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Probiotic properties of *Bacillus* strains isolated from the gastrointestinal tract against pathogenic Vibriosis

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Vibriosis is one of the major diseases leading to massive fish mortality. Probiotics may provide a potential alternative method to protect fish from pathogens and to promote a balanced environment minimizing the use of antibiotics and chemotherapy. The aims of this study were to (i) isolate and purify marine spore-former strains from Sardine and shrimp intestine, (ii) screen for bacteria with potential probiotic properties, and (iii) carry out their *in vitro* safety assessment using a subtractive procedure. Among 108 spore-former strains, five strains exhibited a strong antibacterial activity against Vibriosis such as *Vibrio harveyi* and *Vibrio anguillarum*. These selected strains were unaffected by high-temperature and gastrointestinal conditions; produced amylase, protease, and lipase activities; and showed high percentages of auto-aggregation and co-aggregation with pathogens, as well as a strong adhesion to fish mucus. Partial 16S rDNA gene sequencing and MALDI-TOF MS revealed that isolates are *Bacillus amyloliquefaciens* or *Bacillus subtilis*. All of them were susceptible to antibiotics, while hydrolic enzymes and virulence factors were not detected for *B. subtilis* S17. In conclusion, based on their proprieties and their safety assessment, *B. subtilis* S17 could serve as a potential probiotic candidate for aquaculture.

KEYWORDS

Vibriosis, probiotic, *Bacillus* spp., safety assessment, adhesion

1 Introduction

Intensive aquaculture is known to cause stressful conditions related to stocking densities and high feeding. Such practices enhance the probability of disease outbreaks mainly at larval and early fry stages. Outbreaks are habitually caused by a wide range of pathogenic bacteria. Vibriosis is one of the major diseases leading to poor growth, low

immunity, and high mortality and therefore massive economic losses for the aquaculture sector (Meidong et al., 2017; Kaktcham et al., 2018; Ghanei-Motlagh et al., 2020; Makridis et al., 2021). Several *Vibrio* species have been concerned with marine animals' health problems. Recent studies showed that *Vibrio alginolyticus*, *V. harveyi*, and *V. parahaemolyticus* are the most potent species infecting farms (Daniels and Shafaie, 2000; Yılmaz et al., 2021). To sort out these problems, traditional methods such as vaccines and antibiotics are widely used. Nevertheless, the widespread use of antibiotics leads to the appearance and emergence of resistant bacteria and affect the aquatic environment (water and sediment contamination) (Caruffo et al., 2015; Medina et al., 2020; Makridis et al., 2021). Recently, probiotics have emerged as a good and effective alternative to ban or minimize the antibiotic use and control diseases (Balcazar et al., 2007; Meidong et al., 2017; Emam and Dunlap, 2020). Probiotics are defined as live microorganisms which beneficially affect the health of the host when consumed in adequate concentrations (Fuller, 1989; FAO/WHO/OIE, 2006; Pinto et al., 2020). Their potential benefits on the host are attributed to improve the immune system and enhance digestion and growth promotion by producing enzymes. Other beneficial effects include water remediation and secretion of inhibitory substances which confer resistance to intestinal pathogens and prevent their intestinal adhesion, and lead to better health properties (Verschuere et al., 2000; Balcázar et al., 2006; Balcazar et al., 2007; Fernandes and Kerkar, 2019; Medina et al., 2020; Khan et al., 2021). A large number of bacteria are classified as probiotics, but the most widely used in aquaculture are *Lactobacillus* and *Bacillus*. However, *Bacillus* species have attracted much more attention due to non-toxic and non-pathogenic compound production and their sporulation ability, which gives them protection against stressed conditions (heat, gastrointestinal tract (GIT) compared to other probiotics (Barbosa et al., 2005; Cao et al., 2019; Kuebutornye et al., 2019; Emam and Dunlap, 2020; Amoah et al., 2021). To exert and endure the benefits of probiotics within the host, several properties should be investigated for probiotic strain screening, including antagonism against fish pathogens, being kept alive in GIT, resistance to storage conditions, secretion of exogenous enzymes, and being safe (Fuller, 1989; Muñoz-Atienza et al., 2014; Cao et al., 2019; Zhou et al., 2019; Ghanei-Motlagh et al., 2020). Generally, probiotics are isolated from the indigenous and exogenous microbiota of aquatic animals (Balcázar et al., 2006; do Vale Pereira et al., 2017). However, the search for a putative probiotic should continue as the increase demand for sustainable and environment-friendly aquaculture.

In this study, we used Sardine and shrimp intestine, an untapped source of novel microorganism, as a source of potential probiotic bacteria. Hence, we have isolated, selected, and identified potential probiotic bacteria for aquaculture based on subtractive screening by means of several physiologic criteria and safety assessment.

2 Materials and methods

2.1 Pathogen collection and culture conditions

All *Vibrio* used as indicators (*Vibrio anguillarum* CECT4344; *V. alginolyticus*; *V. fischeri*; *V. harveyi* Lg 10/6, Lg 14/01, Lg 26/01, Lg 48/01, Lg 13/04, Lg 34/04, and Lg 35/03; *V. parahaemolyticus*; *V. proteolyticus*; *V. vulnificus*, and *V. splendidus* provided by The Microbiology Department of the University of Malaga) were isolated from diseased fish. They were potent causative agents of fish infection. Each strain was cultured in Tryptone Soya Agar (TSA) (Oxoid Ltd., Hampshire, UK) supplemented with NaCl 1.5% (w/v) at 28°C. Stock cultures in Tryptone Soy Broth (TSB) were stored at -80°C and -20°C in 1.5% NaCl with 15% glycerol to provide stable inoculum throughout the study (Midhun et al., 2018).

2.2 Sampling procedure and isolation of spore-forming bacteria

Bacteria used in this study were isolated from intestines of Sardine (*Sardina pilchardus*) and shrimp (*Penaeus kerathurus*). Intestines were washed with sterile saline solution, and content was aseptically removed. One gram from each intestine sample was homogenized in 9 ml phosphate-buffered saline (PBS, pH = 7.2). Selection of spore-forming isolates was by heat treatment. The homogenates were diluted in buffered peptone water (up to 10^{-3}) and incubated at 65°C for 30 min. One hundred-microliter aliquots of appropriate serial dilutions were spread onto Difco sporulation medium (DSM) for 48h at 28°C. Colonies obtained were picked and purified. Pure isolates in TSB were stored in 1.5% NaCl with 15% glycerol at -20°C and -80°C until further routine use (Barbosa et al., 2005).

All isolates were then investigated for catalase activity by the suspension of a fresh single colony in 3% of hydrogen peroxide. Air bubble production was considered as a positive result.

2.3 Antimicrobial activity

2.3.1 Colony overlay method

The preliminary screening for antimicrobial activity of spore-forming isolates was done by the colony overlay method described by Alonso et al. (2019). Both indicators and isolates were grown in TSB containing 1.5% NaCl and incubated overnight at 28°C. In order to form a bacterial lawn, 100 µl *Vibrio spp*. Culture (10^5 CFU/ml) was spread in TSA plates using a sterile cotton-tipped swab. Drops of isolate cultures were dispensed onto the plates once dried. After incubation at 28°C for 24 h, antimicrobial activity was assessed by observing the

presence of inhibition zones around colonies and represented as scores as described by Midhun et al. (2017). Based on cumulative scores, promising antagonistic spore-forming bacteria against indicators were selected. Further, the strong *Vibrio inhibitory* spore-forming bacteria were confirmed through cell-free supernatants (CFSs).

2.3.2 Extracellular antimicrobial assay

The extracellular antimicrobial activity of cell-free supernatants was determined using a microtiter plate and an agar well diffusion test (ADT). Briefly, selected strains were cultured in TSB containing 1.5% NaCl incubated at 28°C for 24 h at 150 rpm. CFS was obtained by centrifugation of overnight cultures (10^7 CFU/ml) at 8,000×g at 4°C for 20 min and filter sterilized through 0.22-µm pore-size filters.

