Fate of *Listeria innocua* during production and ripening of smeared hard cheese made from raw milk

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ABSTRACT

The fate of 2 different *Listeria innocua* strains was analyzed during the production and ripening of smeared raw milk Greyerzer cheese (Gruyère). These strains were used as surrogates for the pathogenic *Listeria monocytogenes*, as they are physiologically very similar. Bacterial cells were added to the cheese milk at levels of 10^5 cfu/mL. During the first 24 h of cheese making, the number of the test strains decreased to a level of below 10^2 cfu/g. Obviously, the cooking temperature of 56°C and the subsequent slight temperature decrease to 50°C within 70 min contributed to a distinct reduction of *Listeria* counts. The counts in the cheese cores did not exceed 10^3 cfu/g within 12 wk of cheese ripening and *Listeria* was not detectable after 24 wk. In contrast to the cores of the cheeses of the 4 batches in this study, their rinds always contained a high listerial load of approximately 10^6 to 10^8 cfu/g throughout the entire ripening period. The smeared surface showed an increase of pH to alkaline values, corresponding to smear microbiota development. Coryneforms and *Staphylococcus* counts were stable at $>10^7$ cfu/cm² over 175 d, whereas yeast counts decreased to about 10^5 cfu/cm² at the end of ripening. The study shows that the smear culture had no noticeable anti-listerial potential. When removing the rind or portioning such smeared cheese loaves with a cutting device, a postprocess contamination of the core might occur, thus presenting a major hygienic risk.

Key words: *Listeria innocua*, raw milk cheese, cheese production, smear culture, anti-listerial potential

INTRODUCTION

Microbiological food safety criteria for *Listeria monocytogenes* in ready-to-eat (RTE) foods in Europe were stipulated in regulations (European Union) 2073/2005

and 1441/2007 (Commission of the European Communities, 2005, 2007). Marketed RTE foods that do not support growth of *L. monocytogenes* have to comply with a limit of 100 cfu/g throughout their shelf life. The organism must be absent in 25 g of product at the end of production if the RTE foods are able to support the growth of *L. monocytogenes*.

The behavior of potentially pathogenic *Listeria* in hard and semi-hard cheese during manufacture and ripening has been analyzed in several studies. Ryser and Marth (1987) examined the ability of *L. monocytogenes* to survive the Cheddar cheese making process and persist during foil ripening. Three inoculated strains survived for as long as 254 to 434 d in the cheese. Kaufmann (1989) inoculated *L. monocytogenes* and found that the organism was not detectable in the cheese body and on the surface of the hard cheeses Emmentaler and Greyerzer from the beginning to the end of the ripening period. The results of Bachmann and Spahr (1995) confirmed that Swiss hard cheeses (Emmentaler type) made from raw milk were hygienically safe after 1 wk of fabrication. The semi-hard cheeses (Tilsiter type) were free from inoculated pathogens and their toxic metabolites, except for *L. monocytogenes*, which survived the manufacturing and ripening process and was detectable up to 90 d of ripening. Kovincic et al. (1991) examined the ability of *L. monocytogenes* to survive the cheese-making process of semi-soft Trappist cheese, where the organism persisted during 3 mo of ripening and storage. The counts after 3 mo were in the same range as in the inoculated milk. Northolt et al. (1988) made Gouda and Maasdam cheese from *Listeria*-contaminated milk. During manufacture, *Listeria* was concentrated in the curd by a factor of 10. Over a ripening period of 6 wk, the number of *Listeria* remained approximately constant. Yousef and Marth (1990) found that the Italian hard cheese Parmesan was not a favorable medium for the survival of *L. monocytogenes*. The pathogen was not detected in cheese after 2 to 16 wk of ripening, depending on the strain and batch of cheese. The behavior of inoculated *L. monocytogenes* on the surface of fully ripened Greek Graviera hard cheese was assessed by

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Giannou et al. (2009). *Listeria monocytogenes* did not grow on cheese surfaces, regardless of packaging and storage conditions. However, long-term survival (30–90 d) of the pathogen was noted in all treatments. Overall, the lower the temperature, the higher the survival rate was. Chatelard-Chauvin et al. (2015) investigated the behavior of *L. monocytogenes* in French raw milk Cantal-type uncooked cheese throughout cheese manufacture and storage in different packages. The numbers of the pathogen only increased during the first 24 h of cheese making and decreased significantly in both body and rind during ripening from d 45 to 360.

