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Effects of *Enterococcus faecalis* UGRA10 and the enterocin AS-48 against the fish pathogen *Lactococcus garvieae*. Studies *in vitro* and *in vivo*

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ABSTRACT

The aim of this study was to evaluate the effects of *Enterococcus faecalis* UGRA10 and its enterocin AS-48 against the fish pathogen *Lactococcus garvieae*. The minimum bactericidal concentrations of AS-48 against *L. garvieae* CECT 5807, 5806, and 5274 were 15.62, 15.62, and 7.81 μg/ml respectively. In broth cultures, enterocin at 100, 50, and 25 μg/ml reduced 10^8 CFU/ml lactococci after 2, 5, and 10 h, respectively. In co-cultures of UGRA10/*L. garvieae* at a 1/10 CFU/ml ratio, lactococci were eliminated after 24 h. Studies on UGRA10 biosafety and AS-48 toxicity in R1 cells and in rainbow trout have shown a lack of adverse effects from both the strain and bacteriocin. Trout challenged with *L. garvieae* and UGRA10 administered in diet 30 days before infection had a cumulative survival rate of 50% compared with 0% for control fish. Trout inoculated with the pathogen and treated by regular dipping in AS-48 baths had a survival rate of 60% after 20 days compared with that of untreated fish (0%). These results indicate the protective effect of the UGRA10 strain and the bacteriocin AS-48 against *L. garvieae* and the potential of these natural products as alternatives to antibiotics for controlling diseases in aquaculture.

1. Introduction

Aquaculture is an effective tool for the intensive production of aquatic organisms under controlled conditions. However, extremely dense fish populations in farms lead to severe stress that, in turn, can cause economic and productivity losses due to an increase in infectious diseases (Toranzo et al., 2005).

Lactococcus garvieae is a Gram-positive coccus, aerotolerant, non-spore-former, producing haemolysis on blood agar; it is an emerging zoonotic pathogen that causes serious infections (lactococcosis) in humans and animals (Vendrell et al., 2006). It has been shown to cause fish diseases with a high mortality rate among wild and cultured fish, including rainbow trout (*Oncorhynchus mykiss*). Lactococcosis causes a variety of clinical signs such as anorexia, melanosis, lethargy, conjunctivitis, haemorrhage, and septicemia (Vendrell et al., 2006). This pathology has huge economic and health consequences for the aquaculture sector in Mediterranean countries, particularly in Spain, the leading trout producer in the European Union (FAO, 2018).

In recent years, the prevention and control of fish diseases such as lactococcosis have focused on the use of antibiotics, which generate significant risks to public health by promoting the selection of resistant strains (Aly and Albutti, 2014) and leaving antibiotic residues in water and animal tissues (Vendrell et al., 2012). Furthermore, consumers are increasingly demanding natural products, free of additives such as antibiotics.

Nowadays, vigorous endeavours to prevent these diseases focus on good husbandry techniques and vaccination. Although these methods can improve fish health, they unfortunately appear to be insufficient to prevent disease outbreaks. Furthermore, vaccines are often expensive and highly stressful for the animals (Corripio-Miyar et al., 2007). Consequently, the use of probiotic bacteria is gaining popularity in the aquaculture industry as a sustainable alternative to antibiotic use (Pérez-Sánchez et al., 2013). The majority of probiotics used in aquaculture belong to lactic acid bacteria (LAB) (Gatesoupe, 2008). The species *Pediococcus pentosaceus* (Huang et al., 2014), *Lactobacillus plantarum, Lactococcus lactis*, and *Enterococcus faecium* (Kim et al., 2012; Beck et al., 2015; Dash et al., 2015) are examples of LAB considered for use in aquatic organisms. Probiotic use in aquaculture offers a wide variety of important health benefits for the host (Tinh et al., 2008). Some probiotic mechanisms of action could be explained by

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competitive exclusion (Balcázar et al., 2006), enzymatic contribution to digestion (Musa et al., 2009), immune response enhancement (Huang et al., 2014), or the production of antimicrobial substances such as lysozyme, hydrogen peroxide, or bacteriocins that constitute a barrier against the proliferation of pathogens (Verschuere et al., 2000).

Bacteriocin are ribosomally synthesized antimicrobial peptides produced by bacteria that are active against more or less related bacteria; either in the same species (narrow spectrum) or across genera (broad spectrum) (Cotter et al., 2005). Lantibiotic bacteriocins such as nisin, pediocin AcH, or several enterocins are efficient weapons to protect the intestines from pathogenic invasions, so the production of theses antimicrobial peptides by a strain constitutes a pertinent feature for selecting a probiotic (Desriac et al., 2010). Therefore, bacteriocinogenic strains appear to be excellent candidates since bacteriocins would be used as antimicrobial alternatives, whereas bacteria would be used as probiotics (Gillor et al., 2008).

