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ENTEROCOCCUS FAECALIS CECT712: BIOPRESERVATION OF CRAFTED GOAT CHEESE

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ABSTRACT: ***Poor hygienic practices along manufacture process may result in bacterial contamination of craft cheese. The aim of this work was to assess the biopreservative activity of the probiotic strain Enterococcus faecalis CECT7121 on the indigenous microbiota in crafted goat cheese. A goat milk mixture was divided in two groups: Group A, not inoc ulated with the probiotic strain and Group B, inoculated with E. faecalis CECT7121 (5.0 x 10^4 CFU.mL^{-1}). Small farms` traditional protocol for cheese manufacturing was followed. Viable cell counts of Lactobacillus spp., Lactococcusspp., Enterococcus spp.,Enterobacteriaceae and Staphylococcus aureus were performed. Presence of the probiotic strain in group B was determined by Pulsed-Field Gel Electrophoresis technique. Viable cell counts at T=0 and at the end of the experiment, T=35 days, for the assessment of E. faecalis CECT7121 inhibitory activity were carried out. Bactericidal activity of E. faecalis CECT7121 against Enterobacteriaceae and S. aureus was proven. However, viable cell counts of Lactobacillus spp. and Lactococcuswere spp. were not modified. The biopreservative activity shown by the probiotic strain E. faecalis CECT7121 should be considered as a potential tool for a safer craft goat cheese manufacture.***

KEY WORDS: Biopreservation, Crafted goat cheese, *E. faecalis* CECT7121, Probiotic

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INTRODUCTION

Dairy products are a suitable environment for development of pathogenic bacteria (Barbaros, 2000). Traditionally, physical or chemical methods for preserving these products have been used. Although foodborne outbreaks are more frequently associated with raw-milk cheese, bacterial contamination may occur along pasteurized-milk cheese manufacture (Zhao, 2001).

Craft cheeses made from raw goat`s milk are cherished in several countries, specially for their particular flavor. Manufacture of these products is limited to small family-type farmhouses. Protein, fat and calorie contents make goat cheese a highly nutritious product (Spuergin et al., 1997; Alférez et al., 2001; Barrionuevo et al., 2002). Dairy products account for 2-6% of foodborne outbreaks. However, the lack of standardized protocols hampers their proper monitoring and documentation in different countries (De Buyser et al., 2001). Poor hygienic practices along the cheese manufacturing process represent a relevant concern for Public Health, mainly in developing countries (De Buyser et al., 2001; Espié et al., 2006; Mercado 2007).There is an increased interest related to poor sanitary practices in food preparation and its relationship with the production of food-borne outbreaks. One of the main causes of food poisoning worldwide is *Staphylococcus aureus* (Dinges et al., 2000).

Vasek et al. (2004) assessed the microbiological safety of craft cheeses produced in Corrientes (Argentina) as well as the consumption-related risk on human health. The authors found that 98% of the studied cheeses were not microbiologically safe (ReglamentoTécnico General de Mercosur, GMC Nº69/93). In the South East of the Province of Buenos Aires (Argentina) many farms, among other activities, have developed the manufacture of crafted goat cheese (Gutman et al., 2004)

Enterocins (bacteriocins produced by enterococci) are small, ribosomally synthesized, extracellular released, antibacterial peptides secreted by different strains of *Enterococcus* spp. These peptides show inhibitory activity against other Gram-positive bacteria, food-borne pathogens, and spoilage bacteria (Leroy et al., 2003; Sparo et al., 2009a). Enterocins, usually belong to class II bacteriocins of lactic acid bacteria (LAB) and they

are heat-stable small molecules. They are insensitive to rennet, showing stability over a wide range of pH values, and present a general compatibility with starter LAB species (Ennahar et al., 2001; Giraffa, 2003; Sparo et al., 2008). Enterocins with inhibitory activity against food-borne pathogens (especially *Listeria monocytogenes, S.aureus* and other enterococcal strains) have been characterized (Aymerich et al., 1996; Casaus et al., 1997; Cintas et al., 1997; Floriano et al., 1998). A new enterocin, peptide AP-CECT7121, has recently been reported by Sparo et al. (2009a,b). This peptide is synthesized by an environmental probiotic strain, *Enterococcus faecalis* CECT7121. It is characterized as a low molecular weight and hydrophobic peptide, which is stable at pH values from 4 to 8 (Basualdo et al., 2007; Castro et al., 2007; Castro et al., 2008; Sparo et al., 2008; Sparo et al., 2009a, b).

The aim of this study was to assess the biopreservative activity of *Enterococcus faecalis* CECT7121 on crafted goat cheese.

MATERIALS & METHODS

The study was performed on a small farm located in the South East of the Province of Buenos Aires (Tandil), Argentina. Whole process was carried out by farm workers. The protocol used for regular production was followed. After completing the study, cheese lots (with and without probiotic) were destroyed.

