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Bacillus spp. – A Potential Probiotic

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To determine the probiotic characteristics for selected *Bacillus* spp.
Study Design: The study was aimed for evaluating the in vitro probiotic properties such as acid and bile tolerance, adhesion ability and antibacterial activity of four strains of *Bacillus* spp. such as *Bacillus cereus*, *Bacillus subtilis, Bacillus tropicus* and *Bacillus licheniformis.*
Place and Duration of Study: Department of Dairy Microbiology, Dairy Science College, Hebbal, Bengaluru, Karnataka, India, study conducted between June 2023 to April 2024.
Methodology: Probiotic nature was evaluated by determining the percentage survivability of four strains of *Bacillus* spp. at pH 2.0 and 0.3 % ox bile concentration. Percentage adhesion was determined using xylene hydrocarbon and antibacterial activity of four *Bacillus* spp. against test

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organisms like *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* was tested using agar well diffusion method.

Results: In this present study four strains of *Bacillus* spp. that is *B. cereus*, *B. subtilis, B. tropicus* and *B. licheniformis* exhibited good probiotic characteristics such as acid, bile tolerance, adhesion ability and antibacterial activity against test organisms. Among all the strains *B. subtilis* have exhibited highest survival rate of 88.01 and 74.86 % at pH 2.0 and 0.3% ox bile concentration respectively, whereas *B. tropicus* showed 14.5% adhesion ability to xylene hydrocarbon. Regarding antibacterial activity *B. subtilis* exhibited highest inhibitory zone of 7.1, 6.2 and 4.0 mm against *E. coli*, *Salmonella* spp. and *Stap. aureus* respectively.

Conclusion: In conclusion, the results of present study suggested that *B. subtilis* showed good in vitro probiotic characteristics.

Keywords: Bacillus spp. acid tolerance; bile tolerance; hydrophobicity; antibacterial activity.

1. INTRODUCTION

"Live microorganisms which when administered in adequate amounts confer a health benefits on the host" are termed as probiotics [1]. Today, owing to recognition of health benefits of probiotics as food supplements that include inhibition of intestinal pathogens by promoting the growth of beneficial microflora in GI tract, control of diarrhea, immune response enhancement, reduction in cholesterol level, anti-carcinogenic and antioxidant activity and etc [2]. *Lactobacillus* and *Bifidobacterium* spp. are widely accepted probiotics which confer several therapeutic and commercial advantages, even *Saccharomyces boulardii* and *Saccharomyces cerevisiae* are the most common yeast strains that have desirable probiotic properties and are used in many food products [3]. However, survivability of these organisms in GIT is restricted and bioavailability of these organisms are impacted by numerous factors. Hence spore forming probiotics gaining significance over non-spore forming probiotics. Being heat-stable, *Bacillus* have multiple benefits over other non-spore formers such as *Lactobacillus* spp., the members of the *Bacillus* genus exhibit extremely resistant to heat, UV irradiation, pH conditions, and solvents. Long period storage at low or room temperature, higher stability in heat processing and better survivability under GI tract conditions [4].

Bacillus are rod-shaped, gram-positive bacteria that often occur in chains and have a diameter of 0.5 to 2.5 mm. According to Bergey's Manual of Determinative Microbiology, Bacillus undergo respiratory or fermentative metabolism, that ferment glucose to produce acid, and do not convert sulfates to sulfides and test positive for the production of catalase enzyme. Other biochemical traits of this genus vary and rely on the species [5]. Bacteria of Bacillus genus dominant in soil, but they have been identified in water, air, human and animal gut, vegetables, fermented foods, raw and pasteurized milk and dairy products. Thus, owing to their ubiquitous nature, they could easily find their way into milk and other food products [6]. Several *Bacillus* strains with probiotic potential have been evaluated in various in vitro and in vivo studies but some of them such as *B. subtilis, B. polyfermenticus, B. clausii, B. coagulans, B. licheniformis* and *B. pumilus* have been approved for commercial use as dietary supplements [7].

Many extracellular enzymes, such as lipase, phytase, cellulase, amylase, and protease, are produced by members of *Bacillus* species [8]. Additionally, some *Bacillus* strains have the capacity to lower cholesterol, hydrolyze bile salts, produce antioxidants and also possess pathogen exclusion, antimicrobial, immune-modulatory and food fermentation abilities [9]. Because of their physiological characteristics and their ability to produce a wide range of enzymes, metabolites and antibiotics, which makes them useful for a wide range of applications in the pharmaceutical, medical, and agricultural sectors as well as in industrial and agricultural processes and animal nutrition., etc. [10]. Antibiotics produced by *Bacillus* exhibit broad spectrum of antibacterial activities. For example, bacitracin, laterosporin, gramicidin and tyrocidin are active against Gram-positive bacteria; polymyxin is active against Gram-negative bacteria; and mycobacillin and zwittermicin are antifungal agents. Difficidin is a broad-spectrum antibiotic [11].