Cheeses that are covered with red smear may have a greater risk of containing *Listeria* because it is a suitable medium for growth of *L. monocytogenes* (Terplan et al., 1996). A study of Rudolf and Scherer (2001) determined the incidence of *Listeria* and *L. monocytogenes* in European red smear cheeses. Surprisingly, a higher incidence of *L. monocytogenes* was observed in cheeses from pasteurized milk than in cheeses manufactured from raw milk. It was strongly recommended that red smear cheeses be monitored carefully by cheese-making companies. Detailed studies are limited up to now. Schwartzman et al. (2011) modeled the fate of *L. monocytogenes* during manufacture and ripening of a smeared laboratory cheese made with pasteurized or raw milk. The curd was not pressed before brining and ripening for 28 d. The general results indicate that as the cheese is delivered to the market, the relative risk from cheeses made from pasteurized milk is distinctly less than those made from raw milk. The anti-listerial potential of food-borne yeasts in the red smear of Tilsit and acid-curd cheese was investigated by Goerges et al. (2011). On the Tilsit, but not on the acid-curd cheeses, a decrease of *Listeria* cell numbers by 1 to 2 log units was observed. An inhibitory activity of coryneform bacteria and staphylococci found in the complex surface microbial ripening consortia of red smear cheeses toward *L. monocytogenes* has been reported by Valdés-Stauber et al. (1991) and Carnio et al. (1999). In a study of Pinto et al. (2009), the nonpathogenic *Listeria innocua* was used as a surrogate organism for the pathogenic *L. monocytogenes* because it is physiologically very similar and both can be isolated from cheese produced from raw milk. Kheadr et al. (2002) used *L. innocua* ATCC 33090 instead of *L. monocytogenes* to study the effect of dynamic high pressure on the microbiological quality of Cheddar cheese. Pressurization of milk resulted in a 3- to 4-log cycle reduction of *L. innocua* colony-forming units. After 3 mo of ripening, the Cheddar made of pressurized milk contained 1 to 3×10^2 cfu/g, whereas *L. innocua* was not detected in Cheddar made from pasteurized milk.

The aim of this study was to investigate the fate of 2 inoculated strains of *L. innocua* as surrogates for *L. monocytogenes* during production and ripening of smeared hard cheese made of raw milk. The manufacturing process followed the production of Swiss hard cheese Greyerzer (Gruyère) and the whey/curd mixture was scalded at 56°C. The ripening of the cheeses in this study was performed at high air humidity and at frequent applications of defined smear to analyze the survival of *L. innocua* on the cheese surface and in the interior.

MATERIALS AND METHODS

Microbial Analysis of Cheese Microbiota

Ten grams of cheese core samples were homogenized in a Stomacher blender (Miix 1, Kleinfeld Labortechnik, Gehrden, Germany) for 2 min at maximum speed in 90 mL of a pre-warmed (45°C) aqueous solution of 2% sodium citrate. For analyses of surface microbiota, a rectangular 1- to 2-mm-thick slice from the cheese rind was cut off (10 g of cheese rind) and the size of the surface area was determined. This sample was homogenized as described above and 10-fold dilutions of quarter strength Ringer's solution were plated in duplicate on appropriate selective media. Two dilution steps (10–300 colonies) were used for colony counting. Cell counts were either expressed per centimeter² or per gram of cheese (lactic acid bacteria in the cheese core). If not stated otherwise, growth media were purchased from VWR International (Darmstadt, Germany). Mesophilic and thermophilic lactic acid bacteria were plated on M17 agar (25°C, 45°C, 2 to 3 d) and on de Man, Rogosa, Sharpe agar (MRS; 30°C, 45°C, 2 to 3 d), and the latter was incubated anaerobically. Aerobic surface smear microbiota were analyzed on modified milk agar (mMA, 25°C, 5 to 9 d; Bockelmann et al., 2005), which is based on plate count agar enriched with milk, nitrogen, and vitamins resembling a mature smear cheese surface. Yeasts and molds were analyzed on yeast extract, glucose, chloramphenicol agar (YGC; 25°C, 3 d), enterobacteria on violet red bile dextrose agar (VRBD, 30°C, 24 h), enterococci on kanamycin, esculin, azide agar (KEA, 30°C, 2 d), and pseudomonads on ceftrimid fucidin cephalotin dextrose agar (CFCD, 25°C, 2 d).

Listeria Strains and Inoculum Preparation

For inoculation of cheese milk, 2 strains of *L. innocua* (90, serovar 6a; 114, serovar 6b) were chosen. Both were isolated in our Department of Microbiology

Table 1. Samples, time of sampling, and test applied for the enumeration or detection of *Listeria innocua* during cheese-making

Sample	Time	Colony count	Enrichment ¹
Centrifuge sludge ²	0		x
Cheese milk not inoculated	0		x
Cheese milk inoculated	10 min	x	
Whey before cooking	1.5 h	x	
Curd before cooking	1.5 h	x	
Whey after cooking	2.4 h	x	
Curd after cooking	2.4 h	x	
Cheese before brining	7 h	x	
Cheese after brining	27 h	x	
Cheese rind	wk 3, 6, 12, 24	x	x
Cheese interior	d 1, 2; wk 1, 3, 6, 12, 24	x	x

¹For enrichment, samples of 25 g were used (for wk 24, only 10 g was available); for cheese samples, only applied if no count was obtained.

