Fish and Shellfish Immunology 124 (2022) 244–253

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Dietary supplementation of *Bacillus velezensis* improves *Vibrio anguillarum* clearance in European sea bass by activating essential innate immune mechanisms

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ARTICLE INFO

Keywords:
Candida albicans
Dicentracin
Dicentrarchus labrax
Immunomodulation
Innate mechanisms
LPS
Probiotic

ABSTRACT

Bacillus spp. supplementation as probiotics in cultured fish diets has a long history of safe and effective use. Specifically, *B. velezensis* show great promise in fine-tuning the European sea bass disease resistance against the pathogenicity caused by several members of the *Vibrio* family. However, the immunomodulatory mechanisms behind this response remain poorly understood. Here, to examine the inherent immune variations in sea bass, two equal groups were fed for 30 days with a steady diet, with one treatment supplemented with *B. velezensis*. The serum bactericidal capacity against live cells of *Vibrio anguillarum* strain 507 and the nitric oxide and lysozyme lytic activities were assayed. At the cellular level, the phagocytic response of peripheral blood leukocytes against inactivated *Candida albicans* was determined. Moreover, head-kidney (HK) total leukocytes were isolated from previously *in vivo* treated fish with LPS of *V. anguillarum* strain 507. Mechanistically, the expression of some essential proinflammatory genes (interleukin-1 (*il1b*), tumor necrosis factor-alpha (*tnfa*), and cyclooxygenase 2 (*cox2*) and the sea bass specific antimicrobial peptide (AMP) dicentracin (*dic*) expressions were assessed. Surprisingly, the probiotic supplementation significantly increased all humoral lytic and cellular activities assayed in the treated sea bass. In addition, time-dependent differences were observed between the control and probiotic treated groups for all the HK genes markers subjected to the sublethal LPS dose. Although the *il1b* was the fastest responding gene to a significant level at 48 h post-injection (hpi), all the other genes followed 72 h in the probiotic supplemented group. Finally, an *in vivo* bacteria challenge against live *V. anguillarum* was conducted. The probiotic fed fish observed a significantly higher survival. Overall, our results provide clear vertical evidence on the beneficial immune effects of *B. velezensis* and unveil some fundamental immune mechanisms behind its application as a probiotic agent in intensively cultured European sea bass.

1. Introduction

As the fastest-growing food-producing industrial sector, aquaculture provides almost 50% of the world's edible fish [1]. In this respect, the European sea bass (*Dicentrarchus labrax*) remains a relevant and notable production species in southern Europe, especially in the Mediterranean aquaculture [2]. However, to achieve high production rates, fish are exposed to severe varied stressful conditions with the potential to trigger the emergence of pathogenic diseases such as vibriosis, caused by several Gram-negative *Vibrionaceae* strains. In the European sea bass, the main causative species of this disease is the *Vibrio anguillarum* [3,4]. Vaccines are the gold standard for disease prevention [5]. However, reliable commercial vaccines against the European sea bass vibriosis that may provide extended protection are still limited and require further fine-tuning efforts [6].

In response to the dire consequences caused by pathogens, the last decades have demonstrated the importance of the commensal microbiota for the proper functionality of each organ in the vertebrate host has been studied and demonstrated extensively in the past decades [7,8]. In fish, for example, the early immune priming by the aquatic microbiota

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https://doi.org/10.1016/j.fsi.2022.03.032
Received 29 December 2021; Received in revised form 11 February 2022; Accepted 21 March 2022
Available online 11 April 2022

during hatching and the modulation through the regular diet at later developmental stages is essential and critically determines adult immune function, microbiome status, and overall health [9,10]. As a result, there is an emergence and proliferation of products that claim to affect the functions and composition of the microbiota, particularly those colonizing mucosal tissues, and providing benefits to fish health.

Powered by novel technologies and major international initiatives, most studies suggest that the conversion of dietary components by intestinal bacteria leads to the formation of a large variety of metabolites, which may cause either beneficial outcomes if properly administered or adverse effects if uncontrolled on vertebrate health [11]. Consequently, in the fish culture industry, the microbiota-modulating dietary interventions are included in a myriad of preparations based on probiotics, prebiotics, and synbiotics, the classical representatives of the microbes in the greater functional feed group [12]. For extended definitions and the current findings in the scope of these microbial groups, see the two following excellent reviews: [13,14]. However, in the present research, probiotics are the focus of our attention. Probiotics are live microorganisms conferring health benefits to the host, including proper development, nutritive alteration of raw ingredients and the biosynthesis of bioactive compounds, favorable adjustment of the gut microbiota, and modification of the immune system when administered in adequate amounts [15]. In the fish culture industry, several microorganisms, including algae, yeast, and bacteria are commonly used as probiotics [16].

Among the probiotic bacterial species, numerous reports have been published on the beneficial role of *Bacillus* spp. [17]. Interestingly, all members in the *Bacillus* genus have vast potential to grow in a wide range of environments due to its ability to live either as aerobic or facultative anaerobic. Additionally, their ability to sporulate, forming endospores, increase its survivability in the gastric tract by resisting exposure to gastric acid [18,19]. Moreover, recent advances in genome sequencing have revealed the potential of several species in the genus *Bacillus* to produce a large variety of molecules with antimicrobial properties [20]. In our previous studies, we have characterized the aerobic, Gram-positive, endospore-forming bacterium *B. velezensis* strain D-18 and unequivocally demonstrated that its usage improves the resistance of the European sea bass against *V.anguillarum* by up to 78% [21]. However, the knowledge on the mechanistic effects of *B. velezensis* over the immune parameters in the European sea bass needs to be further elucidated.

In the present study, we explored whether *B. velezensis* strain D-18 improves the disease resistance of the European sea bass through the positive modulation of the innate immune system. To evaluate our hypothesis, we collected blood and hematopoietic tissue from control fish and those fed with the probiotic mixed in the regular diet for 30 continuous days. Then, serum bactericidal and lytic activities, the phagocytic capacity of peripheral blood monocytes, and gene expression of head-kidney (HK) total leukocytes stimulated *in vivo* by intraperitoneal (ip) injection with lipopolysaccharide (LPS) from *V. anguillarum* were screened. Our analyses revealed a comprehensive *B. velezensis* mediated potentiation in all the innate immune mediators tested, including a significant exclusion effect in the primed leukocytes of individuals previously challenged *in vivo* against a sublethal dose of pathogenic bacterial LPS. By exhibiting some indispensable innate immune mechanisms and discovering that leukocytes become polarized toward a proinflammatory phenotype to achieve clearance of pathogenic factors, we provide evidence that this pathogen-exclusion effect in the European sea bass is due to an effective immunological priming mediated mechanism directly associated with the application of a dietary treatment with the probiotic. Indeed, *in vivo*, significant evidence was also achieved when we challenged the treated fish against *V. anguillarum*. Collectively, the results presented may contribute to treating pathogenic diseases in cultured teleost fish.

