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# Evaluation of Bacillus velezensis SM6 isolated from Solea solea: A score-heat map based approach for the selection of probiotic feed additives

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# Abstract

Recently, probiotics have emerged as a promising alternative to antibiotics and chemotherapeutics for protecting fish from opportunistic and pathogenic bacteria while promoting microbial balance. In the present study, the screening of probiotic potential was based on a scoring approach combined with heatmap analysis from the microbiota associated with common sole (*Solea solea*). A total of 87 bacterial strains were isolated from the skin mucus and intestine. Among them, eight strains exhibited strong antibacterial activity (100%) against potent pathogens and subjected to evaluation that combined functional properties (antimicrobial activity, enzymatic profile, and adhesion capacity) with critical safety criteria (hemolytic activity, antibiotic susceptibility, and the presence of virulence genes). Through this score-heatmap analysis, the strain *Bacillus velezensis* SM6 emerged as the superior candidate, achieving the highest rating due to its broad-spectrum inhibition against key pathogens and remarkable adhesion capacity, with 78.28% hydrophobicity and 92% auto-aggregation. Molecular analyses confirmed the absence of enterotoxins and virulence genes, while *in vivo* assay in European seabass (*Dicentrarchus labrax*) validated its total safety, with no recorded mortality or clinical signs post-injection. Our results demonstrate that *Bacillus velezensis* SM6, selected through this multidimensional framework, represents a highly promising probiotic candidate to enhance sustainability in the aquaculture.


# 1 Introduction

Aquaculture has emerged as a vital food production sector to meet rising global demand amid the decline of natural fish stocks [1, 2], providing significant economic benefits over the last six decades [3, 4]. However, modern intensive systems often subject fish to harmful environmental conditions that enhance disease outbreaks [3, 5, 6]. These outbreaks are primarily caused by a wide range of pathogenic bacteria [7, 8] .These pathogens affect both wild and farmed fish globally, leading to significant economic losses due to reduction of growth performance, weakened immunity and elevated mortality rates [7, 9]. To prevent or minimize disease-related losses in aquaculture, the use of chemotherapeutics, antibiotics, and vaccines has become common practice. Nevertheless, the widespread use of antibiotics leads to the appearance and emergence of resistant bacteria and affect the aquatic environment [10]. This residues can foster the development of antibiotic-resistant bacteria, a major concern for both environmental and human health [11]. Additionally, antibiotics can negatively impact beneficial microorganisms within the organism's microbiota, leading to imbalances that affect fish nutrition and immunity [7, 9]. This disturbance can not only impair the host's health but also contribute to the spread of resistance genes [12]. Recently, probiotics have emerged as a sustainable alternative to ban or minimize the antibiotic use and control diseases [11, 13]. Their applications extend beyond boosting fish health [14]. Probiotics improve not only digestion and growth performance through producing enzymes [14] but also enhance immune response[4]. Additionally, some probiotics may contribute to improvement of water quality as well as produce inhibitory compounds which confer resistance to intestinal pathogens and prevent their intestinal adhesion (Gao et al., 2024; Monzón-Atienza et al., 2023).

A wide variety of bacterial species are classified as probiotics, with *Lactobacillus* and *Bacillus* being the most commonly used in aquaculture. *Bacillus* species, however, have garnered more attention due to their ability to produce non-toxic and non-pathogenic compounds. In addition, their capacity for sporulation provides resistance to stressful conditions, such as heat and passage through the gastrointestinal tract (GIT), compared to other probiotics [11, 15].

Additionally, effective Bacillus probiotics should be tested for their ability to inhibit targeted pathogens or protect host organisms during pathogen exposure. To communicate with each other and with their surrounding environment, pathogens use a signaling mechanism known as quorum sensing. This process regulates virulence, biofilm formation and the production of secondary metabolites. *Bacillus spp.* display the ability to disrupts quorum sensing as well as emerged as a promising strategy for bacterial control,thereby limiting pathogenic activity [16, 17].

The aim of this study was to isolate, select and to identify spore-forming strains from the skin mucus and intestine of sole (*Solea solea*), underexplored source of novel microorganisms. Screening of promising probiotic has generally been based either on subtractive methods, which discard strains without considering their overall probiotic potential across all required criteria [10, 18–20], or on unsupervised clustering approaches. In fact, several studies have employed unsupervised clustering methods, such as principal component analysis (PCA), heat-map visualization, and cluster analysis, as major and direct tools for identifying potential probiotic strains [21, 22]. However, these approaches primarily rely on functional parameters, including antibacterial activity, tolerance to pH and bile salts, and adhesion capacity, which are typically expressed as quantitative values [21, 23, 24]. Nonetheless, safety-related criteria are insufficiently considered. To overcome this limitation and enable a comprehensive evaluation of both functional and safety aspects, we invistigated the screening and selection of potential strain using a multi-parameter scoring approach followed by heat-map analysis. Scores were assigned to each parametre, including both functional performance and safety parameters, based on experimental results. Heat-map and cluster analyses were used to enable objective ranking of strains according to their probiotic potential [25]. In this study, the criteria used are antibacterial activity, enzyme production, antibiofilm and anti–quorum sensing activities, pH and bile salt tolerance, aggregation, adhesion, haemolytic activity, antibiotic susceptibility, and the detection of virulence and toxin genes.

## 2 Results

# 2.1 Isolation of strains and screening of antimicrobial activity

A total of 87 colonies were isolated and purified from mucus and intestine of wild-caught Sole (*Solea solea*). Preliminary screening antimicrobial activity was performed using the colony overlay method against *Vibrio anguillarum* (CECT4344), *Vibrio harveyi* (Lg26/01), *Aeromonas hydrophila (*LMG 5734), *Lactococcus garvieae* (CECT 5807), and *Photobacterium damselae* subsp. *damselae* (Lg41/00) (Fig. 1a-c). Out of 87 isolates, only 8 showed antagonistic activities against all tested pathogens (100% activity).

Similarly, 15 isolates showed 87% activity, 20 strains showed 75% activity, another 7 strains showed 50% activity and13 strains showed low inhibition (25% activity). In contrast, 24 isolates didn't inhibit (0% activity) any tested pathogens. Strong pathogen-inhibitory isolates (IM6, IM7, SM4, SM6 I11, I13, I21 and I24) were selected for further antibacterial activity against additional fish pathogens and for various probiotic potentials.

Antibacterial activity of CFS and its nature was shown in Fig. 1d-e. All CFS of selected strains were able to inhibit pathogenic bacteria. Based on IE values, the strain SM6 showed a strong antagonistic activity against all tested pathogens. Moreover, antimicrobial activity remained stable after pH adjustment but was partially or completely lost following heat and proteinase K treatment. Selected isolates exhibited variable antagonistic activity against additional fish pathogens (Supplementary Table S1). SM6 and IM7 showed the highest overall activity, each with a total score of 9, indicating broad-spectrum inhibition. Moderate activity was observed for I11 and I24, while SM4 and I13 showed intermediate inhibition. The lowest inhibition was recorded for IM6 and I21, confirming strain-dependent differences in antibacterial potential.

## 2.2 Morphological and biochemical characterization

Selected strains were characterised based on morphology and biochemical analysis (Table 1). All isolates were Gram-positive, endospore-forming, and catalase- and oxidase-positive. All strains were able to grow at 2% and 4% NaCl, whereas only SM4, SM6, IM6, IM7, and I11 were able to grow at higher salinity levels (≥ 6% NaCl). Sporulation efficiency varied among the selected strains, with spore yields ranging from $10^8$ to $10^9$ spores/mL. After 48 h, SM4 and I24 exhibited the highest sporulation, while IM7 and I2 showed sporulation efficiencies below 90%.

Table 1
morphological and biochemical characterisation of selected strains

| Characteristics | Bacterial Isolates | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **SM4** | **SM6** | **IM6** | **IM7** | **I11** | **I13** | **I21** | **I24** |
| Gram Stain | + | + | + | + | + | + | + | + |
| Sporulation efficiency (%) | 100 | 94 | 97 | 78 | 97 | 94 | 82 | 100 |
| | **Morphology** | | | | | | | |
| Shape | bacilli | | | | | | | |
| Colour | white | | | | | | | |
| Elevation | convex | convex | flat | flat | convex | convex | flat | convex |
| Forms | circular | | | | | | | |
| Catalase | + | + | + | + | + | + | + | + |
| Oxidase | + | + | + | + | + | + | + | + |
| | **Salinity (NaCl)** | | | | | | | |
| 2% | + | + | + | + | + | + | + | + |
| 4% | + | + | + | + | + | + | + | + |
| 6% | + | + | + | + | + | - | - | - |
| 8% | + | + | + | + | + | - | - | - |
| 10% | + | + | + | + | + | - | - | - |

# 2.3 Quorum-Quenching assays

For the agar assay, only SM4 and SM6 among all tested strains exhibited quorum-sensing inhibition through culture and pellet preparations, as demonstrated by the reduction in violacein diameters (5-11mm), while no inhibition was shown at CFS, inactivated CFS and inactivated pellet. SM6 was subsequently selected for further AHL degradation analysis using a liquid assay. The SM6 pellet and CFS exhibited degradation activity against both short-chain (C6-AHL) and long-chain (C12-AHL) molecules, as determined by the percentage of extracted violacein in the presence or absence of the selected strain SM6. AHL degradation was higher in the 48 h CFS for both short- and long-chain AHLs (Fig. 2)

# 2.4 Anti-biofilm activity

- For *Vibrio anguillarum*, all selected strains exhibited inhibitory effects against pre-forming biofilms after 24–48 h, with inhibition percentages ranging from 13% to 76%, while showing lower inhibition (≤ 55%) against established biofilms (Fig. 3a). The strongest biofilm-inhibitory strains were I21 and

I24, whereas IM7 showed the lowest activity.For *Vibrio harveyi*, all tested strains inhibited both pre-forming and established biofilms, with inhibition percentages exceeding 60% after 24–48 h (Fig. 3a).

- Bacterial motility was reduced in the presence of CFS of all tested strains compared to the control after 24–48 h. This reduction was significantly lower at 48 h than at 24 h for both pathogens. SM6 caused the highest reduction in motility in *V. anguillarum*, while I11 showed the strongest reduction in *V. harveyi* (Fig. 3b).In addition, SM6 exhibited AHL-degrading activity against both tested pathogens at 24 and 48 h.

# 2.5 Evaluation of extracellular enzymes

Extracellular enzyme production is wide-ranging among selected strains. Among tested strains, only 4 strains (SM4,SM6, IM6 and I24) exhibited amylase, lipase and protease activities (Table 2). All the remaining strains showed only lipase and protease activities.

Table 2
Extracellular enzymatic profile of selected strains

| Strains | Amylase | Lipase | Protease |
|---|---|---|---|
| SM4 | + | + | + |
| SM6 | ++ | + | ++ |
| IM6 | ++ | + | ++ |
| IM7 | - | + | - |
| I11 | - | ++ | + |
| I13 | - | ++ | + |
| I21 | - | + | + |
| I24 | + | + | + |

Zone of enzyme production was presented as, + indicate 1–10 mm zone of enzyme activity, ++ indicate 10–20 mm zone enzyme activity, – indicate no zone of enzyme activity. All results were assayed at three replications.

# 2.6 Tolerance to pH, bile salts and gastrointestinal conditions

For vegetative cells (vg), all selected strains remained viable (above 75%) after 4h of exposure to acidic conditions (pH 2.0 and pH 3.0) (Fig. 4a). No significant differences were observed between these conditions and control (pH.7) for all tested strains except SM4,IM6,IM7 and I24. Among all tested strains, SM6 showed the highest resistance to acid condition, whereas IM6 exhibited the lowest acid tolerance. Noteworthy, all spores could tolerate exposure to pH 2.0 and pH 3.0 ( viability above 80%) and keep up viable comparing to vg in the same condition (Fig. 4a).

Selected strains showed promising results after exposure to gastrointestinal stress. In the presence of simulated gastric fluid (SGF) or simulated intestinal fluid (SIF), high viability (above 80%) was observed in all vegetative cells after 4 h of exposure, with no significant differences compared to the control, except for SM4, IM6, and IM7. Among the selected strains, IM6 was the most sensitive to both SGF and SIF, showing a 21% reduction in viability after 4 h (Fig. 4b). In contrast, spores of all selected strains remained highly viable (above 85%) after 4 h of exposure to SGF and SIF (Fig. 4b), exhibiting greater resistance than their corresponding vegetative cells.

