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"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

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

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

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

84 Among these, identifying probiotic bacteria that can both produce beneficial compounds and
85 effectively colonize aquaculture environments and antagonize potential pathogens circulating in the
86 system, represents a promising strategy for developing new products tailored to aquaculture (Petit et
87 al., 2024). Concerning the target host, probiotics are living microorganisms that, when administered
88 at appropriate doses, can promote a healthy balance of gut bacteria in animals (and humans). This can
89 result in various benefits such as preventing the invasion of harmful pathogens, improving digestion,
90 promoting growth, and increasing survival rates (Ntakirutimana et al., 2023). Probiotics also support
91 gut microbiome stability, improve water quality through bioremediation, and enrich zooplankton
92 nutrients (El-Saadoni et al., 2021). Various microorganisms, including yeast, fungi, and numerous
93 *Bacillus* species, are recognized for their probiotic functions and potential to replace antibiotics
94 (Iannitti and Palmieri, 2010); administrated via food, they can antagonize intestinal pathogenic
95 bacteria and enhance disease resistance (Li et al., 2019, Khan et al., 2013, Monzón-Atienza et al.,
96 2022). In particular *Bacillus velezensis* was first described twenty years ago during extensive research
97 aimed at discovering bacterial strains capable of synthesizing new lipopeptides with surfactant or
98 antimicrobial properties (Ruiz-García et al., 2005). Concerning its possible application in
99 aquaculture, previous studies have isolated *B. velezensis* from waste-water at a Spanish farm (Ruiz-
100 García et al., 2005) and recent studies demonstrated the positive effects of *B. velezensis* strain D18,
101 (Monzón-Atienza et al., 2021), on the resistance of European seabass against *V. anguillarum* after
102 experimental challenge (Monzón-Atienza et al., 2021, 2022).

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

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

108 2. Materials and methods

109 2.1 Bacterial strains

110 The bacterial strain *Vibrio harveyi* ITT 281/14/B was from the repository of the Fish Pathology Unit
111 of the Department of Veterinary Medical Sciences of the University of Bologna (Unibo) and had been
112 isolated during a mortality outbreak in European seabass (*Dicentrarchus labrax*) farmed in Italy. It
113 had been previously identified by standard microbiological methods, including matrix-assisted laser
114 desorption/ionization time-of-flight mass spectrometry (Maldi-TOF, Bruker) and molecular analyses.
115 The probiotic *Bacillus velezensis* strain D-18 had been isolated, identified, and characterized earlier
116 by the Grupo de Investigación en Acuicultura (GIA), Instituto Ecoaqua, Universidad de Las Palmas
117 de Gran Canaria (ULPGC) (Monzón-Atienza et al., 2021).

118 To conduct the present trial, vials containing *V. harveyi* and *B. velezensis* and stored at -80 °C at
119 UNIBO and ULPGC bacterial collections respectively, were defrosted at about 4 °C on ice. Each
120 strain was aseptically cultured in sterile Erlenmeyer flasks containing 50 ml of brain heart infusion
121 (BHI; Cultimed, Panreac, Spain) supplemented with 1.5% sodium chloride (NaCl). Each flask,
122 inoculated with a single colony-forming unit (CFU) of each bacterial strain, was cultured following
123 classical microbiological techniques at 26 °C ± 1 for 24 hours.

124 2.2 Fish and housing

125 A total of 240 European seabass (*Dicentrarchus labrax*) with an average body weight of 180 ± 10 g
126 were housed at the Marine Science and Technology Park at Universidad de las Palmas de Gran
127 Canaria (ULPGC), Spain. The University of Las Palmas's Ethical Committee accepted all
128 experimental protocol. Specimens selected following gross examination were determined to be
129 clinically healthy, exhibiting normal skin and gill coloration, no external lesions or infections, and no
130 documented history of parasitic infestation. For acclimatization, the experimental fish were randomly
131 distributed into twelve 500 L fiber-reinforced tanks (n = 20 fish/tank) in a closed water system at
132 22°C with continuous aeration, a 12:12 h photoperiod, and a water pH of 8 for two weeks. The fish
133 were fed daily at a regular rate calculated as 2 % of their biomass with a commercial diet (D-4
134 Optibream AE 3 P - Alterna, Skretting, Spain) of 4 mm diameter containing 40.5% fish protein and
135 18% fish oil. Before the beginning of the *V. harveyi* exposure, five individuals were randomly
136 selected to undergo standard microscopic and bacteriological tests to ensure they were not infected
137 with pathogenic bacteria. For each sampling, fish were anesthetized using clove oil overdose at
138 concentration of 0.5 mL/L (Guinama S.L., Spain, Ref. Mg83168), diluted in 100% alcohol (1:1).
139
140
141

142 2.3 Feed preparation and experimental design

143 The commercial European seabass feed served as the experimental control diet and as the basal diet
144 for the supplementation with *B. velezensis* D-18 (10^6 CFU x feed g⁻¹), determined
145 spectrophotometrically at an optical density of 600 nm and by counting colony-forming units (CFU).
146 The incorporation process was conducted as follows. Briefly, the probiotic suspension was applied
147 using a spray bottle with the nozzle adjusted to release mist. The diet was mixed slowly in a drum
148 mixer and then air-dried on a clean bench for 12 hours, ensuring sterile conditions throughout the
149 process. The stock diet was stored at -20 °C, and daily rations were thawed at 4 °C before feeding.
150 The viability of the incorporated *B. velezensis* was assessed by vortexing 10 g of diet in 90 ml of
151 sterile PBS and preparing serial dilutions. 100 µl aliquots were cultured at 26 °C for 24 hours
152

153 following classical microbiological procedures. All fish were fed twice daily by hand for 30 days at
 154 a regular rate calculated as 2% of their biomass.
 155 After the two-week acclimation period, each tank, containing 20 fish fed with commercial diet, was
 156 randomly assigned to one of four experimental groups: Group Ctrl: 3 tanks as a control group - fish
 157 fed commercial diet throughout the trial period and not infected, only inoculated with 0,1 mL of
 158 sterile PBS at the day of the challenge; Group Bv: 3 tanks as another control group - fish fed diet with
 159 probiotics (*B. velezensis*) (10^6 CFU x feed g^{-1}) and not infected, only inoculated with 0.1 mL of sterile
 160 PBS at the day of the challenge; Group Ctrl-Ch: 3 tanks for challenge 1 - fish fed commercial diet for
 161 a month and intraperitoneally (IP) infected with *V. harveyi* and Group Bv-Ch: 3 tanks for challenge
 162 2 - fish fed diet with probiotics for a month and IP infected with *V. harveyi* (2×10^4 CFU x mL) (Fig.
 163 3).
 164 After one month of feeding each group with their own diet as per the experimental protocol, groups
 165 Ctrl-Ch and Bv-Ch were challenged and from this point, all groups were fed only a commercial diet
 166 until the end of the trial (Fig. 1).
 167

