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Microbiota of Minas cheese as influenced by the nisin producer *Lactococcus lactis* subsp. *lactis* GLc05

Running title: *In situ* activity of nisin producer GLc05 on Minas cheese microbiota

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Abstract

Minas cheese is a popular dairy product in Brazil that is traditionally produced using raw or pasteurized cow milk. This study proposed an alternative production of Minas cheese using raw goat milk added of a nisin producer *Lactococcus lactis* subsp. *lactis* GLc05. An *in situ* investigation was carried on to evaluate the interactions between the *L. lactis* subsp. *lactis* GLc05 and the autochthonous microbiota of a Minas cheese during the ripening; production of biogenic amines (BA) was assessed as a safety aspect. Minas cheese was produced in two treatments (A, by adding *L. lactis* subsp. *lactis* GLc05, and B, without adding this strain), in three independent repetitions (R1, R2, and R3). Culture dependent (direct plating) and independent (rep-PCR and PCR-DGGE) methods were employed to characterize the microbiota and to assess the possible interferences caused by *L. lactis* subsp. *lactis* GLc05. BA amounts were measured using HPLC. A significant decrease in coagulase-positive cocci was observed in the cheeses produced by adding *L. lactis* subsp. *lactis* GLc05 (cheese A). The rep-PCR and PCR-DGGE highlighted the differences in the microbiota of both cheeses, separating them into two different clusters. *Lactococcus* sp. was found as the main microorganism in both cheeses, and the microbiota of cheese A presented a higher number of species. High concentrations of tyramine were found in both cheeses and, at specific ripening times, the BA amounts in cheese B were significantly higher than in cheese A ($p < 0.05$). The interaction of nisin producer *L. lactis* subsp. *lactis* GLc05 was demonstrated *in situ*, by demonstration of its influence in the complex microbiota naturally present in a raw goat milk cheese and by controlling the growth of coagulase-positive cocci. *L. lactis* subsp. *lactis* GLc05 influenced also the production of BA determining their amounts in the cheeses were maintained at acceptable levels for human consumption.

Keywords: Minas cheese, raw goat milk, microbial ecology, PCR-DGGE, rep-PCR, biogenic amines

1. Introduction

Minas cheese is a ripened cheese with milky and buttery notes that is produced by enzymatic coagulation of pasteurized cow milk, added or not of starter cultures. Minas cheese is the most traditionally cheese produced in the Minas Gerais state, Brazil, being produced by small farmers and by large dairy industries. Minas cheese can also be produced using raw cow milk in Brazil, since they are subjected to at least 60 days of ripening, unless scientific studies demonstrate that fewer days of ripening do not jeopardize their quality and safety (Brasil, 2013).

Goat milk and its dairy products are widely appreciated due to their nutritional quality, high digestibility, and therapeutic values for human nutrition (Ribeiro & Ribeiro, 2010). Raw goat milk has a rich autochthonous microbiota that develops specific sensory characteristics in fermented products, such as cheeses. Consumers appreciate these characteristics due to the presence of specific flavor nuances present in these products (Bonetta et al., 2008b; Medina et al., 2011; Montel et al., 2014). Due to these advantages, the production of a Minas cheese using raw goat milk can represents a healthier alternative to use raw cow milk.

Despite the sensorial characteristics, the main concern in using raw milk in manufacturing of dairy products is the possible contamination by foodborne pathogens (Brito et al., 2008; Pinto et al., 2009) and the occurrence of spoilage microorganisms that can reduce their shelf life and produce undesirable substances, such as biogenic amines (BA). BA are basic compounds that can occur in fermented foods and once ingested at high levels can cause several toxicological problems in the consumers (Bover-Cid & Holzapfel, 1999).

A natural alternative in cheese production is the use of autochthonous lactic acid bacteria (LAB) strains, which are capable of producing antimicrobial substances, such as bacteriocins. Bacteriocins are antimicrobial peptides, widely produced by several different bacterial

species, that are active against other bacteria (Cotter et al., 2005). However bacteriocinogenic LAB strains have been extensively studied as biological preservatives in food systems as they are Generally Recognized as Safe - GRAS (Pingitore et al., 2012; Schirru et al., 2012; Biscola et al., 2013; Kruger et al., 2013; Perin et al., 2013). However, few studies have investigated the *in situ* interaction between inoculated bacteriocinogenic LAB strains and the autochthonous microbiota of natural matrices, such as raw milk cheeses. LAB may interfere in the quality and variety of dairy products if they are used as starter or co-adjuvants cultures for technological purposes or as biological preservatives (Scintu & Piredda, 2007).

The main problem of applying bacteriocin-producing strains in food fermentation is related to the *in situ* antimicrobial efficacy which can be negatively influenced by various factors, such as binding of the bacteriocins to food components, inactivation by proteases, the chemical and physical properties of the food (pH, proteins, fat and starch), and changes in the cell membrane of the target bacteria (Settanni et al., 2004). Because of these possible limitations, the knowledge of the autochthonous microbiota diversity during cheese production and ripening, as well as the influence of inoculated LAB strains in *in situ* tests need to be enhanced.

These information could be enhanced by using both culture-dependent and independent methods. Many studies have already demonstrated the ineffectiveness of using only conventional culture-dependent methods to understand the ecology of fermented foods. To overcome its limitations, the use of culture-independent methods have been applied to a variety of dairy products (Rantsiou et al., 2008; Dolci et al., 2010; Arcuri et al., 2013; Delgado et al., 2013;) and allowed the simultaneous characterization of whole ecosystems as well as the identification of different species (Cocolin et al., 2013). These methods are usually employed to evaluate the ecological dynamic of artisanal products and are very useful to demonstrate the interactions that might occur due to the addition of strains with technology

or biopreservative interests.

The present study proposed an *in situ* investigation to evaluate the interactions between the nisin producer *L. lactis* subsp. *lactis* GLc05 and the autochthonous microbiota of a Minas cheese produced with raw goat milk after the production and during the ripening; as that cheeses are artisanal products, the production of biogenic amines (BA) was assessed as a safety aspect ensuring its quality for human consumption.

2. Material and Methods

2.1. Cheese production

2.1.1. Preparation of *L. lactis* subsp. *lactis* GLc05 culture

L. lactis subsp. *lactis* GLc05 was previously characterized by Perin & Nero (2014) as able to produce a novel nisin variant. *L. lactis* subsp. *lactis* GLc05 was grown in de Man, Rogosa and Sharpe broth (MRS, Oxoid Ltd., Basingstoke, England) at 35°C for 24 h. The obtained culture was diluted with 0.85% NaCl (w/v) until turbidity equivalent to McFarland scale 1, corresponding to approximately $3x10^8$ colony forming units per mL (CFU/mL). An aliquot of 10 mL of this culture was transferred to 1 L of sterile skimmed milk and incubated at 30°C for 24 h. The obtained culture was used for Minas cheese production.

2.1.2. Minas cheese production

Minas cheese was produced using raw goat milk according to Scholz (1995) and as described in the diagram presented in Figure 1. The cheeses were produced with the same kind of milk from the same origin, in the same period of the year and in three independent batches (R1, R2 and R3). In each batch, the cheeses were produced considering two different treatments (A

and B):

✓ Cheese A: prepared according to Figure 1, by adding the *L. lactis* subsp. *lactis* GLc05 culture to milk before the coagulation step, resulting in a final concentration of 10^6 CFU/mL;

✓ Cheese B: control cheese, prepared according to Figure 1 without adding the *L. lactis* subsp. *lactis* GLc05.