2. Materials and methods

2.1. 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-32/2020). 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 [22].

2.2. Bacterial strains

As described elsewhere, the pathogenic Gram-negative bacteria *Vibrio anguillarum* strain 507 and the probiotic Gram-positive *Bacillus velezensis* strain D-18 have been isolated, identified, and characterized earlier by our group [21]. Briefly, to conduct the present trial, frozen vials from our bacterial collection (stored at −80 °C) containing *V. anguillarum* or *B. velezensis* were defrosted at 4 °C in ice, and every 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). Every flask inoculated with a single colony-forming unit (CFU) of each bacterial strain was cultured following classical microbiological culture at 25 °C for 24 h.

2.3. Fish and housing

138 European sea bass (*Dicentrarchus labrax*) fingerlings (26 ± 0.38 g body weight) were obtained and housed at the Marine Science and Technology Park located in the Universidad de las Palmas de Gran Canaria (ULPGC), Spain. For acclimatization, the experimental fish were randomly allocated in six 500 L fiber-reinforced tanks (n = 15 fish/tank) in a closed water system at 20 °C with continued aeration, 12:12 h photoperiod, and water pH = 8 for two weeks. Fish were fed daily with a commercial diet (Alterna, Skretting, Spain) of 3 mm diameter containing 46% fish protein and 16% fish oil.

2.4. Feed preparation and experimental design

Once the two-week acclimation period elapsed, each tank containing 13 animals was randomly assigned into one of the two experimental groups: Control and *B. velezensis* (probiotic) (n = 3 tanks/group). The commercial sea bass feed was taken as the experimental control diet but also used as the basal diet for the supplementation of *B. velezensis* (10^6 CFU x feed g^{-1}) determined spectrophotometrically at an optical-density of 600 nm. All the procedure was conducted as previously suggested [23]. Briefly, the incorporation was achieved by live spray of the probiotic suspension using a spray bottle with the nozzle adjusted to release mist. The diet was slowly mixed part by part in a drum mixer, after which it was air dried on a clean bench for 12 h. Care was taken to maintain sterile conditions through all the process. The stock diet was kept at −20 °C and the daily rations were thawed at 4 °C prior to feeding. The viability of the incorporated *B. velezensi* was assessed by vortexing 10 g of diet in 90 ml of sterile PBS and preparing serial dilution. 100 μl aliquots were cultured at 25 °C for 24 h following classical microbiological procedures. All the animals were fed twice daily by hand for 30 days at a regular rate calculated as 5% of their biomass (Fig. 1).

2.5. Blood and serum collection

As described in Fig. 1, complete sets of samples were obtained coincidently with the end of the feeding trial on day 30. Briefly, 21 specimens per treatment (seven fish from each triplicated 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

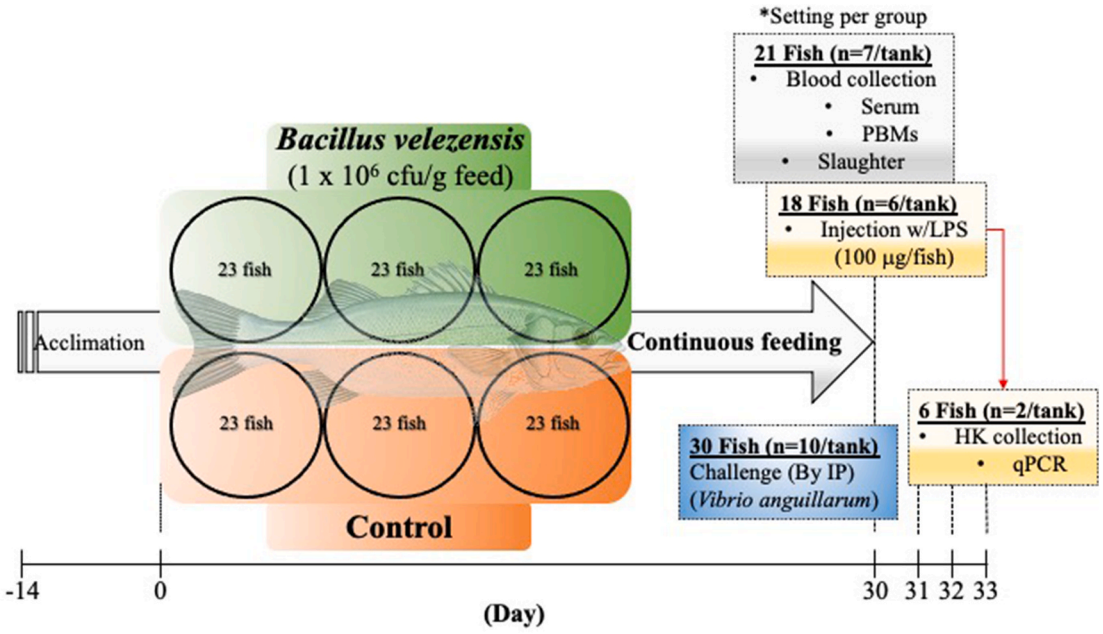

Fig. 1. Experimental setup. After the acclimation period, from day 0, control or *B. velezensis* strain D-18- supplemented diet was orally administered daily within 30 days to the European sea bass. On day 30, 21 animals from each group (n = 7 fish/tank) were aseptically bled. Serum and peripheral blood monocytes (PBMs) were obtained. From serum, bactericidal and lysozyme activities and nitric oxide determination were conducted. The PBMs were incubated with *Candida albicans* (10^9 CFU ml^{-1}), and a classical phagocytic assay was performed. Moreover, 18 fish per treatment were i.p. stimulated with *V. anguillarum*-LPS (100 µg/fish) on the same day. After 24, 48, and 72 h, the head-kidney from six animals per condition (n = 2 fish/tank) were obtained, and the gene expression was analyzed by qPCR. Finally, the remaining 30 animals in each treatment (n = 10 fish/tank) were subjected to a bacteria challenge against (2.7×10^7 CFU ml^{-1}) *V. anguillarum* strain 507.

a 2 ml syringe [24]. One milliliter was collected in a heparinized 1.5 ml Eppendorf tube for monocytes isolation. The remaining 1 mL 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.

