SCREENING OF ANTI-CLOSTRIDIAL LACTIC ACID BACTERIA STRAINS ISOLATED FROM URUGUAYAN DAIRY FARMS

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ABSTRACT

A total of 130 strains of lactic acid bacteria (starter lactic acid bacteria -SLAB- and non-starter lactic acid bacteria -NSLAB-) were isolated from milk, whey and cheese. Anti-clostridial activity was detected in 56 isolates against *Clostridium tyrobutyricum* ATCC 25755. Seven strains with the highest inhibitory activity were selected to determine their inhibitory mechanisms. Anti-clostridial activity of strains *Streptococcus macedonicus* 23, *S. macedonicus* 24, *Lactobacillus casei/paracasei* 29 and *Lactobacillus rhamnosus* 104 was due to organic acids production. Inhibitory mechanisms of strains *L. casei* 26 and 95 were acid production, bacteriocin and hydrogen peroxide. While anti-clostridial activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* 76 was due to acid and hydrogen peroxide production. Cell-free supernatants obtained from strains 26, 95 and 76 conserved their anti-clostridial activity after thermal treatments at 70 and 100 °C. Supernatants obtained from bacteriogenic strains 26 and 95 were analyzed by RP-HPLC. RP-HPLC fractions with anti-clostridial activity were analyzed by mass spectrometry (MALDI-TOF). A peptide with molecular weight of 1162.54 Da was detected in HPLC fraction of *L. casei* 95. No results were obtained for MALDI-TOF analysis of another HPLC fractions. Based on the thermostability and low molecular weight, *L. casei* 95 anti-clostridial peptide would belong to a class I or II bacteriocin according to Gram-positive bacteria antimicrobial peptides classification.

Keywords: LAB, NSLAB, SLAB, *Clostridium*, bacteriocin, inhibition, late blowing

INTRODUCTION

Late blowing defect is one of the major causes of spoilage in semi-hard and hard ripened cheeses. Lactate-fermenting clostridia cause this defect, characterized by excessive gas production and off-flavors. Clostridial spores are able to survive the milk pasteurization and cheese-making processes, remaining in the cheese during ripening. *Clostridium* metabolism of lactate includes the production of organic acids (butyric and acetic), carbon dioxide and hydrogen, resulting in irregular eyes, splits and cracks in the cheese, as well as undesirable aroma and flavors (**Alvenäs, 2015; Calamari *et al.*, 2018**). *Clostridium tyrobutyricum* is considered the primary cause of late blowing in cheese, other clostridial species such as *Clostridium sporogenes, Clostridium beijerinckii* and *Clostridium butyricum* also have been shown to significantly contribute to the appearance of this defect (**Garde *et al.*, 2013; Bermúdez *et al.*, 2016**).

Much work has been undertaken to reduce the number of spores in milk and to prevent spore germination and growth in cheese. Farm-management and good hygienic milking practices are essential to reduce raw milk clostridial contamination (**Gupta and Brightwell, 2017; Komori *et al.*, 2018**). In the cheesemaking process many strategies have been proposed to prevent late blowing defect like addition of lysozyme and physical spores removal by bactofugation or microfiltration. However, the reduction in spore numbers achieved by bactofugation may be insufficient to prevent late blowing defect if spore load is very high. On the other hand, microfiltration can only be applied to skimmed milk. Regarding the use of lysozyme, there is an increasing demand from consumers for foods with low chemical additive content (**Ávila *et al.*, 2014; Garde *et al.*, 2011; Brändle *et al.*, 2016**). As an alternative, addition of lactic acid bacteria (LAB) strains, biologically active against *Clostridium*, as starter (SLAB) or adjunct cultures (non-starter LAB -NSLAB-) in cheese manufacture has been proposed (**Gómez, 2017**).

LAB are considered as natural preservatives due to the production of antimicrobial metabolites such as organic acids (lactic and acetic), hydrogen peroxide, acetaldehyde, diacetyl, acetoin, fatty acids, carbon dioxide, ethanol, D-amino acids, reutericyclin, reuterin and bacteriocins (**Özogul and Hamed, 2018; García-Cano *et al.*, 2019**).

