Behavior of *Escherichia coli* O157:H7 during the manufacture and ripening of Fontina Protected Designation of Origin cheese

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ABSTRACT

This study was conducted to describe the cheese-making procedure of Fontina Protected Designation of Origin (PDO) cheese and to evaluate the behavior of *Escherichia coli* O157:H7 during cheese manufacture and ripening. The study was divided into 2 phases: the production of Fontina PDO cheese was monitored at 3 different dairies in the Aosta Valley and an *E. coli* O157 challenge was conducted at a fourth dairy. The dairies employ different commercial starter cultures for cheese making. The growth of lactic acid bacilli (LAB) and the decrease in pH were slower in the first hours and the LAB concentrations were overall higher in dairy A than in the other 2 dairies. The pH remained substantially unchanged during ripening (range 5.2 to 5.4) in all dairies. Water activity remained constant at around 0.98 until d 21, when it decreased to around 0.97 until d 80 in dairies A and B and 0.95 in dairy E. Whole raw cow milk was used for making Fontina cheese according to the standard procedure. For the experimental production, the milk was inoculated with *E. coli* O157:H7 at a concentration of approximately $5 \log_{10}$ cfu/mL and commercial starter cultures were used according to the Fontina PDO regulation. An increase of $2.0 \log_{10}$ cfu/g in *E. coli* O157:H7 was observed during the first 9.5 h of cheese making, followed by a decrease at 46 h when pH decreased to 5.4 in all trials. Fresh cheeses were salted and held at 10°C for ripening for 80 d. Water activity was decreased to 0.952 at the end of the ripening stage. The LAB concentrations declined gradually; this trend was more marked for the lactobacilli than either the thermophilic or the mesophilic lactococci. The increase in LAB count and the decrease in pH in the first hours did not seem to affect *E. coli* O157 growth. Ripening was found to inhibit pathogen survival, however, as seen in the reduction of $3 \log_{10}$ from the maximum

concentration measured during the earlier stages of production.

Key words: *Escherichia coli* O157, Fontina Protected Designation of Origin, cheese, cheese making, ripening

INTRODUCTION

Domestic animals for milk production are a known major reservoir of verotoxin-producing *Escherichia coli* (VTEC) and are often asymptomatic carriers (Caprioli et al., 2005). Contaminated raw milk and dairy products prepared from contaminated raw milk are a potential source of outbreaks caused by *Escherichia coli* O157:H7 (Honish et al., 2005; Baylis, 2009; Ravel et al., 2009), as also previously reported for Italy (Trevisani et al., 2014; Nobili et al., 2016). The VTEC can cause human infections ranging from uncomplicated diarrhea to such severe diseases as life-threatening hemolytic uremic syndrome and hemorrhagic colitis (Melton-Celsa et al., 2011). In susceptible individuals, the infective dose of VTEC is less than 10 bacteria; to ensure food safety, no VTEC must be present in the cheese (Callon et al., 2016). Behavior of VTEC depends on the temperatures during cheese manufacture and ripening according to the cheese-making method (Farrokh et al., 2013). For these reasons, researchers have examined the behavior of *E. coli* O157:H7 during the manufacture and aging of various cheese varieties such as feta, telemes, Grana Padano Protected Designation of Origin (PDO), Cheddar, Camembert, and Gouda cheese (Govaris et al., 2002; Ercolini et al., 2005; Schlessner et al., 2006; Montet et al., 2009; D'Amico et al., 2010). Furthermore, because many regional cheese specialties are made from unpasteurized milk, they may pose a threat to consumer safety by transmitting pathogens such as *E. coli* O157:H7 (Vernozy-Rozand et al., 2005). Contamination by VTEC is also an important microbiological cause of dairy product recalls in Europe (RASFF, 2017). In 2014, the European Food Safety Authority and the European Centre for Disease Prevention and Control reported that 3.6% of raw cow

Received July 7, 2017.

Accepted February 18, 2018.

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milk samples and 1.2% of milk (excluding raw cow milk) and dairy products tested positive for VTEC, with an increase in the proportion of VTEC-positive samples in the European Union over previous years (EFSA and ECDC, 2016).

No studies to date have investigated the behavior of *E. coli* O157 during the production of Fontina. Fontina is a PDO cheese with a semi-cooked paste made from whole raw cow milk collected from a single milking. Produced in the Aosta Valley in northwest Italy, it is obtained from cow milk from the indigenous valdostana (pezzatarossa, pezzatanera, and castana) breed, which has long been reared for milk production. The milk's characteristics derive from feeding with fodder essences typical of the alpine area; the cheese's characteristics reflect the qualities of raw milk. The cheese must be ripened for at least 80 d according to the Fontina PDO regulation.

