Detection and antimicrobial susceptibility of *E.coli* O157: H7 in raw bovine milk, some of dairy products and water samples

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ABSTRACT

A total of 294 samples of raw-unpasteurized bovine milk and variety of dairy products (hard cheese, sweet cheese, cream cheese and cream), were collected from local markets in Lattakia city as well as 44 samples of used water (clear potable water collected before used in cheese preparing processes) and preserving water (turbid water collected from hard cheese preserving tanks). All samples were analyzed for *E.coli* O157: H7 detection.

A total of 167 isolates *of E.coli* O157 were isolated, of them, 122 were identified as *E.coli* O157: H7 and 45 as *E.coli* O157. Antimicrobial susceptibility tests were carried for all *E.coli* O157: H7 and *E.coli* O157 isolates using the disk diffusion method as described by NCCLS. The antibiotics screened and their resistance levels were as follows: for *E.coli* O157:H7 isolates, ciprofloxacin (0%), gentamicin and chloramphenicol (3.28%) for each, tetracycline (16.39%), amoxicillin/clavulanic acid (92.26%), ampicillin (95.08%), cefoxitin (96.76%), cephazoline (97.54%), cephalothin (98.36%), and sulfamethoxazole/trimethoprim (100%). related values were obtained for *E.coli* O157 isolates.

Key words: *E.coli* O157: H7, Milk, Dairy, Antibiotic, Susceptibility, Resistance.

تحديد الايشريشيا المعوية النزفية *E.coli* O157:H7 في عينات من الحليب البقري الخام وبعض المنتجات اللبنية والماء وحساسيتها للمضادات الميكروبية

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الملخص

جُمعت 294 عينة من الحليب البقري الخام غير المبستر، وتشكيلة من المنتجات اللبنية (جبنة قاسية، وحلاوة بالجبن، وجبنة بالقشطة، وقشطة) بدءاً من الأسواق المحلية في مدينة اللاذقية، بالإضافة إلى 44 عينة من ماء التحضير (ماء نقي صالح للشرب جُمع قبل أن يستخدم في تحضير الجبنة القاسية) ومــاء الحفظ (ماء عكر جُمع من علب حفظ الجبنة القاسية)، حُلِّلت العينات للتحري والكشف عن وجــود *E.coli* O157:H7.

عُزلت 167 عزلة من الايشريشيا المعوية النزفية *E.coli* O157:H7. من هذه العزولات امتلكت 122 عزلة المستضدين O157 وH7، في حين امتلكت 45 عزلة فقط المستضد O157.

أجريت اختبارات التحسس للصادات الحيوية لكل العزلات الجرثومية الناتجة باستخدام طريقة الانتشار من القرص كما وصفت من قبل هيئة المخابر الطبية NCCLS. كانت الــصادات الحيويــة المــستخدمة ومستويات مقاومتها من أجل *E.coli* O157:H7 كمــا يــأتي: سيبروفلوكــساسين (0%)، جينتاميــسين (3.28%)، كلورامفينيكــول (3.28%)، تتراســيكلين (16.39%)، اموكسيــسللين/ كلافولانيــك اســيد (92.26%)، امبيسلين (95.08%)، سيفوكــسيتين (96.76%)، ســيفازولين (97.54%). ســيفالوتين (98.36%)، وسلفاميتاكسازول/ تيميتوبريم (100%). وقد سجلت قيم مشابهة من أجل عــزلات *E.coli* O157.

الكلمات المفتاحية: الايشيريشيا المعوية النزفية، *E.coli* O157:H7، حليب، منتجات لبنية، صادات حيوية، تحسس، مقاومة.