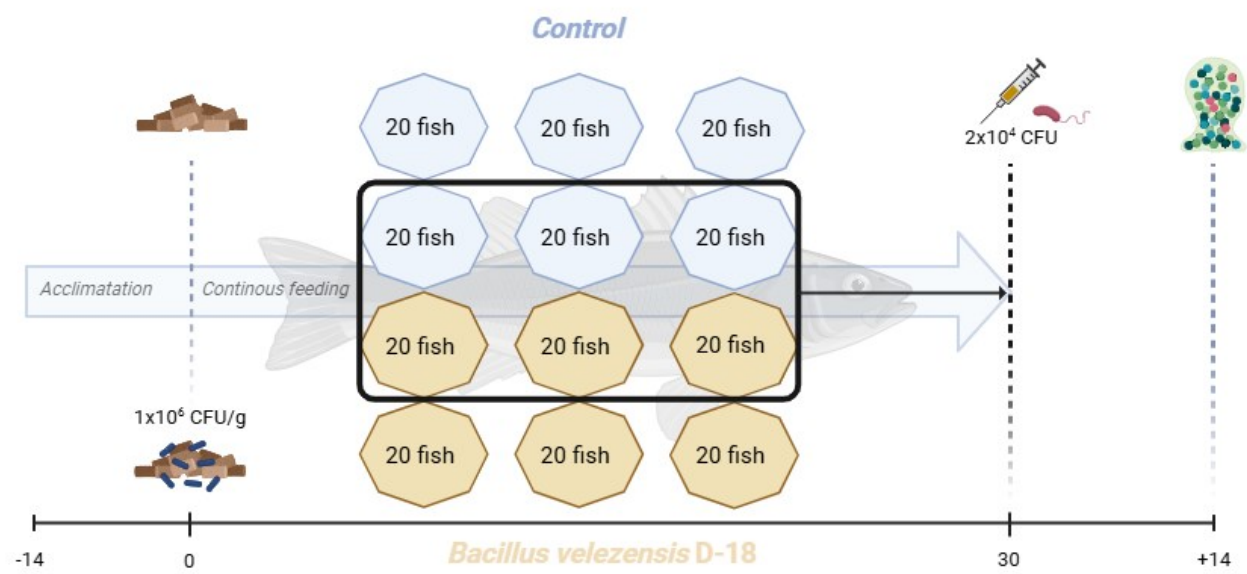


Fig. 1. Scheme of the experimental trial

168
 169
 170
 171 **2.4 Fish sampling**
 172

173 After 6 weeks from the beginning of the experimental trial (2 weeks for acclimation and 4 weeks
 174 of feeding period with experimental diets), all the fish were starved for 24 h. Three fish randomly
 175 collected from the Ctrl and Bv groups (only commercial diet and probiotic diet groups), after 30 days
 176 of feeding with experimental diets, and 14 days after challenge, humanely euthanised by
 177 administering a clove oil overdose at a concentration of 0.5 mL/L (Guinama S.L., Spain, Ref.
 178 Mg83168), diluted in 100% alcohol (1:1), achieving euthanasia within 1 minute and subjected to a
 179 wide set of analyses. After the challenge, the fish were observed and mortality was recorded daily.
 180 After 14 days of observation, the fish were sacrificed with an overdose of anaesthetic and 6 fish per
 181 group were sampled for analysis.
 182

183 **2.5 In vivo challenge test with *Vibrio harveyi***
 184

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

193 With regard to the challenge of fish with bacterial strains, specimens were anesthetized with clove oil
194 (7 mL/100 lt.) and then intraperitoneally injected with 0.1 mL of PBS containing a suspension of *V.*
195 *harveyi* at the LD50 doses indicated by literature (Pujalte et al., 2003; Zhang et al., 2022) and, in the
196 case of the control groups (Ctrl and Bv), only with 0.1 mL of sterile PBS.

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

200

201 **2.6 Serum antibacterial activity**

202

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

209 In a round-bottom 96-well plate, in triplicates, 20 µl of plasma and 20 µl of *V. harveyi* (10^6 CFU/mL)
210 were incubated for 5h at 25°C. Hank's balanced salt solution or sterile PBS instead of plasma was
211 used for positive control. To each well, was added 25 µl of MTT (3-(4,5 dimethyl-2-yl)-2, 5-diphenyl
212 tetrazolium bromide, 1 mg/mL) and incubated for 10 min. at 25°C. After centrifuging at 2,000 x g for
213 10 min, the supernatant was removed and the pellet was dissolved in 200 µl of DMSO (dimethyl
214 sulfoxide). Finally, serum antibacterial activity was evaluated by measuring the absorbance of
215 dissolved format, which is produced by the metabolic activity of *V. harveyi* following the procedure
216 in Chung and Jeffries (1988). The antibacterial activity of plasma is shown in Figure 4. The
217 absorbance was recorded at 570 and 690 nm (final absorbance = Abs. 570 – Abs. 690). The percentage
218 bactericidal capacity is calculated by comparison with the reference sample (positive control).
219 Positive control means 100% of bacterial growth (0% of bactericidal activity).

220

221 **2.7 Gut microbiota and environmental biofilm composition assessment, samples collection,**

222 **DNA extraction and sequencing**

223

224 After sacrifice of the fish, the intestine of 3 fish for group, after 30 days of feeding with experimental
225 diets (before the challenge) at the end of trial (after the challenge), was removed from the visceral
226 mass and approximately 1.5 cm of the medial part was isolated. After opening, it was washed with
227 sterile PBS and the washed content collected in Eppendorf tubes containing 1 mL of RNeasy
228 (Thermo Fisher Scientific, Milan, Italy) for subsequent analyses. At the same time, in addition to the
229 fish gut, the biofilm was sampled from the walls of all the tanks by swabbing them with sterile cotton
230 swab Aptaca. The water level of each tank was briefly lowered by approximately 5 cm. Immediately
231 after taking the sample by swab, it was immersed in 10 mL of sterile PBS with 20 glass beads in the
232 laboratory and subjected to breaking by vortexing. 1 mL of sample was subjected to DNA extraction
233 with DNeasy Power Biofilm kit (QIAGEN, Hilden, Germany), while Total DNA was extracted with
234 briefly adaptation as previously described, using the protocol of DNeasy Blood & Tissue Kit
235 (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 et al., 2005), `cluster` (version 2.1.6, Maechler et 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 et 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 `analisys`® 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.

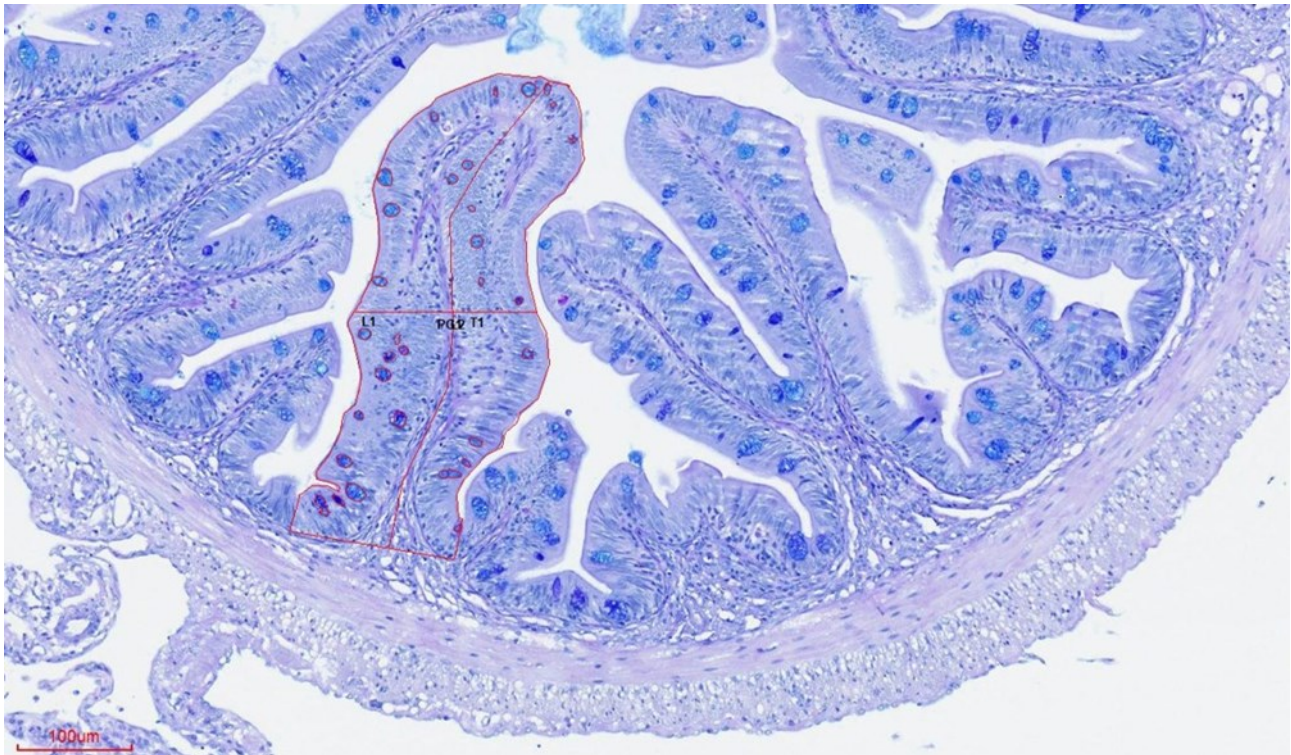


Fig. 2. Representative image of the intestinal measurement process in European seabass fed either a control (Ctrl) or probiotic (Bv) diet, both before and after challenge (Ch). Mucosal fold area, height, and width were manually delineated. Goblet cell number and area within each delineated fold were 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 ($p = < 0.05$).