Raw milk cheese production and commercialization is regulated in Europe under regulation no. 853/2004 (European Union, 2004) which lays down specific hygiene rules for food of animal origin including raw milk cheese. Generally, unsafe food shall not be placed on the European market (European Union, 2002) and microbiological criteria are defined for different kinds of food matrices. Moreover, national laws describe safety criteria related to specific food products: in Italy, VTEC are well regulated in cheese and must be absent in 25 g (Regione Piemonte, 2017).

Fontina was exported to European Union Member States (428 t), the United States (346 t), and Asia (18 t) in 2016 (CLAL, 2017). Some countries pose restrictions on the importation of cheeses made from unpasteurized milk. Food safety regulation requires the demonstration that the level of pathogenic microorganisms in the raw milk cheese does not exceed the level of these microorganisms in the milk from which the cheese was made. Also, it is mandatory that the raw milk cheese does not support the growth of pathogenic microorganisms (Ryser, 2011; Food Standards Australia New Zealand, 2015).

The objectives of this study were to describe Fontina PDO production and to determine the survival of *E. coli* O157:H7 during cheese manufacture and ripening.

MATERIALS AND METHODS

Study Design

The study was divided into a pilot and an experimental phase. The aim of pilot phase was to analyze the entire Fontina PDO production process at 3 dairies. A fourth dairy was selected for the experimental phase in

which the microbial challenge with *E. coli* O157:H7 was performed. For the pilot phase, 3 dairies were selected from among the 20 major Fontina PDO producers in the Aosta Valley.

Temperature was monitored with a data logger (FT-800, Maxim Integrated Products, San Jose, CA) during the production process. Three production batches were monitored at each dairy. Milk, curd, and cheese were sampled to enumerate lactic acid bacilli (LAB) and measure pH and water activity (A_w) at 0, 2, 2.5, 12, and 24 h and at 7, 14, 21, 56, and 80 d. Milk (100 mL) and curd (100 g) samples were collected with a sterile 25-mL serological pipette and a silver spoon, respectively. Each cheese sample (30 g) was obtained from 3 different wheel sites (10 g each) with a sterile silver corer and then pooled.

The aim of the experimental phase was to study the behavior of *E. coli* O157:H7 during cheese production in a fourth dairy. Three batches of Fontina PDO were made from milk spiked with 3 different *E. coli* O157 strains. For the experimental phase, on the selected dairy characteristics, following a repeated ANOVA design, 3 batches of contaminated *E. coli* O157:H7 samples were collected during the entire production process. The batches were uncontaminated and contaminated milk, curd and whey after cutting, whey and curd after cooking, and curd before pressing, after 6 h of pressing, and before and after salting and ripening (7, 14, 21, 28, 56, and 80 d). The following parameters were measured: LAB, pH, A_w , and *E. coli* O157 concentration.

Microbiological Analysis

From each sample collected as described above, 10 ± 0.5 g was homogenized with 90 mL of buffered peptone water (Oxoid, Milan, Italy) for 2 min in a stomacher (LAB Blender 400, PBI, Milan, Italy). Decimal dilutions in sterile peptone water (Oxoid) were prepared, and aliquots of the dilutions were spread on a specific medium according to ISO (2017). Thermophilic and mesophilic lactococci were enumerated with M17 medium (Oxoid) at 45°C for 48 h and at 30°C for 72 h, respectively. Thermophilic streptococci count was performed using de Man, Rogosa, and Sharpe agar (MRS, Oxoid) incubated at 45°C for 48 h (Bellio et al., 2016).

The pH was measured by immersing the probe of the pH meter (GLP 22, Crison, Barcelona, Spain) in milk/whey or in a diluted and homogenized sample containing 10 g of curd/cheese sample and 10 mL of distilled water (MFHPB, 2014). Water activity was measured with a calibrated electric hygrometer (Aqualab, Decagon Devices Inc., Pullman, WA) according to ISO (2004).

Inoculum Preparation

Three different *E. coli* O157 strains were used: *E. coli* O157 (NCTC 12900), *E. coli* O157 (strain EF 390), and *E. coli* O157 (strain EF 430) kindly provided by the European Union Reference Laboratory for *Escherichia coli*, including VTEC. The 2 wild strains were isolated from raw cow milk and carried virulence genes *eae* but not *stx1* or *stx2*. All strains were kept in cryobeads (AEB 400100, AES Laboratories, Combourg, France) and maintained at -20°C . Each strain was grown twice in 10 mL of brain heart infusion broth (Oxoid, Basingstoke, Hampshire, UK) at 37°C for 24 h to revive the culture (1% inoculum) and then incubated at 37°C for 24 h separately. Cultures of each strain were then pooled to obtain a bacterial suspension with a final concentration of about $9 \log_{10}$ cfu/mL.

