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OCCURANCE OF ENTEROBACTERIACEAE IN SKIMMED MILK SOFT CHEESE

AMIRA, O. FARHAT [1]; NAGAH, M. HAFIZ [2]; HALAWA, M.A.[2]; SAAD, M.F. [2] and AYAH, B. ABDEL-SALAM [2]

[1] Veterinary Quarantine, Cairo Airport

[2] Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Cairo University, Giza, P.O Box 12211, Egypt

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ABSTRACT

Fifty samples of skimmed milk soft cheese were randomly collected from different dairy shops and supermarkets in Cairo and Giza Governorates, Egypt. Collected samples were examined microbiologically for enumeration, isolation and identification of Enterobacteriaceae. The results showed that the mean value of Total Enterobacteriaceae Count was $7.49\times10^7 \pm 0.15\times10^7$ cfu/g. *Escherichia coli* could be found in 64% of the examined samples with a mean value of $4.56\times10^7 \pm 0.14\times10^7$ MPN/g. *E.coli* serovars O146, O1, O18, O15, O8 and O78 could be detected in a percentage of 2.90, 2.90, 4.34, 1.45, 1.45 and 1.45, respectively. Salmonella could not be detected. It could be concluded that skimmed milk soft cheese may harbor serious health risk in the study areas.

Key words: Enterobacteriaceae, Skimmed milk soft cheese, *Escherichia coli*.

INTRODUCTION

Milk and dairy foods are nutrient-dense foods, supplying energy, proteins and micronutrients (Park *et al.*, 2007). Skimmed milk soft cheese is one of the most popular local types of fresh soft cheeses in Egypt. That may be attributed to its high protein content and low price (Brooks *et al.*, 2012).

Like most perishable foods, the quality of finished dairy products begins with the used raw milk. Milk contaminated by high levels of spoilage bacteria usually becomes unsuitable for further processing in terms of nutritional value, hygienic quality and sensory attributes. The microbiological flora of cheese depends on the quality of raw milk used in manufacture. Many microorganisms may contaminate cheese from various sources during different stages of cheese production, handling, storage and distribution. Raw milk cheese is more likely to harbor harmful bacteria which may constitute an adverse health effect to the consumers (Smith, 1981; Colak *et al.*, 2006; Nanu *et al.*, 2007; Brito *et al.*, 2008 and Callon *et al.*, 2008). Also neglecting the good hygienic and manufacturing practices (GHPs and GMPs) during the processing, handling and product transportation are considered a main source of product contamination with different pathogens and/ or their toxins (White, 2011).

Enterobacteriaceae have medical and economic importance; their presence in large number indicates faecal contamination, unqualified processing and post processing contamination of food. Enterobacteriaceae spp. has been implicated in many outbreaks (Koneman *et al.*, 1994). This family includes a number of remarkable foodborne pathogens such as Salmonella, pathogenic *E. coli* and Shigella spp. Other family members are regarded as opportunistic pathogens in clinical settings (Klebsiella spp. and Citrobacter spp.). Erwinia spp. is associated with food spoilage and cause a great economic loss (Baylis *et al.*, 2011). Salmonellae are among the most important and common enteric pathogen that may be transmitted to human through consumption of milk and its products. A dose of as little as 15-20 organisms can cause illness (FDA, 2009). The organism is mainly secreted in the faeces and then picked up on hair or teats of animal. Many strains of Salmonella can cause food-borne infection in humans, and all strains exhibit the same disease symptoms (Jantsch *et al.*, 2011). Last reported 2 outbreaks caused by *Salmonella typhimurium* were recorded by CDC in 2013 and it was linked to raw milk and the other for cheese. *E. coli*, *Klebsiella pneumonia*, Enterobacter spp. and Citrobacter spp. were involved in gastrointestinal illness as gastroenteritis, food poisoning, cholera-like syndrome, diarrhea, cystitis, pyelonephritis, appendicitis, pyelitis, peritonitis, lobar pneumonia, and septicemia (Graceleah *et al.*, 2010). *Escherichia*

Corresponding author: Dr. AYAH, B. ABDEL-SALAM
E-mail address: setelkol2003@yahoo.com
Present address: Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Cairo University, Giza, P.O Box 12211, Egypt

coli O_{157}: H_7 is an emerging cause of food borne illness and young dairy cattle are a reservoir for it. Infection with Enterohemorrhagic *Escherichia coli* (EHEC) strains often associated with food borne outbreaks traced to, milk and dairy products, leads to hemorrhagic colitis (bloody diarrhea) and hemolytic uremic syndrome in humans (Desmarchelier and Fegan, 2011).