Enterocin AS-48 is a cationic circular bacteriocin mainly produced by *Enterococcus faecalis* strains with broad bactericidal activity against most Gram-positive bacteria, including pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Mycobacterium* spp., and some Gram-negative bacteria (Gálvez et al., 1989). The target of AS-48 is the bacterial cell membrane, in which the bacteriocin inserts itself and destabilizes the membrane potential, thus leading to pore formation and cell leakage (Gálvez et al., 1991). AS-48 possesses several interesting technological properties (a broad spectrum of antimicrobial activity, stability across a wide range of temperatures and pH, and sensitivity to digestive proteases; Gálvez et al., 1986) that make it a promising alternative to classic chemical preservatives.

In a previous work, an enterocin AS-48 producing strain, *E. faecalis* UGRA10, was isolated from a Spanish sheep's cheese (Cebrian et al., 2012), conducting an extensive study on its *in-vitro* functional, biosafety, and probiotic characteristics. The current study aims to evaluate *in-vitro* antimicrobial effects of the UGRA10 strain and its enterocin AS-48 against pathogenic strains of *L. garvieae* and *in-vivo* effectiveness of both, strain and bacteriocin, to protect rainbow trout (*Oncorhynchus mykiss*) against this pathogen.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Enterococcus faecalis UGRA10 was used as enterocin AS-48 producer. *E. faecalis* S-47 from our collection was used as the standard/control indicator strain for bacteriocin activity assays. *L. garvieae* CECT 5807, *L. garvieae* CECT 5274, and *L. garvieae* CECT 5806 (supplied by the Spanish Type Culture Collection — CECT) were used as target organisms for *in-vitro* studies. For fish infection, *L. garvieae* CECT 5274 was used. All bacterial strains were grown routinely in Brain Heart Infusion broth (BHI, Scharlau, Barcelona, Spain) at 28 °C and were maintained at 4 °C on Brain Heart Infusion agar (BHA) slants. Slants were renewed monthly.

2.2. Cells

R1 cell line (ACC 56), derived from Chinook salmon (*Oncorhynchus tshawytscha*) liver, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), grown at 20 °C in 90% Medium 199 (25 mM HEPES) with Earle's salts and L-glutamine (Sigma-Aldrich, Madrid, Spain), supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% penicillin (100 U/ml), and streptomycin (100 µg/ml). For cell viability assays, cells were seeded in 96-well tissue culture plates at a density of 1×10^4 cells/well in 200 µl medium.

2.3. Preparation of E. faecalis UGRA10 and bacteriocin AS-48

AS-48 was produced by culturing the strain *E. faecalis* UGRA10 in a whey-derived substrate, Esprion 300 (E − 300) (DMV Int., Veghel, Netherlands), supplemented with 1% glucose (Ananou et al., 2008). AS-48 was recovered from cultures by cation exchange chromatography on carboxymethyl Sephadex CM-25 (as described by Abriouel et al., 2003). Eluted fractions were tested for bacteriocin activity against the indicator strain S-47 by the agar well diffusion method (Gálvez et al., 1986). The approximate concentration of AS-48 (in µg/ml) in the preparation was estimated by comparing the diameter of the inhibition halo around the well with a titration curve obtained from purified bacteriocin. Before use, the eluted fractions were dialysed at 4 °C against distilled water through a 2000-Da cut-off membrane to eliminate NaCl, and then sterilized by filtration (0.22 µm, Millipore, Belford, MA, USA).

2.4. Minimum bactericidal concentration (MBC)

A broth microdilution susceptibility assay was used to determine minimum bactericidal concentration (MBC), as recommended by the National Committee for Clinical Laboratory Standards (CLSI, 2012). All tests were performed in Mueller-Hinton broth (Scharlau). Target bacteria were cultured overnight at 25 °C. Enterocin AS-48 dilutions (1:1; v/v) were prepared in 96-well microtiter plates to achieve final concentrations ranging from 0.1 to 500 µg/ml. Afterwards, each well received 10 µl of bacterial suspension with 10^7 CFU/ml and 90 µl of Mueller-Hinton broth. Bacteria were aerobically incubated at 28 °C for 24 h. Concentrations with a complete absence of visual growth were identified, and 100 µl of each culture were transferred onto Columbia blood agar (Scharlau) plates and incubated under the same conditions of time and temperature as mentioned above. The complete absence of growth on the agar surface at the lowest concentration of AS-48 was defined as the MBC. Assays were conducted in duplicate.

2.5. Time-kill curves

Time-kill curves were performed following the procedure described by Guerillot et al. (1993). Each strain was grown overnight in Mueller-Hinton broth (Scharlau). Then, the initial number of bacteria was adjusted to 10^8 CFU/ml. This suspension was used to perform separate assays in sterile glass flasks. Each assay mixture contained bacterial suspension and different concentrations of AS-48 (100, 50, and 25 µg/ml). A flask without AS-48 was used as a control. The flasks were incubated aerobically at 25 °C with constant agitation. Samples were collected at intervals of 1, 2, 3, 4, 5, and 10 h, then serially diluted, plated on Columbia blood agar, and incubated at 25 °C for 24–48 h. Kill curves are represented by expressing the results of log CFU/ml against time.

