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EMERGENCE OF EXTENDED SPECTRUM Β-LACTAMASE (ESBL) PRODUCING AND COLISTIN-RESISTANT *ENTEROBACTERIACEAE* IN MILK AND SOME DAIRY PRODUCTS: MICROBIAL SAFETY AND QUALITY ASSESSMENT

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ABSTRACT

Milk and dairy products are crucial component of the Egyptian daily diet. A major concern in the 21st century is the contamination of dairy products with microorganisms that convey antimicrobial-resistant genes (ARGs), particularly extended spectrum β-lactamases (ESBLs) and colistin genes. Therefore, this study investigated the total viable count, coliforms, *Enterobacteriaceae*, total *Staphylococci*, and aerobic spore formers in 90 samples of raw milk, Baladi yoghurt, and white soft cheese (30 of each). Coupled with the phenotypic and genotypic characterization of the ESBL-producing and colistin resistant *Enterobacteriaceae* strains. The incidences of aerobic spore formers, *Staphylococci*, and *Enterobacteriaceae* were 100, 100, 83% ;100, 67, 100% and 100, 63, 33% in milk, Baladi yoghurt, and white soft cheese, respectively. The phenotypic screening of the identified *Enterobacteriaceae* isolates (n=136) revealed that 80.8% (n=110) were ESBL-producing, while merely 2.9% were colistin resistant. Genotypic analysis revealed that $bla_{CTX\text{-}M}$ and bla_{SHV} were presented in 99% of the identified isolates, although bla_{TEM} was only present in 84.5% of them. Interestingly, the bla_{OXA} gene was not detected. Notably, all phenotypically colistin resistant isolates harboured *mcr-1* gene. Those findings indicated that milk and dairy products can help in spreading of beta-lactam resistant bacteria, emphasizing the importance of further research to mitigate them in the dairy sector.

Keywords: Cheese, Yoghurt, Antimicrobial resistant genes (ARGs), *Enterobacteriaceae*, ESBL, Colistin resistance, *mcr-1gene*

INTRODUCTION

Milk and dairy products, particularly yoghurt and cheese are a vital part of the human diet around the world, since they provide a diverse range of high quality nutrients necessary for sustaining overall nutritional well-being **(Tan *et al*., 2024)**. Yoghurt is regarded as the world's most widely available fermented milk product due to its mildly sour flavour, distinct texture and nutritional value which includes essential nutrients and beneficial probiotics **(Allam *et al*., 2023)**. Cheese, on the other hand, is popular among consumers because of its appealing sensory characteristics and nutritional value. It is an excellent source of various nutrients, especially high-quality protein, carbohydrates, and lipids, as well as vitamins and minerals **(Possas *et al*., 2021)**. Nonetheless, milk and dairy products also provide an ideal environment for the growth of a wide range of spoilage and pathogenic microorganisms **(Owusu-Kwarteng *et al*., 2020)**. The presence of such microorganisms not only impacts the sensory characteristics, nutritional composition and health benefits of milk and dairy products, but it may also result in significant economic losses, foodborne illness, and potential public safety concerns **(Nadi *et al*., 2023)** .
Total viable count and total coliforms are major indicators for assessing the overall safety and quality, as well as monitoring the adoption of sanitary practices during production, storage, and distribution of milk and dairy products. Furthermore, total coliforms are spoilage microorganisms and indicators of faecal contamination **(Sobeih *et al*., 2020)**, Therefore their routine monitoring is essential to assess the sanitary standards in the dairy sector. Additionally, the presence of Aerobic spore formers (ASF) poses another challenge for the dairy industry, since they can survive the pasteurization as well as induce spoilage through their extracellular or intracellular thermo-resistant enzymes (proteases, lipases & phospholipase) that can cause off- flavour, rancidity, and bitterness in the final dairy product **(Taher *et al*., 2023;Finton *et al*., 2024)**.
On the other hand, foodborne diseases continue to be a major worldwide health threat, with over 600 million people experiencing illnesses and 420,000 fatalities due to contaminated food annually **(World Health Organization, 2015)**. *S. aureus* and *E. coli* are two of the most frequently encountered foodborne pathogens in milk and dairy products **(Nadi *et al*., 2023)**. *S. aureus* is considered the third most common cause of foodborne illnesses worldwide, which is capable of surviving in raw milk and different dairy products through several survival mechanisms **(Taher *et al*., 2020; Huang *et al*., 2023)**. Whereas *E. coli* has been associated with a major gastrointestinal disorders and potentially fatal conditions such as haemolytic uremic syndrome **(Fahim *et al*., 2023; Sanjay *et al*., 2024)**.
Another growing food safety concern is the antibiotic-resistant *Enterobacteriaceae*, particularly those producing colistin and extended spectrum β-lactamases (ESBLs), that emerged in recent decades, and continuing to pose a substantial challenge to veterinary and human medicine **(Husna *et al*., 2023)**. ESBL *Enterobacteriaceae* carry a broad spectrum of β-lactamase enzymes that hydrolyse a wide range of penicillin and cephalosporin antibiotics rendering them resistant to almost all available antibiotics **(Cho *et al*., 2023)**. Additionally, those ESBL genes are frequently located on conjugative plasmids that facilitate their horizontal transfer across both similar and diverse bacterial species through the food **(Seethalakshmi *et al*., 2024)**. Colistin, on the contrary, is the last-line defence against infections caused by multidrug-resistant (MDR) Gram-negative bacteria. Nevertheless, the emergence of colistin-resistant strains on a global scale threatens its efficacy **(Hide *et al*., 2024)**.
Certain *Enterobacteriaceae* species, including *Proteus mirabilis* and *Serratia marcescens*, are inherently resistant to colistin. In contrast, other members of *Enterobacteriaceae*, including *E. coli*, *Enterobacter spp.*, *Salmonella spp.*, and *Klebsiella spp.*, as well as non-*Enterobacteriaceae* bacteria such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, can acquire colistin resistance **(Mondal *et al*., 2024)**. From food safety standpoint, the selection and dissemination of such patterns of resistance in food borne bacteria pose a significant hazard.
Hence, this study was initially conducted to evaluate the safety and quality of raw milk, Baladi yoghurt, and white soft cheese sold in the Egyptian dairy market by investigating the prevalence of the main hygienic indicators, spoilage and some pathogenic microorganisms coupled with their biochemical identification. ESBL-producing and colistin-resistant *Enterobacteriaceae* were subsequently further characterized along with assessing the phenotypic and genotypic correlations of their resistance patterns.