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Four *Bacillus* strains *B. cereus*, *B. subtilis*, *B. tropicus*, *B. licheniformis* and test organisms

such as *E. coli*, *Salmonella* spp. and *Stap. aureus* are obtained from Department of Dairy Microbiology, Dairy Science College, Hebbal, Bengaluru were used in the present study. All the *Bacillus* strains and test organisms were maintained on nutrient agar slants at 4°C prior to use. Before evaluating probiotic characteristics all four *Bacillus* spp. were activated by growing them in nutrient broth.

2.2 Evaluation of Probiotic Properties

2.2.1 Acid tolerance test

Tolerance to low pH was tested for the *Bacillus* cultures as described by [12]. Active cultures (incubated for 36± 2 h) were used. Nutrient broth adjusted with pH 2.0 using 1M HCl was prepared and sterilized at 121°C/15 min. Broth was inoculated with fresh *Bacillus* cultures at 1% (10^5 cfu/ml) rate and incubated at 37°C/2 h. Surviving microorganisms were enumerated at 0 and 2 h by plating using sterile molten 2% Nutrient agar and results were expressed as colony-forming units (cfu) per milliliter.

The survival rate was calculated using the formula

Survivors (%) = log number of cells survived / log number of initial cells × 100

2.2.2 Bile tolerance test

Growth in the presence of 0.3% (w/v) ox bile was analyzed as described by [12]. Nutrient broth with 0.3% ox bile was prepared and sterilized at 121°C/15 min. Inoculated with 1% (10^5 cfu/ml) *Bacillus* isolate of 36 h old culture and incubated at 37°C/6 h. Surviving cells were enumerated at 0 and 6 h by plating using nutrient agar and results were expressed as colony forming units per ml.

The survival rate was calculated using the formula

Survivors (%) = log number of cells survived/log number of initial cells x 100

2.2.3 Bacterial adhesion to hydrocarbons

BATH (bacterial adhesion to hydrocarbons) ability of the *Bacillus* cultures was assessed according to the procedure described by [13]. In this test xylene hydrocarbon was used. A 24 h grown *Bacillus* culture (1 ml, approximately 10^7 cfu/ml) was taken and centrifuged at 10000 rpm for 15 min at 4°C. Obtained cell pellet was washed once with phosphate buffer saline and resuspended the cells in phosphate buffer and read absorbance at 600nm. An equal volume of xylene was added and the two phases were completely mixed by vertexing for three minutes. After 1 h of incubation at 27 ± 2°C, the aqueous phase was removed, and the absorbance at 600 nm was recorded.

Adhesion percentage was calculated using the formula:

Adhesion % = [(A0-A)/A0] x 100 where A0 and A are the absorbance (A600) before and after extraction with organic solvents

2.2.4 Antibacterial activity

Antibacterial activity was evaluated against three test organisms using agar well diffusion method as described by [14]. Test organisms (*E. coli*, *Salmonella* spp. and *Stap. aureus*) seeded with 0.2 ml (10^5 cfu/ml) separately and poured into assay plates. Four wells of 7 mm diameter were made on each agar plate and to these wells 50 *µl* of cell free supernatant of selected *Bacillus* spp. obtained by culturing in nutrient broth, centrifuged (10000 rpm for 15 min) and filter sterilized was transferred into the wells using micropipette. The plates were incubated at 37°C for 18-24 h without inverting. Formation of clear zone around the wells indicate antibacterial activity of the selected *Bacillus* isolates against test organisms.

2.3 Statistical Analysis

Statistical analysis was carried out for the obtained data using R software (R. version 4.2.1) to determine the significance and non-significance of the trails and the treatments. Data on the response variables were collected for three replications for each of the treatments. ANOVA tables were prepared to analyze the data and where the F value is significant, the critical difference was calculated (*P*=.05) and used to identify whether significant differences existed and indicated in the table using superscripts.

The formula for the critical difference (CD) is

$$\frac{\sqrt{2} \times \mathrm{MSS(E)} \times \mathrm{t}\alpha \,@\, 0.05}{r}$$

Where;

MSS (E) = Mean Sum of squares of the error, r = number of replications,
tα = table t value of the α level of significance.

