Lactococcus lactis RBT18: from the rainbow trout farm to the lab, the tale of a nisin Z producer [†]

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Abstract: Infectious diseases, such as lactococcosis caused by *Lactococcus garvieae*, are portrayed as critical limiting factors in aquaculture. The antimicrobial properties of Lactic Acid Bacteria (LAB), mainly the production of organic acids and bacteriocins (*e. g.*, the lanthionine containing nisins A and Z; NisA and NisZ, respectively), led to propose LAB as probiotics to be used as an alternative and/or complementary strategy to vaccination and chemotherapy in aquaculture. *L. lactis* RBT18, isolated from cultured rainbow trout (*Oncorhynchus mykiss*, Walbaum)., exerts a strong direct and extracellular antimicrobial activity against *L. garvieae* and other ichthyopathogens, being the latter heat-resistant (100 °C, 10 min), and thus suggesting the involvement of a thermostable antimicrobial compound (*i. e.*, bacteriocin). Cross-immunity tests using the agar-well-diffusion test (ADT) and PCR assays suggested that NisA/Z is the bacteriocin responsible for the extracellular antimicrobial activity exerted by *L. lactis* RBT18. To demonstrate this hypothesis, the bacteriocin was purified to homogeneity by two multi-chromatographic procedures. MALDI TOF-MS analyses of purified samples after the last reverse-phase chromatography step identified the presence of NisZ (3,330 Da), and its oxidized form (3,346 Da), derived from the oxidation of a lanthionine ring. The oxidized NisZ showed a diminished antimicrobial activity which would increase the chances of bacterial pathogens to evade its antimicrobial activity. Further experiments are necessary to assess the *in vitro* and *in vivo* safety and efficiency of *L. lactis* RBT18 as probiotic in aquaculture, but also to optimize the environmental conditions to reduce bacteriocin oxidation and thus bacterial pathogen resistance.

Keywords: aquaculture; probiotics; lactic acid bacteria; antimicrobial activity; bacteriocins; nisin

1. Introduction

Aquaculture is currently a growing worldwide cornerstone of the food supply chain, with an estimated total production of 82 million tonnes in 2018, which constituted a new record [1]. Nevertheless, the required intensification of the sector practices leads to the inevitable emergence of fish diseases, which poses one of the main challenges to the future of aquaculture [2,3]. In respect, lactococcosis, caused by *Lactococcus garvieae*, is regarded as a serious obstacle to freshwater cultures, such as those of rainbow trout (*Oncorhynchus mykiss*, Walbaum). Additionally, *L. garvieae* has also been deemed as an emergent pathogen in human medicine [4]. Lactic

Acid Bacteria (LAB) comprise a vast group of Gram-positive bacteria widely spread in various ecosystems. Generally Regarded As Safe, LAB have been assessed as probiotics in aquaculture for several years, mainly due to their antimicrobial mechanisms, such as the production of organic acids and bacteriocins. The urge to reduce the use of antibiotics in aquaculture and other farming practices has led to an increase in attention towards alternatives such as the bacteriocin-producing bacteria [3,5,6]. Nisin, a lantibiotic (a lanthionine containing bacteriocin) constituted by 34 amino acids residues and five thioether bridges, is by far the most well characterized LAB bacteriocin. Amongst the multiple LAB species assessed, various strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* have been associated with the production of nisin variants, primarily nisin A (NisA), and its most well spread natural variant nisin Z (NisZ) [6-9]. Since bacteriocins are an heterogenous group of proteinaceous compounds that are secreted to the culture medium, bacteriocin purification is regarded as a critical and diverse step for their characterization [5,10,11]. Thus, during the course of this work two different multi-chromatographic procedures were performed in order to purify to homogeneity a putative bacteriocin produced by *L. lactis* RBT18, a strain previously isolated from cultured rainbow trout.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

L. lactis RBT18 was previously isolated from aquacultured rainbow trout. The microorganisms used as indicators included ichthyopathogens, such as *L. garvieae* CF00021, *L. garvieae* JIP29-99 and *L. garvieae* CLG-4, as well as non-pathogenic bacteria such as *Pediococcus damnosus* CECT4797. Additionally, two different nisin producing strains were used as positive controls, namely *L. lactis* BB24 (NisA producer, NisA+) and *L. cremoris* WA2-67 (NisZ producer, NisZ+) [8,9,12]. Unless otherwise stated, all the bacterial strains were aerobically grown in de Man, Rogosa and Sharpe broth (MRS, Oxoid Ltd., UK) and incubated overnight at 30 °C.