Probiotic strain, *E. faecalis* CECT7121

The probiotic strain *E. faecalis* CECT7121 used has been deposited at the Colección Española de cultivosTipo (CECT), Burjasot, Valencia, Spain, with European patent EP07356012.0 and United States Patent 7,927,584 B2. This strain produces an enterocin with a broad inhibitory spectrum over Gram positive bacteria (*Bacillus spp., Clostridium spp., Listeria spp., Staphylococcus spp., Streptococcus spp.,* other enterococcal strains) and some strains of Gram negative bacteria such as *E. coli* and *Shigella spp. E. faecalis* CECT7121 was kept at -70°C in brain heart infusion (BHI) broth plus 20% v/v glycerol. It was utilized after three subsequent overnight passages in BHI agar. Characterization of this strain was done by phenotypic and molecular methods as described below.

Cheese manufacturing process and experimental design

Milk: Twenty liters of goat milk were collected in plastic sterile bottles from the goat milking parlor. Animals were Brucellosis-free and clinically healthy.

Cheese manufacturing: A milk sample was warmed at 37 °C for 30 min and supplemented with $CaCl_2$ (0.02%). Resulting milk mixture was divided in two groups: Group A acted as control and it was not inoculated with the probiotic strain. Group B was inoculated with *E. faecalis* CECT7121, which was diluted in goat milk at a final concentration of 5.0×10^4 CFU/mL.

After incubating for 20 minutes, 0.3 g. L^{-1} of rennet of animal source (clotting activity of 97.54 Rennet Units/ml^{-1}) were added to each group. Once the clotted milk was obtained, curds were separated from the whey using a "cheesecloth" and rinsed under tap water at 40° C. Then curds were placed in rounded molds and pressed for 4 h at 20 °C. After this period, cheeses were removed from the molds and submerged into brine for 3.5 h. Once removed from the brine cheese went through ripening for 35 days at 12 °C and 85% humidity. Eight cheeses per group with an average weight of 143 g each were obtained.

Sampling and sample processing: All the samples were collected from the middle of the cheese at 0, and 35 days (onset, and end of ripening). Samples were immediately processed as described below.

Microbiological Analysis: Samples from both cheese groups (A and B), for assessment of microbiota evolution along ripening, were obtained. Viable cell counts of *Enterobacteriaceae, Lactobacillus* spp., *Lactococcus* spp., *S.aureus* and *Enterococcus* spp. were carried out. Presence of *E. faecalis* CECT7121 in group B from t=0 up to 35 days after inoculation was investigated.

Before sampling, cheeses were washed with water, and their rinds were removed. Ten grams samples from each cheese at Time zero (T=0) and 35 days of ripening were collected. Cheese samples (10 g) were homogenized for 2 min in a Stomacher 400 (Lab. System, UK) with 90 mL of sterile 2% sodium citrate solution pre-warmed at 37 °C (Vanderzant and Splittstoesser, 1992; International Dairy Federation, 1995.) One milliliter of cheese homogenate was collected and 10-fold serial dilutions were prepared using sterile 0.8% NaCl. Each 100 µL aliquot was inoculated by triplicate on agar plates for bacterial counts.

Viability of *Lactobacillus* spp. along ripening was determined in Lactobacillus Selection Agar (LBS agar; BBL™, USA) incubating in anaerobic atmosphere at 30°C for 5 days. Afterwards, Gram staining and catalase reaction were performed.

Viable cell count of *Lactococcuss* pp. was performed after incubation in LM17 agar (Oxoid, UK) at 30 °C for 3 days. Gram staining and catalase reaction were carried out. *Enterobacteriaceae* count was undertaken using crystal violet-neutral red-bile-glucose agar (VRBG, Lab. Britania, Argentina) cultured at 35 °C for 24 h. Ten colonies were picked up from the appropriate agar count dilution at each time and Gram stain and cytochrome oxidase confirmatory tests for Enterobacteriaceaewere done. The presence of *E. coli* was determined using fermentation of lactose, indole production, citrate utilization and decarboxylation of lysine (Sparo and Mallo, 2001; Sparo et al., 2008).

