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Fate of Shiga toxin-producing and generic *Escherichia coli* during production and ripening of semihard raw milk cheese

S. Peng,*†[1] W. Hoffmann,‡ W. Bockelmann,§ J. Hummerjohann,† R. Stephan,* and P. Hammer‡
*Institute for Food Safety and Hygiene, University of Zurich, 8057 Zurich, Switzerland
†Agroscope Liebefeld-Posieux, 3003 Bern, Switzerland
‡Department of Safety and Quality of Milk and Fish Products, Max Rubner-Institut, 24103 Kiel, Germany
§Department of Microbiology and Biotechnology, Max Rubner-Institut, 24103 Kiel, Germany

ABSTRACT

The fate of 5 different *Escherichia coli* strains, including 3 Shiga toxin-producing *E. coli* (STEC) strains, was analyzed during the production and ripening of semihard raw milk cheese. The strains, which were previously isolated from raw milk cheese, were spiked into raw milk before cheese production at 2 different levels (approximately 10^1 and 10^3 cfu/mL, respectively). Two cheese types were produced, which differed in cooking temperatures (40 and 46°C). The cheeses were sampled during manufacture and the 16-wk ripening period. An increase in *E. coli* counts of approximately 3.5 $\log_{10}$ cfu/g occurred from raw milk to fresh cheese at d 1, which was attributed to a concentration effect during cheese production and growth of the strains. During ripening over 16 wk, a slow, continuous decrease was observed for all strains. However, significant differences were found between the *E. coli* strains at the applied spiking levels, whereas the inactivation was similar in the 2 different cheese types. The 2 generic *E. coli* strains survived at higher counts than did the 3 STEC strains. Nevertheless, only 1 of the 3 STEC strains showed significantly weaker survival at both spiking levels and in both cheese types. Six of 16 cheeses made from raw milk at a low spiking level contained more than 10 cfu/g of STEC at the end of the 16-wk ripening process. After enrichment, STEC were detected in almost all cheeses at both spiking levels. Particularly because of the low infectious dose of highly pathogenic STEC, even low colony counts in raw milk cheese are a matter of concern.

Key words: Shiga toxin-producing *Escherichia coli*, raw milk cheese, cheese production, spiking

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (**STEC**) are foodborne pathogens able to cause gastrointestinal diseases, including watery or bloody diarrhea and hemorrhagic colitis. In a proportion of cases, the infection leads to severe complications, including the hemolytic-uremic syndrome (Tarr et al., 2005).

Contaminated raw meat and raw meat products as well as raw milk and raw milk products are the main risk factors considered STEC vectors (Deschênes et al., 1996; Allerberger et al., 2001; Espié et al., 2006; Baylis, 2009). In a Swiss study, STEC were detected in 5.7% of raw milk cheese samples collected at the producer level (Zweifel et al., 2010). Therefore, investigations on the survivability of STEC in raw milk cheese are important in view of food safety and may aid in the development of control strategies for STEC. Previous studies on the survival of STEC in raw milk cheeses mainly focused on serotype O157:H7. Two studies by Schlesser et al. (2006) and D'Amico et al. (2010) indicated a slow decrease in *E. coli* O157:H7 during ripening of Cheddar and Gouda cheese. After 270 d of ripening, *E. coli* O157:H7 were still detected after selective enrichment (D'Amico et al., 2010). In smear-ripened cheese produced from raw milk, between 1 and 10 cfu/g of nontoxigenic *E. coli* O157:H7 was detected after 70 d (Maher et al., 2001). Montet et al. (2009) investigated the growth and survival of acid-resistant and non-acid-resistant non-O157 STEC strains during the manufacture and ripening of Camembert cheese. However, the differences in acid resistance did not result in varied behavior of the strains in the cheese.

In the present study, we used 5 different non-O157 *E. coli* strains, including 3 STEC strains, to spike cheeses similar to Swiss-type semihard raw milk cheese. All strains used were previously isolated from raw milk cheese. The objectives of this study were (1) to investigate the fate of the non-O157 *E. coli* strains during production and ripening of the raw milk cheese, (2) to compare differences in inactivation between the *E. coli* strains, and (3) to examine the effect of 2 different

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[1]Corresponding author: ils@fsafety.uzh.ch

Table 1. Characteristics of the *Escherichia coli* strains used in this study

Strain	Serotype	Virulence gene				RpoS[1] phenotype (catalase test)	Thermal inactivation (55°C, 15 min; $\log_{10}$)	Oxidative AR[2] system (survival, 2 h, pH 2.5; %)
		stx1[3]	*stx2*[3]	*eae*[4]	*hlyA*[5]			
K356	O2:H27	−	+	−	+	+	−1.52	5.2
K303	O9:H21[6]	−	−	−	−	−	−1.89	0.6
N09-1208	O26:H11	+	−	+	+	+	−1.90	7.9
K331/4	O91:H21	+	+	−	+	+	−1.78	12.6
FAM21843	O178:H12	−	−	−	−	+	−0.04	27.6

[1]RPoS = key regulator of general stress response.
[2]Acid resistance.
[3]Shiga toxins 1 and 2.
[4]Intimin.
[5]Hemolysin A.
[6]Strain phenotypically nonmotile.

cooking temperatures during cheese production on the fate of the *E. coli* strains.

