MEDWELL PUBLISHING
Journal of Animal and Veterinary Advances 8 (6): 1124-1130, 2009
ISSN: 1680-5593

Survival and Acid Adaptation Ability of *Salmonella* during Processing and Ripening of *Savak tulumi* Cheese

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Abstract: The present study was undertaken to investigate, the effect of production conditions of *Savak tulumi* cheese, a type of naturally fermented, semi-solid cheese made from raw ewe milk, on the survival and acid adaptation ability of *Salmonella*. Raw ewe milk was inoculated with a 5 strain mixture of *Salmonella* at a level of approximately 7.0 $\log_{10}$ cfu mL^{-1} prior to rennetting. Processing steps included cutting fresh curd into small pieces, pressing, breaking up and mixing with salt then holding at 25°C for 3 days. Next, the resulting cheese was filled in to plastic containers and stored at 6°C for 90 days for ripening. The cheese samples were taken on days 0, 1 and 2 during processing and on days 0, 15, 30, 45, 60 and 90 during ripening for chemical, microbiological analysis and acid challenge tests in synthetic gastric fluid. The results indicated that pH of the curd decreased to 4.65±0.11 by day 90 and the titratable acidity increased to 1.98±0.09 la. The numbers of *Salmonella* decreased significantly as of 2nd of processing and during ripening and finally dropped below detection limit (<1.0 $\log_{10}$ cfu g^{-1}) by day 90. In acid challenge test, in none of the sampling days, *Salmonella* did not survive to 90 min exposure. These results revealed that *Savak tulumi* cheese production yields >7.0 log reduction of *Salmonella* and the resulting intrinsic environment during production do not allow the pathogen to develop any resistance to the acid.

Key words: *Salmonella*, tulumi cheese, ripening, acid resistance, synthetic gastric fluid

INTRODUCTION

Savak tulumi cheese is one of the traditional Turkish tulumi cheese types made from raw ewe milk. The cheese is semisolid, naturally fermented by indigenous microorganisms and ripened at low temperatures. There is no heat treatment involved in production. The cheese is mostly produced in the eastern parts of Turkey where sheep breeding is more common. *Savak tulumi* is produced by small scale processing plants in the region and production is more of an art than a science. Food safety concerns are high due to lack of pasteurization, lack of use of starter culture and poor hygienic practices. Several studies reported the presence of pathogens including *Salmonella*, *Listeria monocytogenes* and *Brucella* sp. in commercial products (Colak *et al*., 2007; Patir *et al*., 2000). Ironically, the cheese remains popular among consumers as a traditional cheese. Turkish Standard (TS 3001) requires a minimum of 90 day ripening for fermented dairy products if made from raw milk (TS 3001, 2006).

During production of a ripened foods, changes in aw, pH, acidity and oxidation-reduction potential of the milk moves from optimal to sublethal/lethal zones for survival of bacteria as the ripening progress (Gahan and Hill, 2003). Such changes are strongly related to the formation of desired characteristics of the cheese as well as setting antimicrobial barriers in the food. However, it is possible that sublethal environments can give bacteria a chance to activate their adaptation mechanisms to survive. For instance, it has been reported that gradual decrease in pH and slow increase in acidity of cheese during production resulted in formation of a sublethal environment in which *L. monocytogenes* activated its acid tolerance system (Faleiro *et al*., 2003). It can be hypothesized that if pathogen bacteria are exposed to sublethal environment for sufficient time, bacteria can develop resistance to the stress factors or barriers in the food. In many case, such resistance can provide pathogens cross-protection against multiple barriers (Faleiro *et al*., 2003; Murphy *et al*., 2003). These bacteria can survive the lethal acidity of the stomach during pass-through so that can cause infection even at low numbers (Jonge *et al*., 2003). Foodborne pathogens are subjected to organic and inorganic acid exposure during not only pass through the stomach, but also during colonization to small intestine

