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Fate of *Listeria monocytogenes* during Ripening of Iranian Traditional Koozeh Cheese Made from Raw Ewe's Milk

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HIGHLIGHTS

- *Listeria monocytogenes* was reduced during ripening days of Koozeh cheese at 4, 9, and 14 °C.
- *L. monocytogenes* was eliminated completely at the end of ripening period.
- All NaCl concentrations (8, 12, and 15%) reduced the *L. monocytogenes* counts.

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Acronyms and abbreviations
CFU=Colony Forming Unit

ABSTRACT

Background: One of the most well-known Iranian traditional cheeses is Koozeh. The aim of present work was to evaluate the survival of *L. monocytogenes* during ripening of Iranian traditional Koozeh cheese made from raw ewe's milk.

Methods: A 2-factor experimental design was applied to study the effect of ripening conditions, including different temperatures (4, 9, and 14 °C) and different concentrations of NaCl (0, 8, 12, and 15%) on the survival of *L. monocytogenes* in the Koozeh cheese. Microbial analysis was carried out over a period of 150 days with sampling in every 10 days. SPSS software (v. 16.0) was used for statistical analysis.

Results: Three NaCl concentrations (8, 12, and 15%) significantly affect the inactivation *L. monocytogenes* ($p<0.05$). After inoculation, *L. monocytogenes* populations were reduced most rapidly during the first ten days of storage (~0.5-1.5 log Colony Forming Unit/g) at three mentioned temperatures; after that, the bacteria were continually decreased, being below the detection limit (1 log CFU/g) at the end of ripening. Numbers of *L. monocytogenes* were reduced more effectively at 14 °C storage temperature than 9 and 4 °C ($p<0.05$).

Conclusion: *L. monocytogenes* was declined drastically during ripening days and eliminated at the end of ripening of Koozeh cheese. *L. monocytogenes* counts were decreased during ripening of Koozeh cheese under adverse conditions such as high salt concentrations and high temperatures. However, since Iranian Koozeh cheese is made from raw and unpasteurized milk, there are still some concerns about health risk of *L. monocytogenes* in this product. Also, the effects of temperature and salting parameters on the sensorial properties of Koozeh cheese should be investigated in future.

Introduction

One of the most well-known Iranian traditional cheeses is Koozeh, which is typically produced in the Urmia region located in West Azarbaijan province, North-West of Iran (Hassanzadazar et al., 2012). It is a high fat, white brined, and semi-hard cheese with sour and slightly acid taste, pleasant flavour, and crumbly texture (Shahbazi et

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al., 2017). The Koozeh cheese is produced from either raw ewe's or goat's milk or mixtures of ewe's and goat's milk without addition of starter (Edalatian et al., 2012). In traditional way, the milk is not heat-treated, coagulated by rennet obtained from abomasum of lamb and veal within 60 min at 33-34 ºC; when the milk just begin to curdle, it is stored at refrigerated condition for 3-12 months in the maturation period (Hassanzadazar et al., 2012). During the ripening process of chesses, due to functions of non-starter lactic acid bacteria, some physicochemical changes are occurred which may decrease the growth of some pathogens (Edalatian et al., 2012).

The microbiological safety of traditional raw milk cheeses is still one of the major problems in the dairy industries in terms of consumers health and financial losses (Yoon et al., 2016). There are numerous studies involving traditional cheeses manufactured with raw milk in different countries that illustrate the presence and/or survival of important food-borne pathogenic bacteria (Bovo et al., 2015; Jakobsen et al., 2011; Pinto et al., 2009). Raw milk and milk products especially cheese made from unpasteurized milk of sheep and goat are widely considered as an important sources of *L. monocytogenes* contamination and a vehicle of listeriosis (Pinto et al., 2009). The presence of *L. monocytogenes* in raw milk and pasteurized cheeses may be related to numerous parameters such as contaminated raw milk, defective pasteurization, and post processing contamination (Dalzini et al., 2017).

In order to improve the safety, quality, and shelf life of raw milk cheeses, various preservation techniques have been previously evaluated such as addition of chemical or natural additives, including essential oils, plant extracts, organic acids, antifungal peptides, bacteriocins, etc. (Kondrotiene et al., 2018). Using hurdles technology, including temperature, salt concentration, and time of ripening may also serve as potential effective methods to eliminate the pathogens in raw milk cheeses (Al-Holy et al., 2012).

