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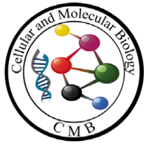

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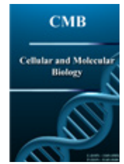

Original Article

Antagonistic and enzymatic activities of Bacillus species isolated from the fish gastrointestinal tract as potential probiotics use in Artemia culture

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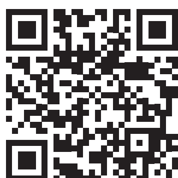

Abstract

Probiotics have been used successfully in aquaculture to enhance disease resistance, nutrition, and/or growth of cultured organisms. Six strains of *Bacillus* were isolated from the intestinal tracts of fish and recognised by conventional biochemical traits. The six isolated strains were *Bacillus cereus* and *Bacillus subtilis* using MALDI-TOF-MS technique. The probiotic properties of these *Bacillus* strains were studied. The tested *bacillus* strains exhibit antibacterial activity against the different pathogens. The strain S5 gave the important inhibition zones against most pathogens (20.5, 20.33, 23, and 21 mm against *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Staphylococcus aureus,* and *Salmonella typhimurium,* respectively). According to our results, all *Bacillus* strains have extracellular components that can stop pathogenic bacteria from growing. The enzymatic characterization showed that the tested strains can produce several biotechnological enzymes such as α-glucosidase, naphtol-AS-BI-Phosphohydrolase, esterase lipase, acid phosphatase, alkaline phosphatase, amylase, lipase, caseinase, and lecithinase. All *Bacillus* strains were adhesive to polystyrene. The adding *Bacillus* strains to the *Artemia* culture exerted significantly greater effects on the survival of *Artemia*. The challenge test on *Artemia* culture showed that the protection against pathogenic *Vibrio* was improved. These findings allow us to recommend the examined strains as prospective probiotic options for the *Artemia* culture, which will be used as food additives to improve the culture conditions of crustacean larvae and marine fish.

Keywords: *Bacillus*, Antagonistic activity, Enzymatic activity, Adherence, *Artemia*

1. Introduction

The human consumption of animal protein has increased quickly that is largely dependent on terrestrial and aquatic farm animals. However, aquaculture is becoming a more significant source for producing animal protein. Fisheries and aquaculture in Tunisia play an important role in socio-economic terms and as a source of food. the gilthead seabream (*Sparus aurata*) and the European seabass (*Dicentrarchus labrax*) are the most important species in terms of farming value. Aquaculture production has increased gradually in recent years, from 2600 tonnes in 2005 to about 21900 tonnes in 2017 [1].

As the aquaculture sector has grown, a wide range of disease-causing pathogens (bacterial, viral, and fungal illnesses) have also emerged. Disease control is a multidisciplinary approach because of interactions among pathogens, the hosts, and their environment [2,3]. For this reason, aquaculture activity requires several alternative strategies to control aquatic disease and promote favorable growth. Some of the most used approaches include chemical additives and microbial drugs, especially antibiotics [4,5]. The massive use of antibiotics for disease control and growth promotion in animals could result in the emergence of resistance among pathogenic bacteria and should

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be avoided in the prevention of disease among fish [6,7]. Therefore, there is a pressing need to develop microbial control strategies. The use of probiotic bacteria as microbial control agents is the main strategy that has emerged in response to disease control issues. Using probiotics is one solution that can reduce the reliance of the aquaculture industry on antibiotics and is used as a crucial component of aquaculture techniques to enhance growth and disease resistance [3,8,9].

Probiotics are living microorganisms that provide the host with health benefits when consumed or applied locally in sufficient quantity. Specifically, they reduce the incidence of disease and improve the host animal's nutritional and intestinal microbial balance [10,11]. Gram-positive and Gram-negative bacteria, bacteriophages, yeasts, and unicellular algae are among the probiotics that have been investigated [12–17]. Several species of the *Bacillus* genus produce spores that are incredibly resistant to a variety of environmental factors. According to reports, the genus *Bacillus* is helpful for aquaculture and has not been linked to any infectious fish diseases [5,18,19]. This bacterium produces several products of industrial interest, including enzymes, antibiotics, amino acids, insecticides, bio-surfactants, and bacteriocins [20,21]. Furthermore, due to their improved tolerance and survival in the harsh environment of the gastrointestinal tract, *Bacillus* strains are attracting attention in dietary studies related to human health. Among the dominant species of the genus *Bacillus* used as a probiotic in feed additives and disease control are *B. subtilis*, *B. licheniformis* and *B. cereus* [22–27]. Probiotic bacteria used in larval cultures of aquatic organisms can be delivered either directly in water or by live carriers such as *Artemia* nauplii used as vectors [28]. *Artemia* is the most common live food used in the larviculture of economically important crustaceans and fish, due to its ease of production and favorable biochemical composition. It contributes significantly to the energy flow of the food chain. This crustacean has a high nutritional value thanks to which it has become the essential trophic link in aquaculture [29].

Thus, the objective of this work is to identify and describe *Bacillus* strains from fish digestive systems for potential use as probiotics. Morphological, biochemical tests and the MALDI-TOF-MS technique were used to identify the *Bacillus* strains. Probiotic effectiveness was evaluated using a number of methods, including tests for antagonistic action against pathogenic microorganisms, hemolysis, enzymatic production, adhesion and effect on *Artemia* culture.