All selected strains (vegetative cells and spores) withstood 10% of fish bile after 6h. For the majority of strains, no significant differences were observed between bile-treated samples and the control (0% bile). Even in strains showing a reduction in viability, the decrease did not exceed 15%, and viability remained above 85% (Fig. 5a). Among the tested strains, IM6 showed the lowest tolerance. Notably, spores of all tested strains demonstrated significantly higher tolerance to fish bile after 6 h of exposure compared to their vegetative cells.In addition, the selected strains exhibited tolerance to 0.3% (w/v) Oxgall after 8 h of incubation (Fig. 5b). The coefficient of inhibition (Cinh) ranged from 0.1 to 0.6, with most strains classified as moderately tolerant, while I13 was sensitive and strains I11 and I21 were classified as highly tolerant. Out of all tested strains, only SM6 exhibited hydrolytic activity of bile salt.

# 2.7 Auto-aggregation and co-aggregation assays

As shown in Fig. 6a-b, auto-aggregation (vegetative cells and spores) increased with incubation period and varied from 3% to 92% for vg and from 3% to 67% for sp respectively. All selected strains (vg; sp) revealed low auto-aggregation ability (< 40%) after 4h, followed by a marked increase at 24 h. Vegetative cells showed higher aggregation than spores, with SM6 displaying the strongest activity (92% and 67% for vg and sp, respectively).

In addition, all strains also demonstrated significant co-aggregation, exceeding 55% for vg and 30% for sp (Fig. 6c-d). Among them, SM6 showed the highest percentage of co-aggregation against *Aeromonas hydrophila LMG* 5734 (86%), *Lactococus garvieae* CECT 5807 (96%) and *Vibrio anguillarum* CECT4344 (97%) after 6h of incubation. SM6 also presented a strong ability to co-aggregate against *V. harveyi* Lg26/01with more than 80%, followed by SM4. Spores also exhibited strong co-aggregation, particularly against *L. garvieae*, *V. anguillarum*, and *V. harveyi* (> 75%).

# 2.8 Hydrophobicity

Cell surface hydrophobicity (Toluene, ethyl acetate and chloroform) varied from 10% to 78% for tested strains (Table 3). Majority of tested strains showed more affinity to ethyl acetate and chloroform than toluene for vegetative cells and spores respectively. Among them, isolates strains SM6 showed the significantly higher hydrophobic percentage (78.3 and 74.2%) for ethyl acetate and chloroform. In addition, strain SM6 showed also the significantly highest percentage (74.2%) for toluene.

Table 3
Hydrophobicity of selected strains

| Strains | Toluene | | Ethyl Acetate | | Chloroform | |
|---|---|---|---|---|---|---|
| | vg | sp | vg | sp | vg | sp |
| IM6 | 18,06 ± 0,73$^{a}$ | 22,58 ± 0,43$^{b}$ | 53,4 ± 1,21$^{c}$ | 48,69 ± 1,96$^{b}$ | 58,71 ± 0,4$^{c}$ | 55,97 ± 0,81$^{c}$ |
| IM7 | 28,89 ± 0,59$^{b}$ | 15,15 ± 0,28$^{a}$ | 22,45 ± 0,43$^{a}$ | 26,5 ± 0,48$^{a}$ | 13,36 ± 0,38$^{a}$ | 13,92 ± 0,31$^{a}$ |
| SM4 | 26 ± 0,97$^{b}$ | 31,88 ± 0,23$^{c}$ | 37,52 ± 0,77$^{b}$ | 58,77 ± 0,25$^{c}$ | 54,71 ± 0,9$^{c}$ | 68,27 ± 0,85$^{d}$ |
| SM6 | 41,57 ± 1,27$^{d}$ | 22,88 ± 0,27$^{b}$ | 78,28 ± 0,38$^{d}$ | 27,75 ± 1,24$^{a}$ | 74,22 ± 1,02$^{d}$ | 13,66 ± 0,23$^{a}$ |
| I11 | 13,8 ± 0,44$^{a}$ | 29,07 ± 0,29$^{c}$ | 48,33 ± 0,79$^{c}$ | 50,96 ± 1,85$^{b}$ | 35,73 ± 0,29$^{b}$ | 40,97 ± 2,05$^{b}$ |
| I13 | 40,9 ± 0,21$^{d}$ | 43,23 ± 0,31$^{d}$ | 77,16 ± 2,86$^{d}$ | 25,95 ± 0,82$^{a}$ | 11,2 ± 0,4$^{a}$ | 14,6 ± 0,65$^{a}$ |
| I21 | 16,08 ± 0,8$^{a}$ | 19,24 ± 0,37$^{a}$ | 39,48 ± 0,68$^{b}$ | 46,72 ± 0,88$^{b}$ | 23,75 ± 1,15$^{ab}$ | 35,89 ± 0,88$^{b}$ |
| I24 | 32,58 ± 0,43$^{bc}$ | 39,32 ± 0,38$^{d}$ | 31,28 ± 1,13$^{ab}$ | 33,22 ± 1,18$^{ab}$ | 10,05 ± 0,45$^{a}$ | 12,4 ± 0,62$^{a}$ |

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

# 2.9 Adhesion assay

The adhesion ability of the tested strains (vegetative cells and spores) to mucus is shown in Fig. 7a and supplementary Figure S1. All strains demonstrated strong adhesion to the intestinal mucus of the sole, with adhesion rates ranging from 7% to 62%. The strains also adhered well to the skin mucus of Mullet (33% to 65%). However, the adhesion to the skin mucus of the sole was lower compared to the intestinal mucus of the same fish. All tested strains exhibited a higher adhesion ability to the skin mucus of Mullet than to the skin mucus of the sole. SM6 showed the highest adhesion ability to both the skin and intestinal mucus of both fish species, with mucus adhesion exceeding 60% for all mucus types, respectively. In addition, adhesion to BSA (36–70%) and polystyrene (38–60%) was lower than mucus

# 2.10 Safety assay *In vitro*

PCR screening for biogenic amine- and toxin-encoding genes revealed that only strains SM4 and SM6 lacked all investigated virulence genes (hdc, tdc, odc, ldc, sfp, cesA, cesB, nheA, nheB, nheC, hblA, hblC, hblD, and entFM).

In contrast, strains I21 and I24 carried 80% and 53.3% of the tested genes, whereas I11and I13 carried 26.7% and 20%, respectively.IM6 and IM7 showed the lowest occurrence (6.7%).

Haemolysis assays revealed that all strains were α- or β-haemolytic, except SM6, which was γ-haemolytic (Fig. 8b). Gelatinase production was detected only in I13 (Supplementary Table S4).

Antibiotic susceptibility testing showed that all strains were susceptible to ampicillin, cefoxitin, nalidixic acid, streptomycin, tetracycline, and vancomycin. Five isolates (62.5%) were resistant to at least one antibiotic, most frequently chloramphenicol (29%), fosfomycin (24%), cefotaxime (18%), kanamycin (12%), clindamycin, erythromycin, and gentamicin (6% each) (Fig. 8c). Notably, strains SM6, SM4, and IM6 were sensitive to all tested antibiotics (Fig. 8c, Supplementary Table S2).

# 2.11 Biosafety assay

No clinical signs, such as swelling, bleeding, lesions, or loss of scales and mucus, were observed in either the injected or control groups. Among all selected strains, only SM6 showed no mortality during the 10-day monitoring period, whereas the remaining strains resulted in some level of mortality.

# 2.12 Selection of potential probiotic

Functional potential was further evaluated using a multi-parameter scoring system combined with hierarchical clustering. Strain SM6 emerged as the strongest candidate, achieving the highest overall score due to its potent antimicrobial activity. In contrast, I24 and I11, despite demonstrating some beneficial functions, showed negative safety scores, indicating potential risks such as virulence or hemolysis.

The heat map analysis was conducted to identify the most suitable probiotic isolates. As shown in Fig. 10, the eight selected strains formed two main clusters, each subdivided into four subclusters, reflecting differences in functional and safety characteristics. Strains I24, I11, I21, and I13 comprised Cluster I, while IM6, SM4, IM7, and SM6 formed Cluster II. Within the two clusters, traits such as adherence (auto-aggregation and adhesion) and Quorum Quenching were consistently observed, highlighting their importance for competitive strains. SM6 was particularly distinguished by broad-spectrum antibacterial activity, strong antibiofilm capacity, quorum-quenching ability, and a favorable safety assessment. For sensitivity analysis, across all scenarios, including the baseline as well as alternative scenarios based on functional or safety-related criteria, SM6 consistently maintained the highest total score (Supplementary Figure S2). In addition, the choice of criteria was based on PCA.

# 2.13 Identification of selected strain

Selected strain SM6 was identified as *Bacillus velezensis*, with MALDI-TOF mass spectrometry providing a score of 1.96. This identification was further supported by 16S rRNA gene sequencing consistent with previous studies. The sequence has been deposited in GenBank under accession number PZ518674.

# 3 Discussion

Probiotics have gained attention as sustainable strategy for controlling fish pathogens [2, 26]. Terrestrial probiotics often show limited efficacy due to poor survival in aquatic hosts and distinct environmental conditions shaping microbial communities [18]. Therefore, the gastrointestinal microbiota of aquatic animals is now considered a more suitable source for isolating probiotic candidates[18, 27]. Probiotics taking from the same host or shared ecological niches with pathogens may exhibit better efficacy [1, 28].

In the present study, 87 spore-forming strains were isolated from mucus and intestine of Sole (*Solea solea*) and evaluated for their probiotic potential and safety in order to identify promising candidates for aquaculture applications. Among them, eight strains displayed complete inhibitory activity against all tested pathogens. Similarly, some *Bacillus* have been reported to inhibit *Vibrio* species including *B. velezensis* STPB10 against *V. anguillarum* [26] and and *B. subtilis* FM2 against *A. veronii*, *A. salmonicida*, *A. sobria*, *Pseudomonas fluorescens*, *P. putida* and *Salmonella Typhimurium* [29]. Selected strains were further evaluated against a panel of pathogens relevant to aquaculture. All tested strains inhibited *Aeromonas salmonicida* CECT 894, *Vibrio alginolyticus* CECT 511, *Vibrio vulnificus* CECT 529 and *Vibrio parahaemolyticus* CECT 5111. SM6 and IM7 showed the highest overall activity (score = 9), indicating broad-spectrum inhibition probiotic potential. For extracellular inhibitory activity, CFS lost antagonistic activity partially or completely after heat or proteinase K treatment, indicating that the active compounds are predominantly proteinaceous and temperature-sensitive. These antibacterial effects likely associated with production of antimicrobial metabolites such as organic acids, hydrogen peroxide, and bacteriocin-like compounds [16, 28, 30]. Furthermore, these antimicrobial factors not only suppress pathogen growth but also enhance host resistance to infectious agents [4, 27].

*Bacillus spp.* Are recognized for their high viability due to spore formation. Their spores enable survival under harsh conditions (heat, UV, acidity, freezing and drought) [15]. In this study, selected strains exhibited sporulation efficiencies exceeding 90% after 48 h incubation, with differences reflecting strain-specific regulatory pathways [15, 31].

Quorum quenching disrupts of pathogen signalling pathways involved in virulence, biofilm formation, and secondary metabolite production [32, 33]. *Bacillus spp.* can produce AHL-degrading enzymes, including lactonases and acylases [32, 33]. In this study, only SM4 and SM6 inhibited QS in culture and pellet suspensions. To investigate QQ mechanism, AHL degradation was examined using short-chain (C6) and long-chain (C12) molecules [34]. In microbial communities, AHL-producing and AHL-degrading bacteria often coexist, competing for ecological advantage [32–34]. Both pellet and CFS of SM6 degraded C6- and C12-AHLs, with activity increasing over 48 h, indicating time-dependent accumulation of QQ enzymes, consistent with Zhang, et al. [35], who reported that the QQ activity of *Pseudomonas nitroreducens* W-7 increased over time due AHL-degrading enzymes accumulation in agreement with [36]. SM6 exhibited quorum quenching in plate assays via both pellet and culture, while in plaque assays, both pellet and CFS degraded AHLs. Differences reflect methodological sensitivity: agar assays detect

strong extracellular activity, whereas 96-well microtiter assays measure total AHL degradation, including weakly secreted or cell-associated enzymes[37].

