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Functional Properties of Spore-Forming *Bacillus* Strains: Pre-requisite for Probiotic Functions

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A B S T R A C T

The survivability of the dietary culture in the presence of acid and bile salts during the passage through the gastro-intestinal tract is the pre-requisite for probiotics to function in the intestine. While the spore-forming *Bacillus* strains have created enormous interest as probiotics in human as well as livestock animals in the recent years, intrinsic functional properties for screening probiotics such as resistance to acid, tolerance to bile salt, cell surface hydrophobicity and rate of acid production were studied with 47 spore-forming *Bacillus* isolates collected from milk, soil and tomato sources. Among 47 *Bacillus* isolates, isolate B9 appeared to be the most acid tolerant, surviving even after 3 h at pH 1.0. Isolate B37 showed the highest tolerance to 2.0% bile salt concentration upto 12th h followed by B48, P3 and B9. Isolate T15 showed the highest adherence ability (48.43%), followed by isolate B37 (42.88%) and isolate B9 (37.43%). *Bacillus* isolates collected from tomato sources have shown to produce lactic acid at the level between 0.98 and 1.23%. Based on multivariate principal component analysis (PCA) with a varimax procedure of factor analysis and weighted linear scoring on the minimum data set (MDS) of variables, first ten *Bacillus* isolates were ranked for the functional properties of probiotics.

Introduction

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). Worldwide, most commonly used microbes as probiotics include many species of the genera, *Lactobacilli* and *Bifidobacterium*, *Enterococcus, Streptococcus* and fungi like *Saccharomyces* (Kumari *et al*., 2011). However, most are unstable at room temperature and need to be freeze dried or encapsulated via special processes to remain viable during manufacturing, storage and exposure to stomach acid and bile (Baick and Kim, 2015). The development and use of spore forming bacteria, *Bacillus* spp. in particular, as probiotic has brought a breakthrough in the probiotic world for both humans and animals (Cutting, 2011). *Bacillus* species have some attractive properties like ability to form spores during adverse conditions, higher resistance to technological

stresses during production and storage processes (Jurenka, 2012) and also higher resistance to gastric (pH, digestive enzymes) and intestinal environmental conditions (Hong *et al*., 2005).

Probiotic microorganisms must have tolerance to acid and bile during passage through the gastro intestinal (GI) tract ensuring that the organisms could adhere within the gut (Shobharani and Halami, 2014). The ability of acid production such as, lactic acid, acetic acid etc. helps probiotic microorganisms to exert many beneficial effects including antimicrobial activity, imparting proper body, texture and flavour in fermented product preparation (Sidira *et al*., 2015). It is thus essential to select probiotic strain on the basis of acid, bile tolerance, adherence property along with acid production ability under *in-vitro* conditions. In the present study, we thus planned to investigate functional properties of some indigenous *Bacillus* isolates for screening the probable probiotic candidates.

Materials and Methods

An array of tests under *in vitro* conditions was conducted as per FAO/WHO (2002) to investigate functional properties of some indigenous *Bacillus* isolates at National Dairy Research Institute, Karnal- 132001, Haryana, India.

Bacterial strains

Forty seven *Bacillus* isolates covering *Bacillus coagulans*, *Bacillus pumilus* and *Bacillus subtilis* were collected 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, as previously reported (Haldar *et al*., 2015). All *Bacillus* isolates were maintained on *Bacillus coagulans* agar (BCA) slants (Atlas, 2004) and sub-cultured after every 25- 30 d period. These forty seven *Bacillus* isolates were screened for functional properties.

Acid and bile tolerance test

The acid (pH to 1.0, 2.0 and 3.0) and bile tolerance (1.0 and 2.0%) of different *Bacillus* isolates were assessed as per the methods described previously (Clark *et al*., 1993; Clark and Martin, 1994).

Surface hydrophobicity

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 (Rosenberg *et al.*, 1980) with slight modification to measure the cell surface hydrophobicity.

Rate of acid production

The freshly grown and active bacterial culture at 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.

