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Evaluation of the Probiotic Characteristics of Newly Isolated Lactic Acid Bacteria

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Abstract Lactic acid bacteria were isolated from fermented vegetables, sour dough, milk products, sheep and human excreta. The newly isolated cultures were evaluated for a number of probiotic characteristics like bile salt resistance, salt tolerance in general, survival in low pH, hydrophobicity of the cell surface, resistance to low phenol concentration, antimicrobial activity and susceptibility pattern against vancomycin and erythromycin. The selected cultures were further screened for their ability to produce the nutraceticals such as folic acid and exopolysaccharide (EPS). Two potent isolates, CB2 (from cabbage) and SD2 (from sour dough) were found to produce both extracellular and intracellular folate. One of the isolates from yogurt (MC-1) and the one from whey (W3) produced significant amount of EPS with a maximum production of 8.79±0.05 g/l by MC-1.

Keywords Lactic acid bacteria · Probiotic · Nutraceutical · Exopolysaccharide · Folic acid

Introduction

Lactic acid bacteria (LAB) comprise a wide range of genera, and they include a considerable number of species with a long history of use by man and have been used for food production and preservation. The role of lactic acid bacteria within the gastrointestinal tract has been one of the most controversial subjects of the area of intestinal microbial ecology. They are able to create a healthy equilibrium between beneficial and potentially harmful microorganism in the gut by competitive exclusion and by the production of organic acids, enzymes, vitamins, antioxidants, exopolysaccharides (EPS) and antimicrobial compounds preventing intestinal infection [1, 2]. To act

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as a probiotic in the gastrointestinal tract, the bacteria must be able to survive the acidic conditions in the stomach and should resist the bile salt effect [3–5]. The employment of antibiotics in animal feeding can be substituted with the development of products with probiotic characteristics.

Lactic acid bacteria produces nutraceuticals, which can be defined as food, or parts of food, that provide medical, or health benefits, including the prevention and treatment of disease [6]. LAB manufactures the essential vitamins B1, B2, B6, B12, niacin, folic acid and pantothenic acid, and synthesises enzymes that increase the digestibility of protein [7]. Folates are essential components in the human diet. They are involved, as cofactor, in many metabolic reactions, including the biosynthesis of nucleotides, the building blocks of DNA and RNA. Folate is known to prevent neural tube defects in newborns. High-folate diets protect against cardiovascular disease and even some forms of cancer. Another nutraceutical, EPS, is used as a food additive in a wide range of products, where they act as viscosifying, stabilising, emulsifying or gelling agents [8]. Some EPS have been shown to have health-beneficial properties such as immune stimulation, antiulcer activities, antitumoral activities and cholesterol-lowering activity.

The present study was carried out to isolate lactic acid bacteria satisfying most of the probiotic criterions and capable of producing nutraceuticals, mainly the folic acid and EPS.

Materials and Methods

Isolation of LAB

Lactic acid bacteria were isolated from different sources like sour dough, whey, fermented vegetables like cabbage, snake gourd, excreta of sheep and human baby. Decimal dilution of these samples was inoculated to de Man–Rogosa–Sharpe (MRS) medium (Himedia, Mumbai) in 250-ml Erlenmeyer flask and incubated at 37 °C for 48 h under anaerobiosis. The cultures were then plated in to MRS agar medium. Pure colonies were picked and maintained in MRS agar at 4 °C for short-term use and made glycerol stocks for preservation. Isolated cultures were identified by morphology, Gram stain [9] and simple physiological tests proposed by Sharpe [10] using morphological, phenotypic and biochemical methods. For general biochemical characterisation, the gas production from glucose in MRS broth was tested using inverted Durham's tubes. Milk agar (Himedia) plates were used for performing casein hydrolysis. After streaking the isolates, the plates were incubated at 37 °C for 24 h to check the casein hydrolysis. Catalase test was carried out by placing a drop of hydrogen peroxide to single colonies of the culture taken in a glass slide.

Production of Lactic Acid

The LAB isolates were checked for the production of lactic acid in MRS broth using an inoculum of each isolates with a cell density of 10^9 cfu ml^{-1}. The amount of lactic acid produced was estimated according to the colorimetric method of Barker and Summerson [11] and was expressed as mg ml^{-1} of the fermentation medium. The amount of reducing sugar was determined by dinitrosalicylic acid (DNS) method [12] using a UV spectrophotometer (Shimadzu, Japan) at 575 nm. All the represented values are means of three replicates ±SD.

