Study of the Properties and Mechanism of Action of *Bacillus velezensis* D-18 for Establishment as a Probiotic Bacterium in Aquaculture

Thesis for the degree of Doctor of Phylosophy
by the University of Las Palmas de Gran Canaria

PhD programme in Sustainable Aquaculture and Marine Ecosystems
ECOAQUA Universitary Institute, Grupo de Investigación en Acuicultura (GIA)

Luis Monzón Atienza

Supervisor:
Prof. Félix Acosta Arbelo

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Título de la tesis: ESTUDIO DE LAS PROPIEDADES Y EL MECANISMO DE ACCIÓN DEL BACILLUS VELEZENSIS D-18 PARA SER ESTABLECIDO COMO UNA BACTERIA PROBIÓTICA EN ACUICULTURA

Tesis doctoral presentada por D. LUIS MONZÓN ATIENZA

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Aún recuerdo aquella tarde, con apenas doce años, cuando mi madre me preguntó qué estudiaría en la universidad. Con la firmeza de la inocencia, respondí que la universidad no era para mí, pues en mi entorno nadie había asistido a una, salvo mi madre. Creía que era un lugar reservado para eruditos un grupo del cual no me considero parte, (especialmente en aquellos días en que dudaba constantemente de mis capacidades) aunque mi madre me llame "pitagorín". Sin embargo, mi madre, con un tono de enfadado característico, me aseguró que, como todos los que estudian, yo también iría a la universidad. Y así fue.

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Ahora, en el punto final de mi periodo predoctoral, mientras redacto esta tesis, reflexiono sobre todo lo aprendido y vivido, y no puedo dejar de agradecer a aquellos que han sido fundamentales en esta andadura:

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ABBREVIATIONS LIST

AHL	N-acyl homoserine lactone	**dNTPS**	2′-deoxynucleoside 5′triphosphate
AI	Autoinducer	**ECPs**	Extracellular products
AMP	antimicrobial peptide	**ELISA**	Enzyme-linked immunosorbent assay
ANOVA	Analysis of variance	**FAO**	Food and Agriculture Organization of the United Nations
ALP	Alkaline phosphatase	**fbl**	Flucose-binding
AP	Acid phosphatase	**FISH**	Fluorescent *in situ* hybridization
AST	Aspartate aminotransferase	**Fw**	Forward primer sequence
β-actin	Beta-actin	**g**	Grams
BHI	Brain heart infusion	**GI**	Gastrointestinal tract
BHIA	Brain heart infusion agar	**GIA**	Research Group in Aquaculture
BHIB	Brain heart infusion broth	**GPX**	Glutathione peroxidase
BLAST	Basic Local Alignment Search Tool	**H**	Heat-inactivated
BSA	Bovine serum albumin	**h**	Hours
Ca^{2+}	Calcium	**Hep**	Hepcidine
CAT	Catalase	**HK**	Head-kidney
cDNA	Complementary deoxyribonucleic acid	**HPB**	High prebiotic level plus probiotic
CD4	Cluster of differentiation 4	**HPLC**	High-performance liquid chromatography
CD8	Cluster of differentiation 8 alpha	**HSP70**	70-kilodalton heat shock protein
CECT	Spanish type culture collection	**hpi**	Hours post-injection
CFU	Colony forming units	**IFN**	Interferon
COX-2	Cyclooxygenase 2	**Ig**	Immunoglobulins
dph	Days post-hatching	**IgM**	Immunoglobulin M
DIC/dic	Dicentracin	**IL-10**	Interleukine 10
DNA	Deoxyribonucleic acid	**IL-1β/il1b**	Interleukine 1β
DNAse	Deoxyribonuclease	**IP**	intraperitoneal

kg	Kilograms	**Mx**	Myxovirus resistance proteins
L	Liters	**NaCl**	Sodium chloride
LAB	Lactic acid-producing bacteria	**NCBI**	National Center for Biotechnology Information
LB	Luria Bertani	**NF-kB**	Nuclear factor kappa beta
LPB	Low prebiotic level plus probiotic	**nm**	Nanometers
LPS	Lipopolysaccharides	**NO**	Nitric oxide
lyz	Lysozyme	**NOD1**	Nucleotide-binding oligomerization domain 1
M	Molar	**OD_{600}**	Optical density 600nm
MAMPs	Microbe-associated molecular patterns	**PAMPs**	Pathogen-associated molecular pattern
Md-2	Myeloid differentiation factor 2	**PBMs**	Peripheral blood monocytes
$MgCl_2$	Magnesium chloride	**PBS**	Phosphate-buffered saline
Mg^{2+}	Magnesium	**PRRs**	Pattern recognition systems
MHCI-α	Major histocompatibility complex class I alpha	**PCTM**	Parque Científico-Tecnológico Marino de Taliarte
MHCII-β	Major histocompatibility complex class II beta	**PCR**	Polymerase chain reaction
min	Minutes	**QQ**	*Quorum quenching*
mL	Milliliters	**qPCR**	Quantitative PCR
mM	Millimolar	**QS**	*Quorum sensing*
mm	Millimeters	**RAS**	Recirculating aquaculture system
MOS	Mannan oligosaccharides	**rbl**	Rhamnose-binding
mRNA	Mitocondrial ribonucleic acid	**RIPK2**	Receptor-interacting protein kinase 2

RNA	Ribonucleic acid	**TLR**	Tolls like receptors
RNase	Ribonuclease	**TNF-α**	Tumor necrosis factor α
rRNA	Ribosomal RNA	**TSA**	Tryptic Soy Agar
ROS	Reactive oxygen species	**TSB**	Tryptic Soy Broth
rps18	Ribosomal protein subunit 18	**U**	Units
RT-qPCR	Retro-transcription real time PCR	**µg**	Micrograms
Rv	Reverse primer sequence	**µL**	Microliters
s	Seconds	**ULPGC**	University of Las Palmas de Gran Canaria
SD	Standard derivation	**UV**	Ultra-violet light
SOD	Superoxide dismutase	**VOCs**	Volatile organic compounds
TAC	Total antioxidant capacity	**WBC**	White blood cells
TCR-β	T-cell receptor β	**WHO**	World Health Organization
TGF-β	Transforming growth factor beta		

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Summary

SUMMARY

Following an exhaustive sampling of wastewater from turbot aquaculture facilities in northern Spain, a meticulous screening process led to the isolation of a unique bacterium, subsequently identified as *Bacillus velezensis* D-18 through molecular sequencing. Drawing upon extensive literature supporting the genus *Bacillus* as beneficial microorganisms for the host, the probiotic potential of *B. velezensis* D-18 in the context of aquaculture was evaluated.

Subsequently to the isolation, a detailed characterization of the strain was conducted. *B. velezensis* D-18 underwent rigorous *in vitro* testing using various selection methods including inhibition of pathogen growth through co-culture, assessment of tolerance to European sea bass bile and pH, and mucus adhesion capacity. Additionally, an *in vivo* evaluation was performed in European sea bass, comprising a biosecurity assay and a challenge against *Vibrio anguillarum* 507 following probiotic administration. Results revealed that *B. velezensis* D-18 can inhibit the growth of pathogenic strains, withstand sea bass gastrointestinal bile and pH, adhere to European se bass mucus, cause no harm to the host, and enhance resistance to *V. anguillarum* 507. These findings raised the central question:

What are the mechanisms of action employed by *B. velezensis* D-18 to confer these benefits to the European sea bass?

To address this question, *B. velezensis* D-18 was administered to European sea bass for a period of 30 days. At the end of this period, blood was extracted to obtain serum and peripheral blood monocytes. The serum was used to evaluate bactericidal activity, lysozyme, and nitric oxide, while peripheral blood monocytes were used to assess phagocytic activity. A specific group of sea bass was inoculated with a sub-lethal dose of *V. anguillarum* 507 lipopolysaccharides (100 µg/µL), and at different time intervals, samples were taken from the anterior kidney to analyze the gene expression of cytokines such as interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), cyclooxygenase (COX-2), and the antimicrobial peptide dicentracin (DIC). These assays were contrasted with an *in vivo* challenge against *V. anguillarum* 507 at the end of the experiment. The

results indicated that *B. velezensis* D-18 enhances the health status of European sea bass after oral administration, by increasing non-specific immune parameters.
However, the possible existence of other mechanisms such as competitive exclusion through the production of inhibitory compounds is suggested.

The concept of *quorum sensing* refers to the intra- and/or interspecies interaction of bacteria through the emission and detection of signaling molecules known as autoinducers (AI). This process triggers gene expression by the receiving bacterium. Therefore, *quorum sensing* is responsible for numerous bacterial activities, such as biofilm formation and other pathogenic activities. Enzymatic inhibition of *quorum sensing* is known as *quorum quenching*. Consequently, the question arises about the *quorum quenching* potential of *B. velezensis* D-18, as well as that of its extracellular products, and its applicability in aquaculture. The assessment of this capacity was carried out through various assays using biomarkers of the *Chromobacterium violaceum* species: MK wild type, CV026, and VIR24. The latter, respectively, detectors of short and long chain acyl homoserine lactones, AI molecules characteristic of Gram-negative bacteria. The results indicated that *B. velezensis* D-18 employs *quorum quenching* as a probiotic action mechanism, by producing lactonases through the ytnP gene, and is capable of controlling biofilm formation and the growth of pathogenic strains such as *V. anguillarum* 507.

In conclusion, *B. velezensis* D-18 has demonstrated its ability to act as a probiotic strain with possible practical application in the aquaculture industry. Based on previous work with other microorganisms considered probiotics (see Chapter VII), *B. velezensis* D-18 possesses the necessary characteristics to be considered a reliable probiotic. This strain is completely harmless to the host and can colonize the fish intestine, resisting gastrointestinal conditions. Additionally, it provides an immunological boost, stimulating the nonspecific immune response and contributing to the control of pathogenic diseases caused by bacteria such as *V. anguillarum*.

Chapter I

Introduction

1.1 AQUACULTURE IN THE GLOBAL AND EUROPEAN CONTEXT

Over the past decades, aquaculture has emerged as the fastest-growing food production sector, becoming a pivotal activity in global food production (Martínez-Porchas et al., 2023). This surge can be largely attributed to the escalating demand for fish and seafood products coupled with declining wild catches (FAO, 2022; Pontecorvo & Schrank, 2012) (Figure 1). Consequently, aquaculture plays a crucial role in ensuring food security while simultaneously fostering economic growth and environmental stewardship. Its nutritional significance cannot be overstated, as it substantially contributes to the provision of protein for the human population (Pradeepkiran, 2019).

Europe stands as a global leader in aquaculture, amalgamating efficient production practices with a sustainable approach and a relentless pursuit of innovation. The aquaculture industry in the region not only meets dietary demands but also charts the course towards more responsible and environmentally respectful practices (FAO, 2022). Within this framework, Europe boasts a diverse aquaculture production comprising various species tailored to the region's specific conditions. Noteworthy species include salmon (*Salmo salar*), trout (*Oncorhynchus mykiss*), sea bream (*Sparus aurata*), mussels (*Mytilus* spp.), oysters (*Crassostrea* spp.), and sea bass (*Dicentrarchus labrax*), among others (APROMAR, 2023; Bostock et al., 2016).

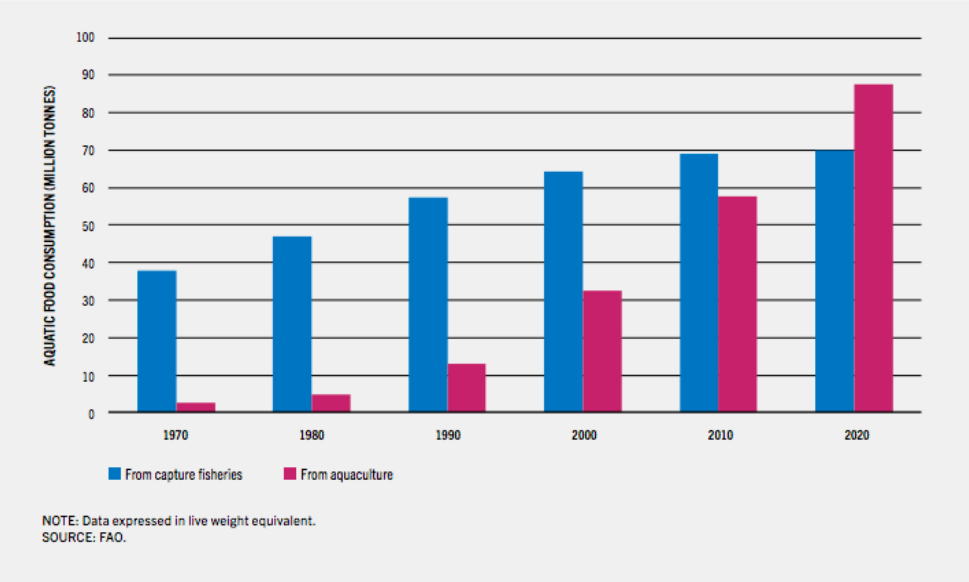

Figure 1. Growth in the consumption of aquaculture products versus fishery products over the last decades (FAO, 2022).

1.2 EUROPEAN SEA BASS (*Dicentrarchus labrax*) AQUACULTURE

The European sea bass (*Dicentrarchus labrax*) emerges as a cornerstone species in European aquaculture, particularly in regions of the Mediterranean and Atlantic, owing to its commercial value and significance in the food chain (Fuentes et al., 2010). The sea bass has garnered recognition for its rapid growth, high feed conversion rate, and adaptability to controlled cultivation conditions (APROMAR, 2023). Consequently, in 2021, it was deemed the second most economically valuable species, with rainbow trout leading the list. By 2022, European sea bass production had reached 301,420 tons, solidifying its status as one of the most relevant species in Mediterranean aquaculture, notably in countries such as Turkey, Greece, Egypt, and Spain (APROMAR, 2023) (Figure 2). Sea bass farming is practiced across virtually all countries in the Mediterranean region. During the first month of life, larvae feed on artemia and rotifers before transitioning to feed formulated from natural raw materials. Rearing systems vary, encompassing floating sea cages, tanks, or land-based ponds (APROMAR, 2023). Commercial sizes range from 250 g to over 2500 g. Typically, the growth process to reach 400 g takes between 20 and 24 months from larval hatching. It is estimated that sea bass production in Spain during 2022 amounted to 23,622 tons, making it the second most produced aquaculture species (APROMAR, 2023). The Canary Islands represent 21% of Spain's total production (APROMAR, 2023) (Figure 3).

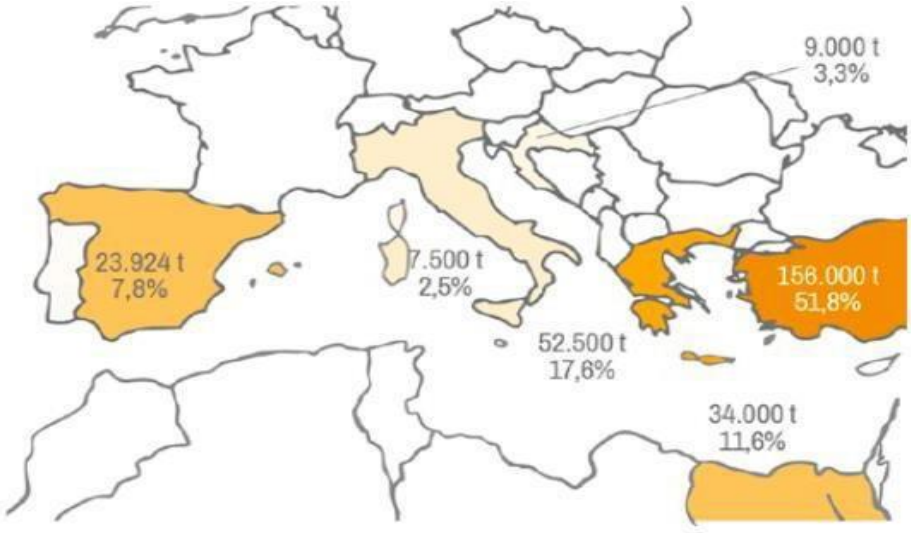

Figure 2. Representation of European sea bass aquaculture production in Europe, expressed in tons (APROMAR, 2023).

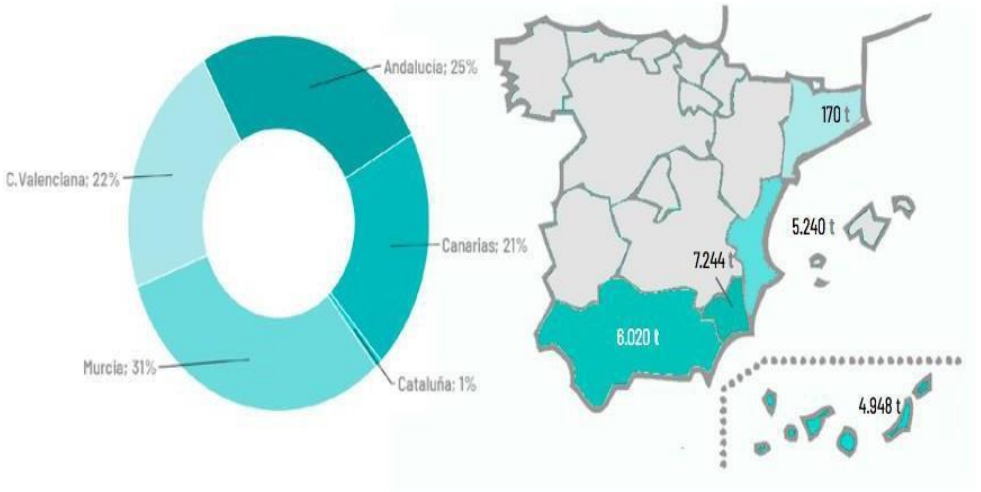

Figure 3. Representation of European sea bass aquaculture production in Spain, expressed in tons (APROMAR, 2023).

1.2.1 The European Sea Bass as a Cornerstone of Aquaculture Research

The sea bass plays a pivotal role in aquaculture research, addressing a wide array of challenges and key aspects in aquaculture production. Its significance lies in its capacity to serve as a valuable model in multiple facets:

- The European sea bass serves as an effective model for examining physiological and metabolic processes linked to growth, reproduction and health in aquaculture environments (Di Marco et al., 2008; Ribas et al., 2019; Stavrakidis-Zachou et al., 2019). Understanding these aspects is essential for enhancing the efficiency of aquaculture production.
- The adaptability of European sea bass to a variety of production systems, from land-based ponds to marine cages (APROMAR, 2023), allows for the investigation of the effects of diverse environmental conditions and management practices on growth and the quality of the final product.
- Research involving European sea bass contributes to the understanding of mechanisms of the immune system and the resistance to common diseases in aquaculture (Miccoli et al., 2024; Valsamidis et al., 2023), thereby informing strategies for disease prevention and control, consequently reducing associated economic losses.

- European sea bass is employed in genomic research and selective breeding, identifying genes related to desirable traits, thereby driving genetic improvement programs for enhanced production (Montero et al., 2023; Vandeputte et al., 2017, 2019).

Consequently, sea bass stands as a fundamental pillar in aquaculture research, providing valuable insights to enhance the efficiency, sustainability, and competitiveness of this constantly evolving industry.

1.3 CURRENT CHALLENGES IN AQUACULTURE

Currently, the success of aquaculture is not without its challenges. Despite its numerous benefits, aquaculture often faces criticism due to its environmental impact, such as water pollution, the introduction of invasive species, degradation of aquatic ecosystems, and the development of antibiotic-resistant bacteria (Martinez-Porchas & Martinez-Cordova, 2012). To address these challenges, the aquaculture industry must focus on implementing sustainable practices, including the establishment of sustainable aquaculture systems with proper waste management (Boyd et al., 2020). Furthermore, the increasing demand for aquaculture products underscores the importance of ensuring food security (Pradeepkiran, 2019). This entails addressing concerns regarding product quality, traceability, and the implementation of stringent standards and regulations.

The social acceptance of aquaculture is also a significant industry challenge. Uncertainties and misconceptions regarding its environmental impact, animal welfare, food safety, and product quality can negatively influence consumer perceptions (Bacher et al., 2014; Schlag, 2010; Whitmarsh & Palmieri, 2009). Raising consumer awareness of the importance of aquaculture in the modern world is essential. Addressing issues such as overfishing, sustainable sources of protein, and product quality and safety should be crucial for industry development.

Regarding animal welfare, the health of aquatic animals is a critical factor directly affecting the production and sustainability of the sector (Franks et al., 2021). Fish handling and manipulation, inadequate environmental conditions, and high stocking densities, prevalent in current European aquaculture, are stress-inducing factors (Bergqvist & Gunnarsson, 2013). Stress is a limiting factor in aquaculture, potentially responsible for reducing growth rates (feed conversion ratio), influencing the quality of

the final product (texture, flavor, and appearance) (Peng et al., 2024), interfering with reproductive processes (egg and larval quality) (Schreck, 2010), and triggering abnormal behaviors such as aggression among fish in cultivation systems (Andersson et al., 2022). Moreover, stress can weaken fish immune systems, making them more susceptible to infectious diseases (Dai et al., 2023; Tort, 2011). Therefore, pathogenic bacteria pose a significant challenge in current aquaculture (Ben Hamed et al., 2018), with particular emphasis on biofilm formation, serving as a pivotal ecological niche for numerous pathogenic microorganisms (De Silva & Heo, 2022).

1.3.1 Biofilm in Aquaculture

Biofilm formation stands out as a prominent concern within the aquaculture industry. Biofilm is a complex microbial structure mainly composed of bacteria adhered to a surface and embedded in a matrix of extracellular polymeric substances (Hobley et al., 2015; Peng et al., 2024). The formation of biofilm (Figure 4), by bacteria, is mediated by the phenomenon described as *quorum sensing* (Hemmati et al., 2024; Peng et al., 2024). The formation of this fascinating structure grants various advantages to the bacteria composing it, such as increased protection against environmental conditions, disinfectants, antibiotics, or other antimicrobial agents (Dufour et al., 2010). It also favors the transmission of antimicrobial resistance genes (Michaelis & Grohmann, 2023). In the intricate scenario of aquaculture, biofilms formed by pathogenic bacteria emerge as a central concern, demanding detailed understanding and effective control strategies (Kilic & Bali, 2023; Mishra et al., 2020). The inherent threat of biofilm from pathogenic bacteria in aquaculture is based on its ability to create favorable microenvironments for the proliferation of harmful microorganisms. This microbial habitat can promote the development and persistence of pathogens, increasing the risk of infectious diseases in aquatic populations.

Given this scenario, different strategies have been proposed to effectively combat biofilm from pathogenic bacteria in aquaculture facilities. However, this tedious problem still persists.

In the academic and research sphere, recent studies have explored innovative strategies for the effective control of biofilm in aquaculture. Recent research includes the application of nanoparticles with antimicrobial properties (Al-Wrafy et al., 2022), bacteriophages (Liu et al., 2022), bactericidal/bacteriostatic coating (Chen et al., 2013), and *quorum quenching* (Paluch et al., 2020).

In the context of European sea bass, literature confirms *Photobacteria* spp., *Tenacibaculum* spp., and *Vibrio* spp. as the predominant bacterial pathogens affecting this species (Muniesa et al., 2020). Specifically, *Vibrio* spp. stand out as a primary concern in the pathology of European sea bass farms in the Canary Islands. In response to the diverse challenges posed by the aquatic environment, *Vibrio* spp. have evolved to utilize biofilm production as a survival strategy (Arunkumar et al., 2020; De Silva & Heo, 2022).

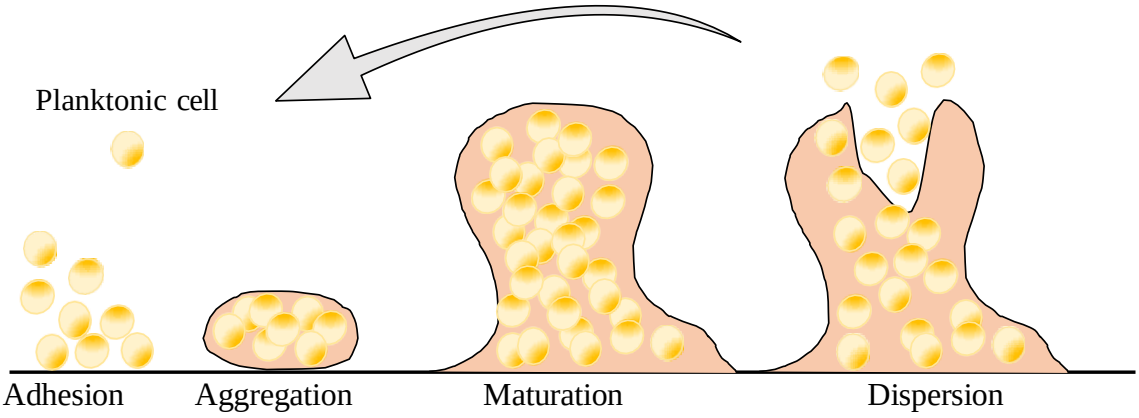

Figure 4. Schematic representation of the process of bacteria biofilm formation. Initially, free-floating planktonic cells adhere to a surface using specific proteins (**Adhesion**). Once attached, these cells start to clump together and initiate the production of extracellular matrix (**Aggregation**). As the cells continue to divide, this aggregation evolves into a mature biofilm (**Maturation**). At the final stage, known as the **dispersion** stage, certain enzymes including protease and nuclease, along with a *quorum sensing* mechanism, facilitate the breakup of the biofilm. This process enables the bacterial cells to release from the biofilm and revert to their planktonic form, thereby spreading to colonize new ecological niches.

1.4 *VIBRIO* SPP. AS A LEADING PATHOGEN IN AQUACULTURE

Vibrio is a genus of Gram-negative bacilli commonly found in aquatic environments, both in saltwater and freshwater (Baker-Austin et al., 2018). *Vibrio* species are characterized by their curved rod-shape, approximately 2-3 µm in length, and a polar flagellum that provides them with mobility (Mittal et al., 2023). Many bacteria within the *Vibrio* genus are pathogenic and cause a disease, vexing aquaculture producers, known as vibriosis (Sanches-Fernandes et al., 2022). The *Vibrio* species most associated with vibriosis in the aquaculture sector include *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio anguillarum* (de Souza Valente & Wan, 2021; Manchanayake et al., 2023). This disease primarily affects the gills, skin, and internal organs of aquatic organisms. Common clinical symptoms include skin ulcers, internal hemorrhaging, inflamed gills, loss of appetite, and lethargy, among others. In more severe cases, vibriosis can lead to mortality (Frans et al., 2011).

The broad range of hosts -such as sea bream (Aly et al., 2023), sea bass (Kapetanović et al., 2022), turbot (Montes et al., 2003), salmon (Benediktsdóttir et al., 1998), shrimp (de Souza Valente & Wan, 2021), and other marine organisms- and its troublesome symptoms underscore the vital importance of its control. While the disease can be suspected based on clinical signs, definitive diagnosis is typically achieved through the following methods: (i) Biochemicals assays, (ii) PCR, (iii) direct microscopy, (iv) ELISA, (v) microarrays (vi) immunoassays and (vii) loop- mediated isothermal amplification, among others (Loo et al., 2022).

The prevention of vibriosis in aquaculture is difficult, and the various treatments for vibriosis have their limitations (Kah Sem et al., 2023). Historically, antibiotics have been relied upon to address this disease (Loo et al., 2020). However, their indiscriminate use has raised significant concerns, including the development of bacterial resistance and the presence of residues in aquaculture products. Consequently, these issues have implications for both human health and aquatic ecosystem health (Bondad-Reantaso et al., 2023). Therefore, aquaculture research prioritizes the pursuit of sustainable and effective alternatives.

1.5 ANTIBIOTICS IN CURRENT AQUACULTURE AND THEIR CHALLENGES

Arguably, one of the most significant historical achievements in science has been the development of antibiotics to combat infectious diseases and problems caused by pathogenic or opportunistic bacteria. The term "antibiotic" was coined last century by the American microbiologist Selman A. Waksman, who described the antagonistic ability of certain microorganisms against others (Waksman, 1947). Antibiotics have been pivotal in saving numerous lives across various organisms. They have also contributed to improvements in animal production systems (Hao et al., 2014), including aquaculture (Adenaya et al., 2023).

In modern aquaculture, the high-density conditions prevalent in ponds and cages create an environment conducive to the rapid transmission of diseases. In these crowded settings, pathogens can easily spread among aquatic organisms, posing a significant threat to production sustainability (Irshath et al., 2023). Therefore, the use of antibiotics is an essential tool to combat bacterial infections that threaten the viability of production. In addition, antibiotics have been used with prophylaxis proposes (Hossain et al., 2022). Aquaculture is not exempt from the challenges posed by antibiotics in contemporary times. In the context of aquaculture, certain regions of the world are deemed "hotspots" for the emergence of antibiotic-resistant bacteria (Cabello et al., 2016). Clear examples of species that readily develop resistance in aquaculture production include *Edwardsiella*, *Vibrio*, *Pseudomonas*, and *Aeromonas* (Dutta et al., 2021; Leung et al., 2019; Nguyen et al., 2014). These multidrug-resistant bacteria, which are resistant to multiple antibiotics, pose significant challenges for companies engaged in fish and mollusk production, making infections harder to control (Bondad-Reantaso et al., 2023). In fact, there is a potential for transferring these resistant strains to human populations through the food chain or environmental pathways (Da Costa et al., 2013). Therefore, there is an urgent need for studies analyzing the presence and transmission of antimicrobial resistance genes and the pursuit of efficient solutions.

The challenges of antibiotic use extend beyond the potential for multidrug resistance. Antibiotics employed in aquaculture production can directly enter the environment, affecting water quality and local biodiversity. These substances can disrupt microbial communities and aquatic ecosystems, potentially causing harm to a wide range of non-target species (González-Gaya et al., 2022; Kraemer et al., 2019).

In addition, there is a growing consumer concern about the presence of antibiotic residues in seafood, which can have health implications and affect market access and product acceptability. In fact, consumer is concern over the antibiotic use in food production and the population is starting to demand for "antibiotic-free" products.
For all these reasons, the search for sustainable alternatives is of paramount importance.

1.6 STRATEGIES TO REDUCE ANTIBIOTIC USE IN AQUACULTURE

Due to the fervent concern over the excessive use of antibiotics in aquaculture, previously described, the search for solutions has become a top priority. In order to prevent antibiotic use, aquaculture facilities implement the following strategies:

i. Reduction of physical (temperature, photoperiod, dissolved oxygen, sound, turbidity, handling), chemical (water quality parameters, pesticides, pollution, diet, metabolic waste), biological (stocking density, micro-organisms, macro-organisms, swimming requirements, predators) stressors (Ciji & Akhtar, 2021). Reducing stress promotes disease resistance, thereby preventing the need for antibiotics.
ii. Disinfection of facilities and tools to prevent the entry and spread of pathogens (Acosta et al., 2021).
iii. Implementation of effective vaccination programs to reduce disease incidence (Du et al., 2022).
iv. Avoidance of the constant use of a single type and instead opting for rotation to prevent the emergence of resistance genes (Brown & Nathwani, 2005). Prior antibiogram testing is always advisable (Truong et al., 2021).
v. Provision of balanced and nutritious diet to strengthen the fish's immune system (Mendivil, 2021).
vi. Implementation of early warning system for fish diseases (Li et al., 2009). Early diagnoses help detect the onset of diseases in their initial stages of infection, enabling timely action to prevent spread within facilities.

Furthermore, raising awareness and educating aquaculture producers about the importance of waste management, antibiotic use, and promoting other sustainable alternatives is crucial. When discussing alternatives to replace antibiotic use in aquaculture, notable options include the use of: vaccines, food additives such as essential

oils and plant extracts, enzymes, bacteriophages, probiotics, prebiotics, postbiotics, and symbiotics, among others (MacNair et al., 2023).

1.7 PROBIOTICS IN AQUACULTURE

In recent years, there has been a significant rise in the utilization of probiotics in aquaculture, owing to their emergence as a promising alternative to conventional antibiotic use (Cruz et al., 2012). Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host (Hill et al., 2014). In the aquaculture context, probiotics can be live bacteria, live yeasts, and live microalgae that usually colonize the gastrointestinal tract of animals (Monzón-Atienza et al., 2023). The application of probiotic components in fish triggers interactions with the host's gut bacteria, resulting in the formation of a wide range of metabolites that could generate positive effects for the host (Ringø et al., 2022). Probiotics enhance various aspects of the host, such as growth, nutrient assimilation, immunomodulation, disease resistance, and survival rates, while mitigating environmental stress (Butt et al., 2021). Additionally, probiotics can modify the relationship between the host and the microbe -including the entire microbial community. They also contribute to optimizing food utilization by increasing its nutritional value and strengthening the host's immune response against various pathogens (Hemarajata & Versalovic, 2013).

1.7.1 Sources of Probiotics

Microorganisms are inherently present in humans, animals, soils, sediments, snow, as well as in fresh-, brackish-, and salt-water environments (El-Saadony et al., 2021). Typically, within the context of aquaculture, these microorganisms are found in the gastrointestinal tract of fish. Through selection methods (See Section 1.7.2) they are isolated and cultured for use as probiotics (Kiron, 2015). *Bacillus* spp. stands out as one of the most employed probiotics (Elshaghabee et al., 2017). This is likely due to its sporulation capability, which enhances survival in the gastric tract by resisting exposure to gastrointestinal acids (Zhang et al., 2020). Furthermore, its dual nature, both aerobic and facultative anaerobic, explains its ability to thrive in various environments (Kuebutornye et al., 2019; Nayak, 2021). In recent years, the most used probiotics in European sea bass are bacteria, particularly *Bacillus* spp., *Pediococcus* spp., *Lactobacillus* spp., *Vibrio* spp., *Shewanella* spp., and *Vagococcus* spp (Monzón-Atienza et al., 2023).

1.7.2 Selection Criteria for Probiotics

The essential characteristics that microorganisms must fulfill to be considered probiotics have been described by numerous authors. The fundamental requirements for a probiotic to be effective and obtain such qualification are detailed as follows (Balcázar et al., 2006; El-Saadony et al., 2021; Hai, 2015; Kesarcodi-Watson et al., 2008; Kiron, 2015; Merrifield et al., 2010):

a. The microorganism must demonstrate the ability to adhere to and grow in the host organism. Therefore, it must be capable of tolerating bile, gastric juice, and the host's pH.
b. The probiotic candidate should lack antibiotic-resistant genes and should not modify the inheritable traits of the host organism.
c. The microbe must benefit the host's system.
d. The probiotic should possess antimicrobial properties against potential pathogens.
e. The probiotic microorganism should not cause harmful effects on the host. The evaluation of probiotic strains is carried out through *in vitro* and/or *in vivo* tests.

1.7.3 Technological Aspects and Routes of Administration of Probiotics

Technological aspects for probiotic production are crucial, as manufacturing conditions and storage can significantly affect the viability of the microorganism. The methods of probiotic administration vary depending on the facility, age, and species of the fish (Cámara-Ruiz et al., 2020). Typically, probiotics are supplied frozen or dried, either in the form of lyophilized or spray-dried powders and encapsulated (Ross et al., 2005). Currently, administration methods in aquaculture include injection, immersion, or incorporation into feed (Amiin et al., 2023; Hai, 2015). However, before choosing the route of administration, certain factors must be considered. Injection induces stress in fish, in addition to being complicated and costly for larval-stage fish (Jahangiri & Esteban, 2018). The advantage of this technique is the assurance that the fish receives the desired dose of probiotic. On the other hand, direct addition of probiotics to water could be applicable at all stages of fish development (Jahangiri & Esteban, 2018). Administration through feed is one of the simplest methods, but the probiotics must be able to withstand pH, temperature, and pressure processes (Kiron, 2015). However, administration via feed faces challenges during larval stages due to the size of fish mouths (Cámara-Ruiz et al., 2020). Regarding research in European sea bass, the most common

routes of administration are through dry feed, vectors, and immersion (Monzón-Atienza et al., 2023).

1.7.4 Modes of Action of Probiotics

For decades, determining the mechanisms of action of probiotics has been a scientific priority. However, accurately pinpointing the mechanisms that probiotic employ to confer a specific benefit to the host is, at the very least, complex. The synergy between multiple modes of action and even interaction with different microbes can be beneficial for the host (Merrifield et al., 2010). In other words, the benefit may not necessarily arise from a direct action of the probiotic. Furthermore, numerous authors disagree on the correlation between results from *in vitro* and *in vivo* trials, as noted by Tinh et al., (2008). Due to the multitude of mechanisms that a probiotic can utilize to exert its action, to date, there is no complete agreement on the outcomes obtained *in vivo*. Therefore, an increase in research by the scientific community is recommended to strengthen understanding of how probiotics function (Bermudez-Brito et al., 2012; Tinh et al., 2008). The most widespread mechanisms of action in fish include (El-Saadony et al., 2021):

i. *Competitive exclusion through the production of inhibitory compounds*. Competitive exclusion through the production of inhibitory compounds is a phenomenon in which an organism (probiotic) competes for available nutrients and mucosal adhesion sites of another organism (pathogen) in a particular environment (Aburjaile et al., 2022). This prevents or limits the growth and/or survival of the pathogenic organism (Knipe et al., 2021). Among the wide variety of exclusion methods available, the production of substances or compounds -including the production of organic acids, inhibitory peptides, inhibitory proteins, bacteriocins, etc- is one of them (Prabhurajeshwar & Chandrakanth, 2017).

   In addition, the inhibition of *quorum sensing* (QS), also called *quorum quenching* (QQ), have been considered as competitive exclusion. *Quorum quenching* involves the inhibition of such inter- and/or intraspecies bacterial communication through chemical or enzymatic means (Sikdar & Elias, 2020).

ii. *Competition for nutrients, chemicals, or energy*.

   Bacteria, encompassing both probiotic and pathogenic strains, rely on nutrients, chemicals and energy for their growth and proliferation. The competition for resources becomes intense when these species utilize similar nutrient sources, leading to a hostile competitive environment (Hoseinifar et al., 2018; Wuertz et

al., 2021). The utilization of available resources by probiotic bacteria serves to limit their accessibility to pathogenic counterparts in the environment (Kuebutornye et al., 2020; Balcázar et al., 2008). By outcompeting pathogens for resources, probiotics can effectively reduce the growth and proliferation of pathogenic bacteria, thereby helping to maintain a healthier microbial community.

iii. *Competition for adhesion sites.*

Bacteria often engage in competitive interactions characterized by exclusion or suppression of the growth of other species. Notably, probiotic bacteria employ various mechanisms and properties to hinder the adhesion of pathogens, primarily resulting in the exclusion of adhesion sites (Balcázar et al., 2008). This competitive exclusion by probiotics effectively impedes the action of pathogenic bacteria by obstructing infection pathways (Raheem et al., 2021).

iv. *Enzymatic contribution.*

The enzymatic contribution of probiotics is essential for maintaining health and balance in fish. Probiotic strains can produce a wide variety of enzymes that aid in nutrient digestion, the degradation of indigestible compounds and intestinal integrity (Assan et al., 2022; Maske et al., 2021; Shekarabi et al., 2022). Therefore, the enzymatic activity of probiotics provides a range of benefits to the host, contributing to its overall well-being and the prevention of various diseases (Assan et al., 2022).

v. *Enhancement of the immune response.*

Broadly, the innate immune response in fish serves as the primary defense mechanism against pathogens. This defense system comprises physical barriers, specialized cells, and effector molecules that swiftly and broadly detect and neutralize infectious threats (Magnadóttir, 2006). At a deeper level, the immune system encompasses pattern recognition systems (PRRs) responsible for identifying pathogen-associated molecular patterns (PAMPs) that breach the host's physical barriers. The interaction between PRRs and PAMPs triggers the activation of the innate immune response (Bermudez-Brito et al., 2012). Among the most extensively studied PRRs are Toll-like receptors (TLRs), which come in various types sharing structural and functional similarities. Notably, type 2 TLRs (TLR-2) specialize in recognizing PAMPs present in bacterial cell walls, particularly those of Gram- positive bacteria (Oliveira-Nascimento et al., 2012). Probiotics contain PAMPs and can thus be detected by the host's PRRs. Upon

detection and binding, this interaction initiates intracellular signaling cascades leading to the expression of effector molecules such as cytokines (Hasan & Banerjee, 2020), nitric oxide (NO) synthesis (Korhonen et al., 2001), production of reactive oxygen species (ROS), and nitrogen (González-Magallanes et al., 2023), which are crucial mechanisms in combating microbial intrusion.

