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Behavior of Four Main Dairy Pathogenic Bacteria during Manufacturing and Ripening of Pecorino Siciliano Cheese

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HIGHLIGHTS

- Lactic acid bacteria populations dominated in control and experimental Pecorino Sicilian cheese productions.
- *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* Enteritidis, as well as *Staphylococcus aureus* were completely disappeared after 60 days of ripening.
- The Randomly Amplified Polymorphic DNA analysis demonstrated the presence of the added strain during production.
- Production conditions of PDO Pecorino Siciliano cheese decreased growth of four main dairy pathogens.

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Acronyms and abbreviations
CFU=Colony Forming Unit
CP=Control Production
EP=Experimental Production
LAB=Lactic Acid Bacteria
PCR=Polymerase Chain Reaction
PDO=Protected Designation of Origin
RAPD=Randomly Amplified Polymorphic DNA

ABSTRACT

Background: Consumption of raw cheese may be associated with different diseases. This study aimed to evaluate behavior of four pathogenic bacteria during manufacture and ripening of Protected Designation of Origin (PDO) Pecorino Siciliano cheese.

Methods: The experimental cheese groups were inoculated with pathogenic bacteria, including *Escherichia coli* O157, *Listeria monocytogenes*, *Salmonella* Enteritidis, and *Staphylococcus aureus*. The cheese making processes were monitored from milk curdling until 3 months ripened cheeses and the levels of Lactic Acid Bacteria (LAB) and the four dairy pathogens were evaluated by plate counts. Randomly Amplified Polymorphic DNA (RAPD)-Polymerase Chain Reaction (PCR) analysis was applied to confirm that the colonies isolated during the several steps of production were the same strains added in milk. Statistical analysis was done using XLStat software.

Results: The levels of mesophilic and thermophilic coccus and rod LAB in curd were comparable in both trials and reached values between 8-9 $\log_{10}$ Colony Forming Unit (CFU)/g in cheeses at 90 days of ripening. The four pathogenic bacteria were found in experimental curd at levels higher than those inoculated in milk and completely disappeared after 60 days of ripening. The RAPD analysis clearly demonstrated the presence of the added strain during production and confirmed the results of plate counts.

Conclusion: This work showed that the production conditions of PDO Pecorino Siciliano cheese decreased growth of *E. coli* O157, *L. monocytogenes*, *S.* Enteritidis, and *S. aureus*.

Introduction

Protected Designation of Origin (PDO) Pecorino Siciliano is a typical Sicilian hard cheese produced on the entire area of Sicily Island. This cheese is obtained from raw ewes' milk subjected to spontaneous fermentation

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of indigenous bacteria of raw milk, animal rennet, and biofilms associated to the wooden equipment (Cruciata et al., 2019). PDO Pecorino Siciliano cheese is generally ripened for at least 4 months (Settanni et al., 2013). Due to the traditional production process, PDO Pecorino Siciliano cheese represents a product well appreciated by consumers who consider foods processed without additives as more natural and safe (Settanni and Moschetti, 2014).

The fermentation of this dairy product relies on the presence of Lactic Acid Bacteria (LAB) with remarkable technological characteristics. So, the LAB, which mostly contribute to the production of PDO Pecorino Siciliano cheese, come from the wooden vat (Gaglio et al., 2019a; Scatassa et al., 2015). The rapid development of the wooden LAB at high levels contrast the development of the undesired microorganisms, such as pathogenic bacteria, acting as starter cultures (Scatassa et al., 2017). Although, dairy products underwent a fermentation process are generally considered safe, some pathogens can also develop at risk levels (Fernández et al., 2015). Indeed, consumption of fresh raw milk cheeses may be associated with different human diseases mainly caused by *Escherichia coli* O157, *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus* (Oliver et al., 2005). The prevalence of these pathogenic bacteria in dairy products is influenced by numerous factors, but the inappropriate handling procedures during manufacturing and storage are considered the main sources of contamination (Fernández et al., 2015; Ortolani et al., 2010).

In the last years, in light of the Commission Regulation (EC) No 2073/2005 on "microbiological criteria for foodstuffs", different Sicilian dairy productions have been also extensively investigated for the presence of the four main dairy pathogens. These studies evidenced that *L. monocytogenes* and *Salmonella* spp. were never found during any step of production of any traditional cheese, while *E. coli* was found at low levels on the surface of a few wooden vats and in raw milk. *S. aureus* were detected in raw milk, even though they were never found after fermentation (Cruciata et al., 2019; Gaglio et al., 2019a; Settanni et al., 2013). These results suggest that the production processes are able to reduce the hygienic risks of the traditional Sicilian cheeses.