Introduction

Since 1982 when it was first identified as human pathogen and implicated in two outbreaks of hemorrhagic colitis (bloody diarrhea), *E.coli* O157:H7 has been considered as a food-born serious pathogen (*Clark et al 2002*). Now, *E.coli* O157:H7 has became important pathogen with worldwide distribution (*Schlundt 2001*). The infected patients are usually vulnerable individuals such as the very younger, very old and immunocompromised ones .The infective dose of *E.coli* O157:H7 is 50-100 organisms and the incubation period to the onset of diarrhea can vary from 1 to 8 days (*Nataro and Kaper1998*) . *E.coli* O157:H7 causes hemorrhagic colitis that is characterized by abdominal pain, watery diarrhea followed by bloody diarrhea and hemolytic uraemic syndrome, notably in infants and young children though it may occur in all age groups (*Griffin and Tauxe 1991, Fitzpatrick 1999*). Infected dairy cattle has been considered as the principle reservoir of *E.coli* O157:H7 (*Borczyk et al 1987, Chapman et al 1997*), with undercooked ground beef and raw milk being the major vehicles of foodborne outbreaks (*Doyle 1991, Griffin and Tauxe 1991*). Although direct transmission of *E.coli* O157:H7 from cattle to man is possible either by direct contact with animals (*Rice et al 1996*) or by contact with animal manure (*Cieslak et al 1993*), *E.coli* O157:H7 usually reaches to human beings by consumption of products contaminated with feces of infected animals, contaminated or undercooked round beef and raw or unpasteurized milk (*Le Saux 1993, Feng et al 2001, Chapman et al 1993, Morgan et al 1988*). Untreated drinking water has also been included as a vehicle of transmission and outbreaks (*Swerdlow et al 1992, Rangel* et al 2005).

Because some antibiotics may cause bacterial lysis and liberate the free Shiga toxins in the intestinal tract (*Karch et el 1986, Wong et al 2000*), and enhance the expression of Shiga toxins genes (*Zhang et al 2000*), the antimicrobial treatment is contraindicated for human *E.coli* O157:H7 infections. However, such treatments may be recommended for cystitis and pyelonefritis other than hemorrhagic colitis all caused by *E.coli* O157:H7 (*Griffin 1995*).

For that limitations of using antimicrobial agents in *E.coli* O157:H7 cases, the general accepted is that the *E.coli* O157:H7 may still susceptible to most of antimicrobial. On the other hand, the usage of antimicrobials for agricultural purposes, particurly, in diseases prevention and growth promotion in animal production is a considerable cause of the selection and prevalence of antibiotic resistant *E.coli* O157:H7 (*Schroeder et al 2002*).

In addition to their epidemiological importance, the studies of antimicrobials susceptibility of *E.coli* O157:H7 may have more therapeutic significance as recent studies have indicated to a possible role of early adminstrated antimicrobials in preventing the progression of hemolytic uraemic syndrome and hemorrhagic colitis both caused by *E.coli* O157:H7 (*Ikeda et al 1999, Shiomi et al 1999*).

The purpose of this study is to monitor the rate of occurrence of *E.coli* O157:H7 in raw milk and variety of dairy products of bovine origin and to study its antimicrobial susceptibility.

Materials and Methods

Samples and culture media: A total of 294 samples of bovine raw milk and variety of its related dairy products were collected from local markets in Lattakia city throughout one year of microbiological surveillance 2005.These samples were distributed as follows: 110 of raw milk, 62 of hard cheese (locally produced, called Akkawi, and prepared by heating the raw milk to no more than 50 C°), 42 of sweet cheese (a kind of Syrian candy called “Halawa with cheese”), 50 of creamed-cheese, and 30 samples of cream. In addition, 44 water samples were collected from used water (clear potable water collected before used in cheese preparing processes) and preserving water (turbid water collected from hard cheese preserving tanks) of hard cheese and analyzed bacteriologically. Samples of 200 to 500gr portions of dairy products were collected using sterile plastic bags. However, milk and water samples were collected by autoclaved glass bottles. All samples were analyzed by no longer than one hour after being collected; Portions of 10 gr of each dairy products sample were homogenized and diluted in 100 ml of saline (0.85%) solution; aliquots of 0.1, 0.5, 1 ml of the homogenized samples were directly plated on two culture media as duplicate. Sorbitol Mac Conky agar: SMAC with CT supplements (cefixim-tellurite) (Merck *Ltd*) was used to discover and enumerate colonies of *E.coli* O157:H7 and Endo agar (Amersham LAB *Ltd*) for fecal coliform counts, plated culture media were aerobically incubated at 37 C° for 24 and 48 hr .