Bacteriocins are ribosomally synthesized peptides of 20 to 60 amino acids length, with antibacterial activity towards closely related microorganisms, although there are bacteriocins with a broad range of antimicrobial activity. The best known mechanism of action of these peptides is the pore formation in the cell membrane of bacteria. As consequence of the loss of essential ions and metabolites through the pores, as well as the dissipation of the proton-motive force, the biosynthesis processes stop which leads to cell death. Bacteriocins of subclass Ia, such as nisin, are able to inhibit clostridial spores outgrowth. They can also inhibit cell wall synthesis by binding to lipid II, responsible for peptidoglycan subunits transfer (**Dickman *et al.*, 2019; Kumariya *et al.*, 2019**). Research on broad spectrum LAB bacteriocins, tolerant to high thermal stress and pH < 6.0, has gained interest for their use in dairy products. A current strategy in some food industrial processes is the addition of bacteriocin produced *ex situ* or the incorporation of LAB strains for *in situ* bacteriocin production. Nisin is the only antimicrobial peptide approved as food preservative worldwide (**Perez *et al.*, 2014; Martínez *et al.*, 2016; Silva *et al.*, 2018**).

The application of bacteriocins for biopreservation of foods usually includes the following approaches: inoculation of food with the bacteriocin-producer strain; addition of purified or semi-purified bacteriocin as food additive; and use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing (**Silva *et al.*, 2018**).

The aim of this study was therefore to isolate anti-clostridial lactic acid bacteria (SLAB and NSLAB) present in dairy products from artisanal cheesemaking farmhouses and to determine the nature of its inhibitory activity against *Clostridium* spp..

MATERIALS AND METHODS

Isolation of anti-clostridial lactic acid bacteria

Thirty raw milk, whey and cheese samples were obtained from ten Uruguayan artisanal cheesemaking farmhouses. Farmers produce cheese with their own milk using non-commercial microbial starters. Decimal dilutions were plated on de

Man, Rogosa and Sharpe agar (MRS, Oxoid Ltd., UK) and incubated at 30 and 42 °C for 48 h under microaerophilic and anaerobic conditions using an Anoxomat Mark II device (Advanced Instruments Inc., USA). Approximately five colonies were picked randomly from each plate and isolated for further analysis. Typical LAB isolates were selected according to colony description, cell morphology, Gram staining and routine biochemical test.

LAB isolates with inhibitory effect against *C. tyrobutyricum* ATCC 25755 were detected by two screening test. In a first screening, 130 LAB isolates were analyzed in cross-streak inhibition assay according to **Christiansen *et al.* (2005)**. Anti-clostridial activity indicator strain *C. tyrobutyricum* ATCC 25755, and LAB isolations were inoculated in Reinforced Clostridium Medium (RCM, Difco, USA) and MRS broth respectively, incubated anaerobically at 37 ºC for 24 - 48 h. Indicator strain was streaked on RCM agar and LAB isolates were streaked across the lane of *C. tyrobutyricum* ATCC 25755. Plates were incubated anaerobically at 37 °C for 48 h. Anti-clostridial LAB strains were detected by the absence of *C. tyrobutyricum* ATCC 25755 growth around the streaks. A second screening test using disc diffusion assay was performed. LAB isolates showing anti-clostridial activity in the first screening test, were grown in 50 mL of MRS broth in a 100 mL Erlenmeyer flask and incubated at 37 ºC during 16 - 18 h. Supernatants were obtained by centrifugation at 10000 rpm for 15 min at 4 ºC, and filtered with 0.22 µm pore size filter (Millipore, MA, USA). A log-phase culture of *C. tyrobutyricum* ATCC 25755 in RCM broth was adjusted to optical density 0.2 - 0.3 at 620 nm and 100 µL were spread on RCM agar plates. For disc diffusion assay, 100 µL of cell-free supernatants were inoculated onto filter paper discs (diameter 6 mm, Whatman, GE Health Care, NJ, USA) placed on RCM plates. Plates were incubated anaerobically at 37 ºC for 48 h (**Jones *et. al.*, 2008; Matamoros *et al.*, 2009**). Antimicrobial activity was evaluated comparing the diameter of the halo around the disc. Commercially available anti-clostridial strain *L. casei* BAL C (LC4P1, Sacco SRL, Cadorago, CO, Italy), was included in the assay. Inhibition results were statistical analyzed by One-way ANOVA. Differences were considered statistically significant at p <0.05.

