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Molecular identification of naturally occurring bacteriocinogenic and bacteriocinogenic-like lactic acid bacteria in raw milk and soft cheese

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ABSTRACT

Lactic acid bacteria (LAB) are currently used by food industries because of their ability to produce metabolites with antimicrobial activity against gram-positive pathogens and spoilage microorganisms. The objectives of this study were to identify naturally occurring bacteriocinogenic or bacteriocinogenic-like LAB in raw milk and soft cheese and to detect the presence of nisin-coding genes in cultures identified as *Lactococcus lactis*. Lactic acid bacteria cultures were isolated from 389 raw milk and soft cheese samples and were later characterized for the production of antimicrobial substances against *Listeria monocytogenes*. Of these, 58 (14.9%) LAB cultures were identified as antagonistic; the nature of this antagonistic activity was then characterized via enzymatic tests to confirm the proteinaceous nature of the antimicrobial substances. In addition, 20 of these antagonistic cultures were selected and submitted to genetic sequencing; they were identified as *Lactobacillus plantarum* (n = 2) and *Lactococcus lactis* ssp. *lactis* (n = 18). Nisin genes were identified by polymerase chain reaction in 7 of these cultures. The identified bacteriocinogenic and bacteriocinogenic-like cultures were highly variable concerning the production and activity of antimicrobial substances, even when they were genetically similar. The obtained results indicated the need for molecular and phenotypic methodologies to properly characterize bacteriocinogenic LAB, as well as the potential use of these cultures as tools to provide food safety.

Key words: lactic acid bacteria, antagonism, bacteriocin, nisin

INTRODUCTION

Lactic acid bacteria (**LAB**) have become very important in the food industry and in public health because of their antagonistic potential against pathogenic and spoilage microorganisms. Such activity is attributed to their growth that competes with other microorganisms in foods. Moreover, their activity is strengthened by the inhibitory effects of their metabolites, such as organic acids, hydrogen peroxide, CO_2, diacetyl, and bacteriocins (Carr et al., 2002; de Martinis et al., 2003). Bacteriocins have been heavily studied because of their significance as biopreservatives (Carr, et al., 2002).

Bacteriocins are biologically active peptides or proteins that present antagonistic activity against specific microorganisms (Lewus and Montville, 1991; Thuault et al., 1991; Carr, et al., 2002). Bacteriocins are sensitive to protease activity because of their proteinaceous nature, which can be identified by specific inhibition tests (Harris et al., 1989; Lewus et al., 1991; Lewus and Montville, 1991; Hoover and Steenson, 1993). Several LAB species have been identified as producers of different bacteriocins (Chen and Hoover, 2003; Cotter et al., 2005).

The expression of bacteriocins is mediated by genes (Chen and Hoover, 2003), many of which have been sequenced (Maldonado et al., 2003; Todokoro et al., 2006). Genetic sequencing is a very important tool for the description of these genes and has been indispensable for the precise identification of new bacteriocinogenic LAB cultures (Holzapfel et al., 2001; Nes and Johnsborg, 2004; Mohania et al., 2008).

Although there is plenty of information on LAB activity in different kinds of foods (Lewus et al., 1991; Thuault et al., 1991; Issa and Ryser, 2000; de Martinis et al., 2003; de Martinis and Freitas, 2003), the identification of new bacteriocinogenic strains is necessary to provide novel tools for pathogen control. Therefore, the objective of this study was to detect LAB cultures naturally present in raw milk and soft cheese, with bacteriocinogenic or bacteriocinogenic-like activity, for future application in the food industry as biopreservatives.

MATERIALS AND METHODS

Sample Collection and LAB Isolation

Samples of raw milk (n = 36) and soft cheese produced with raw milk (n = 18) were collected under

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aseptic conditions from bulk tanks and dairy farms from Viçosa (Minas Gerais, Brazil. The samples remained refrigerated until they were carefully homogenized and submitted to 10-fold dilution using 0.85% NaCl. The dairy farms were selected to represent the microbial profile of the milk produced in the region, according to the dairy production characteristics (Nero et al., 2009a). Sample dilutions were plated on de Man, Rogosa, Sharpe (**MRS**) agar (Oxoid Ltd., Basingstoke, UK) and incubated at 35°C for 48 h under anaerobiosis (Anaerobac, Probac do Brasil, São Paulo, SP, Brazil) for LAB isolation.