2.6. Detection of serum immune parameters

Serum bactericidal activity was assessed by evaluating the effects on the growth curves of *Vibrio anguillarum* strain 507 as described elsewhere [25]. Briefly, the pooled sera from fish (n = 7) in each triplicated treatment were diluted three times with 0.1% gelatin-veronal buffer (pH = 7.5, containing 0.5 mM/ml Mg^{2+} and 0.15 mM/ml Ca^{2+}) and then mixed with *V. anguillarum* (1×10^6 CFU ml^{-1}) suspended in the same buffer at a 1:1 ratio (v/v). The bacterial mixtures were incubated and shaken for 90 min at 20 °C, spread in agar plates, and the number of viable bacteria was calculated by counting the colonies on TSA with 1% NaCl.

Lysozyme activity was measured using a previously described protocol [26]. Briefly, the enzyme activity in the serum was quantified according to a turbidimetric method that uses the lysis of *Micrococcus lysodeitikus* ATCC No. 4698 (Sigma-Aldrich) with hen egg-white lysozyme as the standard. One unit of lysozyme activity was defined as a reduction in absorbance at 450 nm of 0.001/min.

Nitric oxide level was determined by the Griess reaction. Briefly, 100 µl of the pooled fish sera were mixed with the Griess reagent (0.5% sulfanilamide) in 2.5% phosphoric acid and 0.05% N-(1-naphthyl)-ethylenediamine dihydrochloride (all the regents obtained from Merk-Sigma, Spain). The mixture was incubated at 21 °C for 10 min in 96-well plates. The absorbance of the sample and standard wells was measured at 570 nm using an automated ELISA plate reader. The absorbance of test samples was converted to micromolar (µM) concentrations of nitrite by comparison with the absorbance values of sodium nitrite standards within a linear curve fit. Finally, the nitrate concentration in the supernatant was calculated by multiplying the values from the standard curve by the dilution factor and was expressed as µM.

2.7. Mononuclear leukocyte isolation

As previously described, the isolation of peripheral blood monocytes (PBMs) was performed in both the control and *B. velezensis*-treated fish [27]. Briefly, 2 mL of PBS diluted (1:1) heparinized blood was pipetted slowly onto 34–51% discontinuous Percoll density gradients (Sigma Chemical Co, St Louis, MO) and centrifuged (1400 x G; 30 min). Cells at the Percoll interface were collected and washed five times with 5 ml of sterile Hank's buffer by centrifugation (1000 x G; 5 min). The resulting enriched cell pellet was re-suspended in L-15 complete medium (Sigma-Aldrich, USA) supplemented with 15% fetal bovine serum (Gibco, USA) and antibiotics [penicillin-streptomycin (Sigma-Aldrich) at a final concentration of 10 µg mL^{-1}].

2.8. Phagocytosis assay

The phagocytosis assay was performed as previously suggested [28], with slight modifications. PBMs were incubated with 10 ml of 10^9 CFU ml^{-1} (MOI 1:1; inactivated *Candida albicans*/macrophage cell ratio) for 1 h at 22 °C. After washing with PBS, the cells were stained with Diff Quick solution (Panreac, Spain). One hundred macrophages per slide were counted, and the phagocytic activity was determined as the percentage of macrophages containing at least one phagocyted particle per counted cell.

2.9. LPS extraction and purification

LPS was extracted by hot phenol-water method as described previously [29]. In brief, *V. anguillarum* bacterial suspensions (10^8 CFU ml^{-1}) were centrifuged (10,000 x G; 5 min). The pellets were washed twice in PBS (pH = 7.2) (0.15 M) containing 0.15 mM CaCl2 and 0.5 mM MgCl2. Pellets were then resuspended in 10 ml PBS. To ensure complete cell breakage, the cell pellet was sonicated for 10 min on ice. To eliminate

contaminating protein and nucleic acids, treatment with proteinase K, DNase and RNase was performed prior to extraction step. For this purpose, proteinase K (100 μg ml^{-1}) (Roche, Mannheim, Germany) was added to the cell mixture and the tubes were kept at 65 °C for an additional hour. Mixture was subsequently treated with RNase (40 μg ml^{-1}) (Roche, Mannheim, Germany) and DNase (20 μg ml^{-1}) (Roche, Mannheim, Germany) in the presence of 1 μL ml^{-1} 20% MgSO4 and 4 μL ml^{-1} chloroform and incubations were continued at 37 °C overnight. At the next step, an equal volume of hot (65–70 °C) 90% phenol was added to the mixtures followed by vigorous shaking at 65–70 °C for 15 min. Suspensions were then cooled on ice, transferred to 1.5 mL polypropylene tubes, and centrifuged (8500× x G; 15 min). Supernatants were transferred to 15 mL conical centrifuge tubes and phenol phases were re-extracted by 300 μL distilled water. Sodium acetate at 0.5 M final concentration and 10 vol of 95% ethanol were added to the extracts and samples were stored at −20 °C overnight to precipitate LPS. Tubes were then centrifuged (2000 x G; 10 min) at 4 °C. The resulting pellets were resuspended in 1 ml distilled water. Extensive dialysis against double distilled water at 4 °C was carried out until the residual phenol in the aqueous phases was eliminated. Finally, the purified LPS product was lyophilized, weighed to the closest microgram, and stored at 4 °C. At the time of use, it was resuspended in PBS at the desired concentration.

2.10. Fish stimulation with lipopolysaccharide

At the end of the feeding trial (Day 30), the remaining 18 fish from each group were ip stimulated with LPS from *V. anguillarum* 507 at a dose of 100 μg/fish. Samplings were conducted at 24-, 48-, and 72-h post-injection. Each time, two animals from each triplicate tank (n = 6) per treatment were sacrificed within 1 min through anesthetic (clove oil) overdose and sampled as described below.