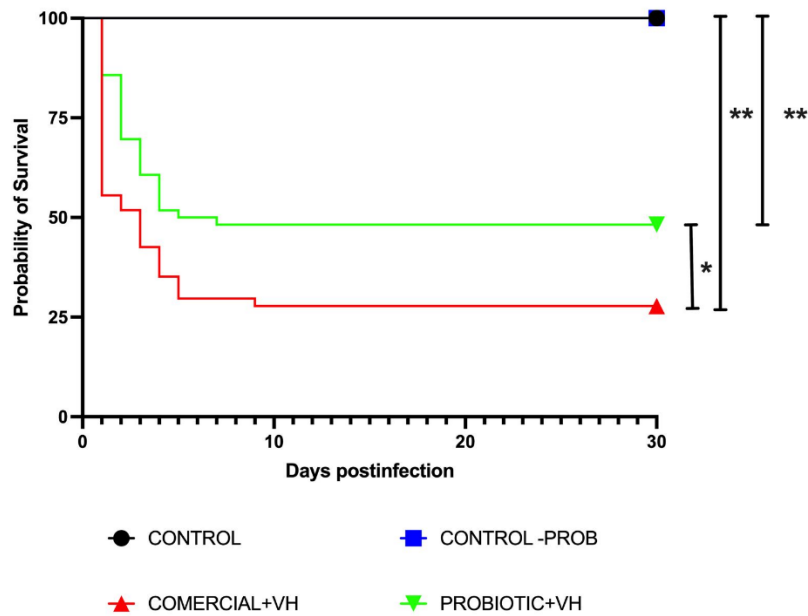


Fig. 3. Effect of the probiotic strain on European seabass survival rate against *V. harveyi*. Asterisks denote significant statistical differences: $p < 0.05$ (*) and $p < 0.001$ (**)

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).

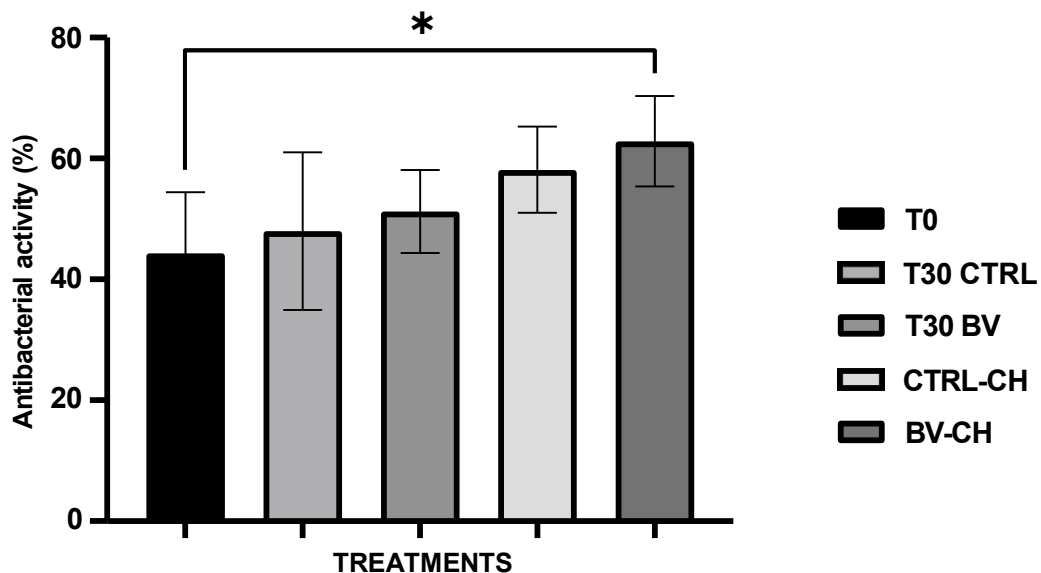
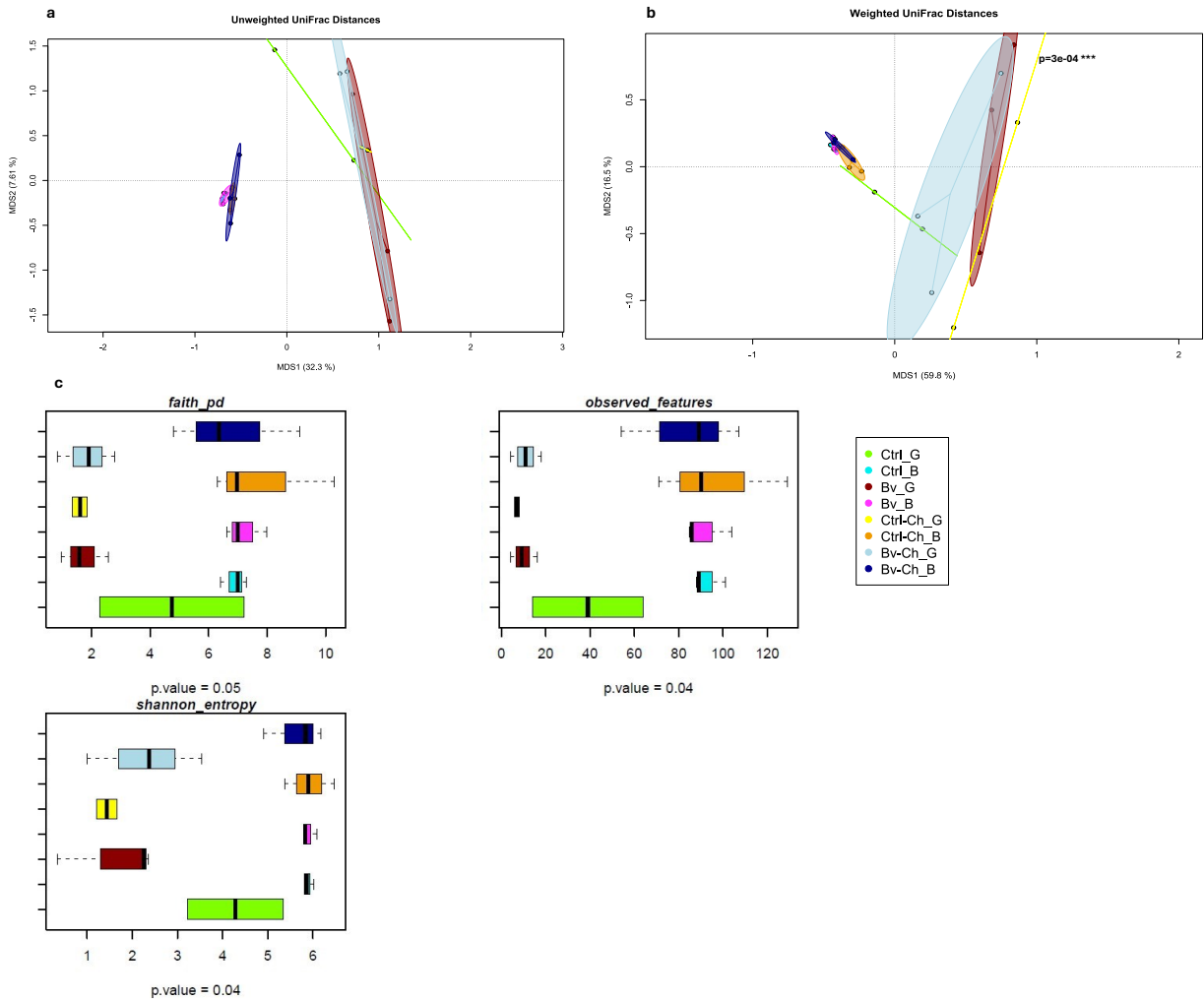


