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FUNCTIONAL AND PROBIOTIC POTENTIAL OF INDIGENOUS *BACILLUS COAGULANS* AND *BACILLUS PUMILUS* STRAINS

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Abstract- The spore forming *Bacillus* species have created enormous interest as probiotics in the recent years, because of it's most important character of thermo-stability that assures its viability in the finished product. Thus, we aimed to explore functional and probiotic properties of indigenous *Bacillus coagulans* (n= 32), *Bacillus pumilus* (n= 2) and *Bacillus subtilis* (n= 2) strains. Intrinsic functional properties like acid and bile salt tolerance, cell surface hydrophobicity and rate of acid production for survival and action in the gut were investigated *in-vitro* for thirty six isolates. Further, we screened the best ten *Bacillus* strains and studied extensively for their different probiotic attributes and made a comparative account. Based on the relative probiotic performances, *Bacillus coagulans* B37 and *Bacillus pumilus* B9 (both isolated from buffalo milk) were appeared to be well adapted to the gastro intestinal environment and found to deliver good probiotic attributes, especially for antibacterial, hypocholesterolemic and β-galactosidase enzyme activities. Though majority of *Bacillus* species still claims better niche and requires Generally Recognized as Safe (GARG) status, these two new indigenous strains, *B. coagulans* B37 and *B. pumilus* B9 might be potential for probiotic use after proper clinical studies and safety assessment in animals and human.

Keywords- Probiotics, *Bacillus coagulans*, *Bacillus pumilus,* acid salt tolerance, bile salt tolerance, cell surface hydrophobicity, antibacterial activity, β-galactosidase activity, hypocholesterolemic activity.

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Introduction

The spore forming lactic acid producing bacteria (SFLAB) holds some promising properties and interesting characteristics for using as probiotic. This group includes many species of the genera, *Bacillus, Sporolactobacillus and Brevibacillus* [1]. The main feature of such type of spore forming organisms is to remain viable for longer duration, because it has natural tendency to transform itself in a dormant endospore in adverse situation. As the viability in the gastro-intestinal tract ecosystem is the primary criteria of any probiotic culture, *Bacillus* species, being spore formers may have the potential possibility to survive during transit through the G.I. tract. The spore-forming *Bacillus* species has the most important character of thermo-stability that attracts for its use as probiotics [2]. However, FDA (Food and Drug Administration) has restricted for granting GRAS (Generally Regarded as Safe) status for any *Bacillus* species for using as probiotic before thorough safety assessment, because of few *Bacillus* species like *B. cereus* are associated with food poisoning [3-5]. Though many probiotic *Bacillus* species like *B. subtilis, B. clausii, B.cereus, B. licheniformis, B. coagulans and B. pumilus* are available in commercial products for human, veterinary use and aquaculture in many developed and developing countries, few *Bacillus* products have been formally approved so far [2, 6]. In 2008, GRAS approved *B. coagulans* GanedenBC^{30} as the first *Bacillus* patented strain in USA [2]. *B. coagulans* GanedenBC^{30} was declared as safe for human consumption [7-8]. *Bacillus coagulans* has been reported to function as a probiotic in pig [9], in cattle [10] and in poultry birds [11]. There is a significant interest in the potential of *Bacillus coagulans* as a probiotic in humans [12- 13]. In the present study, we thus planned to explore functional and probiotic properties of indigenous *Bacillus coagulans* along with *Bacillus pumilus* and *Bacillus subtilis* strains for making comparative accounts and developing a thermo-stable probiotic cultures that might be useful in the sub-tropical country like India.

Materials and Methods

Bacterial strains: Thirty six *Bacillus* strains covering thirty two *Bacillus coagulans*, two *Bacillus pumilus* and two *Bacillus subtilis* were isolated from milk, soil and tomato sources and subsequently characterized morphologically, biochemically and genetically using single strand conformational polymorphism (SSCP) banding patterns and partial 16S rRNA gene sequences [14]. These thirty six *Bacillus* strains were screened for functional properties and probiotic attributes at Dairy Microbiology Division, National Dairy Research Institute, Karnal- 132001, Haryana, India.

Screening for functional properties: To ascertain the functional properties, thirty-six Bacillus strains were subjected to an array of tests under *in vitro* conditions as per FAO/WHO [15] and the same have been described below.