2.2. Antimicrobial activity

Firstly, *L. lactis* RBT18 was assayed for direct antimicrobial activity by a stab-on-agar test (SOAT), against four indicator microorganisms (*L. garvieae* CF00021, *L. garvieae* JIP29-99, *L. garvieae* CLG-4 and *P. damnosus* CECT4797), as previously described by Cintas *et al.* [13]. Briefly, *L. lactis* RBT18 was stabbed onto MRS agar (1.5%, w/v) plates and incubated at 30 °C for 5h. Subsequently, 15 ml of soft agar (0.8%, w/v) MRS medium seeded with the indicator microorganisms cited above (*ca.* 1×10^5 cfu/ml) was added onto the MRS agar plates. After incubation overnight at 30 °C, the plates were analyzed for the presence of inhibition zones (absence of visible microbial growth around the stabbed cultures). Secondly, the extracellular antimicrobial activity of the cell-free supernatants (CFS) of *L. lactis* RBT18 was assayed by an agar well diffusion test (ADT) against two indicator microorganisms (*L. garvieae* CF00021 and *P. damnosus* CECT4797), as previously described by Cintas *et al.* [13]. In brief, CFSs were obtained by centrifugation (12,000 rpm at 4 °C for 10 min) of *L. lactis* RBT18 cultures grown in MRS broth at 30 °C for 16 h. Then, CFSs were filter-sterilized through 0.22 μm-pore-size filters (Millipore Corp., USA). Aliquots of CFSs were heat-treated (100 °C, for 10 min), and, subsequently, 50 μl was placed into wells (5 mm diameter) done in cooled MRS agar (0.8%, w/v) plates, previously seeded with the indicator microorganisms cited above (*ca.* 1×10^5 cfu/ml). After 2 h at 4 °C, the plates were incubated at 30 °C for 16 h, and, subsequently, analysed for the presence of inhibition zones around the wells.

2.3. PCR-amplification of the bacteriocin structural gene

Total bacterial DNA from *L. lactis* RBT18 was extracted by using the InstaGene Matrix (BioRad Laboratories, Inc., USA). PCR reactions were performed using nisin A/Z specific primers (NisF: 5'-CTTGGATTTGGTATCTGT TTCG-3'; NisR: 5'-CAATGACAAGTTGCTGTTTTC A-3'). PCR cycling reactions were conducted in a MJ Mini Gradient Thermal Cycler (BioRad Laboratories, Inc. Spain) as follows: initial denaturation (95 °C for 3 min); followed by 35 cycles of denaturation (94 °C for 45 sec), annealing (57 °C for 50 sec), and elongation (72 °C for 40 sec); and a final extension (72 °C for 5 min). The amplified PCR products were resolved by electrophoresis through agarose (1.5%, w/v) (Pronadisa, Spain) gels stained with the GelRed Nucleic Acid Gel Stain (Biotium, Inc., USA), and analyzed with the GelDoc 1000 documentation system (BioRad Laboratories). The molecular size marker used was the HyperLadder 100-bp (Bioline GmbH, Germany). *L. lactis* BB24 (NisA+) was used as positive control.

2.4. Cross immunity assays

CFSs from *L. lactis* RBT18, *L. lactis* BB24 (NisA+) and *L. cremoris* WA2-67 (NisZ+) cultures grown overnight in MRS broth at 30 °C were obtained as described above. The cross-immunity assays were performed using an ADT, performed as described above. The three bacterial strains were seeded in MRS agar (0.8%, w/v) plates and challenged against the three respective CFSs.