MATERIALS AND METHODS

Study Design

Three STEC and 2 generic *E. coli* strains that were previously isolated from raw milk cheese were selected based on in vitro characterization data, which included phenotypic traits and stress-response abilities relevant to raw milk cheese (Table 1; Peng et al., 2012). Strain FAM21843 was selected based on its high resistance to acid and heat stress. Strain K303 was a catalase-negative strain, which may indicate a defect in RpoS function, a key regulator of the general stress response, and thus affect stress-response abilities (Large et al., 2005). Strain K356 belongs to serotype O2:H27, the most often isolated serotype during a monitoring program in Switzerland (Zweifel et al., 2010). Strain N09-1208 represents serotype O26:H11, 1 of the top 5 STEC serogroups (Bielaszewska et al., 2007). Strain K331/4 belongs to serotype O91:H21, which is one of the most important intimin (*eae*)-negative STEC serotypes associated with severe disease (Mellmann et al., 2009). The *E. coli* strains were split into 2 mixtures for spiking of the raw milk before the cheese production process at 2 different levels to simulate a low and a high contamination level. The raw milk cheeses were produced according to a Swiss recipe for semihard raw milk cheese. In Switzerland, different varieties of raw milk semihard cheeses are produced by using cooking temperatures from 40 up to 46°C. Therefore, 40 and 46°C were selected as cooking temperatures for cheese production. In addition, the fate of the different spiked *E. coli* strains was compared between those 2 different semihard raw milk cheese types, including the effect of the heat shock encountered during cooking at 46°C. During production and the 16-wk ripening period, the cheeses were sampled to investigate the different *E. coli* strains quantitatively and qualitatively. Because of the use of selective media based on inherit properties of the strains, each strain was quantified separately. To assess the cheese production, physicochemical parameters, the behavior of the starter culture, and occurrence of further microbial flora were determined.

Preparation of Spiking and Starter Culture

Escherichia coli strains were grown separately in 10 mL of tryptic soy broth (**TSB**; Oxoid, Wesel, Germany) for 24 h at 37°C. From TSB, 0.1 mL was taken, added to 10 mL of sterile skim milk, and grown for 24 h at 37°C. Cultures were serially diluted in 10 mL of sterile skim milk. For the low spiking level, 10 mL of $1:10^4$ dilutions of strain FAM21843, K303 and K356 (strain mixture 1), or K331/4 and N09-1208 (strain mixture 2) were pooled, and sterile skim milk was added to a total volume of 100 mL. For the high spiking level, 10 mL of $1:10^2$ dilutions of strain FAM21843, K303 and K356, or K331/4 and N09-1208 were pooled, and sterile skim milk was added to a total volume of 100 mL. The 100-mL pooled skim milk contained approximately 5×10^5 or 5×10^3 cfu/mL of each strain in the mixture at the high or low spiking level, respectively. To 1 kg of cold, sterilized milk, 3.6 g of lyophilized starter culture (Choozit Alp D Lyo 100; Danisco, Niebüll, Germany) was added, dispersed, and stored for 12 h at 4°C before use.

Cheese Production and Sampling

Raw cow's milk from the experimental farm of the Max Rubner-Institut (Kiel, Germany) was used for cheese production. From each batch, 25 mL was taken for enrichment and analyzed for the absence of STEC

Table 2. Cheese production process of 2 types of semihard raw milk cheese with different cooking temperatures, 40 and 46°C

Time lapse	Processing step
	50 kg of raw milk (32°C)
0 min	Addition of *E. coli* cultures and water (3.6 kg, pasteurized), stirring
10 min	Addition of starter culture (3.6 g), 12 h earlier dispersed in 1 kg of cold sterilized milk, stirring
1 h 25 min	Addition of rennet and water (0.7 kg, pasteurized)
2 h 5 min	Cutting (cubes with 8 to 10 mm length of an edge), stirring
2 h 35 min	Addition of water (8.2 kg, pasteurized), stirring, cooking (indirect heating)
2 h 50 min	End of cooking (32°C → 40 or 46°C, respectively), stirring
3 h 5 min	Molding
3 h 20 min	Pressing (300 kPa)
3 h 35 min	Turning and pressing (400 kPa)
4 h 5 min	End of pressing, turning
5 h 5 min	Turning
8 h 5 min	Turning
10 h 5 min	Turning (curd temperature approximately 34 and 35°C, respectively)
24 h	Brining of 2 pressed loaves (approximately 2.5 kg, 15°C)
48 h	Drying of cheese surface at 15°C
50 and 74 h	2 coatings at 15°C
80 h	Start of ripening (13 to 14.5°C, 91 to 94% relative humidity)

