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

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ABSTRACT

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

Keywords: Shiga toxin-producing *Escherichia coli* (STEC), raw milk cheese, cheese production, spiking

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are food-borne pathogens able to cause gastrointestinal diseases, including watery or bloody diarrhea and hemorrhagic colitis. In a proportion of cases the infection is leading 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 as STEC vectors (Deschenes et al., 1996; Allerberger et al., 2001; Espie 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 survival abilities 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 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) observed a slow decrease of *E. coli* O157:H7 during ripening of Cheddar and Gouda cheese. After 270 days 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 a non-toxigenic *E. coli* O157:H7 was detected between 1 and 10 CFU/g after 70 days (Maher et al., 2001). Montet et al. (2009) investigated growth and survival of acid-resistant and non-acid-resistant non-O157 STEC strains during manufacture and ripening of Camembert cheese. However, the differences in acid resistance did not result in varied behaviour of the strains in the cheese.

In the present study we used five different non-O157 *E. coli* strains, including three STEC strains, for spiking cheeses similar to Swiss-type semi-hard raw milk cheese. All strains used were previously isolated from raw milk cheese. The objectives of this study were (i) to investigate the fate of the non-O157 *E. coli* strains during production and ripening of the raw milk cheese, (ii) to compare differences in inactivation between the *E. coli* strains, (iii) and to

68 examine the effect of two different cooking temperatures during cheese production on the fate
69 of the *E. coli* strains.
70
71 **MATERIALS AND METHODS**
72 ***Study Design***
73 Three Shiga toxin-producing and two generic *E. coli* strains that were previously isolated
74 from raw milk cheese, were selected based on *in vitro* characterization data which included
75 phenotypic traits and stress response abilities relevant in raw milk cheese (Table 1). Strain
76 FAM21843 was selected based on its high resistance to acid and heat stress. Strain K303 is a
77 catalase negative strain, which may indicate a defect in RpoS function, a key regulator of the
78 general stress response, and thus affect stress response abilities (Large et al., 2005). Strain
79 K356 belongs to serotype O2:H27, the most often isolated serotype during a monitoring
80 program in Switzerland (Zweifel et al., 2010). Strain N09-1208 represents serotype O26:H11,
81 one of the top five STEC serogroups (Bielaszewska et al., 2007). Strain K331/4 belongs to
82 serotype O91:H21 which is one of the most important intimin (*eae*) negative STEC serotype
83 associated with severe disease (Mellmann et al., 2009). The *E. coli* strains were split into two
84 mixtures for spiking of the raw milk prior to the cheese production process at two different
85 levels to simulate a low and a high contamination level. The raw milk cheeses were produced
86 according to a Swiss recipe for semi-hard raw milk cheese. In Switzerland, different varieties
87 of raw milk semi-hard cheeses are produced by using cooking temperatures from 40°C up to
88 46°C. Therefore 40°C and 46°C as cooking temperatures were selected for the cheese
89 production. In addition, the fate of the different spiked *E. coli* strains were compared between
90 those two different semi-hard raw milk cheese types, including the effect of the heat shock
91 encountered during cooking at 46°C. During production and the 16 week ripening period the
92 cheeses were sampled to investigate the different *E. coli* strains quantitatively and
93 qualitatively. Due to the use of selective media based on inherit properties of the strains, each

strain was quantified separately. To assess the cheese production, physicochemical parameters, behaviour of the starter culture and occurrence of further microbial flora were determined.

Preparation of Spiking and Starter Culture

E. coli strains were grown separately in 10 ml tryptic soy broth (TSB, Oxoid, Wesel, Germany) for 24 h at 37°C. From TSB 0.1 ml were taken, added to 10 ml sterile skim milk and grown for 24 h at 37°C. Cultures were serially diluted in 10 ml sterile skim milk. For low spiking level, 10 ml of $1:10^4$ dilutions of either strains 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 high spiking level, 10 ml of $1:10^2$ dilutions of either strains 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 app. 5 x 10^5 or 5 x 10^3 CFU/ml of each strain in the mixture at high or low spiking level, respectively. To 1 kg cold, sterilized milk 3.6 g lyophilized starter culture (Choozit Alp D Lyo 100, Danisco, Niebüll, Germany) were added, dispersed and stored 12 h at 4°C prior to use.