Regarding PDO Pecorino Siciliano cheese, several studies investigated its microbiological, physicochemical, and sensory properties of this cheese (Settanni et al., 2013; Todaro et al., 2011), but so far no study focused on the monitoring of the main dairy pathogens. In the present study, the behavior of four important dairy bacterial pathogens was monitored during the production of PDO Pecorino Siciliano cheese to provide insight on the safety of the production process of this traditional cheese.

Materials and methods

Production of control and experimental cheese

Cheese productions were performed according to the traditional cheese making provided by the production protocol of PDO Pecorino Siciliano cheese (Settanni et al., 2013). The artificial contamination test during PDO Pecorino Siciliano cheese was previously performed in the study of Cruciata et al. (2018). Briefly, the trials were carried out under controlled conditions in a dairy pilot plant (Istituto Zooprofilattico Sperimentale della Sicilia A. Mirri, Palermo, Italy) using chestnut wooden vats. Specifically, four new wooden vats were activated with hot deproteinized whey (about 80 °C) for seven consecutive days (Cruciata et al., 2019), and then filled with 12 L of raw Valle del Belice sheep's milk. Two vats were used for the Control Production (CP) and two vats for the Experimental Production (EP) carried out in presence of pathogenic bacteria. To this purpose, after 15 min of milk in contact with the wooden vat surfaces, the raw bulk milks in EP vats were contaminated with 40 ml of a suspension containing the cocktail of 10^3 Colony Forming Unit (CFU)/ml *E. coli* O157 ATCC 35150 as well as *S. aureus* ATCC 33862 and 30 CFU/ml *L. monocytogenes* ATCC 7644, and *S.* Enteritidis ATCC 13076 prepared in Ringer's solution (Sigma Aldrich, Milan, Italy), while CP vats were supplemented with the same volume of Ringer's solution without bacteria.

The cheese was obtained after coagulation with animal rennet paste, whey draining and molding in plastic moulds. The cheeses were ripened for three months in a storage chamber at 13 °C and 80% of relative humidity. Both EP and CP cheese trials were carried out in duplicate in two consecutive weeks. The following samples were collected during each cheese production: samples from curds, whey, ricotta, deproteinized whey, and cheese at 0, 30, 60, and 90 days of ripening. The experimental design of cheese productions performed at pilot plant scale is reported graphically in Figure 1.

Microbiological analyses

Whey and deproteinized whey were subjected to decimal serial dilutions in Ringer's solution (1:10), while curd, ricotta, and cheese samples were first homogenized by the stomacher (400 Circulator Bags; Seward, AK, USA) for 10 min at 260 rpm in Ringer's solution and then subjected to the serial decimal dilution. Enumeration of LAB and pathogenic bacteria was carried out as follows: mesophilic and thermophilic rod-shaped LAB on de Man-Rogosa-Sharpe (MRS) agar acidified with 5 M lactic acid to pH 5.4 incubated anaerobically at 30 and 44 °C for 48 h, respectively, using the AnaeroGen AN25 (Oxoid, Milan, Italy) in jars closed hermetically;

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mesophilic and thermophilic coccus-shaped LAB on Medium 17 (M17) agar incubated aerobically for 48 h at 30 and 44 °C, respectively (Aureli et al., 2008); *E. coli* O157 on the Chromogenic Media (CHROMagar™ *E.coli*) incubated aerobically for 24 h at 37 °C; *L. monocytogenes* on Agar *Listeria* acc. Ottaviani & Agosti (ALOA) incubated aerobically for 24-48 h at 37 °C; *S.* Enteritidis on Xylose Lysine Deoxycholate (XLD) agar incubated aerobically for 24 h at 37 °C; *S. aureus* on Baird Parker (BP) agar added with rabbit plasma fibrinogen incubated aerobically for 48 h at 37 °C. For the last four microorganisms in order to lowered the limit of enumeration, 1 ml of the initial suspension was spread plated on the surface of three plates (90 mm) of the optimal growth media according to the ISO 7218 (2007). Detection of *E. coli* O157, *L. monocytogenes*, and *S.* Enteritidis was carried out on 25 ml of liquid (whey) samples or 25 g of solid (curd, ricotta and cheese) samples after enrichment on selective broth media as reported by Scatassa et al. (2015). All media were purchased from Oxoid. Microbiological counts were performed in duplicate.