and target *E. coli* strains (enrichment protocol as described in the Microbiological Tests section). The entire cheese production process is summarized in Table 2. For cheese production, 50 kg of raw milk was warmed to 32°C and 100 mL of spiked skim milk was added, which resulted in approximately 10^1 and 10^3 cfu/mL per strain in the mixture at the low and high spiking levels, respectively. After addition of 3.6 kg of pasteurized water and agitation of the milk for 10 min, a sample was taken. Subsequently, 1 kg of milk containing the starter culture was added and the milk was ripened for 75 min before 0.7 kg of pasteurized water and 40 g of rennet (Naturen Premium 145; Chr. Hansen, Nienburg, Germany) were added. The coagulated milk was cut 40 min later into 0.8- to 1.0-cm cubes and agitated for 30 min. After addition of 8.2 kg of pasteurized water, the curd was heated within 15 min from 32 to 40 or 46°C, respectively, and held for 15 min before the curd was filled into 2 rectangular forms (25 × 12.5 × 12.5 cm) per batch. A 10-g curd sample was taken. The curd was pressed, first for 15 min at 300 kPa and then for 30 min at 400 kPa. Between and after the 2 pressing cycles, the cheese loaves were turned. Additional turning occurred 1, 4, and 6 h after pressing. The forms were removed 20 h after pressing, and 10 g of fresh cheese sample was taken from each loaf. The cheese was transferred into brine [20% (wt/vol) sodium chloride, pH 5.1 adjusted with lactic acid] for 24 h at 15°C. The cheese surface was dried at 15°C and coated 2 times with mold-inhibiting plastic dispersion (IP Ingredients, Süderlügum, Germany). Subsequently, the cheese was ripened at 13 to 14.5°C and a relative humidity of 91 to 94% for 16 wk and then sampled after 1, 2, 3, 4, 6, 8, 12, and 16 wk by taking bore samples. The bore holes were filled with wax to avoid dehydration of the cheese at the sampling site. All combinations of cheese type, strain mixture, and spiking level were produced in duplicate (resulting in a sum of 4 cheeses per combination, of which 2 were produced from the same batch of raw milk). In addition, both cheese types were produced without spiking once.

Chemical and Physical Analysis of Cheese

To monitor the cheese production process, acidification of each batch was analyzed during manufacture and ripening by using a pH meter. Before brining, a representative slice of all fresh cheeses was cut out of each loaf on a sterile workbench. Subsequently, samples were prepared for analysis of DM. The remainder of the slice was packaged in aluminum foil and heated in a drying oven for 2 h at 90°C. After this decontamination, the cheese was cooled overnight and further analyzed.

The contents of fat and sodium chloride were determined according to German standard methods (VDLUFA, 2003). DL-Lactic acid and galactose were determined by using enzymatic UV tests (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany). The ratio of DM before and after decontamination was used to calculate the contents of fat, sodium chloride, lactic acid, and galactose before decontamination (VDLUFA, 2003). Each parameter was analyzed in duplicate. The entire procedure was repeated after the 16-wk ripening period.

Microbiological Tests

Generic E. coli and STEC. Cheese samples of 10 g were homogenized with 90 g of dipotassium hydrogenphosphate solution (115 mmol/L of dipotas-

sium hydrogenphosphate, pH 7.5) for 3 min by using a stomacher. Decimal dilution series were made for enumeration of the *E. coli* by spread plating on selective agar plates that use inherited properties of the strains for detection. Strain mixture 1 was spread on adonitol-MacConkey agar (40 g/L of MacConkey Agar Base, Becton Dickinson, Heidelberg, Germany; 10 g/L of adonitol, Sigma-Aldrich, Schnelldorf, Germany) for the enumeration of strain K303, which is not able to ferment adonitol; adonitol-MacConkey agar containing trimethoprim (10 μg/mL; Sigma-Aldrich) for the enumeration of strain FAM21843, which is resistant to trimethoprim; and Rapid *E. coli* 2 agar (Bio-Rad Laboratories, Munich, Germany) for the enumeration of strain K356, which lacks β-glucuronidase activity. Strain mixture 2 was spread on rhamnose-MacConkey agar (40 g/L of MacConkey Agar Base, 10 g of rhamnose; Sigma-Aldrich) for enumeration of strain K331/4, which is able to ferment rhamnose, and strain N09-1208, which does not ferment rhamnose. The Rapid *E. coli* 2 and MacConkey agar plates were incubated at 37°C for 18 to 24 h. For each strain, typical colonies were identified based on colony morphologies and enumerated. At random, isolates were further identified by serogroup-specific tests [monospecific Anti-Coli test sera (Sifin, Berlin, Germany) and serogroup-specific PCR (Liu et al., 2010)].