Experimental Production

Experimental production of Fontina PDO was carried out according to the cheese-making process used at dairy A, which was designated as the worst-case scenario based on slower LAB growth and decreased pH (Table 1). Each batch was prepared from 90 L of raw milk. According to the regulations for Fontina PDO production, raw milk must be used within 2 h after milking without refrigeration. The milk was delivered at 35°C to the experimental dairy (dairy S) and emptied into a stainless-steel container. The batches were artificially contaminated with an inoculum of *E. coli* O157. Bacterial suspension (10 mL) at 37°C was mixed gently and gradually distributed into the raw milk under stirring to achieve uniform distribution of the inoculum and a final concentration of approximately 5 to $6 \log_{10}$ cfu/mL. A freeze-dried starter culture (1.5 g/100 L) containing a LAB mixture of *Streptococcus thermophilus*, *Lactococcus lactis*, and *Lactobacillus delbrueckii* ssp. *Lactis* [FT-1 D, Bioagro, Thiene (VI), Italy] was prepared according to regulations for Fontina PDO production (Repubblica Italiana, 2011) and added to obtain approximately 5 to $6 \log_{10}$ cfu/mL. The milk was stirred, warmed to 37°C , and 1 mg/100 L of powdered calf rennet [Caglifacio Clerici, Cadorago (CO), Italy] was added. After curdling, the curd was cut into pieces 0.5 cm in diameter and heated to 48°C in 1 h. The curd was transferred to molds (50 cm in diameter and 7 cm thick) and mechanically pressed at 300 to 400 kPa for 20 h to drain. The cheese was salted by immersion in 22% (wt/vol) brine in a climate-controlled room for 12 h (temperature 10°C , relative humidity 90%). For each batch, brine was produced with boiling water and salt and then maintained in a climate-controlled room at 10°C . The cheese wheel was transferred to a wooden

board in a climate-controlled room for ripening for 80 d at 10°C and relative humidity of 90%.

For each trial, in conformity with ISO/TS 13136:2012, we checked that *E. coli* O157:H7 was not initially present in the raw milk. Inoculation levels were measured by sampling the inoculated milk. Analyses were performed as described above (LAB, pH, and A_w). *Escherichia coli* O157 was enumerated on CHROMagar O157 (CHROMagar, Springfield, NJ) incubated for 24 h at 37°C .

Statistical Data Analysis

Data were analyzed using SAS software (version 9.2, SAS Institute, Cary, NC). Microbiological counts were converted to $\log_{10}$ colony-forming units. For each parameter (LAB, pH, A_w , *E. coli* O157, and temperature), descriptive statistics of the mean $\pm$ standard deviation, median, minimum, and maximum values were calculated. To select the dairy for the experimental phase, the dairies were compared using nonparametric and parametric ANOVA. To compare the overall effect of LAB and pH between the 3 dairies, the Kruskal-Wallis H test was performed. The effect of time on microbiological flora, A_w , and pH at each dairy was investigated using 1-way ANOVA. The PROC GLM in SAS was used with time as factor. When ANOVA revealed significant differences ($P < 0.05$), the Tukey honestly significant difference test was applied. Differences in *E. coli* O157:H7 concentration between the 3 batches were evaluated by 1-way ANOVA.

To determine *E. coli* O157:H7 survival during production, the difference in *E. coli* O157 counts between whey and curd were compared using the Wilcoxon test. Dairy and time were entered as factors in 2-way ANOVA to compare A_w , pH, and LAB between the 3 dairies (A, B, and E) and the fourth dairy (S) selected for the experimental study. Dairy was entered into the analysis to compare all 4 dairies. Because production times differed between the dairies, 3 time points were used: the beginning (starting time), at 12 h, and at the end of ripening (80 d). Of note, A_w was compared only at 2 time points: 12 h and the end of ripening. Interaction between the factors dairy and time was also compared. When ANOVA showed significant effects, post hoc analysis was done using Tukey's honestly significant difference test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Production Process

The production process at the 3 dairies differed in several ways. The commercial starter culture used at dairy A contained LAB *S. thermophilus*, *Lc. lactis*, and

Lb. delbrueckii ssp. *lactis* (FT-1 D, Bioagro), whereas at the other 2 dairies it contained *S. thermophilus*, *Lc. lactis*, and *Lb. rhamnosus* (FT-N D, Bioagro). None of these strains has been reported to produce bacteriocins against *E. coli* O157. In dairies B and E, 2 batches per day are produced from morning and evening milk collections, respectively. During the pressing stage (12 h), the cheese wheels are overturned to facilitate draining of the cheese mass. The wheel is then placed in brine for 12 h. In dairy A, Fontina PDO is made only from the morning milk collection, while the evening yield is used for other dairy products. In addition, the cheese wheels are pressed for 20 h and then transferred to brine for 12 h.

