"Effects of Bacillus Velezensis D-18 on Health Status of European Seabass (Dicentrarchus labrax) Experimentally Challenged with Vibrio harveyi"

"Effects of *Bacillus velezensis* D-18 on health status of European Seabass (*Dicentrarchus labrax*) experimentally challenged with *Vibrio harveyi*"

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Abstract

In recent years, the use of probiotics as a possible alternative to antibiotics has generated a growing interest in the global aquaculture field. In this study, the probiotic *Bacillus velezensis* D-18 was evaluated for its potential protective effect against the marine pathogen *Vibrio harveyi*. The probiotic was administered through the diet of European seabass (*Dicentrarchus labrax*) for 30 days, followed by an *in vivo* challenge with *V. harveyi* to assess whether the D-18 strain could enhance host resistance to infection. Biofilm formation in tanks was also investigated to analyze its composition and if there are antagonistic interactions between the two bacterial species. From a histological perspective, significant changes were observed in intestinal morphological parameters after infection, the area and base of the villi appeared to increase in the probiotic-fed groups as did the number of goblet cells and in the serum antibacterial activity which was increased in the infected group that received the probiotic compared to baseline levels. The intestinal microbiome was also analyzed to monitor the composition and determine whether different diets before and after infection induced any changes. Although no significant differences were found in the metagenomics of the tank biofilm and the gut microbiome, mortality rates showed that the probiotic provided effective protection against the pathogen. These findings support the potential of *B. velezensis* D-18 as a viable alternative to antibiotics, particularly when included in the diet prior to disease onset.

Keywords: *Bacillus velezensis* D-18, probiotic, *Vibrio harveyi, Dicentrarchus labrax*, biofilm, gut microbiome

1. Introduction

In recent years, aquaculture has become the fastest-growing food production sector globally, playing a vital role in ensuring food safety and nutrition. Remarkably, for the first time in history, in 2022 aquaculture production of aquatic animals (94.4 million tonnes) surpassed capture fisheries, accounting for 51% of total production (FAO, 2024). These trends underscore the critical role aquaculture plays in global food systems and highlight the urgent need for sustainable practices. The FAO has recently called for a "Blue Transformation in action" in the The State of World Fisheries and Aquaculture (FAO, 2024), to enhance the efficiency, inclusiveness, resilience, and sustainability of aquatic food systems worldwide. At present, Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) are the most widely farmed species in the Mediterranean Sea, with a production volume of 520,000 tonnes and a market value of USD 2.58 billion in 2020 (FAO, 2024). Over 95% of the global production of Gilthead seabream and European seabass is derived from aquaculture, with 97% of this production occurring in Mediterranean countries (Zoli et al., 2023).
In this regard, bacterial infections pose serious threats to bass and bream aquaculture (Mougin et al., 2021), with some more recently emerging bacterial diseases due to *Aeromonas veronii* and *Lactococcus garvieae* (Smyrli et al., 2019; Salogni et al., 2024), leading to large-scale mortality

events and severe economic losses. Among the various bacterial diseases affecting European seabass, vibriosis - caused by several *Vibrio* species including *Vibrio anguillarum, V. harveyi, V. alginolyticus, V. ordalii, V. parahaemolyticus* and *V. vulnificus* (Korun and Timur, 2008; Mougin et al., 2021) - stands out as the most significant, impacting fish across all stages of the production cycle (Mougin et al., 2021, Ina-Salwani et al., 2019). However, *V. harveyi* has recently emerged as a major concern in European seabass aquaculture, and currently, no specific or effective prophylactic measures are available to control this pathogen in this fish species (Mougin et al., 2021; Vendramin et al., 2016; Firmino et al., 2019). In cases of severe infection, fish affected by *V. harveyi* display signs of inappetence, lethargy and disorientation. Internally, pathological alterations include meningitis, encephalitis, vasculitis, kidney necrosis, and damage to the liver and kidneys (Mohamad et al., 2019). *Vibrio harveyi* is a Gram-negative, fermentative, rod-shaped bacterium that require sodium chloride for growth and is motile via polar flagella (Farmer et al., 2015; Zhang et al., 2020). It is also a producer of biofilm, a community of microorganisms adhering to and proliferate on biotic or abiotic surfaces encased within extracellular polymeric substances (EPS) (Abebe, 2020; De Silva and Heo, 2023) and able to give the pathogen the ability to persist in the environment, escaping the action of disinfectants and antibiotics.

Historically, the predominant strategies for managing infections have involved chemical antibiotic therapies (Assefa and Abunna, 2018; Torres-Maravilla et al., 2024). As in the case of other fish bacterial infections, even those caused by *V. harveyi* are treated by administration of antibiotics in the feed, but concerns about tissue residues and the emergence and spread of antibiotic resistance have driven research toward alternative approaches to disease control, with a greater emphasis on prevention rather than treatment (Zhang et al., 2020; Chen et al., 2020). Vaccination remains a widely adopted strategy in aquaculture to induce long-lasting immune responses in farmed fish and provide protection against pathogenic microorganisms. Most commercial vaccines are still based on inactivated pathogens and are primarily administered via injection. In the field, autogenous vaccines have been developed to control *Vibrio harveyi* in Gilthed seabream and European seabass production; however, their practical application remain limited, and effective control still largely depends on antimicrobial therapies (Smith et al., 2023).

Consequently, the search for compounds able to offer alternative strategies to combat pathogenic bacteria while reducing antibiotic usage is considered a priority (Petit et al., 2024).

Among these, identifying probiotic bacteria that can both produce beneficial compounds and effectively colonize aquaculture environments and antagonize potential pathogens circulating in the system, represents a promising strategy for developing new products tailored to aquaculture (Petit et al., 2024). Concerning the target host, probiotics are living microorganisms that, when administered at appropriate doses, can promote a healthy balance of gut bacteria in animals (and humans). This can result in various benefits such as preventing the invasion of harmful pathogens, improving digestion, promoting growth, and increasing survival rates (Ntakirutimana et al., 2023). Probiotics also support gut microbiome stability, improve water quality through bioremediation, and enrich zooplankton nutrients (El-Saadoni et al., 2021). Various microorganisms, including yeast, fungi, and numerous *Bacillus* species, are recognized for their probiotic functions and potential to replace antibiotics (Iannitti and Palmieri, 2010); administrated via food, they can antagonize intestinal pathogenic bacteria and enhance disease resistance (Li et al., 2019, Khan et al., 2013, Monzòn-Atienza et al., 2022). In particular *Bacillus velezensis* was first described twenty years ago during extensive research aimed at discovering bacterial strains capable of synthesizing new lipopeptides with surfactant or antimicrobial properties (Ruiz-García et al., 2005). Concerning its possible application in aquaculture, previous studies have isolated *B. velezensis* from waste-water at a Spanish farm (Ruiz-García et al., 2005) and recent studies demonstrated the positive effects of *B. velezensis* strain D18, (Monzón-Atienza et al., 2021), on the resistance of European seabass against *V. anguillarum* after experimental challenge (Monzón-Atienza et al., 2021, 2022).

Main objectives of this study were to verify the effects of the probiotic *B. velezensis* D-18 on the health status of European seabass maintained in a closed recirculation system and intraperitoneally

inoculated with the emerging pathogen *V. harveyi*. Survival rate, plasma antibacterial activity and gut histology/morphology have been investigated. Furthermore, the composition of the tank biofilm and of the fish intestinal microbiome pre- and post-challenge have been studied.

2. Materials and methods

2.1 Bacterial strains

The bacterial strain Vibrio harveyi ITT 281/14/B was from the repository of the Fish Pathology Unit of the Department of Veterinary Medical Sciences of the University of Bologna (Unibo) and had been isolated during a mortality outbreak in European seabass (Dicentrarchus labrax) farmed in Italy. It had been previously identified by standard microbiological methods, including matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Maldi-TOF, Bruker) and molecular analyses. The probiotic Bacillus velezensis strain D-18 had been isolated, identified, and characterized earlier by the Grupo de Investigación en Acuicultura (GIA), Instituto Ecoaqua, Universidad de Las Palmas de Gran Canaria (ULPGC) (Monzón-Atienza et al., 2021).
To conduct the present trial, vials containing *V. harveyi* and *B. velezensis* and stored at -80 °C at UNIBO and ULPGC bacterial collections respectively, were defrosted at about 4 °C on ice. Each strain was aseptically cultured in sterile Erlenmeyer flasks containing 50 ml of brain heart infusion (BHI; Cultimed, Panreac, Spain) supplemented with 1.5% sodium chloride (NaCl). Each flask, inoculated with a single colony-forming unit (CFU) of each bacterial strain, was cultured following classical microbiological techniques at 26 C ± 1 for 24 hours.