Antagonistic Activity of Naturally Occurring LAB

Bacterial Cultures. Reference cultures of *Listeria monocytogenes* ATCC 7644, *Lactobacillus sakei* 2a, and *Lactococcus lactis* ssp. *lactis* DY 13 (Lyofast Dry DY 13, Prime Pharma CC, Gordons Bay, South Africa) were maintained under refrigeration in tubes containing trypticase soy agar (Oxoid) or MRS (Oxoid). Before use, the cultures were grown in trypticase soy broth (Oxoid) or MRS (at 35°C for 24 h) and diluted in trypticase soy broth or MRS broth until they achieved turbidity similar to tube no. 1 on the McFarland scale (3×10^8 cfu/mL).

From the MRS plates, 389 colonies were randomly selected (around 10% of the observed count), purified in MRS agar (at 35°C for 24–48 h), lyophilized, and stored at −80°C. For the antagonism test, the stored cultures were recovered in MRS broth (Oxoid) at 35°C for 24 h; after confirmation of the purity in MRS agar (at 35°C for 24–48 h), a single colony was inoculated into MRS broth (at 35°C for 24 h). The obtained cultures were then diluted in MRS broth until they achieved turbidity similar to tube no. 1 on the McFarland scale (3×10^8 cfu/mL).

Detection of the Antagonistic Activity. Culture aliquots of 2 μL were inoculated onto plates containing 10 mL of brain heart infusion (**BHI**) agar (Oxoid) supplemented with a catalase solution (Sigma, St. Louis, MO) to a final concentration of 100 IU/mL, and the cultures were incubated at 30°C for 24 h. At this stage, the production of acids was minimized by the use of BHI (Harris et al., 1989); the occasional production of hydrogen peroxide was destroyed by the addition of catalase (Corsetti et al., 1996; Moreno et al., 1999). After incubation, each plate received an overlay of 8 mL of semisolid BHI (with agar 0.8%) containing approximately 10^5 cfu/mL of the *L. monocytogenes* culture (sensitive microorganism). After solidification, the plates were incubated at 35°C for 24 h and were examined for the formation of an inhibition halo around the inoculated culture, indicating antagonistic activity by the tested culture. An aliquot of 2 μL of the *Lb. sakei* 2a culture was also plated onto all plates as positive control of antagonistic LAB because of its bacteriocinogenic nature (de Martinis and Franco, 1998). The cultures identified as antagonistic were then further characterized according to morphology (Gram stain) and the production of catalase.

Enzymatic Sensitivity of Antimicrobial Substances. After obtaining the results of the antagonism test, all cultures with antimicrobial activity were submitted to additional assays to confirm the proteinaceous nature of the antagonistic substances according to the methodology described by Lewus et al. (1991), with the following modifications. Colonies isolated from the antagonistic cultures were grown in MRS broth and incubated at 25°C for 24 h. Aliquots of 2 μL of each culture were plated in at least 5 replications onto plates containing 10 mL of modified MRS agar (containing dextrose 0.5%) and were incubated at 25°C for 24 h under anaerobiosis (Anaerobac, Probac do Brasil). After incubation, a well (2 mm diameter) was made in the agar at a distance of 0.5 cm from each formed colony, and each well was filled with 20 μL of a solution (20 mg/mL) of the following enzymes (Sigma): α-chymotrypsin (from bovine pancreas), proteinase K (from *Tritirachium album*), and trypsin (from bovine pancreas). In addition, 20 μL of sterile distilled water was added in 1 of the wells as a negative control. After 30 min at room temperature (absorption and diffusion), each plate received an overlay of 8 mL of semisolid BHI agar (0.8%) containing approximately 10^5 cfu/mL of the *L. monocytogenes* culture (sensitive microorganism) and was incubated at 35°C for 24 h. The production of bacteriocin-like substances by the cultures was confirmed by sensitivity to 1 or more enzymes tested, as identified by the formation of a half moon-shaped halo.