Biofilms play a crucial role in survival and growth by protecting from environmental stresses [4]. *Bacillus* species have been reported to prevent or disrupt biofilm formation by pathogenic bacteria such as Vibrio, Aeromonas, and Pseudomonas [28, 38]. In this study, selected strains more effectively inhibited pre-formed *V. anguillarum* biofilms than established ones. All strains suppressed both pre-formed and mature *V. harveyi* biofilms. Similar studies also reported that *Bacillus subtilis* isolated from the gut of Asian seabass (*Lates calcarifer*) significantly reduced *V. harveyi* biofilm [39].

Probiotics can inhibit pathogen growth and promote fish health. In this study, extracellular enzyme production varied among strains. Four strains (SM4, SM6, IM6, and I24) exhibited amylase, lipase, and protease activities, while the remaining strains showed only lipase and protease activities, in accordance with previous studies [5, 40]. *Bacillus spp.* are known to produce diverse digestive enzymes which enhance nutrient utilization and fish growth [1, 40].

A key requirement for probiotic *Bacillus* strains is the ability to survive gastrointestinal conditions including stomach acidity and bile exposure. Although bile plays an important role in gut defense, it can also disrupt bacterial cell membranes [1, 18, 40].Probiotics are intended to survive and colonize the fish gut and help maintain microbial balance. In this study, vegetative cells and spores were evaluated under simulated gastrointestinal conditions. Most strains maintained high viability (> 75%) at pH 2.0–3.0 while spores showed stronger tolerance, maintaining > 80% viability in agreement with previous studies [30]. A slight reduction may be due to germination [15, 41]. Under simulated gastrointestinal conditions, all strains remained viable (> 80%) with spores exhibiting higher resistance than vegetative cells, in agreement with previous findings [29, 42].

Cell surface properties including auto-aggregation, co-aggregation and hydrophobicity are key indicators of indirect method for probiotic adhesion ability. These traits promote colonization, and contribute to immune modulation [1, 43, 44]. In the present study, auto-aggregation ranged from 3% to 92% for vegetative cells (vg) and 3% to 67% for spores (sp) after 24 h. SM6 showed the highest aggregation in both forms (92% and 67%) in agreement with previous reports [27, 40]. Co-aggregation assays were conducted to evaluate the ability of isolates to interact with pathogenic bacteria [4]. This property may help to limit pathogen colonization [29, 43]. All strains exhibited significant co-aggregation with pathogens (> 55% for vg and > 30% for sp), suggesting potential to limit pathogen colonization by competing for epithelial binding sites. Comparable findings were Chakraborty, et al. [29], who observed high co-aggregation ability of *Bacillus subtilis* FM2(63–52%) against *Aeromonas veronii*, as well as against *Salmonella Typhimurium* and *Escherichia coli*. (Chakraborty et al., 2026). Cell surface hydrophobicity ranged from 10% to 78%, with majority of strains showing greater affinity toward ethyl acetate and chloroform than toluene. SM6 exhibited significantly the highest values for ethyl acetate (78.3%), chloroform (74.2%) and toluene (74.2%), suggesting enhanced adhesion potential, likely linked to electron donor/acceptor properties that promote interaction with epithelial surfaces [20, 45, 46].

Adhesion to the gastrointestinal tract is an important criterion for selecting effective strains, as it enables colonization, immune stimulation, and pathogen exclusion [4, 13, 29, 43]. Cell adhesion involves direct contact between the bacterial cell membrane and the host surface and is influenced by factors such as hydrophobicity, electrostatic interactions, surface components, pH, and environmental viscosity [43, 44]. In this study, all vegetative cells and spores adhered to sole intestinal mucus (7–62%) and mullet skin mucus (33–65%), with lower adhesion to sole skin mucus. SM6 showed the highest adhesion (> 60%) across all mucus types. Adhesion to BSA (36–70%) and polystyrene (38–60%) was lower, suggesting specific mucus interactions. These results aligned with previous studies [16, 44, 47].

Safety assessment is an essential step in evaluating probiotic candidates. Before their application in aquaculture, it is crucial to ensure that the selected strains do not pose any pathogenic or harmful effects to marine hosts [1, 4, 48]. It involves assessment of haemolytic activity, toxin production, and antibiotic susceptibility to confirm the absence of transferable resistance genes that might undermine treatment efficacy in aquaculture or human. In addition, *in vivo* trials in fish and other marine organisms are required also to further confirm host safety [44, 49, 50]. In the present investigation, SM6 did not exhibited show haemolytic activity (γ-haemolytic), gelatinase activity and enterotoxins production. In addition, SM6 was found susceptible to all tested antibiotics, suggesting a low risk of resistance gene and supporting its safety. For biosafety assay, no clinical signs or mortality has been showed no mortality for SM6. This result is in accordance with previous finding[1, 16, 44, 50, 51]. Therefore, SM6 can be considered a safe probiotic candidate.

It is well known that *in vitro* assessment is essential before *in vivo* evaluation to ensure reliable strain selection. Screening probiotic potential strains is a key step for their application in aquaculture [4, 52, 53]. Several studies have used unsupervised clustering approaches, including principal component analysis (PCA), heat-map visualization, and cluster analysis, to divide strains into groups based on their probiotic properties [21, 54]. However, these tools primarily rely on functional parameters, while safety-related criteria are often not adequately integrated [21, 24, 53]. In addition, scoring approach has been employed for various assays in order to rank potential strains for specifics assays [5, 55]. To overcome this limitation, a multi-parameter scoring system combined with hierarchical clustering was applied. In this present study, SM6 emerged as the strongest candidate, achieving the highest overall score and forming a distinct subcluster. It combined effective pathogen inhibition, strong colonization potential, and a favorable safety profile. In contrast, strains I24 and I11showed negative safety scores despite their beneficial functional criteria, indicating potential risks such as virulence or haemolysis. Therefore, SM6 was selected as the most promising probiotic candidate. In order to assess the robustness of score-heatmap approach, sensitivity analysis was performed. Across the alternative scenarios based on functional or safety criteria, SM6 consistently exhibited the highest rank in agreement with previous studies[56–58] .

The promising probiotic strain was identified as *Bacillus velezensis*, with MALDI-TOF mass spectrometry providing a score of 1.9, and the result was further supported by 16S rRNA gene sequencing corroborating previous studies[16].

# 4 Materials and Methods

## 4.1 Indicators and culture conditions

All indicator strains used in this study were kindly provided by Department of Nutrition and Food Science, Complutense University of Madrid, Department of Microbiology, University of Málaga, and Aquaculture Research Group, GIA, ECOAQUA Institute, University of Las Palmas de Gran Canaria. All pathogens employed were recognized as potent causative agents of fish infections. Each strain was cultured on Tryptone Soya Agar (TSA; Oxoid Ltd., Hampshire, UK) supplemented with 1.5% (w/v) NaCl at 28°C (Table 4).

Table 4
Indicators used in the present study

| Abbreviation | Bacterial species | strains | origin/ source |
|---|---|---|---|
| Ah | *Aeromonas hydrophila* | LMG 5734 | L.M.Cintas |
| As | *A. salmonicida* | CECT894 | |
| Lg1 | *Lactococcus garviae* | CECT 5807 | |
| Lg2 | *L. garviae* | JIP29-99 | |
| Va | *Vibrio anguillarum* | CECT4344 | M.A.Morinigo |
| Vh | *V. harveyi* | Lg26/01 | |
| Pd1 | *Photobacterum damselea damselea* | Lg41/00 | |
| Val1 | *V. alginolyticus* | CECT 511 | F.Acosta |
| Val2 | *V. alginolyticus* | Abelon | |
| Vp | *V. parahemolitus* | CECT5111 | |
| Vv | *V. vulnificus* | CECT529 | |
| Pd2 | *P. damselae* subsp. *piscicida* | SABE1 | |

*Chromobacterium violaceum* MK (CECT 494) was employed as a wild-type biosensor for the detection of quorum sensing (QS) inhibitors. *C. violaceum* VIR24 and *C. violaceum* CV026 was used as a biosensor for long-chain AHL and short-chain detection respectively. Both strains were routinely cultured in LB broth at 28°C overnight to ensure optimal growth or violacein production.

## 4.2 Isolation of endospore-forming bacteria

Endospore-forming bacteria were isolated from the intestinal and skin mucus of wild-caught *Solea solea* collected from the Kerkennah Islands, Tunisia.Intestinal mucus was obtained by aseptically removing the intestine and scraping its inner lining, while skin mucus was collected from the dorso-lateral surface

using a sterile scraper. Samples (1 mL) were homogenized in phosphate-buffered saline (PBS, pH 7.2), serially diluted, and subjected to heat treatment at 65°C for 30 minutes to select for spore-forming bacteria. Aliquots were then plated on Difco Sporulation Medium and incubated at 28°C for 48 hours. The resulting isolates were characterized using morphological and biochemical methods. This protocol was performed as previously described by Jlidi, et al. [55]

# 4.3 Antimicrobial activity

- Antimicrobial activity of the isolates was initiallyscreened using the colony overlay method (Husain et al., 2022). Briefly, isolates and indicator strains were cultured overnight in TSB supplemented with 1.5% NaCl at 28°C. TSA plates were swabbed with 100 µL of indicator cultures ($10^5$ CFU/mL), and isolate cultures were spotted onto the surface and incubated at 28°C for 24 h. Inhibition was indicated by clear zones around colonies.

Selected isolates were subsequently evaluated using cell-free supernatants (CFS), obtained by centrifugation of overnight culture at 8,000 × g, 4°C for 20 min folowed by filtration (0.22 µm). Antimicrobial activity was assessed in microtiter plates by mixing TSB, CFS, and pathogen culture, and measuring optical density at 600 nm after 24 h [18]. Inhibition efficiency (IE) was calculated as the ratio of OD in the presence of CFS to the control, where IE < 1 indicates inhibition, IE = 1 no effect, and IE > 1 growth promotion [55].

$$\varvec{IE} = \left(\textbf{OD in presence of isolate CFS}\right) / \left(\textbf{OD of pathogen control}\right)$$

- CFS were neutralized (pH 7), treated with proteinase K (10 mg/mL), and heated at 100°C for 10 min to assess the nature and stability of antimicrobial compounds. Residual antimicrobial activity was evaluated using *Vibrio harveyi* Lg26/01[59] .

# 4.4 Phenotypic characterization of bacterial isolates

Gram staining, oxidase, and catalase tests were performed to characterize isolates [59]. Sporulation efficiency was assessed in DSM broth at 28°C, counting total and heat-resistant spores (80°C, 20 min). Salinity tolerance was tested in TSB with 0–10% NaCl by measuring growth at 600 nm [15, 31].

# 4.5 Quorum quenching assays

# 4.5.1 Anti-quorum sensing potential of selected strains

The quorum sensing (QS) inhibitory activity was conducted according to Monzón-Atienza, et al. [33] with modifications. An overnight culture of *C. violaceum* was mixed with soft LB agar, overlaid onto LB plates, and wells (6 mm) were filled with 50 µL of isolates. After incubation at 26°C for 24 h, QS inhibition was indicated by colorless zones reflecting reduced violacein production without affecting growth. Selected isolates were grown in TSB (1.5% NaCl, 28°C, 24 h, 150 rpm), centrifuged, and supernatants were filtered

(0.22 µm) to obtain CFS, while pellets were resuspended in PBS. Both were heat-inactivated (99°C, 15 min), and quorum-quenching activity was evaluated by well diffusion.

## 4.5.2 AHL-degrading activity of selected strains

AHL-degrading activity was confirmed in liquid cultures by incubating LB broth containing CFS or heat-inactivated pellets with C6- or C12-AHL (10 mg/mL) and the appropriate biomarker *C. violaceum* for 24–48 h. Controls contained only *C. violaceum*. After 24 h, cultures were centrifuged (8,000 × g, 5 min), and the violacein pellet was dissolved in DMSO. Absorbance was measured at 585 nm, and AHL degradation (%) was calculated as:

$$\% \ \mathbf{AHL\ degradation} = (1 - (\mathbf{ODt}/\mathbf{ODc})) * 100$$

Where $OD_c$ represents OD of control (*C. violaceum* without CFS or pellet) and $OD_t$ represents OD of treatment (with pellet or CFS)[60]. Pellets were pre-treated at 60°C for 20 min to inactivate cells while preserving quorum quenching activity [61].

