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Assessment of Preliminary in Vitro Probiotic Characterstics of the Folate Producing Yogurt Starter Culture Streptococcus and Lactobacillus Species

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Abstract: *This study aimed at assessing the probiotic potential of two folate producing strains Streptococcus thermophilus NCIM 2904 and L. helveticus NCIM 2733, yogurt starter culture by in vitro tests. S. thermophilus was better able to survive at pH 1 and 2% bile salt with a positive bile salt hydrolase activity, good cell surface hydrophobicity, moderate antimicrobial activity against almost all the tested clinically important indicator strains except Shigella flexneri and sensitivity to most of the clinically important antibiotics. On evaluation gastric acidity tolerance, S. thermophilus showed viable count of 4.7±0.56 log c.f.u. ml^{-1} after 3 h of incubation at pH low as 1.0 indicated good degree of acid tolerance whereas L. helveticus showed very less tolerance as viable count was found to be 2.35±0.35 log c.f.u. ml^{-1}. For intestinal adaptability, S. thermophilus showed better survival than L. helveticus at 2% bile as viable count was 7.15±0.2 log cfu mL^{-1} and 4.8±0.14 log cfu mL^{-1} respectively. S. thermophilus showed better percentage of cell surface hydrophobicity i.e. 19.2 % with xylene than n-hexadecane indicating ability to adhere to intestinal cells. Although both the strains were observed to possess essential probiotic properties, L. helveticus showed less probiotic potential than S. thermophilus in almost all the in vitro evaluation test. Thus the strains may be used as novel probiotics only after further in vivo tests and clinical trials on animal models.*

Keywords: *Probiotics, acid tolerance, antibiotics susceptibility, cell surface hydrophobicity*.

I. Introduction

The History of probiotics (means "for life") came into the existence first by the Elie Matchnikoff in 1907 as he suggested the longevity of ethnic groups and normal balance of intestinal flora between pathogenic and non pathogenic microorganisms on regular consumption of fermented milk [1]. However, it gained momentum recently after the proper understanding of mechanisms by which it positively affects the human health. According to the definition given by FAO/WHO, "probiotics are live beneficial microorganisms which when administered in adequate amounts confer a health benefit on the host" [2]. Probiotics are the group of the beneficial microorganisms that may enhance the resistance against pathogenic intestinal microflora thus involved in the prevention of diseases. A strain should possesses certain attributes to be known as "Probiotic" like survival through the gastrointestinal tract (GI Tract), human origin, generally recognized as safe (GRAS) status, potential antimicrobial activity by producing either lactic acid, organic acids or bacteriocin (proteinaceous bactericidal compounds), susceptibility for the antibiotics and capacity to adhere to human intestinal cells [3]. For the survival in the gastrointestinal tract, probiotics should possesses certain important features like survival to low pH values of stomach and toleration for the bile salts in the duodenum and transient intestinal colonization for providing the health benefits after consumption [4].

Species belonging to mainly Lactobacillus and Bifidobacterium are considered as probiotics as they are the normal residents of the complex environment of the gastrointestinal tract. However, some species of Enterococcus, Propioniobacterium and Saccharomyces also generally accepted as the probiotics [5, 6, 7]. Some studies were also reported that the probiotic potential exists in the Streptococcus thermophilus strains [8]. Various commercial probiotic preparations available in the market in the may be ingested in the form of as lyophilized powder form or capsules, liquid/gel and functional products that claim for prevention of infectious diseases. Some of the commercially available probiotic preparations include Lactobacillus alone (Lactiflora, LactoBacil, Lactocap, Lactovit, Sporlac) whereas others include combination of Lactobacillus with Streptococcus (Lacticin) or Saccharomyces (Laviest) [9]. Safety and quality of fermented food may be enhanced by some strains of lactic acid bacteria (LAB) due to production of different antimicrobial metabolites such as organic acids, bacteriocins, hydrogen peroxide and diacetyl. Some bacteriocins produced by the Lactobacilli are acidophilin, acidolin, lactocidin, bulgarican, lactolin, lactobacillin and lactobrevin [10]. Recent research on the probiotics is focused mainly to evaluate the potential of probiotics with the immune system as immunomodulator, their anticancer potential, and use of probiotics as biotherapeutics agent for the prevention of certain disease as irritable bowel syndrome and antibiotics associated diarrhea. However the need of the

development of functional food products containing novel active probiotic culture with better probiotic characteristics than those already exists in the market is also emerging to satisfy the increasing demand in the market [11].

