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Study of the inhibitory effect of *Lactobacillus* strains and lysozyme on growth of *Clostridium* spp. responsible for cheese late blowing defect

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Abstract

Three strains of *Lactobacillus* were previously isolated from dairy products, *Lactobacillus casei* 26, *Lactobacillus delbrueckii* subsp. *bulgaricus* 76 and *L. casei* 95. Anti-costridial activities of these strains were evaluated against a collection of *Clostridium* isolates, belonging to species responsible for late blowing defect in cheese. The three strains of *Lactobacillus* inhibited the growth of all *Clostridium* isolates analyzed. The inhibitory effect of cell-free supernatant of each of these *Lactobacillus* strains combined with that of commercial strain *L. casei* BAL C, or with a solution of lysozyme, were compared by disc diffusion assay. Mixtures of cell-free *Lactobacillus* supernatants and lysozyme exhibited higher inhibitory activity than the supernatants and lysozyme solution separately ($P < 0.05$). Additionally, *Lactobacillus* strains were resistant to lysozyme concentrations usually used during cheese making process.

Introduction

Late blowing is the major cause of spoilage in hard and semi-hard cheese, which affects the quality and commercial value of the products. This alteration is due to the butyric acid fermentation produced by *Clostridium* species during the cheese ripening stage (Bermúdez *et al.* 2016; Gómez-Torres *et al.* 2018). The accumulation of gas produced during fermentation causes irregular eyes and cracks in cheese mass. Frequently, unpleasant aromas are also generated by butyrate and acetate production (D'Incecco *et al.* 2018). The studies carried out on the subject indicate that *C. tyrobutyricum* is the primary cause of this defect, while *C. beijerinckii*, *C. sporogenes*, and *C. butyricum* may be also involved (Le Bourhis *et al.* 2007; Bermúdez *et al.* 2016; Storari *et al.* 2016).

Bacteria of the genus *Clostridium* are resistant to extreme conditions including thermal processes, desiccation, UV radiation and chemical agents, persisting in the environment for a long time (Doyle *et al.* 2015; Murphy *et al.* 2016). Consequently, methods used in dairy industry to prevent late blowing defect are designed to avoid spore germination present in milk, and to inhibit the growth of vegetative cells. However, these strategies have disadvantages and are not completely successful (Szabolcs 2014). Lysozyme addition is the most used method for the control of this defect but involves a risk to human health. Commercial lysozyme is obtained from egg white,

one of the eight foods responsible for 90 % of all allergy cases (Garde *et al.* 2011; Titoiu *et al.* 2019). Furthermore, lysozyme can inhibit lactic acid bacteria (LAB) strains used as starter cultures (Licon *et al.* 2020; Morandi *et al.* 2020). Microfiltration and bactofugation of milk are also commonly used. Milk microfiltration retaines about 99 % of spores present but requires the separation of milk fat. Milk bactofugation, although it eliminates about 98.7 % of the spores, may not be effective if spore load is very high. Also, there is a loss of milk components, mainly protein (Ávila *et al.* 2014; Brändle *et al.* 2016). Nitrate salts are used to prevent the spore outgrowth, but they could originate carcinogenic nitrosamines and affect consumer health. Because of this nitrate salts are prohibited in several countries (Licon *et al.* 2020).

Due to these reasons, it is necessary to use alternative control strategies with minimal impact on cheese quality and consumer health (Storari *et al.* 2016; Ávila *et al.* 2020). One of these techniques is to use LAB strains with anti-clostridial activity as starter (Starter Lactic Acid Bacteria; SLAB) or adjunct cultures (Non-Starter Lactic Acid Bacteria; NSLAB). The addition to food of inhibitory metabolites produced *ex situ* by LAB strains has also been investigated, but only the bacteriocin Nisin is approved by WHO/CODEX as a worldwide food preservative (Prudêncio *et al.* 2015; Martínez *et al.* 2016; Collins 2019). In a previous study, a collection of 56 anti-clostridial LAB strains were isolated from dairy products. From this collection, we could identify three strains, *Lactobacillus casei* 26, *L. casei* 95, and *Lactobacillus delbrueckii* subsp. *bulgaricus* 76, with the highest inhibitory activity against *C. tyrobutyricum* ATCC 25755. Inhibition mechanisms of these *Lactobacillus* strains were also established. Anti-clostridial activity of strains 26 and 95 were due to acid production, hydrogen peroxide and bacteriocins. While in strain 76 it was caused by organic acids and hydrogen peroxide (Olivera *et al.* 2020).