Identification of anti-clostridial LAB strains

Anti-clostridial LAB isolates were identified by 16S rRNA sequencing. Preparation of genomic DNA was performed from bacterial overnight cultures in MRS broth, cells were harvested at 10000 rpm for 5 min., and DNA was purified using GeneJet Genomic DNA Purification kit (Thermo Fisher Scientific, Lithuania) following the manufacturer's instructions. Purified DNA was suspended in 100 µL TE buffer and used as template in amplification reactions. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Termo Scientific Incorporation, Wilmington, DE, USA).

For amplification reactions of the 16S rRNA gene, primers rD1 (5'-AAGGAGGTGATCCAGCC-3') and fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') were used according to the protocols of **Weisburg *et al.*, (1991)**. Amplicons were visualized by electrophoresis in 1% agarose gels. Amplified fragments were purified and sequenced by Macrogen Sequencing Service, Korea, in an ABI PRISM 3730XL capillary sequencer, using the primer fD1 already described. DNA sequences were compared by BLAST analysis with those existing in the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Nature of anti-clostridial compounds and sensitivity to heat treatments

Cell-free supernatants of seven LAB strains showing the highest anti-clostridial activity in disc diffusion assay were prepared as previously mentioned. *L. casei* BAL C was included in this study. Nature of anti-clostridial compounds present in the cell-free culture supernatants was elucidated by disc diffusion method. To reject the inhibitory action of organic acids, the cell-free supernatants were adjusted to pH 6.5 using NaOH 1M and concentrated 10X by rotary vacuum evaporation at 270 rpm and 70 ºC. Neutralized supernatants were treated with proteinase K (2.5 mg/mL, Sigma Aldrich, MO, USA) to detect antimicrobial peptide compounds and with catalase (1.0 mg/mL, Sigma Aldrich, MO, USA) for hydrogen peroxide production at 37 ºC for 3 h (**Mathot *et al.*, 2003; Jones *et. al.*, 2008**). Supernatants without enzymatic treatment were included as positive control. Paper discs with 100 µL of each treated cell-free supernatants on RCM agar plates were previously spread with *C. tyrobutyricum* ATCC 25755. Inhibition of growth around the discs was examined after incubation at 37 ºC for 48 h in anaerobic condition. Isolates that produce anti-clostridial compounds other than organic acids, were selected for further analysis

To test heat stability of anti-clostridial compounds, neutralized supernatants were heated at 70 and 100 ºC for 15 min in a temperature-controlled water bath. Remaining anti-clostridial activity was determined by disc diffusion method with *C. tyrobutyricum* ATCC 25755 as indicator microorganism, as described above (**Mathot *et al.*, 2003**).

Purification and characterization of anti-clostridial peptides

Anti-clostridial peptides of *L. casei* BAL C and three bacteriocinogenic LAB isolates were characterized. Cell-free supernatants from 250 mL of MRS culture broth were adjusted to pH 6.5 with NaOH 1M. Ice-cold acetone was added to the extent of 50% saturation under cold stirring for 15 min (**Pal *et al.*, 2010**). Mix was kept at -20 °C overnight and then acetone was discarded. Residual acetone was removed by evaporation at 37 °C. Protein precipitates were resuspended in distilled water and concentrated 10X by rotary vacuum evaporation at 70 °C under agitation at 240 rpm in a Heidolph Laborota 4000 Rotatory Evaporator (Heidolph Instruments GmbH & CO. KG, Schwabach, Germany). The resulting protein crude extracts were filtered with 0.22 µm pore size filter and the anti-clostridial activity was verified.