2.11. RNA extraction and gene expression analysis

Total RNA was aseptically extracted from the HK of both control and probiotic treated (*B. velezensis*) fish with RNeasy mini-Kit (QIAGEN) following the manufacturer's instructions and quantified with a spectrophotometer (NanoDrop, ND-1000). The RNA was treated with DNase I, amplification grade (1 U/mg RNA; Invitrogen), to remove genomic DNA traces that might interfere with the PCRs. Subsequently, the SuperScript IV RNase H reverse transcriptase (Invitrogen, USA) was used to synthesize first-strand cDNA with oligo-dT18 primer from 1 μg total RNA, incubated at 50 °C for 10 min. The b-actin (*actb*) gene was analyzed for sample content standardization using a semiquantitative PCR with an Eppendorf Mastercycle Gradient Instrument (Eppendorf), as previously suggested [7]. In the same samples, the expression levels of the genes coding for the proinflammatory cytokines interleukin-1b (*il1b*), tumor necrosis alpha (*tnfa*), and cyclooxygenase-2 (*cox2*) or the antimicrobial peptide dicentracin (*dic*) were analyzed by real-time PCR performed with a QuantStudioTM 5 Flex instrument (Applied Biosystems) using SYBR Green PCR core reagents (Applied Biosystems), for details see Ref. [7]. After verifying each primer pair amplification efficiency and single peak melting curves presence, appropriate references were selected based on the average M value. Thereafter, the relative expression of each target gene was corrected by the content of two reference genes, the 40S ribosomal protein subunit 18 (*rps18*) and the b-actin (*actb*; reported value) in each sample using the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$) [30]. The European Sea bass specific primers used as targets and reference genes are listed in Table 1. Each PCR was performed in duplicate with three technical replicates each in all cases.

2.12. In vivo challenge test with Vibrio anguillarum

The bacteria challenge was conducted as described elsewhere [3]. Briefly, finalized the probiotic feeding trial, ten individuals in triplicate from control and probiotic (*B. velezensis*) treated groups, were ip injected with (2.7×10^7 CFU ml^{-1}) *V. anguillarum* strain 507 live cells, to assure infectivity. After the injection, fish were monitored every 12 h over a six-day period for clinical signs of disease and mortality recorded.

2.13. Statistical analysis

The results from the humoral activities and the phagocytosis were subjected to a student's t-test, the gene expressions were analyzed by two way-ANOVA and *post hoc* Tukey's, while the survival curve was subjected to a log-rank test to determine the differences among groups. The critical value for statistical significance in all cases was set at $p \leq 0.05$. All statistical analyses were carried out using the GraphPad Prism 8.04 software.

3. Results

The probiotic *Bacillus velezensis* strain D-18 has been proven to be beneficial for treating pathogenic diseases such as vibriosis in cultured marine and freshwater fish [21,31,32]. Our previous study has shown the intimate probiotic characteristics and demonstrated *in vivo* its functional application on the enhancement of fish disease resistance. To further understands some associated innate immune mechanisms, in the present experiment, we orally treated European sea bass fingerlings with *B. velezensis* (10^6 CFU g^{-1} of feed) for 30 days. At the end of the trial, we analyzed the blood serum to search for changes mediated by key humoral mechanisms. Administration of *B. velezensis* did induce significant changes ($p = 0.0012$) in the bactericidal activity against the pathogenic Gram-negative bacteria *Vibrio anguillarum* strain 507 (Fig. 2A). Moreover, the lytic activity of serum lysozyme against *Micrococcus lysodeikticus*, a Gram-positive bacterium, was screened. Lysozyme collected from the probiotic treated European sea bass observed a significant ($p = 0.0006$) shift (Fig. 2B). Likewise, we found that the nitric oxide production in the serum of treated animals was significantly ($p = 0.0231$)

Table 1
Gene primer sequences and NCBI accession numbers used for qPCR analysis.

Gene Name	Gene Symbol	Primer	Primer Sequence (5′ to 3′)	Annealing Temp. (°C)	Accession Number
40S ribosomal protein subunit 18	*rps18*	F1	AGGGTGTTGGCAGACGTTAC	55	AM490061
		R1	CTTCTGCCTGTTGAGGAACC		
B-actin	*bact*	F	ATGTGGATCAGCAAGCAGG	60	AJ537421.1
		R	AGAAATGTGTGGTGTGGTCG		
Dicentracin	*dic*	F	GGCAAGTCCATCCACAAACT	58	AY303949.1
		R	ATATTGCTCCGCTTGCTGAT		
Interleukin-1b	*ilib*	F2	ATCTGGAGGTGGTGGACAAA	58	AJ311925
		R2	AGGGTGCTGATGTTCAAACC		
Tumor necrosis factor-a	*tnfa*	F	AGCCACAGGATCTGGAGCTA	57	DQ200910.1
		R	GTCCGCTTCTGTAGCTGTCC		
Cyclooxigenase-2	*cox2*	F	AGCACTTCACCCACCAGTTC	56	AJ630649.1
		R	AAGCTTGCCATCCTTGAAGA		