Another 25-g cheese sample was taken for the enrichment procedure if an *E. coli* strain was not detected quantitatively. The cheese samples were homogenized with 225 g of modified TSB (Oxoid)-acriflavin (12 mg/L; Sigma-Aldrich) for 3 min by using a stomacher. Enrichment broth was incubated at 37°C for 18 to 24 h. For detection of the target strains, 10 μL of the enrichment broth was streaked onto adonitol-MacConkey agar, adonitol-MacConkey agar containing trimethoprim, Rapid *E. coli* 2 agar, and rhamnose-MacConkey agar. Additionally, for the detection of the Shiga toxin-encoding genes *stx1* and *stx2*, 10 μL of the enrichment broth was streaked onto blood agar [Columbia agar supplemented with 5% (vol/vol) defibrinated sheep blood, both from Oxoid]. The Rapid *E. coli* 2, MacConkey, and blood agar plates were incubated at 37°C for 18 to 24 h. Blood agar plates were washed using 1.5 mL of 0.9% sodium chloride solution. To 200 μL of the eluate, 400 μL of double-distilled water was added. The solution was heated for 5 min at 95°C and centrifuged for 1 min at 10,000 × *g* (microcentrifuge 5415C; Eppendorf, Hamburg, Germany), and the supernatant was transferred to a new tube and used as a template for conventional PCR. Polymerase chain reaction specific for *stx1* and *stx2* was performed according to the methods of Schmidt et al. (1994) and Piérard et al. (1998), respectively.

Starter Culture and Additional Flora. The starter culture and additional cheese flora were examined in fresh cheese and in cheese samples taken after 1, 4, 12, and 16 wk of ripening. Dilution series from homogenized cheese samples were spread on different selective agars and incubated for 18 to 24 h: medium 17 agar incubated at 25 and 42°C for mesophilic and thermophilic lactic *Streptococcus*, respectively (Terzaghi and Sandine, 1975); deMan, Rogosa, and Sharpe agar incubated at 30 and 45°C for mesophilic and thermophilic *Lactobacillus*, respectively; kanamycin esculin azide agar at 37°C for *Enterococcus*; Schleifer-Krämer agar at 30°C for *Staphylococcus*; yeast extract glucose chloramphenicol agar at 25°C for yeasts and molds; and violet red bile dextrose agar at 37°C for *Enterobacteriaceae*. To differentiate the morphology of bacteria on the selective agars, microscopy was performed. All media and supplements were obtained from Merck (Darmstadt, Germany) with the exception of peptone from CN (Sigma-Aldrich), L(+)-ascorbic acid (BDH Prolabo, Darmstadt, Germany), and glycerin (AppliChem GmbH, Darmstadt, Germany).

Statistical Analysis

Colony counts (**CC**) from each batch, consisting of the average of the 2 cheeses produced simultaneously, were logarithmically transformed for statistical analysis. Samples below the limit of quantitative detection (<10 cfu/g) were set at a logarithmized value of 0. Different conditions were compared using repeated-measures ANOVA with Tukey's post hoc test. Decay rates per week were determined by linear regression. Results of physicochemical analysis from the same batch were averaged and compared using ANOVA. For statistical analysis, IBM SPSS Statistics version 19 (IBM Corporation, Armonk, NY) was used.

RESULTS

Raw Milk Before the Cheese-Making Process

The batches of raw milk contained 3.57 to 3.84% fat and 3.02 to 3.09% protein. All samples tested negative for the presence of *stx* genes and target *E. coli* strains (data not shown).

Chemical and Physical Analyses of Cheese

Results of the chemical and physical analyses are summarized in Table 3. The higher cooking temperature resulted in a significantly higher pH after acidification ($P \leq 0.001$; Figure 1). The difference in pH was approximately 0.2 and remained stable over the

Table 3. Results of chemical and physical analyses in fresh and ripened semihard raw milk cheeses[1] made at different cooking temperatures[2]

	Cooking temperature of 40°C, n = 9		Cooking temperature of 46°C, n = 9	
Item	Fresh cheese	Ripened cheese	Fresh cheese	Ripened cheese
DM (%)	52.52 ± 1.82	61.25 ± 1.00	55.60 ± 1.73	63.95 ± 0.86
Fat in DM (%)	51.91 ± 3.84		53.87 ± 2.00	
Moisture on a fat-free basis (%)		56.81		55.00
Sodium chloride (%)	0.11 ± 0.03	2.04 ± 0.17	0.10 ± 0.04	1.69 ± 0.09
Total lactic acid (%)	1.38 ± 0.07	1.55 ± 0.15	1.14 ± 0.04	1.38 ± 0.09
(without inoculation with *E. coli*)	(1.51)	(1.72)	(1.18)	(1.48)
D-Lactic acid (%)	0	0.65 ± 0.14	0	0.54 ± 0.13
L-Lactic acid (%)	1.38 ± 0.07	0.90 ± 0.18	1.14 ± 0.04	0.84 ± 0.16
Galactose (%)	0.14 ± 0.03	0	0.23 ± 0.03	0

[1]Fresh and ripened cheese samples were from d 1 and wk 16, respectively.