2.2 Fish and housing

A total of 240 European seabass (*Dicentrarchus labrax*) with an average body weight of 180 ± 10 g were housed at the Marine Science and Technology Park at Universidad de las Palmas de Gran Canaria (ULPGC), Spain. The University of Las Palmas's Ethical Committee accepted all experimental protocol. Specimens selected following gross examination were determined to be clinically healthy, exhibiting normal skin and gill coloration, no external lesions or infections, and no documented history of parasitic infestation. For acclimatization, the experimental fish were randomly distributed into twelve 500 L fiber-reinforced tanks (n = 20 fish/tank) in a closed water system at 22°C with continuous aeration, a 12:12 h photoperiod, and a water pH of 8 for two weeks. The fish were fed daily at a regular rate calculated as 2 % of their biomass with a commercial diet (D-4 Optibream AE 3 P - Alterna, Skretting, Spain) of 4 mm diameter containing 40.5% fish protein and 18% fish oil. Before the beginning of the *V. harveyi* exposure, five individuals were randomly selected to undergo standard microscopic and bacteriological tests to ensure they were not infected with pathogenic bacteria. For each sampling, fish were anesthetized using clove oil overdose at concentration of 0.5 mL/L (Guinama S.L., Spain, Ref. Mg83168), diluted in 100% alcohol (1:1).

2.3 Feed preparation and experimental design

The commercial European seabass feed served as the experimental control diet and as the basal diet for the supplementation with *B. velezensis* D-18 (10^6 CFU x feed g^{-1}), determined spectrophotometrically at an optical density of 600 nm and by counting colony-forming units (CFU). The incorporation process was conducted as follows. Briefly, the probiotic suspension was applied using a spray bottle with the nozzle adjusted to release mist. The diet was mixed slowly in a drum mixer and then air-dried on a clean bench for 12 hours, ensuring sterile conditions throughout the process. The stock diet was stored at -20 °C, and daily rations were thawed at 4 °C before feeding. The viability of the incorporated *B. velezensis* was assessed by vortexing 10 g of diet in 90 ml of sterile PBS and preparing serial dilutions. 100 µl aliquots were cultured at 26 °C for 24 hours

following classical microbiological procedures. All fish were fed twice daily by hand for 30 days at a regular rate calculated as 2% of their biomass.

After the two-week acclimation period, each tank, containing 20 fish fed with commercial diet, was randomly assigned to one of four experimental groups: Group Ctrl: 3 tanks as a control group - fish fed commercial diet throughout the trial period and not infected, only inoculated with 0,1 mL of sterile PBS at the day of the challenge; Group Bv: 3 tanks as another control group - fish fed diet with probiotics (*B. velezensis*) (10^6 CFU x feed g^{-1}) and not infected, only inoculated with 0.1 mL of sterile PBS at the day of the challenge; Group Ctrl-Ch: 3 tanks for challenge 1 - fish fed commercial diet for a month and intraperitoneally (IP) infected with *V. harveyi* and Group Bv-Ch: 3 tanks for challenge 2 - fish fed diet with probiotics for a month and IP infected with *V. harveyi* ($2x10^4$ CFU x mL) (Fig. 3).

After one month of feeding each group with their own diet as per the experimental protocol, groups Ctrl-Ch and Bv-Ch were challenged and from this point, all groups were fed only a commercial diet until the end of the trial (Fig. 1).

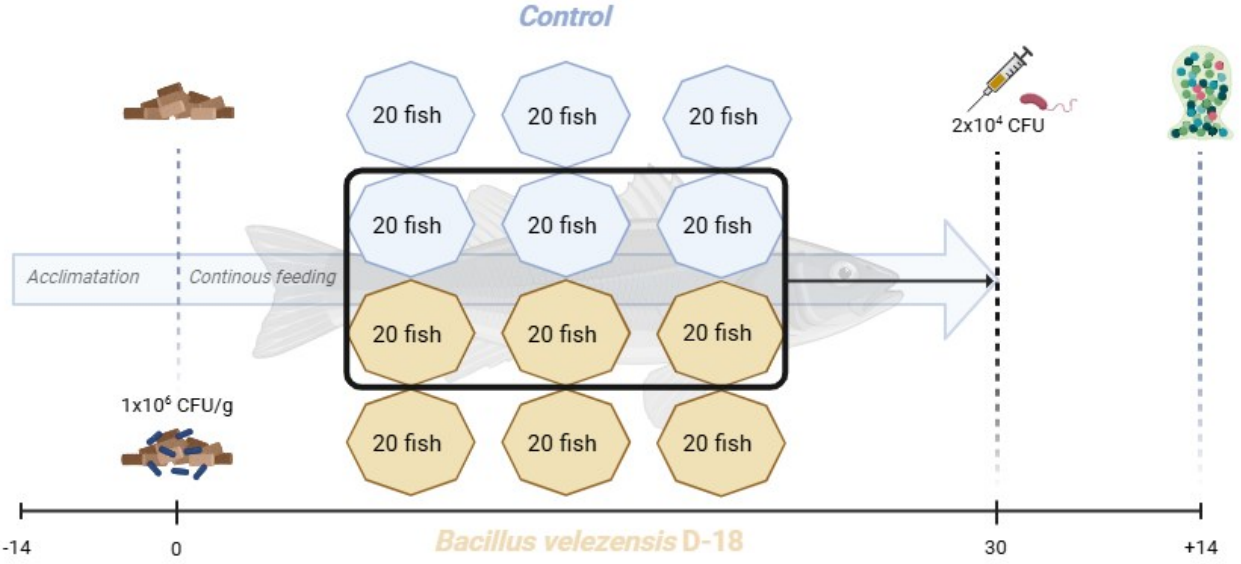

Fig. 1. Scheme of the experimental trial

2.4 Fish sampling

After 6 weeks from the beginning of the experimental trial (2 weeks for acclimatation and 4 weeks of feeding period with experimental diets), all the fish were starved for 24 h. Three fish randomly collected from the Ctrl and Bv groups (only commercial diet and probiotic diet groups), after 30 days of feeding with experimental diets, and 14 days after challenge, humanely euthanised by administering a clove oil overdose at a concentration of 0.5 mL/L (Guinama S.L., Spain, Ref. Mg83168), diluted in 100% alcohol (1:1), achieving euthanasia within 1 minute and subjected to a wide set of analyses. After the challenge, the fish were observed and mortality was recorded daily. After 14 days of observation, the fish were sacrificed with an overdose of anaesthetic and 6 fish per group were sampled for analysis.

2.5 *In vivo* challenge test with *Vibrio harveyi*

The bacterial challenge was conducted as described in Monzón-Atienza et al. (2022). Briefly, finalized the probiotic feeding trial, 3 individuals were ip injected with (2×10^4 CFU mL^{-1}) *V. harveyi* strain live cells, to assure infectivity (Zhang et al., 2020). After the injection, fish were monitored every 12 h over a six-day period for clinical signs of disease and mortality recorded. Upon death of the fish, they were immediately inoculated into brain heart infusion (BHI; Cultimed, Panreac, Spain) supplemented with 1.5% sodium chloride (NaCl) and thiosulfate citrate bile sucrose agar (TCBS, Cultimed) plates from brain, kidney and spleen, to confirm the presence of the pathogen and reisolate it.

With regard to the challenge of fish with bacterial strains, specimens were anesthetized with clove oil (7 mL/100 lt.) and then intraperitoneally injected with 0.1 mL of PBS containing a suspension of *V. harveyi* at the LD50 doses indicated by literature (Pujalte et al., 2003; Zhang et al., 2022) and, in the case of the control groups (Ctrl and Bv), only with 0.1 mL of sterile PBS.

