Selection of putative probiotics based on antigen-antibody cross-reaction with *Photobacterium damselae* subsp. *piscicida* and *Vibrio harveyi* for use in Senegalese sole (*Solea senegalensis*)

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ABSTRACT

Probiotics can be used to reduce disease outbreaks in aquaculture. Some of them are characterised by their antagonistic activity against pathogens or the stimulation of the fish immune response, including the production of specific antibodies. If a probiotic has common antigens with a determined pathogen, it could produce antibodies with a cross-reaction to that pathogen. Thus, a probiotic with these characteristics could be used in a similar way to a live vaccine. The aim of this study was to select bacteria with antigenic similarity and antagonistic activity against the pathogens *Photobacterium damselae* subsp. *piscicida* and *Vibrio harveyi*, and to determine their ability to stimulate the production of antibodies in sole (*Solea senegalensis,* Kaup) with cross-reaction against these pathogens.

Dot blot was used to detect strains with cross-reaction using sera immunized against *P. damselae* subsp. *piscicida* Lg41.01 and *V. harveyi* Lg16.00. A total of 138 strains were selected from 718 strains, based on the intensity of the dot blot reaction. A second selection was performed to detect their ability to inhibit the pathogens growth. Five strains inhibited the growth of *P. damselae* subsp. *piscicida*, four strains inhibited the growth of *V. harveyi*, while two strains inhibited both pathogens. A Western blot confirmed the cross-reactions of the selected strains with the pathogens.

Selected strains were subsequently inoculated into sole specimens by intraperitoneal injection. Four strains produced antibodies with cross-reaction against the pathogens. None mortality was observed in the inoculated fish. Further research demonstrated the storage capability of the selected strains in saline solution and feed, their growth at low pH, and identified their enzymatic characteristics. In conclusion, the selected strains showed antimicrobial activity and capacity to activate a specific immune response against fish pathogens.

1. Introduction

P. damselae subsp. *piscicida* and *V. harveyi* are important pathogens in cultured fish, including Senegalese sole (Magariños et al., 2003; Zorrilla et al., 1999, 2003). Fish pathogens are conventionally eliminated with antibiotics, but these products cause water pollution, accumulate in fish tissues, and have led to bacterial resistance (Karunasagar et al., 1994), so a need for other prophylactic alternatives has been identified. One strategy is the use of probiotics. A probiotic is "a live, dead or component of a microbial cell that, when administered *via* the feed or to the rearing water, benefits the host by improving either disease resistance, health status, growth performance, feed utilization, stress response or general vigour" (Merrifield et al., 2010). Probiotics can act against pathogens *via* different mechanisms that can control and eliminate the pathogen, or enhance the fish immune system

(Nayak, 2010). With respect to the development of antibodies, some probiotics have enabled the production of specific antibodies that had beneficial effects on the animal (Maassen et al., 2003; Abbass et al., 2010).

Immune cross-reactions among phylogenetically-related bacteria are widely documented, and they have an important role in protection against pathogens (Beal et al., 2006). In fact, some vaccines are based on the use of non-pathogenic microorganisms that contain antigens similar to those of pathogenic strains (Brunt and Austin, 2005; Brunt et al., 2007; Arijo et al., 2008; Abbass et al., 2010). Therefore, it might then be possible to identify potential probiotics that have antigens in common with pathogens. These bacteria would act as putative live vaccines, with a double protective effect: first, forming bacteriocins that inhibit pathogen growth, and second, activating the specific immune response with the formation of protective antibodies.

Further testing of the safety of these probiotics (Wang et al., 2008) and their capability to be stored would also be required.

Thus, the aim of this study was to select bacteria with antigenic similarity and antagonistic activity against two fish pathogens (*P. damselae* subsp. *piscicida* and *V. harveyi*) and to study their ability to stimulate the production of antibodies with cross-reaction against these pathogens.

2. Material and methods

2.1. Strains used

A total of 718 strains were used in this research. These strains belong to a collection of the Department of Microbiology the University of Malaga (Spain). They were isolated from sea water, skin mucus and intestine from gilthead bream (*Sparus aurata*), seabass (*Dicentrarchus labrax*), Mediterranean horse mackerel (*Trachurus mediterraneus)*, anchovy (*Engraulis encrasicolus)*, wedge sole (*Dicologlossa cuneata*), Senegalese sole (*Solea senegalensis*), and other flatfish. The pathogens *P. damselae* subsp. *piscicida* Lg41.01 and *V. harveyi* Lg16.00 were isolated from sick Senegalese sole specimens (*S. senegalensis*) (Arijo et al., 2005a,b). All the bacteria were growth aerobically at 22 °C in tryptic soy agar medium, supplemented with 2 % NaCl at final concentration (TSAs), and stored at -80 °C.

2.2. Fish immunization with P. damselae subsp. piscicida and V. harveyi cells

All experiments complied with European Union (2010/63/EU) and Spanish government (RD 53/2013) guidelines for the use of laboratory animals.

Polyclonal antisera against the fish pathogens were obtained from *S. senegalensis* specimens. The fish, weighing about 400 g (10 fish as replicates), were immunized with *P. damselae* subsp. *piscicida* Lg41.01 or *V. harveyi* Lg16.00 bacterin, both supplemented with inactivated extracellular polymeric products (ECP).

