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ARTICLE

Survival Behavior of *Escherichia coli* O157:H7 during over 60-Day Aging of Camembert Cheeses Manufactured from Unpasteurized Raw Milk

Jung-Whan Chon[1,2†], Kun-Ho Seo[1†], Jong-Soo Lim[1], Hye-Young Youn[1], Dongkwan Jeong[3], and Kwang-Young Song[1,4*]

[1]Center for One Health and Department of Public Health, College of Veterinary Medicine, Konkuk University, Seoul, Korea
[2]Department of Companion Animal Health, Inje University, Gimhae, Korea
[3]Department of Food and Nutrition, Kosin University, Busan, Korea
[4]Department of Companion Animal, Seojeong University, Yangju, Korea

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†These authors contributed equally to this study.

*Corresponding author :
Kwang-Young Song
Department of Companion Animal, Seojeong University, Yangju, Korea
Tel : +82-31-860-5075
Fax : +82-31-859-6064
E-mail : drkysong@gmail.com

ORCID
Jung-Whan Chon
https://orcid.org/0000-0003-0758-6115
Kun-Ho Seo
https://orcid.org/0000-0001-5720-0538
Jong-Soo Lim
https://orcid.org/0000-0001-8818-282X
Hye-Young Youn
https://orcid.org/0000-0003-4626-5859
Dongkwan Jeong
https://orcid.org/0000-0002-6305-794X
Kwang-Young Song
https://orcid.org/0000-0002-5619-8381

Abstract

Although many developed countries (USA, Canada, and several EU countries) allow raw milk cheese to be aged more than 60 days, these countries have strict standards for the aging conditions, such as temperature, of raw milk cheese. Spiking experiments were conducted with Camembert cheese made from raw milk, to assess the microbiological safety of raw milk cheese aged for more than 60 days. We spiked *Escherichia coli* O157:H7 into raw milk with different inoculation levels (high, medium, and low). Camembert cheese was prepared from the inoculated raw milk, then aged in an incubator for up to 9 weeks (63 days). There were no significant differences in pH and water activity (a_W) between uninoculated cheese and cheese samples inoculated with *E. coli* O157:H7 ($p<0.05$). The pH and a_W of the Camembert cheese decreased throughout the storage period. In conclusion, *E. coli* O157:H7 did not affect the pH and a_W of the cheese samples. Cell counts were conducted every week using the agar-plating method. Inoculated cells were completely eliminated, especially in Camembert cheese, after 60 days, and the reduction rate of cells was much faster in Camembert cheese.

Keywords

Camembert cheese, unpasteurized raw milk, *Escherichia coli* O157:H7, lactic acid bacteria, water activity

Introduction

As a characteristic of bacteria, enterovirulent *Escherichia coli* in general causes acute gastroenteritis in humans [1-3]. One of the pathogenic *E. coli* is called enterohemorrhagic *E. coli*, of which *E. coli* O157:H7 is the most famous [2,4]. *E. coli* O157:H7 is also commonly referred to as 'O-157' for short because of its long name [1-4]. In addition, to explain the meaning of enterohemorrhagic *E. coli* in detail, entero is translated as 'intestine', hemorrhagic as 'bleeding', and *E. coli* as '*Escherichia coli*', resulting in '*Escherichia coli*' that causes bleeding in the intestine' [4,5]. In other words, enterohemorrhagic *E. coli* is *E. coli* that causes bleeding in the large intestine, and O-157 is one of the bacteria [4-6]. O-157 bacteria produce one or more potent toxins that severely damage the surface of the intestinal tract [6]. The toxin produced by O-157, called verotoxin or shiga-like toxin, is the same as or closely related to the toxin produced by *Shigella dysenteriae* [7,8]. As an acute disease, *E. coli* O157:H7 causes an

acute disease called 'hemorrhagic enteritis' [1-8]. The disease is characterized by severe cramping abdominal pain and diarrhea [1-8]. Diarrhea is initially watery, but changes to bloody stools [1-8]. It is sometimes accompanied by vomiting [1-8]. There may be no fever or a slight fever may occur [1-8]. The disease goes away on its own, but lasts for an average of 8 days [5-8]. Depending on the patient, only watery diarrhea may appear. Infective dose is known to be about 10 CFU like bacillary dysentery [9]. Related foods include alfalfa sprouts, unpasteurized fruit juice, dried salami, wild game meat, lettuce, cheese, etc. This food is known to cause O-157 food poisoning [10-12]. In a school in Canada, unpasteurized milk was the cause of a food poisoning accident [13].

