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Research article

Isolation, Characterization, and Identification of *Bacillus* spp. Strains from the Digestive Tract of Mad Carp (*Leptobarbus hoevenii*) and Their Potential Probiotic Properties

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Abstract: The present study aimed to collect isolates of *Bacillus* spp. from the digestive tracts of mad carps (*Leptobarbus hoevenii*) and to screen these for probiotic properties. In this study, 85 bacterial isolates were obtained from the digestive tracts of mad carps. Of these, 73 isolates were Gram-positive, rod-shaped, and catalase-positive, while only 48 bacterial isolates were endospore-forming. Evaluation of antagonistic effects of *Bacillus* spp. against pathogenic bacteria causing motile *Aeromonas* septicemia (MAS) in fish indicated that a total of 18 out of the 73 isolates exhibited antimicrobial activity, and especially the isolate MCSU14 expressed a significantly enlarged zone of inhibition ($p<0.05$). A study on the hemolytic activity of these 18 antimicrobial isolates revealed that 6 isolates were γ-hemolytic. Furthermore, the 6 γ-hemolytic isolates survived exposure to acidic and bile salt conditions and produced extracellular digestive enzymes. Among these six *Bacillus* spp., the isolate MCSU14 exhibited the most potent probiotic properties. Based on its biochemical characteristics and molecular analyses, the *Bacillus* sp. isolate MCSU14 is closely related to *Bacillus velezensis*. Our findings indicate that *Bacillus* spp. isolates from the digestive tracts of mad carp can be screened to find potent probiotics against MAS in aquaculture.

Keywords: *Bacillus* spp.; Probiotics; *Aeromonas* spp.; Mad carp

1. Introduction

Currently, aquaculture serves a crucial role in food security and nutrition, as it contributes to the production of aquatic animals to meet the growing global demand [1]. Freshwater aquaculture, in particular, is the main production source of aquatic animals, accounting for up to 77% of the world's total aquatic edible animal production [2]. However, infectious diseases remain a significant challenge in freshwater aquaculture, affecting both the cultured aquatic animals and the surrounding environment. In particular, motile *Aeromonas* septicemia (MAS) is a severe disease in fish and aquatic invertebrates, as well as amphibians, reptiles, mammals, and humans [3]. *Aeromonas* species known to cause severe MAS include *A. hydrophila* [4, 5], *A. veronii* [6-9], *A. jandaei* [10, 11] and *A. dhakensis* (formerly known as *A. aquariorum* or *A. hydrophila* subsp. *dhakensis*) [12]. Previously, *A. dhakensis* was often misidentified as *A. hydrophila* due to its close genetic relationship with this species, and was classified as a subspecies before a revision of taxonomy [13]. The use of antibiotics is one

alternative for treating diseases caused by *Aeromonas* spp. However, prolonged and incorrect use of antibiotics leads to bacterial resistance by the selection and development of strains that cause more severe diseases, and the antibiotics leave potential residues in aquatic animals, affecting consumers [14]. However, aquaculture must prioritize sustainability, environmental friendliness, and consumer safety. To achieve this, the use of probiotics is an alternative for supporting aquaculture, helping to replace chemicals and antibiotics [15]. Probiotics are microbial supplements that enhance nutrient digestion, inhibit pathogens, stimulate the immune system in the gut, and improve water quality [16]. These often include strains such as lactic acid bacteria (*Lactobacillus* sp., *Carnobacterium* sp., and *Enterococcus* sp.), yeast (*Saccharomyces cerevisiae*), and *Bacillus* spp. [17]. In particular, *Bacillus* spp. is widely used as a probiotic in aquaculture [18, 19] because this type of species can produce endospores that withstand harsh conditions, enabling survival in the acidic digestive tract [20]. These strains can produce antimicrobial substances against various microorganisms [21, 22], and most strains are not pathogenic to aquatic animals. The sources of potential probiotics in aquaculture, particularly of *Bacillus* spp., are commonly found in various environments, including fermented foods, natural aquatic environments such as sediments and water, as well as in the gastrointestinal tracts of healthy fish [17, 23]. Host-associated probiotics have garnered the most attention and offer a distinct advantage for the specific host, including improved growth performance, enhanced nutritional value, increased digestive enzyme activity, inhibition of pathogenic microorganism colonization, and improved hematological parameters and immune response [24]. Several studies have demonstrated the isolation of potential probiotics from the gastrointestinal tracts of freshwater fishes, such as Nile tilapia (*Oreochromis niloticus*) [25], common carp (*Cyprinus carpio* L.) [26], and snakehead fish (*Channa* sp.) [27]. To date, reports on the isolation of probiotics from the digestive tract of mad carp (*Leptobarbus hoevenii*), an economically important freshwater species native to Malaysia, Cambodia, Indonesia, Laos, Vietnam, and Thailand [28] remain limited. Sunarto *et al.* [29] reported the isolation of the probiotic bacterium *Proteus mirabilis* from the intestinal tract and culture environment of mad carp. Nevertheless, studies on the isolation of probiotics from the digestive tract of mad carp, particularly regarding *Bacillus* spp., are still lacking. Therefore, this study aimed to isolate and characterize *Bacillus* spp. possessing probiotic properties from the intestines of mad carp, which could be used as natural supplements in aquaculture.

2. Materials and Methods

2.1 Ethics statement

The experimental procedures in this study followed the guidelines of the Institute of Animals for Scientific Purposes Development, National Research Council, Thailand. The procedures were approved by the Institutional Animal Care and Use Committee, Prince of Songkla University, under permission number Ref. AQ037/2024.

2.2 Isolation of *Bacillus* spp.

Bacillus spp. were isolated from the digestive tracts of farmed and wild mad carp in southern Thailand using the heat-shock treatment method [30]. The digestive tract was aseptically dissected and washed with 0.85% (w/v) sterile saline. One gram of each sample was finely homogenized, then diluted with 9 ml of 0.85% sterile saline. The homogenates were further diluted to decrease their concentration by a factor of 10^{-3}. Subsequently, the sample was boiled at 80°C for 10 minutes and then immediately soaked in room-temperature water for 3 minutes. A sample volume of 100 µl was spread onto nutrient agar (NA) and incubated at 30°C for 24 to 48 hours. Afterward, morphologically different colonies were selected for re-streaking on NA to obtain pure cultures. The pure cultures were Gram-stained and examined microscopically for their Gram-staining reaction, shape, and endospore formation.