Anti-clostridial peptides purification was realized by analytical RP-HPLC (Shimadzu, LC-20A). All chromatographic processes were performed on a C18 column. The column was equilibrated with solution A (95% ultrapure water, 0.1% trifluoroacetic acid and 5% acetonitrile) and solution B (0.1% trifluoroacetic acid and 100% acetonitrile). Elution program was as follow: 100% solution A for 5 min., solution A linearly decreased to 0% and solution B increased to 100%, finally solution B was maintained at 100% for 30 min. to 40 min. The flow rate was 1 mL/min and elution was monitored by UV detector at 220 nm (**Lü *et al.*, 2014**). HPLC eluted fractions were collected and their anti-clostridial activity was evaluated against *C. tyrobutyricum* ATCC 25755 by disc diffusion assay.

MALDI-TOF mass spectrometry was used to determinate the molecular mass of peptides in HPLC eluted fractions. Mass spectra of digestion mixtures were acquired using a matrix assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF/TOF, 4800Analyzer, ABiSciex) in positive reflector mode. Collision induced dissociation (CID) MS/MS spectra of selected peptides ions were also acquired. Proteins were identified with measured m/z values in MS and MS/MS acquisition modes and using the MASCOT search engine (MatrixScience, http://www.matrixscience.com) in the Sequence Query search mode.

RESULTS AND DISCUSSION

Screening and identification of anti-clostridial LAB strains

A total of 130 LAB were isolated from milk, cheese and whey samples. Isolates were evaluated for their ability to produce anti-clostridial compounds by cross-streak inhibition assay. Inhibitory activity against *C. tyrobutyricum* ATCC 25755 was observed in 56 isolates, 17 were isolated from milk, 29 from cheese and 10 from whey samples (data not shown).

Isolates with anti-clostridial activity were identified by 16S rRNA gene sequencing. Most isolates belonged to the *Lactobacillus casei* Group (66.1%) (*L. casei*, *L. paracasei* and *L. rhamnosus*), followed by *Lactobacillus delbrueckii* (7.1%), *Streptococcus macedonicus* (formally *S. gallolyticus* subsp. *macedonicus*) (7.1%), *Pediococcus pentosaceus* (5.4%) and *Enterococcus faecalis* (5.4%).

L. casei Group comprised most of the isolates with anti-clostridial activity (37 out of 56). The high degree of phenotypic similarity and phylogenetic proximity between species makes it difficult to discriminate between them, both by biochemical tests as well as by molecular biology techniques, such as 16S rRNA gene sequencing (**Iacumin *et al.*, 2015; Huang *et al.*, 2018**). Due to this, 12.5% (7 out of 56) were identified as *L. casei*, while 7.1% (4 out of 56) and 26.8% (15 out of 56) corresponded to *L. paracasei* and *L rhamnosus*, respectively. Unidentified isolates were named as *L. casei* Group strains.

