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From the isolation of bacteriocinogenic LAB strains to the application for fish paste biopreservation

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Abstract

The aims were to isolate, characterize and quantifylactic acid bacteria's (LAB) antimicrobial activity, and evaluate their application on fish paste. One hundred and thirty two LAB were isolated from mussels of the Argentine coast. From all, 22 LAB isolated which's cell-free supernatant (CFS) presented activity against *Listeria innocua* and *Lactobacillus plantarum* were identified as *Enterococcus mundtii* by biochemical tests and 16S rDNA gene phylogenetic analyses. None of selected strains presented virulence factors. All of them presented the gene that codifies either for mundticin KS, or for mundticin KS and enterocin L50A. Their CFS showed activity against Gram positive bacteria. The interaction among binary mixtures of CFS was assessed against *L. innocua*, and an additive response was observed. The strain *E. mundtii* STw38 was selected because of its high capacity to grow and produce bacteriocins at refrigeration temperatures. It was applied as a protective culture to fish paste stored at 4°C. In a first stage fish paste-systems were air-packed, and then, vacuum-packed. In both cases, *E. mundtii* STw38 survived storage conditions and succeeded in reducing the development of native flora of fish paste. Results suggest that *E. mundtii* STw38 is a promising strain to be used for fish biopreservation.

Keywords: bacteriocin, *Enterococcus mundtii*, fish biopreservation, 16S rRNA gene, bioprotective cultures

1. Introduction

Fish is very prone to fast deterioration given its high proportion of free amino-acids, free nitrogenous compounds and high post-mortem pH (dos Reis et al., 2011). Autolytic reactions occur immediately after fish's capture and cause a loss in the characteristic flavor. Additionally, microbial growth also occurs which speeds fish's deterioration (FAO,1995).

Provided fish's highly perishable nature, in last years, many efforts have been done to increase its shelf life (Sivertsvik, Jeksrud, & Rosnes, 2002; Bashir, Kim, An, Sohn, & Choi, 2017). The latter implies the investigation on new alternatives, such as the use of modified atmosphere and the incorporation of natural preservatives responding tocustomers' demand for natural antimicrobial agents. In this context, lactic acid bacteria (LAB) have been applied to improve fish shelf life(Ahmad et al., 2017; Woraprayote et al., 2016). They are Gram positive bacteria which produce ribosomally synthesized peptides called bacteriocins, with antagonist action against a wide range of bacteria, but mainly against other taxonomically close ones (Schelegueda, Vallejo, Gliemmo, Marguet, & Campos, 2015). Additionally, when applied as protective cultures they usually compete against native flora of fish.

Amongst LAB, *Enterococcus* genre is of particular interest since they produce bacteriocins, known as enterocins, which have antagonist activity against a wide range microorganisms (De Kwaadsteniet, Todorov, Knoetze, & Dicks, 2005). Despite this, they have recently been removed from FAO's GRAS list, for being associated with food borne diseases (Khan, Flint, & Yu, 2010). Nevertheless, this behavior is strain related, and does not involve the whole genre (Franz, Stiles, Schleifer, & Holzapfel, 2003). As an example, Eaton and Gasson (2001) determined that food borne *Enterococcus* possessed less virulence determinants than medical strains. On the other hand, enterococci have a long history of safe use. For example, they have been applied in cheese production, due to their capacity to produce desired sensory characteristics and bacteriocins which may act against food-borne pathogens (Giraffa, 2003).Finally, it is important

to highlight that *Enterococcus mundtii*, does not generally present virulence factors (Giraffa, 2003; Schelegueda, Zalazar, Gliemmo, & Campos, 2016). Considering previously cited safety status of enterococci, *E. mundtii* may be a suitable LAB to be applied on food preservation.

Given this panorama, the objective of the present study was to isolate, identify, and assess the safety of LAB from Argentina's Patagonian area to be used as protective cultures on fish paste.

2. Materials and Methods

2.1 Indicator strains and culture conditions

Listeria innocua ATTC 33090 was used as surrogate for pathogen *L. monocytogenes* given its similar response to stress factors (Friedly et al., 2008).In order to represent the deteriorative bacteria present in fish, *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas fluorescens* ATCC 49838, *Lactobacillus plantarum* ATCC 8014, and *Shewanella putrefaciens* ATCC 8071 were used throughout the assays. All of them were stored at -30°C on Tryptein Soy Broth (TSB), supplemented with 10 g/100 g glycerol and 10 g/100 g skim milk. Prior to use, all bacteria were grown twice 18 hours at 30°C on TSB.

2.2 Isolation, phenotypic and genetic identification

Lactic acid bacteria were isolated from different marine species: Tehuelche scallop (*Aequipecten tehuelchus*), Patagonian Argentinean clam (*Ameghinomya antique*), Patagonian blue mussel (*Mytilus edulis platensis*), sea cucumber (*Hemiodema spectabilis*), geoduck (*Panopea generosa*) and razor clam (*Solen tehuelchus*), collected from the marine shore of Chubut, Argentina, from January to March 2016. Shortly, the specimens were aseptically eviscerated, and 0.5 g of gut was transferred to different selective liquid media and incubated for 24-48 hours at 30°C or 37°C. Selective media used were Bile Esculin, Bromocresol Purple and Man Rogosa Sharpe (MRS).Aliquots of all broths on which turbidity was detected were transferred into solid selective agar and incubated under the same conditions. Finally, isolated colonies were transferred to

MRS agar and after 24 hours of incubation at 30°C, they were Gram stained. All homogeneous Gram positive cultures were stored at -30°C as mentioned on 2.1.