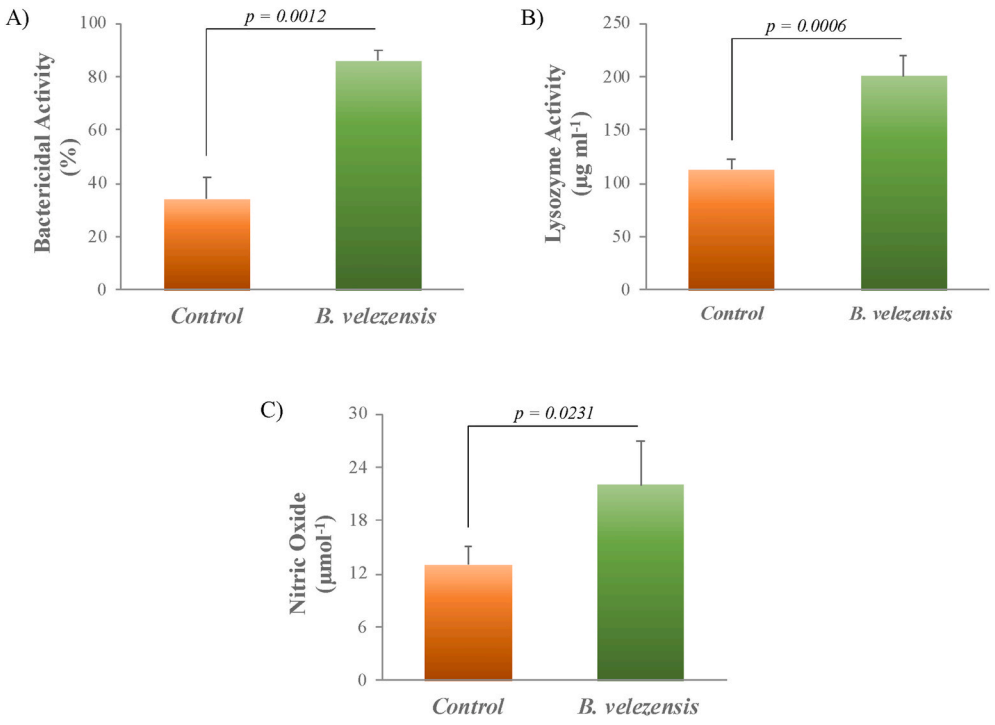

Fig. 2. Probiotic exposure modulates key antimicrobial innate humoral activities in the serum of the European sea bass. The fish had been orally treated with *B. velezensis* strain D-18 (100 µg/g food) or not (Control) within 30 days. (**A**) Bactericidal activity (**B**) Lysozyme, (**C**) Nitric oxide. All data are presented as mean ± the standard deviation (n = 3; seven fish pooled from each triplicated tank per treatment) unless otherwise stated. The student's t-test was used to examine differences in all the parameters tested. The statistically significant *p*-value between groups obtained is shown.

higher than those in the control fish (Fig. 2C). Thus, the present results unveil that oral administration of *B. velezensis* to the European sea bass increased the innate humoral activities without producing any apparent adverse physiological alteration.

Then, we analyzed the effect of the probiotic in the cellular response of the European sea bass. The phagocytic activity of the peripheral blood macrophages to engulf cells of the polymorphic opportunistic fungus *Candida albicans* of fish fed *B. velezensis* was significantly higher (p = 0.0006) after 30 days compared to the control group (Fig. 3).

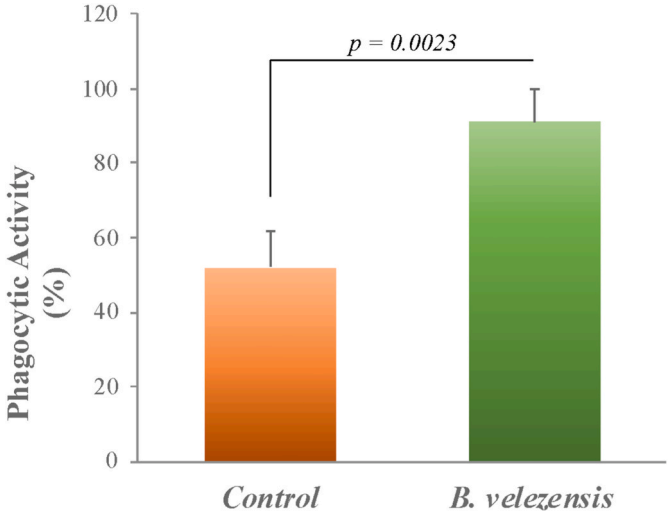

Fig. 3. Phagocytosis of *C. albicans* by activated macrophages from European sea bass is enhanced by the probiotic. PBMs were isolated and cultured overnight with *Candida albicans* from both the control and probiotic-supplemented group at the end of the trial (Day 30). Percentage of phagocytic cells containing at least one phagocyted particle per counted cell are shown. All data are presented as mean ± the standard deviation (n = 7; from each triplicated tank per treatment) unless otherwise stated. The student's t-test was used to examine differences in all the parameters tested. The statistically significant *p*-value between groups obtained is shown.

Subsequently, we examined the quantitative expression of important pro-inflammatory cytokines in total HK leukocytes isolated from fish exposed *in vivo* to an ip injection of pathogenic LPS at the end of the feeding trial (Day 30). A qPCR assay was used to assess the expression of interleukin 1-β (*il1b*), tumor necrosis factor-α (*tnfa*), and cyclooxygenase-2 (*cox2*) every 24 h along a total 72 h period. The levels of three cytokines expression in the treated group showed a time-dependent expression activation along with the trial (Fig. 4). The first significant (p = 0.0132) change was recorded between treated and control fish for *il1b* at 48 h post-injection (Fig. 4A). However, at 72 h post-injection all three genes, *ilib*, *tnfa*, and *cox2* got significantly enhanced expressions (p = 0.0019, p = 0.0026, and p = 0.0106, respectively) compared to the control expression (Fig. 4A, B, C).

Furthermore, we analyzed an ancestral component in the evolution of innate immunity, the endogenous antimicrobial peptide (AMP) dicentracin (*dic*). Several AMPs have been reported in teleost fish. However, *dic* is exclusively expressed only by the European sea bass. Like what was previously observed in the inflammatory cytokines, the probiotic was responsible for the time-dependent enhancement of this AMP in HK total leukocytes isolated from fish exposed *in vivo* to pathogenic LPS at a sublethal concentration through ip injection. However, despite the increasing trend observed in the treated group, it was only after 72 h that a significant (p = 0.0018) enhanced response was recorded (Fig. 5).

We previously demonstrated that injection of LPS in the probiotic treated fish resulted in increased innate effector cytokines expression. Thus, we next wondered whether the addition of this probiotic might also guarantee increased disease protection against *V. anguillarum*. A challenge was conducted after feeding the fish with probiotic for 30 days. Already at day 3 after i.p. infection, mortality in Control group exceeded that recorded in the *B. velezensis* group. At the end of the challenge on day 6, the percent survival by the probiotic group, revealed a significant (p = 0.0011) statistical shift in disease resistance (Fig. 6).