²For fat standardization and cleaning, the cheese milk was centrifuged and the sludge was tested in addition to the raw milk to prove that *Listeria* was not present in the cheese milk.

and Biotechnology from different German smeared soft cheeses. Final identification and serotyping of the strains were performed at the *Listeria* reference laboratory at the University of Wuerzburg, Germany. Culture strains were streaked onto sheep blood agar [Columbia agar base supplemented with 5% (vol/vol) defibrinated sheep blood, both from Oxoid, Wesel, Germany] directly from -80°C stock and incubated for 24 h at 37°C . One colony from each strain was inoculated into 10 mL of trypticase soy broth (Oxoid) and incubated as above. Subsequently, 500 mL of trypticase soy broth was inoculated with 1 mL of the culture and incubated for 24 h at 37°C on a shaker (100 rpm). Of this broth 10 mL was centrifuged for 10 min at $5,000 \times g$ at room temperature to harvest the *Listeria*. The pellet was resuspended in 100 mL of whole UHT milk (3.5% fat). Before adding the inoculum of single strains to the cheese milk, it was stored at room temperature for 30 min for milk adaptation of the strains. To determine the colony count of the inoculum, 1 mL was removed and diluted to 10^{-8} in 0.85% (wt/vol) NaCl (Merck, Darmstadt, Germany) supplemented with 0.1% (wt/vol) tryptone (Oxoid; NaCl-tryptone). Two plates of blood agar were inoculated in spread technique with 0.1 mL each from each dilution step. Colony counts were approximately 10^8 cfu/mL, which resulted in colony counts of the cheese milk of approximately 10^5 cfu/mL (100 mL of inoculum mixed with 100 kg of raw milk).

Detection of *Listeria* During Cheese-Making

For sampling times and the detection methods applied regarding the tested samples, see Table 1. To determine colony counts of *L. innocua* during cheese-making in fluid samples (milk, whey), the method as described above was applied; however, instead of blood agar PALCAM agar (Oxoid) was used. Solid samples

(curd, cheese, centrifuge sludge) were homogenized 1:10 (wt/vol) either in NaCl-tryptone for colony count (see above) or in 1/2 strength Fraser broth (Merck) for enrichment. Detection of the *Listeria* strains via enrichment was performed according to ISO (2005), with the modification that only one solid medium (polymyxin-acriflavine-LiCl-ceftazidime-mannitol-agar) was used for detection. For separate investigation of cheese rind and interior, a slice of approximately 5 cm thickness was cut off the cheese loaf. Using sterile spatulas, approximately 5 mm of the rind was removed from the entire piece and used as a sample. The surface of the remaining part was scraped with a long, sterile knife with one move. This procedure was repeated once, and material of the cheese interior was obtained with sterile spoons from the center of the prepared surface.

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. The contents of DM, fat, protein, and NaCl in cheese after 12 and 24 wk of ripening were determined according to German standard methods (VDLUF, 2003). Each parameter was analyzed in triplicate. For analysis, one-half of a cheese loaf (bread form) was de-rinded (1 cm) and divided into 3 pieces of the same size with a thickness of about 4 cm. The pieces had an increasing distance to the one end of the “bread” and were marked as PA, PB, and PC.

Cheese Production

Raw cow milk from the experimental farm of the Max Rubner-Institut (Kiel, Germany) was used for cheese production. The production facilities are regularly used for production of cheese as food, so for safety reasons

Table 2. Process sheet and sampling during the production of Greyerzer cheese

Time lapse	Processing step	pH value
Baseline	100 kg of raw milk (33°C)	
0 min	Addition of starter culture (in 200 g of sterilized milk) and of 4 individual <i>Listeria innocua</i> strains (in 100 mL of sterilized milk), stirring	6.62–6.65
30 min	Addition of 39 g of rennet	6.57–6.59
1 h 15 min	Cutting (cubes with 4–6 mm length of an edge), stirring, 32°C	6.56–6.58
1 h 35 min	Stirring and cooking (indirect heating to 56°C)	
2 h 05 min	End of cooking (56°C), stirring	
2 h 25 min	Molding (4 Kadova ¹ plastic forms)	6.36–6.38
2 h 35 min	Pressing (50 kPa)	
2 h 45 min	Turning and pressing (100 kPa)	
3 h 15 min	Turning, pressing (150 kPa, curd temperature drop from about 50 to 46°C)	
4 h 15 min	Turning, pressing (200 kPa, curd temperature drop from about 46 to 35°C)	
6 h 45 min	End of pressing (curd about 35°C)	5.11–5.19
7 h	Start of brining (brine 18% NaCl, pH 5.10, 20 h, 15°C)	
27 h	Drying for 2 h at 15°	
29 h	Start of smearing (5 wk) and ripening (temperature drop from 14 to 12°C, 96% relative humidity)	
6 wk	Ripening	5.34–5.42
12 wk	Ripening	5.47–5.57 ²
21 wk	Application of plastic dispersion and cooling at 5°C	
24 wk	End of ripening	5.74–5.93 ²

¹Kadova plastic forms (Be-St Holland-Products, Bergen op Zoom, the Netherlands).

