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Isolation and characterization of a *Bacillus velezensis* D-18 strain, as a potential probiotic in European seabass aquaculture

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Abstract

Within the food producing sectors, aquaculture is the one that has developed the greatest growth in recent decades, currently representing almost 50 percent of the world's edible fish. The diseases can affect the final production in intensive aquaculture, in seabass aquaculture vibriosis is one of the most important diseases producing a huge economical losses in this industry. The usual methodology to solve the problems associated with the bacterial pathology has been the use of antibiotics, with known environmental consequences. This is why probiotic bacteria are proposed as an alternative fight against pathogenic bacteria.

The aim of this study was to analyse a strain of *Bacillus velezensis* D-18 isolated from a wastewater sample collected from a fish farm, for use as probiotics in aquaculture. The strain was evaluated *in vitro* through various mechanisms of selection, obtaining as results for growth inhibition by co-culture a reduction of 30%, *B. velezensis* D-18 was able to survive at 1.5-h exposure to 10% seabass bile and at pH 4 its survival is 5% and reducing by 60% the adhesion capacity of *V. anguillarum* 507 to the mucus of seabass and *in vivo* by performing a challenge. Therefore, in conclusion, we consider *B. velezensis* D-18 isolate from wastewater samples collected from the farms as a good candidate probiotic in the prevention of the infection by *Vibrio anguillarum* 507 in European seabass after *in vitro* and biosafety assays.

Keywords: *Bacillus velezensis* D-18, Probiotic, Vibriosis, Survival, Seabass

Introduction

Aquaculture sector is the one that has developed the greatest growth in recent decades, currently representing almost 50 percent of the world's edible fish, accounting for nearly 50 percent of the world's food fish [1]. Spanish aquaculture production stands out mainly in turbot (*Psetta maxima*), seabass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*) and, especially, in the mussel (*Mytilus galloprovincialis*), being the highest produced species.

Seabass (*Dicentrarchus labrax*) culturing has a great relevance in southern Europe. In 2018, the estimated seabass total production was around 196.573 tons, mainly manufactured in Turkey, Greece, Spain and other Mediterranean countries [2].

Nowadays, in order to optimize benefits, aquaculture carries out intensification. This practice has caused fish to suffer repercussions that end up turning into stress [3]. Catecholamines produced under stress situations cause immune system suppression, creating the ideal environment for bacterial development. Therefore, stress is a determining factor in disease appearance [4].

Bacterial diseases tend to be responsible of high death rates in aquaculture production systems. In seabass, particularly, the most common bacteria affecting fish in marine aquaculture are: *Photobacterium damselae*, *Pseudomonas spp, Aeromonas* and *Vibrio spp.* [5].

Vibrio anguillarum is a Gram-negative bacterium that affects a wide variety of brackish fish and salty waters species, generally shallow. These bacteria cause haemorrhagic septicaemia, which manifest as red ulcers in the mouth, fins, tail and anus, asides from lethargy and anorexia [6].

V. anguillarum is responsible for numerous deaths, and consequently, for great economic losses. Infections take place mainly during seasonal changes, as water temperature fluctuates rapidly [7]. Depending on the water's temperature, the animal's immunological resistance and the agent's virulence, infection periods will oscillate between longer and shorter terms. Prophylaxis against *Vibrio* and other infectious diseases is accomplished using antibiotics, vaccines, management and chemotherapy [8].

The use of antibiotics is one of the most used options to treat aquaculture´s main diseases.

Formerly, the use of antibiotics was higher and uncontrolled, this situation led the European Union to legislate limitations on the use of antibiotics in aquaculture. The use

of vaccines was an aid to limit the use of antibiotics [9], but vaccine prophylaxis is only effective against specific pathogenic bacteria [10]. The use of antibiotics is common practise in aquaculture, however, it creates a selective pressure for emerging drug resistant bacteria, which might be transmitted through food chain from fish to human [11].

The problems presented by the use of antibiotics have led to the development of research in recent decades to establish alternative and environmentally friendly methods to control diseases. Therefore, one of the main goals of aquaculture is researching eco-sustainable options, like probiotics [10].

According to WHO/FAO the probiotics are define as "live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host" [12]. In aquaculture, probiotic utilization increases the nutrients use, therefore increasing fish growth, digestive enzymes and immune system's activity, and improving water quality [10]. It is described that probiotic bacteria find a place to fixate and grow in the intestine of fish, which entails finding a large number of microbial cells in the intestine of these fish [13]. Currently, strains of different genus such as *Arthrobacter* [14], *Bacillus* [15], *Burkholderia* [16], *Enterococcus* [17], *Enterobacter* [18], *Lactobacillus* [19], *Lactococcus* [20], *Micrococcus* [21], *Pediococcus* [22], *Pseudomonas* [21], etc. have been describe as probiotic bacteria.

B. velezensis is an aerobic, Gram-positive, endospore-forming bacterium that for many years were assigned grouping with *B. subtilis* and *B. amyloliquefaciens*, using classic taxonomical parameters [23] and based on the fact that they shared a 99% DNA–DNA percentage phylogenetic similarity [24].