For immunization, inactivated cells (bacterin) were obtained with formalin (Arijo et al., 2005b). The resultant bacterins were supplemented with inactivated ECP obtained by growing the cells on TSAs with a cellophane film (Liu, 1957). Briefly, after incubation, the ECP were harvested in phosphate saline buffer (PBS) and the cell suspensions centrifuged at $13,500 \times g$ for 20 min at 4 °C. The supernatants were filtered through 0.2 μm membrane filters. ECP were inactivated by addition of formalin to achieve a final concentration of 1 % (v/v), followed by heating at 100 °C for 30 min. The protein concentration of ECP was measured using the Bradford method (Bradford, 1976). The inactivated ECP (800 μg proteins/mL) was mixed with bacterin (10^9 inactivated cells/mL) at a proportion of 1:10. The fish were immunized by intraperitoneal injection with 0.2 mL of ECP-bacterin preparation mixed with Freund incomplete adjuvant (FIA; Sigma-Aldrich) at a ratio of 1:1. A group of ten fish were used as negative controls. One month after this first immunization, the fish were inoculated with the same antigens, as described above. One month after the second immunization, the fish were anesthetized and sacrificed. Blood was extracted and allowed to clot to obtain sole antiserum. The sera were stored at −80 °C before use.

2.3. Selection of bacteria according to their cross-reaction with pathogen-immunized fish serum

For the first selection of probiotics, a semi-quantitative dot blot analysis was performed.

The strains used in the screening process were grown in TSAs at 22 °C for 24 h. The bacterial suspensions were centrifuged at $6000 \times g$ for 20 min at 4 °C. The pellets were washed, suspended in PBS, and adjusted to an optical density at 600 nm of 1 ($\sim 10^9$ cfu/mL). The

solution was heated at 60 °C for 60 min, and then cooled to 4 °C before being stored at −20 °C (Helmerhorst et al., 1998).

Duplicate drops of the bacterial suspension were placed on a nitrocellulose membrane (Serva) (Zijun et al., 2004). *P. damselae* subsp. *piscicida* and *V. harveyi* strains were used as positive controls and PBS was used as negative control. After drying, the membranes were blocked for 1 h at room temperature with PBS supplemented with 0.25 % (v/v) Tween 20 (PBS-T) and 3 % (w/v) skimmed milk (PBS-T-M), washed with PBS-T, and incubated for 1 h with sole serum immunized against *P. damselae* subsp. *piscicida* Lg41.01 or *V. harveyi* Lg16.00 (diluted 1:400 in PBS). After incubation, the membranes were sequentially incubated with rabbit anti-Senegalese sole Ig (Sigma) (1:1000 in PBS-T-M) for 2 h and goat anti-rabbit peroxidase-conjugate (Sigma, diluted 1:5000) for 1 h. The membrane was developed with 3-amino-9-ethyl-carbazole (AEC, Sigma) diluted in 0.05 M acetate buffer (pH 5.5) and 0.3 % hydrogen peroxide. The reaction was stopped with distilled water. The membrane was scanned by densitometer (GS-800, Biorad) and the intensity of each strain was analysed using Quantity One software (Biorad). With these data, the percentage of signal similarity was measured between the strains and the positive controls (*V. harveyi* Lg16.00 or *P. damselae* subsp. *piscicida* Lg41.01):

Similarity percentage = 100 × Mean intensity of strain/Mean intensity of the positive control.

The strains with at least 75 % of cross-reaction with pathogens were selected for further analysis.

2.4. Antagonistic ability of selected strains on the growth of P. damselae subsp. piscicida and V. harveyi

The antagonistic ability of the strains that showed cross-reaction with the pathogen-immunized serum was tested by the agar well diffusion method (Rattanachuay et al., 2010). A suspension of *P. damselae* subsp. *piscicida* Lg41.01 or *V. harveyi* Lg16.00 was adjusted to 0.5 McFarland standard ($\sim 10^8$ cells/mL) and then swabbed over the surface of TSAs. Several wells of 5 mm diameter were made on the agar surface. The strains were suspended with an optical density of 1.0 at 600 nm and 60 μL were inoculated into each of the agar wells. The plates were incubated at 22 °C for $24 - 48$ h. After incubation, strains with antagonistic activity showed an inhibition area. The experiments were repeated twice.

2.5. Detection of bacterial proteins antigenically similar to P. damselae subsp. piscicida and V. harveyi proteins

Bacterial outer membrane proteins (OMP) of the antagonist strains were purified according to Lambert (1988) and used for the detection of cross-reaction proteins by electrophoresis. For this, the bacteria were grown overnight in TSAs, suspended in PBS, and washed twice at $3500 \times g$ for 15 min. The cell pellet was suspended in 2 mL PBS containing 10 mM ethylenediaminetetraacetic acid (EDTA; Serva) and 2 mM phenylmethyl-sulphonylfluoride (PMSF; Sigma-Aldrich). The samples were heated at 45 °C for 30 min, and then sonicated for 2 min in a Hielscher UP400S sonicator (Hielscher, Teltow, Germany). Volumes (1 mL) were centrifuged at $3500 \times g$ for 15 min at 4 °C and the pellet discarded. The OMP was purified adding three volumes of ice-cold acetone, incubated overnight and then centrifuged at $4000 \times g$ for 30 min. The OMP were resuspended in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) buffer at 750 μg/mL. The samples were heated at 60 °C for 1 min, and stored at −20 °C until use. The protein samples (25 μL), dissolved in SDS-PAGE buffer, were separated using 4 % stacking gels and 10 % resolving gels with constant voltage of 250 V. The gels were stained with Coomasie blue (Dyballa and Metzger, 2009) or transferred to a nitrocellulose membrane for Western blot (Medina et al., 2015). The membranes were incubated

with sole serum immunized against *P. damselae* subsp. *piscicida* Lg41.01 or *V. harveyi* Lg16.00 (dilution 1:400). The existence of reactive bands was revealed with TMB (tetramethylbenzidine).

