Behaviour of *Listeria monocytogenes* and *Escherichia coli* O157:H7 during the cheese making of traditional raw-milk cheeses from Italian Alps

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Abstract

The behaviour of *Listeria monocytogenes* and *Escherichia coli* O157:H7 was studied during the manufacture and ripening of two traditional Italian Alps cheeses. Each cheese type was manufactured in a pilot plan from raw cow milk (without the addition of starter cultures) artificially inoculated with *L. monocytogenes* and *E. coli* O157:H7 to a final concentration of about 4 log CFU/mL. The pathogens were enumerated throughout the cheese making and ripening processes to study their behaviour. When the milk was inoculated with 4 Log CFU/mL, the pathogens counts increased in the first time during the manufacturing process and then remained constant, until the end of ripening, or decreased significantly. Results indicate that the environment and nature of food borne pathogens affected the concentration of the bacteria during the manufacturing and ripening process. Thus, the presence of low cells numbers of *L. monocytogenes* and *E. coli* O157:H7 in milk destined for the production of raw milk cheeses characterized by a cooking of the curd less than 48°C can constitute a hazard for the consumer.

Introduction

The food business operators (FBOs) have to check the hygiene of their production following the European Commission (EC) Regulation No. 2073/2005 (European Commission, 2005). In the online database, Rapid Alert System for Food and Feed (RASFF), created by the EC, it is published that, in the last ten years, 55 alerts were regarding the presence of *Listeria monocytogenes* and verocytotoxin *Escherichia coli* (VTEC) in raw milk cheeses, mainly produced in France (RASFF, 2007). In fact, among dairy products, the raw milk cheese, characterized by the cooking of the curd at temperature less than 48°C, are known to be the most frequently contaminated (CDSC, 2000; Conedera *et al*., 2004; Bielaszewska *et al*., 1997; EFSA, 2013; Farrokh *et al*., 2013) and it is documented that contaminated raw milk cheeses, with short ripening time (less than 60 days) could generate severe outbreak (Health Canada, 2013).

Many regional cheeses throughout Europe are manufactured from unpasteurized milk, and there is growing concern that fresh cheeses, made by raw milk, could be contaminated by food pathogens (Vernozy-Rozand *et al.,* 2005). Traditional, raw milk cheeses, obtained by the cooking of the curd at temperature less than 48°C, are produced in Alps area, and while more data are available for the French cheeses (Miszczycha *et al*., 2013) few is known about the behaviour of food pathogens during the cheese making of Italian raw milk cheeses produced in Alps area.

The cheese manufacturing process affects strongly the eco-system in which the food pathogen could be present. The cheese making temperature, the pH and a_w reduction, the presence of indigenous bacterial population, are all variables that can modify the behaviour of undesirable bacteria (Buchanan *et al*., 1993). For this reason, many cheese making processes are registered in the Minister of Health web site on quality and safety of Italian food product (www.ars-alimentaria.it).

The purpose of this work was to study the behaviour of *L. monocytogenes* and *E. coli* O157:H7 in two cheeses produced in Alps area, by challenge test performed in a pilot plan at the Veterinary Public Health Institute of Lombardy and Emilia Romagna, Brescia, Italy.

Materials and Methods

Raw milk

A total of 700 L of raw cow milk were collected at different time during the summer season in the Alps in Northern Italy. Milk was collected from the bulk ripening tank and maintained refrigerated at 4±0.5°C for transportation to the pilot plan and processed immediately.

Bacterial cultures

Two multi-strain cocktails of *L. monocytogenes* and *E. coli* O157:H7 were used in this experiment. *L. monocytogenes* ATCC® 19115 and two wild strains (isolated from cheeses; BVR; www.ibvr.org) and *E. coli* O157:H7 ATCC®35150 and two wild strains (isolated from milk; BVR; www.ibvr.org) were used in the challenge test. The bacterial cultures were prepared in agreement with Dalzini *et al*. (2015). Raw milk was separately inoculated with two multi-strain cocktails, with the ratio of 1:100 v/v, in order to obtain an initial milk contamination of about 4 Log CFU/g (contaminated cheese). For the production of not contaminated cheeses the milk was inoculated with sterile peptone water (PW) (CONDA, Madrid, Spain) at the same ratio.