No statistically significant difference in LAB concentration added to the starter milk was found between the 3 dairies (H test = 3.3449, df = 2, $P = 0.1878$): mesophilic and thermophilic lactococci ranged from 5.5 to 6.0 log₁₀ cfu/mL and lactobacilli from 4.5 to 5.0 log₁₀ cfu/mL. At dairies B and E, the LAB concentrations gradually increased during the early stages of process-

ing and reached about 6.5, 7.5, and 7.0 log₁₀ cfu/g for lactobacilli, thermophilic, and mesophilic lactococci, respectively, after 12 h (Table 1).

In dairy A, the effect of time was statistically significant for A_w ($F = 14.1$, $P < 0.0001$), mesophilic lactococci ($F = 42.5$, $P < 0.0001$), thermophilic lactococci ($F = 109.9$, $P < 0.0001$), lactobacilli ($F = 58.3$, $P < 0.0001$), and pH ($F = 587.8$, $P < 0.0001$). In dairy B, the effect of time was statistically significant for A_w ($F = 45.9$, $P < 0.0001$), mesophilic lactococci ($F = 15.3$, $P < 0.0001$), thermophilic lactococci ($F = 21.7$, $P < 0.0001$), lactobacilli ($F = 23.6$, $P < 0.0001$), and pH ($F = 73.4$, $P < 0.0001$). In dairy E, the effect of time was statistically significant for A_w ($F = 48.8$, $P < 0.0001$), mesophilic lactococci ($F = 13.7$, $P < 0.0001$), thermophilic lactococci ($F = 12.9$, $P < 0.0001$), lactobacilli ($F = 17.7$, $P < 0.0001$), and pH ($F = 247.3$, $P < 0.0001$).

In dairy A, slower LAB growth was noted in the first hours but ultimately reached higher concentrations than those observed for the 2 other dairies. This difference was related to the more rapid decrease in pH (5.4

Table 1. Changes in pH, water activity (A_w), thermophilic, and mesophilic lactococci, and lactobacilli counts in Fontina Protected Designation of Origin cheese samples from the 3 pilot study dairies (A, B, and E)¹