Studies on Probiotic Characteristics of the Isolates

Tolerance to Inhibitory Substances

Probiotic features were evaluated by checking the tolerance of the cultures to varying concentrations of acid, salt, phenol and bile salts. Tolerance to the above mentioned inhibitory substances was studied in MRS broth. Growth at an incubation temperature of 37 °C in different medium pH (2.5, 3, 4 and 5), NaCl concentration [4, 5, 8 and 12% (*w*/*v*)], phenol level (0.2–0.5 g phenol/100 ml) and bile salt concentrations (0.3, 0.5 and 0.8 g sodium taurocholate/100 ml) were monitored at 620 nm after 24 h.

Hydrophobicity of Strains

The degree of hydrophobicity of the strains was determined by employing the method described by Thapa et al. [13]. This method was based on adhesion of cells to hexadecane droplets. Cultures were grown in 10 ml MRS broth, centrifuged at 6,000×*g* for 5 min, and the cell pellet was washed and resuspended in 10 ml of Ringer solution (6% NaCl, 0.0075% KCl, 0.01% $CaCl_2$ and 0.01% $NaHCO_3$). The absorbance at 600 nm was measured. Cell suspension was then mixed with equal volume of *n*-hexadecane and mixed thoroughly by vortexing for 2 min. The two phases were allowed to separate for 30 min, and absorbance at 600 nm of the lower phase was recorded. The percentage hydrophobicity of strain adhering to hexadecane was calculated using the equation:

$$\text{Hydrophobicity}(\%) = \frac{\text{OD}_{600}(\text{initial}) - \text{OD}_{600}(\text{with hexadecane}) \times 100}{\text{OD}_{600}(\text{initial})}$$

Antimicrobial Activity

Ten milliliters of sterile MRS broth (pH 6.0) was inoculated with 1% (10^9 cfu ml^{-1}) viable culture of each isolates and incubated at 37 °C for 24 h. The test materials (compounds produced by the microbial cultures having antimicrobial activity) were obtained by centrifuging the broth (20,000×*g* for 15 min). The resulting liquid was dried under vacuum at 45 °C using a rotary evaporator, re-suspended in one fifth of the original volume and filtered through sterile 0.45-mm membrane filters (Millipore). To detect antimicrobial activity of the preparations, the following organisms grown in nutrient broth at 37 °C for 24 h were used: *Escherichia coli* (MTCC 739), *Shigella sonnei* (MTCC 2957), *Shigella flexnerii* (MTCC 1457) and *Staphylococcus aureus* (MTCC 96). Antimicrobial activity was quantitated by a ditch assay using the test organisms. Actively growing culture of the test organisms were mixed at a 2.5% (2.5×10^7 cfu ml^{-1}) with melted nutrient agar poured in sterile petri dishes and allowed to solidify. A 0.5-mm-wide ditch was cut in the agar across the centre of the dish. The test material was diluted in an equal volume of melted bacteriological agar (0.012 g l^{-1}), and then 0.2 ml of the mixture was pipetted into the ditch. When the mixture solidified, the plates were first incubated at 4 °C for 60 min to allow the test material to diffuse in the agar and then incubated at 37 °C for 18 h. After incubation, the diameter of the clear zone was measured in millimeters.

Antibiotic Resistance Study

All the tests were performed in Mueller–Hinton agar (Oxoid). Mueller–Hinton agar was plated evenly over the entire surface with 50 μl (OD was adjusted to 0.6) of the isolates using sterile cotton wool swab. Antibiotic discs of vancomycin (10 μg) and erythromycin (15 μg) were firmly applied to the surface of an agar plate that has previously been air dried. The plates were incubated at 37 °C overnight, and diameters of the zone of inhibition around the discs were measured.

