

REVIEW ARTICLE

Coliforms Contamination in Raw Milk and Some Dairy Products with a Special Reference to Comparative Identification of *Enterobacter* spp.

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Abstract

The current study was undertaken to examine 200 sample of raw milk and some dairy products (Kariesh cheese, plain yoghurt, milk powder and infant formula) for contamination with Coliform group especially *Enterobacter* spp. Coliforms were detected in; 42/50 (84%) raw milk samples from farmers' houses, 25/30 (83.33%) kariesh cheese samples and 23/30 (76.67%) plain yoghurt samples, however, they could not be detected in any of raw milk samples from dairy shops, milk powder and infant formula samples. The mean values of coliforms in the examined samples were $2.80 \times 10^6 \pm 0.73 \times 10^5$, $2.30 \times 10^6 \pm 0.75 \times 10^4$ and $1.08 \times 10^6 \pm 1.50 \times 10^4$ cfu /ml or gm in raw milk from farmers' houses, kariesh cheese and plain yoghurt samples, respectively. The biochemically identified coliforms were *E. aerogenes*, *E. agglomerans*, *E. cloacae*, *C. diversus*, *C. freundii*, *E. coli*, *K. oxytoca* and *K. pneumonia* with respective percentages of; 1.19, 1.19, 2.38, 25.0, 15.48, 26.19, 13.09 and 15.48, in raw milk from farmers' houses, 6.0, 0.0, 2.0, 28.0, 10.0, 22.0, 0.0, and 32.0 in kariesh cheese, 0.0, 0.0, 2.17, 23.91, 0.0, 36.96, 26.09, 10.87 in plain yoghurt. Comparative identification of isolated *Enterobacter* spp. by standard biochemical methods and Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry revealed that the total conformity of identification of *Enterobacter* strains between standard biochemical technique and MALDI-TOF MS technique was 66.6%, where, it was ranged from 50% to 100 for *E. aerogenes* and *E. cloaca*, respectively. However, the only identified *E. agglomerans* isolate from raw milk could not be confirmed by MALDI-TOF MS technique. It has been shown through these results that presence of coliforms and *Enterobacter* bacteria is an evidence of the lack of health requirements and thermal treatments of raw milk and some of its products (kariesh cheese and yoghurt).

Keywords: Milk, Dairy products, Coliforms, *Enterobacter*, MALDI-TOF MS.

Introduction

Good nutrition is the key of the good health which protects human life against diseases. In general, dairy foods are nutritious and typically balanced food stuffs, which become important sources of a healthy food [1]. Nowadays, it's known that "Milk is the most nearly perfect food" [2]. Raw milk is considered as a very good medium for micro-organisms growth due to its high nutrient content [3].

Coliforms is a group of Gram-negative bacteria which are used as indicators for sanitary quality of foods [4], also some of them are responsible for the development of objectionable taints in raw milk and unpasteurized dairy products [5]. Coliforms count above 500 cell / mL in milk indicates poor hygiene [6]. These bacteria cause occasionally food-borne illness [7].

Enterobacter spp. is one of coliform group found in the natural environment in

variable habitats such as water, sewage, vegetables and soil [8]. They are known to act as opportunistic pathogens, which can cause numerous infections, including eye and skin infections, meningitis, bacteremia, pneumonia, urinary tract infections, wound, intestinal infections and surgical site infections [9].

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) as a comparative identification technique. It is an ionization technique that uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation. It has been applied for the analysis of biomolecules (biopolymers such as DNA, proteins, peptides and sugars) [10]. Therefore, the present study was conducted to investigate the public health importance of coliform group through isolation of coliforms from raw milk and some dairy products then biochemical and MALDI-TOF MS identification of the isolated coliforms with special reference to *Enterobacter spp.*

Materials and Methods

Collection of samples

A total of two hundred samples of raw milk and dairy products were examined in this study including 80 raw milk samples (30 from dairy shops and 50 from farmers' houses) and 30 each of kariesh cheese, plain yoghurt, milk powder and infant formula. Samples were collected randomly from Mansoura city, Dakahlia Governorate, Egypt during the period from February to July 2018. All samples were aseptically collected in their original containers or in sterilized capped bottles and transported in an ice box to Animal Health Research Institute laboratory (Mansoura) and analyzed immediately with a minimum of delay.

