LWT - Food Science and Technology 133 (2020) 109944

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Fate and survival of *Listeria monocytogenes* and *Escherichia coli* O157:H7 during ripening of cheddar cheeses manufactured from unpasteurized raw milk

Jung-Whan Chon [a,1], Jhun-Woo Kim [a,1], Kwang-Young Song [a], Jong-Soo Lim [a], Dongryeoul Bae [a], Hyunsook Kim [b], Kun-Ho Seo [a,*]

[a] KU Center for Food Safety and Department of Public Health, College of Veterinary Medicine, Konkuk University, Seoul, 05029, Republic of Korea
[b] Department of Food and Nutrition, College of Human Ecology, Hanyang University, Seoul, 68308, Republic of Korea

ARTICLE INFO

Keywords:
Cheddar cheese
Raw milk
Ripening
Inactivation
Escherichia coli O157:H7
Listeria monocytogenes

ABSTRACT

Cheese products may serve as vehicles for transmission of foodborne pathogens, causing disease outbreaks, particularly when unpasteurized raw milk is used for cheese production. Therefore, the adequacy of a 60-day aging period for raw milk Cheddar cheese to eliminate *Escherichia coli*O157:H7 and *Listeria monocytogenes* was evaluated. The changes in cheese components and characteristics of pH, Aw, coliform, and LAB were examined during the ripening process. Cheddar cheese was manufactured from raw milk artificially spiked with 1, 3, or 5 log CFU/mL *L. monocytogenes* or *E. coli*O157:H7. When compared with the pasteurized samples, the unpasteurized samples exhibited slightly lower pH values and similar aw values. No coliform were observed in the pasteurized cheese, whereas over 5-log CFU/g of coliform were found in the cheese made from unpasteurized milk during the early phases of ripening. However, coliform were completely eliminated from the cheese made from unpasteurized milk within five weeks. In contrast, while the level of LAB was initially 2-log lower in the cheese made from pasteurized milk than that derived with unpasteurized milk, the number of LAB between the two cheese preparations equalized within five weeks. Although *L. monocytogenes* numbers decreased significantly to below the detection limit (1-log CFU/g) during ripening, viable cells were isolated from all the inocula. *E. coli*O157:H7 cells also significantly decreased to below the detection limit during ripening at all the inoculation levels. However, viable cells were still detected. Thus, a 60-day ripening process alone may not be sufficient for elimination of contaminating pathogens.

1. Introduction

Although regarded as highly nutritious in many countries, cheese products have served as a vehicle for many outbreaks of food poisoning (Rodriguez et al., 2005; Schlesser et al., 2006). In particular, cheese manufactured from unpasteurized raw milk has been regarded as an important cause of the transmission of food-borne pathogens, including *Escherichia coli*O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Yersinia enterocolitica*, and *Campylobacter* spp. (Batz et al., 2012; Leong et al., 2014). A total of 87 outbreaks causing 750 laboratory-confirmed illnesses and 215 hospitalizations in the United States (US) between 2009 and 2014 were reported to have been associated with milk and cheese consumption (Costard et al., 2017). *Staphylococcus aureus, Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*O157:H7 and *Campylobacter* account for the most frequent potential pathogens associated with the consumption of milk or dairy products and are the main microbiological hazards linked to raw milk products and raw cheese (Jakobsen, Heggebø, Sunde, & Skjervheim, 2011; Claeys et al., 2013; Kousta et al., 2010; Verraes et al., 2015; Yang et al., 2012). Because dairy cows are a reservoir of *E. coli*, which produce Shiga toxin, raw milk may be contaminated with the enterohemorrhagic serotypes, such as *E. coli*O157:H7, at the farm level (Wells et al., 1991; D'Amico et al.,

* Corresponding author. KU Center for Food Safety and Department of Public Health, College of Veterinary Medicine, Konkuk University, Hwayang-dong, Gwangjin-gu, Seoul, 05029, Republic of Korea.
E-mail addresses: alvarmar@naver.com (J.-W. Chon), acewhip@naver.com (J.-W. Kim), drkysong@gmail.com (K.-Y. Song), limx15@nate.com (J.-S. Lim), dongryeoul@gmail.com (D. Bae), hyunsk15@hanyang.ac.kr (H. Kim), bracstu3@konkuk.ac.kr (K.-H. Seo).
[1] Those authors contributed equally to this study.

https://doi.org/10.1016/j.lwt.2020.109944
Received 16 April 2020; Received in revised form 22 July 2020; Accepted 23 July 2020
Available online 30 July 2020

2010). In addition, *Listeria monocytogenes*, which can grow and survive at low temperatures, is also considered a threat for the raw-milk–based cheese manufacturing (bib_Johnson_et_al_1990Johnson, Nelson, & Johnson, 1990a, 1990b).

