Kafrelsheikh Veterinary Medical Journal
(https://kvmj.journals.ekb.eg/)

Original Article

Received Aug 21, 2020
Accepted Sep 16, 2020
Published Oct 09, 2020

Prevalence of Enterobacteriaceae in raw milk and some dairy products

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Abstract

Objective: This study was performed to detect and identify members of Enterobacteriaceae in raw milk, yoghurt and ice cream to assess their hygienic quality.

Methods: A total number of 200 samples distributed as follows:100 raw milk, 25 small and 25 large scale plain yoghurt, and 25 small and 25 large scale ice cream samples collected from different localities at Kafr El-Sheikh Governorate and examined for Enterobacteriaceae count and identification of members.

Results: Enterobacteriaceae were detected in 84% of examined raw milk samples (with mean count of $1.02x10^6 \pm 1.98x10^5$ cfu/ml), 40% and 32% of examined small and large scale plain yoghurt samples (with mean value of $6.95x10^3 \pm 4x10^3$ and $1.57x10^3 \pm 8.95x10^2$ cfu/g), while detected in 64% and 20% of examined small and large scale ice cream samples (with mean count of $1.02x10^4 \pm 4.13x10^3$ and $1.4x10^3 \pm 8.26x10^2$ cfu/g). The most frequent members isolated were *Hafnia alive* (30.95%), *E. coil* (44.44%), and *Serratia marcescens* (57.14%) from positive raw milk, yoghurt, and ice cream samples, respectively. Other members of Enterobacteriaceae were isolated from positive samples at different percentages.

Conclusion: Presence of members of Enterobacteriaceae is a reliable index of fecal contamination and the possibility of the presence of associated enteric pathogenic organisms. So, it is recommended to specify Enterobacteriaceae as a sanitary index of milk and dairy products in the Egyptian food acts and regulations.

Keywords: Enterobacteriaceae, raw milk, yoghurt, ice cream

1. Introduction

Enterobacteriaceae is a large, heterogeneous group of Gram-negative rods, which naturally inhabit the intestinal tract of both humans and animals. They include many genera (such as *Escherichia coli*, *Shigella*, *Salmonella*, *Enterobacter*, *Klebsiella*, *Serratia*, and *Proteus*). These genera are widespread in the environment and can contaminate milk through feces; bedding, improperly cleaned teats, milk handling, and equipment contaminated with soil or polluted water (Cohen et al., 2017). The presence of Enterobacteriaceae in dairy products induces undesirable changes that render the product of inferior quality, unmarketable, and unfit for human consumption. Moreover, their presence is frequently considered as a reliable index of fecal contamination. Therefore, the presence of Enterobacteriaceae and Coliform are routinely assessed to determine the hygienic quality of foods, particularly dairy products (Martin et. al., 2010).

Food-borne microorganisms are major pathogens affecting food safety and cause human illness worldwide as a result of the consumption of foodstuff, mainly animal products contaminated with vegetative pathogens or their toxins (Abebe et. al., 2020). The outbreak of foodborne illnesses following consumption of raw milk and dairy products made from raw milk may be caused by Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella* spp., and *Yersinia enterocolitica* (Proctor and Davis, 2000; Mazurek et. al.2004; Jayarao et. al.,2006 and Cancino-Padilla et. al. 2017). The primary condition associated with cases of foodborne illness caused by STEC and *Salmonella* spp. is gastroenteritis which is usually self-limiting, while immuno-compromised individuals are at a higher risk of serious illness. The *Yersinia enterocolitica* disease is typically characterized by gastroenteritis and enterocolitis. However, debilitating post-infection immunological sequel, including Guillian-Barré syndrome and reactive arthritis are known to develop in some individuals following an episode of foodborne illness with these pathogens (Altekruse et. al., 1999 and Oliver et. al., 2005).

Yoghurt is the best known and popular cultured milk product concerning nutrition as it contains a high level of protein, calcium, phosphorus, and vitamin B_2 and B_{12} (Piaia, 2001). Yoghurt helps to maintain food and water intake, which are often reduced in older people, moreover, it is a part of the diet designed to promote successful growth. Ice cream is a highly delicious and nutritionally rich frozen milk product

that is widely consumed in Egypt. Microorganisms may gain entrance to such products during processing, handling and distribution also the ingredients used in the manufacture contribute to contamination of the product and consequently lead to public health hazards as gastroenteritis in humans (Yadav et.al. 1993 and Holban and Grumezescu, 2018).