The inhibitory activity was evaluated in a 96-well microtiter plate by inoculation of pathogenic bacteria culture (10 µl, 10^5 CFU/ml) with TSB fresh medium (150 µl) and sterilized CFS (50 µl) in each well. Controls were inoculated with pathogenic bacteria only in 200 µl fresh TSB. The absorbance at 600 nm was used to measure the inhibition efficiency (IE) during 24 h by the following formula:

$$IE = (\textbf{OD in presence of CFS of isolates})/(\textbf{OD of pathogen only})$$

where IE<1 indicates inhibition of pathogenic bacteria by isolates, IE = 1 indicates no inhibition, and IE >1 indicates growth promotion (Mukherjee and Ghosh, 2016).

Besides, antibacterial activity using ADT was assayed as described by Cintas et al. (1995) with some modifications. Plates were seeded with indicators (100 µl, 10^5 CFU/ml). Fifty microliters of CFS was placed into wells (6 mm of diameter) cut in TSB with 1.5% NaCl and 0.8% agar (w/v). After 2 h at 4°C, the plates were incubated under 24 h at 28°C then antimicrobial activity was defined by the presence of a clear inhibitory zone around the wells. Further, neutralization was obtained using 1 N sodium hydroxide to pH = 7. To determine the nature and thermostability of the antimicrobial compounds, the supernatant was treated with proteinase K at 10 mg/ml (AppliChem GmbH, Darmstadt, Germany) and then heated at 100°C for 10 min. After treatments, samples were assayed for residual antimicrobial activity by an ADT using *Vibrio anguillarum* CECT4344 and *V. harveyi* Lg26/01 as indicators.

2.4 Preparation of vegetative cells, spores, and sporulation efficiency

For the preparation of vegetative cells, each selected strain was grown in TSB with 1.5% NaCl and taken from logarithmic-phase cells.

Sporulation of isolates was induced by exhaustion of nutrients in DSM. In fact, overnight cultures of selected strains in TSB with 1.5% NaCl were inoculated to DSM broth and incubated 28°C at 150 rpm for 24 and 48 h. After inoculation, cultures were diluted and plated in TSA in order to determine viable and heat-resistant cells before and after heat treatment (80°C, 20 min), respectively. Sporulation efficiency was determined by quantifying viable cells before and after heating (Barbosa et al., 2005; Prieto et al., 2014; Zhou et al., 2019; Santos et al., 2021).

2.5 Screening of probiotic properties of spore-forming isolates

2.5.1. Evaluation of extracellular enzymes

The extracellular enzyme production of selected bacteria was performed by plate assay according to Hmani et al. (2017) with some modifications. Protease and amylase production was determined using Luria-Bertani (LB) agar plates supplemented with 1% skimmed milk and 1% starch, respectively. Culture plates were spot inoculated and incubated for 24 h at 28°C. The clear zone around the colonies revealed a proteinase production. After plates were flooded with iodine, yellow discoloration appeared, indicating amylase production. For lipase activity, strains were spotted onto LB plates containing 1% olive oil and 1% rhodamine. Lipase activity was observed by zone of clearance surrounding colonies.

2.5.2 Biofilm production

The ability to produce biofilm was determined by the crystal violet assay as described by Midhun et al. (2017) with modifications. Selected strains were inoculated into 10 ml of LB broth containing 1% glucose and incubated at 28°C for 24 h. Cultures were diluted to an OD600 of ~0.1. Each well of a sterile 96-well flat-bottom polystyrene plate (orange scientific) was filled with 200 µl of diluted culture. *Escherichia coli* DH5α and *Staphylococcus aureus* ATCC 6538 were used as negative and positive biofilm-forming strains, respectively, and sterile LB broth was considered as blank to check the sterility and non-specific attachment. After incubation, excess medium from each well was removed by tapping. The wells were washed twice with 250 µl of PBS (pH = 7.2) to eliminate floating bacteria. The adherent bacteria were fixed at 60°C for 1 h and stained with 150 µl of 0.2% crystal violet prepared in 20% EtOH (v/v). The stained bound bacteria were released by adding 200 µl of glacial acetic acid. After incubation for 1 h at room temperature, the absorbance at 570 nm was determined using a microplate reader. The wells of isolates in which OD values are higher than those in blank wells were considered to be biofilm producers.).

2.5.3 Resistance to high temperature, pH, and bile salts

The resistance of selected strains to high temperatures required by the fish feed process was assessed according to Kuebutornye et al. (2020)

with modifications. Briefly, an overnight culture was harvested. The pellets were resuspended in PBS and exposed to elevated temperatures (80°C, 90°C, and 100°C) for 5 and 10 min, respectively. Viable counts were determined by the spread plate method after incubation at 28°C for 24 h.

The tolerance of vegetative cells and spores to low pH and bile salt was assayed as described by Barbosa et al. (2005) and Hmani et al. (2017) with modifications. Essentially, ~10^8 to 10^9 bacterial cells ml^{-1} were resuspended in TSB broth containing bile salts (0%–5%, sodium cholate 50%, sodium deoxycholate 50%) (Bio Basic, Markham, Canada) or in TSB broth adjusted to pH 1, 2, 3, and 7.3 (control) with concentrated HCl. Aliquots were taken immediately and after 3 h for low pH and after 6 h for bile salt tolerance. Viable counts were determined by the spread plate method on TSA after incubation at 28°C for 24 h.

2.5.4 Auto-aggregation and co-aggregation assays

Auto-aggregation assay was performed according to Meidong et al. (2018). Overnight cultures grown were centrifuged, washed twice, and resuspended in PBS followed by turbidity measurement to obtain OD600~1(A0). The bacterial suspensions were vortexed for 10 s and kept undisturbed at 28°C for 5 h and after 24 h. Then each hour OD600 of the upper suspensions (At) was measured. The auto-aggregation percentage (%Agg) was expressed as:

$$\%\,\mathbf{Agg} = 1 - (\mathbf{At}/\mathbf{A0}) \times 100$$

where At represents the OD600 at time t = 1, 2, 3, 4, 5, and 24 h and A0 the OD600 at t = 0.

In the co-aggregation assay, the suspension of isolated strains and fish pathogens was prepared similarly to the auto-aggregation analysis above. Equal volumes of tested strains and pathogens were mixed and incubated at 28°C for 6 h. The OD600 of the mixtures and controls (unmixed cultures of tested strains and pathogens) were measured after incubation. Co-aggregation (Co-agg) was calculated as:

$$\%\ \mathbf{Co-agg} = [(\mathbf{Apat} + \mathbf{Aisolate}) - 2(\mathbf{Amix})/(\mathbf{Apat} + \mathbf{Aisolate})] \times 100$$

where Apat and Aisolate represent the OD600 of each bacterial suspension alone and Amix represents the OD600 of mixed suspension pathogen and isolated strains (Meidong et al., 2018).

2.5.5 Adhesion assay *in vitro*

2.5.5.1 Mucus preparation and characterization

Mucus was isolated from the skin and intestine of gilt-head bream (*Sparus aurata*) according to Balcázar et al. (2008). The skin mucus was collected by scraping the dorsolateral surfaces of fish using a cell scarper. The intestine was removed and scraped to collect intestinal mucus. All mucus were homogenized in PBS and centrifuged three times at 120,000× g for 15 min at 4°C to remove cellular materials and particulate. Then the samples were adjusted to a protein concentration of 1 mg/ml in PBS. The protein concentration was determined according to Beltrán et al. (2017) and Midhun et al. (2018). The mucus preparations were filter sterilized by a 0.22-μm-size Millipore filter and finally stored at -20°C until use.

2.5.5.2 Adhesion and growth on fish mucus

Adhesion ability was evaluated according to Li et al. (2020) with some modifications. Briefly, 100 μl of skin and intestinal mucus was immobilized on a polystyrene microtiter plate by overnight incubation at 4°C. To remove excess mucus, wells were washed twice with 250 μl of PBS. Overnight-grown bacteria (vegetative cells or spores) were centrifuged at 6,000 g for 15 min, washed twice, and resuspended in PBS with fluorescein isothiocyanate (FITC) (100 μg/ml) at 28°C for 1 h in the dark. Labelled bacteria were washed three times with PBS. Then, 100 μl of the labelled bacterial suspension (10^8 CFU/ml) was added to each well. Plates were incubated at 28°C for 1 h and then washed with PBS to remove unbound bacteria. The bound bacteria were lysed with 200 μl of 1% sodium dodecyl sulphate (SDS) in 0.1 M NaOH at 60°C for 1 h. The fluorescence intensity was measured with the excitation and emission spectrum of 485 nm/530 nm by spectrophotometry. Adhesion was calculated using the formula:

$$\%\,\mathbf{Adhesion} = \mathbf{A}/\mathbf{A0} \times 100$$

where A is the fluorescence of adhering bacteria and A0 is the fluorescence of initial bacteria.