The US Food and Drug Administration (FDA) established the cheese standards to define the requirements for pasteurization; as an alternative, cheese made from raw milk could be ripened at least 60 days at 1.7 °C to reduce the number of viable pathogens in the product (Ryser and Marth, 1989; Schlesser et al., 2006). However, cheese made from unpasteurized milk was strictly banned in South Korea until 2015.

After the implementation of the free trade agreement (FTA) between Europe and the USA, the Ministry of Food and Drug Safety (MFDS) of Korea revised the processing standards and ingredient specifications of livestock products. Accordingly, the use of raw milk to be used for cheese ripened over 60 days was excluded from the pasteurization conditions to harmonize with the international standards, eliminate trade frictions related to the exports and imports, and create a basis for the production of diverse products.

However, various studies have suggested that even the minimum 60-day ripening period permitted for the manufacture of unpasteurized raw-milk–based cheese is not sufficient for the elimination or significant reduction of the microbiological hazards (Ryser and Marth, 1989; Reitsma and Henning, 1996; Schlesser et al., 2006; D'Amico et al., 2010). With increasing numbers of studies reporting alternative methods to treat for cheese made from unpasteurized raw milk, it seems necessary to reconsider the requirement of the ripening for 60 days at 1.7 °C. Although several studies have examined reductions in the levels of various pathogens by the 60-day ripening process, few studies have specifically evaluated changes in the levels of *E. coli*O157:H7 and *Listeria monocytogenes* in cheddar cheese (the most frequently consumed cheese in South Korea) made from raw milk that was artificially inoculated with various concentrations of these microorganisms.

Thus, the objective of this study was to evaluate the adequacy of the 60-day aging period to eliminate *E. coli*O157:H7 and *Listeria monocytogenes* from raw-milk–based cheddar cheese. The composition of the cheese, and changes in its pH, water activity (aw), and the levels of non-pathogenic microorganisms, such as coliform bacteria and lactic acid bacteria (LAB), were also examined during the ripening process.

2. Materials and methods

2.1. Manufacturing of raw milk-based cheddar cheese

The raw milk was regularly provided by Konkuk Milk Dairy & Ham Company (Seoul, Korea) and tested for the total number of bacteria, coliforms, generic *E. coli*, and the presence of naturally contaminated *E. coli*O157:H7 or *Listeria monocytogenes* according to the methods described in the MFDS Bacterial Analysis Manual (Ministry of Food and Drug Safety of Korea (MFDS), 2018). Assuming that raw milk could be contaminated with pathogens during the collection step, cheddar cheese was manufactured from raw milk artificially spiked with *E. coli*O157:H7 or *Listeria monocytogenes*. *E. coli*O157:H7 (FS_12) isolated from a dairy food was provided by FDA's Center for Food Safety and Applied Nutrition (College Park, MD, USA), and *Listeria monocytogenes* (ATCC 51776) originated from a dairy product of Belgium was purchased from ATCC (Manassas, VA, USA). *E. coli*O157:H7 (FS_12) or *Listeria monocytogenes* (ATCC 51776) suspensions were incubated overnight in tryptic soy broth (Oxoid) and inoculated into 20 L of raw milk at an approximate density of 10^1–10^5 CFU/mL raw milk. Twenty liters of raw milk artificially inoculated with the pathogen was placed into a 100-L rectangular stainless steel cheese vat (model FT20, Armfield, Hampshire, UK). Cheddar cheese samples were manufactured from both pasteurized and unpasteurized milk and aged up to nine weeks (63 days). For the preparation of cheddar cheese made from pasteurized milk, raw milk was heated at 65 °C for 30 min according to the method recommended by the MFDS (Ministry of Food and Drug Safety of Korea (MFDS), 2018). Milk was heated at 32 °C for 45 min after the addition of 1% commercialized starter culture (Songeun Co., Seoul, Korea) containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophiles*. Liquid animal rennet (19 mL/100 L; MaysaGida San ve Tic, Istanbul, Turkey) diluted 1:9 in sterile deionized water was added and heated at 32 °C for another 45 min. Once curd formation occurred, the coagulum was cut into pieces (0.5 × 0.5 cm) with stainless steel wire knives followed by mild stirring for 15 min. The temperature was increased to a cooking temperature of 39 °C for 30 min. Stirring was continued until the pH reached 6.2, and the whey was then drained. The curd mat was made by turning the curd upside down, and the curd mat was then further cheddared to reach a pH of 5.4. The curd was milled into small pieces (1.5 × 1.5 cm), and the pieces were salted (2%) and mixed for 5 min. The curd was then transferred into cheese molds and pressed overnight on a 24-kg cheese press for 24 h. After pressing, the blocks were dried for 2 days and then placed in a sterilized plastic bag (Kpac Co., Seoul, Korea). The plastic bags were vacuum sealed using a vacuum sealer (Interise, Seoul, Korea), followed by aging at 15 °C for up to nine weeks (63 days). Cheddar cheese samples manufactured from both pasteurized and unpasteurized milk were aged and aseptically collected every week during aging.