Monitoring of the bacterial inoculums

The presence of the dairy pathogen bacteria added in cheese productions was confirmed after colony isolation, purification, and microscopic inspection determined using an optical microscope at 100x (Optika P800 series, Ponteranica, Italy) and Randomly Amplified Polymorphic DNA (RAPD)-Polymerase Chain Reaction (PCR) analysis. For the last analysis, the DNAs from dairy pathogen bacteria cultures were extracted by DNA-SORB-B (Sacace Biotechnologies Srl, Como, Italy) following the manufacturer's instructions. Crude cell extracts were used as template for PCR. RAPD-PCR was performed in 25 µl reaction volume using the single primers M13, AB111, and AB106 (Settanni et al., 2012). PCR mixture included 62.5 ng of target DNA, 2.5 µl of PCR buffer (Fermentas, MMedical, Milan, Italy), 2.5 mM of $MgCl_2$, 250 µM of each dNTP (Life Technologies Italia, Monza, Italy), 0.2 µM of each primer, 2.5 U of Taq DNA polymerase (ThermoFisher Scientific, Monza, Italy), and Milli-Q water to reach the final reaction volume. The PCR program applied for all primers comprised 40 cycles of denaturation for 2 min at 94 °C, annealing for 20 s at 40 °C, and extension for 2 min at 72 °C; the cycles were preceded by denaturation at 94 °C for 2 min and followed by extension at 72 °C for 5 min. The amplifications were performed using a Thermal cycler (Bioer, Hangzhou, China) in order to generate the amplicons which were separated by electrophoresis on 2% (w/v) agarose gels (SeaKem® LE Agarose, Lonza, Rockland, ME, USA), visualized, and acquired by Gel Doc™ XR+ and ChemiDoc ™ XRS+Imaging Systems (Bio Rad, Hercules, CA, USA). In order to perform the recognition of the added pathogenic dairy bacteria, the software package Gelcompare II Version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used.

Statistical analyses

Microbiological data were subjected to One-Way Variance Analysis (ANOVA). Pair comparison of treatment means was achieved by Tukey's test at $p<0.05$. Statistical analysis was conducted using XLStat software version 7.5.2 for excel (Addinsoft, New York, USA).

Results

The viable cell counts for the samples collected in this study are reported in Table 1. The detailed results of microbial loads on ricotta and deproteinized whey are not reported, because all these samples showed undetectable levels for all microbial populations investigated. According to Tukey's test, statistical significant differences were not found for the LAB group object of investigation among the two CP and EP trials.

The levels of mesophilic and thermophilic coccus and rod LAB in curd were comparable at about 5-6 log CFU/ml in both trials. The whey was characterized by a decrease of about 2 log cycles for the mesophilic and thermophilic cocci, while a slight reduction was observed for the other groups. LAB populations dominated in both cheese groups and reached values between 8-9 log CFU/g. These levels remained almost constant in all cheeses until the 90th day of ripening. Statistical significant differences ($p<0.05$) were found for the four main dairy pathogenic bacteria among CP and EP. All four dairy pathogenic bacteria were found in EP curds at the same or higher levels than inoculated in milk, while in CP curds only *S. aureus* was found. After that, the levels of *E. coli* O157, *L. monocytogenes*, and *S.* Enteritidis decreased continuously and were not found from 60 days onward. In particular, *E. coli* O157 was below the detection limit after 30 days of ripening but it was detected in 25 g of cheese after enrichment on selective broth media. Regarding *S. aureus*, this species was found until 30 and 60 days of ripening in CP and EP, respectively.

The RAPD profiles of the isolates were collected at the highest dilutions in CP samples only for *S. aureus* and in EP samples for the four dairy pathogenic bacteria were compared to those of the pure cultures, in order to evaluate their persistence. RAPD profiles were analyzed separately for each pathogenic bacteria resulting in two dendrograms. The direct comparison of the polymorphic profiles by the software Gelcompare II confirmed the results of the plate counts. Indeed, the dendrogram

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obtained with the RAPD profiles of *S. aureus* (Figure 2) showed that this species was present in CP, but the RAPD profiles were different from those of the strain ATCC 33862; while in EP productions clearly demonstrated the presence of the added strain during production. The other dendrogram allowed the only recognition of *E. coli* O157, *L. monocytogenes*, and *Salmonella* spp. added in EP productions (Figure 3).

Discussion

Fermentation of traditional Sicilian cheeses is a biological phenomenon carried out by the indigenous LAB of milk and of the transformation environment (Franciosi et al., 2008; Settanni and Moschetti, 2014). The ability of these microorganisms to growth in dairy products relies on their capacity to compete with other bacteria and overcome the adverse conditions encountered during ripening represented by the physicochemical factors such as low temperature, low pH, and high salt content (Jay et al., 2009). Several studies have been performed on the microbiological, physicochemical, and sensory properties of different cheeses produced in Sicily, Italy (Carpino et al., 2017; Gaglio et al., 2019b; Guarcello et al., 2016; Guarrasi et al., 2017). These studies were mainly focused on the ability of LAB to improve the quality of the final products.