Anti-clostridial activity of LAB isolates and commercial strain *L. casei* BAL C was evaluated by disc diffusion method, sixteen isolates with the highest anti-clostridial activity were selected (Fig. 1 A). The diameters of the inhibition halos generated by these isolates were compared with *L. casei* BAL C (Fig. 1 B). Four LAB isolates that presented lower inhibitory activity (p<0.05) than *L. casei* BAL C were discarded from further analysis. Seven isolates with the highest anti-clostridial activity were selected. Table 1 shows the source and 16S rRNA sequence identity of the selected anti-clostridial LAB isolates, as well as their classification into SLAB and NSLAB strains according to literature criteria (**Reginensi *et al.*, 2016; Blaya *et al.*, 2018**). Except for strain 76, all isolates were classified as NSLAB classified strains. The NSLAB population develops in the cheese ripening stage and becomes the predominant microflora. As at this stage, *Clostridium* also proliferates and causes late blowing defect, several researchers believe that NSLAB strains compatible with the cheese making process would be more suitable for inhibiting *Clostridium* spp. and prevent the appearance of the defect (**Ristagno, 2013; Bowen, 2018; Şatana, 2018**).
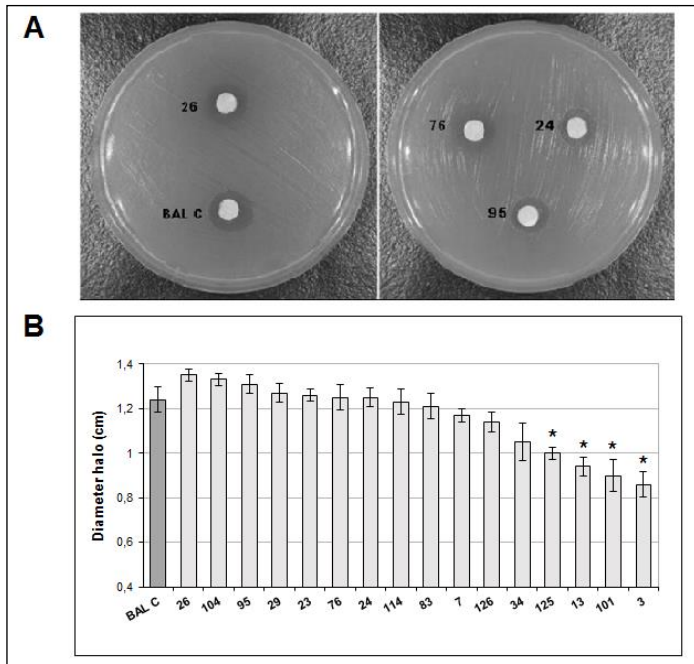
Figure 1 (A) Evaluation of inhibitory activity of anti-clostridial LAB isolates by disc diffusion method against to *C. tyrobutyricum* ATCC25755. (B) Comparison of diameter (cm) of inhibition halos produced by LAB isolates (gray bars) and *L. casei* BAL C (dark gray bar). Asterisks indicate strains with significantly lower inhibitory activity than BAL C (p <0.05).

Table 1 SLAB and NSLAB isolates selected by agar disc diffusion method.

Strain	Identification	Source	LAB type
29	*L. casei* Group	cheese	NSLAB
26	*L. casei*	cheese	NSLAB
95	*L. casei*	raw milk	NSLAB
23	*S. macedonicus*	cheese	NSLAB
24	*S. macedonicus*	cheese	NSLAB
104	*L. rhamnosus*	raw milk	NSLAB
76	*L. delbrueckii* subsp. *bulgaricus*	raw milk	SLAB

Nature of anti-clostridial compounds and sensitivity to heat treatments

Anti-clostridial mechanisms of the selected LAB strains and *L. casei* BAL C were elucidated in neutralized cell free supernatants obtained from the strain cultured in MRS broth. *S. macedonicus* 23, *S. macedonicus* 24, *Lactobacillus* sp. 29 and *L. rhamnosus* 104 supernatants lost anti-clostridial activity after neutralization, hence inhibitory activity was due to organic acids production. Organic acids are products of lactic acid fermentation with antibacterial activity which effectiveness depends on the chemical structure, the amount produced, and the food matrix pH (**Gutierres, 2018; Bushell *et al.*, 2019**). *Clostridium* species responsible for late blowing defect are inhibited at pH 4, but hard and semi-hard cheeses have a pH range of 5.2 - 5.5. Therefore, LAB strains whose anti-clostridial activity is due solely to the organic acids production may not be effective to inhibit *Clostridium* spp. in cheese matrix (**Drouin and Lafrenière, 2012; D'Incecco *et al.*, 2018; Nuñez *et al.*, 2020**).
Supernatants of strains *L. casei* 26, *L. casei* Group 29, *L. delbrueckii* subsp. *bulgaricus* 76 and *L. casei* BAL C retained the inhibitory effect after neutralization. Therefore, supernatants were treated with catalase and proteinase K to determine whether inhibitory activity corresponded to H_2O_2 or bacteriocin production. For all treatments, residual anti-clostridial activity was expressed as a percentage of inhibitory activity produced by neutralized supernatants (Tab. 2).
Hydrogen peroxide was produced by *L. delbrueckii* subsp. *bulgaricus* 76 and responsible for the anti-clostridial activity, as catalase treatment lead to a residual anti-clostridial activity of 5.7%. Peptidic anti-clostridial compounds were not detected for this strain. Hydrogen peroxide generation of by *L. delbrueckii* subsp. *bulgaricus* strains has been frequently reported (**Adesokan *et al.*, 2010; Hertzberger *et al.*, 2014; Selle *et al.*, 2019**). Hydrogen peroxide is an effective bacteriostatic or bactericide agent that acts by oxidizing cellular components such as cell membrane lipids, proteins and DNA. In addition, this compound has a sporicidal effect on *Clostridium* and *Bacillus* spores. Cytotoxic effect of hydrogen peroxide depends on factors such as its concentration, aeration,

