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Characterization of the probiotic strain *Vagococcus fluvialis* in the protection of European sea bass (*Dicentrarchus labrax*) against vibriosis by *Vibrio anguillarum*

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ABSTRACT

Aquaculture is one of the main sources of income in many countries worldwide. Intensive farms are often affected by different infectious diseases that can decrease their final production. To control this situation, several antibiotics are frequently used with known environmental consequences. The aim of this study was to analyze different bacterial strains isolated from of gilthead sea bream, sea bass, sole and meagre guts, for use as probiotics in aquaculture. The strains were evaluated *in vitro* through various mechanisms of selection, such as the production of antagonistic effects against pathogens, production of antibacterial substance, adhesion to the intestinal mucus, competition for nutrients or binding site, and growth in intestinal mucus. A total of 50 bacterial strains were analyzed and only one showed excellent *in vitro* results for consideration as a candidate to be analyzed *in vivo*. The strain, identified as *Vagococcus fluvialis*, showed good protection against *Vibrio anguillarum* 975-1 *in vivo* in the experimental challenge, showing a relative percent survival of 42.3% higher than positive control group. Therefore, in conclusion we consider this strain to be a good candidate for use as a future probiotic in aquaculture.

1. Introduction

The aquaculture industry has been rapidly developing worldwide in the last 30 years. Europe has produced high quality products by developing efficient technology. In southern Europe, the culture of sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*) and sole (*Solea solea*) are of great importance, while the culture of the meagre (*Argyrosomus regius*) has been introduced in recent years. The presence of infectious diseases is inevitable due to the intensive culture conditions, which results in huge economic losses in this sector. Outbreaks of *Vibrio anguillarum* cause acute hemorrhagic septicemia and current control strategies are based on vaccination and chemotherapy (Austin and Austin, 2007).

The use of antibiotics is a very common practice on fish farms, but the negative effects on environmental and public health make it necessary to develop new strategies to control infectious diseases. Due to this reason, the European Union placed restrictions on antibiotic use in aquaculture and to solve this problem, research has been focused in the last decades on alternative environmentally friendly methods to control disease. 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 (Gateusope, 1999).

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To date, a wide range of these bacteria have been proposed for their application as probiotics (Kesarcodi-Watson et al., 2008). However, the search for new microorganisms continues. In this respect, we isolated and evaluated different strains from the gut of different fish species for possible use as probiotics in aquaculture.

2. Materials and methods

2.1. Sampling

A total of 80 cultured gilthead sea bream (*S. aurata*), 60 sea bass (*D. labrax*), 25 sole (*S. solea*) and 30 meagre (*A. regius*), all of different average body weight, were anaesthetized in clove oil and sacrificed in liquid ice to extract the gut. One-gram amounts (wet weight) of the gut content of each fish were homogenized in 9 ml PBS and serial dilutions were spread on marine agar (MA), brain heart infusion agar (BHIA), blood agar base (BAB), trypticase soy agar (TSA) and De Man Rogosa and Sharpe broth (MRS) for 48 h at 25 °C to get as many bacteria as possible, which were stored frozen at −80 °C in BHIB with 15% glycerol.

2.2. Antagonistic effect and production of antibacterial substances of probiotics against pathogens

The production of antagonistic effect was analyzed using several known pathogens of marine and continental aquaculture (Table 1) following the method described by Austin et al. (1992). Briefly, 100 μl of different pathogen were spread on TSA and each isolate was put into the inoculum. Inoculated plates were incubated at 25 °C for 24–48 h and we observed the inhibition halo.

In order to determine the production of antibacterial substances in bacterial supernatants we followed the method described by Nikoskelainen et al. (2001) with modifications (Kim and Austin, 2008). Potential probiotic strains were grown in BHIB for 24 h at 22 °C, centrifuged at 2000 × *g* and the supernatants sterilized through 0.45 μm-pore-size filters, and lyophilized using a freeze dryer (Telstar, Cryodos-50) for 24 h. The freeze-dried supernatant was re-suspended in 100 μl of PBS (10 times concentrated) to be challenged against selected fish pathogen (*V. anguillarum* 975-1). The pathogen were grown in 1 ml of BHIB overnight at 22 °C and transferred evenly onto TSA plates. 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.