The survival of some important food-borne pathogens in several traditional cheeses under various conditions of ripening has been investigated by several authors (Bovo et al., 2015; Hammer et al., 2017; Hanifian and Khani, 2012). However, based on our knowledge, there is no published data about the survival of *L. monocytogenes* in Iranian traditional Koozeh cheese. Therefore, in this context, the aim of present investigation was to evaluate the fate of *L. monocytogenes* during ripening process of Iranian traditional Koozeh cheese made from raw ewe's milk.

Materials and methods

Materials

Rennet casein was obtained from Meito Sngyo Ltd (Tokoyo, Japan). All culture media were purchased from Merck Company (Darmstadt, Germany).

Strain and culture preparation

L. monocytogenes ATCC19118 standard strain was obtained from the culture collection of the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The strain was maintained in Brain Heart Infusion (BHI) broth containing 25% v/v glycerol at -80 °C. The strain was activated by two consecutive subcultures overnight in BHI broth at 37 °C in three replicates. The overnight culture from the second subculture was diluted to achieve an initial inoculation level of 5 log Colony Forming Unit (CFU)/ml of milk.

Cheese manufacturing

A total of 10 kg Koozeh cheese were produced according to the traditional production specification in this experiment (Figure 1). The raw ewe's milk was obtained from a local farm (Kermanshah, Iran) during spring season in 2017. The cheese milk was examined for the absence of *L. monocytogenes* contamination prior to the cheese preparation. For this purpose, *L. monocytogenes* was isolated according to the protocol of Oliveira et al. (2018). For primary enrichment, samples were transferred into 100 ml of Half-Fraser Broth and incubated at 30 °C for 24 h. For secondary enrichment, 0.1 ml aliquots of the primary enrichment were transferred into 10 ml tubes containing Fraser Broth and were incubated at 30 °C for 24 h. An aliquot of 0.1 ml of the broth showing darkening was streaked on the surface of Agar *Listeria* Ottavani and Agosti (ALOA) plates, followed by incubation at 37 °C for 24 to 48 h. From three to five colonies presumed to be *L. monocytogenes* were streaked onto Tryptone Soya Yeast extract agar and incubated at 37 °C for 24 h. After that, the isolates were subjected to Gram stain, catalase, and oxidase tests. Motility at 25 °C in Sulfide, Indole, Motility (SIM) medium, β-hemolysis on Columbia agar supplemented with 5% defibrinated lamb blood, and fermentation of carbohydrates (xylose, mannitol, and rhamnose) in Bromocresol Purple broth were performed for confirmation. A *L. monocytogenes* culture was added to fresh (pH ~6.6) raw whole ewe's milk after the milking stage and before starting the process of Koozeh cheese manufacturing concentration of 5 log CFU/ml.

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Design of ripening conditions

Ripening of the cheese was carried out over a period of 150 days with sampling every 10 days. A 2-factor experimental design was applied to study the effect of ripening conditions, including three different temperatures (4, 9, and 14 °C) and different concentrations of NaCl (0, 8, 12, and 15%) on the survival of *L. monocytogenes* of the cheese.

Microbiological analysis

In general, for each repeat of experiment, 12 batches containing 1000 g cheese were included in this study. Amount of 25 g of each cheese batch was sampled into a sterile stomacher bag with 225 ml of 0.1% peptone water. The sample was then homogenized in a stomacher. The 10-fold dilutions were prepared for plate count enumeration. The PALCAM *Listeria* Selective agar plates were incubated at 35 °C for 48 h. The microbiological data were expressed as a log CFU/g based on Oliveira et al. (2018).

Statistical analysis

All the experiments were carried out in triplicate. The effects of ripening condition, including temperature, salting, and time on the survival of *L. monocytogenes* were evaluated using SPSS software (version 16.0). The *p* value less than 0.05 were considered statistically significant.

Results

In the present study, *L. monocytogenes* was isolated from none of the used milk samples. The effects of ripening condition are exhibited in Figures 2 to 4, including temperature, salting, and time on the survival of *L. monocytogenes* inoculated to the traditional Koozeh cheese. The results obtained from NaCl treatments demonstrated that the NaCl concentrations of 8, 12, and 15% significantly affect the inactivation of *L. monocytogenes* ($p<0.05$). After inoculation, *L. monocytogenes* populations were reduced most rapidly during the first ten days of storage (~0.5-1.5 log CFU/g) at three mentioned temperatures; after that, the bacteria were continually decreased, being below the detection limit (1 log CFU/g) at the end of ripening. The results presented in this work showed that time of ripening significantly influenced the behaviour of *L. monocytogenes* in Iranian traditional Koozeh cheese ($p<0.05$).

