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INFLUENCE OF RIPENING CONDITIONS ON SURVIVAL OF *BRUCELLA MELITENSIS* IN TRADITIONAL LIGHVAN CHEESE (EWE MILK CHEESE)

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ABSTRACT

In developing countries, brucellosis is a reported and in most of cases, consumption of raw milk and traditional cheeses contaminated with *Brucella* spp., especially *B. melitensis*, is the main cause of disease. The aim of this study was to evaluate the effects of ripening conditions (ripening temperatures: 4, 9 and 14°C and salt concentrations: 8, 12 and 15%) on survival of *B. melitensis* in traditional Lighvan cheese (a typical Iranian brine-ripened cheese) manufactured with raw ewe's milk during 150 days of ripening. Results showed that the viable counts of *B. melitensis* changed significantly (p<0.01) as a function of storage temperature. *B. melitensis* survived significantly better at 4°C and 9°C than 14°C (p<0.01). All of salt concentrations (8, 12 or 15% NaCl) significantly (p <0.001) affected the inactivation of pathogen. *B. melitensis* had been completely eliminated at the end of ripening period (150 days). Our findings indicated that the using of hurdle technology (the two limiting factor, namely temperature and salt concentration), is a powerful tool to eliminate *B. melitensis* in Lighvan cheese after at least 5 months of ripening.

1. Introduction

Lighvan cheese is the most popular and commonly consumed Iranian traditional cheese with a long history of manufacturing. It is traditionally manufactured in the Lighvan region located in the province of East Azarbaijan, northern-west of Iran. It is a white brined, semi-hard cheese with sour taste, pleasant and very specific flavor and crumbly texture. The cheese is made from raw ewe's milk or mixtures of ewe and goat milk (70/30). Based on traditional technique, the milk is coagulated by rennet (from abomasum of lambs or kids) and is offered for consumption after undergoing 3-12 months of ripening in brine. During ripening process, some changes in the cheese including physic-chemical changes as well as the production of antagonistic compounds by indigenous microbial flora, organic acid and other compounds, could decrease the growth of pathogens (Hanifian & Khani, 2012).

There are many studies involving traditional cheeses manufactured with raw milk

in different countries that illustrate the presence and/or survival of important pathogenic bacteria such as *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes* and *Escherichia* O157:H7 (Pinto *et al.,* 2009, Jakobsen *et al.,* 2011). The similar pattern of pathogenic bacteria is relevant in Iran, but in our country, raw milk and milk products especially cheese made from unpasteurized milk of sheep and goats is widely recognised as an important source of *Brucella* spp. contamination and a vehicle of brucellosis (Akbarmehr, 2011).

Based on the reports of the Iranian Ministry of Health and Medical Education, brucellosis is a common disease in most regions of Iran (Zamani *et al.,* 2011). Generally, the disease is serious food-borne infection and undulant fever, night sweats, strange odor and severe headache are common symptoms that can observe in patients. Raw milk and cheese manufactured from raw milk of ewe and goats that may harbor *Brucella* spp. (Seleem *et al.,* 2010; Solera, 2010).

Using hurdle technology (temperature, salt concentration and time of ripening) may serve as potential effective methods to eliminate and or reduction of pathogens in raw milk cheeses (Al-Holy *et al.,* 2012). The survival of important food-borne pathogens such as *Yersinia entrocolitica* (Hanifian & Khani, 2012), *Listeria monocytogenes* and *Salmonella typhimurium* (unpublished observations) in Lighvan traditional cheese under various conditions of ripening has been investigated by several authors, but there is little information about the survival of *B. melitensis*. Therefore, in this context, the aim of present work was to evaluate the influence of ripening temperatures (4, 9 or 14°C) and ripening salting (8, 12 and 15%) on survival of *B. melitensis* in traditional Lighvan cheese manufactured with raw milk during 150 days of ripening.

2. Materials and methods

2.1. Materials

Rennet casein was obtained from Meito Sangyo Co., Ltd. (Tokyo, Japan). Brain Heart Infusion (BHI) broth and Peptone Water were used from Merck Company (Darmstadt, Germany). *Brucella* Selective Supplement SR0083, Blood agar, dextrose solution and inactivated horse serum were used to prepare a *Brucella* selective medium. All these media were purchased from Oxoid Co., Ltd. (Hampshire, England).