Acid and bile tolerance: The acid and bile tolerance of different *Bacillus* strains were assessed as per the methods described previously [16- 17]. Different low pH solutions were prepared to stimulate gastric acidic conditions after adjusting the pH to 1.0, 2.0 and 3.0 with 35.8% HCl. Sterile distilled water (pH adjusted to 6.5) was served as control. High bile salt solutions (1.0 and 2.0%) were prepared by dissolving bile salts (Hi-media, India) in distilled water. One ml of fresh culture containing approximately 10^7–10^8 cfu/ ml was added to the different pH solutions (1.0, 2.0, 3.0 and 6.5) as well as bile solutions (1.0% and 2.0%) and mixed thoroughly. One ml of each solution was taken from each tube immediately (0 h) and 10-fold serial dilutions were prepared in 0.1% peptone water. Pour plating

was done on BC agar media. The inoculated pH solutions were incubated at 37°C for a period of 3 h and 1 ml of culture was taken hourly from each tube after an interval of 1, 2 and 3 h of incubation followed by plating. The bile solutions containing cultures were incubated at 37°C for 12 h and 1 ml of bile solution containing culture was taken from each tube after 1, 3 and 12 h of incubation and plated on BC agar. All plates were incubated at 37°C for 24 to 72 h and the colony forming units (cfu) were counted.

Rate of acid production: The freshly grown and active bacterial culture @ 1% was mixed well with 10 ml of sterilized skim milk and incubated at 37°C for 24 h. The aqueous content was titrated against N/10 NaOH with 0.5% phenolphthalein indicator to determine the rate (in percent) of lactic acid production.

Surface hydrophobicity: Any probiotic microorganism could only be effective in the host if it possesses good adherence ability with the inner lining of the gastro intestinal tract. The bacterial adhesion to hydrocarbon like N-hexadecane is considered as a biochemical marker for its adherence ability to the epithelial cells of the gastro intestinal tract. The bacterial adhesion to hydrocarbon like N-hexadecane was determined by employing the standard method [18] with slight modification to measure the cell surface hydrophobicity. The bacterial cells grown in BC broth at 37°C for 16 to 18 h were centrifuged at 10000 × *g* for 10 min for cell separation and the cell pellet was then washed with 10 ml phosphate urea magnesium (PUM) buffer and again centrifuged and washed with 5 ml of PUM buffer. The cell pellet was resuspended in PUM buffer and the absorbance of this aqueous phase at 610 nm as $A_{initial}$ was measured. The cell suspension (4.8 ml) and N-hexadecane (0.8 ml) were mixed by vortexing and incubated at 37°C for 10 min for temperature equilibration. The mixture was again briefly vortexed and incubated at 37°C for 1 h to allow phase separation. The aqueous phase was gently taken out to measure its absorbance at 610 nm as A_{final}. The cell surface hydrophobicity (H%) was calculated as percent decrease in the absorbance of the aqueous phase after mixing and phase separation relative to that of initial suspension as follows: Surface hydrophobicity (H%) = $((A_{initial} - A_{final})/ A_{initial}) \times 100$.

Screening for probiotic attributes: To ascertain the probiotic attributes, the selected ten *Bacillus* strains were subjected to an array of tests under *in vitro* conditions as per FAO/WHO [15] and the same have been described below.

Proteolytic activity: Proteolytic activity of different strains was determined by measuring colorimetrically the quantity of tyrosine liberated according to the modified Hull's method [19]. Different standards of L-tyrosine with concentrations ranging from 10- 100 μg.ml^{-1} were prepared. The freshly grown and active bacterial culture @ 1% was mixed well with 10 ml of sterilized skim milk and incubated at 37°C for 24 h. The curd sample (2.5 g) and 0.4 M tri-chloroacetic acid (5 ml) were mixed well by shaking vigorously and allowed to stand for 10 min at 37°C and then filtered. One milliliter of filtrate or tyrosine standard aliquot was mixed with 0.4 M sodium carbonate reagent (5 ml) and 1N Folin-Ciocalteau reagent (1.5 ml) and incubated at 37°C for 10 min for the development of blue colour which was measured at 660 nm. The concentration of tyrosine (μg.ml^{-1}) was determined using the regression equation as follows: P = 90.90Q + 1.545, where P = concentration of tyrosine in μg.ml^{-1} and Q = absorbance at the respective tyrosine concentration of standard. The quantity of tyrosine liberated by the strain was expressed in terms of tyrosine equivalent (μg.ml^{-1}) as follows: Tyrosine equivalent = P × 10^{-3} μg.ml^{-1}.

Antibiotic susceptibility: The sensitivity of the strains against 15 antibiotics was determined by adopting disc diffusion method [20]. Petri plate containing 15 ml of BC agar overlaid with 4 ml of soft agar (0.75%) was seeded with 200 μl of active culture and allowed to stand for 30 min at room temperature. The bacterial culture was then dispensed to the antibiotic discs (Himedia, Mumbai, India) and incubated at 37°C for 24 h. The diameters of the zone of inhibition around antibiotic discs were measured using slide caliper and results were expressed in terms of resistance (R), moderate susceptible (MS) and susceptible (S) according to the interpretative chart [21].