bacterial sensitivity to oxidation stress, pH and temperature conditions (**Brudzynski *et al.*, 2011; Queiroux *et al.*, 2018; Uwamahoro *et al.*, 2018; Vieco-Saiz *et al.*, 2019**). It is important to note that an essential factor for the hydrogen peroxide production by LAB strains is the presence of O_2, so it is valid to question whether this strategy would be effective in cheese matrix where oxygen diffusion is low (**Brudzynski *et al.*, 2011; Levante, 2017; Lavazani, 2018; Hernandez *et al.*, 2019**).

Table 2 Residual anti-clostridial activity (%) of neutralized cell-free supernatants*

SN treatment	*L. casei* BAL C	*L. casei* 26	*L. casei* 95	*L. delbrueckii* subsp. *bulgaricus* 76
Proteinase K	12,4%	65,2%	80,5%	98,1%
Catalase	71,2%	39,3%	28,3%	5,7%
HT$_1$	97,4%	73,0%	97,3%	98,5%
HT$_2$	73,7%	58,4%	93,8%	90,6%

HT1: Heat treatment at 70 ºC during 15 min.
HT2: Heat treatment at 100 ºC for 15 min.
* Residual anti-clostridial activity was calculated as a percentage of neutralized supernatants without enzymatic or thermal treatment inhibitory activity

Residual inhibitory activity of *L. casei* 26 and 95 supernatants treated with catalase was 39.3% and 28.3%, respectively, while proteinase K treatment preserved 65.2% and 80.5% of the antimicrobial activity (Tab. 2). According to these data, anti-clostridial activity of *L. casei* 26 and 95 is due to the production of hydrogen peroxide and proteinaceous compounds. It is worth mentioning that the production of bacteriocins and hydrogen peroxide are characteristics frequently reported in the genus *Lactobacillus* (**Davoodabadi *et al.*, 2015; Collins *et al.*, 2017; Queiroux *et al.*, 2018; Li *et al.*, 2019**). According to the results obtained, commercial bioprotective *L. casei* BAL C is a bacteriocinogenic strain, but remaining anti-clostridial activity in catalase treated supernatant (71.2%), indicated that hydrogen peroxide was also present.
Thermal stability of neutralized cell-free supernatants was tested at 70 and 100 ºC (Tab. 2). All supernatants conserved their inhibitory activity after the heat treatments performed. *L. casei* 26 supernatant was susceptible to thermal treatments reducing half of its anti-clostridial activity at 100 ºC (58.4%). Supernatants of *L. casei* BAL C and 95 presented higher thermal resistance, retaining a suitable inhibitory activity even after 100 ºC treatment. *L. delbrueckii* subsp. *bulgaricus* 76 supernatant retained most of its anti-clostridial activity after the heat treatments performed.
In this work, the nature of anti-clostridial compounds was elucidated focusing on the search for organic acids, hydrogen peroxide and peptide compounds. Table 3 summarizes the anti-clostridial mechanisms detected in the evaluated LAB strains and the isolation sources from which they were obtained. The presence of other anti-clostridial compounds such as D-amino acids, acetaldehyde, diacetyl or acetoin in the supernatants can not be dismissed.