[2]Mean values and SD of batches made at the same cooking temperature; each batch was the average of the 2 cheeses produced simultaneously.

ripening period. The higher cooking temperature also yielded a higher DM of the semihard cheeses. The difference was approximately 3% in the fresh cheese before brining and in the ripened cheese. Dry matter increased by approximately 8.5% during ripening. Sodium chloride content was at 1.69% after ripening. In contrast, the cheeses cooked at the lower temperature contained 0.35% more sodium chloride after ripening and more total lactic acid (**TLA**) before and after ripening. The TLA content increased during ripening. On average, the 2 batches without addition of *E. coli* contained more TLA than those inoculated with the different strains. However, the difference was significant only in fresh cheeses cooked at the lower temperature ($P \leq 0.05$). Although the cheeses contained only L-lactic acid before ripening, they contained approximately 40% D- and 60% L-lactic acid after ripening.

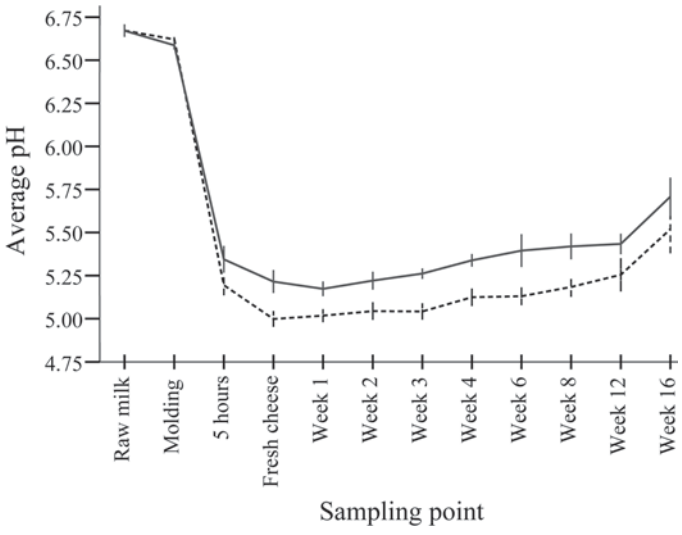

Figure 1. Acidification curves (40°C, dashed line; 46°C, solid line) of the semihard raw milk cheeses produced. Mean values and SD of batches made at the same cooking temperature. Nine batches per cooking temperature, with each batch being the average of the 2 cheeses produced simultaneously.

Fate of E. coli During Cheese Manufacture

Average levels of the *E. coli* strains were 1.42 ± 0.28 $\log_{10}$ cfu/g at the low spiking level and 3.30 ± 0.14 $\log_{10}$ cfu/g at the high spiking level, respectively. Average CC of strains FAM21843, K303, K356, and N09-1208 in fresh cheese were 5.32 ± 0.42 $\log_{10}$ at the low and 6.94 ± 0.19 $\log_{10}$ cfu/g at the high spiking level, respectively. For strain K331/4, a smaller increase from raw milk to fresh cheese was observed, to 4.13 ± 0.37 $\log_{10}$ at the low and 5.73 ± 0.22 $\log_{10}$ at the high spiking level, respectively. The increase in CC during cheese manufacture was lower for strain K331/4 than for the other 4 strains at both spiking levels and in both cheese types (significant only at the high spiking level, $P \leq 0.01$). No significant differences in the increase of CC during manufacture of the cheese were observed between cheeses made at different cooking temperatures.

Inactivation of E. coli During Cheese Ripening at the Low Spiking Level

After the increase in CC during manufacture of the cheese, CC of the *E. coli* strains decreased during cheese ripening, with significant differences between the strains in cheeses cooked at 40 and 46°C ($P \leq 0.01$ each; Figure 2a and 2b). The inactivation of the *E. coli* strains was similar in cheeses made at different cooking temperatures. For strain K303, the highest CC were found in both cheese types. Additionally, strain K303 was inactivated slower than the other strains, at average decay rates of 0.23 ± 0.05 and 0.25 ± 0.03 $\log_{10}$ reduction per week in cheeses made at cooking temperatures of 40 and 46°C, respectively. In contrast, the STEC strain K331/4 showed the highest average decay rates, at 0.58 ± 0.10 and 0.79 ± 0.33 $\log_{10}$ reduction

per week in cheeses cooked at 40 and 46°C, respectively, and was below the limit of detection (<10 cfu/g) in all samples after wk 8. The CC of strains FAM21843, N09-1208, and K356 did not differ from each other and from strain K303 over the ripening period. Their average decay rates were between 0.23 and 0.37 $\log_{10}$ reduction per week in the 2 different cheese types. The *E. coli* strains were detected after enrichment in all but 1 cheese sample after 16 wk, whereas N09-1208 was not detected.