Cheese preparation

Two different cheeses were manufactured in pilot plan. Both types of cheeses were done following specifications of producers (www.ars-alimentaria.it). The manufacturing process were summarized in Table 1 (for cheese A) and in Table 2 (for cheese B). In order to produce cheese A (short ripened cheese), a total of 150 L of raw cow milk was used. During the process, no heat treatment was applied to the curd. Cheeses were ripened on wooden boards at 4-5°C for 60 days with turning over every 1-3 days. A total of 30 cheeses (1 kg each) were obtained: 10 cheeses contaminated with *L. monocytogenes*, 10 cheeses contaminated with *E. coli* and 10 non contaminated cheeses. In order to produce cheese B (long ripening cheese), a total of 150 L were used. The curd was cooked at 45°C for 15 min and then moulded into 80 by 300 mm cylindrical wooden moulds. Cheeses were ripened at 12°C for 4 months. Five cheeses, 8 kg each, were obtained.

Bacterial and physico-chemical analysis

While the milk was not diluted, the solid

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samples were transferred in a sample bags (NEOMED, Milan, Italy) and homogenized 1:3 w/v in sterile PW for 3 min in a Stomacher 400 blender (Seward Medical, London, UK). Serial 10 fold dilutions of control samples test material were prepared in sterile PW. In contaminated samples, the enumeration of *E. coli* O157:H7 was performed in agreement with Vernozy-Rozand *et al*. (2005) and Coscian-Cunico *et al*. (2014), while the *L. monocytogenes* enumeration was carried out according to the standard method ISO 11290-2 (ISO, 1998a). To verify the natural contamination of raw milk, at time zero, the enumeration of pathogens was also investigated in not contaminated samples.

In not contaminated samples, the enumeration of lactic acid bacteria (LAB) was performed according to the standard methods ISO 15214 (ISO, 1998b). All analyses were carried out in milk, curd after the extraction, and in cheese at different sampling times during the ripening step.

The physical-chemical analyses were carried out in not contaminated sample. The pH values (Hanna Instrument, Woonsocket, RI, USA), and the water activity (a_w) (Decagon Devices, Inc., Pullman, WA, USA) were measured.

Statistical analysis

For the short ripened cheese, the average and standard deviations of *L. monocytogenes* and *E. coli* O157:H7 microbial counts were determined from the average of three samples at each sampling time, while the average and standard deviations of LAB microbial counts, as well for the physical-chemical values, were determined from the average of two samples at each sampling time.

For the long ripened cheese, the average and standard deviations of *L. monocytogenes* and *E. coli* O157:H7 microbial counts were determined from the average of two samples at each sampling time, while LAB microbial counts, as well for the physical-chemical values, were determined from a single sample at each sampling time.

Analysis of variance (ANOVA) was carried out. The significance was statistically analysed by Student *t*-test at a 95% confidence interval ($P<0.05$) using R statistical software version 2.7.0 (R Development Core Team, 2008).

Table 1. Conditions of manufacturing process of short ripened cheese (cheese A), made from raw milk, from the cheese making to the end of ripening time.

Manufacturing step	Duration (hours)	Temperature (°C)
Addition of cheese rennet*	0.5 (0.02)	32
Acidification and coagulation	0.75 (0.03)	22-18
Cutting coagulum into 0.5 cm cubes and sineresis	1 (0.04)	22-18
Moulding°	0.5 (0.02)	22-18
Draining and overturning	24 (1)	22-18
Manual salting	0.5 (0.02)	22-18
Draining and inversion	48 (2)	22-18
Ripening	1368-1440 (57-60)	5-6

*1:10000 mL/mL; °40 by 200 mm quadratic wooden moulds. Values in parenthesis represent days.

Table 2. Conditions of manufacturing process of long ripened cheese (cheese B) made from raw milk, from the cheese making to the end of shelf life.