1.8 OBJECTIVES

1.8.1 GENERAL OBJECTIVES

The main objective of this study is to demonstrate the properties of *Bacillus velezensis* D-18, its mechanism of action as a probiotic, and its application in aquaculture.

1.8.2 SPECIFIC OBJECTIVES

- Conduct an *in vitro* analysis to assess the properties of *B. velezensis* D-18 and determine its suitability as a candidate probiotic bacterium.

- Carry out an *in vivo* test in order to establish the safety of *B. velezensis* D-18 and estimate the resistance it can give to European sea bass against infection by *Vibrio anguillarum* 507.

- Conduct an *in vivo* study to investigate the ability of *B. velezensis* D-18 to modulate the innate immune response of European sea bass.

- Perform the gene sequencing of *B. velezensis* D-18.

- Study the *quorum quenching* mechanism of the probiotic *B. velezensis* D-18 for the inhibition of *V. anguillarum* 507.

Chapter II

Materials & Methods

2.1 BACTERIA

2.1.1 Strains

- The probiotic strain *Bacillus velezensis* D-18, previously isolated from wastewater samples of a turbot farm in Cantabria (Spain), underwent routine cultivation at 25ºC/26ºC on Brain Heart Infusion Broth (BHIB; Cultimed, Panreac, Spain) with 1.5% NaCl, Luria Bertani (LB; Condalab, Spain) broth and Tryptic Soy Broth (TSB; Condalab, Spain) -according to the experience conducted in each chapter. To accurately define the concentration of bacteria in each culture, bacterial growth was meticulously monitored at 3, 6, 9, 12, and 24 h using optical density measurements (OD_{600}) and serial dilutions at each time point.
- The fish pathogenic strain *Vibrio anguillarum* 507, isolated within our laboratory, was routinely cultured at 25/26°C on BHIB with 1.5% NaCl supplementation and TSB -according to the experience conducted in each chapter. To precisely ascertain the bacterial concentration within each culture, the growth of *Vibrio anguillarum* 507 was monitored at key time points (3, 6, 9, 12, and 24 h) using optical density measurements (OD_{600}) and serial dilutions.
- *Chromobacterium violaceum* MK wild-type strain [CECT 494, obtained from the Spanish Type Culture Collection (CECT)], was routinely cultured on LB broth at 25°C overnight.
- *Chromobacterium violaceum* CV026, a mini-Tn5 mutant of the wild-type ATCC31532 was cultured on LB broth at 26°C overnight.
- *Chromobacterium violaceum* VIR24, provided by Instituto de Investigación Marqués de Valdecilla – IDIVAL (Cantabria), was routinely cultured on LB broth at 26°C overnight.
- *Bacillus subtilis subsp. subtilis* CECT 39 was routinely cultured on LB broth at 37°C overnight.
- *Bacillus cereus* CECT 148 was regularly overnight cultivated at 37°C on LB broth.

2.2 *IN VITRO* ASSAYS

2.2.1 Isolation of the Probiotic Strain

The candidate strain for probiotic development was isolated from wastewater samples collected from a farm located at the Instituto Español de Oceanografía, Santander, Spain. Isolation of bacterium from water samples was done with serial dilution technique on Brain Hearth Infusion Agar (BHIA; Cultimed, Panreac, Spain) medium supplemented with 1.5% NaCl.

2.2.2 Molecular Identification by Sequencing

The molecular identification of the isolated strain was carried out according to the bases described by Ramlucken et al., (2020) with modifications. The total genomic DNA of the isolated bacteria was extracted and purified using the GeneJET genomic DNA isolation kit (Thermo Scientific, USA). The 16S rRNA gene was amplified by PCR using a pair of universal bacterial 16S rRNA gene primers (Table 1). PCR amplification was carried out in a Mastercycler pro S thermal cycler (Eppendorf, Germany) in a 50 µL reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 nM each 2′-deoxynucleoside 5′-triphosphate (dNTPS), 1 µM each forward and reverse primer, 1.25 U of DreamTaq DNA polymerase (Thermo Scientific, USA), 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 to eliminate unincorporated primers and dNTPs. The cleaned PCR products were sequenced using a BrightDye® Terminator Cycle Sequencing Kit (Nimagen, The Netherlands). Then, Sanger sequencing was performed on the ABI 3130XL DNA sequencer (Applied Biosystems, 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.

2.2.3 Growth Inhibition by Co-culture

For the growth inhibition by co-culture assay (Nikoskelainen et al., 2001), an overnight culture of *V. anguillarum* 507 strain and *B. velezensis* D-18 strain and fish pathogen were washed twice with phosphate-buffered saline (PBS), and cell concentrations were

adjusted to an absorbance of 0.5 at 600 nm. Then, 100 μL of bacterial suspensions of *V. anguillarum* 507 and *B. velezensis* D-18 were added to 1 mL of TSB and then allowed to incubate for 24 h at 25°C. As a comparison, a mixture of 100 μL of PBS and pathogen suspension in TSB at same conditions was employed as a control. Following the incubation period, the cell count in each sample was ascertained by spreading appropriate dilutions on Tryptic Soy Agar (TSA; Condalab, Spain). The outcomes are presented as the percentage of pathogen growth in co-culture with a probiotic strain relative to its growth in isolation (control).

2.2.4 *In Vitro* Screening Tolerance Sea bass Bile and pH

The *in vitro* screening for tolerance to sea bass bile procedures (Sorroza et al., 2012) was performed by added fresh bile diluted at 10% from 48 h starved European sea bass to the probiotic candidate strain tested. Briefly, isolate bacteria from frozen stock were inoculated into the respective broth media and allowed to grow for 24 h at 25°C. Triplicate 500 μL aliquots of each suspension were centrifugated (3000×g at 4°C for 5 min). One pellet was resuspended in 1 mL of 1× PBS and the other in 1 mL of 1× PBS that contained 10% fish bile. Each sample were incubated at 25°C for 1.5 h and subsequently serially diluted and plated on their respective media using the drop plate method and colonies were enumerated after 24 h.

The pH tolerance was tested by the addition of different range of pH (2–8) to the strain. 100 μL of bacterial culture at 10^7 CFU/mL was added to 900 μL of PBS with a pH range 2–8. Then, samples were incubated for 1.5 h at 25°C and serially diluted in PBS and determined by plate counting on TSA.

2.2.5 Adhesion Mucus Assays

For the adhesion mucus assays, intestinal mucus from healthy European sea bass was utilized. Fish with an average body weight of 200 g were subjected to a 48 h starvation period, after which their guts were removed, homogenized in PBS, and the mucus preparations were subsequently centrifuged twice. These solutions were then adjusted to 0.5–1 mg/mL protein in PBS using the Bradford Protein Assay Kit (Merck, Germany). Sterilization was achieved by exposing the solutions to UV light for 30 min, and they were stored at −20°C 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 et al., (2008) and

Sorroza et al., (2012). Briefly, the probiotic strain was stained with 2 μL per 10^9 CFU of green fluorescent nucleic acid stain (SYTO 9) (Invitrogen, Thermo Fisher Scientific, USA). 25 μL of each sample was added to 96-well black polystyrene plates (Nunc) and 75 μL of coating buffer (16.8 g sodium hydrogen carbonate, 21.2 g sodium carbonate per litre, pH 9.6) to each well and incubated overnight at 4 ºC. After washing with saline solution, 25 μL of 10^9 CFU/mL of fluorescently labelled bacterial solution was added and then incubated for 30 min in the dark at room temperature. The plates were washed and liquid was removed. Then 50 μL of saline solution was added to the plate and the fluorescent generated by adherent bacteria was recorded and measure by spectrophotometer (485 nm excitation, 535 nm emissions). 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. Bovine serum albumin (BSA) (1mg/mL) and polystyrene were used as controls.

The test of competitive exclusion was performed to analyze if the probiotic strain was able to compete with analyzed fish pathogen for binding sites. The strain selected (25 μL at 10^9 CFU/mL) was placed with the immobilized mucus for 30 min and washed with saline solution. Then, 25 μL 10^9 CFU/mL of stained fish pathogen cells with SYTO 9 were added and incubated for 30 min in the dark at room temperature. Finally, the wells were washed and 50 μL of saline solution was added to record the stain and measure by spectrophotometer. The competitive exclusion rate was expressed as the ratio between the percentage of adherence of the pathogen with and without the probiotic strain stained with SYTO 9 (Van der Marel et al., 2008).

2.2.6 Genome Sequencing, Assembly and Annotation

B. velezensis D-18 isolated from wastewater samples was sent to Macrogen (South Korea) for Illumina paired-ends Whole Genome Resequencing. The reference genome used was *B. velezensis* CBMB205 (GCF_002117165.1), and the library was prepared using the TruSeq DNA PCR-Free kit. Quality checking was performed using the FastQC tool (V0.11.8), and Trimmomatic (v0.38) was used to remove adapter sequences and low-quality reads (Bolger et al., 2014). The filtered reads were then mapped to the reference genome using BWA (v0.7.17) (Li & Durbin, 2010) and duplicated reads were removed using Sambamba (v0.6.8) (Tarasov et al., 2015). Variants were identified by analyzing the information from aligned reads using SnpEff (v4.3t) (Cingolani et al., 2012). These filtered reads were also introduced into the Read Assembly and Annotation Pipeline Tool

(RAPT) for assembly and annotation. Genomic reads were predicted using the RAST server (Aziz et al., 2008).

2.2.7 *Quorum Quenching* Assay

Quorum quenching assay was carried out in concordance with the protocol outlined by Rehman & Leiknes, (2018), with specific adjustments. Initially, two overnight cultures were initiated: one featuring *B. velezensis* D-18 at 26ºC and 140 rpm in LB broth, and the other with *C. violaceum* MK under identical conditions. Then, 1.5 mL of *B. velezensis* culture was subjected to centrifugation (14000 rpm, 10 min), and the resulting supernatant was filtered through a 0.22 mm membrane to isolate extracellular products (ECPs). Simultaneously, the *B. velezensis* pellet was resuspended in 1.5 mL of PBS. Following this, 1 mL of ECPs and 1 mL of the *B. velezensis* culture were subjected to heat inactivation (99ºC/15 min). A culture medium was prepared by combining 1 mL of *C. violaceum* MK broth with 49 mL (1:50) of LB soft agar (0.4%), which was thoroughly mixed, agitated, and poured into 6-well plates. Once solidified, 10 µL of *B. velezensis* culture, *B. velezensis* pellet, ECPs, heat-inactivated *B. velezensis*, heat- inactivated ECPs, and PBS were added to each respective well. The 6-well plates were then cultured for 24 h at 26ºC, and the entire experiment was conducted in triplicate to ensure experiment repeatability.

2.2.8 AHLs Degradation by *Bacillus velezensis* D-18

The degradation of AHL short (C6AHL) and long (C12 AHL) chains (Sigma-Aldrich, USA) by *B. velezensis* D-18 was assessed with a methodology inspired by Santos et al., (2021), with certain refinements. For each AHL chain degradation, a single colony from a freshly cultivated and uncontaminated *B. velezensis* was cultured overnight in 25 mL of LB at 25ºC with continuous agitation at 140 rpm. From this 25 mL culture, 10 mL were subjected to centrifugation (12000 rpm, 15 min, 4ºC), and the supernatant was filtered through a 0.2 µm membrane to obtain ECPs, which a part was also separated and tested to prevent any interference with violacein production by the biomarkers. Simultaneously, the resulting pellet was resuspended in PBS, constituting the *B. velezensis* pellet. Additionally, 15 mL of the original *B. velezensis* culture were preserved for subsequent use.

For both AHL chains, 1.5 mL each of *B. velezensis* pellet, ECPs, and PBS (as a control) were deposited in three separate 50 mL centrifuge tubes (Falcon®, Corning, USA),

respectively. To each Falcon tube, 0.5 µL of C6 AHL (10 µg/µL) or 0.2 µL of C12 AHL (10 µg/µL) were added. In parallel, 5 µL of C6 AHL (10 µg/µL) or µL of C6 AHL (10 µg/µL) were introduced into the preserved 15 mL *B. velezensis* culture. All Falcon tubes were cultured overnight at 26ºC with continuous agitation at 140 rpm. Following the presumed degradations, the results were transferred to 1.5 mL and were centrifuged (14.000 rpm, 15 min) to eliminate bacteria. On the other hand, 10 mL of both presumed degraded *B. velezensis* cultures with AHL were used for pH reconstitution, as described below.

Subsequently, 100 µL of *B. velezensis* culture, *B. velezensis* pellet, ECPs, PBS, and AHL were individually added to separate wells of a 6-well plate containing soft agar (0,4%) with the respective biomarkers; C. *violaceum* CV026 for AHL short chains and *C. violaceum* VIR24 for AHL long chains. 6-well plates were cultured overnight at 26ºC for 48 h.

2.2.9 AHL Reconstitution via pH Adjustment

The pH reconstitution process was adapted from the methodology outlined by Santos et al., (2021) and Singh et al., (2020). As part of the previous described AHL degradation assay, the overnight cultures resulting from the interaction between *B. velezensis* D-18 and the respective AHLs were subjected to centrifugation to eliminate probiotic bacteria. One hundred microliters aliquot of each presumed degradation was utilized for the degradation assay, previous described, while the remainder were allocated for the pH reconstitution assay.

Subsequently, supernatants were adjusted to pH 2 using hydrochloric acid (HCl). Exactly 100 µL aliquot of both pH 2 supernatants were then carefully added to the wells of the previously prepared 6-well plates containing soft agar (0.4%) with the respective biomarkers, *C. violaceum* CV026 and *C. violaceum* VIR24. The plates were incubated for 48 h at 26ºC.

2.2.10 Genetic Analysis by PCR for Lactonase Genes Detection

To ascertain the presence of lactonase-producing genes within the genome of *B. velezensis* D-18, genomic DNA from *B. velezensis* D-18, *Bacillus subtilis subsp. subtilis* CECT 39, and *Bacillus cereus* CECT 148 were extracted and purified using the GeneJET genomic DNA isolation kit (Thermo Scientific, USA). The experiment utilized specific primers for aiiA (El Aichar et al., 2022; Nusrat et al., 2011) and ytnP (Santos et al., 2021)

(Table 1), which are considered lactonase producer genes. *Bacillus cereus* CECT 39 served as the aiiA control, while *Bacillus subtilis subsp. subtilis* CECT 148 functioned as the ytnP control.

PCR amplification was conducted in a Mastercycler pro S thermal cycler (Eppendorf, Germany) in a 50 µL reaction mixture, comprising 5 µL of Taq PCR buffer (10X), 3 µL MgCl2 (50 mM), 0.2 µL each of 2´-deoxynucleoside 5´-triphosphates (dNTPS) (25mM), 1 µL of each forward and reverse primer (1:10 dilution), 0.25 µL of DreamTaq DNA poly-merase 5U/µL (Thermo Scientific, USA), and 4 µL of genomic DNA. The PCR conditions included an initial denaturation at 95°C for 1 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 10 s. Post-PCR cleanup was accomplished using the ExoSAP-IT enzymatic system to eliminate unincorporated primers and dNTPs. Electrophoresis involved the use of diluted 1/10 PCR products, a 2% agarose gel, GelRed® Nucleic Acid Gel Stain (Biotium, USA), and ran under conditions of 80V for 60 min. The marker employed for reference was DL2000 Plus DNA Marker.

2.2.11 *Vibrio anguillarum* 507 *Quorum Sensing* Signaling Molecules

To elucidate the *quorum sensing* (QS) mechanisms of *V. anguillarum* 507, an assay was conducted employing both short-chain and long-chain AHL biomarkers, *C. violaceum* CV026 and VIR24. Separately, these biomarkers were incorporated into liquid 0.4% LB agar and evenly spread onto Petri dishes. Following solidification, three wells were established in each plate. Subsequently, 10 µL of C12AHL (1 µg/µL) - serving as the positive control -, 10 µL of PBS - serving as the negative control -, and 10 µL of an overnight culture of *V. anguillarum* 507 were added to each respective well. The plates were then incubated overnight at 26°C.

2.2.12 *Bacillus velezensis* D-18 *Quorum Quenching* Effects on *Vibrio anguillarum* 507

To assess the potential *quorum quenching* effects of *B. velezensis* D-18 on the marine pathogenic strain *V. anguillarum* 507, an effective assay was conducted. Briefly, the probiotic strain, the pathogenic strain, and the long-chain AHL biomarker, *C. violaceum* VIR24, were cultured, respectively, at 25°C, 140 rpm overnight. Subsequently, 1 mL of *C. violaceum* VIR24 and another mL of *V. anguillarum* were added to 48 mL of 0.4% soft LB agar. This agar was spread onto a Petri dish. Once solidified, 10 µL of the probiotic strain was added to the centre of the plate. The plate was then incubated at 26°C for 24 h.

2.2.13 Inhibition of Biofilm Formation and Growth of *Vibrio anguillarum* 507 by *Bacillus velezensis* D-18

After monitoring the growth dynamics of the probiotic bacteria and the pathogen, we assessed the capacity to inhibit biofilm formation and growth through the following procedure. The probiotic, *B. velezensis*, underwent a 12 h incubation at 26ºC and 140 rpm in 20 mL of BHIB 1.5% NaCl supplemented, yielding a concentration of 10^8 CFU/mL. Simultaneously, the pathogen, *V. anguillarum*, was cultivated at 26ºC and 140 rpm for 3 h in 20 mL of BHIB 1.5% NaCl supplemented, resulting in a concentration of 10^7 CFU/mL. Serial dilutions were conducted to validate these concentrations.

A 1 mL sample was obtained from each culture, underwent centrifugation, had the supernatant removed, and was resuspended in 100 µL of sterile PBS. For the experiment, a 12-well plate was employed, and an enriched and filtered medium inspired by O'Toole, (2011) (using BHIB 1.5% NaCl supplemented instead of 1 × M63) was prepared to facilitate biofilm formation for both species.

Different well compositions were devised as follows in order to establish the desired concentration of each bacterium for assessing the biofilm formation and growth of both strains. The first comprised 2895 µL of enriched medium, 100 µL of *B. velezensis* (final concentration: 10^8 CFU/mL), and 5 µL of *V. anguillarum* (final concentration: 10^5 CFU/mL). The second consisted of 2900 µL of enriched medium and 100 µL of *B. velezensis* (final concentration: 10^8 CFU/mL). The third included 2995 µL of medium and 5 µL of *V. anguillarum* (final concentration: 10^5 CFU/mL). The last only included 3000 µL of medium and served as a control. The remaining wells of the 12-well plate were used as controls of the different treatments (*B. velezensis* and *V. anguillarum*, *B. velezensis*, *V. anguillarum*, and control) to confirm biofilm formation using crystal violet (0.1%) after incubation.

Subsequently, the plate was cultured at 26°C and 100 rpm for 48 h. After this incubation period, the supernatant was removed, and 1 mL from each well was saved for quantification through serial dilutions. Each well underwent three washes with sterile PBS. The well surfaces were scraped, and the material was resuspended in 1 mL of PBS for further serial dilutions to quantify the biofilm amount in UFC/mL.

Serial dilutions of both biofilm formation and culture growth were plated on oxytetracycline (180 µg/mL) plates to quantify the selective growth of *B. velezensis* and on lincomycin (80 µg/mL) plates for *V. anguillarum*.

2.3 FISH

European sea bass (*Dicentrarchus labrax*) specimens were utilized for the study. Depending on the specific chapter under consideration, the individuals had a body weight between 200 g at most and 26 g at least. The experimentation took place at the Marine Science and Technology Park, situated within the premises of the Universidad de las Palmas de Gran Canaria (ULPGC), Spain. Prior to experimentation, the fish underwent a period of acclimatization. They were housed in tanks with a capacity of 500 L for a duration of 15 days for Chapter III and 14 days for Chapter IV. All tanks were equipped with either continuously running seawater or a closed water system, the selection of which was contingent upon the particular chapter being addressed. Water parameters were maintained at 20 ºC with a pH of 8. Aeration was provided continuously, and the tanks were subjected to a natural photoperiod of approximately 12 h light and 12 h dark cycle. Throughout the acclimatization period, the fish were fed daily with a commercial diet sourced from Alterna, Skretting, Spain. The diet comprised pellets of 3 mm diameter, containing 46% fish protein and 16% fish oil.

2.4 EXPERIMENTAL DIETS

Experimental diets incorporating the probiotic strain were prepared by culturing selected bacteria in BHIB for 24 h at 25°C. The preparation of these diets varied depending on the specific chapter (Irianto & Austin, 2002; Panigrahi et al., 2005; Sorroza et al., 2012).

In the first method, the strain was subjected to centrifugation at 2500 × g for 20 min at 4°C. The resulting cell pellet underwent two washes and was then re-suspended in a saline solution to achieve a concentration of 10^{10} CFU/mL, as determined by plate count on TSA. Subsequently, 25 mL of this concentrated strain suspension was evenly spread onto 120 g of commercial feed (Alterna, Skretting, Spain). The mixture was thoroughly combined and left to dry for 24 h at 37°C, resulting in a final concentration of 10^9 CFU/g of the commercial feed.

In the second method, the incorporation of *B. velezensis* D-18 was achieved by live spraying of the probiotic suspension using a spray bottle with the nozzle adjusted to release mist, resulting in a concentration of 10^6 CFU/g of feed. 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. Sterile conditions were maintained throughout the process.

The viability of the incorporated *B. velezensis* was assessed by vortexing 10 g of diet in 90 mL of sterile PBS and preparing serial dilutions. Aliquots of 100 µL were cultured at 25°C for 24 h following classical microbiological procedures.

The stock diet was stored at -20°C, and daily rations were thawed at 4°C prior to feeding. All animals were fed twice daily by hand for either 20 or 30 days at a regular rate calculated as 2 or 5% of their biomass respectively, depending on the specific chapter.

2.5 GENERAL SAMPLING PROTOCOLS

2.5.1 Euthanasia Protocols

For tissue sampling following the different trials, fish were humanely euthanized by administering an overdose of clove oil at a concentration of 0.5 mL/L (Guinama S.L; Spain, Ref. Mg83168) diluted in 100% alcohol (1:2) within 1 min.

2.5.2 Blood Collection and Component Separation

Blood samples were obtained via caudal sinus puncture using 25 G needles attached to a 2 mL syringe (López-Cánovas et al., 2020). Immediately after extraction, blood intended for plasma samples for monocyte isolation was stored in 1.5 mL Eppendorf tubes coated with heparin to prevent coagulation.

For serum samples, blood was stored in non-coated 1.5 mL Eppendorf tubes and maintained at 4°C for 4 to 24 h to ensure coagulation. Subsequently, the blood was centrifuged at 3000 rpm for 15 min to separate the serum, which was then stored at - 20°C until further use.

2.5.3 Head-kidney Extraction for Relative Gene Expression Analysis

Head-kidney samples were collected using sterilized dissection equipment and carefully stored in 1.5 mL Eppendorf tubes containing 1 mL of RNA later (Invitrogen, Thermo Fisher Scientific, USA) and refrigerated at 4°C until used. To prevent cross-contamination between samples during dissection, the dissection equipment was meticulously cleaned with propane AF and thoroughly washed with Milli-Q water after each fish dissection.

Subsequently, the RNA later was removed and TRI Reagent® (Sigma-Aldrich, USA) was added. Then the samples were stored at -80°C until relative gene expression analysis.

2.6 *IN VIVO* ASSAYS

2.6.1 Bio-safety Assay

To assess the potential harmful effects of *B. velezensis* D-18 in European sea bass, 0.1 mL (10^8 CFU/mL) was intraperitoneally injected into 10 fishes. A control group injected with PBS was used for comparison. Fish were monitored daily for 30 days, and at the end of this period, they were sacrificed with an overdose of clove oil (5 mL/L). A necropsy was performed to evaluate possible lesions in the internal organs, and a histological study was conducted. Additionally, fish internal organs were analyzed by microbiological methods on BHIA to determine the presence or absence of the inoculated *B. velezensis*.

2.6.2 Detection of Serum Immune Parameters

The detection of serum immune parameters included assessing serum bactericidal activity, measuring lysozyme activity, and determining nitric oxide levels through established protocols.

2.6.2.1 Serum Bactericidal Activity

The bactericidal activity of a serum was assessed by examining its impact on the growth patterns of *V. anguillarum* 507 strain, following a methodology outlined in a previous reference (Kajita et al., 1990). To provide a concise overview, sera pooled from seven fish specimens in each of the triplicated treatments were subjected to a threefold dilution using 0.1% gelatin-veronal buffer (pH = 7.5, supplemented with 0.5 mM/mL Mg^{2+} and 0.15 mM/mL Ca^{2+}). Subsequently, the diluted sera were combined with *V. anguillarum* (initial concentration: 1×10^6 CFU/mL) suspended in the same buffer, maintaining a 1:1 ratio (v/v). The resulting bacterial mixtures underwent a 90 min incubation period at 20ºC with continuous shaking, followed by spreading on agar plates. The quantification of viable bacteria was accomplished by enumerating the colonies on TSA supplemented with 1% NaCl.

2.6.2.2 Serum Lysozyme Activity

Lysozyme activity was quantified through a turbidimetric approach, involving the lysis of *Micrococcus lysodeitikus* ATCC4698 (Sigma-Aldrich, USA) by the serum of fish fed with the probiotic diet and control diet. Hen egg-white lysozyme was used as standard. A unit of lysozyme activity was defined as a decrease in absorbance at 450 nm by 0.001 per min (Galindo-Villegas et al., 2019).

2.6.2.3 Serum Nitric Oxide Levels

The determination of nitric oxide levels was carried out through the Griess reaction. Briefly, a pool of fish sera was combined with Griess reagent (0.5% sulfanilamide) in 2.5% phosphoric acid and 0.05% N-(1-naphthyl)-ethylenediamine dihydrochloride, all reagents sourced from Merk-Sigma, Spain. This mixture was incubated at 21°C for 10 min in 96-well plates. Subsequently, the absorbance of both the sample and standard wells was measured at 570 nm using an automated ELISA plate reader. The absorbance values of the test samples were converted to micromolar (μM) concentrations of nitrite by comparing them 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 obtained from the standard curve by the dilution factor and expressed in μM.

2.6.3 Peripheral Blood Monocytes Isolation

Building upon the preceding isolation of peripheral blood monocytes (PBMs), isolation of PBMs was executed in both -the control and *B. velezensis*-treated fish-, following established methods (Sha et al., 2017). In summary, 2 mL of heparinized blood, diluted with PBS at a 1:1 ratio, was slowly pipetted onto discontinuous Percoll density gradients (34–51%, Sigma Chemical Co, USA) and centrifuged (1400 × g; 30 min). Cells situated at the Percoll interface were collected and subjected to five washes with 5 mL of sterile Hank's buffer through centrifugation (1000 × g; 5 min). The resulting enriched cell pellet was then re-suspended in L-15 complete medium (Sigma-Aldrich, USA), supplemented with 15% fetal bovine serum (Gibco, USA), and antibiotics [penicillin-streptomycin (Sigma-Aldrich, USA)] at a final concentration of 10 μg/mL.

2.6.4 Peripheral Blood Monocytes Activity

The phagocytosis assay was conducted following previously recommended procedures (Sorroza et al., 2012), albeit with slight modifications. PBMs were subjected to incubation with 10 mL of 10^9 CFU/mL (MOI 1:1; inactivated *Candida albicans*/macrophage cell ratio) for 1 h at 22°C. Following a wash with PBS, the cells underwent staining with Diff Quick solution (Panreac, Spain). Exactly 100 macrophages per slide were meticulously counted, and the phagocytic activity was determined as the percentage of macrophages containing at least one phagocytosed particle per counted cell.

2.6.5 LPS Extraction, Purification and Fish Stimulation

LPS from *V. anguillarum* -as immunostimulant- was extracted and purified followed a hot phenol-water method (Rezania et al., 2011). Briefly, the bacterial suspensions (10^8 CFU/mL) were initially centrifuged, and the resulting pellets underwent two washes in PBS (pH = 7.2) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The pellets were then resuspended in PBS and subjected to sonication to ensure complete cell breakage. To eliminate protein and nucleic acid contaminants, the cell mixture underwent treatment with proteinase K, DNase, and RNase. Proteinase K was added to the mixture, followed by incubation at 65°C. Subsequently, the mixture was treated with RNase, DNase and the incubation continued at 37°C overnight. Hot phenol was added to the mixtures, vigorously shaken, and then cooled on ice before undergoing centrifugation. The resulting supernatants were subjected to re-extraction, followed by precipitation of LPS with sodium acetate and ethanol. After centrifugation, the pellets were resuspended, extensively dialyzed against double distilled water, and lyophilized. The purified LPS product was then weighed, stored at 4°C, and, when needed, resuspended in PBS at the desired concentration. After feeding trial, fish were intraperitoneally stimulated with LPS at a dose of 100 μg/fish.

2.6.6 RNA extraction and gene expression analysis

RNA extraction from the head kidney (HK) was carried out using the RNeasy mini-Kit (QIAGEN, Germany). The concentration of RNA was determined using a NanoDrop spectrophotometer (ND-1000). Subsequently, the SuperScript IV RNase H reverse transcriptase was employed to synthesize first-strand cDNA from 1 μg total RNA, using an oligo-dT18 primer at 50°C for 10 min. For standardizing sample content, the β-actin gene was assessed through semi-quantitative PCR (Castejón et al., 2021). In the same

samples, the expression levels of genes responsible for proinflammatory cytokines [interleukin-1 beta (IL-1ß), tumor necrosis factor alpha (TNF-α), and cyclooxygenase-2 (COX-2)] and the antimicrobial peptide dicentracin (DIC) were analyzed using real- time PCR with a QuantStudioTM 5 Flex instrument. SYBR Green PCR core reagents were employed for this purpose (Castejón et al., 2021). Primers used are detailed in Table 1. Following verification of primer pair efficiency and melting curves, suitable reference genes [40S ribosomal protein subunit 18 (rps18) and β-actin] were chosen based on the average M value. The relative expression of each target gene was normalized using the content of the reference genes in each sample through the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$) (Pfaffl, 2001). Specific primers for European sea bass were used for both target and reference gene.

Gene		Sequence (5´-3´)	Reference
16S rRNA	*Fw*	AGAGTTTGATCCTGGCTCAG	(Sorroza et al., 2012)
	Rv	GCGCTCGTTGCGGGACT TAACC	
rps18	*Fw*	AGGGTGTTGGCAGACGTTAC	(Sepulcre et al., 2007)
	Rv	CTTCTGCCTGTTGAGGAACC	
β-actin	*Fw*	ATGTGGATCAGCAAGCAGG	(El-Aamri et al., 2015)
	Rv	AGAAATGTGTGGTGTGGTCG	
DIC	*Fw*	GGCAAGTCCATCCACAAACT	(Valero et al., 2020)
	Rv	ATATTGCTCCGCTTGCTGAT	
IL-1β	*Fw*	ATCTGGAGGTGGTGGACAAA	(Sepulcre et al., 2007)
	Rv	AGGGTGCTGATGTTCAAACC	
TNF-α	*Fw*	AGCCACAGGATCTGGAGCTA	(Sepulcre et al., 2007)
	Rv	GTCCGCTTCTGTAGCTGTCC	
COX-2	*Fw*	AGCACTTCACCCACCAGTTC	(Sepulcre et al., 2007)
	Rv	AAGCTTGCCATCCTTGAAGA	
aiiA	*Fw*	CGGAATTCATGACAGTAAAGAAGCTTTA	(El Aichar et al., 2022; Nusrat et al., 2011)
	Rv	CGCTCGAGTATATATTCAGGGAACACTT	
ytnP	*Fw*	ATCGGATAATCATCGTAAGC	(Santos et al., 2021)
	Rv	ATTGAACTAAGAACAGACCC	

Table 1. Primer sequences of the different genes analyzed.

2.6.7 Experimental Challenges

For fish experiment challenges, two different techniques were applied:

For the chapter III, the fish were fed with the experimental diet for 20 days before the experimental challenge. Simultaneously, a control group was fed with the same diet without the presence of the probiotic. After 20 days of feeding, the fish were exposed to live cells of *V. anguillarum* 507 through an 8-hour bath at a concentration of 10^8 CFU/mL. Subsequently, the fish were observed daily for 20 days following the exposure to *V. anguillarum*, and any fish showing signs of illness or deceased individuals underwent necropsy (Sorroza et al., 2012) to complete the assay an analysis of internal organs was performed through histopathology.

In chapter IV, after 30 days of probiotic feeding, fish were intraperitoneally injected with live cells of *V. anguillarum* strain 507 at a concentration of (2.7×10^7 CFU/mL) (Galindo-Villegas et al., 2013). Following the injection, the fish underwent monitoring every 12 h for a six-day duration to observe clinical signs of disease, and any occurrences of mortality were duly recorded.

In both methods, bacteria isolated from the fish were identified biochemically. Experimental designs were in compliance 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).

2.7 STATISTICAL ANALYSIS

Chapter III. *Isolation and Characterization of a Bacillus velezensis D-18 Strain, as a Potential Probiotic in European Seabass Aquaculture.*

The statistical analysis of the data employed Student's t-test, with statistical significance established at a two-tailed threshold ($p < 0.05$). The analysis was conducted using the SPSS Statistics Program 17.0 (SPSS, Inc., USA). In the figures, mean values along with standard deviations represent numerical data and bars. The survival curves were derived using the Kaplan–Meier method and compared through the log-rank test.

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

The humoral activities and phagocytosis results underwent a student's t-test, gene expressions were scrutinized through two-way ANOVA followed by post hoc Tukey's analysis, and the survival curve was assessed using a log-rank test to identify group differences. The threshold for statistical significance in all instances was established at $p \leq 0.05$. All statistical analyses were executed using GraphPad Prism 8.04 software.

Chapter VI. *An In-Depth Study on the Inhibition of Quorum Sensing by Bacillus velezensis D-18: Its Significant Impact on Vibrio Biofilm Formation in Aquaculture.*

Statistical analyses were performed using GraphPad Prism software version 8.4.2 for macOS (GraphPad Software, USA). Unpaired t-test was used to test the differences between the groups. $P < 0.0001$ was defined as statistical significance for all tests that necessitated statistical analyses.

2.8 GRAPHICAL SCHEMES

2.8.1 Chapter III. *Isolation and Characterization of a Bacillus velezensis D-18 Strain, as a Potential Probiotic in European Seabass Aquaculture*.

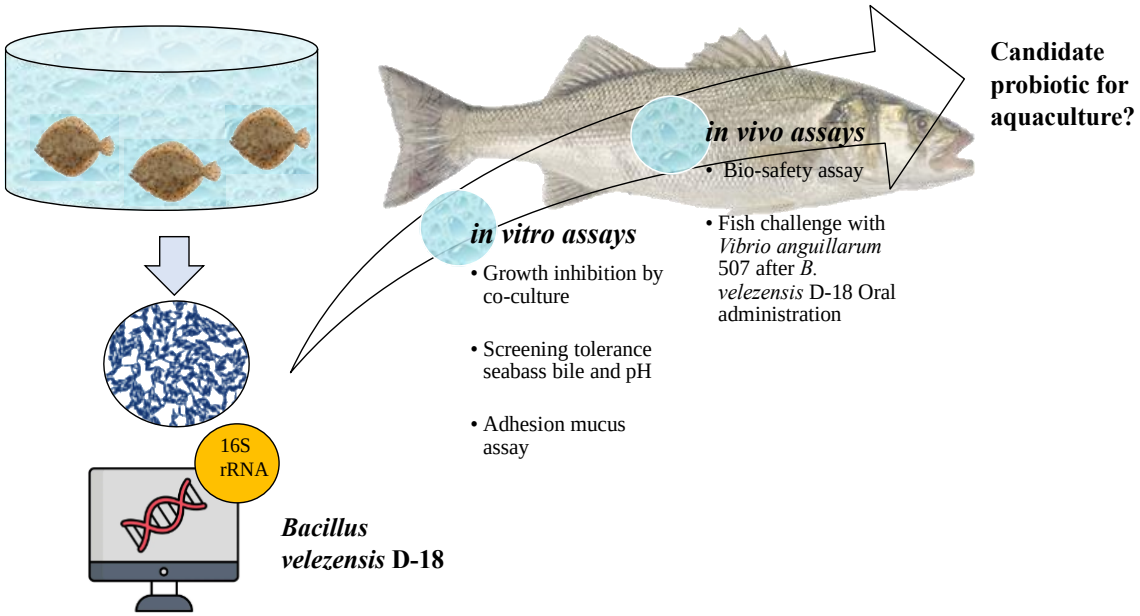

Figure 2.1. Graphical scheme of Chapter III. The aim of this chapter was to analyse a strain of *Bacillus velezensis* D-18 isolated from a wastewater sample collected from a fish farm, for use as probiotics in aquaculture. The strain was evaluated *in vitro* through various mechanisms of selection, as growth inhibition by co-culture, screening tolerance sea bass bile and pH, and adhesion mucus assay. Then an *in vivo* evaluation was followed by a bio-safety assay and by a fish challenge with *Vibrio anguillarum* 507 after *B. velezensis* D-18 oral administration.

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

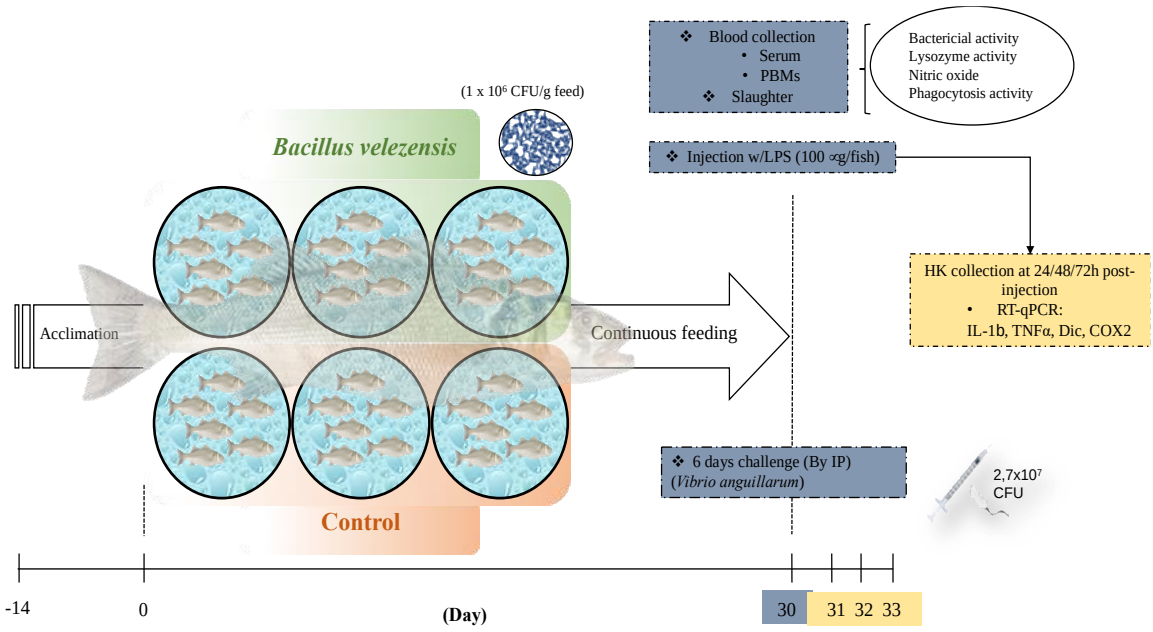

Figure 2.2. Graphical scheme of Chapter IV. After the acclimation period, from day 0, control or *Bacillus velezensis* strain D-18- supplemented diet was orally administered daily within 30 days to the European sea bass. On day 30, animals from each group were aseptically bled. Serum and peripheral blood 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), and a classical phagocytic assay was performed. Moreover, fish per treatment were intraperitoneally. stimulated with *Vibrio anguillarum*-LPS (100 µg/fish) on the same day. After 24, 48, and 72 h, the head-kidney from six animals per condition were obtained, and the gene expression was analyzed by qPCR. Finally, the remaining animals of each treatment were subjected to a bacteria challenge against (2.7×10^7 CFU/mL) *V. anguillarum* strain 507.