2.2. Changes in the pH and aw values of the samples during the ripening step

The aw and pH values of the ripening cheese samples were also measured during all the tested periods. The aw values were measured with AquaLab (Decagon Devices Inc., Pullman, WA, USA), and pH was determined using an Orion 3-star pH-Benchtop (Thermo Fisher Scientific, Hudson, NH, USA). The measurements of aw and pH values were repeated three times and averaged. Enumeration data were log-transformed using Microsoft Excel 2010 in all analyses.

2.3. Quantitative detection of bacteria during the ripening step

For the enumeration of *Listeria monocytogenes* and *E. coli*O157:H7, 25-g cheese samples were cut and suspended in 225 mL of Butterfield's phosphate-buffered water (BPD, Difco) and then homogenized for 2 min using a BagMixer stomacher (Interscience, St. Nom, France). After stomaching, 1 mL of homogenate was serially diluted in BPD, and each dilution was then inoculated onto five Oxford agar plates for *Listeria monocytogenes* or sorbitol MacConkey agar plates (SMAC; Difco) for *E. coli*O157:H7 (0.2 mL for each plate). The plates were incubated at 30 °C for *Listeria monocytogenes* and at 37 °C for *E. coli*O157:H7 for 24 h, and the presumptive positive colonies were enumerated. The enumeration was repeated three times, and the numbers of positive colonies for the three trials were averaged. Enumeration data were log-transformed using SPP software in all analyses (version 17.0.1, SPSS, Inc., Chicago, IL, USA).

2.4. Qualitative detection of pathogens during the ripening step

For the isolation of *Listeria monocytogenes*, 25 g of food was placed in 225 mL of Listeria enrichment broth (Difco) and then homogenized in a BagMixer stomacher (Interscience) for 2 min. After enrichment at 30 °C for 24 h, a 0.1-mL aliquot of the pre-enrichment culture was transferred into 10 mL of a secondary enrichment Fraser broth (Difco) and incubated at 37 °C for 24 h. A loopful of the enrichment broth was streaked on Oxford agar and incubated at 37 °C for 24 h. Suspected colonies with black halos on Oxford agar were selected and biochemically confirmed using a Vitek 2 system GP kit (bioMerieux, France).

For qualitative detection of *E. coli*O157:H7, 25-g samples were mixed with modified tryptic soy broth (mTSB) supplemented with novobiocin (20 mg/L, Oxiod) and homogenized for 2 min. After enrichment at 37 °C for 24 h, the broth cultures were streaked onto Sorbitol MacConkey agar (SMAC: Difco) and incubated at 37 °C for 24 h. Suspected colonies were

identified with colony PCR analysis targeting the stx gene, as previously described (Feng & Monday, 2000).

2.5. Enumeration of coliform bacteria and LAB

One milliliter of each sample homogenized with BPD as described above was serially diluted in BPD, followed by inoculation of 0.2 mL of each sample onto five 3M petrifilms (3M, St. Paul, MN, USA) and MRS agar plates (Difco) for enumeration of coliform bacteria and LAB, respectively. The plates or petrifilms were incubated at 37 °C for 24 h under aerobic or anaerobic conditions, and presumptive positives were then enumerated. The enumeration was repeated three times; the numbers of positive colonies from all the three trials were averaged.

3. Results and discussion

3.1. Changes in the pH and aw values of cheddar cheese samples during ripening

Changes in the aw and pH values of cheddar cheese samples during ripening are summarized in Table 1. All the pH values of the pasteurized and unpasteurized cheeses during this time were consistently between 4.98 and 5.26 or 4.77 and 5.09, respectively (Table 1). The aw values varied little among the cheese samples tested, ranging from 0.95 to 0.98 in both types of cheeses (Table 1). Compared with pasteurized samples, unpasteurized samples showed lower pH values and similar aw values; however, the differences were minimal (Table 1). These ranges were similar to those reported in another study (Leong et al., 2014), in which the pH and aw values of natural cheddar cheese were shown to range from 4.99 to 5.28 and 0.96 to 0.98, respectively.

