Bacteriological quality and safety of four fluid dairy products sold in El Fayoum Governorate, Egypt

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The study was designed to assess the safety and bacteriological quality of 120 samples, including small-scale fluid cream, large-scale Laban rayeb, pasteurised milk, and ultra-high temperature milk (UHT). Thirty samples of each product were collected from different localities in El Fayoum province, Egypt. Samples were analysed for the total bacterial count (TBC), total coliforms, faecal coliforms, *Escherichia coli*, and Staphylococcus aureus. The mean value of TBC in small-scale fluid cream and pasteurised milk were $1.68 \times 10^6 \pm 1.3 \times 10^5$ and $4.30 \times 10^3 \pm 6.66 \times 10^2$ CFU / ml, respectively. The mean value of faecal coliforms in fluid cream was $1.87 \times 10^4 \pm 8.18 \times 10^3$ CFU / ml. *E. coli* was only present in fluid cream with a mean value of $2.25 \times 10^3 \pm 8.63 \times 10^2$ CFU / ml. Isolated *E. coli* strains were serologically identified as O125(16/30), O158 (10/30), O157 (4/30), with a percentage of 53.33, 33.33 and 13.33% respectively. Conventional polymerase chain reaction (PCR) identified the presence of *aroA* and *fimH* but not *Stx1* and *Stx2* genes. *S. aureus* was detected in the examined fluid cream samples, with a mean value of $7.56 \times 10^4 \pm 8.81 \times 10^3$, CFU / ml. High microbial counts of TBC, *E. coli* and *S. aureus* in fluid cream may present a public health hazard to the consumers. Therefore, it is necessary to improve the quality of locally produced cream to diminish the hazard from that product.

1.Introduction

Safe and healthy foods are a mandatory requirement for the maintenance of vital functions. Food safety is a basic concern of both the food industry and consumers because of the dramatically increased number of foodborne diseases. The World Health Organization (WHO) stated that more than 100 million people living in the Eastern Mediterranean region, including countries in the Middle East and North Africa, become ill with a foodborne disease every year (World Health Organization, 2015). Food problems such as, inability to provide food safety are becoming gradually more complex because of the increase in food varieties in the world (Yoruk, 2018).

Milk contains many components that make it a highly nutritious food; equally, it provides a good environment for the growth of a wide variety of microbes. Bacteria can contaminate raw milk through colonisation of the teat canal or an infected udder, or by cross-contamination through the surface of the teats, by air, from manual or mechanical milking contact, water and milk contact surfaces, and storage and transport equipment (Baylis, 2009; Bytyqi et al., 2011).

As a result of the common Egyptian belief that raw milk has health benefits and high nutritional value, the consumption of raw milk is more common than heat-treated milk. However, the consumption of raw milk and its products, such as small-scale cream, have been linked to many forms of bacterial infection, including infection with *Brucella* spp., *S. aureus*, *Salmonella*, tuberculosis, as well as *Yersinia* and pathogenic *E. coli* food poisoning (Baylis, 2009).

Raw milk (or cream) is the basic material from which all dairy products are made. The quality and safety of the final product is greatly affected due to the diversity of microorganisms as well as the level of contamination in the raw materials. Reports outlined by the Centers for Disease Control and Prevention in the period from 1993 to 2006 indicated the risk of causing outbreaks and outbreak-associated illnesses by non-pasteurised milk and milk products exceeded that of pasteurised products by 150 times (Langer et al., 2012).

The current study included some of the dairy products available and circulated in the Egyptian market, including pasteurised milk, sterilised milk and unpasteurised cream. The study is unique in dealing with a product that is only available in the Egyptian market- Laban rayeb, which is a fermented milk made by Egyptian farmers. Fresh milk is placed in an earthenware pot "Matrad" or "Shalia" and left undisturbed in a warm place until the cream rises and the lower, partially skimmed milk coagulates. The layer of cream is removed and made into butter, while the remaining curd 'Laban rayeb' is either consumed as a pasteurised or unpasteurised fermented milk or is converted to a soft acid cheese known as Kareish (El-Gendy, 1983).

Escherichia coli and coliforms in food imply poor hygiene and sanitary practices (Ekici & Dumen, 2019). Their presence increased prevalence is typically due to factors, such as poor sanitation and lack of basic infrastructure like electricity and an adequate water supply (Martin et al., 2016).

E. coli live commensal in the gastrointestinal tract of most mammals, including humans, without causing disease. However, a small fraction of *E. coli* are human pathogens and have been implicated in foodborne illnesses with increasing frequency over the last two decades. Ingestion of enteropathogenic *E. coli* (EPEC)

can result in a mild gastrointestinal disease that is relatively self-limiting. Still, a subset of pathogenic *E. coli*, enterohemorrhagic *E. coli* (EHEC), can cause hemolytic-uremic syndrome (HUS), a serious, potentially fatal illness. *E. coli* has been isolated from a number of food products, including meat, fruits, milk and milk products, which can act as a medium for foodborne disease transmission (Lee et al., 2009; Solomakos et al., 2009; Maffei et al., 2013). The most pathogenic strains are referred to as Shiga toxin-producing *E. coli* (STEC) including *E. coli* O157:H7. Bulk tank milk often contaminated with EHEC through cattle faeces which is considered the major reservoir of this pathogen. Therefore, raw milk poses a risk for STEC, and some outbreaks with dairy products have been recently reported (Dhanashekar et al., 2012; Van Asselt et al., 2017).

An important feature of the STEC group, implicated in many outbreaks, is its ability to resist acidic pH (close to pH of 2.5), which enables it to survive in foods with low pH values and genes that also enable the pathogen to attach to the gastrointestinal tract (Cutrim et al., 2016; Salim et al., 2017). *FimH* is a mannose-specific adhesion protein that is responsible for mediating bacterial attachment and invasive properties of *E. coli* (Chassaing et al., 2011).

S. aureus is an organism that causes foodborne intoxication, usually within four to six hours after eating food containing the enterotoxin (De-Buyser et al., 2001; Zeinhom et al., 2015). *S. aureus* enterotoxins are heat-resistant and not inactivated by the majority of ultra-high temperature (UHT) processes (David et al., 1996). *S. aureus* presence in food is an indicator of its contamination by people during the production and handling of dairy products or the direct shedding into milk from diseased animals.

The objective of this study was to assess the prevalence of some foodborne pathogens as an indicator for food safety and quality in the most popular Egyptian dairy foods, including unpasteurised cream, pasteurised Laban rayeb, pasteurised milk, and UHT milk, address pathogenicity factors and to determine the prevalence, serotypes and virulence genes of isolated *E. coli* strains.