Moreover, to determine whether the observed bacterial adhesion was due to non-specific adhesion or cell surface hydrophobicity, adhesion assays to bovine serum albumin (BSA) and polystyrene were performed as described above.

The ability of the strain to establish and persist in the gut is essential. For this purpose, the growth of the strain in the intestine and skin mucus was evaluated. In fact, the skin and intestinal mucus were inoculated with the bacterial suspension and spores (10^6 CFU/mL) and incubated at 28°C for 24 h. Colony-forming units (CFU/mL) were counted. Sterilized mucus-inoculated plates served as controls (Midhun et al., 2017).

2.6 Identification of selected spore formers

The selected spore formers were identified using molecular identification targeting 16S rRNA genes and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Ultraflex III, Bruker Daltonics, Bremen, Germany).

Total bacterial DNA was extracted using the InstaGene Matrix resin (Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR amplification was performed using primers 27F and 1492R (Table 1). PCR reaction was prepared as follows: 25 µl of MyTaq Mix (Bioline, London, UK), l µl of each primer (0.7 µmol), and l µl of purified DNA (~50 ng). The samples were amplified under the following conditions: initial denaturation at 97°C for 1 min, followed by 35 cycles of denaturation (95°C for 15 s), annealing (48 for 15 s), and elongation (72°C for 10 s), ending with a final extension step at 72°C for 7 min. The corresponding species identity was obtained by comparative sequence analysis against available sequence data in the National Centre for Biotechnology Information (NCBI) database.

A single colony of selected strains was also analysed by MALDI-TOF MS as described by Altakhis et al. (2021). Bruker Flex Control software was used to run measurements of MALDI-TOF MS which were expressed as score. Based on the report of Bruker software, results of identification were reliable only when the scores generated were ≥1.7 (Han et al., 2021).

2.7 *In vitro* safety assay

2.7.1 Antibiotic resistance

The antibiotic susceptibility of selected strains was determined by broth microdilution test as described by Araújo et al. (2015). The antibiotics used were ampicillin (0.12–8 µg/ml), vancomycin (0.5–32 µg/ml), gentamicin (2–128 µg/ml), kanamycin (4–256 µg/ml), streptomycin (4–256 µg/ml), erythromycin (0.12–8 µg/ml), clindamycin (0.25–16 µg/ml), tetracycline (0.5–32 µg/ml), and chloramphenicol (1–64 µg/ml). A single colony was suspended in 5 ml of saline solution (0.85% NaCl) to reach an optical density of 0.5 McFarland Scale (10^8 CFU/ml) and diluted in TSB. Fifty microliters of strain suspension was inoculated with each well containing 50 µl of the different antibiotic concentrations. The inoculated plaques were incubated at 28°C for 18 h; the minimum inhibitory concentrations (MICs) were read as the lowest concentration of antibiotics inhibiting the growth of bacteria and were compared according to EFSA (Muñoz-Atienza et al., 2013). Resistance was defined when the MICs of strains are higher than the respective breakpoint. *Staphylococcus aureus* CECT794 and *Enterococcus faecalis* CECT795 were used as controls.

2.7.2 Production of hydrolic enzymes

Haemolysis activity was investigated as previously described by Muñoz-Atienza et al., 2014 (Eaton and Gasson, 2001). Briefly, selected strains previously cultured in TSB with 1.5%NaCl were streaked on horse blood agar plates (bioMérieux, Marcy l'Etoile, France). After 1–2 days at 28°C, the presence of clear zones or green zones of hydrolysis around the colonies revealed β-haemolysis or α-haemolysis, respectively; the absence of clear zones around colonies means γ-haemolysis. *Enterococcus faecalis* P4 was used as positive control.

Gelatinase production was determined as described by Eaton and Gasson (Eaton et al,. 2001). Selected strains grown in TSB broth at 28 C for 16 h were streaked on Todd–Hewitt (Oxoid) agar plates (1.5%, w/v) with 30 g/l gelatin. After overnight incubation, the plates were placed at 4°C for 5 h before examination. The presence of zone of turbidity (protein hydrolysis) around the colonies revealed the activity. *Enterococcus faecalis* P4 was used as positive control.

The ability of selected strains to hydrolyse primary and secondary bile salts was determined according to Araújo et al. (2015) with some modifications. Briefly, 10 µl of cultures grown

TABLE 1 Oligonucleotide primers used in this study.

Target gene	Primer	5′–3′ sequence	Annealingtemperature	Reference
16S rDNA	27F	AGA GTTTGATCCTGGCTCAG	48	(Araújo et al., 2015)
	1492R	CGGTTACCTTGTTACGACTT		
hdc	CL1	CCWGGWAAWATWGGWAATGGWTA	48	
	JV17HC	AGACCATACACCATAACCTT		
ldc	CAD2-F	CAYRTNCCNGGNCAYAA	53	
	CAS2-R	GGDATNCCNGGNGGRTA		
odc	3	GTNTTYAAYGCNGAYAARACNTAYTTYGT	52	
	16	TACRCARAATACTCCNGGNGGRTANGG		
tdc	TD5	CAAATGGAAGAAGAAGTAGG	48	
	TD2	ACATAGTCAACCATRTTGAA		
sfp	sfp-F	ATGAAGATTTACGGAATTTA	46	(Hsieh et al., 2004)
	sfp-R	TTATAAAAGCTCTTCGTACG		
cesA	CER1	ATCATAAAGGTGCGAACAAGA	52	(Horwood et al., 2004)
	EMT1	AAGATCAACCGAATGCAACTG		
ces B	EM1-F	GACAAGAGAAATTTCTACGAGCAAGTACAAT	60	(Ehling-Schulz et al., 2004)
	EM1-R	GCAGCCTTCCAATTACTCCTTCTGCCACAGT		

in TSB broth was spotted onto TSB agar plates supplemented with 0.5% (w/v) sodium salts of taurocholate or taurodeoxycholate (Sigma-Aldrich, St. Louis, MO, USA) and 0.05% (w/v) L-cysteine (Merck) and incubated at 30°C for 72 h under anaerobic conditions (AnaeroGen, Oxoid). The presence of opaque halos of precipitated deconjugated bile acids around the colonies was considered as a positive result. Fresh faecal slurry of a healthy adult cow was used as the positive control.

2.7.3 PCR detection of potential virulence factors

Detection of genes encoding histidine decarboxylase (*hdc*), tyrosine decarboxylase (*tdc*), ornithine decarboxylase (*odc*), lysine decarboxylase (*ldc*), surfactin synthetase (*sfp*), and creolise synthetase (*cesA*, *cesB*) was carried out by PCR. *Lactobacillus* sp. 30a, *L. brevis* CECT4121, and *Bacillus cereus* 4086 were used as positive controls. PCR amplifications were performed as previously described by Araújo et al. (2015).

2.8 Statistical analysis

Data were expressed as means ± standard error of the mean (SEM). A statistical analysis of the data was performed using the software package SPSS 19.0. One-way analysis of variance (ANOVA) means and Tukey and Duncan's tests were applied. Significant differences were accepted to be significant when $P< 0.05$.

3 Results

3.1 Isolation of spore former strains and screening for antimicrobial activity

A total of 108 colonies were isolated and purified from sardine and shrimp intestine (47 from *Sardina pilchardus* and 61 from *Penaeus kerathurus*). All isolated strains were found to be Gram-positive, catalase positive. Out of 108 isolates, only 20 showed antagonistic activities by the colony overlay method presented as scores (Table 2). Cumulative maximum and minimum scores were 25 and 2, respectively. All 20 exerted direct antimicrobial activity against, at least, two of the indicators (Figure 1). Strain S17 showed a strong antagonistic activity against all tested pathogens.

Based on the total score, the strong pathogen inhibitory strains (Cm4, Cm5, Z1, S17, and C211) were selected and analysed for extracellular antimicrobial activity assay and for various probiotic potentials.