2.6. Identification of the selected strains

For identification, the selected strains (5) were cultured in TSAs at 22 °C for 24 h. After incubation, a colony was suspended in 100 μL of sterile molecular water. The suspension was heated at 100 °C for 15 min, and 900 μL of sterile molecular water were added to each tube. After centrifuging for 5 min at 20,500 × *g*, 5 μL of the supernatant were added for each PCR reaction. The 16S universal primers BACT0008 (5`AGAGTTTGATCCTGGCTCAG 3`) (Hicks et al., 1992) and BACT1492 (5`GGTTACCTTGTTACGACTT 3`) (Kim and Austin, 2006) were used to obtain sequences with approximately 1400 bp. The PCR steps included initial denaturation at 95 °C for 2 min, 35 cycles of denaturation (95 °C, 30 s), annealing (52 °C, 40 s), extension (72 °C, 90 s), and final extension at 72 °C for 5 min. The results were compared with the NCBI data base using the BLAST algorithm (Altschul et al., 1990).

2.7. Ability to produce antibodies in Solea senegalensis and harmlessness of the selected strains

Senegalese soles, ranging from 10 to 50 g, were distributed in groups of 8 fishes per selected strain. A fish group was maintained as control. Each selected strain (only five strains) was grown aerobically in TSAs at 22 °C for 24 h. The biomass was suspended in saline solution at 10^9 cfu/mL. The bacterial suspension was then inoculated intraperitoneally at 10^8 cfu/g of fish. The fish were monitored to detect any signs of sickness or mortality. After 30 days, 3 fishes from each group were sacrificed to measure the antibody titer in serum and the other fishes were reinoculated with the same bacterial solution. The rest of the fish were sacrificed 21 days later to obtain the serum. The sera were used to measure the antibody titer and cross-reactions with pathogens by ELISA. The liver, kidney and spleen were also inspected to evaluate any damage produced by bacteria. Samples from these internal organs were spread in TSAs and incubated aerobically at 22 °C for the detection of colonies. The existence of colonies of the inoculated strains could be considered as a sign of bacterial virulence.

2.8. Supplementary studies of selected strains as potentially probiotics

The selected strains DCF12.2, DCF12.9, DCF12.10 (isolated from *Dicologlossa cuneata*), and PLSW5 (isolated from sea water) were studied under different environmental conditions to evaluate survival in seawater and different pH, storage capability, and enzymatic characteristics.

2.8.1. Survival in sea water

The survival of the strains in sea water was evaluated in order to determine viability for administration by bath. The microorganisms were suspended to a final concentration of 10^7 cfu/mL in 50 mL of sterile seawater and incubated at 22 °C. Samples were taken at the beginning and days 1, 2 and 5. Serial dilutions of the samples were made in saline solution, and cultured in TSAs and incubated for 24 h at 22 °C. After incubation, colony counts were performed to determine the surviving cells in water.

2.8.2. Survival in feed

The study of bacterial survival in the feed is essential to determine the viability of probiotics administered orally and in feed storage.

The bacteria were suspended in saline solution (10^8 cfu/mL) and 6 mL were added per 30 g of feed, stirring until the feed absorbed all the suspension.

The feed was kept at −20 °C, 4 °C and 22 °C, and survival was determined with bacteria counts in TSAs. The feed frozen at −20 °C was

analysed at day 5, and the other feeds were analysed at days 0, 1, 2 and 5. For sampling, 1 g of feed was suspended in 10 mL of sterile PBS. The mixture was then homogenized for 5 min using a homogenizer, and the suspension was centrifuged for 5 min at 300 × *g*. The suspensions were serially diluted in sterile PBS, and incubated on TSAs at 22 °C for 24 h to determine the number of viable cells in the feed.

2.8.3. Determination of enzymatic characteristics related with feed digestion in fish

Some enzymatic activities related with feed digestion were evaluated with the streaking of the strains in different media: amylase (Thomas et al., 2014), caseinase (Chowdhury et al., 2017), gelatinase (Thomas et al., 2014), lecitinase (Thomas et al., 2014), and lipase (Jaiswal et al., 2017) at 22 °C for 48 h.

2.8.4. Growth in media with different pH

The growth of each strain was evaluated in media with different pH. The isolates were grown in TSB and washed twice with sterile saline solution (3000 × *g*, 15 min, 4 °C). The isolates were resuspended to a concentration of 10^6 cfu/mL and incubated in TSB with pH from 1.0 to 9.0 for 24 h. Growth was confirmed when suspension had an optical density greater than 0.5 at 600 nm.

