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Characterization of lactic acid bacteria in Pecorino di Farindola cheese and manufacturing with a *Lacticaseibacillus paracasei* autochthonous culture

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Abstract: This study focused on the characterization of lactic acid bacteria (LAB) in a renowned traditional Italian cheese, Pecorino di Farindola, to select an autochthonous culture and investigate its potential for the improvement of safety and functional properties.

Two hundred and six LAB isolated throughout production and maturation from nine cheese lots of three farms were identified by 16S rRNA gene sequencing and tested for the presence of genes encoding virulence factors, vancomycin resistance (for enterococci), biogenic amines (BAs) and bacteriocin production, for antimicrobial activity, and for the capacity to survive in the gastrointestinal tract (GIT) based on tolerance to low pH and bile salts and adhesion to CaCo-2 cells.

A *Lacticaseibacillus paracasei* isolate was used in cheese making and determined a decline of spiked *Listeria monocytogenes* and *Escherichia coli* O157 faster than in the control cheese. The autochthonous bacterial groups were numerically unaffected, apart from lactobacilli that were recovered in higher numbers in the cheese with addition of the *L. paracasei* strain. Based on Repetitive Extragenic Palyndrome (Rep) polymerase chain reaction (PCR) profiles, the added strain possibly dominated until day 88 in cheese.

Results encourage further trials with autochthonous cultures to efficiently inhibit hazardous bacteria and enrich a functional microbiota in Pecorino di Farindola and similar cheeses.

Keywords: Pecorino di Farindola cheese, lactic acid bacteria (LAB), molecular characterization, *Lacticaseibacillus paracasei* selected culture, cheese making trial

1. Introduction

Traditional cheeses are a food category with economical relevance for defined geographical areas and are appreciated by consumers for their peculiar organoleptic features and genuineness [1]. Most of these products in Italy are obtained from raw milk, added or not with natural whey cultures, following European Union (EU) guidelines to ensure safety [2]. Pecorino di Farindola cheese is an example of dairy product from this category, and it is included in the list of the Italian traditional cheeses held by the Italian Ministry of Agriculture [3]. It is prepared, curiously only by women, from raw, not refrigerated ewe's milk in nine towns of a mountainous district of

Abruzzo region. Animals providing milk are fed exclusively by grazing, cereals and fava beans cultivated locally. The product has an ageing time from three to five months [4].

Previous studies have put in evidence the presence in this cheese of high amounts of biogenic amines (BAs), mainly tyramine, whose formation was primarily attributed to the amino acid decarboxylase activity of enterococci and *Enterobacteriaceae*. However, a correlation between the composition of cultivable microbial groups and BA content could not be established [5]. The high BA content in this cheese is favoured by the use of pig rennet that is unique for its production process [6].

In this and other similar products, the antagonistic actions exerted by naturally occurring lactic acid bacteria (LAB) have a preeminent role in preventing the growth of undesired and pathogenic microorganisms. However, variability in the composition of the indigenous microbiota can cause instability in the safety promoting activities of LAB. The use of added cultures selected among autochthonous LAB could improve safety without modifying the typical manufacturing process and sensory characteristics [7].

Therefore, in this study, the LAB microbiota of Pecorino di Farindola cheese was characterized in order to select strains devoid of hazardous traits, able to persist during ageing and to antagonize pathogens, to be used in cheese making trials. Conventional bacterial counts were used to provide a quantitative estimate of cultivable bacteria that predominated, mostly impacted biochemically changes in the cheese during maturation and were ingested with the product. In addition, this allowed to isolate LAB strains for further characterization. LAB isolated at the end of cheese maturation were also assessed for the prerequisites for survival in the gastrointestinal tract (GIT). In fact, traditional cheese is recognized as a good source of LAB able to colonize GIT that could confer functional properties to the product if able to exert probiotic actions [8].

LAB isolates, including enterococci, were obtained during cheese maturation from nine lots manufactured by three producers. These were screened by polymerase chain reaction (PCR) for genes encoding the production of nisin and other lantibiotics, as a bacteriocin class with broad inhibitory spectrum and therapeutic potential [8], enterocins, enterococcal virulence factors and vancomycin resistance genes and BA production. Moreover, isolates from cheeses at the end of ripening, that are those ingested by consumers, were evaluated for tolerance to low pH and bile salts, adhesion to intestinal CaCo-2 cells, and production of antibacterial substances. Among these, a *Lacticaseibacillus paracasei* isolate was tolerant to low pH and bile salts and was able to inhibit the growth of *Listeria monocytogenes in vitro*. This was used as added culture in a cheese making trial. In particular, the abilities of this autochthonous *L. paracasei* isolate to inhibit *L. monocytogenes* and *Escherichia coli* O157, two pathogens of major concern in this type of products [10,11], and to dominate during cheese maturation were evaluated.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Enterococcus faecalis ATCC 47077, NCTC 13379 (*van*B+), EFS2 ATCC 202014, ATCC 29212, *E. faecium* ATCC 700221, ATCC 19434, ATCC 51559 (*van*A+) and C68 CRA-FLC, *E. gallinarum* ATCC 49573 (*van*C1+), *E. casseliflavus* ATCC 25788, *Lactococcus lactis* subsp. *lactis* NCTC 6681, ATCC 11454, *L. lactis* subsp. *cremoris* ATCC 19257, DSM 20069, *Lacticaseibacillus casei* ATCC 393, *L. paracasei* DSM 5622, *L. rhamnosus* NCTC 12953, *Lactiplantibacillus plantarum* ATCC 55883, *Lactobacillus delbrueckii* subsp. *lactis* NCTC 13361, *L. delbrueckii* subsp. *bulgaricus* DSM 2008, *Lactobacillus* spp. 30a ATCC 33222, *Levilactobacillus brevis* NCTC 13386 and ATCC 367, *Limosilactobacillus fermentum* ATCC 55884, *Listeria monocytogenes* ATCC7644 and EU-RL 12M0B098, *Escherichia coli* O157 VTEC ISS, ATCC 25952 and EURL C07, *Salmonella* Typhimurium ATCC 14028, and *Staphylococcus aureus* ATCC 33862 were used as positive control strains in specific PCR reactions, antimicrobial production tests and cheese making trial. All culture media were provided by Biolife Italiana, S.r.l. (Milan, Italy). Enterococci and rod shaped LAB were

propagated in De Man, Rogosa and Sharpe (MRS) broth (20.0 g/L glucose, 10.0 g/L peptone, 8.0 g/L meat extract, 4 g/L yeast extract, 3 g/L sodium acetate, 2 g/L triammonium citrate, 2 g/L K_2HPO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $MnSO_4$, 1 (mL) sorbitan monooleate, pH 6.5) incubated for 24 – 48 h at 37 °C in anaerobic jars containing Oxoid™ AnaeroGen™ sachets (Thermo Fisher Scientific, Monza, Italy). Lactococci were subcultured on M17 broth or agar in anaerobic conditions at 30 °C for 24–48 h. Coagulase positive cocci, *E. coli*, *L. monocytogenes* and *Salmonella* Typhimurium were propagated in Brain Heart Infusion (BHI) broth at 37 °C in aerobiosis for 24–48 h. Cryopreservation of cultures was done in Microbank tubes (Biolife Italiana) at -80 °C.