Cheese Production and Sampling

Cow's raw milk from the experimental farm of the Max Rubner-Institut (Kiel, Germany) was used for cheese production. From each batch 25 ml were taken for enrichment and analyzed for the absence of STEC and target *E. coli* strains (enrichment protocol as described in paragraph "microbiological tests"). The whole cheese production process is summarized in Table 2. For cheese production, 50 kg raw milk were warmed to 32°C and 100 ml spiked skim milk were added, which resulted in about 10^1 and 10^3 CFU/ml per strain in the mixture at low and high spiking level, respectively. After addition of 3.6 kg pasteurized water and agitation of the milk for 10 min a sample was taken. Subsequently, 1 kg milk containing the

starter culture was added and milk was ripened for 75 min before 0.7 kg pasteurized water and 40 g rennet (Naturen® Premium 145, Chr. Hansen, Nienburg, Germany) were added. The coagulated milk was cut 40 min later into 0.8-1.0 cm cubes and agitated for 30 min. After addition of 8.2 kg pasteurized water the curd was heated within 15 min from 32°C to 40°C or 46°C, respectively, and held for 15 min before the curd was filled into two rectangular forms (25 x 12.5 x 12.5 cm) per batch. A 10 g curd sample were taken. The curd was pressed first for 15 min at 300 kPa then for 30 min at 400 kPa. Between and after the two pressing cycles the cheese loafs were turned. Additional turning occurred 1, 4 and 6 h after pressing. The forms were removed 20 h after pressing and 10 g fresh cheese sample from each loaf was taken. The cheese was transferred into brine (20% (w/v) 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 two times with mould-inhibiting plastic dispersion (IP Ingredients, Süderlügum, Germany). Subsequently the cheese was ripened at 13-14.5°C and a relative humidity of 91-94% for 16 weeks and sampled after 1, 2, 3, 4, 6, 8, 12 and 16 weeks 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 four cheeses per combination of which two 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 using a pH-meter. Before brining, a representative slice of all fresh cheeses was cut out of each loaf under a sterile work bench. Subsequently, samples were prepared for analysis of dry matter. The remaining of the slice was packed in aluminium foil and heated in a drying oven for 2 h at 90°C. After this decontamination, the cheese was cooled overnight and further analyzed.

146 The content of fat and sodium chloride was determined according to German standard
147 methods (VDLUFA, 2003). D-/L-lactic acid and galactose were determined by using
148 enzymatic UV tests (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany). The ratio of
149 dry matter before and after decontamination was used to calculate the content of fat, sodium
150 chloride, lactic acid, and galactose before decontamination (VDLUFA, 2003). Each parameter
151 was analyzed in duplicate. The whole procedure was repeated after the 16 week ripening
152 period.

153

154 ***Microbiological Tests***

155 ***Generic and Shiga Toxin-producing E. coli.*** Cheese samples of 10 g were homogenized with
156 90 g dipotassium hydrogenphosphate solution (115 mmol/l dipotassium hydrogenphosphate,
157 pH 7.5) for 3 min using a stomacher. Decimal dilution series were made for enumeration of
158 the *E. coli* by spread plating on selective agar plates that use inherited properties of the strains
159 for the detection. Strain mixture 1 was spread on adonitol-MacConkey agar (40 g/l
160 MacConkey Agar Base, Becton Dickinson, Heidelberg, Germany; 10 g/l adonitol, Sigma-
161 Aldrich, Schnelldorf, Germany) for the enumeration of strain K303 which is not able to
162 ferment adonitol; adonitol-MacConkey agar containing trimethoprim (10 μ g/ml, Sigma-
163 Aldrich) for the enumeration of strain FAM21843 that is resistant to trimethoprim; and Rapid
164 *E. coli* 2 agar (Bio-Rad Laboratories, Munich, Germany) for the enumeration of strain K356
165 which lacks beta-glucuronidase activity. Strain mixture 2 was spread on rhamnose-
166 MacConkey agar (40 g/l MacConkey Agar Base, 10 g rhamnose, Sigma-Aldrich) for
167 enumeration of strain K331/4 which is able to ferment rhamnose and strain N09-1208 which
168 does not ferment rhamnose. Rapid *E. coli* 2 and MacConkey agar plates were incubated at
169 37°C for 18-24 h. For each strain typical colonies were identified based on colony
170 morphologies and enumerated. At random isolates were further identified by serogroup