Fig. 4. Antibacterial activity (%) of plasma from different experimental groups

3.3 Gut and biofilm bacterial communities

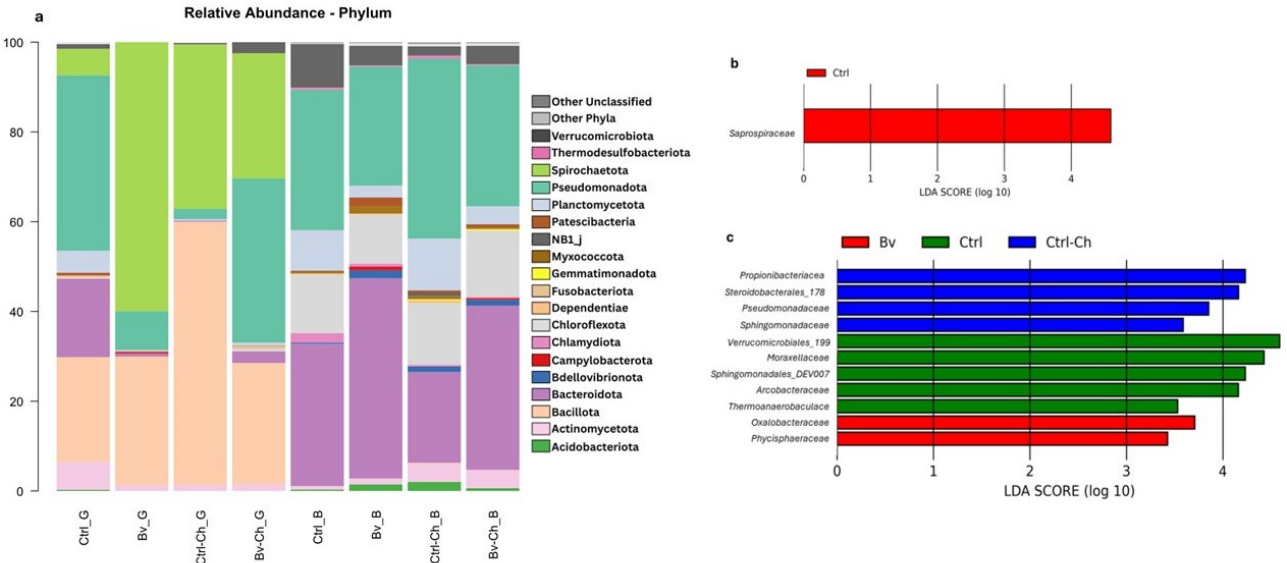
329 To determine whether probiotic supplementation and/or infection influenced the bacterial
 330 composition of the gut and/or biofilm, microbial communities from different sample types were
 331 analysed. Beta diversity was assessed using Principal Coordinate Analysis (PCoA) based on both
 332 unweighted and weighted (Fig. 5. a-b) UniFrac distance metrics. A significant separation in microbial
 333 community structure was observed among all groups ($p < 0.001$). Furthermore, alpha diversity, so the
 334 diversity within groups, differed significantly across groups, as indicated by three diversity metrics:
 335 Faith's Phylogenetic Diversity, Observed Features, and Shannon Entropy (Fig. 5. c). No significant
 336 variations were found for the alpha diversity indices among groups of the same sample types (i.e. fish
 337 gut microbiota or biofilm microbiota assessed in different conditions).
 338
 339



340
 341 Fig. 5. Beta and alpha diversity of gut and biofilm swab microbial community of fishes tank fed with different diet. (a-b) Beta
 342 diversity of biofilm and gut of European seabass fed with different diets. PCoA based on weighted and unweighted UniFrac distances
 343 between microbiota layout of gut content (G) and cage biofilm (B). Samples are significantly separated (permutation test with
 344 pseudo-F ratios Adonis; $p < 0.001$). (c) Boxplots show alpha diversity values measured by Faith's Phylogenetic Diversity (faith_pd),
 345 observed features and Shannon entropy. All metrics displayed significant variation (Kruskal–Wallis test $p < 0.05$) of alpha diversity
 346 among groups [diet Ctrl, Bv, Ctrl-Ch, Bv-Ch, in gut (G) and biofilm (B)].
 347

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

355 Enterobacteriaceae, Alicyclobacillaceae, Flavobacteriaceae, Saprospiraceae, Spirochaetaceae,
 356 Lactobacillaceae, and Streptococcaceae were the mainly present families in Ctrl group. Main families
 357 in Ctrl-Bv group were Enterobacteriaceae, Alicyclobacillaceae, Flavobacteriaceae, Spirochaetaceae,
 358 Lactobacillaceae, Streptococcaceae, Vibrionaceae, and Lachnospiraceae. Ctrl-Ch group was mainly
 359 composed of Spirochaetaceae, Lactobacillaceae, Streptococcaceae, and Lachnospiraceae. Group Ctrl-
 360 Bv-Ch has as main families Enterobacteriaceae, Flavobacteriaceae, Spirochaetaceae,
 361 Lactobacillaceae, Streptococcaceae, Pseudomonadaceae, and Lachnospiraceae, mean and standard
 362 deviation of the most represented families per group with the respective standard deviation are
 363 reported in Supplementary Table 3. Regarding the biofilm microbiota at the family level,
 364 Chitinophagaceae, Flavobacteriaceae, Hyphomonadaceae, Paracoccaceae, Pirellulaceae,
 365 Rubritaleaceae, and Saprospiraceae are the main families present in all groups (Ctrl, Ctrl-Bv, Ctrl-
 366 Ch, Ctrl-Bv-Ch) with the respective mean and standard deviation, described in Supplementary Table
 367 4. To more thoroughly assess taxonomic differences at the family level, Linear Discriminant Analysis
 368 Effect Size (LEfSe) was performed on both intestinal and biofilm samples. In the gut, Saprospiraceae
 369 in the Ctrl group emerged as the only significant discriminant taxon (Fig. 6.b). For the biofilm
 370 samples, Phycisphaeraceae and Oxalobacteraceae were identified as discriminant taxa in the Ctrl-Bv
 371 group. The Ctrl group was characterized by significant higher abundance of
 372 Thermoanaerobaculaceae, Arcobacteraceae, Sphingomonadales_DEV007, Moraxellaceae and
 373 Verrucomicrobiales_199 families. Finally, the Ctrl-Ch group showed significant enrichment of
 374 Propionibacteriaceae, Steroidobacterales_178 family, Pseudomonadaceae, and Sphingomonadaceae
 375 (Fig. 6.c).



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

384 Histological analysis of the midgut samples of European seabass feed experimental diets revealed
 385 significant changes in goblet cells size and density and intestinal fold morphology following probiotic
 386 supplementation and challenge.
 387 The area of goblet cells decreased significantly after the challenge in both the commercial diet group
 388 (Ctrl/Ctrl-Ch; $p = 0.0156$) and the probiotic-fed group (Bv/Bv-Ch; $p = 0.0006$) (Fig. 7). In contrast,
 389 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).

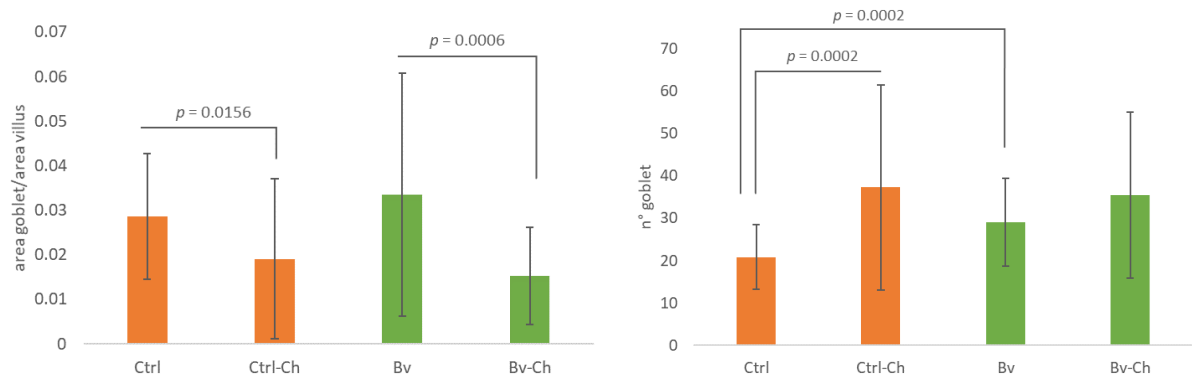


Fig. 7. Goblet cell area and density relative to the fold area in the intestine of European seabass (*D. labrax*) fed control (Ctrl) or probiotic (Bv) diets, pre- and post-challenge.

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 ($p < 0.0001$).