For both cheeses (A and B), 50 L of raw goat milk were heated at 34°C and added to saturated $CaCl_2$ (20%, w/v) and 2.5 mL of commercial rennet (CHY-MAX®M; CHR Hansen, Hørsholm, Denmark). After 30 min, the curd was cut into cubes with a size of 1 cm^3, and slowly mixed for 40 min. Then, the curd was transferred into circular perforated cheese containers (200 g), pressed for 1 h and maintained at 10°C overnight. The cheeses were salted in brine with NaCl (20% w/v) at 10°C for 2 h, left to dry for 5 days, packed into plastic bags under vacuum, and ripened at 15°C for 60 days.

2.2. Evaluation of *in situ* interactions using culture-dependent methods

2.2.1. Microbial analysis and pH values

Samples of cheeses A and B were immediately collected after cheese making (t = 0), after salting (t = 1 day), and during ripening (every 5 days until 30 days, and after 60 days); the samples were subjected to microbial analysis. Samples of 25 g of cheese were homogenized in 225 mL of 0.1% saline peptone solution, using a Stomacher (Seward Ltd., Worthing, England) for 1 min, and plated onto selective media for enumeration of the following microbial groups: mesophilic aerobes on Petrifilm™ Aerobic Count (3M, St. Paul, MN, USA) at 35°C for 48 h, coliforms and *Escherichia coli* on Petrifilm™ *Escherichia coli* (3M) at 35°C for 48 h, *Enterococcus* on Kanamycin Aesculin Azide Agar (KAA, Oxoid) at 35°C

for 48 h, thermophilic and mesophilic LAB cocci on M17 (Oxoid) at 35 and 42°C for 48 h, thermophilic and mesophilic LAB rods on MRS at 35 and 42°C for 48 h under anaerobiosis, coagulase-negative and coagulase-positive cocci (CNC and CPC, respectively) on Fibrinogen Rabbit Plasma agar (bioMérieux, Marcy l'Etoile, France) at 35°C for 48 h and yeast and molds on Petrifilm™ Yeast and Molds (3M) at 25°C for 5 days.

The pH of each sample was measured in the cheese homogenates in 0.1% saline peptone solution, using a pH meter (HI 221, Hanna Instruments, São Paulo, Brazil).

The results were expressed as log CFU/g and the mean counts were compared by analysis of variance (ANOVA; $p < 0.05$), followed by the Fisher test ($p < 0.05$), to identify significant differences between the results obtained for each cheese during the production and ripening steps, using Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA).

After collection of samples for microbiological analysis at each time, the cheese samples were immediately frozen at -80°C.

2.2.2. Extraction of antimicrobial activity from the cheese samples

The presence of antimicrobial substances in the cheese samples was verified according to Ávila et al. (2006), with modifications. Briefly, frozen samples were thawed, and 5 g were homogenized with 5 mL of 0.02 N HCl in a Stomacher and centrifuged (12,000 × *g*, 20 min, 4°C). The supernatants were adjusted to pH 6.0 using 1 N NaOH and then lyophilized. The lyophilized samples were diluted in 200 µL of Ringer solution, and 50 µL of each sample was transferred to a 5 mm well on BHI (Oxoid) semi-solid agar (0.8% w/v agar-agar) inoculated with *Staphylococcus aureus* ATCC 6538 (at a concentration of 10^5 CFU/mL), as the target-microorganism. The samples that presented clear zones around the wells after 24 h at 35°C were considered as positive for the presence of antimicrobial substances.

2.3. Evaluation of *in situ* interactions using culture-independent methods

2.3.1. Nucleic acid extraction

The total DNA was extracted directly from the cheeses samples, in the same times previously described (see section 2.2.1.), according to Rantsiou et al. (2008). Briefly, 10 g of samples were homogenized in 40 mL of Ringer solution, using a Stomacher, for 1 min. Aliquots of 2 mL were centrifuged for 5 min and the pellets were re-suspended in 120 µL of proteinase K buffer [50 mM Tris–HCl, 10 mM EDTA, pH 7.5, 0.5% (w/v) sodium dodecyl sulfate], 25 µL of proteinase K (25 mg/mL, Sigma-Aldrich, St. Louis, MO, USA), and 50 µL of lysozyme (50 mg/mL, Sigma), and incubated at 50°C for 1 h. Samples were transferred to 1.5 mL tubes with glass beads and 150 µL of 2x breaking buffer [4% Triton X-100 (v/v), 2% (w/v) SDS, 200 mM NaCl, 20 mM Tris, pH 8, 2 mM EDTA, pH 8] was dispensed. Phenol-chlorophorm-isoamyl alcohol (300 µL, 25:24:1, pH 6.7; Sigma-Aldrich) was subsequently added before performing three cycles (30 s at 4.5 motion/s) in a bead-beater machine (Fast Prep-24, MP Biomedicals, Solon, OH). Then, 300 µL of TE (10 mM Tris, 1 mM EDTA) was added to the tubes and centrifuged at 20,000 × *g* for 5 min. The aqueous phase was transferred to a new tube and precipitated with ice-cold absolute ethanol. The nucleic acids were obtained after centrifugation at 20,000 × *g* for 10 min, washed briefly in 70% ethanol, and re-suspended in 50 µL of sterile water. NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA USA) was used to quantify the total DNA extracted, which was diluted to the final concentration of 100 ng/µL.

2.3.2. rep-PCR

The rep-PCR analysis was performed using the total DNA extracted directly from the cheese samples. The PCR reactions were performed according to Gevers et al. (2001), with modifications, using a single primer $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3'). PCR final concentration contained 1x PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleoside triphosphates, 0.75 U Taq polymerase (Sigma-Aldrich), 50 pMol of the primer, 2 µL DNA (50 ng/µL), and ultrapure PCR water (Promega Corporation, Madison, WI, USA) was added to a final volume of 25 µL. The PCR conditions were: 5 min at 95°C, 30 cycles of 30 s at 95°C; 1 min at 40°C; 8 min at 65°C; and a final extension of 16 min at 65°C. The PCR products were electrophoresed in 2% (w/v) agarose gels for 2 h at a constant voltage of 120 V in 1x Tris/Borate/EDTA buffer (TBE). A 1 Kb DNA ladder (Sigma-Aldrich) was used as a molecular-size marker. Gels were stained using ethidium bromide (0.5 µg/mL, Sigma-Aldrich) and the images were recorded using transilluminator UVIpro Platinum 1.1 Gel Software (Eppendorf, Hamburg, Germany). Fingerprints were analysed using BioNumerics 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). The similarities between the profiles were calculated using the Pearson correlation and the dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

2.3.3. DGGE

PCR of the extracted DNA was performed using the universal primers 338f (5′-ACT CCT ACG GGA GGC AGC AGCAG-3′) and 518r (5′-ATT ACC GCG GCT GCT GG-3′) (Ampe et al., 1999), annealing to the bacterial V3 region of the 16S rRNA gene. A GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) was attached to the 5′ end of primer 338f for DGGE analysis. The PCR was performed in a final

volume of 25 µL, containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1.25 U Taq polymerase (Eppendorf), 0.2 µM of each primer, and 2 µL of template DNA. PCR conditions were: 10 min at 95°C; 35 cycles of 1 min, at 95 °C; 1 min at 42°C; 2 min at 72°C; and a final extension of 7 min at 72°C. The PCR products were electrophoresed in 2% (w/v) Tris-acetate-EDTA agarose gels.