Recently, the genome of the strain AMB-y1 of *B. velezensis* has been published, showing that the strains of this species present metabolites with antibacterial, antifungal and antibiotic activity and also present tolerance to abiotic stress that could confer probiotic properties [24]. The current use of this bacterium is related to the field of agriculture. Recently it has been shown that *Bacillus velezensis* can be a method of controlling maize against fungal and bacterial pathogens [25], due to the Volatile Organic Compounds (VOCS), siderophore, antibacterial and antifungal molecules that *B. velezensis* produced, which plays a relevant roles in pathogen control and plants growth [23]. There are different pathogenic bacteria of animals (*E. coli, S. aureus* and *Salmonella* spp.) against which *B. velezensis* exhibited good antimicrobial activities [26].

Other studies have applied this bacterium in the field of aquaculture in order to evaluate the effect it had on inflammation and damage to the intestinal mucosa of carp caused by *A. veronii* infection [27] and also *in vitro* demonstrated antibacterial effect against *V. algynoliticus* [28].

These specific characteristics and these previous studies encourage us to investigate the possibility to use a strain of *B. velezensis* isolate form wastewater samples collected from firs farms as probiotic in European seabass to prevent the infection by *Vibrio anguillarum*.

Materials and Methods

Bacterial strains

The strain candidate to probiotic was isolated from wastewater samples collected from a farm located at the Instituto Español de Oceanografía, in Santander, Spain. Isolation of bacteria from water samples was done with serial dilution technique on Brain Hearth Infusion Agar (BHIA; Cultimed, Panreac, Spain) medium supplemented with 1.5% NaCl. The bacterial isolate was routinely cultured on BHIA or brain–heart infusion broth (BHIB; Cultimed, Panreac, Spain) at 25°C and were frozen at -80°C with 20% glycerol.

Vibrio anguillarum 507, a fish pathogenic strain isolated in our laboratory, was routinely cultured at 25 ºC on BHIB medium during 24 h.

Molecular Identification by Sequencing

The molecular identification of the isolated strain was carried out according to the bases described by Ramlucken [29] with modifications. The total genomic DNA of the isolated bacteria was extracted and purified using the GeneJET genomic DNA isolation kit (Thermo Scientific, Waltham, Massachusetts, USA). The 16S rRNA gene was amplified by PCR using a pair of universal bacterial 16S rRNA gene primers, forward 5'-AGAGTTTGATCCTGGCTCAG-3'; reverse: 5'-GCGCTCGTTGCGGGACT TAACC-3'. PCR amplification was carried out in a Mastercycler pro S thermal cycler (Eppendorf, Hamburg, Germany) in a 50µL reaction mixture containing 1×PCR buffer, 1.5mM $MgCl_2$, 200 nM each 2´-deoxynucleoside 5´-triphosphate (dNTPS), 1µM each forward and reverse primer, 1.25U of DreamTaq DNA polymerase (Thermo Scientific), and genomic DNA. PCR conditions were typically as follows: one initial denaturation at

94°C for 3 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Cleanup of PCR products was performed by using the ExoSAP-IT enzymatic system in order to eliminate unincorporated primers and dNTPs. The cleaned PCR products were sequenced using a BrightDye® Terminator Cycle Sequencing Kit (Nimagen, Lagelandseweg, The Netherlands). Then, Sanger sequencing was performed on the ABI 3130XL DNA sequencer (Applied Biosystems, Forester City, CA, USA). Sequence analysis was performed using the BioEdit v7.2.5 sequence alignment editor. Finally, sequences found in the National Center for Biotechnology Information (NCBI) database were compared using the Basic Local Alignment Search Tool (BLAST) program.

Fish

A total of 86 seabass, 10 fishes with 200 g of average body weight for mucus adhesion assays, 10 seabass with an average body weight of 35 g for harmlessness test and 66 seabass with an average body weight of 35 g were obtained from Marine Science and Technology Park of ULPGC. The fish were acclimated in tanks (500 L) for 15 d, all tanks were supplied with continuously running seawater, constant aeration and a natural photoperiod (around 12h:12h L:D). Fish were fed daily with a commercial diet of Skretting (Burgos, Spain).

Growth inhibition by co-culture

Overnight culture of *V. anguillarum* 507 strain and *B. velezensis* D-18 strain and fish pathogen were washed twice with PBS and cell concentrations were adjusted to an absorbance of 0.5 at 600 nm and processed according to [30].

***In vitro* screening tolerance seabass bile and pH**

In vitro intestinal screening methods were performance according other authors protocols [28, 31], adapted by Sorroza [32].

Adhesion mucus assays

Intestinal mucus was isolated from healthy seabass. Fish with 200 g of average body weight were starved for 48h and gut removed and homogenized in PBS. Mucus preparations were centrifuged twice, then, the solutions were adjusted to 0.5–1 mg/mL protein in PBS by Bradford Protein Assay Kit (Merck, Darmstadt, Germany), sterilized

by UV light exposure for 30min and stored at -20 ºC until use. Binding of mucus to plate was confirmed by a lectin-binding assay using ConA and the percentage of adhesion to intestinal mucus was evaluated following the methodology described by Van der Marel [33] and Sorroza [32]. The adhesion was expressed as the percentage of fluorescence of the bound bacteria in relation to the fluorescence of the bacterial suspension added initially to the well.