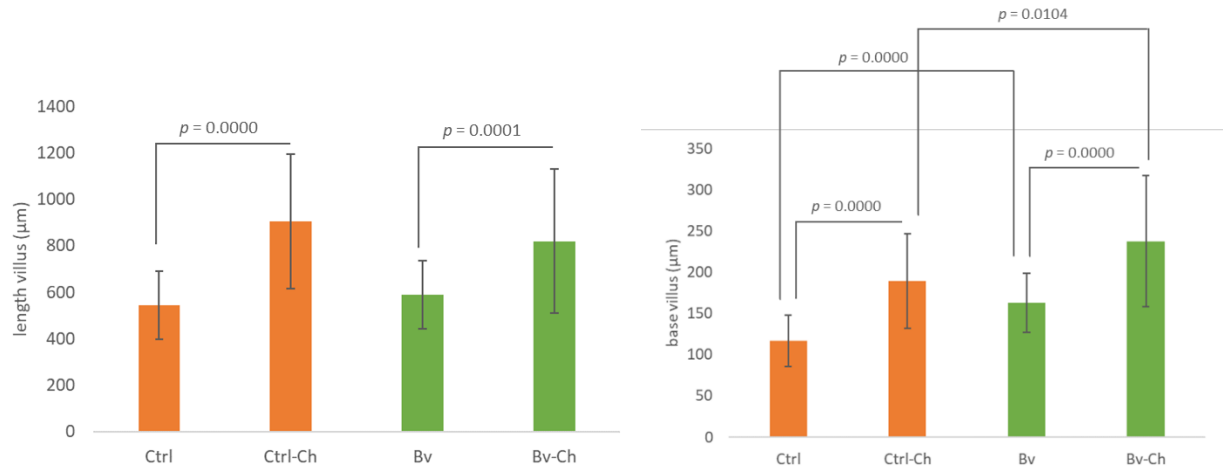


Fig. 8. Fold length and fold base width in the intestine of European seabass (*D. labrax*) fed control (Ctrl) or probiotic (Bv) diets, pre- and post-challenge.

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).

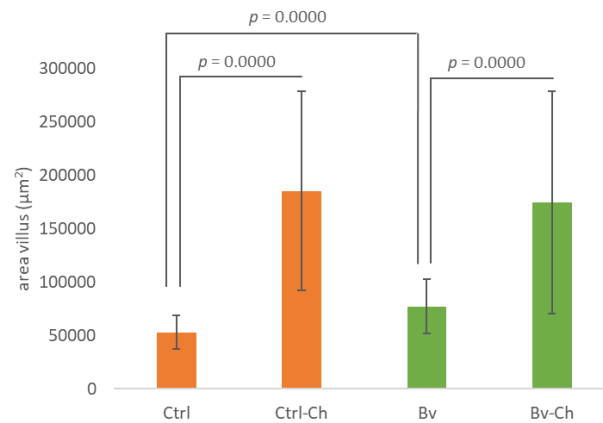


Fig. 9. Fold area in European seabass (*D. labrax*) fed control (Ctrl) or probiotic (Bv) diets, pre- and post-challenge.

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. velezensis* 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

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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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References

- Abdelsamad, A.E.M., Said, R.E.M., Assas, M., Gaafar, A.Y., Hamouda, A.H., Mahdy, A., 2024. Effects of dietary supplementation with *Bacillus velezensis* on the growth performance, body composition, antioxidant, immune-related gene expression, and histology of Pacific white shrimp, *Litopenaeus vannamei*. BMC Veterinary Research 20(1):368. <https://doi.org/10.1186/s12917-024-04207-4>
- Abebe, G.M., 2020. The role of bacterial biofilm in antibiotic resistance and food contamination. International Journal of Microbiology (281). <https://doi.org/10.1155/2020/1705814>
- Acosta, F., Montero, D., Izquierdo, M., Galindo-Villegas, J., 2021. High-level biocidal products effectively eradicate pathogenic γ -proteobacteria biofilms from aquaculture facilities. Aquaculture 532:736004. <https://doi.org/10.1016/j.aquaculture.2020.736004>
- Amoah, K., Dong, X., Tan, B., Zhang, S., Chi, S., Yang, Q., Liu, H., Yang, Y., Zhang, H., 2021. Effects of three probiotic strains (*Bacillus coagulans*, *B. licheniformis* and *Paenibacillus polymyxa*) on growth, immune response, gut morphology and microbiota, and resistance against *Vibrio harveyi* of northern whittings, *Sillago sihama* Forssk'al (1775). Animal Feed Science and Technology 277, 114958. <https://doi.org/10.1016/j.anifeedsci.2021.114958>
- Assefa, A., Abunna, F., 2018. Maintenance of Fish Health in Aquaculture: Review of Epidemiological Approaches for Prevention and Control of Infectious Disease of Fish. Veterinary Medicine International 5432497. <https://doi.org/10.1155/2018/5432497>
- Austin, B., 2010. Vibrios as causal agents of zoonoses. Veterinary Microbiology 140, 310–317. <https://doi.org/10.1016/j.vetmic.2009.03.01>
- Bengtsson, H., Baath R., 2025. matrixStats: Functions that Apply to Rows and Columns of Matrices (and Vectors). R package version 1.5.0. <https://cran.rproject.org/web/packages/matrixStats/index.html>
- Biagi, E., D'Amico, F., Soverini, M., Angelini, V., Barone, M., Turrone, S., Rampelli, S., Pari, S., Brigidi, P., Candela, M., 2019. Faecal bacterial communities from Mediterranean loggerhead sea turtles (*Caretta caretta*). Environmental Microbiology Report 11, 361–371. <https://doi.org/10.1111/1758-2229.12683>
- Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K. Bin, Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciorek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A. V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Priesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W.,

- Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* 2. <https://doi.org/10.1038/s41587-019-0209-9>
- Branda, S.S., Vik, Å., Friedman, L., Kolter, R., 2005. Biofilms: the matrix revisited. *Trends in Microbiology* 13(1):20-6. <https://doi.org/10.1016/j.tim.2004.11.006>
- Cahill, M.M., 1990. Bacterial flora of fishes - A Review. *Microbial Ecology* 19:21-41.
- Cai, W., Arias, C.R., 2017. Biofilm formation on aquaculture substrates by selected bacterial fish pathogens. *Journal of Aquatic Animal Health* 29:95–104 doi: <https://doi.org/10.1080/08997659.2017.1290711>
- Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* 13, 581–583. <https://doi.org/10.1038/nmeth.3869>
- Castejón, P., Cabas, I., Gómez, V., Chaves-Pozo, E., Cerezo-Ortega, I., Morinigo, M.A., Martínez-Manzanares, E., Galindo-Villegas, J., García-Ayala, A., 2021. Vaccination of gilthead seabream after continuous xenoestrogen oral exposure enhances the gut endobolome and immune status via GPER1, *Frontiers in Immunology* 12, 742827, <https://doi.org/10.3389/fimmu.2021.742827>
- Chen, J., Lu, Y., Ye, X., Emam, M., Zhang, H., Wang, H., 2020. Current advances in *Vibrio harveyi* quorum sensing as drug discovery targets. *European Journal of Medicinal Chemistry* 207, 112741. <https://doi.org/10.1016/j.ejmech.2020.1127417>
- Chung, S.G., Jeffries, A.H., 1988. A novel assay to detect macrophage bactericidal activity in fish ; factors influencing the killing of *Aeromonas salmonicida*. *Journal of Fish Diseases*, Wiley Online Library.
- Culhane, A.C., Thioulouse, J., Perrière, G., Higgins, V.J., 2005. made4: Multivariate analysis of gene expression data. R package version 1.78.0. Bioconductor. <http://bioconductor.org/packages/made>
- Cutting, S.M., 2011. *Bacillus* probiotics. *Food Microbiology* 28, 214–220. <https://doi.org/10.1016/j.fm.2010.03.007>
- Dadar, M., Dhama, K., Vakharia, V.N., Hoseinifar, S.H., Karthik, K., Tiwari, R., Khandia, R., Munjal, A., Salgado-Miranda, C., Joshi, S.K., 2017. Advances in aquaculture vaccines against fish pathogens: global status and current trends. *Reviews in Fisheries Science & Aquaculture* 25, 184–217. DOI: <https://doi.org/10.1080/23308249.2016.1261277>
- De Marco, G., Cappello, T., Maisano, M., 2021. Histomorphological Changes in Fish Gut in Response to Prebiotics and Probiotics Treatment to Improve Their Health Status: A Review. *Animals* 13, 2860. <https://doi.org/10.3390/ani13182860>
- De Silva, L.A.D.S., Heo, G-J., 2023. Biofilm formation of pathogenic bacteria isolated from aquatic animals. Vol.:(0123456789)1 3 *Archives of Microbiology* 205:36 <https://doi.org/10.1007/s00203-022-03332-8>