Bacteriocinogenic LAB Molecular Analyses

PCR. Considering the obtained results for morphological characterization and antagonistic activity, 20 LAB cultures were selected for molecular identification and detection of genes related to bacteriocin production.

Initial molecular studies were performed to identify the LAB group through the conserved region of the 16S rRNA gene (Kullen et al., 2000). For these assays, 1 colony of each culture was recovered in MRS broth (at 35°C for 24 h). After incubation, 1 mL of the culture was microcentrifuged for 5 min at 6,000 × g. The supernatant was removed and the pellet was processed for DNA extraction according to Pospiech and Neumann (1995). For PCR, the Illustra PureTaq Ready-to-go

PCR beads kit (GE Healthcare, Chalfont St. Giles, UK) was used, and the oligonucleotides in the assay were plb16 (5′ AGAGTTTGATCCTGGCTCAG 3′) and mlb16 (5′ GGCTGCTGGCACGTAGTTAG 3′), as described by Kullen et al. (2000). The PCR products resulted in a fragment of 500 bp from the conserved region of the 16S rRNA gene. The thermocycler was programmed for 5 min at 94°C, 35 cycles of 15 s at 94°C, 15 s at 55°C, and 1 min at 72°C, and 10 min at 72°C. The products of the reactions were submitted to gel electrophoresis in a 1.0% (wt/vol) agarose gel stained with ethidium bromide and visualized under UV illumination (Kullen et al., 2000).

Sequencing and Sequence Analyses of Nucleotides. After PCR products were purified with the Wizard SV Gel kit and PCR Clean-Up System (Promega Corp., Madison, WI), they underwent automatic sequencing using the methodology adapted from Sanger et al. (1977), performed by Macrogen Inc. (Seoul, South Korea). The obtained nucleotide sequences were compared with sequences previously deposited in the GenBank using the Basic Alignment Search Tool (BLAST) software system (http://www.ncbi.nlm.nih.gov). Next, the sequences were grouped by the neighbor-joining method (with the Poisson correction) according to their genetic profiles using the MEGA 4.0 software system (Tamura et al., 2007). Association analyses were performed using the 1000 bootstrap method. The partial sequence of the 16S rRNA gene from *Lactobacillus acidophilus* ATCC 4356 (access number: M58802) was used in the construction of the external grouping of the phylogenetic tree.

Nisin Genes Detection. The cultures identified as *Lc. lactis* ssp. *lactis* were analyzed to detect the presence of genes encoding nisin. The DNA of the cultures was extracted using the Wizard Genomic DNA Purification kit (Promega). The sequences of oligonucleotides employed (nisAf1: 5′ AAAATGAGTACAAAAGATTTYAAC 3′; nisBr3: 5′ TGCATAACATCATAGAGTTTAGG 3′; nisAf2: 5′ TTCACGTAAGCAAATAACCA 3′; and nisAr2: 5′ TGGTTATTTGCTTACGTGAA 3′) and the amplification cycles of the PCR were according to Espeche et al. (2009). Oligonucleotides were combined as follows: nisAf1/nisAr2 (product ~160 bp), nisAf2/nisBr3 (product ~270 bp), and nisAf1/nisBr3 (product ~430 bp). The Illustra PureTaq Ready-to-go PCR beads kit (GE Healthcare) was then used, with the thermocycler programmed for 4 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 49°C, and 45 s at 72°C, and 5 min at 72°C. The products of the reactions were submitted to electrophoresis in a 1.0% (wt/vol) agarose gel and were visualized using ethidium bromide under UV illumination (Espeche et al., 2009). The strain *Lc. lactis* ssp. *lactis* DY 13 was used as a positive control.