In order to detect strains' antagonist activity, the cell-free supernatants (CFS) were obtained. LAB strains were inoculated to MRS broth, incubated overnight at 30°C and centrifuged at 8000 rpm for 10 minutes at 4°C in a high-speed centrifuge (Eppendorf 5804 R, Germany). Obtained CFS were thermally treated for 5 minutes at 100°C, filtered through a sterilizing syringe filter of 0.20 µm pore size (Sartorius, Stedim Biotech, Germany) and stored at -30°C until use.

Antimicrobial activity of all strains was firstly assessed by the agar well diffusion technique (AWDT) according to Rivas, Castro, Vallejo, Marguet, and Campos (2012).Shortly, an overnight inoculum of *L. innocua* was added to 1.2 g/100 g Tryptein Soy Agar (TSA) to ensure a minimum population of 10^6 CFU/ml. After the agar was poured, 6 mm diameter wells were cut, and 50 µl of CFS were added to each one. All plates were incubated firstly for 2 hours at 4 °C, and later for 24 hours at 30 °C. Translucid areas around the wells were considered as positive results. LAB strains for which CFS showed antagonistic activity against *L. innocua* were chosen for further characterization.

Phenotypic identification of selected strains was done by detection of the enzyme pyrrolidonyl-arylamidase and leucine aminopeptidase, production of yellow pigment and sugar fermentation. All media were from Biokar Diagnostics (Beauvais, France).

The wizard genomic DNA purification kit (Promega Corporation, USA) was used to extract chromosomal DNA of selected isolated strains, according to the manufacturer's instructions. Amplification of the 16S rRNA gene was performed using universal primers 27F (5´AGAGTTTGATCMTGGCTCAG-3´) and 1492R (5- TACGGYTACCTTGTTACGACTT -3´) (Lane, 1991). Purification and sequencing of PCR products were carried out by the sequencing service of Macrogen Inc. (Seoul, Korea). The 16S rRNA gene sequences of strains STw26, STw38, Stw46, STw60, STw66 and STw79 were compared with those of the most closely

related species retrieved from GenBank database, using the BLAST algorithm (Altschul et al., 1997). The phylogenetic tree topology was obtained with the neighbour-joining method using MEGA software, version 7.0 (Kumar et al, 2015). The statistical reliability of phylogenetic tree topology was assessed by bootstrap analysis (1000 replications).

2.3 Determination of virulence factors

2.3.1 Assay of gelatinase and hemolytic activity

Production of gelatinase was determined on TSA plates supplemented with 30 g/l gelatin. Plates streaked with isolates were incubated at 37 °C for 48 hours. Gelatinase production was detected as a clear halo around the colonies after addition of 20 ml/100 mltrichloroacetic acid (Sintorgan S.A., Buenos Aires, Argentina).

Hemolysin production was evaluated on Brain Heart Infusion Agar supplemented with 5 g/100 g human blood. Plates were incubated for at 37 °C for 48 hours under aerobic conditions and clearing zones around colonies indicated hemolysin production.

2.3.2 Antibiotic resistance

For the assessment of bacterial strains susceptibility to ampicillin and vancomycin, serial two-fold dilution procedures were used. The tests were performed according to National Committee for Clinical Laboratory Standards (NCCLS 2015) guidelines.

2.3.3 PCR for the detection of virulence factors

The following virulence genes: gelatinase (*gelE*), enterococcal surface protein (*esp*), aggregation substance (*agg*), cytolysin operon ($cylL_l$/$cylL_s$), hyaluronidase (*hylEfm*) and IS element (*IS16*) were amplified by PCR-specific primers and conditions were those described by the authors mentioned in Table 1. The amplicons were evaluated by 1.5 g/100 g agarose gel electrophoresis followed by staining in 0.5 mg/ml ethidium bromide (Sigma, USA) and were visualized on a UV transilluminator (Labnet, National Labnet Company, USA).

2.4 Characterization of antimicrobial activity

2.4.1 Production of hydrogen peroxide and organic acids

To assess the contribution of possible hydrogen peroxide production to CFS antimicrobial activity, these were treated with 2 mg/ml of catalase (Sigma, USA) for 1 hour at 37 °C. In order to detect the potential contribution of a diminishment of pH due to strains' production of acids, the CFS were alkalinized to pH 7.00 using0.1 N NaOH. Then the residual activity against *L. innocua* was determined by AWDT.

2.4.2 Enzyme sensibility

The CFS were treated with hydrolytic enzymes: lysozyme, pronase, trypsin, pepsin and bromelain (Sigma, USA). Enzymes, in a final concentration of 1.0 mg/ml, were dissolved in the buffers recommended by the suppliers. Enzyme solutions alone and aliquots of CFS were diluted with sterile water and used as negative and positive controls, respectively. The samples were incubated at 30°C for 4 hours, and the residual activity against *L. innocua* was determined by the AWDT.

2.4.3 PCR screening for bacteriocins structural genes

The primers used for the amplification of enterocin A, B, P, L50A, L50B, mundticin KS, bacteriocin 96, bacteriocin 31, 1071 A/B, enterocin Q and HirJM 79 are listed in Table 2. General PCR conditions were those described by the authors mentioned in the Table 2.

2.5 Detection and quantification of antimicrobial activity against the bacterial flora of fish at pH 6.00

In order to dismiss the inhibitory effect of pH on indicator microorganisms and to resemble fish muscle conditions, pH of CFS was adjusted to 6.00 with 0.6 N NaOH. The antagonist activity was tested against all indicator strains mentioned in item*2.1*by AWDT. When inhibition was detected, twofold dilutions of the CFS, ranging from 2^{-1} to 1024^{-1}, were prepared in MRS and the titer was assessed using the AWDT. Bacteriocin titer was defined as the last dilution that produced a perceptible inhibition zone (Delgado, Brito, Fevereiro, Tenreiro, & Peres, 2005).The

strains of which CFS showed greater higher antimicrobial activity at pH 6.00 were selected for further studies.