Sampling was carried out according to the ISO 707:2008 norm [12]. Samples were collected from nine batches of cheese produced in three dairy factories belonging to the producer association "Consorzio di Tutela del Pecorino di Farindola", and comprised milk, curd and cheese at days 7, 14, 21, 35, 49, 63, 77, 91, 121 and 150 of ripening during the period March – September 2018. Samples were transferred into sterile containers, in the manufacturing plant or in the ageing rooms, transported to the laboratory and stored in refrigeration conditions for a maximum of 48 h until analysis. Collected amounts were 1 L of milk, 500 g of curd and a whole piece of cheese. The a_w determination was carried out with the Aqualab 4TE device (Meter Group, Pullman, USA) according to the norm ISO 18787:2017 [13]. The determination of pH was carried out using a Seven Easy pH Meter (Mettler-Toledo S.P.A., Milan, Italy) on a suspension of 10 g of sample homogenized in 90 mL of deionized water for 1 min in a Stomacher 400 (Biosigma, Cona, VE, Italy).

LAB counts were carried out as described by Del Matto et al. [14]. Namely, LAB were counted and isolated on MRS agar medium, M17 agar medium (19.0 Di-sodium-glycerophosphate, 11.0 g/L agar, 5 g/L lactose, 5 g/L meat digest, 5 g/L tryptone, 5 g/L soya peptone, 2.5 g/L yeast extract, 0.5 g/L ascorbic acid, 0.25 g/L $MgSO_4$, pH 6.9) incubated at 37 °C for 72 h in anaerobic jars and Slanetz and Bartley (SB) medium (20.0 g/L tryptose, 13 g/L agar, 5 g/L yeast extract, 2.0 g/L glucose, 4.0 g/L K_2HPO_4, 0.4 g/L sodium azide, 0.2 g/L 2,3,5-Triphenyltetrazolium Chloride, pH 7.2), selective for enterococci, incubated at 37 °C for 24 h in aerobiosis. Presumptive LAB were tested for catalase activity by transferring an individual colony onto a glass slide and adding a drop of 3% (v/v) H_2O_2. Gram staining was also carried out.

Coagulase positive staphylococci were enumerated according to the standard procedure ISO 6888-2 [15]. Absence of *E. coli* O157 and *L. monocytogenes* in milk was tested according to UNI EN ISO 16654:2017 [16] and UNI EN ISO 11290-1:2017 [17], respectively. *E. coli* O157 and *L. monocytogenes* were enumerated according to the ISO 16649-1 [18] and UNI EN ISO 11290-2:2017 [19], respectively.

Tolerance to low pH was tested for strains of lactobacilli grown in MRS broth at 37 °C for 24 h. A 10 μL aliquot of culture was inoculated into 990 μL of phosphate-buffered saline (PBS) (9 g/L NaCl, 9 g/L $Na_2HPO_4.2H_2O$, 1.5 g/L KH_2PO_4) with pH adjusted to 2.5, 3.0 or 6.2 (control). Cell suspensions were incubated for 2 h at 37 °C. Counts were carried out on MRS agar before and after incubation and survival rate was expressed as percentage of Log CFU/ml at the tested pH compared to the initial count.

For the determination of tolerance to bile salts, strains were grown in MRS broth at 37 °C for 24 h and then inoculated at 2% (v/v) ratio into MRS broth containing 0.3%, 0.5% or 1% (w/v) bile salts (Ox-bile, Merck S.p.A., Serono, RM, Italy) and incubated at 37 °C. Optical density at 600 nm (OD_{600}) was measured after 24 h using a BioPhotometer D30 (Eppendorf S.r.l., Milan, Italy) and compared to a control culture grown in MRS broth without bile salts. The results were determined as percentage of OD_{600} in presence of bile salts compared to the control. All experiments were performed in triplicate.

The deferred antagonism test was used to evaluate the *in vitro* production of inhibitory compounds by LAB isolates against pathogenic bacteria. Briefly, 2 μL of a bacterial culture grown for 16–18 h at 37 °C were spotted onto the surface of BHI agar (BHI broth + Bacto agar 1.5% w/v), and incubated at 30 °C for 18-24 h. The plates were than overlaid with 8 mL of BHI soft agar (BHI broth + Bacto agar 0.6%) seeded with 8 μL of indicator strain grown for 16 h at 37 °C in BHI broth. After incubation for 16–18 h at 37 °C, plates were screened for the presence of growth inhibition zones in the indicator layer.

2.2. DNA extraction

DNA was extracted from 300 μL fresh bacterial culture with a Maxwell® Systems-Automated Sample Processing (Promega Italia S.r.l., Milan, Italy) using the Maxwell® 16 Tissue DNA Purification Kit (Promega Italia) according to the manufacturer's instructions.

2.3. PCR reactions

Amplification reactions were performed according to previously reported methods using the PCR 2x Master Mix (Promega Italia) in a GeneAmp PCR System 9700 (Thermo Fisher Scientific). PCR products were analyzed by capillary electrophoresis using the QIAxcel® Advanced System (Qiagen, London, UK). For identification of bacteria, the MicroSEQ® 500 16S rDNA Bacterial Identification System (Thermo Fisher Scientific), amplifying a 500 bp fragment from the 5' end of the 16S ribosomal RNA gene, was used according to the instructions.

L. casei and *L. paracasei* were further differentiated by PCR, according to Ward and Timmins [20], after 16S ribosomal RNA gene sequencing.

Identification of genetic loci involved in the production of lantibiotics (genes *lan*B, *lan*C, and *lan*M), BAs (genes *hdc*1, *hdc*2, *tdc*, *odc*) and enterococcal virulence factors gelatinase (*gel*E), enterococcal surface protein (*esp*), collagen adhesin (*ace*), endocarditis antigen (*efa*A), cytolisin (*cyl*A), hyaluronidase (*hyl*) and aggregation substance (*asa*1) was carried out as reported by Del Matto et al. [12]. The *nis*A gene was amplified according to Li and O'Sullivan [21]. The detection of vancomycin resistance genes was carried out according to Kariyama et al. [22].

Repetitive Extragenic Palyndrome (Rep) PCR was carried out with the GTG$_5$ primer as described by Versalovic et al. [23]. Image analysis and clustering were performed with the BioNumerics V7.5 software (Applied Maths N.V., Sint Martens Latem, Belgium), using the Dice coefficient for pairwise comparison, and the Unweighted Pair Group Method, using Arithmetic Averages (UPGMA) for clustering. Some isolates were analyzed in duplicate in the same experiment to establish a cut off for considering isolates distinct. Consequently, the cut off was set at 90% similarity.

2.4. Sequencing

Purification of PCR fragments for sequencing was performed with the Cytiva Illustra™ ExoProStar™ 1-Step kit (Merck, Paris, France). Sequencing master mix was prepared with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and the final purification step was performed with Sephadex® G-50 (VWR International S.r.l., Milan, Italy). Sequence analysis was performed with ABI-Prism 3500 Genetic Analyzer (Thermo Fisher Scientific). Bacterial isolates were identified to the species level according to the highest score obtained by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) alignment.