Although pasteurization can destroy most of the specific pathogens that pose risk to public health, a potential microbial hazard may be still introduced after pasteurization through adding contaminated ingredients and improper handling process (Marshall, 1998 and Oliver, 2005). Raw milk that consumed raw or used in the manufacturing of yoghurt and ice cream may be produced under poor hygienic status using primitive procedures. Under such circumstances, the finished products would express to harbor a great number and different types of contaminants, particularly those belonging to enteric microorganisms. Therefore, this work was planned to detect and identify members of Enterobacteriaceae in raw milk, yoghurt, and ice cream to assess their quality and sanitation.

2. Materials and methods

This study was conducted after under the ethical approval from the Experimental Animals Care Committee in compliance with guidelines of the University of Kafrelsheikh.

2.1. Collection of samples

A total of 200 random samples of raw milk and some dairy products were collected from small dairies, groceries, and supermarkets from different localities at Kafr El-Sheikh Governorate, Egypt. Of them, 100 raw milk, 50 plain yoghurt (25 small scale and 25 large scale), and 50 ice cream (25 small scale and 25 large scale) samples were obtained in their containers as sold to the consumer or in sterile containers and dispatched directly to the laboratory with a minimum of delay in an insulated icebox at 4ºC to be examined.

2.2. Examination of raw milk for the detection of heat treatment

All collected milk samples were subjected to Storch's test according to A.P.H.A, 2004 to exclude samples proved to be heat treated above 80ºC.

2.3. Preparation of serial dilution

All samples were prepared and serially diluted according to A.P.H.A, 2004.

2.4. Enterobacteriaceae count

According to Harrigan 1998, 2 ml from each of the previously prepared serial dilution milk, yoghurt, or ice cream were transferred into sterile duplicate Petri dishes (1 ml each). About 10-15 ml of violet red bile glucose agar (VRBGA) medium (melted and tempered to 45 $\pm$ 1ºC) were poured into each plate and mixed well with the inoculum and allowed to set. The set medium was overlaid by pouring another 5 ml of VRBGA medium. After solidification, the inoculated plates were incubated at 37ºC for 24 h. All typical colonies, characterized by red or purple color, >0.5 mm in diameter and surrounded by a zone of precipitated bile were counted and recorded as total Enterobacteriaceae count. Five colonies were picked up and transferred to nutrient agar slants, then incubated at 37ºC/24 h. and used for further identification.

2.5. Identification of Enterobacteriaceae

Isolated colonies were purified on plate count agar at 37ºC/24h and identified according to Krieg and Holt, 1984.

3. Results and discussion

Enterobacteriaceae were detected in 84 (84%) of the examined raw milk samples with a mean count of $1.02 \times 10^6 \pm 1.98 \times 10^5$cfu/ml. The highest frequency distribution (48.81%) of the positive samples lies within the range of 10^5: $<10^6$cfu/ml followed by 22.62% in the range of 10^6 : $<10^7$ cfu/ml (Tables 1 and 2).

Enterobacteriaceae members were detected in 40% and 32% of the examined small scale and large scale plain yoghurt samples with a mean value of $6.95 \times 10^3 \pm 4 \times 10^3$ and $1.57 \times 10^3 \pm 8.95 \times 10^2$ cfu/g (Table 1). The highest frequency distribution of positive small scale plain yoghurt samples (70%) lies within the range of 1×10^3:$< 5 \times 10^3$ cfu/g while that of positive large scale plain yoghurt samples (62.5%) lies within the range of >10:$<10^3$cfu/g (Table 3).