Dairy	Time	A_w	pH	Mesophilic lactococci (log ₁₀ cfu/g)	Thermophilic lactococci (log ₁₀ cfu/g)	Lactobacilli (log ₁₀ cfu/g)
A	0 h		6.8 ± 0.1 ^{ab}	5.3 ± 0.6 ^e	5.5 ± 0.5 ^e	5.1 ± 0.5 ^e
	2.0 h		6.7 ± 0.1 ^b	5.3 ± 0.7 ^e	5.6 ± 0.5 ^e	5.0 ± 0.6 ^e
	2.5 h	0.984 ± 0.001 ^a	6.8 ± 0.1 ^a	6.3 ± 0.5 ^d	6.9 ± 0.1 ^d	6.3 ± 0.3 ^d
	12 h	0.984 ± 0.001 ^a	5.7 ± 0.1 ^c	7.7 ± 0.5 ^{bc}	8.1 ± 0.4 ^b	7.2 ± 0.1 ^c
	1 d	0.984 ± 0.001 ^a	5.4 ± 0.1 ^d	8.2 ± 0.4 ^{ab}	8.5 ± 0.1 ^a	7.7 ± 0.3 ^b
	7 d	0.984 ± 0.001 ^a	5.4 ± 0.1 ^d	7.7 ± 0.5 ^{bc}	8.5 ± 0.2 ^a	7.7 ± 0.2 ^b
	14 d	0.984 ± 0.001 ^a	5.4 ± 0.1 ^d	7.7 ± 0.3 ^{abc}	8.4 ± 0.3 ^{ab}	7.5 ± 0.5 ^{bc}
	21 d	0.984 ± 0.001 ^a	5.3 ± 0.1 ^{ef}	8.2 ± 0.5 ^a	8.5 ± 0.5 ^a	7.8 ± 0.8 ^b
	56 d	0.968 ± 0.001 ^b	5.2 ± 0.1 ^f	7.8 ± 0.3 ^{abc}	8.2 ± 0.2 ^{ab}	8.3 ± 0.7 ^a
	80 d	0.974 ± 0.001 ^c	5.3 ± 0.1 ^e	7.5 ± 0.7	7.6 ± 0.4 ^c	7.8 ± 0.2 ^b
B	0 h		6.6 ± 0.1 ^a	5.7 ± 0.3 ^c	5.9 ± 0.4 ^e	4.8 ± 0.4 ^d
	2.0 h		6.5 ± 0.1 ^a	5.8 ± 0.5 ^c	6.3 ± 0.2 ^d	5.3 ± 0.2 ^c
	2.5 h	0.984 ± 0.001 ^a	6.1 ± 0.4 ^b	6.8 ± 0.4 ^b	7.1 ± 0.1 ^{bc}	6.4 ± 0.1 ^b
	12 h	0.984 ± 0.001 ^a	5.4 ± 0.1 ^{cde}	6.9 ± 0.4 ^{ab}	7.5 ± 0.4 ^a	6.7 ± 0.2 ^{ab}
	1 d	0.984 ± 0.001 ^a	5.5 ± 0.2 ^{cd}	6.8 ± 0.4 ^b	7.4 ± 0.1 ^{ab}	6.6 ± 0.5 ^{ab}
	7 d	0.984 ± 0.001 ^a	5.6 ± 0.1 ^c	6.8 ± 0.4 ^{ab}	7.2 ± 0.3 ^{bc}	6.5 ± 0.3 ^{ab}
	14 d	0.982 ± 0.001 ^a	5.3 ± 0.1 ^{de}	7.3 ± 0.7 ^a	7.0 ± 0.3 ^c	6.9 ± 0.9 ^a
	21 d	0.982 ± 0.001 ^a	5.2 ± 0.2 ^e	6.9 ± 0.2 ^{ab}	6.9 ± 0.2 ^c	6.4 ± 0.2 ^b
	56 d	0.966 ± 0.001 ^c	5.3 ± 0.1 ^{de}	7.1 ± 0.5 ^{ab}	7.0 ± 0.6 ^c	6.3 ± 0.2 ^b
	80 d	0.973 ± 0.001 ^b	5.4 ± 0.2 ^{de}	7.3 ± 0.3 ^a	7.0 ± 0.2 ^c	6.7 ± 0.2 ^{ab}
E	0 h		6.7 ± 0.1 ^a	5.7 ± 0.4 ^d	5.7 ± 0.3 ^c	4.6 ± 0.7 ^d
	2.0 h		6.6 ± 0.1 ^a	6.1 ± 0.2 ^d	6.2 ± 0.2 ^c	4.9 ± 0.6 ^d
	2.5 h	0.984 ± 0.001 ^{ab}	6.4 ± 0.2 ^b	7.1 ± 0.2 ^{abc}	7.2 ± 0.1 ^{ab}	5.7 ± 0.7 ^c
	12 h	0.984 ± 0.001 ^a	5.4 ± 0.1 ^{cd}	7.3 ± 0.8 ^a	7.6 ± 0.4 ^a	6.3 ± 0.2 ^{bc}
	1 d	0.984 ± 0.001 ^{ab}	5.5 ± 0.1 ^{cd}	7.1 ± 0.7 ^{abc}	7.6 ± 0.3 ^a	6.0 ± 0.1 ^{bc}
	7 d	0.984 ± 0.001 ^{ab}	5.6 ± 0.1 ^c	7.5 ± 0.6 ^a	7.7 ± 0.3 ^a	6.5 ± 0.2 ^b
	14 d	0.979 ± 0.010 ^{ab}	5.5 ± 0.1 ^e	7.2 ± 0.5 ^{ab}	7.2 ± 0.5 ^{ab}	6.6 ± 1.1 ^b
	21 d	0.977 ± 0.001 ^b	5.2 ± 0.1 ^f	6.7 ± 0.2 ^a	7.2 ± 0.5 ^{ab}	6.3 ± 0.4 ^{bc}
	56 d	0.947 ± 0.001 ^c	5.3 ± 0.1 ^{ed}	6.6 ± 0.3 ^c	6.8 ± 0.9 ^b	7.8 ± 1.0 ^a
	80 d	0.953 ± 0.001 ^c	5.4 ± 0.1 ^d	7.4 ± 0.5 ^a	7.0 ± 0.9 ^b	6.0 ± 0.4 ^{bc}

^{a-f}Within a column, mean values with the same superscript letter are not statistically different ($P > 0.05$).

¹Values are means ± SD. Time was statistically significant within each dairy for each parameter. Post hoc comparison using Tukey's honestly significant difference test is reported.

Table 2. Changes in temperature (°C) in Fontina Protected Designation of Origin cheese samples from 3 pilot study dairies (A, B, and E) and from the experimental study dairy (S)¹

Time	Dairy A	Dairy B	Dairy E	Dairy S
0	25.2 ± 0.8	30.0 ± 2.2	30.3 ± 1.5	25.1 ± 0.6
1.0 h	36.8 ± 0.3	36.3 ± 0.3	36.2 ± 0.3	36.0 ± 1.0
2.0 h	47.5 ± 1.2	47.4 ± 0.4	47.8 ± 0.3	47.9 ± 0.7
2.5 h	46.4 ± 0.6	46.0 ± 0.5	45.8 ± 0.4	44.0 ± 0.8
8.5 h	39.7 ± 0.8	41.5 ± 3.5	39.5 ± 3.5	32.0 ± 0.8
1 d	12.5 ± 0.3	15.5 ± 0.2	12.8 ± 0.4	13.7 ± 0.3
7 d	9.4 ± 0.1	9.3 ± 0.3	8.8 ± 0.3	9.5 ± 0.3
14 d	9.4 ± 0.1	9.3 ± 0.3	8.8 ± 0.3	9.5 ± 0.3
21 d	9.4 ± 0.1	9.3 ± 0.3	8.8 ± 0.3	9.5 ± 0.3
56 d	9.4 ± 0.1	9.3 ± 0.3	8.8 ± 0.3	9.5 ± 0.3
80 d	9.4 ± 0.1	9.3 ± 0.3	8.8 ± 0.3	9.5 ± 0.3

¹Values are means ± SD.