2.2. Strain and culture preparation

Native *B. melitensis* biovar 1 isolated previously from raw milk was obtained from the Razi Institute for Serums and Vaccines (Tehran, Iran). The strain was maintained in BHI broth containing 25% v/v glycerol at -80°C. For activating, two consecutive subcultures were incubated in BHI Broth at 37°C for 24 h. This work was done in 3 replicates. The overnight culture (from the second subculture) was diluted to achieve an initial inoculation level of approximately 10^5 colony forming units per millilitre (CFU/mL) of milk.

2.3. Cheese manufacturing

Lighvan cheese (8.5 kg) was made with raw milk obtained from Lighvan village, as shown Fig. 1. For this, the cheese milk was examined for the absence of *B. melitensis* contamination prior to the cheese preparation. A *B. melitensis* biovar 1 culture was added to Fresh (pH ~6.6) raw whole ewe's milk after the milking stage and before starting the process of cheese manufacturing concentration of 10^5 colony forming units per millilitre (CFU/mL).

2.4. Ripening conditions

Ripening of the cheese was carried out over a period of 150 days during which samples were taken every 10 days. Two factor experimental design was applied to study the effect of ripening conditions (three temperatures: 4, 9 or 14°C), (three concentrations of NaCl: 8, 12 or 15%) on the survival of *B. melitensis* of the cheese. The experiment was done twice.

2.5. Microbiological analysis

25 grams of each cheese was sampled with 225 mL of 0.1% peptone water in a stomacher bag. The sample was then homogenized in stomacher and diluted it ten-fold serial dilution for plate count enumeration. The plates were incubated at 37 °C for 6 days. After which colonies having the 2-7 mm diameter, are spheroid in shape, moist, slightly opalescent and translucent were counted.

2.6. Statistical analysis

The effects of ripening temperature, salting and time on the survival of *B. melitensis* were evaluated using SPSS (version 16.0) and by Analysis of variance (ANOVA) test. P-value less than 0.05 were considered statistically significant.

3.Results and discussions

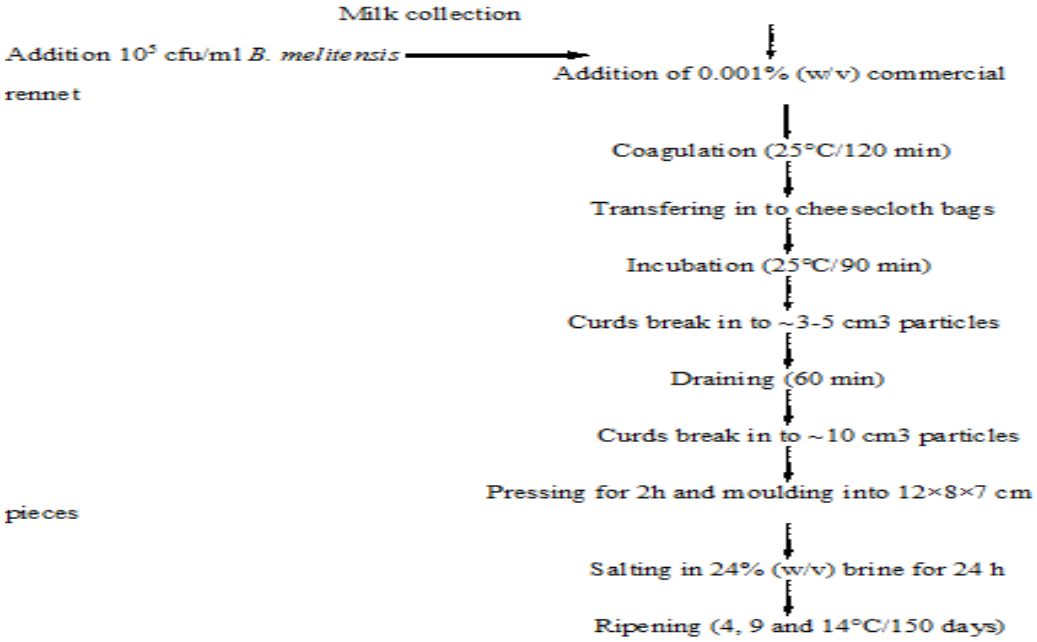

Figure 1. Schematic flowchart of Lighvan cheese production.