The Dcode universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA) was used for DGGE analysis. Electrophoresis was performed in a polyacrylamide gel (8% w/v acrylamide: bisacrylamide 37.5:1) using a denaturing gradient from 25 to 55% of urea/formamide in a 1x TAE buffer (40mM Tris base, 20mM acetic acid, and 1mM EDTA, pH 8). The electrophoresis was performed at a constant voltage of 120 V for 4 h at 60°C, stained in 1x TAE containing 1x SYBR Green I (Sigma-Aldrich), and then analyzed and photographed under UV illumination using UVIpro Platinum 1.1 Gel Software (Eppendorf) (Dolci et al., 2008). Fingerprints were analysed using BioNumerics 6.6 (Applied Maths). The similarities between the profiles were calculated using the Pearson correlation and dendrograms were constructed using UPGMA.

Selected DGGE bands were extracted from the gels, checked by means of DGGE, and sequenced in MWG Biotech in order to identify the species (Dolci et al., 2008). The obtained sequences were compared to the database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/genbank), using the Basic Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) software.

2.4. Quantification of BA by HPLC

Cheese samples were collected after cheese making (t = 0) and after 10, 30, and 60 days of ripening. The BA amounts were quantified after the extraction and derivatization steps as

reported by Innocente et al. (2007), with modifications.

For extraction, the cheese samples (5 g) were added to 10 mL of 0.1 M HCl and 0.5 mL of a 1 g/L solution of 1,7-diaminoheptane (internal standard, IS) in 0.1 M HCl and then homogenized in Stomacher (Seward) for 15 min and centrifuged at 1,400 × *g* for 20 min at 10°C. The supernatant was recovered and the residue was re-extracted using the same procedure. The supernatants were then submitted to the following derivatization process: a 0.5 mL aliquot was added to 150 µL of 0.1 M NaOH, 150 µL of saturated $NaHCO_3$ solution, and 2 mL of 10 mg/mL dansyl chloride solution in acetone and incubated at 40°C for 1 h whilst stirring using a digital pulse mixer (Glas-Col, Terre Haute, USA). At the end of the derivatization reaction, 300 µL of NH_3 were added and the samples were kept at 20°C for 30 min before filtering on PTFE filters (0.45 µm).

BA quantification was performed using a Thermo-Finnigan Spectra System HPLC (Thermo Scientific) equipped with a P2000 binary gradient pump, a SCM 1000 degasser, an AS 3000 automatic injector, and a Finnigan Surveyor PDA Plus detector (PDA, Thermo Scientific). The ChromQuest software 5.0 (Thermo Scientific) was used for instrument control as well as for UV data collection and processing. Separation was achieved on a C18 RP Lichrosphere 250 × 4.6 mm, 5 µm (Merck Millipore, Darmstadt, Germany) column equipped with a C18 RP Lichrosphere guard column 5 µm (Merck Millipore). The mobile phase was composed of solvent A (ultrapure water) and solvent B (acetonitrile) (Moret & Conte, 1996; Moret et al., 2005). The flow rate was set at 1 mL/min and the injection volume was 20 µL. The elution program was as follows: A 35%, kept isocratic for 6 min; A 25% for 1 min, kept isocratic for 13 min; A 0% for 1 min; A 35% for 1 min, kept in isocratic for 10 min. PDA spectra were recorded in full-scan modality over the wavelength range of 200–600 nm, and quantification was performed by recording the peak area at 254 nm. The calibration curves were constructed by plotting the peak area ratios of each external-to-internal standard versus the external

standard concentration. The following external standards were used: 2-phenylethylamine, putrescine, histamine, cadaverine, 1,7-diaminoheptane (IS), tyramine, and spermidine. All standards were of analytical grade and purchased from Sigma Aldrich.

The results were expressed in mg/kg and the mean counts were compared by ANOVA ($p < 0.05$), followed by the Fisher test ($p < 0.05$), to identify significant differences between the results obtained for each cheese during the production and ripening steps, using the software Statistica 8.0 (StatSoft).

3. Results and Discussion

3.1. Evaluation of *in situ* interactions using culture-dependent methods

The mean values of the microbial populations after cheese making and during the ripening of Minas cheese, added (cheese A) or not (cheese B) by nisin producer *L. lactis* subsp. *lactis* GLc05, and the pH values of the samples are presented in Table 1.

The mean counts of mesophilic aerobes, LAB cocci and bacilli at 35 and 42°C were higher ($p < 0.05$) in cheese A than in cheese B at the time of production (t = 0 h, Table 1). After one day of production, the counts of these groups in cheese B were statistically similar to the observed counts in cheese A ($p > 0.05$). The mean counts of mesophilic and thermophilic LAB, as well as presumptive lactococci and lactobacilli, did not present relevant differences in Minas cheeses and after 5 days of ripening reached values between 8 and 9 log CFU/g (Table 1). After 60 days of ripening, enterococci reached counts of approximately 7 log CFU/g in both cheeses (Table 1). Based on these mean values, LAB was the most prevalent microbial group in Minas cheese during ripening.

Coliforms and *E. coli* counts can be considered high in the cheese samples (Table 1). The

values were similar to those observed by Moraes et al. (2009) in raw soft cheese, which indicates the importance of ensuring the microbiological quality of the raw milk employed in the production. Even with an average decrease of 1.0 in the pH value, the mean counts of these groups did not decrease. LAB can be considered as the main group responsible for the pH decrease (Table 1), mainly because of the production of lactic acid, as reported by Dolci et al. (2008).

The mean counts of yeasts and molds started to increase one day after production, until approximately 5 log CFU/g after 60 days of ripening. Yeasts could contribute to the final organoleptic characteristics of the cheese due to the production of volatile compounds; additionally, yeasts can metabolize lactic acid and also produce NH_3 raising the pH value and allowing the growth of salt-tolerant and acid-sensitive bacteria (Montel et al., 2014).

CPC counts in cheese A were significantly lower than in cheese B ($p < 0.05$), after one day of productions and 15 days of ripening; after 60 days of ripening, CPC counts were not recorded in cheese A (counts < 10 CFU/mL) (Table 1). Even though Minas cheese is a typical dairy product in Brazil, there are no standard regulations for its microbiological quality and safety when it is produced with raw milk. The normative instruction n.30 (Brasil, 2013) does not establish maximum limits for microbial counts in these cheeses or the pathogens that must be researched, what can not ensure the safety of this product. A major concern related to cheese production is the poor microbiological quality of the raw milk: inadequate manufacturing practices and improper cold storage during production could also allow the contamination and growth of undesirable microorganisms, such as spoilage and pathogens (Ortolani et al., 2010; Perin et al., 2012). Carmo et al. (2002) described a food-poisoning event from *Staphylococcus* strains present in Minas cheese and raw milk in Brazil, demonstrating the relevance of controlling the growth of this group in dairy products.

L. lactis subsp. *lactis* GLc05 most probably influenced the autochthonous microbiota from

cheese A, determining the decrease in the CPC counts (Table 1). A previous *in vitro* study demonstrated the inhibitory activity of *L. lactis* subsp. *lactis* GLc05 against some *S. aureus* strains (Perin & Nero, 2014), even using the ripening temperature for Minas cheese (15 ºC, data not shown).

In the present study, an *in vitro* test using cheeses A and B samples detected the presence of antimicrobial substance with inhibitory activity against *S. aureus* from the cheese A samples after 5 days of ripening in all repetitions (R1, R2 and R3) and during some ripening steps (variable depending on the repetition, data not shown).