2.6. Antagonism assays in co-cultures

Overnight cultures of *E. faecalis* UGRA10 and *L. garvieae* strains were diluted at 10^4 CFU/ml for UGRA10 and at 10^5 UFC/ml for *L. garvieae* into sterile BHI, individually and in co-culture, and incubated at 25 °C. At selected times of 1, 3, 6, 12, and 24 h, samples were collected and serially diluted into sterile saline solution. Dilutions were plated in triplicate on Slanetz-Bartley agar (Scharlau) and LG-agar (Chang et al., 2014) for quantification of enterococci and lactococci, respectively, and the average numbers of colonies obtained after 48 h incubation at 25 °C were used to establish the growth curves of the bacteria cultured alone or in co-culture.

2.7. Diet preparation

UGRA10 strain was grown in BHI at 30 °C overnight with agitation in a shaking incubator. After incubation, cells were harvested by centrifugation (10,000 × *g* at 4 °C for 5 min), washed twice with phosphate buffer saline (PBS, Sigma-Aldrich, Madrid, Spain), and resuspended in

the same buffer. Then, the bacterial suspension was adjusted to 10^9 CFU/ml. A commercial pelleted high protein feed (Inicio Plus, BioMar Iberia, Spain) was used as the basal diet for supplementation with the UGRA10 strain. Enterococcal suspension was added into the feed, reaching a final concentration of 10^8 CFU/g feed. Bacterial concentration in the diet was determined by plate counting onto Slanetz-Bartley agar.

2.8. Animals

Unvaccinated rainbow trout (*Oncorhynchus mykiss*) with an average weight of approximately 50 g were obtained from a commercial fish farm (Piscifactorías Andaluzas, Granada, Spain). The fish were acclimatized for 2 weeks in 100-l tanks before the start of the trial. All fish were maintained in continuously aerated water at 12 °C with a 12-h light-dark cycle, and 25% water replenishment every day. The fish were hand-fed with a commercial pelleted high protein feed (Inicio Plus, BioMar Iberia) at a rate of 1.5% of biomass per day.

2.9. E. faecalis UGRA10 biosafety assay

Twenty fish were randomly distributed into two 100 l-litre tanks (10 per group). One tank was UGRA10 administered to a final concentration in water of 10^9 CFU/ml. The non-UGRA10 group was the control. For a period of 15 days, the health status of the animals was monitored, recording mortality and identifying visible clinical signs such as anorexia, lethargy, exophthalmia, erratic swimming, gasping at the surface, or skin irritations. Then, fish were sacrificed by immersion in a tricaine methasulfonate (MS-222) overdose (> 300 mg/ml) solution and the heart and spleen were removed in sterile conditions. For *E. faecalis* UGRA10 determination, organs were mixed (1:10; w:v) with PBS and homogenized by forcing tissues up and down through a 1.0 ml syringe. Afterwards, homogenate was enriched in BHI and finally plated on Slanetz-Bartley agar. Plates were incubated at 30 °C for 48 h before colony counting. The assay was performed in duplicate.

2.10. AS-48 toxicity assay

2.10.1. In R1 cell line

An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed to determine the viability of R1 cells exposed to enterocin AS-48, according to Mossman (1983). Cells were seeded in 96-well plates and exposed to enterocin AS-48 at different concentrations (0.5–100 μg/ml). Following 24 h of exposure of the cells to bacteriocin, the medium was discarded and 200 μl of MTT solution (5 mg/ml) was added to the cells. After 4 h of incubation, the MTT solution was removed, and the intracellular purple insoluble formazan was solubilized by the addition of 100 μl/well of DMSO. Optical density (OD) was measured at 570 nm using a microplate reader (Multiskan EX, Thermo Electron Corporation).

2.10.2. In trout

In order to determine whether the lethal concentration of 50 (LC50) was higher than the assayed therapeutic dose of AS-48, a limit test was performed using the procedures described in the guidelines for the testing of chemicals from the Organization for Economic Cooperation and Development (OECD, 2013). To that end, 20 fish were randomly transferred into two continuously aerated 100-l tanks for seven days until acclimatized. Test conditions included a water temperature of 24–25 °C, a pH of 7.5, and 12-h light-dark cycles.

For the limit test, a group of 10 fish was exposed to AS-48 (100 μg/ml) for 96 h. During the exposure time, the fish were not fed. Other tanks without treatment served as the control group. The fish were inspected at a minimum after 24, 48, 72, and 96 h. Visible abnormalities (and mortalities) were recorded. The assay was performed in duplicate.

2.11. Oral administration of E. faecalis UGRA10 to challenged trout

Fish were divided into four different tanks (ten animals per tank). For 30 days, all animals were fed daily with a commercial fish diet, except for a group that was fed with a UGRA10-supplemented diet (described in Section 2.8). Afterwards, the fish were anaesthetized with 100 mg/l of MS-222 and three individual fish of each group (one of them receiving UGRA10 in diet) were challenged by intraperitoneal injection with 20 μl of *L. garvieae* CECT 5274 cells suspended in PBS at a dose of 5×10^8 CFU/fish.