2.9. Statistical analysis

The statistical studies of results obtained were performed by one-way analysis of variance (ANOVA) to determine differences between treatments. The normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Selection of bacteria with common antigens to P. damselae subsp. piscicida and V. harveyi

Of the 718 strains tested, 138 strains showed an antigenic similarity to *P. damselae* subsp. *piscicida* Lg41.01 or *V. harveyi* Lg16.00 greater than 75 %. Forty strains showed a similarity greater than 75 % compared with *P. damselae* subsp. *piscicida* Lg41.01 and 98 strains against *V. harveyi* Lg16.00. Six of these 138 strains showed an antigenic similarity greater than 75 % against *P. damselae* subsp. *piscicida* Lg41.01 and *V. harveyi* Lg16.00 (Fig. 1).

3.2. Antagonist ability of selected strains on the growth of P. damselae subsp. piscicida and V. harveyi

Only 11 of the pre-selected 138 strains showed inhibitory ability: 7 strains inhibited *P. damselae* subsp. *piscicida* Lg41.01 growth and 6 strains inhibited *V. harveyi* Lg16.00 growth. The strains DCF12.2 and DCF12.9 inhibited both *P. damselae* subsp. *piscicida* Lg41.01 and *V. harveyi* Lg16.00 growth (Table 1).

3.3. Selection of strains with proteins antigenically similar to P. damselae subsp. piscicida and V. harveyi

After the inhibition assay against *P. damselae* subsp. *piscicida* Lg41.01 and *V. harveyi* Lg16.00, OMPs of the selected strains were isolated to test their antigenic similarity with the two pathogenic strains.

The Western blot using serum immunized with *P. damselae* subsp. *piscicida* Lg41.01 showed two predominant reactive bands in DCF12.2 and DCF12.10 strains, a band in the strain DCF12.9, and three bands in PLSW5. The bands were contained between 35 to 50 kDa (Fig. 2). Three immunoreactive bands against serum immunized with *V. harveyi*
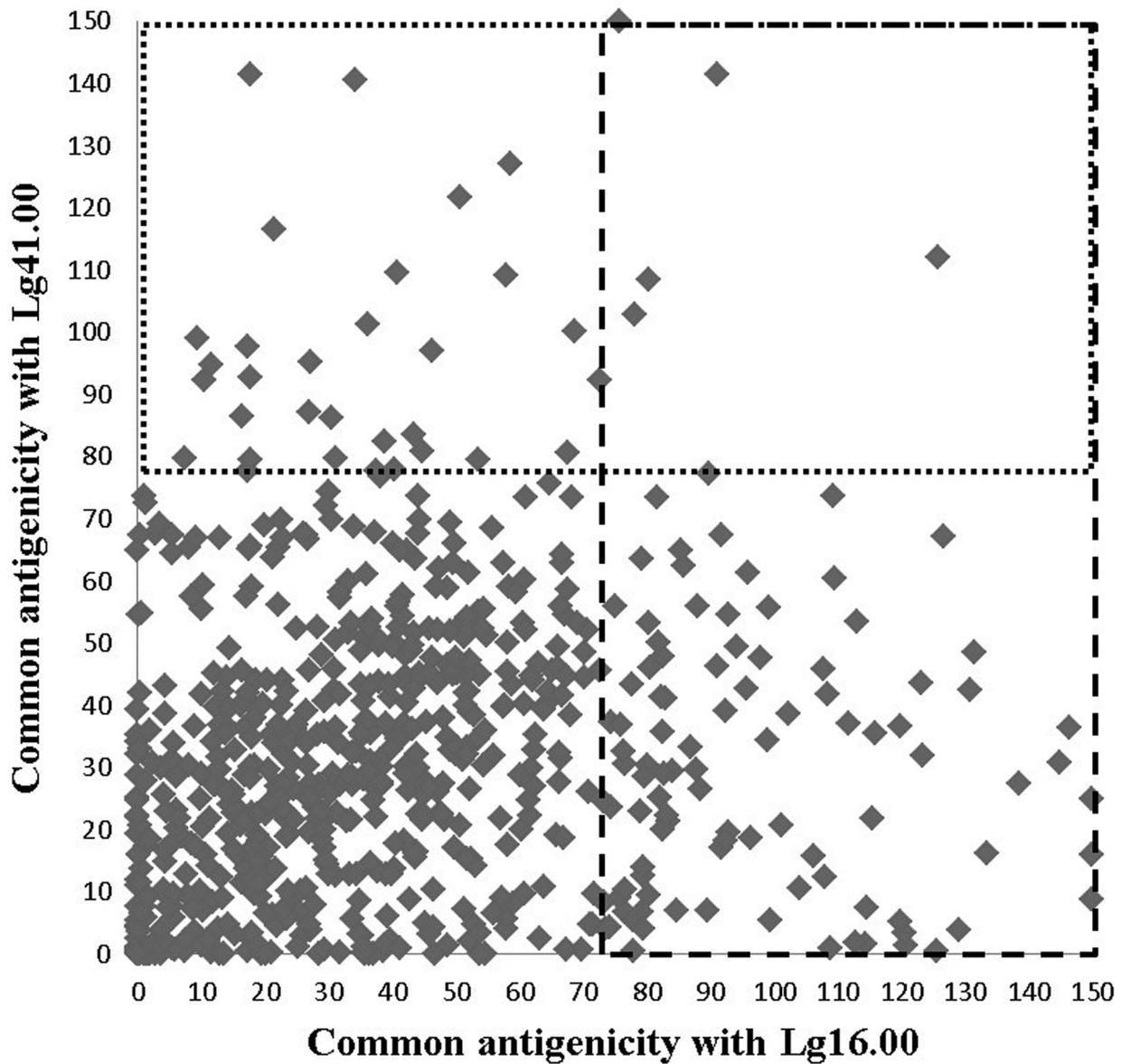
Fig. 1. Percentage of antigenic similarity of the strains for *V. harveyi* Lg16.00 (x-axis) and *P. damselae* subsp. *piscicida* Lg41.01 (y-axis). Each point represents a strain. The percentage (%) of antigenic similarity was obtained by dot blot analysis and processed using *Quantity One* software (Biorad). The squares represent similarity percentages greater than 75 % for Lg41.01 and Lg16.00.