Antibacterial activity: The selected strains were tested for their antibacterial activity against 7 enteric pathogens covering gram positive pathogens, namely *Bacillus cereus* NCDC 240, *Enterococcus feacalis* NCDC-115, *Enterococcus faecium* NCDC-223, *Listeria monocytogenes* Scott A and gram negative pathogens like *Escherichia coli* NCDC 135, *Salmonella* NCDC 113 and *Shigella dysenteriae* NCDC 107, using agar-well-diffusion assay method [22]. The cell-free supernatant of strains was prepared by centrifuging the freshly activated culture at 10000× *g* for 10 min. Different cultures of indicator organisms were activated in nutrient broth at 37°C by three consecutive transfers at 24 h interval. Prepoured nutrient agar plates were seeded with 200μl of freshly activated indicator organisms and allowed to solidify at room temperature for 15 min and thereafter, wells of 6.0 mm diameter were made in agar plates using a sterile cork borer. The base of the well was sealed with melted nutrient agar and 50 μl each of cell-free supernatant of various test strains was filled into each respective well. The plates were subsequently incubated at 37°C for 8- 12 h and the diameter of the zone of inhibition around the well was measured. The antimicrobial arrays of neutralized cell free supernatant were examined as control.

β-Galactosidase enzyme activity: The β-galactosidase enzyme activity of each strain was assessed by measuring colorimetrically the quantity of O-nitrophenol (ONP) released from O-nitrophenol-galactopyranoside (ONPG) following the method described previously [23]. Different standards of ONP with concentrations ranging from 25- 150 μg.ml^{-1} were prepared. The cell-free supernatant of strains was prepared as described earlier. One milliliter of cell-free supernatant or ONP standard aliquot was mixed with O-nitrophenol-galactopyranoside (ONPG) solution made in sodium phosphate buffer, pH 7 and incubated at 37°C for 15 min. The reaction was stopped by adding 2 ml of cold 5.0 mM Na_2CO_3 solution. The absorbance of the solutions was measured at 420 nm. The mole of ONP released was determined using a regression equation as follows: X = 0.0078Y – 0.0025, where X = concentration of ONP in μg.ml^{-1} and Y = absorbance at the respective ONP concentration of standard. The amount of ONP released/ min by the cell-free supernatant was directly proportional to the quantity of enzyme. One unit of enzyme activity was equivalent to 1 mole of ONP liberated from ONPG.min^{-1}. The enzyme activity of cell-free supernatant was expressed as μM of ONP liberated from ONPG.ml^{-1}.min^{-1}.

Hypocholesterolemic activity: The efficiency of different strains to remove cholesterol from the growth media was evaluated by employing the method suggested earlier [24]. Freshly prepared BC media was supplemented with 0.20% sodium thioglycocholate and 0.24% sodium taurocholate and inoculated with 1% of each bacterial culture and incubated at 37°C for 24 h. The cell-free supernatant was prepared as described earlier. To 0.1 ml of cell-free supernatant, 3.0 ml of 95% ethanol and 0.3 ml of 33% potassium hydroxide were added and mixed thoroughly and warmed in a water bath at 60°C for 15 min and then cooled. Thereafter, 10 ml hexane was added to it and mixed well by shaking vigorously for 1 min. Then, 1 ml of hexane layer was taken out and allowed for evaporation of the solvent under a flow of nitrogen gas and 2 ml of O-phthalaldehyde reagent (0.5 mg.ml^{-1} acetic acid) was added and mixed vigorously. After 10 min, 1 ml concentrated sulfuric acid was added and mixed immediately. The absorbance of the solution was recorded at 550 nm to estimate the cholesterol content using the method delineated previously [25]. The coefficient of cholesterol removal (η_{CR}) from the broth was estimated using the equation as follows:

$$\eta_{CR} = 1 - \frac{\text{Cholesterol in the supernatant of the broth inoculated with culture}}{\text{Cholesterol in the supernatant of the broth without any culture}}$$

Statistical analysis: Since the *in vitro* studies of acid and bile tolerance, rate of acid production and surface hydrophobicity ability of 36 strains generated a huge set of data, it was difficult to screen the strains apparently. The data generated

from *in vitro* studies were subjected to multivariate principal component analysis (PCA) with varimax procedure of factor analysis technique based upon correlation matrix using SPSS 10.0 Statistical Software Package, 1997, SPSS, Inc., USA, for primary screening of strains as reported elsewhere [26]. PCA enabled to reduce a large number of variables to a new, smaller, more coherent set of variables, which were principal components or principal descriptors. The correlation matrix analysis allowed to construct minimum data set (MDS) consisting of only those variables, which accounted for maximum variation of total variance of this experiment. The observed values of all strains for only those MDS variables were considered and a criteria like highest value was best for each MDS variable was imposed to score all strains on the basis of weighted linear indexing method, where linear scores were weighted by explained variance of each representative component to total variation explained. The weighted linear scoring was used to rank the strains as this method gave us a clear idea about the relative importance of significant MDS variables due to their contributions towards total accounted for variations of the whole experiment.