Camembert is a small village located in Normandy in northern France [14,15]. Camembert, about 5 km from Vimoutiers, is a small rural village on a hill in Normandy with only about 200 residents running the village [14-16]. Camembert is a white mold cheese made by inoculation with *Penicillium camemberti* [17]. This is because the enzyme produced by the white mold breaks down the protein and the acidity becomes very low [17,18]. *E. coli* O157:H7 bacteria, which are acid-resistant pathogens, are a problem [13,14]. Because these bacteria are acid-resistant, they survive at the pH of the initial stage of cheese production and proliferate when the pH of cheese increases as the ripening progresses, causing food poisoning [19]. Camembert cheese made with unpasteurized raw milk requires special attention to hygiene and safety management compared to hard-ripened cheese [14-19]. In order to prevent the contamination of the Camembert cheese ripening room by residual pathogens, the maturation chamber must be cleaned and disinfected regularly.

In Korea, in the case of cheese manufactured using unpasteurized raw milk, the sale of aged cheese for 60 days is allowed in the manufacturing and processing standards of cheese of the revised Food Code, which is applied from January 1, 2018 [20].

However, milk as a complete food is a very useful food, but it is a food that is easily affected by the external environment due to the quality of milk [20]. For this reason, unpasteurized cheese manufactured without sterilization of raw milk must be supervised according to very strict standards so that Korean consumers can recognize the safety of unpasteurized raw milk cheese. Furthermore, it is believed that it will be able to revitalize the Korean cheese industry.

Furthermore, through free trade agreement (FTA) negotiations between Korea and the United States, Europe, Australia, and Chile, various foreign cheeses are currently being imported [20]. Among imported cheeses, countermeasures such as unpasteurized cheese are urgently required. In addition, it is necessary to prepare for this because the regulations on sterilization of raw milk used in cheese production and the ripening period for cheese made with non-sterilized whey milk differ from country to country.

Therefore, in this study, Camembert cheese made from unpasteurized raw milk was inoculated with *E. coli* O157:H7 strains at various concentrations to evaluate the safety of unpasteurized Camembert cheese during the ripening storage period of more than 60 days.

Materials and Methods

1. Milk, Lactic acid bacteria, Rennet and *Penicillium camemberti*

High-quality unpasteurized raw milk that passed various raw milk tests was purchased from Konkuk Milk and used in this study. *Lactococcus lactis* subsp. *cremoris*, *Laccococcus lactis* subsp. *lactis*, *Lactococcus lasctis* subsp. *lactis* biovar. diacetylactis, *Streptococcus salivarius* subsp. *thermophilus*, and *Lactobacillus helveticus* were purchased from SAMIK Dairy & Food. Each lactic acid bacteria was subcultured three times in 10% sterilized skim milk to improve vitality and then used at the time of inoculation. Standard plus 900 (Chr. Hansen, Denmark) was used as the clotting enzyme, rennet. And *P. camemberti* was purchased from SAMIK Dairy & Food and used in the manufacture of Camembert cheese.

2. *Escherichia coli* O157:H7 strain used for inoculation

E. coli O157:H7 was donated from the US FDA (5100 Paint Branch Parkway, USA). *E. coli* O157:H7 is a strain isolated from food. While stored at −70℃ in the laboratory, the strain was thawed and used whenever necessary. The thawed strain was spread on Nutrient agar (Oxoid, UK) and incubated aerobically at 37℃ for 24 hours. A single colony was selected from the cultured colonies and cultured in Tryptic Soy Broth (Oxoid) at 37℃ for 24 hours, and the bacterial solution subcultured three or more times was diluted and used as an inoculum.

