

Delineation of Beneficial Characteristics of Effective Probiotics

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INTRODUCTION

Researchers have been producing literature on the nutritional and therapeutic benefits of lactic cultures from as early as 1908. Today evidence supported by clinical data documents that consumption of lactic cultures controls gastrointestinal infections,¹ reduces serum cholesterol,² helps in digesting lactose in lactose-intolerant individuals,³ and reduces the incidence of colon cancer⁴ and yeast infections by immunomodulation.⁵ Recent studies also indicate that lactic cultures are useful in the management of food allergy.⁶

However, to be nutritionally and therapeutically beneficial to the host, lactic cultures should not only tolerate and pass through high stomach acidity (low pH), but also grow and proliferate at physiological levels of bile salts and adhere to intestinal epithelial cells.⁷ Further, they should improve mineral absorption, and be good producers of β -galactosidase and vitamins.⁸ Therefore, efforts are being made to isolate strains with the aforementioned properties for maximum health benefits.

In the present study, we screened different lactic cultures, primarily *Lactobacillus acidophilus* and *Bifidobacterium longum*, for their acid/alkaline tolerance, bile tolerance, acid production, antimicrobial property, and β -galactosidase activity, and for their ability to adhere to the intestinal mucosa.

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MATERIALS AND METHODS

Bacterial cultures: *Lactobacillus acidophilus* NRRL 629, NRRL 4495, NRRL 1910 were obtained from the National Center for Agricultural Utilization Research (Peoria, Ill). *L. acidophilus* DDS-1 was obtained from the University of Nebraska, Department of Dairy Science Culture Collection. *Bifidobacterium longum* ATCC 15707 and 15708 are from American Type Culture Collection (Maryland). All cultures were maintained in 10% reconstituted non-fat dry milk (NFDM) supplemented with 0.5% yeast extract from Difco (Detroit, Michigan). Prior to each experiment, the cultures were transferred three times in NFDM at 1% inoculation level and incubated anaerobically at 37°C for 24 h.

Determination of pH and Titratable Acidity: The pH of the fermented bacterial media was determined with a Fisher pH meter model 291 (Fisher Scientific Co, USA). The titratable acidity of the broth was determined before and after fermentation by titrating 10 ml of the broth against 0.1 N sodium hydroxide to a final pH of 7.0 and reported as milliequivalents of sodium hydroxide. The difference in the titratable acidity before and after fermentation was reported as "developed acidity."

Determination of D and L lactic acid: A Boehringer Mannheim Corporation (Indianapolis, Ind) enzyme kit was used to determine D and L lactic acid in the media. Manufacturer's protocol was followed without deviation.

Determination of Antimicrobial activity: The antimicrobial activity of the fermented broths was determined by the disc assay method of Shahani et al.⁹ To 95 ml of sterile antibiotic medium #4 (Difco) tempered at 40°C, actively growing *Bacillus subtilis* ATCC 6633 (18 h culture) was added at 5% v/v (10⁷ CFU/ml) and mixed well.

The medium was immediately poured into sterile petri plates and allowed to solidify. Duplicate sterile 0.5-inch diameter antibiotic assay discs (Schliecher and Schuell, Inc) were picked up aseptically by sterile forceps and touched to the fermented broth so that they absorbed it via capillary action. The discs were immediately placed on the surface of the solidified agar medium containing *B subtilis*. The plates were inverted and incubated aerobically at 37°C for 6 h. The inhibition of *B subtilis* growth (antimicrobial activity) was determined by measuring the diameter of the antimicrobial zone.

Determination of β -galactosidase activity: The β -galactosidase activity of *L acidophilus* and *B longum* strains was determined by the method of Fisher et al.¹⁰ On the day of each experiment, fresh solutions of ONPG containing 100 mg ONPG in 50 ml of 0.1M sodium phosphate buffer (pH 7.0) were prepared and used as the substrate solution. To a suitable volume of the test mixture (0.25 ml to 3 ml), 1.25 ml of ONPG solution was added, and the final volume was made up to 6 ml using 0.1M phosphate buffer. Subsequently, the mixture was incubated at 37°C for 15 minutes in a water bath. At the end of 15 minutes, the reaction was stopped by adding 2 ml of 1.0M cold sodium carbonate solution, and the optical density of the O-nitrophenol produced was read at OD420 using a blank for each sample in a spectrophotometer (Beckman model 25). β -galactosidase activity was reported in micrograms of lactase/ml of fermented broth using a previously generated standard curve for pure *E coli* lactase (Sigma Chemicals, St. Louis, Mo).