Isolation, Purification and Quantification of Exopolysaccharides

MRS medium inoculated with 18 h old culture (10^9 cfu) incubated for 72 h was centrifuged at 11,500×*g* for 15 min at 4 °C. The supernatant was collected, and EPS was precipitated by the addition of double volume-chilled ethanol. The mixture was stored overnight at 4 °C and centrifuged at 2,500×*g* for 20 min. Pellet was collected and dissolved in de-mineralised water, and double-volume cold ethanol was added. It was centrifuged again at 2,500×*g* for 20 min. The pellet was dried, and the dry weight was taken as the amount of EPS produced [14]. The total carbohydrate was estimated by phenol – sulphuric acid method [15].

Folate Production

Isolates were screened for folate production. Cultures were grown on a modified MRS medium containing (g/100 ml): dextrose, 4; ammonium chloride, 0.4; yeast extract, 0.5; dipotassium hydrogen phosphate, 0.2; magnesium sulphate, 0.01 and manganese sulphate, 0.005. The samples were withdrawn at different time intervals (4, 6, 8 and 10 h), and the presence of folate was analysed using HPLC. Folate derivatives such as tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-MeTHF) and 5-formyltetrahydrofolate (5-FmTHF; Sigma, St. Louis, MO, USA) were used as standards. Standard solutions were prepared according to the method described by Holt et al. [16].

Extraction and De-conjugation of Folates from Samples

Extracellular Folate

Six milliliters of the culture or fermented medium was added to 10 ml of extraction buffer (0.5 g sodium ascorbate/100 ml 0.1 M phosphate buffer). The mixture was placed in a boiling water bath for 15 min and then centrifuged at 4,000×*g* for 10 min. To 3 ml of supernatant, 0.4 ml of human plasma was added. Human plasma contains deconjugase, which deconjugates polyglutamic forms of folates to monoglutamic forms [17]. The mixture was incubated at 37 °C for 1 h under continuous rotation. The reaction was stopped by placing the samples in boiling water for 5 min. The extract was centrifuged at 27,000×*g* for 10 min. The supernatant was then filtered through a 0.45 μm filter and used directly or stored at −20 °C until use.

Extraction of Intracellular Folate

Fifty milliliters of fermented medium at different time intervals, as mentioned above, was withdrawn and then centrifuged at 4,000×*g* for 15 min. The pellet was washed in phosphate

buffer (pH 7.4) and treated with lysis buffer [20 mM phosphate buffer (pH 7.4), 0.5M NaCl and 0.1 mg/ml lysozyme] for 10 min and was sonicated in ice. The condition for sonications was standardised at 80 W (nine cycle, 30 s ON pulse and 30 s OFF pulse). Sonicated samples were kept in boiling water bath for 3 min to release folate-binding proteins. The mixture was centrifuged at 27,000×*g* for 10 min. Supernatant was analysed for folate.

HPLC Analysis of Folates

The chromatographic analysis was performed using a Varian HPLC system (USA). The mobile phase was 20 mM phosphate buffer pH 6.2 and acetonitrile in the ratio 39:1. Mobile phase and samples were filtered through 0.45 μm filters (Millipore) before use. The samples were passed though a Nova Pak C18 column 4 μ (4.6×250 mm cartridge) at a flow rate of 1 ml min^{-1} at 25 °C and detected by UV detector (Varian) at 280 nm.

Results and Discussion

Isolation and Characterisation of Lactic Acid Bacteria

Lactic acid bacteria are often associated with animal oral cavities and intestines (e.g. *Enterococcus faecalis*), plant leaves (*Lactobacillus, Leuconostoc*) and decaying plant or animal matter such as rotting vegetables, fecal matter, compost, etc. Around 20 cultures were isolated from different sources that include four from sour dough (SD1, SD2, SD3 and SD4), three from whey (W1, W2 and W3), two from snake gourd (SG1 and SG2), five from cabbage (CB1, CB2, CB3, CB4 and CB5), one from yogurt (MC1), three from sheep excreta (G1, G2 and G3) and two from human baby faeces (WJ1 and MCJ1). All the isolates were found to be Gram-positive bacteria. Most of them were cocci except the isolates from snake gourd and yogurt, which were rods. Except the isolates from goat excreta and snake gourd, all the isolates were catalase negative, which showed no bubbling when hydrogen peroxide was added. Isolates, CB2, MC1, W3, G1, SD3 and SG2, produced gas bubbles in the Durham's tube. Most of the isolates did not hydrolysed casein. Based on the Bergey's manual [18], most of the isolated cultures belong to the genus *Lactococcus*, which is Gram-positive cocci type.