Bio-safety assay

To determine the possible harmful effects of the *B. velezensis* D-18 in seabass, 0.1 mL (10^8 CFU/mL) was injected intraperitoneally into 10 fishes with an average body weight of 10 g by duplicate. As a control we used a group injected with PBS. To evaluate the possible signs of disease, the fish were monitored daily for 30 days after inoculation. At the end of this period, the fish were sacrificed with an overdose of clove oil (5 mL/L) and a necropsy was performed to evaluate possible lesions in the internal organs with a histological study.

The histology protocol consists of several procedures. Once the samples from the necropsy have been obtained, they are stored in buffered formalin until the protocol begins.

The first step consists of drying and fixing the tissues by applying various alcohols: alcohol 70º, 1 h; alcohol 96º, 1 h; alcohol 100º 30 min; alcohol 100º, 1 h; alcohol 100º 1 h; xylene, 30 min; xylene, 1 h; and xylene, 1 h.

The second step is to include the tissue using paraffin during 1 h. After inclusion, the histological cut is made using a microtome at 5 microns, depositing it on the slide.

Must be on the stove for 30 min at 100ºC for subsequent staining with haematoxylin-eosin.

Also, fish internal organs were analysed by microbiological methods on BHIA to determine the presence or absence of the inoculated *B. velezensis*.

Fish challenge with *V. anguillarum* 507 after *B. velezensis* D-18 oral administration

For preparation of the experimental diet with the probiotic strain, selected bacteria were cultured in BHIB for 24 h at 22 ºC following the method by Irianto [34] and Sorroza [32].

For challenge, seabass with an average body weight of 35 g were maintained with a close-water system at 20 ºC with continued aeration and a photoperiod of 12 h. Fish

were fed daily with 2% of body weight, and their health was checked upon arrival and during the 15 days of acclimatization period before starting to feed with the experimental diet containing the probiotic strain selected. Fish were fed during 20 days with the experimental diet including the probiotic before the experimental challenge. The challenge was made in triplicate according Sorroza [32]. The described experiments complied with the European Union (86/609/EU), the Spanish Government and the University of Las Palmas de Gran Canaria (Spain) guidelines for the use of laboratory animals (OEBA-ULPGC 32/2020R1).

Statistical analysis

The data were statistically analysed by using Student's t-test. Statistical significance was set at two-tailed ($p<0.05$), and were examined with SPSS statistics program 17.0 (SPSS, Inc, Chicago, IL, USA). In the figures, numerical data and bars are shown as mean values with standard deviations. The survival curves were estimated by the Kaplan–Meier method and compared by long-rank test.

Results

Bacterial identification

The sequence obtained was analysed with BioEdit v7.2.5 sequence alignment editor and later compared with sequences found in the NCBI database BLAST program, showed a positive result for *Bacillus velezensis* with a homology of 100% compared to *B. velezensis* strains (MT626060.1, MT61167.1, MT611666.1, MT611643.1, MT611594.1) and *Bacillus* sp. (MT605580.1, MT588703.1). After that, this sequence was deposited in the GenBank database and the accession number is MW110900. The strain of *B. velezensis* D-18 has not been deposited in any public or private collection yet.

Growth inhibition by co-culture

After a 24 h growth in co-culture, *B. velezensis* D-18 inhibited 30% of the growth of *V. anguillarum* 507 (Figure 1), this decrease was statistically significant ($p< 0.05$).

In vitro screening tolerance of fish bile and pH

The ability of *B. velezensis* D-18 to inhibit the growth of *V. anguillarum* 507 and its ability to survive or grow in the presence of seabass bile *in vitro* was evaluated. *B. velezensis* D-18 was able to survive a 1.5-h exposure to 10% seabass bile (Figure 2A). Bacteria did not exhibit statistical differences in growth when exposed to PBS with 10% seabass bile and PBS without fish bile added.

At pH below 4, the bacteria do not survive. At pH 4 its survival is 5%. We check better survival at pH 5,6 and 7, this being 35%, 70% and 95% respectively (Figure 2B).

Adhesion mucus assays

Bacillus velezensis D-18 strain showed better adhesion to intestinal mucus (60.33%) than to BSA or polystyrene, with significant differences ($p<0.05$) among the controls (Figure 3). We found similar percentages of adherence of *B. velezensis* D-18 to BSA and polystyrene without statistical differences. The adhesion capacity of *V. anguillarum* 507 to mucus was significantly reduced (60%) after the exposure of the intestinal mucus to the *B. velezensis* D-18 strain (Figure 4).

Bio-safety assay

The strain D-18 tested showed no harmful effects on fish after challenge, and no damage in the internal organs as spleen (Figure 5, A and B), liver (Figure 5, C and D) and kidney (Figure 5, E and F) that were observed at 4x and 10x magnification. Moreover, the inoculated strain was not recovered from internal organs.

