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Prevalence, Molecular Characterization and Antimicrobial Resistance of Vero Toxigenic *E.Coli* in Fresh Soft Cheese, Ice Cream and Yoghurt in Mansoura City

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ABSTRACT

A total of 130 samples of fresh soft cheese (Talaga) (30), ice cream (50) and yoghurt (5(were taken randomly from markets besides small sellers in various regions of Mansoura tow Egypt then examined for the prevalence of cytotoxigenic *Escherichia coli*. The suspecte strains were serological typed then examined for the existence of Vero toxigenic *Escherichi coli* (VTEC) via Multiplex Polymerase Chain Reaction. The exposure to fourteen antibiotic disks was assessed by the disk dispersal way. Results obtained showed that toxigenic *E.co* was discovered in 3% (4/130) of the total examined samples with incidence of 10% (3/30 2% (1/50) and 0% (0/50) in fresh soft cheese, small scale ice cream, traditional yoghur respectively. The serogroups identified were one O26: H11, Three O55: H7 and one O9 H21. *E. coli* O157 was not found in all examined samples. Maximum repeated durability wa detected to the next antibiotics: Streptomycin (100.0%), Nalidixic acid (80.0% Erythromycin (80.0%), Amikacin (60.0%) and Cefotaxim (60.0%). Sulphamethoxaz (60.0%), Tetracycline (60.0%), Cephalothin (40.0%), Chloramphenicol (40.0%), Ampicilli (40.0%), Kanamycin (40.0%), Ciprofloxacin (40.0%), Neomycin (20.0%) and Gentamici (20.0%). Conclusively, some of fresh soft cheese (Talaga) and small scale production ic cream at retail markets located in Mansoura city haven multidrug-resistant VTEC.

1. INTRODUCTION

Verocytotoxin E.coli is a most-identified reason of dangerous illness in people, as hemolytic uremic syndrome (HUS). Although several epidemics have been associated with E. coli serotype O157:H7, non-O157 STEC serotypes relating to other types as O26, O91, O103, O111, O128 and O145 are too identified to reason of serious illness in persons (Johnson et al. 2006). The percentage of STEC infections in Europe produced by non-O157 serotypes amplified from 45.1% to 51.1% in 2013(EFSA 2015). Extra than 40% of the recognized cases of HUS in Italy (Tozzi et al. 2003), Denmark (Piérard et al. 1999), and Germany (Gerber et al 2002), were produced by non-O157 STEC. Additional newly, in 2005, Camembert-type cheeses polluted by E.coli O26 and O80 produced 16 hemolytic uremic conditions (Espié et al. 2008).Although the clinical signs of non-O157 STEC infections may differ; they can be as lethal as O157:H7 infections. Epidemics and periodic conditions of sicknesses were similarly related to ingestion of Verocytotoxin polluted cheese (Deschenes et al., 1996). The development of enterohaemorrhagic Escherichia coli sickness be influenced by the creation of Shiga toxin (Shiga toxin 1 and/or Shiga toxin 2) in mixture with additional risk agents as enterohemorrhagic E.coli usually haven virulence genetic factor for adherence (e.g., intimin genes) and the maximum dangerous shiga toxin E.coli haven an extra risk gene, E-hlyA, that is accountable for the creation of enterohaemolysin toxin (Osek and Gallien 2002).

In most of circumstances, as long as the management remained not adequate, illnesses produced via *Escherichia coli*, in adding toward failing the person resistance besides vulnerability to further infections, this leads to killing. Handling of sicknesses produced through this microorganism frequently needs antibiotic treatment; but antimicrobial-resistant serotypes lead to excessive illnesses into extended interval than their antibiotic-liable complements. Numerous readings revealed that antibiotic durability in *Escherichia coli* is cumulative in these years (Cortés et al. 2010).

Therefore, identification of the susceptibility of virulence *E.coli* to different antibiotics appears to be so vital in decrease of handling prices and time.
So, the aim of the existing research remained to evaluate the existence of Vero toxigenic *Escherichia coli* in fresh soft cheese, traditional ice cream and yoghurt and also detect their virulence genes and antibiotic resistance criteria.

