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Research Article

A Study on Survival of *Listeria monocytogenes* during Manufacture and Ripening of Kashar Cheese

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Abstract: This study was performed to determine the survival of *Listeria monocytogenes* during the manufacture and ripening of Kashar, a popular cheese in Turkey. Raw milk was inoculated at 4 different levels with *L. monocytogenes* serotype 4b (ca. 3, 4, 5, and 6 log cfu ml^{-1} for group 1, 2, 3 and 4 cheeses, respectively) and made into Kashar cheese using the traditional technique. Following dry salting for 10 days at 18 ± 2 ºC, the Kashar cheese was ripened for 20 days at the same temperature and then stored at 5 ± 1 ºC for 3 months. Samples of milk, coagulum, curd and cheese were tested for *Listeria* numbers by surface plating on Oxford Agar. In all groups the numbers of pathogens remained nearly constant during the initial stages of cheese production. However, an approximately 1 log cfu g^{-1} increase was detected in *L. monocytogenes* counts, while the pH decreased from 6.37-6.40 to 5.03-5.07, during curd acidification. Heat treatment of ripe curd at 75 ºC for 5 min greatly influenced viability, but was not enough to destroy all the bacteria, especially when inoculated at high levels. The pathogen was not isolated on the 60^{th} and 120^{th} days of ripening in group 2 and 3 cheeses, respectively. Meanwhile, 1.83 log cfu g^{-1} bacteria remained viable in group 4 even after 120 days of ripening. As a consequence, it was determined that in conjunction with the heat treatment of curd, a ripening period of 4 months was necessary to eliminate *L. monocytogenes* when its levels were lower than 6 log cfu ml^{-1}. It was concluded that the techniques used in the production stages of Kashar cheese are not sufficient to eliminate high contamination (6 log cfu ml^{-1}) by *L. monocytogenes* and the cheese may pose a risk of food-borne listeriosis.

Key Words: Kashar cheese, *Listeria monocytogenes*, survival

Kaşar Peynirinin Üretimi ve Olgunlaşması Sırasında *Listeria monocytogenes*'in Canlılığını Sürdürmesi Üzerine Bir Araştırma

Özet: Bu çalışma, Türkiye'de yaygın olarak tüketilen kaşar peynirinin üretimi ve olgunlaşması sırasında *Listeria monocytogenes*'in canlılığını saptamak amacıyla gerçekleştirildi. Çiğ süt dört farklı düzeyde (1., 2., 3. ve 4. grup peynirler için sırasıyla 3, 4, 5, 6 log kob ml^{-1}) *L. monocytogenes* serotip 4b ile inokule edildi ve geleneksel tekniğe göre kaşar peyniri üretildi. 18 ± 2 ºC'de 10 gün süreyle kuru tuzlamayı takiben aynı ısıda 20 gün olgunlaşmaya bırakılan kaşar peynirleri, daha sonra 5 ± 1 ºC'de 3 ay boyunca depolandı. Süt, pıhtı, teleme ve peynir örnekleri, Oxford agar'a uygulanan yüzey ekim tekniği ile *Listeria* sayıları açısından analiz edildi. Tüm gruplarda, peynir üretiminin başlangıç aşamalarında patojenin sayıları nispeten sabit kaldı. Bununla birlikte pıhtının fermentasyonu sırasında, pH 6,37-6,40'dan 5,03-5,07'ye azalırken, *L. monocytogenes* sayılarında yaklaşık 1 log kob g^{-1}'lık bir artış belirlendi. Telemeye 75 ºC'de 5 dakika süreyle uygulanan ısı işlemi etkenin canlılığını önemli ölçüde etkilemesine rağmen, özellikle yüksek inokulasyonlu peynirlerde bakteriler yeterince inhibe edilmedi. 2. ve 3. grup peynirlerde sırasıyla olgunlaşmanın 60. ve 120. günlerinde izole edilmeyen etken, 4. grup peynirlerde olgunlaşmanın 120. gününde bile 1,83 log kob g^{-1} düzeyinde canlı kaldı. Sonuç olarak, 6 log cfu ml^{-1}'den daha düşük *L. monocytogenes* seviyelerini ortadan kaldırmak için telemeye uygulanan ısı işlemiyle birlikte dört aylık olgunlaşma süresinin gerekli olduğu belirlendi. Ayrıca, kaşar peynirinin üretim aşamalarında yararlanılan tekniklerin yüksek *L. monocytogenes* kontaminasyonunu (6 log cfu ml^{-1}) elimine etmediği ve bu peynirin gıda kökenli listeriosis için bir risk oluşturabileceği sonucuna varıldı.