Data on surface hydophobicity, rate of acid production, proteolytic activity, β-Galactosidase enzyme activity and hypocholesterolemic activity of selected 10 strains were analyzed by one-way Analysis of Variance (ANOVA) using SYSTAT 6.0.1 Statistical Software Package, 1996, SPSS, Inc., USA. Fisher's least significant difference test was applied to compare pair wise mean difference probabilities.

Data are presented as the mean ± the standard error of the mean (SEM). Values are considered statistically significant at P <0.05. The mean (± SEM) of different variables studied are graphically presented using GraphPad Prism 3.02, 1999, GraphPad Software Inc., San Diego CA, USA.

Results and Discussion

Functional properties: Bacterial count at pH 3.0 at 2 h, bacterial count at pH 1.0 at 1 h, bacterial count at 2.0% bile concentration at 1 h, bacterial count at 1.0% bile concentration at 12 h, surface hydrophobicity and rate of acid production were identified as minimum data set (MDS) variables [26]. Considering one criterion that highest value was best for each MDS variable, all the strains were indexed on the basis of total weighted linear scoring as presented in [Table-1]. The maximum value for weighted linear score was found to be 0.92 for strain B9 and minimum value 0.37 for strain S5. We considered first ten *Bacillus* strains (eight *Bacillus coagulans* strains, one *Bacillus pumilus* and one *Bacillus subtilis* strain) for the comparative evaluation of probiotic attributes.

Table-1 *Total scoring of performing Bacillus strains based upon factor analysis linked weighted linear indexing method*

Sl. No.	Strain	Minimum Data Set variables						Total
		pH3/2h	pH1/1h	2%BL/1h	1%BL/ 12h	Acid Prod.	Hydrophobicity	
1	B9	0.34	0.17	0.24	0.09	0.04	0.05	0.92
2	T15	0.29	0.11	0.23	0.06	0.07	0.06	0.82
3	B37	0.31	0.07	0.25	0.10	0.04	0.06	0.81
4	T19	0.27	0.11	0.25	0.07	0.06	0.02	0.78
5	B48	0.34	0.00	0.28	0.10	0.01	0.01	0.75
6	T7	0.23	0.08	0.25	0.10	0.06	0.03	0.75
7	P3	0.29	0.06	0.23	0.08	0.04	0.04	0.74
8	T23	0.29	0.08	0.20	0.05	0.05	0.05	0.73
9	CR2	0.35	0.09	0.21	0.04	0.01	0.04	0.73
10	C8	0.35	0.09	0.16	0.06	0.01	0.05	0.73
11	SM7	0.23	0.00	0.31	0.09	0.04	0.02	0.68
12	B18	0.22	0.06	0.28	0.08	0.02	0.02	0.68
13	B31	0.31	0.00	0.24	0.07	0.04	0.01	0.67
14	C4	0.21	0.05	0.25	0.09	0.03	0.03	0.67
15	B4	0.29	0.07	0.16	0.04	0.04	0.06	0.66
16	B32	0.30	0.00	0.24	0.07	0.03	0.01	0.66
17	CR11	0.28	0.08	0.17	0.09	0.01	0.02	0.64
18	B34	0.29	0.00	0.22	0.06	0.03	0.02	0.62
19	P8	0.33	0.00	0.13	0.05	0.04	0.07	0.61
20	CR5	0.26	0.07	0.22	0.00	0.01	0.02	0.58
21	S13	0.20	0.09	0.17	0.05	0.03	0.02	0.56
22	B30	0.27	0.00	0.15	0.06	0.06	0.02	0.55
23	C6	0.27	0.00	0.14	0.05	0.04	0.04	0.54
24	S9	0.20	0.08	0.16	0.06	0.01	0.04	0.54
25	P12	0.33	0.00	0.12	0.03	0.03	0.03	0.54
26	SM2	0.12	0.00	0.27	0.09	0.03	0.03	0.54
27	B58	0.18	0.00	0.23	0.08	0.03	0.01	0.53
28	B51	0.34	0.00	0.11	0.03	0.04	0.01	0.52
29	CR8	0.28	0.00	0.13	0.08	0.01	0.02	0.51
30	P5	0.23	0.06	0.10	0.05	0.03	0.02	0.50
31	B15	0.24	0.00	0.12	0.05	0.02	0.01	0.44
32	P17	0.22	0.00	0.12	0.04	0.03	0.02	0.43
33	P9	0.29	0.00	0.00	0.05	0.04	0.01	0.40
34	P10	0.22	0.09	0.00	0.00	0.03	0.06	0.40
35	SM5	0.18	0.00	0.14	0.00	0.03	0.04	0.39
36	S5	0.13	0.00	0.15	0.04	0.02	0.02	0.37