Table 1
Inhibition of *P. damselae* subsp. *piscicida* Lg41.01 and *V. harveyi* Lg16.00. The positive sign means existence of inhibition of the pathogen.

Strains	Inhibition against *P. damselae* subsp. *piscicida*	Inhibition against *V. harveyi* Lg16.00
DCF12.2	+	+
DCF12.9	+	+
DCF12.10	+	–
PLSW5	+	–
UMA5	+	–
UMA7	+	–
5-10-11	–	+
23-5-11	–	+
9ACE	–	+
27ACE	+	–
11PINO	–	+

Lg16.00 were detected in the strains 5-10-11, and two bands in the strains DCF12.2 and DCF12.9. These bands were included between 35–50 kD (Fig. 3). The other strains did not show proteins in common with the pathogenic strains, or the reactive bands were weak. These strains were discarded for posterior assays.

3.4. Identification of the potentially probiotic strains

Bacteria were identified by comparing 16S DNA with the NCBI database. All strains were identified as *Vibrio* genus (Table 2). The 5.10.11 strain showed similarity to *Vibrio* sp., DCF12.2 showed similarity to *Vibrio proteolyticus*, while DCF12.9, DCF12.10 and PLSW5 strains showed homology with different strains of *Vibrio alginolyticus*.

3.5. Ability of selected strains to produce antibodies in Solea senegalensis and harmlessness of the selected strains

After fish were inoculated with the selected strains, the highest specific antibody titers were detected in DCF12.2 and DCF12.10 strains
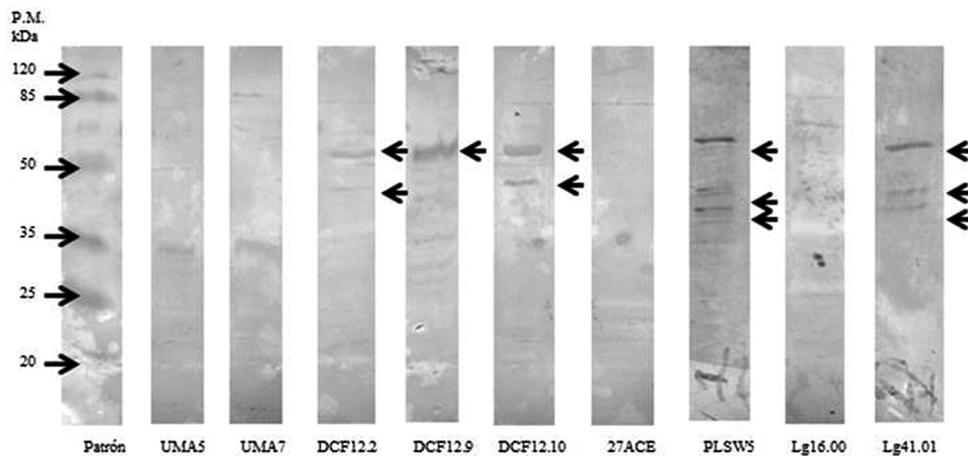
Fig. 2. Western blot using outer membrane proteins of strains with inhibition against *P. damselae* subsp. *piscicida* Lg41.01, and immunized serum against *P. damselae* subsp. *piscicida* Lg41.01. *V. harveyi* Lg16.00 was used as a negative control.
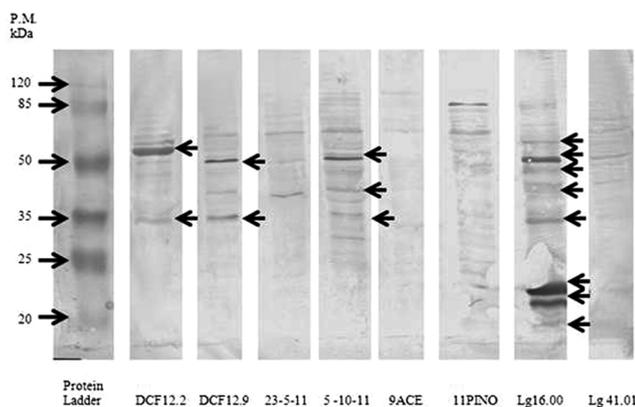

Fig. 3. Western blot using outer membrane proteins of strains with inhibition against *V. harveyi* Lg16.00, and immunized serum against *V. harveyi* Lg16.00. *P. damselae* subsp. *piscicida* Lg41.01 was used as a negative control.

Table 2
Strains identified with 99 % similarity and NCBI access number.