2.8.3 Chapter VI. *An In-Depth Study on the Inhibition of Quorum Sensing by Bacillus velezensis D-18: Its Significant Impact on Vibrio Biofilm Formation in Aquaculture*

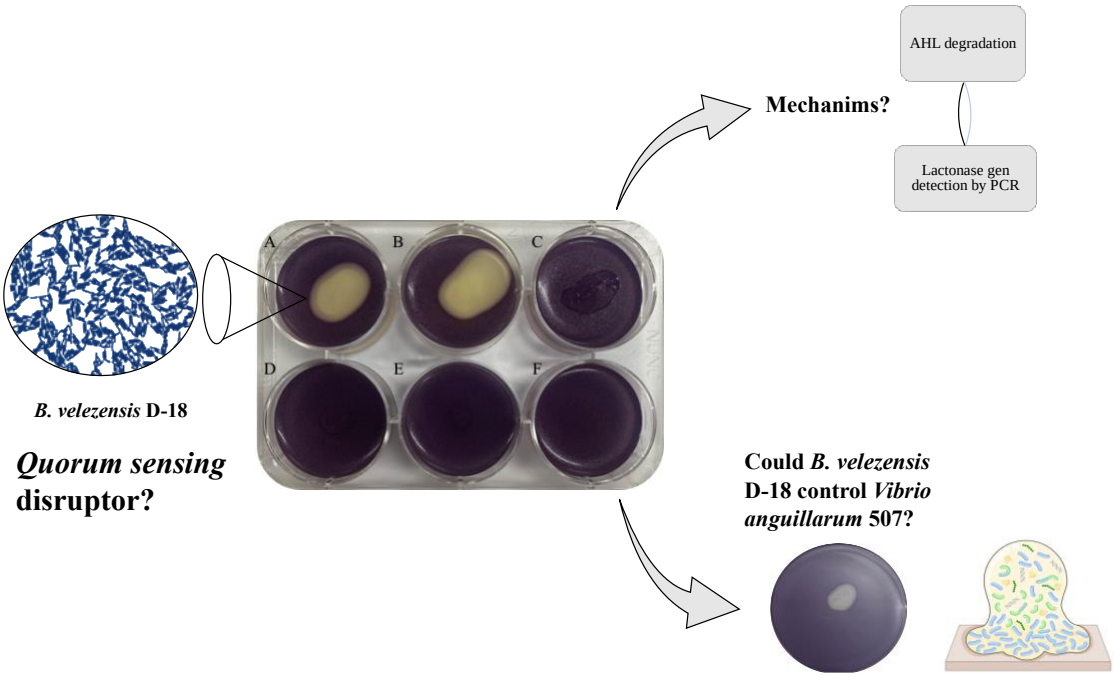

Figure 2.3. Graphical scheme of Chapter VI. *Chromobacterium violaceum* MK serves as the biomarker for evaluating *quorum sensing* (QS) inhibition, testing the *quorum quenching* (QQ) potential of the probiotic strain *Bacillus velezensis* D-18. To assess the inhibitory effects of *B. velezensis* D-18 on QS, a co-cultivation technique was employed, involving the simultaneous growth of the biomarker strain *C. violaceum* MK with *B. velezensis* D-18. Exploration of the QQ mechanism utilized *C. violaceum* CV026 and VIR24. The study assessed *B. velezensis* D-18's ability to degrade both long and short-chain Acyl Homoserine Lactones (AHLs). Additionally, PCR analysis was conducted to identify lactonase-producing genes in *B. velezensis* D-18. Evaluation of the impact of *B. velezensis* D-18 on pathogenic bacteria was performed using *Vibrio anguillarum* 507 as the model organism, focusing on its ability to control biofilm formation and restrain pathogen growth.

2.8.4 Chapter VII. *Current Status of Probiotics in European Sea Bass Aquaculture as One Important Mediterranean and Atlantic Commercial Species: A Review*

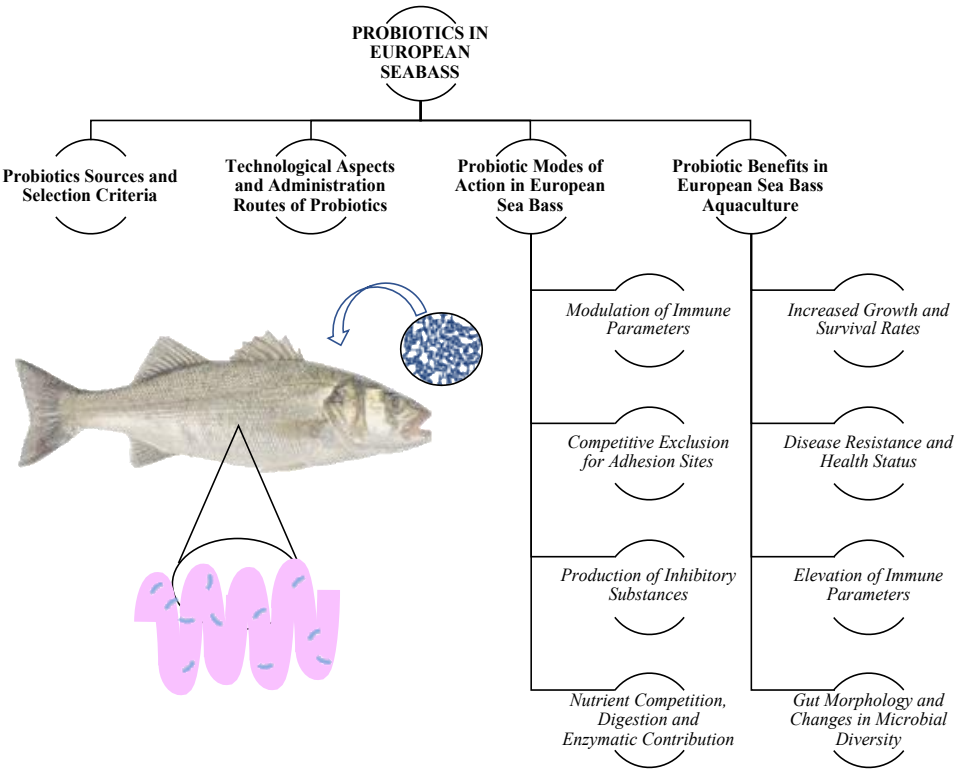

Figure 2.4. Graphical scheme of Chapter VII. Through a comprehensive examination of recent research, this review clarifies the profound impact of probiotics on European sea bass aquaculture. After conducting an overview of probiotics, insights into their mechanisms and benefits in European sea bass are synthesized, highlighting their influence on growth performance, microbial diversity, enzyme production, immunity enhancement, disease resistance, and overall survival, aiming to provide a comprehensive understanding for future research endeavors.

Chapter III

Isolation and Characterization of a *Bacillus velezensis* D-18 Strain, as a Potential Probiotic in European Seabass Aquaculture

Probiotics and Antimicrobial Proteins
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Isolation and Characterization of a *Bacillus velezensis* D-18 Strain, as a Potential Probiotic in European Seabass Aquaculture

Luis Monzón-Atienza[1] · Jimena Bravo[1] · Silvia Torrecillas[1] · Daniel Montero[1] · Ana Franco González-de Canales[2] · Inés. García de la Banda[2] · Jorge Galindo-Villegas[4] · José Ramos-Vivas[1,3] · Félix Acosta[1]

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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% 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 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% of the world's edible fish, accounting for nearly 50% 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 t, mainly manufactured in Turkey, Greece, Spain and other Mediterranean countries [2].

Nowadays, in order to optimise 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 as follows: *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

✉ Félix Acosta
felix.acosta@ulpgc.es

1 Grupo de Investigación en Acuicultura (GIA), Instituto Ecoaqua, Universidad de Las Palmas de Gran Canaria, Las Palmas, Spain

2 Instituto Español de Oceanografía, Centro Oceanográfico de Santander, Santander, Spain

3 Departamento de Biología Molecular, Universidad de Cantabria, Santander, Spain

4 Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway

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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 defined 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 °C 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, MA, 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 50 µL reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 nM each

2′-deoxynucleoside 5′-triphosphate (dNTPS), 1 μM each forward and reverse primer, 1.25 U 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 days; all tanks were supplied with continuously running seawater, constant aeration and a natural photoperiod (around 12 h:12 h 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 48 h 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 30 min and stored at −20 °C 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 70%, 1 h; alcohol 96%, 1 h; alcohol 100%, 30 min; alcohol 100%, 1 h; alcohol 100%, 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 100 °C 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 °C 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 °C 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 log-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, showing 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 (Fig. 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 (Fig. 2a). Bacteria did not exhibit statistical differences in growth when exposed to PBS with 10% seabass bile and PBS without fish bile added.

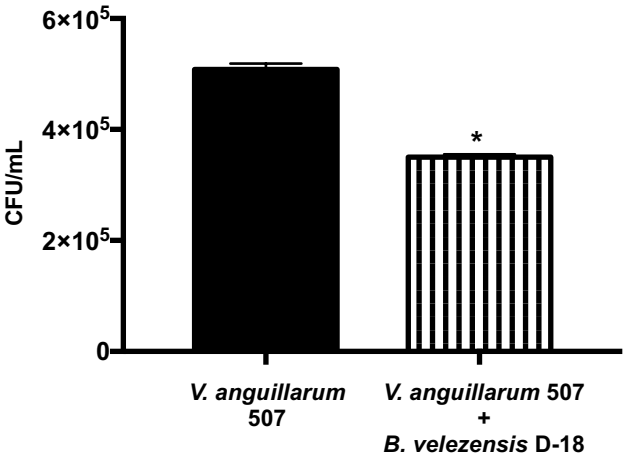

Fig. 1 Growth effect by co-culture between *V. anguillarum* 507 and *B. velezensis* D-18. The asterisk indicates a significant statistical difference ($p < 0.05$) in the reduction of *V. anguillarum* 507 growth when cultured with *B. velezensis*

At pH below 4, the bacteria did 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 (Fig. 2b).

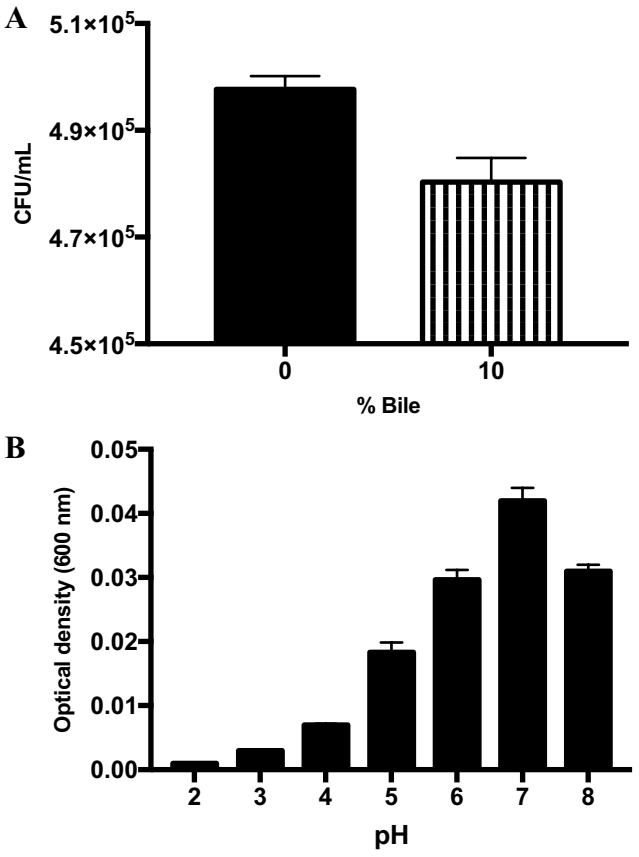

Fig. 2 Tolerance of *B. velezensis* D-18 strain to sea bass bile (**a**) and pH (**b**)

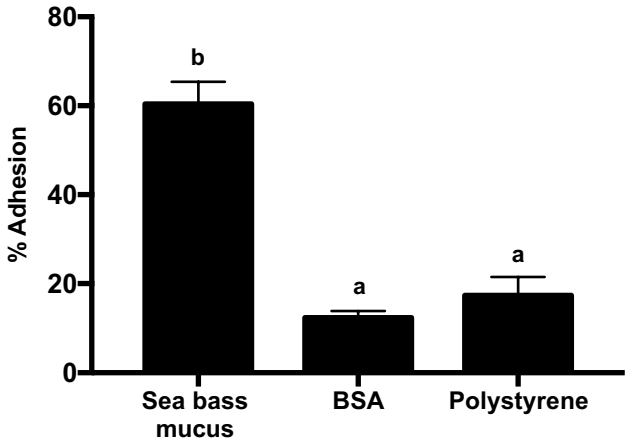

Fig. 3 Percentage of adhesion of the *B. velezensis* D-18 strain to sea bass mucus, BSA, and polystyrene. All data are given as percentage of the absorbance measurements of fluorescent stained bacteria ± SD. Letters indicate significant statistical differences ($p < 0.05$)

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 (Fig. 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 (Fig. 4).

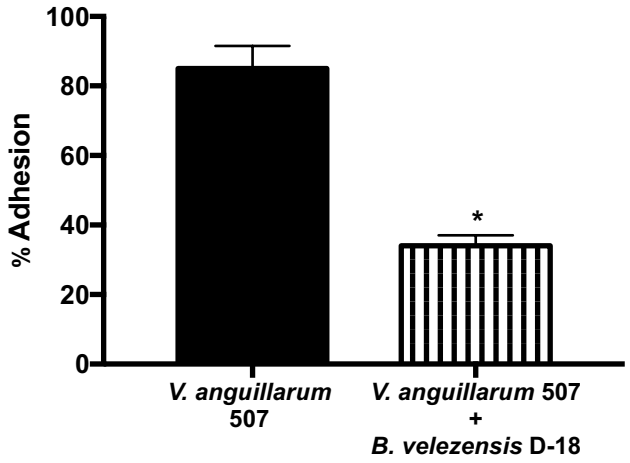

Fig. 4 Percentage of adhesion of the *V.anguillarum* 507 strain to sea bass mucus after exposure of the mucus to *B. velezensis* D-18. All data are given as a percentage of the absorbance measurements of fluorescent stained bacteria ± SD. *Significant statistical differences ($p < 0.05$)

Bio-safety Assay

The strain D-18 tested showed no harmful effects on fish after challenge, and no damage in the internal organs such as spleen (Fig. 5a, b), liver (Fig. 5c, d) and kidney (Fig. 5e, f) that were observed at × 4 and × 10 magnifications. 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 (Fig. 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

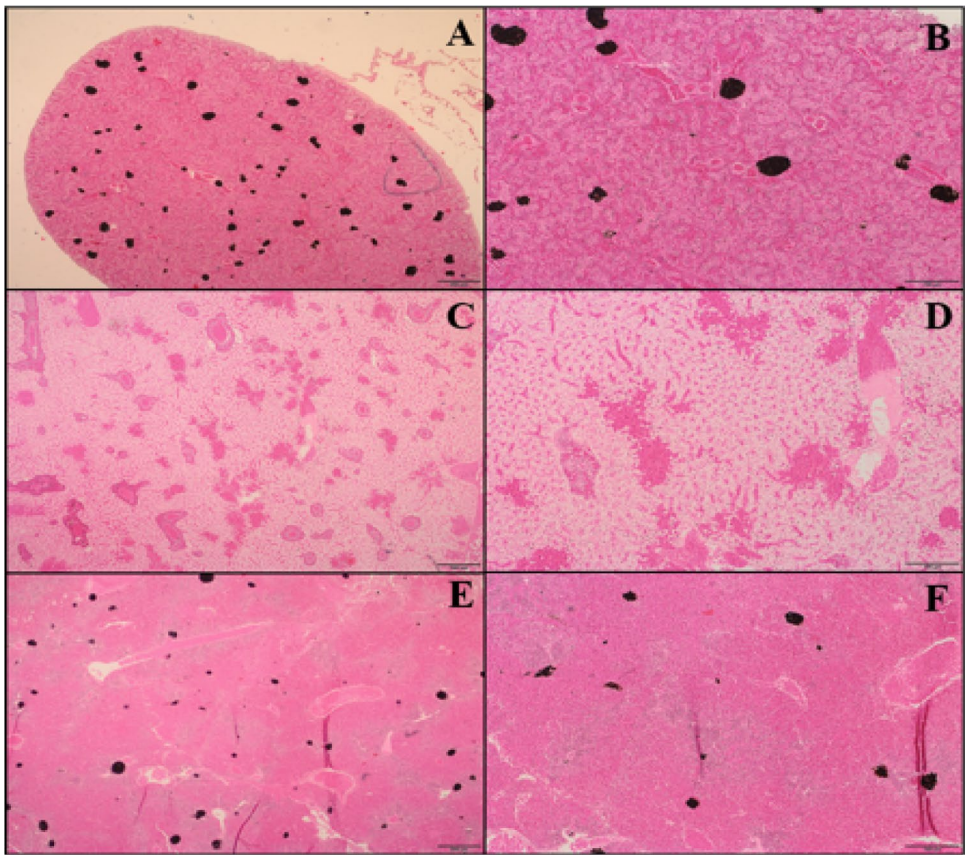

Fig. 5 View of absence of damage after administering *B. velezensis* D-18, showing a normal structure of the spleen (**a**, **b**), liver (**c**, **d**) and kidney (**e**, **f**) at × 4 and × 10, respectively

[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 means that it 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].

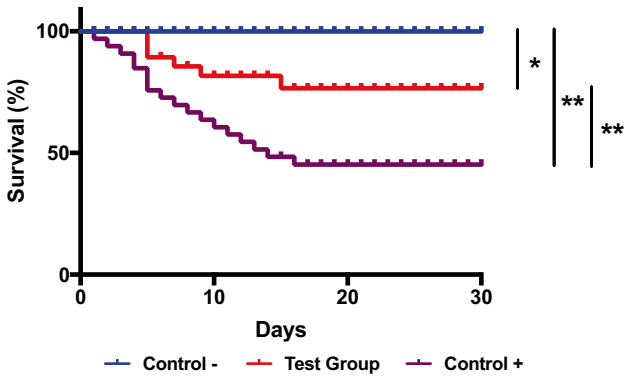

Fig. 6 Effect of the probiotic strain on the survival percentage of seabass against *V. anguillarum* 507. Asterisks indicate a significant statistical difference, *$p < 0.05$ and **$p < 0.001$

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 aware 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 was much higher than that used in the assays with humans (3%).

Like Sorroza [32] for *V. fluvialis*, in this assay, a decrease in the survival of *B. velezensis* D-18 at acid pH was observed, 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 were 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 colonisation 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 tissue 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, 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*.

Author Contribution Conceptualization: Félix Acosta, José Ramos-Vivas. Methodology: Félix Acosta, Luis Monzón-Atienza, Jorge Galindo-Villegas, Jimena Bravo, Silvia Torrecillas, Daniel Montero, Ana Franco González-de Canales, Inés. García de la Banda, José Ramos-Vivas. Formal analysis and investigation: Félix Acosta, Luis Monzón-Atienza, Jorge Galindo-Villegas, Daniel Montero. Writing—original draft preparation: Luis Monzón-Atienza, Félix Acosta, José Ramos-Vivas. Writing—review and editing: Luis Monzón-Atienza, Félix Acosta, Jorge Galindo-Villegas, José Ramos-Vivas.

Availability of Data and Material The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

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).

Conflict of Interest The authors declare no competing interests.

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Chapter IV

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

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

Luis Monzón-Atienza [a], Jimena Bravo [a], Álvaro Fernández-Montero [a], Ives Charlie-Silva [b], Daniel Montero [a], José Ramos-Vivas [a], Jorge Galindo-Villegas [c,*], Félix Acosta [a]

[a] Grupo de Investigación en Acuicultura (GIA), Instituto Ecoaqua, Universidad de Las Palmas de Gran Canaria, Spain
[b] Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo, SP, Brazil
[c] Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway

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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 cyclo-oxygenase 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

* Corresponding author.
E-mail address: Jorge.galindo-villegas@nord.no (J. Galindo-Villegas).

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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

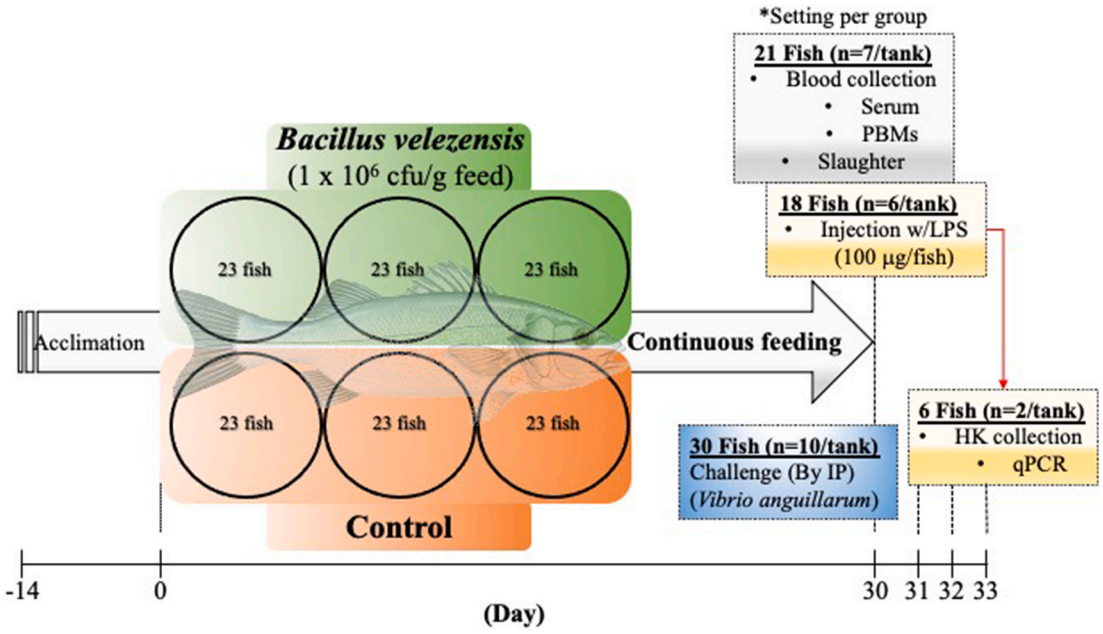

Fig. 1. Experimental setup. After the acclimation period, from day 0, control or *B. velezensis* strain D-18- supplemented diet was orally administered daily within 30 days to the European sea bass. On day 30, 21 animals from each group (n = 7 fish/tank) were aseptically bled. Serum and peripheral blood 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		

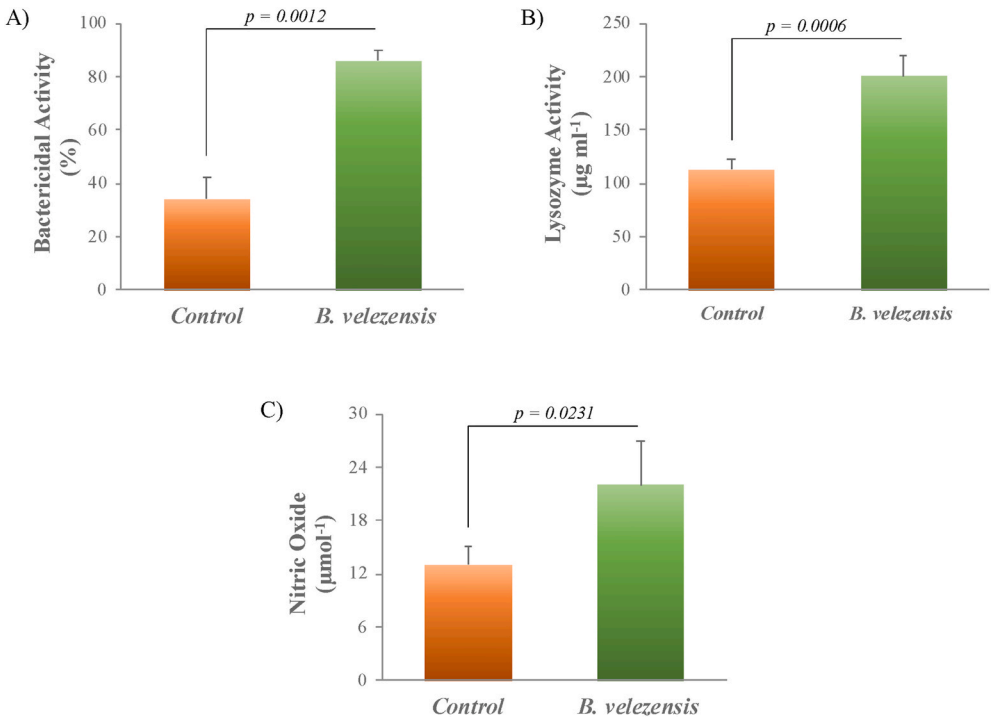

Fig. 2. Probiotic exposure modulates key antimicrobial innate humoral activities in the serum of the European sea bass. The fish had been orally treated with *B. velezensis* strain D-18 (100 µg/g food) or not (Control) within 30 days. (**A**) Bactericidal activity (**B**) Lysozyme, (**C**) Nitric oxide. 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).

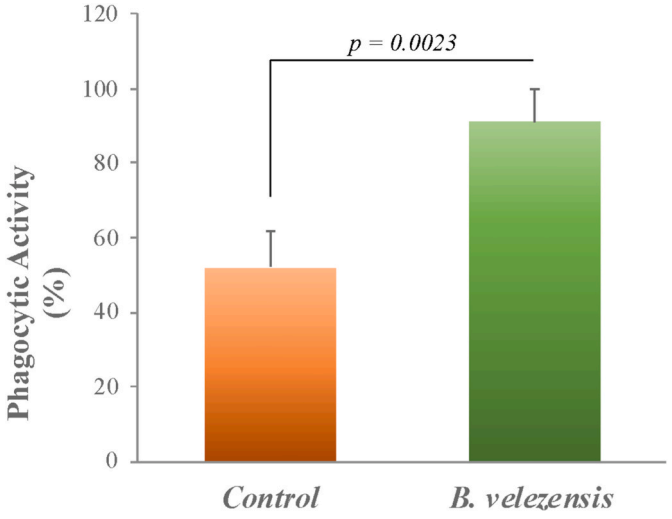

Fig. 3. Phagocytosis of *C. albicans* by activated macrophages from European sea bass is enhanced by the probiotic. PBMs were isolated and cultured overnight with *Candida albicans* from both the control and probiotic-supplemented group at the end of the trial (Day 30). Percentage of phagocytic cells 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

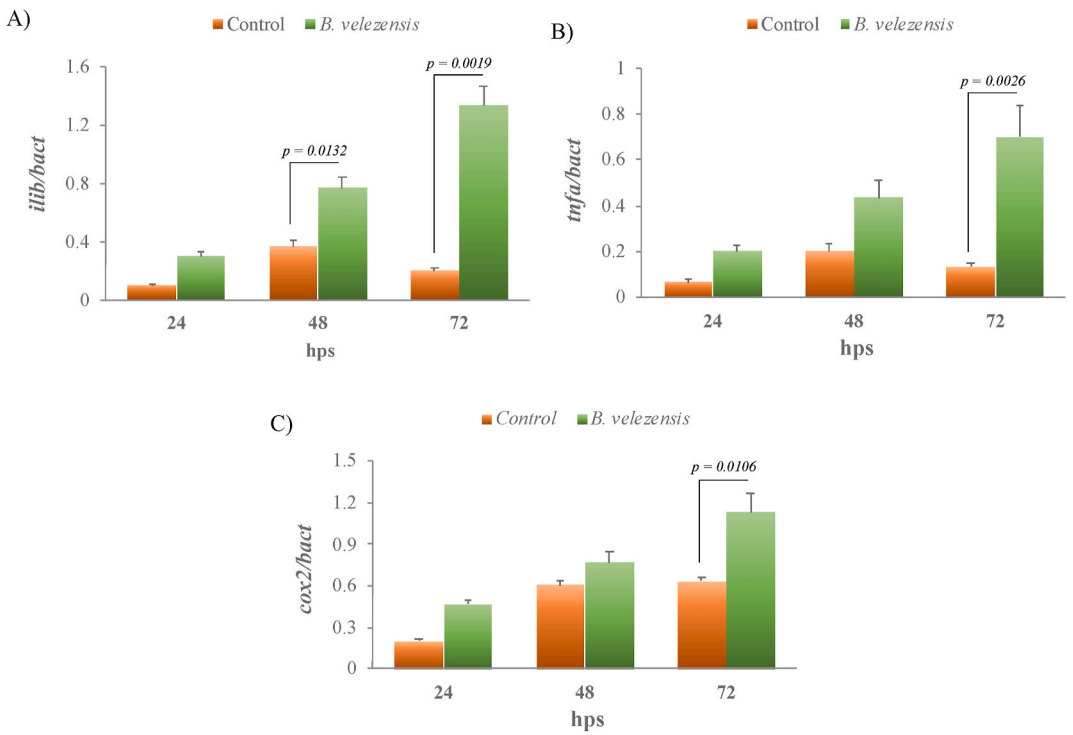

Fig. 4. The relative expression of proinflammatory marker genes in total head-kidney leukocytes from probiotic-treated LPS stimulated European sea bass. On day 30, at the end of the feeding trial with *B. velezensis* or control diets, 18 fish per group (6 fish/tank) were intraperitoneally stimulated 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.

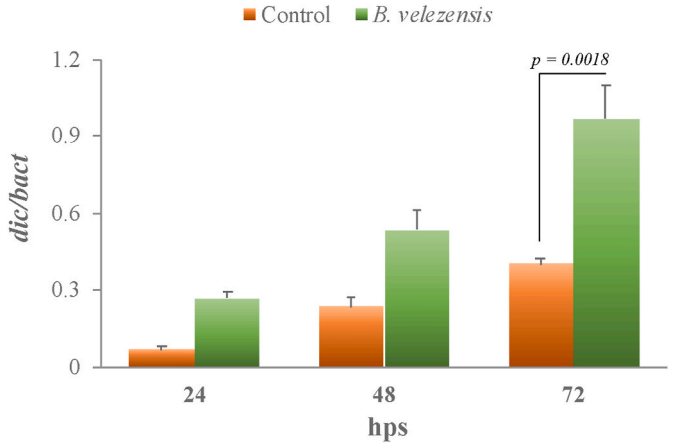

Fig. 5. The species-specific dicentracin mRNA was overexpressed in total head-kidney leukocytes from probiotic-treated LPS stimulated European sea bass. Total leukocytes were isolated from the HK of the fish 24-, 48-, and 72-h post-stimulation *in vivo* with LPS. The *B. velezensis* treated groups showed 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.

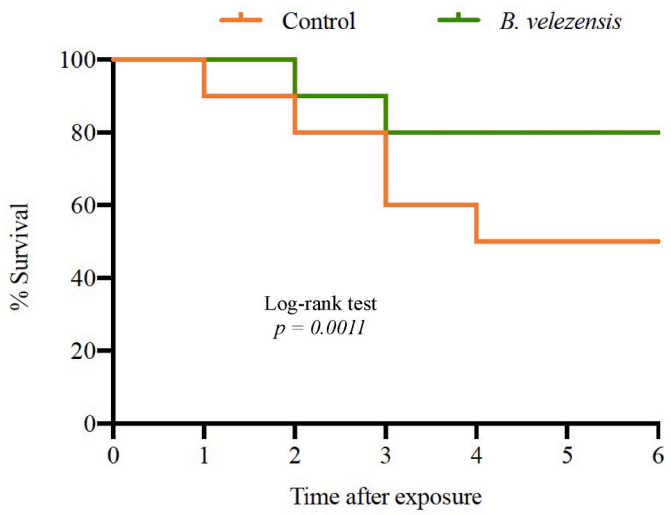

Fig. 6. *In vivo* bacterial challenge. Percentage survival of Control (orange) and *B. velezensis* (green) dietary treated fish experimentally infected by i.p. injection (100 μL of *V. anguillarum* (2×10^6 CFU ml^{-1}). Data are representative of three parallelly repeated trials. The statistically significant 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.

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Update

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Corrigendum to "Dietary supplementation of Bacillus velezensis improves Vibrio anguillarum clearance in European sea bass by activating essential innate immune mechanisms" [Fish Shellfish Immunol. 124 (2022) 244–253]

Luis Monzón-Atienza [a], Jimena Bravo [a], Álvaro Fernández-Montero [a], Ives Charlie-Silva [b], Daniel Montero [a], José Ramos-Vivas [a,c,d], Jorge Galindo-Villegas [e,*], Félix Acosta [a]

[a] Grupo de Investigación en Acuicultura (GIA), Instituto Ecoaqua, Universidad de Las Palmas de Gran Canaria, Spain
[b] Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo, SP, Brazil
[c] Research Group on Foods, Nutritional Biochemistry and Health, Universidad Europea del Atlántico, 39011, Santander, Spain
[d] Department of Project Management, Universidad Internacional Iberoamericana, Campeche, 24560, Mexico
[e] Faculty of Biosciences and Aquaculture, Nord University, 8049 Bodø, Norway

The authors regret the incorrect publication of the actual affiliation for the author José Ramos-Vivas in the original article. The corrected affiliation is provided below:

Luis Monzón-Atienza[a], Jimena Bravo[a], Álvaro Fernández-Montero[a], Ives Charlie-Silva[b], Daniel Montero[a], José Ramos-Vivas[a,d,e], Jorge Galindo-Villegas[c,*], Félix Acosta[a]

[a]Grupo de Investigaci'on en Acuicultura (GIA), Instituto Ecoaqua, Universidad de Las Palmas de Gran Canaria, Spain

[b]Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo, SP, Brazil

[c]Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway

[d]Research Group on Foods, Nutritional Biochemistry and Health, Universidad Europea del Atlántico, 39011 Santander, Spain

[e]Department of Project Management, Universidad Internacional Iberoamericana, Campeche 24560, Mexico

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* Corresponding author.
E-mail address: Jorge.galindo-villegas@nord.no (J. Galindo-Villegas).

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Chapter V

Whole-Genome Sequence of *Bacillus velezensis* D-18, a Probiotic Bacteria for Aquaculture

Whole-genome Sequence of Bacillus Velezensis D-18, a Probiotic Bacteria for Aquaculture

Luis Monzón-Atienza
Grupo de Investigación en Acuicultura, IU_ECOAQUA; Universidad de Las Palmas de Gran Canaria
Alvaro Lorenzo-Felipe
Grupo de Investigación en Acuicultura, IU_ECOAQUA; Universidad de Las Palmas de Gran Canaria
Jimena Bravo
Grupo de Investigación en Acuicultura, IU_ECOAQUA; Universidad de Las Palmas de Gran
Antonio Gómez-Mercader
Canaria Grupo de Investigación en Acuicultura, IU_ECOAQUA; Universidad de Las Palmas de
Silvia Torrecillas
Gran Canaria Grupo de Investigación en Acuicultura, IU_ECOAQUA; Universidad de Las Palmas
Jorge Galindo-Villegas
de Gran Canaria Nord University
Daniel Montero
Grupo de Investigación en Acuicultura, IU_ECOAQUA; Universidad de Las Palmas de Gran Canaria
Felix Acosta
felix.acosta@ulpgc.es

Grupo de Investigación en Acuicultura, IU_ECOAQUA; Universidad de Las Palmas de Gran Canaria

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Abstract

Bacillus velezensis D-18 isolated from a wastewater sample collected from a fish farm, In a previous experiment, *Bacillus velezensis* D-18 had demonstrated its ability to be a good candidate as probiotic in aquaculture, However, information on its genomic content is lacking. This is the complete genome assembly of *Bacillus velezensis* D18 using Illumina paired-ends sequencing, which resulted in a 21 contigs assembly of 3.9 Mb. About 4,179 protein-coding genes, 84 encode RNAs were predicted from this assembly.

BACKGROUND

Aquaculture is the fastest growing food production sector globally, contributes to approximately half of the world's supply of fish and aquatic species. The sector's industrialization and the intensification of production methods have inadvertently facilitated the spread of pathogens. Consequently, infectious diseases have become the most significant impediment to expanding aquaculture production. In recent years, emerging infectious diseases have led to substantial production losses across various species and geographic zones worldwide [1]. To combat this, several innovative approaches to disease control in aquaculture have been developed. These include various treatment methods such as the use of medicinal plants and seaweed extracts, bioactive compounds from actinomycetes, vaccines, probiotic microbes, chemicals, nanoparticles, and the green synthesis of nanoparticles [2]. Probiotics, in particular, are considered non-pathogenic to fish and other aquatic animals, as they are bacterial cultures isolated from bacterial strains. This makes them a safe and effective solution for disease control in the aquaculture industry [3].

Bacillus is a genus of Gram-positive, which are either aerobic or facultative anaerobic and form endospores. For decades, probiotics have been used as feed supplements in animal production to treat various diseases. Among the bacteria used as probiotics, those belonging to the Bacillus genus are some of the most commonly used [4]. In aquaculture, probiotics serve as microbial candidates to maintain health and have been described for numerous fish species [5]. A few Bacillus species, including *B. coagulans*, *B. clausii*, *B. cereus*, *B. subtilis* and *B. licheniformis* are commonly used as probiotics [6]. *B. velezensis* which is not heterotypic synonym of *B. amyloliquefaciens*, is gaining attention among closely related Bacillus species as a valuable biocontrol agent [7]. In aquaculture, it has recently been described as a possible probiotic bacterium in the culture of European seabass (*Dicentrarchus labrax*) [8].

The aim of this study is to investigate the antibacterial mechanisms of action in the D18 strain of B. velezensis through genome sequencing. We also aim to confirm the absence of resistance plasmids, which would validate the potential of this bacteria as a future probiotic for European sea bass.

DATA DESCRIPTION

GENOME SEQUENCING, ASSEMBLY AND ANNOTATION

B. velezensis D18 isolated from wastewater samples collected from a farm at the Instituto Español de Oceanografía in Santander, Spain, was sent to Macrogen (South Korea) for Illumina paired-ends Whole Genome Resequencing. The reference genome used was B. velezensis CBMB205 (GCF_002117165.1), and the library was prepared using the TruSeq DNA PCR-Free kit. *Quality checking was performed using the FastQC tool (V0.11.8), and Trimmomatic (v0.38) was used to remove adapter sequences and low- quality reads (Bolger et al., 2014). The filtered reads were then mapped to the reference genome using BWA (v0.7.17*) [10] and duplicated reads were removed using Sambamba (v0.6.8) [11]. Variants were identified by analyzing the information from aligned reads using SnpEff (v4.3t) [12]. These filtered reads were also introduced into the Read Assembly and Annotation Pipeline Tool (RAPT) for assembly and annotation. Genomic reads were predicted using the RAST server [13].

GENERAL GENOME FEATURES OF B. VELEZENSIS D18

This strain produces quorum quenching signals and forms biofilms. Sequencing resulted in yielded 18.2 million raw reads, with 97.06% and 91.85% having Q2 and Q3 quality, respectively. *B. velezensis* D- 18 genome genome consists of a circular chromosome of approximately 3.9 Mb. The sequencing provided about 94.6% coverage of the entire B. velezensis CBMB205 genome with a mean depth of 511. After adapter removal and quality checking, 17.5 million reads were used for assembly, resulting in 21 contigs, 4,059,220 bp, and N50 and L50 values of 575,178 and 3, respectively. The total number of bases sequenced was 2,746 million bases with a G + C content of 46.60%. After adapter removal and quality checking, 17,5 million reads were used for assembly obtaining 21 contigs, 4,059,220 bp and N50 and L50 values of 575,178 and 3, respectively (Fig. 1). A total of 4,179 protein-coding sequences were predicted, of which 84 encode RNAs using the RAST server (Aziz *et al.*, 2008).

GENES OF B. VELEZENSIS D18 IN GENERAL METABOLISM AND SPECIFIC PROPERTIES AS PROBIOTIC BACTERIA

These coding sequences belong to 324 subsystems, including 217 involved in carbohydrate catabolism, 187 in protein metabolism, 288 in the synthesis of amino acids and derivatives, 77 in cell wall and capsule synthesis, 64 in RNA metabolism, and 71 in DNA metabolism, including 151 in cofactors, vitamins, prosthetic groups, or pigments, 99 in nucleoside and nucleotide synthesis, 54 in fatty acid and lipid synthesis, 38 involved in virulence, disease, and defense, 41 in membrane transport, 46 in stress response, 12 in phosphorus metabolism, 26 in regulation and cell signaling, 6 in secondary metabolism, 5 phages, and 42 in motility and chemotaxis (Fig. 2).

The genes found include the quorum quenching lactonase (ytnP), required for quorum quenching competence, and the yqxM-sipW-tasA operon with its negative regulator SinR and its positive regulator SlR, required for biofilm formation (Fig. 3). These genes showed some differences with the CBMB205 strain used as a reference, and many of them are missense mutations. *B. velezensis* D18 does not contain any antibiotic resistance transfer gene in its genome.