Acid and alkaline tolerance: Ten ml of overnight culture of *L acidophilus* DDS-1 was centrifuged and washed thrice with phosphate buffered saline (PBS, pH 7.0) and re-suspended in 10 ml of PBS. A volume of 200 μ l of this cell suspension was added to 2 ml of sterile 0.2 M glycine-HCl buffer (pH 2.2) for acid tolerance or 2 ml of sterile 0.2 M Tris-HCl buffer (pH 8.0) for alkaline tolerance. The contents were mixed and were incubated at 37°C. Aliquots of 100 μ l of the sample were taken every 15 min from 0 time to 2 h and total viable count was determined in MRS agar (Difco) at each sampling time.

Bile tolerance: MRS agar-containing bile salts with a concentration ranging from 0.05% to 0.2% were prepared. To the MRS agar-containing bile salts, 100 μ l of overnight culture was added and the contents were pour-plated and incubated at 37°C anaerobically. After 48 h of incubation, the total viable count was determined.

In vitro binding to HT-29 cells: HT-29 colonic carcinoma cells (ATCC) were grown in McCoy's 5 A media (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic solution (Life Technologies). The cultures were grown at 37°C in humidified atmosphere with 5% carbon dioxide, and passages

were accomplished using trypsin. For assay, 10⁵ HT-29 cells were grown to confluence in 12-well polystyrene plates. After 48 h, the wells containing confluent cells were washed thrice at 37°C with Earle's Balanced Salt Solution (EBSS). McCoy 5 A medium containing 10% FBS and no antibiotic was added to each cell. Overnight-grown *L acidophilus* and *B longum* strains, each standardized to 10⁶ CFU/ml, were added to the wells separately, in triplicates. The contents were incubated at 37°C. After 1 h incubation, cells were washed six times with Dulbecco's Phosphate Buffered Saline (D-PBS) and were released from wells by adding trypsin. Following trypsinization, 1 ml of sterile cold D-PBS was added to each well and agitated to dissociate bound bacteria from HT-29 cells. The suspension was serially diluted and pour-plated with MRS agar to determine the counts of bound bacteria. For competitive binding studies, a mixture of *L acidophilus* DDS-1 and *E coli* ATCC 25922 was added to confluent HT-29 cells at different times. The ten different time-sequential treatments are described in detail in Table 3.

Statistical analysis: T test for dependent (correlated) variable was done using the computer software *Statistica* (StatSoft, Inc, Tulsa, Okla).

RESULTS AND DISCUSSION

Selection based on acid production: *L acidophilus* and *B longum* cultures showed a time-dependent decrease and increase in pH and titratable acidity, respectively, when grown on MRS medium. As evident from Table 1, the pH for all strains gradually dropped from 6.5 at zero hour to 3.7 after 48 h, except for *L acidophilus* NRRL 1910, where it dropped to 3.2. The production of titratable acid varied widely among the different strains at different times of incubation. After 48h of incubation, the strains *L acidophilus* NRRL 1910 and *L acidophilus* DDS-1 showed the highest acid production of 119.7 and 110 milliequivalents of 0.1N sodium hydroxide, respectively, whereas the other *L acidophilus* and *B longum* strains produced less than 100 mEq of acidity (Table 1). Although all the cultures tested in the present study produced a good amount of acid, a putative probiotic should produce more of L-lactic acid than any other acid.¹¹ Excepting *L acidophilus* NRRL 1910, the other three *L acidophilus* and two *B longum* strains, produced 2.3 to 2.5 g of L-lactic acid and a negligible amount of D-lactic acid per liter of medium after 48 h of incubation (Table 2). *L. acidophilus* NRRL 1910 produced relatively low levels of L and D lactic acid (less than 1 g/L), with approximately similar quantities of D and L lactic acid.