2.6Determination of the interaction among CFS

The interaction among binary mixtures of selected CFS was determined by a microdilution technique, using *L. innocua* as indicator (Schelegueda, Gliemmo, & Campos, 2012).Tested concentrations were chosen using modified Berembaum design (Table 3), which includes the titer of each CFS and combinations of sub-inhibitory concentrations. The titer in liquid media was previously determined using the same technique. In all cases the value obtained was 256^{-1}.Microplatescontaining the concentrations cited on Table 3 were incubated at 30°C for 24 hours. The absorbance of each well was read at 600 nm every hour using a microplate reader commanded by the program Gen5 Data Analysis Software (Reader Control and Data Analysis Software, BioTek Instruments, ELx808, USA).The absorbance of a negative control was used as blank, and a positive control was also included with no presence of CFS. The assay was performed in sextuplicate and replicated twice. Inhibition was considered when a variation of less than 0.1 in the absorbance value was observed. The fractional inhibitory concentration (FIC) was calculated. The latter expresses the value of the titer of a CFS when it is combined ($Titer_{A-B}$ or $Titer_{B-A}$) divided by the titer of this CFS used alone ($Titer_A$ or $Titer_B$), hence the FIC index = $(Titer_{A-B}/Titer_A)+(Titer_{B-A}/Titer_B)$.A FIC close to 1 indicates an additive effect, a value lower to 1 it indicates synergism, and greater than 1 indicates antagonist activity (Schelegueda et al., 2016).

2.7Growth and bacteriocin production from selected LAB during refrigerated storage

Selected LAB in the item *2.5* were adapted to grow at 4°C. For that purpose, they were firstly incubated on MRS broth twice at 30°C for 1 day. Secondly, each inoculum was transferred to a fresh MRS broth and incubated at 15°C for 3 days. Finally, they were inoculated in fresh MRS broth and incubated at 4°C for 10 days. At each temperature, a growth curve was constructed

187 measuring the absorbance at 600 nm periodically. For this purpose, an inoculum of 10^6 CFU/ml
188 was added to each well of a microplate containing MRS broth. Each system was incubated
189 under the time and temperature conditions cited above. For each microorganism and condition,
190 11 replicates were assayed.
191 To determine the ability of selected strains to produce bacteriocin at 4°C, a sample was taken
192 from each culture at the exponential growth stage and CFS were obtained. Detection and
193 quantification of antimicrobial activity against *L. Innocua* and *L. plantarum* were assessed by
194 AWDT.
195 *2.8Application of a selected bacteriocianogenic strain for fish paste preservation*
196 Fresh hake (*Merluccius hubbsi*) was brought to the laboratory preserved on ice. The specimens
197 were washed under tab water, eviscerated, and filleted in sterile conditions. Fillets were washed
198 with sterile chlorinated water (7 mg/l) and processed in a domestic processor for 20 seconds.
199 The obtained paste was separated into 2 different portions and their pH was adjusted to 6.00
200 with 25 g/100 g citric acid. One of the portions was inoculated with10^3 UFC/g of *L. innocua* (L)
201 and used as control system since previous studies demonstrated that *L. innocua* does not affect
202 native flora development in fish(Schelegueda, Delcarlo, Gliemmo, & Campos, 2016). The
203 second one was inoculated with10^3 UFC/g of *L. Innocua* and 10^6 UFC/g of *E. mundtii* STw38 (E).
204 In a first stage, systems were air-packed and stored at 4°C for 6 days. In a second stage,
205 systems were vacuum-packed and stored at 4°C for 10 days (systems VL and VE,
206 respectively).Two samples of each system were analyzed on days 0, 3, 6 and 10 of storage.
207 Preliminary studies showed that *E. mundtii* STw38 is capable of surviving under the two studied
208 storage conditions.
209 Total mesophilic aerobic bacteria were evaluated on Plate Count Agar, *Listeria* spp. was
210 numbered on Palcam Agar supplemented with antibiotics and enterococci population was

counted on Kanamycin Esculin Azide Agar. All plates were incubated at 30°C for 48 hours. Experiments were carried out in duplicate.

In situ bacteriocin production was also assessed by AWDT against *L. innocua* adding a portion of inoculated paste on the wells instead of the CFS. The paste was tested before and after being thermally treated for 1 minute at 100 °C in order to discard the action of the bacteriocianogenic strain producing the bacteriocins when growing on the nutritive agar.

2.9 Data analyses

Data obtained from the detection of antimicrobial activity against the bacterial flora of fish at pH 6.00were analysed using a one-way Analysis of Variance (ANOVA)and all assays were carried out by triplicate, unless otherwise mentioned.

Data obtained from the study of the growth of selected LAB during refrigerated storage were modeled by means of modified Gompertz equation:

$$y = A.exp\{-exp[1 + \frac{\mu}{A}(\lambda - t)]\}$$

This model expresses the change in absorbance (y) -produced by LAB growth-vs time (t). Bacterial growth parameters are the lag phase time (λ), the specific growth rate (μ) and the asymptotic value (A) (Biesta-Peters, Reij, Joosten, Gorris, & Zwietering, 2010).Mentioned parameters were estimated fitting data to nonlinear regression model. The adequacy of growth model was examined by adjusted determination coefficients (R^2adj) and root mean square error (RMSE). Obtained parameters were also analyzed by one-way (strain or temperature, as it corresponds) ANOVA.