CONCLUSION

In conclusion, the genome sequences of *Bacillus velezensis* D18 reveal its potential as a probiotic in aquaculture. Its ability to inhibit pathogenic bacteria, control of biofilms and a posible effects on gut health, make it a promising candidate for further research and development. However, more studies are needed to fully understand the mechanisms of its probiotic action and to assess its safety and efficacy in different applications.

Declarations

Nucleotide sequence accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAWZSS000000000

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

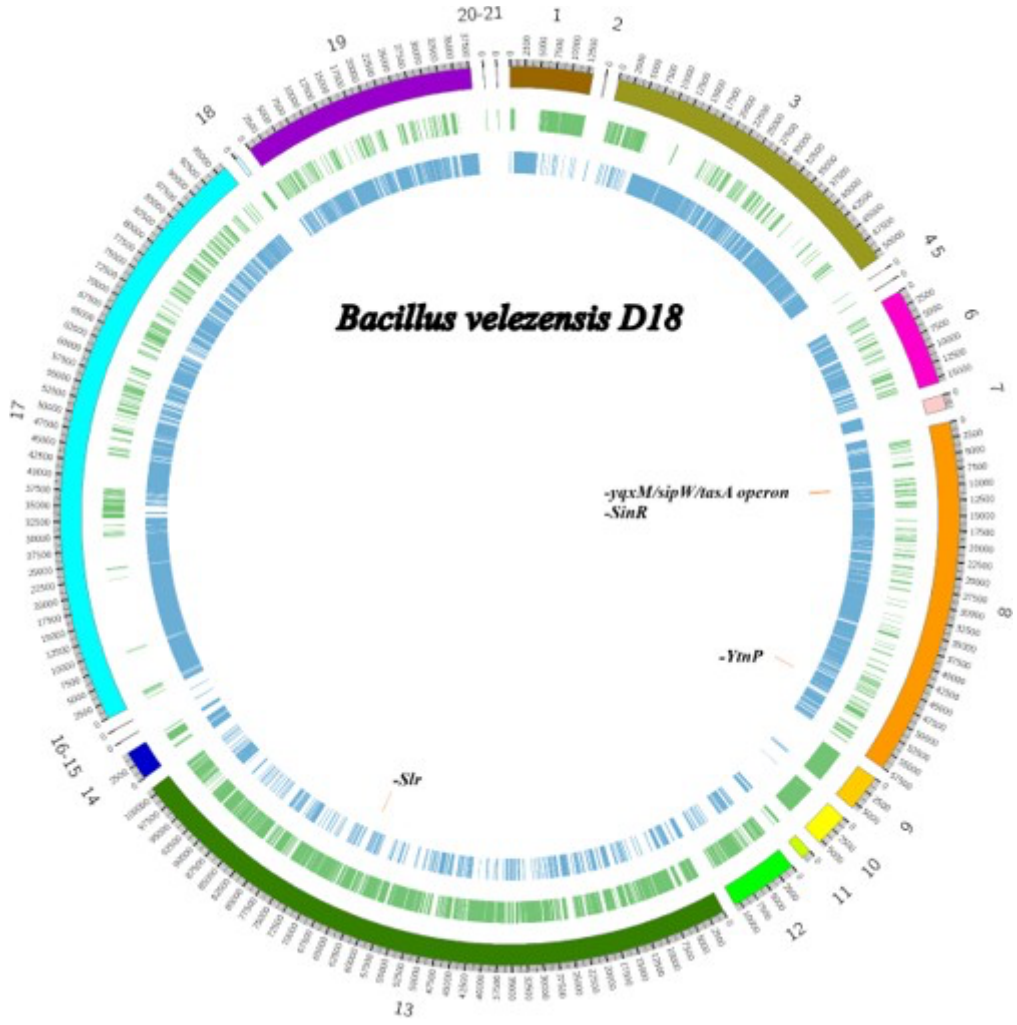

Figure 1

Outer circle represent the 21 contigs assembly of *Bacillus velezensis D18* strain. The next two concentric tracks from outer to inner: sense (green) and antisense (blue) strands, respectively. The inner track (orange) represents genes involve in biofilm formation and quorum quenching competence.

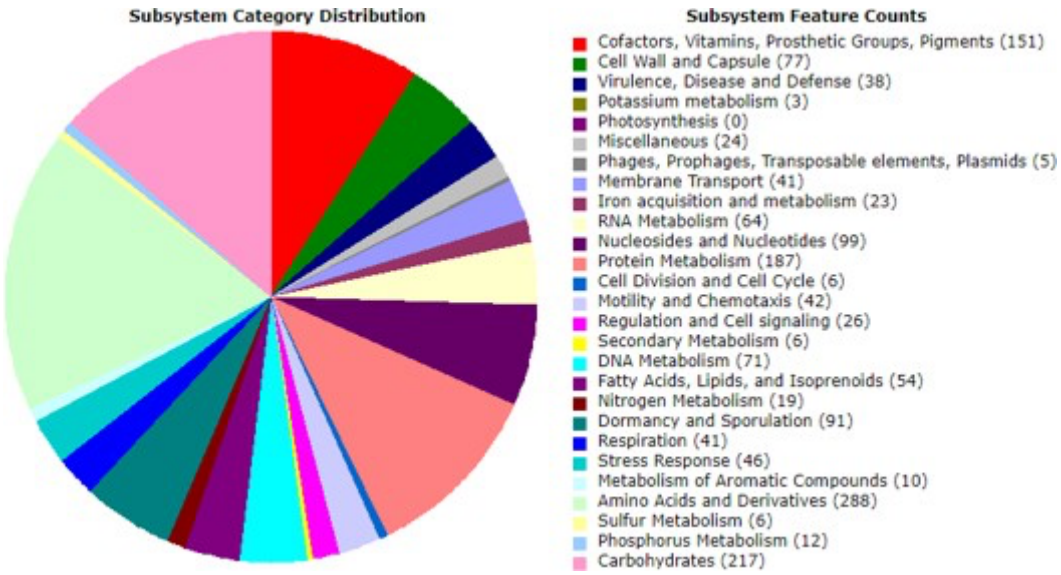

Figure 2

Subsystem category distribution where the 4,179 predicted protein-coding sequences belong and subsystem feature counts.

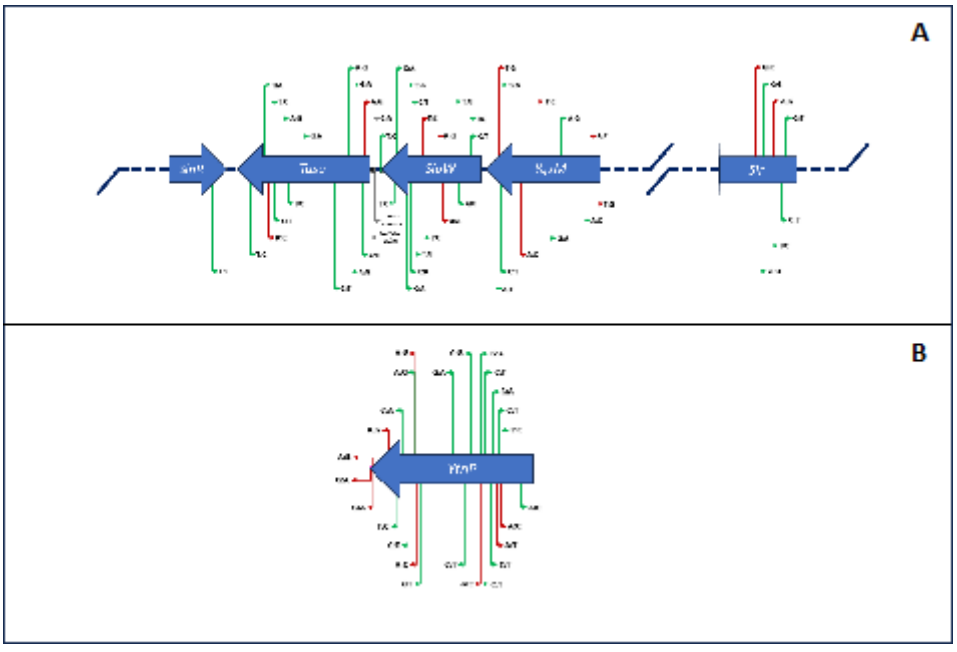

Figure 3

Distribution of *SNPs* and *InDels*in genes involved (**A**) in biofilm formation and (**B**) quorum quenching competence, in *B. velezensis D18* strain in comparison with *CBMB205* strain used as reference. Synonymous variants are marked in green, missense variants in red and variants between genes in gray.

Chapter VI

An In-Depth Study on the inhibition of *Quorum Sensing* by *Bacillus velezensis* D-18: Its Significant Impact on Vibrio Biofilm Formation in Aquaculture

microorganisms

MDPI

Article

An In-Depth Study on the Inhibition of Quorum Sensing by *Bacillus velezensis* D-18: Its Significant Impact on *Vibrio* Biofilm Formation in Aquaculture

Luis Monzón-Atienza [1], Jimena Bravo [1], Silvia Torrecillas [1,2], Antonio Gómez-Mercader [1], Daniel Montero [1], José Ramos-Vivas [1,3], Jorge Galindo-Villegas [4,†] and Félix Acosta [1,*,†]

[1] Grupo de Investigación en Acuicultura (GIA), Instituto Ecoaqua, Universidad de Las Palmas de Gran Canaria, 35001 Las Palmas de Gran Canaria, Spain; luis.monzon@ulpgc.es (L.M.-A.); silvia.torrecillas@irta.cat (S.T.); antonio.gomez@fpct.ulpgc.es (A.G.-M.); jose.ramos@uneatlantico.es (J.R.-V.)
[2] Aquaculture Program, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Centre de Sant Carles de la Rápita (IRTA-SCR), 43540 Sant Carles de la Rápita, Spain
[3] Research Group on Foods, Nutritional Biochemistry and Health, Universidad Europea del Atlántico, 39010 Santander, Spain
[4] Deparment of Genomics, Faculty of Biosciences and Aquaculture, Nord University, 8026 Bodø, Norway; jorge.galindo-villegas@nord.no
* Correspondence: felix.acosta@ulpgc.es
† These authors contributed equally to this work.

Abstract: Amid growing concerns about antibiotic resistance, innovative strategies are imperative in addressing bacterial infections in aquaculture. Quorum quenching (QQ), the enzymatic inhibition of quorum sensing (QS), has emerged as a promising solution. This study delves into the QQ capabilities of the probiotic strain *Bacillus velezensis* D-18 and its products, particularly in *Vibrio anguillarum* 507 communication and biofilm formation. *Chromobacterium violaceum* MK was used as a biomarker in this study, and the results confirmed that *B. velezensis* D-18 effectively inhibits QS. Further exploration into the QQ mechanism revealed the presence of lactonase activity by *B. velezensis* D-18 that degraded both long- and short-chain acyl homoserine lactones (AHLs). PCR analysis demonstrated the presence of a homologous lactonase-producing gene, ytnP, in the genome of *B. velezensis* D-18. The study evaluated the impact of *B. velezensis* D-18 on *V. anguillarum* 507 growth and biofilm formation. The probiotic not only controls the biofilm formation of *V. anguillarum* but also significantly restrains pathogen growth. Therefore, *B. velezensis* D-18 demonstrates substantial potential for preventing *V. anguillarum* diseases in aquaculture through its QQ capacity. The ability to disrupt bacterial communication and control biofilm formation positions *B. velezensis* D-18 as a promising eco-friendly alternative to conventional antibiotics in managing bacterial diseases in aquaculture.

Keywords: *Bacillus*; quorum sensing; quorum quenching; biofilm; *Vibrio*; aquaculture

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1. Introduction

Aquaculture is a vital industry with global significance, providing a substantial source of protein worldwide. However, the imposition of forced practices on aquatic ecosystems can induce stress in fish, detrimentally impacting aquaculture production [1]. Due to their sensitivity to stress factors, fish are highly susceptible to bacterial diseases [2], notably *Vibrio* spp. This vulnerability is a significant concern, considering that *Vibrio* species are responsible for causing vibriosis, a major epizootic disease that affects a broad spectrum of both wild and cultured fish species on a global scale [3]. The clinical presentation of vibriosis in fish encompasses lethargy, anorexia, exophthalmia, hemorrhages, ulcerations, and congestion in internal organs [4].

Vibrio spp. have evolved to employ biofilm production as a survival strategy in response to the diverse challenges posed by the aquatic environment [5]. Biofilm is char-

acterized by a self-produced matrix of extracellular polymeric substances and serves as a bacterial sessile-building mechanism, providing protection against environmental conditions and various agents [6,7]. The formation of biofilm is intricately linked to infection, virulence, and pathogenicity, emphasizing the role of biofilm in bacterial adaptation and resilience [8]. Furthermore, biofilm formation enhances bacterial resistance to different agents, including antibiotics, thereby complicating the effectiveness of conventional treatment methods [7]. Paradoxically, while antibiotics remain the primary treatment for bacterial infections in aquaculture, their indiscriminate use has led to an alarming increase in multidrug-resistant bacteria, necessitating a shift in research focus towards alternative control methods [9].

Bacteria, as remarkable communicators, produce, release, and sense extracellular signaling molecules, facilitating interaction with their environment [10,11]. When the concentration of those signaling molecules reaches a critical threshold, known as a "quorum", bacteria adjust their gene expression to elicit a specific response [12]. This process of cell-to-cell communication, which can occur both within and between species, is referred to as "quorum sensing" (QS) [10]. QS plays a pivotal role in various bacterial activities, including adhesion, biofilm formation, stress adaptation, and the production of virulence factors [13]. The extracellular signaling molecules involved in QS are termed "autoinducers" (AIs) [14]. There are various types of AIs, with N-acyl homoserine lactones (AHLs) being the primary AI for Gram-negative bacteria [15,16].

Recently, the disruption or inhibition of QS has emerged as a viable strategy to counteract the challenges caused by bacteria [17]. This process, known as quorum quenching (QQ), involves the use of chemical or enzymatic means to inhibit QS [16]. The various forms of QQ include the degradation of AI molecules, the inhibition of AI synthesis, and the blocking/inhibition/competition of AI binding to receptors [11,18]. QQ offers a unique perspective compared to conventional treatments, which primarily focus on bacterial growth inhibition. Through QQ, pathogenic bacteria can be transformed into harmless microorganisms, effectively eliminating their pathogenicity [11,19]. As such, QQ serves as an eco-friendly and effective alternative to antibiotics and other chemical control agents for managing bacterial diseases [20] and offers a potential solution to multidrug-resistant pathogens [7].

Remarkably, the disruptive capacity of QQ extends to bacteria classified as probiotics [21]. Probiotics, defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [22], have demonstrated several benefits in aquaculture. These include immunomodulation, increased utilization of digestive enzymes, and improvements in both gut health and water quality [23]. *Bacillus* spp. are among the most widely used probiotic bacteria in aquaculture [24] and exhibit the capacity to degrade AHLs, underscoring their potential as effective agents in managing bacterial diseases [25,26]. As the scientific community strives to address the challenges in aquaculture sustainably, understanding the intricate interplay between forced practices, bacterial communication, and innovative control strategies becomes paramount in shaping the future of this vital industry.

We successfully isolated and characterized the *Bacillus velezensis* D-18 strain in our prior work [27]. We subsequently delved into its advantageous effects on the innate immune status of European seabass in our last study [28]. Despite these advancements, the intricate interaction dynamics between this probiotic bacterium and potential pathogens remain uncertain. Consequently, drawing from the existing literature, the primary objective of the present study was to assess the QQ potential of *B. velezensis* D-18. We also aimed to explore its practical application as a biofilm disruptor specifically targeting *Vibrio* spp. and further enhance our understanding of the multifaceted roles played by *B. velezensis* D-18, particularly in disrupting *Vibrio anguillarum* 507 biofilms, thereby contributing valuable insights to the broader field of probiotic research and aquaculture management.

2. Materials and Methods

2.1. Bacterial Strains

The probiotic strain *Bacillus velezensis* D-18, previously isolated and characterized in our laboratory in prior studies, underwent routine cultivation via two methods: culturing on Luria–Bertani (LB) broth at 26 °C overnight or on brain–heart infusion (BHI) broth with 1.5% NaCl supplementation, following a standardization procedure. Bacterial growth was meticulously monitored at 3, 6, 9, 12, and 24 h using optical density measurements (OD600) and serial dilutions at each time point. These standardization procedures were performed in triplicate to ensure the reliability and reproducibility of the observed growth patterns.

The fish pathogenic strain isolated within our laboratory (*Vibrio anguillarum* 507) was routinely cultured at 26 °C on BHI broth with 1.5% NaCl supplementation. This process adhered to the following standardization: Similar to the *Bacillus velezensis* study, the growth of *Vibrio anguillarum* 507 was monitored at key time points (3, 6, 9, 12, and 24 h) using optical density measurements (OD600) and serial dilutions. This iteration of the experiment was also conducted in triplicate to ensure robustness and consistency in the results.

Chromobacterium violaceum MK, a wild-type strain (CECT494, obtained from the Spanish Type Culture Collection—CECT) producing quorum sensing (QS)-dependent purple pigment violacein, served as a key component in bioassays. This strain was routinely cultured on LB broth at 26 °C overnight.

The biosensor strain *Chromobacterium violaceum* CV026, a mini-Tn5 mutant of the wild-type ATCC31532 deficient in QS-dependent violacein production (from our laboratory collection), was employed to detect exogenous AHLs. *C. violaceum* CV026 produces the purple pigment violacein in response to short-chain AHLs and was cultured on LB broth at 26 °C overnight.

Chromobacterium violaceum VIR24 was provided by Instituto de Investigación Marqués de Valdecilla (IDIVAL, Santander, Cantabria) and was used as a biosensor to detect exogenous long-chain AHLs due to its production of violacein. The strain was routinely cultured on LB broth at 26 °C overnight.

Bacillus subtilis subsp. *subtilis* CECT39 (ytnP—homolog lactonase) and *Bacillus cereus* CECT148 (aiiA—lactonase) were utilized as control strains for lactonase genes. These strains were sourced from the Spanish Type Culture Collection (CECT) (Paterna, Spain) and were routinely cultured on LB broth at 37 °C overnight.

2.2. Quorum Quenching Assay

A quorum quenching assay was carried out in accordance with the protocol outlined in [21], with specific adjustments. Initially, two overnight cultures were initiated: one featuring *B. velezensis* D-18 at 26 °C and 140 rpm in LB broth and the other with *C. violaceum* MK under identical conditions. Then, 1.5 mL of the *B. velezensis* culture was subjected to centrifugation (14,000 rpm, 10 min), and the resulting supernatant was filtered through a 0.22 mm membrane to isolate extracellular products (ECPs). Simultaneously, the *B. velezensis* pellet was resuspended in 1.5 mL of PBS. Following this, 1 mL of ECPs and 1 mL of the *B. velezensis* culture were subjected to heat inactivation (99 °C/15 min). A culture medium was prepared by combining 1 mL of *C. violaceum* MK broth with 49 mL (1:50) of LB soft agar (0.4%), which was thoroughly mixed, agitated, and poured into 6-well plates. Once solidified, 10 µL of the *B. velezensis* culture, the *B. velezensis* pellet, ECPs, heat-inactivated *B. velezensis*, heat-inactivated ECPs, and PBS were added to each respective plate. The 6-well plates were then cultured for 24 h at 26 °C, and the entire experiment was conducted in triplicate to ensure experimental repeatability.

2.3. AHL Degradation by Bacillus velezensis D-18

The degradation of short- and long-chain AHLs (C6 and C12 AHLs, respectively) by *B. velezensis* D-18 was assessed with a methodology inspired by Santos et al. [29], with certain refinements. A single colony from a freshly cultivated and uncontaminated *B. velezensis* was cultured overnight in 25 mL of LB at 26 °C with continuous agitation at 140 rpm. From

this 25 mL culture, 10 mL was subjected to centrifugation (12,000 rpm, 15 min, 4 °C), and the supernatant was filtered through a 0.2 µm membrane to obtain ECPs, some of which were also separated and tested to prevent any interference with violacein production by the biomarkers. The resulting pellet was resuspended in PBS, constituting the *B. velezensis* pellet. Additionally, 15 mL of the original *B. velezensis* culture was preserved for subsequent use.

Then, 1.5 mL each of the *B. velezensis* pellet, ECPs, and PBS (as a control) were deposited in three separate 50 mL centrifuge tubes (Falcon®). Measurements of 0.5 µL of C6 AHLs (10 µg/µL) or 0.2 µL of C12 AHLs (10 µg/µL) were added to each Falcon tube. In parallel, 5 µL of C6 AHLs (10 µg/µL) or 2 µL of C6 AHLs (10 µg/µL) was introduced into the preserved 15 mL *B. velezensis* culture. All Falcon tubes were cultured overnight at 26 °C with continuous agitation at 140 rpm. Following the presumed degradation, the resulting cultures were transferred to 1.5 mL tubes and were centrifuged (14,000 rpm, 15 min) to eliminate bacteria. In addition, 10 mL of the presumed degraded *B. velezensis* cultures were employed for pH reconstitution, as described below.

Subsequently, 100 µL of the *B. velezensis* culture, the *B. velezensis* pellet, ECPs, PBS, and AHLs were individually added to separate wells of a 6-well plate containing soft agar (0.4%) with the biomarkers *C. violaceum* CV026 for short-chain AHLs and *C. violaceum* VIR24 for long-chain AHLs. Six-well plates were cultured overnight at 26 °C for 48 h. The entire experiment was conducted in triplicate to ensure the reliability of the results.

2.4. AHL Reconstitution via pH Adjustment

The pH reconstitution process was adapted from the methodology outlined by Santos et al. [29] and Singh et al. [30]. The overnight cultures resulting from the interaction between *B. velezensis* D-18 and the respective AHLs were subjected to centrifugation as part of the previously described AHL degradation assay to avoid probiotic bacteria. Aliquots measuring 100 mL of both presumed degraded cultures were utilized in the previously described degradation assay, while the remainder was allocated for the pH reconstitution assay.

Supernatants were adjusted to pH 2 using hydrochloric acid (HCl). Then, 100 µL aliquots of both pH 2 supernatants were carefully added to the wells of the previously prepared 6-well plates containing soft agar (0.4%) with the respective biomarkers, *C. violaceum* CV026 and VIR24. The plates were incubated for 48 h at 26 °C.

2.5. PCR Genetic Analysis

Genomic DNA from *B. velezensis* D-18, *B. subtilis* subsp. *subtilis* CECT39, and *B. cereus* CECT148 was extracted and purified using the GeneJET genomic DNA isolation kit (Thermo Scientific, Waltham, MA, USA) to ascertain the presence of lactonase-producing genes within the genome of *B. velezensis* D-18. The experiment utilized specific primers for aiiA (Fw 5′-CGGAATTCATGACAGTAAAGAAGCTTTA-3′; Rv 5′-CGCTCGAGTATATATTCAGGGAACACTT-3′) [31,32] and ytnP (Fw 5′-ATCGGATAATCATCGTAAGC-3′; Rv 5′-ATTGAACTAAGAACAGACCC-3′) [29], which are considered lactonase producer genes. *B. cereus* CECT148 served as the aiiA control, while *B. subtilis* subsp. *subtilis* CECT39 functioned as the ytnP control.

PCR amplification was conducted in a Mastercycler pro S thermal cycler (Eppendorf, Hamburg, Germany) using a 50 µL reaction mixture comprising 5 µL of Taq PCR buffer (10×), 3 µL of MgCl2 (50 mM), 0.2 µL each of 2′-deoxynucleoside 5′-triphosphates (dNTPS) (25 mM), 1 µL of each forward and reverse primer (1:10 dilution), 0.25 µL of DreamTaq DNA polymerase 5 U/µL (Thermo Scientific), and 4 µL of genomic DNA. The PCR conditions included initial denaturation at 95 °C for 1 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 10 s.

Post-PCR cleanup was accomplished using the ExoSAP-IT enzymatic system to eliminate unincorporated primers and dNTPs. Electrophoresis involved the use of diluted 1/10 PCR products, a 2% agarose gel, and GelRed® Nucleic Acid Gel Stain (Biotium, San

Francisco, CA, USA) and was conducted under conditions of 80 V for 60 min. The marker employed for reference was the DL2000 Plus DNA Marker.

2.6. Vibrio anguillarum 507 Quorum Sensing Signaling Molecules

To elucidate the QS mechanisms of *V. anguillarum* 507, an assay was conducted employing both short-chain and long-chain AHL biomarkers, *C. violaceum* CV026, and VIR24. These biomarkers were separately incorporated into liquid 0.4% LB agar and evenly spread onto Petri dishes. Following solidification, three wells were established in each plate. Subsequently, 10 µL of C12AHL (1 µg/µL), serving as the positive control; 10 µL of PBS, serving as the negative control; 10 µL of an overnight culture of *V. anguillarum* 507 were added to each respective well. The plates were then incubated overnight at 26 °C. The experiment was replicated three times to ensure assay reproducibility.

2.7. Bacillus velezensis D-18 Quorum Quenching Effects on Vibrio anguillarum 507

An effective assay was conducted to assess the potential QQ effects of *B. velezensis* D-18 on the marine pathogenic strain *V. anguillarum* 507. Briefly, the probiotic strain, the pathogenic strain, and the long-chain AHL biomarker *C. violaceum* VIR24 were cultured at 26 °C and 140 rpm overnight. Subsequently, 1 mL of *C. violaceum* VIR24 and another mL of *V. anguillarum* were added to 48 mL of 0.4% soft LB agar. This agar was spread onto a Petri dish. Once solidified, 10 µL of the probiotic strain was added to the center of the plate. The plate was then incubated at 26 °C for 24 h. The experiment was performed in triplicate to ensure experimental repeatability.

2.8. Inhibition of Biofilm Formation and Growth of Vibrio anguillarum 507 by Bacillus velezensis D-18

After monitoring the growth dynamics of the probiotic bacteria and the pathogen, we assessed the capacity to inhibit biofilm formation and growth through the following procedure. *B. velezensis* was subjected to a 12-h incubation at 37 °C and 140 rpm in 20 mL of BHI supplemented with 1.5% NaCl, yielding a concentration of 10^8 CFU/mL. Simultaneously, the pathogen *V. anguillarum* was cultivated at 26 °C and 140 rpm for 3 h in 20 mL of BHI supplemented with 1.5% NaCl, resulting in a concentration of 10^7 CFU/mL. Serial dilutions were conducted to validate these concentrations.

A 1 mL sample was collected from each culture and subjected to centrifugation. The supernatant was removed and then resuspended in 100 µL of sterile PBS. A 12-well plate was employed in the experiment, and an enriched and filtered medium inspired by O'Toole [33] (using BHI supplemented with 1.5% NaCl instead of LB) was prepared to facilitate biofilm formation for both species.

Different well compositions were devised as follows in order to establish the desired concentration of each bacterium for assessing the biofilm formation and growth of both strains. The first comprised 2895 µL of enriched medium, 100 µL of *B. velezensis* (final concentration: 10^8 CFU/mL), and 5 µL of *V. anguillarum* (final concentration: 10^5 CFU/mL). The second consisted of 2900 µL of enriched medium and 100 µL of *B. velezensis* (final concentration: 10^8 CFU/mL). The third included 2995 µL of medium and 5 µL of *V. anguillarum* (final concentration: 10^5 CFU/mL). The last only included 3000 µL of medium and served as a control. The remaining wells of the 12-well plate were used as controls of the different treatments (*B. velezensis* and *V. anguillarum*, *B. velezensis*, *V. anguillarum*, and control) to confirm biofilm formation using crystal violet (0.1%) after incubation.

The plate was cultured at 26 °C and 100 rpm for 48 h. After this incubation period, the supernatant was removed, and 1 mL from each well was saved for quantification through serial dilutions. Each well underwent three washes with sterile PBS. The well surfaces were scraped, and the material was resuspended in 1 mL of PBS for further serial dilutions to quantify the biofilm amount in UFC/mL.

Serial dilutions of both biofilm formation and culture growth were plated on oxytetracycline (180 µg/mL) plates to quantify the selective growth of *B. velezensis* and on

lincomycin (80 µg/mL) plates for *V. anguillarum*. The plates were incubated at 26 °C overnight. This entire experiment was conducted in triplicate to ensure the robustness and reliability of the results.

2.9. Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 8.4.2 for macOS (GraphPad Software, San Diego, CA, USA). The unpaired *t*-test was used to test the differences between the groups. $p < 0.0001$ was defined as statistical significance for all tests that necessitated statistical analyses.

3. Results

3.1. Quorum Quenching Assay

The synthesis of violacein by *C. violaceum* MK is a consequence of QS. The QS inhibition by *B. velezensis* (culture and pellet) exhibits an opaque coloration (Figure 1). Importantly, no discernible inhibition was observed in wells containing heat-inactivated *B. velezensis*, PBS, ECPs, heat-inactivated ECPs, and PBS (Figure 1).

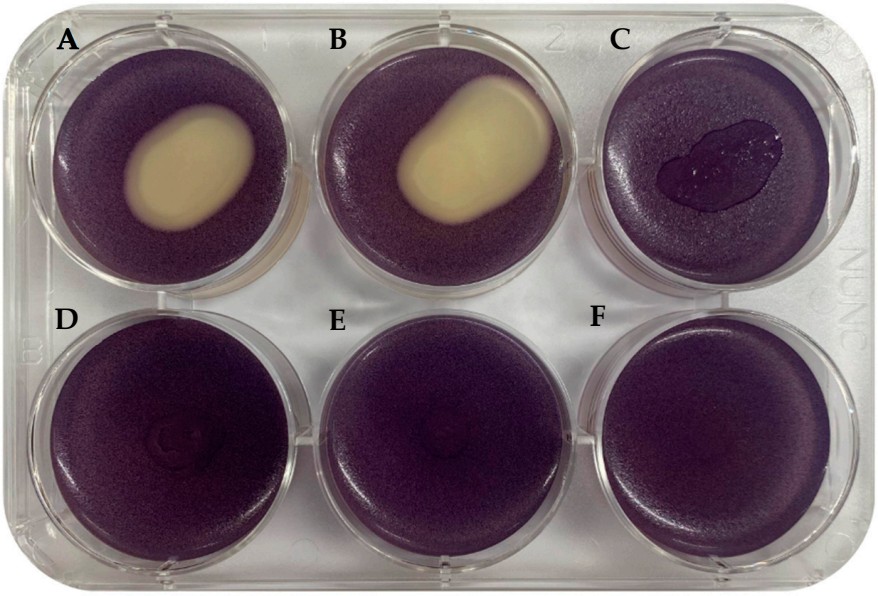

Figure 1. Quorum quenching assay of *Bacillus velezensis* D-18 and its products. *Chromobacterium violaceum* MK produces QS-dependent purple pigment violacein. The lack of production of violacein indicates QQ. (**A**) *B. velezensis* culture. (**B**) *B. velezensis* pellet. (**C**) ECPs. (**D**) Heat-inactivated *B. velezensis*. (**E**) Heat-inactivated ECPs. (**F**) PBS.

3.2. AHL Degradation by Bacillus velezensis D-18 and AHL Reconstitution via pH Adjustment

Following 48 h of growth at 26 °C, the results for the degradation of short-chain AHLs distinctly revealed that wells containing the *B. velezensis* pellet, ECPs, and PBS did not inhibit purple pigment production (Figure 2C,E,F). This implies the absence of inhibitors for short-chain AHL (C6 AHL) components in these conditions. However, the notable inhibition of violacein was observed in the well containing the *B. velezensis* culture (Figure 2A), indicating C6AHL degradation.

Regarding the degradation of long-chain AHLs, no pigment production was observed using *C. violaceum* VIR24 after 48 h in the *B. velezensis* culture well (Figure 2G). The partial degradation of C12AHL was observed in the well containing the *B. velezensis* pellet (Figure 2H). Nevertheless, violacein production was noted in the well containing ECPs, confirming the absence of degradation (Figure 2I).

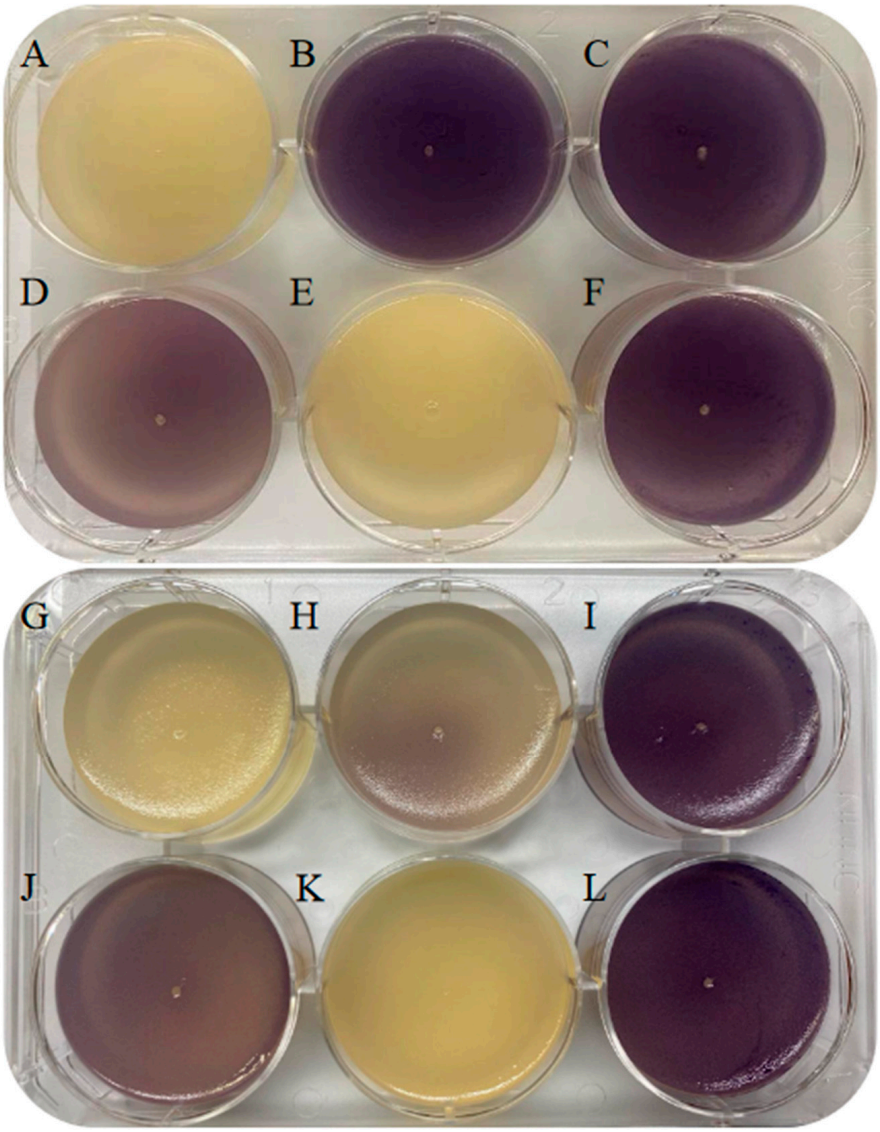

Figure 2. C6 AHL and C12 AHL degradation assay and reconstitution via pH adjustment. C6 AHL degradation assay. (**A**) *B. velezensis* culture. (**B**) *B. velezensis* pellet. (**C**) ECPs of *B. velezensis*. (**D**) Restoration of C6AHL degradation by *B. velezensis* through pH modification. (**E**) PBS. (**F**) C6AHL. C12 AHL degradation assay. (**G**) *B. velezensis* culture. (**H**) *B. velezensis* pellet. (**I**) ECPs of *B. velezensis*. (**J**) Restoration of C12AHL degradation by *B. velezensis* D-18 through pH modification. (**K**) PBS. (**L**) C12AHL.

ECPs previously isolated for both AHL degradation assays to test any possible effects on *C. violaceum* CV026 and VIR24 did not interfere in the production of violacein by both biomarkers (Supplementary Figure S2).

AHL reconstitution via the pH technique serves as a valuable tool for identifying degrading enzymes. This method distinguishes between enzymatic degradation by lactonases, which can be reversed under acidic pH conditions, and by acylases which cannot. Next, 10 mL of both short- and long-chain AHLs degraded by the *B. velezensis* culture were presented to the respective biomarkers, *C. violaceum* CV026 and VIR24. After 48 h, violacein production occurred, confirming the successful reconstitution of short- and long-chain AHLs (Figure 2D,J).

3.3. Genetic Analysis

Conventional PCR and electrophoresis were conducted to confirm the existence of lactonase genes and compare the in vitro findings. The examination of the results on a 2% agarose gel confirmed the absence of the aiiA gene (756 bp) in the probiotic *B. velezensis* D-18. However, *B. velezensis* D-18 exhibited the presence of the homologous lactonase gene ytnP (559 bp) (Figure 3). The identification of lactonase-producing genes justifies the outcomes observed in the AHL reconstitution via acidic pH.

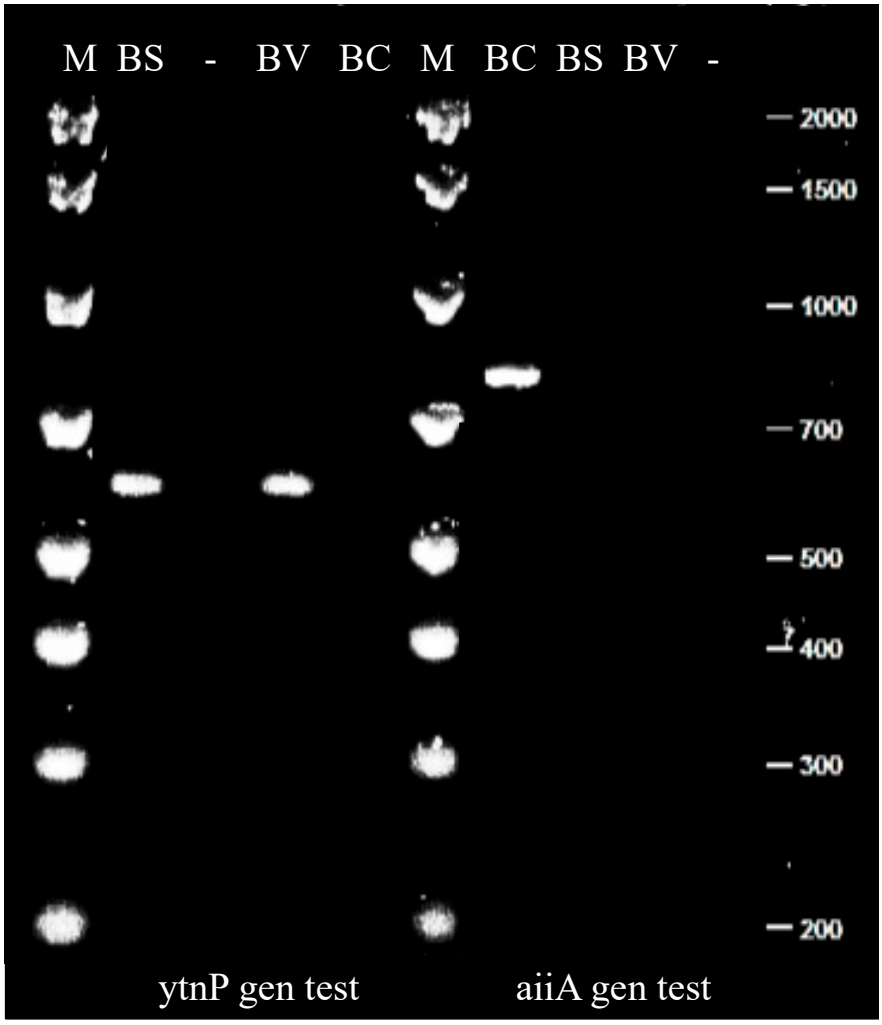

Figure 3. Gene analysis. *B. velezensis* D-18 (BV) DNA was extracted using a commercial kit, and the lactonase genes ytnP (559 bp) and aiiA (756 bp) were tested using PCR. DNA of *B. subtilis* (BS) and *B. cereus* (BC) was used as a control for ytnP and aiiA genes, respectively. (M) DL2000 Marker, (-) Milli-Q water.

3.4. Vibrio anguillarum 507 Quorum Sensing Signaling Molecules

In this experimental study, conspicuous evidence of violacein production, indicative of QS signals, was exclusively observed in the 10 µL *V. anguillarum* 507 overnight culture on the *C. violaceum* VIR24 plate (Figure 4A). Conversely, no discernible violacein signals were detected in the plate containing *C. violaceum* CV026 (Supplementary Figure S3). These observations strongly imply the targeted liberation of long-chain AHLs by V. *anguillarum* 507, thereby reaffirming its pivotal involvement in QS within this bacterial strain.