± 0.1) in dairies B and E (at 12 h) as compared with dairy A (at 24 h). The pH level was higher in dairy A than that measured in either dairy B or E at 2.5 h and the difference was statistically significant (H test 15.1; $P < 0.001$). The pH remained substantially unchanged during the ripening period, with a slight decrease at 21 d (5.1 ± 0.1) in dairy E. The LAB concentrations during the ripening period were constantly high, resulting in a product with a high amount of live LAB. The LAB concentrations were higher in dairy A than in the 2 other dairies; the difference was statistically significant (H test 16.9; $P < 0.001$). This difference could be attributed to the different starter cultures and particularly to pressing the cheese at room temperature for 24 h instead of 12 h (Table 2), which allowed for greater growth of lactobacilli ($7.7 \pm 0.3 \log_{10}$ cfu/g), thermophilic ($8.5 \pm 0.1 \log_{10}$ cfu/g), and mesophilic lactococci ($8.2 \pm 0.4 \log_{10}$ cfu/g) at 24 h. The A_w remained constant at around 0.98 until d 21, when it decreased to around 0.97 until d 80 in dairies A and B and 0.95 in dairy E.

Experimental Production

Analysis of variance comparison between the 4 dairies (A, B, E, and S) at 3 time points was statistically significant for A_w for the 3 factors dairy ($F = 17.7$, $P < 0.0001$), time ($F = 117.9$, $P < 0.0001$), and interaction ($F = 12.2$, $P < 0.001$). The same pattern was observed for pH, dairy ($F = 4.8$, $P < 0.01$), time ($F = 789.6$, $P < 0.0001$), interaction ($F = 3.9$, $P < 0.05$), and lactobacilli. As for lactobacilli, the 3 factors dairy ($F = 14.3$, $P < 0.0001$), time ($F = 106.5$, $P < 0.0001$), and interaction ($F = 3.8$, $P < 0.01$) were statistically significant. However, post hoc analysis showed that dairy S did not differ statistically from dairy E for A_w or from dairies A and B for pH and lactobacilli (Tables 3 and 4). The interaction between the factors time and dairy was not statistically significant for thermophilic and mesophilic lactococci ($F = 0.98$, $P = 0.45$ and $F = 0.96$, $P = 0.47$, respectively).

No studies to date have investigated the behavior of *E. coli* O157 during the making of Fontina PDO.

Table 3. Changes in pH and water activity (A_w) in Fontina-type cheese samples during experimental production¹

Cheese-making stage	Time	A_w	pH
Contaminated milk	0		6.8 ± 0.1
Curd after cutting	1.0 h		6.6 ± 0.1
Curd after cooking	2.0 h		6.5 ± 0.1
Curd before pressing	2.5 h		6.5 ± 0.1
6 h of pressing	8.5 h		6.0 ± 0.3
Before salting	12 h		5.7 ± 0.1
Beginning of ripening	1 d	0.984 ± 0.003	5.4 ± 0.2
7 d after ripening	7 d	0.979 ± 0.004	5.4 ± 0.1
14 d after ripening	14 d	0.973 ± 0.004	5.4 ± 0.1
21 d after ripening	21 d	0.971 ± 0.005	5.3 ± 0.1
28 d after ripening	28 d	0.965 ± 0.006	5.2 ± 0.2
56 d after ripening	56 d	0.950 ± 0.006	5.2 ± 0.2
80 d after ripening	80 d	0.952 ± 0.006	5.3 ± 0.1

¹Values are means ± SD.

Figure 1. Changes in microbial count during Fontina-type cheese making in experimental productions. Ec = *Escherichia coli* O157; Lb = lactobacilli; Lc30 = mesophilic lactococci; Lc45 = thermophilic lactococci. Error bars represent SD.

Nonetheless, the present findings can be compared with those from similar studies performed on other types of cheeses. Our results are shared by previous studies which reported that although *E. coli* O157 is able to survive during cheese production, its concentration decreases during ripening (Vernozy-Rozand et al., 2005; Montet et al., 2009; D'Amico et al., 2010).

In the present study, raw milk was inoculated to obtain a concentration of about $5 \log_{10}$ cfu/mL. No difference in *E. coli* O157 concentration between the 3 batches was observed ($F = 0.45$, $P = 0.63$).