3. Camembert cheese manufacturing

Camembert cheese was prepared according to the method of Ryser & Marth [21] (Fig. 1). In this study, Camembert cheese produced by pasteurized raw milk was used as the control group, and Camembert cheese was manufactured with unpasteurized raw milk as the treated group. Camembert cheese made from unpasteurized raw milk was inoculated with *E. coli* O157:H7, a pathogenic bacterium, at various concentrations, and changes during the storage period of more than 60 days were observed. After draining overnight at room temperature (about 23±2℃), Camembert cheese were dry-salted and then ripened 10 days at 15±1℃ with 90±5% relative humidity so as to enable to desirable growth of *P. camemberti*. And after 10 days, Camembert cheese were wrapped in foil and ripened at 6℃.

4. Microbial measurement for lactic acid bacteria, coliform, and *Escherichia coli* O157:H7

The number of lactic acid bacteria was measured as follows. Add 225 mL of phosphate buffer diluent to 25 g of Camembert cheese sample. Homogenize for 1 minute using a stomacher blender, Bag mixer (Interscience, USA). After serially diluting 100 μL by 10-fold dilution unit, spread on MRS agar (Difco, USA) and incubate at 36℃ for 48 hours. White coli colonies on MRS agar were counted as lactic acid bacteria.

The coliform count was measured as follows. Add 225 mL of phosphate buffer diluted

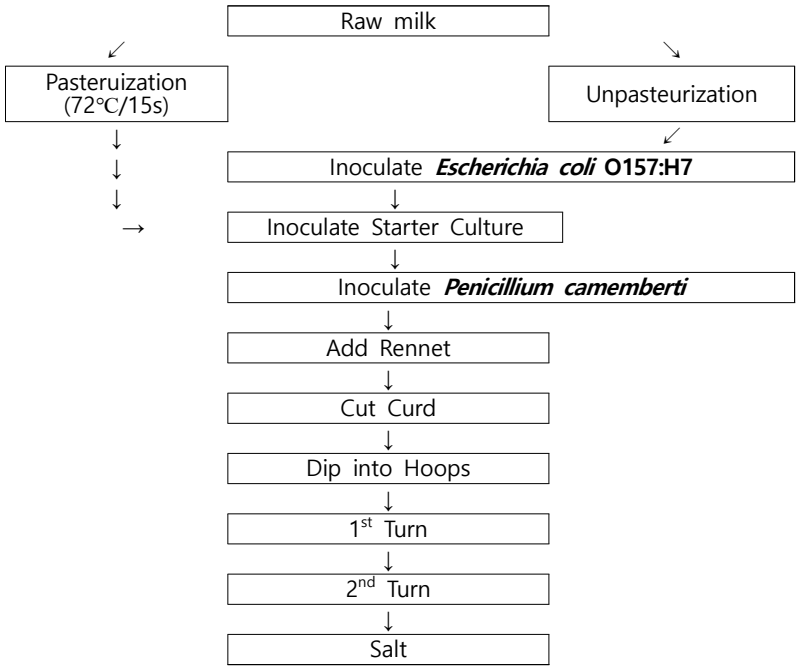

Fig. 1. Typical manufacturing diagram for Camembert cheese using pasteurized raw milk and unpasteurized raw milk with adding 3 different concentrations of *Escherichia coli* O157:H7.

solution to 25 g of camembert cheese sample and homogenize for 1 minute using a stomacher blender, Bag mixer (Interscience). Take 100 μL, dilute by 10-fold dilution unit, spread on coliform dry film (3M Petrifilm, USA) and incubate at 36℃ for 24-48 hours. Among the red colonies on a dry film (3M Petrifilm), colonies forming air bubbles were counted as the number of coliforms.

E. coli O157:H7 measurement is as follows. Add 225 mL of phosphate buffer diluted solution to 25 g of camembert cheese sample and homogenize for 1 minute using a stomacher blender, Bag mixer (Interscience). Then, 100 uL was diluted in a 10-fold unit, spread on sorbitol MacConkey agar (SMAC; BD Difco, USA), and incubated at 37℃ for 24 hours. Colonies formed on SMAC (BD Difco) were counted as *E. coli* O157:H7.