Regarding the artisanal Sicilian cheeses production obtained using wooden equipment, Cruciata et al. (2018) conducted artificial contamination test with *E. coli* O157, *L. monocytogenes*, *Salmonella* spp., and *S. aureus* in order to evaluate their attachment to the wooden vat surfaces during the competition with indigenous LAB. These authors showed that LAB biofilm present on wooden vat surfaces inhibit the adhesion and survival of the main dairy pathogens. However, only limited information is available on the behavior of the main dairy pathogenic bacteria during the manufacture and ripening of traditional cheeses. This is the first work focused on the ability to grow of *E. coli* O157, *L. monocytogenes*, *S.* Enteritidis, and *S. aureus* during the manufacturing process of raw ewes' milk PDO Pecorino Siciliano cheese.

Table 1: Microbial evolution of LAB and pathogenic bacteria during experimental PDO Pecorino Siciliano cheese production

Samples	Microorganisms (log CFU/g)							
	Mesophilic rod LAB	Thermophilic rod LAB	Mesophilic coccus LAB	Thermophilic coccus LAB	*E. coli* O157	*L. monocytogenes*	*S.* Enteriditis	*S. aureus*
Control Production (CP)								
C	6.13±0.25 ^{a}	5.02±0.44 ^{a}	6.37±0.40 ^{a}	5.89±0.28 ^{a}	ND	ND	ND	2.70±0.35 ^{b}
W	3.75±0.22 ^{a}	3.70±0.23 ^{a}	4.56±0.20 ^{a}	4.75±0.27 ^{a}	ND	ND	ND	1.25±0.22 ^{b}
Cht_0	8.14±0.29 ^{a}	8.08±0.31 ^{a}	8.64±0.28 ^{a}	8.47±0.18 ^{a}	ND	ND	ND	3.05±0.30 ^{b}
Cht_{30}	8.64±0.37 ^{a}	8.75±0.45 ^{a}	8.98±0.15 ^{a}	9.02±0.18 ^{a}	ND	ND	ND	1.74±0.36 ^{a}
Cht_{60}	8.70±0.25 ^{a}	8.64±0.33 ^{a}	8.89±0.22 ^{a}	8.95±0.41 ^{a}	ND	ND	ND	<1 ^{b}
Cht_{90}	8.66±0.21 ^{a}	8.55±0.19 ^{a}	8.71±0.39 ^{a}	8.87±0.27 ^{a}	ND	ND	ND	<1 ^{a}
Experimental Production (EP)								
C	6.29±0.39 ^{a}	5.40±0.20 ^{a}	6.49±0.33 ^{a}	6.23±0.36 ^{a}	2.80±0.25	2.38±0.41	2.25±0.33	4.12±0.13 ^{a}
W	4.02±0.27 ^{a}	3.86±0.31 ^{a}	4.85±0.23 ^{a}	4.86±0.31 ^{a}	D	1.46±0.19	D	2.21±0.31 ^{a}
Cht_0	8.39±0.44 ^{a}	7.80±0.16 ^{a}	8.45±0.35 ^{a}	8.34±0.23 ^{a}	3.41±0.12	2.69±0.36	2.53±0.18	4.35±0.20 ^{a}
Cht_{30}	8.65±0.13 ^{a}	8.46±0.40 ^{a}	8.80±0.16 ^{a}	8.85±0.23 ^{a}	D	2.01±0.24	1.12±0.11	2.16±0.42 ^{a}
Cht_{60}	8.81±0.31 ^{a}	8.60±0.17 ^{a}	8.97±0.21 ^{a}	8.77±0.33 ^{a}	ND	ND	ND	1.10±0.21 ^{a}
Cht_{90}	8.71±0.25 ^{a}	8.41±0.40 ^{a}	8.80±0.32 ^{a}	8.70±0.23 ^{a}	ND	ND	ND	<1 ^{a}
Statistical significance (CP * EP)	NS	NS	NS	NS	S	S	S	S

-Units are log CFU/ml for whey samples and log CFU/g for curd and cheese samples. Results indicate mean values±SD of four plate counts (carried out in duplicate for two independent productions).
-Data within a line followed by the same letter in the same sample of CP and EP are not significantly different according to Tukey's test. S: Significant ($p \leq 0.001$); NS: Not Significant.
C: curd; W: whey; Ch: cheese at 0, 30, 60 and 90 days of ripening; ND: Not Detected on 25 g or 25 ml of sample after enrichment on selective broth media; D: detected on 25 ml of whey samples or 25 g of cheese samples after enrichment on selective broth media.