3. RESULTS AND DISCUSSION

3.1 Acid Tolerance Test

B. cereus, B. subtilis, B. tropicus and *B. licheniformis* showed viable log counts of 4.49, 4.42, 4.14 and 3.98 at 0 h and 3.95, 3.89, 3.51 and 3.45 at 2 h respectively. *B. subtilis* was found to be most acid-tolerant exhibiting 88.01% of survivability followed by *B. cereus, B. licheniformis* and *B. tropicus* with 87.97, 86.68, and 84.78% survivability respectively after 2 h of incubation. The results are presented in (Table 1). Statistically significant difference (*P*=.05) was observed between 0 and 2 h at pH 2.0 in all the *Bacillus* spp.

High acid tolerance of *Bacillus* spp. may be due to alleviation of H^+ ATPase activity [15]. Similarly, after 3 h of incubation at pH 2.0, *B. cereus* F20 was determined to be the most acid-tolerant strain, displaying 49.56% survivability, whereas *B. tequilensis* F44 displayed the lowest survival rate (35.92%) which were isolated from Soumbala [16]. Additionally, [6] reported that *B. subtilis* MK559537 and *B. subtilis* MK611084 isolated from camel milk showed 88.58 and 86.64 % survival rate at pH 2.0 respectively after 4 h of incubation.

3.2 Bile Tolerance Test

B. cereus, B. subtilis, B. tropicus and *B. licheniformis* showed viable log counts of 3.11, 3.66, 3.30 and 3.66 at 0 h and 1.84, 2.74, 2.46 and 2.73 at 6 h respectively. The percentage survivability of *B. cereus, B. subtilis, B. tropicus* and *B. licheniformis* after 6 h of incubation were 59.16, 74.86, 74.54 and 74.59 respectively. The results are presented in (Table 2). Statistically significant difference (*P*=.05) was noticed between 0 and 6 h survival counts at 0.3% ox bile concentration in all the *Bacillus* spp.

The presence of bile salt hydrolases that breaks down bile and the modification in the cell wall composition may be the reasons for the survivability of probiotic under bile [17]. However, [16] noticed that *B. subtilis* F24 retained 52.69% viability after 3 h of incubation with 0.3% bile salts, whereas *B. cereus* F20 reported a high level of bile tolerance keeping 87.91% viability these *Bacillus* strains were isolated from Soumbala. *B. subtilis* SM10.1 exhibited 65.35% survivability at 0.3% ox bile concentration after incubation period of 4 h [18].

Table 1. Acid tolerance of probiotic *Bacillus* spp. at pH 2.0

***Bacillus* spp.**		**Acid tolerance Incubation time**		**CD (*P*=.05)**	**% Survivability**
		0 h	**2 h**		
		viable count log_{10} cfu/ml			
Bacillus cereus	**pH 2.0**	4.49^a	3.95^b	**0.467**	87.97
Bacillus subtilis		4.42^a	3.89^b	**0.526**	88.01
Bacillus tropicus		4.14^a	3.51^b	**0.487**	84.78
Bacillus licheniformis		3.98^a	3.45^b	**0.513**	86.68

- *CD= Critical difference; all the values are average of three trails; same superscripts indicate non-significance while different superscript indicates significant difference at P=.05*

Table 2. Bile tolerance of probiotic *Bacillus* spp

***Bacillus* spp.**		**Bile tolerance Incubation time**		**CD (*P*=.05)**	**% Survivability**
		0 h	**6 h**		
		viable count log_{10} cfu/ml			
Bacillus cereus	**0.3% ox bile**	3.11^a	1.84^b	**0.487**	59.16
Bacillus subtilis		3.66^a	2.74^b	**0.500**	74.86
Bacillus tropicus		3.30^a	2.46^b	**0.507**	74.54
Bacillus licheniformis		3.66^a	2.73^b	**0.520**	74.59

- *CD= Critical difference; all the values are average of three trails; same superscripts indicate non-significance while different superscript indicates significant difference at P=.05*

3.3 Bacterial Adhesion to Hydrocarbons

Bacterial adhesion to hydrocarbons was used to determine the adherence capacity of test strains to intestinal cells. *Bacillus* spp. exhibited a varied percentage adhesion to xylene hydrocarbon. Hydrophobic nature of *B. cereus, B. subtilis, B. tropicus* and *B. licheniformis* were 11.9, 13.1, 14.5 and 10.6 % after 1 h of incubation at 27 ± 2°C respectively. The results are presented in (Table 3). Significant difference (*P*=.05) was observed in hydrophobic nature among the *Bacillus* spp. The hydrophobic moieties of surface proteins are one of the factors that lead to extensive adhesion and aggregation of bacteria. Hydrophobic interactions are the strongest long-range non-covalent interactions and are considered as one of the determining factors in microbial adhesion to host epithelial cells [19].