It was found that temperature had significant affect on *L. monocytogenes* counts during ripening period ($p<0.05$). Numbers of *L. monocytogenes* were reduced more effectively at 14 °C storage temperature than 9 and 4 °C ($p<0.05$). Although *L. monocytogenes* numbers were less than amount needed for detection, at the end of period (150 days) at the three studied temperatures, they survived in traditional Koozeh cheese for 120 days at 14 °C and 130 days at both 9 and 4 °C.

Discussion

In Iran, the incidence of *L. monocytogenes* has been reported by some of researchers in different types of milk and dairy products, such as cheese (Akrami-Mohajeri et al., 2018; Jamali et al., 2013; Rahimi et al., 2010; Zamani-Zadeh et al., 2011). To our knowledge, evaluation of potential survival of *L. monocytogenes* has not been evaluated during production and ripening of traditional raw ewe's milk cheeses as well as the influence of ripening conditions on fate of this bacterium in Iranian Koozeh cheese, until now. According to the results of the present study, a significant reduction of the count of *L. monocytogenes* was observed following ripening process of Koozeh cheese. Several factors may contribute to reduction of this pathogen during ripening storage; it could be due to the presence of indigenous lactic acid bacteria, including *Lactococcus lactis* and *Streptococcus thermophilus* (Hanifian and Khani, 2012; Masoud et al., 2012; Navidghasemizad et al., 2009; Ong and Shah, 2009). The progressive production of some compounds is well documented such as bacteriocin, hydrogen peroxide, and volatile compounds by lactic acid bacteria during ripening. Some studies have shown the inhibitory effects of these compounds against of food-borne pathogens (Tamagnini et al., 2005; Tiganitas et al., 2009). On the other hand, the optimal pH range for growth of *L. monocytogenes* is between 6.6 and 7.4; therefore, acidic property would be a key factor in the decrease of survival and growth of *L. monocytogenes* in dairy products such as cheese (Warriner and Namvar, 2009). In a study by Edalatian et al. (2012), the authors showed that the pH reduced from 5.69 in 60^{th} day to 4.65 in 90^{th} day of Iranian Koozeh cheese storage. Hanifian and Khani (2012) revealed that pH reduction at the end of the ripening process could be due to the natural lactic acid bacteria such as mesophilic lactobacilli, thermophilic lactobacilli, and lactococci. The quantity levels of these bacteria were in their maximum levels at the end of ripening period. Therefore, acidic property was associated with a significant decrease of the bacterial count during ripening period of Iranian traditional Koozeh cheese. Previously, some other researchers found that decreasing of pH could inhibit the growth of food-borne pathogenic microorganisms due to generation of acid by lactic acid bacteria present in cheese (Delbes et al., 2006; Öztürkoğlu et al., 2006).

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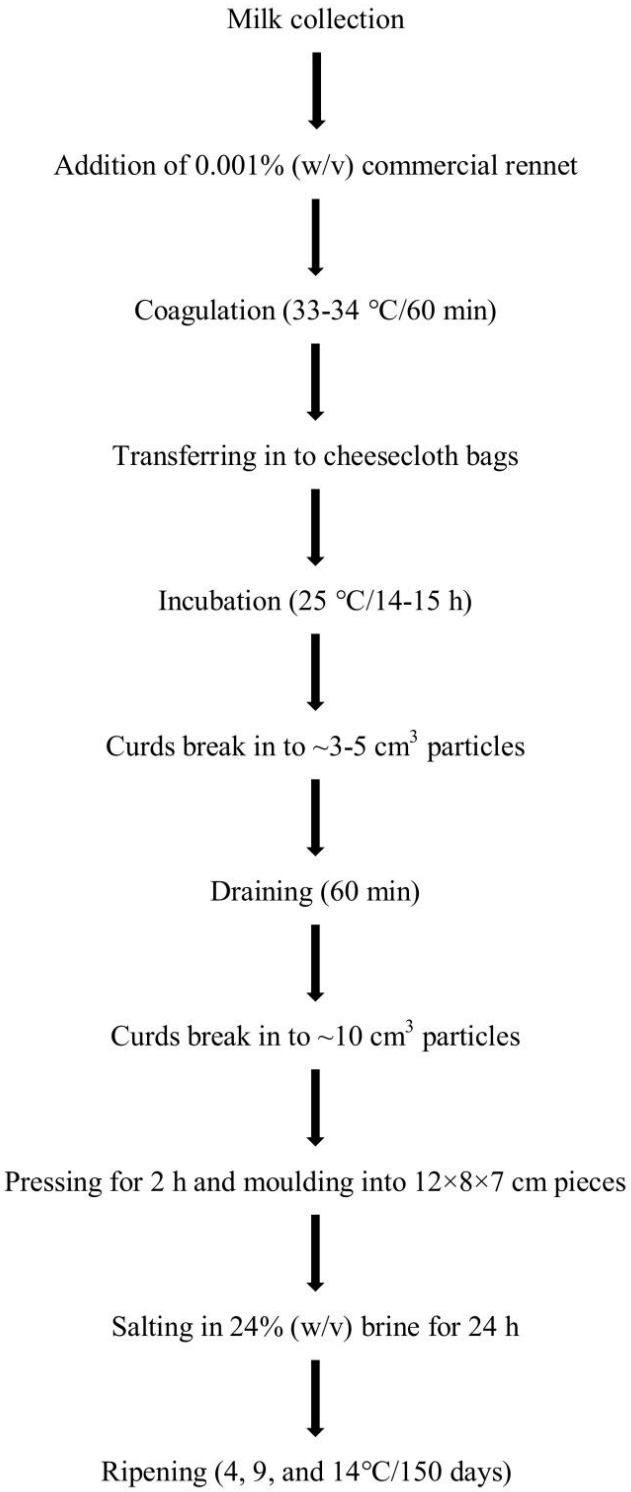