5. Physical and chemical properties of pH and water activity (a_W)

The pH measurement is as follows. For pH change, saline and Camembert cheese are added to a grinding tube in a ratio of 2:1 (saline:cheese=20 mL:10 g). And after homogenizing at 12,000 ×g for 2 minutes, it was measured with a pH meter (Istek Model 720p, Korea).

Water activity (a_W) measurement is as follows. Water activity was measured three times using a Benchtop water activity meter (Aqualab, USA), and the average value was expressed as the water activity value of Camembert cheese.

6. Statistical analysis

For statistical analysis of the results obtained in this study, a statistically significant difference (p<0.05) was analyzed by Fisher's exact test using the statistical program

GraphPad Instat (GraphPad Softwre, USA).

Results and Discussion

1. Physicochemical changes during ripening of Camembert cheese

The pH of Camembert cheese made from pasteurized raw milk changed from 4.86 at 0 week to 5.93 at 8 weeks (Table 1). The pH of Cheddar cheese made from unpasteurized raw milk ranged from 5.04 at 0 week to 5.47 at 8 weeks (Table 1). The pH of Camembert cheese prepared by inoculating unpasteurized raw milk with *E. coli* O157:H7 was varied. When the inoculation amount of *E. coli* O157:H7 was high, the pH was 5.10 at 0 week to 5.49 at 8 weeks (Table 1). When *E. coli* O157:H7 was inoculated with Medium, the pH was 4.93 at 0 week to 5.19 at 8 weeks (Table 1). And when the inoculation amount of *E. coli* O157:H7 was low, the pH was 5.06 0 week at to 5.33 at 8 weeks (Table 1). It was found that the pH value of Camembert cheese was generally inconsistent during the ripening storage period. This is thought to be because the surface of Camembert cheese is covered with white mold by white mold and the inside appears as a typical cheese.

The results of this study were similar to those of Ryser & Marth [21] and Meyrand et al. [22].

The a_W (water activity) of Camembert cheese made from pasteurized raw milk changed from 0.9266 at 0 week to 0.8810 at 8 weeks (Table 2). The a_W (water activity) of Camembert cheese made from unpasteurized raw milk ranged from 0.9225 at 0 week to 0.8976 at 8 weeks (Table 2). The a_W of Camembert cheese prepared by inoculating unpasteurized raw milk with various concentrations of *E. coli* O157:H7 was varied. When the *E. coli* O157:H7 inoculum was high, a_W was 0.9454 at 0 week and 0.9209 at 8 weeks (Table 2). When the *E. coli* O157:H7 inoculum was medium, a_W was 0.9164 at 0 week and 0.9157 at 8 weeks (Table 2). And when the *E. coli* O157:H7 inoculum was low, a_W was 0.9528 at 0 week and 0.9072 at 8 weeks (Table 2). In the case of Camembert cheese, the overall a_W (water activity) decreases slightly as the aging period increases, which is thought to be due to the loss of moisture as Camembert cheese is not vacuum-

Table 1. Change of pH during ripening of Camembert cheese using pasteurized raw milk and unpasteurized raw milk with adding 3 different concentrations of *Escherichia coli* O157:H7

pH	Pasteurization	Unpasteurization			
		Uninoculation	Inoculation of *E. coli* O157:H7		
			High	Medium	Low
0 week	4.86	5.04	5.10	4.93	5.06
1 week	4.93	4.96	5.26	5.20	5.28
2 week	5.33	5.04	5.23	5.13	5.34
3 week	5.03	5.21	5.51	5.02	5.19
4 week	5.25	5.02	5.13	5.01	5.26
5 week	5.28	5.26	5.07	5.18	5.06
6 week	5.71	5.55	5.26	5.25	5.44
7 week	5.53	5.30	5.42	5.23	5.22
8 week	5.93	5.47	5.49	5.19	5.33

Table 2. Change of a_W during ripening of Camembert cheese using pasteurized raw milk and unpasteurized raw milk with adding 3 different concentrations of *Escherichia coli* O157:H7

a_W	Pasteurization	Unpasteurization			
		Uninoculation	Inoculation of *E. coli* O157:H7		
			High	Medium	Low
0 week	0.9266	0.9225	0.9454	0.9164	0.9528
1 week	0.8837	0.8283	0.9331	0.9136	0.9200
2 week	0.8931	0.9172	0.9405	0.8874	0.8796
3 week	0.9207	0.9088	0.9389	0.9284	0.9352
4 week	0.8949	0.9064	0.9414	0.9065	0.9072
5 week	0.8966	0.9182	0.9393	0.9463	0.9132
6 week	0.9001	0.8859	0.9256	0.9162	0.9043
7 week	0.8921	0.8866	0.9219	0.9029	0.8824
8 week	0.8810	0.8976	0.9209	0.9157	0.9072

packed [23].

