Study on the growth and survival of *Escherichia coli* O157:H7 during the manufacture and storage of Iranian white cheese in brine

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Summary

The behaviour of *Escherichia coli* O157:H7 was studied during the manufacture and storage of Iranian white cheese in brine. Cheese was manufactured using pasteurized cow milk and inoculated with *E. coli* O157:H7 with inoculum level of 10^3 cfu/ml. Four treatments were designed. Cheeses were made with or without starter culture and kept immersed in 6 or 8% salt brine during ripening and storage. Cheese samples were analysed for *E. coli* O157:H7 during manufacture and storage period. During cheese manufacture the number of *E. coli* O157:H7 increased by 10^6 cfu/g, but during ripening and cheese storage the number of organism decreased significantly in the cheese samples made with starter culture ($P<0.05$). The results showed an inhibitory effect of starter culture on *E. coli* O157:H7, but the organism can survive in this kind of cheese for up to 60 days of storage, respecting using starter culture, salt brine concentration and cheese storage time.

Key words: *Escherichia coli* O157:H7, Iranian white cheese, Starter culture, Brine salting

Introduction

Since the identification of *E. coli* O157:H7 as a human pathogen in 1982 (Fratamico and Smith, 2006), *E. coli* O157:H7 has become a pathogen of major concern for the food and dairy products because of it's ability to cause severe illness, in particular, haemorrhagic colitis, haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura (Govaris *et al*., 2001; Maher *et al*., 2001). Water and other foodstuffs such as lettuce, alfalfa sprouts and apple juice have also been implicated in outbreaks (Buchanan and Doyle, 1997).

Most of the foodborne outbreaks of *E. coli* O157:H7 have been associated with the consumption of foods originated from cattle, especially foods contaminated with cattle faeces. Because *E. coli* O157 has been found regularly in healthy cattle faeces, this animal is known to be an asymptomatic carrier (Öksüz *et al*., 2004). In 1999, over 11% of the total number of reported cases of infection caused by *E. coli* O157:H7 in England and Wales were due to dairy products (Vernozy-Rozand *et al*., 2005). *Escherichia coli* O157 serotypes are identified as enterohaemorrhagic *E. coli* and categorized as verotoxin-producing *E. coli*. Verotoxin is also known as shiga-like toxin (Jamshidi *et al*., 2008).

The organism is destroyed in pasteurization process, but insufficient heat-treatment of ground meat and raw milk forms a potential infection risk (Betts, 2000; Öksüz *et al*., 2004). The processing

conditions for different milk products are very important from the standpoint of the organism's infection risk. It can grow in trypticase soy broth (TSB) acidified with lactic acid at pH = 4.6 but not at pH = 4.5 (Glass *et al*., 1992).

Cheese made with unpasteurised milk is a potential vehicle for transmission of *E. coli* O157 to the consumer. In Iran, similar to other countries, a large amount of traditional cheeses are manufactured from raw milk and consumed freshly or after ripening in salt brine. The aim of the present study was to determine the effect of pH and different salt brine concentrations of cheese on survival of *E. coli* O157:H7 during manufacture and storage of Iranian white cheese in brine.

Materials and Methods

Cow milk

Pasteurized cow milk was obtained from Iranian Dairy Industries Co., and stored at 4°C. The quality of the milk was within the limits specified in the current Iranian standard for production of cheese (Fat = 2.5%, SNF = 8.9% and pH = 6.7) (Anon. 2002). It was evaluated for the lack of antibiotic residues (copan test).

Bacterial strain and preparation of inocula

Toxigenic strain of *E. coli* O157:H7 (ATCC 25922) was obtained from Faculty of Veterinary Medicine, University of Tehran. This strain was activated during two consecutive cultures in 50 ml brain-heart infusion (BHI) broth for 18-20 h until the optical density reached 0.8 to 0.9 at 600 nm, which corresponded to approximately 1×10^9 cfu/ml. The culture was diluted to obtain a concentration of 10^7 cfu/ml. One ml of this culture was added to 10 L of milk to obtain a 10^3 cfu/ml.

Starter culture

Lyophilized direct vat type thermophilic yoghurt culture containing *Streptococcus thermophilus* and *Lactobacillus delbruekii* subsp. *bulgaricus* (Chr. Hansen's laboratory, FD-DVS CH-1, Denmark) was used to make the Iranian traditional white brined cheese.