2.3 Screening of probiotic properties

2.3.1 Antimicrobial activity

2.3.1.1 Cross-streak method

Seventy-three candidate *Bacillus* spp. isolates were tested for antimicrobial activity using the cross-streak method [31]. *Bacillus* spp. strains were streaked on the center of a Muller-Hinton agar (MHA) plate (Himedia, India) and incubated at 30°C for 48 hours. After incubation, the pathogenic bacteria *A. veronii*,

A. dhakensis, and *A. jandaei* were streaked perpendiculary on the plate. The procedure was performed in triplicate for each bacterial isolate to validate the results. The plates were then incubated at 30°C for 24 hours to observe inhibitory activity. The ability of *Bacillus* spp. to inhibit the pathogenic bacteria was determined by measuring the inhibition zone, and the results are reported in millimeters.

2.3.1.2 Agar well diffusion method

Eighteen *Bacillus* spp. isolates exhibiting the ability to inhibit pathogens, as observed by the cross-streak method, were selected to assess their inhibitory effects against *A. veronii*, *A. dhakensis*, and *A. jandaei* using the agar well diffusion method modified from Baharudin *et al*. [32]. *Bacillus* spp. suspension, cell-free supernatant (CFS), and cell pellet were tested in this study. The suspension of *Bacillus* spp. cultured in nutrient broth (NB) was shaken at 30°C and 160 rpm for 72 hours. Half of the *Bacillus* spp. suspension was then centrifuged at 9,184 ×*g* for 10 min at 4°C. The CFS was collected and filtered using a sterile syringe filter with a 0.22 μm pore size. The pellets were washed twice with 0.85% sterile saline and then resuspended in 0.85% sterile saline. The resuspended cell pellet was adjusted to an optical density of 0.1 at a 600 nm wavelength. The pathogenic bacteria were prepared by adjusting the suspension of each *Aeromonas* spp. isolate with 0.85% sterile saline to achieve a final concentration equivalent to the 0.5 McFarland standard (10^8 CFU/ml). This bacterial suspension was then spread onto MHA and allowed to dry at room temperature. Subsequently, holes were drilled, each with a diameter of approximately 8 mm, at eight positions per plate. Subsequently, 50 μl of each suspension, CFS, or resuspended cell pellet of *Bacillus* spp. was loaded into its well and incubated at 30°C for 24 hours. Oxytetracycline at a concentration of 1.5 mg/ml and NB were used as positive and negative controls, respectively. The assay was conducted in triplicate for each bacterial isolate. The ability to inhibit the pathogenic bacteria was examined by measuring the inhibition zone. Results are reported in millimeters.

2.3.2 Hemolytic activity

Eighteen candidate *Bacillus* spp. isolates with the potential to inhibit pathogenic *Aeromonas* spp. were assessed for hemolytic activity. Each *Bacillus* sp. isolate was streaked on blood agar base containing 5% defibrinated sheep blood and then incubated at 30°C for 24-48 hours to observe the decomposition of red blood cells. The procedure was performed in triplicate for each bacterial isolate. *Bacillus* spp. strains that do not lyse red blood cells (γ-hemolytic *Bacillus* spp.) were selected for further study.

2.3.3 Acid tolerance

Six γ-hemolytic *Bacillus* spp. isolates were tested for pH tolerance using the method modified from Ritter *et al.* [33] and Dabiré *et al.* [34]. Briefly, *Bacillus* spp. strains were inoculated into NB with shaking at 160 rpm and 30°C for 24 hours. Subsequently, 1 mL of suspension was transferred into 10 mL of NB and adjusted to a final pH level of 1, 2, 3, 4, or 5 with 1 N hydrochloric acid and sodium hydroxide. The assay was carried out in triplicate for each bacterial isolate. After incubation at 30°C for 3 hours, the survival rate of bacteria was determined using the drop plating technique at 0 and 3 hours. The suspension of each sample was 10-fold serially diluted, and then 20 μl of the suspension from each dilution was dropped onto NA and incubated at 30°C for 24 hours. The colonies were then counted. The results were calculated in units of CFU/ml, and the survival rate was calculated using the following formula.

$$\text{Survival rate (\%)} = \frac{N_3}{N_0} \times 100$$

Note: N_3 = Number of colonies after incubation for 3 hours (log CFU/ml)

N_0 = Number of colonies at 0 hours (log CFU/ml)

2.3.4 Bile salt tolerance

Six γ-hemolytic *Bacillus* spp. isolates were tested for bile tolerance using the method modified from Dabiré *et al.* [34]. Briefly, *Bacillus* spp. strains were inoculated into NB and incubated at 30°C with shaking at 160 rpm for 24 hours. Subsequently, 1 mL of suspension was transferred into 10 mL of NB supplemented with 0.3% (w/v) bile salt (Oxoid) and incubated at 30°C for 3 hours. The procedure was conducted in triplicate and the survival rate was assessed using the drop plating technique at 0 and 3 hours.

2.3.5 Digestive enzyme production

Six *Bacillus* spp. isolates were tested for the production of digestive enzymes, including amylase, protease, and lipase, using the method modified from Santong *et al.* [35] and Proca *et al.* [36]. Briefly, *Bacillus* spp. strains were point inoculated onto NA supplemented with 1% starch, 2% skim milk, and 1% Tween 80 with 0.1 g of $CaCl_2$ for testing starch, protein, and fatty hydrolyses, respectively. The assay was done in triplicate for each bacterial isolate. The plates were then incubated at 30°C for 24 hours. Subsequently, for starch hydrolysis, Lugol's solution was dropped onto the surface of the starch agar medium and left for 10-15 minutes. The appearance of a clear zone around the *Bacillus* spp. colonies indicated digestive enzyme production. The results are reported as hydrolytic capacity, which was calculated as follows [37].

$$\text{Hydrolytic capacity} = \frac{\text{Diameter of the clear zone around the bacterial colonies (mm)}}{\text{Diameter of the bacterial colonies (mm)}}$$