The aim of this work was to study the anti-clostridial activity of these *Lactobacillus* isolates against native *Clostridium* strains isolated from late blowing affected cheeses. Additionally, compatibility of these anti-clostridial strains with lysozyme treatment was evaluated.

Experimental

Bacterial strains and culture conditions

Native strains of *C. sporogenes*, *C. beijerinckii*, *C. tyrobutyricum*, and *C. butyricum* isolated from dairy products belonging to the strain´s laboratory collection and *C. tyrobutyricum* ATCC 25755 were selected for this work (Bermúdez *et al.* 2016). These strains were recovered from frozen stocks by growing in Reinforced Clostridial Medium (RCM, Difco) broth and then, the cells were transferred to RCM agar plates. Plates were incubated at 37 °C for 48 h under anaerobic conditions using an Anoxomat Mark II device (Advanced Instruments Inc., USA). *Lactobacillus* strains 26, 76 and 95 were grown in MRS broth at 37 °C for 16 to 18 h. Bacterial cultures were spread on MRS agar plates and incubated during 24 h at 37 °C under microaerophilic conditions. Commercially available anti-clostridial strain *L. casei* BAL C (LC4P1, Sacco SRL, Cadorago, CO, Italy) was included in this study.

Inhibitory activity of Lactobacillus isolates against Clostridium strains

Anti-clostridial activities of *L. casei* 26, *L. casei* 95, and *L. delbrueckii* subsp. *bulgaricus* 76 were studied against five isolates of four Clostridium species. The analysis was done on RCM agar plates by disc diffusion assay. Selected LAB isolates and *L. casei* BAL C 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 culture centrifugation (10,000 rpm for 15 min at 4 ºC) and filtrated 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. Discs (diameter 6 mm, Whatman, GE Health Care, NJ, USA) were placed on the plates and 100 µL of cell-free supernatants were applied on the discs. 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 of growth inhibition.

Commercial strain *L. casei* BAL C was included in the assay. All assays were performed in triplicates and the values were expressed in a centimetre (cm) as the mean ± S.D. Statistical analysis was performed using One-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

Evaluation of the sensitivity of Lactobacillus strains to lysozyme

Susceptibility of selected *Lactobacillus* isolates (26, 76, and 95) to lysozyme was evaluated. For each strain, a set of tubes containing 9 mL of MRS broth and tenfold dilution of lysozyme (0.001 to 1,000 µg/mL) were prepared in duplicate. The sets of tubes were inoculated at 1 % v/v with MRS broth *Lactobacillus* cultures ($O.D._{620nm}$ = 0.2). Positive growth control was prepared growing each strain in 9 mL of MRS broth without lysozyme. All tubes were incubated at 37 ºC for 24 h. Bacterial count in each tube was evaluated by streaking 20 µL of the cultures onto MRS agar plates and incubating at 37 ºC for 24 h under microaerophilic condition (Dias *et al.* 2015; Rajoka *et al.* 2018).
Sensitivity to lysozyme concentrations higher than 1.0 $mg.mL^{-1}$ was evaluated in MRS agar plates by disc diffusion assay. Each LAB strain was grown in MRS broth for 7 h at 37 °C. Ten-fold serial dilutions were prepared from these cultures in tubes containing 9 mL of physiological saline solution (0.85 % NaCl). Then, 100 µL of each dilution were spread on MRS agar plates. Lysozyme solutions at final concentrations of 1.88, 3.00, 3.75, 7.50, and 15.00 $mg.mL^{-1}$ were prepared in sodium phosphate buffer (10 mM, pH 7.0). Discs containing 100 µL of each lysozyme solution were placed on MRS agar plates previously spread with a *Lactobacillus* strain. Growth inhibition halo diameter was determined after incubating the plates at 37 ºC during 24 h under microaerophilic conditions (Guariglia-Oropeza and Helmann 2011).