2. Materials and Methods

2.1. Samples

A total of 120 samples, including unpasteurised cream, pasteurised Laban rayeb, pasteurised milk and UHT milk. Thirty samples of each product were collected randomly from different localities in El Fayoum province, Egypt, during the winter season. Unpasteurised cream samples (200 ml) were collected from three separation centres (containing 3 hand-operated separators) from farmer's containers and placed in sterile screw bottles. Other pasteurised samples of dairy products (500 ml each) were purchased in their retail packages from one producer, but with different lot number and from different local grocery stores over several months. Samples were taken to the laboratory in an insulated icebox (3–5°C) within 2 h of purchase for examination (APHA, 1992).

2.2. Determination of acidity%

Titratable acidity (TA) (as lactic acid %) of cream, Laban rayeb, pasteurised milk and UHT milk was measured following the description by AOAC (2000). Briefly, 10 ml of well-mixed samples were placed in a clean porcelain dish and diluted with 20 ml of CO_2 free water. One ml of 1% phenolphthalein (alcoholic solution) was added. After thoroughly mixing, the contents were titrated against N/9 sodium hydroxide solution to the first persistent pink shade. The acidity per cent was calculated according to the following formula:

Acidity % = R/10
R= No. of ml of N/9 NaOH used in titration

2.3. Preparation of samples for microbiological examination

Eleven ml of well-mixed samples was added to 99 ml of sterile peptone water 0.1% to make a dilution of 1/10 from which 10-fold serial dilutions were made (Oxoid, Ltd, Basingstoke, Hampshire, UK)

2.4. Total bacterial count

One ml of each dilution was transferred into duplicated labelled Petri dishes 12–15 ml of liquefied sterile plate count agar at 44°C–46°C were poured into each plate, then incubated at 35°C for 48 h. Negative control sterile plate count agar was used. Plates that had 30 to 300 colonies were counted (Oxoid, Ltd, Basingstoke, UK)

2.5. Total coliforms count (MPN) technique

The test was performed using lauryl sulphate tryptose broth (LST) and Brilliant-green Lactose Bile 2% broth with inverted Durham's tubes according to (APHA, 1992).

2.6. Faecal coliform count (MPN/ml)

A loopful from each positive LST broth was inoculated in sterile tubes of E. coli broth (EC broth). The inoculated tubes, as well as the control ones, were incubated in a thermostatically controlled water bath at 44.5°C for 48 h. Positive tubes showing gas production were recorded. (APHA, 1992).

2.7. E. coli count, biochemical identification, and serology

A loopful from each positive EC broth tubes (showing gas production) was streaked onto Eosin Methylene Blue agar (EMB) (Oxoid, Ltd, Basingstoke, UK). The inoculated plates, as well as the control negative ones, were incubated at 35 + 1°C for 24 h. The plates were examined for typical nucleated, dark centred colonies with or without a green metallic sheen. Two typical colonies were transferred to plate count agar slant. Slants were incubated at 35°C for 18–24 h, and the purified colonies were submitted for further biochemical identification, done using four tests: indole, methyl red, Voges-Proskauer, and citrate utilisation (APHA, 1992).

Serological characterisation of E. coli isolates was carried out using the slide agglutination method with polyvalent and monovalent antisera. The isolates were first tested with OK polyvalent antisera. Briefly, two separate glass slides were used. A saline solution was added to the glass slide, followed by the addition of a portion of a colony from the suspect culture, mixed to form a smooth, dense suspension. To the first glass slide (control) only a drop of saline was added and mixed. To the second, an undiluted antiserum was added then tilted back and forward for one minute. Agglutination was observed using indirect lighting over a dark background. When a colony gave a strongly positive agglutination with one of the pools of poly-

valent serum, a further portion was inoculated onto a nutrient agar slant (Oxoid, Ltd, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 h to grow as a culture for testing with O monovalent antisera for serogroups O26, O55, O86, O111, O114, O119, 125, O126, O127, O142 and O158. The strains belonging to the same serogroups and isolated from the same samples were reported only once. Positive control strains obtained from National Research institute; Cairo were included in each experiment run.

2.8. Detection of virulence genes in *E. coli* using PCR.

2.8.1. Extraction of DNA

DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Briefly, 1.5 ml of an overnight broth culture of *E. coli* grown in MacConkey broth at 37°C was centrifuged in a benchtop centrifuge at 8000 rpm for 5 min and the supernatant discarded. The cell pellet was resuspended in PBS to a final volume of 200 ml. QIAGEN protease (20 ml) was pipetted into the bottom of a 1.5 ml microcentrifuge tube then 200 ml of the sample and 200 ml buffer AL were added and mixed by pulse vortexing for 15 seconds. After that, the mixture was incubated at 56°C for 10 min and centrifugated to remove drops from inside the lid. 200 ml ethanol (96%) were added to the sample and mixed again by pulse vortexing for 15 seconds. After mixing, centrifugation was used to remove drops from inside the lid. The mixture was carefully applied to the QIAamp Mini spin column (in a 2 ml collecting tube) for DNA extraction. The DNA concentration was measured using a spectrophotometer (DU530, Beckman, CA). An average of 10 mg of DNA was obtained.

2.8.2. Cycling conditions of the primers during PCR.

The *fimH*, *aroA, Stx1* and *Stx1* genes for *E. coli* were amplified by a multiplex PCR as described by (Dipineto et al., 2006; Ghanbarpour and Salehi, 2010; La Ragione et al., 2013) as shown in (Table 1). DNA (6 ml of 30–40 ng/ml) was assayed in a 25 μL reaction mixture containing 12.5 μL of Emerald Amp GT PCR master mix (2x premix), 4.5 μL of PCR grade water, 1 ml of forward primer (20 pmol) and 1 ml of reverse primer (20 pmol) according to Emerald Amp GT PCR master mix (Takara), code number RR310AKit. The initial denaturation for *fimH* was for 5 min at 94°C followed by 35 cycles of 94°C for 30s, 50°C for 30s, 72°C for 45s, and a final extension at 72°C for 7 min. The initial denaturation for *Stx1* and *Stx2* was for 5 min at 94°C followed by 35 cycles of 94°C for 30s, 58°C for 40 s, 72°C for 45s, and a final extension at 72°C for 10 min. Lastly, the initial denaturation for *aroA* was for 5 min at 94°C followed by 35 cycles of 94°C for 30s, 50°C for 40 s, 72°C for 1.2 min, and a final extension at 72°C for 12 min. Running gel electrophoresis of 20 ml of the reaction product in a 1.5% agarose gel (AppliChem, Ottoweg 4 Darmstadt, Germany) at 1–5 volts/cm of the tank length for 30 min and the gel was transferred to UV cabinet and photographed by gel documentation system (Alpha Innotech, Biometra, San Francisco, USA), and the data were analysed using computer software. The experiment was repeated three times. The DNA was extracted from the positive control reference strains obtained from the National Research Institute, Cairo, Egypt (Sambrook et al., 1989).

