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Synergistic antimicrobial action and potential application for fish preservation of a bacteriocin produced by *Enterococcus mundtii* isolated from *Odontesthes platensis*

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ABSTRACT

The aims of this study were to characterize an enterococci bacteriocinogenic strain and to evaluate its antimicrobial activity regarding its potential use for fish preservation. The bacterium, isolated from *Odontesthes platensis* in the Patagonian region of Argentine was identified as *Enterococcus mundtii* by means of biochemical test and 16S rDNA gene phylogenetic analyses. Furthermore, the gene that codifies mundticin KS was detected by PCR techniques. The bacteriocin was able to inhibit Gram positive and negative bacteria. In particular at pH 5.50, it was able to control the growth of fish bacterial flora. Construction of isobolograms and value of fractional inhibitory concentrations index showed that its use together with chitosan and sodium lactate (SL) exerted a synergic action on the inhibition of *Listeria innocua*, *Shewanella putrefaciens* and the psychrophilic flora isolated from fish. The use of the mentioned mixture of antimicrobials promoted strong changes on *L. innocua* and *S. putrefaciens* cellular components as shown by differential scanning calorimetry. Results suggest that the combination of the bacteriocin with chitosan and SL would be useful to reduce the amount of antimicrobials added to foods and to prevent the growth of spoilage bacteria and pathogenic microorganisms, such as *Listeria monocytogenes*.
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1. Introduction

Lactic acid bacteria (LAB) are considered safe to consume, as a consequence, they are used with different objectives in food (Deegan, Cotter, Hill, & Ross, 2006). They may be used to develop new flavors, preserve food quality and increase its safety, since LAB exert inhibitory activity against food spoilage and pathogenic bacteria (Calo-Mata et al., 2008). The ability to compete with the native microflora and to produce inhibitory compounds such as bacteriocins, confers to the LAB the potential to be used as bio-preservatives (Tomé, Teixeira, & Gibbs, 2006). Bacteriocins are ribosomally synthesized peptides and proteins with inhibitory activity against bacteria taxonomically close to the producer cell (Deegan et al., 2006; Ness et al., 1996). Among LAB, the genus *Enterococcus* is of special interest. These bacteria are found in the intestine of animals, where they promote many beneficial effects (Khan, Flint, & Yu, 2010). Bacteriocins produced by *Enterococcus* genus are known as enterocins. Their stability under extreme conditions and their activity during extended periods of time are their most notable advantages (Khan et al., 2010).

Since bacteriocin inhibitory action may be limited, their use in the context of hurdle technology is recommended. Usually, bacteriocins exhibit synergistic effects when combined with others treatments, as a consequence, they may be used as an additional hurdle to increase food safety (Matin-Visscher, Yoganathan, Sit, Lohans, & Vederas, 2011; Turgis, Dang Vu, Dupont, & Lacroix, 2012; Vescovo, Scolari, & Zacconi, 2006).

Fish is a highly perishable food due to the high *post-mortem* pH, the presence of large amounts of non-protein nitrogen, the high content of unsaturated fatty acids and the presence of autolytic

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enzymes. The process of degradation is dominated by microorganism's development (Olafsdóttir et al., 1997), therefore the application of a preservation method is essential. The increasing demand for high quality fresh seafood has intensified the search for new methods and technologies; among them, biopreservation has attracted the attention in the last years. It must be noted that fish may act as a source of bacteriocins since many LAB are isolated from these type of food (Campos, Rodríguez, Calo-Mata, Prado, & Barros-Velázquez, 2006; Tomé et al., 2006; Yamazaki, Suzuki, Kawai, Inoue, & Montville, 2003). Therefore, for a possible use in fishery products, bacteriocins would be more adapted to the substrate (Calo-Mata et al., 2008). However, the study of the use of bacteriocins as fish preservatives has been focused on their antilisterial activity and not in their ability to control the growth of spoilage bacteria or their combined use with other antimicrobials (Sarika, Lipton, Aishwarya, & Dhivya, 2012; Vescovo et al., 2006).

Based on the topics discussed, the aims of this study were to characterize an enterococci bacteriocinogenic strain isolated from silverside (*Odontesthes platensis*) and to evaluate its antimicrobial action regarding the potential use for fish preservation in combination with chitosan and sodium lactate.

2. Materials and methods

2.1. Enterococci isolation, phenotypic and genetic identification

Silverside (*O. platensis*) is a marine fish that inhabits the coast of South America. It is found in template or cold water (Sampaio, 2006). It possesses meat of excellent quality. As a consequence, it represents an important resource in the Argentine market (Ministerio de Agricultura, Ganadería y Pesca de la República, Argentina, 2015). Many LAB strains were isolated from silverside intestine sampled in the Patagonian region of Argentine according to Marguet, Vallejo, Sierralta Chichisola, and Quispe (2011). The contents from intestinal tract of silverside specimens were homogenized in 1 ml of sterile saline solution. An aliquot of the homogenates was inoculated into De Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) and incubated at 25 °C for 6 h. Then the enrichments were plated onto MRS supplemented with nalidixic acid (20 mg/l) and cycloheximide (10 mg/l) and incubated at 25 °C for 48 h.