2. MATERIAL AND METHODS

The method that applied for specimens gathering was suggested by American Public Health Association (A.P.H.A. 1992).
One hundred and thirty samples (50small scale production ice cream, 50 plain yoghurt and 30 fresh soft cheese (Talaga) were collected from various areas of Mansoura from the interval among January 2018 to June 2018 .The specimens reserved in an protected ice box (4±10C) to be transported to research workroom for microbiological inspection.

2.1 Microbiological analysis of Vero toxigenic *E.coli*:

2.1.1. Indirect enrichment technique for isolation was according to Robert et al. (1995).
From each 25ml/g sample were aseptically transferred to sterile blender container contained 225ml of sterile Tryptone soya broth (TSB), then these containers were incubated at 37°C for 16-18 hs, then subcultured to Cefixime Tellurite (Oxoid, SR0172E)-Sorbitol MacConkey Agar (Oxoid, CM 0813) .The dishes were incubated at 37°C for 20-24hs. Typical five suspected colorless colonies were selected and streaked onto MacConkey agar and incubated at 37°C for 24hs.The purified pink isolates were then streaked onto nutrient agar slants and incubated at 37°C for 18-24hs for more examination (biochemical, serological and molecular).

2.1.2. All the supposed colonies were biochemically recognized on the foundation of Indole test, Methyl red test, Voges proskauer test, Citrate utilization test and Triple sugar iron agar test according to (ISO 6579 (ISO 2002)).

2.1.3. Biochemically confirmed strains were serologically known agreeing to Kok et al. (1996) by means of fast analytical *E.coli* antisera sets (DENKA SEIKEN Co., Japan) for analysis of the Enteropathogenic kinds .

2.1.4. Discovery of virulence genes by Multiplex PCR:
Serologically identified *E.coli* were suspected to further identification molecularly in Food Analysis Center, Faculty of Veterinary Medicine, Banha University by Multiplex PCR .the primer orders of virulence genes used were *stx1* (F) , *stx1* (R) for gene stx1 at 614 and stx2 (F) , stx2 (R) for gene stx2 at 779 according to Dhanashree and Mallya (2008). *eaeA* (F) , *eaeA* (R) for eaeA gene at 890 according to Mazaheri et al. (2014). *hylA* (F), hlyA (R) for hylA gene at 165 according to Fratamico et al. (1995). All oligonuclueotide primers were obtained from (Pharmacia Biotech). Extraction of deoxyribonucleic acid (DNA) was done by QIA amp kit agreeing to the technique defined by Shah et al. (2009). DNA amplification was achieved on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). The magnification circumstances besides reagents for the PCR examines were those designated via (Fagan et al., 1999). PCR fractions were examined by 2% agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer discolored with ethidium bromide and taken besides imagined on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was utilized to show the piece size.

Primer sequence

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
stx1 (F)	**5′** ACACTGGATGATCTCAGTGG **′3**	614	Dhanashree and Mallya (2008)
stx1 (R)	**5′** CTGAATCCCCCTCCATTATG **′3**		
stx2 (F)	**5′** CCATGACAACGGACAGCAGTT **′3**	779	
stx2 (R)	**5′** CCTGTCAACTGAGCAGCACTTTG **′3**		
eaeA (F)	**5′** GTGGCGAATACTGGCGAGACT **′3**	890	Mazaheri et al. (2014)
eaeA (R)	**5′** CCCCATTCTTTTTCACCGTCG **′3**		
hylA (F)	**5′** ACGATGTGGTTTATTCTGGA **′3**	165	Fratamico et al. (1995)
hylA *(R)*	**5′** CTTCACGTGACCATACATAT **′3**		

2.2 Antimicrobial Sensibility testing:

Antimicrobial sensibility of the detected strains was tested via the single diffusion method according to Mary and Usha (2013) for *E.coli*. the following antimicrobial disks (*Oxoid Limited, Basingstoke, Hampshire, UK*) were used: Erythromycin (15Mg), Cephalothin(30Mg), Kanamycin (30Mg), Gentamicin (10Mg), Amikacin (30Mg), Ciprofloxacin (5Mg), Streptomycin (10Mg), Cefotaxim (30Mg), Neomycin (30Mg), Ampicillin (10Mg), Tetracycline (30Mg),Chloramphenicol(30Mg),Nalidixic acid(30Mg) and Sulphamethoxazol (25Mg).After incubation at 25°C for 2-7 days the vulnerability of the different *Escherichia coli* strain to each antibiotic was specified and the outcomes were tabulated as susceptible, intermediate and resistant agreeing to the rules specified by National Committee for Clinical Laboratory Standards "NCCLS" (2001)