2.9. Enumeration, isolation, and identification of *S. aureus*

One hundred microliters from each dilution were evenly spread over a dry surface of Baird parker agar plates (Oxoid, Ltd, Basingstoke, UK) by using a sterile L-shaped bent glass rod. Streaked plates, as well as the control ones, were incubated at 37°C for 24 h. Suspected colonies (black, shiny with a narrow white margin and surrounded by a clear zone extending into the opaque medium) were counted. The plates were re-incubated for another 24 h before being counted again for further growth. Suspected colonies were picked up and streaked onto agar slants for further identification, which was done using catalase test, citrate utilisation, urease production, and coagulase test (APHA, 1992).

3. Results and Discussion

3.1. Acidity values

The titratable acidity % in the examined fluid cream, Laban rayeb, pasteurized milk, and UHT samples (Table 2) was ranged from 0.01to 0.79, 0.10 to 1.5, 0.1 to

0.25 and 0.13 to 0.17, respectively with a mean value of 0.19 ± 0.04, 0.89± 0.05, 0.15 ± 0.01 and 0.15 ± 0.002, respectively.

Titratable acidity of milk and milk products is considered an index of quality (Griffiths et al., 1988). Other publications reported the acidity percentages in milk and dairy products. In fluid cream, it was 0.20 ± 0.01(Meshref, 2013) and in Laban rayeb, it was 0.68 ± 0.14 (Ahmed et al., 2014). Increased acidity in the fresh cream may be attributed to milk sitting for long periods at room temperature (20–25°C) before the process of separation for long periods, during which lactic acid-producing bacteria and other types of harmful bacteria flourish. The use of dirty utensils and milk pots, in addition to the unclean hands of milkers, constitute the most important sources of milk contamination with lactic acid bacteria (Robinson, 2002). Milk fermentation converts lactose into organic acids, including acetic acid and lactic acid, which decreases the pH of the milk from 6.8 to less than 4.6, thus lowering the risk of fermented milk being contaminated with pathogens (Al-Kadamany et al., 2001).

Table 1. Primers sequences used for detection of *E. coli*

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
fimH	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508 bp	Ghanbarpour and Salehi (2010)
Stx1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614 bp	Dipineto et al. (2006)
Stx2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779 bp	
aroA	ATCCCTGACGTTACAACC TCCGCGCCAGCTGCTCGA	1261 bp	La Ragione et al. (2013)

Table 2. Ttitratable acidity% of dairy samples (n=120) collected from El Fayoum Governorate, Egypt.

Samples	No	Min	Max	Mean ± SE
Cream	30	0.01	0.79	0.19 ± 0.04
Laban rayeb	30	0.10	1.50	0.89 ± 0.05
Pasteurized Milk	30	0. 1	0.25	0.15 ± 0.01
UHT milk	30	0.13	0.17	0.15 ± 0.002

The bacterial count in fluid dairy products potentially reveals the general conditions of sanitation and temperature control under which fluid dairy products were produced, handled, and held. The TBC of fluid cream, pasteurised milk and UHT milk ranged from 2×10^5 to 3×10^6, 1×10^2 to 1.05×10^4 and <10 CFU/ml, with a mean count of $1.68\times10^6 \pm 1.3\times10^5$, $4.3\times10^3 \pm 6.66\times10^2$ and 0 CFU/ml, respectively (Table 3).

In previous studies, the mean values of TBC in fluid cream were 1.8×10^3 CFU/ml (Halpin-Doshnalek & Marth, 1989) and 8.72×10^4 CFU/ml (Meshref, 2013). In pasteurised milk, higher values were reported by Dey & Karim (2013), who mentioned that the values of TBC ranged from 1.8×10^4 to 9.6×10^5 CFU/ml. Additionally, higher levels of TBC in pasteurised milk were reported by Silva et al. (2010), Koushki et al. (2016). They mentioned that 46.5% of the samples had a high total microbial count above 10^6 CFU/ml. The TBC of UHT milk must not be more than 10 CFU/ml (Egyptian Standards, 2005). The TBC of the UHT milk samples was <10, indicating their excellent sanitary quality (Table 3). High levels of TBC in UHT milk were reported by Shojaei & Yadollahi (2008) and Ayad et al. (2009) with mean values of 7.1×10^1 and 3.4×10^1 CFU/ml, respectively.

High TBC counts in fresh unpasteurised cream samples can be attributed to the lack of health knowledge among cream staff, the presence of residues of water on the separator surface that promotes the growth and multiplication of bacteria resulting in milk contamination, the use of dirty utensils, poor personal hygiene and the lack of heat treatment that contributes to the poor hygienic quality of fresh cream (Hayes & Boor, 2001). Although higher counts in pasteurised milk could be attributed to a defect in the pasteuriser, bacteria surviving pasteurisation, and post-pasteurisation contamination due to problems in processing, handling, and bad personnel hygiene (Koushki et al., 2016). Therefore, training and guidance should be given to farmers regarding general milking hygienic practices, pasteurisation of milk used in cream manufacturing, and regular cleaning and sanitation of the separator used for cream production after each production process to avoid microbial growth and lengthen the shelf life.

3.3. Total coliform, Faecal coliform and *E. coli*

Total coliforms were detected in 100, 16.66 and 23.33 % of the examined fluid cream, Laban rayeb and pasteurised milk samples with a mean value of $1.14 \times 10^5 \pm 3.9 \times 10^4$, 1.53 ± 0.79 and 2.53 ± 1.01 MPN/ml, respectively (Table 4). None of the examined UHT samples contained total coliform.

In Egypt, Meshref (2013), found a mean value of $4.21\times10^6 \pm 9.82\times10^5$ in fluid cream. In Hungary, Varga (2007) stated that none of the examined cream samples contained detectable levels of coliforms. There are many reasons for these differences, including different health practices during milking, the difference in climatic changes and lack of hygiene of the herd, polluted water, geographical distribution, equipment that are improperly maintained and washed and unhealthy milking procedures (Hossain et al., 2011).

Table 3. Count of TBC for the examined dairy products (n=120) sampled from El Fayoum Governorate, Egypt.