The bacterial concentration in the inoculum was determined by ten-fold serial dilutions and further plating onto Columbia blood agar (Scharlau). The fourth group of fish was inoculated with 20 μl of sterile PBS as a negative control for infection. After inoculation, the group that previously received UGRA10 supplementation continued with the same diet, whereas another group of 10 animals received a UGRA10-supplemented diet from day 0 of the infection. The control groups (positive and negative for infection) continued on the commercial diet. The fish were observed twice a day for seven days after the infection and mortality was recorded every day for 20 days.

2.12. AS-48 treatments of challenged trout

After the acclimatization period, 80 trout were divided into four two hundred-litre tanks, each containing 20 fish. Three groups of fish were infected by intraperitoneal injections of 20 μl of *L. garvieae* CECT 5274 cells suspended in PBS at a dose of 5×10^8 CFU/fish (a dose established experimentally by the authors). Afterwards, one group received an AS-48 solution intraperitoneally (a total of 100 μg), a second group received regular (every 3 days) baths for 30 min in a solution of AS-48 (12.5 μg/ml), and a third group received no treatment (positive control). A fourth group received the same volume of physiological saline solution (negative control). Mortality was monitored for 20 days, after which no more dead fish were observed.

All animal experimental procedures were conducted in accordance with European Union legislation on animal experimentation and were carried out according to the guidelines of the Helsinki declaration (WMA, 2013).

2.13. Statistical analysis

All results are expressed as the mean ± standard deviation (SD). Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA). The differences between treated and control groups were analysed using the Student's *t*-test. Survival curves were estimated by the Kaplan–Meier method and compared by the log-rank test. All statistics were performed using SPSS-PC 15.0 software (SPSS, Chicago, Ill. USA), and the statistical significance level was accepted at $P < 0.05$.

3. Results

3.1. In-vitro antimicrobial assays

The enterocin AS-48 exhibited potent bactericidal activity with an MBC value of 15.62 μg/ml against *L. garvieae* CECT 5807 and *L. garvieae* CECT 5806. This antimicrobial activity was especially strong against *L. garvieae* CECT 5274, with an MBC of 7.91 μg/ml of AS-48.

The time-kill curves of AS-48 (added at 100, 50, and 25 μg/ml) against *L. garvieae* CECT 5807, *L. garvieae* CECT 5806, and *L. garvieae* CECT 5274 are shown in Fig. 1. The addition of AS-48 to cultures of any *L. garvieae* strain resulted in a drastic mortality of bacteria that was AS-48 concentration-dependent. In fact, after just 60 min of exposure to AS-48 (100 μg/ml), the viability of *L. garvieae* CECT 5807, *L. garvieae* CECT 5806, and *L. garvieae* CECT 5274 declined from 7.8 to 2.2, from 8.1 to 2.5, and from 7.7 to 1.5 log CFU/ml, respectively. After 2 h, the