One strain was selected and its presumptive identification to genus level was carried out by observation of cell pigment production on Brain Heart Infusion (BHI) agar (Biokar Diagnostics, Beauvais, France), morphology, Gram staining, catalase and oxidase production, growth at 10 and 45 °C, growth in presence of NaCl (6.5 g/100 ml) and at pH 9.6, as well as growth and esculin hydrolysis on bile-esculin agar (BEA) (Schleifer & Kilpper-Bälz, 1984). Pyrrolidonyl aminopeptidase activity was tested using a commercial kit (Pyrrolidonyl peptidase strips; BioChemika, Sigma Aldrich, USA). To perform the identification of an *Enterococcus* to species level, sugar fermentation was evaluated according to the Manero and Blanch (1999) scheme.

Genomic DNA was extracted from the pellet of an overnight culture in MRS broth and purified using a purification kit (Wizard, Genomic, Promega, Wisconsin) following manufacturer's recommendations. The 16S rRNA gene sequence (corresponding to positions 27–1492 in the *Escherichia coli* gene) was PCR amplified as described by DeLong (1992), using a DNA thermal cycler Mastercycler (Eppendorf, Hamburg, Germany). Sequencing on both strands of PCR-amplified fragments was performed using the dideoxy chain termination method by the commercial services of Macrogen Inc. (Seoul, Korea). The 16S rRNA homology searches against the NCBI database were carried out using BLAST program (Altschul, Gish, Miller, Myers, & Lipman, 1990). Sequence was deposited at the GenBank database.

2.2. Bacterial strains and culture conditions

Enterococcus mundtii Tw56 was stored in MRS broth. Indicator microorganisms were stored in MRS or Tryptone Soy broth, according to the requirements of each microorganism (Table 1). Both media were supplemented with 10% (w/w) glycerol (Sintorgan S.A., Buenos Aires, Argentina) and 10% (w/w) skim milk. Cultures were stored at −30 °C. Before the use, they were grown twice in the appropriate culture media at 30 °C for 18 h. Mentioned media were purchased from Biokar Diagnostics (Beauvais, France).

2.3. Detection of antimicrobial activity

Cell-free culture supernatant (CFS) was obtained by centrifugation of an overnight culture – grown in MRS broth – of *E. mundtii* Tw56 at 8000 *g* for 10 min at 4 °C. Cell-free culture supernatant was adjusted to pH 6.50 using 0.4 M NaOH and heated at 100 °C for 5 min in order to inactivate endogenous proteases. Afterwards, CFS was filtered through 0.20 μm pore size (Sartorius, Stedim Biotech, Germany) and stored at −20 °C until use. The antimicrobial activity of CFS was determinated by the agar well diffusion assay (AWDA) as previously described by Rivas, Castro, Vallejo, Marguet, and Campos (2012). Culture media used – MRS or Tryptone Soy Agar (TSA), depending on the microorganism – are listed in Table 1.

2.4. Characterization of the antimicrobial activity

2.4.1. Determination of hydrogen peroxide

In order to evaluate the presence of hydrogen peroxide in the CFS, it was treated with 2 mg/ml of catalase (Sigma, USA) for 1 h at 37 °C. Then, the residual antimicrobial activity against *Listeria innocua* ATCC 33090 was determined by AWDA.

2.4.2. Enzyme sensibility

In order to evaluate the chemical nature of the inhibitory compound, the CFS sensitivity to hydrolytic enzymes (using trypsin (Type II-S), lysozyme (Grade III) and lipase (Type VII) at a final concentration of 1 mg/ml) was studied. All enzymes were purchased from Sigma Aldrich (USA) and the assays were carried out at the pH and temperature recommended by the supplier. The CFS was incubated in enzyme solutions for 2 h at 37 °C and the remaining activity was determined by AWDA against *L. innocua* ATCC 33090. Enzyme solutions alone and an aliquot of CFS were used as negative and positive controls, respectively.

2.4.3. Thermal stability and effects of pH

The test of heat sensitivity and the evaluation of the pH effect were carried out as described by Rivas et al. (2012).

The effect of extended storage at low temperature on CFS stability was evaluated by placing CFS in an incubator device at 4 °C and −30 °C for 1 year. CFS was consequently tested for residual activity by the AWDA as described above.

2.4.4. PCR screening for bacteriocins structural genes

The primers used for the amplification of enterocin A, B, P, L50A, L50B were those described by De Vuyst, Foulquié Moreno, and Revets (2003) and mundticin KS gene was investigated using the primers described by Zendo et al. (2005) (Table 2). PCR amplification was performed in a Mastercycler (Eppendorf, Hamburg, Germany) using 20 μl of a reaction mixture containing 50 ng of DNA, 0.5 μM of each primer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1× PCR buffer and 1 U of Taq DNA polymerase (Inbio-Highway, Argentina). The cycles used were 95 °C for 5 min,

Table 1
Culture conditions and antimicrobial activity of cell-free supernatant from *Enterococcus mundtii* Tw56.