Identification and confirmation: For each sample, ten well-isolated colonies that are typical for *E.coli* O157:H7 (i.e.: colorless nonsorbitol fermenters) were selected from the plate of SMAC with the highest dilution ,and first confirmed by agglutination with *E.coli* O157:H7 antiserum coated latex test (Oxoid *Ltd*) to ascertain O157 antigen presence; positive colonies were subcultured on trypticas soy agar (Merck Ltd) and incubated for overnight at 37 C' then screened by H7 latex test (Oxoid *Ltd*). All these typical colonies were biochemically identified to be *E.coli* (*Murray et al 1999*). As parallel, representatives of typical colonies for *E.coli* O157:H7 (i.e.: pink sorbitol fermenters) were routinely serologically confirmed to be *E.coli* O157:H7 .

Antimicrobial susceptibility. Disk diffusion method (Bauer-Kirby methode) as described by (*NCCLS* $2000_{a,b}$) was used to test the senecetivity of all *E.coli* O157:H7 isolates to ten antibiotics (Oxoid *Ltd*). The antibiotics were selected upone distinct criteria i.e.: to represent variety of antibiotics groups and to make our results comparable with other related studies. The antibiotics used and their concentrations were: ampicillin (AMP- 10 mcg), amoxicillin-clavulanic acid (AMC-20 mcg), gentamicin (GEN-10 mcg), sulfamethoxazole-trimethoprim (SXT- 175 + 1.25 mcg), ciprofloxacin (CIP-5 mcg), cefoxitin (CFN-30 mcg), tetracycline (TET-30 mcg),cephazoline (CEZ-30 mcg), cephalothin (CEF-30 mcg), and chloramphenicol (CHL-30 mcg) (Oxoid-Ltd). Results were reported as susceptible, intermediate, and resistant isolate upon criteria outlined by (*Murray et al 1999, NCCLS* 2000_a).

Results. Prevalence of *E.coli* O157:H7. Throughout one year of surveillance, a total of 167 of *E.coli* O157:H7 isolates were obtained from 110 samples of bovine raw milk, 62 hard cheese, 42 sweet cheese, 50 creamed cheese, 30 samples of cream, and 44 water samples. Of these 167 isolates, 122 were serologically and biochemically identified as *E.coli* O157:H7 , and 45 as *E.coli* O157, two hundred forty isolates of *E.coli* other than O157 H:7 were counted and identified from SMAC plates .

As shown in table 1, from sixty two of hard cheese samples, 48 (77.41%) were positive for presence of *E.coli* O157:H7, against of 34 positive samples of raw milk (30.90%); the rates for sweet cheese, creamed cheese, and cream samples were 19%, 18%, and 10% respectively. Similar patterns of distribution of *E.coli* O157 and *E.coli* other than *E.coli* O157:H7 are notable for all samples, so, the positive samples of hard cheese, raw milk, sweet cheese, creamed cheese, and cream samples were as follows: 14 (22.58%), 11 (10%), 3 (7.14%), 8 (16%) and 4 (13.33%) for *E.coli* O157:H7 respectively . These values for *E.coli* rather than *E.coli* O157:H7 were as follows: 52 (83.87%), 39 (35.45), 12 (28.57%), 10 (20%), and 3 (10%) respectively.

Table (1) Numbers of positive samples for presence of different types of *E.coli*. (n: number of samples)

Samples	**n**	**Numbers and (%) of positive samples for presence of:**		
		***E.coli* O157 H:7**	***E.coli* O157**	**Other *E.coli***
Raw milk	110	34 (30.90)	11 (10)	39 (35.45)
Hard cheese	62	48 (77.41)	14 (22.58)	52 (83.87)
Sweet cheese	42	8 (19)	3 (7.14)	12 (28.57)
Cream cheese	50	9 (18)	8 (16)	10 (20)
cream	30	3 (10)	4 (13.33)	3 (10)
Preparing water	20	5(25)	2(10)	7(35)
Preserving water	24	13(54.16)	5(20.83)	21(87.5)
Total	338	120 (35.50)	47 (13.90)	144 (42.60)

The mean values (calculated as medium of the total number of a given bacterium obtained from all analyzed samples) and ranges (the lowest and highest numbers the given bacterium have had for all samples analyzed) of counts concerning *E.coli* O157:H7, other *E.coli*, and fecal coliforms are shown on table (2).