Lactic Acid Production and the Residual Reducing Sugar Level

Lactic acid is the major metabolic end product of carbohydrate fermentation in lactic acid bacteria, and this trait has historically linked LAB with food fermentations as acidification inhibits the growth of spoilage agents. These bacteria can either be homofermentive or herterofermentive. Homofermentative lactic acid bacteria produces more than 85% lactic acid from glucose, and heterofermentative produce only 50% lactic acid and considerable amounts of ethanol, acetic acid and carbon dioxide. The isolates were screened for the production of lactic acid and the remaining reducing sugar (glucose) concentration after 24 h of incubation. The isolates, CB1, G3 and SG2, were found to produce more amounts of lactic acid, 14.49, 13.95, and 16.36 mg/ml, respectively. The initial carbohydrate content was 20 mg/ml. After 24 h, most of the carbohydrate was converted to lactic acid. The lactic acid production profile and remaining reducing sugar level is shown in the Fig. 1. Wherever lactic acid production is high, the remaining residual sugar level is low.

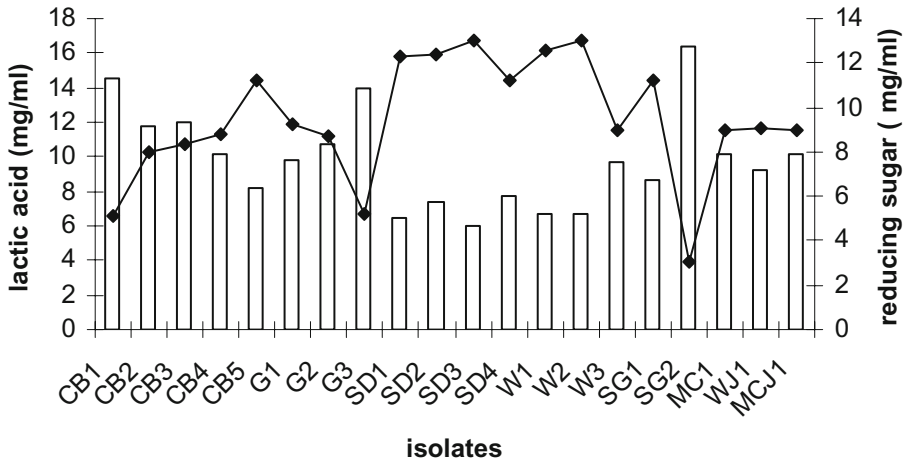

Fig. 1 Lactic acid production (*bar*) profile and the residual reducing sugar level (*line*)

Tolerance to Inhibitory Substances

Notable acid-tolerant bacteria include the *Lactobacillus* and *Streptococcus* species, which play a role in the fermentation of dairy and vegetable products. Most of the isolates grew at pH 5, and the isolates from cabbage, sheep excreta, SG1, SG2, W3, MC1, WJ1, and MCJ1 showed growth in pH 2.5 (Table 1). The growth of LAB lowers the pH due to lactic acid production. The survival of bacteria in gastric juice depends on their ability to tolerate low pH favouring probiotic characteristics. The presence of food raises the pH value to the level of 3 [19], low enough to inhibit the growth of most other microorganisms including the most common human pathogens, thus allowing these foods to have prolonged shelf life.

Lactic acid bacteria generally tolerate high salt concentrations. It allows the bacteria to begin metabolism, which produces acid, that further inhibits the growth of non-desirable organisms. CB1, one of the cabbage isolate, could tolerate up to 12% NaCl. Some isolates such as CB4, CB5, SG1 and SG2 could tolerate 8% NaCl as shown in Table 1.When bacterial cells are grown in medium with salt, they experience a loss in their turgor pressure, which in turn affects the metabolism and their enzyme and water activity. Cells overcome this situation by regulating the pressure inside and outside of the cell by inducing osmolytes, such as glycine betaine, as an adaptive mechanism to withstand increased osmotic potential [20].