Generally it has been observed that probiotics such as Lactobacillus, Bifidobacterium and Streptococcus are usually associated with the milk and milk made products. It is necessary to evaluate the probiotic potential of the microorganisms isolated from the fermented product or the microorganisms producing the fermented product. In our previous study the folate has been produced in fermented milk by the Streptococcus thermophilus NCIM 2904 and Lactobacillus helveticus NCIM 2733, strains isolated from yogurt [12]. So the aim of this study was to assess the probiotic potential of the folate producer strains S. thermophilus NCIM 2904 and Lactobacillus helveticus NCIM 2733. By the assessment of probiotic characteristic, the probiotic potential has been checked to explore the dual advantage of folate enriched probiotic fermented milk which may improve the intestinal microflora [13]. Certain in vitro test are essential to performed to screen the preliminary probiotic potential of the microorganisms such as resistance to gastric acidity, bile resistance, antimicrobial activity, ability to reduce pathogen adhesion on cell surface and bile salt hydrolase activity [14]. These entire tests are considered to be helpful in the selection of strain to be used as probiotic. Based on the results of these tests, preclinical validation upon appropriate animal models should also been subjected before the clinical trials on human subjects.

II. Materials And Methods

2.1 Microorganisms and culture conditions

Streptococcus thermophilus 2904 and Lactobacillus helveticus 2733 for probiotic properties evaluation and Staphylococcus aureus 5021 for antimicrobial activity were procured from the National Collection of Industrial Microorganisms, culture collection of the National culture Laboratory, Pune. However Escherichia coli MTCC 443, Salmonella typhi MTCC 734, Klebsiella pneumonie MTCC 2653, Shigella flexneri MTCC 1457 and Vibrio cholera MTCC 3906 were obtained from Microbial Type Culture Collection of Institute of Microbial Technology, Chandigarh. S. thermophilus and L. helveticus were cultured and maintained in MRS agar (Himedia, India) at appropriate culture conditions and subcultured three times prior to use in experimental studies. Rest of the indicator strains were maintained in nutrient agar and incubated at appropriate culture conditions.

2.2 In vitro Probiotic assessment tests

2.2.1 Resistance to gastric acidity

For the purpose of acid resistance of the strains, MRS broth was adjusted to different pH, i.e. 1, 2, 3, 4 and pH 7 as control by using 0.1N HCl and then sterilized in autoclave at 121ºC for 15 min. Seed culture of the strains S. thermophilus and L. helveticus were inoculated (1% v/v) into the pH adjusted MRS broth and incubated at 37ºC for at least 3 h to simulate the acidic environment of the human stomach. After that cell growth was measured by plating method for the viable cell count.

2.2.2 Resistance to bile salts

Effect of bile salts on the growth of folate producing strains was studied by the method described by Gilliland et.al. [15]. For the purpose, MRS broth having the different concentrations of bile salt (Sigma) (0.5, 1.0, 1.5 and 2.0%) was prepared. MRS media without any bile supplementation is used as control. After this, 0.1 ml inoculum was transferred to MRS broth and incubated for 37ºC for minimum 4 h to stimulate the human intestinal environment. After that cell growth of the strains on the MRS agar plate is indicator for the strain to be bile salt tolerant

2.2.3 Antimicrobial activity against potential pathogenic bacteria

Cell free supernatant (CFS) of the S. thermophilus and L. helveticus were prepared by inoculating the strains in 100 ml of MRS broth at 37ºC till the early stationary phase (8-10 hours). Cells were separated by centrifugation at 10000 rpm for 10 min at 4ºC. The acid present in the supernatant was neutralized to pH 6 or 7 with 1M NaOH and filter sterilized with 0.2 µm filters. For the detection of antimicrobial activity of the substances in the resulting CFS, the agar well diffusion assay was performed [16]. Test pathogens selected for the study were Escherichia coli MTCC 443, Salmonella typhi MTCC 734, Klebsiella pneumonie MTCC 2653, Shigella flexneri MTCC 1457, Vibrio cholera MTCC 3906 and Staphylococcus aureus NCIM 5021. Indicator strains were inoculated and grown in a sterile petri dish containing solidified soft nutrient agar (0.8%, w/v). Wells were made in the nutrient agar and aliquots of 50 µl of supernatant were poured in the wells. After 24 h incubation at the optimal growth temperature of indicator strains, a clear zone of inhibition of at least 2 mm in diameter around the wells was recorded as positive.