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and within the phagozomes of host cells (Gahan *et al*., 1996; Humphrey *et al*., 1996). Likelihood of a resistant pathogen to survive all these host defense systems is higher compared to nonresistant pathogen. Induction of acid resistance in laboratory medium has been demonstrated in many studies for *Salmonella*, *L. monocytogenes* and *E. coli* O157: H7 (Alvarez *et al*., 2009; Foster and Hall, 1991; Koutsoumanis and Sofos, 2004; Lin *et al*., 1995; O'Driscoll *et al*., 1996; Tivari *et al*., 2004). Furthermore, there is some evidence that acid resistant *Salmonella* enteritidis was found more virulent compared to non adapted cells (14). However, the data if such resistance may develop during food production is limited (Cataldo *et al*., 2007; Faleiro *et al*., 2003; Gahan *et al*., 1996; Hsin-Yi and Chou, 2001; Tosun *et al*., 2007; Yousef and Courtney, 2003). *Savak tulumi* cheese is a good model to study stress resistance of pathogens because the production does not involve in any lethal step such as heat treatment and sublethal stress factors may occur during ripening. Therefore, the objective of the current study, was to investigate the survival of *Salmonella* and its adaptation ability to acid conditions in Savak cheese during processing and ripening.

MATERIALS AND METHODS

***Salmonella* strains:** The strains used to inoculate the raw milk were 3 *Salmonella* Enteritidis strains (RSKK 96046, RSKK 91, RSKK 92) and 2 *Salmonella* Typhimurium strains (RSKK 1017, ATCC 14028). The strains were obtained from Institute of Refik Saydam Hifzi Sihha, a National Microbiology Laboratory of Turkey and from American Type Culture Collection.

Preparation of inoculum: The *Salmonella* cultures were stored at -70°C before use. After thawing, each strains were grown separately in 10 mL Tryptic Soy Broth (TSB, LABM-Lancashire, UK) at 35°C overnight. The cultures were passaged in TSB three times. Th final cultures were centrifuged (Nüve NF 800R, Ankara, Turkey) at 4200 rpm for 5 min. The supernatant were removed and the pellets were resuspended and washed with 10 mL sterile 0.9% NaCl before recentrifuging to remove organic residues. The resulting pellets were resuspended using sterile normal saline and all strains were combined in a single tube. The level of the pathogen in the final cocktail was 9.56±0.11 log cfu mL^{-1}.

Production of *Savak tulumi* cheese: The ewe milk was obtained from a local producer. The milk was transferred from farm to the laboratory within 30-40 min after morning milking. Fifteen liters of milk was used for each of three replicates. Approximately, 3.5 kg of tulumi cheese was produced from 15 L milk.

A 500 mL portion of the raw milk was separated and kept at 4°C for chemical and microbiological analysis. The raw milk was then warmed to 32°C and inoculated with *Salmonella cocktail*. Holding for 30 min, rennet was added to the inoculated milk for coagulation within 20 min. The resulting coagulum was cut (5×5 cm) and kept at ambient temperature (ca. 25°C) for 10 min. The curd was divided into 3 portions and each portion was transferred to whey cloths and hanged at ambient temperature for 4 h to remove the excessive whey. Next, the curd portions were piled on top of each other and pressed using a 4 kg metal weight and kept at ambient temperature for 20 h (Processing day 1). On the 2nd day of processing, the curd was broken up to the size of a chickpea by hand. The resulting curd was mixed with 2.5% (w w^{-1}) salt, transferred to whet clothes again, kept under pres using 5 kg weight for 24 h at ambient temperature. On the 3rd day of processing, the curds were filled into plastic containers tightly to remove air as much possible; lips of the containers were covered with stretch film and closed. The containers were stored at 6°C for 90 days for ripening of the *Savak tulumi* cheese. This day were counted as day 0 of ripening.

Analyses: Sampling intervals and analyses performed are presented in Table 1.

Microbiological analyses: On sampling days, 2 of 25 g portions of cheese were taken under aseptic conditions and transferred into sterile stomacher bags. A 225 mL of 0.1% sterile peptone water (LABM, Lancashire, UK) was added prior to pummeling for 2 min (Stomacher 400). The resulting 10^{-1} dilution was serially diluted up to 10^{-8} and double plated onto agar plates.