Statistical analysis

Since the *in vitro* studies of acid and bile tolerance, surface hydrophobicity and rate of acid production ability of 47*Bacillus* isolates generated a huge set of data, it was difficult to screen the desired isolates apparently. The data generated from *in vitro* studies were

subjected to multivariate principal component analysis (PCA) with a 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 the isolates. The correlation matrix analysis allowed to construct minimum data set (MDS) consisting of only those variables, which accounted for maximum variation of the total variance of this experiment. The observed values of all isolates for only those MDS variables were considered and a criteria like highest value was best for each MDS variable was imposed to score all isolates on the basis of the 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 first 10 *Bacillus* isolates.

Results and Discussion

The criteria for screening probiotics such as resistance to acid, bile tolerance, surface hydrophobicity for adhesion property, and acid production potential were studied under *in vitro* conditions with 47 *Bacillus* isolates.

Acid tolerance to simulated pH of the human stomach

The acid tolerance pattern of 47 *Bacillus* isolates is presented in table 1. Isolate B9 appeared to be the most acid tolerant, surviving even after 3 h at pH 1.0, though more than 4 log cycle reduction was evident. Isolates, namely T15, T19, T23, CR2 and C8 survived at pH 1.0 after 2 h of incubation. B48 appeared as the most sensitive among the set which could not survive pH 1.0 at all. In most of the cases, the viable cell count reduced drastically in 1.0 pH even at 0 h. and all the strains showed decrease in viable cell count at pH 1.0 by more than 4-5 log cycles. However, the strains could survive well at pH 3.0 with only 1-2 log reduction, and moderately at pH 2.0 to 2-3 log reduction, except T7 which exhibited more than 4 log cycle reductions at both the pH followed by immediate exposure. The present findings agree well with the earlier report (Fontana *et al*., 2013). In another study (Hyronimus *et al*., 2000), different strains of *B. coagulans, B. laevolacticus* and *B. racemilactiticus* were sensitive to low pH (2.5 and 3) after 3 or 6 h of incubation, while *B. subtilis* survived at pH 2.0 and 3.0 and the survival percentage decreased ($P < 0.05$) when the exposure time progressed from 1 to 4 h (19). In the present study, there was a wide variation in response to acidic environments within different *Bacillus* strains.

Bile tolerance to simulated bile concentrations of the human small intestine

The bile salt tolerance pattern of different *Bacillus* strains is presented in table 2. Bile plays an important role in the digestion of food in the intestine. The rate of secretion of bile and the concentration of bile in different regions of the intestine can range between 0.5 to 2.0 percent during the first hour of digestion; the levels may decrease during the second hour. A period of 12 h has been reported as sufficient time for most foods to pass through the small intestine (Clark and Martin, 1994). Keeping in view these observations, an *in vitro* experiment was designed to examine the tolerance of *Bacillus* isolates in the presence of 1.0 and 2.0 percent of bile during a period of total 12 h of exposure at 37^0C. It revealed considerable changes in count in 1.0 and 2.0 percent bile salt concentrations at different time intervals. Approximately 1-2 log reduction in 1.0% bile salt concentration at 0 h were observed, while exposure to 2.0% bile concentration resulted in about 2-3 log reduction in viable count for most of the isolates. It was recorded that about 10 *Bacillus* isolates could tolerate at 1.0 and 2.0% bile concentrations even after

exposure of 12 h suggesting that these *Bacillus* isolates could survive very well in bile in the small intestine. Around 3-4 log cycle reduction in viable count for most of the isolates was evident, as the exposure time increased upto 12 h. This much reduction appeared to be quite reasonable at higher bile concentrations during long time exposure. Isolate B37 showed the highest tolerance to 2.0 percent bile salt concentration upto 12th h followed by B48, P3 and B9, while CR2 showed no survibility in the same conditions. The present results showed that *Bacillus* isolates survived well even at the higher bile concentration (2.0%) than that of the bile concentrations (0.4, 0.3, 0.2 and 0.1%) used in earlier study (El-Naggar, 2004). The variability in response to bile salt tolerance is in agreement with the earlier report (Nithya and Halami, 2012).