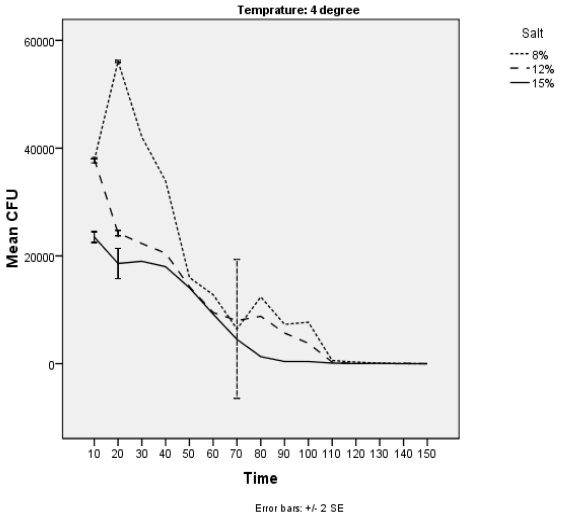

Figure 2. Survival of *B. melitensis* in traditional Lighvan cheese at 4°C during ripening.

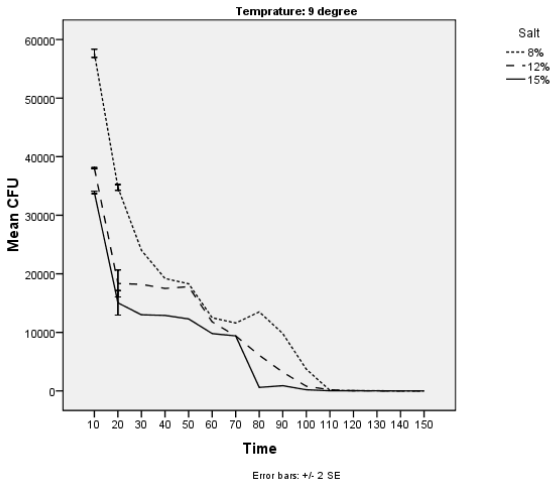

Figure 3. Survival of *B. melitensis* in traditional Lighvan cheese at 9°C during ripening.

Figures 2, 3 and 4 indicated the survival of *B. melitensis* during 150 days of ripening period. *B. melitensis* was not isolated from any of milk samples. In detail, after inoculation, *B. melitensis* populations were reduced most rapidly during the first week of storage (between 34311 (±2624) and 25488 (±2965) log unit) at three temperatures; after that they continually decreased, being below the detection limit (1log CFU/g) at the end of ripening. Temperature had significant effect on *B. melitensis* counts during ripening (Fig 2, 3 and 4). Analysis of variance indicated that numbers of surviving *B. melitensis* differed significantly (p<0.01) with storage temperatures; it was demonstrated that 14°C was more effective than 9°C and 4°C (P<0.01). The mean of the count of *B. melitensis* in traditional Lighvan cheese at 14°C, 9°C and 4°C was 13782, 12794 and 7375 CFU/g, respectively. According to results of Kruskal-Wallis, higher temperature enhanced the sensitivity of *B. melitensis*, suggested that temperature is a crucial factor in decrease of the bacterial populations (P=0.079). Although *B. melitensis* were inhibited at the end of period (150 days) at the three temperatures, they survived in traditional Lighvan cheese for 130 days (14°C) and 140 days (9°C and 4°C). Inhibitory effects of NaCl concentration on the growth of *B. melitensis* are shown in Figures 5, 6 and 7. The results obtained for NaCl concentrations demonstrated that all concentrations (8, 12 and 15% NaCl) significantly (p<0.001) affected the inactivation of pathogen (Fig 5, 6 and 7). Our results indicated that combined effect and interaction of temperature/NaCl significantly (p<0.001) affected the inactivation of pathogen. The results showed that time of ripening significantly influenced (p<0.001) the survival of *B. melitensis* in Lighvan cheese.

In Iran, the incidence of *B. melitensis* in different types of dairy products particularly raw milk and milk products, especially cheese made from raw milk of sheep and goats, has been reported by several of researchers (Akbarmehr, 2011). To our knowledge, evaluation of potential survival of *B. melitensis* during production and ripening of traditional raw ewe's milk cheeses as well as the influence of ripening conditions on the fate of this bacterium in cheese such as Lighvan cheese has never been studied.