2. Materials and Methods

2.1. Fish sampling and bacterial isolation

Ten Samples of healthy wild sea bream (*Sparus aurata*) (about 50 g) were obtained from a fisherman's catch around Monastir coastal waters, Tunisia. The collected samples were placed into a sterile sampling bag and directly transferred to the laboratory for bacterial isolation. For the isolation of bacteria, each fish surface was washed with ethanol solution (75%) in order to prevent bacterial contamination and was dissected aseptically to remove the digestive tracts. The intestine samples were inoculated in Brain Heart Infusion Broth supplemented with 1% NaCl (BHI 1% NaCl) and incubated at 37 °C for 24 h. Then, each sample was streaked onto Tryptone Soya Agar prepared with 1% NaCl (TSA 1% NaCl). The plates were incubated for 24h at 37 °C [30]. Then each isolated colony from TSA 1% NaCl was inoculated in BHI 1% NaCl and incubated at 37 °C. Finally, an antibacterial activity was carried out for the research of probiotic capacity on pathogenic bacteria.

2.2. Screening of antibacterial activity of isolated stra ins

Antibacterial activity was performed for research on the probiotic capacity of isolated strains against seven pathogenic bacteria *Vibrio alginolyticus* (ATCC 177449), *Vibrio parahaemolyticus* (ATCC 17802), *Escherichia coli* (ATCC 35218), *Aeromonas hydrophila* (ATCC 7966), *Salmonella typhimurium* (ATCC 1408), *Staphylococcus aureus* (ATCC 25923), and *Listeria monocytogenes* (ATCC 1915) using the well diffusion agar assay [31]. Briefly, one mL of each pathogen solution (OD_{600} = 0.1) was plated into Muller Hinton agar (MH) and incubated for 30 min at 37 °C. Then, 6 mm-diameter wells were punched into the agar surface and 100 µL of an antagonistic bacterial culture was added to each well. The inhibition zones were detected after 24-48 h of incubation at 37 °C. The presence of clear zones around tested bacteria was considered to indicate antagonistic activity against the pathogen [32]. Sterile BHI 1% NaCl broth was a control. The experiment was performed in triplicate and the ±SD was calculated. Strains with antibacterial activity were considered probiotics and selected for further characterization. Antagonistic strains were kept in Lysogene Broth (LB) with 30% glycerol at -20 °C.

2.3. *In vitro* antibacterial activity of extracellular products (ECPs)

The extracellular product activities of potential probiotics were investigated. After centrifugation at 4500 rpm for 15 min of the bacterial cultures, the extracellular products (ECPs) from antagonistic bacteria were collected and filtered through a 0.22 µm membrane filter for screening of antibiotic or bacteriocin production by *Bacillus* strains. The antibacterial activity of ECPs was determined using the solid MH agar spot assay described by Le Lay *et al*. [33] with slight modifications. Briefly, one mL sample of ECPs from each probiotic strain was then mixed with 15 mL MH agar medium at 45 °C and kept at room temperature for 15-20 min. Ten µL of a suspension containing 10^6 CFU/mL of each pathogen were then dropped (as a spot) onto the solidified MH agar, and the plates were incubated for 24 to 72 h at 37 °C to detect colonies. The absence of colonies was interpreted as antagonistic activity against the pathogen. A plate containing MH without an extracellular product is a positive control. All experiments were performed in triplicate.

2.4. Conventional identification of isolates with antibacterial activity

To identify antagonistic strains, morphological and biochemical identification was performed. After the Gram-staining, spore staining, and morphological characteristics, strains were subjected to phenotypic identification using selective media for *Bacillus cereus*: *Bacillus cereus* agar (BCA). Bacterial strains were grown in BHI 1% NaCl at 37 °C for 24 h. Then each tube was plated onto BCA and incubated at 37 °C. Biochemical identification was

done using the Api 50 CHB (BioMerieux, Marcy-l'Étoile, France) according to the manufacturer's recommendations.

2.5. MALDI-TOF-MS identification of isolates with antibacterial activity

The identification of bacterial strains at the species level was performed using the MALDI-TOF-MS technique (matrix-assisted laser desorption/ionization-time of flight mass spectrometry) as described [34]. After incubation of bacterial strains for 24h–48h at 37°C on TSA, sample preparation was performed on 4 to 5 bacterial colonies which were analyzed using a microflex LT MALDI-TOF MS instrument (Bruker Daltonik GmbH, Bremen, Germany) with Flex Control (version 3.0) software (Bruker Daltonics). Briefly, 1.5 µl of the protein extracts was placed into steel target plates and allowed to dry on air. After drying, each sample was overlaid with 1.5 µl of matrix solution (a saturated solution of α-Cyano-4 Hydroxy Cinnamic Acid: CHCA, in 50 % acetonitrile and 2.5% trifluoracetic acid, Bruker Daltonik) and air dried at room temperature. For identification, all the samples were run in duplicates. A characterization score_cut-off value was attributed to each sample and was interpreted according to the online Bruker database.

2.6. Characterization of extracellular enzymes

The API-ZYM System (Bio-Mérieux, Marcy-l'Étoile, France) composed of 19 enzymatic substrates was used, according to the manufacturer's instructions, to determine the presence of exoenzymes. Additional enzymatic activities were determined following the inoculation of cultures on TSA medium to which these substrates had been added: 0.2% [wt/vol] starch for amylase, 1% [wt/vol] skim milk for caseinase, 1% Tween 80 for lipase, 5% [vol/vol] egg yolk for phospholipase (lecithinase) and 5% [vol/vol]) sheep red blood cells for hemolysin [35]. All experiments were carried out in triplicate.