2.4 Bacterial identification

The candidate *Bacillus* sp. with potential probiotic properties was selected for bacterial identification based on their biochemical characteristics using API 50CHB strips and API 20E strips (BioMérieux, France) according to the manufacturer's instructions. Candidate *Bacillus* sp. was further confirmed by molecular analysis. Bacterial DNA extraction was conducted using a DNA extraction kit (Qiagen). The DNA was then amplified using primers targeting the DNA gyrase subunit A (*gyrA*) and RNA polymerase subunit B (*rpoB*) genes [38]. Polymerase chain reaction (PCR) was performed in a total volume of 50 µL for each sample, consisting of 25 µL of 2x PCR master mix (RBC Bioscience, Taiwan), 1 µL of each primer (0.2 µM), 20.5 µL of distilled water, and 2.5 µL of DNA template. Amplification was done using a thermal cycler (Bio-Rad, USA). The conditions included an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C (for *gyrA*) or 50°C (for *rpoB*) for 30 seconds, and extension at 72°C for 2 minutes. At the end of the cycle, a final extension at 72°C for 5 minutes was performed. The PCR products were analyzed by gel electrophoresis on a 1.8% agarose gel, and the DNA was visualized using gel documentation (Bio-Rad, USA). The amplified DNA was then purified using a gel extraction kit (Qiagen) and subsequently sent to Macrogen (Korea) for sequencing. The resulting sequence was compared to sequences in the GenBank/EMBL/DDBJ database using the Basic Local Alignment Search Tool (BLAST) for comparison. Additionally, phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA 11) software [39].

2.5 Statistical analysis

The results are expressed as an average with standard deviation (SD) from the mean. Percentage data were transformed to arcsine before the variance analysis. The data were analyzed using one-way ANOVA, and comparisons of mean values were analyzed using Duncan's Multiple Range Test (DMRT) at a 95% confidence interval ($p < 0.05$).

3. Results and Discussion

3.1. Isolation of *Bacillus* spp.

Nineteen digestive tract samples of mad carp were subjected to bacterial isolation by heat-shock treatment. A total of 85 isolates were obtained in the present study. Colony morphology was observed after growing colonies on NA and incubating them at 30°C for 48 hours. The colony colors, such as white, cream, pink, and yellow, were observed. The colony shapes varied, including circular, rhizoid, irregular, and filamentous forms. The margins of colonies were noted to be entire, irregular, lobate, or filamentous. It was found that 73 out of the 85 isolates were Gram-positive, rod-shaped, and catalase-positive. Of these, only 48 isolates were capable of forming endospores. *Bacillus* spp. is a group of bacterial strains that has long received attention for its probiotic properties in aquaculture. In particular, the ability of probiotics to control or prevent fish pathogens has been studied. *Bacillus* spp. strains isolated from the digestive tracts of freshwater fish have been effective in inhibiting pathogens in these fish [25, 27, 40].

3.2 Screening of probiotic properties

3.2.1 Antimicrobial activity

3.2.1.1 Cross-streak method

In this study, 73 *Bacillus* spp. isolates from the digestive tract of mad carp were evaluated for their potential to inhibit *A. veronii*, *A. dhakensis*, and *A. jandaei*, the causative agents responsible for MAS in freshwater fishes, using the cross-streak method. The results revealed that a total of 18 out of the 73 isolates exhibited antimicrobial activity against all *Aeromonas* spp. tested in this study. These *Bacillus* spp. isolates showed inhibition zones ranging from 2.17 ± 0.29 mm to 9.00 ± 1.00 mm (Figure 1). Among these, the *Bacillus* sp. isolate MCSU14 exhibited the largest zones of inhibition against *A. veronii* (9.00 ± 1.00 mm), *A. dhakensis* (8.33 ± 0.58 mm), and *A. jandaei* (8.33 ± 0.58 mm).

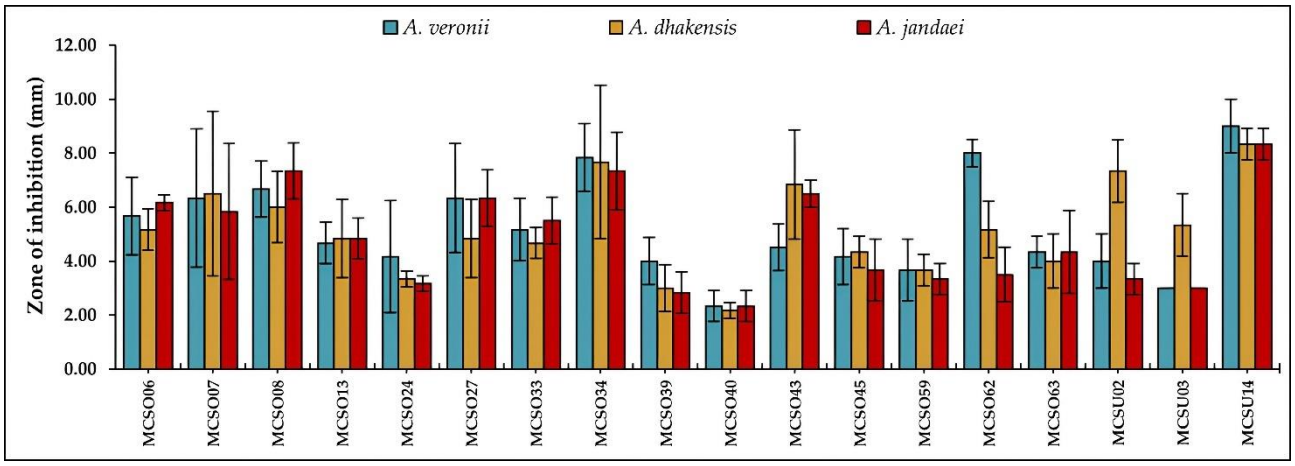

Figure 1. Antagonistic effect of *Bacillus* spp. against *A. veronii*, *A. dhakensis* and *A. jandaei* as determined in a cross-streak assay.