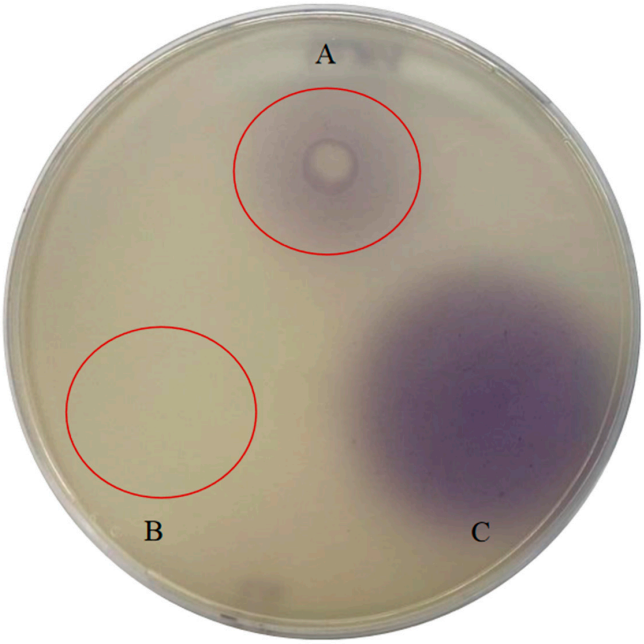

Figure 4. Assay for the demonstration of the presence of long-chain AHLs (QS signaling molecules) via *Vibrio anguillarum* 507. The biomarker *C. violaceum* VIR24 embedded in 0.4% soft LB agar produces violacein pigment upon detecting long-chain AHL molecules. (**A**) *V. anguillarum* 507. (**B**) PBS. (**C**) C12AHL.

3.5. Bacillus velezensis D-18 Quorum Quenching Effects on Vibrio anguillarum 507

Following the incubation of the plate in which *V. anguillarum* was imbibed with the biomarker *C. violaceum* VIR24 with 10 µL of the probiotic added to its surface, an inhibition zone was observed, indicating QQ activity. *C. violaceum* VIR24 did not produce violacein due to the absence of AHL molecules, attributed to the presence of the probiotic (Figure 5).

Figure 5. *Bacillus velezensis* D-18 quorum quenching effects on Vibrio anguillarum 507. *B. velezensis* presented to 0.4% LB agar with *V. anguillarum* 507 and *C. violaceum* VIR24. The inhibition halo generated by *B. velezensis* exhibits an opaque coloration, indicative of QQ activity.

3.6. Inhibition of Biofilm Formation and Growth of Vibrio anguillarum 507 by Bacillus velezensis D-18

Following a 48-h co-culture of probiotic bacteria and the pathogen, the quantification of biofilm formation and culture growth was conducted in terms of CFU/mL. The evaluation of biofilm formation in the co-culture of *B. velezensis* and *V. anguillarum* compared to the control revealed that the presence of the pathogen did not influence *B. velezensis*. The biofilm formation via *B. velezensis* remained comparable to the control, evidencing the probiotic's robust culture. Conversely, the introduction of the probiotic significantly impacted the biofilm formation of *V. anguillarum* (10^3 CFU/mL) in contrast to the control, demonstrating the pathogen's solitary culture (10^6 CFU/mL) (Figure 6A). The assessment of Bacillus velezensis D-18 and Vibrio anguillarum 507 biofilm formation in control wells stained with 0.1% crystal violet is depicted in Supplementary Figure S4.

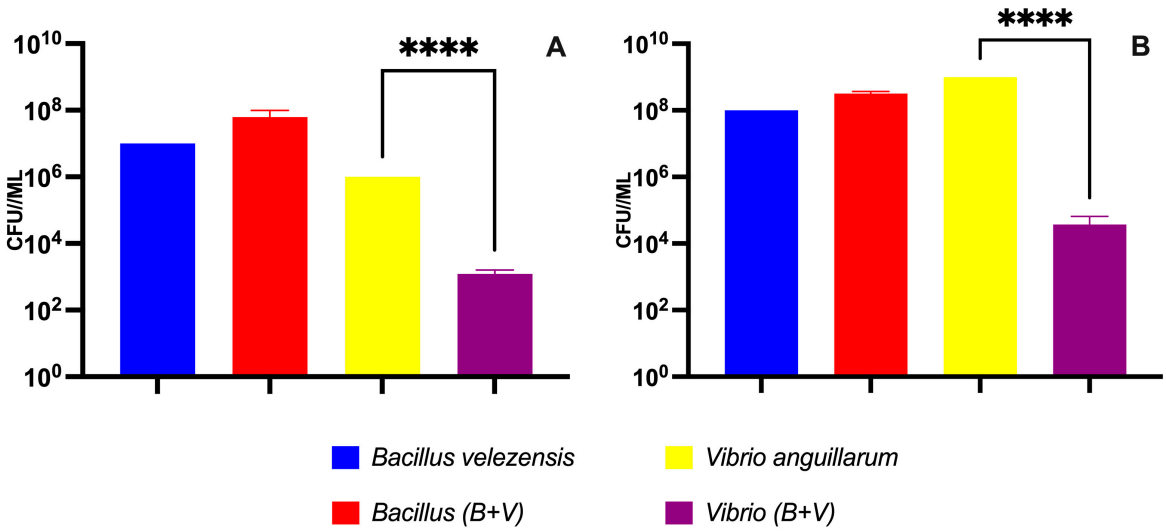

Figure 6. Inhibition of biofilm formation and growth of *Vibrio anguillarum* 507 by *Bacillus velezensis* D-18. (**A**) Biofilm formation following a 48-h co-cultivation of *B. velezensis* (10^8 CFU/mL) and *V. anguillarum* (10^5 CFU/mL), denoted as "B + V". Solitary cultures of *B. velezensis* and *V. anguillarum* were employed as controls. *Bacillus* (B + V) indicates the biofilm formation of *B. velezensis* in the co-culture. *Vibrio* (B + V) indicates the biofilm formation of *V. anguillarum* in the co-culture. (**B**) Bacterial culture after 48 h of co-cultivation of *B. velezensis* (10^8 CFU/mL) and *V. anguillarum* (10^5 CFU/mL), denoted as "B + V". Controls included individual cultures of *B. velezensis* and *V. anguillarum*. *Bacillus* (B + V) indicates the culture growth of *B. velezensis* in the co-culture. *Vibrio* (B + V) indicates the culture growth of *V. anguillarum* in the co-culture. Student's t-test was used to examine differences in all the parameters tested. Asterisks indicate a significant statistical difference, **** $p < 0.0001$.

The culture growth exhibited analogous results. The co-culture data clearly indicate that the growth of *B. velezensis* was unaffected by the presence of *V. anguillarum*. However, the growth of *V. anguillarum* in the presence of the probiotic was significantly inhibited in comparison to the pathogen control (Figure 6B).

4. Discussion

Traditional methods, such as the use of antibiotics, can have detrimental effects on health by fostering the growth of multidrug-resistant bacteria. Therefore, there is an escalating focus on investigating alternative strategies [34]. As described earlier, bacteria employ a signaling process to communicate with each other or with the environment: QS. QS in bacteria is responsible for regulating various processes, including virulence, biofilm formation, sporulation, and the production of secondary metabolites [30,35]. Consequently, QQ, which is the enzymatic disruption of this communication, is emerging as a novel mechanism for bacterial control [19,36].

The application of probiotics with QQ capabilities has recently been on the rise. In this study, we assessed the QQ capacity of the probiotic *B. velezensis* D-18 using a co-cultivation technique with a specific biomarker, *C. violaceum* MK, which produces violacein in response to QS [20]. The disappearance of the purple pigment in *C. violaceum* MK indicated that *B. velezensis* inhibits QS, confirming its QQ ability [30]. Live *B. velezensis* D-18 showed QQ activity, while heat-inactivated *B. velezensis*, ECPs, and heat-inactivated ECPs did not produce an inhibition halo (Figure 2). This suggests that the QQ capacity is associated with live *B. velezensis*, emphasizing the importance of understanding the specific QQ mechanisms involved.

To explore the *B. velezensis* D-18 QQ mechanism, we investigated the degradation of AHLs, the main QS molecules of Gram-negative bacteria, using both short (C6) and long (C12) chains [18]. AHL-producing and AHL-degrading bacteria coexist and employ contrasting strategies to gain a competitive edge over each other [37]. Enzymes catalyzing AHLs can primarily be divided into two groups: (i) those that lead to the degradation of the homoserine lactone ring, known as lactonases, and (ii) those that cause cleavage in the bond between the acyl chain and the homoserine lactone, known as acylases [19]. In the case of acylases, enzymatic degradation is conditioned by the length of the carbon rings, making it highly specific. However, lactonases interact directly with AHLs [19,38]. The *Bacillus* genus is known to degrade AHLs due to the presence of genes that produce degrading enzymes [19,38]. Therefore, the *B. velezensis* culture, the *B. velezensis* pellet, and ECPs were utilized to assess the degradation of long- (C12) and short-chain (C6) AHLs using *C. violaceum* VIR24 and CV026 as biomarkers, respectively [39]. The study revealed that the *B. velezensis* D-18 culture was responsible for the degradation of both forms of AHLs. The *B. velezensis* D-18 pellet exhibited partial degradation of C12AHL. However, it was unable to degrade C6AHL. ECPs showed no capability to degrade any AHL molecules, which contrasts with previous research highlighting the extracellular QQ potential of *Bacillus* spp. mediated by ECPs [29]. Nevertheless, other studies on probiotic candidates have demonstrated intracellular QQ activity [21]. In this study, the majority or sole degradation occurred with the *B. velezensis* culture. There is a strong indication of the presence of an inducible lactonase producer gene in *B. velezensis* D-18 and its subsequent release in the presence of AHL molecules during the biological development of the bacteria. This hypothesis is supported by the well-established communication among bacteria through the emission and uptake of autoinducers (AIs). As previously described, Gram-positive bacteria QS is mediated by autoinducer peptides (AIPs), which are typically released extracellularly and are, therefore, present in the surrounding medium. Gram-positive bacteria can detect and respond to AIPs to regulate their metabolic activities [15]. This feedback loop involving AIPs may enhance QS activity, leading to gene regulation that potentially triggers higher QQ activity, resulting in the detection of AHL molecules and the increase in degradation by *B. velezensis* D-18.

Consequently, the degradation of both long- and short-chain AHLs strongly suggested the presence of lactonase activity. To confirm this, these degraded AHLs were subjected to pH reduction and then exposed to the respective biomarkers. This resulted in the production of violacein, indicating the reconstitution of AHLs. Therefore, the enzyme was confirmed to be a lactonase. Researchers have argued that acylase enzymes have more advantages in practical applications since the AHLs degraded by lactonase could be reconstituted by lowering the pH [17,40]. However, lactonases have a wide range of effects on long- and short-chain AHLs, unlike acylases, which are generally most effective against AHLs with side chains longer than 10 carbon atoms [16,40].

We verified the presence of the lactonase-producing gene in *Bacillus velezensis* D-18 despite confirming that the enzymatic reaction is performed by a lactonase. Several authors support that the aiiA lactonase producer gene is inherent to numerous *Bacillus* spp. [19,25,32]. Recently, researchers have demonstrated the presence of homologous lactonase-producing genes in several *Bacillus* spp., such as the ytnP gene [41]. In this study, PCR was conducted using primers designed and used by previous researchers [29,31,32]

to determine the AHL-producing genes of the probiotic. The PCR results confirmed the absence of the aiiA gene in *B. velezensis* D-18, which aligns with findings in other *Bacillus* spp. [29,32]. However, PCR results confirmed the presence of the homologous lactonase gene ytnP (584 bp) in *B. velezensis* D-18, suggesting an alternative mechanism for QQ activity in accordance with previous studies that identified homologous lactonase-producing genes in *Bacillus* species [25,29,32].

As previously mentioned, pathogenic bacteria form biofilms to adapt to environmental conditions and evade antibacterial agents. This bacterial protection mechanism has led to a decrease in the effectiveness of antibiotic treatments [42]. In the field of aquaculture, biofilms serve as significant reservoirs for pathogenic microorganisms. In particular, the presence of *Vibrio* spp. in aquaculture systems is the cause of numerous economic losses, making its control essential [36,43]. Therefore, the reduction and control of *Vibrio* biofilms contribute to an improvement in animal welfare. Several researchers have offered differing perspectives on the implication of QS in biofilm formation [12,18,42]. QS is one of the mechanisms responsible for many biofilm stages such as bacterial adhesion, biofilm production, and bacterial dispersion [44]. The presence of AIs is also crucial for biofilm formation. In Gram-negative bacteria, AHLs play a crucial role in biofilm development and dispersion [45]. Throughout this study, we have verified that *Vibrio anguillarum* 507 releases long-chain AHLs as QS signal molecules. Numerous investigations have previously delineated the regulatory mechanisms of AHLs in Vibrio spp. and their roles in biofilm formation [12,46,47]. Drawing upon the documented QQ effects of *Bacillus* spp. on *Vibrio* biofilms [48,49], we explored the potential QQ impacts of *B. velezensis* D-18 on *V. anguillarum* 507.

This study unveiled that *B. velezensis* D-18 exerts QQ effects on *V. anguillarum* by degrading AHLs. This was evidenced by the absence of violacein production when the long-chain AHL biomarker *C. violaceum* VIR24 was present. In subsequent assays, the formation of *B. velezensis* D-18 biofilms remained unaffected by the presence of the pathogen *V. anguillarum*. Conversely, the presence of *B. velezensis* significantly impacted the biofilm formation of *V. anguillarum*, suggesting its potential as a biofilm control agent. These findings were mirrored in culture growth, with *B. velezensis* thriving in the co-culture, while the growth of *V. anguillarum* was notably reduced. The evident inhibition of QS in *V. anguillarum* 507, characterized by a lack of observable growth in the medium without a decrease in colonies, underscores the impactful role of *B. velezensis* QQ. The production of lactonase by the probiotic serves as a pivotal factor, actively degrading the signaling molecules, AHLs, crucial for the communication network of the pathogen. This disruption deprives *V. anguillarum* of the vital communication needed to initiate gene expression related to virulence factors, biofilm formation, and growth [19]. Our findings shed light on the multifaceted QQ mechanisms employed by *B. velezensis* D-18. The presence of ytnP further contributes to our understanding of the diverse QQ strategies employed by *Bacillus* species. These results reinforce the position of *Bacillus* spp. as promising candidates for preventing *Vibrio* diseases in aquaculture due to their comprehensive QQ capabilities [24,50].

5. Conclusions

In conclusion, *Bacillus velezensis* D-18 presents a promising avenue for the prevention of *Vibrio anguillarum* 507 diseases in aquaculture due to its quorum quenching capacity as an enzymatic disruptor of AHLs. The ability to disrupt bacterial communication and control biofilm formation positions *B. velezensis* D-18 as a potential eco-friendly alternative to conventional antibiotics in managing bacterial diseases in aquaculture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms12050890/s1, Figure S1: Any interference by *Bacillus velezensis* D-18 Extracellular Products (ECPs) on violacein production by *C. violaceum* CV026. (A). ECPs. (B). Heat-inactivated ECPs. (C). C6AHL (1 ug/uL), Figure S2: Any interference by *Bacillus velezensis* D-18 Extracellular Products (ECPs) on violacein production by *C. violaceum* VIR24. (A). ECPs. (B). Heat-inactivated ECPs. (C). C6AHL (1 ug/uL), Figure S3: Assay for the demonstration of the presence of QS signaling molecules by *Vibrio anguillarum* 507. The biomarker *C. violaceum* CV026, embedded in 0.4% soft LB agar, produces violacein pigment upon detecting short-chains AHL molecules. (A) *Vibrio anguillarum* 507. (B) PBS. (C) C6AHL (1 ug/uL), Figure S4: *Bacillus velezensis* D-18 and *Vibrio anguillarum* 507 biofilms stained with 0.1% Crystal Violet (CV).

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Chapter VII

Current Status of Probiotics in European Sea Bass Aquaculture as One Important Mediterranean and Atlantic Commercial Species: A Review

animals

MDPI

Review

Current Status of Probiotics in European Sea Bass Aquaculture as One Important Mediterranean and Atlantic Commercial Species: A Review

Luis Monzón-Atienza, Jimena Bravo, Antonio Serradell, Daniel Montero, Antonio Gómez-Mercader and Félix Acosta *

Grupo de Investigación en Acuicultura (GIA), Instituto ECO-AQUA (IU-ECOAQUA), Universidad de Las Palmas de Gran Canaria, 35214 Las Palmas de Gran Canaria, Spain; luis.monzon@ulpgc.es (L.M.-A.); jimena.bravo@ulpgc.es (J.B.); tonetser2@gmail.com (A.S.); daniel.montero@ulpgc.es (D.M.); antonio.gomez@fpct.ulpgc.es (A.G.-M.)
* Correspondence: felix.acosta@ulpgc.es

Simple Summary: Probiotic supplementation plays a vital role in European sea bass wellbeing. Accordingly, it is important to increase our knowledge of and experience on their mechanisms of action and host effects. Although information on these aspects is available, further studies are needed to achieve optimal European sea bass aquaculture.

Abstract: European sea bass production has increased in recent decades. This increase is associated with an annually rising demand for sea bass, which encourages the aquaculture industries to increase their production to meet that demand. However, this intensification has repercussions on the animals, causing stress that is usually accompanied by dysbiosis, low feed-conversion rates, and immunodepression, among other factors. Therefore, the appearance of pathogenic diseases is common in these industries after immunodepression. Seeking to enhance animal welfare, researchers have focused on alternative approaches such as probiotic application. The use of probiotics in European sea bass production is presented as an ecological, safe, and viable alternative in addition to enhancing different host parameters such as growth performance, feed utilization, immunity, disease resistance, and fish survival against different pathogens through inclusion in fish diets through vectors and/or in water columns. Accordingly, the aim of this review is to present recent research findings on the application of probiotics in European sea bass aquaculture and their effect on growth performance, microbial diversity, enzyme production, immunity, disease resistance, and survival in order to help future research.

Keywords: probiotic; European sea bass; feed additives; aquaculture; disease; growth

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1. Introduction

Aquaculture is one of the fastest-growing food sectors due to the high population demand for food and the decrease in natural fish stocks [1]. This industry contributes 52% of fish for human consumption and 46% of the total livestock production [2]. Sea bass production in Europe is estimated at 309,226 tons in 2022, and sea bass is one of the most important aquaculture species in Mediterranean countries, especially in Turkey, Greece, Egypt, and Spain [3]. The production of European sea bass is carried out in almost all countries of the Mediterranean. During their first month of life, larvae feed on brine shrimp and rotifers. Afterwards, they begin to consume feed. There are different production methods: floating nurseries at sea, concrete tanks, or ponds on land. Commercial sizes range from 250 g to more than 2500 g. Normally, it takes between 20 and 24 months to reach 400 g from the time the larvae hatch from eggs [3].

Nowadays, aquaculture tends to increase the amount of production to satisfy the food and animal protein human demand through high fish-stock density [4]. To meet this demand, industrial and high-scale aquaculture has to solve many gaps. Overcrowding gives rise to the appearance of diseases due to the stress conditions that fish livestock experience [5]. The main diseases in aquaculture farms are produced by bacteria, which cause great economic losses [6,7]. Bacterial infections dominate the disease reports of European sea bass in the Mediterranean (75%). Reports confirmed *Vibrio* spp., *Photobacteria* spp., and *Tenacibacillus* spp. as the most frequent pathogens in European sea bass [8]. In many cases, antibiotic treatment is beyond the reach of environmental and public health constraints. The administered antibiotics are absorbed at a certain rate, and the unabsorbed treatments go into the environment [9,10] and could promote antibiotic-resistant bacteria [11,12]. Multidrug-resistant bacteria are one of the greatest challenges in public health [13,14].

For this reason, researchers have been looking into new alternative approaches such as probiotics. Probiotics, which comes from the Greek terms *pro* and *bios*, are "live microorganisms which when administered in adequate amounts confer a health benefit to the host" [15,16]. Based on this definition, we considered probiotics as live microalgae, live yeasts, and live bacteria that provide benefits to the host. The use of probiotics in aquaculture production is presented as an ecological, safe, and viable alternative to antibiotics [17]. Moreover, the correct and effective use of probiotics can avoid great economic losses; although their production has certain costs at an industrial level, their application can generate economic benefits [18].

The application of probiotic components on fish causes interactions with host intestinal bacteria. These interactions lead to the formation of a wide variety of metabolites, which could produce beneficial outcomes for the fish [19]. Probiotics enhance host parameters such as growth or nutrient assimilation, immunomodulation, disease resistance, and survival rates and mitigate environmental stress [20]. In addition, probiotics can modify the association between the host and microbe or even the microbial community. They also improve the utilization of feed by increasing its nutritional value and enhancing the host's immune response against different pathogens. Commonly, the application of probiotics in fish industries has been administered via water or feed additives, either singly or in combination with other products or vectors [21,22].

Thus, probiotics have been tested in aquaculture with diverse and interesting results.

Therefore, the aim of this review is to emphasize probiotics' effect and current role on European Sea Bass aquaculture and provide key findings to promote future research.

2. Probiotics Sources and Selection Criteria

2.1. Probiotics Sources

Microbes are generally found naturally in humans, animals, soil, sediment, snow, and fresh, brackish, and salt water [23]. Numerous microorganisms have been used in aquaculture due to their probiotic qualities [24]. Normally, these microorganisms are found in fish gastrointestinal tracts, and, through several selection methods, they are isolated and cultivated for use as a probiotic [25]. *Bacillus* spp. is one of the most frequently used probiotics in aquaculture. This frequency is likely due to its ability to sporulate forming endospores, which increases the survival capacity in the gastric tract by resisting exposure to gastric acid, and to its dual aerobic and facultative anaerobic nature, which explains why it can grow in numerous environments [26–29]. The most common probiotics in European sea bass in recent years are bacteria, specifically *Bacillus* spp., *Pediococcus* spp., *Lactobacillus* spp., *Vibrio* spp., *Shewanella* spp., and *Vagococcus* spp. [30–53]. This commonality stands in contrast to the scarce existing bibliography on live yeast and microalgae probiotics in European sea bass [54–57].

2.2. Selection Criteria for Probiotics

Numerous authors have described the necessary characteristics to qualify a microorganism as a probiotic. Necessary requirements for a probiotic to be effective and qualified as such are listed as follows [23–25,58–60]:

(a) The microorganism should be able to adhere to and grow in the host. Then, it should be able to tolerate the bile, gastric juice, and host pH.
(b) The probiotic candidate must be free of antibiotic-resistant genes and must not modify heritable traits of the host organism.
(c) The microbe should benefit the host system by enhancing the growth or/and development of the immune system against pathogens. It also should have antimicrobial properties.
(d) The probiotic candidate should not have harmful effects on the host.

The evaluation of probiotics is carried out through in vitro or/and in vivo tests. In fact, many assays can be carried out both in vitro and in vivo.

The in vitro evaluation should analyze resistance to bile and pH, adherence factors, anti-pathogenic effect, and non-antibiotic resistance.

On the other hand, the in vivo evaluation of the probiotic candidate must show beneficial effects in the host (increasing the immune response, growth and absorption and utilization of food, modulation of intestinal microbiota, and reducing stress), not have harmful effects—assessed by using a biosafety assay—and improve the diseases resistance with an experimental challenge (see Figure 1).

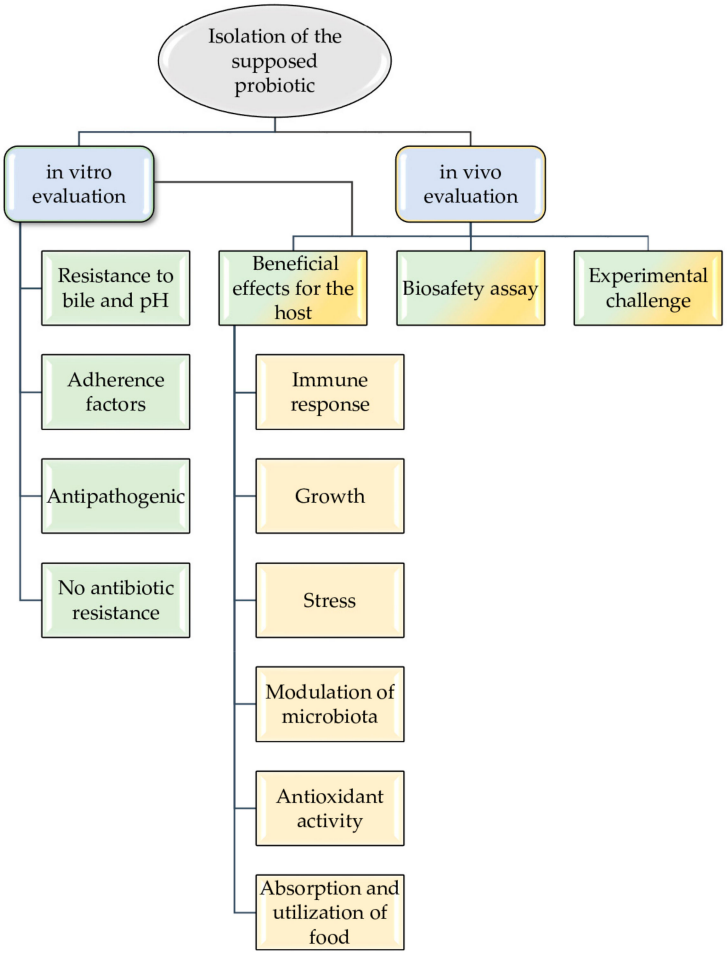

Figure 1. Probiotics selection flow-chart as biocontrol agents in aquaculture.

3. Technological Aspects and Administration Routes of Probiotics

Technological aspects for the production of probiotics must be considered, as their manufacture and storage can affect the stability of the microorganism. The probiotics that are administered through food must be able to withstand processes of pH, temperature, and pressure [25]. Probiotics are generally supplied frozen or dried, either as freeze-dried or spray-dried powders, and encapsulated [61]. Probiotic delivery methods are diverse and often depend on the type of facility, age, and species of fish [62]. Currently, the methods of administration in aquaculture are injection or addition to the water column or feed [24,63]. Certain factors must be taken into account before choosing the route of administration. The injection generates stress for the fish, and it is complicated and expensive in fish in the larval stage [64]. The advantage of this technique is the guarantee that the fish receives the desired dose of the probiotic. On the other hand, the direct addition of probiotics to the water column could be applicable to all stages of fish [64]. Feed administration is one of the simplest methods, although dry food is contraindicated in larval stages due to the size of the larval mouth [62]. Regarding the investigation of European sea bass, the most common routes of administration are dry food [31,36,39,41,47,48,51–56], vectors [30,32–34,37,48,57,65], and addition to the water column [35,38,40,42–46,48,57].

4. Probiotic Modes of Action in European Sea Bass

Probiotics are an effective prophylactic treatment against different diseases in fish. Determining the mechanism of action by which a probiotic benefits the host is complex. The synergy between various modes of action and/or the interaction with different microbes may result in host benefit [59]. In fact, some authors disagree on the correlation between in vitro and in vivo results. Tinh et al. [66] elaborate an interesting review of the mechanisms of action such as colonization of the gut epithelium, production of inhibitory substances, competition for chemicals or available energy, nutritional contribution, green-water effect, interference with quorum sensing, and immunostimulatory function. Based on the large number of mechanisms that a probiotic can use to exert its action, to date, there is no complete agreement on the results obtained in vivo. Therefore, an increase in research is recommended by the research community to reinforce knowledge of how probiotics work [66,67]. Among the several mechanisms used by probiotics in different microorganisms on European sea bass, the most common are the modulation of immune parameters, competitive exclusion for adhesion sites, production of inhibitory substances, and nutrient competition—digestion and enzymatic contribution (see Figure 2).

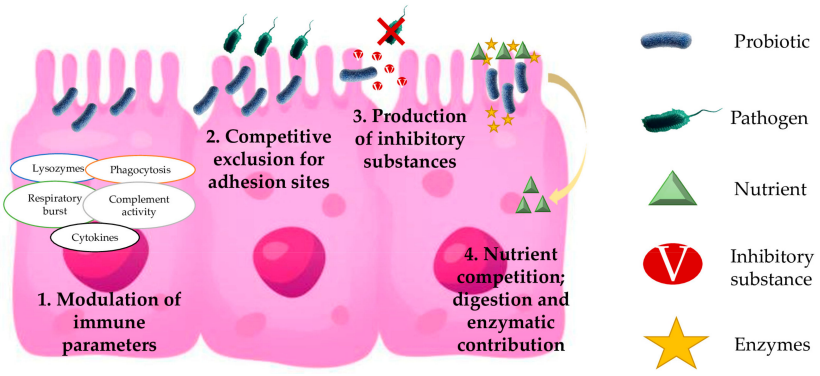

Figure 2. Mechanisms of action of probiotics in European seabass. (1) Modulation of immune parameters—Host immune system responds to microbe-associated molecular patterns (MAMPs) present in probiotics, leading to different intracellular signaling cascades. (2) Competitive exclusion for adhesion sites—Inhibition of pathogen by the colonization of host tissues. (3) Production of inhibitory substances—Production of substances with inhibitory effects on pathogens by probiotics. (4) Nutrient competition (digestion and enzymatic contribution)—Use of nutrients by probiotics, preventing their use by pathogens. Modulation of digestive enzymes that could increase nutrient absorption and improve digestion. Production of beneficial enzymes for the host.

4.1. Modulation of Immune Parameters

The modulation of immune parameters by probiotic bacteria is diverse and complex. The immune system responds to pathogen-associated molecular patterns (PAMPs) present in pathogens. Pattern recognition receptors (PRRs), fundamental in the innate response, attract pathogens and bind to their PAMPs, triggering the activation of the innate immune response. The best-known PRRs are toll-like receptors (TLRs), which are transmembrane proteins expressed in different immune and non-immune cells [68], one of which is toll-like receptor 2 (TLR2). Moreover, researchers have argued that probiotics possess microbe-associated molecular patterns (MAMPs) able to be detected by the host's PRRs, triggering, after detection and binding, an intracellular signaling cascade leading to the expression of effector molecules such as cytokines [69]. TLR2 has the capacity to recognize peptidoglycan, which is a main component of Gram-positive bacteria's cell walls, including lactic acid bacteria (LAB) probiotics [70]. TLR2 stimulation enhances the production of proinflammatory cytokines, such as IL-1β and TNF-α, and induces nitric oxide (NO) synthase. Also, TLR2 stimulation promotes the production of reactive oxygen species (ROS) and nitrogen species, essentials for mechanisms related to host antimicrobial defense. In addition, TLR2 activation has a crucial role in transepithelial resistance against pathogen bacteria [71,72]. Thus, these operations enhance a host's innate immune system in myriad ways such as increasing the production of lysozymes; enhancing phagocytosis and respiratory burst activity; and enhancing complement activity, peroxidase, antiprotease activity, and cytokine production [2,73]. Moreover, some probiotic components contain specific receptors promoting the production of white blood cells (WBCs) [74]. As proof of this immunomodulation in European sea bass, the following results are collected and detailed in Table 1 [33,34,36,38,40,41,44,45,47,50].

Table 1. Effect of probiotics on survival, growth, growth performance, immunity, survival against diseases, enzyme production, gut morphology, microbiota, and other parameters in European seabass. (↑) upregulation/increase, (↓) downregulation/decrease. *TcR-β* T cell receptor β-selection, *IL-1β* interleukin beta, *IL-10* interleukin 10, *COX-2* cyclooxygenase 2, *TGF-β* transforming growth factor beta, *Mx* myxovirus resistance proteins, *CAT* catalase, *HSP70* 70-kilodalton heat shock protein, *TNFα* tumor necrosis factor alpha, *IFN* interferon, *DIC* dicentracin, *fbl* fucose-binding, *SOD* superoxide dismutase, *hep* hepcidine, *rbl* rhamnose-binding, *MHCI-α* major histocompatibility complex class I alpha, *MHCII-β* major histocompatibility complex class II beta, *CD4* cluster of differentiation 4, *CD8-α* cluster of differentiation 8 alpha, *TAC* total antioxidant capacity, *GPX* glutathione, Live (L), heat inactivate (H), UV-Light inactivate (UV), only probiotic bacteria (B), high prebiotic level plus probiotic (HPB), and low prebiotic level plus probiotic (LPB).

Probiotic Bacteria	Doses of Administration and Duration	Observations	References
Lactobacillus delbrueckii* subsp. *delbrueckii	10^5 bacteria/mL **Long treatment:** From 11 to 29 days post-hatching: via *Brachionus plicatilis* From 30 to 70 days post-hatching: via *Artemia nauplii* **Short treatment:** From 30 to 70 days post-hatching: via *Artemia solely*	(↑) Growth performance (↑) Body weight (↓) Cortisol	[30]
***Lactobacillus farciminis* CNCM MA27/6R** **+** ***Lactobacillus rhamnosus* CNCM MA27/6B**	10^8 CFU/g 86 days	(↑) Survival rates (↓) Malformations (↑) Acid phosphatase activity (8 day), trypsin activity (↓) Acid phosphatase activity (23 day), α-amylase activity	[31]
Lactobacillus delbrueckii* subsp. *delbrueckii	10^5 bacteria/mL **Early treatment:** From 11 to 29 days post-hatching: via *Brachionus plicatilis* From 30 to 70 days post-hatching: via *Artemia solely* **Later treatment:** From 30 to 70 days post-hatching: via *Artemia solely*	Modify gut microbiota (↑) Survival (↓) Stress (cortisol)	[32]
Lactobacillus delbrueckii	10^5 bacteria/cm^3 From 11–29 days post-hatching: via *Brachionus plicatilis* From 30–74 days post-hatching: via *Artemianauplii*	(↑) T cells (↑) Acidophilic granulocytes (↑) TcR-β gene expression (↓) L-1β, IL-10, COX-2, and TGF-β gene expression	[33]
Lactobacillus delbrueckii	10^5 bacteria/mL From 11–29 days post-hatching: via *Brachionus plicatilis* From 30–74 days post-hatching: via *Artemia salina*	(↑) T cells (↑) Acidophilic granulocytes (↑) TcR-β gene expression (↓) L-1β, IL-10, COX-2, and TGF-β gene expression	[34]

Table 1. *Cont.*

Probiotic Bacteria	Doses of Administration and Duration	Observations	References
Vagococcus fluvialis	10^6, 10^7, and 10^8 CFU/mL (in vitro) 30 min incubation	10^8 CFU/mL as best results: (↑) phagocytosis (10^8 CFU/mL) (UV>L>H) (↑) Peroxidase (10^8 CFU/mL) (UV>L>H) (↑) Respiratory burst (10^8 CFU/mL) (UV>L>H)	[35]
Vagococcus fluvialis	10^9 CFU/g 20 days	(↑) Survival against *Vibrio anguillarum*	[36]
Bacillus subtilis	7×10^9 CFU/mL For 5 days: via *Artemia nauplii*	(↑) Survival against *Vibrio anguillarum*	[37]
***Vagococcus fluvialis* L-21**	10^8 CFU/mL (in vitro) 1 h incubation	**Mx gene expression:** (↑) 12 h (H), 48 h (L)(H)(UV) (↓) 1 h (L)(H)(UV), 24 h (L)(H)(UV) **IL-1β gene expression:** (↑) 1 h(L)(H)(UV), 48 h (H). (↓) 12 h (L)(H)(UV), 24 h (L)(H)(UV) **IL-6 gene expression:** (↑) 1 h (L), 24 h (H), 48 h (L)(H)(UV) (↓) 12 h (L)(H)(UV) **TNF-α gene expression:** (↑) 1 h (L)(H)(UV) (↓) 12 h (L)(H)(UV), 24 h (L)(H)(UV), 48 h (L)(H)(UV) **IL-10 gene expression:** (↑) 1 h (L)(H)(UV), 12 h (UV), 48 h (L) (↓) 24 h (L)(H)(UV) **COX-2 gene expression:** (↑)1 h (L)(H)(UV), 12 h (L)(H), 24 h (L)(H), 48 h (L)(H)(UV)	[38]
Lactobacillus plantarum	10×10^9 CFU/kg 90 days	(↑) Survival (↑) Blood cholesterol and triglycerides	[39]

Table 1. *Cont.*

Probiotic Bacteria	Doses of Administration and Duration	Observations	References
Lactobacillus casei* X2** ***Pediococcus acidilactici	10^7 CFU/g 40 days	***Lactobacillus casei* X2** (↑) IL-1β gene expression (↑) CAT gene expression (↓) HSP70 gene expression ***Pediococcus acidilactici*** (↑) IL-1β gene expression (↓) CAT gene expression (↑) HSP70 gene expression	[41]
Vibrio lentus	10^6 CFU/mL At 4, 6, and 8 days post-hatching	(↑) Disease resistance against *V. harveyi* SB	[42]
Virgibacillus proomii **+** ***Bacillus mojavensis***	10^6 CFU/mL 60 days	(↑) Growth performance (↑) Phosphatase alkaline, amylase activity (↑) Survival	[43]
Pseudoalteromonas* sp.** ***Alteromonas* sp.** ***Enterovibrio coralii ***Lactobacillus casei***	10^7 cells/mL (in vitro)	***Pseudoalteromonas* sp.** (↑) Mx (3 h), TNF-α (3 h), IL-10 (3 h) gene expression (↓) Mx (12 h), Caspase 3 gene expression (↓) Lysozyme (1–3 h) (↑) Phagocytosis (↑) Respiratory burst ***Alteromonas* sp.** (↓) Lysozyme (1–3 h) (↓) Mx (3–12 h), Caspase 3 gene expression ***Enterovibrio coralii*** (↑) Mx (3–12 h), IL-10 (3 h) gene expression (↓) Caspase 3 gene expression (↑) Respiratory burst ***Lactobacillus casei*** (↓) Mx (1 h), Caspase 3 gene expression (↑) Phagocytosis (↑) Respiratory burst	[44]

Table 1. *Cont.*

Probiotic Bacteria	Doses of Administration and Duration	Observations	References
Vibrio lentus	10^6 CFU/mL At 4, 6, and 8 days post-hatching	(↑) cell proliferation: hematopoiesis, cell death, ROS metabolism, iron transport, and cell adhesion. (↑) Immunomodulatory functions: pathogen recognition, cytokines, chemokines and receptors, humoral and cellular effectors, IFN-mediated response, and cell death	[45]
Vibrio lentus	10^6 CFU/mL 4, 6, and 8 days post-hatching	(↓) Stress	[46]
Lactobacillus rhamnosus	10^6 CFU/mL—Rearing Water or 10^8 CFU/mL From 9 to 50 days post-hatching: via *Artemia nauplii* 10^9 CFU/g From 50 to 125 days post-hatching	(↓) Deformation (↑) Survival rates (↓) *Vibrio* spp. (after probiotic Artemia)	[48]
***Bacillus velezensis* D-18**	10^6 CFU/g 20 days	(↑) Survival against *V. anguillarum* 507	[49]
***Bacillus velezensis* D-18**	1×10^6 CFU/g 30 days	(↑) Serum killing percentages (↑) Phagocytic activity (↑) Lysozyme activity (↑) Nitric oxide (↑) IL-1β, TNF-α, and COX-2 gene expression (↑) DIC gene expression (↑) Survival against *V. anguillarum* 507	[50]
Pediococcus acidilactici	10^{10} CFU/g (2, 2.5, and 3 g) 60 days	(↑) Water quality (↑) Growth performance (↑) Body composition	[51]
Bacillus amyloliquefaciens	10^7 CFU/g 42 days	(↑) Villi length (↑) Goblet cells number (↓) Cyst formation (↓) Actinobacteria phylum and Nocardia genus (↑) Betaproteobacteria and Firmicutes	[52]
***Phaeobacter* sp.**	5×10^7 bacteria/mL From 8 to 14 days post-hatching: via *Brachionus* sp. From 14 to 32 days post-hatching: via *Artemia metanauplii*	(↑) Survival against *Vibrio harveyi*	[64]

Table 1. *Cont.*

Probiotic Bacteria Combinate with Prebiotics	Doses of Administration and Duration	Observations	References
***Shewanella putrefaciens* Pdp11** **+** **Date palm fruits extracts**	10^9 CFU/mL 2 and 4 weeks	***Shewanella putrefaciens* Pdp11:** (↑) Antioxidant potential (2 and 4 weeks) (↓) Respiratory burst (4 weeks) (↑) Phagocytic capacity (2 and 4 weeks) **Head-kidney gene expression:** (↑) fbl (4 weeks) (↑) IL-1β (2 weeks) (↑) hep (2 and 4 weeks) **Gut gene expression:** (↑) SOD (4 weeks) (↑) hep (2 weeks) (↑) Lysozyme (2 weeks) (↓) hep (4 weeks) (↓) rbl (2 weeks) ***Shewanella putrefaciens* Pdp11 + date palm fruits extracts:** (↑) Antioxidant potential (2 and 4 weeks) (↓) Serum antiprotease activity (2 weeks) (↓) Natural hemolytic complement (4 weeks) (↓) Respiratory burst (4 weeks) (↑) Phagocytic ability (4 weeks) (↑) Phagocytic capacity (4 weeks) **Head-kidney gene expression:** (↑) rbl (2 and 4 weeks) (↑) IL-1β (2 and 4 weeks) (↑) SOD (2 weeks) (↑) hep (2 and 4 weeks) **Gut gene expression:** (↓) rbl (2 weeks) (↓) hep (4 weeks)	[40]

Table 1. *Cont.*

Probiotic Bacteria Combinate with Prebiotics	Doses of Administration and Duration	Observations	References
***Pediococcus acidilactici* (Bactocell®)** **+** **Mannanoligosaccharides (MOS)**	MOS(%)/BAC: 0/+ (B) 0.6/+ (HPB) 0.3/+ (LPB) 0/0 Control 90 days	**B:** (↑) TNF-α, IL-1β, COX-2, and IL-10 gene expression (↓) MHCI-α, MHCII-β, CD4, CD8-α, and TCR-β gene expression **HPB:** (↑) TNF-α, COX-2, CD4, and CD8-α gene expression (↓) IL-1β, IL-10, MHCI-α, MHCII, and TCR-β gene expression **LPB:** (↑) TNF-α and IL-1β gene expression (↓) COX-2, IL-10, MHCI I-α, and TCR-β gene expression (↑) Survival against *V. anguillarum* 507	[47]
***Bacillus subtilis* HS1** ***Bacillus subtilis* HS1+** **Chitosan**	10^7 CFU/g From 30 to 45 days post-hatching	**Probiotic:** (↑) Length, weight (↑) Survival (↑) Aspartate aminotransferase specific activity (↓) ALT (↓) SOD, CAT, and TAC **Symbiotic:** (↑) Length, weight (↑) Survival (↑) SOD, CAT, and TAC (↑) Alkaline phosphatase, acid phosphatase enzymes, and total and specific activities	[53]

Table 1. *Cont.*

Probiotic Yeast	Doses of Administration and Duration	Observations	References
***Debaryomyces hansenii* HF1** ***Saccharomyces cerevisiae* X2180**	7×10^5 CFU/g From 10 to 42 days post-hatching	***Debaryomyces hansenii* HF1** **At 27 days post-hatching:** (↑) Amylase (↑) Aminopeptidase N, maltase, and alkaline phosphatase **At 42 days post-hatching:** (↑) Survival (↓) Weight, growth (↓) Malformations ***Saccharomyces cerevisiae* X2180** **At 27 days post-hatching:** (↓) Amylase, trypsin (↓) Aminopeptidase N, maltase, and alkaline phosphatase **At 42 days post-hatching:** (↓) Trypsin (↓) Weight	[54]
***Debaryomyces hansenii* CBS 8339**	10^6 or 6×10^6 CFU/g From 5 to 37 days post-hatching	**10^6 CFU/g** (↑) Survival (↑) Weight/growth (↓) Malformations **At 26 days post-hatching:** (↑) Trypsin activity, lipase activity, and amylase activity (↑) Aminopeptidase N, maltase, and alkaline phosphatase **At 36 days post-hatching:** (↑) Trypsin activity and mRNA expression, lipase activity and mRNA expression (↓) Amylase activity and mRNA expression **6×10^6 CFU/g** **At 26 days post-hatching:** (↑) Trypsin activity, lipase activity (↓) Amylase activity (↑) Maltase, alkaline phosphatase **At 36 days post-hatching:** (↑) Trypsin mRNA expression; lipase activity and mRNA expression (↓) Amylase activity and mRNA expression	[55]

Table 1. *Cont.*

Probiotic Yeast	Doses of Administration and Duration	Observations	References
***Debaryomyces hansenii* CBS 8339**	43 g/kg From 6 to 48 days post-hatching	(↑) Growth performance (↓) GPX, SOD	[56]
Probiotic Microalgae	**Doses of Administration and Duration**	**Observations**	**References**
Tetraselmis chuii ***Nannochloropsis salina*** ***Isochrysis galbana*** ***Chlorella salina***	6 weeks: via water and *Artemia metanuplii*	(↓) Bacterial pathogens (↑) Growth performance	[57]

4.2. Competitive Exclusion for Adhesion Sites

Bacterial adhesion to host tissues is one of the mechanisms that pathogenic bacteria use to establish their infections [75]. The action of probiotics, on many occasions, is to prevent this adhesion of pathogens, and this action can be specific due to the adhesion of probiotics to the pathogen or to its receptor molecules in epithelial cells or non-specific due to the presence of physicochemical agents [17]. Passive and steric forces, lipoteichoic acids, electrostatic interactions, and specific structures such as external appendages covered by lectins can make this adhesion possible [76]. Bacteria tend to compete with each other by the exclusion of or reduction in other species' growth. The exclusion of adhesion sites is the main result of several mechanisms and properties of probiotic bacteria to suppress pathogen adhesion [77]. This competitive exclusion of adhesion sites inhibits the action of pathogenic bacteria by blocking infection pathways [78]. In fact, this ability to compete for the binding site with a pathogen is considered one of the main identification criteria for a probiotic [59,76,79,80]. The interaction between surface proteins, produced by certain probiotic bacteria, and mucins creates specific properties that may inhibit the adhesion of pathogenic bacteria [81]. Regarding European sea bass, the adhesion of probiotics (*Vagococcus fluvialis* and *Bacillus velezensis*) in intestinal mucus showed excellent results compared to a control [36,49].