Finally, data obtained from the application of a selected strain for fish paste preservation were analyzed by a two-way (system and time) ANOVA.

In all cases, ANOVA was followed by Tukey's multiple comparison test. Data were processed using the statistical program Statgraphics (Statgraphics Plus for Windows, version 5.1, 2001, Manugistics, Inc., Rockville, Maryland, USA). The significance level was 0.05%.

3. Results and discussion

3.1Isolation, detection of antimicrobial activity and identification

In total, 132 LAB were isolated from Chubut's coast in Argentina. From all isolated LAB, 22 strains showed antimicrobial activity against *L. innocua* and were selected for further characterization. The species from which these LAB were isolated are shown in Table 4.

All selected strains' phenotypic characterization showed positive presence of the enzymes pyrrolidonyl-arilamydase and leucine aminopeptidase, as well as production of yellow pigment and fermentation of arabinose, mannitol and ribose, which indicates that they all belong to the species *E. mundtii*. Later, the identification was confirmed by PCR amplification of 16s Ribosomal RNA. Results are shown in Figure 1.

3.2 Determination of virulence factors

Gelatinase is an extracellular, zinc-dependent metalloendopeptidase capable of hydrolyzing gelatine, collagen, casein, haemoglobin, and other proteins. This enzyme is the most frequently occurring virulence factor and can be found in isolates from food, environmental and clinical specimens (Eaton&Gasson, 2001). None of the strains studied displayed gelatinase activity in agar plate, results that are in agreement with those obtained by PCR techniques.

Hemolysin, also referred to as cytolysin, is one of the best characterized enterococci virulence factors because contributes to the severity of enterococcal disease in human and animals (Semedo *et al*., 2003). In all studied strains, it was confirmed, by PCR techniques, the absence of $CylL_l$ and $CylL_s$ genes. Both genes encode structural subunits of cytolysin. These results were congruent with the lack of hemolytic activity in human blood agar.

Antibiotic susceptibilities were determined by serial two-fold dilution procedures. The six selected enterococci were susceptible to ampicillin minimum inhibitory concentration (MIC) ≥ 1 µg/ml and vancomycin MIC ≥ 1 µg/ml. Ampicillin resistance is defined by an MIC of >8 µg/ml and vancomycin resistance by >4 µg/ml (http://www.eucast.org). High resistance to ampicillin and vancomycin are usually related to the presence of insertion element IS16 (Werner *et al*., 2011). None of the selected strains displayed amplicons when genomic DNA amplification was performed with specific primers for gene fragment of IS16 element.

Aggregation protein is involved in adherence to eukaryotic cells and mediates cell aggregation between bacteria thereby facilitating conjugation. Enterococcal surface protein (esp) is involved in the formation of biofilm which plays, like aggregation protein, an important role in the exchange of genetic material between cells. No amplification products of the virulent genes *agg* (aggregation protein) and *esp* were observed in all strains tested. The same results were obtained when the *Hyl* gene was amplified with specific primers. Hyl gene encodes hyaluronidase, an enzyme that degrades hyaluronic acid in connective tissue and facilitates bacterial invasion.

3.3Characterization of antimicrobial activity

Selected strains' CFS antagonist activity against *L. innocua* was not affected by treatment with catalase or alkalization, indicating that the inhibition detected was not due to the production of hydrogen peroxide or acidification. Additionally, the antagonist activity was lost after the treatment with trypsin, pronase, bromelin and pepsin, showing a peptidic composition of the antimicrobial substances(Xin et al., 2015). No antimicrobial activity lost was found after treatment with lysozyme which acts on glycosidic bonds, hence, the peptidic compounds present in all enterococci do not present a glycosidic part in their structure.

From structural bacteriocin genes detected by PCR, no strains presented the genes for enterocins A, B, P, L50B or Q,nor bacteriocin 96, 31, 1071 A/B, HirJM 79. Nevertheless, all selected LAB did present bacteriocin structural genes which coded for mundticin KS, and only 17 strains, additionally, presented the genes that coded for enterocin L50A (Table 4),showing that this gene is not present in all bacteriocianogenic enterococci.Ogaki, Rocha, Terra, Furlaneto, and Furlaneto-Maia (2016), tested 138 enteorcocci strains and none of them had the gene that coded for the production ofenterocinL50A,neither.Furthermore, they informed that bacterial strains which presented two genes encoding for different bacteriocins had better antagonist activity against a wider range of pathogens compared to bacteria which only presented one gene.

3.4 Detection and quantification of antimicrobial activity against the bacterial flora of fish at pH 6.00

No isolated enterococci showed antimicrobial activity against Gram negative microorganisms, which is consistent with previous studies (Pinto et al., 2009; Rivas et al., 2012). Additionally,they did show activity against *L. innocua* and *Lb. plantarum* at pH 6.00.Halo's sizes for each indicator strain presented slight differences, where some species showed smaller halos than others (Table 4). Amongst the size of the inhibition halos against *L. innocua* they ranged between 18 and 22.5 mm and *E. mundtii* STw79 produced the biggest halo. Halos against *Lb. plantarum* ranged from 17 to 23 mm, and strain *E. mundtii* STw66 showed the highest one. The titer was assessed against these two indicators by the AWDT. CFS of LAB showed a titer against *L. innocua* and *Lb. plantarum* above 16^{-1}, and the maximum titer obtained was 256^{-1}(Table 4).Halos' size decreased as the strains' CFS were diluted, until they reached a diameter of 10 mm, and this dilution was the informed titer(Figure 2). From all 22 studied strains, *E. mundtii* STw26, *E. mundtii* STw38, *E. mundtii* STw46, *E. mundtii* STw60, *E. mundtii* STw66 and *E. mundtii* STw79 showed the highest titers against *L. innocua* and *Lb. plantarum* and were

selected for further characterization. As it was expected, those strains presented the genes that code for the production of the 2 bacteriocins (mundticin KS and enterocin L50A).

