Investigation on the microbiological hazards in an artisanal soft cheese produced in northern Italy and its production environment in different seasonal periods

Cecilia Crippa,[1] Frédérique Pasquali,[1] Alex Lucchi,[1] Lucia Gambi,[1] Alessandra De Cesare[2]

[1]Department of Agricultural and Food Sciences, and [2]Department of Veterinary Medical Sciences, University of Bologna, Italy

Abstract

The present study aimed at assessing the occurrence of microbiological hazards (*Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella spp*. and *Escherichia coli* O157) in an artisanal soft cheese produced in northern Italy. In the same product total bacterial count, lactic acid bacteria and *Enterobacteriaceae* were enumerated, and pH and water activity measured in two batches sampled in summer and winter. Samples of raw materials, environmental swabs from the production processes and cheese during 15 days of storage at 2 and 8°C as well as dynamic temperature of 2°C for 5 days and 8°C for 10 days were collected and tested. The load of total bacterial count was significantly higher in the winter batch in comparison to the summer one, with a significant increase at the end of the storage period also noticed for lactic acid bacteria. Statistical higher values of pH were registered in raw materials and end of storage in winter batch. *S. aureus* was confirmed only in the winter batch within samples (n=4) of stored cheese. On plates used for *E. coli* O157 detection, colonies of *Klebsiella pneumoniae* and *Klebsiella oxytoca* were isolated. The results suggest that the highest bacterial population in the winter batch was associated to a higher pH in stored cheese and a higher number of biological hazards identified. Their isolation started in the maturation room suggesting this step as relevant for possible cheese contamination.

Introduction

The consumer demand of artisanal foods has been increasing in popularity. These foods are perceived as more genuine and homemade with high quality ingredients. On top of this, these foods are often locally produced with local raw ingredients. The specific local know how, authenticity and agro-environmental conditions of some artisanal foods are protected from imitation and misuse by EU geographical indication schemes such as protected designation of origin (PDO) (EU, 2012). Italy is the EU Country with the highest number of PDOs. A PDO food is a food produced in a specific geographic region following a traditional production process as described in its Product Specification document. Moreover, for PDOs raw ingredients must come from the same region in which the food is produced (EU, 2012). In this research an artisanal soft cheese produced in northern Italy has been tested. The tested cheese is made from pasteurized cow's milk and appears white, shapeless and grainy, with a delicate and sweet taste. From a food safety perspective, soft cheeses can act as a vehicle of transmission to humans, of biological hazard such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella spp*. and verotoxigenic *Escherichia coli* (VTEC) (Choi *et al*., 2016). Because of its tolerance to a wide range of physicochemical conditions, *L. monocytogene*s can grow and survive in the environment of the processing plant and contaminate food during its production. Regarding soft cheese, thirty percent of *L. monocytogenes* outbreaks in US from 1998 and 2014, were associated with soft cheese and resulted in 180 illnesses (Jackson *et al*., 2018). Besides US, fresh and/or soft cheeses have been reported as involved in *L. monocytogenes* outbreaks in Sweden, Canada, Czech Republic, Austria and Portugal (Martinez-Rios and Dalgaard, 2018). *S. aureus* often causes mastitis in cows, leading to milk contamination (Rabello *et al*., 2007). It is also an inhabitant of skin and nasal cavity of warm-blooded animals. Human beings at the operational environment were described as one of the main sources of product contamination. A pH under 5.3, to be reached as soon as possible, is suggested to prevent *S. aureus* growth in cheese. A soft cheese made of raw milk was associated to an outbreak in Sweden in 2014 (Johler *et al*., 2015a).

Several outbreaks of *S. aureus* have been associated to the consumption of contaminated milk or cheese (Johler *et al*., 2015a; Johler *et al*., 2015b). In Italy *S. aureus* was isolated from raw milk and artisanal raw milk cheeses (Johler *et al*., 2018). *Salmonella spp.,* although rarely attributed to dairy products, it has been recently associated to a cheese outbreak in US in 2021. The involved serovar was *Salmonella* Duisburg (CDC, 2021). As a consequence of faecal contamination, *Escherichia coli* VTEC can be isolated from raw milk. VTEC was associated to outbreaks related to the consumption of raw milk and raw milk cheeses. Although inactivated by pasteurization, VTEC can contaminate cheese during manufacturing, being able to persist in the final product (Dos Santos *et al*., 2021).