It has been shown that pH is a critical parameter that affects the textual properties of cheese (Lawrence et al., 1987; Lee & Lee, 2008). The slightly lower pH of cheese made from unpasteurized milk at the early period of aging may be associated with the higher number of LAB which last up to 3 weeks of ripening. Formation of the lactic acid occurs at the beginning of the fermentation during the early phase of the ripening period, whereby the pH value of the cheese is decreased (Lee & Lee, 2008).

3.2. Changes in coliform bacteria and LAB during ripening

Changes in coliform bacteria and LAB are also presented in Table 1. According to the Korea Food Code, coliform bacteria is an important indicator of the quality of processed cheese (Ministry of Food and Drug Safety of Korea (MFDS), 2018). Cheese made from pasteurized milk should be free from coliform bacteria (Khayat et al., 1988). In this study, no coliform bacteria were observed in pasteurized cheese, whereas over 5-log CFU/g coliform bacteria was present in cheese made from unpasteurized milk during the early ripening period (Table 1). However, coliform bacteria in cheese made from unpasteurized milk were completely eliminated within 5 weeks (Table 1).

Although the level of LAB was 2-log lower in the cheese made from pasteurized milk than that in the cheese made from unpasteurized milk, the number of bacteria was similar between the two cheese preparations approximately five weeks later during the ripening stage. The lower number of LAB in the cheese made from pasteurized milk during the first stage of ripening may be due to elimination of the naturally present LAB in the raw milk during the heating step. Leong et al. (2014) have reported that inhibitory compounds generated during manufacturing of cheese, including free fatty acids and metabolites of LAB, result in differences in pathogen growth during unrefrigerated storage.

Table 1
Changes in pH, water activity, lactic acid bacteria, and coliform bacteria during the ripening stage of cheddar cheese manufactured from pasteurized or unpasteurized raw milk.

Days	pH		aw		Lactic acid bacteria (log CFU/g)		Coliform (log CFU/g)	
	P*	U*	P	U	P	U	P	U
0	4.98^{a}	4.86^{a}	0.96^{a}	0.95^{a}	4.65^{b}	6.60^{a}	ND**	5.72^{a}
7	5.15^{a}	4.77^{b}	0.96^{a}	0.97^{a}	4.65^{b}	6.00^{a}	ND	3.28^{a}
14	5.15^{a}	5.09^{a}	0.98^{a}	0.97^{a}	5.45^{b}	6.85^{a}	ND	2.87^{a}
21	5.12^{a}	4.78^{b}	0.97^{a}	0.98^{a}	5.23^{b}	5.99^{a}	ND	1.00^{a}
28	5.20^{a}	4.85^{b}	0.96^{a}	0.98^{a}	6.94^{a}	6.48^{b}	ND	1.78^{a}
35	5.26^{a}	4.91^{b}	0.96^{a}	0.97^{a}	6.99^{a}	6.92^{a}	ND	ND
42	5.14^{a}	4.91^{a}	0.96^{a}	0.96^{a}	7.00^{a}	6.43^{b}	ND	ND
49	5.18^{a}	4.83^{b}	0.95^{a}	0.97^{a}	6.52^{a}	6.51^{a}	ND	ND
56	5.09^{a}	4.90^{a}	0.97^{a}	0.96^{a}	7.28^{a}	6.68^{b}	ND	ND
63	5.13^{a}	5.00^{a}	0.95^{a}	0.96^{a}	6.93^{a}	6.26^{b}	ND	ND

*P, pasteurized raw milk; U, unpasteurized raw milk.
**Under the detection limit (i.e., < 1-log CFU/g).
^{ab}Different letters within a row indicate a significant difference ($p < 0.05$).