Table 1
Pathogens used in testing antagonistic effect.

Pathogen strains	References	Origin
V.anguillarum 4347	CECT	*Anguilla anguilla*
V.anguillarum 975-1	USC	*Psetta maxima*
V. alginolyticus 521	CECT	*Trachurus trachurus*
P. damselae subsp. *piscicida* 94/99	IUSA	*Sparus aurata*
P. damselae subsp. *piscicida* 17911	ATCC	*Perca fluviatilis*
P. damselae subsp. *piscicida* DI-21	ATCC	*Sparus aurata*
P. damselae subsp. *piscicida* C2	IUSA	*Sparus aurata*
Yersinia ruckeri 955	CECT	*Oncorhynchus mykiss*
Lactococcus garvieae 102507	CIP	*Salmo trutta*
Streptococcus iniae 1	IUSA	*Pagrus pagrus*

CECT: Spanish Type Culture Collection; USC: Institute of Aquaculture, Santiago de Compostela Universisy; ATCC: American Type Culture Collection; CIP: Collection Institute Pasteur; IUSA: University Institute of Animal Health.

2.3. Identification of selected strains

The strains that showed antagonistic effect were identified by 16S rRNA gene partial sequencing by BLAST analysis (Altschul et al., 1990). DNA was extracted from bacterial cultures with Genomic DNA Mini Kit (Invitrogen), according to the manufacturer's instructions. PCR by 16S rRNA gene partial sequencing was carried out using a MyCycler thermal cycler (Biorad) with universal primers *PLB* (5′-AGAGTTTGATCCTGGCTCAG-3′) and *MLB* (5′-GGCTGCTGGCACGTAGTTAG-3′) (Hébert et al., 2000).

2.4. Growth inhibition by co-culture

Overnight culture of potential probiotic strain and fish pathogen *V. anguillarum* 975-1 strain were washed twice with PBS and cell concentrations were adjusted to an absorbance of 0.5 at 600 nm (Nikoskelainen et al., 2001). Then, 100 μl of the strains were mixed in 1 ml of trypticase soy broth (TSB) and incubated for 48 h at 22 °C. After incubation, the numbers of cells in each sample were determined by serial dilution in PBS and plated on TSA and thiosulfate citrate bile salts sucrose agar (TCBS).

2.5. Fish bile and pH resistance

A 100 μl aliquot of fresh bile from sea bass was added to 900 μl of strain tested at 10^7 CFU ml^{-1}. 100 μl of same concentrations of strain tested was added to 900 μl PBS with a pH range 3–7, samples were incubated 1.5 h at 22 °C and serially diluted in PBS and determined by plate counting on TSA (Nikoskelainen et al., 2001).

2.6. Adhesion mucus assays

Intestinal mucus was isolated from healthy sea bass. Fish with 400 g of average body weight were starved for 48 h and gut removed and homogenized in PBS. All mucus preparations were centrifuged twice at 12,000 × *g* for 5 min at 4 °C to remove particulate and cellular material (Balcázar et al., 2007). Then, the solutions were adjusted to 0.5–1 mg ml^{-1} protein in PBS by Bradford Protein Assay Kit (Sigma), 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 (Van der Marel et al., 2008).

The percentage of adhesion to intestinal mucus was evaluated following the methodology described by Van der Marel et al. (2008). Briefly, the mucus was prepared as described above and the probiotic strain was stained with 2 μl per 10^9 CFU of green fluorescent nucleic acid (SYTO 9) (Invitrogen). 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 °C. After washing with saline solution, 25 μl of 10^9 CFU ml^{-1} of fluorescently labelled bacterial solution was added and then incubated for 30 min in the dark at room temperature. The plates were washed and 50 μl of saline solution was added to spectrophotometric measurements (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.