BL stands for bile concentration

The performances of ten *Bacillus* strains in respect to six MDS variables are presented in [Table-2]. With a slight variation, most of the first ten *Bacillus* strains showed a considerable viable count at pH 3.0 after 2h of incubation and at pH 1.0 after 1h of incubation except *B. subtilis* B48, *B. coagulans* P3. *B. pumilus* B9 appeared to be the most (P < 0.05) acid tolerant, showing highest bacterial count at pH 1.0 after 1h of incubation. *B. subtilis* B48 and *B. coagulans* P3 were the most (P < 0.05) sensitive strains, as they could not survive at pH 1.0 after 1h of incubation. The viable cell count of *B. subtilis* B48 was least at pH 3.0 after 2h of incubation. The present findings agree well with the earlier report [27] indicating 50 percent survival rate of the vegetative form of *B. coagulans* CNCMI-1061 at low pH. The tolerance and sensitivity against acidic environments within *Bacillus* species have previously been reported [28].

B. pumilus B9, *B. coagulans* B37, *B. subtilis* B48 and *B. coagulans* T7 showed comparatively better (P < 0.05) tolerance both at 2% bile after 1h and 1% bile after 12h of incubation [Table-2]. The variability in response to bile salt tolerance is in agreement with earlier report [29]. All ten *Bacillus* strains could survive at 3- 6 fold

higher (1- 2%) concentration under in vitro conditions than the usual bile salt concentration (0.3%) present in human stomach.

Bacillus strains collected from tomato sources have shown to produce lactic acid at the level between 0.98 and 1.23% [Table-2]. There is a recent evidence of lactic acid production by *B. coagulans* from lignocelluloses [30]. *B. pumilus* B9, *B. coagulans* B37 and *B. coagulans* P3 could produce considerable amount of acid (0.74-0.87%) in growth medium [Table-2]. The lactic acid along with other acids like acetic acid could decrease pH of the intestinal environment making it unsuitable for the survival and growth of different pathogenic and unwanted microorganisms [31]. Acid production by the probiotic microorganisms is also desirable to impart proper body, texture and flavor during the preparation of fermented product.

There was a marked difference in adhesion ability from ~9 to 48% among the tested *Bacillus* strains [Table-2]. *B. coagulans* T15 showed the highest adherence ability (48.43%), followed by *B. coagulans* B37 (42.88%) and *B. pumilus* B9 (37.43%). Our results probably suggest the ability of *Bacillus* strains to adhere to intestinal epithelium for preventing immediate elimination by peristalsis as well as pathogen access by specific blockage on cell receptor or steric interactions [32].

Table-2 *Performances of the selected Bacillus strains in respect to MDS variables*

Sl. No.	Strain	Minimum Data Set variables					
		Bacterial count (cfu/ ml) at pH3for 2h	Bacterial count (cfu/ ml) at pH1for 1h	Bacterial count (cfu/ ml) at 2%BL for 1h	Bacterial count (cfu/ ml) at 1%BL for 12h	Acid Production (%)	Hydrophobicity (%)
1	**B9**	$7.18^{pq} \pm 0.03$	$5.67^{p} \pm 0.02$	$5.36^{pqr} \pm 0.01$	$6.28^{p} \pm 0.02$	$0.74^{st} \pm 0.02$	$37.43^{s} \pm 0.01$
2	**T15**	$7.36^{pq} \pm 0.02$	$3.69^{q} \pm 0.01$	$5.08^{qr} \pm 0.00$	$4.51^{r} \pm 0.01$	$1.23^{p} \pm 0.03$	$48.43^{p} \pm 0.02$
3	**B37**	$6.53^{q} \pm 0.02$	$2.23^{r} \pm 0.02$	$5.62^{pq} \pm 0.02$	$6.81^{p} \pm 0.02$	$0.87^{t} \pm 0.01$	$42.88^{q} \pm 0.01$
4	**T19**	$5.79^{q} \pm 0.01$	$3.72^{q} \pm 0.02$	$5.59^{pq} \pm 0.03$	$5.04^{qr} \pm 0.02$	$1.00^{qr} \pm 0.01$	$16.04^{v} \pm 0.01$
5	**B48**	$4.97^{r} \pm 0.02$	-	$6.32^{p} \pm 0.01$	$7.04^{p} \pm 0.01$	$0.20^{u} \pm 0.00$	$9.13^{w} \pm 0.02$
6	**T7**	$6.23^{q} \pm 0.02$	$2.77^{qr} \pm 0.01$	$5.62^{pq} \pm 0.02$	$6.79^{p} \pm 0.03$	$1.06^{q} \pm 0.01$	$20.22^{u} \pm 0.00$
7	**P3**	$5.95^{q} \pm 0.01$	-	$5.15^{qr} \pm 0.00$	$5.75^{pq} \pm 0.01$	$0.76^{s} \pm 0.02$	$27.38^{t} \pm 0.01$
8	**T23**	$6.28^{q} \pm 0.03$	$2.89^{qr} \pm 0.00$	$4.49^{rs} \pm 0.01$	$3.46^{st} \pm 0.00$	$0.98^{r} \pm 0.03$	$37.42^{s} \pm 0.01$
9	**CR2**	$7.54^{p} \pm 0.03$	$2.95^{qr} \pm 0.01$	$4.63^{r} \pm 0.02$	$2.85^{t} \pm 0.01$	$0.11^{v} \pm 0.01$	$27.42^{t} \pm 0.01$
10	**C8**	$7.58^{p} \pm 0.02$	$3.15^{q} \pm 0.02$	$3.57^{s} \pm 0.01$	$4.28^{rs} \pm 0.03$	$0.19^{u} \pm 0.01$	$38.33^{r} \pm 0.02$