Fish challenge with *V. anguillarum* 507 after *B. velezensis* D-18 oral administration

In the experimental challenge, the survival observed was 35% in group not fed with *B. velezensis* D-18, while this was increased to 78% in the fish previously fed with the D-18 strain (Figure 6). Statistical analysis demonstrated a significant difference ($p<0.05$) in the survival of fish among the different groups analysed. The affected fish showed signs of acute haemorrhagic septicaemia with exophthalmia, corneal opacity and ulcers. The mortality observed in the challenge was attributed to the inoculated pathogen, from each fish killed during the challenge, the inoculated microorganism was isolated from the internal organs in pure culture.

Discussion

In aquaculture, the use of probiotics has different applications as an environmentally friendly antibiotic alternative [35], but in some probiotics the survival rates are low [10]. All presentation of probiotics (live or death) improve fish welfare, although live cells seem to be better than the killed cells [36]. The use of probiotics in the diet has demonstrated their ability to protect different fish species (Hamilton, *Labeo rohita*) [15], tilapia (*O. niloticus*) [21], olive flounder (*Epinephelus bruneus*) [20], rainbow trout (*Oncorhynchus mykiss*) [13], common carp (*Cyprinus carpio*) [37, 38] and seabass [32] against infections by pathogenic microorganisms.

Bacillus sp., *Lactobacillus* sp., and *Saccharomyces* sp. are the most commonly used probiotics in aquaculture [39, 40]. *Bacillus* species are non-pathogenic and non-toxic aerobic Gram-positive bacteria with high survival that are administrated to fish either orally or through the water to enhance body conditions and gastrointestinal (GI) microbial populations. [10, 41, 42]. *Bacillus amyloliquefaciens* has beneficial effects in feed utilization, stress and immune response [10], increasing IgM [15], when added to fish diets [43, 44]. Studies demonstrated that the consumption of *B. velezensis* help the regulation of the innate immune system and decrease the pathogen effects of *A. veronii* in crucian carps [27].

In order to be used, probiotics must meet certain requirements. *In vitro* tests such as inhibitory activity against pathogens or competition for nutrients have been widely reported [45], and it is an important criterion for selecting a probiotic candidate strain [46]. In our study, the *Bacillus velezensis* D-18 is capable of inhibiting *V. anguillarum* 507.

Different authors have reported that the production of volatile organic acid compounds and bacteriocins by probiotics explains the inhibitory effects they present against pathogens [47]. *B. velezensis* produces volatile organic compounds (VOCS) and antimicrobial compounds, such as bacillomycin, surfactins, phengicins, amylocycine, and lipopeptides that exhibit significant antagonistic effects against pathogens [27].

Strain D-18 reduces significantly the growth of *V. anguillarum* 507 after 24 h in co-culture, this mean could be competing for nutrients, or that the probiotic strain inhibits the growth of the *Vibrio* strain by some mechanism (i.e. bacteriocin production) but to select a good probiotic strain this criterion is not essential [48].

Recently, the genome of *B. velezensis* strain AMB-y1 has been published [28]. This genome indicates that strains of this species have some characteristics that could confer

probiotic properties. This reinforces our results. Comparison of this genome with those of our strain and others, could offer a clear insight into the mechanisms by which these bacteria compete with pathogens in the gastrointestinal tract of fish.

New studies showed the antagonistic activity of *B. velezensis* against *L. monocytogenes*, *M. flavus*, *B. cereus* and fungal pathogens. *B. velezensis* shows inhibitory effects against multiple Gram-positive bacteria [25]. Furthermore, good antimicrobial activities against pathogenic bacteria of animals (*E. coli*, *S. aureus* and *Salmonella* spp.) have been described for *B. velezensis* [26].

To simulate the passage of bacteria through the gastrointestinal tract, the effect of bile and pH as a step prior to adhesion were evaluated, showing no statistical differences between the group treated and the PBS group, it should be awared that this assay was carried following the same protocol use by Sorroza [32] where bile concentration was of 10%, and the real concentration in fish is unknown [30]. In this study, the percentage of bile used were much higher than that used in the assays with humans (3%).

Like Sorroza [32] for *V. fluvialis*, in this assay were observed a decrease in the survival of *B. velezensis* D-18 at acid pH, but that does not mean that *B. velezensis* is unable to survive and colonize the intestine because this does not occur *in vivo*, bacteria administered with food will receive an indirect action due to the acidic pH of the gastrointestinal tract [30].

Resistance to acidity is not an essential requirement to select a probiotic, as in the case of marine larvae that in this period of their feeding life with live prey, present an alkaline environment in their digestive tract [49].

Bacillus velezensis D-18 showed the ability to grow and adhere to the intestinal mucus of fish, and these results compared with those obtained in the adhesion to BSA and polystyrene, suggesting that the microbial adhesion process may be due to passive forces, electrostatic interactions, steric forces, lipoteichoic acids and specific structures such as external appendages covered by lectins [47]. This fact is considered as a very important property to enable colonization and persistence in the intestinal tract [50]. In this study, results show a better adhesion in seabass mucus than those obtained by Sorroza for *Vagococcus fluvialis*, and when we perform tests of exclusion, our bacteria also obtain better but not significantly different from those obtained with vagococcus results.