Procedure of making Iranian white cheese in brine

To evaluate the effect of starter on *E. coli* O157:H7, two batches of cheese were prepared, one of them was treated with 0.2 U/L starter (at 35°C) while the other sample was left intact. To speed up the clotting or reducing the amount of rennet needed, $CaCl_2$ (0.02% w/v) was added. Rennet (Chr. Hansen's Laboratory, HANILASE L 3500) was then added to achieve the final concentration of 0.002% (w/v). Cheese was maintained at 35°C for 1 h to curdle. The curd was cut into $2 \times 2 \times 2$ cm^3 and allowed to drain. The mixture was agitated and drained for 1 h. Following drainage, the curd was placed in stainless steel press for 6 h, to fuse the curd grains into a continuous mass (7 h). The moulded cheese was cut into $7 \times 7 \times 7$ cm^3 pieces and kept immersed in 20% solution of pasteurized salt brine for 8 h at 18°C (15 h). After salting, cheese pieces were aseptically packed in 6 and 8% salt brine and hold at 14°C to ripen. The specimens were then kept at 4°C (Hanifian and Karim, 2006). During ripening and storage period, the samples were analysed on dogs 15, 30, 45 and 60.

Enumeration and detection of *Escherichia coli* O157:H7

MacConkey agar containing sorbitol instead of lactose (SMAC) was used for isolation of *E. coli* O157:H7. Due to the fact that these bacteria are unable to ferment sorbitol, non-sorbitol-fermenting (NSF) colonies were potentially considered as *E. coli* O157:H7 (McDonough *et al*., 2000; Meng *et al*., 2001; Jamshidi *et al*., 2008).

At each sampling period, 10 g of cheese was added to a bottle containing 90 ml of 0.1% peptone water and homogenized using a stomacher lab blender for 3 min. Serial 10-fold dilutions of test material were prepared in sterile peptone water, surface spread plated in duplicate on sorbitol MacConkey agar (HIMEDIA M298, India) surfaces containing cefixime (0.05 mg/l) and potassium tellurite (2.5 mg/l) (CT-SMAC), then incubated at 35°C for 24 h. Non-sorbitol-fermenting colonies on CT-SMAC were counted and 5-10 colonies were chosen to confirm by latex-agglutination with the *E.*

coli O157 latex kit (Bahar afshan). Latex agglutinating isolates were further confirmed biochemically in SIM, MR-VP broth, Simon's citrate agar and TSI agar. *E. coli* O157:H7 are glucose, indole and methyl red positive, but negative for lactose, sucrose, Voges-Proskauer, citrate, CO_2 and SH_2 (Meng *et al*., 2001). Then biochemically confirmed *E. coli* O157:H7 colonies were counted.

Physicochemical analysis of cheese

Physicochemical analysis of the samples were made at each sampling time for enumeration of *E. coli* O157:H7. Salt content (Carpenter and Hendricks, 2003), total solid (Bradley, 2003) and pH (Sadler and Murphy, 2003) were determined. The pH of cheese samples was determined using a pH meter (Testo 230, pH-und temperature-mebgerat, EN 50081-1 + EN 50082-1, Gmbh, Germany).

Statistical analysis

A split plot experiment based on completely randomized design (CRD) with three replications was conducted. Factor A included starter (with and without starter), factor B included salt brine concentration (6 and 8%) and factor C was time (15, 30, 45 and 60 days). Data were analysed using the general linear model procedure (SAS, 1992). Analysis of the variance followed by Duncan's multiple range test was employed to find significant differences ($P<0.05$) between the treatments.

Results

The physicochemical properties and counts of *E. coli* O157:H7 in milk and cheeses made with starter and without starter culture, during manufacture, ripening and storage are given in Table 1. *Escherichia coli* O157:H7 was not isolated from the samples of pasteurised milk, starter culture, rennet, $CaCl_2$ or salt brine.

The counts of *E. coli* O157:H7 in all of the cheeses increased continuously from the initial inoculum level by about 3 logs in 7 h during manufacture.

During brine salting (20% solution of NaCl) for 8 h at 18°C, the population of the pathogen remained relatively stable. At the end of 15 h, the NaCl concentration in the cheese was 4%. During ripening, in the cheeses made with starter culture, the pathogen population decreased significantly ($P<0.05$) to 4 log cfu/g, whereas they remained relatively stable (about 10^6 cfu/g) in the cheeses made without starter culture. At those storage times, the pH was dropped to 5.1 and 6.2 in the cheese samples with and without starter, respectively. The pH of the cheeses made with starter dropped gradually to 4.5 on day 60.

At the end of the storage time, survival of *E. coli* was significantly lower ($P<0.05$) in cheese with starter (Fig. 1) compared to that without starter (Fig. 2). However, at 4°C, a rapid decline in *E. coli* O157:H7 population was observed in cheese samples made with starter, but it survived at approximately 80 and 630 cfu/g in 8 and 6% salt brine throughout storage.