Anahtar Sözcükler: Kaşar peyniri, *Listeria monocytogenes*, canlılığını sürdürme

Introduction

Kashar cheese has been one of the most popular semi-hard cheeses produced in the Balkan countries since the 11^{th} and 12^{th} centuries. Kachkaval, Kaser, Kackavaly, Kacekavalo, Dobrogen, Pirdop, Epir, Sarplaninski and Pirotski are some local names for the same product (1). It is produced from either sheep's or cow's milk, or a mixture of the two. It is subjected to a stretching process at 75 ºC during manufacture and a to ripening period of 4 months. Kashar cheese contains an average of 24.2% protein, 4.2% ash, 41.9% moisture, 25.1% fat, and 4.6% salt (2). Its pH and acidity level (LA%) are between

4.9 and 5.4 and 0.8 and 2.3, respectively (3,4). While Kashar is the second most popular cheese in Turkey, there are no standardized techniques used in its manufacture. In traditional techniques, given the fact that no pasteurization process is applied, pathogen microorganisms can be eliminated by heat treatment at 75 ºC during the curd stretching stage as well as by antimicrobials and metabolic acids produced by lactic acid bacteria from the natural flora (4,5).

Several outbreaks and cases associated with the consumption of foods, including milk and dairy products, have provided evidence of the importance of *L. monocytogenes* in the food industry (6,7). In recent years, studies have revealed that soft and semi-soft cheeses provide suitable conditions for the survival and development of this pathogen (8,9). It has been reported that 17 of the 68 traditional hard and semi-hard cheeses (25%) manufactured in Alentejo (a region in Southern Portugal) were contaminated with *Listeria* spp. and 8 of these (11.8%) were identified as *L. monocytogenes* (10).

Outbreaks of cheese-borne listeriosis and conflicting reports regarding its heat resistance (11,12) prompted us to investigate this pathogen in Kashar cheese manufactured according to traditional techniques.

Materials and Methods

Bacterial cultures

L. monocytogenes strain SLCC 2375 serotype 4b used in this study was kindly provided by Dr. Ch. Jacquet, Institut Pasteur, Centre National de Reference des *Listeria*, Paris, France. Stock cultures were maintained on Tryptone Soya Agar (TSA) (no. CM131, Oxoid) slants at 4 ºC, and were subcultured monthly. For intermediate cultures, stock cultures were transferred into Tryptone Soya Broth (TSB) (no. CM129, Oxoid) and incubated at 35 ºC for 24 h.

Inoculation of cheese-milk with *L. monocytogenes* and cheese manufacture

The raw milk (60 l) to be used in the cheese making was first tested for the presence of *L. monocytogenes* before inoculation. Confirmed milk samples were inoculated with *L. monocytogenes* to obtain a final concentration of ca. 3, 4, 5, and 6 log cfu ml^{-1} for groups 1, 2, 3 and 4, respectively. Inoculated milks were heated up to 35 ºC in a stainless steel cheese vat and 4 ml of calf rennet diluted in cold water was added to induce coagulation. The coagulum (usually 45-60 min after rennet addition) was cut into approximately 4 cm cubes and remained quiescent for ca. 15 min. The temperature of the vat content was gradually raised to 41 ºC under continuous stirring. Thereafter, the coagulum was collected in cheesecloth and pressed for syneresis (2 h). Subsequently, the curd was cut into blocks (25-35 cm) and kept at room temperature for 16 h. At the end of this period, the curd assumed an elastic form and its pH reached 5.03-5.07. At this stage, heat treatment of the curd at 75 ± 1 ºC for 5 min was performed. For this purpose, the curd was cut into long strips and placed in a water bath in a stainless steel bucket containing several holes (5 mm diameter). After heat treatment, the curd was manually kneaded for 5 min to eliminate air bubbles and was moulded in plastic moulds (20 cm in diameter and 16 cm in height). The next day, cheese wheels (approximately 3 kg) were removed from the plastic moulds and dry salted. For this, salt (average 13-15 g) was spread on the top surface of the wheels once every 2 days at room temperature for a period of 10 days. The wheels were turned over during each salting process. Thereafter, they were stored at room temperature for 20 days and transferred to the ripening room (5 ± 1 ºC) for 3 months (2).

Sampling

Duplicate samples were taken from inoculated milk, coagulum, acidified curd, heat-treated curd and cheese samples on days 1, 10, 30, 60, 90 and 120 of ripening.

Microbiological analysis

For enumeration of *L. monocytogenes*, samples (10 ml or g) were homogenized in 90 ml of 0.1% peptone water using a Seward Stomacher 80 Lab System (~2 min) and a 0.1 ml portion of the homogenate was plated on the surface of Oxford Agar medium (no. CM856, Oxoid) with supplement (no. SR140E, Oxoid). To increase the detection level to 10 organisms/g, 0.2 ml of the initial 1:10 dilution was surface plated on each of 5 plates of Oxford Agar, and the resulting *Listeria* counts were combined. All plates were incubated aerobically at 37 ºC for 48 h and the typical colonies were counted. Samples in which *L. monocytogenes* was not detected by direct plating were subjected to an enrichment procedure. This included incubation of 25 g cheese samples in 225 ml of Tryptone Soya Broth + 0.6% yeast extract (TSB, no. CM129, Oxoid; YE, no. L21, Oxoid) at 35 ºC for 48 h.