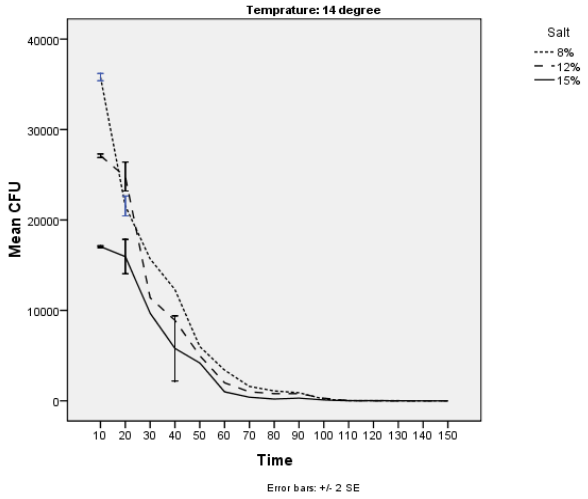

Figure 4. Survival of *B. melitensis* in traditional Lighvan cheese at 14°C during ripening.

According to results of this work, following ripening process, a significant reduction of *B. melitensis* count was observed. Several factors may contribute to reduction of this pathogen during ripening, such as presence of indigenous lactic acid bacteria including *Lactococcus lactis* and *Streptococcus thermophilus* (Hanifian & Khani, 2012, Masoud *et al.,* 2012, Navidghasemizad *et al.,* 2009, Ong *et al.,* 2009). The progressive production of some compounds such as bacteriocin, hydrogen peroxide and volatile compounds by lactic acid bacteria during ripening is well

documented. A number of studies have shown the inhibitory effects of these compounds against food-borne pathogens (Tiganitas *et al.,* 2009, Tamagnini *et al.,* 2008). On the other hand, The pH from 6.6 to 7.4 is the best range for growth and survival of *Brucella* spp., therefore, acidic property (pH) would be a key factor in decrease of survival and growth of *Brucella* spp. in dairy products such as cheese (Ozturkoglu *et al.,* 2005, Zúñiga *et al.,* 2005, Delbes *et al.,* 2006; Falenski *et al.,* 2011). A recent study by Aminifar *et al.* (2010) showed that the pH reduced from 60 to 90 days of Lighvan cheese storage on average from 5.69 to 4.65. Hanifian & Khani (2012), revealed that pH reduction at the end of 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 Lighvan cheese.

This study demonstrated that *B. melitensis* could survive 130 days (at 4 and 9°C) and 140 days (at 14°C) in traditional Lighvan cheese and this survival are regarded as a serious risk for consumer health. Based on our results, it seems that one of the main methods for complete elimination of *B. melitensis* is brining of Lighvan cheese for a long ripening period, at least 5 months.

With regards to the effect of temperature, it was found that 14°C is more effective than 9°C and 4°C (p<0.01). This result is in agreement with those achieved by Ingham *et al.* (2000), Tamagnini *et al.* (2005) and Callon *et al* (2011). Lower growth of most pathogens such as *B. melitensis* at low temperatures is due to alteration of fatty acid components in cell membrane of bacteria that interfere with membrane fluidity and lead to their death (Al-Holy *et al.,* 2012).

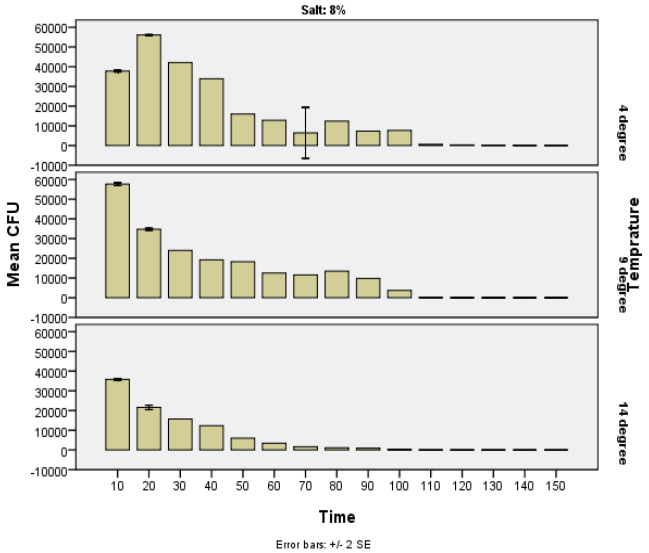

Figure 5. Effect of salt treatment (8%) on the survival of *B. melitensis* in traditional Lighvan cheese at 4, 9 and 14°C during ripening.