Other studies have also demonstrated the effectiveness of bacteriocinogenic LAB strains in raw milk in interfering with the autochthonous microbiota of this product and controlling spoilage and/or pathogenic micro-organisms (Gonzalez et al., 2003; Psoni et al., 2006; Xanthopoulos et al., 2000). This interference can occur in different pathways, such as competition for nutrients and the production of antagonistic substances like lactic acid, diacetyl, hydrogen peroxide, and bacteriocins (Gálvez et al., 2007).

3.2. Evaluation of *in situ* interactions using culture-independent methods

3.2.1. rep-PCR

The dendrograms (for R1, R2, and R3) obtained by rep-PCR clustering of cheeses A and B are presented in Figure 2. rep-PCR is usually employed for clustering bacterial isolates as a previous screening for subsequent identification by sequencing (Cocolin et al., 2011). However, rep-PCR was considered in this study as a culture-independent method, using the total DNA extracted from the samples, to provide an evidence of the *in situ* interactions between the added *L. lactis* subsp. *lactis* GLc05 and the autochthonous microbiota from

cheeses A.

Considering a coefficient of similarity of 80%, two main clusters were obtained for each of the three generated dendrograms, one containing the cheese A samples and other containing the cheese B samples (Figure 2). This result indicates that the microbiota from cheese A, independent of production and ripening step, was different from cheese B based on their molecular profiles. Nevertheless, considering that the only difference between cheeses A and B was the addition of bacteriocinogenic *L. lactis* subsp. *lactis* GLc05, this result supports that *in situ* active bacteriocins can influence the microbial consortium of Minas cheese.

Rep-PCR provided the first evidence of the *in situ* interference in the cheese A microbiota, that is apparently different from cheese B microbiota. However using only rep-PCR is not possible to assess which groups of microorganisms are present or absent in the cheese samples.

3.2.2. PCR-DGGE

Figure 3 presents the dendrograms (for R1, R2, and R3) obtained by DGGE of cheeses A and B. Considering the results obtained by the three repetitions, the similarity between the cheeses A and B was less than 40%, and they were separated into two main clusters (Figure 3). These results confirmed the *in situ* interference of *L. lactis* subsp. *lactis* GLc05 on the cheese A microbiota, as observed by rep-PCR (Figure 2).

The samples were grouped together depending on the production step and the time of ripening, indicating that the microbiota from cheeses A and B changed during the ripening (Figure 3). The obtained results indicate some differences in the microbiota of the cheeses produced in each repetition, indicating that the Minas cheese microbiota and dynamics could change, depending of the microbial consortia present in the raw milk used for production

(data not shown).

The microbiota fingerprints of cheeses A and B obtained by DGGE are shown in Figure 4. The DGGE profile of the *L. lactis* subsp. *lactis* GLc05 was used as a control. Fifteen bands were selected for sequencing (indicated by numbers in Figure 4). The results obtained after the sequencing of bands extracted from the DGGE gels are reported in Table 2. Cheese samples from the third repetition presented a lower diversity of species and bands (Figure 4), again indicating that the microbiota present in Minas cheese are dependent on the microbiota of the milk (data not shown).

Four species were exclusively found in cheese A: *Enterococcus faecalis* (band 4, Figure 4), in all repetitions; *Enterobacter* sp. (band 7, Figure 4), in all ripening steps of the first repetition; *Shigella flexneri* (bands 12, 13, Figure 4), only in the second repetition and after one day of production; and *Acetobacter* sp. (band 9, Figure 4), in all repetitions and only after cheese making (t = 0). Instead, only one genera was exclusively found in cheese B: *Bifidobacterium* sp. (bands 11, 15, Figure 4), except in the third repetition, and only after cheese making (t = 0). Only two species, *L. lactis* and *Propionibacterium* sp., were found in both cheeses A and B in all repetitions (Figure 4). In general, cheese A presented a higher number of bands and greater species diversity; this result in cheese A is an interesting finding, because it indicates that *L. lactis* subsp. *lactis* GLc05 enhanced the microbial diversity in this cheese, determining higher number of species than in cheese B.

The bands identified as *Shigella flexneri* (bands 12, 13, Figure 4) could be considered a concern related to the microbiological quality of this product, but it was identified only in R2 and during the first days of ripening (until 5 days, Figure 4). Also it can not ensure that this DNA came from live cells of *S. flexneri*. None of the bands were identified as *S. aureus*, probably because this microorganism was present in the samples at concentrations lower than 10^4 - 10^5 CFU/g (Table 1); bacterial populations that are present at counts lower than 10^3 or

10^4 CFU/g can not be properly detected by DGGE-PCR (Cocolin et al., 2011). This result demonstrates the relevance of using different culture-dependent and -independent methods to assess the *in situ* interactions and possible influences caused by inoculated strains on the complex microbial ecology of food systems, such as the Minas cheese produced with raw goat milk in the present study.

Some studies have characterized the safety and ecology of Minas cheese produced with pasteurized milk using only culture-dependent methods (Brito et al., 2008; Moraes et al., 2009; Sant'Ana et al., 2013). And the majority of these studies are focused on its technological and sensory characteristics or on the occurrence of specific microorganisms (Nogueira et al., 2005; Brito et al., 2008; Pinto et al., 2009; Sant'Ana et al., 2013). To the best of our knowledge, only one study has investigated the ecology of Minas cheese produced with raw cow milk using PCR-DGGE (Arcuri et al., 2013). The authors identified that *Streptococcus* sp. and *Lactobacillus* sp., followed by *L. lactis*, were the main microorganisms present as autochthonous microbiota. In the present study, *L. lactis* (bands 2, 3, 5, and 6, Figure 4) was the only species present at all ripening times, both in cheeses A and B, and in all repetitions. Bands 2, 5, and 6 (Figure 4) were not present in the *L. lactis* subsp. *lactis* GLc05 profile, indicating the presence of an autochthonous *L. lactis* population coming from the raw goat milk used for cheese production.

The present study represents the first demonstration of *in situ* interference of an artificially added strain on the autochthonous microbiota of a Brazilian artisanal cheese and one of the few to evaluate the effectiveness of a bacteriocinogenic *Lactococcus* in controlling pathogenic micro-organisms in production of fermented foods.

3.3. Quantification of BA by HPLC

BA contents in Minas cheeses A and B are reported in Table 3. Tyramine was present at the highest concentration, with a significant increase ($p < 0.05$) after 30 days of ripening in both cheeses A and B (Table 3). The evidence of a high concentration of tyramine in cheeses, especially in those produced with raw milk, has previously been reported (Bonetta et al., 2008a; Schirone et al., 2011; Spizzirri et al., 2013). Some LAB strains are responsible for tyramine production (Martuscelli et al., 2005; Moraes et al., 2012; Pintado et al., 2008); however, *L. lactis* subsp. *lactis* GLc05 (inoculated in cheese A) is a low tyramine producer (1.19 ± 2.06 mg/kg, data not shown) and cannot be responsible for the observed amounts of tyramine in the samples.

Tyramine and histamine have great impact on human health (Bover-Cid & Holzapfel, 1999) and they are described as the main BA found in cheese produced with goat milk, while 2-phenylethylamine is usually found at low concentrations (Novella-Rodríguez et al., 2004). In the present study, histamine was detected in cheese A, only after 60 days of ripening, but in low concentration; a non significant increase in the histamine concentration was observed after 30 days of ripening of cheeses B ($p > 0.05$) (Table 3). Histamine has already been recorded at high concentrations in cheeses made with raw milk, demonstrating its relevance towards safety (Bonetta et al., 2008a; Ladero et al., 2008).