Tolerance to phenol is a characteristic probiotic property because phenols can be formed in the intestines by bacteria that deaminate some aromatic amino acids delivered by the diet or produced by endogenous proteins [21]. Isolates from sheep excreta G1, from whey W3, MC1 from yogurt and WJ1 from human baby faeces could tolerate up to 0.5% phenol (Table 1). Many of the isolates could tolerate up to 0.5–0.8% oxgall, where as W1, W2 from whey and WJ1 from human baby excreta showed relatively lesser tolerance at 0.8% oxgall (Table 1).

Bile tolerance has been described as an important factor for the survival and growth of LAB in the intestinal tract [21]. The concentration of bile to be used in the selection of probiotic species for human beings must be 0.3% (*w*/*v*).This is so because the isolated

Table 1 Tolerance of newly isolated LAB towards varying growth inhibitory substances.

Isolates	NaCl			pH				Phenol				Bile salt (oxgall)		
	Growth of bacteria (OD at 620 nm)													
	4%	8%	12%	5	4	3	2.5	0.2%	0.3%	0.4%	0.5%	0.3%	0.5%	0.8%
CB1	0.110	0.033	0.560	1.749	0.056	0.012	0.103	0.085	0.061	0.067	0.066	1.514	1.316	1.489
CB2	0.240	0.040	0.065	0.781	0.460	0.002	0.110	1.047	0.31	0.116	0.046	1.475	1.407	1.450
CB3	0.980	0.040	0.065	1.754	0.060	0.002	0.133	1.603	0.378	0.176	0.06	1.815	1.340	1.433
CB4	0.800	0.800	0.051	1.709	0.076	0.017	0.122	1.724	1.597	0.530	0.063	1.455	1.867	1.802
CB5	1.147	1.497	0.041	1.773	0.029	0	0.086	1.397	0.283	0.093	0.043	1.808	1.333	1.489
G1	1.840	0.070	0.058	1.516	0.164	1.112	0.083	1.941	1.925	0.430	0.195	1.757	1.879	1.802
G2	1.614	0.044	0.045	1.492	0.176	0.166	0.165	1.655	1.730	0.156	0.081	1.813	1.841	1.775
G3	1.819	0.077	0.064	1.571	0.078	0	0.086	1.767	1.627	0.232	0.096	1.815	1.853	1.789
SD1	0.035	0.049	0.039	0.758	0.044	0.041	0.026	0.068	0.121	0.007	0.061	1.670	1.603	1.490
SD2	0.045	0.040	0.046	0.823	0.032	0	0	1.298	1.265	0.048	0.079	1.216	0.705	1.231
SD3	0.035	0.032	0.039	0.413	0.045	0.045	0.044	0.068	0	0.002	0.063	1.671	1.421	1.451
SD4	0.033	0.039	0.055	0.746	0.054	0.058	0.049	0.072	0	0.005	0.051	0.803	0.547	0.629
W1	0.538	0.026	0.042	0.003	0.068	0.007	0.017	0.061	0	0.01	0.076	0.600	0.345	0.345
W2	0.036	0.039	0.055	0.537	0.127	0.111	0.029	0.920	0.072	0.092	0.078	0.916	0.723	0.328
W3	1.359	0.035	0.036	1.480	0.330	0.111	0.136	1.961	1.924	0.623	0.325	1.871	1.854	1.846
SG1	2.158	0.806	0.061	1.631	0.027	0	0.09	1.685	0.063	0.061	0.056	1.800	1.724	1.778
SG2	2.114	0.927	0.056	1.696	0.034	0	0.089	1.632	0.122	0.069	0.054	1.843	1.765	1.753
MC1	1.012	0.090	0.076	1.620	0.156	0.132	0.12	0.921	0.441	0.211	0.124	1.755	1.582	1.771
WJ1	1.100	0.118	0.110	1.550	0.125	0.111	0.104	1.120	0.451	0.432	0.144	1.102	0.965	0.206
MCJ1	1.120	0.092	0.080	1.521	0.104	0.1	0.098	1.020	0.321	0.184	0.089	1.023	0.921	1.027

microorganism may present tolerance to high concentrations of bile. According to Pancheniak and Soccol [22], isolates that showed 0.3% tolerance to bile could be used as probiotic for swine. The daily average of biliary flow is very high in swine, around 2 l for each 40 kg of swine [22] as compared to that of an adult human (70 kg) that produces 400 to 800 ml of bile daily.