686 Dragulescu, A.A., Arendt, C., 2020. xlsx: Read, Write, Format Excel 2007 and Excel
687 97/2000/XP/2003 Files. R package version 0.6.5. <https://CRAN.R-project.org/package=xlsx>
688

689 El-Saadony, M.T., Alagawany, M., Patra, A.K., Kar, I., Tiwari, R., Dawood, M.A.O., Dhama, K.,
690 Abdel-Latif, H.M.R., 2021. The functionality of probiotics in aquaculture: An overview. *Fish and*
691 *Shellfish Immunology* 117, 36–52. <https://doi.org/10.1016/j.fsi.2021.07.007>
692

693 EU, 2006. Ban on antibiotics as growth promoters in animal feed enters into effect. Regulation.
694 Brussels, Belgium, 1.
695 [https://ec.europa.eu/commission/presscorner/detail/en/IP_05_1687#:~:text=An%20EU-](https://ec.europa.eu/commission/presscorner/detail/en/IP_05_1687#:~:text=An%20EU-wide%20ban%20on%20the%20use%20of%20antibiotics,phasing%20out%20of%20antibiotics%20used%20for%20non-medicinal%20purposes)
696 [wide%20ban%20on%20the%20use%20of%20antibiotics,phasing%20out%20of%20antibiotics%20](https://ec.europa.eu/commission/presscorner/detail/en/IP_05_1687#:~:text=An%20EU-wide%20ban%20on%20the%20use%20of%20antibiotics,phasing%20out%20of%20antibiotics%20used%20for%20non-medicinal%20purposes)
697 [used%20for%20non-medicinal%20purposes](https://ec.europa.eu/commission/presscorner/detail/en/IP_05_1687#:~:text=An%20EU-wide%20ban%20on%20the%20use%20of%20antibiotics,phasing%20out%20of%20antibiotics%20used%20for%20non-medicinal%20purposes)
698

699 FAO, 2024. The State of World Fisheries and Aquaculture 2024 – Blue Transformation in action.
700 Rome. <http://doi.org/10.4060/cd0683en>
701

702 Farmer, J.J. III, Janda, J.M., Brenner, F.W., Cameron, D.N., Birkhead, K.M., 2005. Genus 1. *Vibrio*
703 Pacini 1854, 411AL. In: Brenner DJ, Krieg NR, Staley JT (eds) *Bergey's manual of systematic*
704 *bacteriology*, 2nd edn. The Proteobacteria part B. The Gammaproteobacteria. Springer, New York,
705 pp 494–546
706

707 Fečkaninová, A., Koščová, J., Mudroňová, D., Popelka, P., Toropilová, J., 2017. The use of probiotic
708 bacteria against *Aeromonas* infections in salmonid aquaculture. *Aquaculture* 469, 1–8.
709 <https://doi.org/10.1016/j.aquaculture.2016.11.042>
710

711 Firimino, J., Furones, M.D., Andree, K.B., Sarasquete, C., Ortiz-Delgado, J.B., Asencio-Alcudia, G.,
712 Gispert, E., 2019. Contrasting outcomes of *Vibrio harveyi* pathogenicity in gilthead seabream, *Sparus*
713 *aurata* and European seabass, *Dicentrarchus labrax*. *Aquaculture* 511, 734210.
714 <https://doi.org/10.1016/j.aquaculture.2019.734210>
715

716 Grimes, D.J., Gruber, S.H., May, E.B., 1985. Experimental infection of lemon sharks, *Negaprion*
717 *brevirostris* (Poey), with *Vibrio* species. *Journal of Fish Diseases* 8:173–180.
718 <https://doi.org/10.1111/j.1365-2761.1985.tb01212.x>
719

720 Guo, Y., Zhou, J., Tang, Y., Ma, Q., Zhang, J., Ji, C., Zhao, L., 2020. Characterization and genome
721 analysis of a zearalenone-degrading *Bacillus velezensis* strain ANSB01E. *Current Microbiology*
722 77:273–278. <https://doi.org/10.1007/s00284-019-01811-8>
723

724 Hrabar, J., Babić, I., Jozić, S., Trumbić, Ž., Pioppi, A., Nielsen, L.J.D., Maravić, A., Tomašević, T.,
725 Kovacs, Á.T., Mladineo, I., 2025. Prospecting microbiota of Adriatic fish: *Bacillus velezensis* as a
726 potential probiotic candidate. *Animal Microbiome* 7(1):64. [https://doi.org/10.1186/s42523-025-](https://doi.org/10.1186/s42523-025-00429-5)
727 [00429-5](https://doi.org/10.1186/s42523-025-00429-5)
728

729 Iannitti, T., Palmieri, B. 2010. Therapeutical use of probiotic formulations in clinical practice.
730 *Clinical Nutrition* 29, 701–725. <https://doi.org/10.1016/j.clnu.2010.05.004>
731

732 Ina-Salwany, M.Y., Al-Saari, N., Mohamad, A., Mursidi, F.A., Mohd-Aris, A., Amal, M.N.A., Kasai,
733 H., Mino, S., Sawabe, T., Zamri-Saad, M., 2019. Vibriosis in Fish: A Review on Disease
734 Development and Prevention. *Journal of Aquatic Animal Health* 31:3–22.
735 <https://doi.org/10.1002/aah.10045>
736

- Islam, S.M.M., Rohani, Md F., Shahjahan, M.D., 2021. Probiotic yeast enhances growth performance of Nile tilapia (*Oreochromis niloticus*) through morphological modifications of intestine. Aquaculture Reports 21, 100800. <https://doi.org/10.1016/j.aqrep.2021.100800>
- Khalid, F., Halid, A., Fu, Y., Hu, Q., Zheng, Y., Khan, S., Wang, Z., 2021. Potential of *Bacillus velezensis* as a probiotic in animal feed: a review. Journal of Microbiology 59(7):627-633. <https://doi.org/10.1007/s12275-021-1161-1>
- Khan, A., Ghosh, K., 2013. Evaluation of phytase production by fish gut bacterium, *Bacillus subtilis*, for processing of ipomea aquatica leaves as probable aquafeed ingredient, Journal of Aquatic Food Product Technology 22, 508–519. <https://doi.org/10.1080/10498850.2012.669032>
- Khojasteh, S.M.B., 2012. The morphology of the post-gastric alimentary canal in teleost fishes: a brief review, International Journal of Aquatic Science 3, 71–88. https://www.journal-aquaticscience.com/article_73560_aa8aabb2621b0eefe2b65afa5746ef2.pdf
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research, Volume 41, Issue 1, Page e1, <https://doi.org/10.1093/nar/gks808>
- Kuebutornye, F.A.K., Wang, Z., Lu, Y., Abarike, E.D., Sakyi, M.E., Li, Y., Xie, C.X., 2020. Effects of three host-associated *Bacillus* species on mucosal immunity and gut health of Nile tilapia, *Oreochromis niloticus* and its resistance against *Aeromonas hydrophila* infection. Fish and Shellfish Immunology 97:83-95. <https://doi.org/10.1016/j.fsi.2019.12.046>
- Korun, J., Timur, G., 2008. Marine vibrios associated with diseased sea bass (*Dicentrarchus labrax*) in Turkey. Journal of Survey of Fisheries Science 2, 66-76. <http://dx.doi.org/10.3153/jfscom.2008007>
- Jacques, M., Aragon, V., Tremblay, Y.D.N., 2010. Biofilm formation in bacterial pathogens of veterinary importance. Animal Health Research Reviews 11:97–121. <https://doi.org/10.1017/s1466252310000149>
- Lee, H.-J., Tran, M.T.H., Le, M.H., Justine, E.E., Kim, Y.-J., 2024. Paraprobiotic derived from *Bacillus velezensis* GV1 improves immune response and gut microbiota composition in cyclophosphamide-treated immunosuppressed mice. Frontiers in Immunology 15:1285063. <https://doi.org/10.3389/fimmu.2024.1285063>
- Li, J., Wu, Z.-B., Zhang, Z., Qu, S.-Y., Qi, X.-Z., Wang, G.-X., Ling, F., 2019. Effects of potential probiotic *Bacillus velezensis* K2 on growth, immunity and resistance to *Vibrio harveyi* infection of hybrid grouper (*Epinephelus lanceolatus* ♂ × *E. fuscoguttatus* ♀). Fish and Shellfish Immunology 93, 1047–1055. <https://doi.org/10.1016/j.fsi.2019.08.047>
- Maechler, M., Rousseeuw, P., Struyf, A., Hubert, M., Hornik, K., 2025. cluster: Cluster Analysis Basics and Extensions. R package version 2.1.6. <https://CRAN.R-project.org/package=cluster>
- Martinez Arbizu, P., 2020. pairwiseAdonis: Pairwise multilevel comparison using Adonis. R package version 0.4.1. <https://cran.r-project.org/web/packages/pairwise/index.html>