Multiple Antibiotic Resistance (MAR) index for every isolate was evaluated agreeing to the way specified by Singh et al. (2010)

MAR index= No. of resistance (strains categorized as intermediate were believed sensitive for MAR index) / Total No. of verified antibiotics

2.3 Determination of Titratable Acidity of yoghurt:

Determination of titratable acidity was agreeing the technique that is used by Caric et al. 2000. Conical flask utilized by a transmission pipette 20ml yoghurt and 1 ml of 2% w/v solution of ph.ph indicator. Then it's titrated with 0.1 M NaOH sol. to presence the faint pink color which shall not develop absent more than 2 minutes. Titratable acidity of yoghurt is evaluated via the equation: K = V·2, wherever V- volume that is used up from alkaline.

Statistical analysis

Data are summarized as mean and standard error which it is quantitative and qualitative data are presented as number and percentage. The investigation is occurring by SPSS (Statistical Set for Social Science) software version 16.

3. RESULTS AND DISCUSSION

Cattle are a main habitant of toxigenic *E. coli*, and it is cause of *E. coli* that passes on a sickness and illness to human beings *(*Hornitzky et al, 2002)

Information recorded in Table (1) showed that the occurrence of Vero toxigenic *E.coli* was 4(3%) in the total examined 130 samples. The highest prevalence was found in fresh soft cheese that was 3 (10%) followed by ice cream samples as the prevalence rate was 1(2%) while yoghurt samples were free from toxigenic *E.coli*. Nearly similar result (3.3%) was stated by Erol et al. 1998 in ice cream samples. while higher results in ice cream were recorded by Kivanc et al. 1994, Toklu and Yaygin 2000, Khalil et al. 2009 and Fadel and Jehan 2009 22%, 70%, 100% and 44.4%, respectively.

Dirty ice-cream resulted in numerous problems of intestinal sicknesses at a numeral of states in Asia, Europe then North America Yaman et al., 2006. Primary causes of bacterial pollution of ice-cream comprise contaminated water besides non-heat treated milk (raw materials),while other causes include additive agents, coloring materials, utensils, management and from dirty air during manufacturing (Khalil et al., 2009) .

Desehenes et al. 1996 and Baylis CL. 2009 are reported that cheeses have been before linked by diseases comprising infective serotypes of Escherichia coli. Also Fegan and Desmarchelier 2010 registered that a variety of dairy products have associated in E. coli sickness including milk, cheese, yoghurt besides ice-cream

Concerning with fresh soft cheese, similar results were reported by Stephan et al. (2008) 5/52 (9.6%) from soft cheese in Switzerland, while nearly similar results were described by Vernozy-Rozand et al. (2005) (13%) in cheese.

On the other hand, lower consequences were recorded via Quinto and Cepeda (1997) (0.4%) within soft cheese at Spain. However, higher outcomes were stated by Araujo et al. (2002) (95.5%) in Brazil.

The Egyptian standards for ice- cream and soft cheese proposed that E. coli should not be detected in the ice-cream and soft cheese (Ministry of Industry and Technological Development, 2000) Contamination represents a public health risk.

Table (1): Prevalence of *Vero toxigenic E.coli* in samples.

Type of samples	Number of inspected samples	Number of positive samples	Percent (%) of Positive samples
Fresh soft cheese Samples	30	3	10
Ice cream Samples	50	1	2
Yoghurt Samples	50	0	0
Total samples	130	4	3%

Table (2): Acidity percent of the examined yoghurt samples.

No. of examined samples	Minimum	Maximum	Mean ± SE
50	0.9	1.82	1.1872 ± 0.2859

Table (3): Serological typing of *the E.coli* isolates.