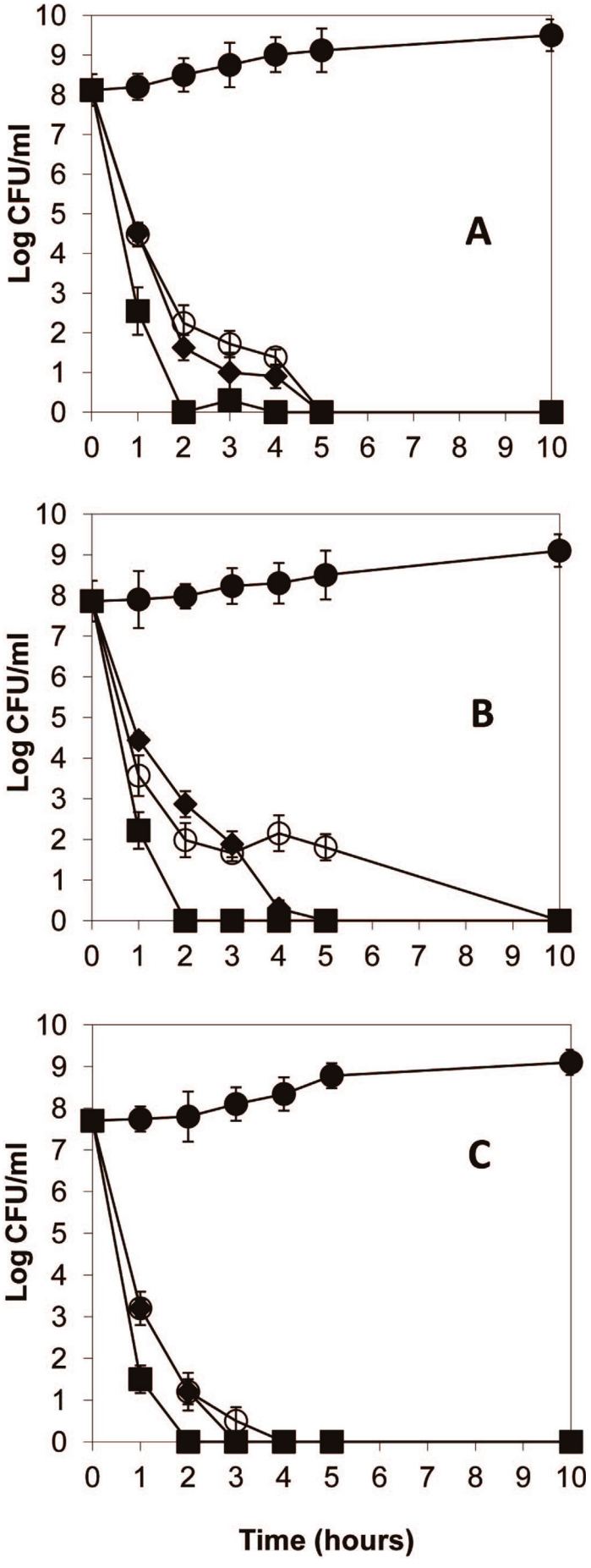

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Fig. 1. Effect of enterocin AS-48 on the viability of *Lactococcus garvieae* CECT 5806 (A), *L. garvieae* CECT 5807 (B), and *L. garvieae* CECT 5274 (C). The bacteriocin was added to cultures of *L. garvieae* in BHI broth at concentrations of 100 (■), 50 (♦), and 25 (○) μg/ml. Values are the average ± standard deviation (error bars) of two independent experiments.

three strains were completely inhibited at this AS-48 concentration. At 50 μg/ml of AS-48, a total inhibition of *L. garvieae* CECT 5274 was observed after 3 h, whereas for *L. garvieae* CECT 5807 and *L. garvieae* CECT 5806, 5 h of exposure were necessary. At 25 μg/ml, the total elimination of theCECT 5274 and CECT 5806 strains occurred after 3 h and 5 h respectively. For L. *garvieae* CECT 5807, complete inhibition occurred after 5–10 h of exposure.

In order to test the antagonism of *E. faecalis* UGRA10 towards *L. garvieae* strains, a co-culture model was established of each *Lactococcus* strain (10^5 CFU/ml initial concentration) with the enterococci (10^4 CFU/ml initial concentration) in BHI broth. The results reported in Fig. 2 reveal that *L. garvieae* strains in pure culture show a normal pattern of growth, and after 12 h, the culture enters the exponential growth phase to reach approximately 9 log CFU/ml in the stationary phase at 24 h. When *E. faecalis* UGRA10 was co-cultivated with any of the *L. garvieae* strains, an evident antagonistic interaction was observed. In all cases, the presence of the bacteriocinogenic strain caused reductions in *Lactococcus* viability from 12 h of co-culturing, when lactococci started exponential growth in pure cultures. The reductions in *Lactococcus* counts caused by the presence of enterococci were significant ($P < 0.01$) after 12 h of co-cultivation, when viable numbers of the pathogen fell dramatically to below detection levels at 24 h, remaining undetectable until the end of the experiment (48 h).

3.2. UGRA 10 safety assays

According to the EFSA approach a great number of LAB species are included in the Qualified Presumption of Safety (QPS) list (EFSA, 2011). However, in the case of *Enterococcus*, a more thorough, strain-specific evaluation is required to assess the risks associated to their intentional use in the food chain (EFSA, 2011). The safety aspects of UGRA10 (extensively investigated by both *in-vitro* studies and *in-vivo* experimental models in mice; Cebrian et al., 2012; Baños et al., 2015) have revealed that this strain can be considered as safe. Nevertheless, to be more certain regarding this controversial aspect of enterococci, the potential capacity of UGRA10 to produce any disease in fish was evaluated, using a trout model. Once again, the results demonstrate the safety of this strain as no deaths or visible signs of disease such as anorexia, lethargy, exophthalmia, erratic swimming, gasping at the surface, or skin irritations were seen in fish. Neither bacteraemia nor translocation of enterococci from the aquatic medium to fish organs (heart and spleen) was observed in any of the experimental groups (results not shown).

3.3. AS-48 toxicity assays

A. In cell line R1. The viability test carried out in the fish cell line R1 showed that enterocin AS-48 did not have cytotoxicity on this cell line for concentrations from 0.5 up to 100 μg/ml. Cell viability (%) of R1 cultures treated with AS-48 was 98.03, 97.06, 97.03, and 90.08 for AS-48 concentrations of 0.5, 1, 10, and 100 μg/ml respectively. In all cases, the differences were significant.

B. In trout. No visible signs of abnormality/toxicity (anorexia, lethargy, exophthalmia, erratic swimming, gasping at the surface, skin irritations) were observed in any of the 10 fish exposed to 100 μg/ml AS-48 for 96 h. In agreement with this finding, the survival rate of fish exposed to AS-48 for 96 h was 100%. According to the OECD (2013), binomial theory dictates that, when 10 fish are used with zero mortality, there is a 99.9% confidence rate that the LC50 is greater than 100 μg/ml and hence greater than the therapeutic dose chosen (12.5 μg/ml).

3.4. Challenge tests

In order to investigate the potential role that *E. faecalis* UGRA10 or its bacteriocin AS-48 may play in the control of lactococcosis in fish, challenge tests were conducted on rainbow trout.