2.2.4 Antibiotic Resistance activity

Antibiotics susceptibility test was performed by the disc diffusion method with standard guidelines of antimicrobial resistance or sensitivity given by the Clinical and Laboratory Standards Institute [17,18]. MRS agar was used instead of Muller Hinton agar for the testing. The antibiotics discs used were ampicillin, amoxycillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, kanamycin, tetracycline, norfloxacin and vancomycin. The results were expressed in term of inhibition zone around the discs and expressed as resistant (0 < 12 mm), intermediate (13 < 16 mm) or sensitive (17 < 33 mm) according to the observed interpretative points issued by CLSI.

2.2.5 Bile salt hydrolase activity

BSH activity was tested by the method described by the Dashkevicz et. al.[19]. 10 mL aliquots of overnight cultures (10^8-10^9) was spreaded on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TCDA) or 0.2% (w/v) glycodeoxycholic acid (GDCA) (Sigma) and 0.37 gL^{-1} $CaCl_2$. Plates were incubated anaerobically by using anaerobic jars for 72 h at 37ºC and strains forming precipitation zones were regarded BSH positive [20].

2.2.6 Cell surface hydrophobicity test

The degree of hydrophobicity of the strains was tested by the Madhvan et.al [21]. Culture to be tested was grown in 10 ml MRS broth, centrifuged at 6,000 rpm for 5 min. The cell pellet obtained was washed and re-suspended 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 to check the turbidity of the suspension. Equal volume of N-hexadecane and xylene was added slowly in the cell suspension and incubated at 37ºC for 10 min followed by vortexing for 2 min. The hydrocarbon layer was allowed to separate and rise completely for about 30 min, than aqueous phase was removed carefully by the pipette and absorbance was measured at 600 nm. The percentage hydrophobicity of strains adhering to n-hexadecane and xylene was calculated using the equation:

$$\text{Hydrophobicity (\%)} = \frac{OD_{600}\ (\text{initial}) - OD_{600}\ (\text{final})}{OD_{600}\ (\text{initial})} \times 100$$

III. Results And Discussion

3.1 Acid tolerance

Probiotic potential of any microorganism is necessarily evaluated by its ability to grow in complex environment of the human digestive tract to impart its health benefits as the pH is too low to inhibit the growth of the most common human pathogens. The bacteria must first survive through the stomach (pH -1.5-2, acidic) before reaching to the intestine [22]. Generally Streptococcus and Lactobacillus species, important in the fermentation of dairy and vegetable products, were found to be acid tolerant. The effect of different pH on the viability of two strains is presented in Table 1. S. thermophilus is able to survive very well at pH 1 however L. helvetics showed very less tolerance to low acidic pH after incubation for 3 h and 24 h. At pH 1.0, the growth of the L. helveticus showed very less viable cells 2.35±0.35 log c.f.u. ml^{-1} was detected after 3 h. The in vitro probiotic evaluation reports of Lactobacillus species are available easily however studies on Streptococcus species are very rare. However this study was supported by the findings of Khalil et al, 2009 in which S. thermophilus CHCC showed better survival at pH 2 however could not grow at pH 1.5 [23]. There was a continuous reduction in viability at pH 1.0 than that in control (pH 7.0). In the present study, survival of S. thermophilus up to 6.0±0.14 log c.f.u. ml^{-1} at 3 h and 7.45±0.49 log c.f.u. ml^{-1} at 24 h at pH 2 and 4.7±0.56 log c.f.u. ml^{-1} and 2.35±0.35 log c.f.u. ml^{-1} at pH 1 indicated good degree of acid tolerance. Vinderola and Reinheimer [24] also found similar results i.e. better survival of S. thermophilus strains at pH 3.0 and 2.0.

Table 1: Resistance of strains to gastric acidity in terms of acidic pH. Values are reported in terms of mean of duplicates ± SD

pH / Strains		7 (Control)	4	3	2	1
		Viable cell count (log cfu/ml)				
S. thermophilus	3 h	8.7±0.3	8.25±0.2	6.15±0.21	6.0±0.14	4.7±0.56
	24 h	10.5±0.07	9.55±0.35	7.0±0.14	7.45±0.49	5.65±0.63
L. helveticus	3 h	8.85±0.07	6.8±0.4	4.8±0.14	3.5±0.4	2.35±0.35
	24 h	9.95±0.5	7.4±0.14	5.25±0.2	3.7±0.84	3±0.14

3.2 Bile Salt tolerance

Bile salt resistance is also required prior condition to be fulfilled by the tested strain to be probiotic as it is important for the metabolic activity and colonization of the strain in the small intestine of the host. Colonization of the strain in the small intestine ultimately leads the balance of intestinal healthy microflora [25, 26]. In this study, a trend of decreasing bacterial viability with increased concentration of bile salt was reported with the both strains. S. thermophilus and L. helveticus showed viable count of 7.15±0.2 log c.f.u. ml^{-1} and 4.8±0.14 log c.f.u. ml^{-1} respectively even on 2% bile salt addition in MRS media after incubation for 4 h (Table 2).