Indicator strains	Growth media and temperature (°C)	Diameter of inhibition zone (mm)
Brochothrix thermosphacta 406*	TSA, 30 °C	–
B. thermosphacta 396*	TSA, 30 °C	–
Enterococcus faecalis Tw27	MRS, 35 °C	18.0 ± 0.1
E. faecium Tw15	MRS, 35 °C	19.0 ± 0.1
E. faecium Tw20	MRS, 35 °C	19.0 ± 0.2
E. faecium Tw22	MRS, 35 °C	18.0 ± 0.2
E. hirae Tw32	MRS, 35 °C	21.0 ± 0.3
E. mundtii Tw22	MRS, 35 °C	19.0 ± 0.2
E. mundtii Tw56	MRS, 35 °C	–
E. faecalis ATCC 29212	MRS, 35 °C	18.0 ± 0.3
Lactobacillus plantarum TwLb3	MRS, 30 °C	13.0 ± 0.1
Lb. plantarum ATCC BAA-171	MRS, 30 °C	11.0 ± 0.2
Lb. casei TwCM 34	MRS, 30 °C	–
Lb. curvatus ACU 1	MRS, 30 °C	23.0 ± 0.2
Lb. delbrueckii ssp *bulgaricus* TwCM 35	MRS, 30 °C	15.0 ± 0.1
Lb. plantarum ATCC 14917	MRS, 30 °C	15.0 ± 0.1
Lb. plantarum ATCC 8014	TSA, 30 °C	15.0 ± 0.1
Lactococcus lactis ATCC 11454	MRS, 30 °C	–
Lc. lactis ssp *cremoris* TwCM6	MRS, 30 °C	–
Lc. lactis ssp *lactis* TwCM7	MRS, 30 °C	–
Lc. lactis ssp *lactis* biovar. *diacetylactis* TwCM8	MRS, 30 °C	–
Leuconostoc mesenteroides ssp. *cremoris* TwCM19	MRS, 30 °C	20.0 ± 0.1
Ln. mesenteroides ssp. *dextranicum* TwCM32	MRS, 30 °C	21.0 ± 0.2
Ln. mesenteroides ssp. *mesenteroides* TwCM30	MRS, 30 °C	–
Listeria innocua 6a ATCC 33090	TSA, 30 °C	16.0 ± 0.2
L. innocua Tw67	TSA, 30 °C	23.0 ± 0.3
L. monocytogenes ATCC 7644	TSA, 30 °C	22.0 ± 0.3
Micrococcus luteus ATCC 15307	TSA, 30 °C	–
Pediococcus pentosaceus Tw224	MRS, 30 °C	23.0 ± 0.1
Pseudomonas aeruginosa ATCC 9027	TSA, 30 °C	17.0 ± 0.2
Ps. fluorescens ATCC 49838	TSA, 30 °C	–
Psychrophilic flora isolated from fish	TSA, 30 °C	–
Shewanella putrefaciens ATCC 8071	TSA, 30 °C	15.0 ± 0.2
Staphylococcus aureus ATCC 25923	TSA, 30 °C	–
S. aureus ATCC 29213	TSA, 30 °C	–
Streptococcus thermophilus TwCM11	MRS, 35 °C	18.0 ± 0.1
St. pyogenes Tw 73	TSA, 35 °C	17.0 ± 0.1
Weissella viridescens Tw235	TSA, 30 °C	–

Negative signs (–) indicate no antimicrobial activity. Tw: National University of the Paragonia San Juan Bosco, Trelew, Chubut, Argentina. ACU: Austral Chaco University (Chaco, Argentina), * strains isolated at the Food Microbiology Laboratory of Austral Chaco University (Chaco, Argentina).

95 °C for 30 s, 56 °C (for the primers of enterocin B, P, L50A/B) or 58 °C (for the primers of the enterocin A) or 55 °C (for the primers of Mundticin KS) for 30 s, and 72 °C for 30 s for the next 30 cycles; 72 °C for 5 min were used for the last cycle. An aliquot of 4-µl of the amplification mixture was combined with 1 µl of loading buffer, and the preparation was electrophoresed on 1.5% agarose gel at 70 V for 1 h.

2.4.5. Detection and quantification of antimicrobial activity against the bacterial flora of fish at pH 5.50

In order to investigate the potential application of the bacteriocin produced by the *Enterococcus* in fish preservation by combined factors, the activity of CFS adjusted to pH 5.50 using 0.4 M NaOH was tested. For that purpose, the antimicrobial activity against *L. innocua* 6a ATCC 33090 (used as an alternative to *Listeria monocytogenes* because of its similar response to stress factors (Friedly et al., 2008)), *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas fluorescens* ATCC 49838, *Lactobacillus plantarum* ATCC 8014, *Shewanella putrefaciens* ATCC 8071 and psychrophilic bacteria isolated from refrigerated vacuum packaged fish was determinated by AWDA.