The described experiments complied with the European Union (86/609/EU), the Spanish Government and the University of Las Palmas de Gran Canaria (Spain) guidelines for the use of laboratory animals (OEBA-ULPGC 11/2024R1).

2.6 Serum antibacterial activity

Complete sets of samples were obtained at the end of the feeding trial on day 30 (Pre Challenge) and at the final sampling (Post Challenge). Briefly, 3 fish for tank were sacrificed through anesthetic (clove oil) overdose within 1 min and blood was collected from the caudal vein using 25 G needles attached to a 2 mL syringe. One milliliter was loaded in a regular 1.5 mL Eppendorf tube and centrifuged at 3000 rpm for 15 min to separate the serum. The collected serum was stored at - 20 ◦C until further use.

In a round-bottom 96-well plate, in triplicates, 20 µl of plasma and 20 µl of *V. harveyi* (10^6 CFU/mL) were incubated for 5h at 25°C. Hank´s balanced salt solution or sterile PBS instead of plasma was used for positive control. To each well, was added 25 µl of MTT (3-(4,5 dimethyl-2-yl)-2, 5-diphenyl tetrazolium bromide, 1 mg/mL) and incubated for 10 min. at 25°C. After centrifuging at 2,000 x g for 10 min, the supernatant was removed and the pellet was dissolved in 200 µl of DMSO (dimethyl sulfoxide). Finally, serum antibacterial activity was evaluated by measuring the absorbance of dissolved format, which is produced by the metabolic activity of *V. harveyi* following the procedure in Chung and Jeffries (1988). The antibacterial activity of plasma is shown in Figure 4. The absorbance was recorded at 570 and 690 nm (final absorbance = Abs. 570 – Abs. 690). The percentage bactericidal capacity is calculated by comparison with the reference sample (positive control). Positive control means 100% of bacterial growth (0% of bactericidal activity).

2.7 Gut microbiota and environmental biofilm composition assessment, samples collection, DNA extraction and sequencing

After sacrifice of the fish, the intestine of 3 fish for group, after 30 days of feeding with experimental diets (before the challenge) at the end of trial (after the challenge), was removed from the visceral mass and approximately 1.5 cm of the medial part was isolated. After opening, it was washed with sterile PBS and the washed content collected in Eppendorf tubes containing 1 mL of RNAlater (Thermo Fisher Scientific, Milan, Italy) for subsequent analyses. At the same time, in addition to the fish gut, the biofilm was sampled from the walls of all the tanks by swabbing them with sterile cotton swab Aptaca. The water level of each tank was briefly lowered by approximately 5 cm. Immediately after taking the sample by swab, it was immersed in 10 mL of sterile PBS with 20 glass beads in the laboratory and subjected to breaking by vortexing. 1 mL of sample was subjected to DNA extraction with DNeasy Power Biofilm kit (QIAGEN, Hilden, Germany), while Total DNA was extracted with briefly adaptation as previously described, using the protocol of DNeasy Blood & Tissue Kit (QIAGEN).

To amplify the V3-V4 hypervariable region of the 16S rRNA gene, the 341F and 785R primers (Klindworth et al., 2013) were used, together with Illumina adaptor overhang sequences and 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems). The thermal cycle for this stage included an initial denaturation phase at 95 °C for 3 minutes, 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds, followed by a final extension step at 72 °C for 5 minutes. To purify PCR products for Illumina sequencing, the Illumina protocol "16S Metagenomic Sequencing Library Preparation" was utilized, as described in earlier publications (Musella et al., 2020; Biagi et al., 2019).

Sequencing was done on an Illumina MiSeq platform with a 2 × 250 bp paired-end technique, following manufacturer's guidelines (Illumina, San Diego, CA). The raw sequences were processed with the QIIME2 pipeline (Bolyen et al., 2019; https://qiime2.org). High-quality readings were obtained by a filtering step with standard quality parameter and settled length parameter (minimum/maximum = 350/550 bp) using DADA2 (Callahan et al., 2016), and also clustered into Amplicon Sequence Variants (ASVs). VSEARCH algorithm (Rognes et al., 2016) was used to assign taxonomy against the SILVA database (v138.2, Quast et al., 2013). The 16S rRNA gene was sequenced from 12 intestinal content and 12 biofilm swabs samples, with three replicates collected per dietary group. Following quality control and reads filtering, 10 intestinal samples were retained for downstream analysis and 12 biofilm samples.

Alpha diversity was measured using faith_pd, observed_features, and shannon_entropy, whereas beta diversity was determined by computing UniFrac distances, both weighted and unweighted, which were then utilized as input for Principal Coordinates Analysis (PCoA). The Wilcoxon rank-sum test was used to examine the significance of alpha diversity, while a permutation test with pseudo-F ratios (adonis function) was utilized for beta diversity, considering the separation of data in principal coordinate analysis (PCoA). Linear discriminant analysis effect Size, LEfSe was then used to identify discriminant taxa per each diet group (Segata, et al., 2011). P-values were considered significant if < 0.05. Statistical analysis of the microbiome were performed using R version 4.4.0 (www.r-project.org) and specifically the following packages were involved: vegan (version 2.6-2, Oksanen et al., 2022), made4 (version 1.78.0, Culhane at al., 2005), cluster (version 2.1.6, Maechler qt al., 2025), pairwiseAdonis (version 0.4.1, Martinez et al., 2020), RcppAlgos (version 2.9.3, Wood et al., 2025), xlsx (version 0.6.5, Dragulescu & Arendt, 2020), matrixStats (version 1.5.0, Bengtsson at al., 2025), RColorBrewer (version 1.1-3, Neuwirth, 2022).

2.8 Histological analysis

Histological examination was conducted on six fish per tank. Intestinal samples were collected from the mid-intestine (three segments) of fish from all the experimental groups. Tissues were fixed in 10% buffered formalin for 24 hours and processed using a Microm STP 120 Spin Tissue Processor (STP120; Thermo Fisher Scientific). Paraffin-embedded sections were cut at 3 µm using a semi-automated microtome (Jung Autocut 2055, Leica, Germany) and mounted on SuperFrost-Plus slides. Sections were stained with Alcian Blue (pH 2.5) – Periodic Acid-Schiff (AB–PAS) following the method of Martoja and Martoja-Pierson (1970), to differentiate between neutral and acidic mucins. This staining protocol was also employed to assess mucosal fold morphology, including fold area, height and width. Stained slides were scanned using a MoticEasyScan Pro digital scanner (Motic, Xiamen, China) operated with Motic DS Assistant software (Motic VM V1 Viewer 2.0). Representative images were selected for analysis. Image analysis was performed using the analySIS® software package (Image Pro Plus® v4.5.0.29; Media Cybernetics, Silver Spring, MD, USA), calibrated using embedded scale bars. Three folds per section (three sections per fish) were analyzed. Fold areas were manually delineated by tracing their perimeters, with the base defined by an imaginary line connecting the junctions of adjacent anterior and posterior folds. Mucosal fold height (from the villus apex to the base line), and fold width, were manually measured using the software's

built-in tools. Goblet cell area within each delineated fold was automatically quantified using the eyedropper tool, and the percentage area was subsequently calculated.

The results from the morphometric analysis of histological sections (villus area, villus length, villus width at base, number of goblet cells and goblet area / villus area ratio) were analysed by ANOVA, with the critical value for statistical significance set at $p \leq 0.05$. Statistical analyses were carried out using the Stata 19 software.