The enrichment culture was then plated on Oxford Agar medium and plates were examined for typical *Listeria* colonies after 48 h incubation at 37 ºC. Selected colonies, which exhibited a gray-black color with a black halo, were tested for morphology, catalase production, hemolysis, motility, xylose, rhamnose and mannitol fermentation (13,14).

Determination of pH

The pH was measured with an Orion pH-meter (Ionalyzer model 399A/F, Cambridge, MA, USA) at 25 ± 1 ºC (15).

Statistical analysis

The experiments were set up in a completely random design with 3 replications. All data were subjected to analysis of variance using MSTAT-C (version 2.1-Michigan State University 1991) and MINITAB (University of Texas at Austin) software. The significance of the differences between groups was determined at the 0.01 probability level, by the F-test. The F-protected least significant difference (LSD) was calculated at the 0.01 probability level.

Results

In the present study the survival and behavior of *L. monocytogenes* during the manufacture and ripening of Kashar cheese, which is usually produced from raw milk according to the traditional technique, are investigated. To test the influence of cheese making technique and ripening period on the survival of *L. monocytogenes*, 4 different inoculation levels were selected arbitrarily. The data on the behavior of *L. monocytogenes* during manufacture and ripening of Kashar cheese are shown in the Table.

When the coagulum was heated at 41 ºC for ca. 30 min, *L. monocytogenes* numbers remained nearly constant. Following syneresis, with a decrease in pH to 5.03-5.07, the population in acidified curd was about 0.73-1.27 log cfu g^{-1} greater compared to that of the inoculated milk. After heat treatment of curd at 75 ºC for approximately 5 min, *L. monocytogenes* inoculated in vat milk at levels of ca. 3 log cfu ml^{-1} was completely eliminated. During heat treatment of curd (ca. 4, 5 and 6 log cfu ml^{-1}) with initial high contamination of the cheese milk, a decrease in *L. monocytogenes* numbers of 1.77-2.34 log cfu g^{-1} was observed in groups 2, 3 and 4. During the ripening period, *L. monocytogenes* was not determined either by direct plating or enrichment procedure in group 1. The pathogen numbers remained nearly unchanged during the first 10 days of ripening in groups 2, 3 and 4. Meanwhile, *L. monocytogenes* counts in the same groups decreased about 0.30-0.58 log cfu g^{-1} on day 30 of ripening compared to populations of the 1-

Table. *L. monocytogenes* counts during manufacture stages and ripening period in Kashar cheese.

		Group 1	Group 2	Group 3	Group 4
		Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Manufacture stages	Inoculated milk	$3.43^{c} \pm 0.12$	$4.71^{b} \pm 0.07$	$5.22^{b} \pm 0.14$	$6.45^{a} \pm 0.12$
	Coagulum	$3.58^{d} \pm 0.20$	$4.72^{c} \pm 0.08$	$5.54^{b} \pm 0.16$	$6.61^{a} \pm 0.20$
	Acidified curd	$4.32^{d} \pm 0.09$	$5.44^{c} \pm 0.19$	$6.49^{b} \pm 0.18$	$7.48^{a} \pm 0.19$
	Heat-treated curd	ND^{c}	$2.48^{b} \pm 0.32$	$3.45^{ab} \pm 0.32$	$4.11^{a} \pm 0.29$
Ripening period (day)	1	ND^{c}	$2.38^{b} \pm 0.32$	$3.45^{ab} \pm 0.31$	$4.14^{a} \pm 0.29$
	10	ND^{c}	$2.52^{b} \pm 0.34$	$3.58^{ab} \pm 0.33$	$4.24^{a} \pm 0.33$
	30	ND^{c}	$1.83^{b} \pm 0.44$	$2.87^{ab} \pm 0.15$	$3.84^{a} \pm 0.14$
	60	ND^{c}	ND^{b}	$2.14^{a} \pm 0.28$	$3.12^{a} \pm 0.34$
	90	ND^{c}	ND^{b}	$1.13^{b} \pm 0.58$	$2.87^{a} \pm 0.15$
	120	ND^{c}	ND^{b}	ND^{b}	$1.83^{a} \pm 0.09$

$^{a, b, c, d}$: Means having different letters within same row differ significantly ($P < 0.01$).
ND: *L. monocytogenes* not detected. SEM: Standard error of means.

day-old cheese. Populations of the pathogen became undetectable on days 60 and 120 of ripening in groups 2 and 3, respectively. Nevertheless, the pathogen counts exhibited a 2.31 log cfu g^{-1} decrease at the end of ripening in group 4, but the organism was still present in cheese at a level of 1.83 log cfu g^{-1}.