MATERIALS AND METHODS

Samples Collection

Ninety random samples of raw milk, Baladi yoghurt, and white soft cheese (30 of each) were collected aseptically from local markets, dairy shops, and supermarkets widely distributed across different districts in Cairo and Giza governorate between November 2023 and June 2024. Samples were identified and promptly transported in an insulated ice box to be analyzed immediately at PC1 Food Hygiene and Control lab, Faculty of Veterinary Medicine, Cairo University, Egypt. Genotyping characterization and microbiological testing of the samples were done in parallel.

Sample preparation and Microbiological examination

Raw milk samples were subjected to Guaiac test to identify and exclude samples that were confirmed to be heat-treated according to **Schonberg (1956)**. The food homogenate and decimal dilutions of the examined samples were carried out in accordance with **APHA (2004)**. Briefly, a well-mixed raw milk samples, Baladi yoghurt, and white soft cheese samples (11 mL/g) were added to sterile peptone water 0.1% (99 mL) and sterile sodium citrate solution 2% for cheese followed by thorough homogenization in a stomacher bag using a Lab-blender 400 (Stomacher; Inter science, France) for 2-4 minutes to to prepare the food homogenate of (1/10 dilution). Then, ten-fold serial dilutions were carried out for the samples. Enumeration and isolation of Total viable count (TVC), Coliforms (MPN), Aerobic spore formers, *Staphylococci* and *Enterobacteriaceae* were done according to **APHA (2004)**.

Biochemical identification of the isolated strains

Biochemical Identification of the Aerobic spore formers and *Staphylococci* isolates were performed according to **Whitman *et al.* (2015)**, while identification of *Enterobacteriaceae* was carried out according to **De Vos *et al.* (2009)**.

Antimicrobial susceptibility testing of *Enterobacteriaceae* isolates

Biochemically identified *Enterobacteriaceae* isolates (n= 136) were examined for susceptibility to different antibiotics (Oxoid, US) using the Kirby-Bauer disk diffusion method using Mueller-Hinton agar (Oxoid, US), according to the Clinical and Laboratory Standards Institute guidelines **(CLSI, 2020)**. The antibiotics included were [Cefpodoxime (CPD, 10 mg), Cefotaxime (CTX, 30 mg), Ceftazidime (CAZ, 30 mg), Ceftriaxone (CRO, 30 mg), Aztreonam (ATM, 30 mg), and Colistin (CLM, 10mg)]. The results were interpreted according to the clinical breakpoints recommended by CLSI (2020),While the interpretation criteria for evaluating colistin resistance were based on breakpoints previously outlined by **Gales *et al.* (2001**).

ESBL detection by double-disk synergy test method

The double-disk synergy test was utilized to confirm the ESBL production in the identified *Enterobacteriaceae* isolates (n=136). A standardized inoculum (0.5 McFarland tube) of every *Enterobacteriaceae* isolates was plated on Mueller-Hinton agar. Cefotaxime (CTX) and ceftazidime (CAZ) alone and in combination with clavulanic acid (CTX/clavulanic acid, 30/10 µg [CTC]; and CAZ/ clavulanic acid, 30/10 µg [CAC]) discs were applied on the plates and incubated overnight at 37°C. *Enterobacteriaceae* isolates with zone diameter >5 mm increase for the combination disk (CAC) or (CTC) or both compared to the single antibiotic were considered phenotypically ESBL-producing *Enterobacteriaceae*, otherwise, the isolates were considered phenotypically non-ESBL-producing **(CLSI, 2020)**.

Genotypic characterization of β-lactamase (ESBL) and colistin encoding genes

In accordance with **(Darwish & Asfour, 2013)**, crude DNA was extracted from bacterial isolates using the boiling method. In brief, 1 mL of broth from each isolate was transferred from the nutrient broth and centrifuged at 5000 rpm for 10 minutes to collect the bacterial pellet. The pellet was then washed twice with Tris-EDTA buffer and subsequently resuspended in 200 µl of lysis buffer [1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA]. Following 10 minutes boiling, the suspension was centrifuged at 10000 rpm for 5 minutes to remove the bacterial debris. The supernatant was carefully removed, and 5 µl of the supernatant was used directly for PCR amplification.

A multiplex PCR protocol was carried out utilizing five pairs of genes targeting primers to detect the presence of following antimicrobial resistance genes (ARGs): ESBL genes (bla_{CTXM}, bla_{TEM}, $bla_{SHV,}$ and bla_{OXA}), plasmid-mediated colistin gene *mcr-1,* and *E. coli isolates* were genotypically confirmed using *16srRNA* gene (Table 1). PCR reactions were performed in a 25 µl volume using 5 µl of template DNA, 5.5 µl of DEPC-treated water, 1 µl from each primer with a concentration of 20 pmol, and 12.5 µl of PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science). The amplifications were carried out in 35 PCR cycles using a PT-100 Thermocycler (MJ Research, USA) and consisted of preheating activation for 5 minutes at 94°C, denaturation at 94°C for 30 seconds, and annealing at 55°C for 1 min for *16srRNA*, 62°C for bla_{CTXM}, bla_{TEM}, $bla_{SHV,}$ and bla_{OXA} and 61°C for *mcr-1* for 60 seconds. The final extension step was performed at 72°C for 10 minutes. The PCR products were electrophoresed in a 1.5% agarose gels visualized by staining with Gel Red and then inspected and photographed under UV light.

Table 1 Sequences of oligonucleotide primers used for PCR amplification of *E. coli*, ESBLs encoding and colistin genes.