Figure 1: Schematic flowchart of Iranian traditional Koozeh cheese production

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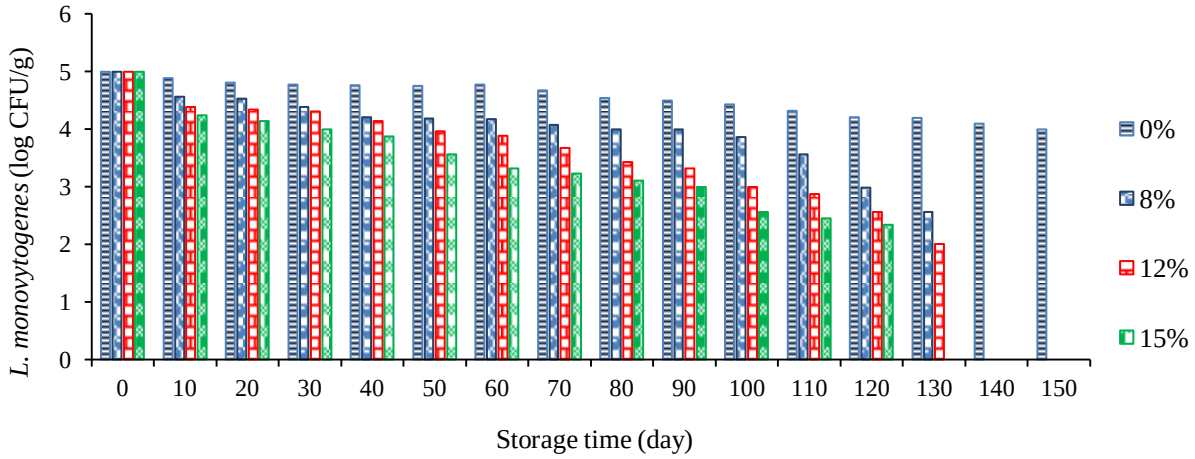

Figure 2: Fate of *Listeria monocytogenes* during ripening of Koozeh cheese containing NaCl (0, 8, 12, and 15%) at 4 °C