²Core of the cheeses (de-rinded piece PC).

L. innocua was used as a surrogate. Three batches of raw milk were processed (each of these tested for the presence of *Listeria*; Table 1). One batch was used for 2 cheese vats that were not inoculated with *Listeria*. The other 2 batches were used for 4 cheese vats that were inoculated either with *L. innocua* 90 or with *L. innocua* 114. Each strain was added to milk of the 2 different batches (A, B). Each batch was processed independently in 2 cheese vats of 50 kg.

The processing steps of cheese manufacture are summarized in Table 2. One hundred kilograms of raw milk was warmed to 33°C in a cheese vat. Two hundred grams of starter culture (FD-DVS RSF-736; Chr. Hansen, Nienburg, Germany; 10 units dispersed for 30 min in 200 g of sterile milk at 30°C) and 100 g of *Listeria* culture was added under agitation, resulting in about 10⁵ cfu/mL of *Listeria* in the cheese milk. After ripening of the milk for 30 min, 40 g of rennet (Naturen Premium 145, Chr. Hansen) diluted in 200 g of water was added. The coagulum was cut into 4- to 6-mm cubes after 45 min. Following agitation for 20 min, the mixture of curd and whey was heated indirectly to 56°C within 30 min. This temperature was held for 20 min under continuous agitation before the pre-pressed curd was transferred into 4 Kadova plastic cheese molds (24.5 × 11.4 × 13.3 cm; Be-St Holland-Products, Bergen op Zoom, the Netherlands). They consisted of the mold with a suitable lining inside, a lid, and a lid lining. The curd was treated with increasing pressure: first 10 min at 50 kPa, then 30 min at 100 kPa, 60 min at 150 kPa, and 150 min at 200 kPa. The curd loaves were turned between the 4 pressing cycles. Dur-

ing pressing, the temperature of the loaves decreased to about 34°C. The forms were removed after 17 h, brined [18% (wt/wt) sodium chloride, pH 5.1 adjusted with lactic acid] for 16 h at 15°C, and dried for 2 h at 15°C. Subsequently, cheeses were ripened for 24 wk at 14 to 12°C and a relative humidity (rh) of 96%. During the first 3 wk of ripening, the loaves were smeared 3 times a week. In the following 2 wk, smearing was applied 4 times. Further surface treatment was limited to an application of a salt solution [5% (wt/wt) sodium chloride] without smear if the surfaces looked too dry. For the last 3 wk of ripening, the cheese surface was coated twice with a plastic dispersion (IP Ingredients, Suederluegum, Germany). This dispersion consisted of fluid plastic, was applied with a brush, and hardened to a flexible coating, which was permeable for gas and humidity.

The manufacturing conditions of the experimental hard cheese were based on recommendations of W. Bisig (Forschungsanstalt Agroscope Liebefeld-Posieux, Bern, Switzerland, personal communication). The homofermentative starter culture contained *Lactococcus lactis* ssp. *cremoris*, *Lc. lactis* ssp. *lactis*, *Lactobacillus helveticus*, and *Streptococcus thermophilus*. The smear culture contained 10⁷ cfu/mL of *Staphylococcus equorum* 91031, *Corynebacterium casei* 95149, *Brevibacterium linens* 95160, and *Microbacterium gubbeenense* 95152, respectively. This smear culture also contained 2 yeasts: 10⁶ cfu/mL of *Debaryomyces hansenii* 6004 and *Geotrichum candidum* 6215, respectively. They were dispersed in sterilized potable water with 5% (wt/wt) sodium chloride. All strains of the smear culture were from the

strain collection of the Department for Microbiology and Biotechnology in Kiel and were originally isolated from smear cheeses.

RESULTS

Chemical and Physical Analysis of Ripened Cheese

Chemical and physical results at 12 wk and at end of ripening are presented in Table 3. At the end of ripening, the fat content in DM was about 50% in all samples and the protein content was about 28%. Dry matter of all pieces was between 61.4 and 64.2% after 12 wk and between 63.3 and 67.3% after 24 wk of ripening. The DM and the content of NaCl decreased from the outer de-rinded pieces (PC) to the inner de-rinded pieces (PA). The content of NaCl was between 1.4 and 1.9% after 12 wk and between 1.5 and 2.0% after 24 wk. The slight increase was a result of the time-dependent increase of DM. The ripening process can be illustrated with the course of the pH values.