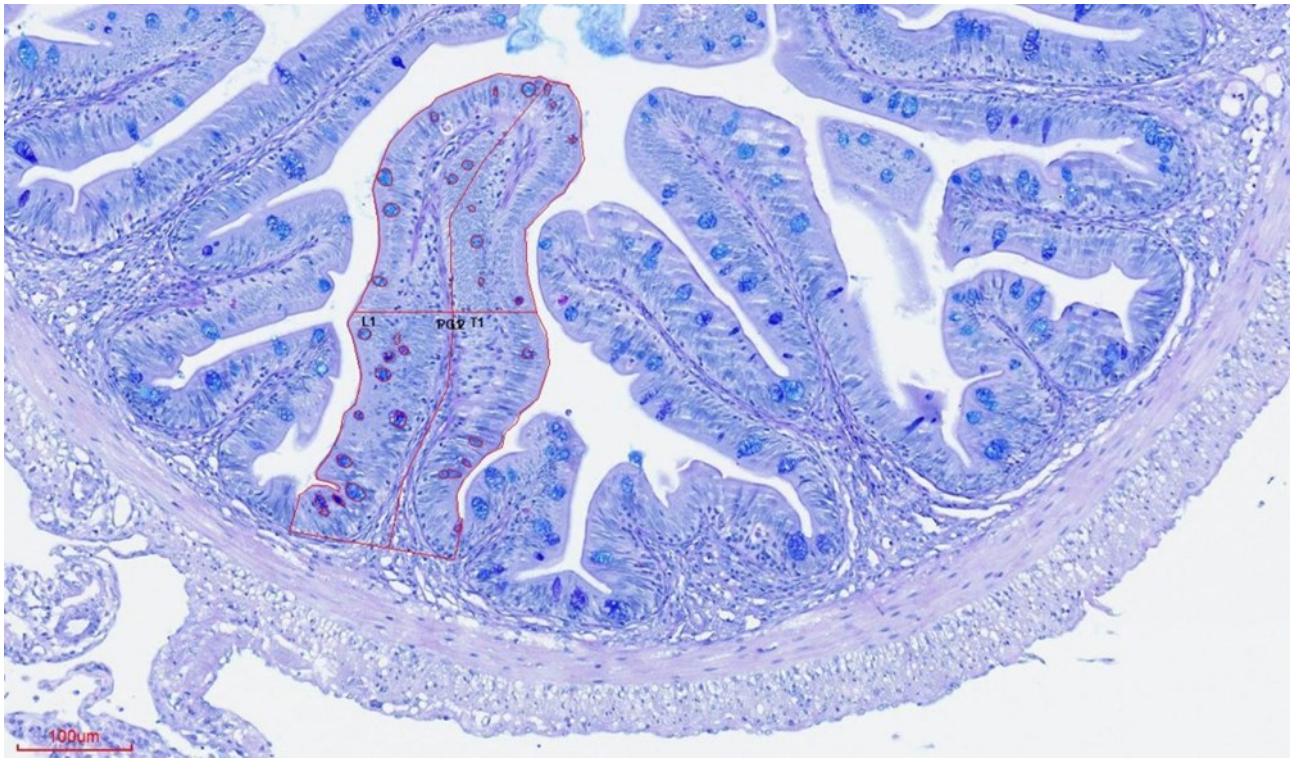

Fig. 2. Representative image of the intestinal measurement process in European seabass fed either a control (Ctrl) or probiotic (Bv) diet, both before and after challenge (Ch). Mucosal fold area, height, and width were manually delineated. Goblet cell number and area within each delineated fold were automatically quantified, and the percentage area was subsequently calculated.

3. Results

3.1 Survival

In the experimental challenge, as shown in figure 3, the relative survival percentage of the group fed with *Bacillus velezensis* D-18 (Ctrl-Bv-Ch) was 50 %, compared to the control group (Ctrl-Ch), which presented 27 % survival, and statistical analysis demonstrated strain D18 significantly increased fish survival ($p = < 0.05$).

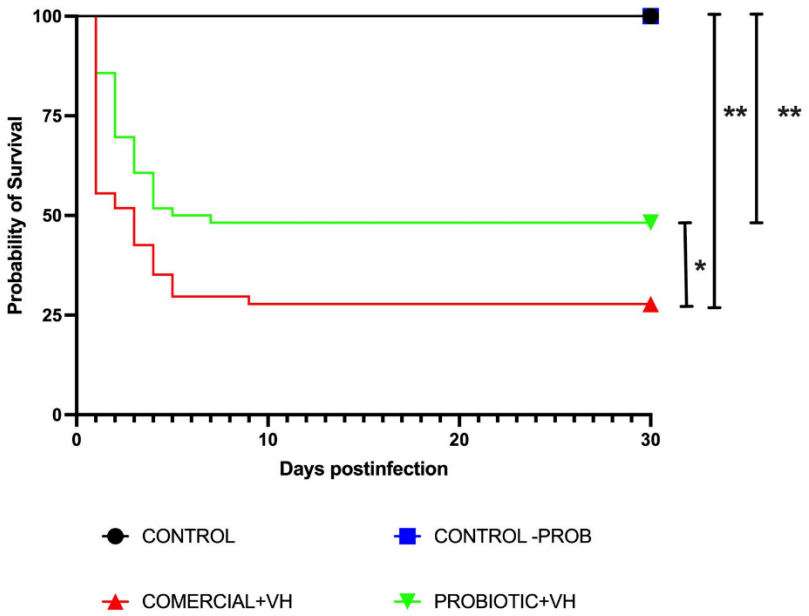

Fig. 3. Effect of the probiotic strain on European seabass survival rate against *V. harveyi*. Asterisks denote significant statistical differences: $p < 0.05$ (*) and $p < 0.001$ (**)

3.2 Antibacterial activity

The result of the antibacterial activity is shown in Figure 4. Briefly, it emerges that, compared to T0, the antibacterial activity increases in the Ctrl, Bv and Ctrl-Ch groups in crescendo. A significant difference is observed in the Bv-Ch group compared to T0 (Fig. 4).

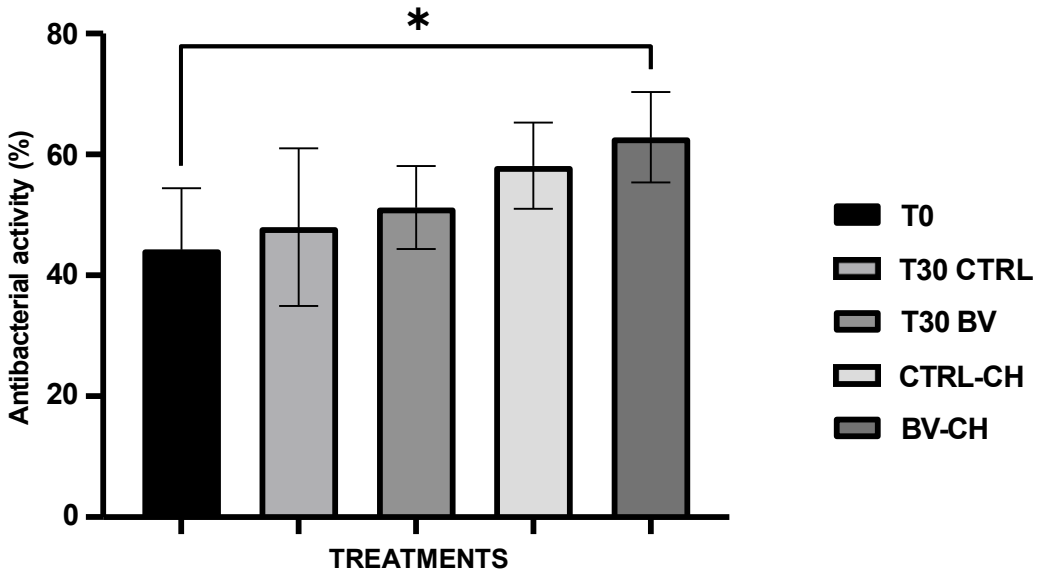

Fig. 4. Antibacterial activity (%) of plasma from different experimental groups

3.3 Gut and biofilm bacterial communities

To determine whether probiotic supplementation and/or infection influenced the bacterial composition of the gut and/or biofilm, microbial communities from different sample types were analysed. Beta diversity was assessed using Principal Coordinate Analysis (PCoA) based on both unweighted and weighted (Fig. 5. a-b) UniFrac distance metrics. A significant separation in microbial community structure was observed among all groups ($p < 0.001$). Furthermore, alpha diversity, so the diversity within groups, differed significantly across groups, as indicated by three diversity metrics: Faith's Phylogenetic Diversity, Observed Features, and Shannon Entropy (Fig. 5. c). No significant variations were found for the alpha diversity indices among groups of the same sample types (i.e. fish gut microbiota or biofilm microbiota assessed in different conditions).