Preparation of samples:

Preparation of serial dilution

Raw Milk

Samples were thoroughly mixed under aseptic condition. A total of 11 mL of well mixed samples were aseptically transferred into sterile bottle containing 99 mL of sterile phosphate buffer saline (PBS) to yield a final dilution of 1:10, from which decimal dilutions were prepared [11].

Kariesh cheese

Each sample was thoroughly mashed in a sterile mortar before being emulsified in a diluent solution. Eleven grams of each prepared sample were transferred into a sterile blender warmed to 40- 45°C, followed by addition of 99 mL of 2% of Sodium citrate phosphate buffer warmed to 40-45°C and then blending for 2 min. at a speed sufficient to emulsify the sample thoroughly to make a dilution of 1:10, from which decimal dilutions were prepared [11].

Yoghurt samples, milk powder and infant formula samples

Yoghurt samples were thoroughly mixed under aseptic condition, however, milk powder and infant formula samples were reconstituted by following reconstitution instruction found on its original packages. Eleven grams of previously prepared sample were transferred into sterile, wide mouth container, containing 99 mL of sterile dilution buffer (40°C to 45°C) and thoroughly mixed until a homogeneous 1:10 solution obtained, from which decimal dilutions were prepared [11].

Enumeration of coliforms

One milliliter of each previously prepared dilutions was transferred into an empty sterile plate to which, 10 to 15 mL of Violet Red Bile Agar (VRBA, Hi Media) tempered to 44-46 °C were added. The mixture was allowed to solidify on a level surface, then an additional 3 to 4 mL of plating medium were distributed as an overlay, completely covered the surface of the solidified medium. The

plates were inverted and incubated for 24 ± 2 hr at $32 \pm 1^\circ\text{C}$. Dark red colonies measuring 0.5 mm or more in diameter on un-crowded plates were counted (15-150 coliforms colonies) and the results were recorded [11].

Confirmed test with colonies from a solid medium

Typical and/or atypical colonies were transferred to tubes of Brilliant green bile (BGB, HiMedia) broth and incubated for 48 ± 3 hr at $35 \pm 1^\circ\text{C}$. The presence of gas in the inverted Durham tube or effervescence after gentle agitation indicates a positive confirmed test. Failure of gas production within 48 hours indicates the absence of coliforms. The confirmed isolates were identified biochemically by Indole test, Methyl Red test, Voges – Proskauer test and Citrate utilization test [12].

Proteomic identification of isolated *Enterobacter* spp. based on MALDI-TOF MS

Nine biochemically confirmed isolates of coliforms (4 *E. aerogenes*, 1 *E. agglomerans* and 4 *E. cloacae*) were subjected to MALDI-TOF MS (MALDI-TOF /MS ultra flex extreme bruker daltonics Germany, flex control, Biotyper RTC) in the Clinical Laboratories, Faculty of Medicine, Alexandria University to calculate the agreement of identification between the biochemical methods and

MALDI-TOF MS, according to the following technique

Preparation of Bacterial Extracts for MALDI-TOF MS

For the MALDI-TOF MS analysis, low-molecular-weight soluble proteins were extracted from intact bacterial cells using the simple and fast method [13].

Spectra Acquisition

The analyte was embedded in a very large excess of a matrix compound deposited on a solid surface called a target, usually made of a conducting metal and having spots for several different samples to be applied. After a very brief laser pulse, the irradiated spot was rapidly heated and became vibrationally excited. The matrix molecules energetically ablated from the surface of the sample absorbed the laser energy and carried the analyte molecules into the gas phase as well. During the ablation process, the analyte molecules were ionized by being protonated or deprotonated with the nearby matrix molecules because the most common MALDI ionization format is for analyte molecules to carry a single positive charge.

Results and Discussion

Raw milk is strongly implicated in human food poisoning (FP) and transmission of various human pathogens [14].