4. Discussion

In the present study, we close the remaining open gap from our prior work. Here, we provide clear evidence on the innate immune

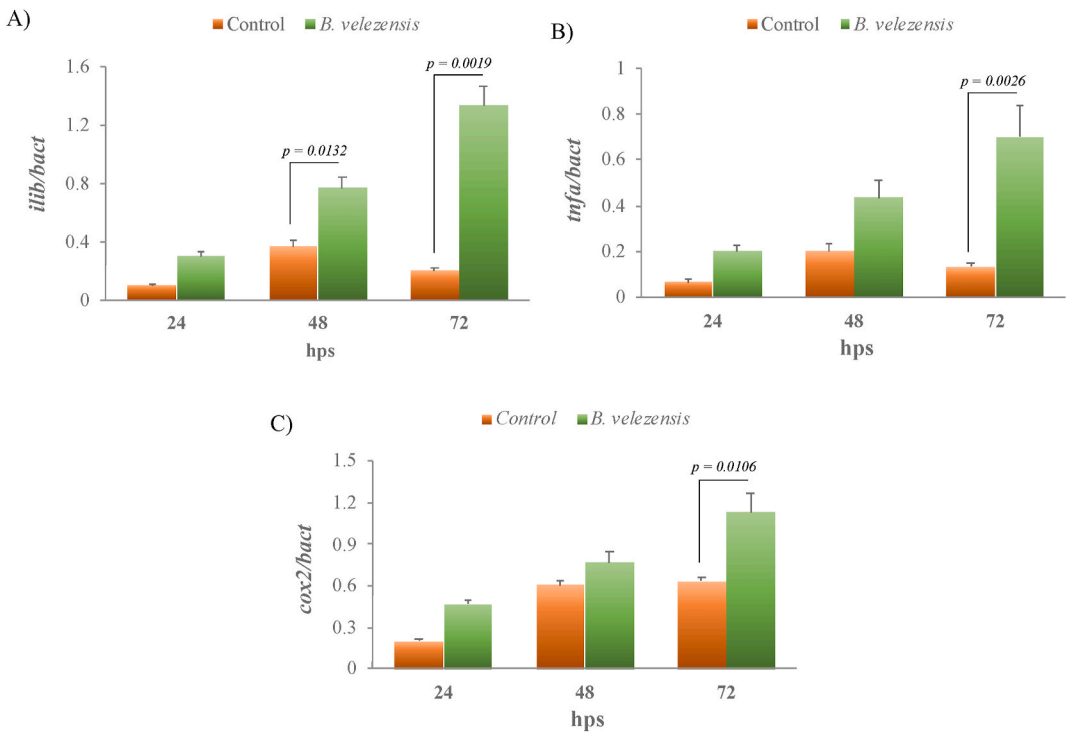

Fig. 4. The relative expression of proinflammatory marker genes in total head-kidney leukocytes from probiotic-treated LPS stimulated European sea bass. On day 30, at the end of the feeding trial with *B. velezensis* or control diets, 18 fish per group (6 fish/tank) were intraperitoneally stimulated with LPS from *V. anguillarum* 507 at 100 μg/fish. After 24, 48, and 72 h, the resulting gene expression of *il1b*, *tnfa*, and *cox2* was quantified by qPCR. The reference gene used for normalization (see section 2.10 for details) was the b-actin (*actb*). All data are presented as mean ± the standard deviation (n = 7; from each triplicated tank per treatment) unless otherwise stated. The statistically significant difference between groups obtained by two-way ANOVA and Tukey *post hoc* is presented as a *p*-value.

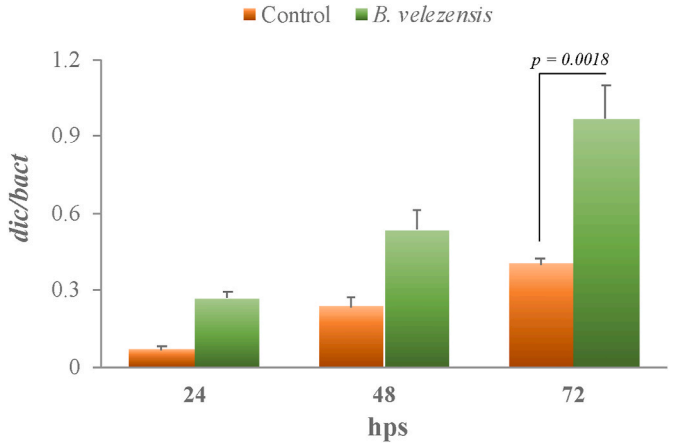

Fig. 5. The species-specific dicentracin mRNA was overexpressed in total head-kidney leukocytes from probiotic-treated LPS stimulated European sea bass. Total leukocytes were isolated from the HK of the fish 24-, 48-, and 72-h post-stimulation *in vivo* with LPS. The *B. velezensis* treated groups showed a gradual time-dependent enhancement in the expression of dicentracin and was statistically significant ($p = 0.0018$) 72 h post-stimulation. The reference gene used for normalization (see section 2.10 for details) was the b-actin (*actb*). All data are presented as mean ± the standard deviation (n = 7; from each triplicated tank per treatment) unless otherwise stated. The statistically significant difference between groups obtained by two-way ANOVA and Tukey *post hoc* is presented as a *p*-value.

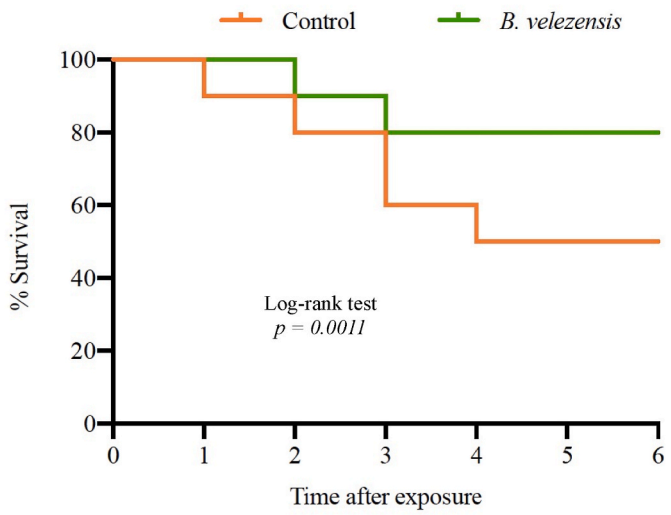

Fig. 6. *In vivo* bacterial challenge. Percentage survival of Control (orange) and *B. velezensis* (green) dietary treated fish experimentally infected by i.p. injection (100 μL of *V. anguillarum* (2×10^6 CFU ml^{-1}). Data are representative of three parallelly repeated trials. The statistically significant difference between groups obtained by the log-rank test is presented as a *p*-value. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mechanisms in the European sea bass fed with a diet supplemented with a specifically designed probiotic for 30 days and challenged with live bacteria or using the crude LPS extracted from a pathogenic strain of *V. anguillarum* injected intraperitoneally to live fish in both cases.

