**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 acid and bile tolerance screening of the *Bacillus* species, nutrient broth adjusted to pH 2.0 and nutrient broth with 0.3 per cent ox bile at pH 7.0 and checking for the viability by pour plating. In order to check the hydrophobic nature which indirectly provides the index of the attachment of *Bacillus* species to intestinal epithelial cells using xylene as a solvent.**Results:** A total of 19 numbers of isolates of *Bacillus* were obtained from the dairy environmental samples included 3 from air sample followed by 2 from each of dung, silage, udder swab, hand swab and can rinse and one isolate from samples of soil, feed, fodder, can milk, aseptic milk and water. Among 19 isolates, B7 and B12exhibited higher percentage of survivability with respect to acid accounting for 96.20, 99.52 and bile of 99.32 and 98.57, respectively. The hydrophobic nature in xylene of B7 & B12 were 37.7 and 38.7%, respectively. **Conclusion:** Among 19 *Bacillus* isolates, B7 and B12exhibited higher of survivability with respect to acid, bile, simulated human gastrointestinal conditions along with good adhesion capacity indicating the two isolates can be further used as probiotics in food or feed for improving the gut health of the hosts. |

***Keywords:*** *Bacillus species*; Screening; Probiotic nature; Dairy environmental samples

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* species as probiotics has gained a lot of attention in recent years. During heat processing, the spores of *Bacillus* species 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; 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). Exposure of 9 test isolates of *B. licheniformis* from fermented mango pickle, to simulated in vitro gastric juice of pH 2, the strain PUFSTP35 revealed higher survivability 99.75 % (8.05 initial to 8.03 log 10 cfu/ml after 2 h of exposure) with a significant variability. PUFSTP35 exhibited comparatively better tolerance to bile accounting for 87.80 % (8.61 initial to 7.56 log 10 cfu/ml after 6 h of exposure). The hydrophobicity of *B. licheniformis* PUFSTP35 to xylene was found to be better among the 9 isolates with 57.33 per cent hydrophobicity (Ragul *et al*., 2017). Among 7 strains of *Bacillus subtilis* isolated from commercial probiotic formulations of CHR01(Gallipro), FTC01 (Biotop), CP01 (Calsporin), KM01(Clostat) and BSN01 (Naatto), and FPR01 and FPR02(Fertitacto) and DSM 4451 (reference strain), FTC01 strain was more tolerant to acid as well as bile exhibiting 96% survivability. KM01 showed around 45% adhesion percent with xylene. The reference strain, DSM 4451 consistently showed all the three probiotic properties (Rritter *et al*., 2018)

# Out of 200 Isolates of *Bacillus* spp from sourdough and chicken gastro-intestinal tract (GIT), 42 of the isolates survived at pH 3 (chicken gizzard pH), while 55 showed the ability to grow on 5 % bile. The percentage of strains isolated from GIT that survived for 3 h at pH 3 was 90% (23/25), whereas those isolated from sourdough was 60% (17/30) However, all the isolated strains from GIT were able to grow in the presence of 5.0% ox bile. Those isolates that showed probiotic nature could be used in poultry feed (Penaloza-Vazquez *et al*., 2019). Amin *et al* (2020) isolated *Bacillus* spp. from Malaysian honey samples and found that *B. amyloliquefaciens* HTI-19 and *B. subtilis* HTI-23 possessed acid (pH 2.0) and bile tolerance (0.3% bile salt solution for 3 h) with the survival rates of >85%. The hydrophobicity percentage of 53.64 and 60.82 was observed in *B. amyloliquefaciens* HTI-19 and *B. subtilis* HTI-23, respectively confirming their adhesion to intestinal epithelial cells. Ramlucken *et al.* (2020) expressed the key advantage of use of *Bacillus* spp. as feed probiotics 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. *Bacillus velezenesis* CGS1.1 isolated from chicken faecal sample when exposed to pH 2.5 showed 75% survivability followed by 73% bile tolerance (0.3 %) and 38% adhesion in xylene (Soni *et al*., 2022). A study by Kostandinovska *et al* (2024), demonstrated that 7 soil isolates of *Bacillus* spp from North Macedonia demonstrated tolerance to 0.3–2% bile salts and survival at pH 3.0, indicating resistance to gastrointestinal conditions expressing them as potential probiotics. In this study attempt has been made to screen the previously obtained *Bacillu*s isolates from dairy environmental samples of university farm for their important probiotic features.