3.3. Reduction and survival of pathogens during ripening

Raw milk was tested for the presence of naturally contaminating *E. coli*O157:H7 or *Listeria monocytogenes*. Only the samples determined to be free of those pathogens were used for the experiments requiring inoculation in order to exclude potential false positives resulting from natural contamination. Changes in *Listeria monocytogenes* and *E. coli*O157:H7 during the ripening are shown in Tables 2 and 3, respectively. Compared with the number of cells of *Listeria monocytogenes* and *E. coli*O157:H7 inoculated in the raw milk samples, that in manufactured cheese on day 0 was increased by approximately 0.32–1.69 log unit (Tables 2 and 3), indicating possible growth near the optimum temperature during the cheese-manufacturing process (Schlesser et al., 2006). Pathogens are known to be able to survive and even grow during the production of cheddar cheese, following concentration and entrapment of bacterial pathogens in the curd (Reitsma & Henning, 1996; Maher et al., 2001; D'Amico et al., 2010).

Artificial inoculation of pathogens indicated that *Listeria monocytogenes* and *E. coli*O157:H7 could survive after a 60-day ripening stage, regardless of the inoculation level (Table 2). Although *Listeria monocytogenes* numbers were significantly decreased to below the detection limit (1-log CFU/g) after 56 days of ripening, viable cells were isolated in all inoculums using the enrichment isolation procedure (Table 2). In the case of *E. coli*O157:H7, cells were significantly decreased to below the detection limit (1-log CFU/g) after 56 days of ripening for all the inoculation levels. However, viable cells were still detectable in the samples with high and medium inoculation levels. No pathogens were observed in the cheese made from pasteurized milk (Table 3).

There have been few studies on the behavior of *Listeria monocytogenes* in Cheddar cheese during ripening. Ryser and Marth (1989) found that *Listeria monocytogenes* can survive during ripening for more than one year. However, this previous study differed from the study presented here because Ryser and Marth (1989) inoculated *Listeria monocytogenes* into pasteurized whole milk, indicating that the contamination of milk had occurred after pasteurization or during cold storage of the pasteurized milk before the cheese was made. Dalmassoand Jordan (2014) have investigated *Listeria monocytogenes* in naturally contaminated cheddar cheese made from raw milk at the farm level. They have found that *Listeria monocytogenes* levels never exceed 20 CFU/g in raw-milk cheeses and could not be detected after aging.

Various levels of *E. coli*O157:H7 for inoculation were tested, and it was found that the pathogens could be completely eliminated if small numbers of cells were contaminated in raw milk. However, *E. coli*O157:H7 was not totally eliminated and could be detected by enrichment culture in the samples inoculated with medium to high numbers of cells. Similar results were reported in previous studies about *E. coli*O157:H7 in cheese. D'Amico et al. (2010) reported that they could still detect viable *E. coli*O157:H7 cells after >270 days of aging in Gouda and stirred-curd cheddar cheeses made from milk inoculated at 20 CFU/mL. Reitsma &

Table 2
Quantitative and qualitative detection of *L. monocytogenes* in cheddar cheese manufactured from unpasteurized raw milk during the ripening stage (unit: log CFU/mL).

Days	The size of initial inoculums					
	High (5-log CFU/mL)		Medium (3-log CFU/mL)		Low (1-log CFU/mL)	
	Quantitative	Qualitative	Quantitative	Qualitative	Quantitative	Qualitative
0	6.69[a]	(+)	3.32[b]	(+)	1.74[c]	(+)
7	5.91[a]	(+)	4.08[b]	(+)	1.94[c]	(+)
14	5.86[a]	(+)	4.32[b]	(+)	2.08[c]	(+)
21	5.81[a]	(+)	3.86[b]	(+)	2.18[c]	(+)
28	5.56[a]	(+)	3.04[b]	(+)	1.99[c]	(+)
35	4.00[a]	(+)	2.71[b]	(+)	1.62[c]	(+)
42	2.23[a]	(+)	1.57[b]	(+)	1.26[c]	(+)
49	ND*	(+)	1.11[a]	(+)	ND	(+)
56	ND	(+)	ND	(+)	ND	(+)
63	ND	(+)	ND	(+)	ND	(+)

*Under the detection limit (i.e., < 1-log CFU/g).
[ab)] Different letters within a row indicate a significant difference ($p < 0.05$).

Table 3
Quantitative and qualitative detection of *E. coli* O157:H7 in cheddar cheese manufactured from unpasteurized raw milk during the ripening stage (unit: log CFU/mL).