2.7. Adhesion assay on polystyrene

Biofilm production by bacterial strains was determined using a semi-quantitative adherence assay on U-Bottomed 96-well polystyrene plates (Nunc, Roskilde, Denmark) according to Chaieb *et al*. [36]. All strains were grown in TSB supplemented with 1% NaCl at 37 °C for 24 h. Bacterial cultures were diluted to 1:100 in the same medium and supplemented with 2% glucose. Then, 200 µl of bacterial suspension was added into each well, and the plates were incubated at 37 °C for 24 h. Wells with sterile TSB 1% NaCl alone served as controls. The wells were subsequently rinsed twice with Phosphate Buffered Saline solution (pH 7.4) to remove non-adherent cells and were dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100 µL of 1% crystal violet for 5 min. The excess stain was poured off, and the wells were washed three times with 300 µL of sterile distilled water. The water was then cleared, and the microplates were air-dried. The bound dye was solubilized with 100 µL of 95% ethanol and the optical density of each well at 570 nm was measured using an automated Multiskan reader (Gio. De Vita EC, Rome, Italy). Adhesion ability was interpreted as strong ($OD_{570} \geq 1$), fair ($0.1 \leq OD_{570} < 1$), or slight ($OD_{570} < 0.1$). All experiments were done in triplicate.

2.8. Effect of candidate probiotic bacteria on *Artemia* culture

2.8.1. Preparation of *Bacillus* strains

Potential probiotic strains were cultured in Nutrient broth prepared with seawater (NBSW) for 24 h at 37 °C. The culture was then centrifuged at 800 rpm for 15 min. The supernatant was poured into a sterile flask and kept at 4 °C, while the bacterial pellet was washed twice by centrifugation to eliminate residues from the culture medium and resuspended in sterile seawater [37].

2.8.2. *Artemia* gnotobiotic culture

Bacteria-free cysts and nauplii of *Artemia salina* were obtained via decapsulation, as described by Sorgeloos *et al.* [38]. Decapsulated cysts were washed with filtered and autoclaved sea water (FASW) over a net 50 µm sterile filter. This procedure was repeated eight to twelve times, using a new FASW each time. After this step, washed decapsulated cysts were transferred to a sterile Falcon containing 30 mL of FASW. The capped Falcons were placed and exposed to constant incandescent light in a shaking incubator (25 °C / 100 rpm). *Artemia* culture containing newly hatched nauplii was obtained after 15-24 h of incubation.

2.8.3. Pathogenicity/toxicity towards *Artemia*

The assay was exhibited in a 96-well polystyrene plate according to Jann-Para *et al.* [39]. From an *Artemia* culture, 100 µL containing 10 newly hatched nauplii were added to each well. Then, 100 µL of each bacterial suspension from each probiotic was added to all the wells. The well-containing FASW served as the negative control. After incubation at 25 °C for 24 h, the number of live *Artemia* was counted in each well. All manipulations were carried out in triplicate under sterile conditions.

2.8.4. *In vivo* tests with *Artemia* nauplii

To assess the impact of bacterial strains on *Artemia* culture, different challenge tests were performed as reported earlier [40] in triplicate under sterile conditions over six days. As described, 10 axenic nauplii instars I obtained from *Artemia* gnotobiotic culture were transferred to sterile falcons containing 30 mL of FASW with the feed scheduled for the first day. All manipulations were performed in a laminar flow hood and all necessary tools were pre-sterilized at 120 °C for 20 min.

The challenge tests were: *Artemia* in anoxic conditions (A); *Artemia* with pathogenic bacteria *V. alginolyticus* VA (A + VA); *Artemia* with beneficial bacterial strains (A+S1; A+S2; A+S3; A+S4; A+S5; A+S6) and *Artemia* with beneficial and pathogenic bacteria *V. alginolyticus* VA (A+S1+VA; A+S2+VA; A+S3+VA; A+S4+VA; A+S5+VA; A+S6+VA). "A" was chosen as a control treatment for experiments when the tested strains were added to the culture medium (A+S1; A+S2; A+S3; A+S4; A+S5; A+S6). Treatment "A+VA" was used as a control when *Artemia* nauplii were challenged by the tested and pathogenic strains (A+S1+VA; A+S2+VA; A+S3+VA; A+S4+VA; A+S5+VA; A+S6+VA). Potential probiotic and pathogenic bacterial suspensions were added at a density of 10^6 CFU/mL. The concentration of each bacterial strain was estimated through a regression analysis of the optical density of the pure culture. The number of CFU/mL was determined using Petri plates with marine agar. The number of swimming larvae was determined daily and the survival percentage was calculated.

2.9. Statistical analysis

All the experiments were performed in triplicate. Statistical analysis of variance was performed using the SPSS 10.05 statistical program (SPSS for Windows; SPSS, Inc., Chicago, IL, USA). Standard deviations (SD) show the variability between samples. Differences in the survival of *Artemia* nauplii cultured under different conditions were presented as mean separated by the least significant difference according to the Student–Newman–Keuls test (at p ≤ 0.05).

3. Results

3.1. Screening of antibacterial activity of isolated strains

In selecting a potential probiotic strain for beneficial health effects on the host, many criteria must be considered. The ability to inhibit pathogenic bacteria is used to select potentially probiotic bacteria. In this experiment, the indicator strains contained Gram-positive bacteria (*S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 1915) and Gram-negative bacteria (*V. alginolyticus* ATCC 17802, *V. parahaemolyticus* ATCC 17802, *E. coli* ATCC 35218, *S. typhimurium* ATCC 1408, and *A. hydrophila* ATCC 7966).

