

Behaviour of *Escherichia coli* O157:H7 during the manufacture and ripening of an Italian traditional raw goat milk cheese

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Abstract

Formagelle di capra is a raw goat cheese produced from whole chilled goat milk; traditional technology involving unpasteurised milk and indigenous lactic starter cultures is employed for its production in Italy. The purpose of this study was to assess the behaviour of *Escherichia coli* O157:H7 during the manufacturing and ripening of this raw goat milk cheese. Raw milk was experimentally inoculated with *E. coli* O157:H7 in a laboratory scale plant and the count was monitored during production and 30 days of ripening required for this cheese. Results showed that *E. coli* O157:H7 count increased to more than 1.5 Log cfu g⁻¹ during cheese production and remained constant until the end of ripening. The evidence that *E. coli* O157:H7 is able to survive during the manufacturing and ripening process suggests that the 30-day ripening period alone is insufficient to eliminate levels of viable *E. coli* O157:H7 in *Formagelle di capra* cheese and that the presence of low numbers of *E. coli* O157:H7 in milk destined for the production of raw goat milk cheeses could represent a potential source of infection for humans and a threat for consumers.

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). Rapid alert system for food and feed (RASFF; European Commission, 2012) reported, in the last ten years, 42 notifi-

cation of non-compliance regarding *Escherichia coli* in dairy products; almost 12 of this belong to verotoxin producing *E. coli* in raw milk cheeses. In fact, among dairy products, the fresh raw milk cheese is known to be the most frequently contaminated (Bielaszewska *et al.*, 1997; EFSA, 2013) and it is documented that contaminated raw milk cheeses, with short ripening time (less than 60 days) could generate severe outbreak (Public Health Agency of Canada, 2013). Many regional cheese specialties throughout Europe are manufactured from unpasteurised milk, and there is growing concern that these products may pose a threat to consumer safety by transmitting pathogens such as *E. coli* O157:H7 (Vernozy-Rozand *et al.*, 2005). For this reason, many cheesemaking processes are registered in the Minister of Health web site on quality and safety of Italian food product (Ars Alimentaria, 2014).

E. coli O157:H7 can survive and may be able to grow in raw milk at low temperatures (Massa *et al.*, 1997; Giacometti *et al.*, 2012) and may not be eliminated from goat lactic cheese made with raw milk because this organism tolerates low pH (Jordan *et al.*, 1999) and low temperatures (Wang *et al.*, 1997; Massa *et al.*, 1999). Therefore, the purpose of this study is to investigate the behaviour of verotoxin producer *E. coli* O157:H7 during production and ripening period of *Formagelle di capra* (a traditional goat cheese of Insubria region, in Northern Italy) by using artificially contaminated raw milk.

Materials and Methods

Bacterial strains

One reference strain (*E. coli* O157:H7 strain ATCC®35150) and two different *E. coli* O157:H7 field isolates (BAC EM SBO 23 and BAC EM SBO 32) isolated from milk and collected in the IZSLER cultures collection Biobanking of Veterinary Resources (BVR) (Biobanking of Veterinary Resources, 2014) were used in the study; they were grown separately on Brain Heart Infusion agar (BHI) (Oxoid, Basingstoke, UK) incubated at 37°C for 24 h. All bacterial colonies present in the plates were collected by a sterile swab and suspended separately in saline (NaCl 0.85%; VWR International, Milan, Italy); the suspensions obtained were cultured separately in BHI (Oxoid), incubated in continuous agitation at 37°C for 24 h, then centrifuged at 4000 x g for 1 h at 4°C and resuspended. For the bacterial inocula, the three isolate suspensions of *E. coli* O157:H7 were quantified spectrophotometrically [8 Log colony-forming unit (cfu) g⁻¹] and serially diluted in sterile physiological solution in order to obtain approximately the

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same concentration (6 Log cfu g⁻¹). An equal volume of the three suspensions was pooled and used to inoculate raw milk in order to obtain a final concentration in milk of about 4.78 Log cfu/mL. The number of viable cells of the three suspensions was verified by 10-fold dilution and direct plating on MacConkey Sorbitol Agar (SMAC) (Oxoid) supplemented with cefixime and potassium tellurite (CT supplement) (Oxoid) incubated at 37°C for 24 h.