Discussion

According to the USA mandates, acidic food processors should guarantee a 5-log reduction of *E. coli* O157:H7 during processing of fermented sausages and fruit juices (because of the survival of *E. coli* O157:H7 in acidic foods) (Getty *et al*., 2000; Samelis and Sofos, 2003). There is no published data on the 5D reduction of the organism for fermented milk products, yet. Validation studies on survival of *E. coli* O157:H7 and other pathogens are also necessary to indicate potential risks and preventive actions to be taken (Leuschner and Boughtflower, 2002; Lekkas *et al*., 2006). Such studies are particularly needed for traditional Iranian white brined cheese, as few data on growth and survival of *E. coli* O157:H7 in this product are currently available.

The results of this study indicated that *E. coli* O157:H7 may have a great potential for survival in Iranian white brined cheese. The lactic acid bacteria in the cheese made with starter culture had an inhibitory effect on *E. coli* O157:H7, since the population of *E. coli* O157:H7 was significantly ($P<0.05$) lower compared to the cheese made without starter culture.

Table 1: Changes in mean of total solid, NaCl content, pH and *E. coli* O157:H7 counts in cheese samples made with starter and without starter

Cheese type	Brine concentration (%)	Time	Total solid (%) ± SEM	NaCl content (%) ± SEM	pH ± SEM	Log *E. coli* O157:H7 ± SEM
With starter	6	0	ND	ND	$6.7^{a} \pm 0.02$	3 ± 0
		7 h	$37.0^{b} \pm 0.60$	ND	$6.1^{b} \pm 0.05$	$6.2^{a} \pm 0.24$
		15 h	$43.3^{a} \pm 0.89$	$3.9^{a} \pm 0.03$	$5.6^{c} \pm 0.03$	$5.8^{a} \pm 0.19$
		15 d	$31.6^{c} \pm 0.25$	$4.0^{a} \pm 0.21$	$5.3^{d} \pm 0.07$	$6.2^{a} \pm 0.06$
		30 d	$35.3^{b} \pm 0.60$	$3.8^{ab} \pm 0.26$	$5.0^{e} \pm 0.09$	$4.1^{b} \pm 0.10$
		45 d	$37.0^{b} \pm 0.31$	$3.6^{b} \pm 0.17$	$4.4^{f} \pm 0.10$	$3.3^{c} \pm 0.20$
		60 d	$43.3^{a} \pm 0.56$	$3.3^{c} \pm 0.29$	$4.4^{f} \pm 0.05$	$2.8^{c} \pm 0.15$
	8	0	ND	ND	$6.7^{a} \pm 0.04$	3 ± 0
		7 h	$37.3^{b} \pm 0.72$	ND	$6.1^{b} \pm 0.05$	$6.2^{a} \pm 0.20$
		15 h	$43.3^{a} \pm 0.87$	$3.9^{c} \pm 0.03$	$5.6^{c} \pm 0.03$	$5.8^{a} \pm 0.39$
		15 d	$32.6^{c} \pm 0.35$	$5.0^{a} \pm 0.25$	$5.4^{c} \pm 0.09$	$5.9^{a} \pm 0.10$
		30 d	$37.0^{b} \pm 0.60$	$5.0^{a} \pm 0.30$	$5.3^{c} \pm 0.13$	$3.9^{b} \pm 0.12$
		45 d	$38.3^{b} \pm 0.35$	$4.4^{b} \pm 0.21$	$4.6^{d} \pm 0.10$	$3.1^{c} \pm 0.10$
		60 d	$45.0^{a} \pm 0.64$	$4.7^{b} \pm 0.31$	$4.6^{d} \pm 0.09$	$1.9^{d} \pm 0.23$
Without starter	6	0	ND	ND	$6.7^{a} \pm 0.03$	3 ± 0
		7 h	$36.6^{cd} \pm 0.42$	ND	$6.7^{b} \pm 0.09$	$6.0^{ab} \pm 0.40$
		15 h	$40.3^{ab} \pm 0.92$	$4.0^{a} \pm 0.15$	$6.5^{bc} \pm 0.04$	$5.8^{b} \pm 0.04$
		15 d	$34.0^{d} \pm 0.89$	$4.1^{a} \pm 0.23$	$6.2^{dc} \pm 0.09$	$6.6^{a} \pm 0.15$
		30 d	$34.3^{d} \pm 0.79$	$4.1^{a} \pm 0.16$	$6.1^{d} \pm 0.06$	$6.3^{ab} \pm 0.10$
		45 d	$38.6^{bc} \pm 0.30$	$4.1^{a} \pm 0.17$	$5.4^{e} \pm 0.09$	$6.2^{ab} \pm 0.05$
		60 d	$42.6^{a} \pm 0.55$	$4.3^{a} \pm 0.19$	$5.7^{e} \pm 0.09$	$5.9^{b} \pm 0.09$
	8	0	ND	ND	$6.7^{a} \pm 0.03$	3 ± 0
		7 h	$36.6^{d} \pm 0.90$	ND	$6.7^{b} \pm 0.07$	$6.0^{a} \pm 0.20$
		15 h	$40.3^{ab} \pm 0.84$	$4.0^{b} \pm 0.19$	$6.5^{bc} \pm 0.06$	$5.8^{a} \pm 0.02$
		15 d	$35.6^{d} \pm 0.85$	$5.0^{a} \pm 0.19$	$6.6^{b} \pm 0.09$	$6.3^{a} \pm 0.11$
		30 d	$37.0^{dc} \pm 0.43$	$4.8^{a} \pm 0.16$	$6.2^{c} \pm 0.04$	$6.1^{a} \pm 0.10$
		45 d	$39.3^{bc} \pm 0.58$	$4.9^{a} \pm 0.21$	$5.8^{d} \pm 0.09$	$6.3^{a} \pm 0.09$
		60 d	$42.0^{a} \pm 0.67$	$5.0^{a} \pm 0.17$	$5.7^{d} \pm 0.09$	$5.7^{a} \pm 0.09$