171 specific tests (monospecific Anti-Coli test sera (Sifin, Berlin, Germany) and serogroup-
172 specific PCR (Liu et al., 2010)).
173 Another 25 g cheese sample was taken for enrichment procedure if an *E. coli* strain was not
174 detected quantitatively. The cheese samples were homogenized with 225 g mTSB (Oxoid) /
175 acriflavin (12 mg/l, Sigma-Aldrich) for 3 min using a stomacher. Enrichment broth was
176 incubated at 37°C for 18-24 h. For the detection of the target strains 10 µl of the enrichment
177 broth was streaked out on adonitol-MacConkey agar, adonitol-MacConkey agar containing
178 trimethoprim, Rapid *E. coli* 2 agar, and rhamnose-MacConkey agar. Additionally, for the
179 detection of *stx1* and *stx2* genes 10 µl of the enrichment broth was streaked out on blood agar
180 (Columbia agar supplemented with 5% v/v defibrinated sheep blood, both Oxoid). Rapid *E.*
181 *coli* 2, MacConkey and blood agar plates were incubated at 37°C for 18-24 h. Blood agar
182 plates were washed off using 1.5 ml 0.9% sodium chloride solution. To 200 µl of the eluate
183 400 µl double-distilled water were added. The solution was heated for 5 min at 95°C,
184 centrifuged for 1 min at 10'000 g and supernatant was transferred to a new tube and used as
185 template for conventional PCR. *Stx1* and *stx2* specific PCR was performed according to
186 (Schmidt et al., 1994) and (Pierard et al., 1998), respectively.
187
188 ***Starter Culture and Additional Flora.*** The examination of the starter culture and the
189 additional cheese flora was made from fresh cheese and from cheese samples taken after 1, 4,
190 12, and 16 weeks of ripening. Dilution series from homogenized cheese samples were spread
191 on different selective agars and incubated for 18-24 h: medium 17 (M17) agar incubated at
192 25°C and 42°C for mesophilic and thermophilic lactic *Streptococci*, respectively (Terzaghi et
193 al., 1975); deMan, Rogosa and Sharpe (MRS) agar incubated at 30°C and 45°C for
194 mesophilic and thermophilic *Lactobacilli*, respectively; kanamycin aesculin azide (KAA) agar
195 at 37°C for *Enterococci*; Schleifer-Krämer (SK) agar at 30°C for *Staphylococci*; yeast extract
196 glucose chloramphenicol (YGC) agar at 25°C for yeasts and moulds; and violet red bile

dextrose (VRBD) agar at 37°C for *Enterobactericeae*. All media and supplements were obtained from VWR International, Darmstadt, Germany.

Statistical Analysis

Colony counts (CC) from the same batch were logarithmized and averaged for statistical analysis. Samples below the limit of quantitative detection (< 10 CFU/g) were set at logarithmized value 0. Different conditions were compared using repeated measurement analysis of variance (ANOVA) with Tukey 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 was used.