3.2.1.2 Agar well diffusion method

The eighteen *Bacillus* spp. isolates that exhibited antimicrobial activity by the cross-streak method were further assessed for antimicrobial activity against *A. veronii*, *A. dhakensis*, and *A. jandaei* using the agar well diffusion method. Using overnight culture suspensions, all *Bacillus* spp. isolates exhibited inhibition zones (Figure 2A), particularly those of *Bacillus* spp. isolates MCSO07, MCSU14, and MCSO34 showed the largest inhibition zones against *A. veronii* (14.67 ± 0.58 mm), *A. dhakensis* (17.33 ± 0.58 mm), and *A. jandaei* (16.00±1.00 mm), respectively. On using the CFS, only 9 *Bacillus* spp. isolates were capable of inhibiting *Aeromonas* spp. (Figure 2B). Notably, CFS from *Bacillus* sp. isolate MCSO34 showed the largest inhibition zones against *A. veronii* (11.00 ± 2.00 mm), *A. dhakensis* (9.33 ± 2.08 mm), and *A. jandaei* (9.67 ± 1.53 mm). Moreover, only three *Bacillus* spp. strains, namely MCSO13, MCSO34, and MCSU14, were capable of inhibiting all three *Aeromonas* spp. Additionally, using only cells at a concentration of 10^8 CFU/ml, *Bacillus* spp. isolates MCSO06, MCSU14 and MCSO34 exhibited the largest inhibition zones against *A. veronii* (12.33 ± 0.58 mm), *A. dhakensis* (14.33 ± 0.58 mm) and *A. jandaei* (13.67 ± 4.04 mm), respectively (Figure 2C). In this study, 18 out of the 73 *Bacillus* spp. isolates showed inhibitory effect against *Aeromonas* spp., as evidenced by the zone of inhibition, preventing the pathogen from growing on the culture medium. Notably, several *Bacillus* spp. isolates from the present study were found to inhibit *A. dhakensis*, a recently recognized species associated with MAS. Previous studies have indicated the activity of *Bacillus* spp. strains against *Aeromonas* spp., such as those of *B. velezensis* [41-42], *B. amyloliquefaciens* [43], *B. subtilis* [44-45], and *B. methylotrophicus* [46]. This is achieved through the function of *Bacillus* spp. in directly inhibiting pathogens, especially by producing pathogen inhibitors. The substances produced, such as bacteriocins, exert their antimicrobial effects by disrupting cell wall synthesis or creating pores in the cell membrane [47]; lipopeptides, including surfactin, iturin, and fengycin, are secondary metabolites known to disrupt bacterial membranes [48]; polyketides exhibit antibacterial activity by inhibiting protein synthesis [49]. In addition, *Bacillus* spp. are capable of producing the enzyme N-acyl homoserine lactones (AHL) lactonase, which plays a critical role in the quorum quenching process. This enzyme degrades

signaling molecules known as AHL, which are essential for quorum sensing, a mechanism used by pathogenic bacteria to coordinate gene expression related to virulence, toxin production, and biofilm formation [50]. Generally, these bioactive substances produced by bacteria are secreted outside the cell as extracellular bacteriostatic substances [51, 52].

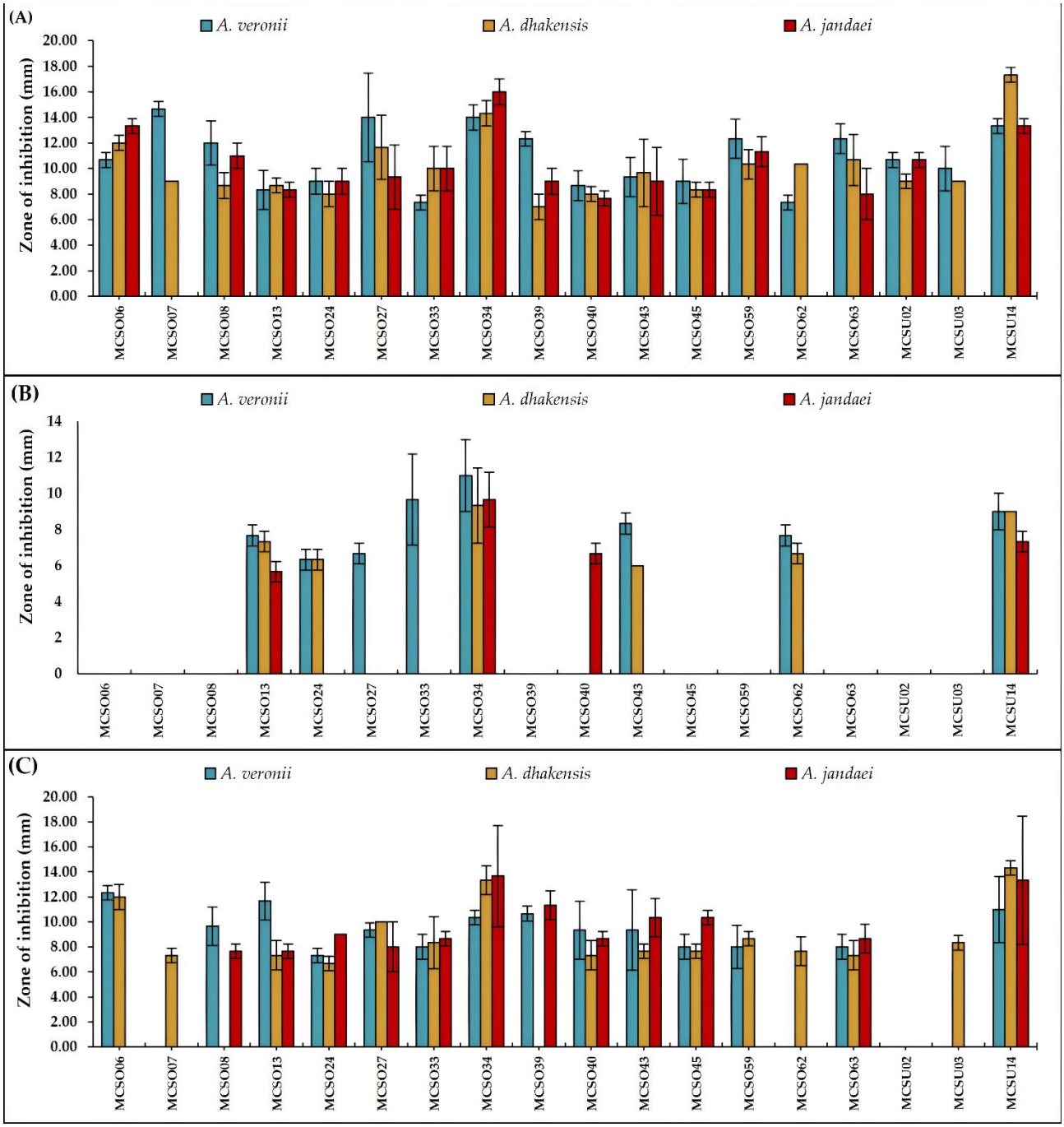

Figure 2. Antagonistic effects of (A) overnight culture suspension, (B) CFS, and (C) cells of *Bacillus* spp. against *A. veronii*, *A. dhakensis*, and *A. jandaei* as determined by agar well diffusion assay.

