**Original Research Article**

**SCREENING OF *BACILLU*S SPECIES OBTAINED FROM DAIRY ENVIRONMENTAL SAMPLES FOR THEIR SALIENT PROBIOTIC FEATURES**

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ABSTRACT

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| **Aims:** To screen the *Bacillus* species for important probiotic features such as acid tolerance, bile tolerance and hydrophobic characteristics. **Study design**: *Bacillus* species (19) obtained from dairy environmental samples were subjected for confirming their acid, bile tolerance with adhesion ability.**Place and Duration of Study**: The study was conducted at Department of Dairy Microbiology, Dairy Science College, KVAFSU, Hebbal, Bengaluru (Karnataka), India for a period of one year.**Methodology**: For screening of *Bacillus* spp. acid and bile tolerance, nutrient broth adjusted to pH 2.0 and nutrient broth with 0.3 per cent ox bile at pH 7.0 which are considered to the simulation of gastrointestinal condition were adopted followed by survivor counts by pour plating. In order to check the hydrophobic nature which indirectly provides the index of the attachment of *Bacillus* spp to intestinal epithelial cells xylene was used as the solvent.**Results:** A total of 19 numbers of isolates of *Bacillus* spp were obtained from the dairy environmental samples which included 3 from air sample followed by 2 from each of dung, silage, udder swab, hand swab & can rinse and one isolate from samples of soil, feed, fodder, can milk, aseptic milk and water. Among 19 isolates, B7 and B12exhibited higher survivability (%) with respect to acid accounting for 96.20, 99.52 and bile of 99.32 and 98.57, respectively. B7 & B12 isolates revealed 37.7 and 38.7% of adhesion ability, respectively. **Conclusion:** Among 19 isolates of *Bacillus* spp., B7 and B12expressed higher survivability with respect to acid as well as bile in simulated human gastrointestinal environment along with good adhesion capacity indicating that these two isolates can be further explored as probiotics in food or feed for improving the gut health of the hosts. |

***Keywords:*** *Bacillus species*; Simulation; Gastrointestinal condition; Hydrophobic nature

1. INTRODUCTION

*Bacillus* species are Gram positive, spore forming, rod shaped, aerobic or facultative anaerobic bacteria, ubiquitously distributed in soil, water, air as well as various food products. By virtue of their multilayer structured endospores, *Bacillus* spp offer high tolerance towards acid, dehydration, γ-ray and ultraviolet radiation which make them stable during heat processing and low-temperature storage. Several *Bacillus* strains have been screened for their potential probiotic functionalities in animal husbandry, bionematicides and antibiotic alternatives. Additionally, they have also been verified to possess pathogen exclusion, anti-oxidant, immuno-modulatory and food fermentation abilities. The use of *Bacillus* spp as probiotics has gained a lot of attention in recent years. During heat processing, the spores of *Bacillus* spp such as *B. clausii, (Alkalihalobacillus clausii*), *B.coagulans* (*Weizmannia coagulans*) *B. licheniformis, B.polyfermenticus (Bacillus velezensis variant polyfermenticus), B. pumilus* and *B.subtilis* have considerably increased resilience and viability in harsher environments with better survival under gastrointestinal tract conditions; possess a long shelf-life and remain viable throughout their shelf life both at room temperature and refrigerated conditions compared to lactic acid bacteria which present them as highly valuable probiotics (Nicholson *et al.*, 2000; Shah *et al*., 2021; Payne *et al*., 2024).

Probiotic *Bacillus* spp have been shown to temporarily reside as symbiotic organisms within the host (Lee *et al.,* 2019; Marzorati *et* *al*., 2020; Choi *et al*., 2021).In order to designate the bacteria as probiotics they should possess the most crucial characteristics such as acid tolerance, bile tolerance, and adhesion ability. These attributes are essential for a probiotic to survive the harsh conditions of the gastrointestinal tract and functionally interact with the host (Vasiljevic and Shah, 2008; Both *et al*., 2010).

The key features of probiotic species of *Bacillus* isolated from various sources like mango pickle, honey samples, chicken faecal samples, commercial probiotic samples and soil samples demonstrated good survivability both acid and bile along with better hydrophobicity indirectly indicating the adhesion capacity (Ragul *et al.*, 2017; Rritter *et al*., 2018; Amin *et al.,* 2020; Soni *et al*., 2022; Kostandinovska *et al*., 2024). These investigations confirmed that *Bacillus* species studied possess probiotic characteristics and further they could be used as prospective industrial probiotics. As stated by Ramlucken *et al* (2020), the key advantage of use of *Bacillus* spp. as feed probiotics was due to their robust nature pertaining to industrial production because of the high-density spore production. Furthermore, spores can retain approximately 90 % viability during the probiotic harvesting process.