Table 3 Nature of anti-clostridial compounds produced by LAB strains.

Strain	Isolation source	Inhibition mechanism
L. casei BAL C	commercial strain	organic acids, hydrogen peroxide, bacteriocin
S. macedonicus 23	cheese	organic acids
S. macedonicus 24	cheese	organic acids
L. casei 26	cheese	organic acids, hydrogen peroxide, bacteriocin
L. casei 95	raw milk	organic acids, hydrogen peroxide, bacteriocin
L. casei/paracasei 29	cheese	organic acids
L. rhamnosus 104	raw milk	organic acids
L. delbrueckii subsp. *bulgaricus* 76	raw milk	organic acids, hydrogen peroxide

Purification and characterization of anti-clostridial peptides

The anti-clostridial peptides present in cell-free supernatants of bacteriocinogenic strains *L. casei* BAL C, 26 and 95 were purified by RP-HPLC. For each supernatant different fractions could be clearly resolved under the conditions employed. Four peaks showing an absorption at 220 nm were individually collected from *L. casei* 26 and 95 supernatants. Only one fraction corresponding

to retention time of 14 - 16 min. was collected from *L. casei* BAL C supernatant. All fractions were tested for anti-clostridial activity by disc diffusion assay (Tab. 4). Fractions P_2 and P_4 of *L. casei* 26, P_1 and P_4 of *L. casei* 95 and P_0 of *L. casei* BAL C were found to have activity against *C. tyrobutyricum* ATCC 25755.

All fractions with anti-clostridial activity were subjected to mass spectrometry analysis for compounds identification. MALDI-TOF analysis only resulted in a suitable mass spectrum for P_0 fraction of *L. casei* BAL C and P_4 of *L. casei* 95 (Fig. 2). The low resolution of mass spectra obtained could be due to background noise produced by ions originated from compounds presents in HPLC fractions. One source of background noise are culture medium components. LAB have harsh nutritional requirements so they require complex culture media, allowing better bacterial growth and increased bacteriocin production. The disadvantage of this type of culture media is the high presence of proteins and other culture medium compounds that are difficult to eliminate in the purification process. Others undesired contaminants could be bacterial metabolic products, degradation of liquid chromatographic column and leachables of recipients (**De Vijlder** *et al.***, 2018; Sidek** *et al.***, 2018**).
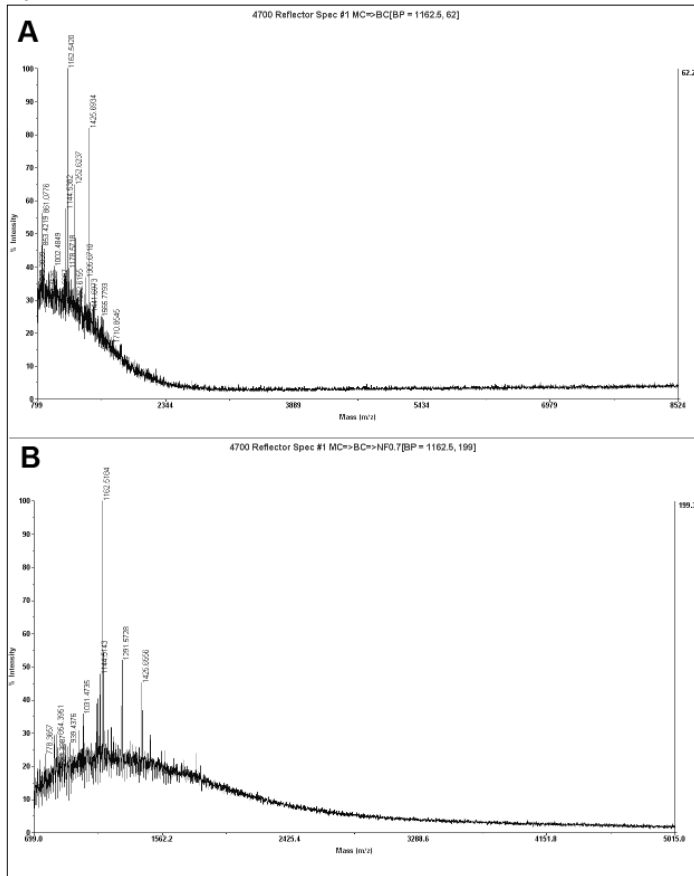
Figure 2 MALDI-TOF mass spectra of *L. casei* BAL C P_0 fraction (A) and *L. casei* 95 P_4 fraction (B).