Table 1. Frequencies of autochthonous cultures isolated from raw milk and soft cheese that demonstrated antimicrobial activity against *Listeria monocytogenes* ATCC 7644

Inhibition halo size (radius, mm)	Target microorganism (*L. monocytogenes*)
Absence	339
<5	8
5 to 10	32
10 to 15	18
>15	0
Total	389

RESULTS

Antagonistic Activity of Naturally Occurring LAB

Antagonistic activity was evaluated according to the production of bacteriocin-like substances by the selected cultures. Of these, 58 (14.9%) cultures presented antagonistic activity against *L. monocytogenes* (Table 1). According to the genuine characteristics of LAB (de Martinis et al., 2003), all antagonistic cultures presented negative results for the catalase test and were classified as gram-positive, with cocci as the prevailing morphology (55 cultures, 94.8%). The observed results were similar to those obtained by Franciosi et al. (2009).

Enzymatic Sensitivity of Antimicrobial Substances

Lactic acid bacteria cultures with antimicrobial activity were submitted to additional tests to confirm the proteinaceous nature of the antagonistic substances and, consequently, their bacteriocinogenic-like nature. Most cultures produced extracts that presented sensitivity to 1 or more enzymes, confirming their ability to produce bacteriocin-like substances (Table 2). The methodology used did not allow for the identification of sensitivity to enzymes in 14 cultures because of the formation of a very small inhibition halo during the analyses. Some antimicrobial activity loss may have occurred in these cultures, as suggested by Hoover and Steenson (1993).

Bacteriocinogenic LAB Molecular Analyses

Although it is impossible to detect sensitivity to proteases in some extracts, 20 cultures were selected, submitted to PCR based on the conserved region of the 16S rRNA gene, and subsequently underwent genetic sequencing (Table 3), confirming the previous identification of the cultures as LAB. Only 2 species, *Lc. lactis* ssp. *lactis* and *Lactobacillus plantarum*, were identified and divided into different strains. A high variability of these strains was observed in the samples of cheese, whereas raw milk samples presented only 1 strain, *Lc. lactis* ssp. *lactis* (Table 3). The phylogenetic analysis of

Table 2. Enzymatic sensitivity patterns of the antimicrobial substances produced by cultures of lactic acid bacteria isolated from raw milk and soft cheese

Enzyme			
Proteinase K	Trypsin	α-Chymotrypsin	n
Sensitive	Sensitive	Sensitive	18
Sensitive	Not sensitive	Sensitive	11
Sensitive	Not sensitive	Not sensitive	5
Not sensitive	Sensitive	Sensitive	4
Not sensitive	Sensitive	Not sensitive	3
Not sensitive	Not sensitive	Sensitive	2
Sensitive	Sensitive	Not sensitive	1
Not sensitive	Not sensitive	Not sensitive	14

the isolates identified the presence of different groupings of cultures with similar genetic characteristics, although they were identified as different strains of *Lc. lactis* ssp. *lactis* and *Lb. plantarum*.

DISCUSSION

It was observed that 58 (14.9%) LAB cultures produced antagonistic strains against *L. monocytogenes*, with 18 presenting inhibition halos greater than 10 mm in radius (Table 1). In a similar study, Coventry et al. (1997) isolated several cultures from milk and cheese that were able to produce bacteriocins with antimicrobial activity against *L. monocytogenes*; these were also identified as nisin and pediocin producers. In addition, several studies have demonstrated the antagonistic activity of autochthonous cultures isolated from dairy and meat products against *L. monocytogenes* (Winkowski et al., 1993; Rodríguez et al., 2000; Nero et al., 2009b), indicating the potential use of these cultures in the control of this pathogen, as observed in the present study (Table 1).

The variation in sensitivity of bacteriocins and bacteriocin-like substances to different enzymes is important for the identification of potential uses of these cultures in the control of foodborne pathogens (Table 2). Similar types of enzyme sensitivities of bacteriocinogenic LAB have been observed in other studies (Tükel et al., 2007; Rashid et al., 2009).