# 4.6 Anti-biofilm activity

## 4.6.1 Anti-adherence assay

Anti-adherence activity was evaluated using a 96-well microtiter plate assay as described by Jardak, et al. [62] and Fahmy, et al. [63] with modifications. *Vibrio anguillarum* CECT 4344 and *V. harveyi* Lg26/01 were grown overnight in TSB at 28°C and adjusted in TSB supplemented with 1% (w/v) glucose to an $OD_{600}$ of approximately 0.1. Each well of a sterile flat-bottom polystyrene microplate was filled with 50 µL of CFS from the selected strains, 100 µL of TSB containing 1% glucose, and 50 µL of the pathogen suspension. Wells containing only the pathogen served as the control. Plates were incubated at 28°C for 24–48 h under static conditions. After incubation, the medium was removed, and wells were washed twice with sterile PBS (pH 7.2) and dried at 60°C for 1 h. Adherent cells were stained with 150 µL of 0.2% (w/v) crystal violet prepared in 20% (v/v) ethanol for 45 min. Excess stain was removed, and wells were rinsed with distilled water. Bound crystal violet was solubilized with 200 µL of 33% (v/v) glacial acetic acid for 1 h at room temperature, and absorbance was measured at 570 nm. Biofilm inhibition (%) was calculated as:

$$\mathbf{Biofilm\ inhibition}\% = (1 - (\mathbf{ODsample}/\mathbf{ODcontrol})) * 100$$

where OD sample represents OD of pathogen with CFS of selected strains.

## 4.6.2 Biofilm eradication assay

Biofilm eradication was assessed according to Jardak, et al. [64] with slight modifications. Briefly, biofilms were established by adding 200 µL of *Vibrio anguillarum* and *Vibrio harveyi* cultures (adjusted as previously described) into sterile 96-well flat-bottom microplates and incubating for 24–48 h at 28°C. Wells were washed with PBS and treated with CFS (200 µL), while PBS served as control, then incubated

overnight. After washing, biofilms were stained with crystal violet and absorbance was measured at 570 nm. Eradication (%) was calculated as:

**Biofilm eradication%= (1- (OD $_{control}$ /OD $_{sample}$ )) *100**

where OD sample represents OD of pathogen with CFS of selected strains.

# 4.6.3 Motility test and visualization of biofilm inhibition

Swarming motility was assessed on LB agar (0.5%) with or without CFS. Plates were incubated at 30°C for 24–48 h, and motility zones were measured [65]. For visualization, *V. anguillarum* biofilms were grown on coverslips in TSB with 1% glucose, washed with PBS, and stained with acridine orange (0.1%) [62].

# 4.7 Screening of Probiotic properties of isolates

# 4.7.1 Evaluation of extracellular enzymes

Extracellular enzyme production was evaluated using a modified plate assay adapted from Gao, et al. [1]. Protease activity was assessed on LB agar supplemented with 1% skimmed milk after incubation at 28°C for 24 h, indicated by clear zones around colonies. Amylase production was tested on LB agar containing 1% starch, with activity revealed by yellow halos after iodine staining [66]. Lipase activity was evaluated on LB agar supplemented with 1% olive oil and Rhodamine, showing fluorescent zones around colonies after incubation [67].

# 4.7.2 Preparation of vegetative cells and spores

Vegetative cells and spores were prepared as previously described by Jlidi, et al. [55]. Briefly, vegetative cells (vg) were harvested during the logarithmic phase in TSB, while spores (sp) were obtained after growth in DSM broth, followed by purification and heat treatment (80°C; 20min).

# 4.7.3 Tolerance to pH, bile salts and gastrointestinal conditions

Tolerance to simulated gastrointestinal conditions was evaluated following Gao et al. (2024) with modifications. Simulated gastric fluid (pH 3) was prepared with HCl and pepsin (1 mg/mL), and intestinal fluid (pH 6.8) with $KH_2PO_4$ and trypsin (10 mg/mL), both filter-sterilized (0.22 µm). Bacterial cultures ($10^8$CFU/mL) were incubated in these media, and viable counts were determined at 0 and 6 h by plating on TSA. Low pH tolerance was assessed as described by Hmani, et al. [19], with cultures exposed to pH 1–3 (control pH 7.3) and viable counts measured after 3 h.

Bile salt tolerance was evaluated using the coefficient of inhibition (Cinh) and tolerance to fish bile juice. Cinh was determined according to Mortezaei, et al. [18], with bacterial suspensions ($10^8$ CFU/mL) inoculated into microplates with or without 0.3% (w/v) Ox-gall. Optical density at 600 nm ($OD_{600}$) was recorded after 8 hours (exponential phase), and Cinh was calculated as

$$\mathbf{Cinh} = 1 - (\mathbf{ODbile} / \mathbf{ODcontrol})$$

where $OD_{control}$ represents the OD of cultures grown in TSB without bile salts and $OD_{bile}$ corresponds to cultures grown in TSB with 0.3% (w/v) Ox-gall. Cinh values were interpreted as follows: >0.4 = sensitive, 0.2–0.4 = moderate, and < 0.2 = resistant.

Colonization ability in fish bile juice was determined by incubating cultures in TSB with or without 10% bile and assessing viable counts after 6 h [18].

## 4.7.4 Auto-aggregation and co-aggregation assays

Auto-aggregation was conducted following Jlidi, et al. [55]. Overnight cultures were centrifuged, washed twice and resuspended in PBS to $OD_{600}$ ≈ 1 ($A_0$). The suspensions were vortexed and incubated undisturbed at 28°C. The $OD_{600}$ of the upper suspension ($A_t$) was measured over time (1–5 and 24 h). Auto-aggregation (%) was calculated as:

$$\%\mathbf{Agg} = (1 - (\mathbf{A}_t / \mathbf{A}_0)) \times 100$$

where $A_0$ represents the initial $OD_{600}$ and $A_t$ is the $OD_{600}$ at time t = 1, 2, 3, 4, 5, and 24 hours.

For the co-aggregation assay, bacterial suspensions of the isolated strains and fish pathogens were prepared similarly to the auto-aggregation method [55]. Equal volumes of the isolated strain and pathogen were mixed and incubated at 28°C for 6 hours. After incubation, the $OD_{600}$ of the mixed suspension ($A_{t,i}$) and the individual controls ($A_{pathogen}$ for the pathogen and $A_i$ for the isolated strain) were measured. The percentage of co-aggregation (%Co-agg) was determined using the formula:

$$\%\mathbf{Co-agg} = [(\mathbf{A}_{pathogen} + \mathbf{A}_{isolated}) - 2\mathbf{A}_{mix}] / (\mathbf{A}_{pathogen} + \mathbf{A}_{isolated}) \times 100$$

## 4.7.5 Hydrophobicity

The surface hydrophobicity of the isolated strains was assessed to determine their adhesion properties to various organic solvents, including chloroform, ethyl acetate and toluene. The assay was performed following Khan, et al. [68]. Overnight cultures were centrifuged, washed twice with PBS, and resuspended in PBS to $OD_{600}$ ≈ 1 ($A_0$). An equal volume of solvent was added, vortexed for 2 minutes, allowed to stand to allow phase separation. The $OD_{600}$ of the aqueous phase was carefully recorded as $A_1$. The percentage of surface hydrophobicity was calculated using the following formula:

$$\%\,\mathbf{Hydrophobicity} = (1 - \mathbf{A}1/\mathbf{A}0) \times 100$$

## 4.7.6 Adhesion *in vitro*

- Mucus preparation and characterization

Mucus was collected from the skin and intestines of Mullet (*Mugil cephalus*), and sole (*Solea solea*), following Guardiola et al. (2014).Skin mucus was obtained by scraping the dorso-lateral surface of the fish while intestinal mucus was collected by scraping the mucosal layer. Samples were homogenized in PBS and centrifuged three times at 120,000 × g for 15 minutes at 4°C. Protein concentration was determined using the Bradford method and adjusted to 1 mg/mL [16, 66]. Mucus preparations were filter-sterilized (0.22 µm) and stored at − 20°C until use.

- Adhesion assay

The adhesion ability was assessed following a modified version described by Yao, et al. [69]. Briefly, intestinal and skin mucus (100 µL) were immobilized in microtiter plates overnight at 4°C and washed with PBS. Overnight cultures (vegetative cells) were centrifuged, washed, and labeled with acridine orange (50 µg/mL) at 28°C for 1 h in the dark, then washed to remove excess dye [70, 71]. Labeled suspensions ($10^8$ CFU/mL) were added to wells and incubated at 28°C for 1 h. After washing, bound vegetative cells were lysed with SDS (1% in 0.1 M NaOH) at 60°C for 1 h, and fluorescence was measured (Ex/Em: 495/520 nm). Adhesion efficiency was calculated using the formula:

$$\% \, \mathbf{Adhesion} \; = \; (\mathbf{A} \; / \; \mathbf{A}_0) \; \times \; 100$$

where A represents the fluorescence of adhering bacteria, and $A_0$ corresponds to the fluorescence of the initial bacterial suspension. To distinguish between specific bacterial adhesion and non-specific interactions due to cell surface hydrophobicity, additional adhesion assays were conducted using bovine serum albumin (BSA) and polystyrene under the same conditions [13]. For spores, adherent cells were detached using 0.1% Triton X-100 in PBS, serially diluted, and plated on DSM for viable counts [72].

# 4.8 Safety assay

# 4.8.1 Antibiotic Resistance

The antibiotic susceptibility of selected strains was determined by the disk diffusion method as described by Husain, et al.[73].The antibiotics used were ampicillin (10 µg), cefotaxime (30 µg), chloramphenicol (30 µg), clindamycin (30 µg), erythromycin (10 µg), fosfomycin (50 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg). 0.1 mL of cultured selected strain ($10^8$CFU/mL) was spread on Muller Hinton agar plate. The disc of each antibiotic was placed on the surface of plate. After incubation for 24h at 28°C, selected strains were classified as resistant (R), Intermediate (I) and sensitive (S), following CLSI 2017 guidelines (Supplementary Table S3).

# 4.8.2 Production of Hydrolic Enzymes

The haemolysis activity was investigated as previously described by Muñoz-Atienza, et al. [74]. Briefly, strains previously cultured in TSB with 1.5%NaCl were streaked on horse blood agar plates (BioMérieux)

and incubated for 24-48h at 28°C. β-, α-, and γ-haemolysis were determined based on clear, green, or absent zones around colonies, respectively. *Enterococcus faecalis* P4 was used as a positive control.

Gelatinase production and bile salt hydrolase were determined as described by Jlidi, et al. [55]. Strains grown in TSB were streaked on Todd-Hewitt agar containing 30 g/L gelatin and incubated at 28°C. Plates were then kept at 4°C for 5 h; turbidity zones around colonies indicated gelatinase activity. *Enterococcus faecalis* P4 was used as positive control. For bile salt hydrolase activity, Cultures (10 µL) were spotted on TSB agar supplemented with 0.5% taurocholate or taurodeoxycholate and 0.05% L-cysteine, then incubated anaerobically at 30°C for 72 h. The formation of opaque halos indicated positive activity. Fresh cow fecal slurry was used as a positive control.

## 4.8.3 PCR detection of potential virulence factors

Genes encoding histidine (*hdc*), tyrosine (*tdc*), ornithine (*odc*), and lysine (*ldc*) decarboxylases, surfactin synthetase (sfp), cereulide synthetase (cesA, cesB), and enterotoxins (*nheA*, *nheB*, *nheC*, *hblA*, *hblC*, *hblD*, and *entFM*) were detected by PCR. *Lactobacillus sp.* 30a, *L. brevis* CECT 4121, and *Bacillus cereus* 4086 were used as positive controls. PCR reactions (50 µL) contained 25 µL MyTaq™ Red Mix (Bioline), 1 µL of each primer (0.7 µM), and 1 µL genomic DNA (50–100 ng). Amplifications were performed following published protocols. Cycling conditions were: 94°C for 2 min; 30 cycles of 94°C for 1 min, gene-specific annealing for 1 min 30 s, and 72°C for 1 min 45 s; with a final extension at 72°C for 10 min as previously described by [75] (Supplementary Table S4).