RESULTS

Raw Milk Prior to the Cheese Making Process

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

Chemical and Physical Analysis of Cheese

The results of the chemical and physical analysis are summarized in Table 3. The higher cooking temperature resulted in a significantly higher pH after acidification ($p \leq 0.001$; Fig. 1). The difference in pH was about 0.2 and remained stable over the ripening period. Higher cooking temperature also yielded a higher dry matter of the semi-hard cheeses. The difference was about 3% in the fresh cheese before brining and in the ripened cheese. Dry matter increased by about 8.5% during ripening. Sodium chloride content was at 1.69% after ripening. In contrast, the lower cooked cheeses contained 0.35% more sodium chloride after ripening and more total lactic acid (TLA) before and after ripening. TLA content increased

223 during ripening. On average, the two batches without addition of *E. coli* contained more TLA
224 than those inoculated with the different strains. However, the difference was only significant
225 in lower cooked fresh cheeses ($p \leq 0.05$). Whereas the cheeses contained only L-lactic acid
226 before ripening, they contained about 40% D- and 60% L-lactic acid after ripening.
227
228 ***Fate of E. coli during Cheese Manufacture***
229 Average level of the *E. coli* strains was 1.42 ± 0.28 $\log_{10}$ CFU/g at low spiking level and 3.30
230 ± 0.14 $\log_{10}$ CFU/g at high spiking level, respectively. Average CC of the strains FAM21843,
231 K303, K356 and N09-1208 in fresh cheese were 5.32 ± 0.42 $\log_{10}$ at low and 6.94 ± 0.19 $\log_{10}$
232 CFU/g at high spiking level, respectively. For strain K331/4 a lower increase from raw milk
233 to fresh cheese was observed to 4.13 ± 0.37 $\log_{10}$ at low and 5.73 ± 0.22 $\log_{10}$ at high spiking
234 level, respectively. The increase in CC during cheese manufacture was lower for strain
235 K331/4 than for the other four strains at both spiking levels and in both cheese types
236 (significant only at high spiking level, $p \leq 0.01$). No significant differences in increase of CC
237 during manufacture of the cheese were observed between cheeses made from different
238 cooking temperatures.
239
240 ***Inactivation of E. coli during Cheese Ripening at Low Spiking Level***
241 After the increase during the manufacture of the cheese CC of the *E. coli* strains decreased
242 during cheese ripening with significant differences between the strains in cheeses cooked at
243 40°C and 46°C ($p \leq 0.01$ each; Fig. 2a and 2b). The inactivation of the *E. coli* strains was
244 similar in cheeses made from different cooking temperatures. For strain K303, the highest CC
245 were found in both cheese types. Additionally, strain K303 was inactivated slower than the
246 other strains at average decay rates at 0.23 ± 0.05 and 0.25 ± 0.03 $\log_{10}$ reduction per week in
247 cheeses made at 40°C and 46°C cooking temperature, respectively. In contrast, the STEC
248 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°C and 46°C, respectively and was below the limit of detection (< 10 CFU/g) in any sample after week 8. The strains FAM21843, N09-1208 and K356 did not differ in CC over the ripening period from each other and from strain K303. Their average decay rates were between 0.23 and 0.37 $\log_{10}$ reduction per week in the two different cheese types. The *E. coli* strains were detected after enrichment in all but one cheese sample after 16 weeks where N09-1208 was not detected.

Inactivation of E. coli during Cheese Ripening at 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°C and 46°C ($p \leq 0.01$ each; Fig. 2c and 2d). The inactivation of the *E. coli* strains was similar in cheeses made from different cooking temperatures. The 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 two STEC strains K331/4 and K356 were significantly stronger reduced in CC than the generic *E. coli* in 40°C cooked cheeses ($p \leq 0.05$). In cheeses cooked at 46°C only K331/4 was significantly different in CC from the two generic *E. coli* strains ($p \leq 0.05$). The *E. coli* strains were detected after enrichment in all but one cheese sample after 16 weeks where K356 was not detected.