The sample dilutions were surface plated on to serial XLD agar (LABM) and incubated at 35°C for 24 h. Typical *Salmonella* colonies were counted from appropriate plates. Three colonies were selected randomly to confirm *Salmonella* using polyvalent *Salmonella* latex agglutination test (Eurobio, 7. Av de Scandinavie- F 91953 Les Ulis Cedex B, France) (2). On days, when numbers of *Salmonella* were expected to drop below detection limit (<1.0 $\log_{10}$ cfu g^{-1}), enrichment procedure were used. The sample were homogenized in 0.1% buffered peptone water and incubated at 35°C for 24 h. A 0, 1 mL of the resulting mixture then was inoculated to 9.9 mL Rappaport-Vasilidas broth (Oxoid) tube and 1.0-9 mL Tetrathionate broth (iodine solution added) (Merck) tube. Both tubes were incubated at 42°C for 24 h and streak plated onto XLD plates. The plates were incubated at 35°C for 24 h. Typical colonies were subjected to agglutination test (Andrews and Hammack, 2003).

Table 1: Sampling intervals during processing and ripening of Savak tulumi cheese and the analyses performed

	Microbiological analyses		Acid challenge (SGF)	Other analyses						
Sampling intervals	*Salmonella* enumeration	Other microbiological analyses[b]	*Salmonella* enumeration	pH	TA	Moisture	Salt	a_w	Fat	Protein
Raw milk	-	+[c]	-	+	+	-	-	-	+	-
After inoculation	+	-	-		-	-	-	-	-	-
Processing										
Day 1	+	+	-	+	+	+	-	-	-	-
Processing day 2	+	+	+	+	+	+	+	+	-	-
0	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+
45	+	+	+	+	+	+	+	+	+	+
60	+[a]	+	+	+	+	+	+	+	+	+
90	+[a]	+	+	+	+	+	+	+	+	+

a : Enrichment was performed; b: Includes enumeration of *Lactobacillus* sp., *Lactococcus* sp., total aerobic mesophile bacteria, aerob psychrophile bacteria *Enterococcus* sp., yeast, *staphylococcus* sp.; c: Coliforms were enumerated in raw milk only

The samples were surface plated onto MRS agar (LABM) for enumeration of *Lactobacillus* sp. (30°C for 72 h), onto M17 agar (LABM) for enumeration of *Lactococcus* sp. (22°C for 72 h), onto Kanamycin Esculin Azide agar (KEAA, LABM) for enumeration of *Enterococcus* sp. (37°C for 48 h), onto Baird-Parker agar (Merck) for enumeration of *Staphylococcus* sp. (35°C for 24 h), onto Rose Bengal Chloramphenicol agar (LABM) for enumeration of yeast (22°C for 5 days) (Rogga *et al*., 2005).

Numbers of total aerobic mesophilic bacteria were determined pour plating on Plate Count Agar (PCA, LABM) and incubating at 25°C for 48-72 h, total aerobic psychrophile bacteria were determined by pour plating onto PCA and incubating at 7°C for 7 days. Numbers of coliforms were determined in raw milk only by double layer pour plating on VRB agar (LABM) and incubating at 35°C for 24 h (Pichhardt, 2004).

Acid challenge tests in synthetic gastric fluid: Synthetic gastric fluid was prepared as described by Molly *et al.* (1994). Briefly, 8.3 g proteose peptone (*E. merck*, Darmstadt, Germany), 3.5 g d-glucose (dextrose, anhydrous) (LABM, Lancashire, UK), 2.05 g sodium chloride (*E. merck*, Darmstadt, Germany), 0.6 g potassium phosphate (monobasic, anhydrous) (*E. merck*, Darmstadt, Germany), 0.11 g calcium chloride (dehydrated) (*E. Merck*, Darmstadt, Germany), 0.37 g potassium chloride (*E. merck*, Darmstadt, Germany), 0.1 g lizozim (egg white origin, crystallize) (Fluka, D-89552 Steinheim, Germany), 50 mg bile salt (cattle origin) (Fluka, D-89552 Steinheim, Germany) and 13.3 mg pepsin (Pepsin A, swine origin) (Sigma, D-89552 Steinheim, Germany) were solved in 1 l distilled water and adjusted to pH 1.0 using 1.0 N HCl (*E. merck*, Darmstadt, Germany).