2.5. Adhesion to CaCo-2 cells

The Human Colon Epithelial Cancer cell line CaCo-2 (ATCC, Rockville, MD, USA) was used as surrogate system to evaluate the strain capacity to adhere to intestinal cells. CaCo-2 cells were seeded in 24-well tissue culture plates at the density of 25,000 cells/cm^2 and grown for 15 days to simulate mature human enterocytes [24]. Bacterial strains were grown for 16–18 h at 37 °C, centrifuged from 1 mL of culture at 10,000 × g for 10 min at 4 °C, and the pellet was resuspended in 1 mL of sterile PBS. The concentration of bacterial cells in these suspensions was determined by plate count. Then, 0.5 mL of this suspension was added to CaCo-2 cell monolayers previously washed five times with sterile PBS. After 1 h of incubation at 37 °C, wells were gently washed several times with PBS to remove unbound bacteria. Adhered bacteria were then detached from CaCo-2 cells using trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05% w/v trypsin, 0.02% w/v EDTA) solution and counted on plate. The adhesion capacity was expressed as percentage of CFU/mL of bacteria detached from cells on the CFU/mL in the inoculum. The experiments were carried out in triplicate.

2.6. Experimental production of Pecorino di Farindola cheese

Two batches of Pecorino di Farindola cheese were prepared from 300 L of raw milk previously tested for the absence of *L. monocytogenes* and *E. coli* O157. Milk was contaminated with *L. monocytogenes* ATCC 7644 and EURL 12M0B098, *E. coli* O157 VTEC ISS, ATCC 25952 and EURL C07 at final concentrations of about 3 Log CFU/mL. The preparation of the inoculum was carried out in accordance with the European Union Reference Laboratory for *Listeria monocytogenes* (EURL) guidelines for *L. monocytogenes* [24] by revitalizing frozen cultures at 37 °C for 18 h in BHI and adapting the strains at the temperature of milk processing (35 °C) in new BHI broth cultures for 24 h. In one batch (E cheese), the isolate *L. paracasei* L11121 was added at a final concentration of about 6 Log CFU/mL using 1.5 L of culture grown in MRS broth for 24 h at 37 °C in aerobic conditions. Bacterial cultures were added after the transfer of the raw milk in the coagulation vat. Cheese manufacturing was carried out according to the procedure adopted by the farms associated to the "Consorzio di Tutela del Pecorino di Farindola". Briefly, milk was heated at 35 °C and pig rennet was added at 2.5 mL/L final concentration. Casein precipitation was obtained in 40 min. The curd was mechanically broken using a lyre and left to rest for about 10 min. Temperature was then raised to 37 °C for 5 min. Curd was transferred into polyethylene molds and, after whey purging and manual pressing, the cheese-wheels were surface salted with coarse salt and left to season on wooden shelves in climatic chambers (Piardi ™ CC2000, Castenedolo, Italy) at 18 °C and 65% relative humidity (RH) for 148 days. Twenty-six cheese wheels of about 2 kilograms (kg) were produced. The spiked pathogens, LAB on MRS medium, LAB on M17 medium, enterococci and coagulase positive staphylococci (CPS) were monitored by plate count at different maturation times.

2.7. Statistical analyses

Two-way analysis of variance (ANOVA) was applied to compare count data of each microbial group during maturation between cheese lots and between E cheese and control cheese (C). The data of isolate tolerance to low pH, bile salts and cell adhesion capacity were analyzed by correlation clustering. Data series were considered significantly different for $P < 0.05$. All statistical analyses were carried out using PAST (Paleontological Statistic Software) V 4.06b, (https://www.nhm.uio.no/english/research/infrastructure/past/) [26].

3. Results

3.1. LAB dynamics in Pecorino di Farindola cheese

MRS and M17 media were used to improve the recovery of LAB. In particular, M17 medium is commonly used to count and isolate lactic cocci. However, this medium also sustains the growth of lactobacilli, so that lactic cocci are not detected when lactobacilli predominate in the sample [27]. Most of the bacteria enumerated on M17 medium were of coccoid shape until day 14. Afterwards prevalently rod shaped LAB grew on this medium. On MRS medium, rod shaped bacteria were isolated at all times, but also coccoid cells were retrieved, that were identified as enterococci by 16S rRNA gene sequencing. In particular, enterococci could be isolated on M17, MRS media and on the more specific SB medium.

The evolution of LAB counted on different culture media in the Pecorino di Farindola cheese lots analyzed in this study is shown in Figure 1.
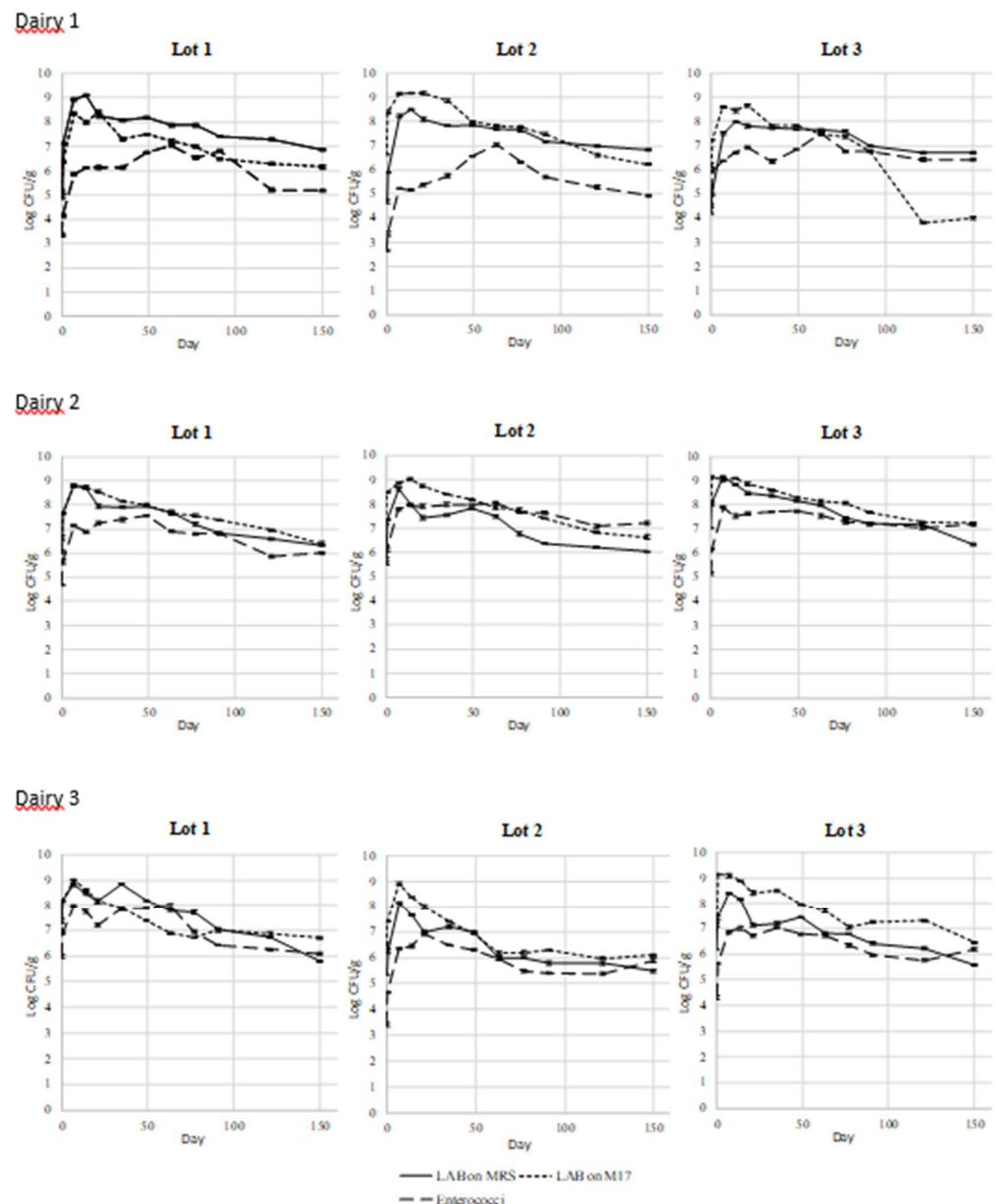