Similarly [20] observed that *B. licheniformis* PUFSTP35 exhibited hydrophobicity of 57.51% and *B. subtilis* PUFSTP39 showed 24.71% hydrophobicity with xylene hydrocarbon. Similarly [21] noticed after 24 h of incubation *B. amyloliquefaciens* HTI-19 and *B. subtilis* exhibited 53.16 and 60.82% hydrophobicity respectively.

3.4 Antibacterial Activity by Agar Well Diffusion Method

The cell free extract of four *Bacillus* spp. was used to test antibacterial activity by agar well diffusion method against *E. coli*, *Salmonella* spp. and *Stap. aureus* as test organisms. *B. cereus* showed inhibitory zone of 5.7, 4.0 and 1.6 mm against *E. coli*, *Salmonella* spp. and *Stap. aureus* respectively. *B. subtilis* exhibited inhibitory zone of 7.1, 6.2 and 4.0 mm against *E. coli*, *Salmonella* spp. and *Stap. aureus* respectively.*B. tropicus* showed inhibitory zone of 5.1, 4.5 and 1.0 mm against *E. coli*, *Salmonella* spp. and *Stap. aureus* respectively. *B. licheniformis* showed inhibitory zone of 6.5, 3.0 and 2.0 mm against *E. coli*, *Salmonella* spp. and *Stap. aureus* respectively. The results are tabulated in (Table 4). Significant difference (*P*=.05) was observed between *Bacillus* spp. with antibacterial activity against test organisms.

B. cereus KY746353.1 showed inhibition zone diameters of 13, 12 and 14.50 mm against *E. coli*, *Klebsiella pneumonia* and *Stap. aureus* respectively, while *B. cereus* KX784915.1 exhibited 12.00, 10.50 and 11.25 mm inhibition against *E. coli*, *K. pneumonia* and *Stap. aureus* respectively [7]. In contrary to present study, [16] observed zero inhibitory zone by 6 strains of *Bacillus* spp. isolated from Soumbala against *E. coli* 12, *Stap. aureus* O10, *Salmonella dysenteriae* 370, but all *Bacillus* strains exhibited > 18mm inhibitory against *B. cereus* 39. Similarly, [22] observed that *B. subtilis* DG101 isolated from Japanese fermented food natto showed inhibition against *Stap. aureus* (16 mm), *Listeria monocytogenes* (15 mm), *B. cereus* (13 mm), *E. coli* (13 mm), *S. typhimurium* (13 mm) and *Vibrio cholerae* (12 mm).

Table 3. Hydrophobic nature of probiotic *Bacillus* spp.

***Bacillus* spp.**	**% Adhesion to Xylene**
Bacillus cereus	11.9[c]
Bacillus subtilis	13.1[b]
Bacillus tropicus	14.5[a]
Bacillus licheniformis	10.6[d]
CD (*P*=.05)	**0.386**

- *CD= Critical difference; all the values are average of three trails; same superscripts indicate non-significance while different superscript indicates significant difference at P=.05*

Table 4. Antibacterial activity of probiotic *Bacillus* spp. by agar well diffusion method

***Bacillus* spp.**	**Inhibitory zone in mm**		
	E. coli	***Salmonella* spp.**	***Stap. aureus***
Bacillus cereus	5.7[c]	4.0[c]	1.6[b]
Bacillus subtilis	7.1[a]	6.2[a]	4.0[a]
Bacillus tropicus	5.1[d]	4.5[b]	1.0[d]
Bacillus licheniformis	6.5[b]	3.0[d]	2.0[c]
CD (*P*=.05)	**0.396**	**0.400**	**0.390**

- *CD= Critical difference; all the values are average of three trails; same superscripts indicate non-significance while different superscript indicates significant difference at P=.05*

4. CONCLUSIONS

Results obtained in the present study showed the survivability of *Bacillus* spp. in the conditions of low pH (pH 2.0) and high bile salt concentration (0.3%), these both features help them to reach and colonize in the small intestine. All the *Bacillus* spp. exhibited good adhesion property and showed antibacterial activity against all the test organisms. Based on the results of our study *B. subtilis* reported remarkable in vitro probiotic properties and can be considered as potential probiotic supplement. This strain should be further subjected for in vivo studies to be used as a probiotic culture in food products.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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