Titers of CFS at pH 5.50 were determinated against indicator microorganisms mentioned in the previous paragraph, by AWDA, placing serial twofold dilutions of CFS in the wells. Bacteriocin titer was defined as the last dilution that produces a perceptible inhibition zone (Delgado, Brito, Fevereiro, Tenreiro, & Peres, 2005).

2.4.6. Determination of the interaction among CFS, chitosan and sodium lactate

As it was mentioned before, the combined use of bacteriocins with natural antimicrobials may increase their antagonistic activity. The evaluation of the interaction among CFS, chitosan and sodium

Table 2
Primer sequences for PCR amplification of enterocin genes in *Enterococcus mundtii* Tw56.

Enterocin	Forward primer	Reverse primer	Reference
A	5-GGTACCACTCATAGTGGAAA-3	5-CCCTGGAATTGCTCCACCTAA-3	De Vuyst, Moreno, and Revets (2003)
B	5-CAAAATGTAAAAGAATTAAGTACG-3	5-AGAGTATACATTTGCTAACCC-3	De Vuyst et al. (2003)
P	5-GCTACGCGTTCATATGGTAAT-3	5-TCCTGCAATATTCTCTTTAGC-3	De Vuyst et al. (2003)
L50A	5-ATGGGAGCAATCGCAAAATTA-3	5-TTTGTTAATTGCCCATCCTTC-3	De Vuyst et al. (2003)
L50B	5-ATGGGAGCAATCGCAAAATTA-3	5-CCTACTCCTAAGCCTATGGTA-3	De Vuyst et al. (2003)
KS	5-TGAGAGAAGGTTTAAGTTTTGAAGAA-3	5-TCCACTGAAATCCATGAATGA-3	Zendo et al. (2005)

lactate (SL), when it comes to the inhibition of *L. innocua* and *S. putrefaciens*, was carried out by a checkerboard microdilution method performed using 96-well microplates according to Schelegueda, Gliemmo, and Campos (2012). The ranges of studied concentrations are shown in Table 3. *L. innocua* 6a ATCC 33090 was selected to model Gram positive and pathogenic flora of fish; while *S. putrefaciens* ATCC 8071 was used as a representative of Gram negative bacteria responsible for fish spoilage (Gram & Dalgaard, 2002).

The minimum inhibitory concentration (MIC) of chitosan and SL, and the titer of CFS were used to graph the isobolograms and to calculate fractional inhibitory concentrations (FIC). The latter are defined as the ratio of the MIC of an antimicrobial when it is combined (MIC_{A-BC} or MIC_{B-AC} or MIC_{C-AB}) divided by the MIC of this antimicrobial when it is used alone (MIC_A or MIC_B or MIC_c). The FIC values were used to calculate the FIC index: $FIC_I = (MIC_{A-BC}/MIC_A) + (MIC_{B-AC}/MIC_B) + (MIC_{C-AB}/MIC_C)$ (López-Malo Vigil, Palou, Parish, & Davidson, 2005). The FIC index value determines the type of interaction among the antimicrobials. A FIC index value near to 1 indicates an additive effect; if it is less than 1 it indicates synergism; and if it is greater than 1, the interaction is antagonic (López-Malo Vigil et al., 2005).

2.4.7. Differential scanning calorimetry (DSC)

The detection of changes in cellular components induced by the use of CFS, chitosan and SL were evaluated by DSC. For that purpose, *L. innocua* and *S. putrefaciens* were grown for 18 h at 30 °C in Mueller Hinton and Tryptone Soy broth (Biokar Diagnostics, Beauvais, France), respectively. After incubation populations were higher than 10^9 CFU/ml. Aliquots of 25 ml of bacterial suspensions were centrifuged (8000 *g*, for 15 min at 4 °C) in order to separate the growth medium. Microorganisms were washed twice and resuspended in 25 ml of Ringer's solution (Biokar Diagnostics, Beauvais, France) at pH 5.50. The CFS was concentrate by lyophilization by means of a freeze dryer (MartinChrist, Alpha 1–4 LD, Denmark). The lyophilized CFS was added, alone or combined with chitosan and SL. The concentrations of each antimicrobial used were equivalent to twice the minimal inhibitory concentration previously determinated (Schelegueda et al., 2012): 200 μg/ml chitosan; 36,000 μg/ml SL. Systems were incubated at 30 °C for 24 h under agitation. After that, cells were pelleted (8000 *g* for 15 min at 4 °C), washed twice with sterile distilled water and centrifuged again. They were weighed (5–15 mg) and transferred into stainless steel DSC crucibles. Pellets water contents were determined drying the samples to constant weight at 105 °C as 80–85% on wet basis. An empty crucible was used as reference. Samples and the reference were heated in the calorimeter (Q100, TA Instruments Waters, USA.) at 3 °C min^{-1} from 5 to 150 °C.