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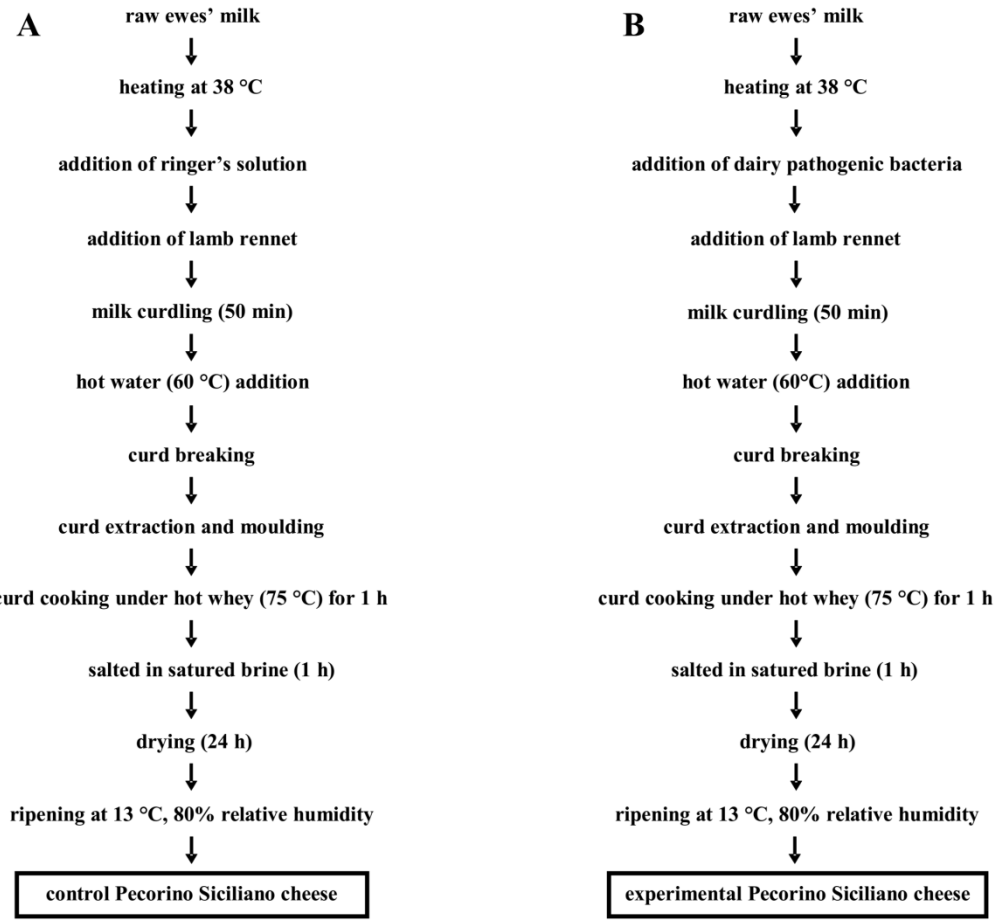

Figure 1: Flow diagram of Pecorino Siciliano cheese production. A: control production; B: experimental production

In the current research, the evolution of LAB populations from curds until the end of ripening was comparable for control production and cheese making artificially contaminated. These microorganisms were found at high numbers in curd and their levels increased during ripening. This trend was observed in the other study carried out on PDO Pecorino Siciliano cheese (Cardamone et al., 2018; Scatassa et al., 2017) as well as on similar Italian (Caridi et al., 2003) and Greek cheeses (Hatzikamari et al., 1999). All these study reported that levels of LAB population increased around 1-2 log cycles from curd to cheese soon after production, and then reached values higher than 8 $\log_{10}$ CFU/g in the final cheese. Vernile et al. (2006) showed lower levels of mesophilic rod and coccus LAB (about 7 $\log_{10}$) for PDO Pecorino Siciliano cheese ripened for 90 days.

The specific investigation of pathogenic bacteria carried out by Scatassa et al. (2018) on ricotta cheese and deproteinized whey did not generate any colony. Their absence could be due to heat treatment (80-84 °C) the whey underwent during ricotta cheese production. Even though, LAB in the present study was below the detection limits, their presence was reported for ricotta cheese (Mancuso et al., 2014; Spanu et al., 2017).

A slight increasing trend for the levels of the four dairy pathogenic bacteria was observed from curd to cheese soon after production, followed by a decrease during ripening. This trend was observed in other reports on the behavior of *E. coli* O157 (Cardamone et al., 2018) and *L. monocytogenes* (Scatassa et al., 2017) during the manufacture and ripening of PDO Pecorino Siciliano cheese. In particular, our data showed higher levels of *L. monocytoges* in PDO Pecorino Siciliano cheese at 30 days of ripening with respect to the work performed by Scatassa et al. (2017) that showed a significant reduction by approximately 3 log cycles of these dairy pathogenic