The ability to compete for the binding site with a pathogen is important for a probiotic, this ability is shown by *Bacillus velezensis* against *V. anguillarum*, a result of which is similar to that of Sorroza with *Vagococcus fluvialis* [31].

This fact is beneficial to the health of the fish due to the presence of probiotic bacteria that may restrict the access of pathogens to tissues receptors by steric hindrance or by blocking the receptor with specific adhesion analogue [51]. To date, it is widely accepted that lactic acid bacteria form part of the normal intestinal microbiota of fish from the first few days of life [36]. *Lactobacillus* and *Bacillus* are considered to be important and more dominant among the gut bacterial flora. Lactic acid bacteria also have a strong antimicrobial activity toward many pathogenic microorganisms and this prevents colonization of pathogenic organisms and helps the optimum utilization of feed [52]. There are no studies analysing this genus as a probiotic in seabass, but in general, it is well documented that many *Bacillus* are harmless and some strains have been reported to have beneficial effects on fish health [10, 15, 35].

In fish, the three major routes of infection are the skin, gills and gastrointestinal tract. Therefore, in the experimental challenge, that the relative survival percentage of the group fed with *Bacillus velezensis* D-18 was 78%, compared to the control group, which presented 35% survival. Many studies in recent years have shown that the administration of bacteria with food can decrease the appearance of diseases or reduce the severity of outbreaks [36].

It is generally accepted that probiotics block the effects of pathogenic bacteria through various mechanisms, enhancing barrier function and stimulating protective responses [53].

Conclusion

All of the parameters that were tested *in vitro* for the strain isolated from wastewater samples collected from the farms and identified as *Bacillus velezensis* D-18 show their ability to remain viable in the extreme conditions of gastrointestinal tract and to compete in such conditions with the pathogen *V. anguillarum*. After feeding the European seabass with *Bacillus velezensis* D-18 they show a high ability to resist infection by *V. anguillarum*, all this suggests that *Bacillus velezensis* D-18 is an optimal candidate for use as a probiotic in the control of infection by *V. anguillarum*.

Declarations

Funding Not applicable

Conflict of Interest. The authors declare that they have no conflict of interest.

Ethical Statement. All procedures 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).

Authors participation statement

Conceptualization: Félix Acosta, José Ramos-Vivas
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Writing - original draft preparation: Luis Monzón-Atienza, Félix Acosta, José Ramos-Vivas
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Consent to participate Not applicable

Consent for publication Not applicable

Availability of data and material (data transparency) The data that support the findings of this study are available from the corresponding author upon reasonable request

Code availability Not applicable

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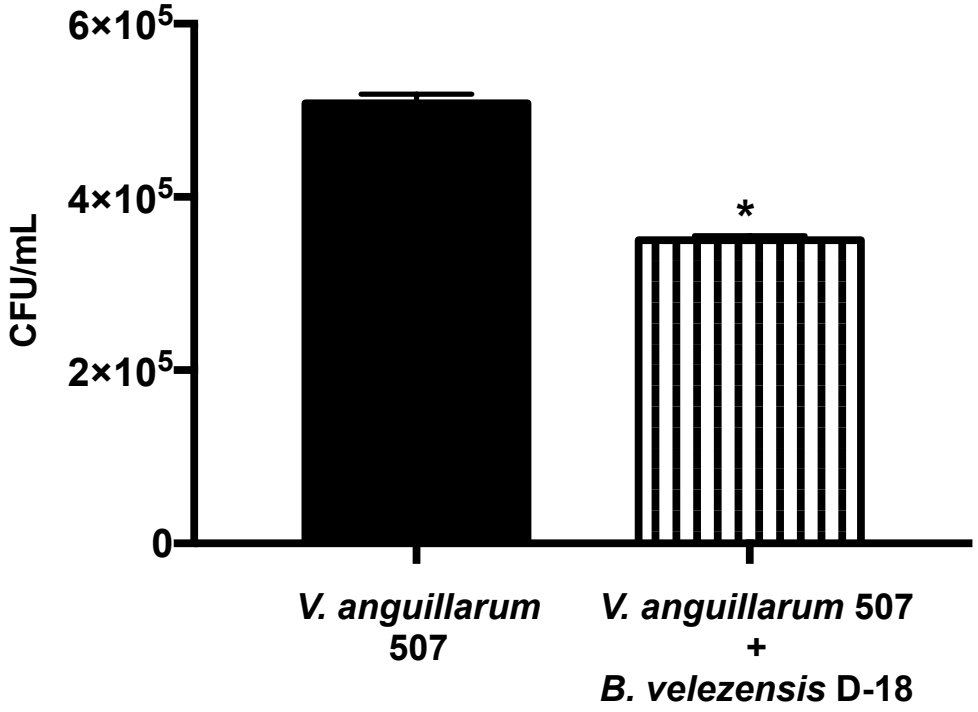

Figure 1. Growth effect by co-culture between *V. anguillarum* 507 and *B. velezensis* D-18. The asterisk indicates a significant statistical difference ($p<0.05$) in the reduction of *V. anguillarum* 507 growth when cultured with *B. velezensis*.