Cheese Microbiota

The microbiota of the cheese core was typical for raw milk cheeses. Starter lactic acid bacteria showed typically high counts (10^9 cfu/g) after 1 wk of ripening and decreased to 10^6 cfu/g over the ripening period of 175 d (Figure 1). The counts of (non-)starter lactic acid bacteria increased within 40 d of ripening and were stable at about 10^8 cfu/g throughout the entire ripening period. *Enterococcus* counts ranged between 10^4 to 10^5 cfu/g, with no substantial deviation during the ripening period; enterobacteria were below the detection limit of 100 cfu/g throughout the ripening period.

The surface showed an increase of pH to alkaline values corresponding to the smear microbiota development (Figure 2). The initially high yeast counts (YGC, $>10^7$ cfu/cm²) decreased during ripening to $<10^5$ cfu/g. Highest bacterial counts ($>10^9$ cfu/cm²) were observed for coryneforms and other rods on mMA. *Staphylococcus* counts (mMA) were stable at $>10^8$ cfu/cm² over 175 d. *Enterococcus* counts (KEA) were between 10^7 to 10^8 cfu/cm² over 175 d; enterobacterial counts dropped from 10^5 to $<10^3$ cfu/cm² (Figure 2). Pseudomonads were only detected on CFCD agar at 2 sampling points in 3 cheese batches (approximately 10^3 cfu/cm², Figure 2).

Survival of *Listeria*

To achieve measurable results, *Listeria* was added to the cheese milk at relatively high levels of 10^5 cfu/mL. Samples of cheese milk and centrifuge sludge (25 mL or

25 g, respectively) from the skimming centrifuge were drawn in advance of inoculation and were tested for the presence of *Listeria*. None of these samples yielded a positive result.

During the first 27 h of cheese-making (until the end of brining), the number of both test strains decreased in the curd to a level $<10^2$ cfu/g (Table 4). Only the first samples were taken in milk after inoculation whereas subsequent analysis was performed in curd/cheese. The whey in our study was removed after 2.4 h and showed colony counts between 3.3×10^2 and 1.8×10^3 cfu/mL. Obviously, the high cooking temperature of 56°C and the subsequent temperature decrease to 50°C within 70 min (see Table 2) contributed to a distinct reduction of *Listeria* counts. This temperature is selective for the microbial flora and inhibits growth of undesired microorganisms. It was applied to determine the survival rate of the 2 inoculated *Listeria* strains. In Figure 3A and 3B the colony counts for both strains are plotted starting with cheese before brining (7 h) and during the entire ripening period. After the development of the cheese rind (wk 3), counts for rind and core were performed separately. The bacterial count of the core was below 10^3 cfu/g after 6 and 12 wk of ripening, and *Listeria* was no longer detectable after 24 wk. Interestingly, colony counts of both *Listeria* strains in the core increased during the first 20 d of ripening.

DISCUSSION

Kaufmann (1989) inoculated *L. monocytogenes* at a comparable level into raw cheese milk for Greyerzer. The cooking temperature was even higher at 57°C. The high thermal input and a decrease to 50°C within 6 h prevented growth of *L. monocytogenes*, with the result that the organism was not detectable until the end of ripening. Bachmann and Spahr (1995) achieved comparable results in Swiss hard cheeses made from raw milk. Inoculated milk for semi-hard Gouda or Maasdam cheese contained only 5×10^2 cfu/mL of *L. monocytogenes* (Northolt et al., 1988). In this study, *Listeria* counts increased to about 2×10^4 cfu/mL during cheese manufacture (maximum temperature 35.5°C) and decreased during ripening at 88 or 92% rh at 13°C to $>10^3$ cfu/g in the interior and to 1.3×10^3 on the surface. The decimal reduction times (D values) for the most heat-resistant strain of *L. monocytogenes* were 17 s at 64°C and 8 s at 66°C. Yousef and Marth (1990) inoculated raw milk for Parmesan cheese with 2 strains of *L. monocytogenes* at a final concentration of 10^4 to 10^5 cells/mL. Curd was cooked at 51°C for 45 min, and *Listeria* counts decreased only slightly. During cheese ripening at 13°C and 70% rh, *Listeria* was not detected after 2 to 16 wk of ripening, depending on the *Listeria*

Table 3. Results of chemical and physical analysis in cheese after 12 and 24 wk of ripening¹