The inhibition of selected strain CFSs was substantiated by IE values varying in reference time (Figure 2). In majority of hours, inhibition of pathogens was more than 20% for *Vibrio anguillarum* (Va) and 30% for *V. harveyi* Lg26/01(Vh3). Moreover, lower inhibition was observed during the initial phase (between 2 and 6 h). On other hand, antibacterial activity of CFS and its nature was as described in Figure 3 and Supplementary Figure S1. All CFSs of selected strains were able to inhibit pathogenic bacteria. The antimicrobial activity of CFS exerted by all these strains withstood after adjustment of pH but disappeared, partially or completely, after heat and proteinase K treatments, thus confirming the proteinaceous nature of the antimicrobial compounds.

3.2 Sporulation efficiency

All selected strains showed different sporulation efficiencies (Figure 4) and showed spore titres in order of 10^7–10^8 spores/ml. The sporulation of S17 and Z1 had the highest rate at 18% and 20% after 24 h and 89% and 92% after 48 h, respectively, while Cm5 had the lowest at 14% and 84% after 24 h and 48 h, respectively.

3.3 Probiotic properties of selected spore-forming bacteria

3.3.1 Evaluation of extracellular enzymes and biofilm production

Extracellular enzyme production is wide-ranging among selected strains. The S17 strain exhibited the highest protease and amylase activities. Lipase activity was detected with Cm5, Z1, and C211 (Supplementary Table S1). Further, lipase activity was not detected in sardine isolates. Finally, all the selected strains were able to form biofilm. Among them, S17 had the strongest ability followed by C211 (Figure 5).

3.3.2 Tolerance to high temperature and pH and bile salt

Vegetative cells and spores were tested for resistance to high temperature, various pH, and bile concentrations.

Selected strains gave promising results after exposure to high temperatures (80°C, 90°C, 100°C for 5 and 10 min). High viability (above 80%) was observed in all selected vegetative cells after exposure to various temperatures compared to control (spore formers without exposure to high temperatures). However, there were no significant differences at different times of exposure among various temperatures for Z1 and S17. At last, the strains Cm4 and C211 had a significant decrease in viability (Supplementary Figure S2).

For the vegetative cells, all selected strains remained viable after 3 h of exposure to pH 2.0 and pH 3.0, but none could tolerate exposure to pH 1.0 (Table 3). Among all tested strains, S17 was found to have the highest resistance to acid conditions, while Cm5 and Z1 showed the lowest acid tolerance. Tested strains also withstood (0.5%–5%) bile concentrations after a 3-h

TABLE 2 Direct antimicrobial activity against fish pathogens.

Biotope	Isolates	Va	Val	Vf	Vh1	Vh2	Vh3	Vh4	Vh5	Vh6	Vh7	Vp	Vpr	Vv	Vs	Total score
Shrimp intestine	**Cm1**	0	0	0	0	0	0	0	0	0	2	0	0	0	0	2
	Cm2	1	0	0	0	0	0	0	0	0	1	0	0	0	0	2
	Cm4	4	0	3	0	0	2	1	1	0	2	2	1	2	0	18
	Cm5	4	0	3	1	2	2	2	1	0	2	2	1	2	0	22
	Cm6	1	0	1	0	0	0	0	0	0	0	0	0	0	0	2
	Z1	4	1	3	0	2	3	1	1	0	1	2	1	2	0	21
	Z3	0	0	1	0	0	0	0	0	0	0	1	1	0	0	3
	C10	0	0	1	0	1	0	0	0	0	0	0	0	0	0	2
	C11	0	0	0	0	0	2	0	0	0	0	0	0	0	0	2
	C20	0	0	0	1	2	1	2	0	0	2	0	0	0	0	8
	C211	3	0	2	0	1	3	2	1	1	0	1	0	3	0	17
Sardine intestine	**S1**	1	0	0	1	1	1	1	1	0	0	0	0	0	0	6
	s2	1	0	0	1	0	1	0	0	0	0	0	0	0	0	3
	S3	1	0	0	1	0	1	0	0	0	0	0	0	0	0	3
	S4	1	0	0	1	0	1	0	0	0	1	0	0	0	0	4
	S6	1	0	0	1	0	1	0	0	0	1	0	0	0	0	4
	S9	0	1	3	0	0	0	0	0	0	0	0	0	0	0	4
	S10	1	0	0	0	0	1	0	0	0	0	0	0	0	0	2
	S14	0	0	2	0	1	0	1	0	0	0	0	0	0	0	4
	S17	3	1	4	1	2	3	2	2	1	1	2	1	2	0	25

Zones of inhibition (halo diameter) were presented as scores; 0 (0–5 mm), 1 (low,6–10 mm), 2 (moderate, 11–20 mm), 3 (high, 21–24 mm) and 4 (very high, ≥25 mm. 0—no antagonistic activity. All indicator strains were assayed at least twice. Va, *Vibrio anguillarum* CECT4344; Val, *V. alginolyticus*; Vf, *V. fischerie*; Vh1, *V. harveyi* Lg 10/6; Vh2, *V. harveyi* Lg 14/01, Vh3, *V.harveyi* Lg 26/01; Vh4, *V. harveyi* Lg 48/01; Vh5, *V. harveyi* Lg 13/04; Vh6, *V. harveyi* Lg 34/04; Vh7, *V. harveyi* Lg 35/03; Vp, *V. parahaemolyticus*; Vpr, *V. proteolyticus*; Vv, *V. vulnificus*; Vs, *V. splendidus*.

exposure. Thus, S17 showed the highest tolerance to 5% of bile salt, whereas strain CJ3 exhibited the lowest tolerance. However, all spores of tested strains demonstrated significantly high tolerance to gastric and intestinal conditions after a 3-h exposure (Figure 6). Noteworthily, all spores could tolerate exposure to pH 1.0 and remain viable compared to vegetative cells in the same condition.

3.3.3 Auto-aggregation and co-aggregation assays

As seen in Table 4, auto-aggregation for selected strains increased with the incubation period and varied from 4% to 92%. All selected strains revealed low auto-aggregation ability (<50%) at the first 3 h. Nonetheless, a significant increase was observed after 24 h. Among them, S17 and C211 exhibited the strongest auto-aggregation ability (92%; 90%, respectively) after 24 h of incubation.

In addition, all tested strains were able to co-aggregate with pathogens significantly, showing co-aggregation percentages above 55% (Figure 7). Among the isolates, S17 showed the highest percentage of co-aggregation against *V. fischeri* (81%), *V. harveyi* Lg48/01 (79%), and *V. harveyi* Lg26/01 (67%) after 6 h of incubation. S17 also presented a strong ability to co-aggregate against *V. anguillarum* with more than 74%, followed by Cm4 and Cm5.

3.3.4 Adhesion and growth on fish mucus

The adhesion ability of the tested strains (vegetative cells) to mucus is shown in Table 5. All tested strains were highly capable of binding to intestinal mucus (44.7 to 58.3%) but less to skin mucus (10.6% to 29.4%). S17 showed the highest, both skin and intestinal mucus adhesion with more than 29% and 58%, respectively. The adhesion ability of spores was found to be lower than that of its vegetative cells for skin and intestinal mucus (Figure 8A). All the spores were more able for adhesion to intestinal mucus than skin mucus, ranging from 8% to 60%. All tested strains (vegetative cells and spores) adhered significantly less to BSA (5% to 37%) and polystyrene (19% to 50%) compared to skin and intestinal mucus.

All tested strains (vegetative cells and spores) were able to grow in fish mucus collected from the skin and intestine of *S. aurata* (Figure 8B). The growth of vegetative cells was found to be higher than that of their spores for skin and intestinal mucus. Interestingly, growth of vegetative cells on intestinal mucus was observed to be higher compared to growth on skin mucus. The significantly highest growth was observed for S17, for both skin and intestinal mucus, respectively.