Figure 1. Evolution of LAB in nine lots of Pecorino di Farindola cheese from three manufacturers (Dairy 1, 2 and 3).

Though with differences in the evolution among manufacturers and, for Dairy 1, among lots, enterococci reached high numbers during maturation in all cheeses and were still high in the products ready for consumption, being in the range 5-7 Log CFU/mL.
LAB showed similar evolution trends among lots and dairies, except for Lot 3 of dairy 1, where LAB grown on M17 medium declined drastically after day 91. Enterococci were often retrieved from all the three isolation media.

3.2. Identification of dominant LAB in Pecorino di Farindola cheese

Predominant LAB involved in the maturation of Pecorino di Farindola cheese were isolated from each growth medium at different maturation times, obtaining 206 isolates. Though the exact load of each isolate in the sample of origin could not be determined, its order of magnitude can be estimated from the count on the respective isolation medium. Isolate identification based on 16S rRNA gene sequences, is reported in Table 1, jointly with source and time of isolation. Isolates of the same species are listed separately according to the detected genes.

Table 1. LAB isolates with detected genes per manufacturer, production lot and time of isolation.

Species, detected genes, source and time of isolation	
Manufacturer 1	
Lactobacilli	*Lacticaseibacillus paracasei*: Lot 1, days 49, 63, 77, 121, 150; Lot 2, days 14, 49, 63, 91, 121, 150; Lot 3, days 49, 77, 150; *Lactiplantibacillus plantarum*: Lot 1, days 14, 21, 49; Lot 2, days 14, 21, 35; *Levilactobacillus brevis:* Lot 1, days 7, 35, 91; Lot 3, day 121; *L. brevis (odc):* Lot 2, day 77; *Leuconostoc mesenteroides*: Lot 2, day 7; *L. pseudomesenteroides*: Lot 3, days 7, 14
Lactic cocci	*Lactococcus lactis* subsp. *Lactis*: Lot 1, milk, curd; *L. lactis* subsp. *lactis* (*nis*A): Lot 2, milk, curd; Lot 3, milk, curd; *Streptococcus infantarius*: Lot 3, day 35; *S. lutetiensis*: Lot 1, day 91
Enterococci	*Enterococcus durans*: Lot 3, day 150; *E. faecalis* (*tdc*A): Lot 2, day 77; *E. faecalis* (*ace, tdc*A): Lot 1, days 77, 121, 150; Lot 3, day 150; *E. faecalis* (*gel*E, *tdc*A): Lot 2, day 35; *E. faecalis* (*gel*E, *ace, tdc*A): Lot 1, milk, day 49; Lot 2, curd; *E. faecalis* (*gel*E, *ace, efa*A): Lot 1, day 63; *E. faecium*: Lot 1, days 28, 35; Lot 2, days 49, 63, 77, 150; Lot 3, days 7, 14, 21, 35, 63, 91; *E. faecium* (*ent*B): Lot 1, day 49; *E. faecium* (*ent*P): Lot 1, day 77; Lot 2, day 91; *E. faecium* (*gel*E): Lot 3, day 121; Lot 2, day 121; *E. faecium* (*gel*E, *tdc*A): Lot 1, day 14; *E. gallinarum*: Lot 2, milk
Manufacturer 2	
Lactobacilli	*L. plantarum*: Lot 1, days 7, 21, 49, 77, 91; Lot 2, days 14, 21, 35, 77, 91, 150; Lot 3, day 77; *L. brevis*: Lot 1, days 121, 150; Lot 2, day 121; Lot 3, days 14, 49, 91, 121, 150; *L. mesenteroides*: Lot 1, day 14; *Loigolactobacillus coryniformis*: Lot 1, day 35
Lactic cocci	*L. lactis* subsp. *Cremoris*: Lot 1, milk, curd; Lot 2, milk, curd, day 7; Lot 3, milk, curd, day 14; *L. lactis* subsp. *lactis*: Lot 3, day 7
Enterococci	*E. casseliflavus*: L1, day 63; *E. durans*: Lot 3, day 121; *E. faecalis* (*gel*E, *tdc*A): Lot 1, milk, day 35; Lot 2, milk, day 35; Lot 3, day 35; *E. faecalis* (*ace, tdc*A): Lot 2, day 21; *E. faecalis* (*gel*E, *ace, tdc*A): Lot 3, day 21; *E. faecium*: Lot 2, days 21, 49, 63, 91; Lot 3, days 14, 21, 49, 91, 121, 150; *E. faecium* (*gel*E): Lot 3, day 63; *E. faecium* (*ent*B): Lot 1, days 35, 63, 121, 150; Lot 2, days 35, 77; Lot 3, days 7, 63, 121; *E. faecium* (*ent*L50AB): Lot 3, day 14; Lot 2, day 150; *E. faecium* (*ent*P): Lot 1, days 21, 49, 63, 121, 150; Lot 3, day 91
Manufacturer 3	
Lactobacilli	*L. paracasei*: Lot 1, days 63, 77, 91; Lot 2, days 14, 77, 91, 121, 150; Lot 3, days 21, 35, 77, 91, 150; *L. plantarum*: Lot 3, days 14, 49; *L. brevis*: Lot 1, days 35, 121, 150; Lot 2, day 121; Lot 3, day 121; *L. mesenteroides*: Lot 3, curd

Lactic cocci	*L. garviae*: Lot 1, days 7, 49; *L. lactis* subsp. *lactis*: Lot 2, milk, curd; Lot 3, milk, curd; *L. lactis* subsp. *lactis* (*nis*I): Lot 1, milk, curd; Lot 2, day 7; Lot 3, day 7
Enterococci	*E. faecalis*: Lot 3, day 14; *E. faecalis* (*tdc*A): Lot 1, day 49; Lot 2, day 21; *E. faecalis* (*ace*, *tdc*A): Lot 1, days 14, 21, 49, 150; Lot 2, days 7, 14; Lot 3, curd, days 7, 21, 49; *E. faecalis* (*asa*1): Lot 3, day 14; *E. faecalis* (*gel*E, *ace*, *tdc*A): Lot 1, day 49; Lot 2, day 35; *E. faecalis* (*ace*, *tdc*A, *asa*1): Lot 1, day 35; Lot 2, curd, day 35; Lot 3, curd, day 21; *E. faecium*: Lot 3, day 63; *E. faecium* (*ent*L50AB): Lot 1, days 77, 121, 150; Lot 2, days 21, 49, 63, 77; Lot 3, days 21, 35, 63, 150