4.3. Production of Inhibitory Substances

The production of inhibitory substances is presented as an absolute advantage of probiotics [82]. There is a wide range of inhibitory substances produced by probiotics. Siderophores, lysozymes, hydrogen peroxides, proteases, and antibacterial peptides—including organic acids, antimicrobial peptides, and bacteriocins—are all responsible for pathogen inhibition [23,67,76]. The organic acids produced by LAB, mainly acetic acid and lactic acid, have the ability to penetrate pathogenic bacteria, reducing their intracellular pH or accumulating and causing the death of the pathogen. Therefore, they are considered the main probiotic antimicrobials against Gram-negative bacteria [83]. In addition, two methods of bacteriocins-mediated pathogen clearance have been demonstrated: one includes cell wall perforation, and the other uses inhibition of cell wall synthesis [84]. Regarding antimicrobial peptides, dicentracin is an antimicrobial peptide exclusively produced by European sea bass. Dicentracin has the ability to lysis a wide range of different pathogens, bacteria being the most known [50,85]. The production of antimicrobial substances is not only directed against the lysis of the pathogen but also may be aimed at modifying the environment to make it less suitable for its competitors [2,86]. Makridis et al. [65] used *Phaeobacter* sp. to improve the rearing of European sea bass larvae, showing an in vitro inhibitory effect against *Vibrio anguillarum*. *Bacillus subtilis* was tested in vitro against vibriosis in European sea bass larvae. Its supernatants presented a significant reduction in pathogen growth [37]. In addition, previous research demonstrated the in vitro antagonistic capacity of *Vibrio lentus* as a probiotic against six sea bass pathogens without pathogenic effects on European sea bass larvae [42]. These facts might be attributed to the production of bacteriocins by probiotics. The same results were obtained by Öztürk and Esendal [48], namely that the presence of *Lactobacillus rhamnosus* through *Artemia nauplii* considerably decreased *Vibrio* spp. in European sea bass cultures. Additionally, El-Sayed et al. [57] demonstrated the antibacterial effects of different probiotic microalgae in water against pathogenic bacteria. On the other hand, Monzón-Atienza et al. [50] showed that the dietary administration of *B. velezensis* D-18 enhanced the dicentracin gene expression. Also, Guardiola et al. [40] showed different modifications of antimicrobial peptide gene expressions after *Shewanella putrefaciens* Pdp11 supplementation.

4.4. Nutrient Competition: Digestion and Enzymatic Contribution

Nutrients are essential for bacterial growth. The use of similar nutrients gives rise to hostile competition among species [87,88]. The utilization of available nutrients in environments by probiotics restricts their use by pathogenic microbes [75,77]. In fact, this

restriction resulting from competition for nutrients is one of the main mechanisms used by probiotics to inhibit pathogens [23,89]. Iron is one of the most important nutrients for pathogenic bacteria since it is related not only to growth but also to virulence [90,91]. For instance, *Bacillus* spp. has shown a capacity to synthase siderophores and also has a higher organic carbon utilization [92,93]. The absence of iron and carbon limits microbes' pathogenic functions. Furthermore, probiotics have the capacity to release a wide range of digestive enzymes. Thus, an increase in digestive enzymes can lead to the degradation of nutrients [94]. This digestive enzyme action can increase host nutrient absorption [95]. Both probiotic actions limit the use of nutrients by pathogenic bacteria.

Several probiotics have been tested in European sea bass and have been observed to enhance the production of enzymes. For one, after the application of *Virgibacillus proomii* and *Bacillus mojavensis*, phosphatase alkaline and amylase presented higher values [43]. Also, the simultaneous administration of *Lactobacillus farciminis* and *Lactobacillus rhamnosus* over 86 days upregulated acid phosphatase activity at day 8 and downregulated acid phosphatase activity at day 23 and a-amylase activity at days 8 and 103 post-administration. Furthermore, trypsin activity presented an increase from days 8 to 103 [31]. In reference to yeasts, various studies by Tovar-Ramírez et al. [54,55] demonstrated the enzymatic modulation capacity of these probiotics in European sea bass. On the other hand, some authors have shown that the application of *Bacillus amyloliquefaciens* for 42 days is capable of modifying the bacterial intestinal flora in European sea bass and reducing the presence of pathogens, surely due to competition for nutrients [52].

In recent years, the study of how probiotics are related to the antioxidant response that occurs in the hosts has had a very important boom, carrying out studies to modulate the redox status of the host via their metal ion chelating ability, antioxidant systems, regulating signaling pathways, enzyme-producing ROS, and intestinal microbiota. The mechanisms of how they act are still not fully understood, and future studies are required to clarify the action of probiotics on the antioxidant response of the hosts [96].

5. Probiotic Benefits in European Sea Bass Aquaculture

5.1. Increased Growth and Survival Rates

Probiotics in aquaculture promote fish growth by improving feed-conversion rates. The survival rate is another parameter that benefits after probiotic implementation [97]. As summarized in Table 1, the application of different probiotics (single or combination) on European sea bass has been reported to promote growth, growth performance, and survival [30–32,36,37,39,42,43,45,47–51,53–57,65].

5.2. Disease Resistance and Health Status

Like other species, European sea bass are susceptible to pathogen bacteria, viruses, fungi, and parasites [98–100]. The application of probiotics in European sea bass has been shown to provide disease resistance. For instance, the administration of *Bacillus velezensis* D-18 at 10^6 CFU/g over 30 days in European sea bass increased survival against *Vibrio anguillarum* [50]. *Bacillus velezensis* also increased the cumulative survival rates against *Vibrio harvey* SB [42]. Similarly, the supplementation of *Phaeobacter* sp. at 5×10^7 CFU/g in European sea bass fed via diets for 60 days increased resistance against *V. harveyi* [65]. Sorroza et al. [36] found a high survival rate against *Vibrio anguillarum* after the application of *Vagococcus fluvialis* at a high concentration (10^9 CFU/g) when compared with a control group. Likewise, both probiotic *Bacillus subtilis* and *Lactobacillus plantarum* at 10^6 CFU/mL demonstrated an increase in disease resistance in European sea bass against *Vibrio anguillarum* [37]. In addition, the presence of *Pediococcus acidilactici* in European sea bass increased survival against *Vibrio anguillarum* [47].

In relation to the health status of the European sea bass after the administration of probiotics, different responses are affected, such as stress modulation, antioxidant status, hematological values, malformations, and parameters of the aquatic environment. Regarding stress, Lamari et al. [41] showed the capacity of *Pediococcus acidilactici* to downregulate

HSP70 at 41 days post-hatching in European sea bass larvae. The HSP70 overexpression gene is considered a sign of improvement in acute stress resistance [101]. Silvi et al. [32] tested the effects of *Lactobacillus delbrueckii subsp. delbrueckii* and found a stress decrease in treated European sea bass larvae. The same results were obtained by Carnevali et al. [30] after the administration of *Lactobacillus delbrueckii subsp. delbrueckii* in European sea bass, showing a decrease in cortisol levels. In addition, the application of *Vibrio lentus* at four, six, and eight days post-hatching (dph) in European sea bass larvae had beneficial effects on stress by reducing glucocorticoids [46].

Free radical formation occurs following different processes such as phagocytic activity as well as cellular metabolism [26], which can lead to loss of biological function, tissue damage, and homeostatic imbalance [102]. The formation of free radicals in fish occurs naturally after different metabolic processes [26]. The presence of antioxidant substances is a fundamental factor in the elimination of free radicals. Antioxidants can be divided into enzymatic and non-enzymatic [96]. It is well known that probiotics have the ability to produce enzymes or antioxidant substances or encourage the host to produce them [26]. In fact, several studies have investigated the modification of the oxidative state after probiotic treatment in European sea bass. In one case, the presence of *Shewanella. putrefaciens* Pdp11 in an experimental diet enhanced the oxidative status and the gene expression of superoxide dismutase (SOD) in European sea bass [40]. Salem and Ibrahim [53] also demonstrated that the sole application of *Bacillus subtilis* HS1 decreased the levels of SOD, catalase (CAT), and total antioxidant capacity (TAC) in European sea bass. In contrast, the symbiotic application of that probiotic with chitosan enhanced SOD, CAT, and TAC. Furthermore, not only does the application of probiotic bacteria have these effects, but also the administration of live yeast—*Debaryomyces hansenii* CBS 8339—showed a considerable decrease in antioxidant status [56].

Regarding other health status parameters, *Vibrio lentus* enhanced cell proliferation (haematopoiesis), iron transport, and cell adhesion in European sea bass larvae [45].

Several authors have described the beneficial effects of probiotics in reducing malformations. In European sea bass, the combination of two different *Bacillus* species—*Lactobacillus farciminis* and *Lactobacillus rhamnosus*—over 86 days at 10^8 CFU/g in feed considerably reduced malformations [31] as well as the probiotic application of *Lactobacillus rhamnosus* in European sea bass [48]. Additionally, live *Debaryomyces hansenii* reduced malformation appearance in European sea bass larvae [54,55].

On the other hand, the surrounding medium is a fundamental factor in fish wellbeing, so water quality is considered an important parameter [103]. Indeed, Eissa et al. [51] demonstrated that the administration of *Pediococcus acidilactici* in European sea bass culture improved water parameters and led to fish welfare as well as the application of live microalgae on water, which reduced the number of different pathogenic bacteria strains [57]. All of these data are summarized in Table 1.

5.3. Elevation of Immune Parameters

The application of probiotics enhances disease resistance by bolstering the immune system as well as general health. It has been demonstrated that probiotics improve different immune parameters in sea bass. In particular, non-specific immune parameters such as lysozyme activity, phagocytic activity, and respiratory burst as well as serum complement activity and the number of macrophages, lymphocytes, erythrocytes, and granulocytes have been modulated after the administration of probiotics in European sea bass [33,34,38,40,44,45,50]. Furthermore, research has shown different modulations in cytokine levels after probiotic supplementation in European sea bass [33,34,38,40–42,44,50]. In fish, an increase in immune parameters is usually related to higher survival rates. Several research studies of European sea bass have verified a high survival rate against pathogens after probiotic applications [36,37,42,47–50,53,65]. All information is summarized in Table 1.

5.4. Gut Morphology and Changes in Microbial Diversity

Symbiotic relationships between host and microbes are present in fish. Host and environment—biotic and abiotic factors, respectively—play a fundamental role in intestinal microbiota modulation [104]. Microbes secrete metabolites, producing effects on intestinal environments and triggering changes in host physiology [2]. Probiotics via intestinal–environment interactions may change host intestinal morphologies, thus increasing the surface absorption area localized in the mucosa and microbial diversity [105]. That results in beneficial changes in host metabolism and energy expenditure [106]. Changes in microbial diversity after probiotic supplementation have been related in European sea bass. Through denaturing gradient gel electrophoresis, Makridis et al. [65] demonstrated an increase in bacterial diversity in European sea bass after the application of *Phaebacter* sp. The dietary administration of *Bacillus amyloliquefaciens* spores at 10^7 CFU/g had implications on gut morphology and microbial diversity in European sea bass. Previously, Silvi et al. [32] showed that the application of *Lactobacillus delbrueckii subsp. delbrueckii* in European sea bass modulated gut microbiota. Moreover, other studies have demonstrated an increase in the number of goblet cells, an increase in the villi length, and the absence of cyst formation, which is a clear indicator of an improvement in gut morphology. Also, after probiotic application, microbial diversity also benefited from a decrease in the Actinobacteria phylum and Nocardia genus. In addition, the number of Betaproteobacteria and Firmicutes—as beneficial bacteria—was higher [52]. All data are summarized in Table 1.

6. Highlight Notes for Further Investigation

Although European sea bass are one of the most used species in European aquaculture, especially in the Mediterranean region, they are surprisingly underexplored in research compared to other global species. Species such as tilapia, carp, trout, and even Asian sea bass are well researched in reference to probiotics [2,107–109]. Apart from the aforementioned European sea bass references, numerous investigations have been described on the use of probiotics in Atlantic and Mediterranean species such as sole [110–112], sea bream [113,114], and turbot [115,116]. However, they are still scarce compared to the global species mentioned above. For instance, the number of microorganisms used as probiotics in Nile tilapia is not nearly comparable to that in European sea bass. This should encourage future research into the framework of this species. Based on the fact that it is a science yet to be investigated, it is possible to delve deeper into probiotic modes of action. Today, it is well known that probiotics have different mechanisms of action as previously described. However, it would be naive to assume that all mechanisms of action are already described. Techniques such as fluorescent in situ hybridization (FISH), different staining methods, and novel microscopy techniques can help to better understand and monitor the behavior of probiotics in hosts and likely identify new mechanisms of action. In fact, the use of European sea bass as a probiotic study model could help to better understand the mechanisms of action in this species. To this end, we recommend the use of germ-free models, as Galindo-Villegas et al. [117] used with zebrafish and Dierckens et al. [118] used with European sea bass, among other studies. Apart from the aforementioned probiotic modes of action in European sea bass, there are other modes that have not been studied in European sea bass such as the inhibition of quorum sensing, also called quorum quenching. Quorum sensing is responsible for several bacterial activities such as biofilm and virulence [119]. However, the literature on quorum quenching by probiotics on European sea bass protection is non-existent. Nonetheless, it is true that quorum quenching of pathogens by probiotics may imply that they can serve as candidates in European sea bass. Other studies have tested it with other aquaculture species such as zebrafish [120] and rainbow trout [121]. The production of inhibitory substances against pathogens is an important probiotic quality [60]. However, studies that describe this production of inhibitory substances by probiotics in European sea bass are scarce. Although the antibacterial activity of probiotics in European sea bass has been published, no reference to antiviral and antifungal probiotic activity has been published yet. The production of these substances by the probiotics, their detection

and identification by techniques such as high-performance liquid chromatography (HPLC), and their application in vitro or in vivo in European sea bass may be of great interest to the scientific community.

Sea bass is a species with a very low stress threshold [122]. Chronic stress is one of the main culprits for the immunosuppression of fish in aquaculture farms [123], causing their death. Therefore, the surrounding environment status is a crucial factor. Improving the water quality is another probiotic mechanism of action that confers benefits to the fish, improving the environmental quality [124]. *Bacillus* spp. has the capacity to convert organic matter into CO_2 and balance phytoplankton production [89]. Certain bacteria are capable of regulating the pH of water in recirculatory aquaculture systems (RASs) by reducing ammonia. The application of novel probiotics in RASs and in biofilters has not been tested in European sea bass. The brief existing literature on water quality improvement after probiotics application in European sea bass comes from Eissa et al. [51] and El-Sayed [57]. However, several studies have demonstrated in other species that the use of probiotics could improve water quality and benefit fish health [23,125].

Future and additional studies about mechanisms of action in European sea bass could focus on profiling the transcriptome and proteome of host gut microbiota; the interactions between host, microbe, and gut; the intestinal epithelium; tissues associated with the immune system; antioxidant status; and the antagonistic and synergistic effects of probiotics.

Probiotic effects on a host depend on the duration and dose of administration. Previous research—described in this review—applied an administration period of fewer than 2 months. However, research in other species such as tilapia used longer time periods of up to 8 months [126]. It would be interesting and novel to study the effects on European sea bass of longer administration times.

In reference to the benefits provided by probiotics in European sea bass, there is a variety of information on immunological parameters, survival, growth, and changes in microbiota diversity, previously described. However, there are alternative benefits that have been studied in other species after the administration of probiotics that have been not studied in European sea bass. As noted above, probiotics have the ability to modulate intestinal morphology and microbial diversity. Numerous probiotics have been studied to evaluate their improvement of intestinal morphology and changes in the microbiota. Nevertheless, research on this field in European sea bass is scant, unlike that for other species. In other species, parameters such as the number and morphology of villi, microvilli, lamina propria, and goblet cells have been described by several studies after the application of probiotics [127–131]. Further research could also examine in greater depth the effects between the different probiotic strains applicable to the European sea bass and the host commensal microbiota.

Overcrowding is one of the main factors responsible for chronic stress in fish. However, to date, no studies on the effects of probiotics on European sea bass have been conducted on this topic. Instead, studies on this topic have focused on other species [132,133].

Positive changes in blood profiles are also considered an improvement in health status, but, again, few studies on this topic with reference to European sea bass after probiotic effects have been conducted, save for the work of Piccolo et al. [39] and Schaeck et al. [45]. In other species, more blood parameters have been tested such as cortisol, glucose, cholesterol, triglyceride, blood urea nitrogen, bilirubin, plasma total protein, and hematocrit value [134,135].

Epithelial surfaces are target areas for possible pathogen invasion [136]. Fish skin abrasions are common injuries in aquaculture, usually due to overcrowded conditions. The skin of the fish acts as a barrier between the host and environment. Additionally, the skin controls homeostasis and provides protection against physical damage [137]. Therefore, the presence of wounds can have a great impact on the economics of aquaculture farms and on animal welfare. Novel research has demonstrated the ability of probiotics to heal wounds [138]. However, no research on this aspect related to European sea bass has been conducted, so these study models could be transferred to European sea bass.

On the other hand, we have been surprised by the few reports we have found regarding the probiotic application of live microalgae or live yeast. Microalgae and yeast have been extended to be used as sustainable feed ingredients for aquaculture. However, the administration of live microalgae or live yeast through vectors—rotifers, *Artemia*, or copepods—in European sea bass larvae could have several beneficial effects not yet described.

Currently, several probiotic studies could be extrapolated to European sea bass. Thanks to novel techniques that describe bacterial genetic affiliations in the case of probiotic bacteria, new candidate probiotic species are emerging, which may be the object of future research in this understudied species. Nevertheless, when carrying out research with probiotic bacteria both in European sea bass and other species, it would be advisable to deepen the presence of genes with antibiotic resistance, which could be transferred to pathogenic bacteria, still under study. Despite this, the current science remains that probiotics generally have a very beneficial effect on European sea bass, but future research will be needed to elucidate novel mechanisms of action and additional beneficial effects.

7. Conclusions

The use of probiotics in European sea bass promotes sustainable production in order to meet the global food demand. The application of these microorganisms improves growth, survival rates, health status, disease resistance, intestinal morphology, and changes in the diversity of the microbiota. Management of doses and duration of administration are essential for the significance of the treatment. Moreover, since the mechanisms of probiotics in aquaculture are not fully understood, the use of probiotics in European sea bass has much room for further study. Investigating the mechanisms of action of probiotics and the effects they produce in European sea bass can provide an invaluable source of knowledge on this species, which, today, is one of the main components of Atlantic and Mediterranean aquaculture.

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Chapter VIII

General Discussion

In contemporary aquaculture, probiotics are increasingly recognized as a fundamental tool for improving fish health, thereby enhancing production, individual health, and promoting the sustainability of the industry. Probiotics play a crucial role in mitigating the adverse impacts associated with antibiotic use, which is linked to the emergence of multi-resistant bacterial strains. Despite the numerous benefits presented by probiotics, their integration into sustainable aquaculture faces several significant challenges that are currently at the forefront of scientific research (Balcázar et al., 2006; Dawood & Koshio, 2016; Hai, 2015; Ringø et al., 2016)

In order to reduce the use of antibiotics and ensure the sustainable application of probiotics in aquaculture, this thesis aims to develop a new strain, *Bacillus velezensis* D-18, that meets the required criteria to be considered a probiotic (Balcázar et al., 2006; El-Saadony et al., 2021; Hai, 2015; Kesarcodi-Watson et al., 2008; Kiron, 2015; Merrifield et al., 2010). The study have examined some of its mechanisms of action and its biological effects on the European sea bass, one of the most important commercial species in the Atlantic and Mediterranean contexts.

Are probiotics an effective measure against the entry of pathogens in aquaculture?

In aquaculture, probiotics have emerged as a promising solution to combat the entry and proliferation of pathogens, offering an eco-friendly and sustainable alternative to traditional antibiotics and chemicals (Heinonen-Tanski & Hancz, 2022). The application of probiotics in aquaculture not only enhances the health and growth of cultured species but also plays a crucial role in disease prevention. Probiotics are able to compete with pathogens for adhesion sites and energy sources and by producing antibacterial substances (Hoseinifar et al., 2018). Additionally, probiotics promote the competitive exclusion of pathogens by effectively colonizing the mucosal epithelium of the gastrointestinal tract of aquatic organisms, thereby preventing pathogen colonization (Sha et al., 2023). Moreover, probiotics can produce various antibacterial compounds, such as organic acids, peroxides, enzymes, and bactericidal proteins, which directly inhibit pathogenic bacteria and enhance the immune response of hosts (Thuy et al., 2024).

Recent research have highlighted the importance of certain probiotics in water quality management. These beneficial microorganisms contribute to the decomposition of organic waste and the cycling of nutrients, creating a healthier and more sustainable environment for aquaculture. The ability of probiotics to transform organic matter into CO_2 and maintain nitrogen balance is particularly valuable in tanks and closed systems (Hasan & Banerjee, 2020).

Based on the results of this thesis, which demonstrate the capability of the *B. velezensis* D-18 strain to increase disease resistance in European sea bass, inhibit the growth of pathogens, interfere with *quorum sensing*, and reduce biofilm formation, it is reasonable to consider probiotics an effective measure against the entry of pathogens in aquaculture.

How do the specific properties of *Bacillus velezensis* D-18 correlate with its potential as a probiotic candidate in aquaculture, and how can these attributes contribute to improving the health and sustainability of cultivated aquatic systems?

Several studies have established the essential characteristics for qualifying an organism as a probiotic through *in vitro* or *in vivo* assays (Balcázar et al., 2006; El-Saadony et al., 2021; Hai, 2015; Kesarcodi-Watson et al., 2008; Kiron, 2015; Merrifield et al., 2010):

a. The strain must demonstrate the ability to adhere to and multiply in the host and be capable of tolerating the adverse conditions of bile and the host's pH.

b. It must lack genes associated with antibiotic resistance and must not induce mutations in the host organism.

c. It must confer benefits to the host, either by improving growth and/or developing the immune system to combat pathogens, or by exhibiting antimicrobial properties.

d. It must be harmless to the host organism.

During the development of this doctoral thesis, it has been established that *Bacillus velezensis* D-18 meets all the necessary criteria to be considered a reliable probiotic in the field of aquaculture:

In Chapter III, through *in vitro* tests, it was demonstrated that *B. velezensis* D-18 adheres to the intestine of the European sea bass and is able to withstand the conditions of bile and pH present in this environment. Subsequently, through intraperitoneal inoculation of the strain in the host, it was verified that *B. velezensis* D-18 is completely harmless, showing no clinical signs or inducing anatomical-morphological changes in the European sea bass (Monzón-Atienza et al., 2021).

The benefits that each probiotic can offer vary depending on the strain used. Therefore, the main criteria for qualifying a microorganism as a guaranteed probiotic include the ability to provide benefits, be safe for the host, and be able to establish itself within the host (Binda et al., 2020). Other studies have addressed various parameters to characterize different probiotic strains, such as the absence of toxins and hemolytic activity (Golnari et al., 2024; Shahbaz et al., 2024). These aspects ensure the strain's safety and biosecurity, a parameter evaluated *in vivo* in Chapter III of this thesis through direct administration to European sea bass, yielding similar results.

It is worth noting that the characterization performed in 2021 (Monzón-Atienza et al., 2021) remains at the forefront, as many of the parameters used in Chapter III continue to be applied in recent research (Elsadek et al., 2023; Golnari et al., 2024; Shahbaz et al., 2024).

In Chapter IV, through *in vivo* trials, the strain's capacity to positively modulate the immune response of the European sea bass was confirmed, which resulted in an increase in the activity of the innate immune system and an improvement in its survival against the pathogen *Vibrio anguillarum* 507 (Monzón-Atienza et al., 2022). These results are consistent with more recent studies demonstrating that the application of *B. velezensis*, specifically the T20 strain, increases disease resistance in turbot (Yu et al., 2024). However, not all strains have the same mechanisms or effects. For example, *B. velezensis* T23 decreases parameters related to the immune system, such as the expression of TNF-α (Yang et al., 2024), whereas in our work, this cytokine is increased in European sea bass following the administration of the D-18 strain. This underscores the importance of bacterial strain characterization, as many bacteria within the same genus and species can

exhibit significant differences in their physiological, pathogenic, and genetic characteristics (Joseph et al., 2012).

In Chapter V, through gene sequencing of the strain, the absence of genes associated with antimicrobial resistance plasmids or potential causes of mutations in the host is verified. While probiotics are generally considered safe and beneficial for health, there is concern that they could serve as reservoirs or conduits for antibiotic resistance. Ensuring that probiotic strains are free of transferable antibiotic resistance genes is vital for their safe and effective use (Doron & Snydman, 2015).

The inhibitory capacity of a microorganism is considered one of the essential criteria for being regarded as a probiotic candidate (Binda et al., 2020). In the penultimate chapter of this thesis (Chapter VI), the antimicrobial activities of *B. velezensis* D-18 were examined, particularly its ability to enzymatically degrade bacterial signaling molecules -*quorum quenching*- associated with Gram-negative pathogens. Consequently, the inhibition of biofilm formation by *V. anguillarum* 507 was evaluated (Monzón-Atienza et al., 2024). The results obtained demonstrate the inhibitory capacity of the D-18 strain, which degrades the long-chain acyl homoserine lactones of *V. anguillarum* 507, a signaling molecule of the pathogen, using lactonases. This inhibition was observed in the co-culture of the probiotic and the pathogen, showing a reduction in growth and biofilm formation by *Vibrio*. The *quorum quenching* activity of probiotics has already been tested in other experiments (Lubis et al., 2024), as well as the ability of *Bacillus* spp. to interfere with biofilm formation through *quorum quenching* (El Aichar et al., 2022; Vinoj et al., 2014; Xu et al., 2024).

B. velezensis D-18 offers benefits that contribute to the sustainability of aquaculture. Frequent use of antimicrobial agents involves high production costs, the presence of antibiotic residues in the muscles of fish, which can have adverse effects on consumer health, and damage to aquatic ecosystems (Okocha et al., 2018; Watts et al., 2017). Therefore, the use of this probiotic can help mitigate the risk of antimicrobial resistance development and can minimize the release of antimicrobial residues into the aquatic environment. Furthermore, *B. velezensis* D-18 improves the well-being of European sea bass through its specific properties, which can contribute to more efficient and sustainable aquaculture production, thus alleviating pressure on natural resources and aquatic ecosystems.

How do the findings of this thesis relate to the existing knowledge on the use of probiotics in aquaculture, particularly in the context of European sea bass?

The findings of this doctoral thesis represent a significant contribution to the field of aquaculture by reinforcing and expanding the existing knowledge on the use of probiotics, specifically in the production of European sea bass.

In particular, this thesis introduces a new probiotic strain to the field of aquaculture, providing concrete evidence of the probiotic efficacy of *Bacillus velezensis* D-18 in European sea bass. Key highlights include its ability to combat pathogenic strains, survive in adverse gastrointestinal conditions, promote mucosal adhesion, and increase resistance to specific diseases caused by *Vibrio* spp. Additionally, this work delves into the mechanisms through which the *B. velezensis* D-18 strain exerts its beneficial effects, exploring its impact on the innate immunity of the fish and its ability to interfere with the bacterial communication -*quorum sensing*- of pathogenic bacteria, thereby preventing biofilm formation.

The current literature supports the benefits of probiotics in this species, showing improvements in growth, survival rates, health, disease resistance, intestinal morphology, and microbiota diversity (Chouayekh et al., 2023; Perdichizzi et al., 2023; Rangel et al., 2024; Serradell et al., 2023). Bacterial probiotic strains such as *Vagococcus fluvialis*, *Bacillus subtilis*, *Vibrio lentus*, and *Phaeobacter* sp. have also demonstrated the ability to improve the survival of European sea bass against the threat of bacteria from the genus *Vibrio* (Schaeck et al., 2016; Sorroza et al., 2012; Touraki et al., 2012). Similar to our *B. velezensis* D-18 strain, other bacterial probiotic strains support the modulation of the immune system in European sea bass (Lamari et al., 2016; Mladineo et al., 2016; Picchietti et al., 2009; Schaeck et al., 2017; Sorroza et al., 2012). Chapter VII of this thesis emerges as a crucial section that encapsulates the significant findings and contributions of probiotic application in European sea bass. This section not only summarizes the efficacy of probiotics in the aquaculture context but also establishes a solid foundation for future research in this area.

How might the *quorum quenching* ability of the *Bacillus velezensis* D-18 strain influence in aquaculture systems?

The bacterial diversity present in seawater is widely recognized, encompassing a variety of microorganisms that can be harmless, pathogenic, or opportunistic (Zinger et al., 2011). In aquaculture environments where fish are directly exposed to seawater, whether in land-based tanks or ocean cages, the presence of microorganisms is inevitable. These microorganisms can colonize the fish's tract, adhere to the surfaces of tanks or cages, or maintain a free-living existence in the aquatic medium (Sehnal et al., 2021). It is crucial to recognize the risk associated with the entry of pathogens into the system, as pathogenic bacteria can form biofilms and adhere to living or inert surfaces. These biofilms not only act as biological niches for pathogens, protecting them from chemicals and environmental conditions but also facilitate their spread (Muhammad et al., 2020).

The concept of *quorum quenching*, which involves the enzymatic inhibition of *quorum sensing* (Sikdar & Elias, 2020), is proposed as an effective tool for addressing problems caused by pathogenic quorum sensing, ranging from infections to the formation of biofilms mentioned earlier (Zhou et al., 2020). Other studies have demonstrated the *quorum quenching* capability of probiotics, particularly of different *Bacillus* spp. (Santos et al., 2021), and specifically their ability to inhibit biofilm formation (Zhou et al., 2019). During the development of this thesis, this disruptive strategy has been demonstrated by the *B. velezensis* D-18 strain. This probiotic, owing to its ability to produce lactonases, degrades acyl homoserine lactones, *quorum sensing* signaling molecules used by numerous pathogenic Gram-negative bacteria to coordinate crucial phenomena such as virulence and biofilm formation (Taghadosi et al., 2015; Xiao et al., 2022). Based on this, incorporating this probiotic into biofilters or tank structures could represent a significant benefit for fish health, while also contributing to the effective control of microbial communities (Ghanei-Motlagh et al., 2019; Heinonen-Tanski & Hancz, 2022; Jahangiri & Esteban, 2018).

Chapter IX

Conclusions

1. The probiotic strain *Bacillus velezensis* D-18 is capable of withstanding the extreme conditions of the European sea bass gastrointestinal tract (pH and bile acids), while also demonstrating adept adherence to the gastrointestinal mucosa and prevention of pathogen adhesion.

2. *Bacillus velezensis* D-18 manifests non-detrimental effects in European sea bass, as evidenced by the absence of any clinical manifestations or anatomopathological changes in this species.

3. The application of the probiotic strain in European sea bass leads to a significant increase in survival against *Vibrio anguillarum* 507.

4. The innate immune response of the European sea bass is strengthened after the probiotic administration of the *Bacillus velezensis* D-18 strain, through the improvement of phagocytic activity, an increase in serum lysozyme, nitric oxide production and bactericidal activity. In addition, an enhancement in the expression of cytokines and the antimicrobial peptide dicentracin was also appreciated.

5. The probiotic strain is confirmed to lack plasmids carrying antimicrobial resistance genes, reinforcing its safety and suitability for use in aquaculture.

6. *Bacillus velezensis* D-18 presents *quorum quenching* demonstrating a remarkable ability to inhibit the growth and biofilm formation of the pathogen *Vibrio anguillarum* 507,

7. The probiotic strain *Bacillus velezensis* D-18 emerges as a promising and sustainable probiotic for the aquaculture industry, providing an effective and safe alternative to antibiotic use and offering significant benefits to the health of European sea bass.

Research Gaps and Future Perspectives

One key area for investigation would be a detailed understanding of the specific mechanisms through which *Bacillus velezensis* D-18 exerts its probiotic effects in aquaculture, including its interaction with the fish's intestinal microbiota and the molecular mechanisms involved in modulating the immune response. There is currently significant uncertainty about these mechanisms, and it is crucial to establish a more accurate correlation between results obtained *in vitro* and *in vivo* (Tinh et al., 2008). The use of germ-free fish may aid this purpose, as previously demonstrated by Galindo-Villegas et al., (2012).

Exploring the dynamic interactions between *B. velezensis* D-18 and the fish's intestinal microbiota will contribute to the advancement of probiotic research. Long- term monitoring to assess the persistence and stability of *B. velezensis* D-18 in the fish intestine, as well as its behavior and distribution, would provide crucial information on its probiotic mechanisms and its competition with other commensal or pathogenic bacteria for nutrients, adhesion sites, and energy sources. The study of gut microbiome after *B. velezensis* D-18 application could be very useful.

On the other hand, various probiotics have been able to positively modulate the production of gastrointestinal enzymes, promoting better digestion and consequently a greater assimilation of nutrients by the host. These benefits are usually reflected in the production and quality of the product. The impact of supplementing with *B. velezensis* D-18 on the production and quality of fish meat, including parameters such as growth, feed conversion rate, and sensory quality, is still a mystery today.

The interaction of *B. velezensis* D-18 with the surrounding aquatic environment, including the impact on microbial diversity and environmental health in aquaculture systems, as well as its ability to maintain its probiotic activity under different conditions, also deserves attention. Previously, the ability of probiotics to improve water quality, a quite relevant condition in current aquaculture, has been described and could be a line of research for this strain.

Exploring into the antimicrobial activity of *B. velezensis* D-18, there is still a knowledge gap in the interaction of the probiotic with pathogenic agents. Although this thesis has emphasized a burgeoning mechanism of bacterial inhibition, such as *quorum quenching*,

further investigation of its effect in regulating the intestinal microbiota and preventing other diseases in European sea bass could be pursued. In addition, various mechanisms have not yet been described in this probiotic strain. Therefore, the study of the production of bacteriocins, organic acids, and lytic enzymes by *B. velezensis* D-18 would also be an interesting line of study for the control of pathogenic agents in aquaculture.

For the realization of this thesis, one of the pathogens currently affecting the Canary coasts, the pathogenic strain *Vibrio anguillarum* 507, was used. However, the antimicrobial evaluation of the probiotic could be extrapolated to other pathogens that also pose a great danger to aquaculture.
On the other hand, it would be novel to explore the potential synergistic effects of *B. velezensis* D-18 in combination with other probiotics, immunostimulants, and even phages, and to assess their long-term effects on fish health, production efficiency, and disease control.

As for practical application, it would be interesting to explore the possibility of using the *B. velezensis* D-18 strain in water recirculation systems as a control measure against *Vibrio* spp. and other pathogenic agents, both in biofilters and on the walls of the tanks for the control of pathogenic bacterial biofilms.