3.4 Identification and *in vitro* safety assay

The selected strains were subjected to partial 16S rDNA gene sequencing and MALDI-TOF MS. According to these methods,

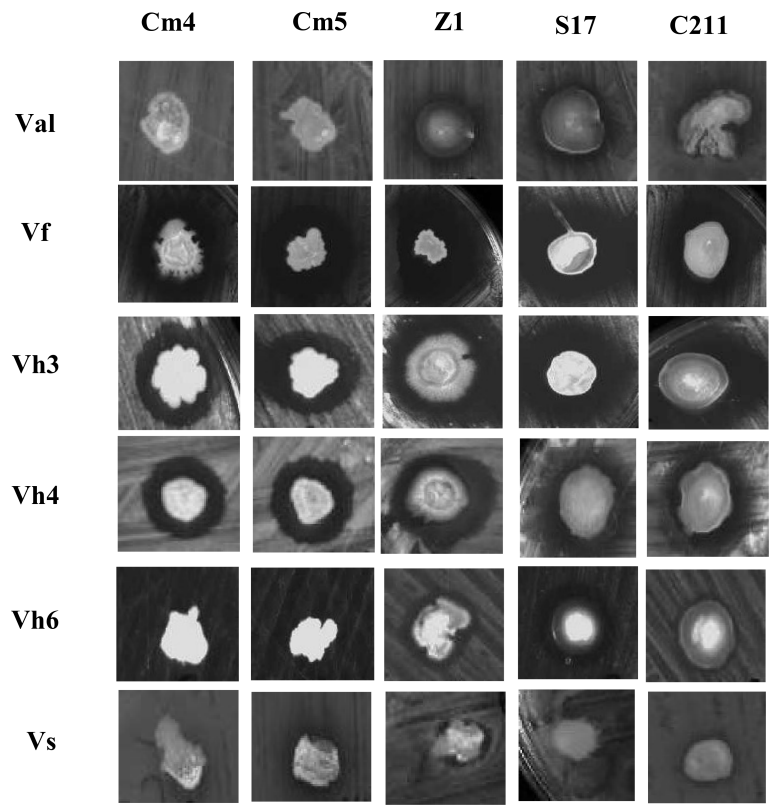

FIGURE 1
Pictorial overview of antimicrobial activities of selected strains against indicators Vibrios (colony overlay method).Val, *V.alginolyticus;* Vf, *V.fischerie;* Vh3, *V. harveyi* Lg 26/01; Vh4, *V. harveyi* Lg 48/01; Vh6, *V. harveyi* Lg 34/04 and Vs, *V. spendidus*.

all selected strains were identified as *Bacillus* spp. The isolates were identified as *B. subtilis* (Cm4, Cm5, S17, and C211) and *B. amyloliquefaciens* (Z1) by MALDI-TOF MS (Table 6) which was confirmed by PCR. In addition, all selected spore formers were found to be susceptible to tested antibiotics.

All strains exhibited haemolysis on blood agar (β-haemolytic) except S17, which was negative for haemolytic activity (γ-haemolytic) (Supplementary Table 2). More analysis revealed that Z1 and C211 were found to exhibit bile salt deconjugation, and all selected isolates showed gelatinase production except S17 (Supplementary Table S2). Furthermore, PCR screening for the presence of biogenic amines and toxins showed that none of the tested strains encoded any of the virulence genes searched *hdc*, *tdc*, *odc*, *sfp*, *cesA*, or *cesB*, with the exception of *ldc* which was detected in Cm4 and C211 (Supplementary Table 2).

4. Discussion

During the past decades, aquaculture production has faced many threats due to the outbreak of diseases, i.e., Vibriosis (Yılmaz et al., 2021). In order to control or prevent fish pathogens, probiotics are used as a viable and friendly alternative (Balcázar et al., 2008; Kavitha et al., 2018; Khan et al., 2021). Gastrointestinal microbiota is considered as the best source to isolate and select potential probiotics. Indeed, fish and shrimp intestine constitute a dynamic ecosystem containing diverse unexplored microorganisms and playing a crucial role in animal health (Romero et al., 2014; Zhou et al., 2014; Kuebutornye et al., 2020; Medina et al., 2020). Numerous studies assumed that probiotics from target marine animals and from the same ecological niche of pathogens would be more effective (Midhun et al., 2017; Kuebutornye et al., 2020; Yamashita et al., 2020; Santos et al., 2021). In the present investigation, spore-former strains were isolated from sardine and shrimp intestine and were evaluated for their probiotic potential and their safety to select the most putative as probiotic for aquaculture.

An initial prescreening was carried out based on antimicrobial activity against Vibriosis which is one of major pathogens in aquaculture. From 108 isolates, only 20 showed antagonistic activity by the colony overlay method against, at least, two of tested Vibriosis such as *V. harveyi*, *V. anguillarum*,

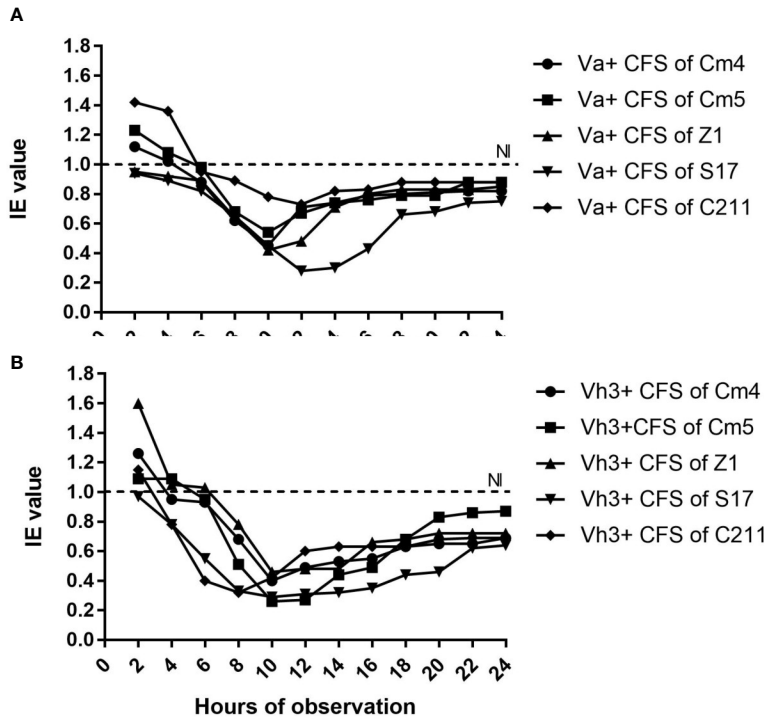

FIGURE 2
Inhibition efficiency (IE) of selected strains against **(A)** *Vibrio anguillarum* CECT4344 (Va) and **(B)** *V.harveyi* Lg 26/01 (Vh3) as a function of time. *IE˃1 indicated growth promotion of pathogen, IE=1indicated no inhibition (NI) and IE<1 indicated inhibition of pathogen by CFS of selected strains.

and *V. fischeri*. Previous studies indicated that *Bacillus* species, including *B. pumilus* (Lei et al., 2015), *B. amyloliquefaciens* (Cai et al., 2019; Medina et al., 2020), and *B. subtilis* (Zhou et al., 2019), exhibited antagonistic activity against Vibriosis (Amoah et al., 2021). Similar studies also reported that some *Bacillus* inhibit the growth of various *Vibrio* species (*V. harveyi*, *V. alginolyticus*, *V. vulnificus*, and *V. parahaemolyticus*) (Prieto et al., 2014; Meidong et al., 2018; Li et al., 2019; Zhou et al., 2019). Later on, selected strains with promising direct antimicrobial activity were tested for extracellular inhibitory activity by ADT and microtiters. In this respect, only five strains were found to produce proteinaceous compounds and *B. subtilis* S17 showed the strongest antagonistic activity against all tested *Vibrio* species. In fact, the antibacterial activity of selected strains is probably explained by the production of inhibitory components such as organic acids, hydrogen peroxide, and bacteriocins or by competition for adhesion sites and/or nutrients in the intestine (Pinto et al., 2020; Santos et al., 2021). Hence, published reports revealed that the antibacterial substances not only inhibit pathogen's growth but also increase the resistance of the host against pathogens (Verschuere et al., 2000; Guo et al., 2016; Midhun et al., 2017; Samson et al., 2020; Khan et al., 2021).