MATERIALS AND METHODS

1. Collection of samples:

Fifty samples of skimmed milk soft cheese (Kariesh) were randomly collected under complete sanitary conditions from different dairy shops and supermarkets in Cairo and Giza Governorates, Egypt. Collected samples were transferred to laboratory in an insulated ice-box with minimum of delay to be examined.

2. Bacteriological examination:

2.1 Preparation of decimal serial dilutions according to (APHA, 2004).

2.2 Enumeration of Total Enterobacteriaceae Count according to (ISO, 2004).

One ml of each dilution was transferred into each of appropriately marked duplicate dishes, then approximately 10 ml of the violet red bile glucose medium (Oxoid, CM0485) was poured into each dish. After solidification of the mixture, a covering layer of approximately 15 ml of the violet red bile glucose medium was added to prevent spreading growth and to achieve semi-anaerobic conditions, and then the prepared dishes were incubated at 37˚C/24 hours. Characteristic colonies were purple or pink to red (with or without precipitation haloes). Such colonies were randomly selected for subculturing for the confirmatory tests (glucose fermentation and oxidase test), then Total Enterobacteriaceae Count was calculated CFU/g.

2.3 Enumeration and isolation of *Escherichia coli* (MPN/g) according to (BAM, 2002).

One ml from each previously prepared decimal dilution was inoculated into a series of 3 fermentation tubes containing Lauryl Sulphate Tryptose (LST) broth (Oxoid, CM0451) supplemented with inverted Durham's tubes for collection of gas. Inoculated tubes, as well as, the control were incubated at 35^{o}C for 48 hours and examined for gas production. From each gassing LST tube (presumptive test), a loopful of each suspension was transferred to *Escherichia coli* (EC) broth tube (Oxoid, CM0853), EC tubes were incubated for 48 hours at 45.5°C and examined for gas production. From the results obtained, MPN/g of *E. coli* was calculated. Loopful from positive tube was streaked on the surface of petri dish containing Levine Eosin Methylene Blue Agar (Oxoid, CM0069) before being incubated at 35º C for 48 hours. The plates were examined for suspected colonies of *E. coli*.

2.4 Isolation of Salmonella according to (ISO, 2002)

Twenty-five g of skimmed milk soft cheese sample were aseptically added to two hundred and twenty-five ml buffered peptone water and incubated at 37˚C for 18 hours. 0.1 ml of the previous culture was transferred to 10 ml of Rapport-Vassiliadis (RVS) medium (Oxoid, 0669) tube. The inoculated RVS broth was incubated at 41.5˚C for 24 hours, then loopful of the culture obtained in the RVS broth was streaked on the surface of one large size petri dish containing Xylose Lysine Deoxycholate (XLD) agar (Oxoid, 0469). The dishes were inverted and placed in the incubator set at 37˚C for 24 hours. After incubation, the plates were examined for typical and atypical colonies.

2.5 Identification of Enterobacteriaceae.

2.5.1 Morphological and Biochemical examination according to (Bergey's manual, 2005).

2.5.2 Biotyping of some *Escherichia coli* isolates using VITEK 2 compact system according to (Pincus, 2005).

2.5.3 Serological identification of the isolated *Escherichia coli* according to (BAM, 2011).

Slide agglutination technique, at the Animal Health Research Institute, El- Dokki, Giza, was adopted for serotyping of the same isolates of *E. coli*, which identified by VITEK 2 compact system, using available coli antisera of BEHRING WEKE AG, MARBURG W., Germany.

RESULTS

Table 1: Statistical analytical results of Total Enterobacteriaceae Count (CFU/g) and *E.coli* Count (MPN/g) of the examined skimmed milk soft cheese (Kariesh) samples.

Parameter	Total no. of samples	Positive samples		Min.	Max.	Mean	±S.E.M
		No.	%				
Total Enterobacteriaceae Count	50	50	100	7.0×10	2.2×10^{9}	7.49×10^{7}	0.15×10^{7}
E. coli Count	50	32	64	10^{2}	9.46×10^{8}	4.56×10^{7}	0.14×10^{7}

Table 2: Frequency distribution of Total Enterobacteriaceae and *E. coli* count in examined samples.