3.2.2 Hemolytic activity

In this study, eighteen *Bacillus* spp. isolates that exhibited antimicrobial activities were evaluated for hemolytic activity. The results showed that 6 out of these 18 isolates, namely MCSO07, MCSO13, MCSO33, MCSO45, MCSO62, and MCSU14, displayed γ-hemolysis, defined by the inability to lyse red blood cells. On the other

hand, twelve isolates exhibited α-hemolytic activity, with a slight ability to lyse red blood cells. A good probiotic bacterium should possess qualities beneficial to the host organism. Additionally, it must not cause disease or be toxic to the host [53]. The hemolytic activity test is a crucial examination in this regard. If the chosen probiotic exhibits hemolytic activity, it is considered a pathogen [54], as it may cause anemia and edema in the host [55].

3.2.3 Acid tolerance

The six γ-hemolytic *Bacillus* spp. isolates were evaluated for their survival under acidic conditions at pH levels ranging from 1 to 5 for 3 hours, indicating their potential as effective probiotics. It was found that all the isolates exhibited viability under acidic conditions (Figure 3). At pH 1 and 5, no significant difference ($p > 0.05$) was observed in the survival rate for any of the isolates tested in this study. Moreover, *Bacillus* spp. isolates MCSO45 and MCSU14 showed the highest survival rates at pH 2 and pH 3, respectively. Additionally, *Bacillus* sp. isolate MCSO45 demonstrated the highest survival rate at pH 4. The ability of probiotics to tolerate a wide range of environmental conditions is a crucial property that enables them to be well tolerated in the digestive tract [56]. Further investigation into their survival under a wide range of temperatures and salinities may provide more insight into understanding the adaptability and probiotic efficacy of the isolates within the complex gastrointestinal environments of aquatic animals.

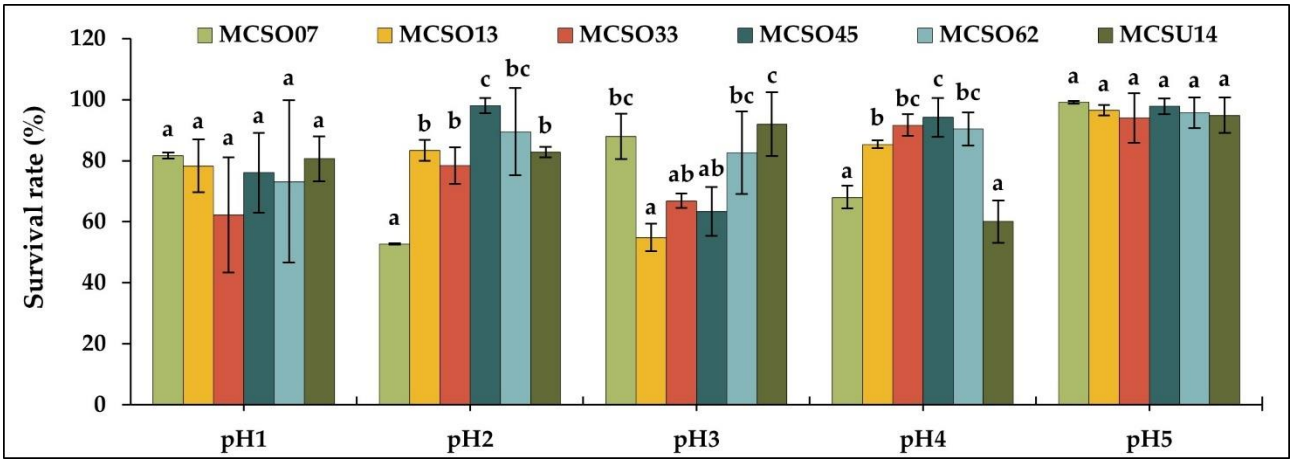

Figure 3. Survival rates of *Bacillus* spp. cultured under various acidity levels for 3 hours. Different letters indicate significant differences between the *Bacillus* sp. strains when compared at the same pH level ($p < 0.05$).

3.2.4 Bile salts tolerance

Evaluation of survival under exposure to bile for the six γ-hemolytic *Bacillus* spp. strains indicated that all these isolates exhibited viability, with a survival rate of over 90% after 3 hours (Figure 4). *Bacillus* sp. isolate MCSO33 showed the significantly lowest survival rate ($p < 0.05$). In this study, six isolates of *Bacillus* spp. were capable of enduring bile exposure for three hours, with a survival rate exceeding 90% suggesting that they have the potential for stability and survivability within the gastrointestinal tract. The resistance to stressful conditions by *Bacillus* spp. strains are attributed to their endospores, which can endure harsh environmental conditions [54]. Yousuf *et al.* [27] reported that *B. paramycoides* demonstrated tolerance to both acidic and alkaline pH levels, bile salts, and exhibited strong adhesion capacity. Therefore, further evaluations of persistence within the gastrointestinal tract, such as adhesion capacity, cell surface hydrophobicity,

autoaggregation, and coaggregation of *Bacillus* spp. from the present study, are necessary to understand better their functional properties and ability to colonize the host's gastrointestinal environment.

