

Characterization *in vitro* of new bacterial strains showing potentially probiotic crossed effect against vibriosis in relevant fish species for marine aquaculture

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ABSTRACT

Intensive fish farms are often affected by different organisms that produce infectious diseases. To control this situation, antibiotics have been used with negative repercussions for the environment and health. As an alternative to this, probiotics are used that show more effective and respectful results with the environment. The aim of this project is to obtain new potentially probiotic strains against one of the most relevant pathogens of marine aquaculture, vibrios. These bacterial strains were isolated from the gills and intestines of European seabass, meagre and common sole. Later, were evaluated *in vitro* against 6 pathogenic strains of the genus *Vibrio* to demonstrate the production of antagonistic effects, production of antibacterial substances, resistance to bile, resistance to pH gradients, adhesion and growth to mucus, competition for nutrients and hydrophobicity. A total of 156 bacterial strains were isolated, but only 7 strains of the genera *Alcaligenes*, *Pseudomonas*, *Shewanella* and *Proteus*, showed excellent *in vitro* results to be considered as candidates to be reevaluated by *in vivo* tests, to determine their harmlessness and protective effect after challenge, and elucidating in future studies their use as possible probiotic strains for aquaculture, highlighting the results obtained with the strain *Alcaligenes faecalis* subsp. *faecalis*-1.

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Introduction

Aquaculture has been considered as the fastest growing food production system in the world, however, it has been affected by the onset of infectious diseases, that suppose a limitation to the expansion of this sector because many of these pathogens are part of the normal flora of the water (Pillay 2004; Austin and Austin 2012; Sharifuzzaman and Austin 2017). The importance of aquaculture products will increase as a result of illegal and overfishing in the world and the increasing demand for seafood with the ever-increasing population (Kesarcodei-Watson et al. 2008). In southern Europe, the culture of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*, Linnaeus, 1758) and common sole (*Solea solea*) are of high importance.

Growth of aquaculture industry is hampered by unpredictable mortality, many of them caused by pathogenic microorganisms, with a less importance of non-infectious diseases (Ibrahem 2015). Bacterial diseases are very common in intensive aquaculture producing a huge economic loss (Sica et al. 2012). However, a major drawback in aquaculture is the sudden outbreak of diseases, especially those caused by different species of genus *Vibrio*, which are considered as major pathogenic bacteria in aquaculture (Bergh et al. 2001). The disease caused by pathogenic species of the Genus *Vibrio* affects different species of farmed marine fish of commercial

interest throughout the world and is widely responsible for mortality in cultured aquaculture systems (Chen et al. 2000).

Antibiotics are the first option to farmers to control infectious diseases, due to rapid action and availability. However, despite of being an effective strategy in the beginning, the negative effects on environmental and public health justifying the need of developing new strategies (Hagi and Hoshino 2009). In order for the aquaculture sector to expand, it is necessary to find alternatives to antibiotics, safe and effective (Abdelkhalek et al. 2017; Yan et al. 2017). Due to this reason, the European Union placed restrictions on antibiotic use in aquaculture and to solve this problem, research has been focused on alternative environmentally friendly methods to control disease (Sorroza et al. 2012).

The use of probiotics is gaining interest in the aquaculture industry as an environmentally friendly management alternative to the use of antibiotics and other antimicrobials for disease prevention and reduce the incidence of fish diseases (Etyemez and Balcázar 2016). Most probiotics used in aquaculture are lactic acid bacteria or bacterial strains that belong to the genus *Vibrio*, *Bacillus* and *Pseudomonas* (Balcázar et al. 2007). These have been tested in food or added to water, and the most studied aspect has been on the improvement in animal health (Gatesoupe 1999). Today, there are many commercial probiotics available for mono or multi-strain

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bacteria (Van Doan et al. 2017). When selecting a new microorganism for testing as an effective probiotic, a number of properties need to be considered. To colonize the gastrointestinal tract, potential probiotics should express high tolerance to acid and bile, have the ability to adhere to intestinal surfaces and, immune modulation (Hagi and Hoshino 2009; Sica et al. 2012). Among them, potential probiotics should provide protection through the creation of a hostile environment for pathogens by the production of inhibitory compounds and by competing for adhesion sites (Etyemez and Balcázar 2016). To date, a wide range of bacteria proposed for their application as probiotics (Kesarcodi-Watson et al. 2008). However, this area is not so explored and a few probiotics are commercially available to market (Banerjee and Ray 2017). The use of probiotics is regarded as a very promising strategy and their wide acceptance for use in aquaculture is evidently shown in the number of research studies published over the last 10 years (Lazado and Caipang 2014). The aim of the present work has been to carry out the *in vitro* evaluation of bacterial strains isolated from the gut and gills of seabass, meagre and common sole, looking for those showing wider activity against different pathogen species of the genus *Vibrio* in marine aquaculture.