788 Meidong, R., Khotchanalekha, K., Doolgindachbaporn, S., Nagasawa, T., Nakao, M., Sakai, K.,
789 Tongpim, S., 2018. Evaluation of probiotic *Bacillus aerius* B81e isolated from healthy hybrid catfish
790 on growth, disease resistance and innate immunity of *Plamong Pangasius bocourti*, Fish and Shellfish
791 Immunology 73, 1–10. <https://doi.org/10.1016/j.fsi.2017.11.032>
792
793 Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bøgwald, J., Castex, M.,
794 Ringø, E., 2010. The current status and future focus of probiotic and prebiotic applications for
795 salmonids. Aquaculture 302, 1–18. <http://dx.doi.org/10.1016/j.aquaculture.2010.02.007>
796
797 Mohamad, N., Amal, M.N.A., Yasin, I.S.M., Zamri Saad, M., Nasruddin, N.S., Al-saari, N., Mino,
798 S., Sawabe, T., 2019. Vibriosis in Cultured Marine Fishes: A Review. Aquaculture 512, 734289.
799 DOI: 10.1016/j.aquaculture.2019.734289
800
801 Monzón-Atienza, L., Bravo, J., Torrecillas, S., Montero, D., González-de Canales, A. F., García de
802 la Banda, I., Galindo-Villegas, J., Ramos-Vivas, J., Acosta, F., 2021. Isolation and Characterization
803 of a *Bacillus velezensis* D-18 strain, as a Potential Probiotic in European Seabass Aquaculture.
804 Probiotics and Antimicrobial Proteins 13:1404–1412. <https://doi.org/10.1007/s12602-021-09782-8>
805
806 Monzón- Atienza, L., Bravo, J., Fernández-Montero, A., Charlie-Silva, I., Montero, D., Ramos-
807 Vivas, J., Galindo-Villegas, J., Acosta, F., 2022. Dietary supplementation of *Bacillus velezensis*
808 improves *Vibrio anguillarum* clearance in European sea bass by activating essential innate immune
809 mechanisms. Fish and Shellfish Immunology 124, 244–253. <https://doi.org/10.1016/j.fsi.2022.03.032>
810
811 Monzón-Atienza, L., Bravo, J., Torrecillas, S., Gómez-Mercader, A., Montero, D., Ramos-Vivas, J.,
812 Galindo-Villegas, J., Acosta, F., 2024. An In-Depth Study on the Inhibition of Quorum Sensing by
813 *Bacillus velezensis* D-18: Its Significant Impact on *Vibrio* Biofilm Formation in Aquaculture.
814 Microorganisms 12(5):890. <https://doi.org/10.3390/microorganisms12050890>
815
816 Mougín, J., Roquigny, R., Flahaut, C., Bonnin-Jusserand, M., Grard, T., Le Bris, C., 2021. Abundance
817 and spatial patterns over time of Vibrionaceae and *Vibrio harveyi* in water and biofilm from a seabass
818 aquaculture facility. Aquaculture 542, 736862. <https://doi.org/10.1016/j.aquaculture.2021.736862>
819
820 Musella, M., Wathsala, R., Tavella, T., Rampelli, S., Barone, M., Palladino, G., Biagi, E., Brigidi, P.,
821 Turróni, S., Franzellitti, S., Candela, M., 2020. Tissue-scale microbiota of the Mediterranean mussel
822 (*Mytilus galloprovincialis*) and its relationship with the environment. Science of the Total
823 Environment 717. <https://doi.org/10.1016/j.scitotenv.2020.137209>
824
825 Neuwirth, E., 2022. RColorBrewer: ColorBrewer Palettes. R package version 1.1-3. [https://CRAN.R-](https://CRAN.R-project.org/package=RColorBrewer)
826 [project.org/package=RColorBrewer](https://CRAN.R-project.org/package=RColorBrewer)
827
828 Ntakirutimana, R., Syanya, F.J., Mwangi, P., 2023. Exploring the Impact of Probiotics on the Gut
829 Ecosystem and Morpho-Histology in Fish: Current Knowledge of Tilapia. Asian Journal of Fisheries
830 and Aquatic Research 25(3), pp.93-112. <https://doi.org/10.9734/ajfar/2023/v25i3670>
831
832 Oksanen, J., Blanchet, F.G., Friendly, M., De Cáceres, M., Legendre, P., McGlinn, D., Minchin, P.R.,
833 O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2022. vegan:
834 Community Ecology Package. R package version 2.6-2. <https://CRAN.R-project.org/package=vegan>
835
836 Petit, C., Caudal, F., Taupin, L., Dufour, A., Le Ker, C., Giudicelli, F., Rodriguez, S., Bazire, A.,
837 2024. Antibiofilm Activity of the Marine Probiotic *Bacillus subtilis* C3 Against the

- Aquaculture-Relevant Pathogen *Vibrio harveyi*. Probiotics and Antimicrobial Proteins 17:1551–1562. <https://doi.org/10.1007/s12602-024-10229-z>
- Pirarat, N., Pinpimai, K., Endo, M., Katagiri, T., Ponpornpisit, A., Chansue, N., Maita, M., 2011. Modulation of intestinal morphology and immunity in Nile tilapia (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG. Research in Veterinary Science 91(3): e92-7.doi: 10.1016/j.rvsc.2011.02.014
- Pujalte, M.J., Sitjà-Bobadilla, A., Macián, M.C., Belloch, C., Álvarez-Pellitero, P., Pérez-Sánchez, J., Uruburu, F., Garay, E., 2003. Virulence and Molecular Typing of *Vibrio harveyi* Strains Isolated from Cultured Dentex, Gilthead Sea Bream and European Sea Bass. Systemic and Applied Microbiology 26, 284–292. <https://doi.org/10.1078/072320203322346146>
- Quast, C., Klindworth, A., Pruesse, E., Schweer, T., Horn, M., Glo, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies 41, 1–11. <https://doi.org/10.1093/nar/gks808>
- Rahman, Md.A., Ashrafudoulla, Md., Akter, S., Park, S.H., Ha, S.D., 2023. Probiotics and biofilm interaction in aquaculture for sustainable food security: A review and bibliometric analysis. Critical Reviews in Food Science and Nutrition 1–17. <https://doi.org/10.1080/10408398.2023.2249114>
- Ramos, M.A., Batista, S., Pires, M.A., Silva, A.P., Pereira, L.F., Saavedra, M.J., Ozório, R.O.A., Rema, P., 2017. Dietary probiotic supplementation improves growth and the intestinal morphology of Nile tilapia. Animal 11, Issue 8, Pages 1259-1269. <https://doi.org/10.1017/S1751731116002792>
- Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: A versatile open source tool for metagenomics. PeerJ 2016, e2584. <https://doi.org/10.7717/peerj.2584>
- Ruiz-García, C., Béjar, V., Martínez-Checa, F., Llamas, I., Quesada, E. 2005. *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Vélez in Málaga, southern Spain. International Journal of Systematic Evolutionary Microbiology 55, 191–195. <https://doi.org/10.1099/ijs.0.63310-0>
- Salogni, C., Bertasio, C., Accini, A., Gibelli, L.R., Pigoli, C., Susini, F., Podavini, E., Scali, F., Varisco, G., Alborali, G.L., 2024. The Characterisation of *Lactococcus garvieae* Isolated in an Outbreak of Septicaemic Disease in Farmed Sea Bass (*Dicentrarchus labrax*, Linnaeus 1758) in Italy. Pathogens 13, no. 1: 49. <https://doi.org/10.3390/pathogens13010049>
- Santos, L., Ramos, F., 2018. Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. International Journal of Antimicrobial Agents 52, 135–143. <https://doi.org/10.1016/j.ijantimicag.2018.03.010>
- Sarkar, P., Issac, P.K., Raju, S.V., Elumalai, P., Arshad, A., Arockiaraj, J., 2021. Pathogenic bacterial toxins and virulence influences in cultivable fish. Aquaculture Research :1–16. <http://dx.doi.org/10.1111/are.15089>
- Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, E., Garrett, W. S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biology 12(6), R60. <https://doi.org/10.1186/gb-2011-12-6-r60>