Sample type	Positive samples %	Min	Max	Mean ± SE
Cream	100	2×10^5	3×10^6	$1.68\times10^6 \pm 1.3\times10^5$
Pasteurized milk	100	1×10^2	1.05×10^4	$4.3\times10^3 \pm 6.66\times10^2$
UHT milk	0	<10	<10	0

Table 4. Incidence and mean count of Total coliform, Fecal coliform and *E. coli* in the examined dairy products (n=120) sampled from El Fayoum Governorate, Egypt.

Sample type	Total coliform count (MPN/ ml)		Fecal coliform count (MPN/ ml)		*E. coli* count (MPN/ ml)	
	Positive %	Mean ± SE	Positive %	Mean ± SE	Positive %	Mean ± SE
Cream	100	$1.14 \times 10^5 \pm 3.9 \times 10^4$	100	$1.87 \times 10^4 \pm 8.18 \times 10^3$	100	$2.25 \times 10^3 \pm 8.63 \times 10^2$
Laban rayeb	16.66	1.53 ± 0.79	0	0 ± 0	0	0
Pasteurized milk	23.33	2.53 ± 1.01	0	0 ± 0	0	0
UHT milk	0	0 ± 0	0	0 ± 0	0	0

Higher results of coliforms in Laban rayeb were reported by Ahmed et al. (2014), who revealed coliforms were present in 17.5% of the examined samples with a mean value of $2.5 \times 10^4 \pm 4.5 \times 10^4$ CFU/ml. The high number of coliform is a sign of poor hygienic processing conditions and post-processing contamination. Higher results of coliforms in pasteurised milk were reported by Koushki et al. (2016), who revealed that 76.6% of samples had coliform. Similar results for coliforms in UHT were reported by Al-Tahiri (2005) and Banik et al. (2014). Still, a higher result of total coliforms in UHT milk was obtained by Shojaei & Yadollahi (2008), which could be attributed to secondary bacterial contamination, and or the type of packaging (Tortorello, 2003).

According to Robinson (2002), the total coliform count in fresh cream should be less than 30 CFU/ml. This study found that all fresh cream samples (100%) were above this limit. The contamination of samples with coliform is due to many reasons, including direct faecal pollution, lack of personal hygiene and poor sanitary practices during the milking and handling process, which poses a potential hazard to people consume these products.

Unfortunately, there are no regulations about the production or consumption of unpasteurised dairy products in Egypt; however, Egyptian Standards mention the limits of some pathogens in raw milk and some dairy products. According to Egyptian Standards (2005), the permissible limit of coliforms in fresh cream, Laban rayeb, and pasteurised milk should be no more than10 MPN/ml, 100% of examined cream was incompatible with the legal standard (Table 5).

On the other hand, 16.66% of Laban rayeb was incompatible with the legal standard, 23.33% of pasteurised milk was incompatible with the legal standard, and all UHT milk samples were compatible with the standards. Pasteurised milk should not contain any coliform bacteria because it cannot withstand the pasteurisation temperature. The occurrence of bacterial contamination in pasteurised milk may be attributed to a defect in the pasteuriser or post pasteurisation contamination due to bad handling conditions (Banik et al., 2014).

Faecal coliforms and *E. coli* were detected in 100% of the examined fluid cream samples with a mean value of $1.87 \times 10^4 \pm 8.18 \times 10^3$ and $2.25 \times 10^3 \pm 6.63 \times 10^2$ MPN/ml, respectively. Faecal coliforms and *E. coli* of each of the Laban rayeb, pasteurised milk and UHT-processed milk samples were <3 CFU/ml (Table 4). Failure to find *E. coli* in the examined Laban rayeb samples may be attributed to the lower pH value. Several studies were able to detect and isolate *E. coli* from Laban rayeb (Olasupo et al., 2002) and pasteurised milk (El Zubeir et al., 2008; Vahedi et al., 2013).

Egyptian Standards (2005) stated that milk products should be free from any *E. coli* yet, 100% of the examined cream was incompatible with the legal standard. On the other hand, Laban rayeb, Pasteurised milk, and UHT milk samples were compatible with the standard. Coliform count and *E. coli* contamination are indicators of faecal contamination caused by using unpasteurised milk in the manufacturing of dairy products, pasteurisation deficiency, secondary contamination, and type of packaging (Tortorello, 2003).

Table 5. Acceptability of the examined dairy products (n=120) sampled from El Fayoum Governorate, Egypt based on *E. coli* count according to Egyptian standards (2005)

Product	Permissible limit of *E. coli*	Acceptable samples %	Unacceptable Samples
Cream	0 CFU/ml	0	100
Laban rayeb	0 CFU/ml	100	0
Pasteurized milk	0 CFU/ml	100	0
UHT milk	0 CFU/ml	100	0

3.4. Virulence genes and the serological analysis in *E. coli*

Serological identification of 30 isolates of *E. coli* was serotyped to O125, O157and O158 with an incidence of 53.33%, 33.33%, and 13.33%, respectively (Table 6). Four virulent genes were identified in all *E. coli* strains (Table 6 and Figure 1). Each of the *aroA* gene and *fimH* gene were detected in all serotyped *E. coli* with incidences 100%, 100% and 100% for O125, O157and O158. Moreover, the *Stx1* and *Stx2* genes were not detected in all serotyped *E. coli.*

Shiga toxin-producing *E. coli* (STEC) are considered the main causative agents of hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (Aidar- Ugrinovich et al., 2007). Consumption of undercooked meat, non-pasteurised dairy products and vegetables, and water contaminated with faeces are some of the possible routes for STEC human exposure (Hussein and Bollinger, 2005). Similar results were detected by Carneiro et al. (2006). They reported that the *Stx1* and *Stx2* genes were not detected in *E. coli* isolated from pasteurised cow milk sold in Rio de Janeiro, Brazil. Various studies have investigated the virulence genes of STEC in unpasteurised milk and its derivatives (Paneto et al., 2007; Solomakos et al., 2009; Islam et al., 2010; Rantsiou et al., 2012).

A study by Khairy et al. (2019) mentioned that the *fimH* gene was the most prevalent virulent gene (66.9% and 91.4%) in uropathogenic *E. coli* (UPEC) and diarrheagenic *E. coli* (DEC) strains, respectively. Moreover, Biscola et al. (2011) showed that in EHEC strains, the *fimH* gene was conveyed by the majority of non O157:H7 *E. coli* strains (97%) and by all the O157:H7 *E. coli* strains. Attachment of *E. coli* to the urothelial cell surface is mediated by *fimH* adhesion leading to urinary tract infections (Wojnicz, 2007; Kaczmarek et al., 2012). All these findings indicate that *E. coli* strains isolated in this study from cream samples might contribute to public health hazards due to the presence of such virulence genes. Therefore, strict hygienic measures should be taken to mitigate and prevent such contamination, for example, using large scale products which are recommended to reduce such risk.