Hydrophobicity of Isolates

Bacterial surface characteristic is one of the *in vitro* properties, which were studied for knowing the probiotic nature. Bacterial surface properties have been associated with attachment to a variety of substrate, which in turn is associated with hydrophobicity. Bacterial adhesion can also determine the colonisation capability of a microorganism. Through adhesion ability and colonisation of tissues, probiotic microorganisms can prevent pathogen access by steric interactions or specific blockage on cell receptors [23]. As the hydrophobicity of the cell increases, the level of adhesion also increases [24]. According to Gilbert et al. [25], only for hydrophobic microorganisms surface hydrophobicity is correlated to adhesions. Among the isolates, isolates of sour dough SD2, SD3 and SD4 showed hydrophobicity values 52.4, 24 and 73% respectively; snake gourd isolates SG1, SG2 had values 65 and 40% and MC1, 23% hydrophobicity. Hydrophobicities of some known cultures like *Lactobacillus plantarum* ATCC8014, *Lactobacillus pentosus* ATCC8041, *Lactobacillus casei* NCIMB 3254, and *Lactobacillus delbrueckii* NCIM

Table 2 Antimicrobial activity of the LAB isolates.

Cultures	Clear zone formed in antimicrobial activity (mm)			
	Staphylococcus aureus	*Escherichia coli*	*Shigella sonnei*	*Shigella flexneri*
CB2	–	5	–	–
CB3	–	6	5	–
CB4	4	7	5	–
CB5	–	10	–	–
G1	5	5	4	–
G2	–	5	–	–
G3	–	7	5	–
SD1	–	–	–	4
SD2	–	7	5	5
SD3	5	8	6	–
SD4	–	6	–	6
W1	–	6	–	4
W2	–	–	–	–
W3	6	6	6	5
SG1	6	6	5	5
SG2	5	6	6	6

2025 were found to be 5.5, 6.5, 6.2 and 3.7%, respectively, and all are very less than the isolates.

Antimicrobial Activity

Lactic acid bacteria have been useful at promoting bacterial interference by the formation of inhibitory substances like organic acids, free fatty acids, ammonia, diacetyl, hydrogen peroxide and bacteriocins. Hydrogen peroxide is produced by many of the lactics. The antimicrobial effect is based on the oxidative properties that results in irreversible changes in the microbial cell membrane. Isolates from snake gourd, W3, G2, G3 and CB4, showed antimicrobial activity against potential human pathogens such as *Shigella sonnei*, *Shigella flexneri*, *Staphylococcus aureus* and *E. coli*. The zone of inhibition is tabulated (Table 2).

Table 3 Antibiotic resistance shown by LAB isolates.

Isolates	Diameter of the zone of inhibition (cm)	
	Vancomycin	Erythromycin
G3	0.9	3
MC1	–	1.2
WJ1	1.3	2
MCJ1	–	1.1
CB1	–	–
CB2	1.9	–
SD1	2.5	–
SD2	2.0	–
W3	2.3	3