Serial No.	Examined product	Identified Bacterium	Serodiagnosis
1	Ice cream	*Escherichia coli*	O26 : H11
2	Fresh soft cheese	*Escherichia coli*	O55 : H7
3		*Escherichia coli*	O55 : H7
4		*Escherichia coli*	O91 : H21
5		*Escherichia coli*	O55 : H7

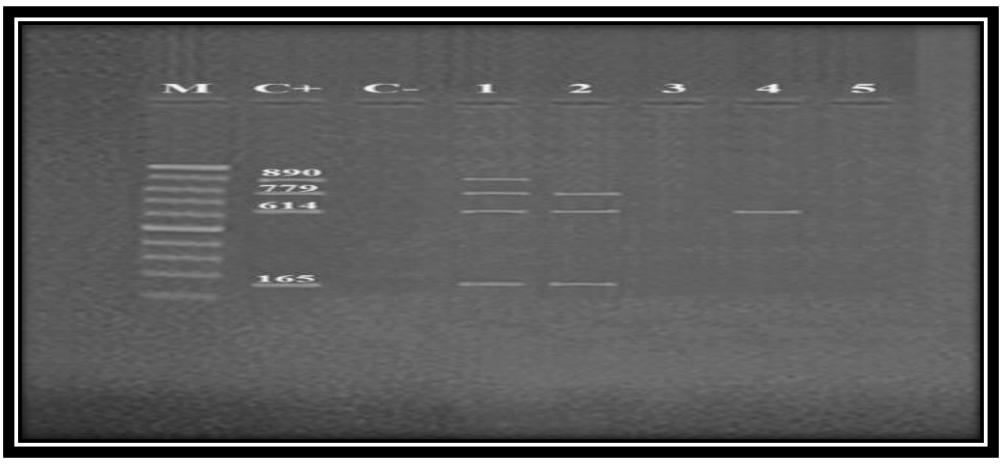

Figure (1): Agarose gel electrophoresis of multiplex PCR of *stx1* (614 bp), *stx2* (779 bp), *eaeA* (890 bp) and *hlyA* (165 bp) virulence genes for description of *Vero toxigenic E. coli.*
Lane M: 100 bp ladder as molecular size DNA marker.
Lane C+: Control positive *Escherichia coli* for *stx1*, *stx2*, *eaeA* and *hlyA* genetic factors.
Lane C-: Control negative.
Lanes 1 (O26) that was isolated from ice cream: Positive *Escherichia coli* strain for *stx1*, *stx2*, *eaeA* and *hlyA* genetic factors.
Lane 2 (O91)that was isolated from fresh soft cheese: Positive *Escherichia coli* strain for *stx1*, *stx2* and *hlyA* genes.
Lane 4 (O55) that was isolated from fresh soft cheese : Positive *Escherichia coli* strain for *stx1* gene.
Lanes 3 (from ice cream) & 5 (from fresh soft cheese)(O55): Negative *Escherichia coli* strains for *stx1*, *stx2*, *eaeA* and *hlyA* genes.

Table (4): Antimicrobial susceptibility of *E. coli* (n=5).

Antimicrobial agent	S		I		R	
	NO	**%**	**NO**	**%**	**NO**	**%**
Streptomycin (S)	-	-	-	-	5	100
Nalidixic acid (NA)	-	-	1	20	4	80
Erythromycin (E)	1	20	-	-	4	80
Amikacin (AK)	-	-	2	40	3	60
Cefotaxim (CF)	1	20	1	20	3	60
Sulphamethoxazol (SXT)	1	20	1	20	3	60
Tetracycline (T)	2	40	-	-	3	60
Cephalothin (CN)	-	-	3	60	2	40
Chloramphenicol (C)	1	20	2	40	2	40
Ampicillin (AM)	2	40	1	20	2	40
Kanamycin (K)	3	60	-	-	2	40
Ciprofloxacin (CP)	3	60	-	-	2	40
Neomycin (N)	3	60	1	20	1	20
Gentamicin (G)	4	80	-	-	1	20

Table (5): Antimicrobial resistance outline of *E. coli* (n=5).

NO	Examined product	*E. coli* Strains	Antimicrobial resistance outline	MAR index
1	*Ice cream*	O26 : H11	S, NA, E, AK, CF, SXT, T, CN, C, AM, K, CP, N, G	1
2	*Fresh soft cheese*	O91 : H21	S, NA, E, AK, CF, SXT, T, CN, C, AM, K, CP	0.857
3	*Ice cream*	O55 : H7	S, NA, E, AK, CF, SXT, T	0.500
4		O55 : H7	S, NA, E	0.214
5	*Fresh soft cheese*	O55 : H7	S	0.0.71
			Average	***0.528***

S: Streptomycin E: Erythromycin CF: Cefotaxim NA: Nalidixic acid
SXT: Sulphamethoxazol AM: Ampicillin C: Chloramphenicol AK: Amikacin CN: Cephalothin
T: tetracycline N: Neomycin K: Kanamycin
CP: Ciprofloxacin G: Gentamicin

In table (1) my yoghurt examined samples were free from E.coli. In the contract Darma et al., 2016 and Tankoano et al., 2016 detected E.coli in 26% and 6.67%, respectively.