Triplicate samples of 10 g each was used in the tests. The samples were added with 30 mL of sterile 0.9% NaCl and homogenized for 3 min using a stomacher resulting in a ¼ dilution. An aliquot of 1 mL were separated for enumeration of *Salmonella* for 0 min. Next, 40 mL synthetic gastric fluid (pH adjusted to 1.0 with HCl) was added to the homogenate, mixed by shaking and incubated at 37°C. One mL samples were taken at 30, 60 and 90 min intervals for enumeration of *Salmonella*. Due to buffering effect of the cheese, pH of the SGF increased up to 2.0, depending on the sampling day. Those exceeding pH 2.0 were not evaluated and the test was repeated with another sample.

Other analyses: pH of the samples were determined using a digital pH m (Selecta pH 2001, Spain). Fat level in raw milk and cheese samples were determined using procedures by Turkish Standards Institute TS 8189 (TS 8189, 1990) and TS 3046 (TS 3046, 1978), respectively. Water activity was determined using an a_w m (Testo 650, USA). Titratable acidity, protein content, moisture level and salt level were determined using AOAC procedures (AOAC, 1990).

Statistical analysis: The numbers of bacteria were converted to $\log_{10}$ cfu g^{-1}. The data were subjected to Analysis of Variance (ANOVA) appropriate to replicate x subsamples x time to determine fixed effects and interactions between variables. The means were separated using Fisher's Least Square Differences method according to the General Linear Models (GLM). Statistical significance level was accepted as 0.05. Data obtained from acid challenge in SGF were compared between sampling days and exposure time. Statistical analyses were performed using Statistical Analysis System Software (SAS, 1999).

RESULTS

The effect of processing and ripening time was found significant ($p<0.001$) on the survival of *Salmonella*. The initial level of *Salmonella* in the raw milk was 6.63 $\log_{10}$ cfu g^{-1}. After the milk was converted to curd

Table 2: Mean numbers of microorganisms during processing and ripening of Savak cheese made from raw sheep milk inoculated with *Salmonella* (log_{10} cfu g^{-1}±SD) (n:6)

	Processing			Ripening (days)					
Microorganisms	Raw milk*	Day 1	Day 2	0	15	30	45	60	90
Salmonella	6.63±0.32^{ab}	7.95±0.24^{a}	3.82±1.12^{bc}	5.29±1.56^{b}	2.24±0.95^{cd}	2.54±1.32^{c}	1.03±0.20^{d}	1.02±0.02^{d}	<1.0
Enterococcus sp.	5.68±0.24^{b}	6.66±0.24^{a}	6.78±0.31^{a}	6.65±0.37^{a}	6.61±0.36^{a}	6.42±0.32^{a}	6.38±0.26^{a}	6.58±0.23^{a}	6.50±0.19^{a}
Lactococcus sp.	7.60±0.38^{c}	9.47±0.30^{a}	9.49±0.07^{a}	9.21±0.20^{ab}	9.25±0.38^{ab}	8.53±0.18^{b}	8.67±0.28^{b}	8.70±0.19^{b}	8.90±0.24^{b}
Lactobacillus sp.	7.39±0.54^{c}	9.01±0.12^{ab}	9.55±0.06^{a}	9.00±0.20^{ab}	8.59±0.45^{b}	8.41±0.35^{b}	8.45±0.31^{b}	8.56±0.42^{b}	8.42±0.13^{b}
Yeast	2.04±0.24^{c}	3.98±0.95^{b}	5.37±0.32^{a}	5.83±1.36^{a}	5.13±0.57^{ab}	6.08±0.95^{a}	5.61±0.92^{ab}	5.14±0.82^{ab}	5.83±0.19^{a}
Staphylococcus sp.	5.61±0.24^{ab}	6.23±0.34^{a}	5.13±0.28^{b}	5.87±0.44^{ab}	4.10±0.60^{c}	4.28±0.73^{bc}	4.34±0.73^{bc}	4.04±0.18^{c}	4.36±0.33^{bc}
Total mesophile aerobic bacteria	8.29±0.19^{c}	9.28±0.09^{b}	10.2±0.54^{a}	9.10±0.15^{bc}	8.46±0.29^{c}	8.40±0.24^{c}	8.49±0.25^{c}	8.65±0.36^{c}	8.61±0.40^{c}
Psychrophile bacteria	7.04±0.08^{b}	7.69±0.45^{ab}	7.74±0.29^{ab}	8.13±0.65^{a}	6.28±0.41^{b}	6.42±0.47^{b}	6.44±0.35^{b}	6.68±0.29^{b}	6.65±0.31^{b}