In milk, curd and cheese in early ripening the dominant microorganisms present in all three dairies, though not detected in all lots, were *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides*. In particular, *L. lactic* subsp. *lactis* with the nisin structural gene *nis*A was predominant for five lots from two dairies. The heterofermentative species *Levilactobacillus brevis* was the only microorganism isolated from all cheese lots, generally retrieved until advanced maturation, while other LAB species predominated according to the dairy. The *L. brevis* isolates did not possess amino acid decarboxylase encoding genes except for an isolate from Dairy 1, in which an ornithine decarboxylase gene, *odc*, was identified. *Lactiplantibacillus plantarum* was also isolated from all three dairies but not from all cheese lots. *L. plantarum* isolates from Dairy 2 were obtained from late ageing cheese, thus indicating their capacity to persist during maturation. The presence of *Lacticaseibacillus paracasei* was demonstrated only in two dairies. Other species found to predominate in particular maturation phases, i.e. *Loigolactobacillus coryniformis*, *L. pseudomesenteroides*, *L. garviae*, *Streptococcus infantarius* and *S. lutetiensis*, appeared to be specific for the manufacturing plant.

Enterococci exhibited high diversity in the endowment of virulence, bacteriocin and *tdc* genes. The tyrosine decarboxylase gene *tdc*, suggested to be a species-specific character in *E. faecalis* and *E. faecium* [28], was detected in all the *E. faecalis* isolates but not in *E. faecium*, except for one isolate belonging to this species. All isolates were negative for genes encoding vancomycin resistance. Different species or biotypes of enterococci codominated or alternated as dominant at different maturation times. In two cases *E. faecalis* isolates with virulence characters were still present in the product ready for consumption. Enterococci with bacteriocin encoding genes and devoid of virulence genes were isolated from all three dairies also in late ageing.

3.3. Evaluation of the prerequisites for survival in GIT

LAB isolated at days 121 and 150, i.e. those that are ingested in high numbers with the product, including the enterococcal isolates devoid of virulence characters, were tested for their ability to survive in acidic conditions (pH 2.5 and 3.0), in the presence of bile salts (1%, 0.5% and 0.3%), and to adhere to CaCo-2 cells. These cells are generally used as a model of the intestinal epithelial cells [29]. *L. casei* ATCC 393, a strain isolated from cheese and already tested for tolerance to acidity, bile salts and adhesion properties [30], was used as a reference to interpret the characteristics in the isolates examined in this study by comparison with previous work. Results are reported in Table 2.

Table 2. LAB tolerance to pH 2.5 and 3.0, to bile salts 0.3, 0.5 and 1 % (w/v) and adhesion capacity to CaCo-2 cells. In isolate labels "L" or "E", stand for lactobacilli and enterococci, respectively, the first following number indicates the dairy, the second the lot and the three last numbers the day of isolation (121 or 150). Letters at the end distinguish isolates obtained from the same sample.

Isolate	pH tolerance (% survival after 2 h)		Bile tolerance (% growth vs control)			Adhesion to CaCo-2 cells (%)
	2.5	3	0.3	0.5	1	
Lacticaseibacillus casei ATCC 393	0	94.60±2.12	27.00±0.71	12.00±0.97	5.00±1.22	0.42±0.04
Enterococcus faecium E12150	47.22±1.34	90.82±1.45	96.00±3.48	95±4.37	80.01±2.43	0.52±0.07
E. durans E13150	0	93.76±2.67	63.42±1.34	35.03±2.14	20.46±1.76	0.22±0.02
E. faecium E21121a*	0	92.80±2.13	31.93±4.22	23.39±1.36	12.79±1.46	0.34±0.09
E. faecium E21121b†	0	85.10±1.17	41.50±3.12	32.81±1.44	12.71±1.24	0.35±0.11
E. faecium E21150a*	42.62±1.25	83.96±2.46	93.01±2.33	78.79±2.65	73.74±1.96	0.61±0.08
E. faecium E21150b†	0	95.40±2.21	51.69±4.32	32.14±1.87	22.19±1.47	0.71±0.02
E. faecium E22150‡	0	87.08±1.27	41.37±1.22	32.31±2.98	12.33±0.98	0.97±0.18
E. durans E23121	0	91.67±1.65	39.66±2.09	35.43±3.16	33.34±1.33	0.30±0.05
E. faecium E23121*	0	77.59±2.14	74.87±1.76	57.04±2.56	52.16±2.06	0.52±0.07
E. faecium E23150	0	96.52±2.56	35.63±1.09	24.41±2.16	20.68±1.67	0.38±0.03
E. faecium E31121	0	90.38±1.68	39.10±1.99	38.76±1.03	29.18±2.12	0.21±0.01
E. faecium E31150	0	98.05±1.35	34.63±1.47	30.9±1.76	26.05±1.09	0.59±0.02
E. faecium E33150‡	0	90.96±2.22	36.76±1.76	17.24±1.33	16.56±1.23	0.47±0.03
L. paracasei L11121	0	95.44±2.13	80.23±2.46	45.88±2.46	16.32±1.76	0.03±0.01
Levilactobacillus brevis L21121	0	80.37±2.97	48.02±1.96	24.79±1.22	23.36±1.33	0.45±0.02
L. brevis L31121	0	94.15±4.22	97.00±2.65	72.83±2.09	65.28±2.95	3.2±0.12
L. paracasei L12121	0	97±1.76	52.75±2.06	28.72±1.67	13.16±1.22	0.03±0.01
L. brevis L22121	73.75±2.11	92.62±2.56	58.92±1.99	19.1±1.09	9.28±0.97	1.22±0.03
L. brevis L32121	0	80.28±1.48	83.62±2.55	33.85±2.98	29.59±1.96	1.05±0.02
L. brevis L13121	48.07±1.63	93.33±2.77	51.87±2.46	24.83±1.24	14.78±1.03	0.96±0.02
L. brevis L23121	42.68±1.25	97±2.55	43.55±1.33	11.54±1.76	9.93±0.98	1±0.01
L. brevis L33121	0	92.45±3.15	74.58±2.16	51.55±1.99	49.43±2.16	1.58±0.07
L. paracasei L11150	0	95.59±3.11	17.71±1.34	18.14±0.98	10.02±1.22	0.03±0.00
L. brevis L21150	0	97.36±1.47	8.73±1.03	6,89±0.03	3.38±0.04	0.28±0.03
L. brevis L31150	0	91.62±1.27	80.06±2.67	41.84±2.05	26.3±1.33	1.54±0.02

L. paracasei L12150	33.09±1.34	98±1.03	36.69±1.22	18.81±1.33	8.34±0.96	0.08±0.02
Lactiplantibacillus plantarum L22150	90.87±1.98	97.81±2.19	50.42±2.09	30.04±1.47	21.77±1.99	1.33±0.07
L. paracasei L32150	42.18±1.55	95±4.21	46.72±2.06	34.56±2.16	14.48±1.67	0.03±0.01
L. paracasei L13150	78.57±1.58	94.22±2.23	9.56±0.98	4.61±0.78	4.78±0.06	0.03±0.01
L. brevis L23150	54.09±1.44	73.4±2.67	53.3±1.34	45.00±1.33	12.00±1.24	0.02±0.00
L. paracasei L33150	45.05±1.77	72.05±1.33	97.42±2.55	96.13±3.13	68.46±2.05	0.01±0.00

*isolates with an *ent*B gene;

†isolates with an *ent*P gene;

‡isolates with an *ent*L50AB gene.