Since lyophilized CFS contains MRS media, an additional system of each microorganism was prepared in order to study its effect on the growth of *L. innocua* and *S. putrefaciens*. In this case, Ringer's solution was added with MRS media.

2.5. Statistical analyses

The AWDA were conducted in triplicate. Data obtained were analyzed by a variance analysis (ANOVA), 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%.

Regarding to microdilution method, each mixture of antimicrobials was tested by quintuplicate, and the same results were found for each replicate.

DSC and PCR analysis were conducted in duplicate. Same results were obtained in each trial.

Table 3
Range of concentrations and tested microorganisms.

Microorganisms	CFS (dilution)	Chitosan (μg/g)	SL (μg/g)
L. innocua 6a ATCC 33090	4^{-1}–256^{-1}	0–100	0–18,000
S. putrefaciens ATCC 8071	4^{-1}–256^{-1}	0–125	0–19,600

3. Results and discussion

3.1. Identification of the isolate

The studied strain produced yellow pigment in BHI agar, microscopy observation revealed Gram-positive cocci arranged in pairs or short chain, displayed pyrrolidonyl aminopeptidase activity but no catalase activity, showed the ability to grow at 10 and 45 °C, in media containing 6.5 g/100 ml NaCl, at pH 9.6, and in BEA medium; all features were in agreement with the genus *Enterococcus*. Further biochemical test suggested by Manero and Blanch (1999) allowed classifying the strain as *E. mundtii*.

Phylogenetic analysis based on 16S rRNA gene sequence (1396 bp) was performed. The fragment of the 16S rRNA gene amplified from strain *E. mundtii* Tw56 exhibited 100% homology with respect to *E. mundtii* ATCC 43186 and the entire sequence was deposited in the GenBank database under the accession number KC346258.

3.2. Detection of antimicrobial activity

Cell-free culture supernatant from *E. mundtii* Tw56 was able to inhibit the growth of different genera of Gram positive bacteria such as *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria*, *Pediococcus* and *Streptococcus* (Table 1). Bacteriocins antilisterial activity has been widely reported (Pinto et al., 2009; Tomé et al., 2006). Moreover, it has been observed that bacteriocins are able to inhibit other Gram positive bacteria genera including *Bacillus* and *Staphylococcus*. However, no inhibitory activity against Gram negative bacteria was found in these studies (Pinto et al., 2009; Rivas et al., 2012; Sequeiros, Vallejo, Marguet, & Olivera, 2010). It must be noted that CFS from *E. mundtii* Tw56 showed antibacterial activity against *Ps. aeruginosa* and *S. putrefaciens*. These bacteria are more resistant to bacteriocins due to the protection conferred by the outer membrane of Gram negative bacteria (Deegan et al., 2006). Previously, Kwaadsteniet, Todorov, Knoetze, and Dicks (2005) and Todorov, Wachsman, Knoetze, Meincken, and Dicks (2005) reported that some bacteriocins produced by *E. mundtii* inhibited the growth of Gram negative bacteria. Regarding to antimicrobials produced by other LAB genus, Ghanbari, Jami, Domig, and Kneifel (2013) observed that bacteriocins produced by *Lactobacillus casei* AP8 and *Lb. plantarum* H5 had antibacterial activity against *E. coli*, *Salmonella* spp., *Aeromonas hydrophila* and *Vibrio anguillarum*. Furthermore, Sarika et al. (2012) reported that bacteriocins produced by different subspecies of *Lactococcus lactis* were able to inhibit the growth of *Acinetobacter* sp., *E. coli* and *Ps. aeruginosa*. Obtained results suggest that the application of the bacteriocin like substance produced by *E. mundtii* Tw56 as a biopreservative may be more effective than the use of those bacteriocins that act exclusively against Gram positive bacteria.

3.3. Characterization of the antimicrobial activity

Treatment of CFS with trypsin resulted in loss of activity, confirming its proteinaceous nature. Resistance to treatment with

lysozyme suggests that the bacteriocin is not glycosylated. Same result was obtained when the CFS was treated with lipase, confirming that no lipid was involved in the inhibitory activity. Catalase test did not affect the antibacterial activity of strain under investigation; this result suggests that the inhibitory activity could not be due to hydrogen peroxide.

No change in inhibitory activity against *L. innocua* was recorded when the CFS was treated at pH settings between 2.0 and 10.0. Heating progressively reduced the inhibitory activity of the supernatant; the inhibition was completely lost after treatment at 121 °C for 15 min. As a consequence it cannot be used in foods submitted to thermal sterilization.