Even though we did not detect STEC genes, the presence of *E. coli* in fluid cream indicates inadequate sanitary hygiene practices. It does not exclude the possibility that other pathotypes of *E. coli* are present which can present a health risk to consumers.

3.5. Incidence of *S. aureus*

In the present study, 30 (100%) of fresh unpasteurised cream samples were contaminated with *S. aureus* (Table 7) with a mean count of $7.56 \times 10^4 \pm 8.81 \times 10^3$ CFU/ml, but was not detected in the Laban rayeb, pasteurised milk and UHT milk samples (< 10 CFU/ml). All positive samples were above the limits (100 CFU/ml) established by Robinson's standards (2002). Egyptian Standards (2005) stated that the permissible limit of *S. aureus* to be no more than 10^2 CFU/ml; therefore, 100% of the examined cream samples were incompatible with the legal standard. On the other hand, Laban rayeb, pasteurised milk, and UHT milk samples were compatible with the standard.

The incidence of *S. aureus* in unpasteurised cream in this study was higher than those reported by Varga (2007), who failed to detect *S. aureus* in the examined cream samples and Meshref (2013), who reported

Table 6. Prevalence of some virulence genes among *E. coli* strain (N=30) isolated from the examined dairy products sampled from El Fayoum Governorate, Egypt.

E. coli strain	Sample NO	(%)	No of detected genes			
			Stx1	*Stx2*	*fimH*	*aroA*
O125	16	53.33	0	0	16/16 (100%)	16/16 (100%)
O157	10	33.33	0	0	10/10 (100%)	10/10 (100%)
O158	4	13.33	0	0	4/4 (100%)	4/4 (100%)
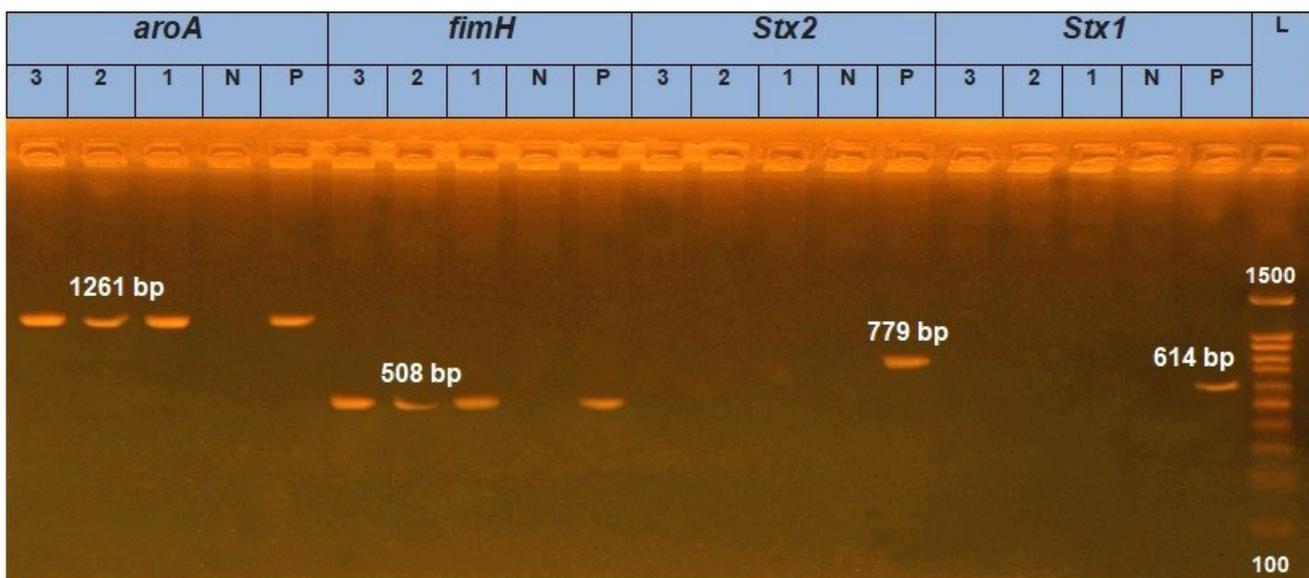
Figure 1. Agarose gel electrophoresis of PCR products after amplification of: 1- *fimH*, *aroA*, *Stx1* and *Stx2* genes for serologically identified *E. coli* strains, MWM-molecular weight marker (100 – 1500 bp DNA ladder), control (Positive, Negative), and different strains of *E. coli*. *fimH* gene products at 508 bp, *aroA* gene products at 1261 bp.

Table 7. Statistical analytical results of *S. aureus* in the examined dairy products (n=120) sampled from El Fayoum Governorate, Egypt.

Sample type	Positive %	Mean ± SE	Permissible limit of *S. aureus*	Acceptable samples %	Unacceptable Samples %
Cream	100	$7.56 \times 10^4 \pm 8.81 \times 10^3$	Not more than 10^2 CFU/ml	0	100
Laban rayeb	0	0 ± 0	Not more than 10^2 CFU/ml	100	0
Pasteurized milk	0	0 ± 0	Not more than 10^2 CFU/ml	100	0
UHT milk	0	0 ± 0	Not more than 10^2 CFU/ml	100	0

that 12 % of the examined fluid cream samples were contaminated with *S. aureus*. On the other hand, Halpin-Dohnalek & Marth (1989) tested the ability of different strains of *S. aureus* on the growth and production of enterotoxin in sweet and sour cream samples. They concluded that sweet and fresh cream with varying fat percentages supports the growth of *S. aureus* to a level sufficient for enterotoxin production. Several publications approved that at growth rate above 10^3 CFU/ml, *S. aureus* was probably able to produce enterotoxins. The count of $7.56\times10^4 \pm 8.81\times10^3$ CFU/ml reported in this study is considered dangerous and supposes the production of enterotoxins in the samples, which could create a public health hazard (Tebaldi et al., 2008; Zeinhom et al., 2015).

Higher results in Laban rayeb was recorded by Ahmed et al. (2014) who reported that 93.3 % of the examined Laban rayeb samples were contaminated with *S. aureus* with a mean count of $11.9\times10^3 \pm 39.5\times10^2$ CFU/ml. Additionally, higher *S. aureus* count in pasteurised milk was recorded by Vahedi et al. (2013). Similar results of *S. aureus* in UHT milk samples were recorded by Al-Tahiri (2005).