## 4.8.4 Biosafety

The biosafety of the tested strains was assessed as described by Monzón-Atienza, et al. [16]in European sea bass (*Dicentrarchus labrax*). Ten fish (60 g) were intraperitoneally injected with 0.1 mL of bacterial suspension ($10^8$ CFU/mL). Each treatment was performed in duplicate, and control fish received sterile PBS. Fish were monitored daily for 10 days for mortality and abnormal behaviour. At the end of the experiment, fish were euthanized using an overdose of clove oil (5 mL/L). Internal organs were examined for lesions, and samples were cultured on tryptic soy agar (TSA) to detect the presence of the tested strains.

## 4.9 Selection of safe and potential strains using score system

The selection of candidate strains was performed using a point-based scoring system integrating both functional performance and safety assessment parameters. Each strain was evaluated for antibacterial activity, extracellular enzyme production, antibiofilm formation, and anti–quorum-sensing activity, reflecting its probiotic potential. In addition, key probiotic-related properties were assessed, including tolerance to low pH and bile salts, auto-aggregation, co-aggregation, cell surface hydrophobicity, and adhesion capacity. In parallel, a comprehensive safety assessment was conducted, including hemolytic activity, antibiotic susceptibility and detection of virulence and toxin genes. Scores were assigned to each parameter based on the experimental results, with higher points indicating more desirable traits.

Scores were assigned based on the experimental results obtained for each parameter (Table 5). Heat map analysis was subsequently used to facilitate data visualization and enable objective ranking of the strains according to their probiotic potential [21, 23, 76–78]. To validate the score–heatmap approach, we applied principal component analysis and performed sensitivity analysis to assess robustness.

Table 5
Summary of total score determination

| Parametres | Criteria | | Point value |
|---|---|---|---|
| Functional Criteria | **Direct Antibacterial activity (halo diameter)** | **No activity (0-5mm)** | 0 |
| | | Low (6–10 mm) | 1 |
| | | Moderate (11–20mm) | 2 |
| | | High (21–24 mm) | 3 |
| | | Very high (≥ 25 mm) | 4 |
| | **Broad-spectrum activity** | Number of pathogens affected | 1/pathogen |
| | | Score of activity | |
| | **Nature of activity** | Protease K effect | 2 |
| | | pH effect | 1 |
| | | Combined effect | 4 |
| | **Quorum Quenching (QQ) (per halo diameter)** | - (< 5 mm). | -4 |
| | | + (5–10 mm) | 2 |
| | | ++ (11–20 mm) | 4 |
| | | +++ (≥ 20 mm) | 6 |
| | **Antibiofilm activity** | No effect (< 10%) | 0 |
| | | Moderate effect (25–50%) | 1 |
| | | Strong effect (50–75%) | 2 |
| | | Very strong effect (> 75%) | 3 |
| | | antibiofilm activity persist 24h-48h | 1 |
| | **Enzymes production (per each enzyme)** | presence | 1 |
| | | Absence | 0 |
| | **Tolerance to pH and bile salts** | Non-Viable (0–39%) | -1 |
| | | Low (40–59%) | 0 |
| | | Moderate (60–80%) | 1 |
| | | High (80–100%) | 2 |
| | **Coefficient of inhibition (Cinh)** | Sensitive (> 0,4) | -2 |

| Parametres | Criteria | | Point value |
|---|---|---|---|
| | | Moderate (0,2 – 0,4) | 2 |
| | | Resistant (< 0,2) | 4 |
| | | Sum score of bile salt and Cinh | |
| | **Autoaggregation - Coaggregation** | Very Low (0–19%) | -4 |
| | | Low (20–39%) | -2 |
| | | Moderate (40–59%) | 0 |
| | | High (60–79%) | 1 |
| | | Very High (80–100%) | 2 |
| | **Hydrophobicity** | Non-Hydrophobic (0–20%) | -4 |
| | | Low (21–50%) | -2 |
| | | Moderate (50–69%) | 1 |
| | | High (70–100%) | 2 |
| | **Adhesion to mucus** | Very Low (0–19%) | -4 |
| | | Low (20–39%) | -2 |
| | | Moderate (40–59%) | 0 |
| | | High (60–79%) | 1 |
| | | Very High (80–100%) | 2 |
| | | Sum score of mucus Adhesion | |
| | **Adhesion to BSA- polystyrene** | Low (10–29) | 1 |
| | | Moderate (30–50) | 2 |
| Safety criteria | **Hemolytic activity** | α | -10 |
| | | β | -20 |
| | | γ | 10 |
| | **Antibiotics susceptibility** | Sensitive | 1 |
| | | Resistant | -5 |
| | **virulence and toxin genes** | - | 0 |
| | | + | -1 |
| Biosafety | Death 0 | | |

| Parametres | Criteria | Point value |
|---|---|---|
| | Alive 1 | |

## 4.10 Identification of selected strains

Selected strains were identified by 16S rRNA gene sequencing and MALDI-TOF MS (Ultraflex III, Bruker Daltonics, Germany) as previously described by Jlidi, et al. [55]. Briefly, genomic DNA was extracted using InstaGene Matrix (Bio-Rad), and the 16S rRNA gene was amplified using primers 27F and 1492R. PCR conditions and sequencing were performed as previously reported, and sequences were compared against the NCBI database. MALDI-TOF MS identification was conducted from a single colony using Bruker Flex Control software, with scores ≥ 1.7 considered reliable for species-level identification.

## 4.11 Ethical statement

All experimental procedures involving fish were conducted in accordance with the European Union Council Directive 86/609/EEC and Spanish legislation (Royal Decree 53/2013). All procedures were approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (OEBA-ULPGC-01/2025).

## 4.12 Statistical analysis

Results are presented as means ± standard error of the mean (SEM). Statistical analyses were conducted using SPSS 25.0, applying one-way analysis of variance (ANOVA) followed by Tukey's or Duncan's post hoc tests. A significance level of $p < 0.05$ was considered statistically significant. Hierarchical clustering based on Euclidean distance and the complete linkage approach was carried out, and the results were visualized as a heat map using Origin 2018.

# 5. Conclusion

In this study, 87 strains were isolated from *Solea solea*. Among them only *Bacillus velezensis* SM6 displayed a suitable probiotic potential for aquaculture use based on multi-parametric scoring and heat map approach. This method overcome the previous limitations and enable a more comprehensive evaluation of both functional and safety aspects. *Bacillus velezensis* SM6 exhibited a superior balance of high adhesive capacity, broad-spectrum pathogen inhibition, absence of harmful traits and validated *in vivo* safety in European seabass (*Dicentrarchus labrax*). Subsequently, *in vivo* studies are required to validate its efficacy and assess its potential for application in aquaculture.

# Declarations

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All authors have read and agreed to the published version of the manuscript.

**Supplementary material**

The Supplementary Material for this article can be found online.

**Conflict of Interest**

The authors declare that they have no conflicts of interest regarding the publication of this paper since it is their original work.

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**Data Availability**

The 16S rRNA gene sequence generated in this study has been deposited in the NCBI GenBank database under accession number PZ518674 and is available at https: https://www.ncbi.nlm.nih.gov/search/all/?term=PZ518674

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# Figures

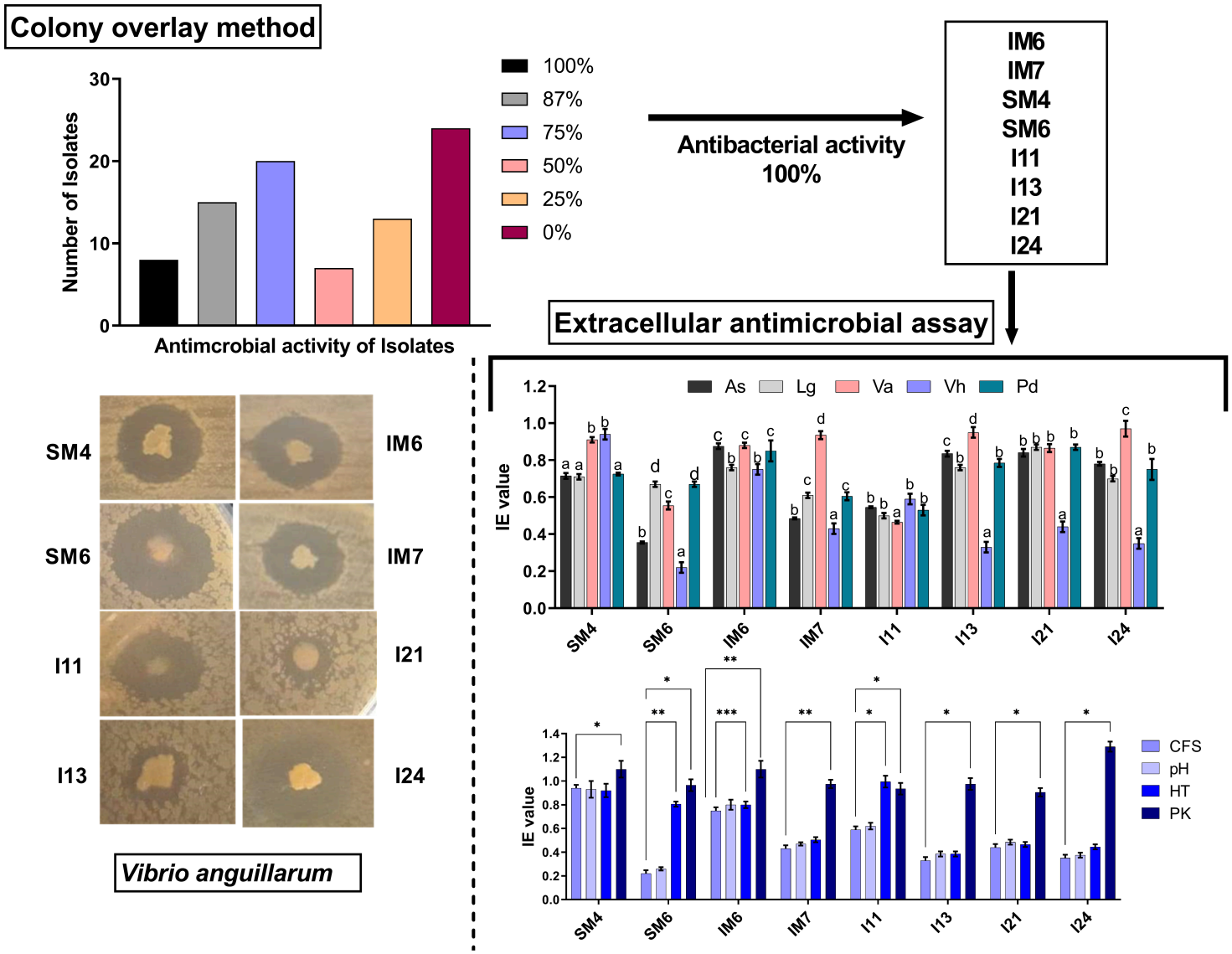



**Figure 1**

Antibacterial activity of isolates against fish pathogens. (a) Distribution of antimicrobial activity among isolates using *method of* colony overlay*;* (b) antimicrobial activity pictorial overview of selected strains against *Vibrio anguillarum;* (c) Antagonistic activity of selected strains against indicators*;* (d) Antimicrobial activity of CFS against fish pathogens*;* (e) Nature of antagonistic activity. Ah, *Aeromonas hydrophila* (LMG 5734); Lg1, *Lactococcus garvieae* (CECT 5807); Va, *Vibrio anguillarum* (CECT 4344); Vh, *Vibrio harveyi* (Lg 26/01); and 8Pd2, *Photobacterium damselae subsp. damselea* (Lg41/00).*CFS: untreated cell-free supernatant; pH: pH-neutralized CFS; HT: heat-treated CFS at 100 °C; pK: proteolytic enzyme-treated CFS. *IE≤1 indicated growth promotion of pathogen, IE=1indicated no inhibition (NI) and IE<1 indicated inhibition of pathogen by CFS of selected strains. Data are presented as (mean ± SD, n=3). Statistical significance was determined by one-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001)