The risk of contamination is of particular concern in artisanal foods for which the

Correspondence: Cecilia Crippa, Department of Agricultural and Food Sciences, Alma Mater Studiorum – University of Bologna, Via del Florio 2, 40064 Ozzano dell'Emilia (BO), Italy.
Tel.: +39.051.2097900.
E-mail: cecilia.crippa2@unibo.it

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control of the process is often challenging due to the lack of a full automation of the production process and control of environmental parameters. In this framework, monitoring the microbial quality of the food and the environment is important to minimise safety risks for the consumers. In the milk and cheese industry, standard microbial testing focuses on enumeration of total mesophilic bacteria, as well as enterobacteria as indicator bacteria for contamination (Fusco *et al*., 2020).

The present study aimed to assess the occurrence of *L. monocytogenes*, *S. aureus*, *Salmonella spp*. and *E. coli* O157, as well as the enumeration of total bacterial count, lactic acid bacteria and *Enterobacteriaceae* along the analysis of pH and water activity,

Table 1. Lactic acid bacteria and total bacteria count in samples of raw materials, the environment and final products of soft cheese in the two tested batches.

Sample	N° tested samples	Summer batch	Winter batch
Lactic acid bacteria (mean log_{10} CFU/g)			
Raw materials and cheese during processing			
Milk post pasteurisation	5	1.00±0.22	<1.00±0.00
Cheese at the end of the storage in warm room	5	2.50±0.82	<1.00±0.00
Cheese at the end of the maturation process	5	1.67±0.19	1.35±0.41
Final product			
Cheese after packaging	5	1.64±0.27	1.34±0.60
Cheese packed day 1 at °2C	5	2.02±0.48	1.28±0.43
Cheese packed day 1 at °8C	5	1.35±0.77	2.07±1.22
Cheese packed day 1 at 2/8°C	5	1.75±0.23	1.26±0.40
Cheese packed day 4 at °2C	5	1.95±0.14	1.33±0.76
Cheese packed day 4 at °8C	5	1.79±0.35	1.14±0.72
Cheese packed day 4 at 2/8°C	5	1.80±0.17	1.24±0.69
Cheese packed day 8 at °2C	5	2.19±0.57	1.25±0.71
Cheese packed day 8 at °8C	5	3.62±0.27	3.70±0.19
Cheese packed day 8 at 2/8°C	5	1.91±0.42	2.13±0.56
Cheese packed day 11 at °2C	5	2.17±0.41	1.27±0.76
Cheese packed day 11 at °8C	5	3.06±0.46a*	4.76±0.18b
Cheese packed day 11 at 2/8°C	5	2.47±0.39a	3.94±0.58b
Cheese packed day 15 at °2C	5	2.11±0.23a	4.86±0.03b
Cheese packed day 15 at °8C	5	4.31±0.46	5.31±0.18
Cheese packed day 15 at 2/8°C	5	2.40±0.61a	4.99±0.10b
Total bacteria count (mean log_{10} CFU/g)			
Raw materials and cheese during processing			
Milk before pasteurisation	5	6.28±0.22	6.56±0.11
Milk post pasteurisation	5	3.52±0.13	2.36±0.30
Calf rennet	5	0.90±0.83	2.76±0.56
Cheese at the end of the storage in warm room	5	5.03±0.09	5.27±0.07
Cheese at the end of the maturation process	5	5.59±0.09	5.87±0.05
Environmental samples			
Warm room environmental swabs	5	4.32±0.46	2.86±0.43
Water drainage channel swab within the warm room	5	7.35±0.28	6.88±0.44
Maturation room environmental swab	5	2.52±1.90	3.75±0.15
Water drainage channel swab within the maturation room	5	4.70±1.55	5.40±0.51
Worker gloves swab	5	2.61±0.45	2.69±0.75
Water drainage channel swab within the packaging room	5	5.24±0.27	4.64±0.68
Final product			
Cheese after packaging	5	5.63±0.06	5.90±0.16
Cheese packed day 1 at °2C	5	5.48±0.09	6.00±0.12
Cheese packed day 1 at °8C	5	5.50±0.04	5.87±0.09
Cheese packed day 1 at 2/8°C	5	5.48±0.06	5.96±0.09
Cheese packed day 4 at °2C	5	5.50±0.12	5.93±0.12
Cheese packed day 4 at °8C	5	5.59±0.05	5.92±0.03
Cheese packed day 4 at 2/8°C	5	5.55±0.09	5.92±0.07
Cheese packed day 8 at °2C	5	5.46±0.11	6.21±0.06
Cheese packed day 8 at °8C	5	5.51±0.07	6.48±0.43
Cheese packed day 8 at 2/8°C	5	5.52±0.08	6.14±0.08
Cheese packed day 11 at °2C	5	5.53±0.08	6.22±0.05
Cheese packed day 11 at °8C	5	5.61±0.13	7.33±0.10
Cheese packed day 11 at 2/8°C	5	5.64±0.22[a]	7.71±0.17[b]
Cheese packed day 15 at °2C	5	5.59±0.04	6.84±0.34
Cheese packed day 15 at °8C	5	5.72±0.07[a]	8.03±0.43[b]
Cheese packed day 15 at 2/8°C	5	5.64±0.03[a]	7.71±0.61[b]