Materials and methods

Ethics statement

The experimental procedure with fish collected in this study fulfils the requirements contained in the Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, on the protection of animals used for scientific purposes.

Sampling and isolation of potential probiotic strains

A total of 12 European seabass (*Dicentrarchus labrax*), 19 meagre (*Argyrosomus regius*) and 12 common sole (*Solea solea*) were slaughtered by immersion in anaesthetic solution with clove oil and sacrificed in liquid ice and kept in refrigeration conditions. The intestinal content of each fish (one-gram amounts) was homogenized in phosphate buffered saline (PBS), and serial dilutions (1/10–1/1000) were spread on Brain Heart Infusion Agar (BHIA), Marine Agar (MA) and Blood Agar (BA) for 48 h at 25°C. In addition, gill samples were taken directly by means of a sowing handle and spread in the same culture media. All isolates strains were tested for antagonistic effects as potential probiotic strains.

Table 1. Pathogenic strains used in testing antagonistic effect.

Pathogen strains	Reference
<i>Vibrio anguillarum</i> 112P	CECT
<i>Vibrio anguillarum</i> 975-1	CECT
<i>Vibrio anguillarum</i> 507	CECT
<i>Vibrio anguillarum</i> 4347	CECT
<i>Vibrio harveyi</i> 525	CECT
<i>Vibrio alginolyticus</i> 521	CECT

Note: CECT: Spanish type culture collection.

Antagonistic effect of potential probiotic strains against *Vibrio*

The production of antagonistic effect was analysed using different species and strains of the genus *Vibrio* (Table 1) following the method described previously by Austin et al. (1992). *Vibrio* strains and potential probiotic strains were grown in Brain Heart Infusion Broth (BHIB) for 24 h at 25°C to obtain an exponential phase culture. Then, 100 µl of different pathogen strains (10⁷ CFU/ml) were spread on BHIA and BA, and each isolated strain of the fish was put into the plate. Inoculated plates were incubated at 25°C for a maximum of 48 h until a halo of inhibition is observed. Strains with positive halo production were identified by MALDI-TOF mass spectrometry system (Autoflex III, Bruker Daltonics GmbH) (Croxatto et al. 2012; Bizzini and Greub 2010).

Production of antibacterial substances of probiotics against pathogens

In order to determine the production of antibacterial substances in bacterial supernatants we followed the well diffusion method described by Nikoskelainen et al. (2001) with modifications (Kim and Austin 2008). Potential probiotic strains were cultured in BHIB 24 h at 22°C, centrifuged at 2000 g (Sigma, 4-16KS) for 10 min and the supernatants sterilized through 0.45 µm-pore-size filters and lyophilized using a freeze dryer (Telstar, Cryodos-50) for 18 h. The freeze-dried supernatant was re-suspended in 100 µl of PBS (10 times concentrated) to be challenged against pathogens. *Vibrio* strains were grown in BHIB overnight and transferred evenly onto TSA and BA plates. Then, 10 µl of lyophilized sample was added to each well, made with sterile Pasteur pipette, and the inhibition zone was observed after incubation for 24 h.

Fish bile and pH resistance

Fish bile was obtained from sea bass (average 400 g body weight) with a previous 24 h fast. Once sacrificed with oil of clove and immersion in liquid ice, we proceed to the extraction of the biliary secretion by direct puncture under sterile conditions with a fine needle. To analyse the resistance to fish bile, a 100 µl aliquot of fresh bile of seabass was added to 900 µl of each potential probiotic strain analysed at 10⁷ CFU/ml. In order to determine the resistance at acid pH, 100 µl of same concentrations of strains tested was added 100 ml of PBS with a pH range 3–7. In both test, samples were incubated for 90 min at 22°C and serially diluted in PBS and determined by plate counting on TSA (Nikoskelainen et al. 2001).