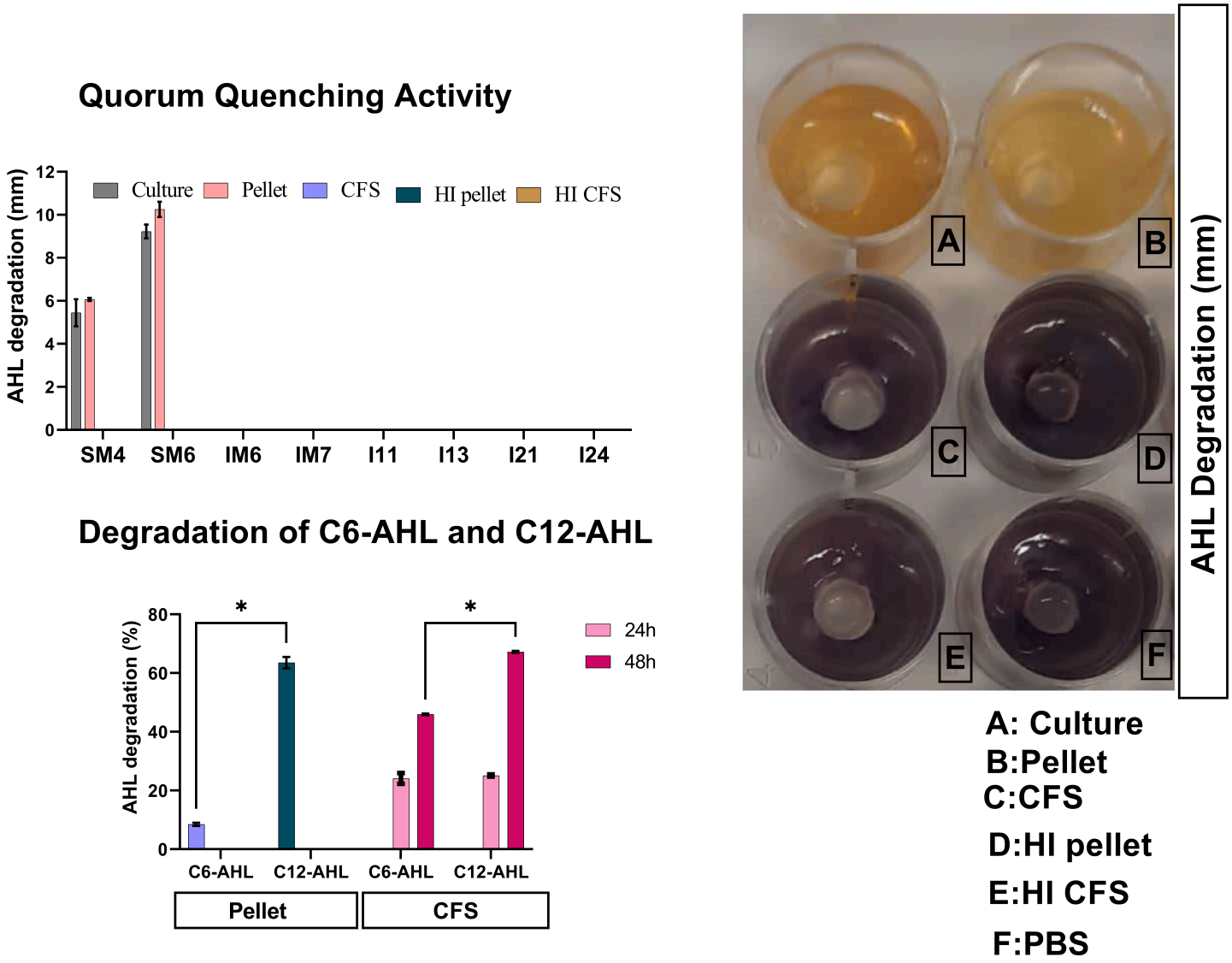
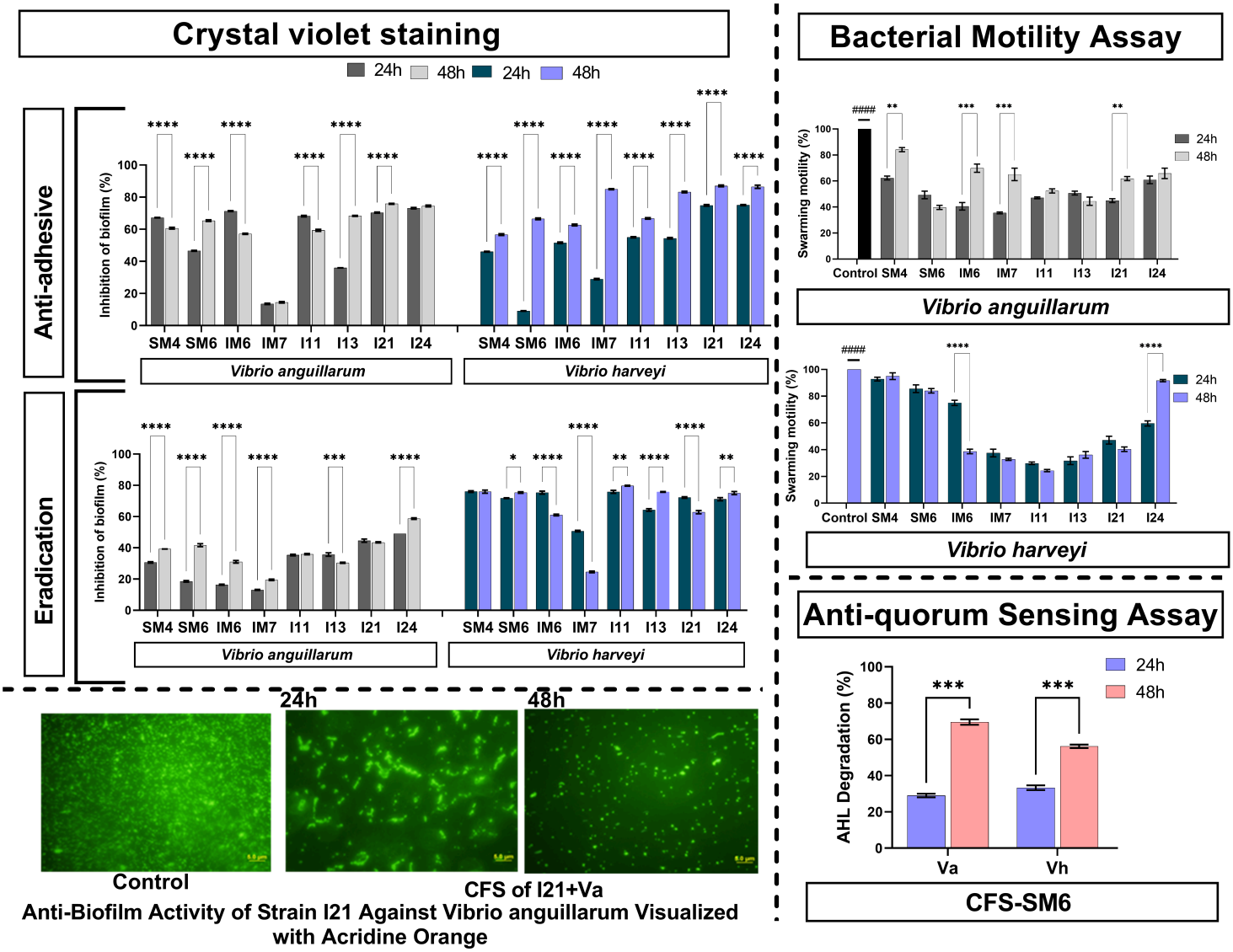



**Figure 2**

(a) Quorum quenching (QQ) activity of the selected strains against Chromobacterium violaceum CV-MK, (b) AHL degradation capacity of the SM6 using both short-chain (C6-AHL) and long-chain (C12-AHL) molecules and (C) Representative biosensor assay images showing AHL degradation of SM6. * CFS; cell-free supernatant, untreated, pellet: cell bacterial pellet untreated, HI-CFS: heat-inactivated supernatant, HI-pellet: heat-inactivated pellet.Data are presented as (mean ± SD, n=3) with different superscript letters denoting a significant difference ($p<0.05$).




## Figure 3

Anti-biofilm activity of selected strains against *Vibrio anguillarum* CECT 4344 and *Vibrio harveyi* Lg 26/01. (a) Crystal violet assays showing anti-adhesive and eradiction activities of tested pathogens, (b) Bacterial motility assay, (c) anti-quorum assay using SM6 CFS and (d) Fluorescence microscopy images illustrating the anti-adherence activity of *Vibrio anguillarum* biofilms: control (untreated) and biofilms treated with CFS of I21 and SM6 after 24h.

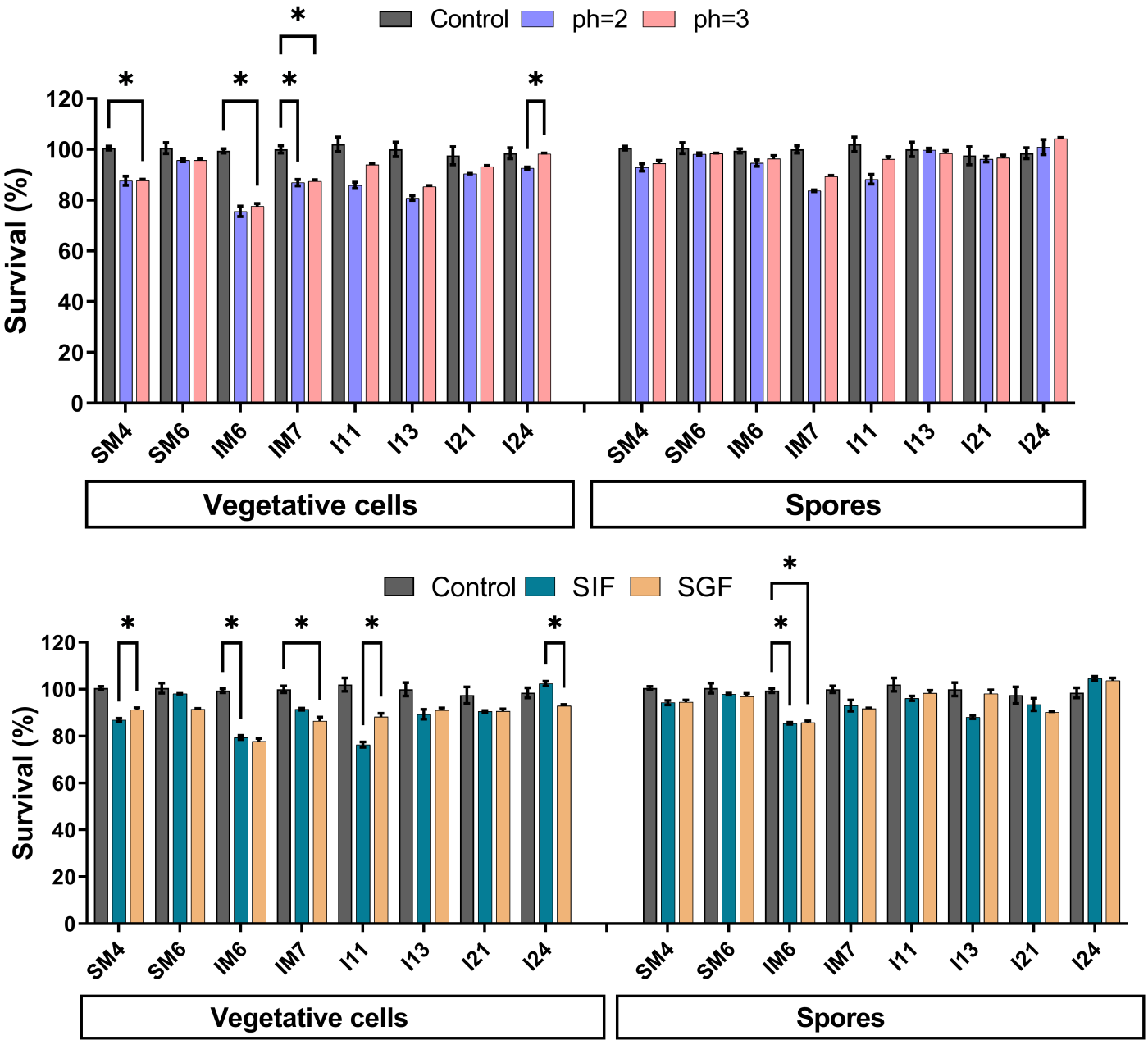



**Figure 4**

Survival percentages of vegetative cells and spores of selected strains under different stress conditions. (a) *S*urvival at pH 2 and pH 3 compared with the control (pH 7), (b) survival in simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) compared with the control (TSB without SIF or SGF). Data are mean ± SD (n = 3). *p < 0.05 vs control.