ND: Not determined. Values are means of triplicate experiments (n = 3). The means with different letters in each column are significant at $P<0.05$, using Duncan's multiple range test. SEM: Standard error of the mean

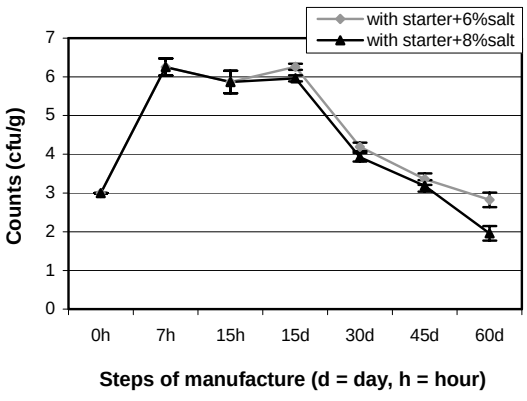

Fig. 1: *Escherichia coli* O157:H7 counts during the manufacture and storage of cheese made with starter

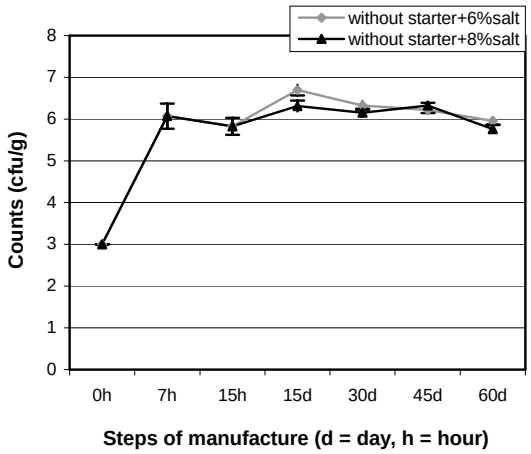

Fig. 2: *Escherichia coli* O157:H7 counts during the manufacture and storage of cheese made without starter

A similar antagonistic effect of lactic acid bacteria on food-borne pathogens was reported by Larson *et al*. (1993) in whey. They reported a significant growth of *Listeria monocytogenes* and *salmonella Heidelberg* in whey containing no lactic culture compared to the samples containing lactic acid bacteria.

A study by Glass *et al*. (1992) showed that *E. coli* O157:H7 can grow in TSB

containing ≤6.5% NaCl or at a pH of 4.5 to 9.0, adjusted with HCl. When TSB was acidified with lactic acid, the organism grew at pH 4.6 but not at pH 4.5.

According to a study by Leyer *et al*. (1995), the organism was adapted to acid by culturing for one to two doubling at pH 5.0. Acid adapted cells had an increased resistance to lactic acid, and their survival was better than non-adapted cells during the fermentation of sausage. Also, there were no differences in the salt content of the cheeses, given that increased salt (4%) may protect *E. coli* O157:H7 from acid (Casey and Condon, 2002; Lekkas *et al*., 2006).

Since the populations of *E. coli* O157:H7 was later decreased during storage period, one of the main factors causing the initial decrease in *E. coli* O157:H7 might be attributed to the storage temperature of 4°C. Optimum growth temperature of *E. coli* O157:H7 is around 37°C in several foods (Govaris *et al*., 2001) and incubating the milk at this temperature during preripening procedures contributed to the excessive growth rate.

Results presented in this study may suggest that the manufacturing procedure of Iranian white cheese in brine do not eliminate *E. coli* O157:H7, emphasizing the tolerance of this pathogen to acid produced by starter culture. Although the population of *E. coli* O157:H7 was high (2.3 log cfu/g), no signs of spoilage were observed from the day of inoculation until day 60 of storage at 4°C. Since no spoilage was visible in the cheese containing high numbers of the *E. coli* O157:H7, consumer health could be endangered.

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