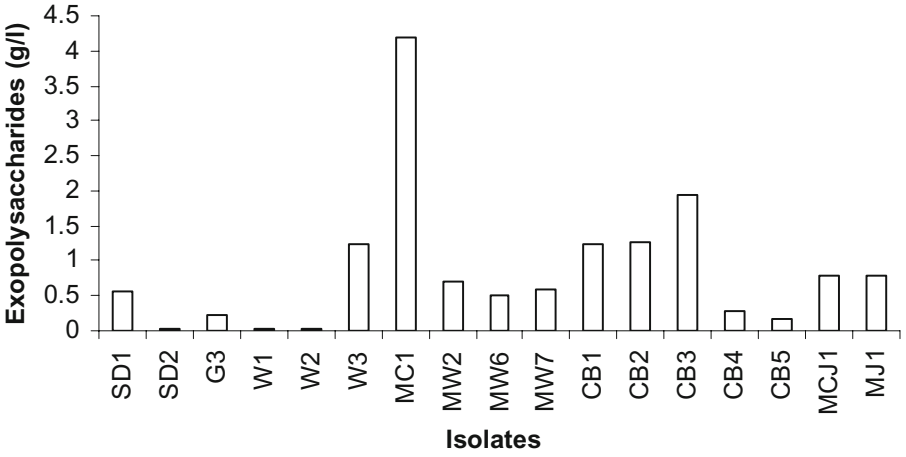

Fig. 2 EPS production of isolates

Antibiotic Resistance

This work reports the susceptibility patterns of a number of LAB species against vancomycin and erythromycin. MC1, MCJ1 and CB1 were resistant to vancomycin, and the other isolates studied were sensitive to vancomycin. CB1, CB2, SD1 and SD2 were resistant to erythromycin. According to Klein et al. [26], the resistance of *Lactobacillus* and *Leuconostoc* spp. to vancomycin may be due to the presence of D-Ala-D-Lac as the normal dipeptide in their peptidoglycan. Lactic acid bacteria (LAB) from fermented products may act as a reservoir of antimicrobial-resistance genes that could be transferred to pathogens, either in the food matrix or, more importantly, in the gastrointestinal tract [27, 28]. Table 3 summarises the results of antibiotic resistance study.

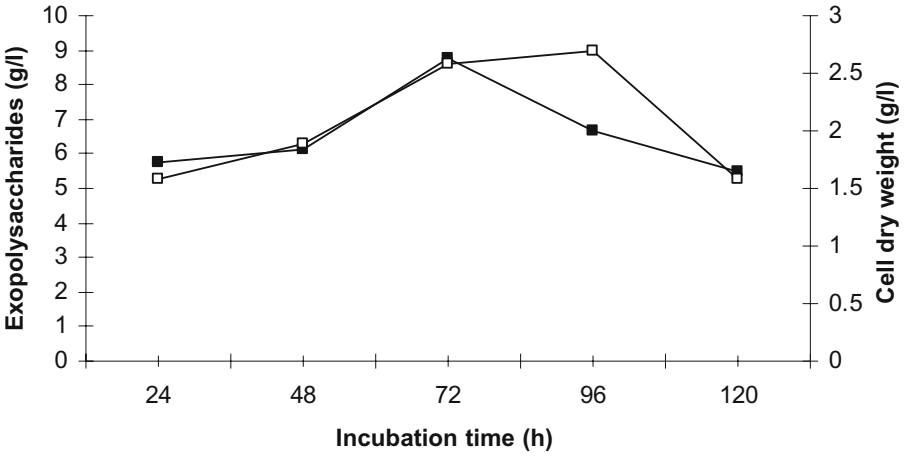

Fig. 3 Time course study of exopolysaccharide production by MC1: cell dry weight (*empty square*), *exopolysaccharide* (*filled square*)

Screening for Nutraceuticals

Exopolysaccharides

Extracellular polysaccharides (EPS) produced by LAB increase the total amount of solids, improve the consistence and avoid the gel fracture in fermented milks. EPS have the ability to lower blood cholesterol. EPS are primarily composed of carbohydrates, but in addition to the various sugars such as D-glucose, D-galactose and D-mannose [29], there are organic (pyruvate and acetate) and inorganic (phosphate and divalent cations) substituents. Several amino sugars such as *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and some rare ones such as fucosamine and talosamine are also present. One of the isolates from yogurt