Six strains were isolated from the fish intestine samples and showed antagonistic activity against the bacterial pathogens tested. Potential probiotic strains have an inhibitory effect against pathogenic strains, as mentioned in Table 1. The inhibitory zones were about 9.16 to 24.5 mm in diameter.

The strains S2, S3, and S5, gave the best results. Specifically, they are effective against *V. parahemolyticus* (23.5 ± 0.5 mm), *E. coli* (24 ± 0.57 mm), and *S. aureus* (23 ± 0.2 mm) respectively (Table 1). The strains S1 produced significant inhibition zones for *V. parahemolyticus* and *S. aureus* (20.83 ± 0.28 and 22.36± 0.15 mm, respectively). *L. monocytogenes* was strongly inactivated by S3 and S4 with an inhibition zone of 21.5 and 21 mm, respectively. In addition, *S. typhimurium* was strongly inactivated by S5 and S6 with an inhibition zone of 21 and 20 mm, respectively. Our results showed that *A. hydrophila* was weakly inactivated by all strains.

3.2. *In vitro* antibacterial activity of extracellular products (ECPs)

After 24 h of incubation of the MH agar plates mixed with the ECPs seeded by the pathogenic bacteria, we observed no colony on the surface of the agar for the strains S1, S3, S4, S5, and S6 (Table 2). For strain S2 we observed that the number of colonies of the pathogens (*V. alginolyticus, V. parahemolyticus* and *S. aureus*) are fewer than that of the positive control. While for *E. coli* and *A. hydrophila*, we observed no colonies on the MH medium (Table 2).

3.3. Conventional identification of isolates with antibacterial activity

The isolated strains showed cellular morphology typical of spore-forming Gram-positive rods and tested positive for catalase and negative for oxidase. The metabolism was facultatively anaerobic.

The results of API-50-CHB indicated that the isolates were similar to *Bacillus* spp. S1 and S2 strains were plated on a selective medium (BCA agar), and results showed reddish colonies with a clear white halo. We also observed a change in the colour of the agar to dark pink. In contrast, S3, S4, S5, and S6 colonies were yellow with a clear halo and a change in the colour of the agar to yellow. According to our results, strains S1 and S2 produced reddish colonies

Table 1. Antibacterial activities of the isolated strains towards indicator bacteria.

Indicator bacteria	Isolated strains					
	S1	S2	S3	S4	S5	S6
V. alginolyticus	17.33 ± 0.57	14.83 ± 0.28	24.5 ± 0.5	17.83 ± 0.28	20.5 ± 0.5	18 ± 0.00
V. parahémolyticus	20.83 ± 0.28	23.5 ± 0.5	18.33 ± 0.57	12.16 ± 0.28	20.33 ± 0.28	18.83 ± 0.15
L. moncytogenes	15 ± 0.57	14.66 ± 0.57	21.5 ± 0.70	21 ± 0.88	19.5 ± 0.70	18 ± 0.00
S. aureus	22.36 ± 0.15	17.26 ± 0.2	15 ± 0.00	14.5 ± 0.5	23 ± 0.2	9.16 ± 0.28
S. typhimurium	18.66 ± 0.57	17.33 ± 0.57	17.50 ± 0.70	19 ± 0.41	21 ± 0.41	20 ± 0.00
E. coli	1 6.23 ± 0.25	23 ± 0.57	24 ± 0.57	20.43 ± 0.4	16.76 ± 0.25	16.16 ± 0.28
A. hydrophila	15 ± 0.00	13.5 ± 0.5	14.9 ± 0.1	11.5 ± 0.5	11.66 ± 0.28	18.56 ± 0.1

± : Standard Deviation

Table 2. Effect of extracellular products of six probiotics against pathogenic bacteria.

Strains	*V. alginolyticus*	*V. parahémolyticus*	*S. aureus*	*E. coli*	*A. hydrophila*
S1	-	-	-	-	-
S2	+/-	+/-	+/-	-	-
S3	-	-	-	-	-
S4	-	-	-	-	-
S5	-	-	-	-	-
S6	-	-	-	-	-
posititive control	+	+	+	+	+

(-) : absence of colonies ; (+) : presence of colonies; (+/-) : colonies of pathogenic bacteria are less numerous than those of the positive control.

are *B. cereus*. The strains S3, S4, S5, and S6 showing up as yellowish colonies are *B. subtilis*.

3.4. MALDI-TOF MS identification of isolates with antibacterial activity

The bacterial isolates were confirmed as belonging to the *Bacillus* genus using the MALDI-TOF-MS technique. Two distinct *Bacillus* species were identified: *B. cereus* (S1 et S2) and *B. subtilis* (S3, S4, S5 et S6). These results confirm those obtained with the API test and BCA selective medium.

3.5. Characterization of extracellular enzymes

Tested strains produced exoenzymes such as amylase, lipase, caseinase, and lecithinase. In addition, all strains were non-hemolytic. Analysis of the enzyme activity API-ZYM assays revealed that all strains produced biotechnological enzymes (Table 3). Results showed that all investigated bacteria except S2 can assimilate naphthol-AS-BI-phosphohydrolase. The strains S1 and S3 can produce α-glucosidase and acid phosphatase. Esterase lipase (C8) and N-acetyl-β-glucosaminidase were produced by S1, S2, S3, and S6 strains. Esterase (C4) was produced by S2, S4 and S6 strains. While alkaline phosphatase was produced by S5 and S6, α-galactosidase produced by S1 and S3, and α-glucosidase produced by S2 and S3. Lipase (C14) was only produced by strain S3. Likewise, β-galactosidase and α-mannosidase were only produced by strain S2.