2. material and methods

 **2.1 Preliminary identification of *Bacillus* species 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}

3. results and discussion

**3.1 Preliminary Identification of the isolates of *Bacillus* species 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 preliminary identification tests in order to place the isolates under the genus of *Bacillus*. The tests included Gram’s staining, spore staining, catalase test and oxidase tests as per the standard procedures of Harrigan (1998). Altun and Erginkaya (2021) isolated 4 strains of *Bacillus* spp from various food samples like potato, pickle, corn and tomato. The four isolates from vegetables were identified as *Bacillus species* through phenotypic identification that resulted in Gram-positive, spore-forming rods, catalase and oxidase positive. Sireesha *et al* (2024), noticed the acid tolerant as well as bile tolerant *Bacillus* counts in dairy environmental samples.

 **Table 1: Isolates of *Bacillus* species 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 were subjected to acid tolerance, Bile tolerance, and hydrophobicity to validate for the salient features of probiotics.

**3.2.1 Acid tolerance**

 All most all the isolates showed survival at pH 2 for 2 h of incubation, a simulation of gastric or stomach condition of 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 sterile nutrient agar medium with survivor percentage of 76.19 to 99.52. Very slight reduction in viable count of the isolates after incubation in pH 2 was noticed, indicating the isolates can tolerate harsh acidic condition 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). Thankappan *et al.* (2015) also found among the three crab (*Labeo rohita*) isolates, KADR1 showed highest per cent of viability of 90.90 % after 3 h of exposure compared to 85.71 and 84.61 % demonstrated by the isolates, KADR3 and KADR4, at highly acidic pH 2 at 37 °C. Similar to the present study Ritter *et al.* (2018) investigated acid tolerance property of *Bacillus subtilis* strains obtained from probiotic formulations and found that after 2 h of incubation at pH 2, the FTC01 isolate was more tolerant to acid exhibiting 96 per cent survivability while FPR01 and FPR02 were the cultures showing lower resistance to pH 2, whereas Soni *et al.* (2022) observed 75 % survivability of *Bacillus velezenesis* CGS1.1 isolated from chicken faecal sample when exposed to pH 2.5.

 **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). On par with the present study done at 0.3 per cent bile for 6 h exposure, according to Thankappan *et al* (2015), among the three crab isolates, KADR3 showed higher bile tolerance with 41.12 per cent followed by KADR1(27.35 %) and KADR4 (25.71 %). Amin *et al* (2020) found the survival rates of *B. amyloliquefaciens* HTI-19 and *B. subtilis* HTI-23 from honey of the stingless bee *Heterotrigona itama* as >85 %, after exposure to 0.3% bile salt solution for 3 h at 37 °C. Soni *et al* (2022) found the survivability of 73 % bile tolerance (0.3 %) was by *Bacillus velezenesis* CGS1.1 isolated from chick faces sample.

**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. Similarly, Pełka *et al* (2023) also found BB19.21 and BP20.9 of bee pollen and bee bread from Polish apiaries had the highest survival rates in the harsh conditions of GIT among ten isolates of *Bacillus* spp tested for pH 2 and 0.3% bile salt solution. According to Anyairo *et al* (2024), out of 17 Bacillus spp. obtained from Miang, a fermented tea in north Thailand, the isolates K29.2 and K15.4 showed the highest survival rate of 72.9 and 64.7% in gastric acidic environment as well as 99 and 98%, for bile respectively.

**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. In the present study, hydrophobic nature of 19 isolates of *Bacillus* spp ranged from 0.60 to 38.7 per cent, when xylene was used as hydrocarbon solvent for the test. Out of 19 isolates only 4 isolates such as B12, B13, B14 and B16 exhibited more than 34 percent adhesive ability, and B12 showed higher hydrophobic nature of 38.7 percent (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 a better 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). Similar to the present study Ragul *et al.* (2017) evaluated hydrophobicity with xylene of *B. licheniformis* PUFSTP35 isolated from fermented mango pickle with and the isolate demonstrated 57.33 percent hydrophobicity. Ritter *et al.* (2018) also used the same method of hydrophobicity using xylene which was highest for *Bacillus subtilis* strain DSM 4451(reference strain) accounting for 56.4 percent while KM01(Clostat) and FPR02 (Fertitacto) isolated from commercial probiotic products presented 35 percent BATH (Bacterial Adhesion to Hydrocarbon) value. Soni *et al* (2022) observed 38 percent hydrophobic nature of the probiotic *Bacillus velezenesis* CGS1.1 isolated from chick faces in xylene. Hydrophobicity for the ten isolates of *Bacillus* spp from bee pollen and bee bread samples derived from Polish apiaries varied from 5.69 to 61.08%, while the maximum affinity toward xylene was exhibited by strain PG10.5 (Pełka *et al*., 2025).

 **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 percentage of survivability with respect to acid, bile with good hydrophobic nature indicating their probiotic nature which were isolated from dairy environmental samples such as silage and can rinse of the dairy farm, respectively. Except one isolate almost 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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