Surface characteristics: hydrophobicity

The hydrophobicity is used as an indicator of the ability of the bacteria to adhere to tissues, and was analysed by the microbial adhesion to hydrocarbons method (Ocaña et al. 1999). 600 µl of xylene (Panreac) is added to 2.4 ml of bacterial suspension with absorbance from 0.4 to 0.6 (OD₆₀₀) and vortexed vigorously for 90 s. They were kept still to allow the immiscible solvent and

aqueous phase to separate for 30 min. The aqueous layer was carefully removed, transferred to clean tubes and measured the absorbance by spectrophotometry (Selecta, V-1100). The percent of hydrophobicity (H%) was obtained from the following formula:

$$H = \left[\frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \right] \times 100$$

The cellular hydrophobicity is classified as high (71–100%), medium (36–70%) and low (0–35%).

Adhesion in intestinal and skin mucus

Intestinal and skin mucus was isolated from healthy sea bass of 400 g of average body weight. All mucus was centrifuged twice at 12,000 g for 5 min to remove particulate and cellular material, and adjusted to 0.5 mg/ml protein in PBS by Bradford Protein assay. The percentage of adhesion to intestinal and skin mucus was evaluated following the methodology described by Etyemez and Balcázar (2016). Briefly, 100 µl of each mucus was deposited in polystyrene plates overnight at 4°C. The wells were washed twice with sterile PBS and 100 µl of a bacterial suspension of each potentially probiotic strain is added at a concentration of 10⁹ CFU/ml in PBS and then incubated for 1 h at 22°C. The plates were washed to release the bacteria that remained attached to the polystyrene plate, 0.5% Triton X-100 was added to each well for 5 min. Adhesion was expressed as the percentage of bacteria recovered after adhesion in relation to the number of bacteria initially added to each well.

Growth in intestinal and skin mucus

The intestinal and skin mucus isolated from sea bass was diluted in sterile PBS to a final protein concentration of 0.5 mg/ml. Then, 3 ml of diluted mucus was added to 10 ml of an overnight culture of each potentially probiotic strain, and incubated at 22°C for 22 h in a shaking incubator. Samples with BHIB and PBS were used as negative controls. The growth rate of each strain in intestinal and skin mucus, BHIB and PBS was measured by monitoring the optical

density at 540 nm and serial dilutions on TSA (Olsson et al. 1992).

Inhibition of growth in co-culture

Potential probiotics and *Vibrio* strains were cultured overnight in trypticasein soybean medium (TSB), centrifuged at 2000 g for 10 min, performing two washes with sterile PBS. Bacterial suspension was adjusted by spectrophotometry at 0.5 absorbance at 600 nm. Then, 100 µl of each bacterial suspension (probiotic and pathogen), were mixed in 1ml of TSB medium and incubated at 22°C for 48 h, and serially diluted in PBS and determined by plate counting on TSA (Nikoskelainen et al. 2001).

Statistical analysis

All experiments were carried out in triplicated using the univariate general model. Data were analysed by two-way analysis of variance (ANOVA) using the statistical package SPSS for Windows version 22.0 (SPSS). Differences were considered statistically significant when *p* < 0.05.

Results

A total of 156 bacterial strains were recovered from the intestinal gut and gills of the different species of fish sampled, but only 7 strains showed inhibitory effect against at least, one of the pathogens tested of the genus *Vibrio*. Table 2 shows the identification of these potential probiotic strains by the MALDI-TOF mass spectrometry system, as well as their profiles of growth inhibition against the vibrio strains analysed. Of the 7 strains that have presented inhibition of growth against pathogens used, the strain *Alcaligenes faecalis* subsp. *faecalis*-1 is the only one that has an inhibitory effect against all the vibrio strains tested. Conversely, the strains *Proteus penneri*-1 and *Proteus penneri*-2 only have an inhibitory effect against a single strain, *Vibrio anguillarum* 4347.

Table 2. *In vitro* antagonistic effect and identification of potential probiotic strains isolated from in seabass, meagre and common sole against *Vibrio*.

Pathogen strains tested	Potential probiotic strains (No.)						
	1	2	3	4	5	6	7
<i>Vibrio anguillarum</i> 112P	+	+	+				
<i>Vibrio anguillarum</i> 975-1	+			+	+		
<i>Vibrio anguillarum</i> 507	+	+	+				
<i>Vibrio harveyi</i> 525	+	+	+				
<i>Vibrio alginolyticus</i> 521	+				+		
<i>Vibrio anguillarum</i> 4347	+			+	+	+	+

Notes: Source of pathogens strains tested is showed in Table 1.