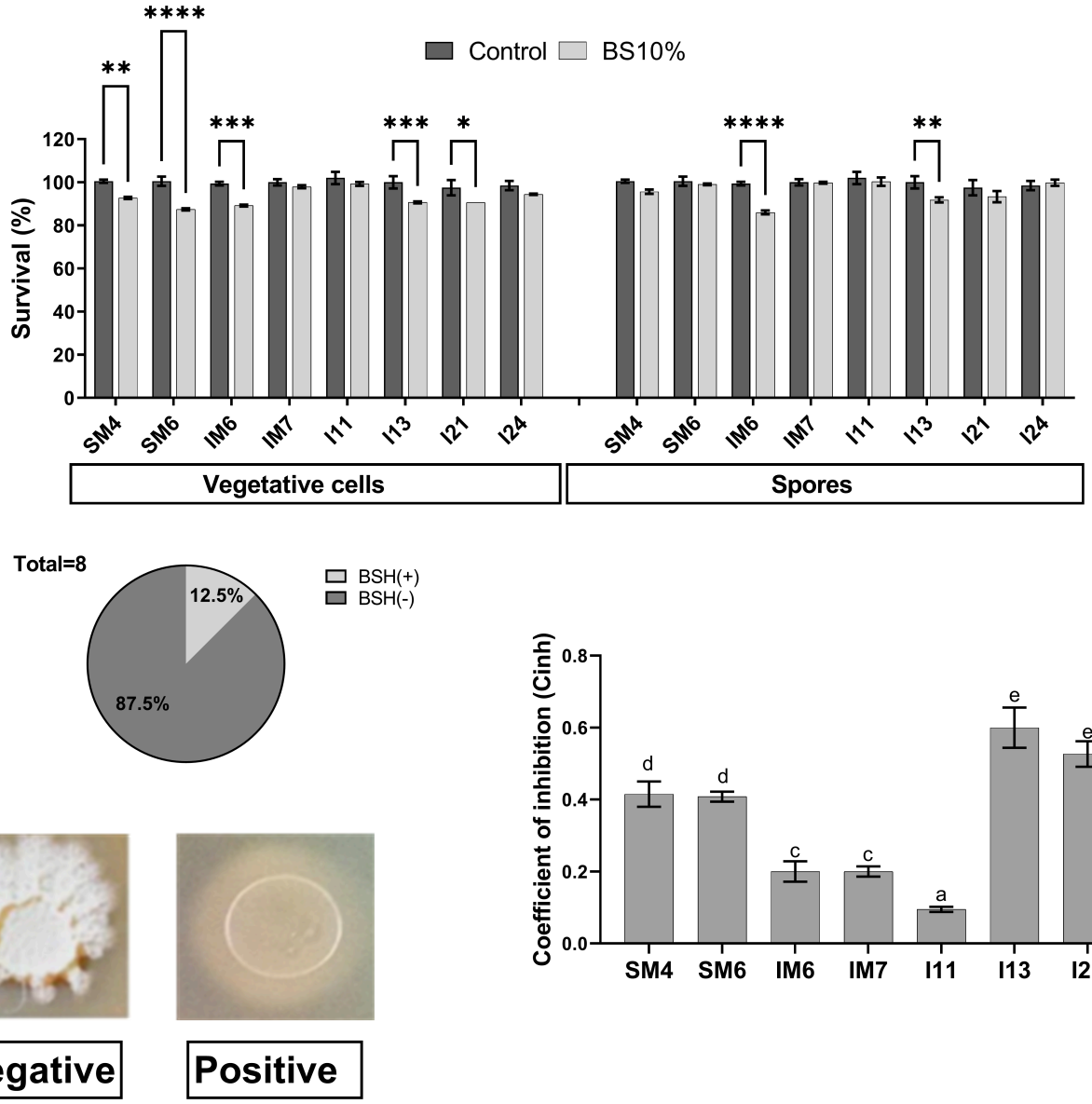
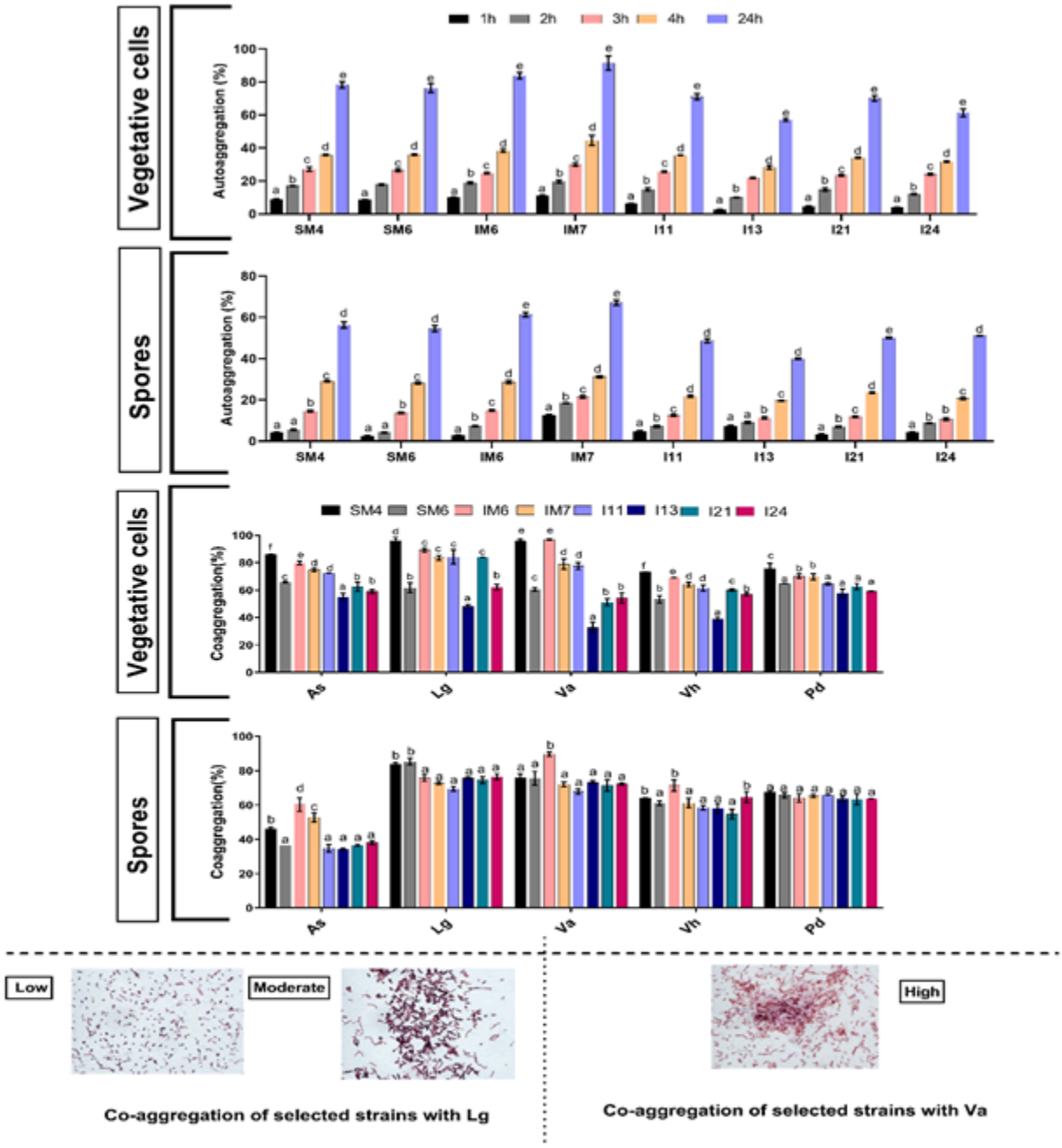



**Figure 5**

(a) Tolerance of selected strains (vegetative cells and spores) to bile exposure. Cells were exposed to 10% (v/v) fish bile juice for 4 h at 28 °C. (b) The coefficient of inhibition (Cinh) was also determined after exposure to 0.3% (w/v) Ox gall at 28 °C for 8 h. Cinh values were interpreted as follows: >0.4 = sensitive, 0.2–0.4 = moderate, and <0.2 = resistant. (c) bile salt hydrolase of tested strains. Data are presented as mean ± SD (n = 3). Statistical significance was determined by one-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001) vs control (0% bile salt)




## Figure 6

Autoaggregation of vegetative cells (a) and their spores (b); Coaggregation of vegetative cells (c) and spores (d). (e) Representative Gram-stained image of SM6 coaggregating with Lg and Va. Ah, Aeromonas hydrophila (LMG 5734); Lg1, Lactococcus garvieae (CECT 5807); Va, Vibrio anguillarum (CECT 4344); Vh, Vibrio harveyi (Lg 26/01); and Pd2, Photobacterium damselae subsp. damselea(Lg41/00). Data are presented as (mean ± SD, n=3) with different superscript letters denoting a significant difference between selected isolates (*p < 0.05).

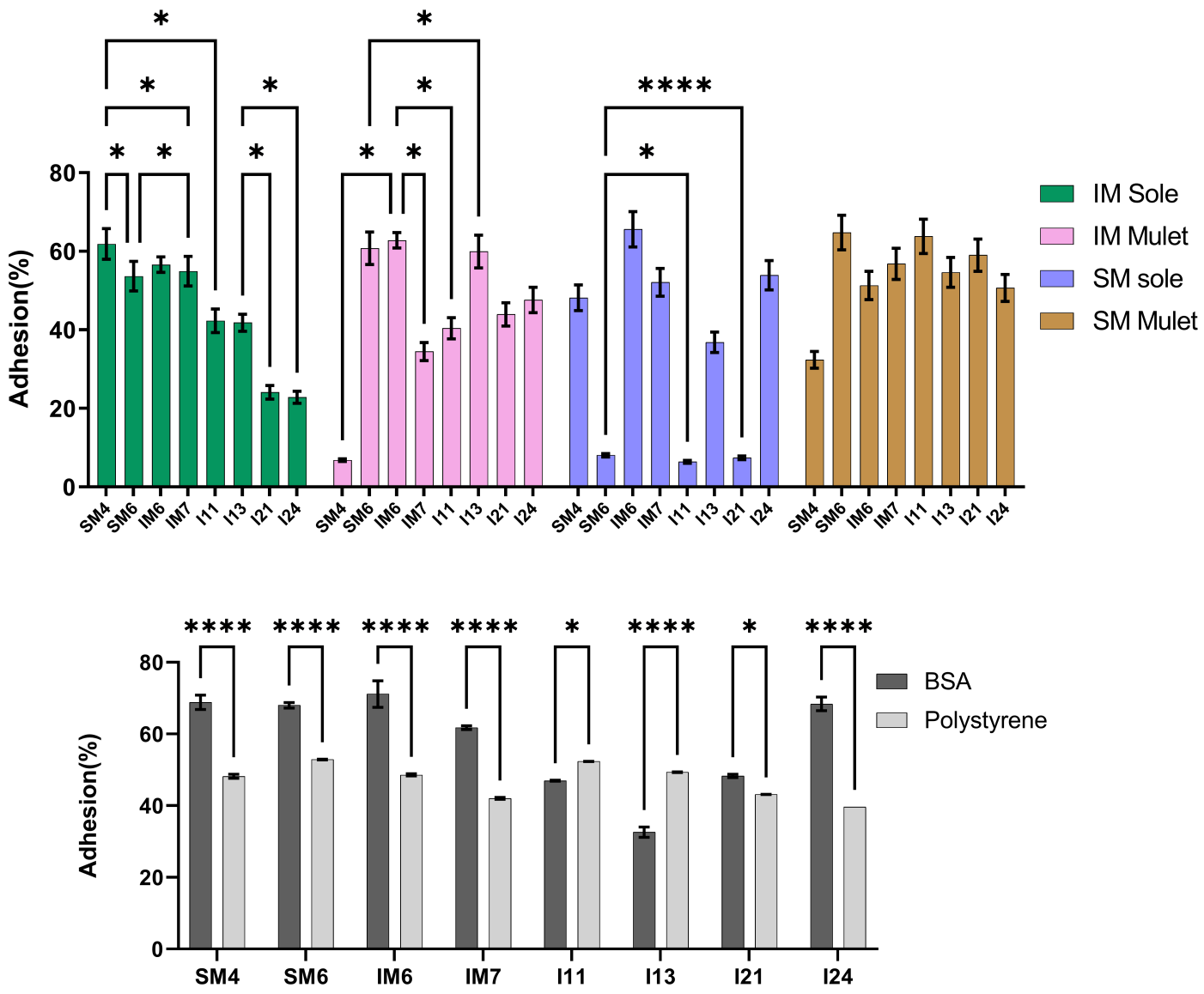



**Figure 7**

(a) Adhesions of selected strains (vegetative cells) to fish mucus (b) Adhesion to BSA and polystyrene* IM, intestinal mucus; SM, skin mucus. Statistical significance was determined by one-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001).