The results of bacteriocin stability throughout the storage time showed that the maximum inhibitory activity remained constant up to 6 months when the supernatant was stored at 4 °C, but after 6 months of storage there was a decrease of 50% in the activity. In contrast, 100% of the initial activity was observed after 1 year storage at −30 °C. Therefore, the bacteriocin can be applied to foods preserve by freezing.

3.3.1. PCR screening for bacteriocins structural genes

In order to identify the genes involved in the bacteriocin synthesis, PCR amplifications were performed on *E. mundtii* Tw56 genomic DNA using primer pairs based on *Enterococcus* bacteriocin structural genes detailed in Table 2. A PCR product of about 380 bp was obtained when specific primers for the detection of mundticin KS structural gene were used (Zendo et al., 2005). On the contrary, none of the enterocin A, B, P and L50A/B structural genes could be amplified suggesting that the corresponding gene clusters were not present in *E. mundtii*. In particular, only a few studies reported the inhibitory action of bacteriocins produced by *E. mundtii* (Pingitore, Todorov, Sesma, & Franco, 2012; Settanni et al., 2014). It must be highlight that mundticin KS produced by *E. mundtii* Tw56 was active against Gram negative bacteria, an unusual characteristic for bacteriocin produced by LAB.

3.3.2. Detection and quantification of antimicrobial activity against the bacterial flora of fish at pH 5.50

As it is shown in Table 4, CFS from *E. mundtii* Tw56 at pH 5.50 was able to inhibit the growth of bacterial flora of fish. Antibacterial effect was greater at pH 5.50 than at pH 6.50, since in the first case inhibitory activity against *Ps. fluorescens* and psychrophilic microflora isolated from fish was observed. Furthermore, diameters of inhibition zones were slightly higher at pH 5.50 than at 6.50. The greater inhibitory action of bacteriocins at acidic pH was previously reported. As an example, Campos et al. (2006) found that the bacteriocin produced by *E. mundtii* USC-51 inhibited *L. monocytogenes* and that its effect increased as pH decreased. Moreover, Khan et al. (2010) observed that the bacteriocin produced by *Enterococcus faecium* 7C5 and a pH decrease caused a synergistic effect. According to Gálvez, Abriouel, López, and Omar (2007) an acidic pH causes an increase in the net charge of bacteriocins which might promote their translocation through the cell wall. In addition, bacteriocin solubility could be increased with the decrease in pH. When titers were determined, it was observed that diameters of inhibition zone decreased as the CFS was diluted (Fig. 1). The larger titers were observed for *Ps. aeruginosa* and for psychrophilic flora isolated from fish (Table 4).

3.3.3. Determination of the interaction among CFS, chitosan and SL

For each bacterium, the mixture containing the least amount of preservatives that exhibited antimicrobial activity was selected. On this base, the FIC_I were calculated and the isobolograms were constructed (Fig. 2). Results showed that studied antimicrobials acted cooperatively on the inhibition of *L. innocua* and *S. putrefaciens*, since FIC_I were less than 1. Furthermore, points representing the antimicrobials mixtures on the isobolograms are below the lines of additivity, suggesting the presence of a synergistic effect (Berembaum, 1978). The interaction between chitosan and SL against *L. innocua* and *S. putrefaciens* has been previously determined (Schelegueda et al., 2012). On this base, the results indicate that the presence of the bacteriocin increases the synergistic effect which had been already observed. Moreover, synergistic effects between bacteriocins and other preservation factors have been reported (Deegan et al., 2006). Regarding SL, it has been reported that its combination with nisin produces an increase of antibacterial activity in several food systems (Gálvez et al., 2007). When it comes to chitosan, it is known that chelating agents can enhance the inhibitory effect of bacteriocins on Gram positive bacteria or allow its action on Gram negative ones. The latter effect is related to the fact that chelating agents make the outer

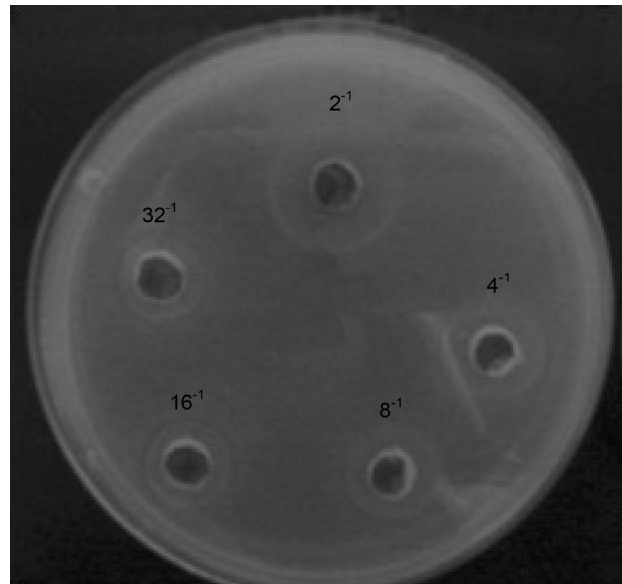

Fig. 1. Inhibition zones obtained by agar well diffusion assay against *Ps. aeruginosa* ATCC 9027. Represented numbers indicate assayed dilutions of CFS at pH 5.50.