The identified strains (Table 3) were often described as bacteriocin or bacteriocin-like substance producers (Chen and Hoover, 2003), suggesting their possible applicability as biopreservatives in the food industry. In addition, these cultures are typically present in dairy products (Rodríguez et al., 2000; Dolci et al., 2008). *Lactobacillus plantarum* is able to produce different kinds of plantaricins, which are bacteriocins that present antimicrobial activity against several microorganisms such as LAB, *Staphylococcus aureus*, *Bacillus cereus, Clostridium sporogenes*, and some species of the Enterobacteriaceae family (Chen and Hoover, 2003; Hernández et al., 2005). *Lactococcus lactis* ssp. *lactis* is able to produce several bacteriocins including nisin, the best characterized and only bacteriocin considered safe for consumption (GRAS, generally regarded as safe) (Chen and Hoover, 2003; Cotter et al., 2005). Nisin is used as a preservative by the food industry (Cotter et al., 2005) and presents a broad spectrum of activity against different gram-positive microorganisms (Chen and Hoover, 2003).

Molecular methodologies are extremely important in studies of antagonism because they allow for the precise identification of LAB isolates (Mohania et al., 2008), genes known as codifiers of already described bacteriocins, and new sequences of genes that codify new antimicrobial metabolites (Nes and Johnsborg, 2004). From the 18 cultures identified as *Lc. lactis* ssp. *lactis*, 7 tested positive for nisin genes by PCR (Figure 1). Nisin

Table 3. Identification of bacteriocinogenic or bacteriocinogenic-like lactic acid bacteria cultures isolated from raw milk and soft cheese, as well as genetic information obtained by sequencing of the 16S rRNA gene

Origin	No. of identified cultures	Species	Strain	Identity[1]	GenBank no.
Raw milk	5	*Lactococcus lactis* ssp. *lactis*	RO37	482/485	AF515226.1
	1	*Lactococcus lactis* ssp. *lactis*	RO37	471/485	AF515226.1
	1	*Lactococcus lactis* ssp. *lactis*	RO37	481/485	AF515226.1
	1	*Lactococcus lactis* ssp. *lactis*	RO37	466/485	AF515226.1
	1	*Lactococcus lactis* ssp. *lactis*	0711XYBLS	480/484	EU869288.1
		Lactococcus lactis	SL3	480/484	AY675242.1
Soft cheese	7	*Lactococcus lactis* ssp. *lactis*	RO37	482/485	AF515226.1
	1	*Lactococcus lactis* ssp. *lactis*	RO37	464/485	AF515226.1
	1	*Lactococcus lactis* ssp. *lactis*	IL1403	484/491	AE006456.1
	1	*Lactobacillus plantarum*	WCFS1	498/505	AL935261.1
	1	*Lactobacillus plantarum*	L5	496/501	DQ239698.1
		Lactobacillus plantarum	L2	496/501	DQ239695.1
		Lactobacillus plantarum	LP3	496/501	AY675256.1
		Lactobacillus plantarum	WCFS1	496/501	AL935261.1

[1]Identity = number of identical bases/total number of analyzed bases.

is not degraded by trypsin, α-chymotrypsin, catalase, or pepsin (Cholett et al., 2008), and the obtained results (Figure 2) indicated that only culture 32M3 expressed nisin genes and produced this bacteriocin. Based on the results of the enzymatic tests, the other isolates with positive results for nisin (31M7, 17M2, 48M5, 54M2, 22M8, and 19M3) did not demonstrate the production of either this substance or other bacteriocins (Figure 2).

The results of the phylogenetic analysis by genetic sequencing, together with the enzymatic profile of the bacteriocins and bacteriocin-like substances and the positive genetic variations for nisin codification, allow for the observation of wide variability among the isolates (Figure 2). The 11 cultures of *Lc. lactis* ssp. *lactis* that belonged to the largest phylogenetic grouping presented relevant differences in the enzymatic sensitivity of the bacteriocins and bacteriocin-like substances and also carried genes for nisin.