2.5. Bacteriocin purification procedures

Procedure 1. Firstly, the putative bacteriocin produced by *L. lactis* RBT18 was purified by a modification of the multi-chromatographic procedure described by Cintas *et al.* [13]. Briefly, CFS from a 1-l *L. lactis* RBT18 culture, grown in MRS broth overnight at 30 °C, was obtained through centrifugation (8,000 rpm, 4 °C, for 20 min). Then, ammonium sulphate (50%, w/v; saturation) (Merck, Germany) was added to the supernatant and the sample was kept with slow stirring at 4 °C for 2 h, followed by a second centrifugation at 8,000 rpm for 30 min. This resuspended fraction was subsequently desalted by gel filtration, by using PD-10 columns (GE Healthcare Life Sciences, Spain). Following gel filtration, a cation-exchange chromatography was performed by using a column containing SP Sepharose Fast Flow resin (GE Healthcare Life Sciences). Subsequently, a hydrophobic interaction chromatography was performed using a smaller column, this time filled with Octyl-Sepharose CL-4B resin (GE Healthcare Life Sciences).

Procedure 2. Additionally, the putative bacteriocin produced by *L. lactis* RBT18 was purified by a modification of the multi-chromatographic procedure described by Suda *et al.* [14]. In brief, *L. lactis* RBT18 was cultured in GM17 [M17 broth supplemented with 0.5% (w/v) glucose] (Difco, USA) overnight at 30 °C. A 1.4 l of Tryptone Yeast Extract (TY) broth was vacuum-filtered through a 22 μm filter and passed through a column packed up to one third with Amberlite XAD-16 beads (Sigma Aldrich Co., Ltd., USA), where 0.5 l was retained in the column. 50 ml of glucose [at 20% (w/v)] and 50 ml of β-glycerophosphate (Sigma Aldrich Co., Ltd.) were added to the resulting 900 ml of TY broth and this medium was inoculated with a *L. lactis* RBT18 culture. The culture was then centrifuged at 7,000 g for 15 min, and the resulting cell pellet was resuspended in 300 ml of 70% (v/v) isopropanol with 0.1% (v/v) trifluoroacetic acid (TFA), and gently stirred at room temperature for 3 h. Meanwhile, the overnight cultured supernatant was loaded into a smaller column previously packed with 60 g of Amberlite XAD-16 beads and eluted by 70% (v/v) isopropanol/0.1% (v/v) TFA. After stirring for 3 h, the cell pellet was re-centrifuged at 7,000 g for 15 min. After being combined, the propanol from the two split samples was evaporated by using a Rotavapor R-210 (Buchi, Switzerland) at 42 °C with an initial pressure of 120 mBar, that was slightly decreased throughout the process. Subsequently, the sample pH was adjusted to 4.0 with NaOH and then applied to a 10 g (60 ml) Mega BE-C18 column (Agilent, USA), previously equilibrated with methanol and deionized water. The column was subsequently washed with 30%(v/v) EtOH, and finally eluted with 70% (v/v) isopropanol/0.1% (v/v) TFA. Finally, aliquots of 10 ml were re-applied to the rotary evaporator until each aliquot reached a final volume of, approximately, 2 ml.

2.5.1. Reversed Phase-Fast Protein Liquid Chromatography (RP-FPLC)

The final fractions from both purification procedures were applied to a reversed-phase (RP) chromatography column (Source 5RPC ST 4.6/150) (GE Healthcare Life Sciences) in an ÄKTA purifier fast protein liquid chromatography (RP-FPLC) system (GE Healthcare Life Sciences). The bacteriocins were eluted with a linear gradient of 70% (v/v) isopropanol with 0.1% (v/v) TFA. The resulting fractions were assayed for antimicrobial activity against *L. garvieae* CF00021 by a microtiter plate assay (MPA), as previously described by Cintas *et al.* [13]. The microtiter plates were incubated at 30 °C for 16 h, and the growth inhibition of the indicator microorganism was assessed spectrophotometrically (620 nm) with a microtiter plate reading system (FLUOstar Optima, BMG Labtech, Germany). The antimicrobial activity was expressed as bacteriocin units (BU), defined as the reciprocal of the highest dilution of purified bacteriocin causing 50% growth inhibition.