The current study was designed to screen the isolated *Bacillus* spp from dairy environmental samples for their preliminary probiotic criteria, such as acid tolerance, bile tolerance and adhesion ability under simulated conditions, taking into account all the available data on the species. The suitability of these bacteria as probiotics that can withstand harsh gastrointestinal environment are gradually making their way into the probiotic field alongside lactic acid bacteria.

2. material and methods

 **2.1 Preliminary identification of *Bacillus* spp from dairy environmental samples**

 The isolates of *Bacillus* spp previously obtained from the dairy environmental samples such as air, dung, silage, soil, feed, fodder, can milk, aseptic milk, water, udder swab, hand swab and can rinse samples of university dairy farm are used in this study. The isolates were purified by streaking, confirmed for genus level identification through Gram’s staining, spore staining, catalase and oxidase test by standard procedures given by Harrigan (1998).

 **2.2 To screen the *Bacillus* species for probiotic properties**

 The confirmed isolates of *Bacillus* spp for probiotic properties as acid tolerance, bile tolerance slightly modified method given by Ritter et al (2018) and hydrophobic nature as per Nayarisseri et al (2018) were followed.

 **2.2.1 Acid tolerance test**

Sterile pre-adjusted Nutrient broth to pH 2.0 was inoculated individually with 19 *Bacillus* isolates and incubated at 37°C. Samples drawn immediately after inoculation considered as 0 h and after 2 h of incubation were plated. Surviving bacteria were enumerated at 0 and 2 h by plating using sterile molten nutrient agar and expressed as colony-forming units (cfu) per millilitre. The survival rate was calculated using the formula:

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

* + 1. **Bile tolerance test**

Nutrient broth with 0.3 % ox bile was prepared and sterilized at 121 °C /15 min. All the 19 isolates grown in nutrient broth were inoculated at 1 % to bile broth (nutrient broth with 0.3 % ox bile) and incubated at 37oC/6 h. Viable counts at 0 and 6 h of incubation was carried out using nutrient agar and expressed as colony forming units per ml. The survival rate was calculated using the formula:

Survivors (%) = log no of cells survivors/log no of initial cells x 100

# Hydrophobic nature

 BATH (bacterial adhesion to hydrocarbons) test was conducted using hydrocarbon xylene. A 18 h grown culture (1 ml, approximately 107 CFU/ml) was taken and centrifuged at 10000 rpm for 15 min at 4°C. The collected pellet was washed with phosphate-buffered saline, suspended in the same buffer and read absorbance (600 nm). An equal volume of xylene was added and the two-phase system was thoroughly mixed by vertexing for 3 min. The aqueous phase was removed after 1 h incubation at 27 ± 2°C and the absorbance measured at 600 nm.

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

 solvent}

1. results and discussion

#  In the present study isolates of Bacillus spp. were confirmed as Bacillus by preliminary identification tests and further subjected them to acid tolerance, bile tolerance and hydrophobicity test. These conducted tests initially helped to place the isolates as probiotics. The results obtained in the study are discussed along with the studies of other authors in this section

**3.1 Preliminary Identification of the isolates of *Bacillus* spp from dairy environmental samples**

 The dairy environmental samples such as dung, soil, feed, fodder, silage, udder swab, hand swab, can rinse, can milk, aseptic milk, water and air samples were the sources for the isolation of *Bacillus* spp. A total of 19 numbers of previously obtained isolates of *Bacillus* such as 3 from air sample followed by 2 from each of dung, silage, udder swab, hand swab and can rinse samples, while one isolate from samples of soil, feed, fodder, can milk, aseptic milk and water samples (Table 1) were subjected for Gram’s staining, spore staining, catalase test and oxidase test (Harrigan,1998). According to the results obtained, all the 19 isolates were Gram positive rods with spores and showed positive for both catalase and oxidase test which placed the isolates under the genus *Bacillus*. Similarly, Altun and Erginkaya (2021) who isolated 4 bacterial strains from vegetables such as potato, pickle, corn and tomato, which were Gram-positive, spore-forming rods, catalase and oxidase positive. And placed them under genus *Bacillus*. Sireesha *et al* (2024), also noticed the acid tolerant as well as bile tolerant *Bacillus* counts in dairy environmental samples.