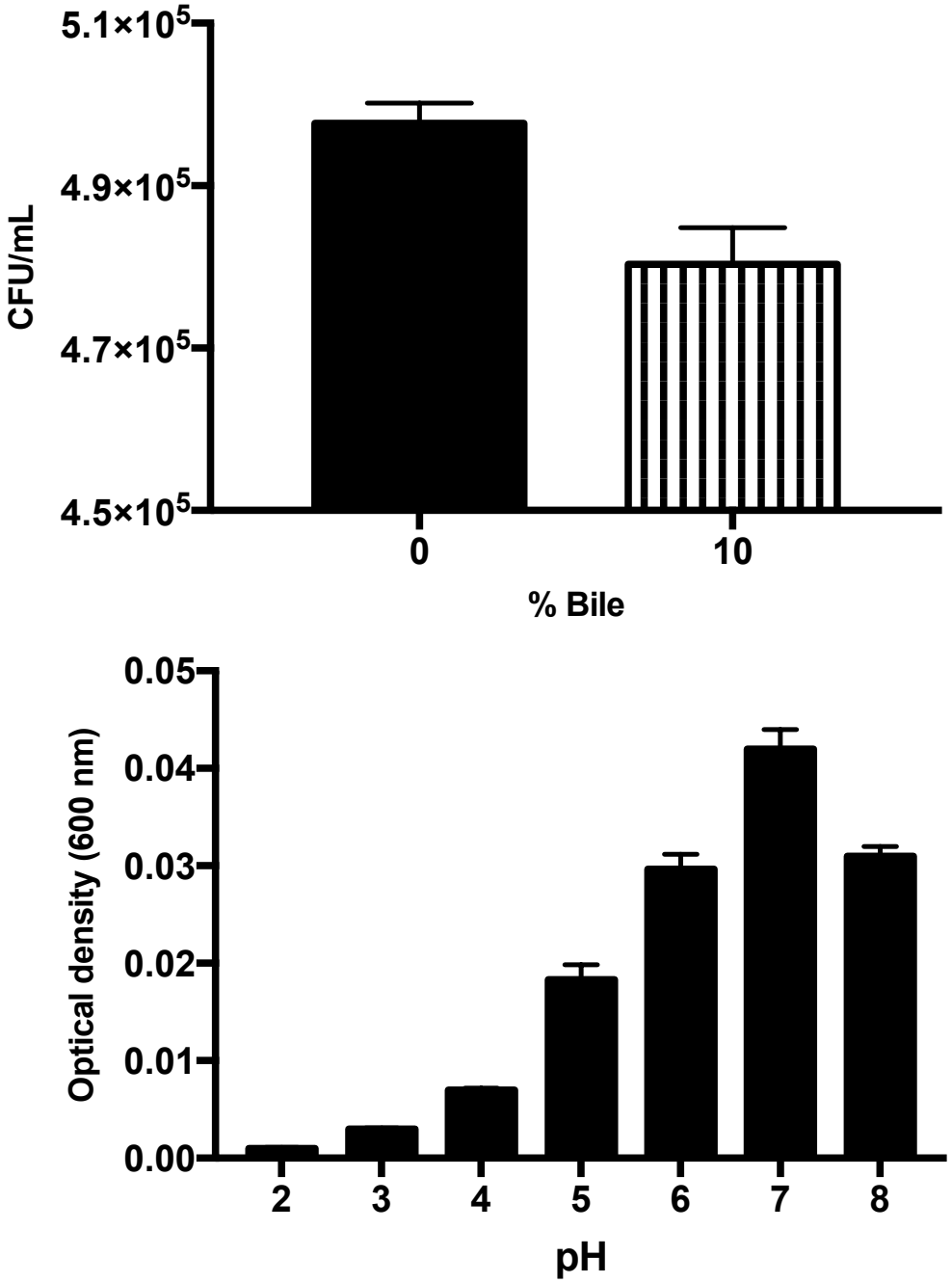

Figure 2. Tolerance of *B. velezensis* D-18 strain to sea bass bile (A) and pH (B).