Strain	Specie	Similarity (%)	Access number
5-10-11	*Vibrio sp*	100	KU667081.1
DCF12.2	*Vibrio proteolyticus*	99	KP640643.1
DCF12.9	*Vibrio alginolyticus*	100	KY229850.1
DCF12.10	*Vibrio alginolyticus*	99	KX108994.1
PLSW5	*Vibrio alginolyticus*	99	KR347232.1

after one and two immunizations (1/256.000). These strains also showed a cross-response against *P. damselae* subsp. *piscicida* Lg41.01 and *V. harveyi* Lg16.00. In the case of DCF12.2, a second immunization

produced the highest increase in cross-reaction titer for *V. harveyi*, whereas the strain PLSW5 produced the highest cross-reaction titer with *P. damselae* subsp. *piscicida* (Table 3). The strain 5-10-11 was discarded for subsequent assays due to the low specific humoral response (Table 3).

No mortality was observed in inoculated fish after 30 days, nor was any anomaly detected in internal organs, and the inoculated strains were not isolated from the internal organs of the fish.

3.6. Supplementary studies of strains pre-selected as potential probiotics

3.6.1. Survival in feed
The results showed a decrease in the bacteria viability. All strains survived in feed stored at −20 °C (Table 4), 4 °C (Fig. 4) and 22 °C (Fig. 5), although they showed a loss of viability of several orders of magnitude. The strain DCF12.9 showed the highest decrease in the number of viable bacteria in all conditions. Frozen DCF12.10 strains showed greater viability (Table 4).

3.6.2. Determination of enzymatic characteristics related with digestibility
All strains assayed showed lecitinase, lipase, gelatinase, caseinase, amylase and lipase activities.

3.6.3. Growth in media with different pH
All strains grew overnight in broths with acidity between pH 5.0 and pH 9.0. When the pH was lower than 5, the tubes did not show turbidity in the medium (optical density lower than 0.5 measured at 600 nm) due to absence of bacterial growth.

4. Discussion

The dot blot technique was used as a first step to identify bacteria

Table 3
ELISA results obtained from fish inoculated once and twice with potentially strains. Data are expressed as the inverse (1/n) of the limiting dilution (maximum dilution of serum from immunized fish with average value higher than control sera plus twice the standard deviation). NT = Not tested. Bold numbers show the highest titers.

Strain	ELISA with inoculated strains as antigen		ELISA with *P. damselae* subsp. *piscicida* as antigen		ELISA with *V. harveyi* as antígen	
	First Inoculation	Second inoculation	First Inoculation	Second inoculation	First Inoculation	Second inoculation
5-10-11	1000	1000	< 500	NT	< 500	NT
DCF 12.2	**256,000**	**256,000**	2000	8000	**16,000**	**64,000**
DCF 12.9	4000	**32,000**	2000	4000	2000	1000
DCF 12.10	**256,000**	**256,000**	4000	4000	8000	**16,000**
PLSW5	**64,000**	**256,000**	2000	**16,000**	4000	2000

Table 4

Strain survival administered in feed and stored at −20 °C. Values expressed in log cfu/g of feed. The tables show the mean and the standard deviation of the survival capability.

Strain	Initial value	Final value
DCF12.2	6.75 ± 0.67	4.99 ± 0.09
DCF12.9	6.70 ± 006	2.72 ± 0.06
DCF12.10	7.30 ± 0.16	6.01 ± 0.53
PLSW5	7.17 ± 0.87	5.99 ± 0.44

with greater antigenic similarity against studied pathogens. Dot blot is used routinely for detection of antibodies (Wang and Zhan, 2006; Longyant et al., 2008) and this technique has been used for the rapid detection of *Vibrio cholerae* (Qadri et al., 1994).

In this study we used a new semi-quantitative method: the signal intensity of each strain in the dot blot was measured with image analysis software (Quantity One software, Biorad). This method differentiated between mildly positive results (similar to the negative results) and clearly positive results (very similar to positive control results). The mean intensity of each strain was relativized with its corresponding positive control. Strains with an average intensity greater than 75 % with respect to the positive control were selected. This limit was established to reduce the number of strains to be tested. The measurement of signal intensity is a new application of dot blot in the selection of large quantities of antigens. Thus, 138 strains were selected from 718 strains tested.

The next selection criterion of the 138 strains with common antigens to *P. damselae* subsp. *piscicida* Lg41.01 and *V. harveyi* Lg16.00 was their inhibitory effect against the growth of these pathogens. Growth inhibition is a common phenomenon among bacteria, and it is one of the first characteristics determined in the selection of a probiotic (Saint-Cyr et al., 2016). Of the 138 strains used in inhibition tests, 7 inhibited the growth of *P. damselae* subsp. *piscicida* Lg41.01 and 6 inhibited the growth of *V. harveyi* Lg16.00. Two of these strains inhibited both pathogens. This inhibitory ability is associated with several factors, such as the production of bacteriocins, hydrogen peroxide, proteases, short-chain organic acids (Verschuere et al., 2000) and acidification of media. Further research is necessary to characterise their inhibitory mechanisms.

Western blot of bacterial proteins was used to confirm the cross-reactivity of preselected strains. OMP are highly immunogenic components with exposed epitopes on the cell surface. Homology among OMP may explain the cross-reactions between Gram negative bacteria (Lun et al., 2014).

The potentially probiotic strains were identified as *Vibrio* genus. DCF12.9, DCF12.10 and PLSW5 were identified as *Vibrio alginolyticus* strains. Despite other strains of these species having been reported as pathogenic in crustaceans, fish and others species (Zorrilla et al., 1999, 2003; Lee et al., 1996), *V. alginolyticus* strains have also been proposed as probiotics (Verschuere et al., 2000). Finally, DCF12.2 was identified as *Vibrio proteolyticus*. According to Schrijver and Ollevier (2000), the ingestion of *V. proteolyticus* stimulates protein digestibility in turbot (*Scophthalmus maximus*) and for this reason it can be considered as a potential probiotic.