2-phenylethylamine was present at higher levels in cheese B than in cheese A after 60 days of ripening ($p < 0.05$, Table 3). The presence of this BA at high concentrations in cheese was previously described (Martuscelli et al., 2005; Schirone et al., 2011). Cadaverine was present at lower levels in the cheese A than in cheese B during the production and ripening ($p < 0.05$; Table 3). Spermidine and putrescine were found in cheeses A and B at low concentrations (Table 3). *L. lactis* subsp. *lactis* GLc05 influenced the production of BA in cheese A determining lower amounts of 2-phenylethylamine, cadaverine, and histamine ($p < 0.05$ by comparing cheeses A and B, Table 3) and also controlling its production at acceptable levels

for human consumption.

The presence of BA in cheeses can vary, depending on the type of cheese, precursor amino-acid availability, the ripening time and temperature, the manufacturing process, the quality of raw material used for production and the microbial ecology of the food matrix (Schirone et al., 2011). The higher BA content in ripened cheeses, compared to fresh ones, is commonly evidenced due to their accumulation over time (Loizzo et al., 2013). However, Buňková et al. (2013) compared the amount of BA in cheeses produced with both raw and pasteurized milk, and did not find significant differences among them.

High amounts of BA in cheeses can be originated by both starter and non-starter LAB that are used in the manufacture of these products or that can get into products during their processing, respectively. But BA can also be produced in contaminated microflora, especially by the Enterobacteriaceae and other Gram-negative bacteria, mainly observed when cheeses are manufactured with raw milk (Coton et al., 2012).

The safety concentration of BA in foods was not being determinate yet in any regulation, but considering their toxicity to humans, their investigation and control is of extreme importance.

4. Conclusions

The importance to use a novel strain nisin producer *L. lactis* subps. *lactis* GLc05 to offset the possible risks related to the use of raw milk, was demonstrated by a significant decrease of coagulase-positive cocci in the cheeses A. Moreover, by culture-independent methods (rep-PCR and PCR-DGGE) it was possible to clearly demonstrate the differences between the microbiota from cheese A and B. *L. lactis* subsp. *lactis* GLc05 also influenced the production

of BA determining that their amounts in the cheeses were maintained at acceptable levels for human consumption.

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Table 1. Mean counts (log CFU/g) and standard deviation of the main microbial populations in Minas cheese inoculated (A) or not (B) with *L. lactis* subsp. *lactis* GLc05 during the production and ripening.

Step	Time (days)	Cheese	pH	Mesophilic aerobes	Coliforms	*Escherichia coli*	Enterococci	Coagulase positive cocci	Coagulase negative cocci	Yeasts and Molds	LAB cocci 35 °C	LAB cocci 42 °C	LAB bacilli 35 °C	LAB bacilli 42 °C
production	0	A	6.1 ± 0.5	8.0 ± 0.0 Aa	4.2 ± 0.5 Ba	4.1 ± 1.5 Aa	6.0 ± 1.2 Aa	4.3 ± 0.8 Aa	2.3 ± 0.0 Aa	2.7 ± 0.4 Ba	7.6 ± 0.7 Ba	7.0 ± 1.9 Ba	7.6 ± 0.6 Ba	6.9± 0.6 Aa
		B	7.0 ± 0.1	5.4 ± 0.3 Bb	4.4 ± 0.4 Ba	3.0 ± 0.3 Aa	4.4 ± 1.3 Ba	4.7 ± 0.7 Aa	3.6 ± 0.40 Aa	2.8 ± 0.3 Ba	5.3 ± 0.4 Cb	4.9 ± 0.0 Cb	4.8 ± 0.5 Cb	4.6 ± 0.6 Cb
salting	1	A	5.7 ± 0.4	8.3 ± 0.5 Aa	6.3 ± 0.8 ABa	5.7 ± 1.4 Aa	6.6 ± 0.6 Aa	2.9 ± 1.3 Ab	4.5 ± 0.8 Aa	4.0 ± 1.1 ABa	8.2 ± 0.5 ABa	7.9 ± 1.0 Aa	8.0 ± 0.9 ABa	7.2 ± 0.4 Aa
		B	6.2 ± 0.7	7.8 ± 0.2 Ca	6.3 ± 0.5 aAB	5.4 ± 0.7 Aa	5.8 ± 0.4 ABa	5.3 ± 0.2 Aa	4.6 ± 1.3 Aa	4.6 ± 1.0 ABa	7.3 ± 0.7 Ba	7.2 ± 0.7 Ba	7.6 ± 0.4 Ba	6.5 ± 1.4 BCa
ripening	5	A	5.7 ± 0.1	8.7 ± 0.5 Aa	6.9 ± 0.4 Aa	5.7 ± 1.3 Aa	7.0 ± 0.4 Aa	3.3 ± 0.0 Aa	4.4 ± 0.5 Aa	4.0 ± 1.1 ABa	8.5 ± 0.1 ABa	8.6 ± 0.2 Aa	8.4 ± 0.3 ABa	7.5 ± 0.6 Aa
		B	5.5 ± 0.2	8.6 ± 0.4 ACa	7.4 ± 0.4 Aa	6.1 ± 0.9 Aa	6.4 ± 0.2 ABa	5.3 ± 0.3 Aa	5.1 ± 0.7 Aa	4.5 ± 1.6 ABa	8.8 ± 0.5 Aa	8.9 ± 0.3 ABa	9.2 ± 0.2 Aa	7.9 ± 0.5 ABa
	10	A	5.6 ± 0.2	8.8 ± 0.4 Aa	7.1 ± 0.7 ABa	5.8 ± 1.8 Aa	7.1 ± 0.4 Aa	3.2 ± 0.2 Aa	4.8 ± 0.8 Aa	5.4 ± 0.8 Aa	8.5 ± 0.1 ABa	8.5 ± 0.1 Aa	8.5 ± 0.0 ABa	7.6 ± 0.5 Aa
		B	5.4 ± 0.2	8.6 ± 0.4 ACa	7.3 ± 0.7 Aa	6.3 ± 0.8 Aa	6.7 ± 0.5 ABa	5.3 ± 0.4 Aa	5.8 ± 0.2 Aa	5.0 ± 0.3 ABa	9.2 ± 0.2 Aa	8,9 ± 0.7 Aa	9.3 ± 0.1 Aa	8.4 ± 0.8 ABa
	15	A	5.4 ± 0.0	8.8 ± 0.5 Aa	6.7 ± 0.8 ABa	6.3 ± 1.8 Aa	7.4 ± 0.1 Aa	3.0 ± 0.0 Ab	4.9 ± 0.4 Aa	5.7 ± 0.3 Aa	8.8 ± 0.5 Aa	8.6 ± 0.3 Aa	8.7 ± 0.4 Aa	8.2 ± 1.0 Aa
		B	5.4 ± 0.0	9.0 ± 0.5 Aa	7.2 ± 0.7 Aa	6.4 ± 0.8 Aa	7.1 ± 0.5 Aa	5.2 ± 0.0 Aa	5.9 ± 0.5 Aa	5.5 ± 0.4 Aa	9.0 ± 0.5 Aa	9.0 ± 0.3 ABa	9.0 ± 0.5 Aa	8.6 ± 0.4 Aa
	20	A	5.4 ± 0.0	8.7 ± 0.4 Aa	6.2 ± 0.7 ABa	5.8 ± 1.6 Aa	6.5 ± 1.6 Aa	3.8 ± 0.0 Aa	4.5 ± 0.4 Aa	5.4 ± 0.5 Aa	9.0 ± 0.2 Aa	8.6 ± 0.2 Aa	8.9 ± 0.2 Aa	8.4 ± 0.6 Aa
		B	5.4 ± 0.1	9.0 ± 0.2 Aa	7.2 ± 0.6 Aa	6.1 ± 1.1 Aa	7.2 ± 0.5 Aa	5.3 ± 0.5 Aa	5.5 ± 0.6 Aa	5.3 ± 0.5 Aa	9.1 ± 0.3 Aa	8.8 ± 0.3 ABa	9.2 ± 0.3 Aa	8.4 ± 0.7 ABa
	25	A	5.4 ± 0.0	8.9 ± 0.3 Aa	6.4 ± 1.0 ABa	5.3 ± 1.5 Aa	7.7 ± 0.5 Aa	4.2 ± 1.0 Aa	4.8 ± 0.4 Aa	5.1 ± 0.5 Aa	9.0 ± 0.2 Aa	8.5 ± 0.2 Aa	9.1 ± 0.2 Aa	8.6 ± 0.6 Aa
		B	5.4 ± 0.0	8.7 ± 0.2 ACa	7.0 ± 0.8 Aa	5.9 ± 1.3 Aa	7.2 ± 0.3 Aa	5.8 ± 0.5 Aa	5.3 ± 0.8 Aa	5.8 ± 0.4 Aa	8.7 ± 0.1 Aa	8.3 ± 0.1 ABa	8.7 ± 0.2 ABa	8.4 ± 0.3 ABa
	30	A	5.4 ± 0.1	8.9 ± 0.2 Aa	6.2 ± 1.0 ABa	5.5 ± 1.6 Aa	7.6 ± 0.3 Aa	3.5 ± 0.0 Aa	4.4 ± 0.2 Aa	5.4 ± 1.0 Aa	9.1 ± 0.1 Aa	8.6 ± 0.2 Aa	9.0 ± 0.2 Aa	8.4 ± 0.7 Aa
		B	5.4 ± 0.1	8.8 ± 0.4 ACa	7.0 ± 1.0 Aa	5.8 ± 1.0 Aa	7.3 ± 0.7 Aa	4.7 ± 0.3 Aa	5.3 ± 1.0 Aa	5.5 ± 0.8 ABa	8.8 ± 0.3 Aa	8.4 ± 0.4 ABa	8.8 ± 0.4 Aa	8.5 ± 0.7 Aa
	60	A	5.6 ± 0.1	8.2 ± 0.4 Aa	5.6 ± 0.8 ABa	4.6 ± 1.3 Aa	7.0 ± 0.4 Aa	ND	4.5 ± 0.9 Aa	5.3 ± 0.1 ABa	8.3 ± 0.3 ABa	7.7 ± 0.1 Aa	8.4 ± 0.2 ABa	7.9 ± 0.1 Aa
		B	5.6 ± 0.1	8.4 ± 0.3 ACa	5.9 ± 0.5 ABa	5.1 ± 1.1 Aa	7.0 ± 0.4 Aa	3.0 ± 0.0 Aa	5.1 ± 0.5 Aa	5.2 ± 1.1 ABa	8.5 ± 0.2 Aa	7.9 ± 0.4 ABa	8.5 ± 0.1 ABa	8.2 ± 0.4 ABa

