1 h in PBS (pH 7.3) which was higher than strains reported by Collado et al. (2008). The values of microbial adhesion to xylene (22.27%) at a high ionic strength of 0.1 M KNO₃ (pH 6.2) reflects cell surface hydrophobicity due to absence of electrostatic interactions. Many other studies on the microbial cell surface chemistry have shown that the presence of (glycol-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides (Collado et al. 2008). Furthermore, it was able to bind mucin coated microtitre plate which was determined as 0.077 $\pm$ 0.003 at 450 nm reflecting colonization capability of a microorganism in the gastrointestinal tract preventing their immediate elimination by peristalsis and providing a competitive advantage in this ecosystem (Alander et al. 1997).

Isolate exhibited good antimicrobial activity against *S. typhimurium*, *S. pyogenes* and *S. aureus*. These results indicate the ability of culture to reduce food-borne outbreaks or sporadic cases caused by these enteric pathogens. The culture CD6 was sensitive to variety of antibiotics tested. The degree of sensitivity was quite high (≥ 10 mm zone of inhibition) to amoxycyclav (10 μ g), clindamycin (2 μ g), tetracyclin (30 μ g), and amoxycillin (10 μ g). Moderate (≥ 5 –9 mm zone of inhibition) to cephalixin (10 μ g), cloxacillin (1 μ g), co-trimoxazole (25 μ g), erythromycin (15 μ g), chloramphenicol (30 μ g), and gentamycin (10 μ g). It showed low resistance to ciprofloxacin (10 μ g). These results are in agreement with various reports indicating that LAB are normally resistant to the principal antibiotics, such as penicillin G, ampicillin, vancomycin, chloramphenicol or ciprofloxacin (Coppola et al. 2005). CD6 showed non-hemolytic activity on sheep blood, which ensures its safety.

Several LAB have been reported to synthesize B vitamins in fermented dairy products (Naidu et al. 1999). The strains of *Lactococcus* and *Lactobacillus* have been reported earlier

Fig. 3 Native gel electrophoresis and SOD staining activity. Lanes a–b: stained for SOD activity (a intracellular cell free extract of isolate; b SOD from bovine erythrocytes) Lanes c–e: stained with coomassie brilliant blue for protein pattern (c SOD from bovine erythrocytes; d & e intracellular cell free extract of isolate)

(Gangadharan et al. 2010; Shane et al. 1983) for FA production. FA is used, in many countries as nutritional supplement or for fortification of cereals and their products. It is also used in vitamin pills and widely reported as antioxidants (Gliszczynska-Świgło and Muzolf 2007; Rezk et al. 2003). In this study isolate CD6 showed production of biologically active form of folate 5-MeTHF. The THF and 5-MeTHF are known for its strong antioxidative activity than unreduced form of FA, since it was suggested that an electron donating effect of the five amino group is of major importance in the antioxidant activity (Rezk et al. 2003). The studies of Gliszczynska-Świgło and Muzolf (2007) indicated that possible health effects of FA and its reduced forms associated with their radical scavenging activity is dependent on the pH of body fluids or tissue. In this context, folate producer CD6 can serve the purpose along with its nutritional effect.

Conclusion

Based on these findings, the folate producing isolate *Lactobacillus helveticus* CD6 from fermented milk demonstrated antioxidative ability, although significant SOD activity and its responsible enzyme were not found. The culture showed its ability to chelate metal ions, scavenging reactive oxygen species, reducing activity and required probiotic characteristics. The survival of strain at high bile salt concentrations, low pH values, autoaggregation, cell hydrophobicity, mucin binding revealed its effectiveness in colonization of intestine contributing towards sustenance in intestine. In addition, folate production can serve the purpose of nutrition as well as natural antioxidative supplement. Incorporation of such potential probiotic culture in commercial functional foods holds promise to reach masses to confer health benefits to the consumers.

Acknowledgement The authors are thankful to University Grants Commission, New Delhi for financial support.