Cows with mastitis are the reservoir of toxigenic *S. aureus* strains in raw milk. Keeping raw milk at high room temperatures before separating the cream contributes strongly to the growth and multiplication of *S. aureus*, which secretes enterotoxin. This result highlights the unhygienic handling and inadequate personal hygiene. This study has found that the count of *S. aureus* in fresh cream was less than 10^6 - 10^8 CFU/ml, noting that this number is required for food poisoning to occur (Kerouanton et al., 2007). Moreover, the finding still presents a public health hazard, as sufficient toxins remain to elicit symptoms of staphylococcal food poisoning (Bennett and Monday, 2003). Therefore, we recommend the application of general health practices for local cream manufacturers to reduce bacterial contamination of cream and milk products, consistent food safety education, and the application of public health standards that would make a significant positive impact on food safety knowledge and hygiene practices of food handlers (Mgqibandaba et al., 2020).

4. Conclusion

The fresh cream produced by farmers in El Fayoum province, Egypt, is unsuitable for human consumption due to high microbial counts of SPC, *E. coli* and *S. aureus*. Using raw milk in cream manufacturing and separators are the main sources of cream contamination and frequently, the principal causes of high bacterial counts. The presence of pathogenic bacteria such *E. coli* and the prevalence of virulence genes specially *fimH* gene and *S. aureus*, may pose a risk for public health. Therefore, it is desirable to improve the hygienic status of locally produced fresh cream through educating the farmers in general hygienic practices and handling and storage of products to protect them from infection and deterioration. Also, information on health hazards associated with consumption of raw unpasteurised milk should be publicised, so that consumption of untreated raw milk and its products can be minimised. We plan to conduct a more extensive survey of cream samples to better assess the overall quality and variability in the quality of fresh cream.

Conflict of interest

The authors declare no conflict of interest.

References

Ahmed, L.I., Morgan, S.D., Hafez, R.S. & Abdel-All, A.A.A. (2014). Hygienic quality of some fermented milk products. International Journal of Dairy Science, 9, 63–73. https://doi.org/10.3923/ijds.2014.63.73.

Aidar-Ugrinovich, L., Blanco, J., Blanco, M., Blanco, J. E., Leomil, L., Dahbi, G., Mora, A., Onuma, D.L., Silveira, W.D., & Pestana de Castro, A.F. (2007). Serotypes, virulence genes, and intimin types of Shiga toxin-producing *Escherichia coli* (STEC) and enter pathogenic *E. coli* (EPEC) isolated from calves in São Paulo, Brazil. International Journal of Food Microbiology, 115, 297–306. https://doi.org/10.1016/j.ijfoodmicro.2006.10.046

Al-Kadamany, E., Toufeili, I., Khattar, M., Abou-Jawdeh, Y., Harakeh, S., & Haddad, Y. (2002). Determination of shelf life of concentrated yogurt (labneh) produced by in-bag straining of set yogurt using hazard analysis. Journal of Dairy Science, 85(5), 1023-1030. https://doi.org/10.3168/jds.S0022-0302(02)74162-3

Al-Tahiri, R. (2005). A Comparison on microbial conditions between traditional dairy products sold in

Karak and some products produced by modern dairies. Pakistan Journal of Nutrition, 4, 345–48. https://doi.org/10.3923/pjn.2005.345.348.

AOAC (2000). Official Methods of Analysis Association of Official Agriculture Chemists (17th Ed.). Wisconsin: Georgea Banta Co. Inc, Association of official Analytical Chemists.

APHA. (1992). Standard methods for the examination of dairy products (3rd Ed.). Washington. D.C.: American Public Health Association.

Ayad, E.H.E., Darwish, S.M., & Abu-Elreesh, S.M.I. (2009). The quality of market milk and some Egyptian dairy products collected from Alexandria City. Egyptian Journal of Dairy Science, 37, 139-159. https://www.cabdirect.org/cabdirect/abstract/20103015650.

Banik, S.K., Das, K.K., & Uddin, M.A. (2014). Microbiological quality analysis of raw, pasteurised, UHT milk samples collected from different locations in Bangladesh. Stamford Journal of Microbiology, 4, 5-8. https://doi.org/10.3329/sjm.v4i1.22753 .

Baylis, C.L. (2009). Raw milk and raw milk cheese as vehicles for infection by Verocytotoxin-producing Escherichia coli. International Journal of Dairy Technology, 62, 293–307. https://doi.org/10.1111/j.1471-0307.2009.00504.x

Bennett, R.W., & Monday, S.R. (2003). Staphylococcus aureus. In M.D. Miliotis, J.W. Bier (Eds.), International handbook of foodborne pathogens (pp.240-58). New York: Marcel Dekker. https://doi.org/10.1201/9780203912065.ch4.

Biscola, F.T., Abe, C.M., & Guth, B.E.C. (2011). Determination of adhesin gene sequences in, and biofilm formation by, O157 and non-O157 Shiga toxin-producing Escherichia coli strains isolated from different sources. Applied and environmental microbiology, 77, 2201-2208. https://doi.org/10.1128/aem.01920-10

Bytyqi H, Bigler S, Muji S, Jahja A, & Zaugg U. (2011). Survey on raw milk quality in Kosovo. Food and Nutrition Sciences, 2, 414–421. https://doi.org/10.4236/fns.2011.25058.

Carneiro, L.A., Lins, M.C., Garcia, F.R., Silva, A.P., Mauller, P.M., Alves, G.B., Rosa, A.C., Andrade, J.R.,

Freitas-Almeida, A.C., & Queiroz, M.L. (2006). Phenotypic and genotypic characterisation of Escherichia coli strains serogrouped as enteropathogenic   E. coli (EPEC) isolated from pasteurised milk. International Journal of Food Microbiology, 108, 15-21. https://doi.org/10.1016/j.ijfoodmicro.2005.10.010

Chassaing, B., Rolhion, N., de Vallée, A., Salim, S.Y., Prorok-Hamon, M., Neut Campbell, B. J., Soderhom, J.D., Hugot, J.P., Ugot, J.P., Colombel, J.F., & Darfeuille-Michaud, A. (2011). Crohn's disease associated adherent- invasive   E. coli bacteria target mouse human Peyer's patches via long polar fimbriae. The Journal of Clinical Investigation, 121(3), 966-975. https://doi.org/10.1172/jci44632

Cutrim, C.S., de Barros, R.F., da Costa, M.P., Franco, R.M., Conte-Junior, C.A., & Cortez, M.A.S. (2016). Survival of Escherichia coli O157: H7 during manufacture and storage of traditional and low lactose yogurt. LWT 70, 178-184. https://doi.org/10.1016/j.lwt.2016.02.047

David, J.R., Graves, R.H., & Carlson, V.R. (1996). Aseptic Processing and Packaging of Food: A Food Industry Perspective. New York: CRC Press, Inc.