888 Smith, P., Cortinovis, L., Pretto, T., Manfrin, A., Florio, D., Fioravanti, M.L., Baron, S., Le
889 Devendec, L., Jouy, E., Le Breton, A., et al., 2023. Setting epidemiological cut-off values for *Vibrio*
890 *harveyi* relevant to MIC data generated by a standardised microdilution method. Diseases of Aquatic
891 Organisms 155, pp.35 - 42. <http://dx.doi.org/10.3354/dao03740>
892
893 Smyrli, M., Triga, A., Dourala, N., Varvarigos, P., Pavlidis, M., Quoc, V.H., Katharios, P., 2019.
894 Comparative Study on A Novel Pathogen of European Seabass. Diversity of *Aeromonas veronii* in
895 the Aegean Sea. Microorganisms 7, 504; <https://doi.org/10.3390/microorganisms7110504>
896
897 Tasneem, U., Yasin, N., Nisa, I., Shah, F., Rasheed, U., Momin, F., Zaman, S., Qasim, M., 2018.
898 Biofilm producing bacteria: a serious threat to public health in developing countries. Journal of Food
899 Science & Nutrition 01:25–31. <https://doi.org/10.35841/food-science.1.2.25-31>
900
901 Thurlow, C.M., Williams, M.A., Carrias, A., Ran, C., Newman, M., Tweedie, J., et al., 2019. *Bacillus*
902 *velezensis* AP193 exerts probiotic effects in channel catfish (*Ictalurus punctatus*) and reduces
903 aquaculture pond eutrophication, Aquaculture 503, 347–356,
904 <https://doi.org/10.1016/j.aquaculture.2018.11.051>
905
906 Tonetti, F.R., Eguielor, A., Llorente, C., 2024. Goblet cells: guardians of gut immunity and their role
907 in gastrointestinal diseases. eGastroenterology;2(3): e100098. [https://doi.org/10.1136/egastro-2024-](https://doi.org/10.1136/egastro-2024-100098)
908 [100098](https://doi.org/10.1136/egastro-2024-100098)
909
910 Torres-Maravilla, E., Parra, M., Maisey, K., Vargas, R.A., Cabezas-Cruz, A., Gonzalez, A., Tello,
911 M., Bermúdez-Humarán, L.G., 2024. Importance of Probiotics in Fish Aquaculture: Towards the
912 Identification and Design of Novel Probiotics. Microorganisms 12, 626.
913 <https://doi.org/10.3390/microorganisms12030626>
914
915 Triga, A., Smyrli, M., Katharios, P., 2023. Pathogenic and Opportunistic *Vibrio* spp. Associated with
916 Vibriosis Incidences in the Greek Aquaculture: The Role of *Vibrio harveyi* as the Principal Cause of
917 Vibriosis. Microorganisms 11, 1197. <https://doi.org/10.3390/microorganisms11051197>
918
919 Vargas-Albores, F., Martínez-Cordova, L.R., Hernandez-Mendoza, A., Cicala, F., Lago-Leston, A.,
920 Martínez-Porchas, M., 2021. Therapeutic modulation of fish gut microbiota, a feasible strategy for
921 aquaculture? Aquaculture 544, 737050. <https://doi.org/10.1016/j.aquaculture.2021.737050>
922
923 Vazquez, F.J.S., Munoz-Cueto, J.A., 2014. Biology of European Sea Bass. CRC Press. 1st Edition.
924
925 Vendramin, N., Zrncic, S., Padros, F., Oraic, D., Le Breton, A., Zarza, C., Olesen, N.J., 2016. Fish
926 health in Mediterranean aquaculture past mistakes and future challenges. Workshop. Bulletin of the
927 European Association of Fish Pathologists 36, 38–45
928
929 Wang, C., Liu, Y., Sun, G., Li, X., Liu, Z., 2019. Growth, immune response, antioxidant capability,
930 and disease resistance of juvenile Atlantic salmon (*Salmo salar* L.) fed *Bacillus velezensis* V4 and
931 *Rhodotorula mucilaginosa* compound, Aquaculture 500, 65–74,
932 <https://doi.org/10.1016/j.aquaculture.2018.09.052>
933
934 Wood, T., 2025. RcppAlgos: High Performance Tools for Combinatorics. R package version 2.9.3.
935 <https://CRAN.R-project.org/package=RcppAlgos>
936

937 Ye, M., Tang, X., Yang, R., Zhang, H., Li, F., Tao, F., Li, F., Wang, Z. 2018. Characteristics and
938 application of a novel species of *Bacillus*: *Bacillus velezensis*. ACS Chemistry & Biology 13, 500–
939 505. <https://doi.org/10.1021/acscchembio.7b00874>
940

941 Yi, Y., Zhang, Z., Zhao, F., Liu, H., Yu, L., Zha, J., Wang, G., 2018. Probiotic potential of *Bacillus*
942 *velezensis* JW: antimicrobial activity against fish pathogenic bacteria and immune enhancement
943 effects on *Carassius auratus*, Fish and Shellfish Immunology 78, 322–330.
944 <https://doi.org/10.1016/j.fsi.2018.04.055>
945

946 Zhang, X.-H., He, X., Austin, B., 2020. *Vibrio harveyi*: a serious pathogen of fish and invertebrates
947 in mariculture. Marine Life Science & Technology <https://doi.org/10.1007/s42995-020-00037-z>
948

949 Zoli, M., Rossi, L., Bibbiani, C., Bacenetti J., 2023. Life cycle assessment of seabass and seabream
950 production in the Mediterranean area: A critical review. Aquaculture 573, 739580.
951 <https://doi.org/10.1016/j.aquaculture.2023.739580>
952

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
<i>Enterobacteriaceae</i>	16.23 ± 22.95	0.82 ± 1.42	0 ± 0	8.85 ± 15.33
<i>Alicyclobacillaceae</i>	10.25 ± 7.53	1.202 ± 2.08	0 ± 0	0 ± 0
<i>Flavobacteriaceae</i>	8.77 ± 3.13	0.66 ± 1.14	0 ± 0	2.02 ± 2.82
<i>Saprospiraceae</i>	6.06 ± 3.48	0 ± 0	0 ± 0	0 ± 0
<i>Spirochaetaceae</i>	5.90 ± 8.35	59.95 ± 35.92	36.64 ± 51.82	27.92 ± 43.22
<i>Lactobacillaceae</i>	4.59 ± 6.49	1.64 ± 1.43	6.15 ± 8.69	1.80 ± 3.12
<i>Streptococcaceae</i>	4.02 ± 5.68	12.95 ± 15.99	31.80 ± 16.69	12.68 ± 3.12
<i>Vibrionaceae</i>	0 ± 0	4.37 ± 4.01	0 ± 0	0 ± 0
<i>Pseudomonadaceae</i>	0 ± 0	0 ± 0	0 ± 0	15.96 ± 15.74
<i>Lachnospiraceae</i>	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
<i>Chitinophagaceae</i>	0.38 ± 0.34	6.39 ± 3.78	0.16 ± 0.28	0.27 ± 0.47
<i>Flavobacteriaceae</i>	16.61 ± 1.33	10.82 ± 5.36	14.59 ± 7.20	19.29 ± 16.37
<i>Hyphomonadaceae</i>	2.51 ± 0.76	1.91 ± 1.90	4.37 ± 2.05	4.43 ± 1.89
<i>Paracoccaceae</i>	12.08 ± 1.16	7.98 ± 3.93	10.66 ± 3.04	7.81 ± 0.34
<i>Pirellulaceae</i>	6.77 ± 1.95	1.31 ± 0.71	4.81 ± 1.47	2.08 ± 0.19
<i>Rubritaleaceae</i>	9.62 ± 0.25	4.37 ± 0.66	1.80 ± 1.40	3.72 ± 4.09
<i>Saprospiraceae</i>	9.89 ± 2.83	21.86 ± 3.79	2.89 ± 0.58	13.61 ± 10.05