Viable counts of *S. aureus* were performed in Baird Parker agar (Lab. Britania, Argentina) incubating at 35 °C for 48 h. For species-level confirmation, manitol fermentation,

coagulase and DNase production were carried out (Collins et al., 1991; Vanderzant and Splittstoesser, 1992). Viable counts of *Enterococcus* spp. were assessed in Kenner fecal agar (KFA; Difco, USA) after incubation at 35 ºC for 2 days. Phenotypic characterization of *E. faecalis* in cheese samples was performedaccording Sparo and Mallo (2001) and Sparo et al. (2008). Briefly, *Enterococcus* isolates were confirmed by: catalase production (hydrogen peroxide 3%), hydrolysis of pyrrolydonil-beta-naphtilamide (PYR-A-ENT, Lab. Britania, Argentina), esculin hydrolysis in presence of 40% bile (bile-esculin agar, Difco), leucinearylamidase activity (LAP, Lab. Britania, Argentina), hemolysis in Columbia base agar (Oxoid) containing 5% (v/v) sheep blood, resistance to vancomycin (30 µg), acetoine production, motility, pigment production, pyruvate utilization, tolerance to **0.04**% potassium telurite, arginine hydrolysis, acidification of 1% methyl-alpha-**d**-glucopyranoside (Sigma Co., USA). Fermentation studies: glucose, melibiose, adonitol, D-rafinose, lactose, melezitose, ramnose, mannitol, sorbitol, L-arabinose, L-sorbose, trehalose, D-xylose, ribose, sacarose and D-arabitol (Casaus et al. 1997; Cintas et al., 1997). The strains were maintained in brain heart infusion (BHI) broth with glycerol 20% (v/v) at – 70º C.

SDS-PAGE studies

As an additional test for phenotypic characterizationof *E. faecalis* CECT7121, Whole Cell Protein (WCP) profiles was performed according to Merquior et al., 1994; modified by Sparo et al., 2008. Briefly, enterococcal test isolates were grown for 18 hours at 35° C on trypticase soy agar with 5% sheep blood. Samples were prepared by collecting bacteria grown on the surface of the agar plate using a sterile disposable loop. Then, they were inoculated in 5 mL of sterile saline solution until reaching an equal turbidity to Nº 8 of McFarland´s scale tube. Samples were centrifuged, and re-suspended in 0.5 mL of an aqueous lysozyme solution (10 mg. mL^{-1}). Suspensions were incubated in a water bath at 37°C for 2 h. WCP extracts were obtained mixing one part of WCP with one part of sample loading buffer, then the mixture was boiled for 5 min and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. For molecular weight estimation of electrophoretic bands, a molecular weight marker was added (Prestained SDS-PAGE Standard, Bio-Rad, California, USA).

SDS-PAGE was performed applying samples to wells in a 5% stacking gel over a 10% separating gel, at a constant current of 20 mA, in a mini-gel electrophoresis system (Bio-Rad Mini Protean II, Richmond, California, USA) and stained with Coomassie brilliant blue. Profiles of enterococcal strains isolated from cheese were compared with the profiles of reference strain E. faecalis ATCC 29212 and E. faecalis CECT7121. Densitometric analysis using Image Pro and Origin 6.0 software (Germany) was carried out. Homology percentage was assessed by the Dice's coefficient (Dice, 1945).

Pulsed-field gel electrophoresis

Bacterial isolates were grown overnight in BHI broth. Chromosomal DNA was prepared in agarose plugs and endonuclease digestion with *SmaI* (New England Biolabs, Beverly, MA, US) was performed, as previously described (Chung et al., 2000). DNA fragments were separated in 0.8% agarose using a CHEF-DRIII apparatus (Bio-Rad Laboratories, Richmond, CA,US), run at 6 $V.cm^{-1}$ for 26 h at 7°C, with intermittent pulses ranging from 5 to 35 secs (Corso et al., 2007). A molecular size standard, Lambda ladder (New England Biolabs), and a reference strain, *S. aureus* NCTC8325, were used. Gels were stained with 1 $\mu g.mL^{-1}$ of ethidium bromide and photographed under UV illumination. Similarity between isolates was determined by visual comparison of isolate banding patterns. Interpretation of the band patterns was carried out according to previously published guidelines (Tenover et al., 1995). If the pulsed-field gel electrophoresis (PFGE) patterns differed by more than 6 bands, the bacterial isolate was defined as a different strain type, or unrelated to the others. When strains differed by 2 - 6 bands, isolates were considered as subtypes, possibly related among them. Isolates whose restriction patterns had the same number and band size were considered genetically indistinguishable and were then assigned to the same strain type and subtype.

pH assessment

The pH values were assessed inserting a special pH meter probe (Crison Instruments S.A., Spain). Duplicate cheese samples from both groups (A and B) were evaluated. Samples were initially taken from the raw milk and over the cheese ripening period, at 16 days and 35 days.

Sensory analysis

A group of 13 volunteers adults (belonging to the University), trained in the methodology of work and very frequent consumers of this type of cheese, performed the evaluation on cheeses samples from groups A (control) and B (inoculated with *E. faecalis* CECT7121 strain). Organoleptic features such as flavour, acidity, texture and general acceptance were evaluated using a scoring scale from 1 to 10 (FIL-IDF 99C:1997). For each sample, a cheese slice was placed in a dish and equilibrated at room temperature before serving.