Raw goat milk cheese production

Raw goat milk was purchased and delivered to the laboratory scale plant of the Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna (IZSLER), Brescia, Italy, in bulk tank at 4±0.5°C within 6 h of production and artificially contaminated. Indigenous lactic starter cultures were prepared from raw goat milk (1 L) incubated at 45°C until reaching pH 4.8. The experimental design consisted of six trials: three replicates of inoculated batches and three noninoculated control batches.

Raw goat milk cheese, namely *Formagelle di capra*, was produced in the laboratory scale plant according to producer's specifications (www.ars-alimentaria.it), using 50 L of unpas-

teurised milk, indigenous starter cultures (500 mL) and liquid veal rennet (15 mL). Briefly, the raw milk was heated to 32°C, indigenous starter cultures and rennet were added and were left to rest at 30°C for 30 min. Afterwards, the curd was cut twice, into 4 cm cubes at the beginning and then in smaller cubes of about 0.5 cm, the curd was stirring heated at 38°C for about 5 min, left to rest for 10 min and then molded into the traditional cylindrical wooden moulds, which have an internal diameter of 102 mm with a height of 50 mm. The moulds were left to drain at room temperature for 4 h during which they were turned three times. Cheeses were removed from the moulds, dry cured for 2 days with overturning every 12 h and ripened on wooden boards at 12°C for 20-30 days with turning over every 1-3 days.

E. coli O157:H7 count

For each inoculated batch, count of *E. coli* O157:H7 was performed in triplicate from raw milk (before and after *E. coli* O157:H7 inoculation), curd immediately after the extraction, curd after drying, and cheese during ripening after 1, 4, 9, 11, 16, 21, 24 and 30 days. The count was performed by 10-fold dilution and direct plating (0.1 mL in duplicate) on sorbitol MacConkey agar plates (Microbiol Diagnostici, Cagliari, Italy) containing cefixime (0.05 mg L⁻¹) and potassium tellurite (2.5 mg L⁻¹) (CT-SMAC) (Microbiol Diagnostici), and incubated at 37°C for 24 h. Non-sorbitol-fermenting colonies on CT-SMAC, were tested by latex-agglutination with the *E. coli* O157 latex kit (DR620; Thermo scientific Inc., Waltham, MA, USA). Latex agglutinating isolates were confirmed biochemically as *E. coli* by API 20^B (20100; bioMérieux, Marcy l'Etoile, France). Presumptive *E. coli* O157:H7 colonies were counted.

Lactic acid bacteria count, pH, and a_w determination

The following analyses were additionally made for each sample of both inoculated and uninoculated batches: for mesophilic lactic acid bacteria (LAB) count, 10-fold dilution were prepared, pour plated (1 mL) in duplicate in MRS agar (Microbiol Diagnostici) and the plates were incubated under microaerobic condition at 37°C for 48-72 h; the water activity (a_w) and pH were measured in uninoculated batches. All analyses were performed in triplicate.

Statistical analysis

Counting results were expressed as colony forming unit (cfu) per gram and reported in terms of Log cfu g⁻¹. The average and standard deviations of microbial counts and physico-chemical values were determined from the average of three samples at each sampling time. Analysis of variance (ANOVA) was carried out to evaluate the difference of microbial counts during production and ripening; 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.

Results

E. coli O157:H7 was not detected in uninoculated raw milk, or in any samples of the uninoculated batch. The change in *E. coli* O157:H7 count, pH, and LAB population count during *Formaggelle di capra* production are summarised in Table 1. The pH decreased during coagulation and during all the ripening days (from 6.80 to 5.03) as well the a_w, whereas the LAB

count increased from 4.97 Log cfu mL⁻¹ to 7.93 Log cfu g⁻¹ in the first ripening day, reached at about 9 Log ufc g⁻¹ in the second ripening day and remained substantially unchanged until the end of ripening. Statistical analysis showed that not significant difference was between LAB concentrations on control and contaminated samples (P>0.05) (data not shown). The *E. coli* O157:H7 increased during manufacturing from 4.78 Log ufc mL⁻¹ to 6.57 Log ufc g⁻¹, reached 7.36 Log ufc g⁻¹ in the ninth ripening day, and remained substantially unchanged until the end of ripening.