[a,b]Values with different letters within a row are significantly different (P<0.05).

of two batches belonging to summer and winter season respectively, in order to compare the occurrence of biological hazards along with the microbiological quality and physicochemical parameters of cheese and environmental samples belonging to the two batches and collected along the production process as well as during 15 days of storage at 2°C, 8°C and dynamic temperature of 2°C for 5 days and 8°C for 10 days. Static and dynamic-abuse temperature were tested, besides refrigeration temperature, in order to test whether changes in storage temperature conditions might affect bacterial growth.

Materials and methods

In the present study, two batches of an artisanal soft cheese produced in northern Italy were examined, respectively produced in two different seasons: July 2020 (summer batch) and January 2021 (winter batch). Cheese was manufactured in an artisanal small-scale plant from pasteurized cow milk added by salt and calf rennet, with no preservatives added. The maturation of cheese lasted 3-4 days and, after being packed in air, each single cheese unit was stored up to 15 days at three different temperatures: 2°C (standard refrigeration tem-

Table 2. pH and water activity in raw materials and final products of soft cheese in the two tested batches.

Sample	N° tested samples	Summer batch	Winter batch
pH mean value			
Raw materials and cheese during processing			
Milk before pasteurisation	5	6.78±0.01	6.76±0.01
Milk post pasteurisation	5	6.71±0.03	6.72±0.02
Calf rennet	5	5.09±0.00[a]	5.30±0.04[b]
Cheese at the end of the storage in warm room	5	5.87±0.04	5.81±0.04
Cheese at the end of the maturation process	5	5.27±0.03[a]	5.54±0.05[b]
Final product			
Cheese after packaging	5	5.24±0.02[a]	5.47±0.03[b]
Cheese packed day 1 at °2C	5	5.36±0.02	5.32±0.08
Cheese packed day 1 at °8C	5	5.35±0.01	5.36±0.02
Cheese packed day 1 at 2/8°C	5	5.37±0.02	5.26±0.03
Cheese packed day 4 at °2C	5	5.37±0.04	5.36±0.03
Cheese packed day 4 at °8C	5	5.29±0.02	5.31±0.02
Cheese packed day 4 at 2/8°C	5	5.28±0.02	5.36±0.02
Cheese packed day 8 at °2C	5	5.41±0.13b	5.23±0.03[a]
Cheese packed day 8 at °8C	5	5.24±0.01	5.25±0.02
Cheese packed day 8 at 2/8°C	5	5.28±0.01	5.29±0.02
Cheese packed day 11 at °2C	5	5.23±0.09	5.27±0.01
Cheese packed day 11 at °8C	5	5.12±0.01	5.12±0.01
Cheese packed day 11 at 2/8°C	5	5.21±0.01	5.21±0.01
Cheese packed day 15 at °2C	5	5.24±0.08[a]	5.44±0.10[b]
Cheese packed day 15 at °8C	5	5.17±0.01[a]	5.35±0.06[b]
Cheese packed day 15 at 2/8°C	5	5.16±0.01[a]	5.43±0.03[b]
Water activity, mean value			
Raw materials and cheese during processing			
Milk before pasteurisation	5	0.9966±0.0009	1.0016±0.0018
Milk post pasteurisation	5	0.9972±0.0005	1.0026±0.0009
Calf rennet	5	0.8262±0.0007	0.8663±0.0043
Cheese at the end of the storage in warm room	5	0.9911±0.0021	0.9957±0.0012
Cheese at the end of the maturation process	5	0.9921±0.0007	0.9962±0.0030
Final product			
Cheese after packaging	5	0.9916±0.0004	0.9949±0.0020
Cheese packed day 1 at °2C	5	0.9914±0.0004	0.9942±0.0011
Cheese packed day 1 at °8C	5	0.9909±0.0004	0.9944±0.0016
Cheese packed day 1 at 2/8°C	5	0.9913±0.0003	0.9941±0.0011
Cheese packed day 4 at °2C	5	0.9908±0.0004	0.9945±0.0030
Cheese packed day 4 at °8C	5	0.9912±0.0002	0.9950±0.0015
Cheese packed day 4 at 2/8°C	5	0.9919±0.0007	0.9982±0.0038
Cheese packed day 8 at °2C	5	0.9917±0.0020	0.9963±0.0013
Cheese packed day 8 at °8C	5	0.9896±0.0025	0.9969±0.0018
Cheese packed day 8 at 2/8°C	5	0.9922±0.0003	1.0016±0.0040
Cheese packed day 11 at °2C	5	0.9950±0.0005	1.0014±0.0027
Cheese packed day 11 at °8C	5	0.9952±0.0002	1.0017±0.0011
Cheese packed day 11 at 2/8°C	5	0.9948±0.0006	1.0005±0.0068
Cheese packed day 15 at °2C	5	0.9935±0.0009	0.9949±0.0005
Cheese packed day 15 at °8C	5	0.9956±0.0011	0.9959±0.0004
Cheese packed day 15 at 2/8°C	5	0.9970±0.0008	0.9965±0.0006