Inactivation of E. coli During Cheese Ripening at the High Spiking Level

Colony counts of the *E. coli* strains decreased during cheese ripening, with significant differences between the strains in cheeses cooked at 40 and 46°C ($P \leq 0.01$ each; Figure 2c and 2d). The inactivation of the *E. coli* strains was similar in cheeses made at different cooking temperatures. Strains FAM21843, K303, and N09-1208 were similarly inactivated in CC and in rates of decay (0.16 to 0.29 $\log_{10}$ reduction per week) in both cheese types. The CC of STEC strains K331/4 and K356 showed greater reductions compared with the generic *E. coli* in cheeses cooked at 40°C ($P \leq 0.05$). In cheeses cooked at 46°C, the CC of only strain K331/4 was significantly different from those of the 2 generic *E. coli* strains ($P \leq 0.05$). The *E. coli* strains were detected after enrichment in all but 1 cheese sample after 16 wk, whereas K356 was not detected.

Starter Culture and Additional Flora

Average CC of the starter culture and the additional flora were not significantly different between the spiked

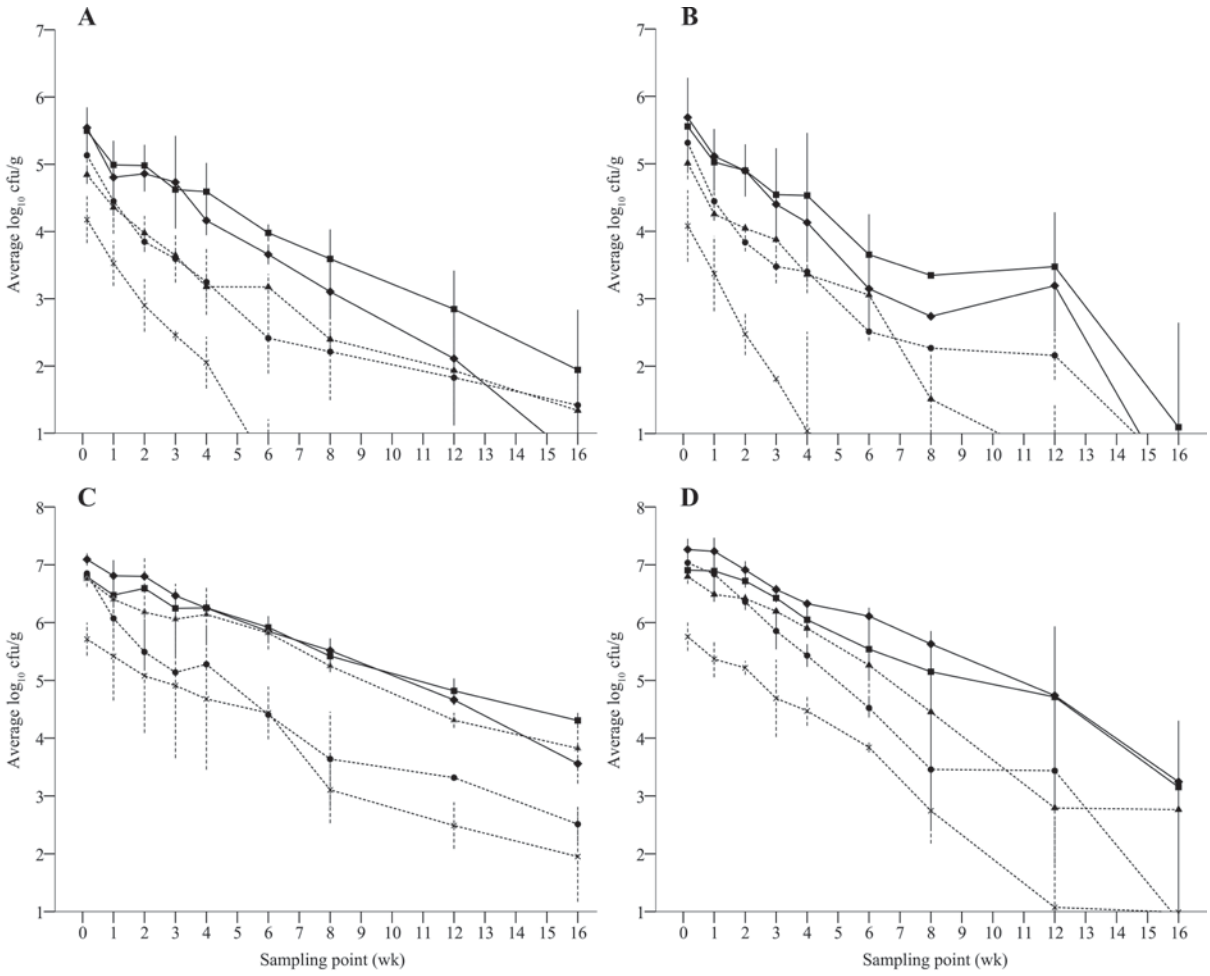

Figure 2. Average colony counts of *Escherichia coli* strains (generic *E. coli*, solid lines; Shiga toxin-producing *E. coli*, dashed lines) during ripening of semihard raw milk cheese. Mean values and SD of batches. Two batches per combination of strains, cooking temperature, and spiking level, with each batch being the average of the 2 cheeses produced simultaneously. K303 (■), FAM21843 (◆), N09-1208 (▲), K356 (●), K331/4 (×). A) 40°C cooking temperature, low spiking level; B) 46°C cooking temperature, low spiking level; C) 40°C cooking temperature, high spiking level; D) 46°C cooking temperature, high spiking level.

and unspiked cheeses (Table 4). Thermophilic and mesophilic *Streptococcus* decreased by approximately 1.2 $\log_{10}$ cfu/g during ripening, whereas thermophilic and mesophilic *Lactobacillus* were able to grow in the cheese. The CC of *Enterococcus* remained stable over the ripening period. The CC of *Staphylococcus* decreased by more than 2 $\log_{10}$. Small amounts of yeasts were found, which decreased during ripening. Only for *Staphylococcus* was a significant difference in CC ($P \leq 0.05$) observed between the 2 cooking temperatures, with counts being higher in cheeses cooked at 46°C than in those cooked at 40°C at all sampling points. However, the inactivation was similar in both cheese types. The average counts of *Enterobacteriaceae* on violet red bile dextrose were mainly due to the spiked *E. coli* strains, and they did not differ significantly from the sum of average counts of the strains (data not shown).

DISCUSSION