Surface hydrophobicity

The observations on surface hydrophobicity of different *Bacillus* isolates are presented in table 2. The results revealed a wide variation in hydrophobic interaction within *Bacillus* isolates. There was a considerable variation in the hydrophobicity of spores. Isolate T15 showed the highest adherence (48.43%), followed by B37 (42.88%) and B9 (37.43%), while B48 had the least adherence ability (9.13%). These findings are in agreement with the previous findings (Andersson *et al*., 1998). The variation in hydrophobicity to N-hexadecane among different isolates could be explained by the fact that adhesion depended upon the origin of isolates as well as surface properties (Thwaite *et al*., 2009). The information regarding the hydrophobic interaction as well as adherence ability of the *Bacillus* spores and vegetative cells are very sparse, and it is also a contentious issue. Experiments on chicks revealed that after being given a single dose of *B. subtilis* spores, spores could persist for upto 36 d in the avian intestine (La Ragione and Woodward, 2003). The S-layer proteins showed to function as adhesins to human epithelial cells and fibronectins (Hynonen *et al*., 2002), mouse ileal-epithelial cells (Frece *et al*., 2005).

The present study results probably suggest the ability of *Bacillus* isolates 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 (Otero *et al*., 2004).

Rate of acid production

The amount of lactic acid production (expressed in % lactic acid) of different *Bacillus* isolates is documented in table 2. *Bacillus* isolates collected from tomato sources have shown to produce lactic acid at the level between 0.98 and 1.23%. B9, P3, and B37 were also capable of producing a considerable amount of acid (0.74 - 0.87%) in growth medium within 24 h, while C8 and B48 were very poor in this regard. There is a recent evidence of lactic acid production by *B. coagulans* from lignocelluloses (Ou *et al*., 2011). The lactic acid along with other acids like acetic acid could decrease the pH of the intestinal environment making it unsuitable for the survival and growth of different pathogenic and unwanted microorganisms (Lankapurtha and Shah, 1998).

Comparative accounts on functional properties of *Bacillus* isolates

The present study registered comparative accounts on functional properties of *Bacillus* isolates. 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.

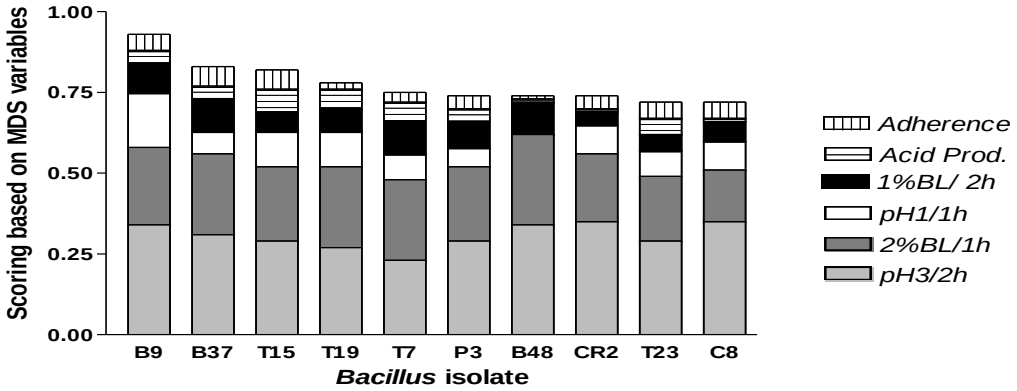
Table.1 Acid tolerance ability of different *Bacillus* isolates