Manufacturing step	Duration (hours)	Temperature (°C)
Addition of cheese rennet*	0.5 (0.02)	36
Acidification and coagulation	0.5 (0.02)	21
Cutting of the coagulum in 4 cm cubes and then in grains of rice	1 (0.04)	21
Cooking of the curd, sineresis	0.25 (0.01)	45-48
Moulding°	0.5 (0.02)	38
Pressing of the curd	3 (0.125)	21
Draining and overturning	36 (1.5)	20-18
Manual salting	192 (8)	12
Ripening	1488 (120)	12

*1:10000 mL/mL; °80 by 300 mm cylindrical wooden moulds. Values in parenthesis represent days.

Table 3. p_H, aw values and lactic acid bacteria concentration in milk, curd and cheeses during manufacturing and ripening.

Cheese type	Test material	pH	a_w	LAB (Log CFU/mL, CFU/g)
Cheese A	Milk	6.75±0.03	ND	4.66±0.01
	Curd	6.5±0.08	0.967±0	8.38±0.05
	Cheese (1)°	5.56±0.3	0.981±0.002	9.22±0.11
	Cheese (15)°	5.24±0.09	0.983±0.01	9.42±0.08
	Cheese (30)°	5.39±0.06	0.971±0.006	9.2±0.08
	Cheese (60)°	5.4±0.15	0.947±0.007	8.93±0.25
Cheese B	Milk	6.73	ND	4.07
	Curd	6.68	0.991	6.81
	Cheese (1)°	6.03	0.994	8.57
	Cheese (3)°	5.12	0.993	8.77
	Cheese (7)°	5.24	0.989	8.99
	Cheese (10)°	5.22	0.997	9.07
	Cheese (20)°	5.11	0.968	8.96
	Cheese (30)°	5.11	0.96	8.83
	Cheese (60)°	5.02	0.95	8.77
	Cheese (90)°	5.04	0.956	8.65
	Cheese (120)°	5.12	0.951	8.67

LAB, lactic acid bacteria; ND, not determined; CFU, colony forming unit. °Day of ripening. Values are represented as mean±standard deviation of two replicates samples for the short ripened cheese (A); while as a single value for long ripened cheese (B). Values in parenthesis represent days.

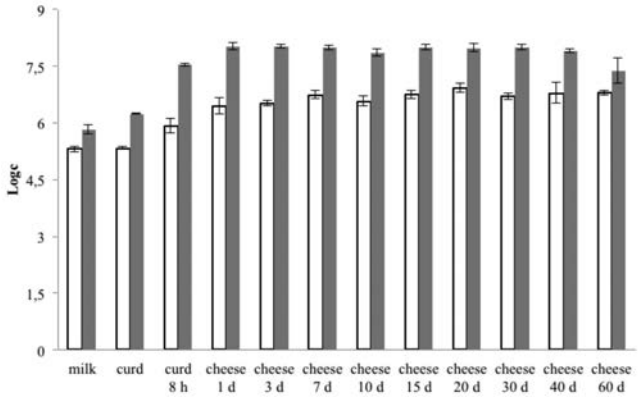

Figure 1. Logc concentration of *Listeria monocytogenes* (white bars) and *Escherichia coli* O157:H7 (grey bars), throughout the cheese making and ripening of short ripened cheese. Values are obtained from the average and standard deviations of three samples at each sampling time.

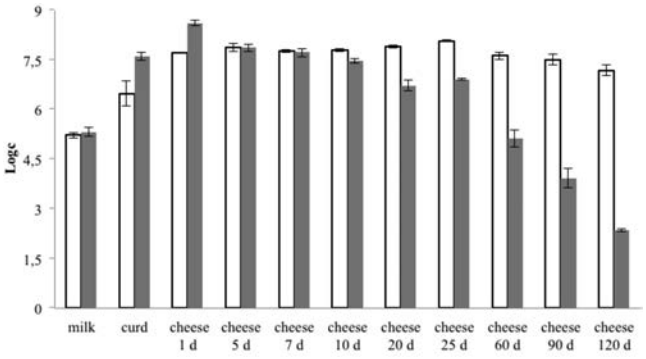

Figure 2. Logc concentration of *Listeria monocytogenes* (white bars) and *Escherichia coli* O157:H7 (grey bars), throughout the cheese making and ripening of long ripened cheese. Values are obtained from the average and standard deviations of two samples at each sampling time.