Selection based on β -galactosidase activity: It has been suggested that most of the *L acidophilus* and *B longum* cultures are not efficient probiotics to alleviate the symptoms of lactose maldigestion due to their low produc-

Time (h)	<i>Lactobacillus acidophilus</i>								<i>Bifidobacterium longum</i>			
	NRRL 629		NRRL 4495		NRRL 1910		DDS-1		15707		15708	
	Titratable acid	pH	Titratable acid	pH	Titratable acid	pH	Titratable acid	pH	Titratable acid	pH	Titratable acid	pH
0	10.5	6.5	11.3	6.5	10.8	6.5	10	6.5	9.8	6.5	10.7	6.5
12	10.5	6.5	14.5	6.4	45.0	5.7	18	6.5	14.9	6.3	10.7	6.5
18	16.8	6.3	32.4	5.8	69.0	4.9	35	5.3	16.2	6.3	16.4	6.3
24	29.6	5.9	67.6	4.7	99.0	3.9	70	4.1	32.2	5.8	29.8	5.9
30	77.4	4.4	83.6	4.2	111.0	3.5	92	3.9	70.6	4.6	64.9	4.8
36	83.8	4.2	90.0	4.0	112.2	3.5	110	3.8	86.6	4.1	87.2	4.1
48	96.6	3.8	96.4	3.8	119.7	3.2	110	3.7	93.0	3.9	92.9	3.9

Table 1. Changes in pH and titratable acidity during growth of *Lactobacillus acidophilus* and *Bifidobacterium longum* cultures in MRS broth

Time (h)	<i>Lactobacillus acidophilus</i>								<i>Bifidobacterium longum</i>			
	NRRL 629		NRRL 4495		NRRL 1910		DDS-1		15707		15708	
	LACTIC ACID (g/L)											
	D-	L-	D-	L-	D-	L-	D-	L-	D-	L-	D-	L-
0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0.3	0.0	0.5	0.29	0.27	0	0.75	0	0.46	0	0.45
18	0	0.5	0.1	0.9	0.50	0.43	0.02	1.45	0	0.49	0	0.48
24	0	0.8	0.2	1.8	0.76	0.62	0.14	2.38	0.04	0.89	0.04	0.87
30	0.2	2.0	0.3	2.2	0.86	0.69	0.3	2.3	0.21	1.84	0.21	1.79
36	0.3	2.2	0.3	2.3	0.88	0.71	0.35	2.25	0.28	2.26	0.28	2.21
48	0.3	2.5	0.3	2.5	0.95	0.76	0.38	2.5	0.31	2.41	0.31	2.35

Table 2. Production of D- and L- lactic acid during growth of *Lactobacillus acidophilus* and *Bifidobacterium longum* cultures in MRS broth

tion of β -galactosidase.¹² However, while screening lactic cultures for probiotic use, it is always beneficial to select a strain that produces high levels of β -galactosidase for maximum therapeutic effects. We observed that *B longum* strains produced less than 1 unit of β -galactosidase, whereas *L acidophilus* cultures produced activity up to 6 units (Figure 1). Amongst the strains studied, *L acidophilus* DDS-1 and *L acidophilus* NRRL 629 produced significant higher levels ($P < 0.05$) of 6 and 5.5 units of β -galactosidase activity/ml of broth, respectively, at 30h of fermentation compared to other cultures. Although the activity remained the same for DDS-1 even after 48h of incubation, it dropped to 4 units by 48 h in the case of *L acidophilus* NRRL629.

Selection based on antimicrobial activity: All six strains of *L acidophilus* and *B longum* varied significantly in their antimicrobial activity against *B subtilis*. The

antimicrobial activity increased with increase in incubation time (Figure 2). Within *L acidophilus*, the strain NRRL 1910 showed significantly ($P < 0.05$) higher antimicrobial activity than the strains NRRL 629 and 4495. However, the difference in antimicrobial activity compared to that of *L acidophilus* DDS-1 was insignificant ($P = 0.18$). Although both *B longum* strains demonstrated similar antimicrobial activity on *B subtilis*, their magnitudes were significantly lower than those of *L acidophilus* strains ($P < 0.05$) (Figure 2). The antimicrobial property of lactic cultures is attributed to the production of natural antibiotics, hydrogen peroxide and organic acids.¹³ The high degree of antimicrobial activity of *L acidophilus* 1910 and *L acidophilus* DDS-1 may be partly attributed to their higher acid production.