Sl. No.	Isolate	Bacterial spore count (cfu) at pH 1.0				Bacterial spore count (cfu) at pH 2.0				Bacterial spore count (cfu) at pH 3.0			
		0h	1h	2h	3h	0h	1h	2h	3h	0h	1h	2h	3h
1	B1	1.48	0.00	0.00	0.00	5.71	3.57	3.15	2.61	6.69	5.86	5.84	4.51
2	B4	3.61	2.51	2.11	0.00	6.86	5.49	4.18	3.85	7.90	6.54	6.11	4.60
3	B7	2.11	1.60	0.00	0.00	6.54	5.32	4.23	2.85	6.97	6.59	5.61	5.36
4	B9	6.32	5.67	3.51	2.11	7.86	7.36	6.23	5.85	7.96	7.59	7.18	6.87
5	B13	2.61	1.85	0.00	0.00	5.15	4.59	4.11	3.85	7.85	6.99	6.49	6.28
6	B15	1.85	0.00	0.00	0.00	4.91	4.69	4.18	3.90	5.88	5.67	5.11	4.83
7	B18	2.67	2.18	0.00	0.00	5.80	4.67	3.86	3.23	5.97	5.51	4.62	4.71
8	B30	0.00	0.00	0.00	0.00	4.23	2.95	3.71	3.95	6.63	5.49	5.69	8.90
9	B31	2.65	0.00	0.00	0.00	4.59	3.63	3.23	3.28	6.86	5.96	6.64	6.88
10	B32	2.97	0.00	0.00	0.00	5.92	4.65	3.30	4.32	6.18	5.90	6.49	6.65
11	B34	0.00	0.00	0.00	0.00	4.64	3.96	3.23	2.67	5.54	5.88	6.11	6.63
12	B37	3.69	2.23	0.00	0.00	6.18	5.85	5.28	5.36	6.81	6.73	6.53	6.11
13	B48	2.97	0.00	0.00	0.00	6.63	6.90	6.92	6.96	7.97	7.61	7.36	6.85
14	B51	4.23	0.00	0.00	0.00	6.68	5.90	5.79	5.11	6.72	6.97	7.23	7.36
15	B58	0.00	0.00	0.00	0.00	4.97	3.62	2.97	0.00	5.59	4.67	3.86	2.61
16	C3	0.00	0.00	0.00	0.00	5.26	5.73	5.97	5.90	6.95	7.11	6.93	6.99
17	C4	2.60	1.85	0.00	0.00	4.20	4.28	4.32	4.73	6.73	5.86	4.57	4.97
18	C6	0.00	0.00	0.00	0.00	4.90	4.23	3.91	3.73	5.18	5.43	5.80	5.95
19	C8	3.81	3.15	2.70	0.00	7.28	7.36	6.23	6.28	7.48	7.69	7.58	7.94
20	C14	2.15	0.00	0.00	0.00	3.95	4.23	3.90	4.38	5.15	4.97	4.94	4.85
21	P3	3.56	2.18	0.00	0.00	6.62	6.28	5.48	5.32	6.90	6.57	6.15	5.95
22	P4	3.88	2.73	0.00	0.00	4.65	3.40	2.84	0.00	4.84	4.66	3.40	3.11
23	P5	3.15	1.99	2.05	0.00	3.97	3.15	2.73	1.60	5.32	5.64	4.97	4.95
24	P8	0.00	0.00	0.00	0.00	4.67	4.97	5.02	5.60	6.63	6.87	6.97	7.32