Discussion

In this study, the behaviour of *E. coli* O157:H7 was examined during the manufacture and ripening of *Formaggelle di capra* by contaminating raw goat milk. The results demonstrated that *E. coli* O157:H7 is able to survive during the cheese manufacturing and also that its concentration increased more than 1.5 Log cfu g⁻¹ during the first ripening days and remained substantially unchanged until the end of ripening.

Several challenge studies have reported that *E. coli* O157:H7 can survive the processing conditions for a variety of cheeses (Ramsaran *et al.*, 1998; Schlessler *et al.*, 2006; Spano *et al.*, 2003) but only one study was in literature on raw goat cheese (Vernozy-Rozand *et al.*, 2005). Results of our study are similar to that reported by Ramsaran *et al.* (1998) that observed, in an artificially contaminated Camembert cheese, a comparable *E. coli* O157:H7 increase during manufacturing; on the contrary, Vernozy-Rozand *et al.* (2005) observed that, in a French goat cheese made with raw milk, after an increase found on day 1, the *E. coli*

Table 1. Evolution of *E. coli* O157:H7 and mesophilic lactic acid bacteria count, pH and a_w on milk and curd.

Sample	Parameter			
	a _w	pH	LAB	<i>E. coli</i> O157:H7
Milk	ND	6.80±0.01	4.97±0.66	4.78±0.1
Curd just after extraction	ND	6.73±0.06	5.09±0.13	ND
Curd just after drying	0.992±0.001	6.39±0.06	5.06±0.07	5.2±0.03
Cheese (1) [°]	0.976±0.018	5.79±0.04	7.93±0.09	6.57±0.02
Cheese (4) [°]	0.955±0.016	5.22±0.07	9.04±0.06	6.8±0.01
Cheese (9) [°]	0.965±0.004	5.24±0.04	9.27±0.23	7.36±0.13
Cheese (11) [°]	ND	5.15±0.04	8.81±0.17	ND
Cheese (16) [°]	0.941±0.012	5.01±0.04	8.88±0.04	6.88±0.09
Cheese (21) [°]	0.943±0.009	5.05±0.06	9.31±0.10	7.11±0.06
Cheese (24) [°]	0.940±0.002	5.07±0.06	9.24±0.05	6.79±0.15
Cheese (30) [°]	0.938±0.002	5.03±0.10	8.68±0.11	6.7±0.17

LAB, lactic acid bacteria; ND, not determined. Values are reported as mean of three replicates samples expressed as Log cfu/g±standard deviation. °Day of ripening.

O157:H7 count level decreases gradually during the ripening phase; this is probable due to the fact that the manufacturing process and the physico-chemical variables of the two cheeses are different. In fact, the acidification of the French cheese is faster and the pH value is lower (pH 4.8) than the Italian one. Another hypothesis could be found in the different strains used as starter cultures; in the French study was used a commercial selection of thermophilic LAB while in the Italian study was preferred a natural starter cultures obtained by the fermentation of raw goat milk, in order to simulate a realistic cheesemaking scenario. The *E. coli* O157:H7 strains used in our study also confirm the acid tolerance and good competitor ability and the fact that pH is positively correlated with levels of *E. coli* O157:H7 in fresh cheese. Furthermore, our results indicate that 30-day ripening period is not adequate for eliminating *E. coli* O157:H7.

Conclusions

Although a high number of *E. coli* O157:H7 are unlikely to be present in milk used to produce *Formaggelle di capra*, these observations suggest that even low numbers of *E. coli* O157:H7 in raw goat milk destined for the production of this traditional raw goat milk cheese, characterised by a short ripening period, may represent a potential source of infection for humans and a threat for consumers.

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