 **Table 1: Isolates of *Bacillus* spp obtained from dairy environmental**

 **samples**

|  |  |  |
| --- | --- | --- |
| **Sources of the isolates** | **Code of *Bacillus* isolates** | **Number of isolates obtained** |
| Soil | B1 | 1 |
| Feed | B2 | 1 |
| Fodder | B3 | 1 |
| Dung | B4, B5 | 2 |
| Silage | B6, B7 | 2 |
| Udder swab | B8, B9 | 2 |
| Hand swab | B10, B11 | 2 |
| Can rinse | B12, B13 | 2 |
| Water | B14 | 1 |
| Aseptic milk | B15 | 1 |
| Can milk | B16 | 1 |
| Air | B17, B18, B19 | 3 |
| Total | 19 |

 **Note: All the 19 isolates were Gram positive rods with spores and showed**

 **positive for both catalase and oxidase test.**

**3.2 Screening of *Bacillus* isolates for important probiotic nature**

 All the 19 isolates of *Bacillus* confirmed to the genus *Bacillus* were subjected to acid tolerance, Bile tolerance and hydrophobicity to validate for the salient features of probiotics.

**3.2.1 Acid tolerance**

 All the isolates showed survival at pH 2 for 2 h of incubation, a simulation of gastric condition in human beings. The viable counts of the isolates of *Bacillus* spp at 0 h ranged 4.60 to 6.70 log10 cfu/ml while after 2 h of incubation at 37°C, the counts were 4.30 to 6.27 log10 cfu/ml on nutrient agar medium with survivor of 76.19 to 99.52%. The reduction in viable count of the isolates after incubation in pH 2 was very meager indicating that the isolates can tolerate harsh hydrochloric acid condition (pH 2) of stomach. A good viable count with survivor rate was noticed in B12 (isolate of can rinse) accounting for 6.27 log10cfu/ml which was initially having viability of 6.30 with percent survivor of 99.52 (Table 2). The acid tolerant effect of the *Bacillus* isolates may be due to the urea hydrolysis that neutralized gastric environment for making the bacteria to survive (Liu *et al*., 2015).

 **Table 2: Acid tolerance of probiotic *Bacillus* isolates from dairy environmental**

 **samples**

|  |  |  |
| --- | --- | --- |
| **Isolate code** | **Acid tolerance** **Incubation time** | **% Survivor** |
| **0 h** | **2 h** |
| **viable count (log10 cfu/ml)** |
| B1 | 6.70 | 5.42 | 80.89efghij |
| B2 | 5.78 | 5.53 | 95.67abc |
| B3 | 5.96 | 5.92 | 99.32ab |
| B4 | 6.36 | 4.31 | 67.76kl |
| B5 | 5.93 | 5.38 | 90.72abcde |
| B6 | 5.93 | 5.73 | 96.62abc |
| B7 | 5.63 | 5.36 | 95.20abc |
| B8 | 6.32 | 5.62 | 88.92bcdefg |
| B9 | 6.26 | 4.77 | 76.19hijkl |
| B10 | 4.60 | 4.30 | 93.47abcd |
| B11 | 5.34 | 5.0 | 93.63abcd |
| B12 | 6.30 | 6.27 | 99.52ab |
| B13 | 5.78 | 5.70 | 98.61ab |
| B14 | 6.02 | 5.53 | 91.86abcde |
| B15 | 6.23 | 6.10 | 97.91abc |
| B16 | 5.70 | 5.07 | 88.94bcdefg |
| B17 | 6.42 | 5.96 | 92.83abcd |
| B18 | 5.34 | 5.23 | 97.94abc |
| B19 | 6.23 | 6.12 | 98.23abc |
| **Critical Difference****(*P*=.05)** | **3.02** |

**Note**: Different superscripts with in the column are compared with other values

**3.2.2 Bile tolerance**

 The bile salt tolerance of all the 19 *Bacillus* isolates were tested in the presence of 0.3 per cent ox bile at pH 7.0 because of its similarity to human bile juice after an exposure of 6 h which was similar to exposure of food in duodenum of human beings. Viability of isolates at 0 h was between 5.59 and 6.76 log10 cfu/ml, while after 6 h of exposure of isolates to bile, reduction in viability was noticed with counts ranging from 4.80 to 6.24 log10 cfu/ml while survivor rates were 76.67 to 99.32 percent. The trend observed among the isolates with respect to bile exposure was nearly similar to the gastric simulation condition. Out of 19 *Bacillus* isolates, B7(silage isolate) showed best viability which was initially 5.90 log10 cfu/ml that reduced very minimally accounting for 5.86 log10 cfu/ml with percent survivability of 99.32 (Table 3). The bile tolerance of the isolates may be due to presence of bile salt hydrolase (BSH) that hydrolysis the bile to nontoxic form (Joyce *et al.,* 2014).