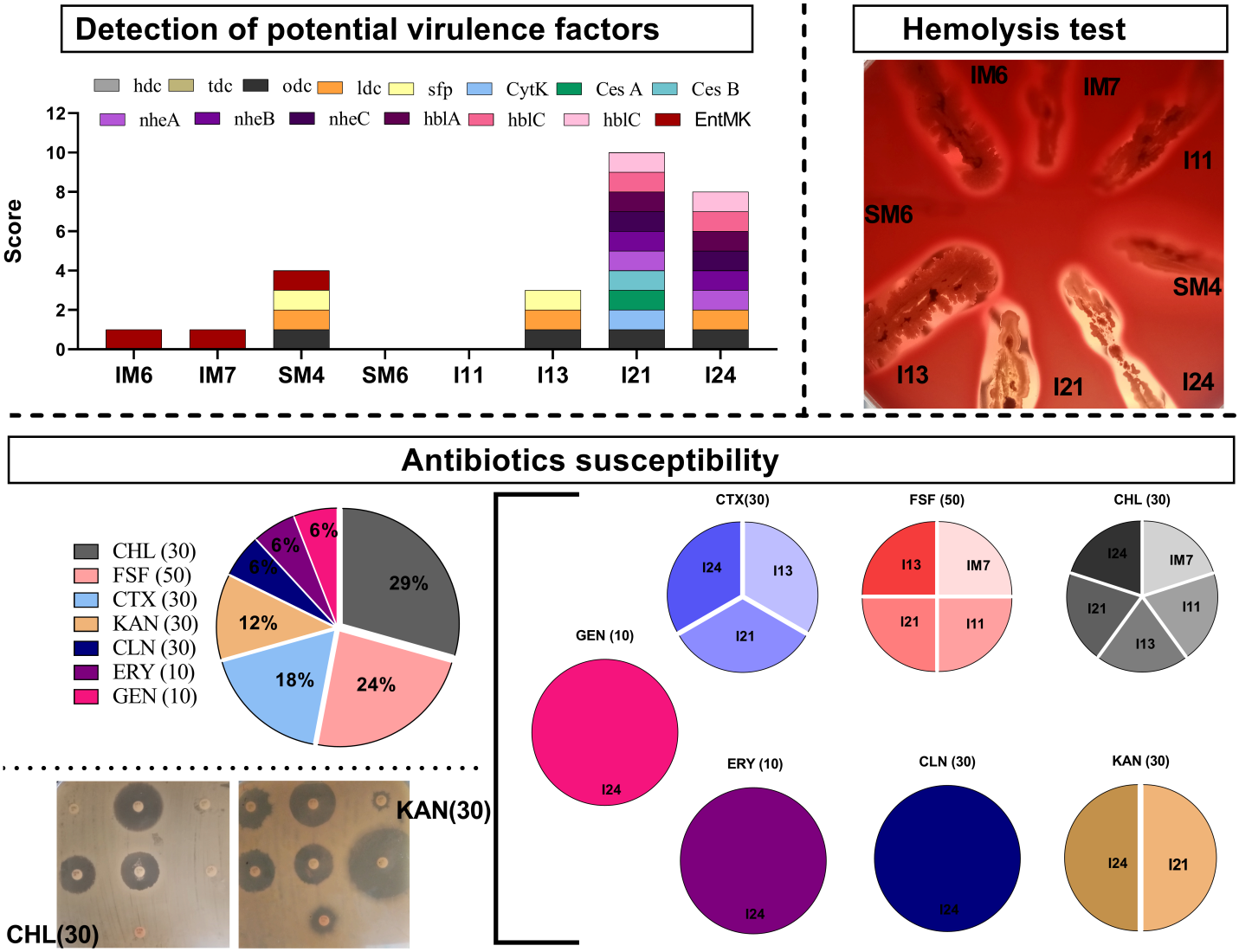



**Figure 8**

In vitro safety characterization of selected strains, including (a) the detection of potential virulence-associated genes, (b) hemolytic activity on blood agar and (c) antibiotic susceptibility profiles. Biogenic amines and toxins are: hdc (histidine decarboxylase); tdc (tyrosine decarboxylase); odc (ornithine decarboxylase); ldc (lysine decarboxylase); sfp (surfactin synthetase); nheABC (non-hemolytic enterotoxin complexe); hblACD (hemolytic enterotoxin complexe); entFM enterotoxin FM; cytK (cytotoxin K); and CesA, CesB (cereulide synthetase genes). Antibiotics are: ampicillin (AMP, 10μg), cefotaxime (CTX, 30μg), chloramphenicol (CHL, 30 μg), clindamycin (CLN, 30 μg), erythromycin (ERY, 10 μg), fosfomycin (FSF, 50 μg), gentamicin (GEN, 10 μg), kanamycin (KAN, 30 μg), Nalidixic acid (NAL, 30 μg), streptomycin (STR, 10 μg), tetracycline (TET, 30 μg) and vancomycin (VAN,30 μg).

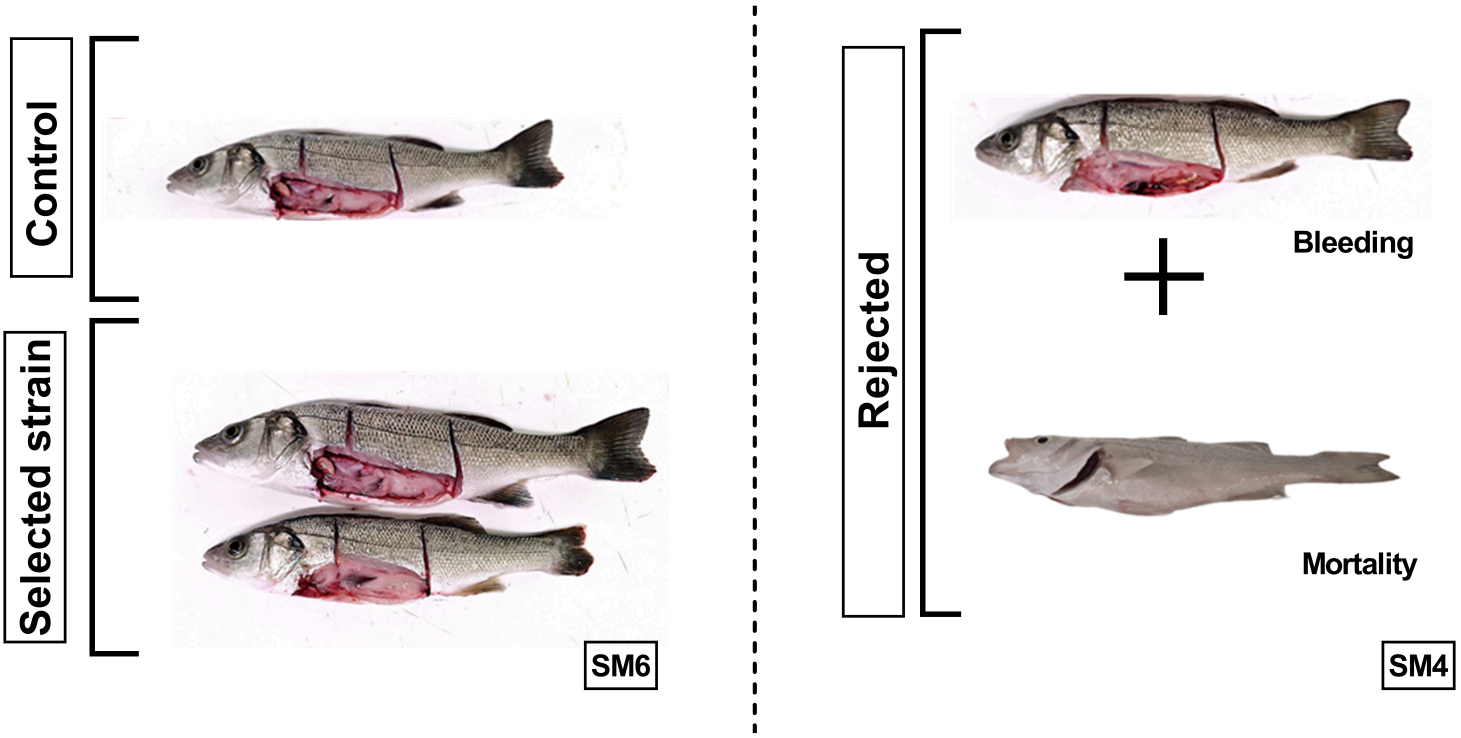



**Figure 9**

Biosafety test of selected strains: No clinical signs were observed in either the control fish or fish injected with the tested strain, and no abnormalities were detected in the internal organs.

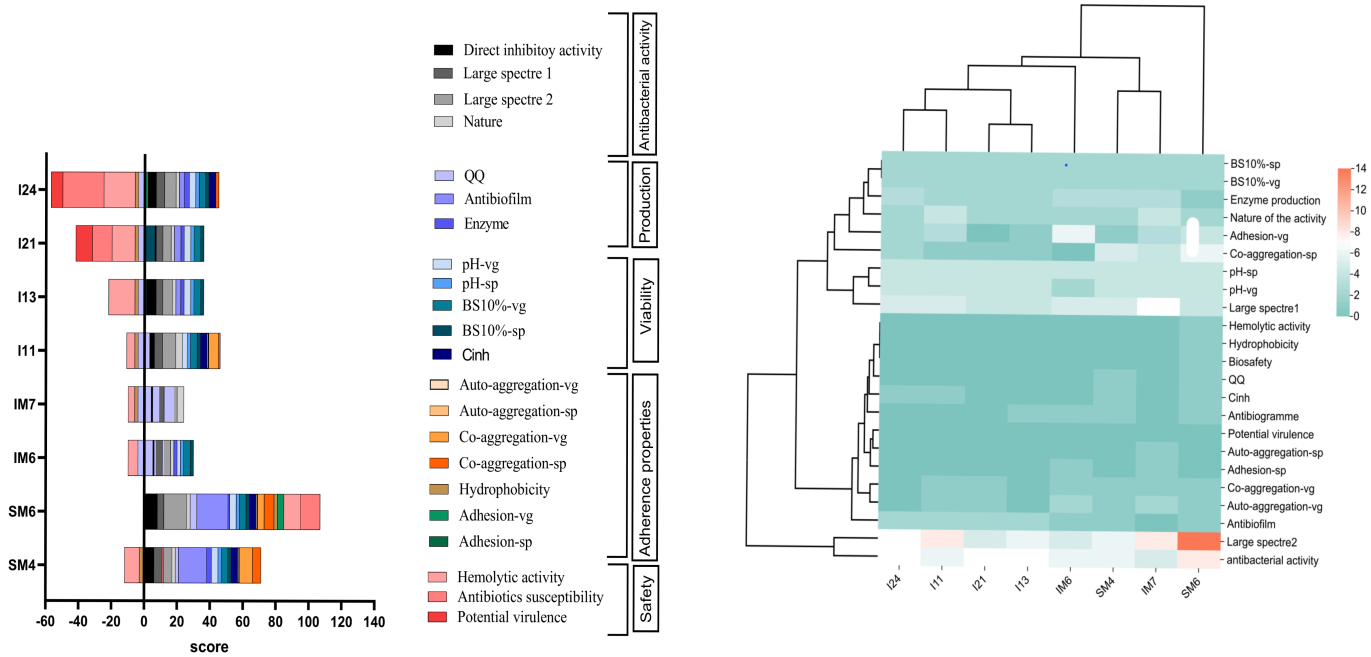



**Figure 10**

Scoring-Based Evaluation of fish-derived strains (a) Scores by criteria (b) Heat-map and cluster analysis.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- highlights.docx