Unlike lactic acid bacteria, *Bacillus* sp. is considered to be a very stable strain due to its sporulation ability. Their endospores are resistant to harsh conditions, including high temperature, UV and acidity, drought, freezing, radiation, and rising oxygen levels. Relatively, *Bacillus* spores are able to survive throughout the simulation of GIT (Barbosa et al., 2005; Kuebutornye et al., 2020). In this present work, the sporulation rates of selected strains were more than 80% after 48 h of incubation. However, the differences in sporulation rates observed among the bacilli tested may be due to a different mechanism of sporulation process (Barbosa et al., 2005; Prieto et al., 2014; Banerjee and Ray, 2017).

Probiotics not only reduce the risk of fish infection in aquaculture by inhibiting the growth of pathogens but also could promote animal health. Several studies report that *Bacillus* sp. is able to produce digestive enzymes, including amylase, protease, lipase, cellulose, and xylanase (Hmani et al., 2017; Midhun et al., 2017). The present study confirms the ability of selected bacterial strains to produce extracellular enzymes including amylase, protease, and lipase. Indeed, these enzymes facilitate the digestion of fish diet and can help and improve the growth rate. Therefore, application of probiotics capable of producing enzymes is gaining attention to promote a

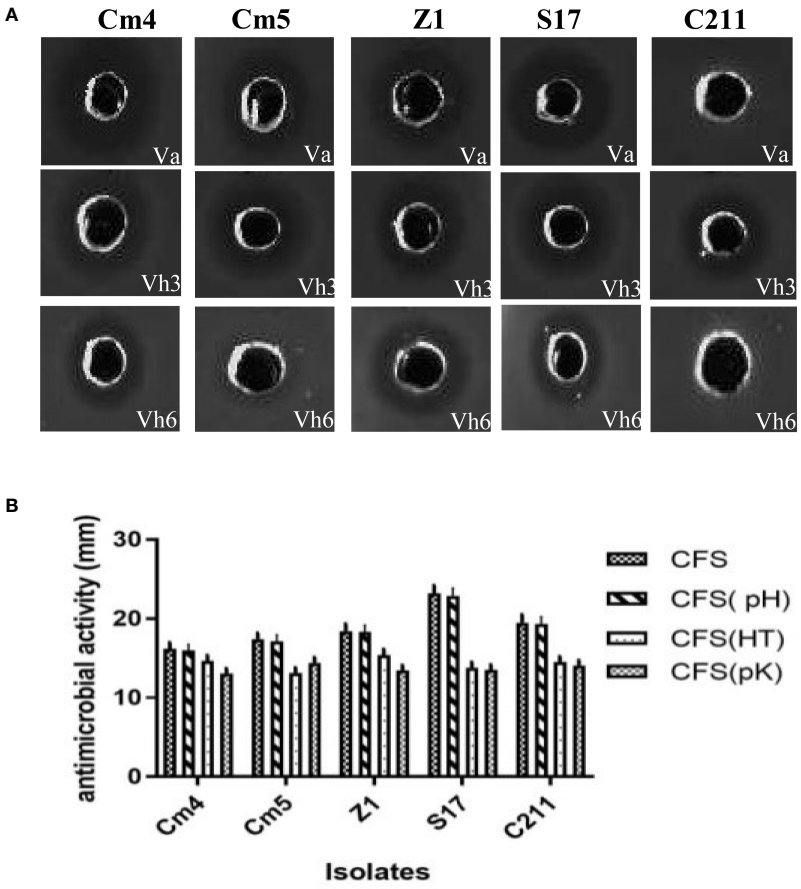

FIGURE 3
(A) Pictorial overview of antibacterial activity of selected strains against indicators (VA, *Vibrio anguillarum* CECT4344; Vh3, *Vibrio harveyi* Lg 26/01and Vh4, *V. harveyi* Lg 34/04) and **(B)** nature of their antagonistic activity against *Vibrio anguillarum* CECT4344 using agar well-diffusion method. *CFS: supernatant without any treatment, CFS (pH): pH neutralization treatment, CFS (HT): heat treatment at 100 ° C, CFS (pK): proteolytic enzyme treatment.

nutritional benefit in cultivable fish (Benhamed et al., 2014; Sharifuzzaman et al., 2018).

Moreover, biofilm is a complex and heterogenic aggregation of microorganisms. The probiotic biofilm has many benefits to host intestinal health including increasing the gut resistance by producing secondary metabolites, increasing the colonization efficiency, protecting the gut epithelium by competing with pathogenic strains for adherence sites, and inducing immune reactions to the gut pathogens in the host (Midhun et al., 2017; Banerjee and Ray, 2017; Midhun et al., 2017; Chauhan and Singh, 2019). In this study, all selected strains were capable of forming biofilm at high levels. Among them, strains S17 and C211 had the strongest ability to form biofilm.

During the production of animal feed production, heat treatment is considered as an essential process to get rid of vegetative cells (Banerjee and Ray, 2017; Kuebutornye et al., 2020). In this report, all tested strains showed higher survivability percentages above 80% during heat treatment (80°C, 90°C, 100°C) which were mentioned on previous findings (Kuebutornye et al., 2020; Amoah et al., 2021). Furthermore, probiotics are intended to colonize the GIT of fish and to restore the balance of intestinal microflora. The candidate strains should tolerate GIT conditions. In fact, ingested probiotic strains need to survive the acidity of stomach and fish bile in the small intestine. Moreover, bile plays an important role in the mechanism (specific and non-specific) of defence in the gut. Hence, probiotic strains are required to tolerate bile salts which are so toxic for bacteria due to their effect on the structure of the membrane (Ramesh et al., 2015; Egerton et al., 2018; Feng et al., 2019; Samson et al., 2020). In the present study, the vegetative cells and spores of selected strains were tested in GIT conditions. No vegetative cells could tolerate pH 1.0 while the majority of tested cells withstand a low pH above 1.0 and a wide range of bile concentrations up to 5%. Previous studies have reported the ability of some strains of *Bacillus* to survive the stomach and

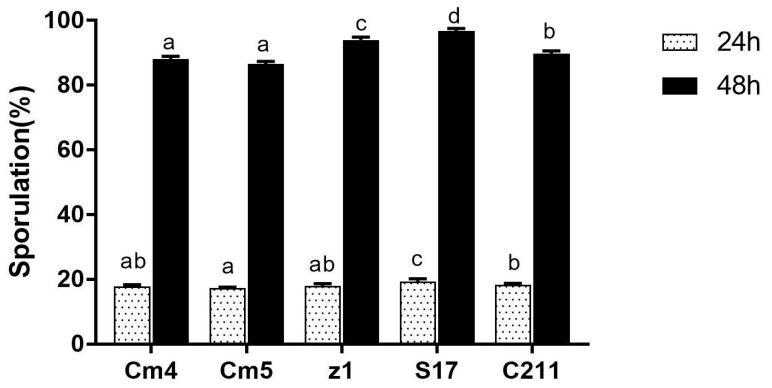

FIGURE 4
Sporulation efficiency of selected strains. The sporulation is expressed as the percentage of spores relative to vegetative cells after 24 h (white bar) and 48 h (black bar). Data are presented as (mean ± SD, n=3) with different superscript letters denoting a significant differences ($p<0.05$).

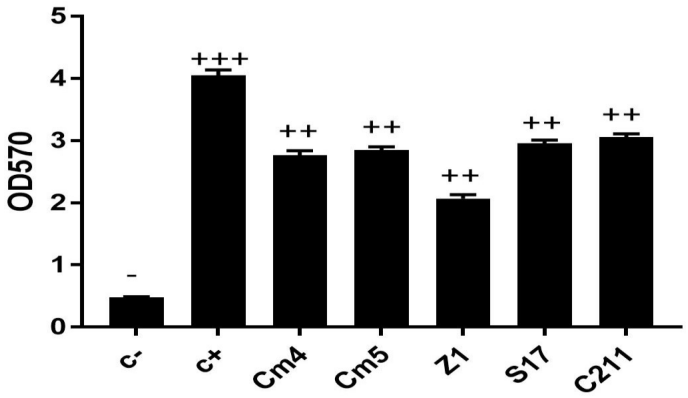

FIGURE 5
Biofilm production of selected strains. The ability of biofilm production is classified as follows: OD570 < 1, non-biofilm (-); 1< OD570 < 2, weak (+); 2< OD570 < 4, medium (+ +) and OD570>4, strong biofilm (+ + +).