Lastly, research on the industrial application of *B. velezensis* D-18 would be an important step, and market studies and commercial viability analyses would be required to assess its potential as a probiotic product in the aquaculture industry.

This set of research would address fundamental aspects of the probiotic potential of *B. velezensis* D-18 and would significantly contribute to the advancement of knowledge in the field of aquaculture.

Resumen en español

1. INTRODUCCIÓN

1.1 Acuicultura en el Contexto Global y Europeo

Durante las últimas décadas, la acuicultura se ha proclamado como el sector de producción de alimentos de más rápido crecimiento, convirtiéndose en una actividad fundamental en la producción mundial de alimentos (Martínez-Porchas et al., 2023). Este aumento puede atribuirse en gran medida a la creciente demanda de productos pesqueros y mariscos por parte de la población, junto con la disminución de las capturas de especies salvajes (FAO, 2022; Pontecorvo & Schrank, 2012) (Figura 1). La acuicultura desempeña un rol fundamental en la seguridad alimentaria, al mismo tiempo que fomenta el crecimiento económico y el cuidado del medio ambiente. Su importancia nutricional no debe subestimarse, dado que desempeña un papel crucial en la provisión de proteínas esenciales para la población humana (Pradeepkiran, 2019).

Europa se erige como líder mundial en acuicultura, amalgamando prácticas de producción eficientes con un enfoque sostenible y una búsqueda incesante de innovación. La industria acuícola no solo satisface las demandas nutricionales de la población, sino que también marca el rumbo hacia prácticas más responsables y respetuosas con el medio ambiente (FAO, 2022). Dentro de este marco, Europa cuenta con una producción acuícola diversa que comprende diversas especies adaptadas a las condiciones específicas de la región. En el contexto europeo, las especies destacadas son: el salmón (*Salmo salar*), la trucha (*Oncorhynchus mykiss*), la dorada (*Sparus aurata*), mejillones (*Mytilus* spp.), ostras (*Crassostrea* spp.) y la lubina (*Dicentrarchus labrax*), entre otras (APROMAR, 2023; Bostock et al., 2016).

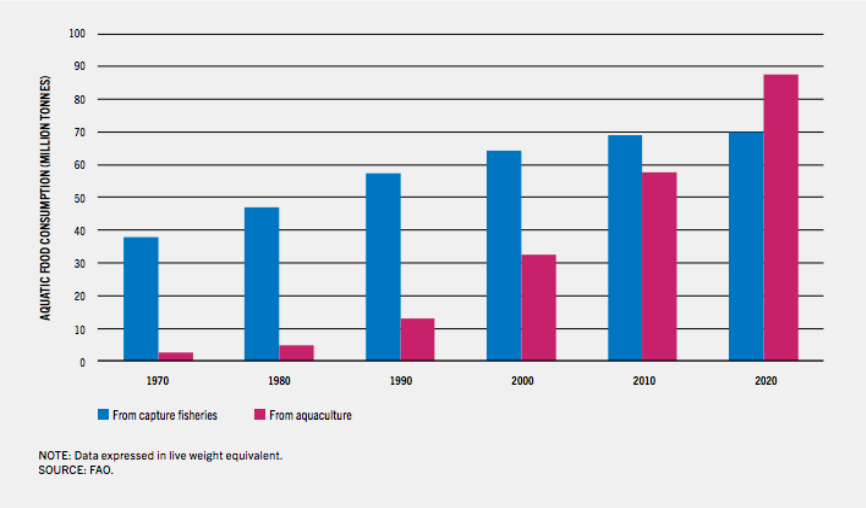


Figura 1. Crecimiento en el consumo de productos de acuicultura versus productos pesqueros durante las últimas décadas (FAO, 2022).

1.2 Acuicultura de la Lubina Europea (*Dicentrarchus labrax*)

La lubina europea (*Dicentrarchus labrax*) emerge como una especie fundamental Europa, especialmente en regiones del Mediterráneo y del Atlántico, debido a su valor comercial y su importancia en la cadena alimenticia (Fuentes et al., 2010). La lubina es una especie de rápido crecimiento, alta tasa de conversión de alimento y una alta adaptabilidad a condiciones de cultivo controladas (APROMAR, 2023). En 2021 fue considerada la segunda especie más valiosa económicamente, después de la trucha arcoíris. Ya en 2022, su producción alcanzó las 301.420 toneladas, consolidando su estatus como una de las especies más relevantes en la acuicultura mediterránea, especialmente en países como Turquía, Grecia, Egipto y España (APROMAR, 2023) (Figura 2). La cría de lubina se realiza en prácticamente todos los países de la región mediterránea. Durante el primer mes de vida, las larvas se alimentan de artemia y rotíferos antes de pasar a consumir pienso. Los sistemas de cría son diversos, incluyendo jaulas flotantes en el mar, tanques o estanques en tierra (APROMAR, 2023). Los tamaños comerciales van desde los 250 g hasta más de 2500 g. Normalmente, el proceso de crecimiento para alcanzar los 400 g lleva entre 20 y 24 meses desde la eclosión de las larvas. Se estima que la producción de lubina en España en el año 2022 alcanzó las 23.622 toneladas, convirtiéndola en la segunda especie de acuicultura más producida (APROMAR, 2023). Las Islas Canarias representan el 21% de la producción total de España (APROMAR, 2023) (Figura 3).

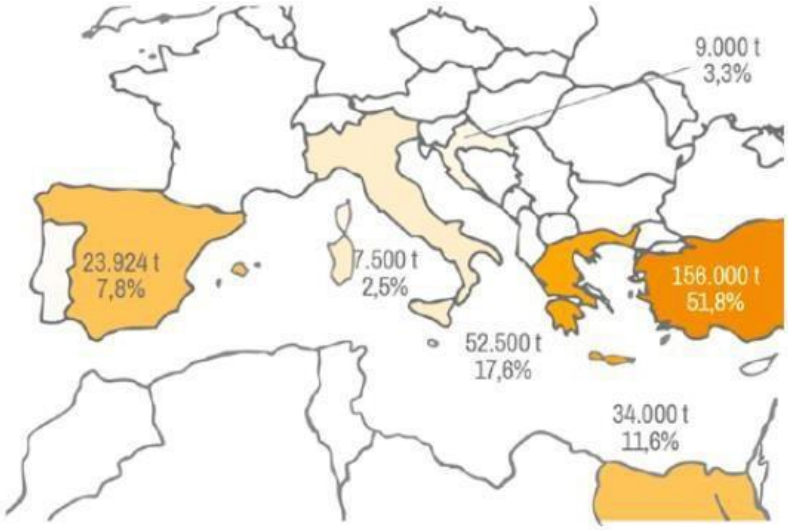


Figura 2. Representación de la producción acuícola de la lubina europea en Europa, expresada en toneladas (APROMAR, 2023).

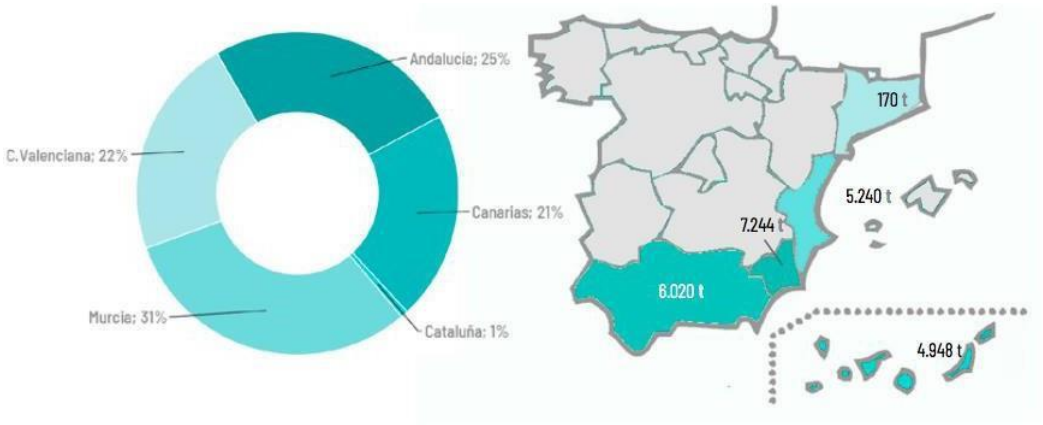


Figura 3. Representación de la producción acuícola de lubina europea en España, expresada en toneladas (APROMAR, 2023).

1.2.1 La Lubina Europea como Piedra Angular de la Investigación en Acuicultura

La lubina juega un papel fundamental en la investigación en acuicultura, abordando una amplia gama de desafíos y aspectos clave en la producción acuícola:

- La lubina europea es un modelo efectivo para el estudio de procesos fisiológicos y metabólicos vinculados al crecimiento, la reproducción y la salud en la acuicultura (Di Marco et al., 2008; Ribas et al., 2019; Stavrakidis-Zachou et al., 2019). A día de hoy, comprender estos aspectos es esencial para mejorar la eficiencia de la producción acuícola.
- La gran adaptabilidad de la lubina europea a los diversos sistemas de producción acuícola, desde estanques en tierra hasta jaulas marinas (APROMAR, 2023), permite investigar los efectos bajo diferentes condiciones ambientales y prácticas de gestión en el crecimiento y la calidad del producto final.
- La investigación utilizando lubina europea como modelo de estudio, contribuye a la comprensión de los mecanismos del sistema inmunológico y la resistencia a enfermedades comunes en acuicultura (Miccoli et al., 2024; Valsamidis et al., 2023). De esta forma, es posible la elaboración de diferentes estrategias para la prevención y el control de enfermedades, reduciendo consecuentemente las pérdidas económicas asociadas.
- La lubina europea se utiliza en la investigación genómica y la cría selectiva, identificando genes relacionados con rasgos deseables, impulsando así programas de mejora genética para una producción mejorada (Montero et al., 2023; Vandeputte et al., 2017, 2019).

En consecuencia, la lubina se erige como un pilar fundamental en la investigación en acuicultura, proporcionando conocimientos valiosos para mejorar la eficiencia, sostenibilidad y competitividad de esta industria en constante evolución.

1.3 Desafíos Actuales en la Acuicultura

Actualmente, el éxito de la acuicultura no está exento de desafíos. A pesar de sus numerosos beneficios, la acuicultura a menudo enfrenta críticas debido a su impacto ambiental, como la contaminación del agua, la introducción de especies invasoras, la degradación de los ecosistemas acuáticos y el desarrollo de bacterias resistentes a los antibióticos (Martinez-Porchas & Martinez-Cordova, 2012). Para abordar estos desafíos, la industria acuícola debe centrarse en la implementación de prácticas sostenibles (Boyd

et al., 2020). Además, la creciente demanda de productos acuícolas subraya la importancia de garantizar la seguridad alimentaria (Pradeepkiran, 2019). Esto implica abordar preocupaciones relacionadas con la calidad del producto, la trazabilidad y la implementación de estándares y regulaciones estrictas.

La aceptación social de la acuicultura también es un desafío significativo para la industria. Incertidumbres y conceptos erróneos sobre su impacto ambiental, bienestar animal, seguridad alimentaria y calidad del producto pueden influir negativamente en las percepciones del consumidor (Bacher et al., 2014; Schlag, 2010; Whitmarsh & Palmieri, 2009). Concienciar al consumidor sobre la importancia de la acuicultura en el mundo moderno es esencial, haciéndole entender los problemas derivados de la sobrepesca, la búsqueda de fuentes sostenibles de proteínas, además de la importancia de la calidad y la seguridad del producto.

En cuanto al bienestar animal en la acuicultura, la salud de los animales es un factor crítico que afecta directamente a la producción y sostenibilidad del sector (Franks et al., 2021). La manipulación y manejo de peces, condiciones ambientales inadecuadas y una alta densidad de población -situaciones prevalentes en la acuicultura europea actual- son factores estresantes (Bergqvist & Gunnarsson, 2013). El estrés es un factor limitante en la acuicultura, potencialmente responsable de reducir las tasas de crecimiento (tasa de conversión alimenticia), influir en la calidad del producto final (textura, sabor y apariencia) (Peng et al., 2024), interferir con los procesos reproductivos (calidad de los huevos y larvas) (Schreck, 2010) y desencadenar comportamientos anormales como la agresión entre peces en sistemas de cultivo (Andersson et al., 2022). Además, el estrés puede debilitar el sistema inmunológico de los peces, haciéndolos más susceptibles a enfermedades infecciosas (Dai et al., 2023; Tort, 2011). En estas condiciones, las bacterias patógenas representan un desafío significativo en la acuicultura actual (Ben Hamed et al., 2018), con un énfasis particular en la formación de biopelículas, que sirven como nicho ecológico para numerosos microorganismos patógenos (De Silva & Heo, 2022).

1.3.1 Biopelículas Bacterianas en la Acuicultura

La formación de biopelículas destaca como una preocupación prominente dentro de la industria acuícola. Las biopelículas son una estructura microbiana compleja compuesta principalmente por bacterias adheridas a una superficie e incrustadas en una matriz de sustancias poliméricas extracelulares (Hobley et al., 2015; Peng et al., 2024). La

formación de biopelículas (Figura 4) está mediada por el fenómeno descrito como "*quorum sensing*" (Hemmati et al., 2024; Peng et al., 2024). La formación de esta fascinante estructura otorga diversas ventajas a las bacterias que la componen, como una mayor protección contra condiciones ambientales, desinfectantes, antibióticos u otros agentes antimicrobianos (Dufour et al., 2010). En ocasiones, también favorece la transmisión de genes de resistencia antimicrobiana (Michaelis & Grohmann, 2023). En el intrincado escenario de la acuicultura, la formación de biopelículas por parte de bacterias patógenas emerge como una preocupación capital, exigiendo una comprensión detallada y estrategias de control efectivas (Kilic & Bali, 2023; Mishra et al., 2020). La amenaza inherente de las biopelículas provenientes de bacterias patógenas en la acuicultura se basa en la creación de microentornos favorables para la proliferación de dichos microorganismos patógenos. Es decir, este hábitat microbiano puede promover el desarrollo y la persistencia de patógenos, aumentando el riesgo de enfermedades infecciosas en las poblaciones acuícolas. Ante este escenario, se han propuesto diferentes estrategias para combatir eficazmente las biopelículas formadas por patógenos en instalaciones acuícolas. Sin embargo, este tedioso problema sigue persistiendo.

En el ámbito académico y de la investigación, estudios recientes han explorado estrategias innovadoras para su control efectivo en la acuicultura. La investigación reciente incluye la aplicación de nanopartículas con propiedades antimicrobianas (Al-Wrafy et al., 2022), bacteriófagos (Liu et al., 2022), utilización de bactericidas/bacteriostáticos (Chen et al., 2013) y la inhibición de *quorum sesing* (Paluch et al., 2020).

En el contexto de la lubina europea, la literatura confirma a *Photobacteria* spp., *Tenacibaculum* spp. y *Vibrio* spp. como los patógenos bacterianos más frecuentes (Muniesa et al., 2020). En concreto, el género *Vibrio* sobresale como una preocupación principal en la patología de las granjas de lubina europea en las Islas Canarias. En respuesta a los diversos desafíos planteados por el ambiente acuático, este género ha evolucionado para utilizar la producción de biopelículas como estrategia de supervivencia (Arunkumar et al., 2020; De Silva & Heo, 2022).

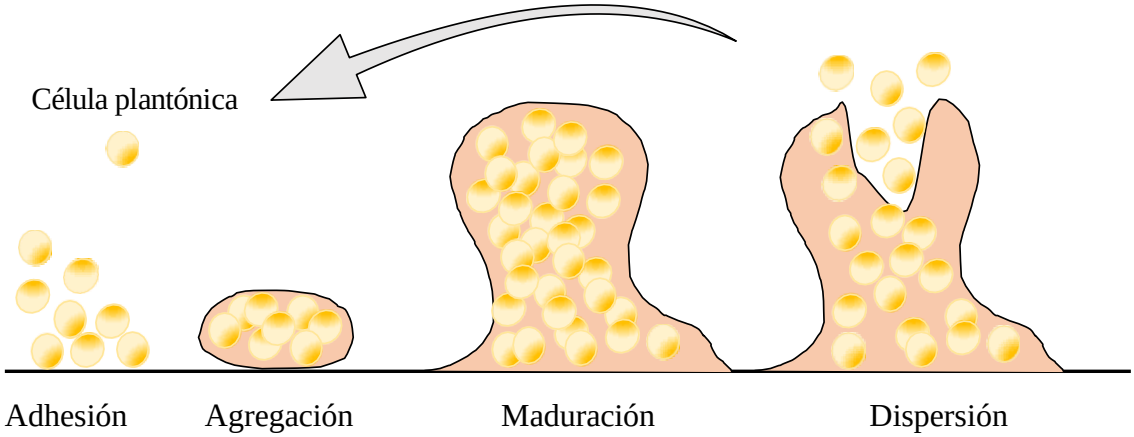


Figura 4. Representación esquemática del proceso de formación de biopelículas bacterianas. Inicialmente, las células planctónicas se adhieren a una superficie utilizando proteínas específicas (**Adhesión**). Una vez adheridas, estas células comienzan a agruparse y a iniciar la producción de la matriz extracelular (**Agregación**). A medida que las células continúan dividiéndose, esta agregación evoluciona hacia una biopelícula madura (**Maduración**). En la etapa final, conocida como **dispersión**, ciertas enzimas, incluidas proteasas y nucleasas, junto con un mecanismo de *quorum sensing*, facilitan la ruptura de la biopelícula. Este proceso permite que las células bacterianas se liberen de la biopelícula y vuelvan a su forma planctónica, propagándose así para colonizar nuevos nichos ecológicos.

1.4 *Vibrio* spp. como Patógeno en la Acuicultura.

Vibrio es un género de bacilos Gram negativos comúnmente presentes en ambientes acuáticos, tanto en agua salada como en agua dulce (Baker-Austin et al., 2018). Las especies de *Vibrio* se caracterizan por su forma curva, con aproximadamente 2-3 µm de longitud, y un flagelo polar que les proporciona movilidad (Mittal et al., 2023). Muchas bacterias dentro del género *Vibrio* son patógenas y causan una enfermedad conocida como vibriosis, que afecta a la producción acuícola (Sanches-Fernandes et al., 2022). Las especies de *Vibrio* más comúnmente asociadas con la vibriosis en la acuicultura mundial incluyen *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio parahaemolyticus y Vibrio anguillarum* (de Souza Valente & Wan, 2021; Manchanayake et al., 2023). Esta enfermedad afecta principalmente a las branquias, piel y órganos internos de los hospedadores. Los signos clínicos comunes incluyen úlceras en la piel, hemorragias internas, inflamación de las branquias, pérdida de apetito y letargo, entre otros. En casos más graves, la vibriosis puede provocar la muerte (Frans et al., 2011).

La amplia gama de hospedadores a los que afecta, como la dorada (Aly et al., 2023), la lubina (Kapetanović et al., 2022), el rodaballo (Montes et al., 2003), el salmón (Benediktsdóttir et al., 1998), el camarón (de Souza Valente & Wan, 2021) y otros organismos marinos, junto con sus síntomas problemáticos, subrayan la importancia vital de su control.

Si bien la enfermedad puede sospecharse en función de los signos clínicos, el diagnóstico definitivo generalmente se logra a través de los siguientes métodos: (i) ensayos bioquímicos, (ii) PCR, (iii) microscopía directa, (iv) ELISA, (v) *microarrays*, (vi) otros inmunoensayos, y la (vii) amplificación isotérmica mediada por bucle (Loo et al., 2022). La prevención de la vibriosis en la acuicultura es difícil, además sus diversos tratamientos para la tienen limitaciones (Kah Sem et al., 2023). Históricamente, los antibióticos han sido utilizados para tratar esta enfermedad (Loo et al., 2020). Sin embargo, su uso indiscriminado ha generado importantes preocupaciones, destacando el desarrollo de resistencia bacteriana y la presencia de residuos en los productos de acuicultura. En consecuencia, estos problemas tienen implicaciones tanto para la salud humana como para la salud del ecosistema acuático (Bondad-Reantaso et al., 2023). Por lo tanto, la investigación en acuicultura prioriza la búsqueda de alternativas sostenibles y eficaces.

1.5 Antibióticos en la Acuicultura Actual y sus Desafíos

Quizás uno de los logros históricos más significativos en la ciencia ha sido el desarrollo de antibióticos para combatir enfermedades infecciosas y otros problemas causados por bacterias patógenas u oportunistas. El término "antibiótico" fue acuñado el siglo pasado por el microbiólogo estadounidense Selman A. Waksman, quien describió la capacidad antagonista de ciertos microorganismos contra otros (Waksman, 1947). Los antibióticos han sido fundamentales para salvar numerosas vidas en diversas especies. También han contribuido a mejoras en los sistemas de producción animal (Hao et al., 2014), incluida la acuicultura (Adenaya et al., 2023). En la acuicultura moderna, las condiciones de alta densidad prevalentes en estanques y jaulas crean un entorno propicio para la rápida transmisión de enfermedades. En estos entornos abarrotados, los patógenos pueden propagarse fácilmente entre los organismos acuáticos, representando una amenaza significativa para la sostenibilidad de la producción (Irshath et al., 2023). Por lo tanto, el uso de antibióticos es una herramienta esencial para combatir infecciones bacterianas que amenazan la viabilidad de la producción. Además, los antibióticos se han llegado a utilizar con fines profilácticos (Hossain et al., 2022).

La acuicultura no está exenta de los desafíos que plantean los antibióticos en la actualidad. En el contexto de la acuicultura, ciertas regiones del mundo se consideran "puntos críticos" para la aparición de bacterias resistentes a los antibióticos (Cabello et al., 2016). Claros ejemplos de especies que desarrollan fácilmente resistencia en la producción acuícola incluyen *Edwardsiella* spp., *Vibrio* spp., *Pseudomonas* spp. y *Aeromonas* spp. (Dutta et al., 2021; Leung et al., 2019; Nguyen et al., 2014). Estas bacterias multi-resistentes, es decir, resistentes a varios antibióticos, plantean desafíos significativos para las empresas dedicadas a la producción de peces y moluscos, dificultando el control de las enfermedades (Bondad-Reantaso et al., 2023). De hecho, se corre el riesgo de transferir estas cepas resistentes a la población humana a través de la cadena alimentaria o mediante vías ambientales (Da Costa et al., 2013). Por lo tanto, urge la necesidad de estudios que analicen la presencia y transmisión de genes de resistencia antimicrobiana y la búsqueda de soluciones eficientes. Los desafíos del uso de antibióticos van más allá del potencial de resistencia a varios fármacos. Los antibióticos empleados en la producción acuícola pueden llegar al medio ambiente, afectando la calidad del agua y la biodiversidad local. Estas sustancias pueden alterar las comunidades microbianas y los ecosistemas acuáticos, causando daño a una amplia gama de especies no objetivo (González-Gaya et al., 2022; Kraemer et al., 2019). Además, existe una creciente preocupación del consumidor sobre la presencia de residuos antibióticos en los productos acuícolas, lo que tendría implicaciones para la salud y en la aceptabilidad del producto, que afectaría al mercado. De hecho, los consumidores están preocupados por el uso de antibióticos en la producción de alimentos y la población está comenzando a demandar productos "libres de antibióticos". Por estas razones, la búsqueda de alternativas sostenibles es de suma importancia.

1.6 Estrategias para Reducir el Uso de Antibióticos en la Acuicultura.

Debido a la preocupación ferviente por el uso excesivo de antibióticos en la acuicultura, descrito anteriormente, la búsqueda de soluciones se ha convertido en una prioridad máxima. Con el fin de prevenir el uso de antibióticos, las instalaciones acuícolas implementan las siguientes estrategias:

i. Reducir factores estresantes físicos (temperatura, fotoperiodo, oxígeno disuelto, sonido, turbidez, manipulación), químicos (parámetros de calidad del agua, pesticidas, contaminación, dieta, desechos metabólicos) y biológicos (densidad de siembra, microorganismos, macroorganismos, requisitos de natación,

depredadores) (Ciji & Akhtar, 2021). La reducción del estrés promueve la resistencia a enfermedades, evitando así la necesidad del uso de antibióticos.

ii. Desinfectar instalaciones y aplicar herramientas para prevenir la entrada y propagación de patógenos (Acosta et al., 2021).

iii. Implementar programas efectivos de vacunación para reducir la incidencia de enfermedades (Du et al., 2022).

iv. Evitar el uso constante de un solo tipo y optar en su lugar por la rotación, para prevenir la aparición de genes de resistencia (Brown & Nathwani, 2005). Siempre es recomendable realizar pruebas de antibiograma previas (Truong et al., 2021).

v. Proporcionar una dieta equilibrada y nutritiva para fortalecer el sistema inmunológico de los peces (Mendivil, 2021).

vi. Implementar sistemas de alerta temprana para detectar enfermedades en los peces (Li et al., 2009). Los diagnósticos tempranos ayudan a detectar el inicio de las enfermedades en sus etapas iniciales de infección, permitiendo una acción oportuna para prevenir su propagación dentro de las instalaciones.

Además, crear conciencia y educar a los productores acuícolas sobre la importancia del manejo de desechos, el uso de antibióticos y la promoción de otras alternativas sostenibles es crucial. Cuando se discuten alternativas para reemplazar el uso de antibióticos en la acuicultura, opciones destacadas incluyen: vacunas, el uso de aditivos alimentarios como aceites esenciales y extractos de plantas, enzimas, bacteriófagos, probióticos, prebióticos, postbióticos y simbióticos, entre otros (MacNair et al., 2023).

1.7 Probióticos en la Acuicultura

En los últimos años, ha habido un aumento significativo en la utilización de probióticos en la acuicultura, como una alternativa prometedora al uso convencional de antibióticos (Cruz et al., 2012). Los probióticos son microorganismos vivos que, cuando se administran en cantidades adecuadas, confieren beneficios para la salud al hospedador (Hill et al., 2014). En el contexto de la acuicultura, los probióticos pueden ser bacterias vivas, levaduras vivas y microalgas vivas, que normalmente colonizan el tracto gastrointestinal de los animales (Monzón-Atienza et al., 2023). La aplicación de componentes probióticos en los peces desencadena interacciones con las bacterias intestinales del huésped, lo que resulta en la formación de una amplia gama de metabolitos que podrían generar efectos positivos para el huésped (Ringø et al., 2022). Los probióticos mejoran diversos aspectos del huésped, como el crecimiento, la

asimilación de nutrientes, la inmunomodulación, la resistencia a enfermedades y las tasas de supervivencia, al tiempo que mitigan el estrés ambiental (Butt et al., 2021). Además, los probióticos tienen la capacidad de modificar la relación entre el huésped y el microbio, incluida toda la comunidad microbiana. También contribuyen a optimizar la utilización de los alimentos al aumentar su valor nutricional y fortalecer la respuesta inmunitaria del huésped contra varios patógenos (Hemarajata & Versalovic, 2013).

1.7.1 Fuentes de Probióticos

Los microorganismos están inherentemente presentes en humanos, animales, suelos, sedimentos, nieve, así como en ambientes de agua dulce, salobre y salada (El-Saadony et al., 2021). Normalmente, en el contexto de la acuicultura, estos microorganismos se encuentran en el tracto gastrointestinal de los peces. A través de métodos de selección (ver sección 1.7.2) se aíslan y cultivan para su uso como probióticos (Kiron, 2015). El género *Bacillus* spp. se destaca como uno de los probióticos más empleados (Elshaghabee et al., 2017). Esto se debe probablemente a su capacidad de esporulación, que mejora la supervivencia en el tracto gastrointestinal al resistir la exposición a los ácidos (Zhang et al., 2020). Además, su naturaleza dual, tanto aeróbica como anaeróbica facultativa, explica su capacidad para prosperar en diversos entornos (Kuebutornye et al., 2019; Nayak, 2021). En los últimos años, los probióticos más comúnmente utilizados en la lubina europea son bacterias, particularmente *Bacillus* spp., *Pediococcus* spp., *Lactobacillus* spp., *Vibrio* spp., *Shewanella* spp. y *Vagococcus* spp. (Monzón-Atienza et al., 2023).

1.7.2 Criterios de Selección de Probióticos

Las características esenciales que los microorganismos deben cumplir para considerarse probióticos han sido descritas por numerosos autores. Los requisitos fundamentales para que un probiótico sea efectivo y obtenga tal calificación se detallan a continuación (Balcázar et al., 2007; El-Saadony et al., 2021; Hai, 2015; Kesarcodi-Watson et al., 2008; Kiron, 2015; Merrifield et al., 2010):

a. El microorganismo debe demostrar la capacidad para adherirse y crecer en el hospedador. Por lo tanto, debe ser capaz de tolerar la bilis, el jugo gástrico y el pH del organismo hospedador.
b. El candidato a probiótico debería carecer de genes de resistencia a antibióticos y no modificar los rasgos heredables del hospedador.

c. El microbio debe beneficiar al sistema inmunológico.
d. El probiótico debe poseer propiedades antimicrobianas contra patógenos potenciales.
e. El microorganismo probiótico no debe causar efectos nocivos al hospedador.

La evaluación de las cepas probióticas se lleva a cabo mediante pruebas *in vitro* y/o *in vivo*.

1.7.3 Aspectos Tecnológicos y Rutas de Administración de Probióticos:

Los aspectos tecnológicos de la producción de probióticos son cruciales, ya que las condiciones de fabricación y almacenamiento pueden afectar significativamente la viabilidad del microorganismo. Los métodos de administración de probióticos varían según la instalación, la edad y la especie de los peces (Cámara-Ruiz et al., 2020). Generalmente, los probióticos se suministran congelados o secos, ya sea en forma de polvos liofilizados o atomizados y encapsulados (Ross et al., 2005). Actualmente, los métodos de administración en acuicultura incluyen la inyección, la inmersión o la incorporación en el alimento (Amiin et al., 2023; Hai, 2015). Sin embargo, antes de elegir la ruta de administración, se deben considerar ciertos factores. La inyección induce estrés en los peces, además de ser complicada y tediosa para los peces en etapa larvaria (Jahangiri & Esteban, 2018). La ventaja de esta técnica es asegurar que los peces reciban la dosis deseada de probiótico. Por otro lado, la adición directa de probióticos al agua podría ser aplicable en todas las etapas del desarrollo de los peces (Jahangiri & Esteban, 2018). La administración a través del alimento es uno de los métodos más simples, pero los probióticos deben ser capaces de resistir procesos de pH, temperatura y presión (Kiron, 2015). Sin embargo, la administración a través del alimento se enfrenta a desafíos durante las etapas larvarias debido al tamaño de las bocas de los peces (Cámara-Ruiz et al., 2020). En cuanto a la investigación en lubina europea, las rutas de administración más comunes son a través del alimento seco, vectores e inmersión (Monzón-Atienza et al., 2023).

1.7.4 Modos de Acción de los Probióticos

Durante décadas, determinar los mecanismos de acción de los probióticos ha sido una prioridad científica. Sin embargo, señalar con exactitud los mecanismos que los probióticos emplean para conferir un beneficio específico al huésped es, como mínimo, complejo. La sinergia entre múltiples modos de acción e incluso la interacción con diferentes microbios puede resultar en beneficios para el hospedador (Merrifield et al., 2010). En otras palabras, el beneficio puede no surgir necesariamente de una acción directa del probiótico. Además, numerosos autores discrepan sobre la correlación entre los resultados de los ensayos *in vitro* y los *in vivo*, como ha señalado Tinh et al., (2008) previamente. Debido a la multitud de mecanismos que un probiótico puede utilizar para ejercer su acción, hasta la fecha, no existe un acuerdo completo sobre los resultados obtenidos *in vivo*. Por lo tanto, se recomienda un aumento en la investigación por parte de la comunidad científica para fortalecer la comprensión de cómo funcionan los probióticos (Bermudez-Brito et al., 2012; Tinh et al., 2008). Los modos de acción más extendidos en los peces incluyen (El-Saadony et al., 2021):

i. *Exclusión competitiva mediante la producción de compuestos inhibitorios*.

   La exclusión competitiva mediante la producción de compuestos inhibitorios es un fenómeno en el que un organismo (probiótico) compite por los nutrientes disponibles y sitios de adhesión con otro organismo (patógeno) (Aburjaile et al., 2022). Esto previene o limita el crecimiento y/o la supervivencia del organismo patógeno (Knipe et al., 2021). Entre la amplia variedad de métodos de exclusión existentes, la producción de sustancias o compuestos como ácidos orgánicos, péptidos inhibidores, proteínas inhibidoras, bacteriocinas, etc., es uno de ellos (Prabhurajeshwar & Chandrakanth, 2017). Además, la inhibición del *quorum sensing*, también conocido como *quorum quenching*, está reconocido como un método de exclusión competitiva. El *quorum quenching* implica la inhibición de dicha comunicación bacteriana a través de medios químicos o enzimáticos (Sikdar & Elias, 2020).

ii. *Competencia por nutrientes, productos químicos o energía*.

   Las bacterias, incluyendo tanto cepas probióticas como patógenas, dependen de nutrientes, productos químicos y energía para su crecimiento y proliferación. La competencia por recursos se intensifica cuando estas especies utilizan fuentes de nutrientes similares, lo que lleva a un entorno competitivo hostil (Hoseinifar et al., 2018; Wuertz et al., 2021). La utilización de recursos disponibles por parte de

las bacterias probióticas sirve para limitar su accesibilidad a los patógenos del entorno (Kuebutornye et al., 2020; Balcázar et al., 2008). Al competir con los patógenos por recursos, los probióticos pueden reducir efectivamente el crecimiento y la proliferación de bacterias patógenas, ayudando así a mantener una comunidad microbiana más saludable.

iii. *Competencia por sitios de adhesión*.

Las bacterias a menudo participan en interacciones competitivas caracterizadas por la exclusión o supresión del crecimiento de otras especies. Las bacterias probióticas emplean varios mecanismos y propiedades para obstaculizar la adhesión de patógenos, lo que resulta principalmente en la exclusión de sitios de adhesión (Balcázar et al., 2008). Esta exclusión competitiva por parte de los probióticos impide efectivamente la acción de bacterias patógenas al obstaculizar las vías de infección (Raheem et al., 2021).

iv. *Contribución enzimática*.

La contribución enzimática de los probióticos es esencial para mantener la salud y el equilibrio en los peces. Las cepas probióticas son capaces de producir una amplia variedad de enzimas que ayudan en la digestión de nutrientes, la degradación de compuestos no digeribles y la integridad intestinal (Assan et al., 2022; Maske et al., 2021; Shekarabi et al., 2022). Por lo tanto, la actividad enzimática de los probióticos proporciona una gran variedad de beneficios al huésped, contribuyendo a su bienestar general y la prevención de diversas enfermedades (Assan et al., 2022).

v. *Mejora de la respuesta inmunitaria*.

En general, la respuesta inmunitaria innata en los peces sirve como mecanismo de defensa primario contra patógenos. Este sistema de defensa comprende barreras físicas, células especializadas y moléculas efectoras que detectan y neutralizan rápidamente amenazas infecciosas. A un nivel más profundo, el sistema inmunitario abarca sistemas de reconocimiento de patrones (PRRs, de sus siglas en inglés) responsables de identificar patrones moleculares asociados a patógenos (PAMPs, de sus siglas en inglés) que violan las barreras físicas del huésped. La interacción entre PRRs y PAMPs desencadena la activación de la respuesta inmunitaria innata (Bermudez-Brito et al., 2012). Entre los PRRs más estudiados se encuentran los receptores tipo Toll (TLRs, de sus siglas en inglés), que vienen en varios tipos compartiendo similitudes estructurales y funcionales. Los TLR

tipo 2 (TLR-2) se especializan en reconocer PAMPs presentes en paredes celulares bacterianas, especialmente las de bacterias Gram positivas (Oliveira-Nascimento et al., 2012). Los probióticos contienen PAMPs y, por lo tanto, pueden ser detectados por los PRRs del huésped. Tras la detección y unión, se inician cascadas de señalización intracelular que conducen a la expresión de moléculas efectoras como citoquinas (Hasan & Banerjee, 2020), síntesis de óxido nítrico (NO) (Korhonen et al., 2001), producción de especies reactivas de oxígeno (ROS) y nitrógeno (González-Magallanes et al., 2023), que son mecanismos cruciales en la lucha contra la intrusión microbiana.

1.8 OBJETIVOS

1.8.1 Objetivo General

El objetivo principal de este estudio es demostrar las propiedades del *Bacillus velezensis* D-18, su mecanismo de acción como probiótico y su aplicación en la acuicultura.

1.8.2 Objetivos Específicos

- Realizar un análisis *in vitro* para evaluar las propiedades de la cepa bacteriana *B. velezensis* D-18 y determinar su idoneidad como candidato probiótico.

- Efectuar un ensayo *in vivo* con el fin de establecer la inocuidad del *B. velezensis* D-18 y estimar la resistencia que puede otorgar a la lubina europea frente a la infección por *Vibrio anguillarum* 507.

- Llevar a cabo un estudio *in vivo* para investigar la capacidad del *B. velezensis* D-18 para modular la respuesta inmunológica innata de la lubina europea.

- Realizar la secuenciación génica de la cepa *B. velezensis* D-18

- Estudiar el mecanismo de *quorum quenching* del probiótico *B. velezensis* D-18 para la inhibición del *Vibrio anguillarum* 507.

2. MATERIALES & MÉTODOS

Capítulo III. "*Isolation and Characterization of a Bacillus velezensis D-18 Strain, as a Potential Probiotic in European Seabass Aquaculture*".

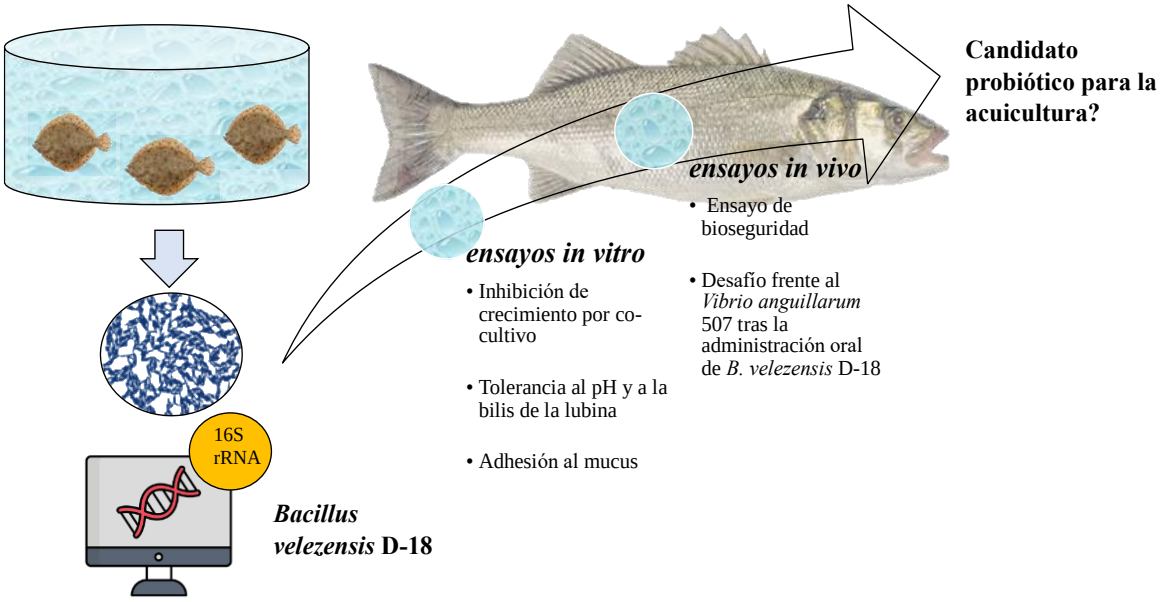


Figura 2.1. Esquema gráfico del Capítulo III. El objetivo de este capítulo fue analizar la cepa de *Bacillus velezensis* D-18 aislada de muestras de aguas residuales recolectadas de una piscifactoría, para su uso como probiótico en acuicultura. La cepa fue evaluada *in vitro* a través de varios mecanismos de selección, como la inhibición del crecimiento por co-cultivo, la tolerancia a la bilis y al pH de la lubina Europea, y la capacidad de adhesión al moco intestinal de la misma. Luego se realizó una evaluación *in vivo* mediante un ensayo de bioseguridad y un desafío frente al patógeno *Vibrio anguillarum* 507 después de la administración oral de la cepa probiótica en la lubina.