Anti-clostridial activity of Lactobacillus strains supernatants, lysozyme, and mixture of both

The study was done on RCM agar plates by disc diffusion assay. Anti-clostridial activities of cell-free supernatants prepared from strains 26, 76, 95, and *L. casei* BAL C against *C. tyrobutyricum* ATCC 25755 were compared to that produced by lysozyme solution and by mixtures of the supernatants and lysozyme. Cell-free supernatants from LAB strains were obtained following the procedure performed in the study of inhibitory activity of *Lactobacillus* strains against *Clostridium* strains isolated from dairy products. A culture of the inhibition indicator strain in RCM broth was adjusted and spread on plate as describe in the mentioned section. Lysozyme solution was prepared at 0.75 $mg.mL^{-1}$ final concentration with sodium phosphate buffer (10 mM, pH 7.0). Lysozyme concentration used in this study is higher than that added in milk for cheese production (0.020 to 0.035 $mg.mL^{-1}$) (D'Amato *et al.* 2010; Gobbetti *et al.* 2018; Junior *et al.* 2019). The mixtures of lysozyme and cell-free supernatants were prepared by mixing the supernatants with a 1.5 $mg.mL^{-1}$ lysozyme solution at 1 : 1 ratio. A volume of 100 µL of each mixture was dispensed on a sterile paper disc placed on RCM agar spread with a suspension of *C. tyrobutyricum* ATCC 25755. The plates were incubated at 37 ºC for 48 h under anaerobic conditions and the presence of inhibition halos was evaluated. All experimental trials were performed in triplicate. Inhibition results were statistical analyzed by the One-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Inhibitory activity of Lactobacillus isolates against Clostridium *native strains*

Anti-clostridial activity of strains 26, 76, 95, and commercial inhibitory strain BAL C against native *Clostridium* species was studied. The diameter of inhibition halos produced by the cell-free supernatants of these strains, were compared for each *Clostridium* species under study (Fig. 1).
According to data obtained in this study, cell-free supernatants of LAB isolates 26, 76 and 95, had inhibitory activity against all native *Clostridium* strains. *C. tyrobutyricum* was inhibited by all LAB strains at the same extent (Table 1). However, *C. tyrobutyricum* strains showed variations on the sensitivity to LAB cell-free supernatants (Fig. 1A). *C. tyrobutyricum* 23.4 was the most sensitive strain

($P < 0.05$). In the case of *C. butyricum* the strain BAL C and isolate 26 had the highest inhibitory activity (Table 1), and not difference was observed between *C. butyricum* strains (Fig. 1B). None significant difference was observed on *C. beijerinckii* strains sensitivity (Fig. 1C), as well as for *C. sporogenes* strains (Fig. 1D). It is noteworthy that *L. casei* BAL C produced the maximum inhibition on *C. beijerinckii* (Table 1). Regarding *C. sporogenes*, cell-free supernatants of *L. casei* 95 exerted the maximum anti-clostridial activity, while *L. casei* 76 showed the minimal inhibitory activity (Table 1).