Table (2) Mean values and ranges of counts of *E.coli* O157 H: 7 (including *E.coli* O157), other *E.coli*, and Fecal coliform in different samples.(n: number of samples)

Samples	**n**	**Counts as c.f.u / 1 ml (gr)**					
		***E.coli* O157 H:7**		**Other *E.coli***		**Fecal coliform**	
		Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**
Raw milk	110	2.1x10	5-9.2x10	$3.9x10^2$	$9x10-8.8x10^2$	$8.1x10^2$	$1.2x10^2-4x10^3$
Hard cheese	62	3.1x10	$2-1.4x10^2$	$4.4x10^2$	$3.6x10-1.1x10^3$	$9.3x10^2$	$4.2x10^2-1.4x10^4$
Sweet cheese	42	6	1-2.8x10	2.2x10	$9-5.2x10^2$	7.5x10	$4x10-7.7x10^2$
Cream cheese	50	4	1-1.1x10	8	2-2.2x10	3.3x10	$4-1.1x10^2$
cream	30	3	1-9	3	1-1.8x10	2.1x10	2-8.1x10
Preparing water	20	1.1x10	1-3 x10	7.7x10	$3.1x10-1.1x10^2$	$1.2x10^2$	$4.3x10^2-8.5x10^2$
Preserving water	24	2.2x10	1-9.1x10	$1.5x10^2$	$9.1x10-6.7x10^2$	$7.7x10^3$	$3.4x10^2-5.5x10^5$

First to note, is the associating of the highest counts for all above bacteria with the hard cheese samples, so for *E.coli* O157:H7 the mean value is 3.1x10 and range is $2-1.4x10^2$. However, the counts were high in raw milk samples, the mean and range values were 2.1x10 and 5-9.2x10 respectively. Similar distribution is notable for fecal pollution indicators *E.coli* other than O157:H7 and fecal colifrom since the counts were also high in all samples with the highest values for hard cheese and raw milk. As regards water samples, we can recognize two main points, first is the relatively higher numbers of *E.coli* O157:H7 and fecal coliform in the preserving water compared with preparing ones; second is being these numbers lower than those in the hard cheese itself that preserved in such waters. This certify that the fecal contaminated hard cheese is the main source of *E.coli* O157:H7 rather than water although the latter may serve as temporary or transient environment for *E.coli* O157:H7.

Antimicrobial susceptibility. As shown from table (3), there is prevalence of high levels of antimicrobial resistance among *E.coli* O157:H7 and *E.coli* O157 isolates for most of the antibiotics screened, however, all isolates of *E.coli* O157:H7 and *E.coli* O157 were susceptible to ciprofloxacin. Slight differences were observed between *E.coli* O157:H7 and *E.coli* O157 isolates in susceptibility to the rest of nine screened antibiotics.

As regards *E.coli* O157:H7 isolates, all but four of 118 (96.76%) isolates were susceptible to both of gentamicin and chloramphenicol, and high susceptibility were also found to tetracycline 100 (81.97%).

However, the rest six antibiotics were found to be ineffective against *E.coli* O157:H7, so, the resistance ranged between 113 (92.26%) for amoxicillin/clavulanic acid and 122 (100%) for sulfamethoxazole/tremethoprim. Intermediate susceptibilities were found to ampicillin and amoxicillin, 6 (4.94%) for each, to cefazolin 3(2.46%), and to tetracycline 2 (1.64%).

For *E.coli* O157, the susceptibility levels to the three active antibiotics chloramphenicol, gentamicin, and tetracycline were 44 (97.77%), 43 (95.55%) and 34 (75.55%) respectively. Compared with *E.coli* O157:H7, little increases in susceptibility of *E.coli* O157 to the rest six antibiotics, since the resistance ranged between 35 (77.77%) to cephalothin and 43 (95.55%) to cefoxitin. Intermediate susceptibilities were reported to tetracycline 4 (8.89%), to amoxicillin 2 (4.45%) and to ampicillin and sulfamethoxazole/trimethoprim 1 (2.22%) for each.

Table (3) Antimicrobial susceptibility of isolates of *E.coli* O157 H: 7 and *E.coli* O157.