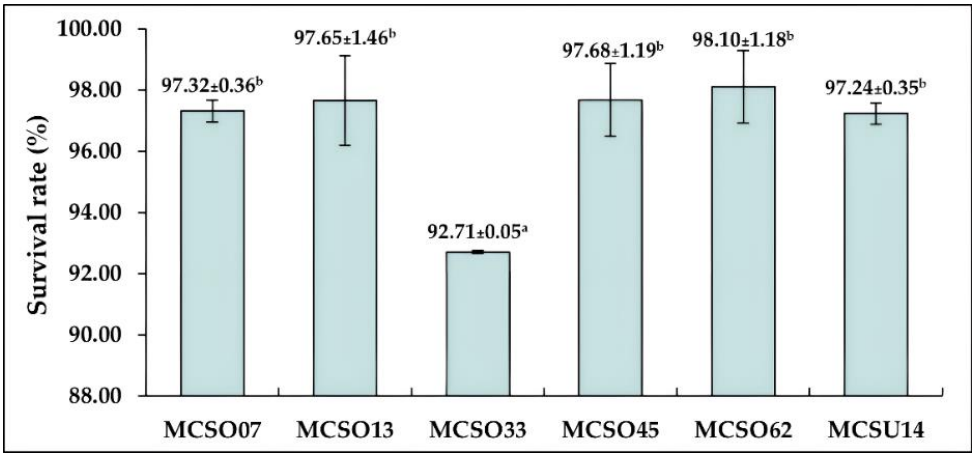

Figure 4. Tolerance of *Bacillus* spp. cultured in NB supplemented with 0.3% bile for 3 hours. Different superscripts indicate significant differences ($p < 0.05$).

3.2.5 Digestive enzyme production

The results revealed that six *Bacillus* spp. isolates produced extracellular digestive enzymes (Figure 5). All isolates showed hydrolysis capacity to digest starch in the range from 1.37 ± 0.38 to 3.53 ± 0.42, protein in the range from 1.17 ± 0.07 to 2.17 ± 0.48, and fat in the range from 1.12 ± 0.07 to 1.56 ± 0.38 (Figure 6). Additionally, it was found that *Bacillus* spp. isolates MCSO07, MCSO13, and MCSO33 exhibit the highest hydrolysis capacities for digesting starch, protein, and fat, respectively ($p < 0.05$). Important properties of probiotics for the host include helping to balance the intestinal microbiome and aiding in the digestion of food. Some probiotic bacteria can produce digestive enzymes that assist digestion, such as amylase for carbohydrate digestion, protease for protein digestion, and lipase for fat digestion [37]. In this study, six isolates of *Bacillus* spp. were found to be capable of producing digestive enzymes, specifically extracellular enzymes. Liu *et al.* [57] reported that *B. subtilis* HAINUP40 increased the protease and amylase activities in the digestive tract of Nile tilapia.

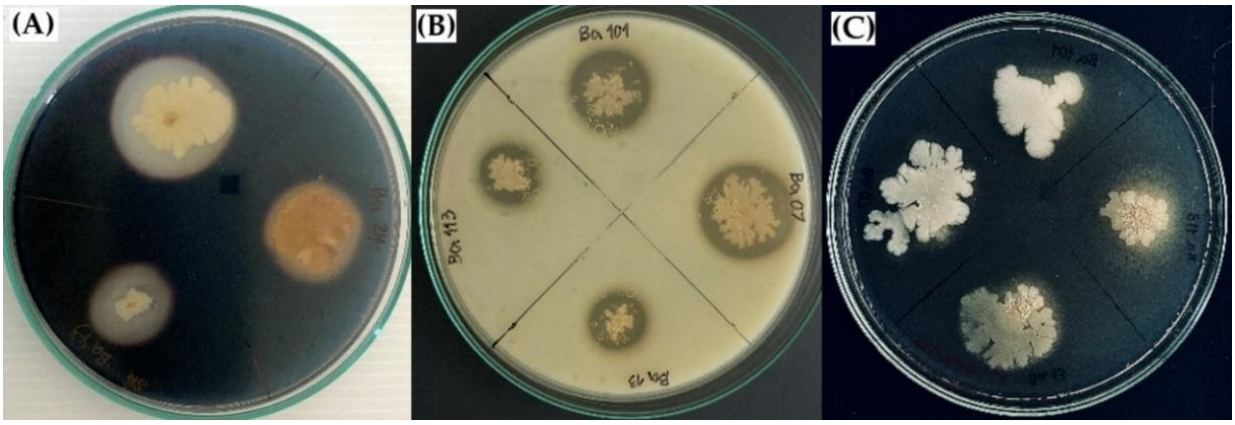

Figure 5. Zones of hydrolysis for (A) starch, (B) protein, and (C) fat, by *Bacillus* spp. isolates tested in the present study.

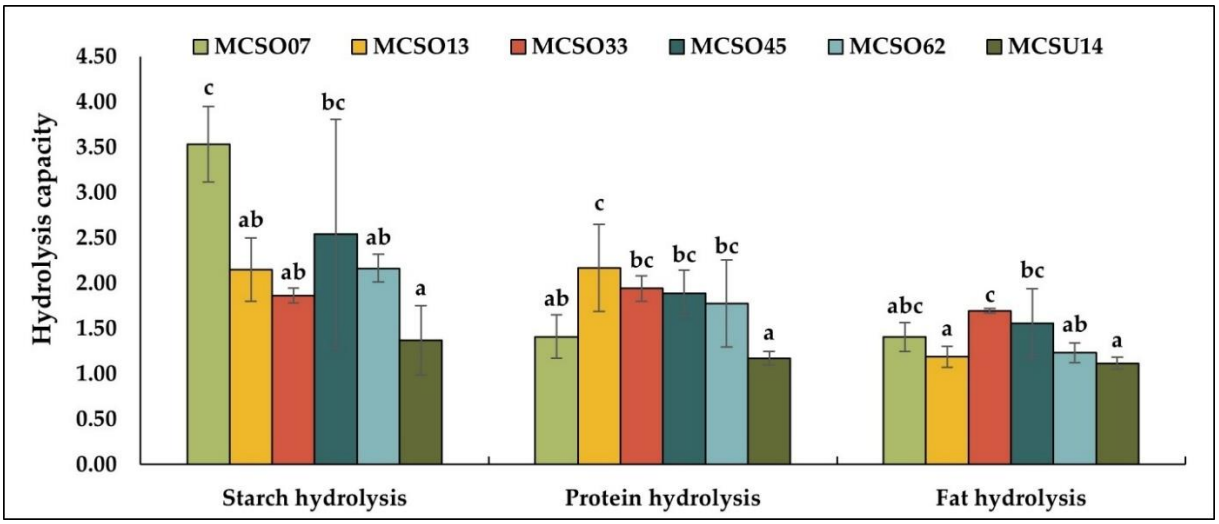

Figure 6. The capacity to hydrolyze starch, protein, and fat by six *Bacillus* spp. isolates tested in the present study. Different letters indicate significant differences between the *Bacillus* sp. strains with the same substrate ($p < 0.05$).