The results show that the *E. coli* O157 concentration increased during the early stages of the cheese-making process (Figure 1). At the end of the pressing stage,

the *E. coli* O157 count was increased by $2 \log_{10}$ cfu/g. The increase in *E. coli* O157:H7 after expulsion of whey and contraction of the curd during heating and pressing may have been due to entrapment of bacterial cells in the curd and population growth after a lag phase during the heating step. Most of the cells are trapped in the curd and only some are released into the whey (D'Amico et al., 2010).

The difference in *E. coli* O157 concentration between whey and curd was statistically significant (Wilcoxon test = 462.5, $Z = 4.08$, $P < 0.001$): *E. coli* concentration in whey was $4.7 \pm 0.6 \log_{10}$ cfu/mL and $4.5 \pm 0.4 \log_{10}$ cfu/mL, after cutting and after cooking, respectively, and appeared lower than that measured in the

Table 4. Changes in pH, water activity (A_w), thermophilic and mesophilic lactococci, and lactobacilli count in Fontina Protected Designation of Origin and Fontina-type cheese samples from pilot study dairies (A, B, and E) and from the experimental study dairy (S)¹

Item	Time	Dairy A	Dairy B	Dairy E	Dairy S
A_w	12 h	0.984 ± 0.001^a	0.984 ± 0.001^a	0.984 ± 0.001^a	0.984 ± 0.001^a
	80 d	0.974 ± 0.008^a	0.973 ± 0.001^a	0.947 ± 0.012^b	0.952 ± 0.006^b
pH	0 h	6.8 ± 0.1^{ab}	6.6 ± 0.1^a	6.7 ± 0.1^a	6.8 ± 0.1^{ab}
	12 h	5.7 ± 0.1^a	5.4 ± 0.1^b	5.4 ± 0.1^b	5.7 ± 0.1^a
Mesophilic lactococci ($\log_{10}$ cfu/g)	80 d	5.3 ± 0.1^a	5.4 ± 0.2^b	5.4 ± 0.1^{ab}	5.3 ± 0.1^a
	0 h	5.3 ± 0.6^a	5.7 ± 0.3^a	5.7 ± 0.4^a	6.0 ± 0.4^a
	12 h	7.7 ± 0.5^a	6.9 ± 0.4^a	7.3 ± 0.8^a	8.2 ± 0.5^a
Thermophilic lactococci ($\log_{10}$ cfu/g)	80 d	7.5 ± 0.7^a	7.3 ± 0.3^a	7.4 ± 0.9^a	8.0 ± 0.2^a
	0 h	5.5 ± 0.5^a	5.9 ± 0.4^a	5.7 ± 0.3^a	6.1 ± 0.5^a
	12 h	8.1 ± 0.4^a	7.5 ± 0.4^a	7.6 ± 0.4^a	8.3 ± 0.5^a
Lactobacilli ($\log_{10}$ cfu/g)	80 d	7.6 ± 0.4^a	7.0 ± 0.2^a	7.0 ± 0.9^a	8.1 ± 0.2^a
	0 h	5.1 ± 0.5^a	4.8 ± 0.4^{ab}	4.6 ± 0.7^b	5.3 ± 0.2^a
	12 h	7.2 ± 0.1^a	6.7 ± 0.2^b	6.3 ± 0.2^c	7.6 ± 0.2^a
	80 d	7.8 ± 0.2^a	6.7 ± 0.2^b	6.0 ± 0.4^c	6.5 ± 0.3^b

^{a-c}Within a column, mean values with the same superscript letter are not statistically different ($P > 0.05$).

¹Values are means $\pm$ SD. Post hoc comparison using Tukey's honestly significant difference test is reported.

respective curd samples (after cutting: $5.5 \pm 0.8 \log_{10}$ cfu/g, after cooking: $5.8 \pm 0.4 \log_{10}$ cfu/g).

In the present study, the production temperature favored pathogen growth, which reached $7.2 \log_{10}$ cfu/g in the cheese at the end of the pressing stage. Heating stress during curd cooking did not affect *E. coli* O157 viability. In contrast, *E. coli* O157:H7 grew by approximately $2 \log_{10}$ in fresh cottage cheese but was completely inactivated during the heating phase (57°C for 1.5 h; Arocha et al., 1992). High temperatures are unable to inactivate *E. coli* O157:H7 in Grana Padano PDO (up to 55°C) and Indian paneer (62°C for 5 min; Ercolini et al., 2005; Wahi et al., 2006).

Pressing overnight at room temperature appeared to have an effect on *E. coli* O157 counts in our samples. The population in curd was $6.0 \pm 0.2 \log_{10}$ cfu/g before and $7.2 \pm 0.3 \log_{10}$ cfu/g after pressing. Similarly, Reitsma and Henning (1996) reported increases in *E. coli* O157:H7 populations after pressing (overnight at 2.5 kg/cm^2) in Cheddar made from high inoculation milk ($3 \log_{10}$ cfu/mL) and decreases when the cheeses were inoculated with a lower concentration ($1 \log_{10}$ cfu/mL).