The obtained results indicate a great amount of variability among the bacteriocin or bacteriocin-like substances produced by LAB isolates that were identified

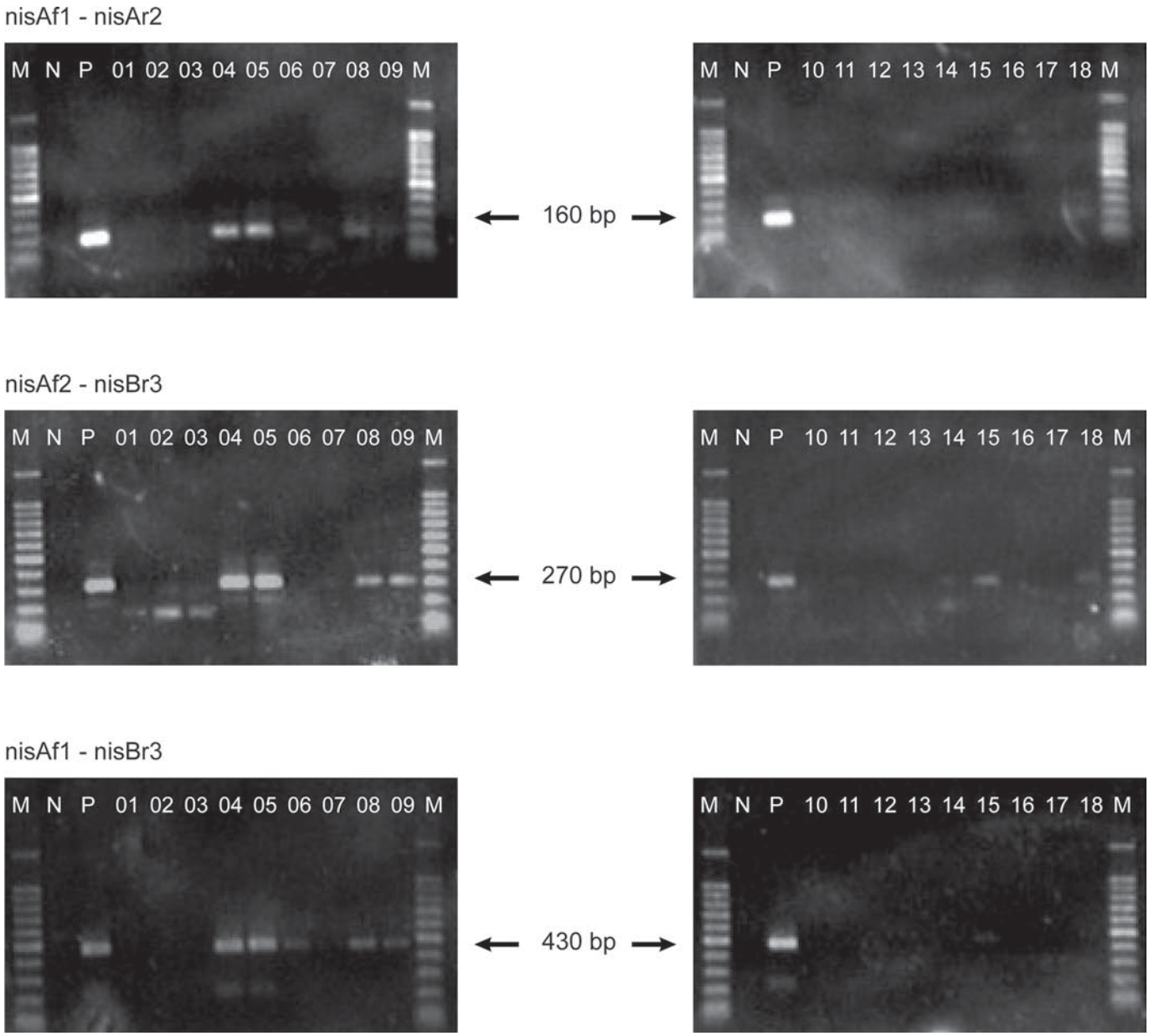
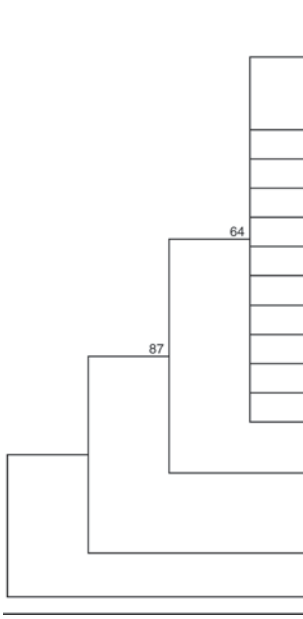