At pH 3.0, all isolates tested exhibited good tolerance, while at pH 2.5, only ten were able to survive. Most isolates showed significantly higher tolerance than the reference strain to all tested bile salt concentrations. In the cell adhesion test, 6 enterococci, 7 *L. brevis* isolates and *L. plantarum* L22150 showed better adhesion capacity than the reference strain. All the *L. paracasei* isolates showed a low cell adhesion capacity.

Patterns of tolerance to GIT conditions and adhesion capacity were not species-specific, as shown by correlation clustering of average data series for the isolates listed in Table 2 (Figure 2).
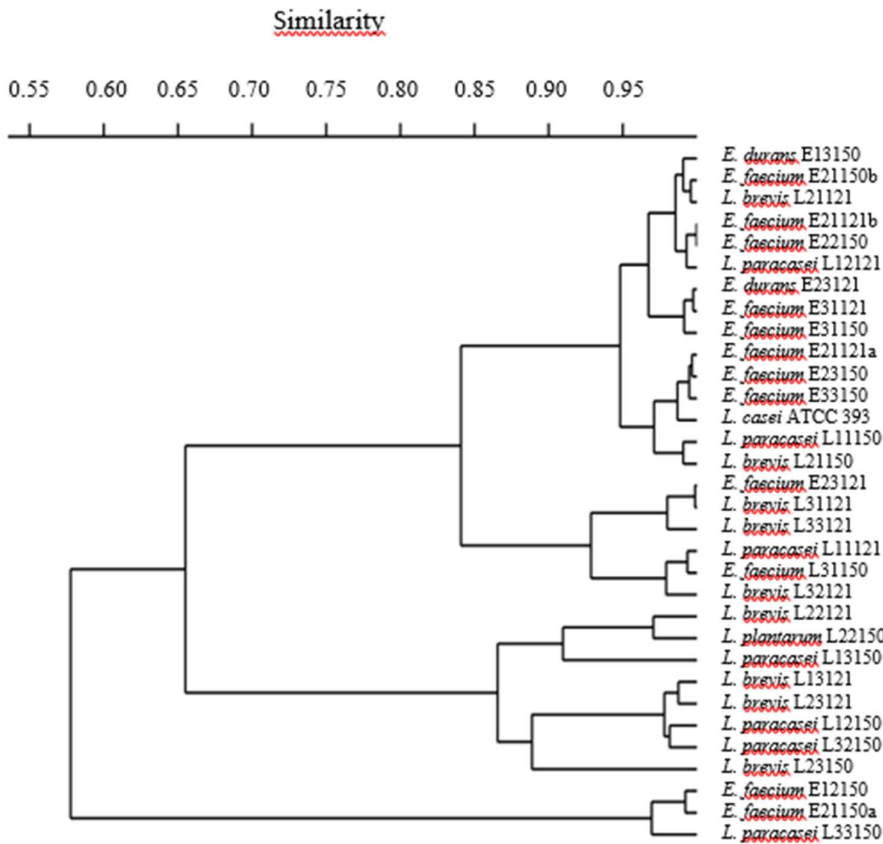

Figure 2. Correlation clustering based on average values of tolerance to low pH and bile salts and adhesion capacity reported in Table 2 for LAB isolates at 121 and 150 days .

3.4. LAB activity against pathogenic bacteria

The same LAB isolated at days 121 and 150 were also tested for the production of antimicrobial compounds active against *L. monocytogenes* ATCC 7644, *Salmonella* Typhimurium ATCC 14028 and *Staphylococcus aureus* ATCC 33862 by the deferred antagonism test. Seven enterococcal isolates, coinciding with those in which enterocin genes were detected, and *L. paracasei* L11121 showed activity against *L. monocytogenes* ATCC 7644. In particular, the enterococcal isolates produced inhibition halos with diameters, extending from the spot margin, between 4 and 10 mm, while *L. paracasei* L11121 produced an inhibition halo of 4 mm. The latter autochthonous strain was selected as culture to be used in a cheese making trial because of the recognized safety status of the species [31].

3.5. Cheese making trial with the addition of L. paracasei *L11121 isolate*

Count values for all the microbial groups assessed in the cheese making trial with use of *L. paracasei* L11121 are reported in Table 3.

Table 3. Evolution of a_w, pH and microbial groups in Pecorino di Farindola produced with (E) or without (C) addition of *Lacticaseibacillus paracasei* L11121 isolate.

	Batch	Milk	Curd	Day 1	Day 7	Day 17	Day 23	Day 28	Day 37	Day 58	Day 88	Day 118	Day 148
a_w	C		0.994±0.004	0.965±0.000	0.952±0.000	0.916±0.001	0.867±0.000	0.859±0.001	0.856±0.002	0.828±0.004	0.822±0.000	0.827±0.001	0.819±0.001
	E		0.992±0.005	0.957±0.000	0.951±0.001	0.916±0.001	0.895±0.003	0.868±0.002	0.861±0.001	0.827±0.002	0.820±0.02	0.822±0.000	0822±0.001
pH	C	6.85±0.05	6.74±0.01	6.60±0.01	6.01±0.08	5.80±0.01	5.71±0.01	5.67±0.02	5.74±0.01	5.43±0.02	5.38±0.02	5.41±0.01	5.46±0.03
	E	6.85±0.05	6.74±0.01	6.60±0.01	6.01±0.08	5.80±0.01	5.65±0.04	5.58±0.02	5.75±0.06	5.45±0.05	5.43±0.05	5.39±0.00	5.26±0.01
LAB on MRS (Log CFU/g)	C[a]	5.33±0.02	5.44±0.04	7.63±0.02	7.53±0.03	7.54±0.04	7.85±0.02	7.63±0.02	7.85±0.02	6.48±0.03	6.41±0.05	4.72±0.09	4.58±0.06
	E[a]	6.18±0.05	6.33±0.04	8.73±0.02	8.71±0.03	8.66±0.03	8.56±0.02	8.03±0.00	7.89±0.02	6.56±0.08	6.42±0.08	5.14±0.05	5.11±0.03
LAB on M17 (Log CFU/g)	C[c]	5.55±0.03	5.61±0.02	8.72±0.02	8.72±0.01	8.55±0.03	8.51±0.02	7.69±0.01	7.97±0.02	6.41±0.04	6.40±0.10	5.35±0.06	5.27±0.07
	E[c]	6.33±0.01	6.53±0.02	8.64±0.02	8.70±0.02	8.73±0.02	8.60±0.02	8.78±0.05	7.96±0.03	6.51±0.07	6.21±0.01	5.74±0.07	5.68±0.06
Enterococci (Log CFU/g)	C	3.62±0.02	3.77±0.03	3.80±0.02	4.94±0.01	5.42±0.05	5.37±0.02	5.96±0.03	5.91±0.03	5.37±0.03	4.86±0.08	4.66±0.14	4.45±0.10
	E	3.35±0.03	3.49±0.03	3.91±0.01	5.03±0.02	5.77±0.02	5.93±0.02	5.88±0.02	5.87±0.02	5.58±0.01	4.74±0.09	4.86±0.08	4.50±0.15
Coagulase-positive staphylococci (Log CFU/g)	C	4.48±0.00	4.51±0.04	5.02±0.03	5.01±0.02	4.93±0.01	4.81±0.02	4.72±0.01	4.63±0.02	3.45±0.04	3.47±0.04	2.34±0.12	nd
	E	4.42±0.03	4.51±0.04	5.02±0.03	5.01±0.02	4.93±0.01	4.81±0.02	4.63±0.02	4.40±0.07	3.60±0.03	3.30±0.08	2.54±0.11	nd