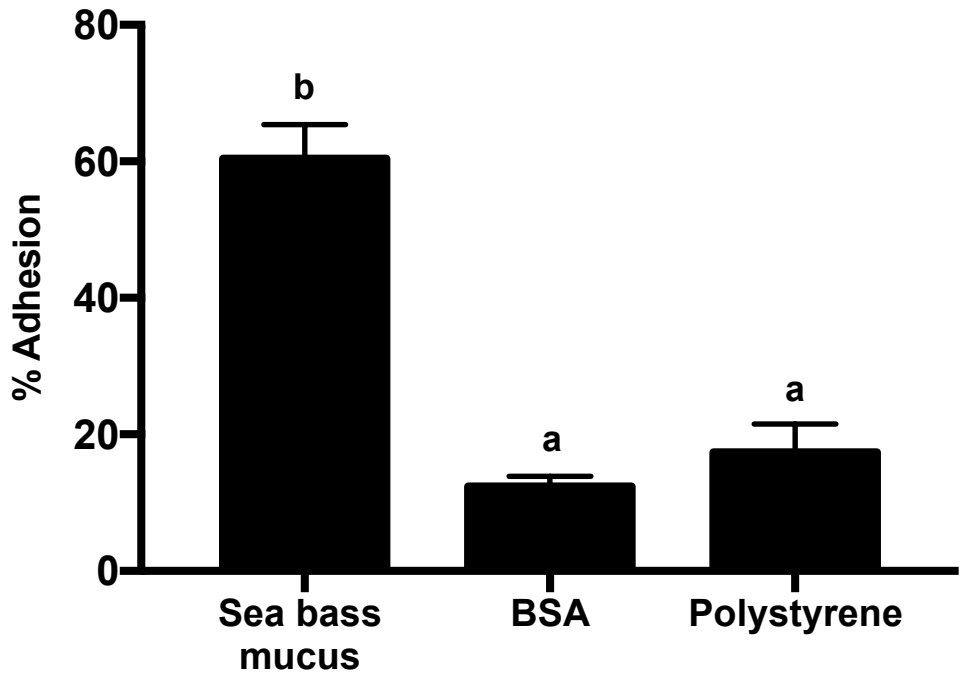

Figure 3. Percentage of adhesion of the *B. velezensis* D-18 strain to sea bass mucus, BSA and polystyrene . All data are given as percentage of the absorbance measurements of fluorescent stained bacteria ±SD. Letters indicate significant statistical differences ($p<0.05$).

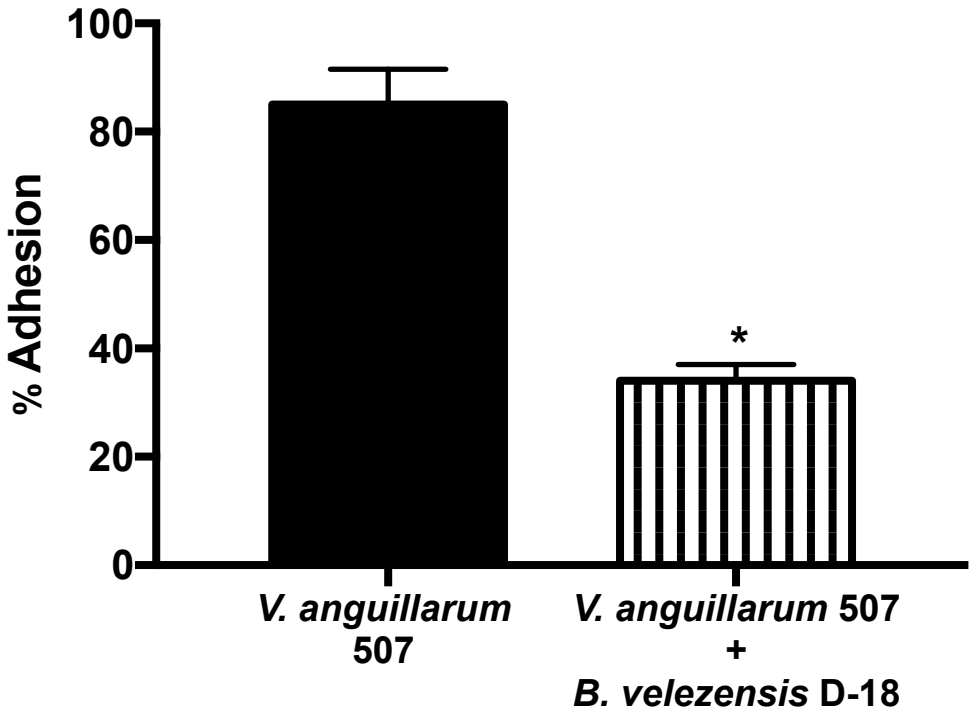

Figure 4. Percentage of adhesion of the *V.anguillarum* 507 strain to sea bass mucus after exposure of the mucus to *B. velezensis* D-18. All data are given as a percentage of the absorbance measurements of fluorescent stained bacteria ±SD. * indicates significant statistical differences ($p<0.05$).