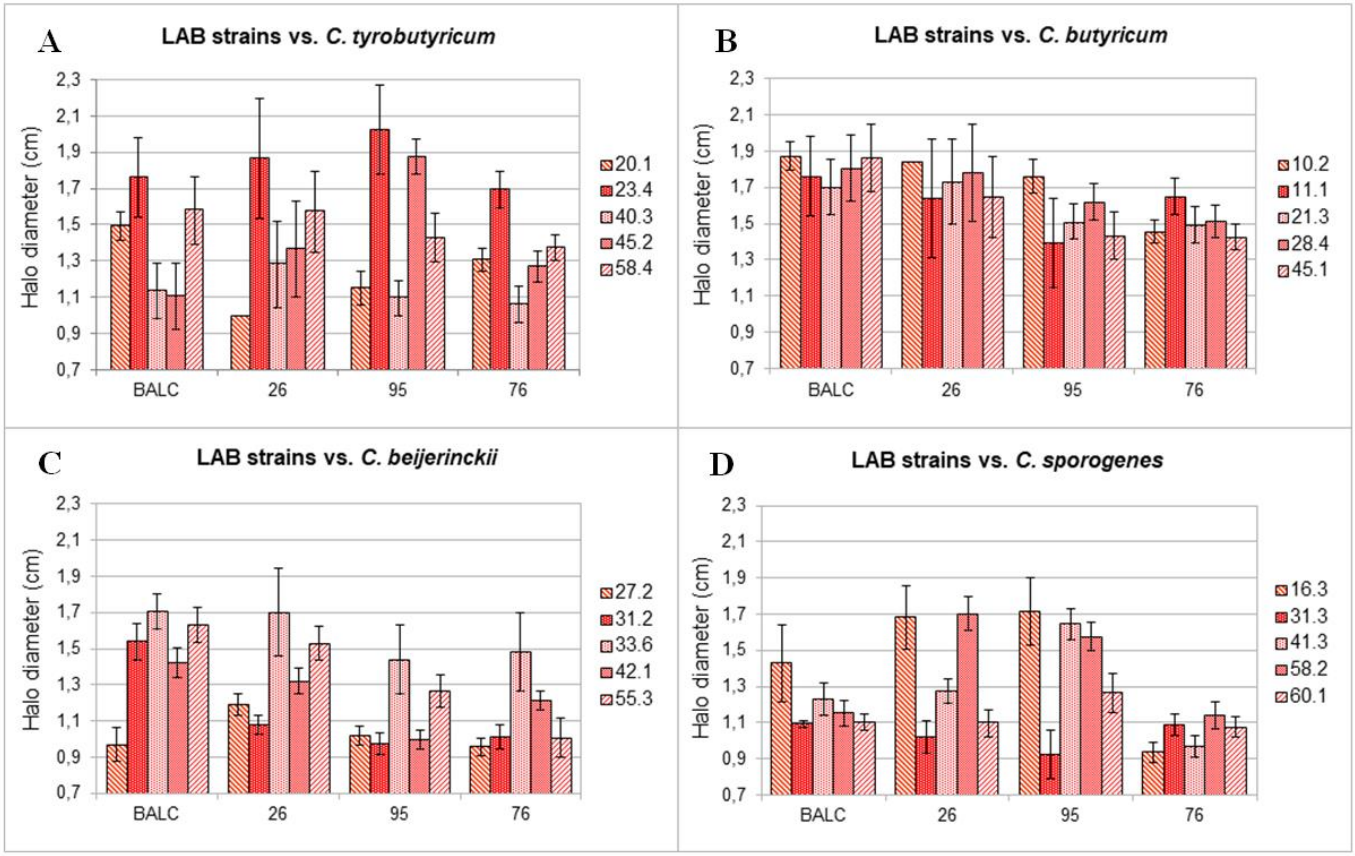

Fig. 1. Comparison of the diameter of the inhibitory halos produced by cell-free supernatants of BAL C, 26, 95 and 76 strains against strains of *C. tyrobutyricum* (**A**), *C. butyricum* (**B**), *C. beijerinckii* (**C**), and *C. sporogenes* (**D**). Legend to the right of each graph indicates the *Clostridium* strains considered in the study.

Table 1. Anti-clostridial activity of LAB strains for the different *Clostridium* species (diameter halo of growth inhibition, mean ± S.D.).

	BAL C [Ø cm]	**26 [Ø cm]**	**95 [Ø cm]**	**76 [Ø cm]**	***P*-value**
C. tyrobutyricum	1.413 ± 0.285	1.416 ± 0.325	1.514 ± 0.420	1.338 ± 0.230	0.860
C. butyricum	1.800 ± 0.072	1.727 ± 0.081	1.543 ± 0.150	1.507 ± 0.088	0.001
C. beijerinckii	1.575 ± 0.123	1.364 ± 0.252	1.139 ± 0.205	1.134 ± 0.218	0.020
C. sporogenes	1.200 ± 0.138	1.355 ± 0.322	1.548 ± 0.199	1.039 ± 0.086	0.013

Bermúdez *et al.* (2016) analyzed the seasonal dynamics of strict anaerobic sporulated bacteria present in Uruguayan cheese making plants. According to that study *C. tyrobutyricum* and *C. sporogenes* were the predominant species. Previous studies have also found that *C. tyrobutyricum*, *C. sporogenes* and *C. beijerinckii* are the predominant species in cheeses with late blowing defect and there is synergism between them, while *C. butyricum* has lower incidence (Le Bourhis *et al.* 2007; Gómez-Torres *et al.* 2015; Brändle *et al.* 2016; Gupta and Brightwell 2017). According to

the results obtained in the analysis performed, *L. casei* strains 95 and 26 and *L. delbrueckii* subsp. *bulgaricus* 76 would be candidate strains to evaluate their ability to control the main *Clostridium* species responsible for late blowing defect in Uruguayan cheese making plants.

Evaluation of the sensitivity of Lactobacillus strains to lysozyme

Lysozyme is a food preservative widely used in cheese making, but it can affect LAB strains (Licon *et al.* 2020; Morandi *et al.* 2020). The effect of lysozyme solutions, up to 15.00 mg.mL^{-1}, on *Lactobacillus* strains 26, 76 and 95 growth was evaluated. LAB strains were not sensitive to lysozyme at the concentrations analysed.