Treatment No.	Particulars	Harvest time
1	At 0 hours 100 µl of 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 was added to the well containing confluent HT-29 cells	4 hours
2	At 0 hours 100 µl of 10 ⁶ CFU of <i>E coli</i> ATCC 25922 was added to the well containing confluent HT-29 cells	4 hours
3	At 0 hours 100 µl of 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 and 100 µl of 10 ⁶ CFU of <i>E coli</i> ATCC 25922 were added to the well containing confluent HT-29 cells	4 hours
4	At 0 hours 100 µl of 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 was added to the well containing confluent HT-29 cells; after 1 hour medium was aspirated and fresh medium along with 100 µl of 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 and 100 µl of 10 ⁶ CFU of <i>E coli</i> ATCC 25922 were added	4 hours
5	At 0 hours 100 µl of 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 was added to the well containing confluent HT-29 cells; after 1 hour the medium was aspirated and fresh medium along with 100 µl of 10 ⁶ CFU <i>L acidophilus</i> DDS-1 was added; and further, after 1 hour the medium was aspirated and fresh medium along with 100 µl of 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 and 100 µl of 10 ⁶ CFU of <i>E coli</i> ATCC 25922 were added	4 hours
6	At 0 hours 100 µl of 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 was added to the well containing confluent HT-29 cells; after the 1st and 2nd hour the medium was aspirated and fresh medium along with 100 µl of 10 ⁶ CFU <i>L acidophilus</i> DDS-1 was added; and at the 3rd hour after aspirating the medium, fresh medium along with 100 µl of 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 and 100 µl of 10 ⁶ CFU <i>E coli</i> ATCC 25922 were added	4 hours
7	At 0 hours 100 µl of 10 ⁶ CFU of <i>E coli</i> ATCC 25922 was added to the well containing confluent HT-29 cells; after 1 hour the medium was aspirated and fresh medium and 100 µl of 10 ⁶ CFU <i>L acidophilus</i> DDS-1 were added	4 hours
8	At 0 hours 100 µl of 10 ⁶ CFU of <i>E coli</i> ATCC 25922 was added to the well containing confluent HT-29 cells; after the 1st and 2nd hour the medium was aspirated and fresh medium and 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 were added	4 hours
9	At 0 hours 100 µl L of 10 ⁶ CFU of <i>E coli</i> ATCC 25922 was added to the well containing confluent HT-29 cells; after the 1st, 2nd, and 3rd hour the medium was aspirated and fresh medium and 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 were added	4 hours
10	At 0 hours 100 µl of 10 ⁶ CFU of <i>E coli</i> ATCC 25922 was added to the well containing confluent HT-29 cells; after the 1st, 2nd, 3rd and 4th hour the medium was aspirated and fresh medium and 10 ⁶ CFU of <i>L acidophilus</i> DDS-1 were added	4 hours

Table 3: Competitive binding of *L acidophilus* and *E coli* to HT-29 cells: details of the treatments

Selection based on acid, alkali, and bile stability:

Due to the secretion of gastric juice, stomach normally has a pH of approximately 2.0. The highly acidic pH acts as a defense mechanism by killing pathogenic microorganisms entering the body with the food we eat.¹⁴ The pH in the small intestine is generally 8.0 due to the secretion of pancreatic juices. To aid in the emulsification of fats ingested with food, a concentration of 0.05 to 0.1% of bile is secreted into the intestine.¹⁵ Thus for any bacterium to survive in the stomach and proliferate in the intestine, it should be stable at low and high pH levels, and be tolerant to bile. Data on acid stability of *L acidophilus* and *B longum* cultures, in the present study, indicated that *L acidophilus* strains DDS-1 and NRRL4495 were fairly stable up to 120 min at pH 2.2. Their

viability decreased only by 1 log at the end of 240 min. The strains NRRL 1910 and 629 showed a gradual decrease in viability, a loss of about 3 log CFU/ml within 240 min. Amongst the *B longum* strains, strain 15708 was more acid-tolerant than *B longum* 15707 (Figure 3). The stability of *L acidophilus* DDS-1 was found to be significantly higher (P<0.05) than all cultures under study except for LA 4495 which showed no significant (P=0.4) difference in stability at acidic pH when compared to LA DDS-1.