Table (1): Statistical analytical results of total coliforms count of examined samples:

Samples	No. of examined samples.	Positive samples		Count CFU /ml or gm.		
		No	%	MIN.	MAX.	Mean \pm S.E.M*
Raw milk (dairy shops)	30	0	0.00	0.00	0.00	0.00
Raw milk (dairy farmers)	50	42	84.00	6.00×10^5	8.09×10^6	$2.80 \times 10^{6a} \pm 0.73 \times 10^5$
Kariesh cheese	30	25	83.33	2.00×10^5	4.04×10^6	$2.30 \times 10^{6a} \pm 0.75 \times 10^4$
Plain yogurt	30	23	76.67	1.87×10^5	2.26×10^6	$1.08 \times 10^{6b} \pm 1.50 \times 10^4$
Milk powder	30	0	0.00	0.00	0.00	0.00
Infant formula	30	0	0.00	0.00	0.00	0.00

*SEM = Standard error of mean.

^a and ^b: Mean within the same column bearing different superscript letters are significantly different at $P < 0.05$ according to tukey-kramer honestly significant difference tests.

In the current study, a total of 200 milk and dairy products samples were analyzed for the presence of coliforms. The results illustrated in Table (1) reveal that 42 (84%) out of 50 raw milk samples from farmers' houses, 25 (83.33%) out of 30 kariesh cheese samples and 23 (76.6%) out of 30 plain yoghurt samples were contaminated with coliforms. Coliforms failed to be detected in raw milk samples collected from dairy shops, while, they were ranged from 6.00×10^5 to 8.09×10^6 with a mean value of $2.80 \times 10^6 \pm 0.73 \times 10^5$ in raw milk samples collected from dairy farmers' houses. Nearly similar results were obtained by Sobeih et al. [16] who found that, the overall average count of coliforms was $2.5 \times 10^6 \pm 3.9 \times 10^5$ cfu/ml. Lower findings were obtained by Ibrahim et al. [17] who found that, the logarithmic average of coliforms was 2.5×10^3 cfu/ml. On the other hand, higher counts were obtained Chye et al. [18]. These results may be attributed to manual milking, and bad

handling, using unsterilized utensils as well as using inferior quality water.

While, coliforms count in kariesh was ranged from 2.00×10^5 to 4.04×10^6 with Mean value of $2.30 \times 10^6 \pm 0.75 \times 10^3$. Nearly similar results were obtained by Abd El-Latif [19].

Many sources lead to microbial contamination of kariesh cheese as using inferior quality raw milk for manufacture of Kariesh cheese, processing under unorganized environments [7].

In plain yoghurt coliforms were ranged from 1.87×10^5 to 2.26×10^6 with Mean value of $1.08 \times 10^6 \pm 1.50 \times 10^4$. These results were nearly similar to El-Diasty et al. [20] and lower than El-Biaa [21]. On the other side, coliforms were absent in the examined yoghurt samples [22]. The presence of coliforms in milk and milk products is an indication of unsanitary production and or improper handling of either milk or milk utensils [23].

Table (2): Incidence of identified coliforms in examined samples (identified by standard biochemical methods).

Identified strains*	Raw milk (dairy farmers)		Kariesh cheese		Plain Yogurt	
	No.	%	No.	%	No.	%
<i>E. aerogenes</i>	1	1.19	3	6.00	0	0.00
<i>E. agglomerans</i>	1	1.19	0	0.00	0	0.00
<i>E. cloacae</i>	2	2.38	1	2.00	1	2.17
<i>C. diversus</i>	21	25.00	14	28.00	11	23.91
<i>C. freundii</i>	13	15.48	5	10.00	0	0.00
<i>E. coli</i>	22	26.19	11	22.00	17	36.96
<i>K. oxytoca</i>	11	13.09	0	0.00	12	26.09
<i>K. pneumoniae</i>	13	15.48	16	32.00	5	10.87
Total	84	100.0	50	100.0	46	100.0

*Two colonies were selected from each positive sample.