Figure 1. Molecular identification of nisin coding genes by PCR: nisAf1-nisAr2 (product ~160 bp), nisAf2-nisBr3 (product ~270 bp), and nisAf1-nisBr3 (product ~430 bp). M = 100-bp ladder; N = negative control; P = positive control; 01 to 18 = tested cultures.



Culture	Identification	Sensitivity of the antagonistic substances to enzymes			PCR for
		proteinase K	trypsin	α-chymotrypsin	nisin
48M3	*Lactococcus lactis* subsp. *lactis*	S	NotS	S	-
31M7	*Lactococcus lactis* subsp. *lactis*	NotS	S	S	+
17M2	*Lactococcus lactis* subsp. *lactis*	S	S	S	+
54M1	*Lactococcus lactis* subsp. *lactis*	S	S	S	-
03M4	*Lactococcus lactis* subsp. *lactis*	S	NotS	NotS	-
48M5	*Lactococcus lactis* subsp. *lactis*	S	S	S	+
38M2	*Lactococcus lactis* subsp. *lactis*	NotS	NotS	NotS	-
04M2	*Lactococcus lactis* subsp. *lactis*	NotS	S	S	-
54M2	*Lactococcus lactis* subsp. *lactis*	NotS	NotS	NotS	+
22M8	*Lactococcus lactis* subsp. *lactis*	NotS	NotS	NotS	+
53M3	*Lactococcus lactis* subsp. *lactis*	S	S	S	-
51M8	*Lactococcus lactis* subsp. *lactis*	S	S	S	-
46M6	*Lactococcus lactis* subsp. *lactis*	NotS	NotS	S	-
03M3	*Lactococcus lactis* subsp. *lactis*	NotS	S	NotS	-
19M3	*Lactococcus lactis* subsp. *lactis*	NotS	NotS	S	+
24M2	*Lactococcus lactis* subsp. *lactis*	S	S	S	-
52M8	*Lactococcus lactis* subsp. *lactis*	S	S	S	-
32M3	*Lactococcus lactis* subsp. *lactis*	S	NotS	NotS	+
43M3	*Lactobacillus plantarum*	NotS	S	S	NP
53M6	*Lactobacillus plantarum*	S	NotS	NotS	NP
ATCC 4356	*Lactobacillus acidophilus*	NP	NP	NP	NP

Figure 2. Genetic grouping based on the partial sequence of nucleotides of the *Lactobacillus acidophilus* ATCC 4356, identification by genetic sequencing, sensitivity to enzymes, and presence of genes related to the encoding of nisin from cultures of bacteriocinogenic or bacteriocinogenic-like lactic acid bacteria isolated from raw milk and soft cheese. The length of the horizontal bar of the groups is proportional to the genetic distance between the isolates. The numbers next to the lines indicate the percentage of repetitions of the bootstrap analysis in which ramifications were observed. S = sensitive to the enzyme solution; NotS = not sensitive to the enzyme solution; NP = test not performed; + = positive; – = negative.

as antagonistic against *L. monocytogenes*. These characteristics suggest that these cultures could potentially be used as biopreservatives in dairy products and diverse processing situations. Further studies are necessary for a better understanding of the antimicrobial potential of these cultures.

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