**Table 3: Bile tolerance of probiotic *Bacillus* isolates from dairy environmental samples**

|  |  |  |
| --- | --- | --- |
| **Isolate code** | **Bile tolerance** **Incubation time** | **% Survivor** |
| **0 h** | **6 h** |
| **viable count (log10 cfu/ml)** |
| B1 | 6.29 | 5.13 | 81.55klmnop |
| B2 | 6.26 | 5.43 | 86.74cdefghijklmno |
| B3 | 5.90 | 5.40 | 91.52abcdefghijk |
| B4 | 6.26 | 4.80 | 76.67mnop |
| B5 | 6.11 | 5.17 | 84.61ghijklmnop |
| B6 | 6.00 | 5.86 | 97.6ab |
| B7 | 5.90 | 5.86 | 99.32ab |
| B8 | 6.05 | 5.96 | 98.51ab |
| B9 | 6.39 | 6.28 | 98.27ab |
| B10 | 6.42 | 5.04 | 78.5nop |
| B11 | 5.77 | 5.66 | 98.09ab |
| B12 | 6.32 | 6.23 | 98.57ab |
| B13 | 6.41 | 6.12 | 95.47abcdef |
| B14 | 6.38 | 6.13 | 96.08abcd |
| B15 | 6.44 | 6.23 | 96.73abcd |
| B16 | 5.63 | 5.51 | 97.86ab |
| B17 | 6.76 | 5.63 | 83.2ijklmnop |
| B18 | 5.59 | 5.17 | 92.48abcdefghij |
| B19 | 6.36 | 6.24 | 98.11ab |
| **Critical Difference****(*P*=.05)** | **2.84** |

**3.2.3 Effect of acid and bile on the isolates**

When the results of both acid and bile tolerance of the *Bacillus* isolates were compared, except B4 (isolate of cow dung), all the 18 isolates exhibited a good survivability as evident in Table 4, indicating their resistance to both the major harsh conditions of gastro-intestinal tract. *Bacillus* spp. isolated from different sources also exhibited acid and bile tolerance, key features of probiotics. Ragul *et al* (2017) who isolated *B. licheniformis* PUFSTP35 obtained from fermented mango pickle, manifested higher survivability of 99.75 and 87.80% in acid and bile among 9 isolates. While Ritter *et al* (2018) found *B. subtilis* FTC01 strain from commercial probiotic product was more tolerant to both acid (96%) and bile accounting for 96% survivability out of 7 strains.About90% and 60% of *Bacillus* spp from sourdough showed acid and bile tolerance, respectively as mentioned by Penaloza-vazquez *et al* (2019). Similar trend of acid and bile tolerance was noticed in case of *B. amyloliquefaciens* HTI-19 and *B. subtilis* -23 isolated from Malaysian honey samples accounting for more than 85% (Amin *et al.,* 2022). Similarly, Pełka *et al* (2023) also found BB19.21 and BP20.9 of bee bread and bee pollen from Polish apiaries had the highest survival rates in the harsh conditions of gastrointestinal tract among 10 isolates of *Bacillus* spp. Even soil isolates (7) of *Bacillus* spp from North Macedonia tolerated both acid and bile indicating resistance to gastrointestinal conditions expressing them as potential probiotics according to Kostandinovska *et al* (2024). The same authors declared that those strains can be used in poultry feed for extending probiotic benefits to the poultry.

On the contrary *B. velezenesis* CGS1.1 isolated from chicken faecal sample showed survivability of 74% on an average in acid and bile when tested separately and was accounted lesser compared to present study (Soni *et al*., 2022). Anyairo *et al* (2024), also could able to establish lesser survival of 72.9 and 64.7% for K29.2 and K15.4 isolates of *Bacillus* spp., out of 17 isolatesobtained from miang, a fermented tea of North Thailand, towards gastric acid whereas higher survivability of 99 and 98% for bile was noticed, respectively. The results of these authors when compared to present study acid tolerance was lesser whereas bile was almost the same with respect to the isolates of *Bacullus* spp.