As a quality control for cheese production and ripening, the starter culture, natural cheese flora, and physicochemical parameters were examined during the process. The acidification of the cheeses as well as further physicochemical parameters were in the expected range and did not differ significantly between unspiked and all spiked cheeses. The behavior of the lactic acid bacteria was as expected for both *Streptococcaceae* and *Lactobacillus*.

In contrast to other studies, the *E. coli* strains used in this study for the spiking experiments were isolated from raw milk cheese and the strains were precultured in milk before spiking. Therefore, the *E. coli* strains were adapted to the cheese production environment.

From raw milk to fresh cheese, *E. coli* counts increased within the first day of cheese production. This effect was also observed in other cheese-spiking studies and has been attributed to the entrapment of bacteria in the curd and the draining of whey (Schlesser et al., 2006; Montet et al., 2009). The physical concentration effect was expected to correlate with the mass ratio between raw milk used and cheese produced. Therefore, an increase of approximately 1 $\log_{10}$ attributable to the physical concentration was estimated, and the additional increase was attributed to the growth of the *E. coli* strains. This growth was supported by the slow temperature decrease in the cheese loaves while being stored at room temperature for pressing and turning (Table 2), which reflects the situation in practice. The increase for 4 *E. coli* strains was similar, whereas it was significantly lower for STEC strain K331/4. While growing in milk simultanously at 30 or 37°C, the STEC strains showed similar growth patterns and no strain competition (data not shown). Therefore, the lower increase of K331/4 is most probably due to the stresses occurring during cheese production, which affected this strain more than the other 4 *E. coli* strains.

During the ripening period, a slow, continuous decrease in colony counts was observed for all strains at both spiking levels, which was attributed to the sum of stresses in the raw milk cheese (Peng et al., 2011). The decrease occurred similarly to other challenge test studies, which examined the behavior of *E. coli* in different cheese types (Maher et al., 2001; Schlesser et al., 2006; Montet et al., 2009; D'Amico et al., 2010). The inactivation of the *E. coli* strains during the ripening period was not significantly different with regard to the varying cooking temperature and the resulting difference in acidification of the cheese. Although the higher cooking temperature was expected to cause a heat shock response, it did not result in a significant reduction of the *E. coli* strains. The difference in pH between cheeses cooked at 40 and 46°C was probably too small to cause a significant difference in inactivation of the *E. coli* strains. However, the decay rates in the 40°C cooked cheese type were by trend lower than for the cheeses cooked at 46°C.