Statistical analysis

A two way ANOVA followed by Simple Effects tests were performed to evaluate microbiological analysis and the pH value differences along sampling times and between the two kinds of cheese (with and without inoculation of *E. faecalis* CECT7121 (Winer, 1991). McNemar paired-sample test was used to evaluate sensorial analysis data (Zar, 1999). Software used: SPSS 12.0 (Chicago, Ill), Excel XP (Microsoft Corporation) and InfoStat (2006 Version). A probability less than $p<0.05$ was considered significant.

RESULTS

Inhibitory activity on indigenous microbiota

The initial *Lactobacillus* spp. viable counts obtained in

control (group A: 6.23 ± 0.78 log CFU.g^{-1}) and treated with probiotic strain (group B: 6.52± 0.62 log CFU.g^{-1}) were similar to those ones at the end of ripening process (group A: 7.26± 0.64 log CFU.g^{-1}; group B: 7.64± 0.58 log CFU.g^{-1}). A similar trend in growth pattern over time followed the values obtained for *Lactococcus* spp.in both, control and experimental probiotic inoculated, groups. Therefore *Lactobacillus* spp. and *Lactococcus* spp. viable counts were not affected by the presence of E. faecalis CECT7121 (Table 1).

TABLE 1. Mean ± Standard Deviation of crafted goat cheese microbiota evolution (log CFU g $^{-1}$) at the beginning and end of the maturation process. ND: not detectable; Group A: control; Group B: treated with *E. faecalis* CECT7121. The results represent the average of determinations for three independent experiments. (*)Values are statistically different to the control group and between 0 and 35 days at $p< 0.05$

MICROBIOTA	DAY 0	DAY 35
***Lactobacillus* spp.**		
Control	6.23 ± 0.78	7.26± 0.64
+ *E. faecalis* CECT7121	6.52 ± 0.62	7.64 ± 0.58
***Lactococcus* spp.**		
Control	7.19 ± 0.57	8.37 ± 0.75
+ *E. faecalis* CECT7121	7.38 ± 0.61	8.21 ± 0.69
***Enterococcus* spp.**		
Control	3.47 ± 0.53	6.82 ± 0.63*
+ *E. faecalis* CECT7121	7.51 ± 0.61	4.36 ± 0.56 *
S. aureus		
Control	3.45 ± 0.54	6.14 ± 0.54*
+ *E. faecalis* CECT7121	3,52 ± 0.51	ND *
Enterobacteriaceae		
Control	4.79 ± 0.68	6.78 ± 0.73
+ *E. faecalis* CECT7121	4.86 ± 0.72	ND *

The initial viable counts obtained for *Enterococcus* spp. in group A (control) achieved a concentration of 3.47 ± 0.53 log CFU.g^{-1}. Higher levels of *Enterococcus* were initially isolated from group B (sum of probiotic + indigenous enterococci), however a significant ($p<0.05$) decrease to 4.36 ± 0.56 log CFU.g^{-1} viable counts were found at 35 days post-probiotic inoculation (Table 1).

S. aureus initial viable counts at t=0 were similar in both series (group A: 3.45 ± 0.54 log CFU.g^{-1}, group B: 3.52 ± 0.51 log CFU.g^{-1}); although at 35 days of ripening viable cells were not detected in group B, where *E. faecalis* CECT7121 strain was added. In the control group the levels of viable counts reached 6.14 ± 0.54 log CFU.g^{-1}.

Enterobacteriaceae population obtained in both series, control and inoculated with the probiotic strain, at the onset of the experiment (time 0), resulted in a viable count of 4.79±0.68 log CFU.g^{-1} and 4.86 ± 0.72 log CFU.g^{-1}; achieving no detectable levels at 35 days in those cheeses inoculated with *E. faecalis* CECT7121. However, the concentrations obtained for the control group increased up to 6.78 ± 0.73 log CFU.g^{-1} at time 35 days. The predominant species identified was *E. coli*.

SDS-PAGE and PFGE studies

At the end of ripening, all colonies characterized as *E. faecalis* by PFGE (from samples of group B), showed an identical profile to the obtained by the probiotic strain (*E. faecalis* CECT7121). These results indicate that the probiotic strain was viable up to 35 days in cheese (end of ripening). This pattern was not related to those observed by different *E. faecalisstrains* isolated from cheese, which presented high clonal diversity, 8 clonal types or 12 clonal subtypes (Fig, 1, lanes 3 to 13, 15 and 16). Band pattern of *E. faecalis* CECT7121 in SDS-PAGE correlated with those obtained by PFGE at the onset and the end of cheese ripening.

pH assessment

Along cheese ripening, evolution of pH values showed no statistical differences ($p<0.05$) when control (A, without probiotic) and experimental group (B, probiotic treated) were compared. Values: Cheese batches control at 16 days (5.58 ± 0.07) vs. Cheese batches + *E. faecalis* CECT7121 at 16 days (5.58 ± 0.11), Cheese batches control at 35 days (5.58 ± 0.09) vs. Cheese batches+*E. faecalis* CECT7121 at 35 days (5.59 ± 0.13).