De-Buyser, M.L., Dufour, B., Maire, M., & Lafarge, V. (2001). Implication of milk and milk products in foodborne diseases in France. International Journal of Food Microbiology, 67(1-2), 1-17. https://doi.org/10.1016/S0168-1605(01)00443-3

Dey, S., & Karim, M.H. (2013). Study on physicochemical and microbial quality of available raw, pasteurised and UHT milk during preservation. International Journal of Science Inventions Today, 2(2), 150-157.

Dhanashekar, R., Akkinepalli, S., & Nellutla, A. (2012). Milk-borne infections. An analysis of their potential effect on the milk industry. Germs 2, 101–109. https://doi.org/10.11599/germs.2012.1020 .

Dipineto, L., Santaniello, A., Fontanella, M., Lagos, K., Fioretti, A., & Menna, L.F. (2006). Presence of Shiga toxin-producing Escherichia coli O157: H7 in living layer hens. Letters in Applied Microbiology, 43, 293-295. https://doi.org/10.1111/j.1472-765x.2006.01954.x.

Egyptian Standards (2005). ES: 1623/ 2005, long life sterilised milk. Egyptian Organization for Standardization and Quality, Egypt.

Ekici, G., & Dumen, E. (2019). *Escherichia coli* and Food Safety. In The Universe of *Escherichia coli*. IntechOpen. https://doi.org/10.5772/intechopen.82375.

El-Gendy, S.M. (1983). Fermented foods of Egypt and the Middle East. Journal of Food Protection, 46, 358–367. https://doi.org/10.4315/0362-028X-46.4.358

El Zubeir, I.E., Gabriechise, V., & Johnson, Q. (2008). Comparison of chemical composition and microbial profile of raw and pasteurised milk of the Western Cape, South Africa. International Journal of Dairy Science, 3(3), 137-143. https://doi.org/10.3923/ijds.2008.137.143.

Ghanbarpour, R., & Salehi, M. (2010). Determination of Adhesin Encoding Genes in *Escherichia coli* Isolates from Omphalitis of Chicks. American Journal of Animal and Veterinary Sciences, 5, 91-96. https://doi.org/10.3844/ajavsp.2010.91.96.

Griffiths, M.W., Phillips, J.D., West, I.G., & Muir, D.D. (1988). The effect of extended low-temperature storage of raw milk on the quality of pasteurised and UHT milk. Food Microbiology, 5(2), 75-87. https://doi.org/10.1016/0740-0020(88)90025-1

Halpin-Dohnalek, M.I., & Marth, E.H. (1989). Growth and production of enterotoxin A by Staphylococcus aureus in cream. Journal of Dairy Science, 72, 2266-2275. https://doi.org/10.3168/jds.s0022-0302(89)79357-7

Hayes, M.C., & Boor, K. (2001). Raw milk and fluid milk products. In: Marth EH, Steele JL, eds. Applied dairy microbiology (2nd ed.) (59-76). New York: Marce l Dekker, Inc. USA.

Hossain, T.J., Alam, K., & Sikdar, D. (2011). Chemical and microbiological quality assessment of raw and processed liquid market milks of Bangladesh. Continental Journal of Food Science and Technology, 5(2), 6-17. https://doi.org/10.3923/rjdsci.2010.28.34.

Hussein, H.S., & Bollinger, L.M. (2005). Prevalence of Shiga toxin-producing *Escherichia coli* in beef cattle. Journal of Food Protection, 68(10), 2224-2241. https://doi.org/10.4315/0362-028x-68.10.2224.

Islam, M.A., Mondol, A.S., Azmi, I.J., de Boer, E., Beumer, R.R., Zwietering, M.H., Heuvelink, A.E., & Talukder, K.A. (2010). Occurrence and characterisation of Shiga toxin–producing *Escherichia coli* in raw meat, raw milk, and street vended juices in Bangladesh. Foodborne Pathogens and Disease, 7(11), 1381–1385. https://doi.org/10.1089/fpd.2010.0569.

Kaczmarek, A., Budzyńska, A., & Gospodarek, E. (2012). Prevalence of genes encoding virulence factors among *Escherichia coli* with K1 antigen and non-K1 *E. coli* strains. Journal of Medical Microbiology, 61(10), 1360-1365. https://doi.org/10.1099/jmm.0.044263-0.

Kerouanton, A.1., Hennekinne, J.A., Letertre, C., Petit, L., Chesneau, O., Brisabois, A., & De Buyser, M.L. (2007). Characterisation of Staphylococcus aureus strains associated with food poisoning outbreaks in France. International Journal of Food Microbiology, 115, 369-375. https://doi.org/10.1016/j.ijfoodmicro.2006.10.050.

Khairy, R.M., Mohamed, E.S., Abdel Ghany, H.M., & Abdelrahim, S.S. (2019). Phylogenic classification and virulence genes profiles of uropathogenic *E. coli* and diarrhegenic *E. coli* strains isolated from community acquired infections. PloS One, 14: e0222441. DOI: 10.1371/journal.pone.0222441

Koushki, M., Koohy-Kamaly, P., Azizkhani, M., & Hadinia, N. (2016). Microbiological quality of pasteurised milk on expiration date in Tehran, Iran. Journal of Dairy Science, 99(3), 1796-1801. https://doi.org/10.1371/journal.pone.0222441.

Langer, A.J., Ayers, T., Grass, J., Lynch, M., Angulo, F.J., & Mahon, B.E. (2012). Non pasteurised Dairy Products, Disease Outbreaks, and State Laws—United States, 1993–2006. Emerging Infectious Diseases, 18(3), 385-391. https://dx.doi.org/10.3201/eid1803.111370.

La Ragione, R.M., Woodward, M.J., Kumar, M., Rodenberg, J., Fan, H., Wales, A.D., & Karaca, K. (2013). Efficacy of a live attenuated *Escherichia coli* O78: K80 vaccine in chickens and turkeys. Avian Diseas-

es, 57(2), 273-279. https://doi.org/10.1637/10326-081512-Reg.1

Lee, G.Y., Jang, H.I., Hwang, I.G., & Rhee, M.S. (2009). Prevalence and classification of pathogenic *Escherichia coli* isolated from fresh beef, poultry, and pork in Korea. International Journal of Food Microbiology, 134(3), 196-200. https://doi.org/10.1016/j.ijfoodmicro.2009.06.013.