Starter Culture and Additional Flora

Average CC of the starter culture and the additional flora were not significantly different between spiked and unspiked cheeses (Table 4). Thermophilic and mesophilic *Streptococci* decreased approximately 1.2 $\log_{10}$ CFU/g during ripening while thermophilic and mesophilic *Lactobacilli* were able to grow in the cheese. The CC of *Enterococci* remained stable over the ripening period. CC of *Staphylococci* decreased for more than 2 $\log_{10}$. Small amounts of yeasts were found which decreased during ripening. Only for *Staphylococci* a significant

275 difference in CC ($p \leq 0.05$) was observed between varying cooking temperatures as counts
276 were higher in 46°C cooked cheeses than in 40°C cooked cheeses at all sampling points.
277 However the inactivation was similar in both cheese types. The average counts of
278 *Enterobacteriaceae* on VRBD were mainly due to the spiked *E. coli* strains and did not differ
279 significantly from the sum of average counts of the strains (data not shown).
280

281 **DISCUSSION**

282 As quality control for the cheese production and ripening, the starter culture, natural cheese
283 flora and physicochemical parameters were examined during the process. The acidification of
284 the cheeses as well as further physicochemical parameters were in the expected range and did
285 not differ significantly between unspiked and all spiked cheeses. The behaviour of the lactic
286 acid bacteria was as expected for both, *Streptococcaceae* and *Lactobacilli*.
287 In contrast to other studies, the *E. coli* strains used in this study for the spiking experiments
288 were isolated from raw milk cheese and the strains were pre-cultured in milk before spiking.
289 Therefore the *E. coli* strains were adapted to the cheese production environment.
290 From raw milk to fresh cheese *E. coli* counts increased within the first day of the cheese
291 production. This effect was also observed in other cheese spiking studies and attributed to the
292 entrapment of bacteria in the curd and the draining of whey (Schlesser et al., 2006; Montet et
293 al., 2009). The physical concentration effect was expected to correlate with the mass ratio
294 between raw milk used and cheese produced. Therefore an increase of about 1 $\log_{10}$ due to the
295 physical concentration was estimated and the additional increase was attributed to the growth
296 of the *E. coli* strains. This growth was supported by the slow temperature decrease in the
297 cheese loafs while stored at room temperature for pressing and turning (Table 2), which
298 reflects the situation in practice. The increase for four *E. coli* strains was similar while it was
299 significantly lower for STEC strain K331/4. While growing in milk simulteanously at 30°C or
300 37°C the STEC strains showed similar growth curves and no strain competition (data not

shown). Therefore, the lower increase of K331/4 is most probably attributed to the stresses occurring during cheese production which affected this strain more than the other four *E. coli* strains.

During the ripening period a slow continuous decrease in colony counts was observed for all strains at both spiking levels which is attributed to the sum of stresses in the raw milk cheese (Peng et al., 2011). The decrease occurred similar to other challenge test studies, which examined the behaviour 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 to 40°C and to 46°C cooked cheeses was probably too small to cause a significant difference in inactivation of the *E. coli* strains. However, the decay rates in the to 40°C cooked cheese type were by trend lower than for the to 46°C cooked cheeses.

The differences in inactivation between the two generic *E. coli* and three STEC strains were significant. It is important to use different strains and evaluate each strain individually for spiking and challenge tests to include variations that potentially affect the survival of the strains..The results presented here indicate that the differences in heat shock response and oxidative acid response system that were used for selection of the strains (Table 1) are not a major factor contributing to survival of *E. coli* in raw milk cheese. Although the generic *E. coli* strain K303 was highly susceptible to heat and acid stress and additionally has a potential defect in RpoS-function, the lowest rate of decay and 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 five strains were identical on 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 the strains FAM21843 and N09-1208 in both cheese types.

The strongest inactivation was found for strain K331/4, which already during the production exhibited a lower increase than it was observed for 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 one *stx* bacteriophage, but increase in a strain harboring more than one bacteriophage - as K331/4 which harbors two *stx* bacteriophages.This hypothesis has to be tested by further experiments as well as other possible factors influencing the fate of K331/4 during cheese making, e.g. starter culture.