As previously mentioned, CFS titers obtained on liquid media were$256^{-1}$for the 6 selected enterococci, which differs slightly from the ones obtained by the AWDT. Aspri et al. (2017)also assessed the antagonist activity of 3 different strains of *E. faecium* which presented activity against *L. monocytogenes* by the AWDT. When the same tested bacteriocin concentration was assayed on liquid media, the bacteriocin only had a bacteriostatic effect for less than 24 hours, which would indicate that the titer on liquid media may vary from the one obtained by the AWDT.

Given the presumed similarity between the bacteriocins of *E. mundtii* selected, the type of interaction was assessed, expecting a synergistic effect such as it occurs with Type IIb bacteriocins. There is previous evidence of enterocins presenting a synergistic activity with other bacteriocins. For example, higher antagonist activity was detected when enterocin 1071 was combined with the complementary lactococcin G peptides(Nissen-Meyer, Oppegård, Rogne, Haugen, & Kristiansen, 2010). On the assessment of the type of interaction of the CFS on liquid media, a total of 15 binary mixtures was tested. In all cases, an additive effect was observed. These results indicated that the combination of two CFS would not provide an improvement in antimicrobial activity against *L. innocua*. Hence, further studies were required to choose one strain to be used on fish preservation.

3.5 Development of selected LAB and bacteriocin production during refrigerated storage

Growth curves were satisfactorily modeled at30°C, 15°C and 4°C using Eq (1), obtaining R^2adj values between 0.98 and 0.99 and low RMSE. Parameters λ (lag phase time), μ (growth rate) and A(asymptotic value) were very close for all strains at a given temperature, except for *E. mundtii* STw79 which had a significantly longer λ and smaller A when grown at 4°C (Table 5). As it was expected, all six selected enterococci showed longer λ and slower μ as the growth temperature diminished (data not shown).As an example, Figure 3 shows experimental data and

modeled growth curves for strain *E. mundtii* STw38, and Table 5 shows its obtained parameters at all studied temperatures. A values were similar at all temperatures indicating that *E. mundtii* STw38 can grow satisfactory at refrigeration temperature.

Additionally, when strains were grown at 4 °C, CFS' inhibition halos were detected against *L. innocua* and *Lb. plantarum* for all of them. Titers from different CFS assessed ranged from $128^{-1}$to 512^{-1} for *L. Innocua* and from 8^{-1} to $64^{-1}$for *Lb. Plantarum* (Table 5). *E. mundtii* STw38 showed the highest titer against these indicator strains and was chosen for further tests. It is important to highlight, that numerous studies have assessed different LAB's capacity to produce bacteriocins directly on different food matrixes (Aspri et al., 2017; Lianou, Kakouri, Pappa, & Samelis, 2017; Coelho, Silva, Ribeiro, Dapkevicius, & Rosa, 2014), but preliminary tests on bacteriocin production under unfavorable conditions, such as refrigeration, is not usually tested before *in situ* assays.

3.6 Application of a selected bacteriocinogenic strain for fish paste preservation

Considering previous results, *E. mundtii* STw38 was chosen to be applied as a protective culture on Argentine hake paste stored air-packed for 6days or vacuum-packed for 10 days, at 4°C.

Among air-packed systems, those inoculated with *E. mundtii* STw38 (E), an increase of total enterococci was found until day 3, and then their population remained constant. Regarding *L. innocua*, no significant differences were found among systems E and the control one (L). This population increased throughout the storage, double folding their initial counts on day 6. When it comes to total mesophilic aerobic bacteria, a diminishment of 3 log cycles was observed on system E compared to system L during the entire storage. These results show that the application of *E. mundtii* STw38 could reduce the growth of native flora of fish for 6 days on air-packed hake paste (Figure 4, panel A).

When the paste was vacuum-packed, in systems inoculated with *E. mundtii* STw38 (VE), the enterococci population decreased after 3 days of storage, and then, increased to the previously

357 inoculated level. Regarding *L. innocua*, no significant differences were found on its growth
358 between the control system (VL) and the one of interest (VE).In both cases, *L. innocua*
359 population decreased after 3 days of storage, and then, increased slightly reaching a population
360 1 log cycle greater than the inoculated level (data not shown). Both the enterococci and *L.*
361 *innocua* populations decreased at the beginning of storage. This trend is related to the time
362 required for microorganisms to adapt to adverse growth conditions. Finally, the population of
363 mesophilic aerobic bacteria in the control samples (VL) increased 1 log cycle during the first 3
364 days of storage. However, in those samples inoculated with the strain of interest (VE), the
365 population of mesophilic bacteria decreased slightly (Figure 4, panel B).Then, both populations
366 grew until they reached their maximum value after 10 days of storage. It should be noted that in
367 the VL system, mesophilic aerobic bacteria increased 4 log cycles, while in the VE system the
368 increase was 1.5 log cycles. From that point until the end of the storage, the populations
369 remained constant (Figure 4, panel B).
370 Results of total mesophilic counts are in line with Boulares, Mankai, Sadok, and Hassouna
371 (2017), who applied *Carnobacterium piscicola* and *Lactococcus lactis* to fresh farm sea bass,
372 under vacuum, and found a diminishment of the growth of total mesophilic count for 21 days.
373 Additionally Ananou et al. (2014), found that there was a synergistic action between the
374 application of semipurified bacteriocin AS-48 and vacuum storage, which is similar to results
375 herein obtained. Furthermore, Gómez-Sala et al. (2016b), applied *Lb. curvatus* as a protective
376 culture on megrim and young hake under refrigeration. They found a reduction of mesophilic
377 bacteria population for 14 days.
378 Finally, hake paste analyzed by the AWDT (both packed in air and vacuum) showed activity
379 against *L. innocua* before the thermal treatment. These results show that enterococci population
380 maintained their viability and capacity to produce bacteriocins when inoculated in fish paste
381 even in presence of fish native flora. Additionally, no inhibitory action was found after thermal