Antibiotics	No. and (%) of isolates with susceptibility					
	***E.coli* O157 H:7 (n = 122)**			***E.coli* O157 (n = 45)**		
	S	**I**	**R**	**S**		**R**
Ampicillin	0 (-)	6 (4.92)	116 (95.08)	4 (8.89)	1 (2.22)	40 (88.89)
Amoxicillin/ clavulanic acid	3 (2.46)	6 (4.92)	113 (92.26)	5 (11.11)	2 (4.44)	38 (84.44)

Cephalothin	2 (1.64)	0 (-)	120 (98.36)	10 (22.22)	0 (-)	35 (77.77)
cefazolin	0 (-)	3 (2.46)	119 (97.54)	6 (13.33)	0 (-)	39 (86.67)
cefoxitin	4 (3.46)	0 (-)	118 (96.76)	2 (4.44)	0 (-)	43 (95.55)
Sulfamethoxazol/ trimethoprim	0 (-)	0 (-)	122 (100)	3 (6.67)	1 (2.22)	41 (91.11)
Ciprofloxacin	122 (100)	0 (-)	0 (-)	45 (100)	0 (-)	0 (-)
Gentamicin	118 (96.76)	0 (-)	4 (3.28)	43 (95.55)	0 (-)	2 (4.44)
Tetracycline	100 (81.97)	2 (1.64)	20 (16.39)	34 (75.55)	4 (8.89)	7 (15.55)
Chloramphenicol	118 (96.72)	0 (-)	4 (3.28)	44 (97.77)	0 (-)	1 (2.22)

S: Sensitive, I: Intermediate, R: Resistant

As relates to the multiple resistance, isolates of *E.coli* O157:H7 and *E.coli* O157 have showed high levels of multiresistance. All isolates of *E.coli* O157:H7 122 (100%) were resistant to one, two, three, and four antibiotics, 110 (90.16%) were resistant to five, 103 (84.43%) were resistant to six, 20 (16.39%) were resistant to seven and 6 (4.92%) were resistant to eight. Similar pictures were observed for *E.coli* O157 isolates.

The most frequent multiresistance that has been detected among either *E.coli* O157:H7 or *E.coli* O157:H7 isolates was to sulfamethoxazole/trimethoprim, ampicillin, cefazolin, and cefalothin together.

Discussion

Prevalence of *E.coli* O157:H7. In consideration of the satisfactory microbiological quality for *E.coli* O157:H7 that is < 20 c.f.u. g^{-}1 with the acceptable range being 20 to < 100 c.f.u g^{--1} (*Gilbert et al 2000*), 86 (29%) samples will be classified as unacceptable, 16 (5.44%) as satisfactory, and the rest 192 (65.30%) as free of *E.coli* O157:H7.

Although dairy products and unpasteurized milk have been implicated in outbreaks of *E.coli* O157:H7, (*Chapman 2000*), little is known about occurrence of *E.coli* O157:H7 in variety of local prepared, produced and consumed food items especially dairy products, and their potential role in inducing infections and outbreaks.

In general, all kinds of dairy products and raw milk analyzed have had *E.coli* O157:H7, however, there were some differences in rate of positive samples, frequency of isolation, and counts of *E.coli* O157:H7 among these products. The measurements were the highest in hard cheese samples, significant numbers were obtained from samples of sweet and cream cheese but the lowest values were reported in cream samples.

As regard raw milk samples, previous reports have showed that milk appears to be favorite for the survival of *E.coli* O157:H7 *(Massa et al 1997*) although their growth in unpasteurized milk may be slower than that in pasteurized milk due to the presence of other microorganisms (*Wang et al 1997*). In our study, presence of *E.coli* O157:H7 in high rate in milk samples indicates heavy contamination of milk with feces of infected dairy animals , this can be attributed to closed contacts with feces matters or manure of infected cows, as *E.coli* O157:H7 has high rate of survival in feces (*Kudva et al 1998*).

There were increases in numbers of *E.coli* O157:H7 in hard cheese compared with raw milk, this result and the presence of *E.coli* O157:H7 in a significant numbers in all kinds of cheese tested do not agree with the data obtained by some of the studies which showed that there were reductions in numbers of *E.coli* O157:H7 during preparation and storage of yoghurt (*Massa et al 1997*) and cheddar cheese (*Reitsma and Henning 1996*) and inactivation of *E.coli* O157:H7 cells in commercial products including sour cream and buttermilk that were inculcated with *E.coli* O157:H7 (*Dineen et al 1998*). However, in other studies, growth of *E.coli* O157:H7 has been recorded during manufacture of some dairy products such as cottage cheese, and soft Hispanic cheese (*Arocha et al 1992, kasrazadeh and Genigeorgis 1995*)