Primer name	Target gene	Oligonucleotide primer sequences (5′-3′)	Product size (bp)	PCR condition	References
***E. coli* identification**	***16srRNA***	**F:** CGGTGAATACGTTCCCGG **R:** GGTTACCTTGTTACGACTT	142	1 cycle (95 °C, 5 min)30 cycle (94 °C, 30 s/55 °C, 1 min / 72 °C, 1 min)1 cycle (72 °C, 10 min)	**(Suzuki *et al*., 2000)**
ESBLs	*SHV* (bla_{SHV})	**F:** CTT TAT CGG CCC TCA CTCAA **R:** AGG TGC TCA TCA TGG GAA AG	237	1 cycle (95 °C, 5 min)30 cycle (94 °C, 30 s/62 °C, 1 min / 72 °C, 1 min)1 cycle (72 °C, 10 min)	**(Fang *et al*., 2008)**
	TEM (bla_{TEM})	**F:** CGC CGC ATA CAC TAT TCT CAG AAT GA **R:** ACG CTC ACC GGC TCC AGA TTT AT	445		**(Monstein *et al*., 2007)**
	CTXM (bla_{CTXM})	**F:** ATG TGC AGY ACC AGT AAR GTK ATG GC **R:** TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593		**(Boyd *et al*., 2004)**
	OXA (bla_{OXA})	**F:** ACA CAA TAC ATA TCA ACTTCG C **R:** AGT GTG TTT AGA ATG GTG ATC	813		**(Ouellette *et al*., 1987)**
Colistin	MCR-1 (*mcr-1*)	**F:** AGT CCG TTT GTT CTT GTG GC **R:** AGA TCC TTG GTC TCG GCT TG	320	1 cycle (95 °C, 5 min) 30 cycle (94 °C, 30 min/ 61 °C,1 min/72 °C, 1 min) 1 cycle (72 °C, 10 min)	**(Rebelo *et al*., 2018)**

Statistical analysis

The statistical analysis was carried out using IBM SPSS Statistics (version 27.0) for Windows. The results were conducted as mean ± SEM, A one-way analysis of variance (ANOVA) with Post hoc analysis was employed for multiple comparisons of the means across all tested parameters. Significance was determined at $P < 0.05$. Pearson correlation was constructed using R version (4.3.2), heat map (version 1.0.12) to detect correlation between phenotypic-genotypic variables among *Enterobacteriaceae* isolates.

RESULTS

Milk and dairy products are highly perishable foods due to their high nutrients and water content that render them prone to both pathogenic and spoilage microorganisms, putting consumer's health at risk **(Ismail, 2021)**. Data presented in (Table 2) revealed that all examined samples were contaminated with microorganisms. The raw milk and Baladi yoghurt samples showed the highest TVC with mean values of 8.52±0.13 and 8.34±0.10 log_{10}cfu/mL/g, respectively. Meanwhile the white soft cheese exhibited the lowest TVC with mean value of 7.14±0.38 log_{10}cfu/g, with presence of statistically significant difference ($P<0.05$). Such high TVCs in the examined samples indicated poor sanitary standards throughout the production, processing, and storage, contributing to the decline in their quality and safety.

Table 2 Descriptive statistical analysis (Mean± S.E.M) of the tested microbial parameters of the examined raw milk, Baladi yoghurt and white soft cheese samples.

Parameters (log10 cfu/mL org)	Raw milk			Baladi Yoghurt			White soft Cheese		
	No.	Prevalence%	Mean± S.E.M.	No.	Prevalence%	Mean± S.E.M.	No.	Prevalence%	Mean± S.E.M.
Total viable count	30	100	8.52±0.13*b	30	100	8.34±0.10*b	30	100	7.14±0.38*a
Aerobic spore formers	30	100	4.79±0.10*b	30	100	4.12±0.09*b	30	83	2.47±0.22*a
Total *Staphylococci*	30	100	6.31±0.08*c	30	67	3.01±0.40*a	30	100	4.64±0.35*b
Total *Enterobacteriaceae*	30	100	6.83±0.08*c	30	63	2.60±0.42*b	30	33	1.34±0.36*a
Total coliforms (MPN/ mLorg)	30	100	5.40±0.10*b	30	100	1.06±0.22*a	30	100	1.18±0.30*a

No.: number of samples. The variance among values within the same row, denoted by different superscripts, is statistically significant ($P < 0.05$)

Aerobic spore formers (ASF) counts demonstrated that raw milk and Baladi yoghurt exhibited the highest mean counts of 4.79±0.10 and 4.12±0.09 $\log_{10}$cfu/g, respectively (Table 2), while cheese had mean count of 2.47±0.22 $\log_{10}$cfu/g ($P<0.05$). Interestingly, *B. subtlis* (14 isolates;25.9%) and *B. cereus* (8 isolates;14.8%) were the most prevalent biochemically identified strains from the examined milk samples (Fig. 1). However, the most abundant species isolated from Baladi yoghurt and white soft cheese were *B. infernus* (18 isolates; 33.9%) and *B. pumilus* (14 isolates; 20.5%), respectively.

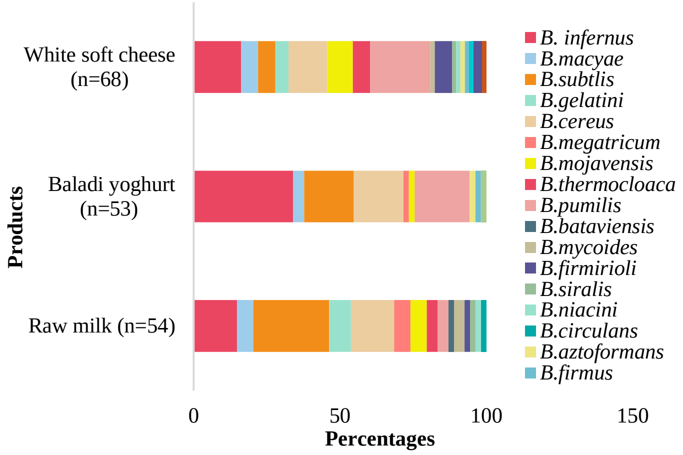

Figure 1 Incidences of the biochemically identified Aerobic spore formers (n=175) in the examined samples.