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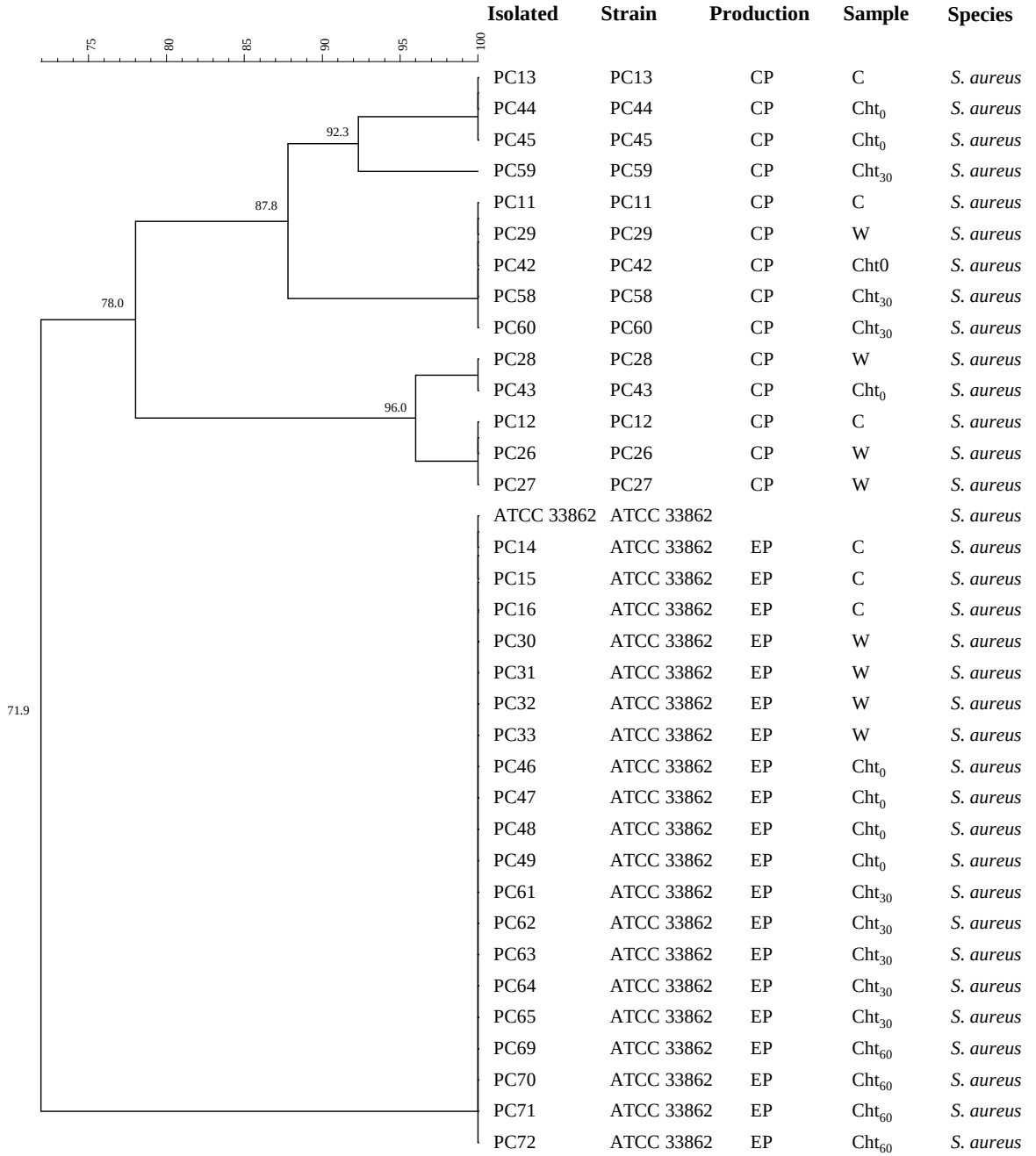
bacteria from inoculated milk to cheese at 15 days of ripening. We found that *E. coli* O157 was completely disappeared at 30 days of ripening, while Cardamone et al. (2018) denoted this pathogen ability to survive until 60 days of ripening of PDO Pecorino Siciliano cheese.