Table 2: Bile Salt tolerance study of strains. Values are reported in terms of mean of duplicates ± SD

Bile Salt concentration (%w/v) / Strains		Control (0%)	0.5%	1.0%	1.5%	2%
		Viable cell count (log cfu/ml)				
S. thermophilus	4 h	8.95±0.2	8.3±0.14	7.75±0.2	7.55±0.4	7.15±0.2
	24 h	13.9±0.3	9.6±0.4	8.5±0.2	8.3±0.28	7.7±0.14
L. helveticus	4 h	8.85±0.5	7.05±0.2	6.8±0.14	5.5±0.4	4.8±0.14
	24 h	11.7±0.3	8.15±0.07	7.95±0.35	6.95±0.35	5.05±0.07

3.3 Antimicrobial activity against potential pathogenic bacteria

Antimicrobial activity of the strains was checked by the agar well-diffusion method. Results (Table 3) showed that the CFS of S. thermophilus culture had moderate activity against most of the indicator strains tested. Maximum antimicrobial activity was observed with the Escherichia coli and Staphylococcus aureus, since these strains were strongly inhibited (zone of inhibition between 6 to 8 mm). Moderate activity of CSF of S. thermophilus was observed with the Klebsiella pneumonie and Vibrio cholera while no activity was recorded against the Shigella Flexneri. However L. helveticus showed moderate or less antimicrobial activity against only three indicator strains Escherichia coli, Salmonella typhi and Staphylococcus aureus. No activity had been observed with other pathogenic indicator strains. Impact of antimicrobial activity depends on the strain, media components and physical parameters. Generally it had been suggested that growth of the pathogenic microorganisms is restricted by the production of inhibitory compounds such as lactic acid and organic acids and some kind of bacteriocins produced by the probiotic strains.

Table 3: Antimicrobial activity of strains against potential pathogenic bacteria

Strains	Indicator strains					
	Escherichia coli MTCC 443	Salmonella typhi MTCC 734	Klebsiella pneumonie MTCC 2653	Shigella flexneri MTCC 1457	Vibrio cholera MTCC 3906	Staphylococcus aureus NCIM 5021
S. thermophilus	+++	+	++	_	++	+++
L. helveticus	++	+	_	_	_	+

Diameters of inhibition zone are the mean of duplicates: + diameter of inhibition zone <2 mm, ++ diameter of inhibition zone between 2 and 5 mm, +++ diameter of inhibition zone between 6 and 8 mm, - no effect detected

3.4 Antibiotic Resistance activity

Due to the adequate use of antibiotics in humans, generally microorganisms possesses antibiotic resistance gene. For the strain to be used as probiotics, presence of antibiotic resistant gene is major issue in terms of safety aspects due to transferrable genes mainly. Generally antibiotic susceptibility test is used to evaluate the presence of antibiotic resistance gene that could transfer to the human or animal and became a potential threat for the life. S. thermophilus were found either sensitive or moderately sensitive to ampicillin, amoxicillin, chloremphenicol, ciprofloxacin, gentamicin, kanamycin and vancomycin whereas resistant against erythromycin, tetracycline, norfloxacin and streptomycin and L. helveticus was found to be sensitive or moderate sensitive to ampilcillin, amoxicillin, chloremphenicol and ciprofloxacin whereas resistant against rest of the antibiotics (Table 4). It has been reported that Lactobacilli generally had antibiotic resistance gene naturally [27]. Strains which are safe in the sense of antibiotic resistance may be used as a potential candidate in the development of future probiotics. In this study S. thermophilus showed sensitivity to most of the antibiotics studied thereby showing probiotic potential.