	Total Enterobacteriaceae		*E. coli*	
Intervals	**no. of samples**	**%**	**no. of samples**	**%**
$< 10^2$	2	4.00	18	36.00
$10^2 - < 10^4$	4	8.00	8	16.00
$10^4 - < 10^6$	14	28.00	10	20.00
$10^6 - < 10^8$	26	52.00	14	28.00
$10^8 - \leq 10^{10}$	4	8.00	0	0
Total	**50**	**100.00**	**50**	**100.00**

Table 3: Incidence of Enterobacteriaceae spp. in examined samples (total no. of isolates=180).

Type of isolates	**no.**	**%**
Escherichia coli	69	38.33
Klebsiella pneumoniae subsp.*ozaenae*	28	15.55
Morganella morganii subsp. *morganii*	21	11.66
Serratia fonticola	18	10.00
Edwardsiella tarda	10	5.56
Citrobacter diversus	8	4.44
Citrobacter freundii	7	3.89
Proteus vulgaris	4	2.22
Enterobacter cloacae	3	1.67
Klebsiella oxytoca	3	1.67
Klebsiella pneumoniae subsp. *pneumoniae*	3	1.67
Enterobacter aerogenes	2	1.11
Enterobacter intermedius	2	1.11
Serratia liquefaciens	1	0.56
Proteus penneri	1	0.56
Total	**180**	**100.00**

Table 4: Incidence of *E.coli* serovars in the examined isolates (no. = 69).

***E.coli* serovars**	**no. of isolates**	**%**
O 146	2	2.90
O 1	2	2.90
O 18	3	4.34
O 15	1	1.45
O 8	1	1.45
O 78	1	1.45
Non typed	59	85.51
Total	**69**	**100.00**

DISCUSSION

Manufacture and handling techniques of skimmed milk soft cheese in Egyptian markets are still primitive and unhygienic. Many contaminants find their way to raw milk, from which they gain access to dairy products (Al-Khatib and Al-Mitwalei, 2009). Results presented in (Table 1) revealed that, the Enterobacteriaceae were present in 100% of the examined skimmed milk soft cheese (Kariesh) samples, which agreed with Hanaa and Jehan (2009) and Sherein *et al.* (2014). El-Bakery (2004); Ibtisam

et al. (2006); Dina (2008); Ibrahim *et al.* (2008); Gihan *et al.* (2013) and Jehan *et al.* (2015) recorded lower incidence rate of Enterobacteriaceae than that obtained in this study.

The results registered in (Table 1) revealed that the count of Enterobacteriaceae ranged from 7.0×10 CFU/g to 2.20×10^9 CFU/g and a mean value of $7.49\times10^7 \pm 0.15\times10^7$ CFU/g. Enterobacteriaceae count is taken as an index for the probable presence of enteric pathogens, which may constitute public health hazards (Eman and El- Kaseh, 2008). These results were lower than that obtained by El-Bakery (2004) and higher than that obtained by Ibtisam *et al.* (2006); Dina (2008); Hanaa and Jehan (2009); Omar *et al.* (2011); Abd El-Fattah (2013); Sherien *et al.* (2014) and Jehan *et al.* (2015). Nearly similar results were obtained by Ebrahim (1998) and Tirloni *et al.* (2014). The high rate of contamination with Enterobacteriaceae in skimmed milk soft cheese (Kariesh) may be due to handling, transportation or storage in a place contaminated with these types of bacteria or during processing of cheese which contains low salt (He and Macgregor, 2007). The highest frequency distribution of Enterobacteriaceae count in examined samples lies within the range of 10^6-$<10^8$ CFU/g in a percentage of 52 as showed in Table (2). This result was higher than that obtained by (Sherein *et al.,* 2014) who found that the highest frequency distribution of Enterobacteriaceae count in examined skimmed milk soft cheese samples lies within the range of 10^5-$<10^6$ CFU/g in a percentage of 41.67.