Incidence of *E. aerogenes*, *E. agglomerans*, *E. cloacae*, *C. diversus*, *C. freundii*, *E. coli*, *K. oxytoca* and *K. pneumoniae* in raw milk samples collected from farmers' houses were 1.19%, 1.19%, 2.38%, 25.0%, 15.48%, 26.19%, 13.09% and 15.48 %, respectively (Table 2). These results differ from those recorded by Bahout and Moustafa [24] who found that the prevalence of *E. aerogenes*, *E. agglomerans*, *E. cloacae*, *C. diversus*, *C. freundii*, *E. coli*, *K. oxytoca* and *K. pneumoniae* were 38.89%, 34.34%, 28.77%, 31.34%, 47.77%, 18.89%, 36.78% and 20.00%, respectively. Enterobacter species is a member of the coliform group. There are two clinically important species from this genus, *E. aerogenes* and *E. cloacae* [25]. On the other hand the prevalence of *E. aerogenes*, *E. cloacae*, *C. diversus*, *C. freundii*, *E. coli*, and *K. pneumoniae* in kariesh cheese isolates were 6.00%, 2.00%, 28.00%, 10.00%, 22.00%, and 32.00%, respectively, but *E. agglomerans* and *K. oxytoca* failed to be detected in these samples. These results were less than those reported by El-Bagory et al. [26]. The high prevalence of coliforms in kariesh cheese samples indicated bad quality and neglected sanitary measures of these products. Detection of coliforms often reflects faecal

contamination [27]. Enterobacter species are known to act as opportunistic pathogens; they can cause numerous infections, including eye and skin infections, meningitis, bacteremia, pneumonia, urinary tract infections, wound, intestinal infections and surgical site infections [28]. The occurrence of *E. cloacae*, *C. diversus*, *E. coli*, *K. oxytoca* and *K. pneumoniae* in plain yoghurt samples were 2.17%, 23.91%, 36.96%, 26.09% and 10.87%, respectively, while *C. freundii*, *E. aerogenes* and *E. agglomerans* could not be detected in these isolates (Table 2). El-Ansary [29] found that the incidence of *E. freundii*, *E. aerogenes*, *E. cloacae*, *E. coli*, and *K. pneumoniae* in yoghurt samples was 2 (6.89%), 3 (10.34%), 3 (10.34%), 4 (13.79%) and 13 (44.82%), respectively.

E. aerogenes have taken on clinical significance as an opportunistic bacteria and have been emerged as nosocomial pathogen from intensive care patients, especially to those who are on mechanical ventilation [30]. *K. pneumoniae* constitutes a part of the pneumonia-causing microorganisms worldwide [31]. *K. oxytoca* causes meningitis, severe neurological complications such as hydrocephaly, empyema, and brain abscesses [32].

Table (3): Incidence of Enterobacter spp. in examined samples (identified by MALDI -TOF MS)

<i>Enterobacter spp.</i>	Raw milk		Kariesh		Yoghurt	
	No.	%	No.	%	No.	%
<i>E. aerogenes</i>	1	100.0	1	33.3	0	0.0
<i>E. agglomerans</i>	0	0.0	0	0.0	0	0.0
<i>E. cloacae</i>	2	100.0	1	100.0	1	100.0

The results indicated in Table (3) revealed that *E. aerogenes* was confirmed by MALDI-TOF MS technique in one isolate each of raw milk and Kariesh cheese out of 1 and 3 of biochemically identified *E. aerogenes* isolates, respectively.

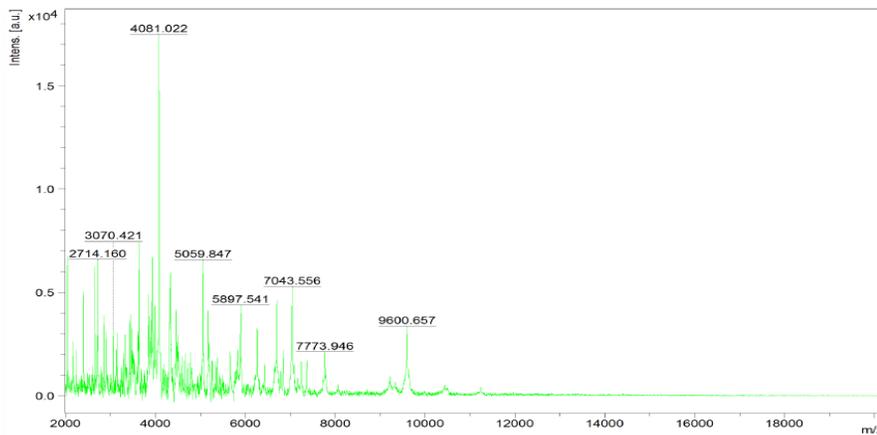


Figure (1): Spectrum view of *E. aerogenes* indicating the pattern of the conserved ribosomal protein in *E. aerogenes* by MALDI-TOF MS.