Staphylococci were found in all the examined raw milk and white soft cheese samples with mean counts of 6.31±0.08 and 4.64±0.35 $\log_{10}$cfu/g, respectively ($P<0.05$), contrarily, it was only prevalent in 67% of the examined Baladi yoghurt samples, with mean count of 3.01±0.40 $\log_{10}$cfu/g. The biochemical identification of the isolated strains indicated that *S. aureus* was identified with percentages of 22.8 (19/83 isolates), 7.5 (3/40 isolates), and 13.7 (7/51 isolates) in raw milk, Baladi yoghurt, and white soft cheese, respectively (Table 2; Fig. 2).
Enterobacteriaceae and coliforms were prevalent in all the examined raw milk samples with mean value of 6.83± 0.08 $\log_{10}$ /mL and 5.40±0.10 MPN/mL, respectively, while Baladi yoghurt exhibited the lowest coliforms count with mean value of 1.06±0.22 MPN/g. Notably, the lowest *Enterobacteriaceae* count was recorded in white soft cheese samples with mean of 1.34±0.36 $\log_{10}$cfu/g (Table 2).

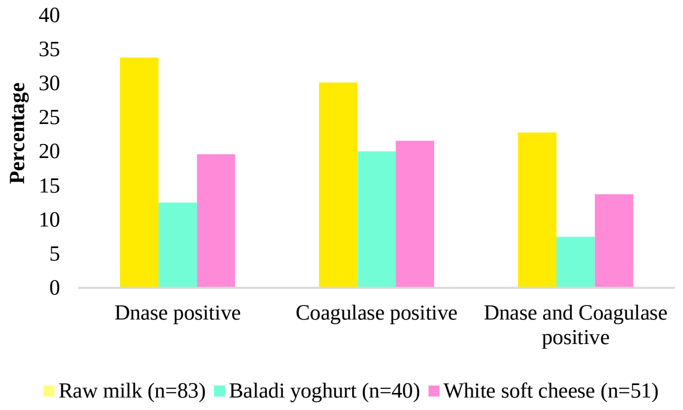

Figure 2 Biochemical identification of *Staphylococci* (n=174) isolated from the examined raw milk, Baladi yoghurt and white soft cheese samples

Remarkably, the biochemical identification of the isolated *Enterobacteriaceae* strains (n= 136) demonstrated that *K. oxytoca* was the most abundant strain (31/70 isolates; 44.2%) in raw milk, whereas *S. marscenes* predominated (9/32 isolates;28.1%) in Baladi yoghurt samples and *E. aerogene* (12/34 isolates;35.2%) in white soft cheese. *Shigella*, *C. amalonaticus*, *P. stuartii*, *Salmonella*, *Y. pestis*, *H. alvei*, *E. greoviae and p. mirabilis* were commingled since there were less than five isolates per species. Interestingly, *E. coli* was only detected in yoghurt and cheese (3 isolates; 9.3% and 2 isolates; 5.8%), respectively (Fig. 3**)**, meanwhile all the biochemically identified *E. coli* isolates were genotypically confirmed.
The antimicrobial susceptibility of the isolated *Enterobacteriaceae* isolates (n=136) to the tested antibiotics (Table 3) indicated that majority of the isolates were resistant to cefotaxime (CTX), with the *E. cloaca* isolates displaying the highest resistance (94.1%). Nonetheless, 65.6% of the isolated *S. marscenes* and 78.9% of the *K. oxytoca* isolates were resistant to ceftriaxone (CRO). According to the results depicted in Table 3, *S. marscenes*, *K. oxytoca*, *E. cloaca*, and *E. coli* were the isolates that showed resistance to all tested cephalospories. Additionally, *K. oxytoca* was particularly notable for its extensive resistance to four cephalosporins: aztreonam (ATM), ceftriaxone (CRO), ceftazidime (CAZ), and cefotaxime (CTX). In contrary, the majority of the isolates were susceptible to cefpodoxime (CPD). The confirmatory test (CDT) indicated that 80.8% of the identified *Enterobacteriaceae* isolates were ESBL, with *K. oxytoca* and *E. coli* had the highest occurrences (89.5 and 100%, respectively). On the other hand, only 2.9% of the *Enterobacteriaceae* isolates exhibited phenotypic colistin (CLM) resistance, which were predominantly observed in *K. oxytoca*, *P. mirabilis*, *S. liquifaciens*, and *E. coli* isolates.
The genotypic characterization of the ESBL-encoding genes in all identified *Enterobacteriaceae* isolates (n= 110) revealed that bla_{SHV} *and* bla_{CTXM} were *the most* prevalent identified genes with percentages of 99 followed by Bla_{TEM} gene with 84.5%. Nonetheless, bla_{OXA} was not detected in all the identified isolates (Fig. 4). In addition, the study revealed that all the isolates that showed phenotypic resistance to colistin (CLM) were harbouring the plasmid mediated *mcr-1* gene (Table 3; Fig. 4).
In this study, Pearson's correlation analysis was employed to explore the relationships between the phenotypic and genotypic resistance patterns of the isolated ESBLs *Enterobacteriaceae spp*. As illustrated in the correlation matrix (Fig. 5), weak positive associations were observed between the two variables. There were no correlations observed between bla_{SHV} and $bla_{CTX\text{-}M}$ genes with any of their corresponding phenotypic tested antibiotics (aztreonam (ATM), cefpodoxime (CPD), ceftriaxone (CRO), cefotaxime (CTX), and ceftazidime (CAZ)). However, a weak positive correlation (r=0.26) was detected between the bla_{TEM} gene and resistance to aztreonam (ATM). Conversely, a weak negative correlation was observed between bla_{TEM} and cefotaxime (CTX) (r=−0.24) and ceftazidime (CAZ) (r=−0.12), as well as between $bla_{CTX\text{-}M}$ and ATM (r=−0.13).

Table 3 Phenotypic and genotypic antibiogram of the identified *Enterobacteriaceae* spp.