Enterobacteriaceae was used as indicator organisms for assessing hygienic quality of food; because the enteric bacteria that fail to ferment lactose are of more public health importance than those that ferment lactose and their presence could be related to direct or indirect faecal contamination of food (Morales *et al.,* 2003). The data summarized in Table (3) revealed that the identification of Enterobacteriaceae members isolated from the examined samples were *Klebsiella pneumoniae subsp.ozaenae*, *Klebsiella oxytoca* and *Klebsiella pneumoniae subsp.pneumoniae* in percentages of 15.55, 1.67 and 1.67, respectively. *Morganella morganii subsp. morganii, Edwardsiella tarda, Citrobacter diversus* and *Citrobacter freundii* were isolated in percentage of 11.66, 5.56, 4.44 and 3.89, respectively. Also *Enterobacter cloacae, Enterobacter aerogenes* and *Enterobacter intermedius* were present in percentages of 1.67, 1.11 and 1.11, respectively. *Serratia fonticola, Serratia liquefaciens, Proteus vulgaris* and *Proteus penneri* were present in percentages of 10.00, 0.56, 2.22 and 0.56, respectively. The highest percentage of Enterobacteriaceae isolates was identified as *E. coli* in a percentage of (38.33%), which is higher than that obtained by Ibtisam *et al.* (2006) and Omar *et al.* (2011) and lower than that obtained by Baraheem *et al.* (2007) and Uraz *et al.* (2008).

Klebsiella pneumoniae is a world wide spread bacteria that could be responsible for arthritis, meningitis, appendicitis, cystitis, pneumonia and septicemia in newborns (Bernabe *et al.,* 1998). Results of Klebsiella spp. isolated from samples were higher than that obtained by Falegan and Akere (2014) and lower than that obtained by Ogbolu *et al.* (2014) and Uraz *et al.* (2008). *Klebsiella pneumoniae subsp. ozaenae* isolated from samples was lower than that obtained by Aya (2014) and higher than that obtained by Elbagory *et al.* (2016). *Klebsiella oxytoca* isolated from cheese samples was lower than that obtained by Tornadijo *et al.* (2001) and Abd El-Fattah (2013). *K. pneumonia* and *K. oxytoca* cause community-acquired meningitis and brain abscesses (Janda and Abbott, 2006). *Citrobacter freundii* isolated from cheese samples was lower than obtained by (Ibtisam *et al.,* 2006 and Abd El-Fattah, 2013). Results of Serratia spp. isolated from cheese samples were lower than that obtained by (Uraz *et al.,* 2008); also results of Proteus spp. isolated were lower than that obtained by (El Sayed *et al.,* 2011).

Morganella morganii is a Gram-negative rod which could be found naturally in the environment and as normal flora in the intestinal tracts (Miller, 2015). *Morganella morganii* isolated from cheese samples was lower than that obtained by (Uraz *et al.,* 2008). Salmonella failed to be isolated from all examined samples neither by using violet red bile glucose agar nor by selective (XLD medium). The absence of Salmonella in the examined samples makes these samples complied with the Egyptian standards in this point. *Escherichia coli* are representing a portion of the intestinal tract normal microflora of animals and human. Also it is used as indicator microorganism to detect and measure fecal contamination in the evaluation of food safety (Borgatta *et al.,* 2012). *E. coli* presence in food not only indicates contamination, poor hygiene and sanitary practice but also it could be hazardous for consumers (Hahn, 1996). In Table (1), it was obvious that *E. coli* incidence rate in the examined samples was 64% either by selective EMB or Violet Red Bile Glucose agar medium, which was higher than that obtained by Walaa (2008); Hanaa and Jehan (2009); Omar *et al.* (2011); Eman (2012); Abd El-Fattah (2013); El nahas *et al.* (2015) and Elbagory *et al.* (2016), while Najand and Ghanbarpour (2006); Omar *et al.* (2007); Campos *et al.* (2009) and Enas (2015) reported higher incidence. *E. coli* were found in the examined samples with a minimum count of 10^2 MPN/g, maximum count of 9.46×10^8 MPN/g and a mean value $4.56 \times 10^7 \pm 0.14 \times 10^7$ MPN/g Table (1). The count of *E. coli* in this study was lower than that obtained by Alper and Nesrin (2013). Bahout and moustafa (2006); El nahas *et al.* (2015) and Enas (2015) could enumerate *E. coli* in lower count. The highest frequency distribution of *E. coli* count in the examined samples was $<10^2$ MPN/g in percentage of 36 as showed in Table (2). *E. coli* is one of the most