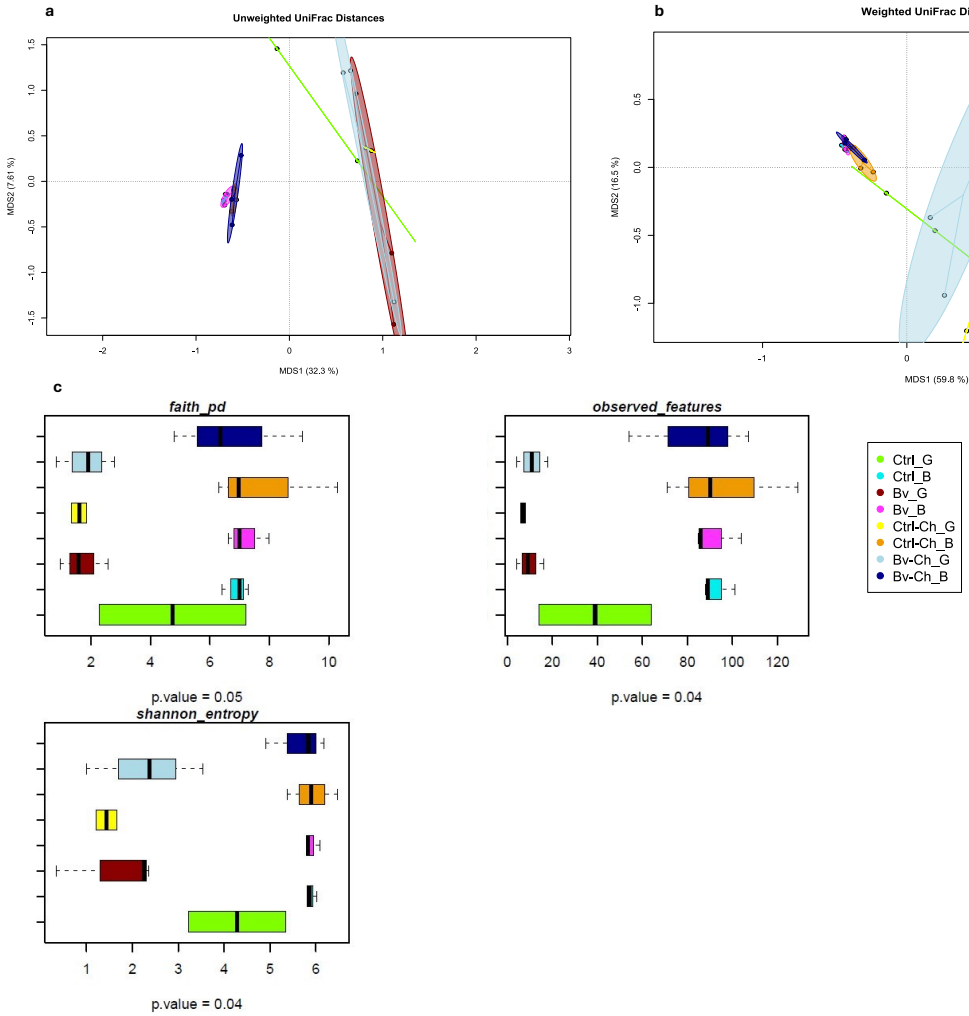

Fig. 5. Beta and alpha diversity of gut and biofilm swab microbial community of fishes tank fed with different diet. (a-b) Beta diversity of biofilm and gut of European seabass fed with different diets. PCoA based on weighted and unweighted UniFrac distances between microbiota layout of gut content (G) and cage biofilm (B). Samples are significantly separated (permutation test with pseudo-F ratios Adonis; $p < 0.001$). (c) Boxplots show alpha diversity values measured by Faith's Phylogenetic Diversity (faith_pd), observed features and Shannon entropy. All metrics displayed significant variation (Kruskal–Wallis test $p < 0.05$) of alpha diversity among groups [diet Ctrl, Bv, Ctrl-Ch, Bv-Ch, in gut (G) and biofilm (B)].

To further investigate the microbial composition, different phylogenetic levels were analyzed. As shown in figure 6.a, at the phylum level, the gut microbiota was predominantly composed by Pseudomonadota, Bacillota, Bacteroidota, Actinomycetota, and Spirochaetota phyla, mean abundance and standard deviation are reported in Supplementary Table 1. In contrast, the biofilm swabs exhibited a higher relative abundance of Bacteroidota, Pseudomonadota, Chloroflexota, Verrucomicrobiota, and Planctomycetota (Supplementary Table 2). Analysis was also conducted at the family level for both intestinal and biofilm samples. Concerning gut composition,

Enterobacteriaceae, Alicyclobacillaceae, Flavobacteriaceae, Saprospiraceae, Spirochaetaceae, Lactobacillaceae, and Streptococcaceae were the mainly present families in Ctrl group. Main families in Ctrl-Bv group were Enterobacteriaceae, Alicyclobacillaceae, Flavobacteriaceae, Spirochaetaceae, Lactobacillaceae, Streptococcaceae, Vibrionaceae, and Lachnospiraceae. Ctrl-Ch group was mainly composed of Spirochaetaceae, Lactobacillaceae, Streptococcaceae, and Lachnospiraceae. Group Ctrl-Bv-Ch has as main families Enterobacteriaceae, Flavobacteriaceae, Spirochaetaceae, Lactobacillaceae, Streptococcaceae, Pseudomonadaceae, and Lachnospiraceae, mean and standard deviation of the most represented families per group with the respective standard deviation are reported in Supplementary Table 3. Regarding the biofilm microbiota at the family level, Chitinophagaceae, Flavobacteriaceae, Hyphomonadaceae, Paracoccaceae, Pirellulaceae, Rubritaleaceae, and Saprospiraceae are the main families present in all groups (Ctrl, Ctrl-Bv, Ctrl-Ch, Ctrl-Bv-Ch) with the respective mean and standard deviation, described in Supplementary Table 4. To more thoroughly assess taxonomic differences at the family level, Linear Discriminant Analysis Effect Size (LEfSe) was performed on both intestinal and biofilm samples. In the gut, Saprospiraceae in the Ctrl group emerged as the only significant discriminant taxon (Fig. 6.b). For the biofilm samples, Phycisphaeraceae and Oxalobacteraceae were identified as discriminant taxa in the Ctrl-Bv group. The Ctrl group was characterized by significant higher abundance of Thermoanaerobaculaceae, Arcobacteraceae, Sphingomonadales_DEV007, Moraxellaceae and Verrucomicrobiales_ 199 families. Finally, the Ctrl-Ch group showed significant enrichment of Propionibacteriaceae, Steroidobacterales_178 family, Pseudomonadaceae, and Sphingomonadaceae (Fig. 6.c).