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^{-1}) was placed with the immobilized mucus for 30 min and washed with saline solution. Then, 25 μl 10^9 CFU ml^{-1} 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 the spectrophotometric measurements. 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).

2.7. Growth in intestinal mucus

The intestinal mucus of each fish species was diluted in PBS to a final protein concentration of 0.5 mg ml^{-1}. 10 μl of an overnight culture of each strain tested was inoculated in 3 ml of diluted mucus and incubated at 22 °C in a shaking incubator. Samples with BHIB and PBS were included as negative controls. The growth rate of each strain in mucus, BHIB and PBS was measured by monitoring the optical density at 540 nm and serial dilutions on TSA (Olsson et al., 1992).

2.8. Harmlessness test

To determine the possible harmful effects of the probiotic strain in sea bass, 0.1 ml (10^8 CFU ml^{-1}) was injected intraperitoneally into two separated groups of 20 sea bass with an average body weight of 10 g. A control group was injected with the same volume of PBS. Fish were monitored daily to detect any adverse clinical signs for 30 days after inoculation, and sacrificed with an overdose of clove oil and necropsied to evaluate any possible lesions in the internal organs by histopathology. Also, fish were analyzed by microbiological methods on BHIA from internal organs to determine the presence or absence of the inoculated strain.

2.9. Probiotic administration and fish challenge with V. anguillarum 975-1

For preparation of the experimental diet with the probiotic strain, selected bacteria were cultured in BHIB for 24 h at 25 °C following the method by Irianto and Austin (2002). Briefly, the strain was centrifuged at 2500 × *g* for 20 min at 4 °C, and cell pellet was washed twice and re-suspended in saline solution to 10^{10} CFU ml^{-1} by plate count on TSA. 25 ml of this selected strain were spread on 120 g of commercial feed (BioMar YM 558; Dueñas, Spain), mixed and dried for 24 h at 37 °C, to obtain a final concentration of 10^9 CFU per gram of the commercial feed. The viability of the probiotic strain in feed was assessed by colony counts on TSA following storage of the diet at room temperature for 3 weeks.

For challenge, sea bass with an average body weight of 18 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 with 25 fish per tank. A negative control group (not fed with probiotic and not challenged with *V. anguillarum* 975-1), and a positive control group inoculated with the pathogen and never previously fed with probiotic, were also included. 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.

V. anguillarum 975-1 was passaged in sea bass three times before performing the challenge by the intraperitoneal injection route to activate the bacteria. Twenty days after feeding, the positive control and probiotic test groups were exposed to *V. anguillarum* 975-1 with 10^8 CFU ml^{-1} in a bath for 8 h. The water temperature was raised and maintained from 21 °C to 24 °C to increase the effect of the experimental infection. Fish were observed everyday during 20 days after exposure to *V. anguillarum*, and each moribund or dead fish was necropsied. Isolated bacteria from fish were biochemically identified and internal organs analyzed by histopathology.

2.10. Statistical analysis

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

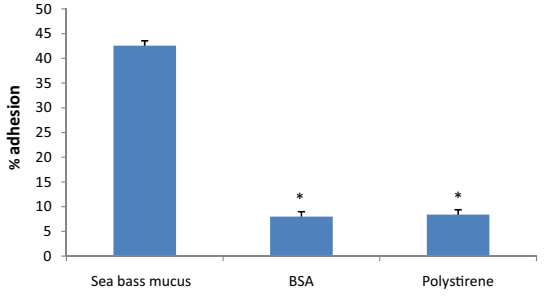
3. Results

50 bacterial strains were recovered from the guts of gilthead, sea bream, meagre, sea bass and sole, but only one strain from sole showed inhibitory effect against at least one of the pathogens tested. This strain showed inhibitory effect against *V. anguillarum* 4347 and 975-1, *Photobacterium damselae* subsp. *piscicida* 94/99, C2 and DI-21, and the strain *Yersinia ruckeri* 955. None of the analyzed strains showed inhibitory effects against *Streptococcus iniae*, *Vibro alginolyticus* or *Lactococcus garvieae*. The strain that showed inhibitory effect was identified as *Vagococcus fluvialis*, showing a similarity of 1051/1056 base pair