important food-borne pathogens and has been involved in many outbreaks associated with severe symptoms and highly fatality rate (Doyle and Beuchat, 2007). The serological typing of the *E. coli* isolated from skimmed milk soft cheese samples were identified as O146, O1, O18, O15, O8 and O78 in percentage of 2.90, 2.90, 4.34, 1.45, 1.45 and 1.45, respectively, and non-typed isolates could be detected from the samples in a percentage of 85.51 as shown in (Table 4). Shiga Toxigenic *Escherichia coli* (STEC) are known as important pathogens and have been associated with diarrhea and Hemolytic Uremic Syndrome (Paton and Paton, 1998). *E. coli* O146 strains belong to this group and have been implicated in human diseases (Beutin *et al.,* 2004). Also *E. coli* O78 belongs to STEC group and associated with enteritis, newborn meningitis and sepsis (Gophna *et al.,* 2001). The Enterotoxigenic *E. coli* such as O8, O27 and O78, secretes toxins, which lead to the production of a watery diarrhea and severe dehydration in children (Cohen and Gianella, 1995 and Fratamico *et al.,* 2002).

CONCLUSION

The results in this study revealed that the Egyptian skimmed milk soft cheese samples were heavily contaminated with Enterobacteriacae spp. and the isolated strains constitute public health hazard to consumer. Bad hygienic quality of raw milk used in cheese manufacturing could be considered as the potential source of cheese contamination. The objectionable heavy contamination of cheese samples with different types of Enterobacteriacae may result into serious changes in the product rendering it of inferior quality. Moreover, being an important vehicle for the transmission of milk-borne pathogens to consumers. Routine assessment of skimmed milk soft cheese quality produced by small-scale livestock keepers has to be mandatory in order to safeguard the public from milk-borne zoonotic infections. Strictly hygienic measures should be applied during the whole chain of cheese production starting from milking of milk, all manufacturing steps, storage and distribution of cheese. The behavior of consuming raw milk and products made from raw milk should be discouraged. Good Manufacturing Practices (GMP), Good Hygienic Practices (GMP) and strict personal hygiene are the way to ensure safety and high quality of dairy products. It is concluded that a gradual move to total Enterobacteriacae (measure of food quality and spoilage) to improve assessment of food safety and quality rather than limiting examination for coliforms and fecal coliforms.

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مدى تواجد الميكروبات المعوية فى الجبن الطرى منزوع الدسم

أميره فرحات ، نجاح مصطفى حافظ ، محمدى أحمد حلاوه ، مينا فؤاد سعد ، ايه عبد السلام

Email: setelkol2003@yahoo.com Assiut University web-site: www.aun.edu.eg

يعتبر الجبن الطرى منزوع الدسم (الجبن القريش) من أشهر أنواع الجبن الطرى فى مصر لقيمته الغذائية العالية وسعره المنخفض. يوجد العديد من الكائنات الحية الدقيقة تستطيع أن تلوث الجبن خلال المراحل المختلفة للتصنيع أو التخزين والتوزيع. أجريت الدراسة على خمسين عينة من الجبن الطرى منزوع الدسم جمعت عشوائيًا من الأسواق وبعض محلات الألبان المختلفة بالقاهرة والجيزة. وقد تم فحص العينات ميكروبيولوجيا لعد وعزل وتصنيف الميكروبات المعوية وأظهرت النتائج أن متوسط العد الكلى للميكروبات المعوية كالتالى $7.49 \times 10^7 \pm 0.15 \times 10^7$ مستعمرة بكتيرية/ جرام. وقد تواجد ميكروب الايشيريشيا كولاى بنسبة ٦٤% من العينات المفحوصة بمتوسط قدره $4.56 \times 10^7 \pm 0.14 \times 10^7$/ جرام وأسفر التحليل السيرولوجى عن تواجد العترات , O15, O18, O1 O78, O8, O146 بنسبة ٢.٩٠ , ٢.٩٠ , ٤.٣٤ , ١.٤٥, ١.٤٥ و ١.٤٥% على التوالى. بينما خلت جميع العينات المفحوصة من ميكروب السالمونيلا. وقد خلصت الدراسة الي أن الجبن الطرى منزوع الدسم يمثل خطرا صحيا فى مناطق الدراسة.

الكلمات الدالة: الانتيروباكترياسى، الجبن الطرى منزوع الدسم و الايشيريشيا كولاى.