In the last years, probiotics have been developed and fine-tuned to provide a sustainable and innovative oral functional element that may promote disease prevention in several vertebrates. To this end, we have already reported the isolation of *B. velezensis* strain D-18 from the wastewater in an experimental fish farm and proposed it as a suitable

probiotic candidate for orally treating the European sea bass. Indeed, the proposal was supported after conducting a detailed biochemical characterization, providing biosafety evidence, and demonstrating the functionality by testing its efficacy in live fish against the pathogenic bacterium *Vibrio anguillarum* [21]. In the aquaculture industry, the farming of European sea bass suffers from significant loss due to diseases that generate severe mass mortalities [3,33]. Therefore, our efforts were focused on *V. anguillarum* since it is the leading causative agent of seasonal vibriosis, a deadly hemorrhagic septicemia disease. Historically, vibriosis has strongly hampered the biosecurity protocols and developmental plans in most farms culturing the European sea bass [34,35]. Consequently, vibriosis prevention and control are pivotal for this species' thriving culture and development.

In many vertebrates, the use of bacterial species belonging to the genus *Bacillus* as probiotics have been associated with protection against pathogen outbreaks, enhancement of inflammatory processes, and improved gut health [36–38] [36–38] [36–38]. In conjunction with recent efforts [39], our findings clearly show that teleosts are not an exception. A similar protective effect against pathogens has been shown in numerous fish species which received treatment using several species under the *Bacillus* genus. Among them, the list comprises the Nile tilapia (*Oreochromis niloticus*) [40], Atlantic salmon (*Salmo salar*) [32], Crucian carp (*Carassius carassius*) [41], Pangasius (*Pangasius pangasius*) [42], and the hybrid grouper (*Epinephelus fuscoguttatus*) [43]. In the present study, the use of *B. velezensis* strain D-18 as probiotic exhibited enhanced activity of crucial innate immune killing mediators targeting a selected panel of pathogens with opposing structural and biological characteristics but with a similar extended capacity to negatively impact the European sea bass health status. Nevertheless, these results are not fully surprising since fish live in aquatic media and are continuously challenged by many infectious agents including viruses, bacteria, fungi, other protists, and metazoan that can potentially cause diseases [44,45]. Here, the panel of selected pathogenic microorganisms was composed of two bacterial species representing the Gram-negative and Gram-positive classification (*V. anguillarum* and *M. lysodeitikus*, respectively), and one fungus (*C. albicans*). The panel of pathogens was utilized to quantify the humoral and cellular activation in the European sea bass after feeding daily with *B. velezensis* strain D-18 for one month.

The serum of probiotic-treated European sea bass showed an enhanced transition to a hyperactive innate immune state, resulting in the significantly effective killing of *V. anguillarum*. In agreement with our results, previous studies have reported that members of the *Bacillus* genus such as *B. subtilis* used as dietary probiotics in the Japanese red sea bream (*Pagrus major*), possess the beneficial capacity to enhance the pathogen-killing activity of the serum [46]. However, the application of these probiotics needs to be properly assessed. A very recent *in vitro* study evaluating 13 different *Bacillus* strains against *V. vulnificus*, *V. parahemolyticus*, and *V. anguillarum* in the European sea bass put forward the importance of evaluating each strain to be considered as a potential probiotic [47]. Consequently, only strain PJ_11 presented a reliable and consistent antibacterial activity in the European sea bass among the 13 tested strains. Mechanistically, it has been demonstrated in humans that the administration of probiotics belonging to the *Bacilli* class resulted in a potent increased bactericidal activity achieved through the production of bacteriostatic molecules, including hydrogen peroxide and lactic acid with a strong killing capacity against a wide range of pathogens, even including several species resistant to multiple antibiotics [48]. Thus, we speculate that the oral administration of *B. velezensis* in the European sea bass may also provide and follow similar mechanisms to promote the effective innate growth inhibition and multiplication of *V. anguillarum*. However, further experimental evidence using the genus *Vibrio* and other variated pathogens is still required.

In this paper, our observations of the enhanced activity of the peptidoglycan recognition protein (i.e., lysozyme) in the Gram-positive bacteria *M. lysodeitikus*, suggest this protein as a critical host factor mediating the probiotic function in the European sea bass. The mechanisms of action of probiotics are multiple. However, lysozyme seems to respond generically [49]. Under physiological conditions, lysozyme is a vital immune system activator possessing a natural broad-spectrum bactericidal profile. In mammals, the intestinal Paneth cells secrete lysozyme via secretory autophagy during the activated state to achieve intestinal homeostasis [50]. Autophagy is a conserved process that occurs in all eukaryotic cells, and it has been repeatedly proposed as one of the primary mechanisms induced by probiotics [51]. Although fish lacks Paneth cells, in mammals, it was demonstrated that they possibly monitor and direct the intestinal type 1 immunity via lysozyme while goblet cells coordinate with type 2 immunity. In doing so, the Th-1 Paneth cell axis is balanced by goblet-Th2 circuits to maintain gut homeostasis [52]. Moreover, in an activated state, as the one induced by probiotics, the goblet cells increase their number and size, and the lysozyme uses to increase [7]. Similar mechanisms of the goblet cells can also play a central mediator role in the positive immune effects recorded in the European sea bass. Likewise, as in the current experimental setting, dietary *B. velezensis* strain AP193 in channel catfish (*Ictalurus punctatus*) [31], *B. licheniforims* strain Dahb1in the tilapia mossambica (*Oreochromis mossambicus*) [53], or even two mixed-species *Bacillus pumilus* strain 47B and *B. amyloliquefaciens* strain 54A in striped catfish (*Pangasianadon hypophthalmus*) [54] produced an enhanced serum lysozyme response. Despite the potent lytic capacity of lysozyme and its direct antimicrobial capacity, it can also act as a potent opsonin, promoting the phagocytosis process in the fish intestine and contributing to the innate defense against bacterial infection [55,56].