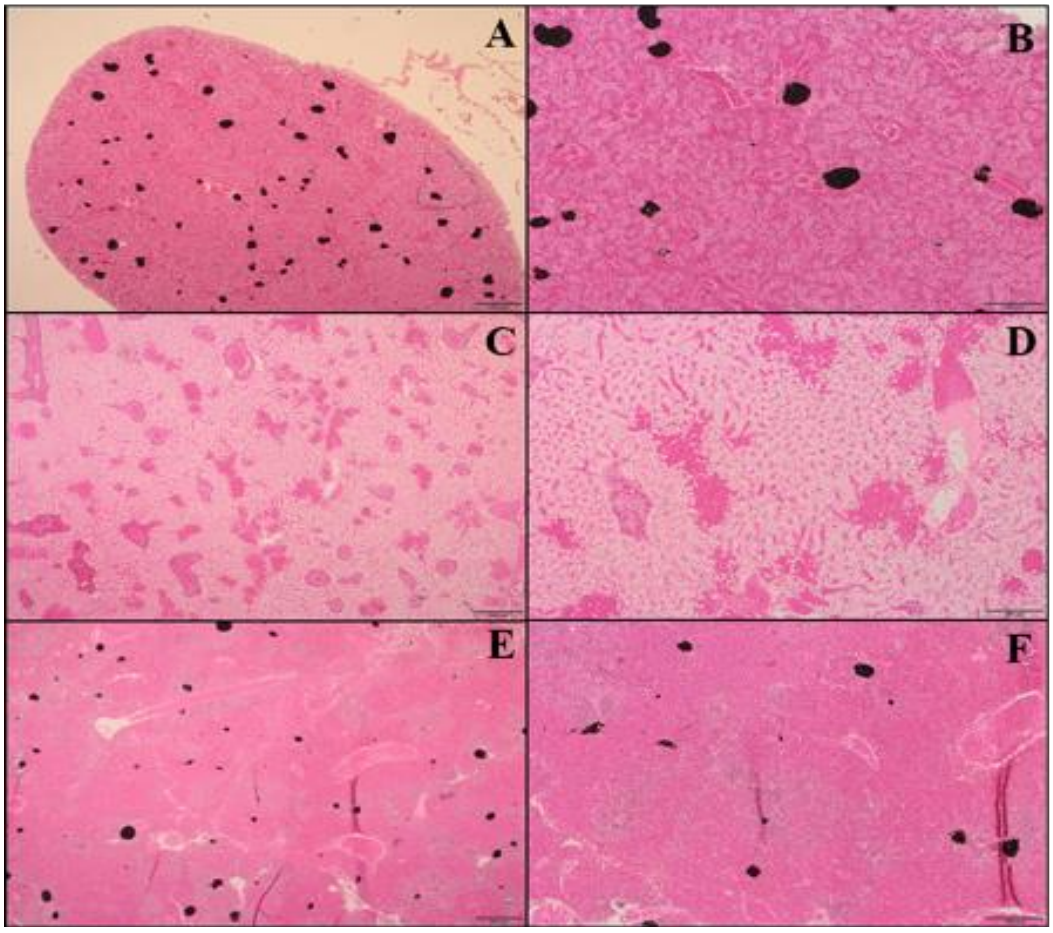

Figure 5. View of absence of damage after administering B. velezensis D-18, it showing a normal structure of the spleen (A and B), liver (C and D and kidney (E and F) at 4x and 10x, respectively.

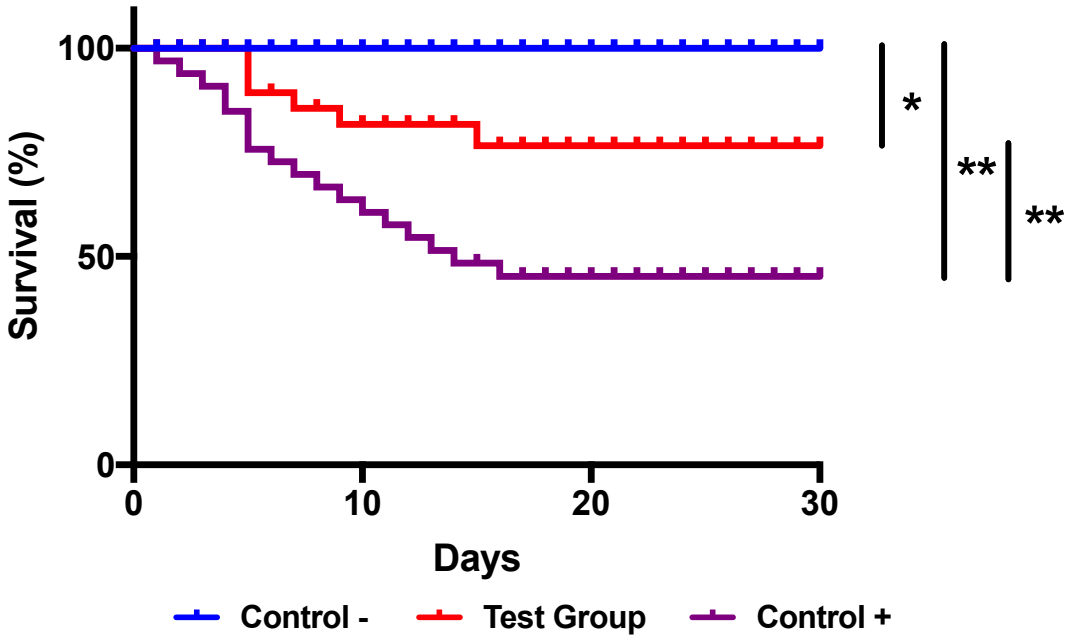

Figure 6. Effect of the probiotic strain on the survival percentage of seabass against *V. anguillarum* 507. Asterisks indicate a significant statistical difference * ($p<0.05$) and ** ($p<0.001$).