On the other hand, *E. cloacae* was confirmed by MALDI-TOF MS in all of biochemically identified *E. cloacae* isolates.

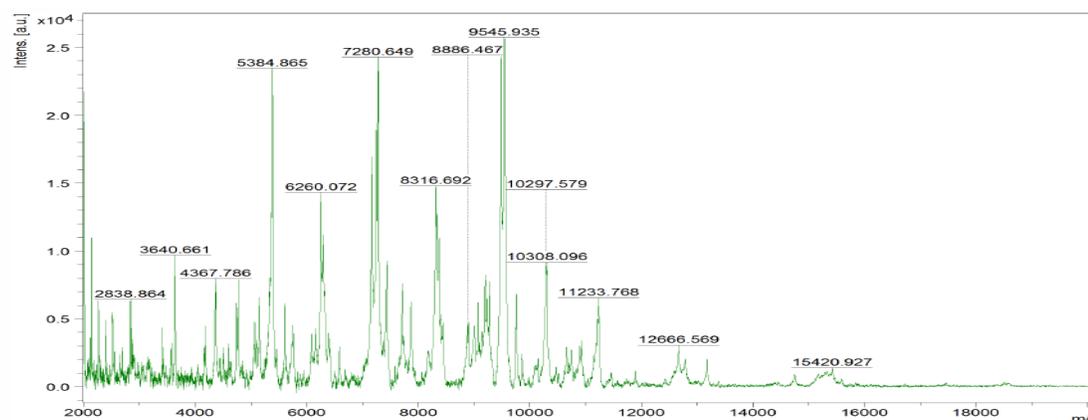


Figure (2): Spectrum view of *E. cloacae* indicating the pattern of the conserved ribosomal protein in *E. cloacae* by MALDI-TOF MS

However, *E. agglomerans* could not be confirmed by MALDI-TOF MS technique in the single biochemically identified isolate from raw milk. *E. agglomerans* is known today as *Pantoea agglomerans*, occasionally reported to be an opportunistic pathogen in immunocompromised patients, causing wound, blood, and urinary-tract infections. Infections are typically acquired from

infected vegetation parts penetrating the skin. Contaminated intravenous fluids or blood products are rarely the causative agent [33]. Blood stream infection can lead to disseminated disease and end-organ infection, mainly septic arthritis, endophthalmitis, periostitis, endocarditis and osteomyelitis in humans [34].

Table (4): Agreement of identification between standard biochemical methods and MALDI-TOF MS for Enterobacter isolates.

Isolates	No. of isolates identified by biochemical methods	No. of Identical identified isolates by MALDI-TOF MS	AI* %
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<i>E. aerogenes</i>	4	2	50.00
<i>E. agglomerans</i>	1	0	0.00
<i>E. cloaca</i>	4	4	100.0
Total AI			66.66

AI*: agreement of identification

The stated findings in Table (4) showed a total conformity of identification of *Enterobacter* strains between standard biochemical technique and MALDI-TOF MS technique was 66.6%, where, it was 50% and 100% for *E. aerogenes* and *E. cloaca*, respectively. However, the only identified *E. agglomerans* isolate from raw milk could not be confirmed by MALDI-TOF MS technique.

This result was lower than that revealed by Van Veen *et al.* [35] who found that the correct species identification by MALDI-TOF MS was 97.7%. Meanwhile, Cherkaoui *et al.* [36] evaluated the agreement of identification between MALDI-TOF MS system (Bruker MS) and conventional biochemical test, which was 99.1%, while Rodrigues *et al.* [37] found that the matching in identification of *Enterobacter spp.* biochemically and by MALDI-TOF MS was 92.9%. Also Singhal *et al.* [38] compared the identification by the burker MALDI-TOF MS and conventional phenotypic and found an agreement with a percentage of 95.4%.