In table (2) the mean of acidity% in the observed yoghurt samples was 1.1872. I measured it in yoghurt because I observed sour flavor from some samples although I was collected its fresh .the great sourness at yoghurt specimens probably because of accumulative the quantity of starter, extensive incubation interval or inadequate refrigeration next clotting. Additional, great degree of the sourness in the inspected specimens stayed adequate to prevent or inhibit greatest microbes. The inactivation of these bacteria was covered through the creation of antimicrobial materials by lactic acid microorganisms at yoghurt (Arnott et al., 1974).

Recent readings about probiotics exposed that the fermented foodstuffs of probiotics have powerful anti-bactericidal impacts opposite foodborne illness Yesillik et al. 2011

The Vero toxigenic Escherichia coli that is usually mentioned to Shiga-toxin generating Escherichia coli includes above four hundreds strains of Escherichia coli (Scheutz and Strockbine 2005). Clinical signs of VTEC illness variety from slight diarrhea till life-intimidating circumstances like hemorrhagic colitis, serious problems of hemolytic uremic syndrome and thrombotic thrombocytopenic purpura, that can end in nephritic destruction, renal failure then even killing (Tarr et al. 2005).

Strains of pathogenic E.coli that are isolated from examined fresh soft cheese and ice cream were illustrated as one O91: H21 isolate (from one fresh soft cheese sample), three O55: H7 isolates (from one ice cream and two fresh soft cheese samples) and one O26: H11 isolate (from the same one ice cream sample) (Table 3).

These are approved by Vernozy-Rozand et al. 2005 and Farrokh et al 2012 as they recorded that Shiga toxin Escherichia coli serotypes have been detect in milk and cheeses

Araujo et al. 2002 detected E.coli O26 plus O55 in soft cheese in Rio de Janeiro, Brazil, also the occurrence of E. coli O26 illness at France was happened in 2005 by ingestion of cheese from raw milk (Espie et al., 2006).

While Stephan et al. (2008) found E.coli O91: H21 in soft cheese in Switzerland.

On the other hand, Fadel and Jehan (2009) isolated O26:H11 from ice cream in different localities in Ismailia city and A 5 infected cases with HUS-outbreak caused by O26:H11 that related to ice-cream prepared by pasteurized milk (Buvens et al., 2011), while Martin and Beutin (2011) isolated O91:H21 from dairy products and Momtaz et al. (2012) isolated O26 and O91 from dairy products in Iran.

Enter pathogenic Escherichia coli (EPEC) strains as E.coli O55 is source of severe diarrhea between children in developing states through the countries and have been insulated from an extensive extent of food and milk products (Levine 1987) also Nataro and Kaper, 1998 recorded that O55 commonly involved in human diseases.

E.coli O157:H7 cannot found in the present research**.** Similar result reviewed by Stephan et al. (2012**)** as they recorded that in their examined dairy samples, O157:H7 developed fewer powerfully besides was lower tenacious than different strains.

The maximum significant virulence features of Shiga toxin *Escherichia coli* are the two phage toxins, named Shiga toxin 1 and Shiga toxin 2, the protein intimin (eae), besides the plasmid-encoded enterohaemolysin (ehly) (Law 2000).

In my research *E.coli* O26 that was isolated from ice cream sample was positive to all previous virulence genes so it is high pathogenicity also *E.coli* O91 that was isolated from fresh soft cheese was Positive for stx1, stx2 and hlyA genes that indicated strongly dangerous (Figure 1). Many researches as DebRoy et al. 2004, Heijnen and Medema 2006, Erickson and Doyle 2007, and Lin et al. 2011 were indicated that many *E. coli* serogroups such as O26, O91 were produce Shiga toxins.