TABLE 3 Tolerance of vegetative cells and spores at different pH conditions for 3 h at 28°C (log CFU/mL, SD*).

Viable count (log CFU/ml) Strains	Vegetative cells				spores			
	7.3	1	2	3	7.3	1	2	3
Cm4	9.84 ± 0.01^{c}	ND	6.85 ± 0.02^{c}	6.94 ± 0.01^{c}	7.92 ± 0.01^{b}	7.84 ± 0.01^{b}	7.84 ± 0.01^{b}	7.86 ± 0.03^{b}
Cm5	9.46 ± 0.01^{b}	ND	5.65 ± 0.03^{b}	5.61 ± 0.01^{a}	7.47 ± 0.01^{a}	7.29 ± 0^{a}	7.36 ± 0.02^{a}	7.41 ± 0.05^{a}
Z1	8.78 ± 0^{a}	ND	5.32 ± 0.02^{a}	5.69 ± 0.09^{a}	8.5 ± 0.02^{c}	8.31 ± 0.05^{c}	8.38 ± 0.03^{c}	8.41 ± 0.05^{c}
S17	9.63 ± 0.07^{b}	ND	8.46 ± 0.01^{d}	8.85 ± 0.01^{d}	8.56 ± 0.08^{c}	8.53 ± 0.05^{d}	8.55 ± 0.05^{d}	8.55 ± 0.05^{d}
C211	9.89 ± 0.01^{c}	ND	5.51 ± 0.03^{b}	6.13 ± 0.13^{b}	8.42 ± 0.02^{c}	8.28 ± 0^{c}	8.3 ± 0^{c}	8.32 ± 0.02^{c}

**Bacterial levels were determined by plate count on TSB. Data are presented as (mean ± SD, n = 3) with different superscript letters denoting a significant difference ($p< 0.05$). ND, not detected.*

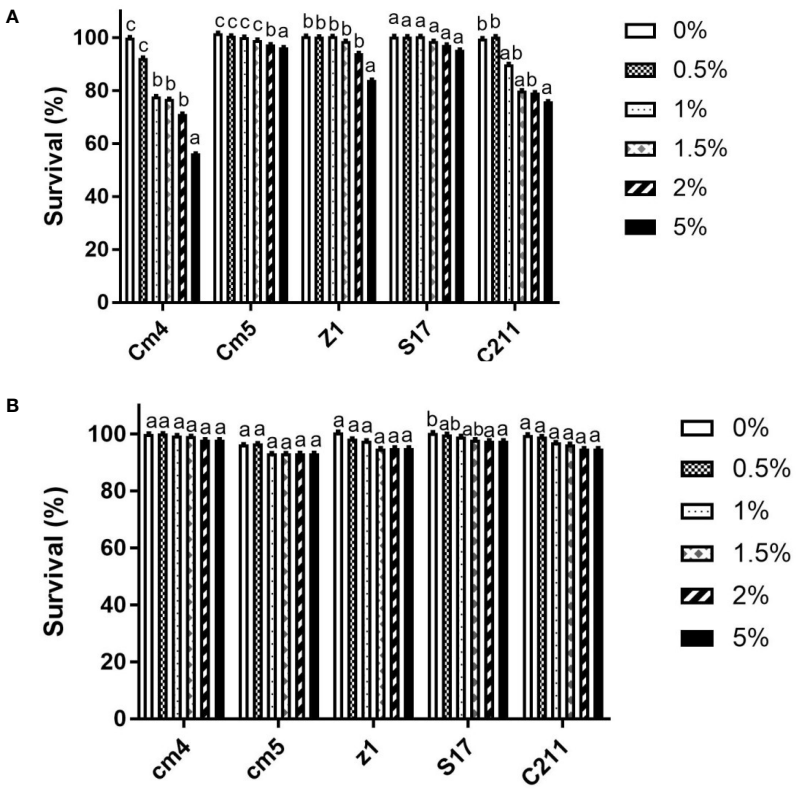

FIGURE 6
Tolerance of vegetative cells **(A)** and spores **(B)** at different bile concentrations for 3h at 28°C (log CFU/mL, SD*). Data are presented as (mean ± SD, n=3) with different superscript letters denoting a significant differences ($p<0.05$).

intestinal environment where they could work effectively (Shinde et al., 2019; Medina et al., 2020), whereas a small reduction of spore count of tested strains was observed and could be due to their germination (Barbosa et al., 2005; Sahoo et al., 2015). This finding suggests that those can be used as probiotic as they could tolerate the harsh gastric acid and toxic bile.

Determination of bacterial cell surface properties, i.e., hydrophobicity, auto-aggregation, and co-aggregation, is an indirect method for the adhesion ability of probiotic strains. Further, aggregating probiotic bacteria may help not only adhesion and colonization of the GIT but also the modulation of the immune system (Balakrishna, 2013; Dutta et al., 2018; Kaktcham et al., 2018; Khan et al., 2021). In the current study, all tested strains showed auto-aggregation ability ranging from 4% to 92% after 24 h of incubation. Among them, S17 and C211 exhibited high auto-aggregation ability (92%; 90%, respectively), in accordance with previous findings (Meidong et al., 2017; Khan et al., 2021; Amoah et al., 2021). Moreover, a co-aggregation assay was developed to quantify

TABLE 4 Auto-aggregation of the isolate strains after 24 h.

Strains	Auto-aggregation					
	1 h	2 h	3 h	4 h	5 h	24 h
Cm4	4.02 ± 0.18^{a}	41.77 ± 2.03^{b}	45.86 ± 2.16^{a}	53.68 ± 2.45^{a}	58.54 ± 2.65^{a}	60.52 ± 2.76^{a}
Cm5	11.4 ± 0.51^{b}	19.81 ± 0.89^{a}	47.04 ± 2.27^{a}	78.95 ± 3.82^{b}	79.48 ± 3.76^{b}	88.74 ± 4.4^{b}
Z1	17.21 ± 0.72^{c}	34.04 ± 1.57^{b}	42.98 ± 2.09^{a}	54.95 ± 2.49^{a}	77.46 ± 3.68^{b}	83.93 ± 4.08^{b}
S17	21.43 ± 0.65^{c}	36.8 ± 1.67^{b}	44.33 ± 1.97^{a}	47.04 ± 2.19^{a}	77.63 ± 3.51^{b}	91.94 ± 4.21^{b}
C211	28.79 ± 1.31^{d}	37.43 ± 1.69^{b}	41.48 ± 1.69^{a}	81.01 ± 3.87^{b}	87.37 ± 4.13^{b}	90.14 ± 4.36^{b}

Data are presented as (mean ± SD, n = 3) with different superscript letters denoting a significant difference ($p< 0.05$).

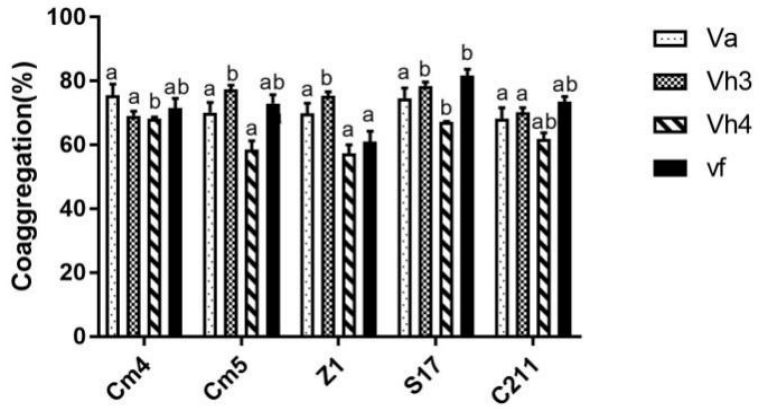

FIGURE 7
Co-aggregation percentages of selected strains with pathogens after 6h.Va, *Vibrio anguillarum* CECT4344; Vh3, *V. harveyi* Lg 26/01; Vh4, *V. harveyi* Lg 48/01; Vf, *V. fischerie*. Data are presented as mean ± SD (n=3) with different superscript letters denoting a significant differences ($p<0.05$).

inter-bacterial adherence (Dutta et al., 2018; Rungsirivanich et al., 2020). Co-aggregation capacity is considered as a way to exclude pathogens from the host. According to different investigations, co-aggregation ability can increase the competition of an epithelial cell's receptor and may protect GIT against undesired microorganisms (Balakrishna, 2013; Dutta et al., 2018; Kaktcham et al., 2018; Meidong et al., 2018; Rungsirivanich et al., 2020). In the present study, all strains tested were able to co-aggregate with pathogens, showing inhibition percentages above 55%. The result also points out that strain S17 showed the highest percentage of co-aggregation against *Vibrio fischeri* (81%), *V. harveyi* Lg48/01 (79%), and *V. harveyi* Lg26/01 (67%) after 6 h of incubation. Dutta **et al.** found that *B. subtilis* LR3H1A and *B. tequilensis* LR3F3P showed a strong co-aggregation with several pathogens (Khan et al., 2021).