Oral administration of E. faecalis UGRA10. Results of mortality rates for a 30-day period point to a protective effect of the UGRA10 strain against infection caused by *L. garvieae* CECT 5274. Results comparing the survival rate of the two groups fed the UGRA10 strain with that of the control group that received no treatment (positive control) and with the uninfected control group (negative control) are presented in Fig. 3 as the cumulative survival rate. As can be observed, 100% of fish in the control group that received no treatment died. In contrast, infected fish that received UGRA10 strain (10^8 CFU/g feed) for 30 d before the infection appeared to be significantly more protected ($P < 0.05$) than the controls, with these animals having a cumulative survival rate of 50%. The animals that started the diet after infection showed a slight delay in the onset of the disease although mortality at 15 days was 100%. These results confirm the usefulness of applying the UGRA10 strain for preventive treatment against infections caused by *L. garvieae*.

Bath treatment and intraperitoneal administration of enterocin AS-48. This test was intended to investigate the potential role of enterocin AS-48 (produced by *E. faecalis* UGRA10) in controlling lactococcosis in fish. In this test, AS-48 was administered in two different ways, either by intraperitoneal injection (100 μg) or by regular bathing with an AS-48 solution (12.5 μg/ml). Both treatments were applied just after infection with *L. garvieae*. The survival rates for both AS-48 treated groups were compared with a control group that received no treatment (positive control) and with an uninfected control group (negative control). For a 20-day period, mortality rates were recorded for each group, and the results are presented as the cumulative survival rate (Fig. 4). Three days after infection, fish survival rate in the control group (inoculated with *L. garvieae* and untreated with the UGRA10 strain) was 80% compared to a 100% survival rate for the group that received AS-48 intraperitoneally and to an 90% survival rate for the group of fish infected after the first AS-48 bath. After one week, the control group showed a 30% survival rate compared to a 80% survival rate for the group that received regular AS-48 baths (after 2 baths), and a 100% survival rate for the group treated intraperitoneally with AS-48. Twelve days after infection, all animals in the control group had died. The group of animals treated with regular AS-48 baths had a survival rate of 70% (after 3 baths), and the survival rate for the group of fish that received a single dose of AS-48 intraperitoneally was 60%. At the end of the study (day 20), the survival rate of the group treated with AS-48 baths was 60% (after 6 baths) compared with a 30% survival rate for the group that received AS-48 intraperitoneally.

Statistical analysis shows that fish that received the treatment based on immersion baths of AS-48 experienced a significantly higher survival rate (60%, $P < 0.01$) than the control group. The intraperitoneal application of AS-48 also exerted a protective effect, showing a survival rate of 30% by the end of the test. This method of application delayed the onset of infection by about one week, but once unleashed, the mortality rates were quite similar to those of the control group.

4. Discussion

In aquaculture, lactococcosis produced by *L. garvieae* is one of the most serious diseases for both marine and freshwater cultured species, including rainbow trout (Vendrell et al., 2006). Since antibiotic use has been shown to generate significant risks to public health (selection of resistant strains, presence of antibiotic residues in water and animal