1. – *Alcaligenes faecalis* subsp. *faecalis*-1
2. – *Alcaligenes faecalis* subsp. *faecalis*-2
3. – *Pseudomonas viridiflava*
4. – *Shewanella putrefaciens*
5. – *Alcaligenes faecalis* subsp. *faecalis*-3
6. – *Proteus penneri*-1
7. – *Proteus penneri*-2

+ potential probiotic strain isolated shows antagonistic effect against any pathogen strains tested.

Table 3. Percentage of survival to pH gradients tested for potential probiotic strains isolated from seabass, meagre and common sole.

pH	Probiotic strains tested (No.) % survival†† (mean ± sd)						
	1	2	3	4	5	6	7
pH 7	100%	100%	100%	100%	100%	100%	100%
pH 6	91.6 ± 3.3	71.8* ± 2.2	85.7 ± 3.5	81.4 ± 2.5	81.3 ± 8.0	89.5 ± 3.3	74.1* ± 4.4
pH 5	74.3* ± 4.5	41.5* ± 3.5	65.5* ± 1.2	65.4* ± 3.4	62.5* ± 3.2	72.7* ± 2.0	69.6* ± 2.9
pH 4	50.4* ± 10.6	25.2* ± 2.7	55.6* ± 0.8	35.5* ± 2.5	43.6* ± 2.6	66.5* ± 3.5	43.0* ± 2.0
pH 3	6.3* ± 3.8	4.9* ± 1.84	11.5* ± 0.9	11.1* ± 1.3	2.9* ± 0.2	63.5* ± 3.0	18.8* ± 1.8

Notes: 1. – *Alcaligenes faecalis* subsp. *faecalis*-1

2. – *Alcaligenes faecalis* subsp. *faecalis*-2

3. – *Pseudomonas viridiflava*

4. – *Shewanella putrefaciens*

5. – *Alcaligenes faecalis* subsp. *faecalis*-3

6. – *Proteus penneri*-1

7. – *Proteus penneri*-2

††percentage of survival for each pH value was carried out in triplicated.

*statistically significant differences were found for the percentage of survival of a pH value with regard to that showed for the same strain at pH 7 (*P* < 0.05).

Only the strain *Shewanella putrefaciens* produced a zone of inhibition around the well on BHIA by well diffusion method against the tested pathogens where it showed an antagonistic effect, showing that the inhibitory effect was due to the production of antimicrobial substances in its metabolism.

As the pH decreases, the bacterial viability is reduced (Table 3), showing values below 50% at pH 3, except for the strain *Proteus penneri*-1 that shows a viability of 63.5%. The viability of strains *Proteus penneri*-1 and *Alcaligenes faecalis* subsp. *faecalis*-1 are not affected significantly ($P < 0.05$) by the action of bile, showing survival rates of 99.2% and 95.7%, respectively. The other probiotics tested are affected significantly by the action of the bile, showing values below 50%.

A total of 4/7 probiotic strains have hydrophobic capacity. The strains *Shewanella putrefaciens* and *Pseudomonas viridiflava* shows high hydrophobicity, with values of 86% and 79%, respectively. The strains *Alcaligenes faecalis* subsp. *faecalis*-1 and *Alcaligenes faecalis* subsp. *faecalis*-2 shows medium hydrophobicity (38% and 68%). The other potential probiotic strains tested show hydrophilic capacity.

All strains tested have the ability to use sea bass intestinal and skin mucus as a source of nutrients, producing a statistically significant growth after 24 h of incubation with increments greater than 1 logarithm, highlighting the *Alcaligenes faecalis* subsp. *faecalis*-1 and *Proteus penneri*-1. In the adhesion test, the strain *Alcaligenes faecalis* subsp. *faecalis*-3 shows the highest adhesion in the intestinal mucus (16.8%) and in the skin mucus (26.08%). The rest of the strains have an average adherence under 10%.

All potential probiotic strains tested produced a decrease in the growth of vibrio strains analysed in the test of inhibition of growth in co-culture after 24 h of incubation, but this decrease was only statistically significant for the probiotic *Proteus penneri*-2 against *Vibrio anguillarum* 4347 (78.07%) and *Alcaligenes faecalis* subsp. *faecalis*-1 against *Vibrio alginolyticus* (40.27%). The rest of the probiotic strains tested produce a decrease in the growth in a range between 20 and 30%.