Isolates (N)	**Antibiogram (%) – Antimicrobials**																		**CDT (%)**		***Antimicrobial resistance genes* N (%)**				
	ATM			**CRO**			**CPD**			**CTX**			**CAZ**			**CLM**					***ESBLs-encoding genes***				***Colistin gene***
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**ESBL**	**Non ESBL**	***bla_{TEM}***	***bla_{CTXM}***	***bla_{SHV}***	***bla_{OXA}***	***mcr-1***
Enterobacter cloaca(n=17)	29.4	52.9	17.6	23.5	5.8	70.5	94.1	5.8	-	-	5.8	94.1	11.7	11.7	76.4	100	-	-	88.2	11.8	12(80)	15(100)	15(100)	-	-
Enterobacter aerogene (n=22)	86.3	-	13.6	45.5	9.09	45.5	100	-	-	13.6	18.1	68.1	18.1	31.8	50	86.3	13.6	-	72.7	27.3	13(81.2)	16(100)	16(100)	-	-
Enterobacter intermedius (n=8)	50	-	50	12.5	25	62.5	100	-	-	12.5	12.5	75	12.5	25	62.5	75	25	-	62.5	37.5	3(60)	5(100)	5(100)	-	-
Serratia marscenes (n=32)	53.1	28.1	18.7	28.1	6.25	65.6	96.8	3.1	-	12.5	9.3	78.1	12.5	28.1	59.3	68.7	28.1	3.1	75	25	24(100)	24(100)	24(100)	-	1(3.1)
Klebsiella oxytoca (n=38)	31.5	42.1	26.3	18.4	2.6	78.9	97.3	2.6	-	2.6	10.5	86.6	10.5	18.4	71.05	84.2	115.7	2.6	89.5	10.5	34(100)	33(97)	33(97))	-	1(3.1)
E. coli (n=5)	60	-	40	20	40	40	80	20	-	-	40	60	60	-	40	80	-	20	100	-	5(100)	5(100)	5(100)	-	1(3.1)
Others[b] (n=14)	71.4	14.2	14.2	42.8	35.7	21.4	100	-	-	21.4	42.8	35.7	14.2	57.1	28.5	78.5	7.1	7.1	78.6	21.4	2(18.1)	11(100)	11(100)	-	1(3.1)

[a]The tested antimicrobials ATM, aztreonam; CAZ, ceftazidime; CDT, combinational disk test in the presence of clavulanic acid; CPD, cefpodoxime; CRO, ceftriaxone; CTX, cefotaxime; CLM, colistin. Antibiogram: S, sensitive; I, intermediate; R, resistant. [b]Others are commingled *Enterobacteriaceae* species with less than five isolations per species, including *Campylobacter amalonaticus (n=3), Providencia stuartii (n=4), salmonella (n=2) , Yersinia pestis (n=2), Hafnia alvei (n=1) , Proteus mirabilis (n=1), and Enterobacter greoviae (n=1).*

Table 4 Microbiological acceptability (%) of the examined raw milk, Baladi yoghurt, and white soft cheese samples according to the Egyptian standards–2005 and 2016.

Parameters	Products	Permissible limits	Non compatibility	
			No.	%
Total viable count	Raw milk	$<50\times10^{4}$ (reduction of Methylene blue at not less 4.5 hours)	30	100
	Yoghurt	NM*	-	-
	Cheese	(10^{4} - 10^{5}) cells/g.	20	66.6
S. aureus	Raw milk	not more than 100 cell/mL.	NC*	NC*
	Yoghurt	Nil	3	7.5
	Cheese	Nil	7	13.4
B. cereus	Raw milk	not more than one cell/mL	8	26.6
	Yoghurt	NM*	-	-
	Cheese	NM*	-	-
Coliforms	Raw milk	NM*	-	-
	Yoghurt	Not more than 10 cell/g	16	53.3
	Cheese	Not more than 10 cell/g	18	60
E. coli	Raw milk	NM*	-	-
	Yoghurt	Nil	3	10
	Cheese	Nil	2	6.6

(ES 154/2005), ES (2016/8042), ES (2005/1-1008), ES Guidelines for Soft Egyptian Cheese, 2001. NM*: Not Mentioned. NC*: Not counted

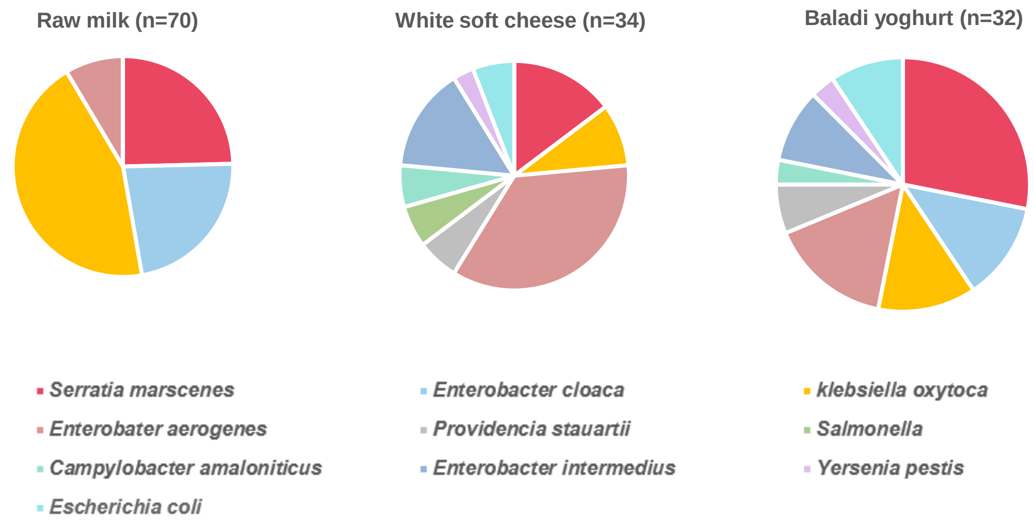

Figure 3 Incidence of the biochemically identified *Enterobacteriaceae* (n=136) strains in the examined samples.