According to Table 3, we can observe that strain S3 could produce most enzymes, and strain S4 produced only two enzymes. Undesirable activities, such as trypsin, α-chymotrypsin, and β-glucuronidase activities, were not detected in the selected strains.

3.6. Adhesion assay on polystyrene

All isolated *Bacillus* strains were screened for their adherence to polystyrene microplates. The results of the OD_{570} presented in Table 4 show that all *Bacillus* strains were fairly adhesive to polystyrene with values ranging from 0.11 to 0.39.

3.7. Effect of candidate probiotic bacteria on *Artemia* culture

3.7.1. Pathogenicity/toxicity of candidate probiotics

After incubation of the plate at 25°C for 24h, we counted the number of live nauplii in each well and calculated their survival rate. The results reveal that the tested strains were not pathogenic or toxic to *Artemia* nauplii (Fig. 1). No significant difference occurred in the survival rate of *Artemia* between the treatments. The survival rate of *Artemia* in the control treatment was 89.5% and the survival rate of *Artemia* nauplii treated with probiotic bacterial suspension ranged from 90% (A+S1, A+S2, and A+S5) to 96.67% (A+S4 and A+S6).

3.7.2. Effect of candidate probiotics on *Artemia* survival

During the follow-up of the survival of *Artemia* nau-

Table 4. Measurement of the adhesion of the six probiotic strains.

Strain	Adhesion to polystyrene (OD at 570 nm) ± SD
S1	0.205 ± 0.01
S2	0.11 ± 0.05
S3	0.26 ± 0.02
S4	0.29 ± 0.16
S5	0.39 ± 0.09
S6	0.17 ± 0.25

Table 3. Extracellular enzymatic activity of the tested strains using API-ZYM galerie.

Enzyme	S1	S2	S3	S4	S5	S6
Alcaline phosphatase	-	-	-	-	+	+
Esterase (C4)	-	+	-	+	-	+
Esterase Lipase (C8)	+	+	+	-	-	+
Lipase (C14)	-	-	+	-	-	-
Leucine arylamidase	-	-	-	-	-	-
Valine arylamidase	-	-	-	-	-	-
Cystine arylamidase	-	-	-	-	-	-
Trypsin	-	-	-	-	-	-
α-Chymotripsin	-	-	-	-	-	-
Acid phophatase	+	-	+	-	+	-
Naphtol-AS-BI-phosphohydrolase	+	-	+	+	+	+
α-galactosidase	+	-	+	-	-	-
β-galactosidase	-	+	-	-	-	-
β-glucoronidase	-	-	-	-	-	-
α-glucosidase	-	+	+	-	-	-
β-glucosidase	+	-	+	-	-	-
N-acetyl-β-glucosaminidase	-	+	+	-	-	+
α-mannosidase	-	+	-	-	-	-
α-fucisidase	+	-	+	-	-	+

(-) : absence of enzyme; (+) : enzyme production

plii, we found that they could not withstand the anoxic conditions, as we noticed a low survival rate that reached death on the fifth day of treatment (Fig. 2).

Thus, adding *Bacillus* strains to the *Artemia* culture showed an improved survival rate. The strains S1, S2, S3, and S5 exerted similar effects on the survival of *Artemia* with a rate ranging from 90 % to 10 % at the end of the treatment (Fig. 2a and 2b). However, the strains S4 and S6 exerted significantly greater effects on the survival of *Artemia* with 63.33 % and 70 %, respectively, at the end of the treatment (Fig. 2b). If compared these last results with the control ones, we could notice a significant difference ($P < 0.05$) with a net survival improvement in the presence of *Bacillus* strains, especially in the presence of strain S4 or strain S6.

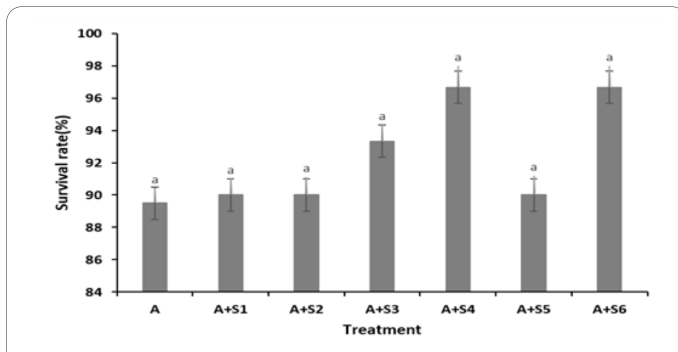

Fig. 1. Survival rate of *Artemia* nauplii after 24 h exposure to *Bacillus* strains. Controls were incubated in sterile seawater (A). A+S1 : *Artemia* + strain S1 ; A+S2 : *Artemia* + strain S2 ; A+S3: *Artemia* + strain S3 ; A+S4 : *Artemia* + strain S4 ; A+S5 : *Artemia* + strain S5 ; A+S6 : *Artemia* + strain S6. Means (n = 3) followed by the same lower-case letter are not significantly different according to the Student–Newman–Keuls test ($p \leq 0.05$).