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

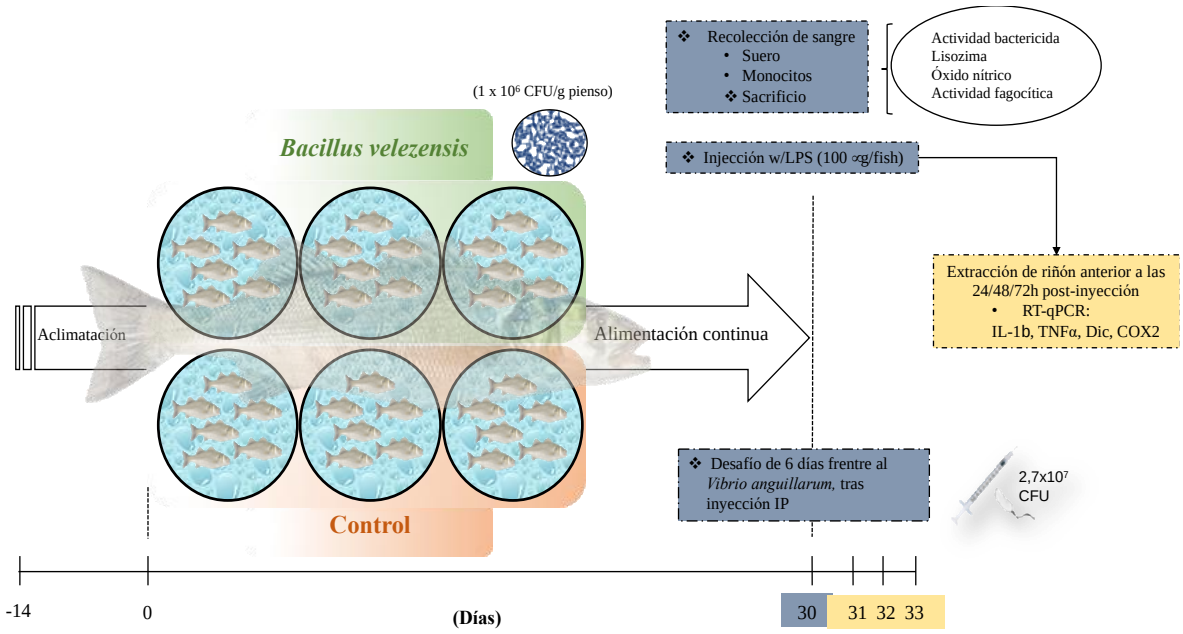


Figura 2.2. Esquema gráfico del Capítulo IV. Tras período de aclimatación (día 0), se administró de forma diaria y de manera oral, a la lubina Europea, una dieta control y otra suplementada con la cepa *Bacillus velezensis* D-18. Después de 30 días, se obtuvo sangre de los animales de cada grupo, con el fin de obtener suero y monocitos de sangre periférica (PBMs, de sus siglas en inglés). A partir del suero, se comprobó la actividad bactericida y de lisozima del suero, así como la determinación de óxido nítrico. Los PBMs se incubaron con *Candida albicans* (10^9 UFC/mL), con el objetivo de realizar un ensayo fagocítico clásico. Además, los peces de cada tratamiento fueron estimulados intraperitonealmente con lipopolisacáridos (LPS) de *V. anguillarum* (100 µg/pez). A las 24, 48 y 72 h de la estimulación, se obtuvo el riñón craneal de seis animales por condición, y se analizó la expresión génica mediante qPCR. Finalmente, los animales restantes de cada tratamiento fueron sometidos a un desafío bacteriano frente a la cepa *V. anguillarum* 507 (2.7×10^7 UFC/mL).

Capítulo VI. "*An In-Depth Study on the Inhibition of Quorum Sensing by Bacillus velezensis D-18: Its Significant Impact On Vibrio Biofilm Formation in Aquaculture"*

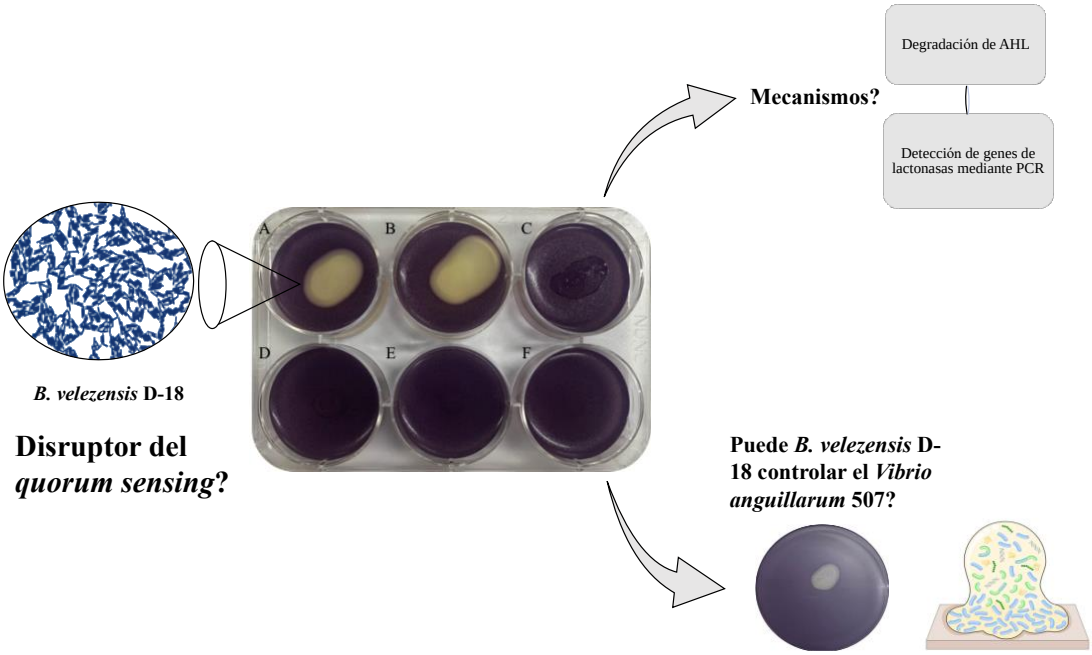


Figura 2.3. Esquema gráfico del Capítulo VI. Con el fin de evaluar el potencial *quorum quenching* (QQ), es decir la disrupción del *quorum sensing* (QS), de la cepa probiótica *Bacillus velezensis* D-18, se empleó una técnica de co-cultivo, que implicaba el crecimiento simultáneo de la cepa biomarcadora *Chromobacterium violaceum* MK, detectora de QQ, con el *B. velezensis* D-18. Una vez demostrado la capacidad QQ de la cepa, con el fin de explorar el mecanismo, se valoró la capacidad de degradación de las moléculas de señalización acil homoserina lactonas (AHLs, de sus siglas en inglés) de cadena corta y larga. Posteriormente, se realizó una PCR para identificar genes productores de lactonasas en *B. velezensis* D-18. La evaluación del impacto del *B. velezensis* D-18 sobre bacterias patógenas se realizó utilizando *Vibrio anguillarum* 507, centrándose en su capacidad para controlar la formación de biopelículas y restringir el crecimiento del patógeno.

Capítulo VII. "*Current Status of Probiotics in European Sea Bass Aquaculture as One Important Mediterranean and Atlantic Commercial Species: A Review*"

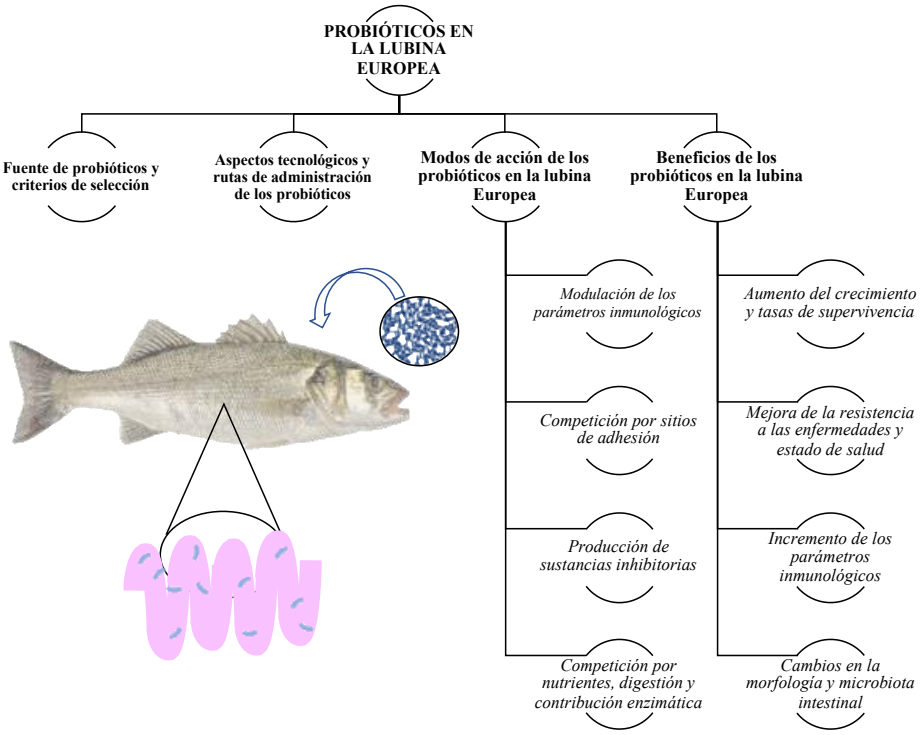
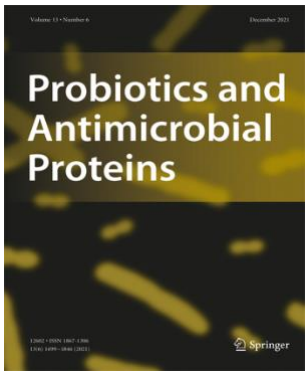


Figura 2.4. Esquema gráfico del Capítulo VII. A través de un examen exhaustivo de las investigaciones recientes, esta revisión aclara el profundo impacto de los probióticos en la acuicultura de la lubina europea. Después de realizar un análisis general de los probióticos, se sintetizan las perspectivas sobre sus mecanismos y beneficios en la lubina europea, destacando su influencia en el rendimiento del crecimiento, la diversidad microbiana, la producción de enzimas, el aumento de la inmunidad, la resistencia a enfermedades y la supervivencia general, con el objetivo de proporcionar una comprensión integral para futuras investigaciones.

3. CAPÍTULOS

CAPÍTULO III. "*Isolation and Characterization of a Bacillus velezensis D-18 Strain, as a Potential Probiotic in European Seabass Aquaculture*"

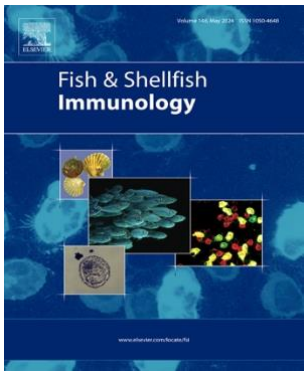


Probiotics and Antimicrobial Proteins. Volume 13, pages 1404–1412, (2021).

Dentro de los sectores de producción de alimentos, la acuicultura ha experimentado el mayor crecimiento en las últimas décadas, representando actualmente casi el 50% del pescado consumible del mundo. Sin embargo, las enfermedades pueden impactar significativamente la producción final en la acuicultura intensiva, siendo la vibriosis una de las enfermedades más perjudiciales en la acuicultura de la lubina. Tradicionalmente, se han utilizado antibióticos para abordar las patologías bacterianas, a pesar de sus conocidas consecuencias ambientales. Por lo tanto, las bacterias probióticas se están considerando como un enfoque alternativo para combatir las bacterias patógenas.

El objetivo de este estudio fue analizar una cepa de *Bacillus velezensis* D-18 aislada de una muestra de aguas residuales recogida de una granja piscícola para su posible uso como probiótico en acuicultura. La cepa fue evaluada *in vitro* a través de diversos mecanismos de selección. Los resultados mostraron una reducción del 30% en la inhibición del crecimiento por co-cultivo, una supervivencia del *B. velezensis* D-18 a una exposición de 1.5 horas al 10% de bilis de lubina, y una supervivencia del 5% a pH 4, junto con la reducción del 60% en la capacidad de adhesión de *Vibrio anguillarum* 507 al moco intestinal de lubina. La evaluación *in vivo* involucró un ensayo de inocuidad y un desafío frente al *V. anguillarum* 507.

En conclusión, basándonos en los resultados de los ensayos *in vitro* y de seguridad biológica, consideramos a la cepa de *B. velezensis* D-18, recogida de muestras de aguas residuales de una granja piscícola, como un prometedor candidato probiótico para prevenir la infección por *V. anguillarum* 507 en la lubina europea.



CAPÍTULO IV. "*Dietary supplementation of Bacillus velezensis improves Vibrio anguillarum clearance in European sea bass by activating essential innate immune mechanisms*"
Fish and Shellfish Immunology Volume 124, Pages 244-253, May 2022.

Basándonos en la literatura, la suplementación con *Bacillus* spp. como probiótico en dietas en acuicultura es segura y efectiva. En concreto, el *Bacillus velezensis* D-18 muestra un gran potencial en la mejora de la resistencia a enfermedades de la lubina europea contra la patogenicidad causada por el género *Vibrio*. Sin embargo, los mecanismos inmunomoduladores detrás de esta respuesta aún no se comprenden completamente. Con el fin de examinar las variaciones inmunológicas producidas por el probiótico, se alimentaron diariamente dos grupos de lubinas durante 30 días, un grupo con una dieta control y el otro con una dieta suplementada con *B. velezensis*. Tras 30 días, se analizó la capacidad bactericida frente a células vivas de la cepa *Vibrio anguillarum* 507 y la actividad de óxido nítrico y de lisozima del suero. A nivel celular, se estudió la respuesta fagocítica de los leucocitos de sangre periférica contra la cepa *Candida albicans* inactivada. Además, los leucocitos totales del riñón craneal (HK, de sus siglas en inglés) fueron aislados de peces previamente tratados *in vivo* con lipopolisácaridos (LPS) del *V. anguillarum* 507. Posteriormente, se evaluó la expresión de algunos genes proinflamatorios esenciales [interleucina 1 beta (IL-1β), factor de necrosis tumoral alfa (TNFα) y ciclooxigenasa 2 (COX-2)] y la expresión de un péptido antimicrobiano (AMP) específico de la lubina, dicentracina (DIC). La suplementación con el *B. velezensis* aumentó significativamente todas las actividades líticas humorales y celulares testadas en la lubina. Además, se observaron diferencias dependientes del tiempo entre los grupos de control y el tratamiento para todas las expresiones de los genes proinflamatorios analizados. Finalmente, se realizó un desafío bacteriano *in vivo* frente a la cepa viva de *V. anguillarum*. Los peces alimentados con *B. velezensis* mostraron una supervivencia significativamente mayor. En general, nuestros resultados proporcionan una clara evidencia de los efectos inmunológicos beneficiosos de *B. velezensis* y revelan algunos mecanismos inmunológicos fundamentales detrás de su aplicación como probiótico en la acuicultura de la lubina europea.

CAPÍTULO V. "*Whole-Genome Sequence of Bacillus velezensis D-18, a Probiotic Bacteria for Aquaculture*"

Pre-print: DOI: https://doi.org/10.21203/rs.3.rs-3812427/v1

Bacillus velezensis D-18, una cepa aislada de una muestra de aguas residuales tomada en una piscifactoría, mostró ser prometedora como probiótico en acuicultura en un experimento previo. Sin embargo, existe una falta de información sobre su contenido genómico. En este trabajo, presentamos el ensamblaje completo del genoma del *B. velezensis* D-18 obtenido mediante la secuenciación de extremos pareados de Illumina, lo que resultó en un ensamblaje de 21 contigs que totalizan 3.9 Mb. Este ensamblaje arrojó predicciones de aproximadamente 4,179 genes codificadores de proteínas y 84 ARN codificados.

CAPÍTULO VI. "*An In-Depth Study on the Inhibition of Quorum Sensing by Bacillus velezensis D-18: Its Significant Impact On Vibrio Biofilm Formation in Aquaculture*"

Microorganisms 2024, 12(5), 890.

En medio de crecientes preocupaciones sobre la resistencia a los antibióticos, se vuelven imperativas estrategias innovadoras para abordar las infecciones bacterianas en la acuicultura. El *quorum quenching* (QQ), es decir, la inhibición enzimática del *quorum sensing* (QS), ha surgido como una solución prometedora. Este estudio profundiza en las capacidades QQ de la cepa probiótica *Bacillus velezensis* D-18 y sus productos, particularmente en la comunicación y formación de biopelículas del *Vibrio anguillarum* 507. Utilizando el biomarcador *Chromobacterium violaceum* MK, el estudio confirmó que la cepa de *B. velezensis* D-18 inhibe efectivamente el QS. Una exploración adicional del mecanismo de QQ reveló la presencia de actividad lactonasa por parte del *B. velezensis* D-18, degradando tanto las acil homoserina lactonas (AHLs, de sus siglas en inglés) de cadena larga como corta. El análisis de PCR demostró la presencia de un gen homólogo productor de lactonasa, ytnP, en el genoma del *B. velezensis* D-18.

El estudio evaluó el impacto de la cepa *B. velezensis* D-18 en el crecimiento y la formación de biopelículas de *V. anguillarum* 507. El probiótico no solo controla la formación de biopelículas de *V. anguillarum*, sino que también restringe significativamente el crecimiento del patógeno.

Por lo tanto, *B. velezensis* D-18 demuestra un potencial sustancial para prevenir enfermedades causadas por *V. anguillarum* en la acuicultura a través de su capacidad de QQ. La capacidad para interrumpir la comunicación bacteriana y controlar la formación de biopelículas posiciona a la cepa *B. velezensis* D-18 como una alternativa prometedora y respetuosa con el medio ambiente a los antibióticos convencionales en el manejo de enfermedades bacterianas en la acuicultura.

CAPÍTULO VII. "*Current Status of Probiotics in European Sea Bass Aquaculture as One Important Mediterranean and Atlantic Commercial Species: A Review*"

Animals 2023, 13(14), 2369

La producción de lubina europea ha aumentado en las últimas décadas. Este incremento está asociado con una mayor demanda de esta especie, lo que anima a las industrias acuícolas a aumentar su producción para satisfacer esa demanda. Sin embargo, esta intensificación tiene repercusiones en los animales, causando estrés que suele ir acompañado de disbiosis, bajos índices de conversión alimenticia e inmunodepresión, entre otros factores. Por lo tanto, la aparición de enfermedades patógenas es común en estas industrias. Buscando mejorar el bienestar animal y disminuir el uso de antibióticos, los cuales tienden a favorecer la aparición de bacterias multi-resistentes, los investigadores se han centrado en enfoques alternativos como la aplicación de probióticos. El uso de probióticos en la producción de lubina europea se presenta como una alternativa ecológica, segura y viable además de mejorar diferentes parámetros del huésped como el rendimiento del crecimiento, la utilización del alimento, la inmunidad, la resistencia a enfermedades y la supervivencia de los peces contra diferentes patógenos mediante su inclusión en las dietas de los peces, a través de vectores y/o en columnas de agua. Por lo tanto, el objetivo de esta revisión es presentar hallazgos de investigaciones recientes sobre la aplicación de probióticos en la acuicultura de lubina europea y su efecto en el rendimiento del crecimiento, la diversidad microbiana, la producción de enzimas, la inmunidad, la resistencia a enfermedades y la supervivencia con el fin de ayudar a investigaciones futuras.

4. DISCUSIÓN GENERAL

En la acuicultura contemporánea, los probióticos son cada vez más reconocidos como una herramienta fundamental para mejorar la salud de los peces, aumentando así la producción, la salud de los individuos y promoviendo la sostenibilidad de la industria. Los probióticos desempeñan un papel crucial en aliviar los impactos adversos asociados con el uso de antibióticos, lo cual está vinculado con la aparición de cepas bacterianas multi-resistentes. A pesar de los numerosos beneficios que presentan los probióticos, su integración en la acuicultura sostenible enfrenta varios desafíos significativos que actualmente están en la vanguardia de la investigación científica (Balcázar et al., 2006; Dawood & Koshio, 2016; Hai, 2015; Ringø et al., 2016).

Con el fin de disminuir el uso de antibióticos y garantizar la aplicación sostenible de probióticos en la acuicultura, esta tesis pretende el desarrollo de una nueva cepa, *Bacillus velezensis* D-18, que cumpla los requisitos requeridos para ser un probiótico (Balcázar et al., 2006; El-Saadony et al., 2021; Hai, 2015; Kesarcodi-Watson et al., 2008; Kiron, 2015; Merrifield et al., 2010), estudiando parte de sus mecanismos de acción y sus efectos biológicos en la lubina Europea, una de las especies comerciales más importantes en el contexto Atlántico y Mediterráneo.

¿Son los probioticos una medida efectiva contra la entrada de patógenos en el ambito de la acuicultura?

En el contexto de la acuicultura, los probióticos han emergido como una solución prometedora para combatir la entrada y proliferación de patógenos, representando una alternativa eco-amigable y sostenible frente al uso tradicional de antibióticos y productos químicos (Heinonen-Tanski & Hancz, 2022). De esta forma, la aplicación de probióticos en acuicultura no solo mejora la salud y el crecimiento de las especies cultivadas, sino que también juega un papel crucial en la prevención de enfermedades al competir con patógenos por sitios de adhesión y fuentes de energía, y al producir sustancias antibacterianas (Hoseinifar et al., 2018), además promueven la exclusión competitiva de patógenos al colonizar eficazmente el epitelio mucoso del tracto gastrointestinal de los organismos acuáticos, impidiendo así la colonización de patógenos (Sha et al., 2023).

Por otra parte, los probióticos pueden producir una variedad de compuestos antibacterianos (como ácidos orgánicos, peróxidos, enzimas y proteínas bactericidas), los cuales inhiben directamente a las bacterias patógenas y mejoran la respuesta inmunológica de los hospedadores (Thuy et al., 2024).

Recientemente se ha investigado la importancia de algunos probióticos en la calidad del agua. Estos microorganismos beneficiosos contribuyen a la descomposición de residuos orgánicos y al ciclo de nutrientes, lo que resulta en un ambiente más saludable y sostenible para el cultivo acuícola. La capacidad de los probióticos para transformar la materia orgánica en CO_2 y mantener el equilibrio de nitrógeno es especialmente valiosa en tanques y sistemas cerrados (Hasan & Banerjee, 2020).

En base a los resultados de esta tesis, que demuestran la capacidad de la cepa *Bacillus velezensis* D-18 para aumentar la resistencia a enfermedades en la lubina Europea, inhibir el crecimiento de patógenos, interferir con el *quorum sensing* y disminuir la formación de biopelículas, es razonable considerar a los probióticos como una medida efectiva frente a la entrada de patógenos en el ámbito de la acuicultura.

¿Cómo se correlacionan las propiedades específicas de *Bacillus velezensis* D-18 con su potencial como candidato probiótico en acuicultura, y cómo pueden estas atribuciones contribuir a mejorar la salud y sostenibilidad de los sistemas acuícolas?

Diferentes estudios han establecido las características esenciales para calificar a un organismo como probiótico, a través de pruebas *in vitro* o *in vivo* (Balcázar et al., 2006; El-Saadony et al., 2021; Hai, 2015; Kesarcodi-Watson et al., 2008; Kiron, 2015; Merrifield et al., 2010):

a. La cepa debe demostrar la capacidad de adherirse y multiplicarse en el hospedador, y ser capaz de tolerar las condiciones adversas de la bilis y el pH del mismo.

b. Debe carecer de genes asociados con la resistencia a los antibióticos y no debe inducir mutaciones en el organismo hospedador.

c. Debe conferir beneficios al hospedador, ya sea mejorando el crecimiento y/o desarrollando el sistema inmunológico para combatir patógenos, o exhibiendo propiedades antimicrobianas.

d. Debe ser inofensivo para el organismo hospedador.

Durante el desarrollo de esta tesis doctoral, se ha establecido que *Bacillus velezensis* D-18 cumple con todos los criterios necesarios para ser considerado un probiótico fiable en el ámbito de la acuicultura:

En el Capítulo III, a través de pruebas *in vitro*, se demostró que *B. velezensis* D-18 se adhiere al intestino de la lubina Europea y es capaz de soportar las condiciones de bilis y pH presentes en este ambiente. Posteriormente, mediante la inoculación intraperitoneal de la cepa en el hospedador, se verificó que el *B. velezensis* D-18 es completamente inofensivo, no mostrando signos clínicos ni induciendo cambios anatómico-morfológicos en la lubina Europea (Monzón-Atienza et al., 2021).

Los beneficios que cada probiótico puede ofrecer varían según la cepa utilizada. Por lo tanto, los criterios principales para calificar a un microorganismo como probiótico de calidad incluyen la capacidad de proporcionar beneficios, ser inocuo para el huésped y establecerse en él (Binda et al., 2020). Otros estudios han abordado diversos parámetros para caracterizar distintas cepas probióticas, como la ausencia de toxinas y la capacidad hemolítica (Golnari et al., 2024; Shahbaz et al., 2024), aspectos que garantizan la inocuidad y bioseguridad de la cepa, parámetro que en el Capítulo III de esta tesis se ha valorado *in vivo* tras su administración directa en la lubina Europea, obteniendo resultados similares.

Cabe destacar que la caracterización realizada en 2021 (Monzón-Atienza et al., 2021) sigue estando en vanguardia, ya que muchos de los parámetros utilizados en el Capítulo III continúan siendo aplicados en investigaciones recientes (Elsadek et al., 2023; Golnari et al., 2024; Shahbaz et al., 2024).

En el Capítulo IV, mediante ensayos *in vivo*, se confirmó la capacidad de la cepa para modular positivamente la respuesta inmunológica la lubina Europea, lo que resultó en un aumento en la actividad del sistema inmunológico innato y una mejora en su supervivencia contra el patógeno *Vibrio anguillarum* 507 (Monzón-Atienza et al., 2022). Estos resultados son compatibles con estudios más recientes que demuestran que la aplicación del *B. velezensis*, en este caso la cepa T20, incrementa la resistencia a enfermedades en el rodaballo (Yu et al., 2024). Por otro lado, no todas las cepas tienen

los mismos mecanismos ni efectos, por ejemplo, el *B. velezensis* T23 disminuye parámetros relacionado con el sistema inmunológica como la expresión de TNF- α (Yang et al., 2024), mientras que en nuestro trabajo dicha citoquina se ve incrementada en la lubina europea tras la administración de la cepa D-18. Esto destaca la importancia de la caracterización de la cepa bacteriana, debido a que muchas bacterias dentro del mismo género y especie pueden presentar diferencias significativas en sus características fisiológicas, patogénicas y genéticas (Joseph et al., 2012).

En el Capítulo V, a través de la secuenciación génica de la cepa, se verifica la ausencia de genes asociados con plásmidos de resistencia antimicrobiana o potenciales causas de mutaciones en el hospedador. Si bien los probióticos generalmente se consideran seguros y beneficiosos para la salud, existe la preocupación de que puedan servir como reservorio o conducto para la resistencia a los antibióticos. Asegurar que las cepas probióticas estén libres de genes de resistencia a los antibióticos transferibles es vital para un uso seguro y eficaz (Doron & Snydman, 2015).

La capacidad inhibitoria de un microorganismos se considera uno de los requisitos para ser considerado candidato probiótico (Binda et al., 2020). En el penúltimo capítulo de esta tesis (Capítulo VI), se examinó la presencia de actividades antimicrobianas del *B. velezensis* D-18, en particular, su capacidad para degradar enzimáticamente moléculas de señalización bacteriana -*quorum quenching*- asociadas con patógenos Gram negativos y, en consecuencia, valorar la inhibición en la formación de biopelículas de *Vibrio anguillarum* 507 (Monzón-Atienza et al., 2024). Los resultados obtenidos demuestran la capacidad inhibitoria de la cepa D-18, degradando mediante la utilización de lactonasas las acil homoserina lactonas de cadena larga del *V. anguillarum* 507, molécula de señalización del patógeno. Esta inhibición se apreció en el co-cultivo entre el probiótico y el patógeno, observando una inhibición en el crecimiento y en la formación de biopelículas por parte del *Vibrio*. La actividad *quorum quenching* de los probióticos ya ha sido testada en otros experimentos (Lubis et al., 2024), además de la capacidad de las especies del género *Bacillus* en interferir la formación de biopelículas mediante *quorum quenching* (El Aichar et al., 2022; Vinoj et al., 2014; Xu et al., 2024)

La cepa *B. velezensis* D-18 ofrece beneficios que contribuyen a la sostenibilidad de la acuicultura. El uso frecuente de agentes antimicrobianos implica altos costos de

producción, la presencia de residuos de antibióticos en los músculos de los peces -que pueden tener efectos adversos en la salud del consumidor- y daños a los ecosistemas acuáticos (Okocha et al., 2018; Watts et al., 2017). Por lo tanto, el uso de este probiótico puede ayudar a mitigar el riesgo de desarrollo de resistencia antimicrobiana y puede minimizar la liberación de residuos antimicrobianos en el medio acuático. Además, *Bacillus velezensis* D-18 mejora el bienestar de la lubina Europea a través de sus propiedades específicas, lo que puede contribuir a una producción acuícola más eficiente y sostenible, aliviando así la presión sobre los recursos naturales y los ecosistemas acuáticos.

¿Cómo se relacionan los hallazgos de esta tesis con el conocimiento existente sobre el uso de probióticos en la acuicultura, especialmente en el contexto de la lubina Europea?

Los hallazgos de esta tesis doctoral representan una contribución significativa en el campo de la acuicultura al reforzar y expandir el conocimiento existente sobre el uso de probióticos, específicamente en la producción de la lubina Europea.

En particular, esta tesis aporta una nueva cepa probiótica en el campo de la acuicultura proporcionando la evidencia concreta de la eficacia probiótica del *Bacillus velezensis* D-18 en la lubina Europea, destacando: su capacidad para combatir cepas patógenas, sobrevivir en condiciones gastrointestinales adversas, promover la adhesión a la mucosa y aumentar la resistencia a enfermedades específicas como las causadas por especies de *Vibrio*. Además, esta se profundiza en los mecanismos a través de los cuales la cepa *B. velezensis* D-18 ejerce sus efectos beneficiosos, explorando su impacto en la inmunidad innata del pez y su capacidad para interferir con la comunicación bacteriana -*quorum sensing*- de bacterias patógenas, evitando la formación de biopelículas.

La literatura actual respalda los beneficios de los probióticos en esta especie, mostrando mejoras en el crecimiento, las tasas de supervivencia, la salud, la resistencia a enfermedades, la morfología intestinal y la diversidad de la microbiota (Chouayekh et al., 2023; Perdichizzi et al., 2023; Rangel et al., 2024; Serradell et al., 2023). Cepas probióticas bacterianas como *Vagococcus fluvialis*, *Bacillus subtilis*, *Vibrio lentus* y *Phaeobacter* sp. también han demostrado la capacidad de mejorar la supervivencia de la

lubina Europea frente a la amenaza de bacterias del género *Vibrio* (Schaeck et al., 2016; Sorroza et al., 2012; Touraki et al., 2012). Al igual que nuestra cepa de *B. velezensis* D-18, otras cepas probióticas bacterianas sostienen la modulación del sistema inmunológico en la lubina Europea (Lamari et al., 2016; Mladineo et al., 2016; Picchietti et al., 2009; Schaeck et al., 2017; Sorroza et al., 2012). El Capítulo VII de la tesis se revela como una pieza fundamental que recoge los hallazgos y contribuciones significativas de la aplicación probiótica en la lubina Europea. Esta sección no solo resume la eficacia de los probióticos en el contexto acuícola, sino que también establece una base sólida para futuras investigaciones en esta área.

¿Cómo podría influir la capacidad de *quorum quenching* de la cepa *Bacillus velezensis* D-18 en sistemas de acuicultura?

La amplia diversidad bacteriana presente en el agua de mar abarca una variedad de microorganismos que pueden ser inofensivos, patógenos u oportunistas (Zinger et al., 2011). En entornos de acuicultura donde los peces están directamente expuestos al agua de mar, ya sea en tanques en tierra o jaulas oceánicas, la presencia de microorganismos es inevitable. Estos microorganismos pueden colonizar el tracto de los peces, adherirse a las superficies de los tanques o jaulas o mantener una existencia de vida libre en el medio acuático (Sehnal et al., 2021). Es crucial reconocer el riesgo asociado con la entrada de patógenos a los sistemas, ya que las bacterias patógenas tienen la capacidad de formar biopelículas y adherirse a superficies vivas y/o inertes. Estas biopelículas no solo actúan como nichos biológicos para los patógenos, protegiéndolos de productos químicos y condiciones ambientales, sino que también facilitan su dispersión (Muhammad et al., 2020).

El concepto de *quorum quenching*, que implica la inhibición enzimática de *quorum sensing* (Sikdar & Elias, 2020) se postula como una herramienta eficaz para lidiar con los problemas ocasionados por el *quorum sensing* patógeno, desde infecciones hasta la formación de las biopelículas mencionadas anteriormente (Zhou et al., 2020). Otros estudios han demostrado la capacidad *quorum quenching* de los probióticos, en particular de diferentes especies de *Bacillus* (Santos et al., 2021), y en concreto, su capacidad de inhibir la formación de biopelículas (Zhou et al., 2019). Durante la elaboración de esta tesis, esta estrategia disruptiva ha sido demostrada por la cepa de *Bacillus velezensis* D-18. Este probiótico, gracias a su capacidad para producir lactonasas, degrada las moléculas

de señalización de *quorum sensing* de acil homoserina lactonas utilizadas por numerosas bacterias patógenas Gram negativas para coordinar fenómenos cruciales como la virulencia y la formación de biopelículas (Taghadosi et al., 2015; Xiao et al., 2022). Basándonos en esto, la incorporación de este probiótico en biofiltros o a las estructuras de los tanques podría representar un beneficio significativo para la salud de los peces, al tiempo que contribuye al control efectivo de las comunidades microbianas (Ghanei-Motlagh et al., 2019; Heinonen-Tanski & Hancz, 2022; Jahangiri & Esteban, 2018).

5. CONCLUSIONES

1. La cepa probiótica *Bacillus velezensis* D-18 es capaz de soportar las extremas condiciones del tracto gastrointestinal de la lubina europea (pH y ácidos biliares) además de ser capaz de adherirse con solvencia a la mucosa gastrointestinal y prevenir la adhesión de patógenos.

2. *Bacillus velezensis* D-18 no presenta efectos perjudiciales en la lubina europea, evidenciado por la ausencia de cualquier manifestación clínica o cambio anatomopatológico en dicha especie.

3. La aplicación de la cepa probiótica en la lubina europea conlleva un aumento en la supervivencia frente al *Vibrio anguillarum* 507.

4. La respuesta inmunitaria innata de la lubina europea se ve reforzada tras la administración probiótica de la cepa *Bacillus velezensis* D-18, mediante la mejora de la actividad fagocítica, un aumento de la lisozima sérica, la producción de óxido nítrico y la actividad bactericida. Además, también se apreció una mejora en la expresión de citocinas y del péptido antimicrobiano dicentracina.

5. Se confirma que la cepa probiótica carece de plásmidos transmisores de resistencia antimicrobiana, reforzando su seguridad e idoneidad para su uso en la acuicultura.

6. *Bacillus velezensis* D-18 presenta el mecanismo de acción el *quorum quenching* que demuestra su capacidad para inhibir el crecimiento y la formación de biofilm del patógeno *Vibrio anguillarum* 507.

7. La cepa probiótica *Bacillus velezensis* D-18 emerge como un probiótico prometedor y sostenible para la industria acuícola, ofreciendo una alternativa eficaz y segura al uso de antibióticos, otorgando grandes beneficios a la salud de lubina europea.

6. LAGUNAS EN LA INVESTIGACIÓN Y PERSPECTIVAS FUTURAS

Las posibles investigaciones futuras sobre el probiótico *Bacillus velezensis* D-18 abarcan diferentes líneas de estudio.

Una de las áreas clave para investigar sería la comprensión en detalle de los mecanismos específicos mediante los cuales el *B. velezensis* D-18 ejerce sus efectos probióticos en la acuicultura, incluida su interacción con la microbiota intestinal del pez y los mecanismos moleculares involucrados en la modulación de la respuesta inmunológica. Actualmente, existe una incertidumbre significativa sobre estos mecanismos, y es fundamental establecer una correlación más precisa entre los resultados obtenidos *in vitro* e *in vivo* (Tinh et al., 2008). La utilización de peces gnotobióticos (*germ-free*) pueden ayudar a este propósito, como demostró previamente Galindo-Villegas et al., (2012).

Explorar las interacciones dinámicas entre *B. velezensis* D-18 y la microbiota intestinal del pez contribuirá al avance de la investigación probiótica. Realizar un seguimiento a largo plazo para evaluar la persistencia y estabilidad de *B. velezensis* D-18 en el intestino de los peces, así como su comportamiento y distribución, proporcionaría información crucial sobre sus mecanismos probióticos y su competencia con otras bacterias comensales o patógenas por nutrientes, sitios de adhesión y fuentes de energía. El estudio del microbioma tras la administración probiótica podría ser de gran utilidad.

Por otro lado, diversos probióticos han sido capaces de modular positivamente la producción de enzimas gastrointestinales, favoreciendo una mejor digestión y por consiguiente una mayor asimilación de nutrientes por parte del hospedador. Estos beneficios se suelen ver reflejados en la producción y calidad del producto. El impacto de la suplementación con *B. velezensis* D-18 en la producción y calidad de la carne de pescado, incluyendo parámetros como el crecimiento, la tasa de conversión alimenticia y la calidad sensorial, a día de hoy es todo una incógnita.

La interacción del *B. velezensis* D-18 con el entorno acuático circundante, incluyendo el impacto en la diversidad microbiana y la salud ambiental en sistemas de acuicultura, así como su capacidad para mantener su actividad probiótica en distintas condiciones, también merece atención. Previamente, se ha descrito la capacidad de los probióticos para mejorar la calidad del agua, condición bastante relevante en la acuicultura actual y que podría ser una línea de investigación para esta cepa.

Profundizando en la actividad antimicrobiana del *B. velezensis* D-18, aún existe una brecha de conocimiento en la interacción del probiótico con agentes patógenos. Pese a que esta tesis ha hecho énfasis en un mecanismo de inhibición bacteriana en auge, como es el *quorum quenching*, se podría indagar más su efecto en la regulación de la microbiota intestinal y la prevención de otras enfermedades en la lubina europea. Además, existen diversos mecanismos que aún no han sido descritos en esta cepa probiótica. Por ello, el estudio de la producción de bacteriocinas, ácidos orgánicos y enzimas líticas por parte del *B. velezensis* D-18, también serían una línea de estudio interesante para el control de agentes patógenos en la acuicultura.

Para la realización de esta tesis se ha utilizado uno de los patógenos que actualmente azora las costas canarias, la cepa patógena *Vibrio anguillarum* 507. Sin embargo, la evaluación antimicrobiana del probiótico puede ser extrapolada a otros patógenos que también presentan un gran peligro para la acuicultura.

Por otro lado, sería novedoso explorar los efectos sinérgicos potenciales de *B. velezensis* D-18 en combinación con otros probióticos, inmunoestimulantes e incluso con fagos, y evaluar sus efectos a largo plazo en la salud de los peces, la eficiencia de producción y el control de diferentes enfermedades.

En cuanto a la aplicación práctica, sería interesante explorar la posibilidad de utilizar la cepa *B. velezensis* D-18 en sistemas de recirculación de agua como medida de control frente a *Vibrio* spp. y otros agentes patógenos, tanto en biofiltros como en las paredes de los tanques para el control de biopelículas de bacterias patógenas.

Por último, la investigación sobre la aplicación industrial de *B. velezensis* D-18 sería un paso importante, y se requerirían estudios de mercado y análisis de viabilidad comercial para evaluar su potencial como producto probiótico en la industria de la acuicultura.

Este conjunto de investigaciones abordaría aspectos fundamentales sobre el potencial probiótico de *B. velezensis* D-18 y contribuiría significativamente al avance del conocimiento en el campo de la acuicultura.

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This thesis establishes *Bacillus velezensis* D-18 as a promising probiotic candidate for aquaculture. It emphasizes the strain's safety, its ability to enhance the host's immune response, and its potential to control pathogenic diseases through mechanisms like *quorum quenching*. This work adds valuable insights into the use of probiotics in aquaculture, proposing *Bacillus velezensis* D-18 as a reliable option for improving the health and disease resistance of European sea bass.