Mean (± SEM) with different superscripts within the same column are significantly different ($P < 0.05$).

Probiotic attributes: In the present study, the selected ten *Bacillus* strains were subjected to screen for the following probiotic attributes.

Proteolytic activity: The proteolytic activities of the selected *Bacillus* strains are presented in [Fig-1]. The proteolytic activities of seven *Bacillus* strains were ~0.80- 0.88 mg tyrosine/ ml. The variation in proteolytic activity of the selected *Bacillus* strains suggests the difference in ability of hydrolyzing the complex protein moiety into easily digestible form of small peptides and amino acids in the gut by different strains.

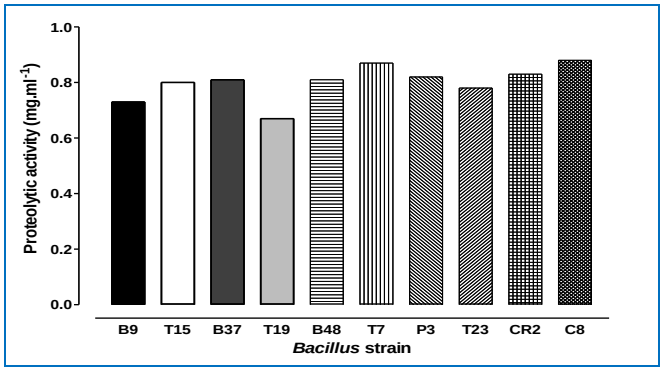

Fig-1 Proteolytic activity of the selected *Bacillus* strains

β-Galactosidase enzyme activity: There was a significant variation ($P < 0.05$) in β-galactosidase activity among the strains [Fig-2]. *B. coagulans* B37 exhibited highest β-galactosidase activity followed by *B. coagulans* T7, *B. coagulans* T19 and *B. pumilus* B9. The ability of β–galactosidase enzyme activity of any probiotic microorganism is one of the most promising applications of probiotic bacteria in food and dairy industries [23].

Antibiotic susceptibility: As shown in [Table-3], the selected *Bacillus* strains were highly sensitive to seven tested common antibiotics, namely ampicillin, chloramphenicol, ciprofloxacin, cloxacillin, gentamycin, rifampicin and tetracycline. Some strains were moderate to highly sensitive to kanamycin, streptomycin, vancomycin and a combination of amoxicillin + clauvulanic acid. Majority of the strains were susceptible in varying degrees to nalidixic acid, bacitracin and erythromycin. The variable susceptibility profiles of *Bacillus* strains to antibiotics agree well with the previous findings [28, 33]. These findings largely support the ideal probiotic characteristics of the selected *Bacillus* strains. *B. pumilus* B9 exhibited resistance against bacitracin, kanamycin and nalidixic acid, while *B. coagulans* B37 was resistant to erythromycin and nalidixic acid. It is reported that probiotic Biosporine ® contains antibiotic resistant probiotic *B. subtilis* 3 and *B. licheniformis* 31 strains [34]. Hence, the antibiotic resistance pattern and its related mechanism need to be tested for safety assessment before these strains can be considered as probiotics.

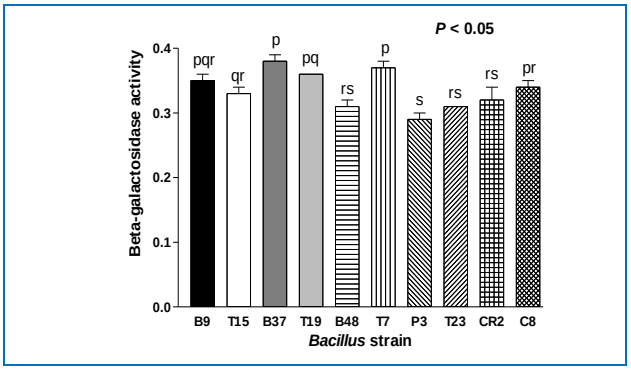

Fig-2 β-galactosidase enzyme activities of the selected Bacillus strains. Bars with different letters are significantly different (P < 0.05).