Obs.: Capital letters: mean differences of each treatment in different days of production/ripening (Fisher test, $p < 0.05$); lowercase letters: mean difference between treatments, inoculated or not with GLc05 (ANOVA, $p < 0.05$); ND: not detected, counts lower than 10 CFU/g.

Table 2. Identification of bacterial species present in Minas cheese from the DGGE bands based on the BLAST sequence comparison in GenBank.

Band[a]	Closest sequence relative	% identity[b]	GenBank accession no.
1	*Lactobacillus plantarum*	98%	KF682392.1
2	*Lactococcus lactis*	97%	KF623100.1
3	*Lactococcus lactis*	99%	KF623100.1
4	*Enterococcus faecalis*	99%	AB761302.1
5	*Lactococcus lactis*	99%	KF623100.1
6	*Lactococcus lactis*	99%	KF673548.1
7	*Enterobacter* sp.	99%	AJ564061.1
8	*Lactobacillus* sp.	99%	JX520291.1
9	*Acetobacter* sp.	99%	HF969863.1
10	*Propionibacterium freudenreichii* subsp. *shermanii*	99%	NR_102946.1
11	*Bifidobacterium psychraerophilum*	99%	NR_029065.1
12	*Shigella flexneri*	100%	AM777394.1
13	*Shigella flexneri*	99%	AM777394.1
	Escherichia coli	99%	GU646146.1
14	*Propionibacterium freudenreichii* subsp. *shermanii*	100%	NR_102946.1
15	*Bifidobacterium* sp.	99%	EF990663.1

[a] The numbers correspond to the band numbers in Figure 4.

[b] Percentage of similarity between the sequences obtained from the DGGE band and the sequence of the closest species in the GenBank database.

Table 3. Mean concentrations (mg/kg) and standard deviation of the biogenic amines in Minas cheese inoculated (A) or not (B) with *L. lactis* subsp. *lactis* GLc05 during the production and ripening.

Biogenic Amine	Cheese	Production and ripening steps (days)			
		0	10	30	60
2-Phenylethylamine	A	45.3 ± 5.2 Ba	135.1 ± 55.8 Aa	94.8 ± 42.2 Aa	135.4 ± 24.9 Ab
	B	203.6 ± 186.7 Ba	135.4 ± 54.6 Ba	244.7 ± 160.7 Ba	592.8 ± 314.0 Aa
Putrescine	A	5.3 ± 8.2 Ab	5.8 ± 5.0 Aa	10.3 ± 8.1 Aa	11.0 ± 10.5 Aa
	B	64.4 ± 48.1 Aa	25.7 ± 24.8 Aa	40.3 ± 46.3 Aa	55.9 ± 63.4 Aa
Histamine	A	ND	ND	ND	25.4 ± 39.4 a
	B	24.7 ± 38.3 ABa	ND	325.8 ± 503.7 Aa	32.9 ± 25.5 ABa
Cadaverine	A	42.9 ± 27.0 Bb	172.8 ± 92.6 Ab	130.3 ± 42.2 Ab	156.2 ± 84.8 Ab
	B	104.9 ± 11.7 Ba	370.9 ± 160.4 ABa	354.8 ± 215.2 ABa	565.4 ± 366.6 Ba
Tyramine	A	1,719.1 ± 110.2 Ba	1,855.5 ± 233.5 Ba	2,523.2 ± 515.9 Aa	2,846.0 ± 547.2 Aa
	B	1,626.9 ± 196.9 Ba	1,780.6 ± 269.3 Ba	2,637.6 ± 162.0 Aa	2,956.9 ± 461.6 Aa
Spermidine	A	45.1 ± 35.2 Ba	68.6 ± 5.1 Aa	75.8 ± 9.5 Aa	81.1 ± 10.5 Aa
	B	56.0 ± 16.9 Ba	70.1 ± 8.6 ABa	75.0 ± 11.2 Aa	77.8 ± 14.1 Aa

Obs. Capital letters: mean differences of each treatment in different days of production/ripening (Fisher test, $p < 0.05$); lowercase letters: mean difference between treatments, inoculated or not with GLc05 (ANOVA, $p < 0.05$); ND: not detected.