Sensory evaluation

Average ± SD results obtained from 13 tasters evaluating organoleptic characteristics of the goat-cheeses with or without probiotic, using the 1-10 score scale, indicated a non significant statistical difference, although a positive trend was observed in favor of the cheese probiotic addition.

DISCUSSION

Use of raw milk and failures in manufacture, transportation and storage, allow to understand why these products are hazardous to consumers' health and are involved in foodborne illness-outbreaks in the Province of Buenos Aires, Argentina (Manfredi et al., 2010). This investigation showed a significant ($p<0.05$) initial levels of *Enterobacteriaceae* (10^4-10^5 CFU. g^{-1}), with E. coli as predominant species, and *S. aureus* (10^3-10^4 CFU.g^{-1}) population, although a high viable cells count at the end of the experiment (T=35 days) was only seen in the group A without treatment with *E. faecalis* CECT7121 strain.

The broad inhibitory spectrum of this probiotic strain on Gram positive and Gram-negative bacteria offers advantages for the preservation of fermented products, where no inhibitory activity against *Lactobacillus* spp. and *Lactococcus* spp. was observed.

González et al. (2007) when studied the Genestoso cheese, produced on a purely craft basis, recognized

that the main problem was the difficulty of ensuring an adequate level of hygiene. In the same report, the authors found antimicrobial activity in twenty four strains of lactic acid bacteria (*Lactobacillus, Lactococcus, Leuconostoc* and *Enterococcus*) isolated from Genestoso cheese. Interestingly, the inhibitory activity of E. faecalis CECT7121 used in this assay correlates with the spectrum of the antimicrobial peptide AP-CECT7121, secreted by this strain (Sparo et al., 2009a; 2009b).

Lactic acid bacteria (*Lactobacillus* spp., *Lactococcusspp.*) are responsible for organoleptic characteristics of cheese (Sánchez et al., 2006). The viable counts obtained for *Lactobacillus* and *Lactococcus* from craft cheese made with raw goat milk without starter cultures ranged 10^8-10^9 CFU.g^{-1}. These values correlate with those ones obtained by Fox et al. (2000).

At the end of the experiment, a group of 13 volunteers tested the organoleptic characteristics of the cheeses inoculated with *E. faecalis* CECT7121 in a blind fashion and a positive acceptance trend of the product was observed. These results are in agreement with those of a previous study with fermented craft sausages, where it was reported that the taste of the fermented food products, with addition of E. faecalis CECT7121, was not adversely modified (Sparo et al., 2008). Enterococccal counts between <10^1 CFU. mL^{-1} and 10^4 CFU. mL^{-1} in raw cow's milk from New Zeeland were observed (Hill et al, 1997). In European raw milk, cell counts between 10^3 CFU. mL^{-1} and 10^5 CFU. mL^{-1} or more were reported, without any species being markedly represented (Pérez et al, 1982).

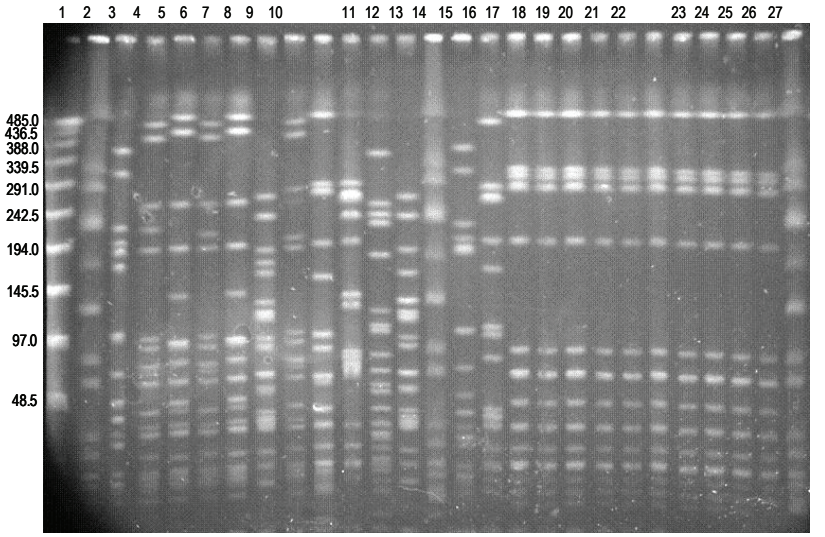
On the experimental group inoculated with *E. faecalis* CECT7121, a 3 log viable count decrease of enterococci was achieved at 35 days post-inoculum ($p<0.05$). These results supported evidence for the bactericidal activity of the probiotic against other enterococcal strains. This inhibitory activity was observed as well in craft-dry fermented sausages inoculated with *E. faecalis* CECT7121 (Sparo et al., 2008). Enterococci are prevalent among the species found in the raw milk microbiota. Therefore, there are not available standardized minimum and maximum viable count levels for this species since they are not normally counted in microbiological analyses.