3.3 Bacterial identification

Among the six *Bacillus* spp. isolates, namely MCSO07, MCSO13, MCSO33, MCSO45, MCSO62, and MCSU14, which had exhibited probiotic properties, *Bacillus* sp. isolate MCSU14 demonstrated outstanding probiotic characteristics, including the highest inhibitory activity against *Aeromonas* spp. Furthermore, *Bacillus* sp. isolate MCSU14 did not hydrolyze red blood cells, exhibited survival in acidic and bile salt conditions, and produced extracellular digestive enzymes. As a result, *Bacillus* sp. isolate MCSU14 was chosen as the superior candidate for serving as a probiotic. *Bacillus* sp. isolate MCSU14 was identified based on its biochemical characteristics using API 50 CHB and API 20E strips. The results showed that *Bacillus* sp. isolate MCSU14 was capable of fermenting 27 carbohydrates, including glycerol, L-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, D-sorbitol, methyl α-D-glucopyranoside, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, inulin, D-raffinose, starch, glycogen, gentiobiose, and D-turanose (Table 1) Additionally, the isolate was capable of producing acetoin through glucose fermentation and exhibited gelatinase enzyme activity (Table 2) Based on the APIweb™ software, *Bacillus* sp. isolate MCSU14 was identified as *Bacillus subtilis/amyloliquefaciens* with a 99.6% similarity. The results do not clearly distinguish between the two species using the API 50CHB. This could be due to having only insufficient data for these species, or to difficulties in classification within the API 50CHB system. Moreover, the APIweb™ database does not contain data for other members of the *Bacillus subtilis* species complex, such as *B. velezensis, B. siamensis, B. tequilensis, B. valismortis*, and *B. mojavensis* [58]. Therefore, the classification of *Bacillus* sp. based only on the biochemical characteristics appears insufficient for accurate identification. As a result, additional molecular identification methods were necessary for proper classification. Moreover, the carbohydrate fermentation profile of *Bacillus* sp. isolate MCSU14 was compared with closely related strains, including *B. velezensis* CR-502 [59], *B. amyloliquefaciens* PMC-80 [60], and *B. siamensis* PD-A10 [61] (Table 1). The results indicate that *Bacillus* sp. isolate MCSU14 is closely related to these three species, suggesting that it may belong to the operational group of *B. amyloliquefaciens*.

The operational group of *B. amyloliquefaciens* (OGBa) consists of *B. amyloliquefaciens*, *B. velezensis*, *B. siamensis*, and *B. nakamurai* [62], all of which are part of the *Bacillus subtilis* species complex group [63]. Identification of *Bacillus* species using 16S rRNA gene sequencing may not provide clear differentiation for closely related species [64]. Therefore, *gyrA* and *rpoB* gene sequences were used to assess the identity of the chosen *Bacillus* sp. within the closely related *Bacillus* group. The *gyrA* gene sequence from *Bacillus* sp. isolate MCSU14 showed 99.89% similarity to *B. velezensis* PFX12 (accession number: MZ027153), *B. velezensis* gjfn4 (accession number: MW316630), and *B. velezensis* 26.3 (accession number: CP115185). The *rpoB* gene sequence

from *Bacillus* sp. isolate MCSU14 showed 100% similarity to *B. velezensis* AP45 (accession number: CP160218), *B. velezensis* R-71003 (accession number: CP092446), and *B. velezensis* SRCM102741 (accession number: CP028205). The phylogenetic trees based on the *gyrA* gene (Figure 7A) and *rpoB* gene (Figure 7B) sequences for the *Bacillus* sp. isolate MCSU14 revealed that it is closely related to *B. velezensis* in both genes. Therefore, it can be concluded that *Bacillus* sp. isolate MCSU14 is closely related to *B. velezensis*. However, it should be noted that additional techniques, such as whole-genome sequencing, fatty acid methyl ester profiling, or DNA G+C content analysis, could support a more precise identification. Previously, Kang *et al.* [26] reported the isolation of *B. velezensis* R-71003 from the intestine of common carp, while Wu *et al.* [40] reported the isolation of *B. velezensis* B8 from the gut of grass carp (*Ctenopharyngodon idella*). Our findings highlight the significance of *Bacillus* sp. isolate MCSU14, which is closely related to *B. velezensis* isolated from the intestine of mad carp, a novel source of *Bacillus* spp.

Table 1. Characterization of *Bacillus* sp. isolate MCSU14 by using API 50CHB strip. Comparison of the phenotypic characteristics of the present isolate with the reference strains.

No.	Biochemical test	1	2	3	4	No.	Biochemical test	1	2	3	4
0	Control	-	-	-	-	25	Esculin	+	+	+	+
1	Glycerol	+	+	+	+	26	Salicin	+	+	+	+
2	Erythritol	-	-	-	-	27	D-Cellobiose	+	+	+	+
3	D-Arabinose	-	-	-	-	28	D-Maltose	+	+	+	+
4	L-Arabinose	+	+	+	+	29	D-Lactose	+	+	-	+
5	D-Ribose	+	+	+	+	30	D-Melibiose	+	-	+	-
6	D-Xylose	+	+	-	+	31	D-Saccharose	+	+	+	+
7	L-Xylose	-	-	-	-	32	D-Trehalose	+	+	+	-
8	D-Adonitol	-	-	-	-	33	Inulin	+	-	+	-
9	Methyl-ß-D-xylopyranoside	-	-	-	-	34	D-Melezitose	-	-	-	-
10	D-Galactose	-	-	+	-	35	D-Raffinose	+	+	+	+
11	D-Glucose	+	+	-	+	36	Starch	+	ND	+	+
12	D-Fructose	+	+	+	+	37	Glycogen	+	+	+	+
13	D-Mannose	+	+	-	-	38	Xylitol	-	-	-	-
14	L-Sorbose	-	-	+	-	39	Gentiobiose	+	-	-	+
15	L-Rhamnose	-	-	-	-	40	D-Turanose	+	-	-	-
16	Dulcitol	-	-	-	-	41	D-Lyxose	-	-	-	-
17	Inositol	+	+	-	+	42	D-Tagatose	-	-	+	-
18	D-Mannitol	+	+	+	+	43	D-Fucose	-	-	-	-
19	D-Sorbitol	+	+	+	+	44	L-Fucose	-	-	-	-
20	Methyl α-D-mannopyranoside	-	-	-	-	45	D-Arabitol	-	-	-	-
21	Methyl α-D-glucopyranoside	+	+	+	+	46	L-Arabitol	-	-	-	-
22	N-Acetylglucosamine	-	-	-	-	47	Potassium gluconate	-	-	-	-
23	Amygdalin	+	+	+	+	48	Potassium 2-ketogluconate	-	-	-	-
24	Arbutin	+	+	+	+	49	Potassium 5-ketogluconate	-	-	-	-

Remark: 1; *Bacillus* sp. isolate MCSU14 (this study), 2; *B. velezensis* CR-502 [59], 3; *B. amyloliquefaciens* PMC-80 [60], 4; *B. siamensis* PD-A10 [61], +; Positive result, -; Negative result, and ND; Not determined.