Raw goat milk (50 L)

↓

Addition of $CaCl_2$, rennet and GLc05 (34°C) - Cheese A

or

Addition of $CaCl_2$ and rennet (34°C) - Cheese B

↓

Coagulation step (35°C/30 min)

↓

Curd cutting and mixing (40 min)

↓

Transfer to perforated containers

↓

Pressing and dripping (room temperature/1 h)

↓

Storage (10°C/overnight)

↓

Salting (10°C/2 h)

↓

Drying (10°C/5 days)

↓

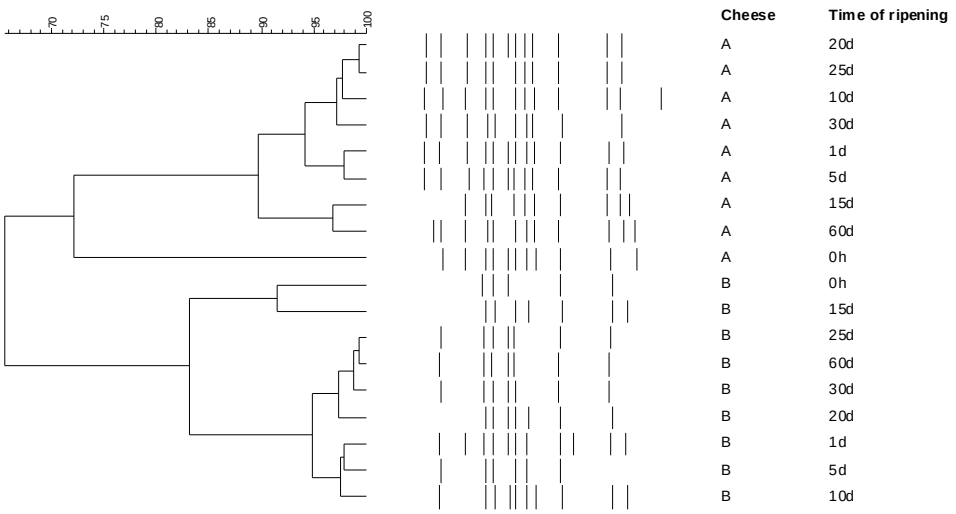
Transfer to plastic bags

↓

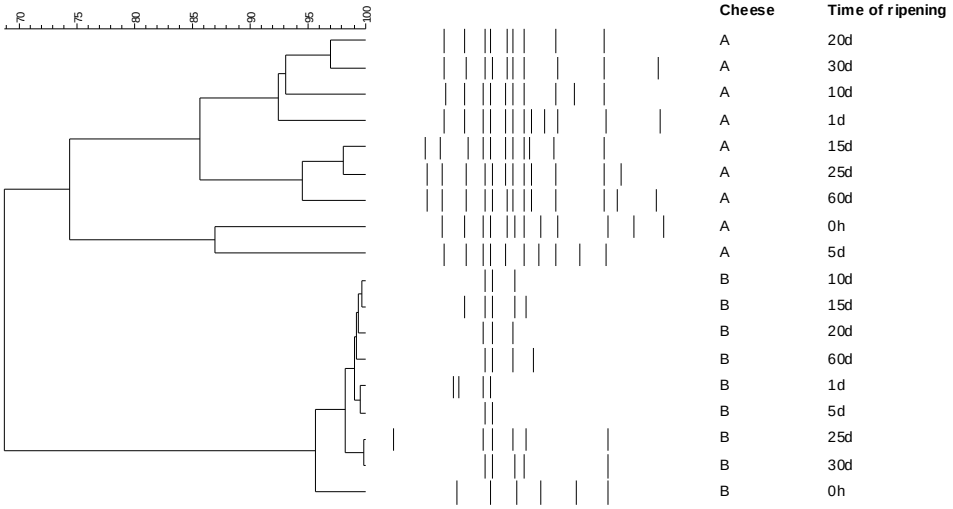
Ripening (15°C/60 days)

Figure 1.