The differences in inactivation between the 2 generic *E. coli* and 3 STEC strains were significant ($P \leq 0.01$ in both cheese types at both inoculation levels). It is important to use different strains and evaluate each strain individually for spiking and challenge tests to

Table 4. Average colony counts of additional flora[1]

Item	d 1	wk 1	wk 4	wk 12	wk 16
Lactic *Streptococcus*, mesophilic	9.21 ± 0.20	9.19 ± 0.25	9.08 ± 0.25	8.31 ± 0.29	8.03 ± 0.36
Lactic *Streptococcus*, thermophilic	8.71 ± 0.35	8.75 ± 0.33	8.60 ± 0.33	7.56 ± 0.86	7.49 ± 0.36
Lactobacillus, mesophilic	ND[2]	6.48 ± 0.47	7.53 ± 0.55	7.95 ± 0.15	7.83 ± 0.29
Lactobacillus, thermophilic	4.70 ± 0.45	5.56 ± 0.47	7.19 ± 0.62	7.24 ± 0.18	7.08 ± 0.26
Enterococcus	4.11 ± 0.39	4.09 ± 0.36	4.09 ± 0.36	4.19 ± 0.26	4.23 ± 0.30
Yeasts and molds	3.66 ± 0.42	2.36 ± 1.04	2.35 ± 1.04	0.96 ± 1.60[3]	0.81 ± 1.59
Staphylococcus	5.39 ± 0.42	4.78 ± 0.41	4.58 ± 0.43	3.16 ± 0.66	3.15 ± 0.70

[1]Mean values and SD of all batches ($\log_{10}$ cfu/g); 18 batches, each of which was the average of the 2 cheeses produced simultaneously.

[2]Not determined (ND) because of strong growth of coccoid flora.

[3]One batch was excluded from statistical analysis because of the growth of molds on the surface of the cheese.

include variations that potentially affect the survival of the strains. The results presented here indicate that the differences in the heat shock response and oxidative acid response system that were used for selection of the strains (Table 1) were not a major factor contributing to the survival of *E. coli* in raw milk cheese. In the cheeses, it is probably more important to cope with the sum of the relatively mild stresses than with the stronger stresses applied for selection of the strains. The generic *E. coli* strain K303 was highly susceptible to heat and acid stress and additionally has a potential defect in RpoS-function. Nevertheless, the lowest rate of decay and a higher CC for this strain were observed than for the STEC strains at both spiking levels and in both cheese types. The *RpoS* gene of the *E. coli* strains used were sequenced to investigate the potential defect in RpoS function of strain K303. However, the *RpoS* genes of the 5 strains were identical at the protein level. This does not exclude a potential regulatory defect in RpoS function of strain K303, but even then the survival of the strain was similar to strains FAM21843 and N09-1208 in both cheese types.

The greatest inactivation was found for strain K331/4, which, already during the production, exhibited a lower observed increase compared with the other strains. The stresses occurring during production and ripening could lead to a higher induction rate of prophages and therefore accelerate the reduction of the STEC strains. This effect could be small in STEC strains harboring 1 *stx* prophage but could increase in a strain harboring more than 1 prophage, such as K331/4, which harbors 2 *stx* prophages. This hypothesis, as well as other possible factors influencing the fate of K331/4 during cheese making (e.g., starter culture), will need to be tested by further experiments

Even at the low spiking level and without enrichment, strains K303, FAM21843, N09-1208, and K356 were detected in several cheeses after the 16-wk ripening period. Only STEC strain K331/4 declined below the limit of quantitative detection (<10 cfu/g) during cheese ripening in all cheeses made at the low spiking level. However, strain K331/4 was still detectable after enrichment in all cheeses. Detection after enrichment past a long cheese-ripening period (e.g., 270 d in Cheddar and Gouda) was also shown in other studies (Schlesser et al., 2006; D'Amico et al., 2010). After 4 mo of ripening, STEC strains were still quantified (>10 cfu/g) from 6 of 16 cheeses made at the low spiking level and from 13 of 16 cheeses made at the high spiking level, whereas detection after enrichment was possible in almost all cheeses. Particularly because of the low infectious dose of highly pathogenic STEC (estimated at <100 cells; Kaper et al. 2004), even low CC in raw milk cheese pose a potential health risk. The 2 generic *E. coli* strains survived at higher counts than the STEC strains and therefore may be considered model organisms for further studies. If these 2 strains were inactivated during raw milk cheese production, the process would be expected to reduce the STEC strains as well. The use of the 2 generic *E. coli* strains as model organisms in further challenge tests would allow the production of raw milk cheese to move even closer to reality, namely, in the size and form of the cheese and in the use of the typical red smear instead of a wax coating. The use of red smear, which is very common in Swiss semihard cheeses, could not be applied in this study because of biosafety restrictions.

In summary, it was possible to show differences in the fate of 5 *E. coli* strains, which included 3 STEC strains, during the production and ripening of semihard raw milk cheese. Both generic *E. coli* and STEC strains were detected in almost all cheeses at the end of the 16-wk ripening period, which is a considerable food safety issue. Therefore, additional research is necessary to understand which factors are contributing to the fate of diverse *E. coli*, particularly STEC, in raw milk cheese.

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