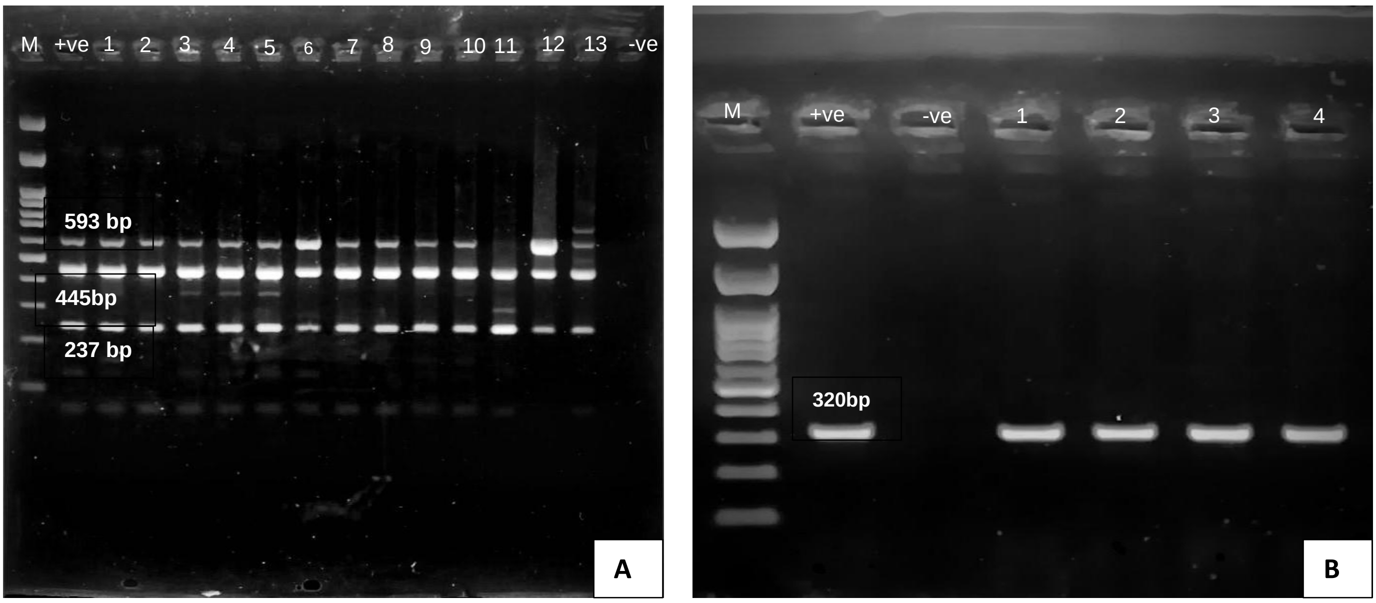

Figure 4 Multiplex PCR for detection of **(A)** bla_{SHV} (237bp), bla_{TEM} (445bp), bla_{CTXM} (593bp) and bla_{OXA} (813bp) genes in the confirmed ESBL isolates (n=110). **(B)** *mcr-1* gene (320bp) for detection of colistin resistant isolates (n=4). M: molecular size marker (100bp DNA ladder), Lane +ve: Positive control for bla_{SHV}, bla_{TEM}, bla_{CTXM} and *mcr-1* genes, Lane -ve: Negative control, **(A)** Lanes 1-10 and 12-13 positive bla_{CTXM}, bla_{TEM} and bla_{SHV}, lanes 11 positive bla_{TEM} and bla_{SHV}. **(B)** Lanes (1-4) positive *mcr-1* gene

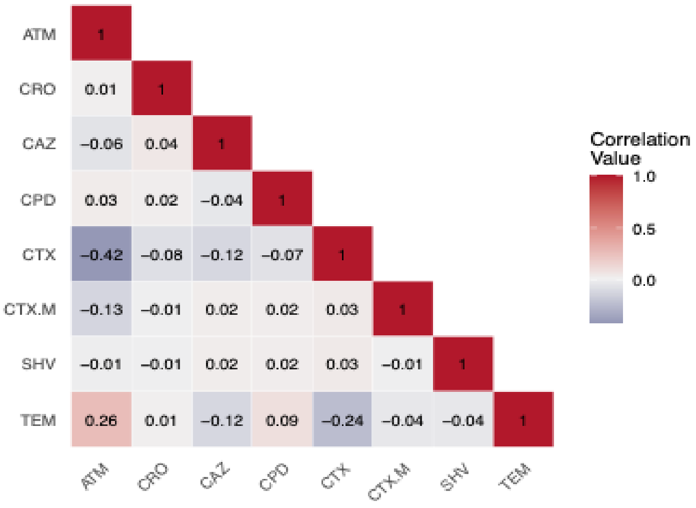

Figure 5 Pearson's correlation between the phenotypic-genotypic resistance among *Enterobacteriaceae* isolates. The heatmap showed Pearson's correlation coefficients (r) represented with a red gradient showing the strength of the correlation, where darker red indicated stronger positive correlations, lighter red indicated weaker correlations, and white represented negative correlation

DISCUSSION

The objective of this study was to evaluate the safety and quality of milk and some dairy products in the Egyptian market through investigating the presence of different deteriorating and pathogenic microorganisms coupled with phenotypic and genotypic assessment of the potential emergence of extended spectrum β-lactamase (ESBL) producing and colistin-resistant *Enterobacteriaceae* in the examined samples.

TVC is one of the main utilized techniques for initial ensuring product hygienic quality throughout production, processing and handling **(Markusson, 2021)**. The TVC of all examined samples in this study was relatively high (Table 2), where all raw milk and Baladi yoghurt samples exceeded the acceptable limits of Egyptian standards (ES 154/2005) and FSSAI regulations for fermented milk **(Pal *et al.*, 2015)**, respectively (Table 4). While 66.6% of white soft cheese samples failed to comply with the **Egyptian Standard (2001)**. These findings were consistent with the results reported by **Hasan *et al*. (2016)** and Ndahetuye *et al.* (2020). While they were comparatively higher than the counts observed by **Taiwo *et al*. (2018)**, **Tamam *et al*. (2021)** and **El-Dougdoug *et al*. (2022)**. The TVC of the white soft cheese samples were lower than those recorded by **Nadi *et al*. (2023)**, yet they were higher than the findings of **Mohamed *et al*. (2019)**. Such high total viable counts could be attributed to numerous variables, including the high initial microbial loads in the raw milk due to unsanitary/manual milking, the resistance of some spore-forming bacteria to heat treatment, potential post-pasteurization contamination, and poor storage and/or handling **(Machado *et al*., 2017)**. Reflecting an underlying problem in the handling and processing in the Egyptian dairy sector and emphasizing the need for more robust control measures to satisfy the regulatory standards.