Table 4
Antibacterial activity and titers of CFS from *E. mundtii* Tw56 against bacterial flora of fish.

Indicator microorganism	Diameter of inhibition zone (mm)	Titer (dilution)
L. innocua 6a ATCC 33090	16.0 ± 0.2^a	32^{-1}
Ps. aeruginosa ATCC 9027	18.0 ± 0.1^b	128^{-1}
Ps. fluorescens ATCC 49838	16.0 ± 0.1^a	2^{-1}
Lb. plantarum ATCC 8014	17.0 ± 0.2^c	32^{-1}
S. putrefaciens ATCC 8071	16.0 ± 0.1^a	32^{-1}
Psychrophilic flora isolated from fish	22.0 ± 0.2^d	64^{-1}

Superscript letters indicates no statistical difference.

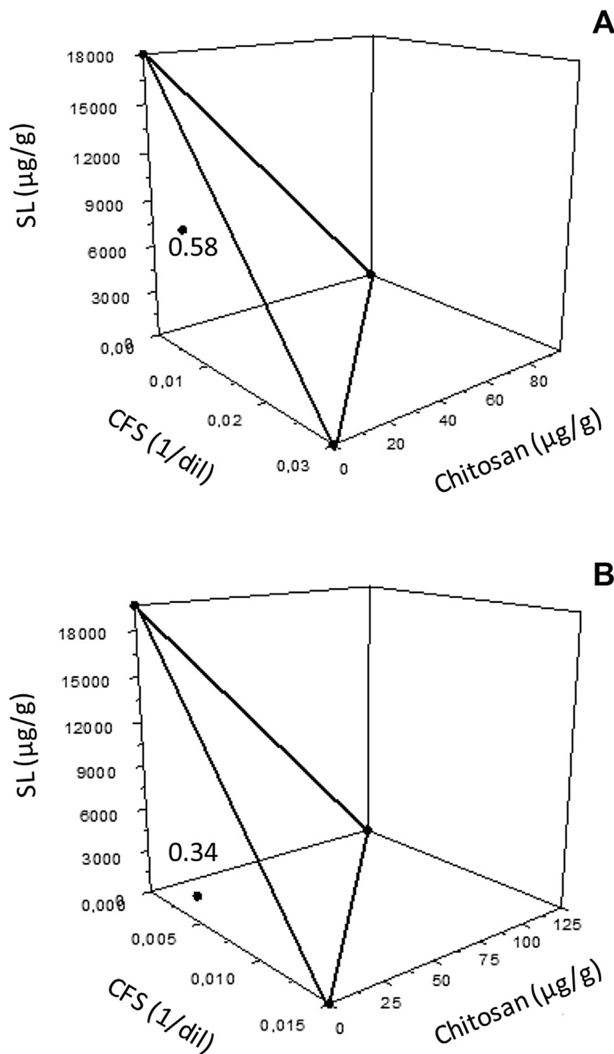

Fig. 2. Isobolograms of CFS titers and chitosan and SL MIC against *L. innocua* (panel A) and *S. putrefaciens* (panel B). Numbers represented near experimental points are FIC indexes.