2.6. Mass Spectrometry Analysis (MALDI-TOF/TOF)

MALDI-TOF/TOF (Time of Flight) was performed with a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, MDS Sciex, Canada) equipped with a pulsed nitrogen laser emitting at 337 nm, at the Unidad de Proteómica, Facultad de Farmacia (UCM).

3. Results and Discussion

3.1. Antimicrobial activity

L. lactis RBT18 displayed a strong direct and extracellular antimicrobial activity against all the microorganisms tested as indicators (results not shown). The extracellular antimicrobial activity was heat-resistant (100 °C, 10 min), thus suggesting the involvement of a thermostable antimicrobial compound (*i. e.*, bacteriocin) [5-7].

3.2. PCR-amplification of the extracellular antimicrobial compound (bacteriocin) structural gene

The pair of specific primers for the amplification of NisA/Z structural gene allowed to amplify in *L. lactis* RBT18 a fragment of the expected size (132-bp) (results not shown).

3.3. Cross immunity assay

The cross-immunity assay revealed that none of the three tested CFSs (*L. lactis* RBT18, *L. cremoris* WA2-67 and *L. lactis* BB24) exerted antimicrobial activity against the three respective producer strains. Taken all together, results from PCR and cross-immunity assays, strongly indicated that the antimicrobial activity exerted by *L. lactis* RBT18 might be due to the production of NisA/Z or other nisin-variant bacteriocin. With regard to this, lactococci producing nisin and nisin-variants are widespread in nature [6-9].

3.4. RP-FPLC bacteriocin purification and MALDI-TOF/TOF mass spectrometry analyses

The last RP-FPLC performed at the end of the first purification procedure resulted in a single absorbance peak with a high antimicrobial activity (Fraction I), which eluted at 58% (v/v) elution buffer (results not shown). This fraction represented a yield of 77% and a 348,000-fold increase in specific antimicrobial activity (Table I). MALDI-TOF/TOF mass spectrometry analysis allowed the identification of a peptide with a molecular mass of 3,332.17 Da, which fits with that of NisZ (3,331 Da), and a second peptide with a molecular mass of 3,348.97 Da (Figure 1), suggesting the presence of a NisZ oxidized form (16 Da added), likely derived from the oxidation of a lanthionine ring. The RP-FPLC performed at the end of the second purification procedure resulted in two distinctive absorbance peaks with antimicrobial activity (Fractions I and II), which eluted at 55 and 58% (v/v) elution buffer, respectively (Figure 2). These fractions represented a yield of 0.4 and 3.8%, and a 2,700- and 35,000-fold increase in specific antimicrobial activity, respectively (Table 1). MALDI-TOF/TOF mass spectrometry analyses of these samples allowed the identification of two peptides with molecular masses of 3,330.09 Da (Fraction II), which, once again, fits with that of NisZ (3,331 Da) [9] and 3,346.26 Da (16 Da added) (Fraction I), which corresponds to an oxidized form of NisZ (results not shown). Our results showed a diminished specific antimicrobial activity of oxidized NisZ as compared to that the non-oxidized form (Table I). The decrease in specific antimicrobial activity of the oxidized form might be related to the nisin mechanism of bactericidal action. In this respect, the interaction between nisin and a peptidoglycan precursor named lipid II, not only allows the formation of a highly specific pore, but also interferes with the bacterial cell wall synthesis. Therefore, the diminished specific antimicrobial activity of the NisZ oxidized form might be related to its lack of ability to properly bind to and sequester the lipid II, which is a crucial step of the bactericidal process [7,15].

Table 1. using two multi-chromatographic purification procedures.