2. Microbiological changes during ripening of Camembert cheese

Fig. 2 shows changes in coliform counts during ripening storage of Camembert cheese made from pasteurized and unpasteurized raw milk.

In the case of Camembert cheese made from unpasteurized raw milk, it was observed that the number of coliforms gradually decreased during the aging storage period, and from the 5th week, it was observed that the coliforms completely disappeared and were not detected. On the other hand, in the case of Camembert cheese made from pasteurized raw milk, no coliforms were detected from the beginning to the 8th week (Fig. 2).

Fig. 3 shows the changes in the number of lactic acid bacteria during the ripening

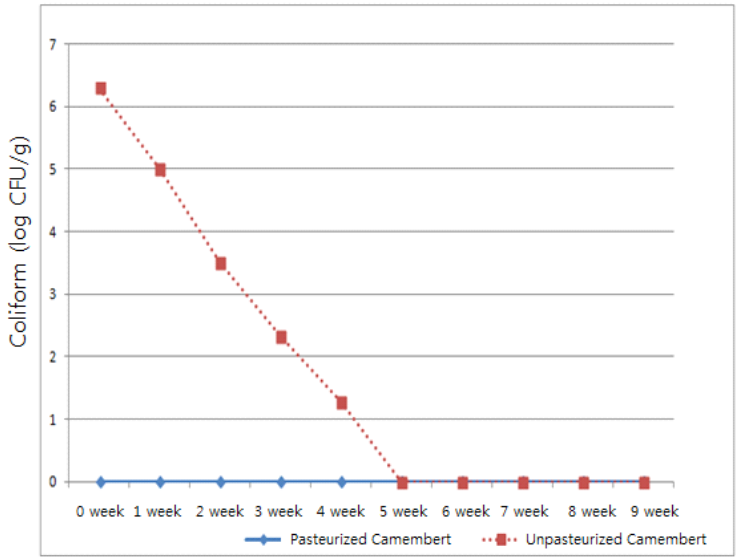

Fig. 2. Change of coliform population during ripening of Camembert cheese using pasteurized raw milk and unpasteurized raw milk without adding 3 different concentrations of *Escherichia coli* O157:H7.

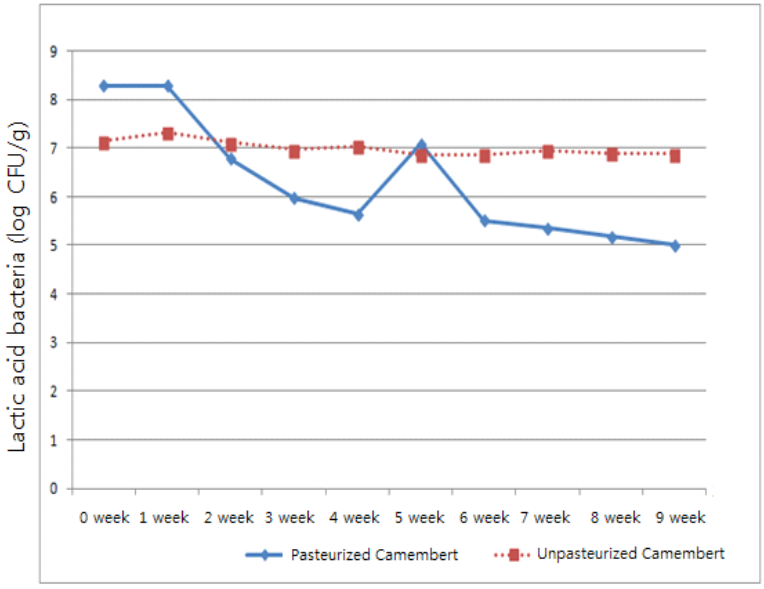

Fig. 3. Change of lactic acid bacteria population during ripening of Cheddar cheese using pasteurized raw milk and unpasteurized raw milk without adding 3 different concentrations of *Escherichia coli* O157:H7.

storage period of Camembert cheese made from pasteurized and unpasteurized raw milk.