From the other side, one of the three *E.coli* O55 that were separated from fresh soft cheese was Positive for stx1 gene only (Figure 1)

This result is similar to (Campos et al., 1994) as they reported that Various serotypes of EPEC leading to a various degrees of illness , and it has been exposed that these serotypes are usually altering and their genetic factors come to be like to hemolytic serotypes. And also Neill 1997 said that certain Vero toxigenic *Escherichia coli* serotypes missing intimin and enterohaemolysin genes have in some time been exposed to cause human disease, so those genetic factors may be not essentially for Vero toxigenic *Escherichia coli* to create sickness, even though Burland et al. 1998 reviewed that serotypes having eaeA are frequently linked with more intensive forms of sickness

Commensal *E. coli* strains were vulnerable to a great amount of antimicrobial agents (Kumar et al 2014), but, due to the wide use and unrestrained treatment of farm animals (Van et al. 2007 and 2008), antimicrobial-resistant strains have develop a serious problem (Van et al. 2008)

From tables 4 and 5, in all detected five isolates the maximum resistance (100%) was detected against the Streptomycin followed by Nalidixic acid (80%) and Erythromycin (80%) while the least resistance was observed against Neomycin (20%) and Gentamicin (20%).The highest sensitivity was recorded for Gentamicin (80%) and no sensitivity was recorded against Streptomycin.

In this research the highest resistant (100%) was in streptomycin. In contrast with present work low resistance to streptomycin was described (57.89%) by Thaker et al. 2012 and Virpari et al. 2013(23.91%) Also in the current research the least resistance was observed against Neomycin (20%) and Gentamicin (20%). Nearly similar results were reported by Virpari et al. 2013 as they recorded that no resistance was observed against Gentamicin. While Ranjbar et al. 2018 recorded that the highest resistant (100%) was against gentamicin.

On the other hand, 60% of E. coli isolates were withstands Cefotaxim, in difference with current result lower confrontation was registered via Virpari et al. 2013 (6.52 %).While 40% of isolates were reported strength to the Ampicillin .The corresponding result was recorded via Virpari et al. 2013. But Ranjbar et al. 2018 recorded STEC serotypes had the extreme incidence of confrontation in contrast toward ampicillin (100%). On the other hand 60% of isolates in my study were resistance against tetracycline. Higher resistance (96.87%) was registered by Ranjbar et al. 2018

The higher multiple antibiotic resistance index (MAR) (1) was in E.coli O26 that was resistant to all fourteen using antibiotic while MAR was 0.857 in O91: H21 as it was resistant to twelve antibiotics and susceptible to Neomycin and Gentamicin.

on the other hand E.coli O55 that was isolated from fresh soft cheese and was Positive for stx1 gene was resistant to Streptomycin, Nalidixic acid, Erythromycin and it's MAR was 0.214 but E.coli O55: H7 that was insulated from ice cream and was negative to all examined virulence genes was resistant to Streptomycin, Nalidixic acid, Erythromycin, Amikacin, Sulphamethoxazol and

tetracycline and it's MAR was 0.500.This mean it is not a condition that E.coli have the virulence genes to become multiple drug resistance.

These previous Multiple drug resistance were resulted from The worldwide overdoing or ill use of antibiotics in varied fields, comprising human drugs and veterinary drugs as protective additions or development-stimulating means in the animals fodder, have produced antimicrobial resistance between microbial pathogens and endogenous micro flora (WHO, 2000).

5. CONCLUSION

Outcomes of this work record that some of fresh soft cheese and small scale production ice cream at retail markets located in Mansoura city haven multidrug-resistant VTEC and several researches register that VTEC is very dangerous and can cause severe disease and even death especially in children and old people.

So application of hygienic practices during production, transportation, besides storage is very important as Infection of ice cream either originate from polluted water, absence of individual cleanliness of the ice cream producer, tools utilized for ice cream, distribution besides surroundings. Also fresh cheese may be produced from raw milk or also from previous contamination sources

The protection of dairy products will be confirmed by continuous awareness of the threats and managing of the food safety hazards over the dairy product supply chain from farm to consumer as application of HACCP, good hygienic systems in addition to perfect manufacturing systems and also More researches are wanted to evaluate the prevalence of VTEC in various dairy products besides how we can control and eliminate of these pathogens from dairy products by using natural ways.

DECLARATION OF INTEREST

The author records no fights of benefit. The author only is accountable for the content and lettering of this paper

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