*Level of pathogen was determined after inoculation; a,b,c,d: Means within a row lacking a common superscript letter are different ($p<0.05$)

Table 3: Changes in chemical parameters occurred during processing and ripening of Savak cheese made from raw sheep milk inoculated with *Salmonella* (n:6)

	Processing			Ripening (days)					
Parameter	Raw milk	Day 1	Day 2	0	15	30	45	60	90
pH	6.51±0.45^{a}	4.73±0.21^{b}	4.57±0.13^{b}	4.60±0.12^{b}	4.72±0.06^{b}	4.70±0.12^{b}	4.68±0.17^{b}	4.75±0.13^{b}	4.65±0.11^{b}
Acidity (% la)	0.17±0.01^{c}	1.05±0.03^{b}	1.39±0.26^{b}	1.72±0.07^{ab}	1.8 1±0.24^{ab}	1.82±0.21^{ab}	1.85±0.13^{ab}	1.93±0.25^{a}	1.98±0.09^{a}
Moisture (%)	nt	58.95±3.4^{a}	46.65±1.79^{b}	42.63±3.45^{b}	44.96±2.49^{b}	43.03±2.55^{b}	42.14±2.27^{b}	44.33±3.38^{b}	43.65±3.05^{b}
a_w	nt	nt	0.92±0.005^{a}	0.916±0.006^{ab}	0.908±0.005^{b}	0.899±0.001^{b}	0.904±0.004^{b}	0.901±0.003^{b}	0.902±0.006^{b}
Salt (%)	nt	nt	nt	2.88±0.13^{c}	3.12±0.14^{bc}	3.51±0.01^{b}	3.67±0.27^{ab}	3.89±0.13^{ab}	4.05±0.14^{a}
Fat (%)	nt	nt	nt	32.33±3.51^{b}	34.00±1.73^{b}	36.67±0.58^{b}	37.00±2.64^{ab}	40.33±0.58^{ab}	42.33±0.58^{a}
Protein (%)	nt	nt	nt	22.87±0.83^{b}	23.3±0.87^{ab}	24.70±0.17^{ab}	24.13±1.68^{ab}	25.33±0.96^{ab}	25.77±0.50^{a}

a,b,c,d: Means within a row lacking a common superscript letter are different ($p<0.05$); nt: not tested

and processed, the level was increased to 7.95 log_{10} cfu g^{-1} on the 1st day probably due to loss of whey. As shown in Table 2, however, on the second day of processing, the numbers of *Salmonella* significantly ($p<0.001$) decreased to 3.82±1.12 log_{10} cfu g^{-1}. On day 1 of ripening, the pathogen numbers were found 5.29±1.56. The difference in numbers between day 2 of processing and day 0 of ripening was not significant probably due to large variation. During ripening, numbers of the pathogen continuously decreased. On day 60 of ripening, the numbers of *Salmonella* dropped below detection limit (<1.0 lof_{10} cfu g^{-1}) (Table 2). After enrichment, no viable cells were recovered in 2 trials while survivors were detected in 1 trial. However, all samples on 90 day were found negative for viable *Salmonella* after enrichment.

pH of the curd significantly decreased to 4.72 on the 1st day of processing ($p<0.05$) (Table 3). However, no significant ($p>0.05$) change was observed in pH during the remaining part of processing or ripening. By the end of ripening period, the final pH was 4.65. Similarly, total acidity increased to 1.05% la at the beginning of processing and showed gradual but not significant ($p>0.05$) change until day 60. The final level of acidity on day 90 was 1.98% la which was significantly ($p<0.05$) higher than the acidity of processing day 1. Significant changes was also observed in moisture, salt, protein, fat and water activity values during as the production period progressed (Table 3).