(99.5%) with sequence NR-026489 (type strain M-29c) by BLAST analysis against the NCBI database. This strain produced a zone of inhibition around the well on TSA by well diffusion method, showing that the inhibitory effect was due to this strain producing antimicrobial substances in its metabolism. After a 48-h growth in co-culture, the selected strain inhibited 10% of the growth of *V. anguillarum*, but this decrease was not significant ($P < 0.05$). The strain showed 66.7% and 53.5% survival in 10% of sea bass bile and at pH <5, respectively.

In the adhesion assay, this strain showed better adhesion to intestinal mucus (42.56%) than to bovine serum albumin or polystyrene, with significant differences ($P < 0.05$) among the controls (Fig. 1). The strain adhered to BSA and polystyrene at similar percentages. In the competitive adhesion assay, the adhesion capacity of *V. anguillarum* to mucus was significantly reduced (54.54%) after the exposure of the intestinal mucus to the probiotic strain. Furthermore, the selected strain showed the ability to use the mucus as a sole nutrient source and grew significantly in the intestinal mucus of sea bass (2.4×10^7 CFU ml^{-1}) compared with the control (1.5×10^6 CFU ml^{-1}) in PBS.

Evaluation of possible harmful effects by fish challenge showed that the strain tested was harmless to sea bass since no mortality or damage in the internal organs were observed. Moreover, the inoculated strain was not recovered from internal organs. In the experimental challenge to evaluate the level of protection of this probiotic against infection by *V. anguillarum* in sea bass, the mortality observed was 30% in the positive control fish group, while this was reduced to 17.3% in the fish previously fed with the probiotic strain (Fig. 2), representing a relative percent of survival of 42.3%. Statistical analysis demonstrated a significant difference ($P < 0.05$) in the survival of fish among the different groups analyzed. The affected fish showed signs of acute hemorrhagic septicemia with exophtalmia, corneal opacity and ulcers. Mortalities were attributed to the inoculated pathogen since the inoculated microorganism was recovered from the internal organs of dead fish in pure culture.



* Denotes a statistically significant difference ($P < 0.05$) compared with sea bass mucus

Fig. 1. Adhesion of selected strain to intestinal mucus from sea bass, bovine serum albumin and polystyrene. All data are given as percentage of the absorbance measurements of fluorescent stained bacteria ± SD. * Denotes a statistically significant difference ($P < 0.05$) compared with sea bass mucus.

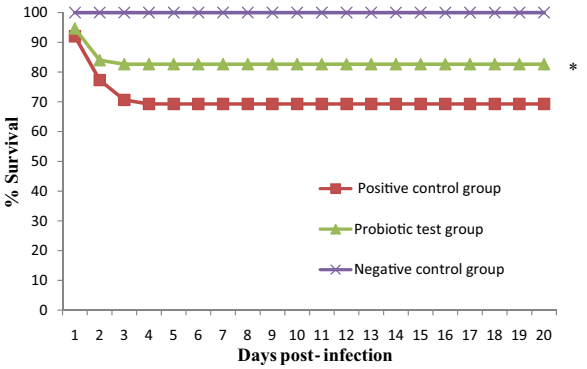


Fig. 2. Percentage of survival of sea bass previously fed with selected probiotic and challenged with *V. anguillarum*.