	Volume (ml)	Total $A_{280}{}^a$	Total activity (10^3 BU)[b]	Specific activity[c]	Increase in specific activity[d]	Yield (%)[e]
			Procedure 1			
Fraction I	1.5	0.031	983	31,700,000	348,000	77
			Procedure 2			
Fraction I	0.5	0.010	10	975,000	2,700	0.4
Fraction II	0.6	0.007	98	12,600,000	35,000	3.8

[a]Absorbance at 280 nm (A280) multiplied by the volume (ml). [b]Antimicrobial activity in bacteriocin units per milliliter (BU/ml) and multiplied by the total volume (ml). [c]Specific antimicrobial activity expressed as the total

antimicrobial activity (BU) divided by total A280. [d]Specific antimicrobial activity of a fraction (BU/A280) divided by the specific antimicrobial activity of the first supernatant (BU/A280). [e]Yield expressed as the total antimicrobial activity (BU) of a fraction multiplied by 100 and divided by the total antimicrobial activity (BU) of the CFS.
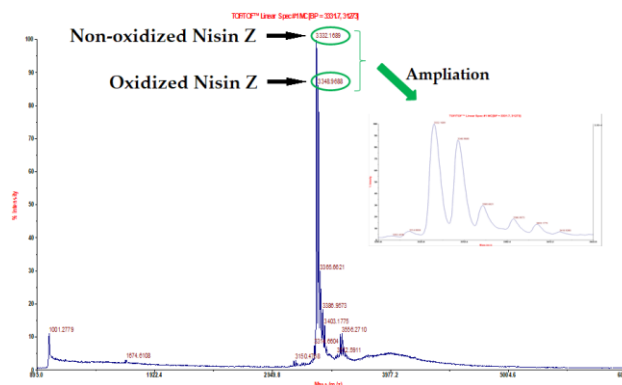

Figure 1. MALDI-TOF/TOF mass spectrometry analysis of NisZ from *L. lactis* RBT18 purified after the final RP-FPLC using the multi-chromatographic procedure 1.
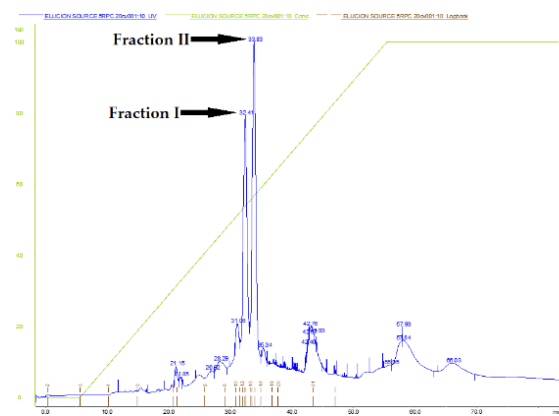

Figure 2. Final RP-FPLC of the multi-chromatographic procedure 2 used to purify NisZ from *L. lactis* RBT18.

4. Conclusions

The PCR analysis and cross-immunity assays carried out in this work constitute an appropriate strategy for the preliminary identification of lactococcal strains producing NisA/Z or other nisin-variant bacteriocins. Moreover, the two multi-chromatographic purification procedures used in this work were found to be suitable for the purification of NisZ, both in native and oxidized forms, being the procedure 1 much more appropriate and effective for this goal, since the antimicrobial activity yield and the increase in specific antimicrobial activity were 18-times higher. On the other hand, the diminished antimicrobial activity of oxidized-NisZ would increase the chances of bacterial pathogens to evade its antimicrobial activity, being thus necessary a better understanding of the bacteriocin oxidation process and its implications on pharmacokinetics/pharmacodynamics and development of pathogen resistances. Moreover, further experiments are required to assess the *in vitro* and *in vivo* safety and efficiency of the bacteriocinogenic strain *L. lactis* RBT18 as probiotic in aquaculture, but also to optimize the environmental conditions leading to reduce bacteriocin oxidation and thus bacterial pathogen resistance.

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