[a,b]Values with different letters within a row are significantly different (P<0.05).

perature), 8°C (static-abuse temperature) and 2°C for 5 days and 8°C for the residual storage (dynamic-abuse temperature). Samples (n=5) of cheese were collected at 1, 4, 8, 11 and 15 days of storage along with raw materials (pasteurized and non-pasteurized milk and calf rennet) and environmental samples (swabs from walls, surfaces and water drainage channel located in each processing area). A total of 280 samples were collected. Samples of cheese, raw materials and the environment were submitted to enumeration of *Enterobacteriaceae* (ISO 21528-2:2017), lactic acid bacteria (ISO 15214:1998) and total bacteria count (ISO 4833-2:2013). Moreover, on the same samples, the detection of *Listeria monocytogenes* (ISO 11290-1:2017), *Salmonella spp*. (ISO 6579-1:2017), *Staphylococcus aureus* (ISO 6888-1/A1:2004) and *E. coli* O157 (ISO 16654:2001) was performed. Isolates were confirmed by biochemical test (RapID™ ONE System and RapID™ STAPH PLUS System, Thermo Scientific™) and PCR (Wesley *et al*., 2002; Perelle *et al*., 2004, Chander *et al*., 2011, Brakstad *et al*., 1992). Samples of raw materials and final products were also submitted to physicochemical analyses of pH (ISO 2917:1999) and water activity (ISO 21807:2004). Data obtained were submitted to analysis of variance (ANOVA test) followed by a Scheffé test for post-hoc comparative analysis in order to detect any significant differences ($p<0.05$) between batches, days and temperatures of storage.

Results

In Table 1, enumeration of lactic acid bacteria and total bacteria count are reported for both summer and winter batches. In both batches, the load of lactic acid bacteria increased during the shelf life of the cheese from day 0 to day 15 (Table 1). This increase was statistically significantly higher in the winter batch at the end of the storage period (days 11 and 15) especially at 8°C and 2/8°C. Similarly, the load of total bacteria counts in the winter batch increased significantly in comparison to the summer batch at the end of the storage period. No statistically significant differences were reported for raw materials and environmental samples between batches. All samples showed values of *Enterobacteriaceae* under the detection limit (<10 $\log_{10}$ CFU/g) in both batches (data not shown). Concerning pH, statistical higher values were registered in calf rennet, cheese in the maturation room and packed cheese of winter batch. Statistically significant differences were registered also at the end of storage with higher pH values in winter batch (Table 2).