E. coli O157 (Log CFU/g)	C	3.77±0.02	3.49±0.04	4.03±0.01	4.04±0.01	4.02±0.01	3.92±0.01	3.65±0.11	3.38±0.02	3.38±0.08	2.62±0.04	nd	nd
	E	3.63±0.02	3.40±0.05	4.01±0.02	4.00±0.00	3.64±0.03	3.06±0.05	3.08±0.05	3.06±0.05	3.05±0.05	nd	nd	nd
L. monocytogenes (Log CFU/g)	C[b]	3.32±0.02	3.34±0.06	3.95±0.02	3.94±0.01	3.37±0.08	3.31±0.03	3.30±0.03	3.32±0.02	3.27±0.03	2.25±0.06	2.03±0.11	nd
	E[b]	3.44±0.05	3.37±0.06	3.47±0.03	3.48±0.04	3.36±0.04	3.33±0.02	1.88±0.03	1.71±0.05	1.68±0.05	nd	nd	nd

Nd; not detected

[a]significantly different for $P < 0.001$

[b]significantly different for $P < 0.01$

[c]significantly different for $P < 0.05$

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According to two-way ANOVA analysis, the numbers of LAB grown on MRS and 8
M17 media were significantly higher in E cheese (with addition of *L. paracasei* L11121) 9
compared to C cheese (control) for P < 0.001 and P < 0.05, respectively. The decrease of *E.* 10
coli O157 and *L. monocytogenes* in E cheese was more rapid, becoming evident from day 11
17 for *E. coli* O157 and from day 28 for *L. monocytogenes*. The count values of *L. monocyto-* 12
genes in E and C cheeses were significantly different (P < 0.01). In E cheese, this pathogen 13
remained at levels below the admitted limit of 100 CFU/g [10] since day 28. 14

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3.6. Rep PCR for L. paracasei *L11121 tracing during cheese maturation* 16
The persistence of *L. paracasei* L11121 in cheese was determined by isolating LAB at 17
each time interval and identifying *L. paracasei* isolates by species-specific PCR. Ten *L.* 18
paracasei isolates were examined for each cheese at each time of analysis for a total of 260 19
isolates that were submitted to genotyping by Rep PCR. In the dendrogram reported in 20
Figure 3 one representative profile for each group of isolates separated at 90% similarity 21
from each sample is included, so that all the profiles representative of each cheese batch 22
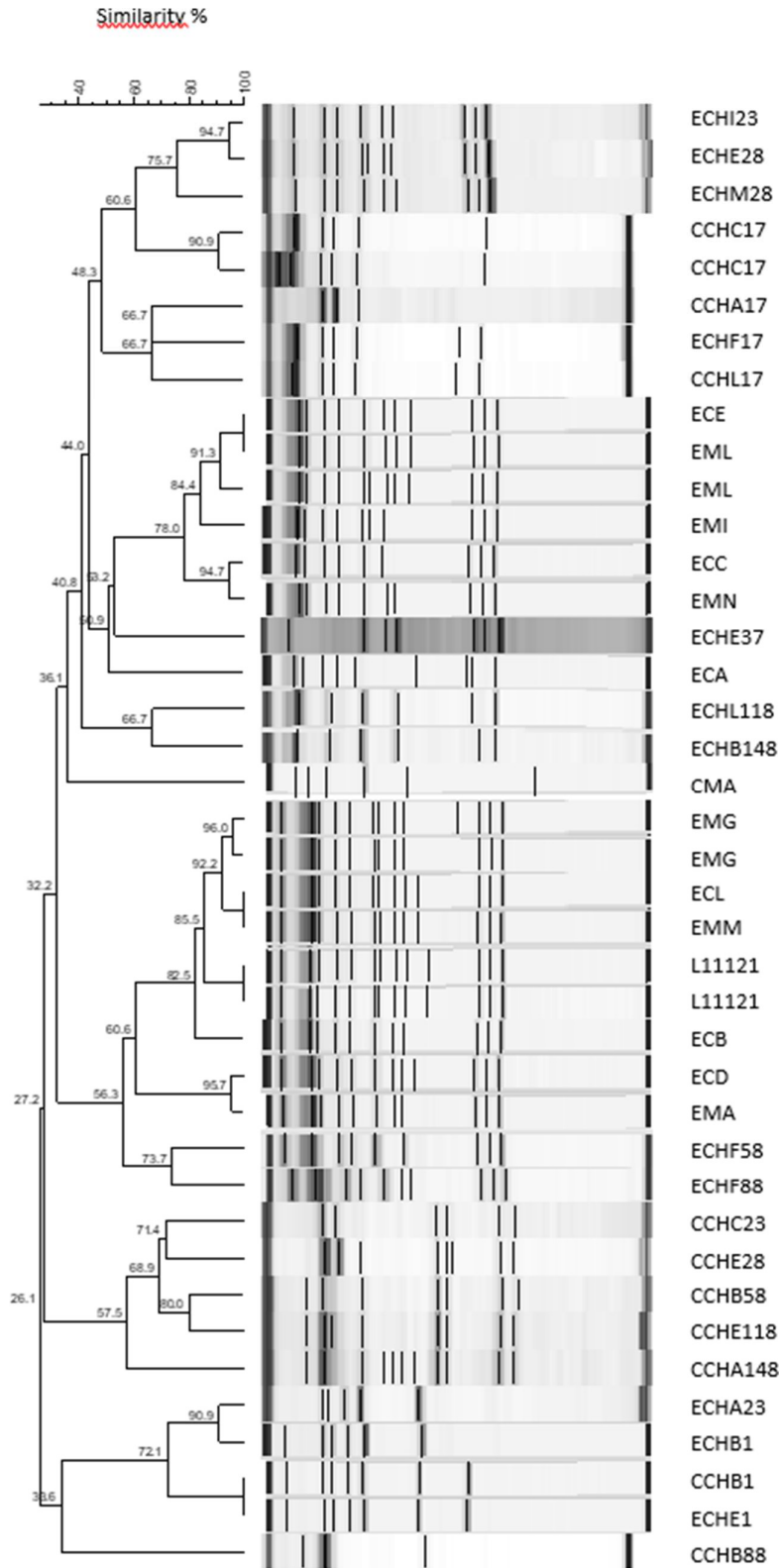and time of isolation are shown. 23

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Figure 3. Clustering of Rep PCR profiles for *L. paracasei* isolates from the cheese production trial
for cheese with (E) or without (C) addition of *L. paracasei* L11121. The isolate labels indicate the
cheese batch of origin, E or C, the sample type, i.e. milk (M), curd (C) or cheese (CH), isolate iden-
tity, defined with a letter, and the day of isolation.