The increases in numbers of *E.coli* O157:H7 in hard cheese compared with raw milk can be explained by hypothesis that *E.coli* O157:H7 cells become free of antimicrobial effects resulted from presence of lactoperoxidase thiocyanate-hydrogen peroxide system in raw milk (*Heuvelink et al 1998*), however, the bad heat treatment (usually, milk is only heated to 50 C˚ or lower), preservation and handling conditions of hard cheese (usually, preserved in containers filled with turbid milky water resulted from process of cheese preparation) could contribute to the resuscitation of *E.coli* O157:H7 stressed and injured cells.

Sweet cheese and creamed cheese have showed close counts and frequency of isolation of *E.coli* O157:H7. Differences between three kinds of cheese analyzed can be partially attributed to differences in their compositions and the ways by which they are prepared. Lowest values obtained from cream samples ascertain the efficacy of heat treatments in elimination of *E.coli* O157:H7 from the given product as this bacterium is somewhat considered susceptible to heat treatments (*D Aoust et al 1988*).

Notable association between fecal colifroms and *E.coli* O157:H7 counts in all positive samples (table 2) might suggest the direct effect of fecal contamination on occurrence of *E.coli* O157:H7 in those high quantities in the variety of the tested food products (*Wang et al 1997*).

The low numbers of *E.coli* O157:H7 and so the positives samples for it in preparing water of hard cheese compared with hard cheese itself indicates that water is not the main source of this organism though it can be considered as tool of transmission. On the other hand, the slight arising of numbers of *E.coli* O157:H7 in the preserving water can be explained by the washing action from hard cheese preserved in it; But some thing else can be concluded here; the water is not the suitable environment for surviving and offspring the *E.coli* O157:H7 (*Lisle et al 1998)*. This may explain why these numbers were not superior or even equal to those found in hard cheese.

Finally, the potential risk associated with products made from unpasteurized milk appears to be high, and these products must be a serious cause for concern.

As shown from the study and related worldwide studies, fecal contamination of food items is a likely route of transmission of *E.coli* O157:H7 to humans.

Antimicrobial susceptibility, the complete activity demonstrated by ciprofloxacin makes it, and to some extent the florequinolones, the drug of choice in killing of the *E.coli* O157:H7. Any way, high but not perfect susceptibility of *E.coli* O157:H7 isolates to ciprofloxacin has been reported (*Schroeder et al 2002*). On the other hand, as the other authors (*Farina et al 1996*, *Kim et al 1994*) have concluded that there are increases in resistance of *E.coli* O157:H7 isolates to tetracycline and sulfonamides, this may be true in our study for sulfamethoxazole (in sulfamethoxazole-trimethoprim), but not for tetracycline, since high susceptibility to tetracycline was found among our isolates. As many previous studies have indicated to gradual decreases in susceptibility of *E.coli* O157:H7 to defined antibiotics namely cephalosporins (*Galland et al 2001, Schroeder et al 2002*), we have obviously observed that decreases since the resistance was to be complete for cephalothin, cefazolin and cefixitin.

In general, the high levels of antimicrobial resistance and multiresistance among isolates of *E.coli* O157:H7 and *E.coli* O157 obtained in our study are complete, but a few exceptions different from that were obtained by other authors **(***LukaSova et al 2004***,** *Galland et al 2001, Schroeder et al 2002***)**

Conclusion

In our study, contaminated or infected hard cheese has demonstrated the high frequency of harboring the *E.coli* O157:H7 cells and consequently was responsible for most of the infection cases and illnesses that have been reported during the surveillance. Absence or appropriate heat treatment of the used raw milk during cheese preparing was the main cause responsible for infectious state of produced cheese.

Further studies are needed to investigate the fate of *E.coli* O157:H7 in such products under variety of conditions.

High and unexpected antibiotic resistance and prevalence of multiresistant strains of *E.coli* O157:H7 in food products should be a reason of concern in future studies.

High contents of fecal coliforms detected in the majority of analyzed samples may be as real evidence of the responsibility of fecal contamination as the main factor affecting the presence of *E.coli* O157:H7 in milk and dairy products.

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