Concerning tested biological hazards, *L. monocytogenes, Salmonella spp*. and *E. coli* O157 were never detected. Four *Staphylococcus aureus* isolates were collected from cheese of the winter batch during storage at day 1, 4, 8 and 11 at 2°C. (Table 3). In all samples but the four in which *S. aureus* was detected the number of coagulase positive staphylococci was lower than 100 CFU/g. In the four named samples this value reached approx 1000 CFU/g (data not shown). On plates used for the isolation of *E. coli*, colonies of *Klebsiella pneumoniae* and *Klebsiella oxytoca* were identified and confirmed by biochemical tests and PCR. Two isolates of *Klebsiella spp*., 2 and 9 of *K. oxytoca* were collected from cheese and maturation room in the summer and winter batches respectively (Table 3). Among final products samples, only one positive sample of *K. oxytoca* occurred for summer batch in cheese packed at day 0, whereas for winter batch positive samples were identified at day 0, 1 (2°C and 8°C), 4 (2°C and 2/8°C) and 11 (2°C and 8°C). *Klebsiella pneumoniae* occurred in both batches (n=1) on final product samples of cheese packed at 2°C, day 4 and 15 for summer and winter respectively (Table 3).

Discussion

The identification of *K. pneumoniae* and *K. oxytoca* is of particular relevance since these bacteria are pathogens of clinical importance. Both species are generally acquired by environmental sources and are often associated to bronchopneumonia, urinary tract infection and septicaemia in hospitalized patients. In caws, they are often associated to mastitis. Being the species *K. pneumoniae* the most frequently identified, *K. oxytoca* occurrence is emerging (Singh *et*

Table 3. Biological hazard identified in raw material, environment and final products of soft cheese in the two tested batches.

Sample	*Klebsiella oxytoca*	*Klebsiella pneumoniae*	*Staphylococcus aureus*
	Summer batch		
Environmental samples			
Maturation room wall swab	Detected		
Final product			
Cheese packed day 0	Detected		
Cheese packed day 4 - 2° C		Detected	
	Winter batch		
Cheese during processing			
Cheese at the end of the maturation process	Detected		
Environmental			
Water drainage channel swab within the maturation room	Detected		
Final product			
Cheese packed day 0	Detected		
Cheese packed day 1 - 2° C	Detected		Detected
Cheese packed day 1 - 8° C	Detected		
Cheese packed day 4 - 2° C	Detected		Detected
Cheese packed day 4 - 2/8° C	Detected		
Cheese packed day 8 - 2° C			Detected
Cheese packed day 11 - 2° C	Detected		Detected
Cheese packed day 11 - 8° C	Detected		
Cheese packed day 15 - 2° C		Detected	
Total positive samples	11	2	4

al., 2016). Both are clinical pathogens of concern due to their frequently identified character of antimicrobial resistance. Publications on the isolation of *Klebsiella spp*. in milk and cheese are available. Both species were isolated from spoiled Italian mozzarella, as well as from milk in Spain, from a ripened cheese made of raw caw's milk in Portugal and from soft cheese in India (Massa *et al*., 1992; Kongo *et al*., 2008; Vaishnavi *et al*., 2001; Tornadijo *et al*., 1993). Nonetheless, to the best of author's knowledge, no *Klebsiella* human infection has been associated to consumption of contaminated cheese so far, although this possibility cannot be ruled out. In the present study, *Klebsiella oxytoca* positive samples belonged to environmental and final product categories as well as cheese during processing. Regarding environmental samples, the maturation room represented the processing area where strains were detected, in swabs picked from walls and water drainage channel. Accordingly, *Klebsiella oxytoca* was identified also in cheese at the end of the maturation process in winter batch (Table 3). No *Klebsiella spp*. or *Staphylococcus aureus* were isolated from milk before pasteurisation, suggesting raw milk contamination as unlikely. The higher number of *K. oxytoca* isolates in comparison to *K. pneumoniae* ones might be related to the higher tolerance of *K. oxytoca* to pH values below 6.0 (Ajayasree *et al*., 2018).

Conclusions

Results of the present study suggest that the level of total bacteria count, and lactic acid bacteria was significantly higher in the winter batch in comparison to the summer one, with a higher increase at the end of storage at 8°C and dynamic-abuse (2/8°C) temperature. The highest bacterial population in the winter batch was associated to a higher pH in packed cheese which might also explain the isolation of *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Staphylococcus aureus*. The isolation of bacterial hazards in both batches started from the maturation room (from both cheese and environmental samples) suggesting this step at high risk for cheese contamination.

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