Table 2. Characterization of *Bacillus* sp. isolate MCSU14 by using API 20E strip.

No.	Biochemical test	Result	No.	Biochemical test	Result
1	O-nitrophenyl-ß-D-galactopyranoside	-	7	Urease	-
2	Arginine dihydrolase	-	8	Tryptophan deaminase	-
3	Lysine decarboxylase	-	9	Indole	-
4	Ornithine decarboxylase	-	10	Voges-Proskauer	+
5	Citrate	-	11	Gelatinase	+
6	Hydrogen sulfide	-	12	Nitrate	-

Remark: +; Positive result and -; Negative result

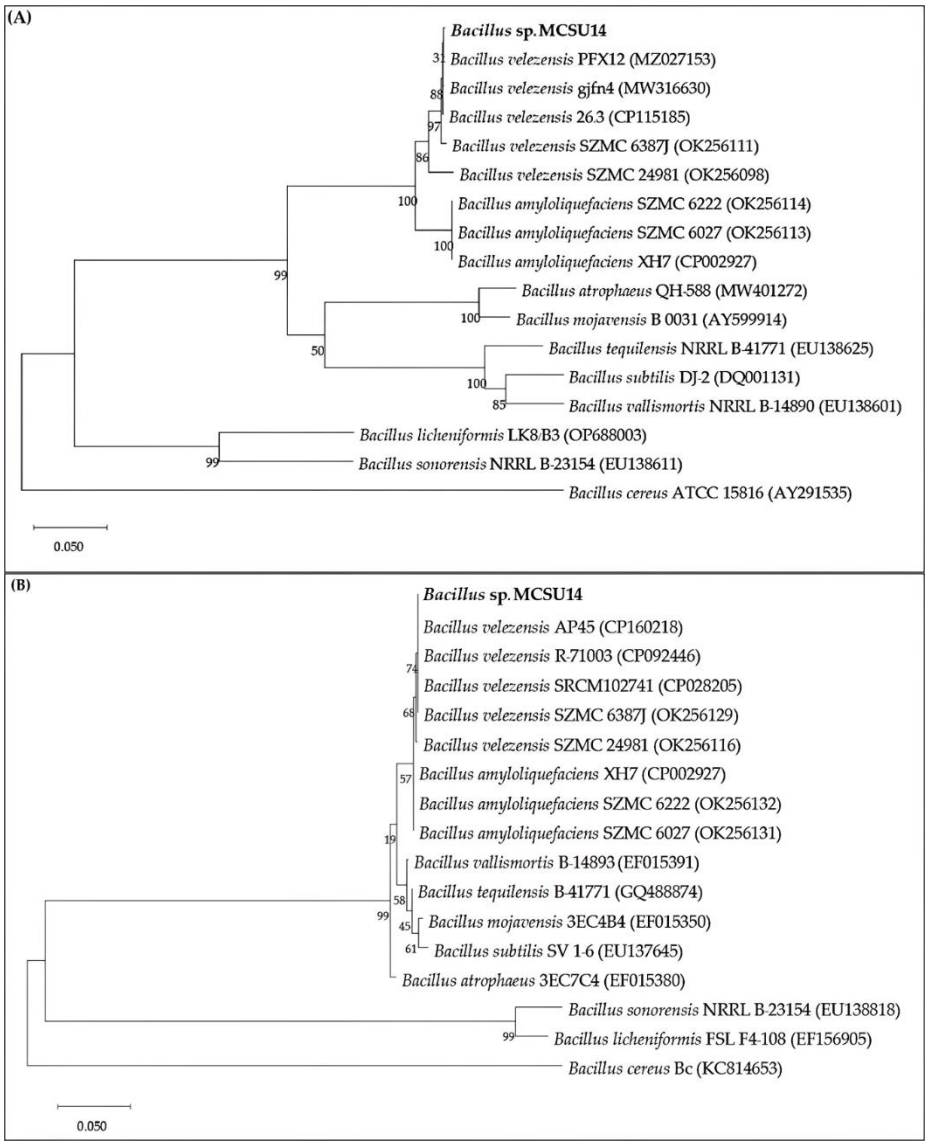

Figure 7. Neighbor-joining phylogenetic tree based on *gyrA* gene (A), and on *rpoB* gene (B) sequences of *Bacillus* species, showing positions of *Bacillus* sp. isolate MCSU14 and closely related members of the genus *Bacillus* sp. Bootstrap values (in percent) calculated from 1000 replications are shown at branch nodes. The evolutionary distances were computed using the Maximum Composite Likelihood method. The scale bar represents 0.05 nucleotide substitutions per nucleotide position. *B. cereus* ATCC 15816 and *B. cereus* Bc were used as the outgroups.

4. Conclusions

In the present study, 73 *Bacillus* spp. isolates from the gastrointestinal tracts of mad carps were examined for potential probiotic properties. Only 18 isolates of *Bacillus* spp. demonstrated antagonistic activity against *A. veronii*, *A. dhakensis*, and *A. jandaei*. Additionally, *Bacillus* spp. isolates MCSO07, MCSO13, MCSO33, MCSO45, MCSO62, and MCSU14 exhibited non-hemolytic activity, tolerance to acids and bile salts, and the production of digestive enzymes. Identification of the *Bacillus* isolate MCSU14, based on biochemical characteristics and molecular analysis, indicated that this bacterial isolate is closely related to *B. velezensis*. This study highlights that *Bacillus* spp. isolates from the gastrointestinal tracts of mad carps have significant potential to be screened for potent probiotics.

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