References

- Agerholm-Larsen L, Raben A, Haulik N, Hansen AS, Manders M, Astrup A (2000) Effects of 8 weeks intake of probiotic milk products on risk factors for cardiovascular diseases. *Eur J Clin Nutr* 54:288–297
- Ahire JJ, Patil KP, Chaudhari BL, Chincholkar SB (2010) *Bacillus* spp. of human origin: a potential siderophoregenic probiotic bacteria. *Appl Biochem Biotechnol*. doi:10.1007/s12010-010-9142-6
- Ahire JJ, Patil KP, Chaudhari BL, Chincholkar SB (2011) A potential probiotic culture ST2 produces siderophore 2, 3-dihydroxybenzoylserine under intestinal conditions. *Food Chem*. doi:10.1016/j.foodchem.2010.12.126
- Alander M, Korpela R, Saxelin M, Vilpponen-Salmela T, Matilla-Sandholm T, Wright A (1997) Recovery of *Lactobacillus rhamnosus* GG from human colonic biopsies. *Lett Appl Microbiol* 24:361–364
- Anonymous (1990) Probiotics: a review of some of the products currently available to compound feed manufacturers. *Feed Compd* 5:58–64
- Axelsson L (2004) Lactic acid bacteria microbiological and functional aspect. In: Salminen S, Wright A, Ouwehand A (eds) (3rd ed.). Marcel & Dekker, New York USA, pp 1–30
- Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and assay applicable to acrylamide gels. *Anal Biochem* 44:276–287
- Brashears MM, Jaron D, Trimble J (2003) Isolation, selection and characterization of lactic acid bacteria for a competitive exclusion product to reduce shedding of *Escherichia coli* O157:H7 in cattle. *J Food Prot* 66:355–363
- Brosius JM, Palmer L, Kennedy P, Noller HF (1978) Complete nucleotide sequence of the 16S ribosomal DNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* 75:4801–4805
- Buettner GR (1990) Use of ascorbate as test for catalytic metals in sample buffers. *Methods Enzymol* 186:125–127
- Collado MC, Meriluoto J, Salminen S (2008) Adhesion and aggregation properties of probiotic and pathogen strains. *Eur Food Res Technol* 226:1065–1073
- Coppola R, Succi M, Tremonte P, Reale A, Salzano G, Sorrentino E (2005) Antibiotic susceptibility of *L. rhamnosus* strains isolated from Parmigiano Reggiano cheese. *Lait* 85:193–204
- Del Re B, Sgorbati B, Miglioli M, Palenzona D (2000) Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Lett Appl Microbiol* 31:438–442
- Diplock AT, Aggett P, Ashwell M, Borner F, Fern E, Roberfroid M (1999) Scientific concepts of functional foods in Europe: consensus document. *Br J Nutr* 81:S1–S27
- Duhutrel P, Bordat C, Wu TD, Zagorec M, Guerquin-Kern JL, Champomier-Verges MC (2010) Iron sources used by the nonpathogenic lactic acid bacterium *Lactobacillus sakei* as revealed by electron energy loss spectroscopy and secondary-ion mass spectrometry. *Appl Environ Microbiol* 76:560–565
- Duthie SJ, Narayanan S, Brand GM, Pirie L, Grant G (2002) Impact of folate deficiency on DNA stability. *J Nutr* 132:2444S–2449S
- Evans P, Halliwell B (2001) Micronutrients: oxidant/antioxidant status. *Br J Nutr* 85(S2):S67–S74
- FAO/WHO (2002) Guidelines for the evaluation of probiotics in food, report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food, London, Ontario, Canada, pp 1–11
- Gangadharan D, Sivaramakrishnan S, Pandey A, Nampoothiri MK (2010) Folate-producing lactic acid bacteria from cow's milk with probiotic characteristics. *Int J Dairy Technol* 63:339–348
- Gardner HW (1975) Decomposition of linoleic acid hydroperoxides. *J Agric Food Chem* 23:129–136
- Ghosh D, Chattopadhyay P (2010) Preparation of idli batter, its properties and nutritional improvement during fermentation. *J Food Sci Technol*. doi:10.1007/s13197-010-0148-4
- Gliszczynska-Świgło A, Muzolf M (2007) pH-dependent radical scavenging activity of folates. *J Agric Food Chem* 55:8237–8242
- Grice HC (1988) Safety evaluation of butylated hydroxyanisole from the perspective of effects on forestomach and oesophageal squamous epithelium. *J Food Chem Toxicol* 26:717–723
- Halliwell B, Chirico S (1993) Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr* 57:715S–725S
- Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine, vol 3. Oxford University Press, New York
- Holt JH, Kreig NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology. In: WR Hensley