Discussion

As shown in the Table, we could not detect a clear difference in bacterial counts when the coagulum was heated to 41 ºC. This can be explained by the prolonged lag phase of the bacteria in these conditions. In a previous study, the authors determined that the lag phase for *L. monocytogenes* strains Scott A, V7 and CA in sterile whole milk incubated at 35 ºC was about 2 h (16). Therefore, growth during the early stages of cheese manufacture would not be expected.

An approximately 1 log cfu g^{-1} increase was detected in *L. monocytogenes* counts in the curd kept at room temperature for 16 h. The increase in *L. monocytogenes* counts can be correlated with the combination of a high moisture content of curd and a pH value of around 6.0 at the beginning that decreased to 5.0 during curd acidification. Similarly, in studies on Camembert (17) and Cheddar cheeses (18) *L. monocytogenes* counts increased by 0.38-0.71 and 0.1-0.8 log cfu g^{-1}, respectively, in the curd after pressing overnight at room temperature.

Heat treatment of the curd in hot water (75 ºC) was found to constitute the most important barrier in the elimination of the pathogen. In group 1, where the initial inoculation level was ca. 3 log cfu ml^{-1}, no viable *L. monocytogenes* was found after this stage. In contrast, this heat treatment was not sufficient to eliminate the pathogen in groups 2, 3 and 4. This can be explained by the fact that although the temperature of the water used in heat treatment of curd is high enough to kill all the bacteria, heat transfer towards the curd core might be impeded by the massive structure of the curd block. In studies on Mozzarella cheese the authors reported that even at high inoculation levels *L. monocytogenes* could be eliminated during the stretching process at 77 ºC for 1-4 min (19,20). On the other hand, Villani et al. (14), working on the elimination of *L. monocytogenes* during the stretching of traditional Mozzarella cheese, reported a decrease of about 2 log at 95 ºC.

During the ripening period, no bacteria were isolated on days 60 and 120 in groups 2 and 3, respectively, whereas in group 4 even after 120 days of ripening 1.83 log cfu g^{-1} bacteria remained viable. Decreases in viability may be due to heat-injured *L. monocytogenes* becoming more susceptible to the low pH of the cheese, which is between 5.13 and 5.43 during the entire ripening period. This phenomenon was also observed previously during the ripening of several cheese varieties. Patır and Güven (21) indicated the survival of *L. monocytogenes* for 120 days in brined Şavak cheese (pH 5.5 to 5.7) produced from raw ewe's milk, although the numbers of the organism decreased gradually during cheese ripening at 4 ºC. Ryser and Marth (17) also observed similar findings for Camembert cheese stored for 18 days at 6 ºC. In that study, reductions in *Listeria* counts were attributed to the pH of the cheese varying around 5.5. Glass et al. (22) reported that *L. monocytogenes* counts decreased by only 0.6 $\log_{10}$ cfu/g in cheese (pH 5.6 to 5.84, 2.3 to 2.6% salt) stored for 4 days at 30 ºC. However, Buchanan et al. (23) reported that the organism exhibited a decrease of 4 $\log_{10}$ cfu/ml in brain heart infusion broth (pH 5.0, acidified with 50 mM acetic acid) over 4 days at 28 ºC. These findings indicate that *L. monocytogenes* viability is dependent on incubation temperature and medium composition; so the rate of inactivation of the pathogen may also be affected by a combination of factors in cheese such as proteins, lipids, water content, and pH. In addition, lactic flora during fermentation plays an important role in flavor, aroma and texture formation (24,25) and also in the microbiological quality of cheese. Numerous reviews (26-31) have suggested that pathogens and spoilage microorganisms in fermented foods may be inhibited during the growth of lactic acid bacteria. The major cause of this inhibition is suggested as being the production of organic acids and, additionally, the secretion of metabolic compounds such as hydrogen peroxide, diacetyl (26,32), bacteriocins (33-36) and other low molecular weight products (37).

In conclusion, it can be stated that the temperature of curd heat treatment during Kashar cheese production does not represent a real control point for the complete demise of the pathogen, especially when it is at the >3 log cfu ml^{-1} level, and a combination of curd heat treatment and a ripening period of at least 4 months is necessary to

eliminate *L. monocytogenes* when the cheese is produced from milk containing approximately 4-5 log cfu ml^{-1}. Furthermore, it is concluded that 4 months of ripening for Kashar cheese is not sufficient to eliminate the high contamination (6 log cfu ml^{-1}) of *L. monocytogenes* and that such cheese may pose an important risk to public health.

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