Sl. No.	**Isolate**	**Bacterial spore count (cfu) at pH 1.0**				**Bacterial spore count (cfu) at pH 2.0**				**Bacterial spore count (cfu) at pH 3.0**			
		0h	**1h**	**2h**	**3h**	**0h**	**1h**	**2h**	**3h**	**0h**	**1h**	**2h**	**3h**
25	P9	2.81	0.00	0.00	0.00	4.86	4.77	4.23	4.67	5.91	5.97	6.23	6.08
26	P10	5.96	3.23	2.59	0.00	4.83	3.97	3.73	3.51	6.93	6.54	4.60	5.26
27	P12	3.20	0.00	0.00	0.00	6.73	6.51	5.67	4.08	7.59	6.86	6.99	6.26
28	P17	2.98	0.00	0.00	0.00	5.19	4.28	3.64	2.93	5.80	5.76	4.78	2.23
29	SM2	0.00	0.00	0.00	0.00	2.97	2.08	0.00	0.00	4.11	3.30	2.66	0.00
30	SM5	2.62	0.00	0.00	0.00	3.56	3.15	2.95	2.26	3.88	3.18	3.90	3.65
31	SM7	2.65	0.00	0.00	0.00	4.90	4.68	4.41	4.96	5.91	5.77	4.86	4.61
32	CR2	3.20	2.95	2.66	0.00	6.75	6.63	6.28	6.23	7.91	7.86	7.54	6.60
33	CR5	2.23	2.45	0.00	0.00	4.65	4.97	3.57	3.85	5.90	5.71	5.51	4.48
34	CR8	0.00	0.00	0.00	0.00	3.73	3.97	4.04	4.56	5.18	5.86	5.97	5.75
35	CR10	0.00	0.00	0.00	0.00	5.32	4.51	3.63	2.28	6.85	5.95	5.74	5.20
36	CR11	2.70	2.60	0.00	0.00	4.87	4.97	5.06	4.99	5.43	5.85	6.04	6.51
37	T4	0.00	0.00	0.00	0.00	6.81	6.51	5.30	5.61	7.97	7.85	6.28	6.32
38	T7	2.95	2.77	0.00	0.00	4.43	3.65	3.97	3.45	4.23	4.73	4.97	4.08
39	T15	3.87	3.69	2.54	0.00	5.18	4.28	4.38	4.99	6.91	6.61	6.23	5.49
40	T19	3.23	3.72	2.90	0.00	5.73	5.89	5.91	5.20	6.96	6.56	5.79	4.52
41	T23	3.73	2.89	2.28	0.00	5.62	4.97	4.43	4.08	6.76	6.91	6.28	6.38
42	S1	0.00	0.00	0.00	0.00	3.65	2.51	0.00	0.00	4.23	4.63	3.89	3.61
43	S2	2.88	0.00	0.00	0.00	4.69	2.89	2.28	0.00	6.04	5.93	4.23	3.62
44	S5	0.00	0.00	0.00	0.00	4.04	3.61	0.00	0.00	5.86	4.97	2.76	0.00
45	S9	2.90	2.61	0.00	0.00	4.92	3.85	2.28	0.00	5.26	4.64	4.28	3.61
46	S10	2.96	0.00	0.00	0.00	4.07	3.64	2.96	0.00	4.57	4.62	3.15	0.00
47	S13	4.73	2.99	2.59	0.00	4.63	4.28	3.34	2.61	5.59	4.63	4.23	3.28