R1



R2



R3

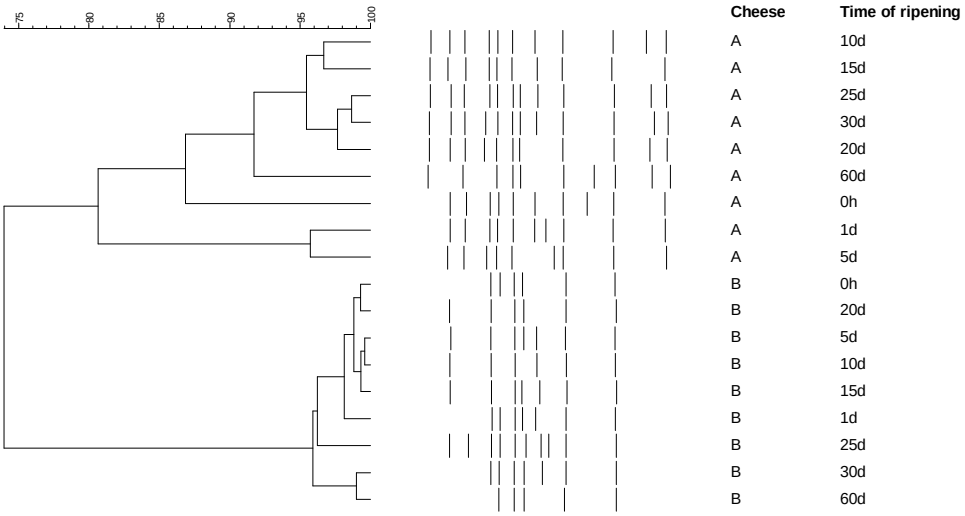


Figure 2

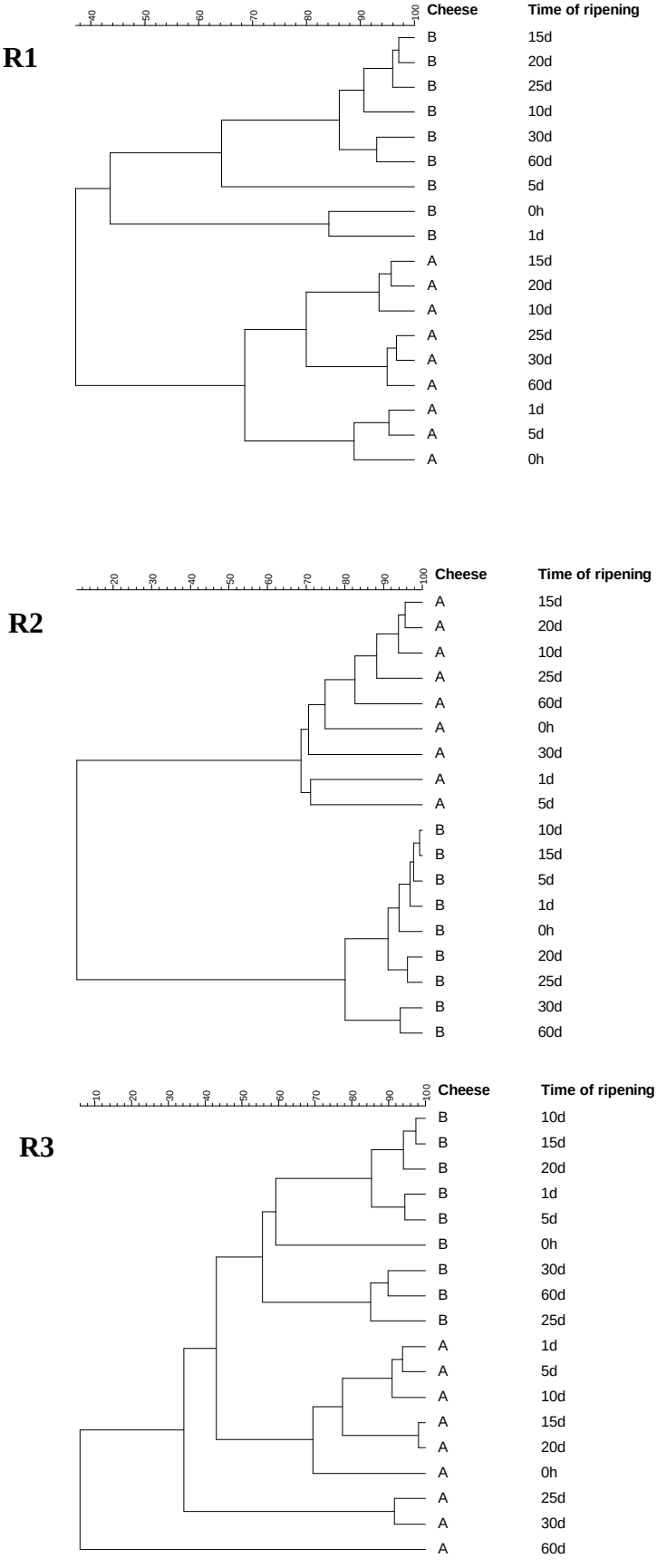
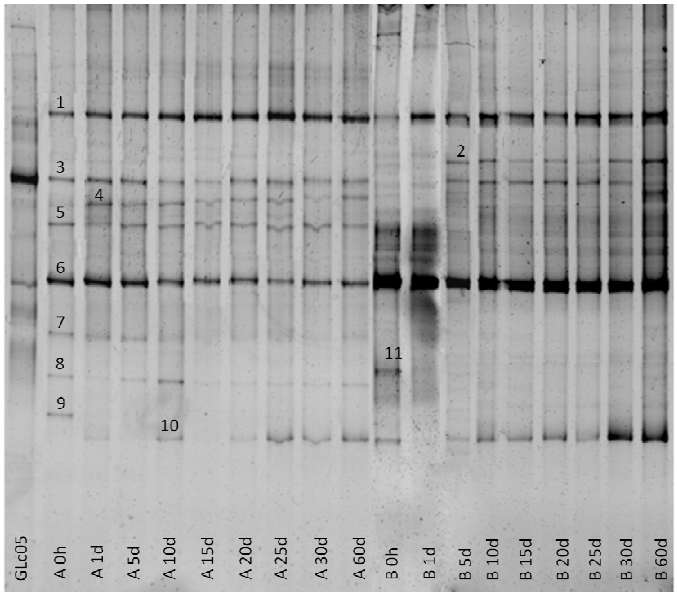
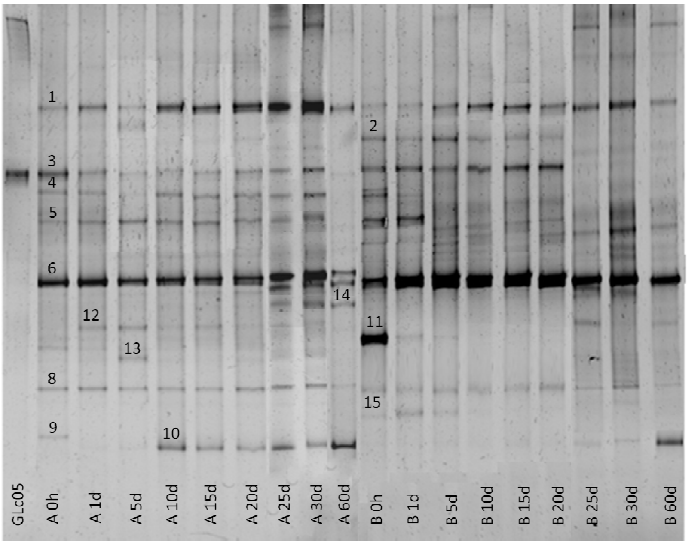

R1
40
50
60
70
80
90
100
Cheese
Time of ripening
B 15d
B 20d
B 25d
B 10d
B 30d
B 60d
B 5d
B 0h
B 1d
A 15d
A 20d
A 10d
A 25d
A 30d
A 60d
A 1d
A 5d
A 0h
R2
20
30
40
50
60
70
80
90
100
Cheese
Time of ripening
A 15d
A 20d
A 10d
A 25d
A 60d
A 0h
A 30d
A 1d
A 5d
B 10d
B 15d
B 5d
B 1d
B 0h
B 20d
B 25d
B 30d
B 60d
R3
10
20
30
40
50
60
70
80
90
100
Cheese
Time of ripening
B 10d
B 15d
B 20d
B 1d
B 5d
B 0h
B 30d
B 60d
B 25d
A 1d
A 5d
A 10d
A 15d
A 20d
A 0h
A 25d
A 30d
A 60d

Figure 3

R1



R2



R3

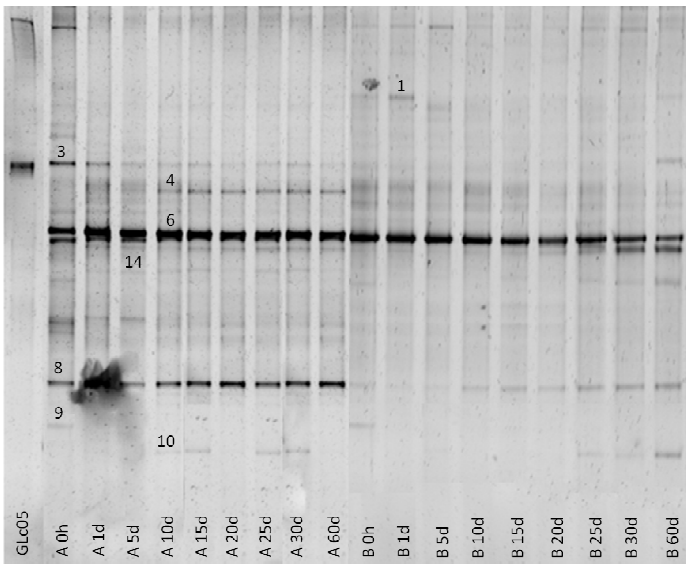


Figure 4

Figure 1. Diagram for Minas cheese production, demonstrating the differences for cheese A and cheese B production in the second step of processing. GLc05: nisin producer *Lactococcus lactis* subsp. *lactis.*

Figure 2. Cluster analysis of rep-PCR fingerprints obtained from Minas cheese produced with raw goat milk inoculated (A) or not (B) with nisin producer *L. lactis* subsp. *lactis* GLc05. The dendrograms were generated for each cheese production (R1, R2 and R3) after cluster analysis of the digitized fingerprints and were derived from UPGMA linkage of Pearson correlation coefficient.

Figure 3. Cluster analysis of PCR-DGGE fingerprints obtained from Minas cheese produced with raw goat milk inoculated (A) or not (B) with nisin producer *L. lactis* subsp. *lactis* GLc05. The dendrograms were generated for each cheese production (R1, R2 and R3) after cluster analysis of the digitized fingerprints and were derived from UPGMA linkage of Pearson correlation coefficient.

Figure 4. DGGE fingerprintings of the bacterial ecology of the tree production of Minas cheese (R1, R2 and R3) manufactured with raw goat milk and inoculated (lines indicated as "A") or not (lines indicated as "B") with the nisin producer *L. lactis* subsp. *lactis* GLc05 from 0 day of production to 60 days of ripening. The numbers indicate the bands sequenced for molecular identification. These results are reported in Table 2.