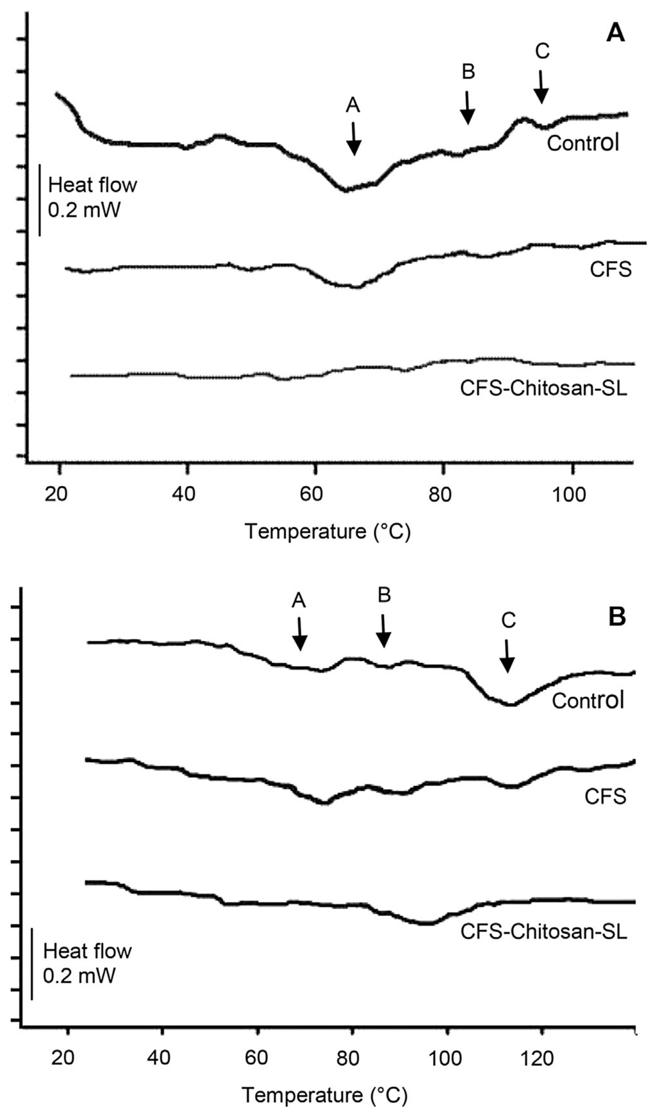

Fig. 3. Thermograms of *L. innocua* cells (panel A) and *S. putrefaciens* cells (panel B).

membrane of Gram negative bacteria leaky by extracting divalent cations that confer stability, allowing bacteriocins to reach the cytoplasmic membrane (Gálvez et al., 2007). In addition, Turgis et al. (2012) observed that the use of different essential oils in combination with nisin, pediocin or other bacteriocins produced by *E. faecium* MT 104 and MT 162 produced a synergistic effect on the inhibition of pathogenic microorganisms, such as *L. monocytogenes* or *E. coli* O157:H7. The existence of a synergic action allows the use of lower concentrations of the antimicrobials.

3.3.4. Differential scanning calorimetry (DSC)

Thermograms obtained by DSC were studied to identify sites of injury in *L. innocua* and *S. putrefaciens* (Fig. 3). When it comes to *L. innocua*, three major peaks were observed when untreated samples were heated. One of them (peak A) corresponds to thermal denaturalization of ribosomes. The second one (peak B), is consequence of the melting of DNA; while the last one (peak C), is associated with cell wall components (Lee & Kaletunc, 2002). Although a fourth peak, associated with the outer membrane, was reported when Gram negative bacteria were studied by DSC (Lee & Kaletunc, 2002), three peaks were observed on untreated *S. putrefaciens* samples. It must be noted that the third peak spanned a wider temperature range than the one obtained in the case of *L. innocua*.

Thermograms obtained from microorganisms treated with CFS, alone or in combination with chitosan and SL, showed changes on temperature and/or area of mentioned peaks. The temperature range and the percentage reduction of the peaks area are summarized in Table 5. MRS broth did not affect the thermal transitions of the studied bacteria.

Treatment with CFS reduced the area of the three identified peaks. In both microorganisms, the greater reduction was observed in the peak corresponding to cell envelope (C). These results are in concordance with Martinez, Rodriguez, and Suarez (2000), who report that once bacteriocins enter to a cell, through the pores formed in the cell membrane, may affect the DNA and RNA.

The joint use of CFS with chitosan and SL produced strong changes in *L. innocua* and *S. putrefaciens* thermograms. Regarding to *L. innocua*, the reduction of peaks A and B was greater than 90%, while peak C could not be detected. In the case of *S. putrefaciens*, peaks A and C could not be detected, and peak B showed an increase of its area. Considering the range of temperature of the latter peak, there may be an overlap between peaks B and C, which could justify the increase of enthalpy released.

Table 5
Effect of CFS alone and combined with chitosan and SL on the thermal transitions of *L. innocua* and *S. putrefaciens*.

	System	Peak A		Peak B		Peak C	
		T (°C)	Area reduction (%)	T (°C)	Area reduction (%)	T (°C)	Area reduction (%)
L. innocua	Control	58–72		80–91		92–99	
	CFS	56–76	45	82–89	80	97–100	82
	CFS-Chitosan-SL	54–60	98	70–78	92	ND	
S. putrefaciens	Control	50–73		78–86		101–119	
	CFS	60–74	55	81–89	64	105–113	98
	CFS-Chitosan-SL	ND		81–100	*	ND	

ND: not detectable.
*An increase of the peak area was observed.

The analysis of the thermograms shows that cellular components are more severely affected when CFS if combined with chitosan and SL, than when it is used alone. This would explain the synergistic behavior among the antimicrobials observed in the previous item.

4. Conclusions

From the results obtained in this study, it can be concluded that mundticin KS produced by *E. mundtii* Tw56, strain isolated from *O. platensis* intestine, exhibited a broad spectrum of activity being able to act on *Ps. aeruginosa* and *S. putrefaciens*. In consequence, it could be used as an additional hurdle in the preservation of minimally processed fish and/or other sea products. The combination of the bacteriocin with chitosan and SL induced a synergic action on *L. innocua* and *S. putrefaciens* inhibition. Mentioned action can be linked with strong changes produced on cellular components as it was demonstrated by DSC. The studies about the effect of the bacteriocin in combination with chitosan and SL in fish are in progress in order to confirm their ability to control bacterial growth.

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