Days	The size of initial inoculum					
	High (5-log CFU/mL)		Medium (3-log CFU/mL)		Low (1-log CFU/mL)	
	Quantitative	Qualitative	Quantitative	Qualitative	Quantitative	Qualitative
0	5.56[a]	(+)	3.85[b]	(+)	2.78[c]	(+)
7	5.38[a]	(+)	3.77[b]	(+)	1.23[c]	(+)
14	3.83[a]	(+)	3.08[b]	(+)	ND	(+)
21	4.04[a]	(+)	1.56[b]	(+)	ND	(+)
28	4.89[a]	(+)	1.38[b]	(+)	ND	(+)
35	3.40[a]	(+)	ND	(+)	ND	(+)
42	3.28[a]	(+)	ND	(+)	ND	(+)
49	1.99[a]	(+)	ND	(+)	ND	(+)
56	ND*	(+)	ND	(+)	ND	(+)
63	ND	(+)	ND	(+)	ND	(−)

*Under the detection limit (i.e., < 1-log CFU/g).
[ab] Different letters within a row indicate a significant difference ($p < 0.05$).

Henning (1996) showed similar results in their study, where viable *E. coli*O157:H7 was detected in cheddar cheese made from milk that had been inoculated at 10^3 CFU/mL for up to 158 days using an enrichment procedure. The differences between the results of this study and those of previous studies may be due to the variations in the strains used, ripening temperatures, and cheese manufacturing processes.

The observed reductions in pathogen numbers during the ripening period may result from the extended exposure to low pH, the starter culture, low temperatures, and high salt concentrations (Hudson et al., 1997; D'Amico et al., 2010). However, high acid tolerance, resistance to fermentation by-products, survival during storage at 4 °C, and high salt tolerance may allow these pathogens to survive in cheddar cheese (Reitsma & Henning, 1996). Moreover, expression of virulence factors within the cheese matrix may be altered over time by prolonged exposure to salt, acid, low temperatures, low moisture, and the presence of a starter culture (D'Amico et al., 2010). Further studies are needed for a better understanding of these factors.

Despite the survival of pathogens during the ripening stage, the reported incidences of illness outbreaks linked to raw-milk cheeses are low in comparison with those linked to cheeses made with pasteurized milk (D'Amico et al., 2010). Recently, various pathogens such as *Salmonella* spp., *Listeria monocytogenes*, and *E. coli*O157:H7 have been implicated in food poisoning outbreaks related to cheeses produced from pasteurized milk (Centers for Disease Control and Prevention, 2013; Leong et al., 2014). The infrequent occurrence of pathogens in raw milk products may explain this observation. Indeed, several studies have reported that *Listeria monocytogenes* and *E. coli*O157:H7 are rarely found in raw milk products. Previous studies have reported that the incidence of *E. coli*O157:H7 in raw milk used for raw milk cheese is much lower (<1%) than that reported for milk in general (D'Amico et al., 2008, 2009, 2010). The number of pathogens present at levels below the infectious dose after ripening may also explain the low incidence of foodborne illnesses from raw milk products. In this study, the numbers of *Listeria monocytogenes* and *E. coli*O157:H7 were less than 10 cells after a 60-day ripening period, indicating that the number of cells was too low to cause food poisoning (Table 3). In previous studies, researchers have shown that *Listeria monocytogenes* does not pose a public health risk up to 100 CFU/g, even in young, old, pregnant, or immunosuppressed individuals (Dalmasso & Jordan, 2014; Ross, 2011, pp. 25–81).

This study indicated that the 60-day ripening process alone may not be sufficient to effectively eliminate contaminating pathogens, i.e., *Listeria monocytogenes* and *E. coli*O157:H7, in cheddar cheese made from unpasteurized raw milk, particularly at medium (10^3 CFU/g) or high (10^5 CFU/g) contamination levels. Some researchers have suggested effective alternative methods for pathogen removal. For example, subpasteurization heat treatment may be applied as an additional method for the elimination of pathogens (Reitsma & Henning, 1996; Schlesser et al., 2006). A minimum heat treatment of 64.48 °C for 16 s in combination with the 60-day aging would be an alternative option to eliminate vegetative pathogens effectively without any adverse effects on the quality of the product (Johnson et al., 1990a, 1990b; Reitsma & Henning, 1996). Using bacteriocin or nisin-producing starter cultures or high-pressure treatments would also be an effective control method for the inhibition of undesirable pathogens in cheeses made from raw milk (Ramasaran et al., 1998; Rodriguez et al., 2005).