The incidence of total spore formers in raw milk and Baladi yoghurt was rather high, whereas white soft cheese recorded relatively lower counts. Alarmingly, biochemical identification revealed that *B. cereus* was considerably abundant in all examined samples (Fig. 1). Raw milk samples showed the highest prevalence for both *B. subtlis* and *B. cereus* isolates with 26.6% of non-compliance with the Egyptian standards (ES 154/2005) (Table 4). On the contrary, *B. pumilu*s and *B. infernus* were the most abundant strains in the white soft cheese and Baladi yoghurt samples, respectively (Fig. 1). These findings aligned with those reported by **Khater & Abdella, (2017)** and **Nazem *et al.* (2020)**. However, they were relatively higher than **El-Dougdoug *et al*.(2022)** findings. The existence of such organisms could be explained by the extremely resistant endospores that have the potential to withstand pasteurization then germinate and grow under favourable conditions leading to dairy products deterioration and potential foodborne illnesses, putting customers' health at risk **(Tirloni *et al*., 2022)**.

Despite the advancements on the food safety practices, *Staphylococci*, particularly *S. aureus* food poisoning still posing a perpetual public health concern worldwide. A high incidence of total *Staphylococci* was observed in raw milk and white soft cheese samples, which could be attributed to the poor hygienic practices among the food handlers **(Yosry Abdel Halim *et al*., 2021)**. On the contrary, it was considerably low in Baladi yoghurt, suggesting that the production of organic acids during fermentation with the high acidity of such products inhibited the growth of some of the *Staphylococci* spp. **(Feyissa *et al*., 2023)** . Interestingly, *S. aureus* was isolated from almost 50% of the raw milk samples (46.6%) whereas Baladi yoghurt and white soft cheese showed non-compliance with the Egyptian standards by 7.5 and 13.4%, respectively, where the *S. aureus* should be null in these products (Table 4). These results shared similarities with the results obtained by **Lemma *et al*. (2021)** and **Ahmed *et al*. (2022)**, but it was in contrast to **Saad *et al*. (2023).**

Enterobacteriaceae and coliforms have a significant impact on milk and dairy products, affecting the safety aspects of the dairy industry. Their presence in large quantities not only indicates faecal contamination but also poses serious health risks, potentially leading to hospitalizations and complicating medical treatment. **(Elsherbeny *et al*., 2024)**. *Enterobacteriaceae* and coliforms counts were relatively high in raw milk, while Baladi yoghurt and white soft cheese counts were comparatively lower. Over 50% of Baladi yoghurt and white soft cheese samples exceeded the permissible limits set by the Egyptian standards ES (2016/8042) and ES (2005/1-1008) (Table 4). It is noteworthy that *K. oxytoca* was the most prevalent isolated strains from the raw milk emphasizing its role as a multidrug-resistant pathogen capable of causing severe infections such as bronchopneumonia, colitis, and sepsis **(Song *et al*., 2023)**. while *S. marcescens* and *E. aerogenes* were the most prevalent species in Baladi yoghurt and white soft cheese, respectively their presence raises significant public health concerns (Fig. 3). While *S. marcescens*, once thought to be a harmless microorganism, is now identified as a clinically significant pathogen with the potential to cause serious infections, particularly in neonates and immunocompromised individuals **(Melo *et al*., 2018)**. On the other hand, *E. aerogenes* has evolved into a multidrug-resistant opportunistic pathogen, often associated with a diverse range of nosocomial infections, including those involving the urinary and respiratory infections **(Farag *et al*., 2023)**.

E. coli was not isolated from any of the examined raw milk samples, yet it had been isolated from both Baladi yoghurt and white soft cheese indicating that it might be introduced through the non-hygienic manufacturing and processing steps leading to non-compliance with the Egyptian standards ES (2016/8042) and ES (2005/1-1008) in the latter two products by 10 and 6.6%, respectively (Table 4). These results correlated favourably to the results obtained by **Sobeih *et al.* (2020)**, **Lotfy *et al.* (2022)**, and **Mohamed *et al*.(2022)**. While they were in contrast with the high incidence reported by **Fathi *et al*. (2019)** and **El-Dougdoug *et al.* (2022)**.

The growing problem of antimicrobial resistance among *Enterobacteriaceae* poses a serious threat for both human and veterinary medicine in developing countries. Asia, Africa, and the Middle East have emerged as hotspots for ESBL (Extended-Spectrum Beta-Lactamase) and colistin resistance **(Badri *et al*., 2017)**. Without an effective action plan to control the antibiotic usage and mitigate the antimicrobial resistance (AMR) problem, the death rate is anticipated to rise to 10 million per year by 2050 **(Samreen *et al*., 2021)**.

Antibiogram of the identified *Enterobacteriaceae* isolates revealed that the majority of the examined isolates showed significant resistance to (cefotaxime) and (ceftazidime) antibiotics while none of them were resistant to (cefpodoxime). The double disc synergy test confirmed that 80.8% of the identified isolates were phenotypically ESBL positive (Table 3). All *E coli*, 89.5% of *K. oxytoca*, and 88.2% of *E. cloaca* isolates were ESBL-positive. Intriguingly, over 25% of the *Enterobacter* spp. (*E. aerogene* and *E. intermedius*) were non-ESBL. These results were relatively close to the results reported by **Odenthal *et al.* (2016)** and **Gucukoglu *et al.* (2022)**, while they were in contrast with the results obtained by **El-Halem *et al*.(2021)**.