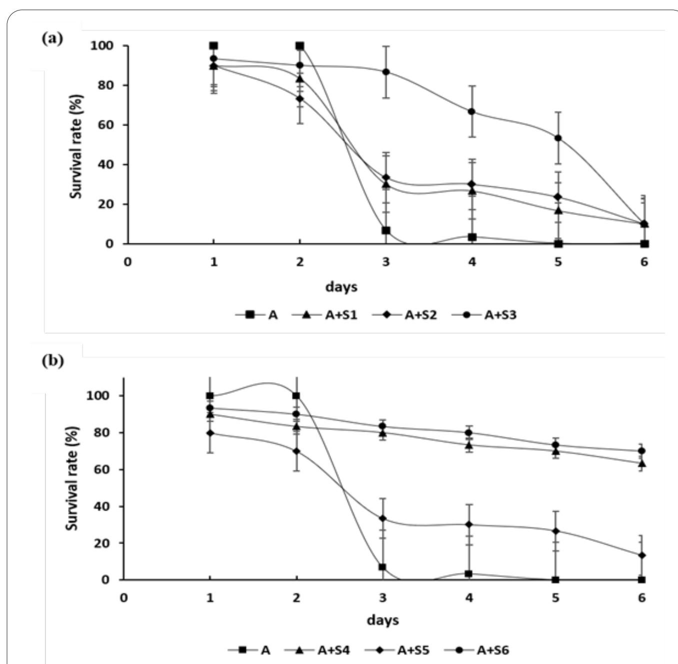

Fig. 2. Survival rate (mean + SD) of *Artemia* nauplii in the presence of *Bacillus* strains for 6 days. (a) : A : *Artemia* ; A+S1 : *Artemia* + strain S1 ; A+S2 : *Artemia* + strain S2 ; A+S3: *Artemia* + strain S3 ; (b) : A : *Artemia* ; A+S4 : *Artemia* + strain S4 ; A+S5 : *Artemia* + strain S5 ; A+S6 : *Artemia* + strain S6.

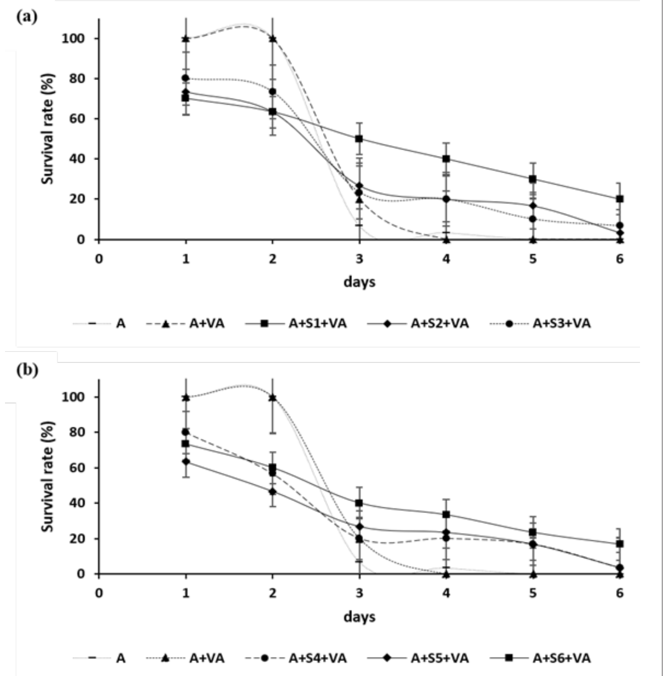

Fig. 3. Survival rate of *Artemia* nauplii in the presence of *V. alginolyticus and Bacillus* strains for 6 days. Means (n = 3). (a) A : *Artemia* ; A+VA : *Artemia* + *V. alginolyticus* ; A+VA+S1 : *Artemia* + *V. alginolyticus* + strain S1 ; A+VA+S2 : *Artemia* + *V. alginolyticus* + strain S2 ; A+ VA+S3: *Artemia* + *V. alginolyticus* + strain S3 ; (b) A : *Artemia* ; A+VA+S4 : *Artemia* + *V. alginolyticus* + strain S4 ; A+VA+S5 : *Artemia* +*V. alginolyticus* + strain S5 ; A+VA+S6 : *Artemia* + *V. alginolyticus* + strain S6.

3.7.3. *In vivo* interaction between probiotic bacteria and the pathogenic strain *V. alginolyticus*

According to the results reported in Figure 3, we could notice that *Artemia* nauplii reached 94% of mortality on the third day of treatment with the presence of *V. alginolyticus* (A+VA). However, the survival rate of *Artemia* nauplii showed a significant improvement ($P < 0.05$) an improvement after adding the different tested *Bacillus* strains. In particular, strain S1 raised the *Artemia* survival to 50% around the third day and 20% around the sixth day (Fig. 3a). Likewise, strain S6 maintained a growth rate of 40% on the third day and 16.66 % on the sixth day (Fig. 3b). Still, no significant difference in comparison with control treatment was noted ($P > 0.05$).

4. Discussion

4.1. Identification of isolates with antibacterial activity

Among the probiotic properties of a bacterium is its antagonistic effect against pathogens. The culture and the extracellular products of the six *Bacillus* strains were investigated against seven pathogens (five Gram-negative and two Gram-positive bacteria). We found that the isolated strains inactivated all the pathogenic strains tested. The diameters of the inhibition zones varied from 11.5 to 24.5 mm (Table 1). Our results showed that the extracellular products of all *Bacillus* strains inhibited the growth of pathogenic bacteria (Table 2).