The obtained molecular mass of the *L. casei* BAL C P_0 fraction was 1162.54 (retention time: 14 - 14 min.), while for *L. casei* 95 P_4 fraction it was 1162.52 Da (retention time: 22 - 26 min.). The low intensity of the mass spectrum signals did not allow to obtain fragmentation spectra (MS/MS) from the resulting peptide ions and therefore structural information of the compound was not found. No results were obtained for MALDI-TOF analysis of *L. casei* 95 P_4 fraction and *L. casei* 26 P_2 and P_4 fractions. This may be due to the degradation of anti-clostridial peptides presents in the supernatants by proteases from bacteriocinogenic strains or the low concentration of the bacteriocins produced. The production of bacteriocins depends on several parameters such as the composition and pH of the culture medium, temperature and the growth kinetics of the producing microorganism. In many cases the maximum production of bacteriocin occurs under conditions other than optimal for bacterial growth (**Spivak, 2010; Saavedra and Sesma, 2011; Relloso** *et al.***, 2015; Sidooski** *et al.***, 2019**). In spite of not having obtained structural information about the anti-clostridial compound present in P_0 *L. casei* BAL C and P_4 *L. casei* 95 fractions, thermotolerance and low molecular weight (<10 kDa) of both compounds would imply that they are class I or II bacteriocins (**da Costa** *et al.***, 2019; Hols** *et al.***, 2019**).

CONCLUSION

From raw milk, artisan cheese and whey samples analyzed in this work, 56 LAB strains with inhibitory activity against *C. tyrobutyricum* ATCC 25755 were isolated. Seven of these isolates had anti-clostridial activity similar to the commercial-strain *L. casei* BAL C and their inhibition mechanisms were studied. Inhibitory action of four isolates tested (23, 24, 29 and 104) were due only to acid production, whereas antimicrobial activity of isolates 26, 76 and 95 was due to the production of other antimicrobial metabolites besides organic acid production. In the case of *L. casei* strains 26 and 95, organic acid, hydrogen peroxide and bacteriocins production was observed, while the inhibition developed by *L. delbrueckii* subsp. *bulgaricus* 76 was due to its acidifying activity and hydrogen peroxide generation. *L. casei* strains 26, 95 and BAL C anti-clostridial peptides were thermo-resistant. Mass spectrometry analysis MALDI-TOF allowed to detect inhibitory peptide compounds produced by *L. casei* strains 95 and BAL C with a molecular weight of 1.2 kDa. Due to the thermostability and low molecular weight of these compounds, they would be bacteriocins of the classes I or II. The characterization of bacteriocins produced by *L. casei* 26 and *L. casei* 95 is an issue that should be addressed in the future.

Table 4 Retention time and anti-clostridial activity of HPLC fractions.

HPLC fraction	*L. casei* 26				*L. casei* 95				*L. casei* BAL C
	P_1	P_2	P_3	P_4	P_1	P_2	P_3	P_4	P_0
retention time (min.)	3 - 6	7 - 9	10 - 14	14 - 21	3 - 4	4 - 6	14 - 20	22 - 26	14 - 16
anti-clostridial activity	-	+	-	+	+	-	-	+	+

(+/-): presence/absence of inhibition halo in disc diffusion assay

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