On the other hand Enterobacteriaceae members were detected in 64% and 20% of the examined small scale and large scale ice cream samples with a mean count of $1.02 \times 10^4 \pm 4.13 \times 10^3$ and $1.4 \times 10^3 \pm 8.26 \times 10^2$ cfu/g (Table 1). The highest frequency distribution of both small scale ice cream samples (43.75%) and large scale ice cream samples (60%) lie within the range of >10:$<10^3$ cfu/g (Table 3). According to Egyptian Standards for yoghurt and ice cream, Enterobacteriaceae count must not exceed 10 cell/g (E.S.2005, a and b), consequently all positive yoghurt and ice cream samples (>10 cfu/g) (Table 3) exceed the permissible limit of Egyptian Standards, 2005.

It is evident that the most frequent members isolated from the examined raw milk samples were *Hafnia alive* (30.95%), *Serratia liquefaciens* (25.0%), *and Klebsiella pneumonia* (15.48%). *Klebsiella pneumoniae* found in soil, water, sewage and constitute a part of the flora of the mouth and intestinal tract of human and animals. It responsible for pneumonia and upper respiratory tract infection and may be responsible for meningitis, pyaemia, and cystitis (Martin and Bachman, 2018).The prevalence of *Klebsiella oxytoca*, *Enterobacter aerogenes* and *Proteus vulgaris* was 13.1% (of the positive samples). Both *Serratia marcescens* and *Proteus rettgeri* were isolated at percentage of 11.9%, while, *E. coli*, *Providencia rettgeri* and *Citrobacter diversus* could be identified in 9.52, 9.52, and 3.57% of the positive samples, respectively. Enterobacteriaceae members which isolated at low percentage were *Proteus morganii*, *Providencia alcalifaciens*, *Citrobacter freundii*, *Shigella flexneri* and *Shigella sonnei* each at 2.38% of the positive samples, while *Enterobacter cloacae* and *Yersinia enterocolitica* isolated only from 1.19% of the positive samples. Most of these groups were isolated by many investigators (Saleh, 2000; Nyein et. al. 2002 and Jayarao et. al. 2006).

The presence of a large number of Enterobacteriaceae in raw milk indicate unsafe raw milk for human consumption (Nyein et. al. 2002) unless it is pasteurized as Enterobacteriaceae in raw milk were completely removed by pasteurization and not appear during the manufacturing process of dairy product (Branciari et. al. 2004) so milk for human consumption must be pasteurized. The mean Enterobacteriaceae count in small scale plain yoghurt was significantly ($P<0.01$) higher than that of large scale plain yoghurt (Table 1). These results declare neglected sanitary measures in small-scale plain yoghurt and un-heat treated milk may be used for the preparation of yoghurt. The presence of Enterobacteriaceae in yoghurt is considered as an index of unsatisfactory sanitation and the possible presence of enteric pathogens.

In normal fermentation, a final pH of <4.5 is developed in cultured milk products. Low pH generally prevents the growth of most spoilage and pathogenic organisms despite interference with acid development may allow the growth of undesirable microorganisms (A.P.H.A, 1992).

Regarding yoghurt samples, *E. coil* is the most frequent member isolated (44.44% out of 18 positive samples examined) (Table 5). *E. coli* is the most common aerobic bacterium of the large bowel of man and

animal, thus its presence in food generally indicates direct or indirect fecal pollution, so that it has been implicated in cases of gastroenteritis, epidemic diarrhea in infants, sporadic cases of summer diarrhea in children and cases of food poisoning (Bennett et. al., 2015). It is important to conclude that the detection of even low numbers of *E. coli* in foods reveals public health risk (I.C.M.S.F, 1978). According to Egyptian Standards yogurt must be free from *E.coli* (E.S. 2005, a), thus confirm public health risk for yoghurt consumers.

Serratia liquefaciens could be isolated from 27.78% of the positive samples examined while *Shigella sonnei, Shigella flexneri, Klebsiella pneumoniae,* and *Hafnia alive* isolated at 22.22%. On the other hand, *Serratia marcescens, Enterobacter aerogenes, Providencia staurtii, Edwardsiella hoshinae,* and *Proteus morganii* were detected in 11.11%. Citrobacter diversus, *Proteus rettgerii,* and *Enterobacter cloacae* were detected in 5.56% of the positive samples. No salmonellae could be detected in any of the examined yoghurt samples. Similar results were recorded by many authors (El-Kasas, 2004; Braun and Preuss, 2007 and Rotar et.al., 2007).