In the case of Camembert cheese made by pasteurized raw milk, it was observed that the number of lactic acid bacteria decreased during the aging period. However, in the case of Camembert cheese made from unpasteurized raw milk, the number of lactic acid bacteria remained constant during the maturation period without significant change (Fig. 3).

Qualitative tests for *E. coli* O157:H7 were simultaneously conducted in Camembert cheese made from unpasteurized raw milk, but all were negative during the aging period (Data not shown).

3. Changes of *Escherichia coli* O157:H7 during the ripening period of Camembert cheese made by inoculating the pathogenic bacteria *Escherichia coli* O157:H7 at various concentrations

Table 3 and Fig. 4 show the changes in *E. coli* O157:H7 during the ripening period of Camembert cheese prepared after inoculating unpasteurized raw milk with the pathogenic bacteria *E. coli* O157:H7 at various concentrations.

A variety of Camembert cheeses were prepared by inoculating *E. coli* O157:H7 into unpasteurized raw milk. For High, 4.3×10^5 CFU/g of *E. coli* O157:H7 was inoculated, for Medium, 2.0×10^3 CFU/g of *E. coli* O157:H7 was inoculated, and for Low, <10 CFU/g of *E. coli* O157:H7 was inoculated, respectively (Table 3).

The results of *E. coli* O157:H7 quantitative detection are as follows. High with 4.3×10^5 CFU/g of *E. coli* O157:H7 inoculation amount showed 0 CFU/g at 5 weeks,

Table 3. Change of *Escherichia coli* O157:H7 during ripening of Camembert cheese using pasteurized raw milk and unpasteurized raw milk with adding 3 different concentrations of *E. coli* O157:H7

Storage	Quantitative and qualitative analysis of *E. coli* O157:H7					
	Inoculation of *E. coli* O157:H7					
	High		Medium		Low	
	4.3×10^5 CFU/g		2.0×10^3 CFU/g		<10 CFU/g	
	Quantitative analysis	Qualitative analysis	Quantitative analysis	Qualitative analysis	Quantitative analysis	Qualitative analysis
0 week	4.3×10^5	(+)	2.0×10^3	(+)	<10	(+)
1 week	7.0×10^4	(+)	1.2×10^3	(+)	<10	(+)
2 week	4.3×10^3	(+)	5.5×10	(+)	<10	(+)
3 week	<10	(+)	<10	(+)	<10	(+)
4 week	<10	(+)	<10	(+)	0	(−)
5 week	0	(−)	0	(−)	0	(−)
6 week	0	(−)	0	(−)	0	(−)
7 week	0	(−)	0	(−)	0	(−)

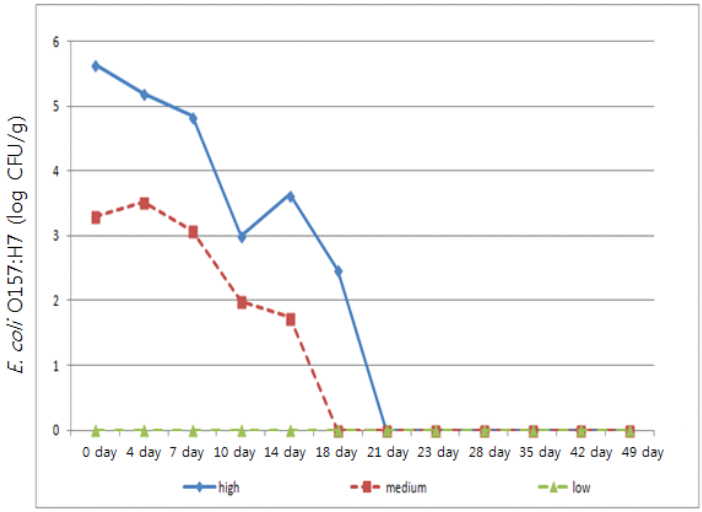

Fig. 4. Change of *Escherichia coli* O157:H7 during ripening of Camembert cheese using pasteurized raw milk and unpasteurized raw milk with adding 3 different concentrations of *E. coli* O157:H7.