We observed a slight reduction from 7.2 ± 0.3 to $6.8 \pm 0.3 \log_{10}$ cfu/g after brining, which may be attributed to the increased susceptibility of *E. coli* O157:H7 to the antimicrobial effects of salt (Spano et al., 2003). At the end of the production process, the *E. coli* O157:H7 concentration was $4.0 \pm 0.3 \log_{10}$ cfu/g (Figure 2) as compared with an inoculum of approximately $5.0 \log_{10}$ cfu/mL. Its concentration decreased by about $3 \log_{10}$ cfu/g during the ripening period from the maximum concentration of $7.2 \pm 0.3 \log_{10}$ cfu/g. In their study on Cheddar cheese, Reitsma and Henning (1996) reported

that *E. coli* O157:H7 grew and survived during the ripening stage. When the milk was initially inoculated with $3 \log_{10}$ cfu/mL, viable bacteria were still present in the cheese after 158 d. A significant increase in *E. coli* O157:H7 count was reported during the manufacture of Camembert cheese (Ramsaran et al., 1998). Unlike both Camembert and Cheddar, cottage cheese undergoes extensive heat treatment (57°C over a 1.5-h period) during curd cooking (Vernozy-Rozand et al., 2005). Govaris et al. (2002) observed that *E. coli* O157:H7 inoculated in pasteurized milk for making Teleme cheese decreased during ripening and that viable bacteria were no longer present after 40 d. Viable *E. coli* O157:H7 cells were detected for up to 158 d (using an enrichment procedure) in Cheddar cheese made from milk inoculated at $3 \log_{10}$ cfu/mL (Reitsma and Henning, 1996). After an initial raw milk low inoculation (1 cfu/mL), *E. coli* O157:H7 was detected in Cheddar cheese at 180 d (D'Amico et al., 2010) and at 240 d (Schlesser et al., 2006) of aging. Schlesser et al. (2006) reported that viable *E. coli* O157:H7 cells were detected after more than 1 yr of aging in cheese made from milk with high inoculation levels (3 to $5 \log_{10}$ cfu/mL).

In our study, the LAB concentration increased during the first hours of processing and reached maximum values already at 6 h when the cheese wheel was in the press (lactobacilli, $7.6 \pm 0.2 \log_{10}$ cfu/g; thermophilic lactococci, $8.3 \pm 0.3 \log_{10}$ cfu/g; and mesophilic lactococci, $8.2 \pm 0.2 \log_{10}$ cfu/g; Figure 1). During the ripening stage, the concentrations declined gradually: this trend was more marked for the lactobacilli ($6.5 \pm 0.2 \log_{10}$ cfu/g) than either the thermophilic ($8.1 \pm 0.2 \log_{10}$ cfu/g) or the mesophilic lactococci ($8.0 \pm 0.2 \log_{10}$ cfu/g).

Figure 2. Changes in microbial count during Fontina-type cheese ripening in experimental production. Lc30 = mesophilic lactococci; Lb = lactobacilli; Lc45 = thermophilic lactococci; Ec = *Escherichia coli* O157. Error bars represent SD.

Water activity decreased gradually during the ripening stage and reached 0.95 at d 80 (Table 3). The pH decreased rapidly during the first 24 h (average pH 5.3 ± 0.1), after which it remained constant during ripening up to d 80.

In conclusion, *E. coli* O157 can grow during the early stages of processing of Fontina PDO. Curd cooking (48°C for 20 min) and preceding progressive heating to reach this temperature are insufficient to inactivate *E. coli* O157. The increase in LAB and the decrease in pH during the first hours do not seem to be effective in controlling *E. coli* O157 growth. Ripening is able to inhibit its survival, however, as observed in the reduction by 3 log from the maximum concentration during the earlier stages of production. The gradual reduction in A_w , associated with constantly high LAB concentration, could play an important role in reducing the concentration of *E. coli* O157. Its potential presence in the production of raw milk cheese should be kept in mind because the infectious dose of *E. coli* O157 is only a few cells. To the best of our knowledge, no scientific studies reporting *E. coli* O157 in Fontina PDO cheese have been published to date, nor have samples been found positive during official control analyses in our food laboratory over the years. This could be linked to either its low incidence in the milk used for making Fontina PDO or a low level of contamination that is eliminated during cheese ripening. It is essential that the milk used for raw milk cheese making be of the highest bacteriological quality.

ACKNOWLEDGMENTS

The present study received funding from the Italian Ministry of Health in the form of a centre grant: “Sviluppo e validazione di modelli matematici di microbiologia predittiva per la documentazione scientifica della sicurezza igienico sanitaria dei prodotti tradizionali italiani.” The authors thank the European Union Reference Laboratory for kindly providing *Escherichia coli*, including verotoxigenic *E. coli* for the *E. coli* O157 strains used in this study.

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