treatment since bacteriocin producer bacteria were inactivated, and/or the remaining bacteriocin concentration was not enough to inhibit *L. innocua*. Finding LAB capable of producing bacteriocins in complex food matrixes is a real challenge, since both the native flora and the matrix of the food can affect their capacity to produce bacteriocins (Aspri et al., 2017; Lianou et al., 2017).

4. Conclusions

Novel bacteriocianogenic strains of *E. mundtii* were characterized showing no presence of virulence factors, and capability to grow and produce bacteriocins at refrigeration temperatures. Additionally, one of these strains, *E. mundtii* STw38 could grow on fish paste and control native fish flora, when packed in air or vacuum, showing that it may be a promising bacterium to be applied for fish preservation. Further studies should be carried out combining the strain together with additional stress factors to enhance its antagonist activity.

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550 **Figure captions**

551 Figure 1: Phylogenetic tree constructed by Neighbor-Joining method based on the relationship
552 between the 16S rRNA gene sequences of strains isolated of *Enterococcus* species (▲). The
553 numbers at internal nodes are bootstrap support values. GenBank accession numbers are given
554 in parentheses. The 16S rRNA sequence of *L. plantarum* was chosen arbitrarily as the outgroup
555 sequence. (bar, 0.01 substitution per nucleotide position)

556 Figure 2: Inhibition halos of *E. mundtii* STw38 against *L. innocua.*

557 Figure 3: Growth curvesof*E. mundtii* STw38. Dots represent experimental data at 30°C (•), 15°C
558 (•) and 4°C (◦), and gray lines show fitting modelled curves.

559 Figure 4: Air and vacuum-packed hake paste total mesophilic counts: (Panel A) Air packed: (■)
560 Control, (■) Fish paste inoculated with *L. innocua* and *E. mundtii* STw38 (LEA).(Panel B)
561 Vacuum packed: (■) Control, (■) Fish paste inoculated with *L. innocua* and *E. mundtii* STw38.

562

Table 1: PCR amplification of potential enterococcal virulence factors.

Virulence Factor	Sequence (5` - 3`)	Size (pb)	Reference
Agg	f: AAGAAAAAGAAGTAGACCAAC		
	r: AAACGGCAAGACAAGTAAATA	1553	Eaton&Gasson, 2001
gelE	f: ACCCCGTATCATTGGTTT		
	r: ACGCATTGCTTTTCCATC	419	Eaton&Gasson, 2001
Esp	f: TTGCTAATGCTAGTCCACGACC		
	r: GCGTCAACACTTGCATTGCCGAA	933	Eaton &Gasson, 2001
hylEfm	f: GAGTAGAGGAATATCTTAGC		
	r: AGGCTCCAATTCTGT	661	Rice et al., 2003
IS16	f: CATGTTCCACGAACCAGAG		
	r: TCAAAAAGTGGGCTTGGC	547	Werner et al., 2011
$CylL_l$	f: GATGGAGGGTAAGAATTATGG		
	r: GCTTCACCTCACTAAGTTTTATAG	253	Semedo et al., 2003
$CylL_s$	f: GAAGCACAGTGCTAAATA AGG		
	r: GTATAAGAGGGCTAGTTTCAC	240	Semedo et al., 2003

Table 2: Primer sequences for PCR amplification of enterocin genes in *E. mundtii* strains.

Enterocin	Sequence (5´- 3´)	Size (pb)	Reference
Ent A	f: GGTACCACTCATAGTGGAAA r: CCCTGGAATTGCTCCACCTAA	138	De Vuystet al.,2003
Ent B	f: CAAAATGTAAAAGAATTAAGTACG r: AGAGTATACATTTGCTAACCC	201	De Vuystet al., 2003
Ent P	f: GCTACGCGTTCATATGGTAAT r: TCCTGCAATATTCTCTTTAGC	87	De Vuystet al., 2003
Ent LB50A	f: ATGGGAGCAATCGCAAAATTA r: TTTGTTAATTGCCCATCCTTC	274	De Vuystet al., 2003
Ent LB50B	f: ATGGGAGCAATCGCAAAATTA r: TAGCCATTTTTCAATTTGATC	274	De Vuystet al., 2003
Bact 96	f: GTGGAGAGGACGAAAGGAGA r: TTGATTAGTGGAGAGGACGGATTA	291	Henning et al., 2015
Bact 31	f: CCTACGTATTACGGAAATGGT r: GCCATGTTGTACCCAACCATT	130	Özdemiret al., 2011
1071 A/B	f: GGGGAGAGTCGGTTTTTAG r: ATCATATGCGGGTTGTAGCC	273	Martín et al., 2006
EntQ	f: ATGAATTTTCTTCTTAAAAATGGTATCGCA r: TTAACAAGAAATTTTTTCCCATGGCAA	105	Belgacemet al., 2010
mun KS	f: TGAGAGAAGGTTTAAGTTTTGAAGAA r: TCCACTGAAATCCATGAATGA	379	Zendo et al., 2005
HirJM 79	f: ATGAAAAAGAAAGTATTAAAACATTGTGTTATTCTAGG r: ATAAGTTAAGCTTGTACTACCTTCTAGGTGCCCATGGACC	408	Almeida et al., 2011

Table 3: Berembaums' modified design used to test interactions between LAB's CFS.