Gram-positive bacteria isolated from the intestines of fish and having an antagonistic effect were identified as *Bacillus* spp., by biochemical identification. In addition, the MALDI-TOF-MS technique showed that the strains

S1 and S2 are *B. cereus* and the strains S3, S4, S5, and S6 are *B. subtilis*.

Bacillus strains are suitable as probiotics for aquaculture because they are frequently found as part of the microorganisms in fresh and seawater as well as in the gastrointestinal tract of animals [41]. Already, several studies have shown that *Bacillus* probiotic have been isolated from a healthy fish gastrointestinal tract [42–47]. Thus, it is believed that they are well-adapted and are more colonizing than transient in the gastrointestinal tract. Moreover, several studies have shown the antibacterial effect of *Bacillus* strains against several pathogens [48–52]. We have suggested the inhibitory effects of *Bacillus* spp. maybe because of the alteration in the pH of the growth medium or the production of antimicrobial proteins and chemical compounds synthesized by secondary metabolic pathways [53]. Similarly, genes encoding pre-subtilisin (sboA), sublichenin (spaS), malonyl CoA transacylase (ituD), and the putative transcriptional terminator of surfactin (sfp) were detected in *Bacillus* spp. suggesting the production of the bacteriocin subtilisin A and the lipopeptides iturin A and surfactin by this strain [54,55]. In addition, Yahav *et al.* [56] have shown that *B. subtilis* produces an extracellular matrix that protects it from stressful environments. They suggest that the extracellular matrix produced by *B. subtilis* could protect other probiotic bacteria and therefore could be a vehicle for delivering viable probiotic cells to humans. Piewngam *et al.* [57] have shown that a widespread class of *Bacillus* lipopeptides, the fengycins, eliminates *S. aureus* by inhibiting *S. aureus* quorum sensing, which is a process used by bacteria to respond to their population density by altering gene regulation. Likewise, the mechanisms (production of bacteriocins, suppression of virulence gene expression, competition for adhesion sites, production of lytic enzymes, production of antibiotics, immunostimulation, competition for nutrients and energy, and production of organic acids) used by *Bacillus* probiotics in mitigating fish pathogens ranging from *Aeromonas*, *Vibrio*, *Sreptococcus, Yersinia, Pseudomonas, Clostridium, Acinetobacter, Edwardsiella, Flavobacterium,* white spot syndrome virus, and infectious hypodermal and hematopoietic necrosis virus have been proven to be mitigated by *Bacillus* as shown by Kuebutornye *et al.* [58].

4.2. Characterization of extracellular enzymes

Probiotics can not only inhibit the growth of pathogens in aquaculture but can also benefit animal health. In our study, the enzymatic characterization showed that all *Bacillus* strains isolated from the intestinal tracts of fish did not produce β-glucuronidase, a carcinogenic enzyme, and other undesirable activities, such as trypsin, α-chymotrypsin (Table 3).

The *Bacillus* strains can produce enzymes that play an important role in digestion such as the alcaline phosphatase, acid phosphatase, Naphthol-AS-BI-phosphohydrolase, esterase, esterase lipase, N-acetyl-β-glucosaminidase, α-fucisidase amylase, and lipase. These activities are necessary for probiotics with digestive effects, as these enzymes generate amino acids, sugars, organic acids, and diverse small compounds [59,60]. Therefore, application of probiotics capable of producing enzymes is gaining attention to promote a nutritional benefit in aquaculture [52,61,62]. *B. subtilis* has previously been shown to produce large levels of extracellular proteases (exoproteases), which degrade proteins from the environment and are mainly encoded by two genes; aprE (subtilisin) and bpr (bacillopeptidase) [63]. Diverse studies have reported the ability of *Bacillus* to produce digestive enzymes, including amylase, protease, lipase, cellulose, and xylanase [64,65].

The present study confirmed that the isolated *Bacillus* strains did not show any haemolytic activity on sheep blood agar and hence it can be used as probiotic for better health. Similarly, B. clausii ATCC and B. subtilis did not show hemolysis on sheep blood agar [59]. Likewise, B. polyfermenticus CJ6 does not cause hemolysis on horse blood agar [66]. The haemolytic activity of the selected bacterial strains of *Bacillus* spp. isolated from the digestive tract of freshwater fish did not show any haemolytic activity against human blood [60].

4.3. Adhesion capability in polystyrene surfaces

The adhesive capacity of the isolated strains was then tested. Indeed, the semi-quantitative study of adhesion on polystyrene plates revealed that all strains are adherent to polystyrene micro-plates (Table 4). This property allows these microorganisms to persist in the intestine for several days and to be active during intestinal transit. Thus, they can eliminate potential pathogens and participate in the creation of a healthy environment. This agrees with the results published by Mahdhi *et al.* [67], which have shown that potential probiotic candidates based on *Bacillus* with moderate adhesion capacity can improve rearing conditions and protection against pathogens often dreaded in aquaculture. Selected probiotic bacteria, *B. licheniformis,* and *Bifidobacterium breve*, have adhesive properties that enable them to decrease the colonization of pathogens [68,69]. Also, following adhesion to a surface, spores may germinate, and vegetative cells may multiply. This helps this bacterium to compete with pathogens and ensures protection for the host [70]. Microbial cell surface hydrophobicity is one of the determining factors in microbial adhesion to bioremediation surfaces, a phenomenon commonly observed in natural and engineered systems [71]. It is generally accepted that hydrophobic interactions play an important role in bio adhesion.