Table 4: Antibiotic susceptibility profiles

Strains	A	Am	C	Cf	E	G	K	T	N	Va	S
S. thermophilus	S	S	MS	S	R	S	S	R	R	S	R
L. helveticus	S	S	S	MS	S	R	R	R	R	R	R

Antibiotics: ampicillin-A; amoxicillin-Am; chloramphenicol-C; ciprofloxacin-Cf; erythromycin-E; gentamicin-G; kanamycin-K; tetracycline-T; norfloxacin-N; Vancomycin- Va; streptomycin-S
S: sensitive i.e. inhibition >50%; MS: moderately sensitive i.e. inhibition 10–30%; R: resistant i.e. no inhibition. Zone of inhibition calculated according to the table given by NCCLS.

3.5 Bile salt hydrolase activity

Bile salt hydrolase (BSH) activity is a revalant characterstics of probiotics by which they can grow in and colonize the intestine and eliminating the toxicity of conjugated bile salts readily excreted from GI tract by deconjugating them in the duodenum [28]. In our study, both strains were able to grow in the presence of bile salts even after 24 h of incubation. However, both the strains showed the hydrolase activity with only with sodium salt of taurodeoxycholic acid (TCDA). L. helveticus showed no hydrolase activity with glycodeoxycholic acid (GDCA) as no precipitate was found around the colonies as indicated by the BSH test on MRS agar plates. Thus L. helveticus hydrolysed only the sodium salt of taurodeoxycholic acid (TCDA) and S. thermophilus had the ability to show hydrolase activity with both TCDA and GDCA (Table 5). BSH activity is desirable properties of a probiotic strain since it enhances the survival and persistence in the gastrointestinal tract however it had been observed that most frequently used probiotics genera Lactobacillus and Bifidobacterium did not have the ability to hydrolyse the conjugated bile salts [29, 30].

Table 5: Bile salt hydrolase activity and cell surface hydrophobicity tests of the strains

Strains	Bile salt hydrolase activity		% hydrophobicity	
	TCDA*	GDCA	n-hexadecane	xylene
S. thermophilus	+	+	15.95±0.9	19.2±0.42
L. helveticus	_	+	3.35±0.2	5.6±1.5

* TCDA-Sodium salt of taurodeoxycholic acid, GDCA- Glycodeoxycholic acid

3.6 Cell surface hydrophobicity test

Adherence of bacteria to intestinal epithelial cells is determined by the degree of hydrophobicity. Cell surface hydrophobicity reflects the physical and chemical characteristics of the cell surface. As microbial adhesion is a combined impact of long-range Vanderwaal forces and electrostatic forces and various other short-range interactions, the strains adhering well to the hydrocarbons could be considered as positive probiotic candidate. Cell surface hydrophobicity may be influenced by the incubation time, growth conditions, and growth medium. In this study, both the strains were evaluated for their cell surface hydrophobicity towards hydrocarbons i.e. n-hexadecane and xylene. Both strains showed some extent of hydrophobicity with both the hydrocarbons (Table 5). As evident from the table, it has been observed that both S. thermophilus and L. helveticus have relatively more affinity towards the xylene than n-hexadecane. The percent hydrophobicity values observed with xylene were 19.2±0.42% and 5.6±1.5% for S. thermophilus and L. helveticus respectively while it was less in case of n-hexadecane, 15.95±0.9 and 3.35±0.2 respectively. The results were in accordance with that of reported by Iyer et al 2010 in which cell surface hydrophobicity of S. thermophilus with xylene and n-hexadecane was found in the range of 18.3 to 24.5%. Different strains may show variation in hydrophobicity with different solvents due to the fact the adhesion is dependent on both the origin of strains as well as surface properties [31]. Flint et al also reported that hydrophobicity of S. thermophilus may vary from 24% to 98% depending on their source [32].

IV. Conclusion

In conclusion, the present study revealed that folate producing strain S. thermophilus was exhibited good probiotic potential by in vitro tests in terms of acid, bile tolerance, antimicrobial activity, antibiotic resistance, bile salt hydrolase activity and cell surface hydrophobicity. However, L. helveticus also showed very less potential of probiotic attributes. On the basis of in vitro test performed, these strains could be considered as appropriate probiotics candidate however, additional ratification is required in terms of in vivo and clinical studies on animal models as well as to estimate the strains stability during the manufacturing processes. Furthermore these strains might be used in adjunct culture in food and dairy fermentation industry to prepare functional quality food products that can also contribute health benefits to the consumers. Study also suggested that these cultures can also be used in milk or milk made products due to presence of folate binding proteins in milk and provide double advantage of folate consumption along with maintenance and restoration of normal intestinal microbial flora thus prevents the pathogen adhesion to intestinal cells.

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