The ability to produce antibodies and induce cross-reaction with the antigens of the pathogens was also tested. DCF12.2, DCF12.10 and PLSW5 strains had the ability to induce antibody production in fish, 30 days after initial intraperitoneal inoculation. Antibodies are produced by the interaction of the probiotic strains with the immune response of fish (Korkea-aho et al., 2011), an effect that may help protect the fish against pathogens (LaPatra et al., 2014). The antibody titer obtained against *P. damselae* subsp. *piscicida* Lg41.01 and *V. harveyi* Lg16.00 was due to cross-reaction in the fish between probiotics and pathogenic strains, suggesting that the titer of antibodies observed 28 days after immunization could have an important protective role (LaPatra et al., 2014).

One of the main criteria to confirm the safety of probiotics in animal health is the absence of pathogenicity. All selected strains in this study could be considered non-pathogenic for sole, because no fish died due a high dose of probiotic (Aly et al., 2008). Although harmlessness has been demonstrated in sole, this does not imply that the strains are not pathogenic to other species, so complementary studies would be required to test the strains in other fish species (Pandiyan et al., 2013).

Once the strains were selected on the basis of their common antigens and antagonistic capacity, further studies were conducted on the potential probiotics.
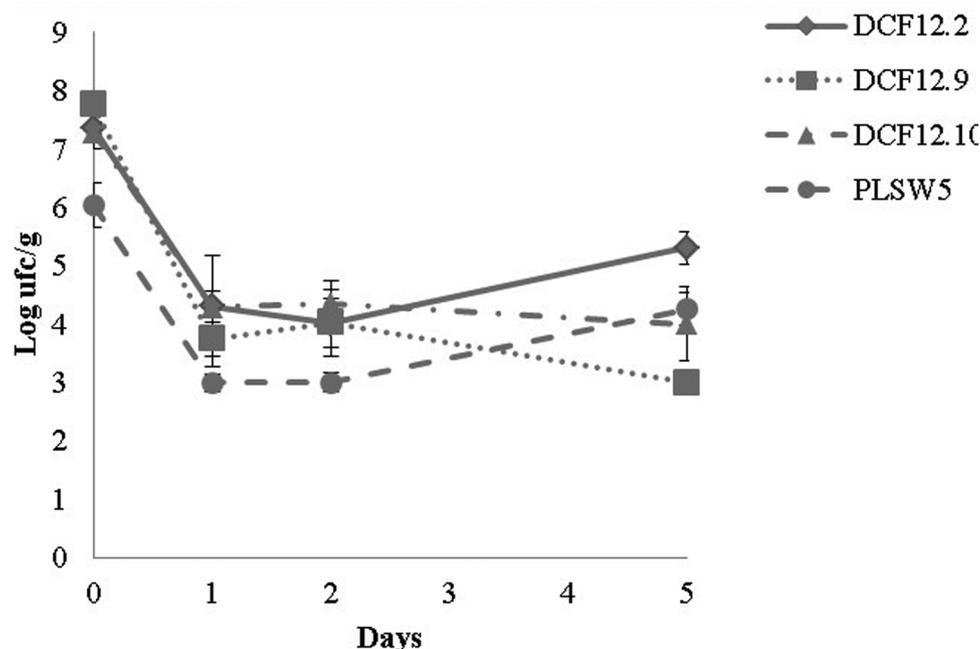

Fig. 4. Strain survival in feed stored at 4 °C for 5 days. Values expressed in log cfu/g of feed.
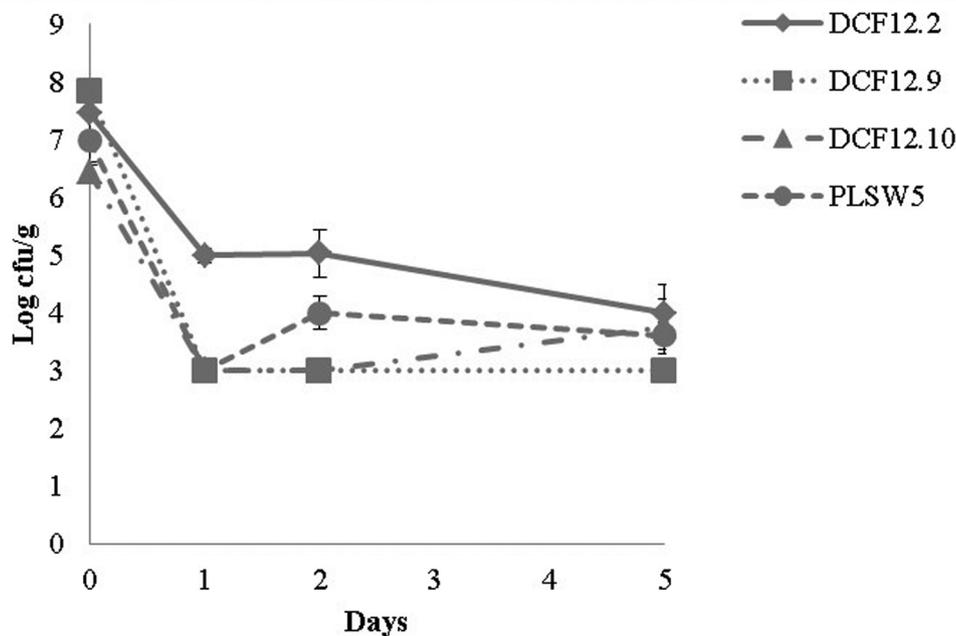
Fig. 5. Strain survival in feed stored at 22 °C for 5 days. Values expressed in log cfu/g of feed.