The strains K303, FAM21843, N09-1208 and K356 were even at low spiking level and without enrichment detected in several cheeses after 16 week ripening period. Only STEC strain K331/4 went below the limit of quantitative detection (< 10 CFU/g) during cheese ripening in all cheeses made at low spiking level. However, strain K331/4 was still detectable after enrichment in all cheeses. The detection after enrichment past long cheese ripening period, e.g., 270 days in Cheddar and Gouda, was also shown by other studies (Schlesser et al., 2006; D'Amico et al., 2010) . After four months of ripening, STEC strains were still quantified (> 10 CFU/g) from 6 of 16 cheeses made at low spiking level and from 13 of 16 cheeses made at high spiking level, while detection after enrichment was possible in almost all cheeses. Particularly due to 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 two generic *E. coli* strains survived in higher counts than the STEC strains and therefore may be considered as model organisms for further studies. If these two strains would be inactivated during raw milk cheese production the process is expected to reduce the STEC strains, too. The use of the two generic *E. coli* strain as model organisms in further challenge

353 tests would allow the production of raw milk cheese even closer to reality, namely in size and
354 form of the cheese and in the use of typical red smear instead of wax coating. The use of red
355 smear, which is very common in Swiss semi-hard cheeses, could not be applied in this study
356 due to biosafety restrictions.
357 In summary, it was possible to show differences in the fate of five *E. coli* strains, which
358 include three STEC strains, during the production and ripening of semi-hard raw milk cheese.
359 Both generic and Shiga toxin-producing *E. coli* strains were detected in almost all cheeses at
360 the end of the 16 week ripening period, which is a considerable food safety issue. Therefore
361 additional research is necessary to understand which factors are contributing to the fate of
362 diverse *E. coli* and in particular STEC in raw milk cheese.
363

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Fig. 1. Acidification curves of the semi-hard raw milk cheeses produced. Mean values and standard deviation of batches made from the same cooking temperature. Nine batches per cooking temperature, each batch comprises the average of the two cheeses produced simultaneously. 40°C dashed line, 46°C solid line.

Fig. 2. Average colony counts of *E. coli* strains during ripening of semi-hard raw milk cheese. Mean values and standard deviation of batches. Two batches per combination of strains,

cooking temperature and spiking level, each batch comprises the average of the two cheeses produced simultaneously. K303 (■), FAM21843 (◆), N09-1208 (▲), K356 (●), K331/4 (X). Generic *E. coli*: solid lines, STEC: dashed lines. 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.

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

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

[1]Acid resistance.

[2]Intimin.

[3]Hemolysin A.

[4]Strain phenotypically non-motile.

457

458 **Table 2.** Cheese production process of two types of semi-hard raw milk cheese with different cooking temperatures at 40°C

459 and 46°C

Time lapse	Processing step
	50 kg 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 ago dispersed in 1 kg cold, sterilized milk, stirring
1 h 25 min	Addition of rennet and water (0.7 kg, pasteurized)
2 h 5 min	Cutting (cubes with 8-10 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 °C resp. 46 °C), stirring
3 h 5 min	Moulding
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 about 34 °C and 35 °C, resp.)
24 h	Brining of two pressed loafs (about 2.5 kg, 15 °C)
48 h	Drying of cheese surface at 15 °C
50 h and 74 h	2 coatings at 15 °C
80 h	Start of ripening (13-14.5 °C, 91-94% relative humidity)

460
461
462 **Table 3.** Results of chemical and physical analysis in fresh and ripened semi-hard raw milk cheeses made from different
463 cooking temperatures; mean values and standard deviations of batches made from the same cooking temperature, each batch
464 comprises the average of the two cheeses produced simultaneously

	Cooking temperature 40 °C, n = 9		Cooking temperature 46 °C, n = 9	
	Fresh cheese[1]	Ripened cheese[1]	Fresh cheese[1]	Ripened cheese[1]
Dry matter (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
(w/o inoc. *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

465 [1]Fresh and ripened cheese samples were from day 1 and week 16, respectively.

Table 4. Average colony counts of additional flora; mean values and standard deviation of all batches (log_{10} CFU/g). 18 batches of which each comprises the average of the two cheeses produced simultaneously

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

[1]Not determined because of strong growth of coccoid flora.

[2]One batch excluded from statistical analysis because of growth of moulds on surface of the cheese.