Batch no.: cheese with or without <i>Listeria innocua</i> ²	After 12 wk				After 24 wk			
	DM (%), n = 3	NaCl (%), n = 3	pH	DM (%), n = 3	Fat in DM (%), n = 2	Moisture on a fat-free basis (%), n = 2	Protein (%), n = 9	NaCl (%), n = 3
Batch 1: without								
PA	62.05 ± 0.04	1.60 ± 0.02	5.45	64.28 ± 0.14		52.1		1.74 ± 0.02
PB	63.12 ± 0.07	1.59 ± 0.01	5.52	64.45 ± 0.08	49.6 ± 0.5	52.5	27.88 ± 0.38	1.78 ± 0.01
PC	63.34 ± 0.10	1.85 ± 0.02	5.50	66.51 ± 0.08		50.0		1.92 ± 0.00
Batch 1: with								
PA	62.05 ± 0.09	1.47 ± 0.01	5.47	65.36 ± 0.04		51.7		1.77 ± 0.03
PB	62.42 ± 0.07	1.52 ± 0.01	5.48	66.04 ± 0.08	50.3 ± 0.3	50.7	27.81 ± 0.45	1.90 ± 0.03
PC	62.49 ± 0.03	1.73 ± 0.01	5.47	67.31 ± 0.15		49.5		2.00 ± 0.01
Batch 2: with								
<i>L. innocua</i> 90 A								
PA	62.49 ± 0.16	1.57 ± 0.02	5.56	63.31 ± 0.09		54.2		1.66 ± 0.03
PB	62.91 ± 0.08	1.61 ± 0.01	5.55	64.26 ± 0.07	50.3 ± 1.0	52.2	27.25 ± 0.31	1.71 ± 0.02
PC	63.36 ± 0.13	1.84 ± 0.00	5.57	64.78 ± 0.18		52.6		1.87 ± 0.01
Batch 2: with								
<i>L. innocua</i> 114 A								
PA	61.44 ± 0.07	1.49 ± 0.01	5.56	63.55 ± 0.09		54.4		1.68 ± 0.03
PB	62.21 ± 0.08	1.64 ± 0.02	5.57	64.29 ± 0.18	51.1 ± 1.0	55.5	27.16 ± 0.32	1.81 ± 0.03
PC	62.87 ± 0.08	1.86 ± 0.01	5.59	65.56 ± 0.02		52.0		1.89 ± 0.01
Batch 3: with								
<i>L. innocua</i> 90 B								
PA	63.29 ± 0.16	1.48 ± 0.01	5.54	64.51 ± 0.04		52.6		1.59 ± 0.03
PB	63.43 ± 0.15	1.56 ± 0.01	5.50	65.06 ± 0.02	50.1 ± 0.7	51.4	28.41 ± 0.35	1.73 ± 0.04
PC	63.44 ± 0.09	1.75 ± 0.01	5.54	66.27 ± 0.07		50.7		1.85 ± 0.01
Batch 3: with								
<i>L. innocua</i> 114 B								
PA	63.55 ± 0.08	1.36 ± 0.01	5.58	64.38 ± 0.06		52.8		1.52 ± 0.02
PB	63.57 ± 0.03	1.46 ± 0.02	5.62	64.77 ± 0.09	50.5 ± 1.0	51.8	27.96 ± 0.11	1.59 ± 0.02
PC	64.24 ± 0.04	1.64 ± 0.02	5.55	64.85 ± 0.06		52.9		1.74 ± 0.00

¹Results of ingredients are mean values ± SD.²PC, PB, and PA = de-rinded pieces of the same size (thickness 4 cm) with increasing distance from the one end of the cheese bread.

strain and the batch of cheese. During production of Grana Padano cheese, Ercolini et al. (2005) modeled the efficacy of scalding. Keeping the curd for 1 h at 55°C did not result in a reduction of *L. monocytogenes* (initial count approximately 10^4 cfu/mL). However, the data indicated that a first thermal stress induced by the cooking temperature was able by itself to affect the subsequent viability of *Listeria* and other pathogens. After further keeping of the curd at 55°C, *Listeria* remained undetectable after the first hour level. In contrast, subsequent keeping at 45 or 38°C for 15 min simulating the faster cooling of the developing rind did not lead to a decrease. The cooking temperature in our experiments was 56°C and the decrease in temperature after draining during the next 5.5 h was about 4.5°C per hour in the curd interior (Table 2). Before brining, the curd interior was cooled to 35°C at a room temperature of about 26°C. At this time the level of *L. innocua* was below or near the detection limit. Obviously, this course of temperature was also sufficient to inactivate the *Listeria* in the core of our small loaves. Comparing the survival of *Listeria* in our study and in other cited experiments, it must be considered that 2

different species were used. It was already mentioned that Kheadr et al. (2002) and Pinto et al. (2009) used *L. innocua* as a surrogate for *L. monocytogenes* because it is physiologically very similar and both can be isolated from cheese produced from raw milk. Boyer et al. (2009) compared the survival of both species in chill brines (7.9 and 13.2% NaCl) containing lactic acid bacteria. The cell membrane function of *L. innocua* is more electronegative and variable in its Lewis acid-base characteristics as a function of temperature. Membrane function is essential in the mechanism for surviving environmental stressors, such as salt, pH, and water activity (Russell et al., 1995). The results of Boyer et al. (2009) showed that *L. innocua* was less resilient than *L. monocytogenes* after some days in the brine. The behavior against thermal input was not analyzed.