Identification of Enterobacteriaceae isolated from ice cream samples as recorded in Table (6). Serratia marcescens had the highest isolation percentage (57.14% out of 21 positive samples). It was reported that Serratia organisms have been implicated in human infection, including pulmonary and urinary tract infections as well as septicemia (Mahlen, 2011). *E.coli* could be isolated at 33.33% which is nearly similar to results recorded by (Yaman et.al. 2006). Both *Klebsiella pneumoniae* and *Hafnia alvie* were isolated from 23.81% while both *Citrobacter diversus* and *Enterobacter aerogenes* isolated with a percentage of 19.05%. On the other hand, *Klebsiella ozaenae, Citrobacter freundii, Proteus rettgerii,* and *Shigella flexneri* were detected in 9.52% of positive samples and *Serratia odorifera, Enterobacter cloacae, Shigella sonnei,* and *Proteus vulgaris* were isolated only from one sample at a percentage of 4.76 of the positive samples. This study showed that *Salmonella* spp. could not be detected in any of the examined ice cream samples as reported by many previous authors (Fadel and Ismail, 2009 and El-Bana, 2011).

The high Enterobacteriaceae count in the examined ice cream samples obtained in this investigation reflects unhygienic practices during manufacture and distribution. This may be due to poor ingredients, carelessness of the employer, who may lack the necessary knowledge of personal hygiene, product handling, and sanitation routines. So, this indicates a need for more concern on the part of the dairy industry to prevent contamination of ice cream. Ensure ingredients of good quality and especially those added after heat treatment should be free from pathogens and conform to microbiological criteria. Sufficient heat treatment to ice cream mix, prevention of post heat treatment contamination, and special attention should be given to utensils and equipment which come in contact with ice cream also the packaging used.

Enterobacteriaceae are sometimes contaminants of pasteurized dairy products. Their presence is indicative of unsanitary methods of manufacture, inadequate pasteurization, or post-pasteurization contamination. This indicates a need for more concern on the part of the dairy industry to prevent contamination with Enterobacteriaceae and thus prevent additional outbreaks of food-borne illness caused by their members.

Table 1. Statistical analytical results of Enterobacteriaceae count in the examined samples

Type of samples	No. of examined samples	Positive samples		Enterobacteriaceae count (cfu/ml or g)		
		No.	%	Minimum	Maximum	Mean ± SE
Raw milk	100	84	84	3×10^2	1.1×10^7	$1.02 \times 10^6 \pm 1.98 \times 10^5$
Yoghurt						
-Small scale	25	10	40	$9x10^2$	$4.3x10^2$	$6.95x10^3 \pm 4x10^3$
-large scale	25	8	32	5x10	$6.5x10^3$	$1.57x10^3 \pm 8.95x10^2$ *
Ice cream						
-Small scale	25	16	64	5x10	$5x10^4$	$1.02 \times 10^4 \pm 4.13 \times 10^3$*
-Large scale	25	5	20	3×10^2	$4.65x10^3$	$1.4 \times 10^3 \pm 8.26 \times 10^2$

cfu = colony forming unit, SE= Standard error of mean
* Small and large scale (yoghurt and ice cream) samples differed significantly at P <0.01

Table 2. Frequency distribution of positive raw milk samples based on their Enterobacteriaceae count

Interval (cfu/ml)	No. of samples	%
$>10 : < 10^4$	7	8.33
$10^4 : < 10^5$	16	19.05
$10^5 : < 10^6$	41	48.81
$10^6 : < 10^7$	19	22.62
$\geq 10^7$	1	1.19
Total	84	100

Table 3. Frequency distribution of positive yoghurt and ice cream samples based on their Enterobacteriaceae count

Interval (cfu/g)	Yoghurt samples				Ice cream samples			
	Small-scale		Large-scale		Small-scale		Large-scale	
	No.	%	No.	%	No	%	No	%
>10 : < 10^3	1	10	5	62.5	7	43.75	3	60
10^3 : < 5 x 10^3	7	70	2	25.0	3	18.75	2	40
5 x 10^3 : < 10^4	1	10	1	12.5	1	6.25	0	0
10^4: < 5x 10^4	1	10	0	0	5	31.25	0	0
Total	10	100	8	100	16	100	5	100