The adhesion of probiotic strains to mucus is required and is considered as an important criterion for probiotic selection. Adherence to GIT is the first step in probiotic strains for colonization, stimulation of the immune system, and antagonistic activity. Upon ingestion in GIT, probiotic strains have to attach to the border of microvilli or to the mucus to prevent the colon sweep. Cell adhesion is a complex process which involves contact between the membrane of bacterial cell and surface (Mbozo et al., 2017; Dutta et al., 2018; Rungsirivanich et al., 2020; Gutiérrez Falcón et al., 2021). In fact, many factors including electrostatic interactions, hydrophobicity, steric forces, components of the surface, pH of the milieu, and viscosity are involved (Balcázar et al., 2008; Muñoz-Atienza et al., 2014; Chauhan and Singh, 2019). In this study, all tested strains (vegetative cells and spores) were highly capable of binding to the intestinal mucus but less to skin mucus. This higher adhesion capacity may be due to the presence of specific receptors on the intestinal mucus or may be related to mucus composition (Balcázar et al., 2008). However, all tested *Bacillus* adhered less to polystyrene and BSA compared to intestinal mucus. This binding may involve specific interactions. Similar results were reported by Balcázar et al. and Muñoz-Atienza et al. (Balcázar et al., 2008; Muñoz-Atienza et al., 2014). Once probiotic strains adhered, the next step involved their mucus growth in order to establish in the

TABLE 5 Adhesion of selected strains to fish mucus.

Strains	% adhesion			
	SM	IM	BSA	Polystyrene
Cm4	13.88 ± 0.53^{b}	58.33 ± 1.19^{b}	7.32 ± 0.18^{b}	29.89 ± 0.35^{a}
Cm5	17.04 ± 0.81^{c}	53.25 ± 2.22^{b}	19.55 ± 0.28^{c}	45.34 ± 2.2^{c}
z1	15.4 ± 0.44^{bc}	44.74 ± 1.15^{a}	4.71 ± 0.18^{a}	31.21 ± 1.2^{a}
S17	29.41 ± 0.59^{d}	55.56 ± 2.6^{b}	36.58 ± 0.4^{d}	40.74 ± 0.87^{b}
C211	10.58 ± 0.37^{a}	56.2 ± 1.03^{b}	16.56 ± 0.63^{c}	50.05 ± 0.51^{c}

Data are presented as mean ± SD (n = 3) with different superscript letters denoting a significant difference ($p< 0.05$). SM, skin mucus; IM, intestinal mucus.

TABLE 6 Identification of selected spore formers.

Isolate	Top best matched species	MALDI-TOF MS	Score value
Cm4	*Bacillus subtilis*	*Bacillus subtilis*	1.78
Cm5	*Bacillus subtilis*	*Bacillus subtilis*	1.8
Z1	*Bacillus amyloliquefaciens*	*Bacillus amyloliquefaciens*	1.84
S17	*Bacillus subtilis*	*Bacillus subtilis*	2.10
C211	*Bacillus subtilis*	*Bacillus subtilis*	1.96

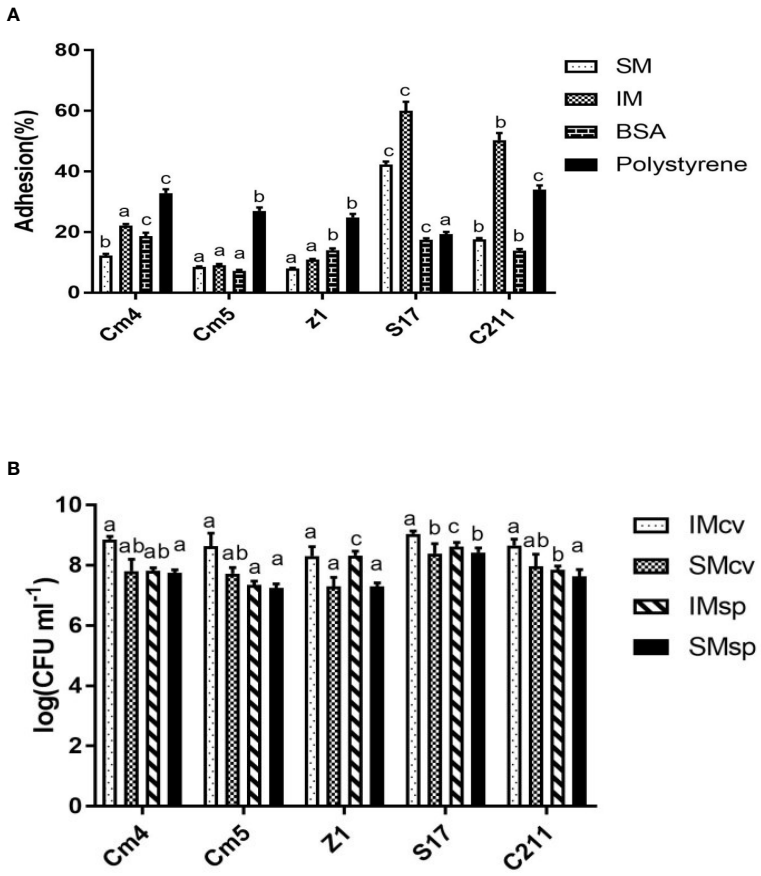

FIGURE 8
(A) Adhesions of spores to fish mucus, BSA and polystyrene and **(B)** Growth of selected strains on intestinal and skin mucus of *Sparus aurata*. Data are presented as mean ± SD (n=3) with different superscript letters denoting a significant differences ($p<0.05$). * IM, intestinal mucus; SM, skin mucus; cv, vegetative cells; sp, spores.

intestine of marine animals (Mbozo et al., 2017). In the present investigation, all vegetative cells and spores of tested strains grew well in the skin and intestine mucus of *S. aurata*, which indicates that our probiotic strains will be able to bind, establish, and remain in the intestine.

Safety assessment is an important precondition for probiotic evaluation. Before the use of probiotics, it is mandatory to confirm that no pathogenic and harmful effects may occur in marine hosts (Verschuere et al., 2000; Medina et al., 2020; Pinto et al., 2020). In the current investigation, all selected stains were found to be susceptible to tested antibiotics demanded by EFSA. This result may ensure the inability and scarcity to transfer antibiotic resistance genes to gut bacteria, thus assuring their safety for aquaculture application (Gutiérrez Falcón et al., 2021). Regarding hydrolic enzymes and virulence factors, S17 did not show haemolytic activity (γ-haemolytic), gelatinase production, and bile salt deconjugation. In addition, S17 appeared to be free of studied virulence factors. Our results are in accordance with the previous study (Hsieh et al., 2004; Shinde et al., 2019). With this regard, candidate probiotic S17 may be considered as a safe strain.

5 Conclusions

In the present study, 108 spore-former strains were isolated from sardine and shrimp intestine. Among all tested strains, only *B. subtilis* S17 possesses suitable probiotic potential for Vibriosis control based on antimicrobial activity, heat and GIT tolerances, extracellular enzyme production, adhesion ability, and safety assessment. Afterward, *in vivo* evaluation studies are required to determine its real-life applications.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, MZ378829.

Author contributions

MJ, IA and MamBA designed the study. MJ conducted the study, analysed the data, and wrote the manuscript. AH, WB, and ManBA participated in its organization and helped to draft the manuscript. MJ and MamBA revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.884244/full#supplementary-material

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