Medium with 2.0×10^3 CFU/g of *E. coli* O157:H7 inoculation amount showed 0 CFU/g at 5 weeks, and Low with<10 CFU/g of *E. coli* O157:H7 inoculation amount showed 0 CFU/g at 5 weeks, respectively (Table 3).

The result of *E. coli* O157:H7 qualitative detection is as follows. In the case of *E. coli* O157:H7 inoculation amount High, (+) at 0 week was observed as (−) at 5 weeks. In the case of *E. coli* O157:H7 inoculation amount Medium, (+) at 0 week was observed as (−) at 5 weeks. And in the case of *E. coli* O157:H7 inoculation amount Low, (+) at 0 week was observed as (−) at 5 weeks (Table 3).

In the case of Camembert cheese, the number of *E. coli* O157:H7 tended to decrease rapidly. When the inoculation amount was High or Medium, the Camembert cheese was negative from the 5th week in the qualitative test, whereas in the Camembert cheese with the low inoculation amount, *E. coli* O157:H7 was not found in the qualitative test

from the 4th week (Fig. 4).

According to a research report by Ramsaran et al. [24], Camembert cheese and Feat cheese were prepared after inoculating 10^4 CFU/mL of *E. coli* O157:H7 into pasteurized and unpasteurized raw milk respectively. After manufacturing, the survival of microorganisms was investigated after storage at 2℃ for 65 and 75 days [24]. It was reported that *E. coli* O157:H7 was detected in both Camembert cheese and Feat cheese. Furthermore, *E. coli* O157:H7 was detected in the brine used for Feta cheese on 75 days [24].

In this study, in order to experimentally verify the safety of cheese aged for more than 60 days manufactured with unpasteurized raw milk, this study was conducted to compare and verify cheese made through aging as in Europe and cheese made with pasteurized raw milk. Camembert cheese, a soft cheese, was selected. As a result of this study on microbiological properties and safety, there was no significant difference in the number of lactic acid bacteria in cheese between unpasteurized raw milk-manufactured cheese and pasteurized milk-manufactured cheese. In addition, in the case of coliforms, a large number of 6 Log CFU or more was present in unpasteurized cheese, but all of them were removed after the 5th week, and it is considered that there is no big problem in terms of hygiene when ripening. Considering the infection amount of the pathogen, it is evaluated that it cannot necessarily be seen that food poisoning is caused below the infection amount. However, considering the risk of bacteria, it is absolutely necessary to control proliferation through proper storage and aging process after cheese production. However, since this study was conducted under specific conditions by manufacturing Camembert cheese with pathogens that are problematic in certain cheeses, then it seems that there is a need for a wide range of additional experiments applying various conditions, cheeses, and pathogens in the future.

In the 2000s, several studies were published that showed that aging of unpasteurized cheese for 60 days was not safe [25–27]. Also, in 2010, special attention should be paid to the fact that the issue of cheese safety is being reviewed at the US FDA level [28]. In other words, it is considered that additional scientific data should be observed and judged. Currently, in Korea, *E. coli* is a bacterium to which negative standards are applied, and it is an important food poisoning causative agent in cheese [20]. It is necessary to establish reasonable standards for cheese at the level of developed countries in preparation for FTA by conducting additional scientific risk assessment studies on the pasteurization equivalence of these bacteria. Internationally, many countries allow unpasteurized aged cheeses longer than 60 days. However, it is premised that items equivalent to pasteurization are strictly applied, such as storage temperature regulations, aging conditions subdivided for each cheese, and labeling regulations specifying that they are manufactured with unpasteurized milk. Therefore, it is expected that the cheese manufacturing process and ripening period can have the same effect as cheese using unpasteurized raw milk only when hygiene management guidelines such as HACCP are properly applied and thoroughly managed during the production process and ripening period. Therefore, research on this is also urgently required.

Conflict of Interest

The authors declare no potential conflict of interest.

Acknowledgements

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