Table 4. Incidence of Enterobacteriaceae members isolated from the examined raw milk samples

Isolates	Number	% *	Positive samples	
			No.	% **
Hafnia alive	54	17.65	26	30.95
Serratia liquefaciens	34	11.11	21	25.00
Klebsiella pneumoniae	18	5.88	13	15.48
Klebsiella oxytoca	30	9.80	11	13.10
Enterobacter aerogenes	20	6.54	11	13.10
Proteus vulgaris	12	3.92	11	13.10
Serratia marcescens	44	14.38	10	11.90
Proteus rettgerii	28	9.15	10	11.90
Escherichia coli	20	6.54	8	9.52
Providencia rettgeri	10	3.27	8	9.52
Citrobacter diversus	4	1.31	3	3.57
Proteus morganii	3	0.98	2	2.38
Providencia alcalifaciens	4	1.31	2	2.38
Citrobacter freundii	8	2.61	2	2.38
Shigella flexneri	6	1.96	2	2.38
Shigella sonnei	6	1.96	2	2.38
Enterobacter cloacae	4	1.31	1	1.19
Yersinia enterocolitica	1	0.33	1	1.19

* Percent calculated to total No. of isolates (306) ** Percent calculated to number of positive samples (84)

Table 5. Incidence of Enterobacteriaceae members isolated from the examined yoghurt samples

Isolates	Number	%*	Positive sample	
			No.	%**
E. coli	42	26.58	8	44.44
Serratia liquefaciens	20	12.66	5	27.78
Shigella flexneri	14	8.86	4	22.22
Shigella sonnei	22	13.92	4	22.22
Klebsiella pneumoniae	12	7.59	4	22.22
Hafnia alvie	10	6.33	4	22.22
Serratia marcescens	10	6.33	2	11.11
Enterobacter aerogenes	8	5.06	2	11.11
Providencia staurtii	4	2.53	2	11.11
Edwardsiella hoshinae	2	1.27	2	11.11
Proteus morganii	2	1.27	2	11.11
Proteus rettgerii	2	1.27	1	5.56
Enterobacter cloacae	8	5.06	1	5.56
Citrobacter diversus	2	1.27	1	5.56

* Percent calculated to total No. of isolates (158) ** Percent calculated to number of positive samples (18)

Table 6. Incidence of Enterobacteriaceae members isolated from the examined ice cream samples

Isolates	Number	%*	Positive samples	
			No.	%**
Serratia marcescens	24	21.82	12	57.14
Escherichia coli	16	14.55	7	33.33
Klebsiella pneumoniae	14	12.73	5	23.81
Hafnia alive	12	10.91	5	23.81
Citrobacter diversus	8	7.27	4	19.05
Enterobacter aerogenes	8	7.27	4	19.05
Klebsiella ozaenae	4	3.64	2	9.52
Citrobacter freundii	4	3.64	2	9.52
Shigella flexneri	6	5.45	2	9.52
Proteus rettgerii	6	5.45	2	9.52
Serratia odorifera	2	1.82	1	4.76
Enterobacter cloacae	2	1.82	1	4.76
Shigella sonnei	2	1.82	1	4.76
Proteus vulgaris	2	1.82	1	4.76

* Percent calculated to total No. of isolates (110) ** Percent calculated to number of positive samples (21)

4. Conclusion

The results obtained allow concluding that the sanitary measures adopted during production, handling, and distribution of the examined milk and milk products (yoghurt and ice cream) are neglected in most cases as Enterobacteriaceae members existed in most samples examined. Also, the presence of these members of Enterobacteriaceae is a reliable index of fecal contamination and the possibility of the presence of associated enteric pathogenic organisms. So, Good hygiene, GMP, sanitation in operating procedures, and implementation of standardized HACCP and pasteurization procedures are effective methods for control and prevention. Therefore, it is recommended to specify the Enterobacteriaceae as a sanitary index of milk and dairy products in the Egyptian food acts and regulations.

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