Maffei, D.F., Silveira, N.F.D., & Catanozi, M.D.P.L.M. (2013). Microbiological quality of organic and conventional vegetables sold in Brazil. Food Control, 29(1), 226-230. https://doi.org/10.1016/j.foodcont.2012.06.013

Martin, N.H., Trmčić, A., Hsieh, T.H., Boor, K.J., & Wiedmann, M. (2016). The evolving role of coliforms as indicators of unhygienic processing conditions in dairy foods. Frontiers in Microbiology, 7,1549. https://doi.org/10.3389/fmicb.2016.01549

Meshref, A.M.S. (2013). Bacteriological quality and safety of raw cow's milk and fresh cream. Slovenian Veterinary Research, 50(1), 21-30.

Mgqibandaba, P.Z., Madilo, F.K., Du-Preez, C.J., Mjoka, J., & Unathi, K. (2020). Evaluating food safety and hygiene knowledge and practices among foodservice staff of feeding scheme in the primary schools in Soweto, South Africa. Journal of Food Safety, e12792 . https://doi.org/10.1111/jfs.12792

Paneto, B.R., Schocken, R.P.I., Macedo, C., Santo, E., & Marin, J.M. (2007). Occurrence of toxigenic *Escherichia coli* in raw milk cheese in Brazil. Brazallian Journal of Veterinary and Animal Science, 59(2), 508–512. http://dx.doi.org/10.1590/S0102-09352007000200035.

Olasupo, N.A., Smith, S.I., & Akinsinde, K.A. (2002). Examination of the microbial status of selected indigenous fermented foods in Nigeria. Journal of Food Safety, 22, 85-93. https://doi.org/10.1111/j.1745-4565.2002.tb00332.x.

Rantsiou, K., Alessandria, V., & Cocolin, L. (2012). Prevalence of Shiga toxin-producing *Escherichia coli* in food products of animal origin as determined by molecular methods. International Journal of Food Microbiology, 154(1-2), 37–43. https://doi.org/10.1016/j.ijfoodmicro.2011.12.010

Robinson, R.K. (2002). Dairy microbiology handbook: The microbiology of milk and milk products (3rd ed.). New York: Wiley-Interscience. https://doi.org/10.1002/0471723959

Salim, A.P.A., Canto, A.C., Costa-Lima, B.R., Simoes, J.S., Panzenhagen, P.H., Franco, R.M., Silva, T.J., Conte-Junior, C.A. (2017). Effect of lactic acid on *Escherichia coli* O157: H7 and on color stability of vacuum-packaged beef steaks under high storage temperature. Journal of microbiology, biotechnology and food sciences, 6(4), 1054-1058. https://doi.org/10.15414/jmbfs.2017.6.4.1054-1058

Sambrook, J., Fritsch, E.F., & Maniatis, T. (1989). Molecular coloning: A laboratory manual (2nd ed.). New York: Cold spring Harbor Laboratory Press.

Shojaei, Z.A., & Yadollahi, A. (2008). Physicochemical and microbiological quality of raw, pasteurised and UHT milks in shops. Asian Journal of Scientific Research, 1, 532–538. https://doi.org/10.3923/ajsr.2008.532.538

Silva, R., Cruz, A.G., Faria, J.A., Moura, M.M., Carvalho, L.M., Water, E.H., & Sant'Ana, A.S. (2010). Pasteurised milk: efficiency of pasteurisation and its microbiological conditions in Brazil. Foodborne pathogens and Disease, 7(2), 217-219. https://doi.org/10.1089/fpd.2009.0332

Solomakos, N., Govaris, A., Angelidis, A.S., Pournaras, S., Burriel, A.R., Kritus, S.K., & Pupageorgiou, D.K. (2009). Occurrence virulence genes and antibiotic resistance of *Escherichia coli* O157 isolated from raw bovine, caprine and ovine milk in Greece. Food Microbiology, 26, 865-571. https://doi.org/10.1016/j.fm.2009.06.002

Tebaldi, V.M.R., Oliveira, T.L.C., Boari, C.A., & Piccoli, R.H. (2008). Isolation of coliforms, staphylococci and enterococci in raw milk from communitarian expansion refrigeration tanks: identification, lipolytic and proteolytic action. Food Science and Technology, 28, 753–60. https://doi.org/10.1590/S0101-20612008000300036.

Tortorello, M.L. (2003). Indicator organisms for safety and quality—uses and methods for detection: mini-review. Journal of AOAC International, 86(6), 1208-1217.

Vahedi, M., Nasrolahei, M., Sharif, M., & Mirabi, A.M. (2013). Bacteriological study of raw and unexpired pasteurised cow's milk collected at the dairy farms and supermarkets in Sari city in 2011. Journal of preventive medicine and hygiene, 54, 120-123.

Van Asselt, E.D., van der Fels-Klerx, H.J., Marvin, H.J.P., van Bokhorst-van de Veen, H., & Groot, M.N. (2017). Overview of Food Safety Hazards in the European Dairy Supply Chain. Comprehensive Reviews in Food Science and Food Safety, 16(1), 59–75. https://doi.org/10.1111/1541-4337.12245

Varga, L. (2007). Microbiological quality of commercial dairy products. In Mendez-Vilas, A. (Eds), Communicating Current Research and Educational Topics and Trends in Applied Microbiology. Formatex Microbiology Series (1). Formatex, Badajoz, pp. 487-494. http://real.mtak.hu/id/eprint/6206

Wojnicz, D. (2007). Virulence factors of uropathogenic *Escherichia coli* strains isolated from children with chronic pyelonephritis. Advances in Clinical and Experimental Medicine, 16(5), 651-657.

World Health Organization. (2015). WHO's first ever global estimates of foodborne diseases find children under 5 account for almost one third of deaths. World Health Organization, Geneva.

Yoruk, N.G. (2018). Most probable number technique in *Escherichia coli* count using ISO 16649 3, ISO 7251, and rapid test enumeration device (TEMPO EC) methods in milk and dairy products. Journal of Food Safety 38(5), e12502. https://doi.org/10.1111/jfs.12502

Zeinhom, M.M.A., Abdel-Latef, G.K., & Jordan, K. (2015). The Use of Multiplex PCR to Determine the Prevalence of Enterotoxigenic Staphylococcus aureus Isolated from Raw Milk, Feta Cheese, and Hand Swabs. Journal of Food Science, 80, M2932–M2936. https://doi.org/10.1111/1750-3841.13147.