Giannou et al. (2009) used pieces of fully ripened Greek hard Graviera cheese that were inoculated with a 5-strain cocktail of *L. monocytogenes* at approximately $3 \log \text{ cfu/cm}^2$ on the surface. These experiments simulated a postprocess contamination during retail operations. *Listeria* did not grow on the surface up to 90 d, regardless of packaging conditions and different storage

Figure 1. Bacterial growth in the cheese core of 6 different cheese batches produced from raw milk. Mesophilic and thermophilic cocci were plated onto M17 agar; mesophilic and thermophilic lactobacilli onto de Man, Rogosa, and Sharpe agar (MRS); and enterococci onto kanamycin, esculin, azide agar (KEA). The counts of enterobacteria (violet red bile dextrose agar; VRBD) were below the detection limit of 100 cfu/g. Results are mean values and SD of batches.

Figure 2. Development of the cheese smear: modified milk agar (mMA) was used for enumeration of cocci, coryneforms, and rods; yeast extract, glucose, chloramphenicol agar (YGC) for yeasts; violet red bile dextrose agar (VRBD) for enterobacteria; and kanamycin, esculin, azide agar (KEA) for enterococci. Pseudomonads plated on cetrimid fucidin cephalotin dextrose agar (CFCD) were detected at 2 sampling points only. Results are mean values and SD of the 6 experimental cheese batches.

Table 4. Colony counts of *Listeria innocua* strains 90 and 114 per mL or g during cheese-making and in the cheese interior (A, B: results from independent experiments)

Sample	90 A	90 B	114 A	114 B
Cheese milk	1.8×10^5	1.3×10^5	1.9×10^5	1.3×10^5
Curd before cooking	1.3×10^4	5.4×10^5	3.3×10^5	6.3×10^5
Curd after cooking	4.5×10^2	2.8×10^3	4.7×10^2	3.1×10^3
Cheese before brining	2.0×10^1	3.0×10^1	4.3×10^1	2.0×10^1
Cheese after brining	$<10^*$	$<10^*$	2.0×10^1	$<10^*$
Cheese wk 1	$<10^*$	$<10^*$	1.8×10^2	$<10^*$
Cheese wk 3	1.7×10^2	4.0×10^3	4.2×10^2	3.4×10^3
Cheese wk 6	1.0×10^1	5.0×10^1	2.1×10^2	1.1×10^2
Cheese wk 12	3.0×10^2	1.0×10^1	1.1×10^2	1.3×10^2
Cheese wk 24	$<10^\dagger$	$<10^\dagger$	$<10^\ddagger$	$<10^\ddagger$

*Enrichment of 25 g positive.

†Enrichment of 10 g positive.

‡Enrichment of 10 g negative.

Figure 3. Colony counts of *Listeria innocua* after inoculation in cheese milk and on the rind and in the interior of cheese during ripening (starts with cheese before brining): (A) strain 90, and (B) strain 114 (A and B were independent experiments). For graphical reasons, values for 24 wk were set at 1.0 if enrichment of 10 g was positive and at 0.1 if enrichment was negative.

temperatures. Interestingly, colony counts of both *Listeria* strains in the core increased during the first 20 d of ripening. Ryser and Marth (1987) previously reported that the initial *Listeria* count in cheese milk for Cheddar after inoculation of 3 different strains was 2 to 3×10^2 /mL and remained almost constant in the Cheddar curd (cooking temperature 39°C) until pressing. All 3 *Listeria* strains survived for as long as 251 to 434 d at 6 and 13°C in the experimental Cheddar. During the first 14 to 28 d, counts of all strains increased 0.1 to 0.8 log cfu/g in cheese at the 2 ripening temperatures. These surprising data are in contrast to the results of Kaufmann (1989) and Bachmann and Spahr (1995), but confirm our results during the first 20 d of ripening (Table 4). Ryser and Marth (1987) did not discuss possible reasons for the occurrence of these peaks. One reason could be that the hygienic “hurdles” present in the core (salt, pH, water activity value, competitive flora) took a long time to work effectively. In addition, the relatively small loaves in our study resulted in a faster decline of core temperature than in the original size Greyerzer loaves. However, the rind temperature was only affected a little by the loaf size.