4. Discussion

The use of probiotics in aquaculture is a very recent development, but it has been used in terrestrial animals and humans for years, and must satisfy certain requirements before being used in aquaculture. *In vitro* tests to assess probiotic strains allowed us to determine whether these strains could be used for *in vivo* tests. The inhibitory activity against pathogens or competition for nutrient has been widely described and discussed (Kesarcodi-Watson et al., 2008), and it is an important criterion to select a probiotic candidate strain (Pan et al., 2008). In our study, only 1 out of the 50 isolated strains tested produced extracellular substances capable of inhibiting fish pathogens. Previous studies have suggested that inhibitory effects could be caused by volatile compound production of organic acid and bacteriocins (Balcázar et al., 2007). Our strain, identified as *V. fluvialis*, did not significantly reduce the growth of *V. anguillarum* after 24 h in co-culture, this mean it does not compete for nutrients, but this criterion is not necessary for the pre-selection of a good probiotic strain (Chabrillón et al., 2005).

In this study, we evaluated the effect of bile and pH as a prior step to adhesion, trying to simulate the passage of the bacteria through the gastrointestinal tract. The strain was affected by the bile, showing a survival of 66.7%, but we must bear in mind that this assay was carried out with a bile concentration of 10% since its real concentration in fish is unknown (Nikoskelainen et al., 2001), a much higher percentage than that used in the assays with humans (3%). We also observed a decrease in the survival of the strain in acid pH, but this does not mean that this strain is unable to survive and colonize the intestine because this does not occur *in vivo*. Bacteria will mix with food and the action of the acid pH will not be direct (Nikoskelainen et al., 2001). Also, tolerance to acidic conditions is not always a prerequisite for selecting a candidate probiotic aimed at marine larvae since their digestive system is alkaline during the live feed period (Hoehne-Reitan et al., 2001). The strain tested showed the ability to grow and adhere to the intestinal mucus of fish, and these results compared with those obtained in the adhesion to BSA and polystyrene, suggesting that the microbial adhesion process

may be due to passive forces, electrostatic interactions, steric forces, lipoteichoic acids and specific structures such as external appendages covered by lectins (Balcázar et al., 2007). This fact is considered as a very important property to enable colonization and persistence in the intestinal tract (Verschuere et al., 2000).

The strain tested also showed the ability to compete for attachment site with *V. anguillarum*. This fact is beneficial to the health of the fish due to the presence of probiotic bacteria that may restrict the access of pathogens to tissues receptors by steric hindrance or by blocking the receptor with specific adhesion analog (Tuomola et al., 1999). To date, it is generally accepted that lactic acid bacteria form part of the normal intestinal microbiota of fish from the first few days of life (Ringo et al., 2010). The genus *Vagococcus* belong to lactic acid bacteria and can be found in different environments, being part of the microbiota of several fish species, especially in freshwater fishes (González et al., 2000) although some species of these genera have been isolated from diseased fish (Michel et al., 2007). However, this strain was harmless in sea bass. There are no studies analyzing this genus as a probiotic, but in general, it is well documented that many lactic acid bacteria are harmless and some strains have been reported to have beneficial effects on fish health (Gatesoupe, 2008).

In fish, the three major routes of infection are the skin, gills and gastrointestinal tract. Therefore, in the experimental challenge, we observed that relative percent survival with *V. fluvialis* was 42.3%, respect control group (infected with *V. anguillarum* and not fed probiotic), without assessing the mode of action. However, many studies in recent years have shown that dietary administration of lactic acid bacteria may reduce the incidence of diseases or lessen the severity of outbreaks (Ringo et al., 2010).

It is generally accepted that probiotics block pathogenic bacterial effects by various mechanisms, enhancing barrier function and stimulating protective responses (Vanderpool et al., 2008).

5. Conclusion

In conclusion, our data show that the strain isolated from sole gut and identified as *V. fluvialis* could be used as probiotic bacteria to protect sea bass against infection by *V. anguillarum*, and may be an important management tool for the control of this disease in marine culture.

Conflict of interest

This research does not present conflicts of interest.

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