The amount of lysozyme usually added to milk for cheese making reaches final concentrations in the range of 20 to 30 mg.L^{-1} of milk (D'Amato *et al.* 2010; Gobbetti *et al.* 2018; Junior *et al.* 2019). Results obtained in this study indicate that BAL strains 26, 76 and 95 would be compatible with the lysozyme amounts used in cheese production. Several authors have reported the presence of lysozyme-resistant strains of LAB and NSLAB in cheeses, probably because lysozyme acts as a selection factor that favours the proliferation of bacteria compatible with this enzyme (Brändle *et al.* 2016). Although LAB lysozyme resistance varies at species or strain level, tolerance detected in the strains evaluated is consistent with previous studies carried out on strains of *L. delbrueckii* and *L. casei* Group (D'Incecco *et al.* 2016). Also, several studies agree that heterofermentative mesophilic *Lactobacillus* species, for example *L. casei*, are often more resistant to lysozyme than thermophilic species of the same genus (Carminati *et al.* 2014; Aspri *et al.* 2016; Riaz *et al.* 2017; de Mattos *et al.* 2020).

Anti-clostridial activity of Lactobacillus strains supernatants, lysozyme, and mixture of both

Anti-clostridial activity of cell-free supernatants of isolates 26, 95, 76 and the commercial strain BAL C, was compared with that produced by lysozyme. The diameters of growth inhibition halos of LAB cell-free supernatants, lysozyme, and mixture of both are shown in Fig. 2.

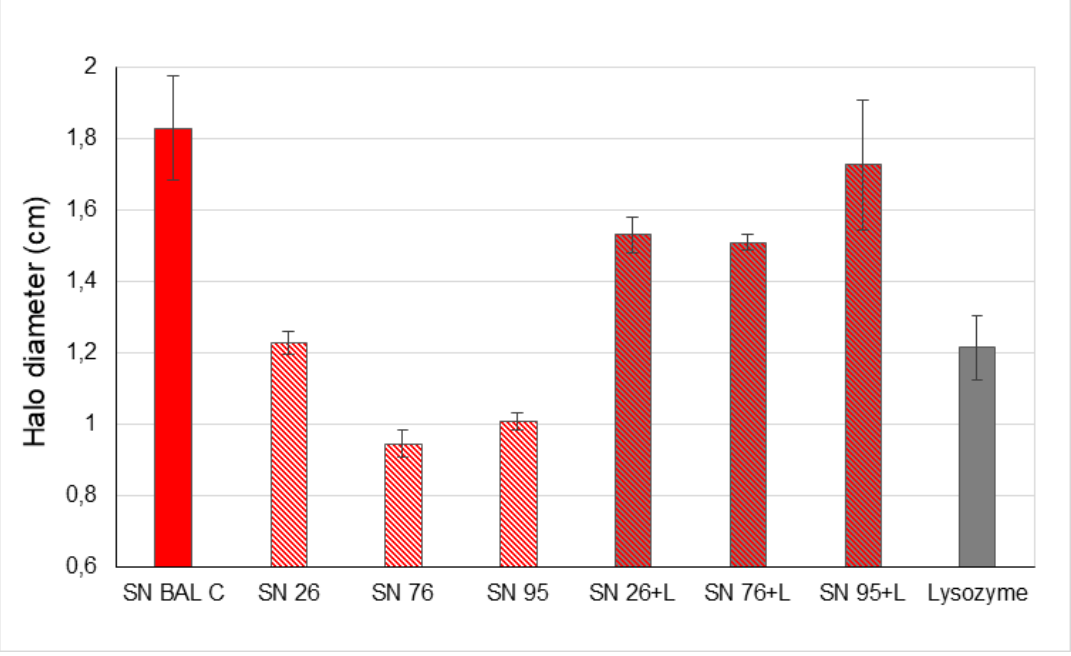

Fig. 2. Comparison of growth inhibition halos produced by cell-free supernatants (SN) of strains 26, 76, 95 (red and white striped bars), BAL C (red bar), 1 : 1 mixture of each cell-free supernatants with lysozyme (red and grey striped bars), and lysozyme solution (0.75 mg.mL^{-1}) (grey bar). The inhibition indicator strain was *C. tyrobutyricum* ATCC 25755.