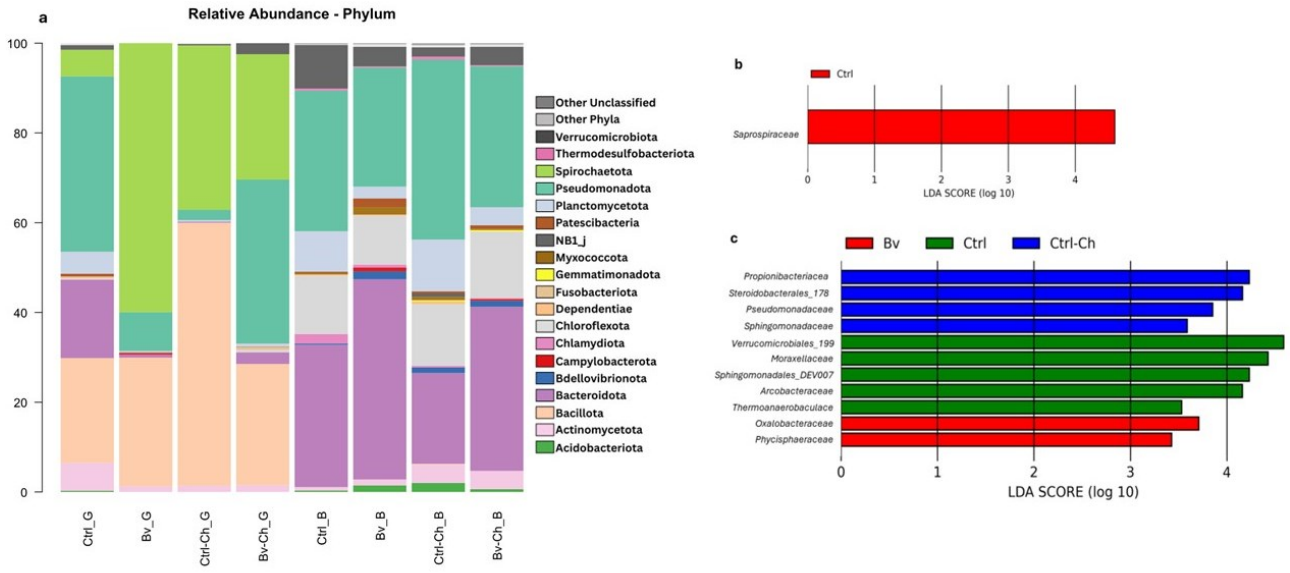

Fig. 6. Microbiota composition of tank biofilm and gut samples of European seabass fed with different diet. (a) Barplot summarizing the microbiota composition at phylum level of tank biofilm swab (B) and fishes' gut (G) divided in study groups (Ctrl, Bv, Ctrl-Ch, Bv-Ch). (b-c) Lefse analysis performed on gut (b) and biofilm (c) composition with LDA score threshold of 1.5 and a $p < 0.05$.

3.4 Histological analysis

Histological analysis of the midgut samples of European seabass feed experimental diets revealed significant changes in goblet cells size and density and intestinal fold morphology following probiotic supplementation and challenge.

The area of goblet cells decreased significantly after the challenge in both the commercial diet group (Ctrl/Ctrl-Ch; $p = 0.0156$) and the probiotic-fed group (Bv/Bv-Ch; $p = 0.0006$) (Fig. 7). In contrast, the number of goblet cells increased post-challenge only in the commercial diet group (Ctrl; p =

0.0002), while the probiotic-fed group (Bv) exhibited a higher baseline count compared to Ctrl (p = 0.0002) (Fig. 7).

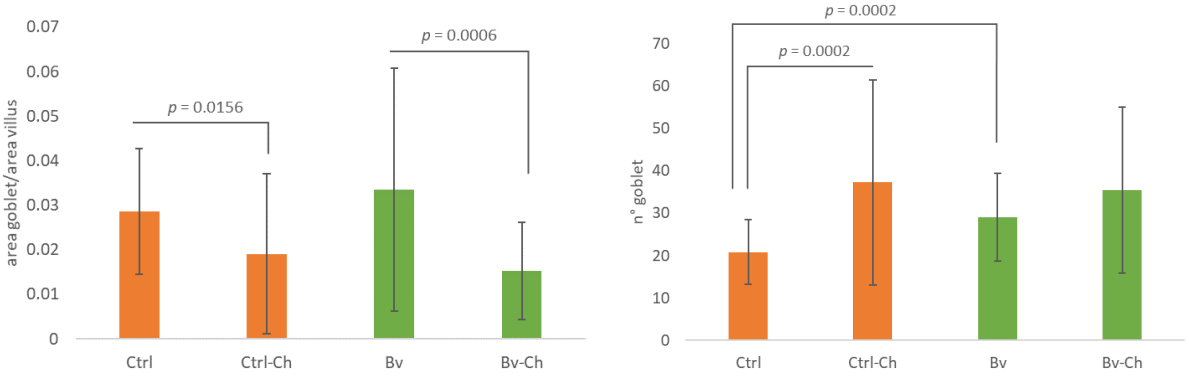

Fig. 7. Goblet cell area and density relative to the fold area in the intestine of European seabass (*D. labrax*) fed control (Ctrl) or probiotic (Bv) diets, pre- and post-challenge.

Fold measurements showed consistent post-challenge increases: length expanded in both the commercial diet (Ctrl-Ch; p < 0.0001) and probiotic-fed (Bv-Ch; p = 0.0001) groups (Fig. 8), while base width increased in all challenged groups (Ctrl-Ch/Bv-Ch; p < 0.0001), with Bv-Ch maintaining a significantly wider base than Ctrl-Ch (p = 0.0104) (Fig. 8). Notably, the probiotic-fed group (Bv) displayed wider fold bases at baseline compared to Ctrl (p < 0.0001).

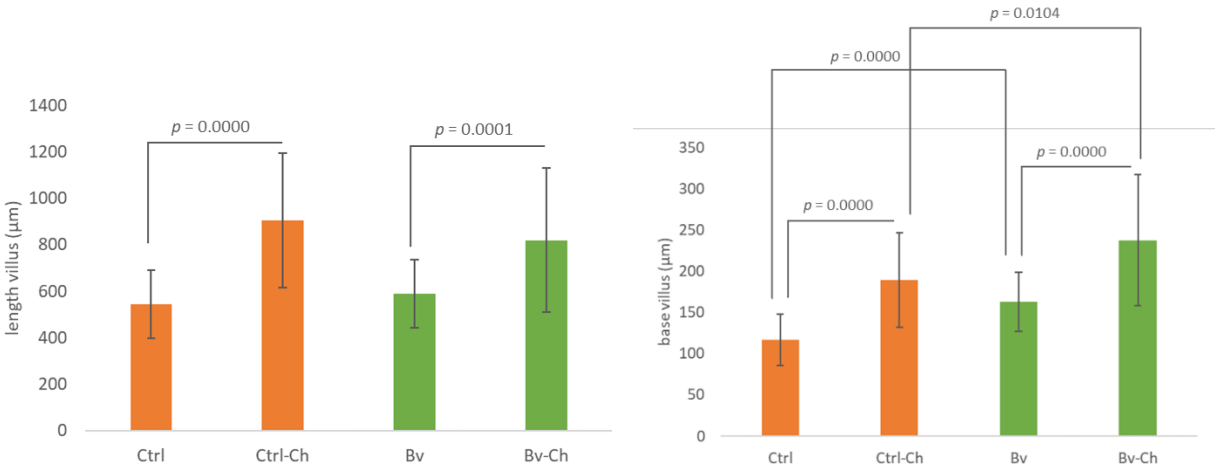

Fig. 8. Fold length and fold base width in the intestine of European seabass (*D. labrax*) fed control (Ctrl) or probiotic (Bv) diets, pre- and post-challenge.

Fold area similarly increased post-challenge in all groups (p < 0.0001), with Bv showing larger baseline areas than Ctrl (p < 0.0001) (Fig. 9). Regarding the fold area, after the challenge it was increased in both cases (without probiotics and with probiotics) (p = 0.0000). The group fed with commercial diet (Ctrl) were found to have a smaller fold area compared to the groups fed only with probiotic diet (Ctrl-Bv) (p = 0.0000) (Fig. 9).

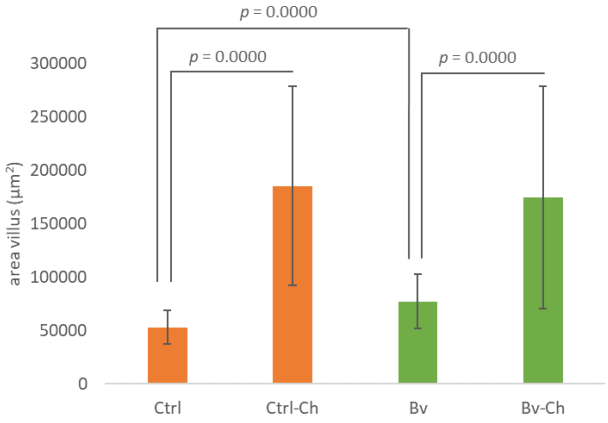

Fig. 9. Fold area in European seabass (*D. labrax*) fed control (Ctrl) or probiotic (Bv) diets, pre- and post-challenge.