TABLE 2. Average ± SD results obtained from 13 tasters evaluating the organoleptic characteristics of the goat-cheeses treated either without or with probiotic using the 1-10 score scale. No statistical differences were obtained between groups

ORGANOLEPTIC FEATURE	Without E. Faecalis CECT7121	With *E. Faecalis* CECT7121
Flavour	5.93 ± 1.00	7.06 ± 1.30
Acidity	6.14 ± 0.50	6.41 ± 0.70
Texture	6.53 ± 0.80	7.26 ± 0.70
Acceptance	7.89 ± 0.3	8.67 ± 0.5

The final concentration of *S.aureus* population (10^6 CFU.g^{-1}) detected in the control group in this study, would allow to achieve enterotoxin levels hazardous for human health. Interestingly, *S. aureus* was the most sensitive bacteria to the bactericidal activity of *E. faecalis* CECT7121, since at the end of the experiment (T=35 days) no viable *S. aureus* were detected. These findings correlate with previous *in vitro* studies that have shown the bactericidal activity of the peptide AP-CECT7121 against human multi resistant *S. aureus* strains as well as other etiological agents of bovine mastitis (Sparo et al., 2009a,b). These results have encouraged the study of *E. faecalis* CECT7121 as a biological preservative (biopreservative) in craft goat cheese. In addition, when the probiotic strain was inoculated undesired changes on the organoleptic characteristics or pH values were not observed (Table 2).

FIGURE 1. PFGE banding patterns of SmaI-digested chromosomal DNAs of *E. faecalis* strains. Lane 1 lambda ladder; lanes3 to 7 *E. faecalis* isolated at T=0; lanes 2, 14 and 27 reference strain S. aureus NCTC 8325; lanes 8 to 13, 15 and 16 *E. faecalis* strains isolated from other artisanal cheeses; lane 17 *E. faecalis* CECT7121, lanes 18 and 19 strains isolated from the inoculated cheese at T=0, lanes 20 to 26 strains isolated from the inoculated cheese at 35 days. The size of the DNA fragments (Kb), are shown to the left of the picture.



In a previous study, the usefulness of PFGE for identifying different strains of vancomycin-resistant enterococci isolated from human clinic samples was proved (Corso et al., 2007). PFGE is a sensitive and reproducible method for the genetic identification of E. faecalis CECT7121 in crafted goat cheese, since E. faecalis CECT7121 showed a different electrophoretic pattern than other enterococcal strains (Fig.1). This

methodology identified unrelated enterococcal clones along the ripening. In this context, we may assert that the probiotic was effectively implanted in the food product. The presence of *Enterobacteriaceae*, *S. aureus* and other enterococci in cheese, seems to vary depending upon the type of cheese, and the use of raw milk (Coppola et al., 2000; Marino et al., 2003). The obtained results in this study have proven that goat artisanal cheese is a suitable environment for growth of the probiotic strain *E. faecalis* CECT7121.

Finally, the present study demonstrated that food biopreservation with *E. faecalis* CECT7121 is a viable alternative to conventional chemical and physical methods. Inhibitory activity of *E. faecalis* CECT7121 against food contaminants and food-borne pathogens turn this strain or its enterocin AP-CECT7121 into interesting natural food additives for safe management and preservation of craft goat cheese.

REFERENCES

Alférez, M.J., Barrionuevo, M., López-Aliaga, I., Sanz-Sampelayo, M.R., Lisbona, F., Robles, J.C., Campos, M.S. (2001). Digestive utilization of goat and cow milk fat in malabsorption syndrome. *Journal of Dairy Research* **68**: 451-461.

Aymerich, T., Holo, H., Havarstein, L.S., Hugas, M., Garriga, M., Nes, I.F. (1996). Biochemical and genetic characterization of enterocin A from *Enterococcus faecium,* a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Applied and Environmental Microbiology* **62**:1676-1682.

Barbaros, H.O. (2000). Encyclopedia of Food Microbiology (Oxford, UK: Academic Press).

Barrionuevo, M., Alférez, M.J.M., López-Aliaga, I., Sanz-Sampelayo, M.R., Campos, M.S. (2002). Beneficial effect of goat milk on nutritive utilization of iron and copper in malabsorption syndrome. *Journal of Dairy Science* **85**:657-664.