Table.2 Bile tolerance ability, adherence and acid production performance of *Bacillus* isolates

Sl. No.	Isolate	Bacterial spore count (cfu) at 1.0% bile				Bacterial spore count (cfu) at 2.0% bile				Adherence (%)	Acid Production (%)
		0h	1h	3h	12h	0h	1h	3h	12h		
1	B1	6.46	6.11	4.86	3.81	5.28	4.62	3.48	2.53	19.48	0.63
2	B4	5.86	5.40	4.69	2.51	4.71	3.65	2.91	0.00	49.19	0.71
3	B7	5.11	3.87	2.94	0.00	2.82	0.00	0.00	0.00	07.02	0.51
4	B9	6.43	5.95	5.64	6.28	5.70	5.36	4.59	4.87	37.43	0.73
5	B13	5.11	5.62	4.54	3.38	3.46	3.60	3.41	3.08	44.42	0.67
6	B15	5.51	4.32	3.96	3.18	3.11	2.61	2.34	2.37	08.18	0.43
7	B18	6.60	6.51	5.90	5.43	6.53	6.23	5.60	5.51	14.37	0.37
8	B30	5.28	4.86	4.28	3.95	4.30	3.32	2.30	2.32	12.27	1.13
9	B31	6.59	5.86	5.76	4.93	5.18	5.40	5.30	4.38	06.04	0.80
10	B32	7.48	6.45	5.08	5.26	7.85	5.30	4.48	4.18	10.58	0.54
11	B34	7.63	5.49	4.62	4.15	5.23	4.86	4.23	4.32	16.12	0.62
12	B37	7.46	6.11	6.86	6.81	6.28	5.62	5.48	5.53	42.88	0.67
13	B48	7.49	6.60	7.23	7.04	6.18	6.32	5.85	5.30	09.13	0.19
14	B51	5.46	3.75	3.08	1.95	3.65	2.36	0.00	0.00	06.73	0.71
15	B58	6.99	6.91	6.38	5.60	5.49	5.18	5.63	4.46	09.10	0.47
16	C3	5.69	4.52	4.86	4.95	4.51	4.60	4.76	4.85	51.20	0.19
17	C4	6.59	6.11	6.15	6.49	6.15	5.62	5.59	5.99	19.81	0.62
18	C6	5.48	5.38	4.71	3.85	4.23	3.08	2.32	0.00	32.90	0.67
19	C8	4.30	4.73	4.18	4.28	3.70	3.57	3.86	3.23	38.33	0.19
20	C14	6.08	5.11	6.04	6.11	5.20	5.00	5.95	5.85	17.29	0.72
21	P3	6.65	6.43	7.66	5.75	6.57	5.15	6.18	4.99	27.38	0.76
22	P4	3.65	2.48	3.23	4.59	0.00	0.00	0.00	0.00	19.10	0.60
23	P5	4.32	4.85	3.64	3.73	2.64	2.29	2.43	2.78	18.40	0.58
24	P8	5.87	4.62	3.58	3.28	3.56	2.89	2.08	0.00	51.50	0.72
25	P9	3.87	3.23	3.48	3.65	0.00	0.00	0.00	0.00	11.01	0.79
26	P10	4.79	2.60	2.20	0.00	3.65	0.00	0.00	0.00	42.37	0.57
27	P12	5.75	3.88	3.53	2.26	4.59	2.61	2.08	0.00	22.44	0.59
28	P17	6.65	4.20	3.38	2.90	3.11	2.63	0.00	0.00	15.58	0.57
29	SM2	8.61	8.77	8.38	6.00	7.56	6.00	7.73	6.48	22.91	0.51
30	SM5	5.48	3.30	0.00	0.00	5.60	3.11	0.00	0.00	27.93	0.60
31	SM7	7.52	6.28	6.85	6.23	8.64	6.95	7.57	7.28	14.89	0.65
32	CR2	6.28	5.38	4.45	2.85	5.85	4.63	2.86	0.00	27.42	0.11
33	CR5	6.65	5.49	3.11	0.00	6.53	4.99	2.32	0.00	16.84	0.13
34	CR8	4.48	4.15	4.57	5.38	3.08	2.85	3.65	3.95	17.81	0.12
35	CR10	3.20	3.15	2.86	2.60	0.00	0.00	0.00	0.00	12.19	0.60
36	CR11	4.95	4.49	5.73	6.00	3.62	3.85	4.49	4.97	15.04	0.11
37	T4	6.28	5.60	5.78	5.51	4.63	3.95	3.70	3.00	06.94	0.49
38	T7	7.75	7.57	6.90	6.79	5.97	5.62	4.53	4.15	20.22	1.06
39	T15	6.95	6.73	6.86	4.51	7.73	5.08	3.90	2.96	48.43	1.22
40	T19	7.23	6.65	5.85	5.04	6.85	5.59	5.18	3.70	16.05	0.99
41	T23	5.70	6.18	5.45	3.46	5.49	4.49	3.11	2.71	37.42	0.98
42	S1	6.59	5.69	4.97	0.00	3.30	2.60	0.00	0.00	08.40	0.46
43	S2	4.85	3.60	2.30	1.23	4.15	3.36	0.00	0.00	18.53	0.11
44	S5	5.70	5.23	4.43	3.15	4.04	3.30	2.95	0.00	17.89	0.43
45	S9	7.23	4.97	4.23	3.95	6.49	3.61	2.73	2.38	28.33	0.12
46	S10	6.36	5.23	4.96	3.23	4.61	3.40	2.18	0.00	13.42	0.76
47	S13	6.86	5.64	4.67	3.23	5.57	3.85	3.56	2.15	14.21	0.60

Fig.1 A comparative accounts for resistance to acid, tolerance to bile salt, cell surface hydrophobicity and rate of acid production of the selected *Bacillus* isolates



Considering one criterion that highest value was best for each MDS variable, first ten *Bacillus* isolates were ranked on the basis of total weighted linear scoring as presented in Fig. 1. Based on weighted linear scoring on the minimum data set (MDS) of variables, isolate B9, isolate B37 and isolate T15 were ranked first, second and third, respectively.

In conclusion, the present study revealed a considerable variability among different *Bacillus* isolates for acid tolerance, bile salt tolerance, cell surface hydrophobicity and rate of acid production potential. A multivariate principal component analysis with a varimax procedure of factor analysis and weighted linear scoring on the minimum data set of variables registered a ranking of *Bacillus* isolates for the functional properties of probiotics.

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