Results

Microbiological analysis in not contaminated milk revealed the absence (<0.47 log CFU/mL) of *E. coli* O157 and *L. monocytogenes* (data not shown). The LAB concentration and the physical-chemical properties of milk, curds and cheeses measured on not contaminated cheeses, were shown in Table 3. In the cheese with a short ripening time (cheese A) the pH decreased during the coagulation step, reaching pH 5.56±0.3 at the end of the draining, and remained almost stable until the end of ripening (pH 5.4±0.15). In the cheese with a long ripening time (cheese B) the pH reached, after 3 days, the pH 5.12 and remained constantly low until the end of the ripening. The a_w decreased gradually during the ripening until it reached almost 0.95 in both cheeses. Analyses of raw milk indicated that, before the cheese making, the LAB concentration was 4.66 and 4.07 Log CFU/mL respectively in cheese A and B. During the process, LAB concentration increased up to 9.22 and 8.57 Log CFU/g in both cheeses and remained relatively stable during the ripening step. Different *E. coli* O157:H7 and *L. monocytogenes* behaviour were observed during both manufacturing processes. In both contaminated cheese types, the pathogen concentrations increased significantly during the first hours of the manufacturing process (Figures 1 and 2). This can be due to the concentration of milk proteins in the curd (Miszczycha *et al.*, 2013). While *L. monocytogenes* concentration remained stable during the ripening time in both cheeses, the concentration of *E. coli* O157:H7 remained stable during the ripening time of short ripening cheese, but decreased significantly (P>0.05, more than 6 Log CFU/g) in long ripening cheese (Figures 1 and 2).

Discussion

In the present study we observed as the manufacturing process of raw milk cheese can affect the behaviour of two important food pathogens such as *L. monocytogenes* and *E. coli* O157:H7 in different ways. In fact, the cheese-making process and the physical chemical variables of the two cheeses are different and this seems to affect more the behaviour of the Gram negative *E. coli* than the Gram positive *L. monocytogenes* (El-Ziney *et al*., 1998). In particular, the *L. monocytogenes* concentration remained stable during the type manufacturing process of both cheeses and showed a low variability during the ripening step. The pathogen reached the apparent stationary phase at the beginning of ripening time and remained constant. This can be explained considering that the amount of organic acid, produced by the lactic acid bacteria, inhibited the growth of *L. monocytogenes* (Le Marc *et al*., 2002; Mellefont *et al*., 2008; Cornu *et al*., 2011). During the short ripened cheese type process, even the *E. coli* O157:H7 concentration did not change, as previously reported in a *Formaggella* produced in the northwest area of Lombardy region (Cosciani-Cunico *et al*., 2014). Conversely, in the long ripened cheese, the pathogen concentration decreased significantly, which can be due to the lower pH of cheese during the ripening step (under 5.3 value) in agreement with Buchanan (1993). The same result was previously observed in a French goat cheese made with raw milk in which *E. coli* O157:H7 concentration decreased gradually during the ripening phase (Vernozy-Rozand *et al.,* 2005).

Conclusions

The present study confirms the influence of the cheese-manufacturing environment on the *L. monocytogenes* and *E. coli* O157:H7 growths, in particular during the first days of the process as reported by others authors in different cheese types (Ryser and Marth, 1987; Ramsaran *et al*., 1998; www.combase.cc). Results indicate that *L. monocytogenes* and *E. coli* O157:H7 can survive the manufacturing process. Thus, the presence of low numbers of these pathogens in milk, destined for the production of raw milk cheeses characterized by a short ripening time, can constitute a threat to the consumer. Even *L. monocytogens* did not decrease during the ripening of the long ripening time cheese, considered in this study, while *E. coli* O157:H7 decreased significantly. Knowing the intrinsic end extrinsic variables of these two traditional Italian Alps cheeses, observed data reported in this study could be used to validate dynamic predictive mathematical model published in the literature (Baranyi and Roberts, 1995).

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