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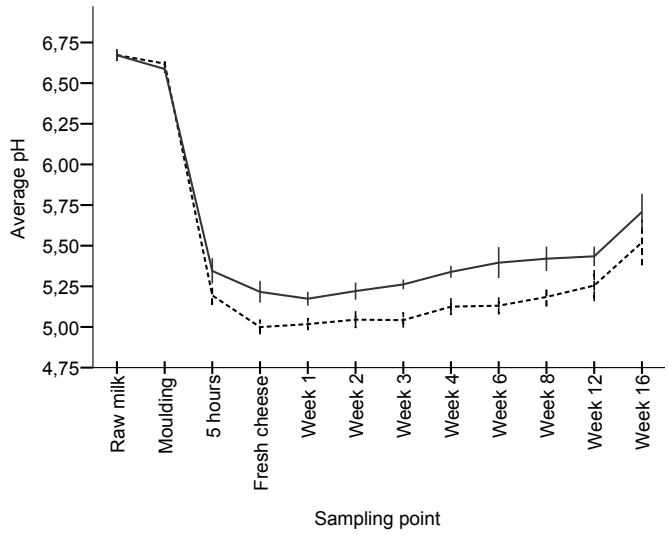

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473 Peng, Figure 1

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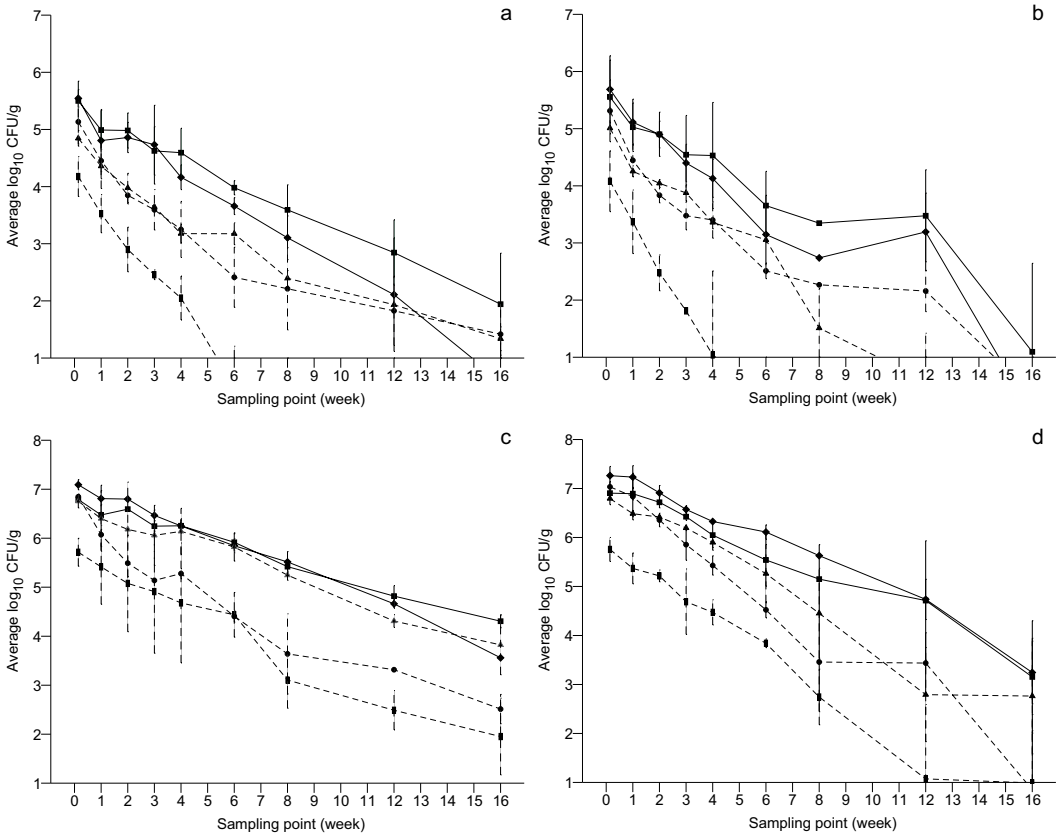

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476 Peng, Figure 2

477 add new figure, other symbol for k331/4