**Table 4: Comparison of survivor rates of *Bacillus* isolates in acid and bile environment**

|  |  |
| --- | --- |
| **Isolate code** | **% Survivors** |
| **Acid** | **Bile** |
| B1 | 80.89 | 81.55 |
| B2 | 95.67 | 86.74 |
| B3 | 99.32 | 91.52 |
| B4 | 67.76 | 76.67 |
| B5 | 90.72 | 84.61 |
| B6 | 96.62 | 97.60 |
| B7 | 95.20 | 99.32 |
| B8 | 88.92 | 98.51 |
| B9 | 76.19 | 98.27 |
| B10 | 93.47 | 78.50 |
| B11 | 93.63 | 98.09 |
| B12 | 99.52 | 98.57 |
| B13 | 98.61 | 95.47 |
| B14 | 91.86 | 96.08 |
| B15 | 97.91 | 96.73 |
| B16 | 88.94 | 97.86 |
| B17 | 92.83 | 83.20 |
| B18 | 97.94 | 92.48 |
| B19 | 98.23 | 98.11 |

**3.2.4 Hydrophobic nature**

Cell surface hydrophobicity plays a key role in the attachment of bacterial cells to epithelial cells (Kos *et al*., 2003). The test was conducted using xylene as it is a non-polar solvent which was also followed by many of the authors (Ritter *et al*., 2018; Soni *et al.,* 2022; Pelka *et al.,* 2025). In the present study, also xylene was used as the hydrocarbon for testing the hydrophobic nature of 19 isolates of *Bacillus* spp that ranged from 0.60 to 38.7%. Out of 19 isolates only 4 isolates such as B12, B13, B14 and B16 exhibited more than 34% adhesive ability. Out of the 4 isolates, B12 showed higher hydrophobic nature of 38.7% (Table 5). B12 and B13 were isolated from can rinse which have the better ability to adhere to can used to store raw milk, whereas B14 from water and B16 from can milk also showed abetter adhesion ability. The adhesion ability in general may be attributed to peritrichous flagellation and exopolysaccharide production from *Bacillus* isolates (Guttenplan & Kearns, 2013; Yang et al., 2025).

On par with the present study, Soni *et al* (2022) also observed 38%t hydrophobic nature of the probiotic *Bacillus velezenesis* CGS1.1 isolated from chick faces. Similarly Ritter *et al.* (2018) found hydrophobicity of 35% in *B.subtilis* strains KM01(Clostat) and FPR02 (Fertitacto) isolated from commercial probiotic products, the value was near to the present study while reference strain DSM 4451 accounted 56.4%.

On the contrary to the current study, Ragul *et al.* (2017) experienced better hydrophobicity in *B. licheniformis* PUFSTP35 isolated from mango pickle accounting for 57.33 percent compared to present study. Maximum affinity of 53.64 and 60.82% towards xylene was noticed in *B. amyloliquefaciens* HTI-19 and *B. subtilis* HTI-23 of Malaysian honey, respectively confirming their adhesion to intestinal epithelial cells (Amin *et al*., 2020). Even Pełka *et al* (2025), also demonstrated higher hydrophobicity of 61.08% in case of *Bacillus* spp PG10.5 isolated from Polish bee pollen and found to be maximum affinity towards xylene out of 10 isolates. These three studies showed good hydrophobic nature of two times more in the isolates of *Bacillus* spp. compared to the present study.

 **Table 5: Hydrophobic nature of *Bacillus* spp using xylene**

|  |  |
| --- | --- |
| **Isolate code** | **% of Adhesion**  |
| B1 | 0.60p |
| B2 | 8.00 lmnop |
| B3 | 9.00 lmnop |
| B4 | 17.70 hijklmn |
| B5 | 19.00 ghijklm |
| B6 | 28.00 bcdefgh |
| B7 | 37.50 abc |
| B8 | 24.50 efghij |
| B9 | 8.24 lmnop |
| B10 | 29.64 abcdefg |
| B11 | 2.52 p |
| B12 | 38.70 ab |
| B13 | 37.46 abc |
| B14 | 33.90 abcde |
| B15 |  3.00 p |
| B16 | 36.07 abc |
| B17 | 3.00 p |
| B18 | 3.20 p |
| B19 | 2.30 p |
| **Critical Difference (*P*=.05)** | **3.14** |

 **4. CONCLUSION**

Among 19 *Bacillus* isolates, B7 and B12exhibited higher survivability with respect to acid, bile with good hydrophobic nature indicating their fundamental probiotic nature which were isolated from dairy environmental samples such as silage and can rinse, respectively. Except one isolate, all the *Bacillus* isolates showed a better basic probiotic nature which can be exploited either in feed or food by confirming few more tests, as they are easy to grow and store.

**DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

**Option 1:**

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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