On the other hand, the colistin (CLM) resistance was only noticed in 2.9% of the *Enterobacteriaceae* isolates, particularly in *E. coli, S. marscenes, and K. oxytoca.* These results were relatively low compared to the results obtained by **Tartor *et al*. (2021)**. Meanwhile they were in accordance with the results observed by **Koriem *et al*. (2024)**. The variations in ESBL and colistin prevalence could be attributed to species, geographic location, infection control practices, and antibiotic use patterns **(Mohamed *et al.*, 2020)**. However, the limited information in Egypt about the ESBL-producing bacteria in these products, resulted in minimal updates on their monitoring.

Genotypic characterization revealed that more than 80% of the identified *Enterobacteriaceae* isolates harboured the $bla_{CTX\text{-}M}$, bla_{TEM} *and* bla_{SHV} genes, however, bla_{OXA} was not detected. Interestingly, more than 50% of the ***K. oxytoca*** isolates were carrying three of the β-lactamase genes. Unfortunately, *Klebsiella spp.* is among the most highly recognized antimicrobial-resistant pathogens and a key member of the widely recognized ESKAPE group, which has been reported by the World Health Organization (WHO) as a global priority pathogen due to its significant public health impact. This group comprises *E. faecium, S. aureus, A. baumannii, P. aeruginosa, K. pneumoniae*, and *Enterobacter spp.* (Khasapane *et al*., 2024). They play a crucial role in the dissemination of antimicrobial resistance genes (ARGs) among various bacterial species, thereby exacerbating the global antimicrobial resistance crisis (Malekjamshidi *et al*., 2019). These results were consistent with those reported by **Badri *et al*. (2017)**. While it was in contrast with the data revealed by **Majoie *et al*. (2021)** who reported lower incidences of $bla_{CTX\text{-}M}$. Such high prevalence of the β-lactamase ARGs in the *Enterobacteriaceae* isolates could be attributed to their plasmid-mediated nature, allowing their potential horizontal gene transfer (HGT) between various bacterial species **(Gelalcha & Kerro Dego, 2022)**.

On the other hand, genotypic characterization of colistin resistance confirmed that all phenotypic resistant isolates were carrying the *mcr-1* gene (Fig. 4). Among all the identified *Enterobacteriaceae*, the *mcr-1* gene was primarily present in *E. coli*, *P. mirabilis, S. marscenes*, and *K. oxytoca* species. Notably, all the prior mentioned colistin resistant strains were co-carrying both ESBL and *mcr-1*genes. These findings were comparable with those described by **Sabala *et al*. (2021)** and **Zaatout *et al.* (2023)**, who similarly identified both *mcr-1* and ESBL genes in three isolates from different food commodities.

Pearson's correlation analysis (Fig. 5) demonstrated that the majority of phenotypic and genotypic data showed no correlation, as exemplified by the negligible correlations observed with bla_{SHV} and $bla_{CTX\text{-}M}$. This observation aligns with the concept of silent antimicrobial resistance genes (cryptic genes), which are carried by bacteria either on plasmids or chromosomal DNA without conferring corresponding phenotypic resistance to antibiotics **(Deekshit & Srikumar, 2022)**. A similar phenomenon was reported by **Son *et al*. (2021)**, who revealed that the $bla_{CTX\text{-}M}$ gene was present in some bacterial isolates without conferring phenotypic resistance, highlighting the complexity of antimicrobial resistance mechanisms. The same study suggested that silent ESBL genes may act as reservoirs of latent resistance, which can only be activated under specific conditions such as antibiotic pressure or environmental stress. Another study conducted by **Askari *et al.* (2024)** reported that while phenotypic detection identified a portion of isolates as ESBL producers, genotypic analysis revealed the presence of at least one ESBL gene in all tested isolates. These findings suggested that discrepancies between phenotypic and genotypic results may be attributed to factors such as low levels of gene expression or the masking effect of coexisting of other β-lactamase genes leading to false-negative phenotypic outcomes.

A weak positive correlation was observed between the bla_{TEM} gene and resistance to aztreonam (ATM), suggesting that the presence of this gene likely contributed to the observed resistance patterns for these antibiotics. Conversely, a weak negative correlation was detected between aztreonam (ATM) with $bla_{CTX\text{-}M}$, as well as between cefotaxime (CTX) and ceftazidime (CAZ) with bla_{TEM}. This explained the observed susceptibility to some tested antibiotics despite the presence of their corresponding antimicrobial resistance gene indicating the potential presence of non-expressive ARGs for instance $bla_{CTX\text{-}M}$ and bla_{TEM} in some of the sensitive strain. Nevertheless, more RNA analysis would be necessary to fully establish their expression levels. These findings align with those of **Galal *et al*. (2019)**, who reported no correlation between $bla_{CTX\text{-}M}$ and either ceftazidime (CAZ) or cefotaxime (CTX). However, this contrasts with observations reported by **Somily *et al*. (2015)**, who noted a positive correlation between $bla_{CTX\text{-}M}$ presence with the high phenotypic resistance to ceftazidime (CAZ) in the isolated strains. These findings highlighted the intricacy of the antimicrobial resistance mechanisms and the necessity for further research including plasmid extraction and sequencing as well as RNA genes expression levels.

CONCLUSION

This study provided valuable insights into microbial safety of raw milk, Baladi yoghurt and white soft cheese in the Egyptian market, through investigating the prevalence of various spoilage and pathogenic microorganisms as well as shedding more light on the emergency of the extended spectrum β-lactamases (ESBLs) and colistin resistant *Enterobacteriaceae* species. The findings demonstrated the significant economic and food safety challenges posed by contamination with spore formers, *Staphylococci*, coliforms, as well as *Enterobacteriaceae* which reflected a significant degree of non-compliance with the Egyptian standards in all examined samples. The phenotypic and genotypic characterization of the *Enterobacteriaceae* spp. revealed notable prevalences of the ESBLs, and quite lower incidence of colistin resistance as well as co-existence of both ESBL and *mcr-1*genes in some spp. These findings highlighted the significant importance of regular microbial safety assessments and the need for implementing strong quality control and food safety measures throughout the dairy food chain. As well as the pressing need for action plans to control and mitigate the antimicrobial use in dairy sector and assess the potential dissemination of ARGs through the food chain in Egypt.

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