- (ed) (9th ed). Williams & Wilkins Baltimore, MD, USA, pp 1208–1234
- Jin M, Cai Y, Li J, Zhao H (1996) 1, 10-Phenanthroline Fe^{2+} oxidative assay of hydroxyl radical produced by $\text{H}_2\text{O}_2/\text{Fe}^{2+}$. *Prog Biochem Biophys* 23:553–555
- Kaizu H, Sasaki M, Nakajima H, Suzuki Y (1993) Effect of antioxidative lactic acid bacteria on rats fed a diet deficient in vitamin E. *J Dairy Sci* 76:2493–2499
- Kuhle A, Skovgaard K, Jespersen L (2005) In vitro screening of probiotic properties of *Saccharomyces cerevisiae* var. *boulardii* and food borne *Saccharomyces cerevisiae* strains. *Int J Food Microbiol* 101:29–39
- Lin MY, Yen CL (1999) Antioxidative ability of Lactic acid bacteria. *J Agric Food Chem* 47:1460–1466
- Looyenga R, Boltz WDF (1971) Spectrophotometric study of the determination of copper with ammonium 1-pyrrolidinedithiocarbamate. *Talanta* 19:82–87
- Metchnikoff E (1908) Prolongation of life, optimistic studies. Putnam, New York, pp 161–183
- Mishra OP, Kovachich GB (1984) Inhibition of the autoxidation of ascorbate and norepinephrine by extracts of *Clostridium butyricum*, *Megasphaera elsdenii*, and *Escherichia coli*. *Life Sci* 35:849–854
- Naidu AS, Bidlack WR, Clemens RA (1999) Probiotic spectra of lactic acid bacteria (LAB). *Crit Rev Food Sci Nutr* 38:13–126
- Na-kyoung L, Yun CW, Kim SW, Chang HI, Kang CW, Paik HD (2008) Screening of Lactobacilli derived from chicken feces and partial characterization of *Lactobacillus acidophilus* A12 as animal probiotics. *J Microbiol Biotechnol* 18:338–342
- Oyaizu M (1986) Antioxidative activity of browning products of glucosamine fractionated by organic solvent and thin-layer chromatography. *Nippon Shokuhin Kogyo Gakksishi* 35:771–775
- Patel AK, Ahire JJ, Pawar S, Chaudhari BL, Shouche YS, Chincholkar SB (2010) Evaluation of the probiotic characteristics of siderophoregenic *Bacillus* spp. isolated from dairy waste. *Appl Biochem Biotechnol* 160:140–155
- Pedersen C, Lindberg E, Roos S (2004) Microbiological characterization of wet wheat distillers' grain with focus on isolation of Lactobacilli with potential as probiotics. *Appl Environ Microbiol* 70:1522–1527
- Rezk BM, Haenen GRMM, van der Vijgh WJF, Bast A (2003) Tetrahydrofolate and 5-methyltetrahydrofolate are folates with high antioxidant activity. Identification of the antioxidant pharmacophore. *FEBS Lett* 555:601–605
- Rosenberg M, Gutnick D, Rosenberg E (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* 9:29–33
- Salminen S, Bouley MC, Boutron-Ruault MC, Cummings J, Franck A, Gibson G, Isolauri E, Moreau MC, Roberfroid M, Rowland I (1998) Functional food science and gastrointestinal physiology and function. *Bri J Nutr (suppl 1)*:147–171
- Sanders JW, Leehouts KJ, Haandrikman AJ, Venema G, Kok J (1995) Stress response in *Lactococcus lactis*: cloning, expression analysis and mutation of the lactococcal super oxide dismutase gene. *J Bacteriol* 177:5254–5260
- Sarri JT (1989) Chronic treatment with dimethyl sulphoxide protects against cardiovascular defects of copper deficiency. *Proc Soc Exp Biol Med* 190:121–124
- Seifried HE, Anderson DE, Fisher EI, Milner JA (2007) A review of the interaction among dietary antioxidants and reactive oxygen species. *J Nutr Biochem* 18:567–579
- Shane B, Bogner AL, Goldfarb RD, LeBowitz JH (1983) Regulation of folylpolygamma-glutamate synthesis in bacteria: *in vivo* and *in vitro* synthesis of pteroylpoly- gamma-glutamates by *Lactobacillus casei* and *Streptococcus faecalis*. *J Bacteriol* 153:316–325
- Shimada K, Fujikawa K, Yahara K, Nakamura T (1992) Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J Agric Food Chem* 40:945–948
- Simic MG (1988) Mechanisms of inhibition of free-radical processed in mutagenesis and carcinogenesis. *Mutat Res* 202:377–386
- Smith MA, Harris PLR, Sayre LM, Perry G (1997) Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc Natl Acad Sci USA* 94:9866–9868
- Stanger O (2002) Physiology of folic acid in health and disease. *Curr Drug Metabol* 3:211–223
- Sun M, Zigman S (1978) An improved spectrophotometric assay for superoxide dismutase based on epinephrine autoxidation. *Anal Biochem* 90:81–89
- Vakil JR, Shahani KM (1968) Disc assay method for determination of folic acid content of milk, cheese, and other foods. *J Dairy Sci* 52:325–328
- Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39:44–84
- Vanaja G, Gotcheva V, Angelov A, Agrawal R (2011) Formation of volatiles and fattyacids of therapeutic importance in the probiotic *Lactobacillus plantarum* LPcfr adapted to resist GIT conditions. *J Food Sci Technol* 48:110–113
- Verma AK, Banerjee R (2010) Dietary fibre as functional ingredient in meat products: a novel approach for healthy living—a review. *J Food Sci Technol* 47:247–257
- Wang AN, Yi XW, Yu HF, Dong B, Qiao SY (2009) Free radical scavenging activity of *Lactobacillus fermentum* in vitro and its antioxidative effect on growing–finishing pigs. *Appl Microbiol* 107:1140–1148
- Wolff T, Witkop TC, Miller T, Syed BS (2009) Folic acid supplementation for the prevention of neural tube defects: an update of the evidence for the U.S. preventive services task force. *Ann Intern Med* 150:632–639
- Yamauchi R, Goto Y, Kato K, Ueno Y (1984) Prooxidant effect on dihydroxyacetone and reducing sugars on the autoxidation of methyl emulsions. *J Agric Biol Chem* 48:843–848