Therefore, the adequacy of a 60-day aging period for raw milk Cheddar cheese to eliminate *Escherichia coli*O157:H7 and *Listeria monocytogenes* was evaluated. The changes in cheese components and

characteristics of pH, Aw, coliform, and LAB were examined during the ripening process. Cheddar cheese was manufactured from raw milk artificially spiked with 1, 3, or 5 log CFU/mL *L. monocytogenes* or *E. coli*O157:H7. When compared with the pasteurized samples, the unpasteurized samples exhibited slightly lower pH values and similar aw values. No coliform were observed in the pasteurized cheese, whereas over 5-log CFU/g of coliform were found in the cheese made from unpasteurized milk during the early phases of ripening. However, coliform were completely eliminated from the cheese made from unpasteurized milk within five weeks. In contrast, while the level of LAB was initially 2-log lower in the cheese made from pasteurized milk than that derived with unpasteurized milk, the number of LAB between the two cheese preparations equalized within five weeks. Although *L. monocytogenes* numbers decreased significantly to below the detection limit (1-log CFU/g) during ripening, viable cells were isolated from all the inocula. *E. coli*O157:H7 cells also significantly decreased to below the detection limit during ripening at all the inoculation levels. However, viable cells were still detected. Thus, a 60-day ripening process alone may not be sufficient for elimination of contaminating pathogens.

In conclusion, although naturally present coliform bacteria were completely inactivated as early as 5 weeks of aging for raw milk Cheddar cheese, *L. monocytogenes* or *E. coli*O157:H7 that were artificially inoculated at three different levels survived the entire 60-day aging period indicating the ripening process alone may not be sufficient for complete inactivation of contaminating pathogens.

CRediT authorship contribution statement

Jhun-Woo Kim: carried out the experiment, wrote the draft manuscript, All authors read and approved the final manuscript. **Jung-Whan Chon:** carried out the experiment, wrote the draft manuscript, All authors read and approved the final manuscript. **Kwang-Young Song:** carried out the experiment, All authors read and approved the final manuscript. **Jong-Soo Lim:** carried out the experiment, All authors read and approved the final manuscript. **Dongryeoul Bae:** contributed to the interpretation of the results and data analysis. All authors read and approved the final manuscript. **Hyunsook Kim:** contributed to the interpretation of the results and data analysis. All authors read and approved the final manuscript. **Kun-Ho Seo:** supervised the project, All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no potential conflict of interest.

Acknowledgement

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1D1A1B03035427), and this study was also supported by the National Research Foundation of Korea grant funded by the Korean government (MSIP; No. 2018R1A2A2A14021671). We would like to thank Editage (www.editage.co.kr) for English language editing.

References

Batz, M. B., Hoffmann, S., & Morris, J. G., Jr. (2012). Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. *Journal of Food Protextion, 75*, 1278–1291.

Centers for Disease Control and Prevention. (2013). *Foodborne outbreak*. Online Database http://www.cdc.gov/foodborneoutbreaks/ Accessed date: 4 November 2019.

Claeys, W. L., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., & Dierick, K. (2013). Raw or heated cow milk consumption: Review of risks and benefits. *Food Control, 31*, 251e262.

Costard, S., Espejo, L., Groenendaal, H., & Zagmutt, F. J. (2017). Outbreak-related disease burden associated with consumption of unpasteurized cow's milk and cheese, Untied States, 200-2014. *Emerging Infectious Diseases, 23*, 957–964.

Dalmasso, M., & Jordan, K. (2014). Absence of growth of *Listeria monocytogenes* in naturally contaminated Cheddar cheese. *Journal of Dairy Research, 81*, 46–53.

D'Amico, D. J., Druart, M. J., & Donnelly, C. W. (2010). Behavior of *Escherichia coli*O157:H7 during the manufacture and aging of Gouda and stirred-curd Cheddar cheeses manufactured from raw milk. *Journal of Food Protection, 73*, 2217–2224.

D'Amico, D. J., Groves, E., & Donnelly, C. W. (2008). Low incidence of foodborne pathogens of concern in raw milk utilized for farmstead cheese production. *Journal of Food Protection, 71*, 1580–1589.

Feng, P., & Monday, S. R. (2000). Multiplex PCR for detection of trait and virulence factors in enterohemorrhagic *Escherichia coli* serotypes. *Molecular and Cellular Probes, 14*, 333–337.

Hudson, L. M., Chen, J., Hill, A. R., & Griffiths, M. W. (1997). Bioluminescence: A rapid indicator of *Escherichia coli*O157:H7 in selected yogurt and cheese varieties. *Journal of Food Protection, 8*, 891–897.

Jakobsen, R. A., Heggebø, R., Sunde, E. B., & Skjervheim, M. (2011). Staphylococcus aureus and Listeria monocytogenes in Norwegian raw milk cheese production. *Food Microbiology, 28*, 492–496.