Antibacterial activity: The antibacterial activity of the selected strains against some enteric pathogens is presented in [Table-4] and [Fig-3]. Except *B. subtilis* B48 and *B. coagulans* C8, eight strains showed varying zones of inhibition from 7.5 mm to 16.5 mm depending upon the tested pathogens. The antagonistic effects against gram positive bacteria were much more prominent as compared to the gram negative organisms in most cases. Previously, the growth of various gram positive and gram negative pathogenic bacteria was inhibited by *B. coagulans* [35], *B. pumilus* [36] and *B. subtilis* [33]. *B. coagulans* B37 exhibited largest antibacterial spectrum with highest zone of inhibition against *B. cereus* followed by *E. faecalis*, *E. Faecium, E. coli* and *Salmonella* spp. *B. coagulans* B37 and T7 strains exhibited antimicrobial activity against *L. Monocytogenes* and *Shigella dysenteriae*, respectively. *B. coagulans* T7 and T19 ranked second and third, respectively, in respect to produce zone of inhibition against most of the tested pathogens. *B. coagulans* T15 and *B. pumilus* B9 showed zone of inhibition against five and four tested pathogens, respectively. In the present study, antibacterial activity of *B. coagulans* and *B. pumilus* strains could be due to

Table-3 *In-vitro antibiotic susceptibility profile of the selected Bacillus strains*

Antibiotic	Conc. (µg)	Diameter of clear zone (mm) for different *Bacillus* strains									
		B9	T15	B37	T19	B48	T7	P3	T23	CR2	C8
Ampicillin	30	23 (S)	22 (S)	22 (S)	21 (S)	21 (S)	21 (S)	20 (S)	19 (S)	29 (S)	21 (S)
Amoxycillin	10	15 (R)	18 (R)	15 (R)	13 (R)	14 (R)	14 (R)	21 (MS)	15 (R)	19 (MS)	18 (R)
Amoxicillin + Clauvulanic acid	30	24 (S)	31 (S)	19 (MS)	18 (R)	21 (S)	24 (S)	21 (S)	20 (MS)	22 (S)	20 (MS)
Bacitracin	10	11 (R)	15 (R)	18 (S)	16 (MS)	18 (S)	19 (S)	18 (S)	21 (S)	21 (S)	22 (S)
Chloramphenicol	30	21 (S)	28 (S)	20 (S)	21 (S)	21 (S)	25 (S)	24 (S)	20 (S)	26 (S)	34 (S)
Ciprofloxacin	10	30 (S)	27 (S)	26 (S)	30 (S)	28 (S)	29 (S)	29 (S)	24 (S)	20 (S)	29 (S)
Cloxacillin	15	26 (S)	19 (S)	22 (S)	26 (S)	24 (S)	27 (S)	22 (S)	24 (S)	23 (S)	22 (S)
Erythromycin	15	14 (MS)	14 (MS)	12 (R)	15 (MS)	18 (S)	21 (S)	18 (S)	16 (MS)	13 (R)	16 (MS)
Gentamycin	10	17 (S)	19 (S)	13 (S)	15 (S)	15 (S)	18 (S)	15 (S)	13 (S)	14 (S)	15 (S)
Kanamycin	30	13 (R)	20 (S)	15 (MS)	24 (S)	15 (MS)	21 (S)	21 (S)	26 (S)	16 (MS)	15 (MS)
Nalidixic Acid	30	13 (R)	15 (MS)	12 (R)	21 (S)	10 (R)	19 (S)	16 (MS)	20 (S)	26 (S)	26 (S)
Rifampicin	5	23 (S)	28 (S)	21 (S)	20 (S)	26 (S)	27 (S)	21 (S)	24 (S)	23 (S)	18 (S)
Streptomycin	10	13 (MS)	14 (MS)	13 (MS)	12 (MS)	10 (R)	16 (S)	14 (MS)	19 (S)	17 (S)	13 (MS)
Tetracyclin	30	28 (S)	26 (S)	33 (S)	31 (S)	32 (S)	27 (S)	29 (S)	30 (S)	29 (S)	31 (S)
Vancomycin	5	15 (MS)	15 (MS)	15 (MS)	16 (MS)	16 (MS)	18 (S)	14 (R)	21 (S)	21 (S)	24 (S)

S- Sensitive, R- Resistant, MS- Moderate Sensitive

Table-4 *Antimicrobial activity of selected Bacillus strains against enteric pathogens*