Anti-clostridial activity of *L. casei* BAL C SN was higher than that produced by lysozyme solution and cell-free supernatants of the remaining strains evaluated ($P < 0.05$). On the other hand, lysozyme solution showed similar inhibitory activity to *L. casei* 26 ($P > 0.05$), but it was higher than that produced by the SN of *L. casei* 95 and *L. delbrueckii* subsp. *bulgaricus* 76 ($P < 0.05$). Mixture of lysozyme and SN of strains 26, 76 and 95, inhibitory activity did not differ between strains ($P > 0.05$). It was also determined that SN and lysozyme combinations showed higher inhibitory activity than SN and the enzyme separately for all strains ($P < 0.05$). In a previous work it was determined that anti-clostridial activity of the strains of *L. casei* 26 and 95 is due to organic acids, bacteriocins and hydrogen peroxide, while in *L. delbrueckii* subsp. *bulgaricus* 76 is due to acid production and hydrogen peroxide (Olivera *et al.* 2020). The results obtained in the study suggest that the anti-clostridial compounds present in cell-free supernatants together with lysozyme act additively.

Different studies have reported the existence of compatibility between anti-clostridial compounds produced by LAB strains and lysozyme. Some of them have even reported synergistic interactions between lysozyme and other antimicrobial compounds, such as bacteriocins, hydrogen peroxide and organic acids. In the case of bacteriocins, it has been suggested that synergistic effect is because lysozyme breaks down the bacterial cell wall, allowing greater bacteriocin access to the cell membrane, and/or facilitating their interaction with existing receptors (Grande Burgos *et al.* 2011; Maliničová *et al.* 2011; Cabrefiga and Montesinos 2017; Hernandez *et al.* 2017; Ananou *et al.* 2018; Lopes *et al.* 2019). On the other hand, some studies report that lysozyme present higher inhibitory activity in the presence of organic acids. In addition, compared to neutral pH, the enzymatic activity of lysozyme is more stable under acidic conditions (Johansen *et al.* 1994; Oh *et al.* 2016; Sheng *et al.* 2017; Dong *et al.* 2020).

Previous studies confirm the presence of synergism between hydrogen peroxide and lysozyme (Losso *et al.* 2000; Roller and Board 2011). The synergism between both compounds could be due to the cell wall rupture by lysozyme. This allows higher access of hydrogen peroxide to the cell membrane to which it damages and increases its permeability (Rao *et al.* 2011; Şanlıbaba and Güçer 2015; Bermúdez *et al.* 2016). Hydrogen peroxide oxidizes cell membrane lipids, proteins, and DNA. In addition, this compound has a sporicidal effect on *Clostridium* and *Bacillus* spores (Kenters *et al.* 2017; Uwamahoro *et al.* 2018; Porras 2019; Vieco-Saiz *et al.* 2019). Likewise, Bukharin and Sgibnev (2013) have determined that metabolites resulting from hydrogen peroxide modify the lysozyme increasing its bactericidal activity.

Conclusion

This work describes the inhibitory activity of *L. casei* 95, *L. casei* 26, and *L. delbrueckii* subsp. *bulgaricus* 76, against isolates of the most frequent *Clostridium* species found in cheese with late blowing defect. The results indicate that LAB strains used in the study inhibited all *Clostridium* isolates analyzed. Compared to the other *Lactobacillus* strains, *L. casei* 95 exhibited the highest inhibitory activity on *C. tyrobutyricum*. Likewise, this strain and *L. casei* 26 had the maximum anti-clostridial action against *C. sporogenes* under the conditions of an assay. In addition, anti-clostridial activities of *L. delbrueckii* subsp. *bulgaricus* 76 and the commercial inhibitory strain *L. casei* BAL C against *C. sporogenes* were similar.

The results obtained in this work allow us to affirm that the inhibitory compounds produced by the *Lactobacillus* strains evaluated are compatible with lysozyme. It was also found that the mixtures of cell-free supernatants of LAB strains and lysozyme showed higher anti-clostridial activity than supernatants and lysozyme separately. Although additional studies should be carried out to determine whether or not there is a synergistic interaction between these antimicrobial compounds.

L. casei 26, *L. delbrueckii* subsp. *bulgaricus* 76, and *L. casei* 95 would be promising candidates for the control of the main species of *Clostridium* in cheese making. However, it would be necessary to evaluate the compatibility of these strains with LAB starters frequently used in the dairy industry.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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