Experiment	Titer Fractions		FIC
	CFS Strain A	CFS Strain B	
1	1	0	1
2	0	1	1
3	1/2	1/2	1
4	1/3	1/3	0.66
5	1/3	1/6	0.5
6	1/6	1/3	0.5
7	1/6	1/6	0.33
Control	0	0	-

Table 4: Isolation species of selected LAB. Inhibition halo size and titer against *L. innocua* and *L. plantarum*. Assays were done by triplicate and expressed ± Standard deviation. Strains indicated with an asterisk (*) presented the genes that coded both for the production of mundtiicin KS and enterocin L50A.

		L. innocua		*Lb. plantarum*	
Strain	Marine species	Inhibition halo (mm)	Titer	Inhibition halo (mm)	Titer
E. mundtii STw3*	Tehuelche scallop	19.0 ± 1.0 ^{a}	32^{-1}	17.0 ± 1.0 ^{a}	32^{-1}
E. mundtii STw13*	Patagonian Argentinean clam	18.0 ± 1.0^{a}	32^{-1}	19.0 ± 1.0^{ab}	64^{-1}
E. mundtii STw26*	Patagonian Argentinean clam	20.5 ± 0.5 ^{ab}	128^{-1}	21.0 ± 1.0 ^{ab}	64^{-1}
E. mundtii STw33*	Tehuelche scallop	19.5 ± 1.5 ^{ab}	32^{-1}	20.5 ± 0.5 ^{ab}	128^{-1}
E. mundtii STw35	Patagonian Argentinean clam	19.5 ± 0.5^{ab}	64^{-1}	20.5 ± 0.5^{ab}	128^{-1}
E. mundtii STw38*	Patagonian Argentinean clam	19.0 ± 1.0^{a}	256^{-1}	20.5 ± 0.5^{ab}	256^{-1}
E. mundtii STw39	Razor clam	18.5 ± 1.5 ^{a}	32^{-1}	20.0 ± 1.0^{ab}	64^{-1}
E. mundtii STw40	Razor clam	19.0 ± 1.0 ^{a}	32^{-1}	18.0 ± 1.0^{a}	16^{-1}
E. mundtii STw42	Patagonian blue mussel	20.0 ± 1.0 ^{ab}	16^{-1}	19.0 ± 1.0^{ab}	16^{-1}
E. mundtii STw46*	Patagonian Argentinean clam	19.5 ± 0.5 ^{ab}	32^{-1}	19.5 ± 0.5 ^{ab}	32^{-1}
E. mundtii STw47*	Patagonian Argentinean clam	19.0 ± 1.0 ^{a}	16^{-1}	21.0 ± 1.0 ^{ab}	64^{-1}
E. mundtii STw49*	Patagonian blue mussel	20.0 ± 1.0 ^{ab}	32^{-1}	18.0 ± 1.0^{a}	64^{-1}
E. mundtii STw51*	Sea Cucumber	19.0 ± 1.0 ^{a}	64^{-1}	20.0 ± 1.0^{ab}	32^{-1}
E. mundtii STw54*	Sea Cucumber	20.5 ± 0.5 ^{ab}	64^{-1}	19.5 ± 0.5 ^{ab}	64^{-1}
E. mundtii STw55*	Patagonian blue mussel	21.0 ± 1.0^{ab}	32^{-1}	19.0 ± 1.0^{ab}	32^{-1}
E. mundtii STw60*	Sea cucumber	21.0 ± 1.0^{ab}	128^{-1}	20.0 ± 1.0^{ab}	128^{-1}
E. mundtii STw66*	Razor clam	21.0 ± 1.0 ^{ab}	128^{-1}	23.0 ± 1.0^{b}	128^{-1}
E. mundtii STw68*	Patagonian blue mussel	21.0 ± 1.0^{ab}	64^{-1}	21.5 ± 0.5 ^{ab}	64^{-1}
E. mundtii STw70*	Geoduck	20.0± 1.0 ^{ab}	64^{-1}	20.3 ± 0.5^{ab}	64^{-1}
E. mundtii STw77*	Tehuelche scallop	20.5 ± 1.5^{ab}	32^{-1}	20.5 ± 0.5^{ab}	128^{-1}
E. mundtii STw78	Patagonian Argentinean clam	21.0 ± 1.0 ^{ab}	16^{-1}	18.5 ± 0.5 ^{a}	32^{-1}
E. mundtii STw79*	Tehuelche scallop	22.5 ± 0.5^{b}	64^{-1}	18.5 ± 0.5^{a}	64^{-1}