Acid challenge data are presented in Table 4. Effect of sampling day and exposure time on the survival of the pathogen was significant ($p<0.05$). Beginning from the 1st day of processing, *Salmonella* did not survive (<1.0 lg_{10} cfu mL^{-1}) 90 min exposure to SGF during the processing and ripening. The longest survival which was 60 min was observed only on the 2nd day of processing.

Table 4: Survival of *Salmonella* in synthetic gastric fluid in savak tulum cheese during processing and ripening (log_{10} cfu mL^{-1}±SD) (n: 9)

	Exposure time to SGF (min)			
Sampling interval	0*	30**	60**	90**
Ripening period				
Processing day 2	4.11±1.22^{az}	1.52±1.08^{bz}	1.18±0.52^{b}	<0.9
Day 0	3.57±1.10^{az}	1.05±0.31^{bz}	<0.9	<0.9
Day 15	2.05±1.30^{ay}	0.90±0.02^{bz}	<0.9	<0.9
Day 30	1.85±1.07^{ayx}	<0.9	<0.9	<0.9
Day 45	1.25±0.96^{ax}	<0.9	<0.9	<0.9
Day 60	0.63±0.04^{ax}	<0.9	<0.9	<0.9
Day 90	<0.6	<0.9	<0.9	<0.9

a,b,c,d: Means within a row lacking a common superscript letter are different ($p<0.05$); x,y,z: Means within a column lacking a common superscript letter are different ($p<0.05$); *Calculated by decimal dilutions of 1/4 initial dilution; **Calculated by decimal dilutions of 1/8 initial dilution

DISCUSSION

It has been reported that nonacid adapted cells gain adaptation/resistance to acid conditions during fermentation or in acidic foods (Gahanand Hill, 2003; Hsin-Yi and Chou, 2001; Tosun *et al*., 2007) because they survive equal to or longer than acid adapted cells. It has been our hypothesis that nonadapted *Salmonella* could activate its tolerance mechanisms and survive throughout the ripening. Even if the numbers of pathogen decreased

to certain extend, the survivors could be more resistant to acid challenge. However, in the current study, none of these occurred. The numbers of *Salmonella* dropped below detection limit (<1.0 $\log_{10}$ cfu g^{-1}) by day 90 of ripening. This finding indicates that although made from raw milk, production of raw milk yields at least 7.0 $\log_{10}$ reduction in numbers of the pathogen. Although, *Salmonella* is known as relatively resistant to hard conditions such as dry conditions (Christian, 2000), rapid decrease of the numbers of the pathogen during processing and ripening can be explained by significant changes occurred in pH, acidity and aw within the first 2 days of production. In addition, as seen in Table 4, the microbial flora was predominated by lactic acid bacteria including *Lactobacillius* sp., *Lactococcus* sp. and *Enterococcus* sp., resulting in an increased competition in favor of lactic bacteria. All of these changes might cause *Salmonella* to metabolically exhaust and die by time (Frank and Marth, 1988; Gahan and Hill, 2003; Hsin-Yi and Chou, 2001; Leistner, 2000; Rogga *et al*., 2005). However, Colak *et al*. (2007) reported that *Salmonella* was present in 6 of 250 *Savak tulum* cheese samples collected from retail stores. One reason for this could be that some producers may not be ripening the Savak cheese for 90 days as required by Turkish Standard (TS 3001, 2006) if the market demand for the product is high.