Basualdo, J.A., Sparo, M., Chiodo, P., Ciarmela, M., Minvielle, M. (2007). Oral treatment with a potential probiotic (*Enterococcus faecalis* CECT 7121) appears to reduce the parasite burden of mice infected with Toxocaracanis. *Annals of Tropical Medicine and Parasitology* **101**:559-562.

Casaus, P., Nilsen, T., Cintas, L.M., Nes, I.F., Hernandez, P.E., Holo, H. (1997). Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. *Microbiology* **143**:2287-2294.

Castro, M., Sparo, M., Molina, M., Andino, J., Manghi, M. (2007). *Enterococcus faecalis* CECT7121 induces systemic immunomodulatory effects and protects from *Salmonella* infection. *International Journal of Probiotics and Prebiotics* **2**:215-224.

Castro, M., Molina, M., Sparo, M., Manghi, M. (2008). Effects of *Enterococcus faecalis*CECT7121 on the specific immune response after DTPw vaccination. *International Journal of Probiotics and Prebiotics* **3**: 25-30.

Chung, M., de Lencastre, H., Matthews, P., Tomasz, A., Multilaboratory Project Collaborators: Adamsson, I., Aires de Sousa, M., Camou, T., Cocuzza, C., Corso, A., Couto, I., Dominguez, A., Gniadkowsky, M., Goering, R., Gomes, A., Hortal, M., Kikuchii, K., Marchese, A., Mato, R., Melter, O., Oliveira, D., Palacio, R., Sá-Leão, R., Santos Sánchez I., Song, J., Tassios, P., Villari, P. (2000). Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. *Microbial Drug Resistance* (Larchmont, N.Y.) 6: 189-198.

Cintas, L.M., Casaus, P., Havarstein, L., Hernandez, P.E., Nes, I.F. (1997). Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from Enterococcus faecium P13 with a broad antimicrobial spectrum. *Applied and Environmental Microbiology* **63**: 4321-4330.

Collins, C.H., Lyne, P.M., Grange, J.M. (1991). Microbiological Methods, 6th ed. (London, UK: Butterworths-Heinemann).

Coppola, R., Nanni, M., Succi, M., Sorrentino, A., Iorizzo, M., Chiavari, C., Grazia, L. (2000). Microbiological characteristics of Parmigiano-Reggiano cheese during the cheese making and the first months of the maturation. *Lait.* **80**:479-490.

Corso, A., Gagetti, P., Rodríguez, M., Melano, R., Ceriana, P., Faccone, D., Galas, M.F. (2007). VRE Argentinian Collaborative Group. Molecular epidemiology of vancomycin-resistant *Enterococcus faecium* in Argentina. *International Journal of Infectious Diseases* **11**: 69-75.

De Buyser, M.L., Dufour, B., Maire, M., Lafarge, V. (2001). Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *International Journal of Food Microbiology* **67**: 1-17.

Dice, L.R. (1945).Measures of amounts of ecologic association between species. *Ecology* **26:** 297-302.

Dinges, M., Orwin, P., Schlievert, P. (2000). Exotoxins of Staphylococcus aureus. *Clinical Microbiology Reviews* **13**:16-34.

Ennahar, S., Asou, Y., Zendo, T., Sonomoto, K., Ishizaki, A. (2001). Biochemical and genetic evidence for production of enterocins A and B by Enterococcus faecium WHE 81.

International Journal of Food Microbiology **70**: 291- 301.

Espié E. Vaillant V. Mariani-Kurkdjian P. Grimont F. Martin-Schaller R. De Valk H. Vernozy-Rozand C (2006). *Escherichia coli* O157 outbreak associated with fresh unpasteurized goats` cheese. *Epidemiology and Infection* **134**:143–146.

Floriano, B., Ruiz-Barba, J.L., Jimnez-Diaz, R. (1998). Purification and genetic characterization of enterocin I from *Enterococcus faecium* 6T1a, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocins.*Applied and Environmental Microbiology* **64:** 4883 - 4890.

Fox, P.F., Guinee, T.P., Cogan, T.M., Mc Sweeney, P.L.H. (2000). Cheese: Historical Aspects. In: Fundamentals of Cheese Science. AN Aspen Publication.

Giraffa, G. (2003). Functionality of enterococci in dairy products. *International Journal of Food Microbiology* **88**:215-222.

González, L., Sandoval, H., Sacristán, N., Castro, J.M., Fresno, J.M., Tornadijo, M.E. (2007). Identification of lactic acid bacteria isolated from Genestoso cheese throughout ripening and study of their antimicrobial activity. *Food Control*. **18**:716-722.