Figure 1

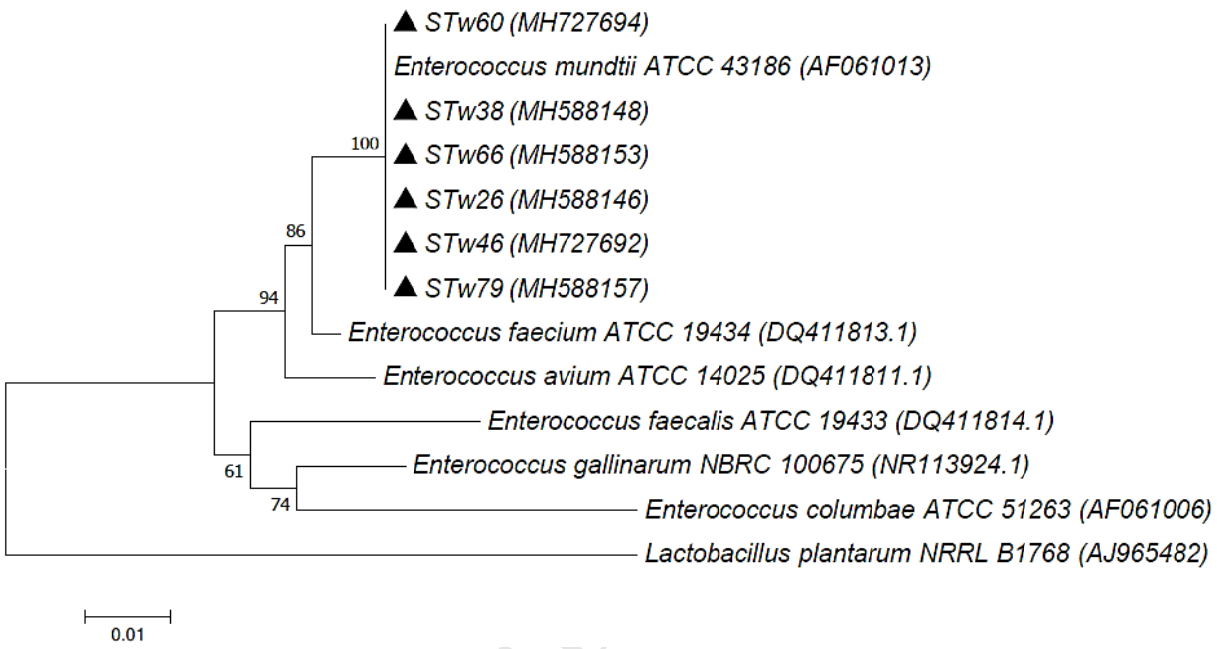


Figure 2

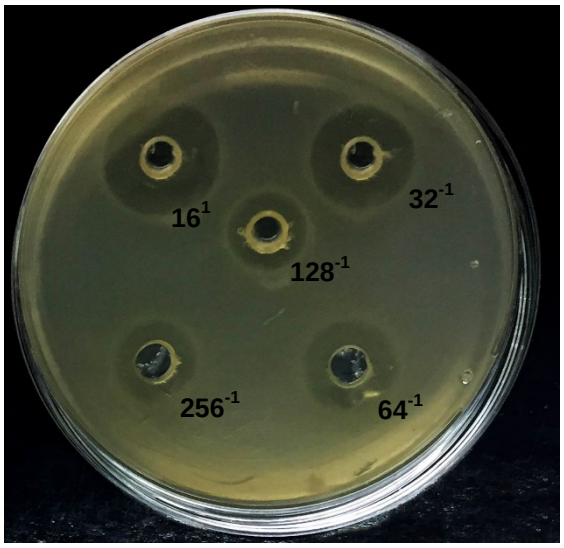


Figure 3

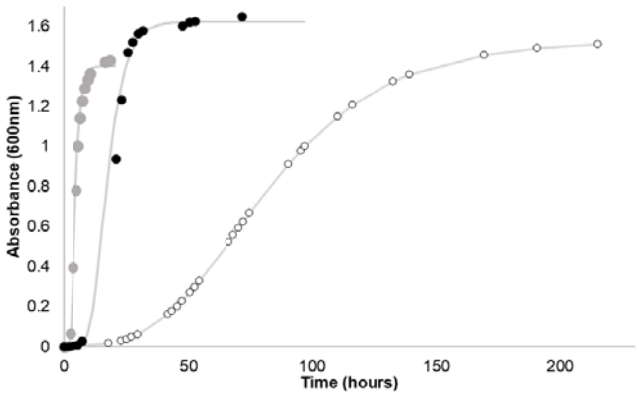
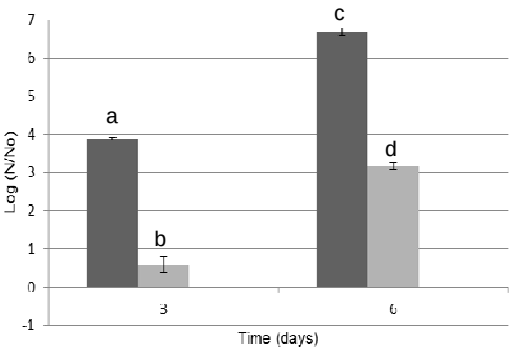
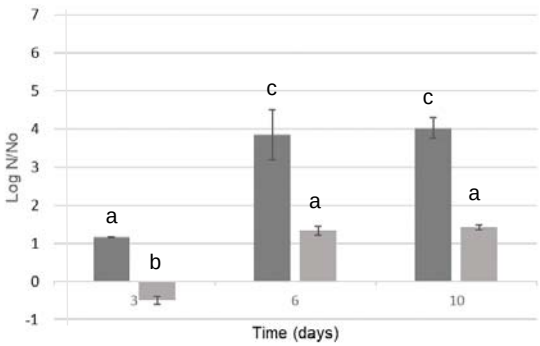


Figure 4



Panel A



Panel B

1 LAB Bacteriocinogenic strains possess mundticin and enterocin L50A structural genes

2 High capacity to grow and produce bacteriocins at refrigeration temperatures

3 Mixtures of cell-free supernatants act additive on *L. innocua* inhibition

4 Enterococci presented no virulence factors and diminished native biota in fish paste

5 Protective cultures were promising as an additional hurdle in fish preservation