4.4. Pathogenicity/toxicity of candidate probiotics and their effect on Artemia culture

To complete the screening of the isolated bacteria and to attribute the probiotic character, we tested their potential for negative effects. Indeed, we found that the strains tested did not have pathogenic or toxic effects on the *Artemia* culture. But they improved the survival rate (exceeding 90%) (Fig. 1 and 2). Moreover, we noted abrupt mortality of *Artemia* incubated alone (control treatment (a)) (Fig. 2 and 3). This allows us to confirm that our strains can be a source of nutrients [72]. Laranja *et al.* [73] have shown that the *Bacillus* spp., JL47 is an important determinant for the increased survival of challenged *Artemia*. Also, the adhesive capacity of tested *Bacillus* strains helps them to adhere to the intestinal tract, and their effect on the digestive process of aquatic animals can improve *Artemia* culture and protect against other pathogenic microorganisms and significantly increase the survival rate.

Our results can also be explained by bacteria being the major sources of proteins, fatty acids, and amino acids for the development of *Artemia* larvae. In addition, the attachment surface between the bacteria and the nutrient allows

the formation of particles rich in protein and increases its nutritional value for this small crustacean [74]. Likewise, a study carried out by Ofelio *et al.* [75] has shown that the bacterial biomass of probiotic *Lactobacillus rhamnosus* can be used by nauplii as a source of nutrients. This bacterial biomass also helps improve the nutritional value of *Artemia* nauplii and stimulates their immune system to defend against pathogens often feared in aquaculture. The work of Arig *et al.* [76] showed that the administration of *Bacillus* spp. in the food of sea bream *Sparus aurata* influenced growth parameters and improved digestion by stimulating acid and alkaline proteases activity in this fish. The improvement observed during the addition of these potential probiotics may be because of the antitoxic role of bacteria which have a positive effect intervening in the neutralization of toxic products by causing attenuation of intra-digestive catabolism and orientation of the intestinal microflora for reducing the absorption of toxic substances. It has been shown that the survival and growth rate of shrimp (*Litopenaeus vannamei*) were influenced by *B. subtilis* and *B. lichenformis* added to the diet as probiotics, with better growth performance and survival [77].

Bacteria of the genus *Vibrio* are ubiquitous members of marine ecosystems. *Vibrio* species are reported as opportunistic pathogens to aquatic animals and are associated with high mortality throughout the world [78]. Therefore, Among the tested bacteria, *V. alginolyticus* was evaluated for *in vivo* tests with *Bacillus* strains. *In vivo* tests of most *Bacillus* strains on *Artemia* nauplii in the presence and absence of the pathogen have revealed a high survival rate (Fig. 3). Our study focused on the interaction between probiotic bacteria and the pathogen *V. alginolyticus* has shown that probiotics confer protection for *Artemia* nauplii against this pathogen. This may be due to competition for nutrients or to binding at adhesion sites on the larval body and in the intestinal epithelium, thus reducing the pathogenicity of *V. alginolyticus*. Several studies showed that *Bacillus* strain was tested for its protective effects against vibriosis in Artemia culture [73]. Similarly, Ahmadifard *et al.* [79] showed the beneficial effect of enriched *Artemia* with *B. subtilis* on growth performance, reproductive factors, proximate composition, intestinal microflora, and resistance to *A. hydrophila* of ornamental fish (*Poecilia latipinna*). Furthermore, the addition of *B. subtilis* to the rotifer culture water resulted in an increase in rotifer numbers. This fact could be related to the production of bacteriocins by the probiotic bacteria that inhibit or regulate the growth of harmful bacteria, and enzymes that improve the digestion and utilization of nutrients by rotifer [80]. The use of a strain of *B. subtilis* in a culture of shrimp improved the survival rate and growth as well as protecting against *Vibrio* spp. stimulating their immune system, and enhancing their resistance to disease [48,81]. Likewise, the probiotics *B. subtilis*, *L. plantarum,* and *L. lactis* protect *Artemia* against a *V. anguillarum* challenge by enhancing its immune responses thus contributing to reduced oxidative damage and increased survival [82]. Moreover, fish fed with *B. velezensis*-supplemented diets showed a significantly improved survival rate after *A. hydrophila* infection [83]. *B. amyloliquefaciens* and *B. pumilus* could stimulate growth performance, innate immunity, and stress tolerance of striped catfish [84]. In this context, Olmos *et al.* [85] considered *B. subtilis* to be an ideal multifunctional probiotic bacterium. This bacterium prevented pathogens' development, enhanced nutrient assimilation, improved environmental parameters, and increased aquaculture profitability.

5. Conclusion

In this study, we isolated six strains of *Bacillus* with antibacterial activity against several pathogens. The production of several extracellular enzymes by the studied strains has an important advantage. They can participate in the stimulation of the digestive and immune systems and reinforce resistance to diseases. Adhesive ability to abiotic surfaces has been demonstrated, which may enhance antibacterial activity by promising longer residence in the digestive tract of the hosts. The bacterial strains isolated have exhibited promising results in the ability to improve *Artemia* cultures and protect them against the pathogenic strain *V. alginolyticus,* which can be used for the improvement of crustaceans or fish larvae. However, further research is still ongoing to clarify the exact mode of action of the observed beneficial and pathogenic effects of probiotic bacteria.

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Interest conflict

The authors declare no competing interests.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article.

Authors' Contribution

All authors had equal role in study design, work, statistical analysis and manuscript writing.

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