Chatelard-Chauvin et al. (2015) prepared a cocktail of 5 strains of *L. monocytogenes*. This cocktail was inoculated in raw cheese milk for the manufacture of French Cantal type uncooked semi-hard cheeses. However, the milk contained only 6 cfu/mL of *Listeria* after inoculation. The cheeses were ripened at 9°C and 96% rh for 360 d. After various stages in the ripening process, portions of the cheeses were stored in different packages and storage temperatures for up to 45 d. Counts of *L. monocytogenes* only increased during the first 24 h of cheese making to 10^3 to 10^4 cfu/mL and decreased significantly in both cores and rinds during ripening at 9°C from d 45 to d 360. The decrease continued in the cores during storage in all 3 package types. In the rind, counts of *L. monocytogenes* decreased most significantly in nonpermeable kraft paper (cheese wrapped in kraft paper + plastic stretch film covering core) at low water activity (<0.90) compared with plastic packs. Kovincic et al. (1991) inoculated pasteurized cheese milk for manufacture of semi-soft Trappist cheese and found initial counts between 2.9×10^2 and 2.4×10^5 cfu/mL. The curd was scalded at 39°C for 45 min. After ripening and storage of the waxed cheeses at 10°C for 90 d, *Listeria* counts ranged from 4.4×10^2 to 4.4×10^5 cfu/g.

In contrast to the cores of the 4 cheese batches in our study, the smeared rind always contained a high listerial load of approximately 10^7 and up to 10^8 cfu/g for up to half of the ripening period (Figure 3). *Listeria* counts decreased slightly at the end of ripening to 10^6

cfu/g. This might be a result of the applied plastic dispersion 3 wk before the end of ripening.

Schvartzman et al. (2011) studied brined cheese curds after laboratory-scale production that were manually smeared one time and subsequently ripened at 13°C and 90% rh for 2 wk, followed by another 2 wk at 8°C. The raw or pasteurized cheese milk used was inoculated with a single strain of *L. monocytogenes* at a final concentration of about 5×10^2 cfu/mL. Results indicated that in cheeses made from pasteurized milk, less *Listeria* (almost 2 log) survived than in cheeses made from raw milk. During the short ripening period of 28 d, the listerial load in the core and on the rind was at the same level after smearing. Goerges et al. (2011) investigated the anti-listerial potential of foodborne yeasts in red smear cheese. Their screening list of 175 strains also contained 2 strains that were part of the smear culture in this study. It was reported that the reduction of *L. monocytogenes* (WSLC 1364) was mostly 1 log/cm² in the presence of *D. hansenii* and 4 log/cm² for *G. candidum*. Carnio et al. (1999) analyzed the bacterial ripening flora of German smeared cheeses with respect to their anti-listerial potential and found that *Corynebacterium* strains such as *C. casei*, which was also used in our study, have anti-listerial properties. However, their observations also indicated that selective pressure within the flora of the cheese surface might have resulted in the development of resistance mechanisms by micro-evolutionary adaptations of *L. monocytogenes*.

Our results indicate that a possibly existing anti-listerial potential of *D. hansenii*, *G. candidum*, or *C. casei* was not perceptible, or at a very low level. As the *L. innocua* strains were isolated from the surface of red smeared soft cheese, it cannot be excluded, however, that they had already developed resistance mechanisms as observed by Carnio et al. (1999). The relatively high salt content (5% NaCl) of the smear culture also did not decrease the listerial load in the cheese rind. This is somewhat disappointing as hurdles such as competitive flora and salt should be effective against *Listeria* on the rind irrespective of whether the contamination originates from the cheese milk or from recontamination via the environment.

CONCLUSIONS

The produced raw milk Greyerzer cheeses showed typical microbiota in the cheese core and on the surface. Apart from the desirable starter and nonstarter lactic acid bacteria, smear bacteria (coryneforms and staphylococci), and yeasts, a typical contamination with enterobacteria and enterococci was also detected. The cheese surface also contained about 10^6 to 10^8 cfu/g of the 2 inoculated *Listeria innocua* strains throughout the

entire ripening period despite the high concentration of smear bacteria. Obviously, the moist smear surface permitted an almost constant *Listeria* level, whereas in the core no *Listeria* was detectable at the end of ripening. The study shows that the smear culture developed no considerable anti-listerial potential. Considering that *L. innocua* is possibly even less resilient than *L. monocytogenes* against environmental stressors such as water activity, lactic acid bacteria, and dissolved NaCl, a postprocess contamination by *L. monocytogenes* might be even more likely, posing a major hygienic risk when the rind is removed or such smeared cheese loaves are portioned with a cutting device.

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