Sublethal conditions can be defined as those conditions that do not allow bacteria to grow but do not kill immediately either like heat does. It has been known that when exposed to sublethal conditions, bacteria express a variety of stress shock proteins and become more resistant to extreme conditions (Foster, 1999). In another word, stress resistance is an induced defense system for bacteria to survive against unfavorable conditions. In the current study, *Salmonella* was inoculated to the raw milk without a prior adaptation step. *Salmonella* did not survive to 90 min exposure to SGF (pH 1.5-2.0) during production and ripening (Table 4). The longest survival in SGF was observed on the 2nd day of production when the numbers of the pathogen in cheese were the highest. Initial population density is expected to increase the survival time. In a comparative study, the highest resistance of *Salmonella* was induced after exposing the pathogen cell to pH 4.5 (Koutsoumanis and Sofos, 2004). The pH of the *Savak tulumi* was close to this inductive pH. However, findings of the current study indicated that there was no evidence that production and ripening conditions of the *Savak tulumi* cheese resulted in an increase in resistance of the pathogen to SGF exposure. This can be explained by several factors. First, pH of Savak cheese did not decrease gradually during the ripening but decreased rapidly in the first day of production and remained almost the same during the ripening. Such decrease, in combination with other factors including decrease in water activity and domination of lactic acid bacteria in the flora could not have given a chance to *Salmonella* to activate its tolerance mechanism effectively. Secondly, in most of the previous experiments (Alvarez *et al*., 2009; Berk *et al*., 2005; Koutsoumanis and Sofos, 2004; Maurer and Lee, 2005; Tivari *et al*., 2004) demonstrating acid adaptation, pathogen bacteria were exposed to a fixed pH for a certain period of time, which was no longer than 24 h and then subjected those pathogens to lethal pH. However, in the current study, *Salmonella* were in a continuously changing environment for extended period of time. This could have confused *Salmonella* cells to perceive the cheese environment accurately resulting in failure in expressing the gene sets necessary for protection. A third explanation could be differences in the methods of measurement of acid resistance used in the literature. The variation in challenge media and pH used in previous studies yields contradictory results. For instance, *Salmonella* were challenged to pH 1.5 (Yuk and Schneider, 2006), pH 2.5 (Berk *et al*., 2005), pH 3 (Alvarez *et al*., 2009) and pH 3.3 (Tivari *et al*., 2004) in laboratory media, SGF or juice. In the present study, samples were directly exposed to SGF simulating the digestion of contaminated food. If the samples were challenged at a higher pH such as at 3.5, *Salmonella* cells could have responded differently. It is our thought that use of SGF for evaluating the acid resistance level of a pathogen is more realistic. However, it should underlined that buffering capacity of cheese increased as the proteolysis progressed during ripening and final pH of the SGF increased up to pH 2.0.

Response of foodborne pathogenic bacteria against various stress types, particularly to acid, has been widely studied (Bunnig *et al*., 1990; Dykes and Morrhead, 2000; Wang and Doyle, 1998). Majority of the studies were performed in laboratory media modified by acidifying, or adding relatively high levels of salt. Heat stress was also applied by heating bacteria in culture media. Although, such experiments are essential for understanding the behavior of pathogens under stress, or understanding the genetic mechanism of the response given by bacteria, they are not sufficiently simulating the food environment, especially the fermented foods. One reason for that is food environment may harbor more stress factors than any laboratory medium made stressful for bacteria. In several studies, survival and/or acid tolerance of acid adapted and nonadapted foodborne pathogenic bacteria were investigated during the production and storage of various foods were compared (Catalgo *et al*., 2007; Sing *et al*., 2006; Tosun *et al*., 2007). There are some

conflicting results reported, as it might be expected because severity of stress factors varies from food to food. In addition, tolerances of bacteria also vary greatly from species to species.

CONCLUSION

Results of the present study indicate that conditions of *Savak tulumi* cheese production described herein do not allow *Salmonella* to survive. The changes in the intrinsic factors occurring during cheese production act as if natural refinement system to eliminate *Salmonella* by the end of ripening. Although, exposed to an ideal pH for activating acid resistance mechanism (Koutsoumanis and Sofos, 2004) in the cheese, no indication of increased resistance of *Salmonella* to acid was observed as measured by SGF. Microbial risks associated with the occurrence of acid resistant bacteria during food production should be evaluated based on data produced from food inoculation studies. *Savak tulum* cheese was used in the present study as a model food only. Our results do not support the idea that cheese made from raw milk is safe to consume because other pathogenic bacteria might behave differently under the conditions of production of *Savak tulumi* cheese.

ACKNOWLEDGEMENTS

The present study was supported by TUBITAK (project number: 106O330). The authors thank Dr. Pinar Þeker for assistance during cheese production and Kaplan Gida for providing raw sheep milk.

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