In consequence, using serum as the liquid matrix, we also studied the biological activity of nitric oxide (NO). We found that NO product formation in the *B. velezensis* strain D-18-treated group was significantly higher than the basal generation in the control group. Previously, it has been shown that feeding *B, licheniformis* strain Dahb1 to Pangasius (*P. pangasius*), and *B. amyloliquefaciens* strain FPTB16 in Nile tilapia (*O. niloticus*) and Catla (*Catla catla*) produced a significant positive shift in the NO production [40,42,57]. Interestingly, the generation of humoral NO in all the vertebrate lineage is perceived as a conserved feature in the anti-microbial activity of activated macrophages against various intracellular pathogens, particularly fungus [58,59]. Therefore, we tried to analyze the fungicidal capacity of macrophages. The results revealed that macrophages from the *B. velezensis* strain D-18-treated European sea bass were capable of engulfing more cells of *C. albicans* when compared to the macrophages obtained from the control fish. Several studies using diverse dietary probiotics in fish have reported increased phagocytosis activity against several pathogens at different timepoints after treatment [49]. Mainly, phagocytic enhancement in fish fed with members of the genus *Bacillus* tested at similar periods like the one we used here has been previously shown in *B. subtilis* 7k in Hulong hybrid grouper (*Epinephelus fuscoguttatus* x *E. lanceolatus*) [60], *B. pumilus*, or *B. clausii* in orange-spotted grouper (*E. coioides*) [61], and *B. circulans* in Catla (*C. catla*) [62]. Overall, our results provide evidence that the probiotic has remarkable immune functions in the European sea bass macrophages. However, we hypothesize that granulocytes are likewise affected and significantly contribute to the response. Nevertheless, this hypothesis still needs further investigation.

Until this point, we have shown that the European sea bass humoral and cellular immune defense mechanisms express significant enhancements underlying marked changes between treatments after stimulation with *B. velezensis* as a dietary probiotic. Moreover, we explored relevant immunological mechanisms at the genetic level by qPCR to expand our knowledge. In our model, the exposure of the fish to *B. velezensis* significantly augmented transcript levels of three master inducers of inflammation (*il1b*, *tnfa*, and *cox2*) and one peculiar species-specific anti-microbial effector (*dic*). Although the probiotic treated fish always dominated the observed responses along with the trial, in a global context, it was only after 72 h of LPS treatment that a significantly marked capacity of mounting an immune defensive mRNA strategy

through diverse inducible pathways was recorded. In support of our findings, Nile tilapia (*O. niloticus*) fed with *B. amyloliquefaciens* for one month enhanced the capacity to modulate the production of *il1b* and *tnfa* [40]. However, dietary supplementation of *B. licheniformis* FA6 down-regulated the expression of the same two cytokine transcripts while increasing the anti-inflammatory cytokine *il10* as a homeostatic countermeasure [63]. This apparent contradictory behavior is not surprising since the dual functional role of cytokines is supported by several different molecular investigations utilizing diverse biological models [64,65]. Mechanistically, in amniotes, the protein complex formed by TLR4 and myeloid differentiation factor 2 (Tlr4/Md-2) recognizes the bacterial molecule LPS and triggers an inflammatory response. On the contrary, fish are much less sensitive to LPS, and the induction of cytokines with this component remains ambiguous, even with the recent proposal that fish retain an ancestral Tlr4/Md-2 complex that confers the LPS responsiveness [66]. However, a recent study indicates that NOD1 could identify LPS and activate the NF-κB signal pathway by recruiting RIPK2 and promoting proinflammatory cytokine expression to induce resistance of a representative marine Sciaenidae the miiuy croaker (*Miichthys miiuy*) against bacterial infection [67]. Whatever the case, understanding the intimate synergies between the LPS and the *B. velezensis* requires further studies. Finally, the expression of two major components in the European sea bass leukocytes was also recorded. As we observed here, the inducible inflammatory gene *cox2* has several possible probiotics (eg., *B. subtilis*, *Ecklonia cava*, and *Lactobacillum plantarum*) modulators acting in several fish species [68,69]. More importantly, we have shown that the expression of *dic* was significantly enhanced by the dietary supplementation of the probiotic *B. velezensis* strain D-18. In the European sea bass, *dic* is a potent antimicrobial peptide with broad killing and lytic capacities and has been reported to be present in granulocytes, macrophages, and monocytes from peripheral blood, HK, and peritoneal cavity [33,70]. Therefore, due to the wide scope of *dic*, this last finding is crucial in the examination of the immune mechanisms associated with the use of *B. velezensis* as a probiotic.

By the end of the trial, we conducted an *in vivo* challenge to determine whether feeding the sea bass with *B. velezenesis* strain D-18 for short periods may improve the fish disease resistance. The results obtained provide a good overview of the significant enhancement achieved in the probiotic group. This enhancement is consequent with all the findings presented in the present research. The increase in innate humoral and cellular parameters fully backs up the resistance of sea bass against *V. anguillarum* infection. Moreover, these results support our previous findings [21] and support the inclusion of this probiotic as a modern preventive solution in the marine fish feed industry.

5. Conclusion

In summary, our present work has complemented a comprehensive analysis of the probiotic *B. velezensis* strain D-18, ranging from the previous essential characterization to demonstrating here direct evidence of the operating mechanisms that potentiate the animal's health status after orally receiving the preparation described herein. However, several other complex mechanisms of pathogen elimination by the probiotic (e.g., signaling interference by quorum quenching or the exclusion by overarching the intestinal microbiota) may exist that require further detailed investigation. Nonetheless, the results we have presented so far are clear evidence on the beneficial effects of *B. velezensis* strain D-18 in fish immunity, as well as unveil some fundamental immune mechanisms behind its application as a probiotic agent in the intensively cultured European sea bass.

CRediT authorship contribution statement

Luis Monzón-Atienza: Methodology, Data curation, Writing – original draft, preparation. **Jimena Bravo:** Methodology. **Álvaro Fernández-Montero:** Data curation. **Ives Charlie-Silva:** Data curation. **Daniel Montero:** Data curation. **José Ramos-Vivas:** Methodology, Writing – original draft, preparation. **Jorge Galindo-Villegas:** Conceptualization, Methodology, Writing – review & editing. **Félix Acosta:** Conceptualization, Methodology, Data curation, Writing – original draft, preparation, Writing – review & editing.

Declaration of competing interest

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

Availability of data and material (data transparency)

The data that support the findings of this study are available from the corresponding author upon request.

Acknowledgements

We thank J. L. Superio for proofreading the article. Nord University Access Fund covers the OA publication cost.

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