Johnson, E. A., Nelson, J. H., & Johnson, M. (1990a). Microbiological safety of cheese made from heat-treated milk. Part 1.Executive summary, introduction and history. *Journal of Food Protection, 53*, 441–452.

Johnson, E. A., Nelson, J. H., & Johnson, M. (1990b). Microbiological safety of cheese made from heat-treated milk, Part III. Technology, discussion, recommendations, bibliography. *Journal of Food Protection, 53*, 610–623.

Khayat, F. A., Bruhn, J. C., & Richardson, G. H. (1988). A survey of coliforms and *Staphylococcus aureus* in cheese using impedimetric and plate count methods. *Journal of Food Protection, 51*, 53–55.

Kousta, M., Mataragas, M., Skandamis, P., & Drosinos, H. (2010). Prevalence and sources of cheese contamination with pathogens at farm and processing levels. *Food Control, 21*, 805–815.

Lawrence, R. C., Creamer, L. K., & Gilles, J. (1987). Texture development during cheese ripening. *Journal of Dairy Science, 70*, 1748–1760.

Lee, M. R., & Lee, W. J. (2008). Understanding of development of cheese texture during ripening. *Korean Journal of Dairy Technology Science, 26*, 57–60.

Leong, W. M., Geier, R., Engstrom, S., Ingham, S., Ingham, B., & Smukowski, M. (2014). Growth of *Listeria monocytogenes, Salmonella* spp., *Escherichia coli*O157:H7, and *Staphylococcus aureus* on cheese during extended storage at 25 °C. *Journal of Food Protection, 77*, 1275–1288.

Maher, M. M., Jordan, K. N., Upton, M. E., & Coffey, A. (2001). Growth and survival of *E. coli*O157:H7 during the manufacture and ripening of a smear-ripened cheese produced from raw milk. *Journal of Applied Microbiology, 90*, 201–207.

Ministry of Food and Drug Safety of Korea (MFDS). (2018). *Food code.* http://www.law.go.kr/ Accessed date: 4 March 2019.

Ramsaran, H., Chen, J., Brunke, B., Hill, A., & Griffiths, M. W. (1998). Survival of bioluminescent *Listeria monocytogenes* and *Escherichia coli*O157:H7 in soft cheeses. *Journal of Dairy Science, 81*, 1810–1817.

Reitsma, C. J., & Henning, D. R. (1996). Survival of enterohemorrhagic*Escherichia coli*O157:H7 during the manufacture and curing of cheddar cheese. *Journal of Food Protection, 59*, 460–464.

Rodriguez, E., Arques, J. L., Nuñez, M., Gaya, P., & Medina, M. (2005). Combined effect of high-pressure treatments and bacteriocin-producing lactic acid bacteria on inactivation of *Escherichia coli*O157:H7 in raw-milk cheese. *Applied and Environmental Microbiology, 71*, 3399–3404.

Ross, T. (2011). *Challenge testing of microbiological safety of raw milk cheeses: The challenge trial toolkit- challenge testing of cheese*. MAF Technical Paper No: 2011/51. Ministry of Agriculture and Forestry. New Zealand Food Safety Authority; 2011.

Ryser, E. T., & Marth, E. H. (1989). Behavior of *Listeria monocytogenes* during manufacture and ripening of brick cheese. *Journal of Dairy Science, 72*, 838–853.

Schlesser, J. E., Gerdes, R., Ravishankar, S., Madsen, K., Mowbray, J., & Teo, A. Y. (2006). Survival of a five-strain cocktail of *Escherichia coli*O157:H7 during the 60-day aging period of cheddar cheese made from unpasteurized milk. *Journal of Food Protection, 69*, 990–998.

Verraes, C., Vlaemynck, G., Van Weyenberg, S., De Zutter, L., Daube, G., M. Sindic, M., Uyttendaelec, M., & Herman, L. (2015). A review of the microbiological hazards of dairy products made from raw milk. *International Dairy Journal, 50*, 32–44.

Wells, J. G., Shipman, L. D., Greene, K. D., Sowers, E. G., Green, J. H., Cameron, D. N., … Ostroff, S. M (1991). Isolation of Escherichia coli serotype O157:H7 and other Shiga-like-toxin-producing E. coli from dairy cattle. *Journal of Clinical Microbiology, 29*, 985–989.

Yang, B., Shi, Y., Xia, X., Xi, M., Wang, X., Ji, B., … Meng, J. (2012). Inactivation of foodborne pathogens in raw milk using high hydrostatic pressure. *Food Control, 28*, 273–278.