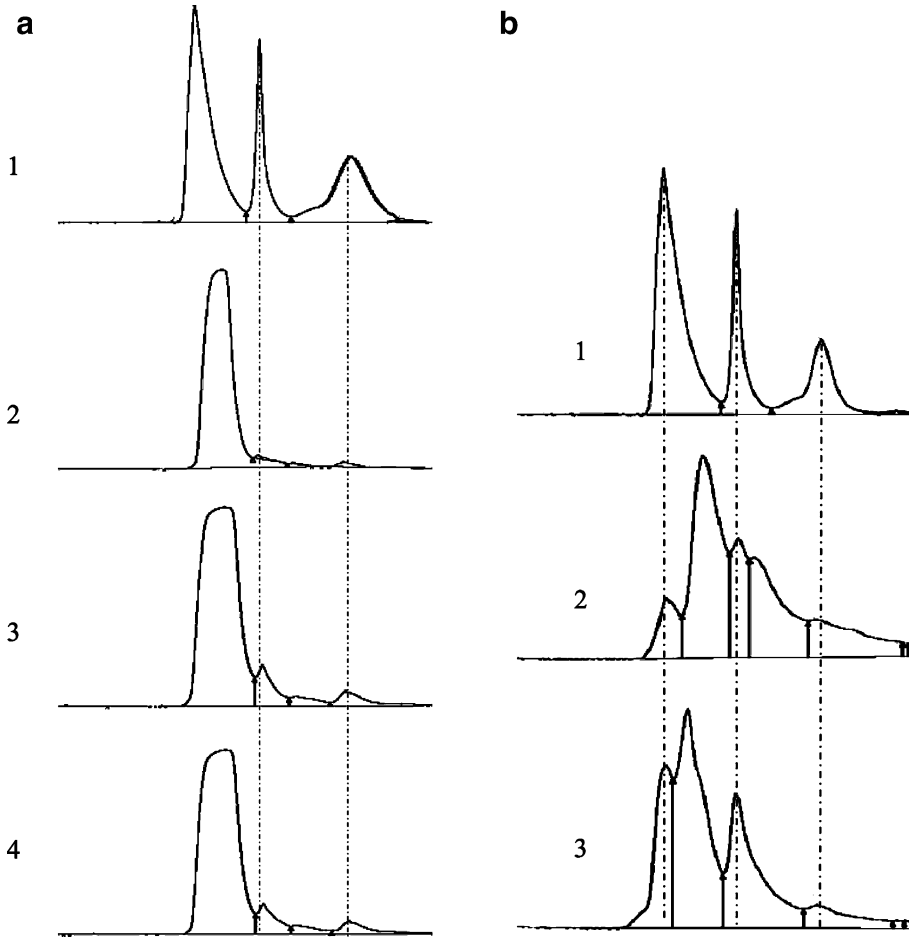

Fig. 4 Screening of folate derivatives. **a** 1 Standards (peaks for tetrahydrofolate, 5-formyl-tetrahydrofolate and 5-methyl-tetrahydrofolate with a retention time of 2.2, 3.2 and 4.3 respectively) *2–4* Extracellular samples (MC1, G3 and CB2, respectively); **b** 1 Standards (peaks for tetrahydrofolate, 5-formyl-tetrahydrofolate and 5-methyl-tetrahydrofolate with a retention time of 2.2, 3.2 and 4.3, respectively) *2*, *3* Intracellular samples (CB2 and SD2, respectively)

(MC1) and whey (W3) showed promising growth with EPS of dry weight greater than 1 g/l and can be used for EPS production (Fig. 2).

A time-course study using the MC1 culture showed a maximum EPS production of 8.79±0.05 g/l after 72 h fermentation as shown in Fig. 3. The data shows a growth-dependent EPS production.

Folate

Folates are essential components in the human diet. The preliminary screening results were compared with the retention time of folate standards such as tetrahydrofolate (2.2 min), 5-formyl-tetrahydrofolate (3.2 min) and 5-methyl-tetrahydrofolate (4.3 min) under the given conditions. The results indicate the presence of both the derivatives 5-formyl-tetrahydrofolate and 5-methyl-tetrahydrofolate in G3 and CB2 and 5-methyl-tetrahydrofolate in MC1. Corresponding peaks for the derivatives were not obtained for SD2 and W3 extracellular samples. Even though the level of folate derivatives were very low, the peak with retention time 3.2 and 4.3 min indicates the presence of 5-formyl-tetrahydrofolate and 5-methyl-tetrahydrofolate in the extracellular samples and can be considered as a qualitative method of folate detecting. Similarly, the intracellular samples of CB2 and SD2 showed the presence of tetrahydrofolate with retention time 2.2 min as the prominent derivative in addition to the other two derivatives (Fig. 4).

Conclusion

LAB cultures are generally designed to meet food safety, shelf life, technological effectiveness and economic feasibility criteria. Specially selected cultures may also provide probiotic benefits, and, if properly modified, they may even be endorsed with nutraceutical traits. Not all desirable probiotic characteristics were present in a single isolate, where many isolates displayed varying individual but promising capabilities. Very interestingly, the experimental results indicated that some of the isolates can be used to produce nutraceuticals such as folic acid and EPS.

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