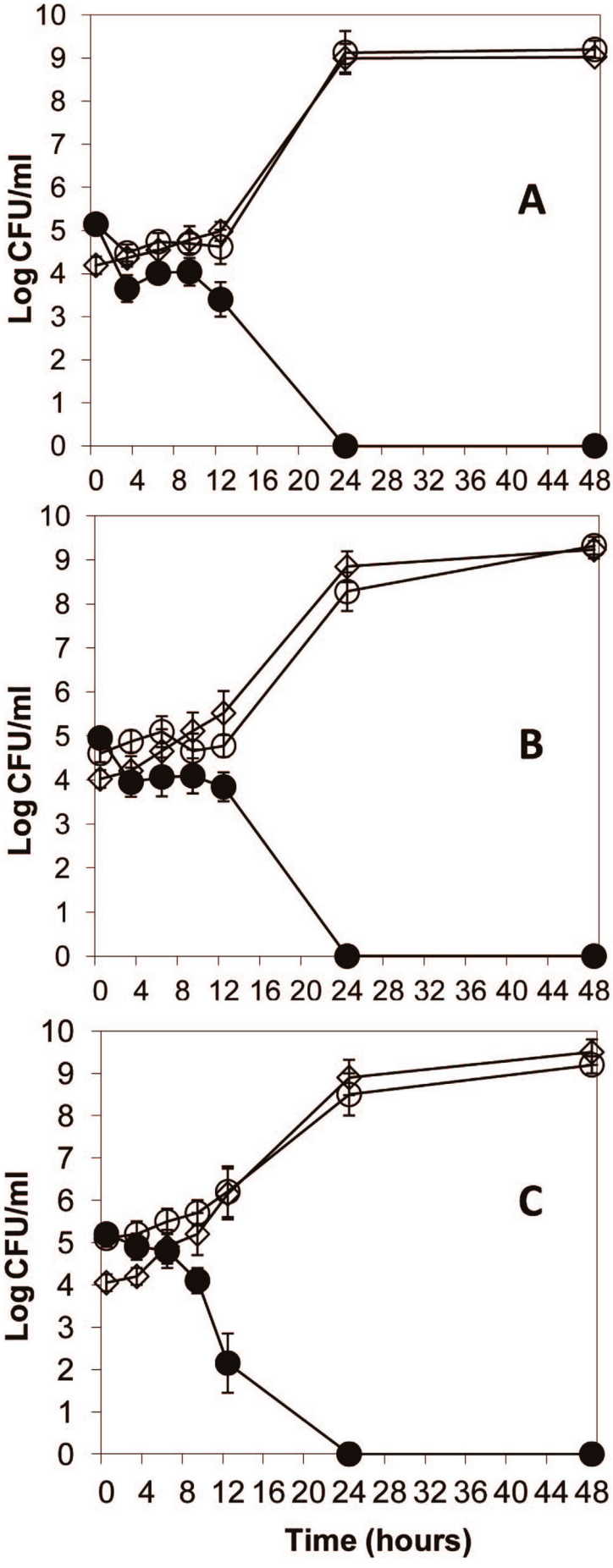

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Fig. 2. Growth of *Lactococcus garvieae* CECT 5806 (A), *L. garvieae* CECT 5807 (B), and *L. garvieae* CECT 5274 (C) in BHI broth, in pure cultures, and in co-cultures with *Enterococcus faecalis* UGRA10. *L. garvieae* in co-culture (•), *L. garvieae* in solitary (o), and *E. faecalis* UGRA10 in co-culture (⋄). Values are the average ± standard deviation (error bars) of two independent experiments.

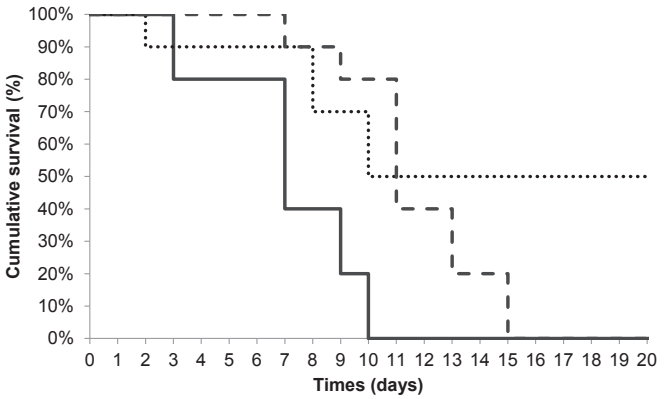

Fig. 3. Survival curves of rainbow trout challenged with *Lactococcus garvieae* CECT 5274 and administered *Enterococcus faecalis* UGRA10 (10^8 CFU/g feed) in different conditions. Control not administered with UGRA10 (−), administered with a UGRA10 supplemented diet for 10 d before the challenge (·····), and administered with a UGRA10 supplemented diet just after the challenge (—). The mortality rate was calculated according to the Kaplan–Meier method.

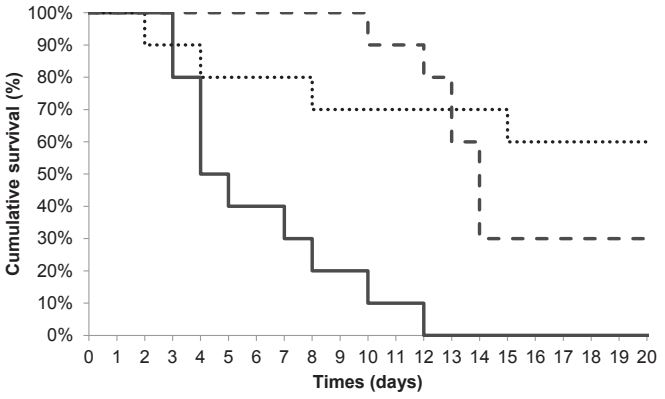

Fig. 4. Survival curves of rainbow trout challenged with *Lactococcus garvieae* CECT 5274. Control (−), regular bathing with AS-48 solution (12.5 µg/ml) (·····), and intraperitoneal AS-48 (100 µg) (—). The mortality rate in the group that received regular AS-48 baths was significantly lower ($P < 0.01$) than that of the control group, according to the Kaplan–Meier method.

tissues), prevention of this disease has focused on complementary approaches such as good husbandry techniques and vaccination. Unfortunately, these methods appear to be insufficient to completely prevent disease outbreaks. For all these reasons, the use of probiotic bacteria is gaining popularity in the aquaculture industry as a sustainable alternative to antibiotic use (Balcázar et a., 2006; Pérez-Sánchez et al., 2013). Moreover, the use of bacteriocins as a natural alternative to classic antibiotics in veterinary medicine and/or aquaculture has also been highlighted by some authors (Montalbán-López et al., 2011; Nishie et al., 2012).