MALDI-TOF MS is a rapid diagnostics technique with low costs and accurate method of identification. It may be considered as an alternative technique for conventional biochemical methods for correct bacterial identification. MALDI-TOF MS has been successfully and extensively applied for the identification and typing of microorganisms implicated in clinical sector [39] and recently in food sector [40], proving higher identification and discrimination potentials, less-costiveness, rapidity and labor-saving compared to traditional tools [41].

In microbiology, MALDI-TOF MS allows the identification of microorganisms such as yeasts and bacteria. This identification is based on the analysis of the peptidic spectra (also called protein fingerprint signature) which is specific of each species and family [38]. Nowadays, MALDI-TOF MS can be used as a sensitive, reliable and rapid procedure for identification of various clinical bacterial isolates such as Enterobacteriaceae [42].

MALDI-TOF MS involves an ionization of the sample covered of an excess of matrix by using a laser which form protonated molecules, an acceleration of molecules by an electric field until a detector trough a vacuum flight tube, and a mass spectrum obtained from data analysis [43].

Conclusion

The current results allowed to assume that the sanitary measures adopted during production, handling and processing of raw milk from farmers' houses, kariesh cheese and plain yoghurt were neglected and this represent a public health hazard for humans. This was contrary to what the results showed for dairy shops raw milk, milk powder and infant formula. MALDI-TOF MS has a high discrimination and identification potentials, rapidity, less- costiveness and labor-saving compared to traditional methods for bacterial identification.

Conflict of interest

There is no conflict of interest.

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الملخص العربي

مدي تواجد القولونيات في اللبن الخام وبعض منتجاته مع اشارة الى التصنيف المقارن للانتيروباكتري

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أجريت الدراسة الحالية لفحص 200 عينة من الحليب الخام وبعض منتجات الألبان (الجبن القريش، زبادي الساده، مسحوق الحليب وحليب الأطفال) للتلوث بمجموعة الكوليفورم خاصة الانتيروباكتري. تم اكتشاف مجموعة الكوليفورم في 50/42 (84%) من عينات الحليب الخام المجمعة من منازل المزارعين، 30/25 من عينات الجبن القريش (83.33%) و 30/23 من عينات الزبادي الساده (76.67%)، في حين لم يتم اكتشافها في أي من عينات الحليب الخام المجمع من محلات الألبان ومسحوق الحليب وعينات حليب الأطفال. أظهرت النتائج ان متوسطات أعداد بكتريا الكوليفورم في اللبن الخام والجبن القريش و الزبادى الساده هي $610 \times 2.80 \pm 510 \times 0.73$ و $610 \times 2.30 \pm 410 \times 0.75$ و 610×1.08 و 410×1.50 على التوالي. تم تصنيف العزلات بالأختبارات البيوكيميائية الى السيتروباكتري دايفرساس والانتيروباكتري ايروجينيز والانتيروباكتري ارجلومييرانس والانتيروباكتري كولواكي والكليسيلا اوكسى توكا والكليسيلا نيموني واليشيريشيا كولاي والستروباكتري فرويندي بنسب مختلفة من اللبن الخام والجبن القريش و الزبادي الساده. تم تصنيف ميكروبات الانتيروباكتري بأستخدام تقنية المالدى توف ومقارنتها بنتائج الأختبارات البيوكيميائية واسفرت النتائج عن وجود توافق بين نتائج الأختبارات بنسبة 66.6% حيث تراوحت هذه النسبة بين 50% لعزلات الانتيروباكتري ايروجينيز و 100% لعزلات الانتيروباكتري كلواكي. وقد اتضح من خلال هذه النتائج ان وجود ميكروبات الكوليفورم وخاصة الانتيروباكتري دليل على نقص الاشرطاطات الصحية والمعاملات الحرارية للبن الخام وبعض منتجاته من الجبن القريش و الزبادى الساده.