Gutman, G., Iturregui, M.E., Filadoro, A. (2004). Propuestas para la formulación de políticas para el desarrollo de tramas productivas regionales. El caso de la lechería caprina en Argentina. Oficina de la Comisión Económica para América Latina y el Caribe CEPAL - SERIE Estudios y perspectivas – Oficina de la CEPAL en Buenos Aires N° 21: 7-47. NacionesUnidas.

Hill, B.M., Smythe, B.W. (1997). A study of the microbiological quality of raw milk from individual farms. New Zealand Dairy Research Institute. 1-35.

International Dairy Federation.Milk and milk products -detection of *Listeria monocytogenes.* International IDF Standard No. 143A.International Dairy Federation, Brussels, Belgium. 1995.

International Standard FIL-IDF. International Dairy Federation, Brussels, Belgium.(1997). 99C. Sensory evaluation of dairy products by scoring.

Leroy, F., Foulquie Moreno, M.R., De Vuyst, L. (2003). *Enterococcus faecium* RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. *International Journal of Food Microbiology* **88**:235-240.

Manfredi, E.A., Leotta, G.A., Rivas, M. (2010). PCR múltiple para la detección de los genes sea,seb, sec, sed y see de *Staphylococcus aureus.*Caracterización de aislamientos de origen alimentario. *Revista Argentina de Microbiología* **42**:212-215.

Marino, M., Maifreni, M., Rondinini, G. (2003). Microbiological characterization of artisanal Montasio cheese: analysis of its indigenous lactic acid bacteria. *FEMS Microbiology Letters* **229**:133-140.

Mercado, C. (2007). Los ámbitos normativos, la gestión de la calidad y la inocuidad alimentaria: una visión integral. *Agroalimentaria* **24**:119-131.

Merquior, V.L.C., Peralta, J.M., Facklam, R.R., Teixeira, L.M. (1994). Analysis of electrophoretic whole-cell protein profiles as a tool for characterization of *Enterococcus species*. *Current Microbiology* **28**:149 - 153.

Pérez, B.S., Lorenzo, P.L., Garcia, M.L., Hernandez, P.E., Ordonez, J.A. (1982). Heat-resistance of Enterococci. *Milchwissenschaft*. **37**:724-726.

Sánchez, I., Seseña, S., Poveda, J.M., Cabezas, L., Palop, L. (2006). Genetic diversity, dynamics, and activity of *Lactobacillus* community involved in traditional processing of artisanal Manchego cheese. *International Journal of Food Microbiology*. **107**:265-273.

Sparo, M., Mallo, R. (2001). Evaluation of the bacterial flora in natural corn silage. *Revista Argentina de Microbiología* **33**:75-80.

Sparo, M., Nuñez, G.G., Castro, M., Calcagno, M.L., García Allende, M.A., Ceci, M., Najle, R., Manghi, M. (2008). Characteristics of an environmental strain, *Enterococcus faecalis* CECT7121 and its effects as additive on craft dry-fermented sausages. *Food Microbiology* **25**: 607- 615.

Sparo, M.D., Jones, D.G., Sánchez Bruni, S.F. (2009a). In vitro efficacy of the novel peptide CECT7121 against bacteria isolated from mastitic dairy cattle. *Letters in Applied Microbiology* **48**:187-192.

Sparo, M.D., Jones, D.G., Sánchez Bruni, S.F. (2009b). Assessment of the in vitro efficacy of the novel antimicrobial peptide CECT7121 against human Gram-positive bacteria from serious infections refractory to treatment.*Chemotherapy* **55**:270-277.

Spuergin, P., Walter, M., Schiltz, E., Deichman, K., Forster, J., Mueller, H. (1997). Allergenicity of a-caseins from cow, sheep, and goat. *Allergy* **52**:293-298.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., Swaminathan, B. (1995).

Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* **33**: 2233-2239.

Vanderzant, C., Splittstoesser, D.F. (1992). Compendium of methods for the microbiological examination of food, 3rd edn. American Public Health Association, Washington, D.C.

Vasek, O., Cabrera, R., Coronel, G., de Giori, G., Fusco, A. (2004). Análisis de riesgos en la elaboración de queso artesanal de Corrientes (Argentina). *Facena.* **20**:13-22.

Winer, B.J., Brown, D.R., Michels, K.M. (1991). Statistical Principles in Experimental Design, 3rd ed. (New York, New York: McGraw-Hill, Inc.).

Zar, J.H. (1999). Biostatistical Analysis, 4th edn. (Upper Saddle River, New Jersey: Prentice Hall, Inc.).

Zhao, Y. (2001). Development and delivery of risk reduction strategies for New England cheese manufacturers. Durham, New Hampshire: New England Cooperative Extension Consortium. http://www.umass.edu/umext/consortium/cheese.htm