In most cases, profiles from the same sample, always obtained in the same analysis session, shared more than 90% similarity and were considered duplicates of identical isolates. For this reason, with a few exceptions, only one isolate per sample is shown in the dendrogram (Figure 3). It can be observed that *L. paracasei* L11121 shared highly similar profiles with isolates derived from milk and curd of E cheese. Profiles that clustered together with those of *L. paracasei* L11121, milk and curd isolates from E cheese, but showed lower similarity with the latter, possibly as a consequence of the not complete reproducibility known to occur among different Rep PCR runs [32], were retrieved until day 88 from E cheese. This observation suggests that the added culture was able to persist until the minimum maturation period of Pecorino di Farindola cheese.

4. Discussion

In this study, variability was observed in the identity of dominant bacteria in Pecorino di Farindola cheese among producers and among lots from the same producer during the whole maturation process. Nevertheless, constant microbiological features could be defined, i.e. the predominance of *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* in curd and in cheese in early ripening and the predominance of *Levilactobacillus brevis* and enterococci throughout ripening.

An unusual observation was that the species *Lacticaseibacillus paracasei*, found to be the most persistent in many other traditional cheeses [14], was not isolated from all manufacturers.

L. brevis, present in all cheese lots, was found to persist in the product ready for consumption. This species has been little characterized for its role in cheese flavor formation and safety improvement, though its presence has been documented also in other cheeses from raw ewe and goat milk [33]. In addition, strains of *L. brevis* with probiotic properties have been described [34,35]. These aspects should be addressed in further studies on *L. brevis* isolates from Pecorino di Farindola cheese, together with the safety concern of BA formation and horizontal gene transfer (HGT) of the genetic determinants involved reported for this species [36]. The fact that no amino acid decarboxylase genes, except an *odc* gene found in one isolate, were detected in this study, indicates that this LAB species is not frequently responsible for BA formation in Pecorino di Farindola cheese.

Enterococci were found to be abundant in this cheese, as in other cheeses made from raw milk [33,37]. A high diversity was observed for these bacteria, on the basis of presence of genes encoding beneficial or undesired traits. It was surprising that the *tdc* gene was not detected in most of the *E. faecium* isolates examined in this study, since it was suggested that the presence of this gene is a species-specific trait [29]. This finding is in favor of the safety status of the *E. faecium* isolates from Pecorino di Farindola cheese, in which the presence of virulence characters was not frequent and bacteriocin genes were detected. Therefore, it would be interesting to investigate the ability of these *E. faecium* isolates to outcompete enterococci with risk characters, such as the examined *E. faecalis* isolates.

Since individual strains of enterococci can present risk characters, the Qualified Presumption of Safety (QPS) status cannot be conferred to the whole genus or species [37]. On the other hand, the flavor formation ability and probiotic prerequisites, demonstrated also in this study, as well as the production of broad spectrum bacteriocins [37,38], represent desirable properties for autochthonous microbiota components of traditional cheeses. Therefore, standardized procedures should be fixed to individually

evaluate enterococci for safety-related aspects to allow their use as autochthonous cul-   77
tures. These procedures must include the demonstration that these bacteria are not   78
prone to acquire unwanted genetic traits by HGT in the cheese environment as it was   79
reported for some strains [39,40].   80

Analogous issues regard other dominant bacterial species found in this study, e.g.   81
L. garviae, *Streptococcus infantarius* and *S. lutetiensis*, that can cause infections in humans   82
[41,42] but can carry beneficial characters and be devoid of virulence traits at the strain   83
level [43,44].   84

The assessment of the capacity of isolates obtained at the end of maturation to   85
withstand the harsh conditions of GIT indicated that enterococci, *L. brevis* and *L. planta-*   86
rum isolates from Pecorino di Farindola cheese are potentially able to survive in GIT   87
upon consumption of the product. This observation encourages future investigations of   88
the probiotic characteristics of the isolates to enrich the product with autochthonous cul-   89
tures able to enhance its functional properties.   90

The cheese making trial was carried out with an autochthonous added culture, a *L.*   91
paracasei isolate, since this species has a proven suitability for use as adjunct culture in   92
cheese [45] and a recognized safety status [31]. The added culture possibly dominated in   93
the experimental cheese until day 88, that is the minimum maturation time for Pecorino   94
di Farindola cheese. Indeed, Rep PCR profiles clearly similar to that of *L. paracasei*   95
L11121 were obtained for *L. paracasei* isolates only from E cheese until advanced matura-   96
tion. These should be considered different from the added strain because they show less   97
than the 90% profile similarity fixed in this study for replicate profiles of the same strain.   98
However, this value regarded genotypic profiles obtained in the same experiment, while   99
low reproducibility among different experiments was reported for this typing method   100
[32]. Therefore, the fact that *L. paracasei* L11121 was able to persist until the minimum   101
maturation period of Pecorino di Farindola cheese cannot be ruled out.   102

The favorable effects observed from the use of the *L. paracasei* culture, i.e. the en-   103
richment in lactobacilli and faster pathogen decline, can be considered relevant func-   104
tional and safety improvements. Indeed, *L. monocytogenes* was below the tolerance limits   105
in E cheese already at day 28 and was absent at the minimum ripening time of 90 days.   106
In a previous investigation on the same cheese with no added cultures, the pathogen   107
was undetectable after the minimum ripening period only in one out of three batches   108
examined [46]. A variability in the presence of an anti-listerial microbiota probably de-   109
termined the different behavior of the pathogen in different cheese batches, confirming   110
the necessity to use added cultures to ensure its absence before product commercializa-   111
tion.   112

Results suggest further developments of mixed cultures able to enhance safety im-   113
provements, by inhibiting enterococci with risk characters and CPS. CPS in this cheese   114
appeared to be of particular concern since they reached numbers that could cause staph-   115
ylococcal toxin accumulation during early ripening. These bacteria exceeded the critical   116
limit [10,47] until day 88 also in E cheese, thus requiring the selection of autochthonous   117
LAB strains able to antagonize them.   118

In particular, lactococci with the nisin structural gene, found to naturally dominate   119
in some lots of Pecorino di Farindola cheese during early ripening, should be further   120
investigated for the capacity to inhibit detrimental microorganisms. Indeed, it was   121
demonstrated that nisin is produced in cheese and that its production can continue in   122
GIT with consequent health promoting effects [48].   123

124

5. Conclusions

In this study, a "cultivable" core microbiota of Pecorino di Farindola cheese was defined based on the presence of some LAB species in all cheese lots examined. It was put in evidence that this cheese can be considered a source of microorganisms that could exert beneficial effects, being potentially able to establish in GIT and to express antimicrobial activity. On the other hand, enterococci with virulence characters and CPS were found to be present in the product at high levels and were not affected by addition of an autochthonous *L. paracasei* culture. Therefore, the use of native cultures in association should be further investigated to ensure safety and increase health promoting features of Pecorino di Farindola and other similar cheese types.

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