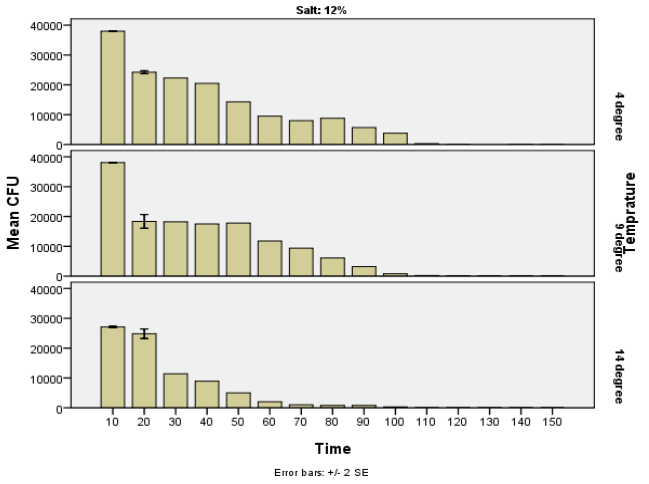

Figure 6. Effect of salt treatment (12%) on the survival of *B. melitensis* in traditional Lighvan cheese at 4, 9 and 14°C during ripening.

In addition to the temperature, the concentration of NaCl could affect the survival of pathogens during ripening period. Therefore, in the present study, effects of ripening salting (8, 12 or 15%) on

survival of B. melitensis also were evaluated. The results showed that the number of B. melitensis reduced drastically by all of concentrations (8, 12 and 15% NaCl) (p<0.001). In addition, Aminifar *et al.* (2010) showed that the salt concentration was increased (approximately 3%) during ripening of Lighvan cheese.

The observed trends for inactivation of pathogens with increasing of osmotic stress posed by NaCl in the present study are in agreement with those achieved by Ozturkoglu *et al.* (2006) on *Listeria innocua* during manufacture and storage of Turkish White Cheese in which the authors indicated the number of *listeria* cells drastically reduced following salt treatment.

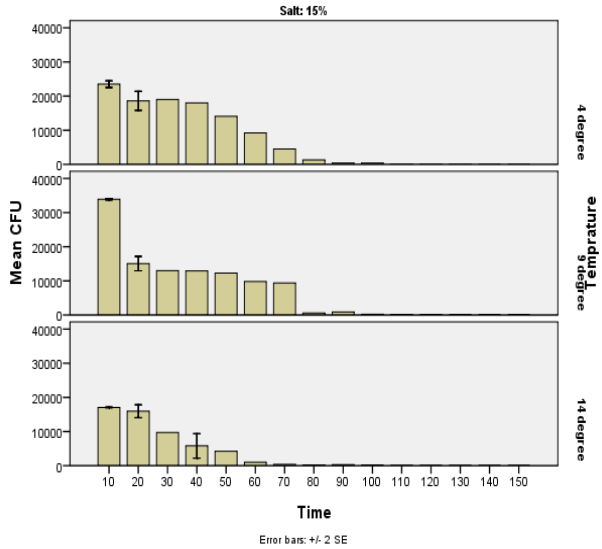

Figure 7. Effect of salt treatment (15%) on the survival of *B. melitensis* in traditional Lighvan cheese at 4, 9 and 14°C during ripening.

4. Conclusions

It can be concluded that the number of *B. melitensis* cells declined drastically during ripening days and eliminated at the end of ripening, but survival of *B. melitensis* during ripening under adverse conditions such as high salt concentrations and high temperatures along with high contamination of raw milk and consequently traditional Lighvan cheese with mentioned bacteria could be a risk for consumer health. Therefore, additional studies are needed to develop rapid methods for detection of *B. melitensis* in such products. In addition, molecular studies are required to fully understand the survival of *B. melitensis* in order to complete elimination of this pathogen. Further investigations are also required to assess the effects of ripening temperatures and ripening salting on sensorial properties of Lighvan cheese.

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