4. Discussion

Recent studies have further highlighted the probiotic potential of Bacillus velezensis in aquaculture systems. For instance, Abdelsamad et al. (2024) demonstrated that dietary supplementation with B. velezensis significantly enhanced growth performance, antioxidant enzyme activity, and immune-related gene expression in Pacific white shrimp (*Litopenaeus vannamei*). These findings reinforce the immunomodulatory role of B. velezensis across diverse aquaculture species. Moreover, Hrabar et al. (2025) isolated B. velezensis from the gut microbiota of Mediterranean fish and confirmed its biosynthetic potential through whole genome sequencing. The isolate induced a strong pro-inflammatory cytokine response in peripheral blood leukocytes, suggesting its capacity to prime host immunity. This aligns with our findings of increased goblet cell density and antibacterial activity in European seabass fed with *B. velezensis* D-18. In addition, paraprobiotic forms of *B. velezensis* have shown promise in modulating both innate and adaptive immune responses in vitro and in vivo (Lee et. Al., 2024). These heat-killed preparations may offer a safe and effective alternative in scenarios where live probiotics are not feasible. Collectively, these recent advances support the broader application of *B. velezensis* as a multifunctional probiotic in aquaculture, capable of enhancing host immunity, improving gut morphology, and potentially reducing reliance on antibiotics.

The probiotic *B. velezensis* strain D-18 has been shown to effectively combat pathogenic diseases such as vibriosis in both marine and freshwater fish (Thurlow et al., 2019; Wang et al., 2019; Monzón-Atienza et al., 2021; 2022). Several review articles have highlighted that the use of probiotics in aquaculture offers numerous benefits, such as enhanced growth performance, disease resistance, immune response, overall health, and balanced fish physiological functions (El-Saadony et al., 2021). With the increasing demand for environmentally friendly additives, *Bacillus* spp., along with other probiotics used in aquaculture, have attracted growing interest from researchers due to their significant immunomodulatory effects and role in disease prevention (Amoah et al., 2021; Monzón-Atienza et al., 2022), also because, in recent years, the use of antibiotic treatments has come under scrutiny and been restricted in several countries due to their bioaccumulative properties and the growing issue of antimicrobial resistance, which poses significant risks to both human and animal health (Santos and Ramos, 2018). It is therefore urgent to find alternatives to antibiotics that can fight pathogens. Some of the alternatives to the excessive use of antibiotics in order to deal with pathogens in aquaculture are vaccination and the use of probiotic strains (Torres-Maravilla et al., 2024). In fish, probiotics can be administered through various methods, including bath immersion, suspension, and dietary supplementation. Oral delivery of probiotics has been found to enhance immunity and provide better protection compared to bath immersion (Taoka et al., 2006). In fact, the effectiveness of probiotic microorganisms largely depends on their ability to survive and multiply within the host. For optimal impact, the bacteria should remain metabolically stable and active in the product and endure passage through the intestinal tract in substantial numbers. A feed's probiotic effect is likely to achieve the desired results only if it contains at least 10^6 to 10^7 live probiotic bacteria per gram or

milliliter (Fečkaninová et al., 2017). In this study, every two days, *B. velezensis* D-18 inoculum was prepared and aseptically supplemented to the commercial diet. There are numerous studies on probiotics used in aquaculture, *Bacillus* spp. have seen growing and widespread application in aquaculture as probiotics. Their innate advantages are more pronounced compared to non-spore-forming bacterial strains (Meidong et al., 2018). Most *Bacillus* strains are both aerobic and facultatively anaerobic, allowing them to thrive in diverse environments and effectively compete with potential pathogens (Cutting, 2011). The ability of *Bacillus* species to form endospores enables them to survive under extreme environmental stresses and offers biological solutions to formulation and preservation challenges in large-scale industrial production. This study investigated how the probiotic *B. velezensis* affects European seabass by feeding it for 30 days and then challenging the fish with *V. harveyi*—an emerging pathogen in Mediterranean mariculture (Vendramin et al., 2016; Zhang et al., 2020). The survival rates we recorded at the end of the trial demonstrate that the probiotic *B. velezensis* D-18 has a positive effect on the fish infected by the pathogen, in fact the group fed with this probiotic had a survival of 50%, statistically higher than the survival of 27% registered in the group fed only with commercial diet (fig. 2), as also indicated in a recent study by Li et al. (2019), who used a different strain of *B. velezensis* against *V. harveyi* as a pathogen evaluating the efficacy of this probiotic as resistance, growth and immunity to the hybrid fish *Epinephelus.* Probiotics can modulate various innate immune mechanisms in fish, including enhancing phagocytic activity, stimulating the production of antimicrobial peptides, and promoting the expression of cytokines involved in inflammation and immune regulation (Monzón-Atienza et al., 2022). These effects contribute to a more responsive and balanced immune system, improving the host's ability to resist pathogenic infections. Our results suggest that *B. velezen*sis D-18 may act through some of these pathways; however, the precise mechanisms remain to be elucidated. Further studies are needed to fully characterize how this probiotic interacts with host immune components and to explore its long-term effects under commercial aquaculture conditions.

A fundamental aspect concerns the ability of this pathogen to produce biofilm, in fact the release of *V. harveyi* from biofilms into the rearing water or its contact with the walls of aquaculture tanks may elevate bacterial prevalence in fish, increasing the risk of vibriosis outbreaks. Therefore, monitoring *V. harveyi* abundance in the surrounding environment, such as rearing water and biofilms, could serve as an indicator of European seabass contamination and the likelihood of vibriosis outbreaks. For this reason, in this study we considered both the survival of the fish after the challenge and the composition of the intestinal microbiome and the bacterial composition of the tank biofilm. These types of pathogenic bacteria also can survive independently of a host and readily proliferate within aquaculture systems. Although their transmission strategies are not yet fully understood, it is estimated that approximately 90% of bacteria persist through biofilm formation. This process has been widely recognized as a key mechanism for the survival of pathogenic bacteria (Cai and Arias, 2017; Tasneem et al., 2018). In this study we also wanted to analyze environmental biofilm as both probiotics and pathogenic bacteria can produce it as a survival strategy in the aquaculture environment. Identifying probiotic bacteria capable of both producing bioactive compounds and colonizing aquaculture environments represents a promising strategy for developing new products specifically designed for use in aquaculture. Bacteria from the *Bacillus* genus are well known for their antibacterial and antibiofilm properties, rapid growth rate, low nutritional requirements, and tolerance to anaerobic conditions (Petit et al., 2024). The metagenomic results of the biofilm removed from the walls of the tanks show no differences in the different tanks/groups, the Phylum Bacteroidota is present both in the intestine and in the tanks. This shows that in a short period of treatment as in our case, the biofilm remains a dangerous reservoir of potentially pathogenic bacteria that can remain in the environment in a stable way over time.

A strong correlation exists between gut microbiota composition and the overall health status of fish. An early theory suggested that probiotics played a role in competitively excluding infectious pathogens. Upon entering the host's digestive tract, these beneficial microorganisms were thought to either produce inhibitory compounds or compete with pathogens for adhesion sites, nutrients,

chemicals, or energy sources, thereby hindering their growth or activity (Merrifield et al., 2010; El-Saadony et al., 2021). The gut microbiota—widely recognized for its crucial role in immune function, maintaining homeostasis, enhancing intestinal health, and promoting growth and overall well-being—has become a central focus of research. Cahill (1990) reported that bacteria present in the aquatic environment influence the composition of gut microbiota in aquatic organisms, and this interaction is reciprocal. The gastrointestinal tract of fish harbors a diverse community of resident microorganisms, collectively known as the microbiota, which have co-evolved with the host in a symbiotic relationship, contributing to metabolic functions and defense against pathogenic infections (Vargas-Albores et al., 2021). Numerous studies have linked beneficial shifts in the gut microbiota to probiotic supplementation (Amoah et al., 2021). Our results show that there are no major changes in the composition of the intestinal microbiome after administration of probiotic for a month, nor for the biofilm of the tanks (Fig. 5-6). This aspect is very interesting because it shows that *B. velezensis* works, since the fish is more resistant after having taken it, despite the fact that the microbiome is almost stable. Probably results that indicate more important changes can be obtained after a more prolonged administration over time. In the present study, since the fish showed a higher survival after the challenge, it can be deduced that the probiotic *Bacillus* strains improve and maintain a balanced intestinal microflora, thus enhancing the health and well-being of European seabass as already demonstrated in a work with Nile tilapia (Kuebutornye et al., 2020).