Our study shows the anti-infective activity against *L. garvieae* of *E. faecalis* UGRA10 and the bacteriocin produced by this enterococcal strain, the enterocin AS-48. In addition, in order to confirm their usefulness in the prevention of lactococcosis, possible adverse effects of the UGRA10 strain and AS-48 administration were investigated in rainbow trout. Knowledge about LAB strains with antagonistic activity against fish pathogens like *L. garvieae* is still new and limited (Araújo et al., 2015b; Ben Braïek et al., 2017; Lin et al., 2013; Muñoz-Atienza et al., 2013). Due to the inherent difficulty of experiments in aquatic environments, *in-vivo* assays showing the anti-infective role of selected probiotic bacteria against lactococcosis are also scarce. Vendrell et al. (2008), for instance, reported three protective effects of *Lb. plantarum* CLFP 238 against *L. garvieae* infections in rainbow trout. In addition, Pérez-Sánchez et al. (2011) showed that supplementing fish feed with a bacteriocinogenic strain of *Lb. plantarum* reduced the mortality rate in rainbow trout infected with this pathogen. Another bacteriocin with potential applications in aquaculture due to its activity against *L. garvieae* is garvicin A (Maldonado-Barragán et al., 2013). Nevertheless, while it has been suggested that LAB bacteriocins may be one of the most powerful weapons in the fight against these fish pathogens (Desriac et al., 2010), there are still few fish studies investigating the relationship between bacteriocin production by a strain and its role as a defence mechanism against infections. Worth noting are the *in-vivo* studies carried out by Araújo et al. (2015a,b) that showed the role of nisin Z production as the main anti-infective mechanism of *Lb. cremoris* WA2-67 against *L. garvieae.*

The results of this study shown the ability of *E. faecalis* UGRA10 to inhibit *L. garvieae in vitro* in co-culture and *in vivo* in experimental infections, significantly reducing mortality in rainbow trout previously fed a diet supplemented with UGRA10 strain for 30 d before infection. The continued administration of UGRA10 strain seems to enable better colonization in the animal gastrointestinal tract, exerting a protective role against pathogen invasion. The mechanisms involved could be related to the ability of UGRA10 to produce AS-48, and even with immunomodulatory effects that could help to improve the animal's natural defence mechanisms (Baños, 2015).

The effectiveness of purified enterocin AS-48 against different strains of *L. garvieae* has been also shown through *in-vitro* and *in-vivo* studies. *In-vivo* AS-48 showed anti-lactococcal activity preventing the lethal effects of the pathogen in trout when applied by immersion in an AS-48 bath or by intraperitoneal injection. This is the first time that the effect of the UGRA10 strain and the bacteriocin AS-48 has been described in the protection against an infectious disease in animals. These results suggest that treatments of bath immersions in AS-48 solutions sustained over time might become an efficient therapy for the control of lactococcosis in rainbow trout although the most efficient means of application remains to be more accurately determined. The application of AS-48 in this field is supported by the fact of having a simple, two-step method to obtain semi-purified AS-48 from an economic food-grade substrate, Esprion 300, a whey derivative (Ananou et al., 2008). This procedure has been now scaled to the pilot plant level, which will undoubtedly make obtaining and applying AS-48 more feasible. The role of AS-48 in the preventive anti-infective effect of UGRA10 have not determined since it not known whether AS-48 is produced in the gut of animals administered this strain. At this respect, AS-48 is produced in a wide-ranging types of media (broths, whey derivatives, dairy and meat products), so it is probable (although speculative) that the production of the bacteriocin also occurs in the gut. If this is so, AS-48 would play an important role in the lactococcosis prevention exerted by UGRA10 strain.

Moreover, with an eye to the application of the UGRA10 strain and its bacteriocin AS-48 in the prevention of lactoccocosis, possible toxic effects of both agents were investigated. Although bacteriocins are generally considered safe and harmless, very little is known about their application in aquaculture, as well as possible adverse effects on the general health of fish. UGRA10 is a lactic acid bacterium, which are recognized as QPS, but since it belong to the genus *Enterocccus*, a specific study was deemed advisable. AS-48 applied in bath treatments using concentration limit values (up to 100 µg/ml) established according to the guidelines for the testing of chemicals (OECD, 2013) had

no toxic effect on the trout. According to the method cited, DL50 for AS-48 would be significantly higher than the therapeutic dose chosen (12.5 μg/ml). UGRA10 did not produce any visible toxic effect nor abnormalities. Neither were bacteraemia nor translocation of enterococci from the aquatic medium to fish heart and spleen observed.

Therefore, *E. faecalis* UGRA10, an AS-48 producer strain with some proven probiotic properties, represents a promising alternative for application as an additive in fish feed for prevention of disease caused by *L. garvieae*. Furthermore, the application of fully or partially purified AS-48 in baths could be a safe and sustainable tool to prevent and control lactococcosis in fish farms. Overall, the results presented in this work provide a new testing ground to develop novel experiments that confirm the safety and usefulness of UGRA10 and its bacteriocin AS-48 in aquaculture for the control of fish pathogens and establish the most appropriate means of application.

5. Conclusions

The bacteriocin AS-48 administered in trout challenged with *L. garvieae* has a protective effect against lactococcosis. Likewise, the supplementation of diet with *E. faecalis* UGRA10 protects the animals from infection by this pathogen. These results, together with the lack of adverse effects of both the bacteriocin and UGRA10 strain in trout, support their use in the treatment/prevention of diseases caused by this bacterial pathogen.

Conflicts of interest

The authors declare no conflict of interest.

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