Strain	Inhibition Zone Diameter (mm) for different pathogens						
	E. coli	*Salmonella* spp.	*Shigella dysentariae*	*E.faecalis*	*E.faecium*	*B.cereus*	*Listeria monocytogenes*
B9	10.00	0.0	0.0	14.50	15.50	15.50	0.0
T15	9.50	9.0	0.0	9.50	15.00	11.50	0.0
B37	12.50	8.5	0.0	16.00	14.50	16.50	10.5
T19	11.00	7.5	0.0	14.00	15.00	14.00	0.0
B48	0.00	0.0	0.0	0.00	0.00	0.00	0.0
T7	9.50	9.5	9.5	15.00	11.00	13.00	0.0
P3	0.00	0.0	0.0	11.50	9.50	14.50	0.0
T23	11.00	0.0	0.0	11.50	13.00	12.00	0.0
CR2	7.50	7.5	0.0	9.50	12.00	10.50	0.0
C8	0.00	0.0	0.0	0.00	0.00	0.00	0.0

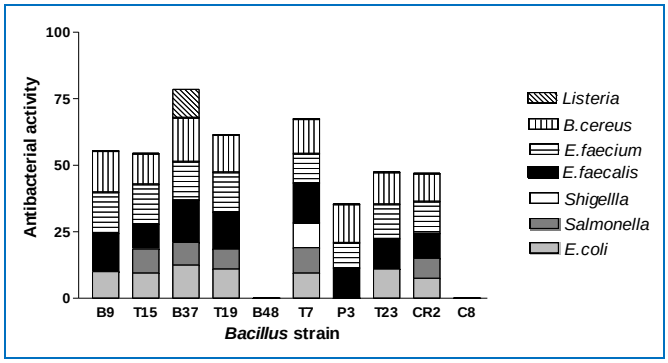

Fig-3 Antimicrobial activities of the selected Bacillus strains against enteric pathogens

lowering of pH by producing various organic acids including lactic acid [37]. Besides, *Bacillus* strains could give less access to pathogenic bacteria for binding with G.I. tract lining in the competitive exclusion mode by specific blockage on cell receptor or steric interactions [32]

Hypocholesterolemic activity: To date, there is no report on hypocholesterolemic activity of *Bacillus* species. As shown in [Fig-4]. *B. coagulans* B37 and *B. pumilus* B9 were equally effective to remove cholesterol from the medium. The cholesterol-reducing coefficient for *B. pumilus* B9 was more ($P < 0.05$) as compared to other eight strains. Dropping of pH of the media due to acid production during bacterial growth leads to the de-conjugation of bile salts and thereby results an increase demand for cholesterol as a precursor of bile salts [38]. This may be one of the reasons for lowering cholesterol from the broth when cultured with *Bacillus* strains.

Comparative accounts of probiotic Bacillus species: The present study reveals comparative accounts on functional and probiotic potential of *Bacillus* strains. The results indicate that *B. coagulans* B37 and *B. pumilus* B9 strains isolated from buffalo milk are equally good for probiotic attributes, especially for antibacterial, hypocholesterolemic and β-galactosidase enzyme activities. *B. subtilis* B48 and *B. coagulans* C8 could not be considered as suitable candidates for probiosis, as they failed to show antibacterial activity against the tested pathogens. Moreover, *B. subtilis* B48 could not survive at pH 1.0 for 1h of incubation and it showed the lowest cell surface hydrophobicity. *B. coagulans* CR2 and P3 showed poor probiotic characteristics. Possibly, all four strains collected from tomato, namely T7, T15, T19 and T23 could be the second choice of probiotic *Bacillus coagulans* strains.

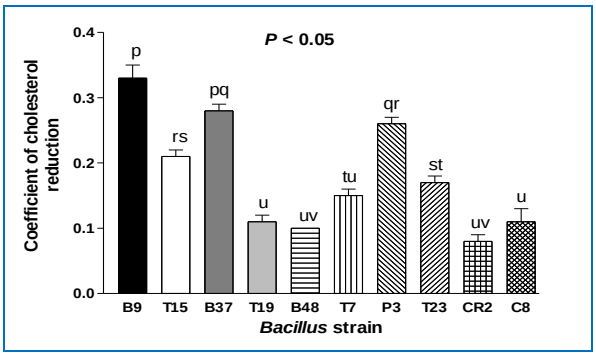

Fig-4 *In vitro* cholesterol reduction performance of the selected *Bacillus* strains. Bars with different letters are significantly different ($P < 0.05$)

Conclusions

Our studies suggest that *B. coagulans* B37 and *B. pumilus* B9 could have potential for using as probiotic candidates. Since FDA has some restrictions for granting GRAS status for any *Bacillus* species, two indigenous strains, *B. coagulans* B37 and *B. pumilus* B9 could be considered for probable probiotics after proper clinical studies and safety assessment in animals and human.

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