Discussion

Use of living organisms that may show an antagonistic effect is a very useful tool to prevent against different pathogens of aquaculture (Hjelm et al. 2004; Makridis et al. 2005). Probiotics have been used in terrestrial animals and in humans for many years (Irianto and Austin 2002). Nowadays, the application of probiotics is used as a control method and also as a new mechanism of natural prevention against diseases of fish caused by pathogenic bacteria (Nikoskelainen et al. 2001).

Three relevant fish species for marine aquaculture of Spain, seabass (*D. labrax*), meagre (*A. regius*) and common sole (*S. solea*) were used in this work with the aim of isolate and selecting different strains with probiotic activity. The evaluation and characterization of the potential probiotic strains were carried out through the analysis of its mechanism of action *in vitro*.

The fact that the isolated strains produces an inhibitory effect on the growth of different marine fish pathogens of the genus *Vibrio* is the method that allowed us the initial pre-selection, to continue later with a battery of complementary

tests, methodology indicated by previous works (Pan et al. 2008). A total of 7/156 isolates showed inhibitory capacity against different vibrio strains, highlighting the strain *Alcaligenes faecalis* subsp. *faecalis*-1, which produced inhibition of bacterial growth of all pathogenic strains analysed (Table 2). Production of antibacterial substances allows us to determine if the inhibitory effect on pathogens observed in previous tests, is produced by extracellular substances. In our study, only 1/7 isolated strains analysed (*Shewanella putrefaciens*) produced extracellular substances able of inhibiting all fish pathogens tested. Previous studies have suggested that the inhibitory effects could be caused by the production of volatile organic acid compounds and bacteriocins (Balcázar et al. 2007).

In this study, the effect of bile and pH as a prior step to adhesion were evaluated, trying to simulate the passage of the bacteria through the gastrointestinal tract for subsequent colonization in the intestines (Robertson et al. 2000; Irianto and Austin 2002; Nikoskelainen et al. 2003), in order its long-term effect was observed, or add it continuously in the diet (Duwat et al. 2000; Nikoskelainen et al. 2001). The viability of our potential probiotic strains analysed has been affected by the action of sea bass bile. However, the real concentration of bile in fish is unknown, therefore an overestimation has been made, using 10% fresh bile, a much higher concentration than used in the assays with humans (3%) (Sorrosa et al. 2012; Nikoskelainen et al. 2001). Low pH values affect the viability of the strains analysed, showing rates below 50% at pH 3, except the *Proteus penneri*-1 (63.5%), but this does not mean that these strains are unable to survive and colonize the intestine. We must consider that the strains are not directly exposed to stomach secretions since the bacteria will mix with food, and in addition, other elements are involved in digestion, such as the number of daily food intakes (Nikolopoulou et al. 2011; Nikoskelainen et al. 2001). In fact, the tolerance limit to acid medium is not always a previous condition of bacterial strains to be selected as probiotics.

Selected probiotics are able to increase their initial concentration, after 24 h of incubation using intestinal and skin mucus of sea bass as the sole source of nutrients. This means that the probiotic could be kept in the intestine and colonize (Ringø et al. 2010). The capacity of adhesion to the mucosa and its subsequent growth in the mucus of the preselected probiotics is a very important property to enable colonization and persistence in the intestinal tract (Collado et al. 2007; Verschuere et al. 2000). We analysed the adhesion of the strains both to the intestinal mucus and to the mucus of skin of sea bass, showing very high values for the strain *Alcaligenes faecalis* subsp. *faecalis*-3, and values below 10% for the rest of the strains. The hydrophobicity of the cell surface is related with the adherence of microorganisms beneficial to the intestinal tract of fish (Vázquez-Juárez et al. 1994), and in our study, 5/7 strains showed hydrophobicity activity.

Before beginning with subsequent studies, it is absolutely necessary to have a good enough collection of new bacterial strains showing some particular probiotic effect in order to test all together at the same conditions by *in vivo* studies. Here is where the importance of the present study lies.

In conclusion, in this work we have performed a screening in different marine species of fish with interest for aquaculture,

looking for bacterial strains with possible probiotic effect. By *in vitro* tests 7 strains were selected with inhibitory activity against different pathogenic strains of the genus *Vibrio*, which could be suitable to carry out the *in vivo* studies. We also highlight the results obtained with strain *Alcaligenes faecalis* subsp. *faecalis*-1 isolated from meagre, which has a marked inhibitory effect against all the vibrio strains analysed, and shows the adequate conditions for adhesion and colonization in fish intestines.

Disclosure statement

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