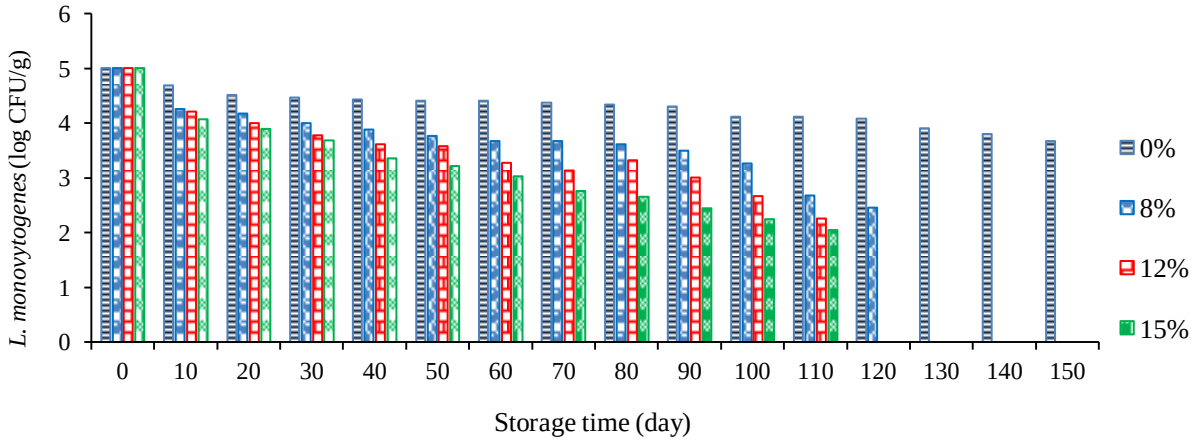

Figure 3: Fate of *Listeria monocytogenes* during ripening of Koozeh cheese containing NaCl (0, 8, 12, and 15%) at 9 °C

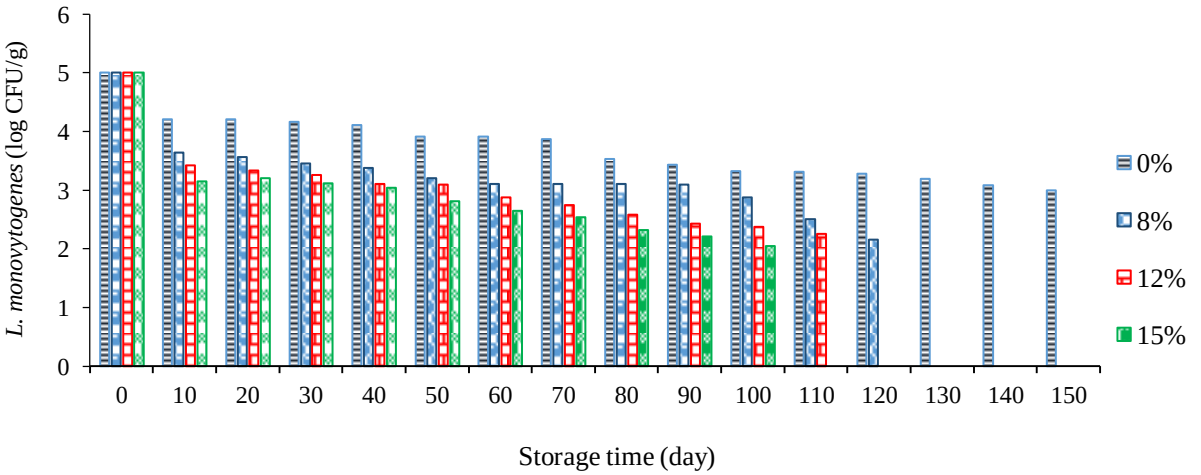

Figure 4: Fate of *Listeria monocytogenes* during ripening of Koozeh cheese containing NaCl (0, 8, 12, and 15%) at 14 °C

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With regards the effect of temperature, we found that 14 °C is more effective than 9 and 4 °C that is in agreement with those achieved by Callon et al. (2011) and also Tamagnini et al. (2005). Lower growth of most pathogens such as *L. monocytogenes* is probably due to alternation of fatty acid components of lipids in cell membrane of bacteria that cause to interfere with membrane fluidity and lead to death of the bacteria (Al-Holy et al., 2012).

In addition to the temperature, the concentration of NaCl could affect on the survival of some pathogens during ripening period (Jay et al., 2005). Our results showed that the number of *L. monocytogenes* reduced drastically by all studied NaCl concentrations (8, 12, and 15%). The observed trends for inactivation of pathogens with increasing of osmotic stress posed by NaCl in the present study are similar with those achieved by Öztürkoğlu et al. (2006), who indicated that the *Listeria innocua* counts were drastically reduced following salt treatment of Turkish white cheese.

Conclusion

The *L. monocytogenes* were declined drastically during ripening days and eliminated at the end of ripening of Koozeh cheese. *L. monocytogenes* counts were decreased during ripening of traditional Koozeh cheese under adverse conditions such as high salt concentrations and high temperatures.

However, since Iranian Koozeh cheese is made from raw and unpasteurized milk, there are still some concerns about health risk of *L. monocytogenes* in this product. Therefore, further studies will be necessary to fully understand the survival of *L. monocytogenes* in cheese that can help in finding effective treatments to complete elimination of this pathogen. Also, the effects of various ripening parameters such as temperatures and salting on the sensorial properties of Koozeh cheese should be investigated in future.

Author contributions

Y.Sh. and N.Sh. designated the study and conducted the experiments. Y.Sh. wrote the manuscript and analyzed the data. Both authors read, revised, and approved the manuscript.

Conflicts of interest

All authors confirmed that there is no conflict of interest in this study.

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