In the alkaline buffer (pH 8.0), *L acidophilus* DDS-1 and NRRL 1910 revealed significantly higher stability (P<0.05) with no substantial loss of viability up to 240 min; other cultures showed a 3 or 4 log CFU/ml decrease within 240 min (Figure 4). The fairly acid-stable strain *L aci-*

dophilus NRRL 629, was in fact, rather unstable in alkaline pH, whereas *L acidophilus* NRRL 1910 was unstable at acid pH, but stable at alkaline pH. *L acidophilus* DDS-1 was stable under both acid and alkaline conditions. In comparison to *L acidophilus* strains, both *B longum* strains were less stable at alkaline pH with a gradual loss in viability of up to 4 log CFU/ml within 240 minutes.

During exposure to increasing bile salt concentrations, the viability of all cultures decreased gradually, with a total loss at 0.25% bile salt concentration (Figure 5). Though the overall difference in stability was not significant, *L acidophilus* DDS-1 appeared to be the most resistant to bile salts amongst all strains and could withstand even 0.15% bile concentration with a viability of 27% compared to the control. At 0.1% bile salt concentration, more than 80% of *L acidophilus* DDS-1 survived in comparison to the 73, 58 and 42% viability in the case of *L acidophilus* strains NRRL 4495, NRRL 629, and NRRL 1910, respectively. *B longum* strains showed a viability up to 30% at 0.15% bile salt concentration and more than 65% viability at 0.1%.

Selection based on binding to intestinal cell lines: To become established as a successful inhabitant, bacteria must adhere strongly to the intestinal mucosal surface to not be dislodged by the contraction of the gut. The adhesive property of bacteria is generally measured by cell culture studies on intestinal cells, extracellular matrix, or basement membrane material. In the present study, the human colon tumor cell line HT-29 was used.¹⁶ The binding capacity of *L acidophilus* strains DDS-1 to HT-29 cells was significantly higher ($P < 0.05$) than all other strains studied. For example, 14 CFU were bound to a single cell of HT-29 cell line, whereas NRRL 1910, NRRL 629, and NRRL 4495 bound at 6, 3, and 2 CFU/cell of HT-29, respectively, after 1h of incubation at 37°C.

Based on the above observations, *L acidophilus* DDS-1 was further studied to determine its ability to scavenge or prevent adhesion of *E coli* to HT-29 cells, to determine whether DDS-1 could alleviate or reduce the incidence of diarrhea. Competitive studies revealed that *L acidophilus* DDS-1 replaced *E coli* in a time dependent manner. *L acidophilus* DDS-1 and *E coli* bound 14 and 15 CFU/cells of HT-29, respectively, when incubated separately. However, when HT-29 was incubated with approximately the same population of *L acidophilus* DDS-1 and *E coli*, *L acidophilus* DDS-1 bound in higher proportion than *E coli* on a per cell basis (Figure 6, treatment 3). Continuous-addition of *L acidophilus* DDS-1 to already bound *E coli*, effectively displaced *E coli* from HT-29 cells (Figure 6 treatment 7-10). Addition of *E coli* along with *L acidophilus* DDS-1 did not inhibit or decrease the binding of *L acidophilus* DDS-1 to HT-29 cells nor did it show any higher counts of *E coli* binding (Figure 6, treatment 4-6). This data indicates *L acidophilus* DDS-1 could potentially reduce binding of *E coli* to HT-29 cells.

CONCLUSIONS

Our screening studies revealed that *L acidophilus* DDS-1 is a putative probiotic strain. Its acid, bile, and alkaline stability will allow it to survive in the stomach and proliferate in the intestines, producing more of L-lactic acid than D-lactic acid, thus reducing the possibility of development of acidosis in the intestine. *L acidophilus* DDS-1 also demonstrated excellent binding to intestinal cell lines with a capability to displace *E coli*. This corroborates our earlier findings that *L acidophilus* DDS-1 inhibited the initiation of small tumors induced by N-nitrosobis (2-oxopropyl) amine in rats,¹⁷ and that such inhibition may have been immunologically augmented.¹⁸ The strain also possessed high antimicrobial activity, thus might potentially help to alleviate diarrhea and other intestinal infections. It also produced the highest quantity of β -galactosidase and may be useful in reducing the symptoms of lactose maldigestion.

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LEGENDS TO THE FIGURES

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