Isolated	Strain	Production	Sample	Species
PC13	PC13	CP	C	*S. aureus*
PC44	PC44	CP	Cht_0	*S. aureus*
PC45	PC45	CP	Cht_0	*S. aureus*
PC59	PC59	CP	Cht_{30}	*S. aureus*
PC11	PC11	CP	C	*S. aureus*
PC29	PC29	CP	W	*S. aureus*
PC42	PC42	CP	Cht0	*S. aureus*
PC58	PC58	CP	Cht_{30}	*S. aureus*
PC60	PC60	CP	Cht_{30}	*S. aureus*
PC28	PC28	CP	W	*S. aureus*
PC43	PC43	CP	Cht_0	*S. aureus*
PC12	PC12	CP	C	*S. aureus*
PC26	PC26	CP	W	*S. aureus*
PC27	PC27	CP	W	*S. aureus*
ATCC 33862	ATCC 33862			*S. aureus*
PC14	ATCC 33862	EP	C	*S. aureus*
PC15	ATCC 33862	EP	C	*S. aureus*
PC16	ATCC 33862	EP	C	*S. aureus*
PC30	ATCC 33862	EP	W	*S. aureus*
PC31	ATCC 33862	EP	W	*S. aureus*
PC32	ATCC 33862	EP	W	*S. aureus*
PC33	ATCC 33862	EP	W	*S. aureus*
PC46	ATCC 33862	EP	Cht_0	*S. aureus*
PC47	ATCC 33862	EP	Cht_0	*S. aureus*
PC48	ATCC 33862	EP	Cht_0	*S. aureus*
PC49	ATCC 33862	EP	Cht_0	*S. aureus*
PC61	ATCC 33862	EP	Cht_{30}	*S. aureus*
PC62	ATCC 33862	EP	Cht_{30}	*S. aureus*
PC63	ATCC 33862	EP	Cht_{30}	*S. aureus*
PC64	ATCC 33862	EP	Cht_{30}	*S. aureus*
PC65	ATCC 33862	EP	Cht_{30}	*S. aureus*
PC69	ATCC 33862	EP	Cht_{60}	*S. aureus*
PC70	ATCC 33862	EP	Cht_{60}	*S. aureus*
PC71	ATCC 33862	EP	Cht_{60}	*S. aureus*
PC72	ATCC 33862	EP	Cht_{60}	*S. aureus*

Figure 2: Dendrogram obtained with combined RAPD-PCR patterns generated with three primers for *S. aureus* strains isolated from samples collected during cheese productions. The line at the top indicates percentages of similarity. CP: Control Production; EP: Experimental Production; C: Curd; W: Whey; Ch: Cheese at 0, 30 and 60 days of ripening

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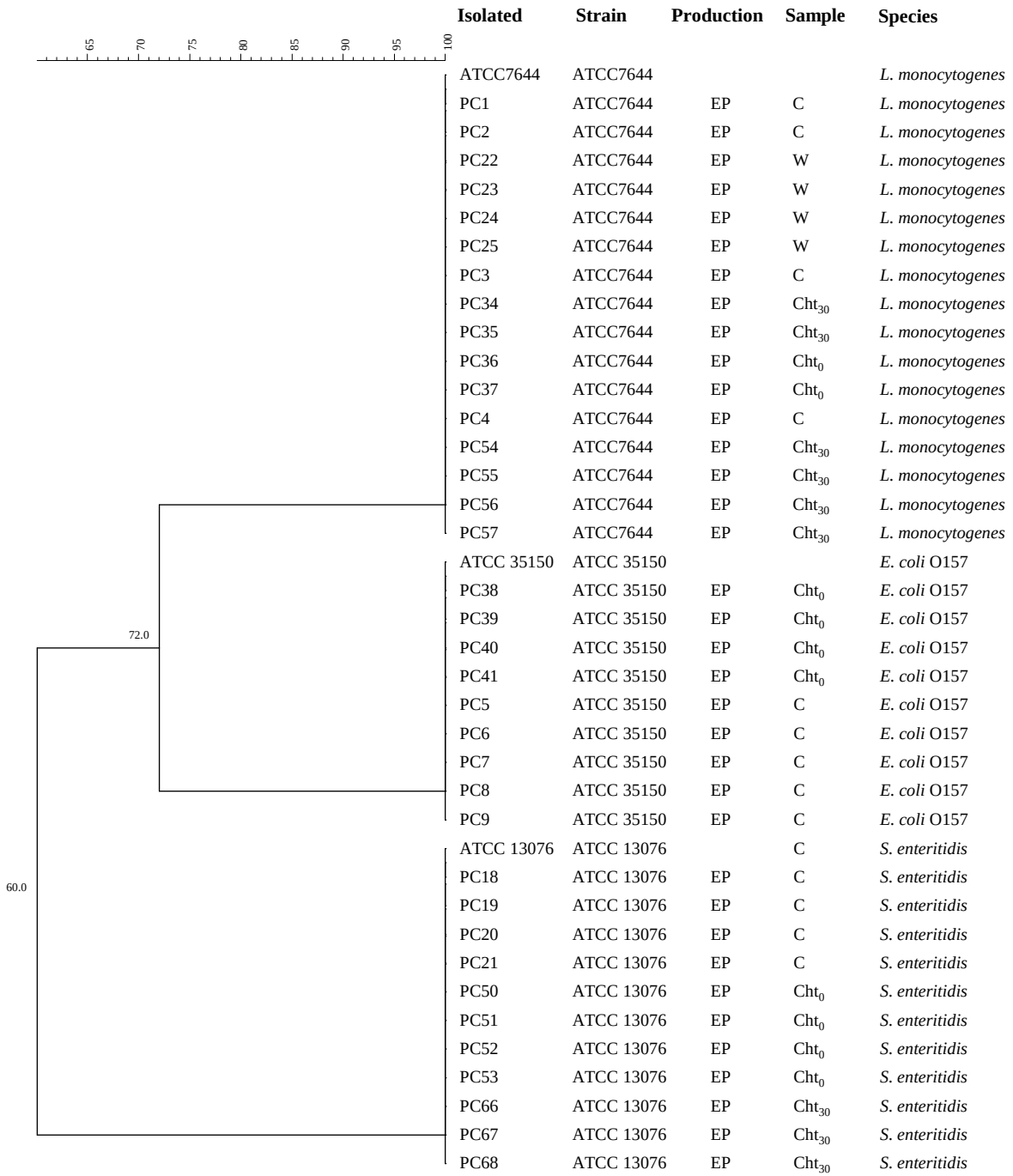