We also evaluated the plasma bactericidal activity and histologically analyzed the intestinal villi and related goblet cells. Regarding the immune parameters analyzed, our results about bactericidal activity show that there is a statistically significant difference between the initial group (T0) and the probiotic-fed and infected group, this confirms what was stated in studies in humans that have demonstrated that administering probiotics from the *Bacilli* class significantly enhances bactericidal activity by promoting the production of bacteriostatic molecules such as hydrogen peroxide and lactic acid (Abdul-Rahim et al., 2021). These molecules exhibit potent antimicrobial effects against a broad spectrum of pathogens, including various multi-antibiotic-resistant species. The bactericidal activity (Fig. 4) observed after the challenge confirms a greater activity of the infected group fed with *B. velezensis* compared to the control, as also demonstrated by the study by Monzón-Atienza et al. (2022) who carried out a test to verify the effects of *B. velezensis* with a challenge with *V. anguillarum*. Moreover, in an activated state, such as that induced by probiotics, goblet cells increase in both number and size, and bactericidal activity levels also tend to increase (Castejón et al., 2021). The modulation of goblet cell dynamics—where probiotic-fed fish maintained higher baseline cell counts but exhibited reduced cell area post-challenge—may indicate a strategic trade-off between mucus secretion efficiency and epithelial coverage. This aligns with findings that *Bacillus*-derived probiotics stimulate goblet cell proliferation while optimizing mucus composition for pathogen exclusion (Khojasteh, 2021; Tonetti et al., 2024). Such adaptations are particularly relevant in RAS, where high stocking densities and biofilm formation increase *Vibrio* transmission risks (Pirarat et al., 2011; Monzón-Atienza et al., 2024). Additionally, the application of histological assays is discussed as a reliable method for assessing fish welfare, with particular attention given to gut morphology as an indicator of nutrient absorption capacity and immune responsiveness (De Marco et al., 2023). The increase in fold dimensions (length and base width) aligns with previous studies demonstrating that probiotics like *B. velezensis* promote intestinal absorptive surface area, thereby improving nutrient utilization and disease resistance (Ramos et al., 2017). Notably, the wider fold base in probiotic-fed fish post-challenge could reflect an adaptive response to *V. harveyi* infection, as intestinal structural integrity is critical for mitigating pathogenic colonization (Kuebutornye et al., 2020).

Collectively, these findings support the potential of *B. velezensis* to enhance European seabass health in RAS environments by reinforcing intestinal morphology and goblet cell-mediated defenses. Future studies could explore the probiotic's impact on *V. harveyi* biofilm disruption and immune gene expression to elucidate additional protective mechanisms.

5. Conclusion

In conclusion, this study provides an assessment of the characteristics of the European seabass gut microbiome and environmental biofilm composition after one month of administration of *Bacillus velezensis* strain D-18, contributing to provide data that extend previous studies. Although further complex mechanisms, such as quorum sensing interference and competitive exclusion within the gut microbiota, our results provide compelling evidence of the increased resistance of European seabass to *V. harveyi* and highlight its potential as an effective probiotic agent to improve disease resistance in intensively farmed European seabass, as confirmed by the increase in the number of goblet cells and antibacterial activity in the probiotic-fed group. Although no changes occurred in the gut microbiome and bacterial content of the biofilm in the tanks, the challenge is to be able to prepare fish from intensive farms through a change in the intestinal microbiome to be able to achieve such resistance that when the disease arrives they can survive without the use of emergency interventions with antibiotics. Further studies will be needed to investigate the mechanisms of the probiotic at the intestinal and environmental biofilm levels, to verify the actual link between increased resistance and changes in the intestinal and environmental microbiome.

Ethics approval

All procedures conducted with the fish agreed to the guidelines of the European Union Council (86/609/EU) and Spanish legislation (RD 53/2013) and were approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (OEBA-ULPGC-11/2024R1). Notably, the number of animals used was determined following a highly restricted f-size a priori effect established at the 0.05 α-error probability on the power analysis accomplished.

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Supplementary tables

Supplementary Table 1				
Mean relative abundance and standard deviation of the most abundant phyla in gut				
	Ctrl	Ctrl-Bv	Ctrl-Ch	Ctrl-Bv-Ch
Pseudomonadota	39.09 ± 6.37	8.63 ± 9.13	2.29 ± 1.85	36.56 ± 17.69
Bacillota	23.36 ± 6.84	28.69 ± 32.66	58.61 ± 52.51	27.05 ± 20.67
Bacteroidota	17.46 ± 1.51	0.65 ± 1.14	0.25 ± 0.35	2.57 ± 2.62
Actinomycetota	6.23 ± 8.81	1.26 ± 1.52	1.39 ± 1.97	1.47 ± 2.14
Spirochaetota	5.901 ± 8.35	59.95 ± 35.92	36.64 ± 51.82	27.92 ± 43.22

Supplementary Table 2				
Mean relative abundance and standard deviation of the most abundant phyla in biofilm				
	Ctrl	Ctrl-Bv	Ctrl-Ch	Ctrl-Bv-Ch
Bacteroidota	31.69 ± 3.21	44.59 ± 5.12	20.22 ± 7.85	36.56 ± 6.99
Pseudomonadota	31.36 ± 3.34	26.56 ± 5.67	40.16 ± 12.94	31.42 ± 9.55
Chloroflexota	13.005 ± 0.34	10.87 ± 5.52	13.77 ± 9.94	14.81 ± 10.19
Verrucomicrobiota	9.72 ± 0.41	4.37 ± 0.66	2.078 ± 1.27	4.09 ± 3.69
Planctomycetota	9.02 ± 2.54	2.62 ± 0.86	11.53 ± 4.55	4.04 ± 2.35

Supplementary Table 3				
Mean relative abundance and standard deviation of the most abundant families in gut				
	Ctrl	Ctrl-Bv	Ctrl-Ch	Ctrl-Bv-Ch
Enterobacteriaceae	16.23 ± 22.95	0.82 ± 1.42	0 ± 0	8.85 ± 15.33
Alicyclobacillaceae	10.25 ± 7.53	1.202 ± 2.08	0 ± 0	0 ± 0
Flavobacteriaceae	8.77 ± 3.13	0.66 ± 1.14	0 ± 0	2.02 ± 2.82
Saprospiraceae	6.06 ± 3.48	0 ± 0	0 ± 0	0 ± 0
Spirochaetaceae	5.90 ± 8.35	59.95 ± 35.92	36.64 ± 51.82	27.92 ± 43.22
Lactobacillaceae	4.59 ± 6.49	1.64 ± 1.43	6.15 ± 8.69	1.80 ± 3.12
Streptococcaceae	4.02 ± 5.68	12.95 ± 15.99	31.80 ± 16.69	12.68 ± 3.12
Vibrionaceae	0 ± 0	4.37 ± 4.01	0 ± 0	0 ± 0
Pseudomonadaceae	0 ± 0	0 ± 0	0 ± 0	15.96 ± 15.74
Lachnospiraceae	0 ± 0	9.62 ± 16.66	19.92 ± 28.17	4.97 ± 8.61

Supplementary Table 4				
Mean relative abundance and standard deviation of the most abundant families in biofilm				
	Ctrl	Ctrl-Bv	Ctrl-Ch	Ctrl-Bv-Ch
Chitinophagaceae	0.38 ± 0.34	6.39 ± 3.78	0.16 ± 0.28	0.27 ± 0.47
Flavobacteriaceae	16.61 ± 1.33	10.82 ± 5.36	14.59 ± 7.20	19.29 ± 16.37
Hyphomonadaceae	2.51 ± 0.76	1.91 ± 1.90	4.37 ± 2.05	4.43 ± 1.89
Paracoccaceae	12.08 ± 1.16	7.98 ± 3.93	10.66 ± 3.04	7.81 ± 0.34
Pirellulaceae	6.77 ± 1.95	1.31 ± 0.71	4.81 ± 1.47	2.08 ± 0.19
Rubritaleaceae	9.62 ± 0.25	4.37 ± 0.66	1.80 ± 1.40	3.72 ± 4.09
Saprospiraceae	9.89 ± 2.83	21.86 ± 3.79	2.89 ± 0.58	13.61 ± 10.05