4.1. Supplementary studies of strains selected as potential probiotics

The selected strains are viable in seawater at 22 °C for several days, so they can be administered to the fish by bath treatment. Bath administration is a good therapeutic route, because the fish are not handled, decreasing stress considerably (Parra et al., 2015).

The evaluation of probiotic survival in fish feed is essential in order to determine the feasibility of oral administration and storability. One of the most important factors to ensure beneficial effect of probiotics is to maintain viability in fish feed (Merrifield et al., 2010), but probiotic viability could decrease with storage temperature (Newaj -Fyzul et al., 2007). The results showed a loss of several orders of magnitude in the viability of bacteria in the first 24 h, both at 22 °C and 4 °C. A decrease of viability was also observed when the strains were frozen. However, a percentage of cells remained viable for several days. The loss of viability in frozen food could be due to the effect of the formation of ice crystals (Kirsop and Doyle, 1991).

Enzymatic activities were studied to determine if the probiotics could facilitate the assimilation of nutrients. Amylase and lipase are enzymes responsible for carbohydrate and fat digestion, so when probiotics colonize the intestine, they improve feed digestibility and facilitate absorption in the intestine (Ray et al., 2012), making them an important factor in the selection of probiotics. These enzymes are also considered a virulence factor (Quesada-Herrera et al., 2004), since various lipases, such as glycerophospholipid-cholesterol acyltransferase (GCAT) and lecithinase-phospholipase C (PLC), have been associated with pathogenicity in fish (Su et al., 2004). The strains selected in this study showed caseinase and gelatinase activity. Caseinase activity has been detected in probiotic bacteria (Mahdhi et al., 2011; Martínez-Hidalgo et al., 2014), especially in bacteria related to the *Bacillus* genus (Mahdhi et al., 2012; Ambas et al., 2015; David et al., 2016). Mahdhi et al. (2012) concluded that caseinase activity is a resistance strategy in the absence of nutrients. Caseinase activity is positive in lactic acid bacteria in dairy products (Cebrian et al., 2012). Gelatinase activity has been identified in probiotic bacteria of the *Bacillus* genus, in bacteria with industrial interest (Su et al., 2017), inhibiting pathogenic bacteria (Banerjee et al., 2007) or enhancing the growth of species with economic interest (Boonthai et al., 2011). However, caseinase and gelatinase activities facilitate infection by pathogens (Ruwandeepika et al., 2012). Lecithinase activity is responsible for phosphatidylcholine hydrolysis and is found in bacteria of the genus *Vibrio* and other genera (Esselmann and Liu, 1961). Although lecithinase activity has been reported as a virulence factor (Koo et al., 2007), it is widely distributed in environmental bacteria (Bumpa et al., 2016; Galach'yants et al., 2016). For example, Bumpa et al. (2016) described lecithinase, caseinase and gelatinase activities in strains of *V. alginolyticus* from seawater and marine sediment. In conclusion, these strains potentially have positive activity in the digestibility of nutrients in feed, one of the most important parameters when selecting probiotics (Ray et al., 2012).

Probiotics must survive in the gastrointestinal system, so acid tolerance is one of the properties to analyse in probiotic bacteria (Perez -Sanchez et al., 2014). The selected strains were cultured in broths with different acidity levels to evaluate the growth at different pH. All strains were affected in media with a low pH, as described in previous studies with various vibrionaceae, such as *Vibrio angillarum* (Frans et al., 2011), *V. cholerae* (Labas et al., 2002), *V. harveyi* (Prayitno and Latchford, 1995), *V. parahaemolyticus* (Wong and Wang, 2004) and *V. vulnificus* (Bang and Drake, 2005). The main digestive activity in sole occurs in the intestine, unlike other fish which have strong acid activity in their stomach (Saenz de Rodrigañez et al., 2005). Yufera and Darias (2007) concluded that the gastric pH of S. *senegalensis* is above 6.0, a value attributable to the omnivorous diet of these fish. The selected strains could then be viable under these pH conditions.

In conclusion, we applied a rapid technique, based on semi-quantitative dot blot, to select bacteria with cross-reaction with the two pathogens studied. Four strains (DCF12.2, DCF12.9, DCF12.10 and PLSW5) were finally selected. These strains showed both the ability to inhibit bacterial growth and the ability to produce antibodies that could react with the pathogens. Moreover, they showed characteristics that make them feasible for use as probiotics, such as avirulence, viability after storage in feed and survival in the pH conditions of fish gut. All these features confer on these bacteria a great potential for dual use as both probiotics and live vaccines that interact with the immune system of fish, generating antibodies that protect against pathogens. Future investigation is needed to verify these characteristics in *in vivo* experiments.

CRediT authorship contribution statement

Alberto Medina: Conceptualization, Methodology, Validation,

Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Miguel Ángel Moriñigo:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Visualization. **Salvador Arijo:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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