Isolated	Strain	Production	Sample	Species
ATCC7644	ATCC7644			*L. monocytogenes*
PC1	ATCC7644	EP	C	*L. monocytogenes*
PC2	ATCC7644	EP	C	*L. monocytogenes*
PC22	ATCC7644	EP	W	*L. monocytogenes*
PC23	ATCC7644	EP	W	*L. monocytogenes*
PC24	ATCC7644	EP	W	*L. monocytogenes*
PC25	ATCC7644	EP	W	*L. monocytogenes*
PC3	ATCC7644	EP	C	*L. monocytogenes*
PC34	ATCC7644	EP	Cht_{30}	*L. monocytogenes*
PC35	ATCC7644	EP	Cht_{30}	*L. monocytogenes*
PC36	ATCC7644	EP	Cht_{0}	*L. monocytogenes*
PC37	ATCC7644	EP	Cht_{0}	*L. monocytogenes*
PC4	ATCC7644	EP	C	*L. monocytogenes*
PC54	ATCC7644	EP	Cht_{30}	*L. monocytogenes*
PC55	ATCC7644	EP	Cht_{30}	*L. monocytogenes*
PC56	ATCC7644	EP	Cht_{30}	*L. monocytogenes*
PC57	ATCC7644	EP	Cht_{30}	*L. monocytogenes*
ATCC 35150	ATCC 35150			*E. coli* O157
PC38	ATCC 35150	EP	Cht_{0}	*E. coli* O157
PC39	ATCC 35150	EP	Cht_{0}	*E. coli* O157
PC40	ATCC 35150	EP	Cht_{0}	*E. coli* O157
PC41	ATCC 35150	EP	Cht_{0}	*E. coli* O157
PC5	ATCC 35150	EP	C	*E. coli* O157
PC6	ATCC 35150	EP	C	*E. coli* O157
PC7	ATCC 35150	EP	C	*E. coli* O157
PC8	ATCC 35150	EP	C	*E. coli* O157
PC9	ATCC 35150	EP	C	*E. coli* O157
ATCC 13076	ATCC 13076		C	*S. enteritidis*
PC18	ATCC 13076	EP	C	*S. enteritidis*
PC19	ATCC 13076	EP	C	*S. enteritidis*
PC20	ATCC 13076	EP	C	*S. enteritidis*
PC21	ATCC 13076	EP	C	*S. enteritidis*
PC50	ATCC 13076	EP	Cht_{0}	*S. enteritidis*
PC51	ATCC 13076	EP	Cht_{0}	*S. enteritidis*
PC52	ATCC 13076	EP	Cht_{0}	*S. enteritidis*
PC53	ATCC 13076	EP	Cht_{0}	*S. enteritidis*
PC66	ATCC 13076	EP	Cht_{30}	*S. enteritidis*
PC67	ATCC 13076	EP	Cht_{30}	*S. enteritidis*
PC68	ATCC 13076	EP	Cht_{30}	*S. enteritidis*

Figure 3: Dendrogram obtained with combined RAPD-PCR patterns generated with three primers for *L. monocytogenes*, *E. coli* O157, and *S.* Enteritidis strains isolated from samples collected during cheese productions. The line at the top indicates percentages of similarity. EP: Experimental Production; C: Curd; W: Whey; Ch: Cheese at 0, 30 and 60 days of ripening

Conclusion

This study demonstrated that the production conditions of PDO Pecorino Siciliano cheese decreased growth of *E. coli* O157, *L. monocytogenes*, *S.* Enteritidis, and also *S. aureus*. However, *E. coli* O157, *L. monocytogenes*, and *S.* Enteritidis were able to survive in PDO Pecorino Siciliano cheese until the 30 days of ripening, while, *S. aureus* until the 60 days of ripening. Further studies are needed in order to better investigate the contribution of other bacterial related factors such as organic acid production and bacteriocin generation by the indigenous LAB populations.

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Author contributions

R.G. and M.L.S. designed the project of study; F.C., F.D., S.S., and V.P. conducted the experiments; I.M., C.C., and M.L.S. analyzed the data; R.G. and M.L.S. wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

There was no conflict of interest in this study.

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