JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Analysis of antimicrobial resistance and virulence of pathogens in dairy products from Egypt by whole genome sequencing

Dina Saleh[1], Nora Mahmoud[2,3], Shymaa Enany[2,4], Samira Zakeer[2]

[1] *National Food Safety Authority, Damietta, Egypt*
[2] *Department of Microbiology and Immunology, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt*
[3] *Department of Microbiology and Immunology, Faculty of Pharmacy, Sinai University – Kantara Branch, Ismailia, Egypt*
[4] *Biomedical Research Department, Armed Force College of Medicine, Cairo, Egypt*

Abstract

Introduction: Raw milk and dairy products can cause a variety of diseases due to environmental pollution. The objectives of this study were to identify the genetic variables, evaluate antibiotic resistance, and isolate microorganisms from raw milk and dairy products.
Methodology: Three hundred samples of raw milk were collected from farmers and marketplaces in Gamasa, Mansoura, and Damietta cities in Egypt. Various bacterial species were isolated using selective culture media, of which 101 (33.6%) were *Staphylococcus* species, 11 (3.6%) were *Escherichia coli*, and 1 (0.3%) was *Salmonella*. The isolates were confirmed by biochemical tests. The disk diffusion method was used to test antibiotic susceptibility. Additionally, resistance genes and virulence factors in 4 randomly selected *Staphylococcus* species samples were identified through MinION nanopore sequencing (Oxford Nanopore Technologies, Oxford, UK).
Results: *Staphylococcus* species showed resistance to antibiotics, including ampicillin (89%) and colistin (94%). Whole genome sequencing was used to identify the molecular traits of antibiotic-resistant *Staphylococcus* species. The genes associated with resistance to antibiotics in *Staphylococcus* species were examined. Four *Staphylococcus* samples were randomly selected for extracting DNA and nanopore sequencing. *Staphylococcus* species exhibited remarkable resistance to colistin because of the presence of putative serine protease proteins (colistinase) or resistance-nodulation-division (RND) efflux pumps (*ACRB* gene).
Conclusions: The identification of resistant bacterial strains in unpasteurized milk and dairy products highlights the critical need for strict regulations to limit the overuse of antibacterial treatments in dairy herd management.

Key words: whole genome sequencing; antimicrobial resistance; virulence, milk infection.

J Infect Dev Ctries 2025; 19(4):544-552. doi:10.3855/jidc.20845

(Received 11 November 2024 – Accepted 17 November 2024)

Introduction

Milk is regarded as the primary food source for numerous minerals and nutrients. However, raw milk contains bacteria that pose a serious risk to public health, and it has been identified as the cause of a number of disorders [1]. The informal dairy sector, characterized by unregistered milk suppliers and processors who do not implement food safety management systems, dominates the dairy industry in many developing countries, particularly in Africa [2]. Egypt is recognized as Africa's largest producer of cow milk, and relies heavily on smallholder farmers who produce 70% of the country's milk. This milk is typically sold in informal markets or directly to consumers and milk collectors without undergoing any heat treatment [3]. *Salmonella* spp., *Escherichia coli*, and *Staphylococcus aureus* are common pathogens detected in raw milk and its products. These bacteria can infect animals that produce dairy; leading to illnesses including mastitis, which is considered one of the most prevalent illnesses affecting dairy-producing animals; as well as infections of the uterus, feet, and respiratory tract [4]. Mastitis affects the glandular tissue of the udder, and results in chemical, microbiological, and physical changes in milk; thus, leading to large financial losses. In addition to having a detrimental effect on the health of cows; mastitis in food-producing animals increases the likelihood of transmission of harmful pathogen species, their spores, and/or toxins to humans through food. Foodborne illness outbreaks and the spread of mastitis pathogens are associated with improper handling, processing, and storage of milk and dairy products, particularly in developing countries [5].

Food-borne illnesses are frequently caused by *S. aureus*, *Salmonella*, and *E. coli*. Antibiotics such as β-lactams, tetracyclines, and sulfadimethoxine can be used to treat infections in animals that produce dairy products [6]. In addition to treating mastitis, beta-lactams and macrolides (such as erythromycin) can also be used to prevent infections. Occasionally, there may be a need for ceftriaxone when an infection occurs in the bloodstream. Tetracycline can be used preventively to treat bacterial infections in dairy animals; however, it leads to development of resistance in the bacteria. Some bacteria developed resistance to colistin due to its extensive use as a mastitis treatment in the past, resulting in an exceptionally high degree of colistin resistance [7]. The production of colistinase, a serine alkaline protease that breaks down colistin, is one of the enzymatic strategies used by bacteria to resist colistin. Other strategies include overexpressing the efflux pump system, altering their outer membrane to reduce its negative charge, and producing excessive amounts of capsule polysaccharide [8].

This study aimed to comprehensively investigate the pathogens contaminating milk and dairy products collected from different locations in Egypt. We employed advanced nanopore sequencing to characterize the resistance profiles of these pathogen strains, and elucidate their population structure and genetic composition. This research provided critical insights into the public health risks associated with these pathogens and informed strategies for improving food safety and public health interventions.

Methodology

Samples collection

Three hundred samples of raw milk, traditionally made yogurt, and kariesh cheese were collected from several farms in Damietta, Mansoura, and Gamasa cities in Egypt; between January 2018 and February 2019. The samples were collected in sterile containers, labeled, and kept in an icebox at 4 °C. They were then promptly transported to the microbiology laboratories of the Ministry of Health in Damietta for bacteriological analysis.

Bacterial isolation and identification

Staphylococci were isolated from 10 mL samples of raw milk, or 10 g samples of cheese or yogurt. The samples were mixed thoroughly with a stomacher to create an initial suspension. These samples were then added to 90 mL of maximum recovery diluent (MRD; Oxoid, Basingstoke, UK) and placed in aseptic bags under sterilized conditions. A 0.1 mL portion of this initial suspension was applied to mannitol salt agar (MSA) plates (Oxoid, Basingstoke, UK). The plates were incubated at 37 °C for 48 hours. The colonies that developed on MSA were observed under a microscope, and identified as Gram-positive cocci with structure resembling grapes and producing a strong effervescence when activated with hydrogen peroxide. The colonies were kept at 5 °C on nutrient agar slants at – 80 °C in a broth glycerol medium and on semisolid nutrient agar.

A different method was used for *Salmonella* spp. isolation and detection. Twenty-five mL of raw milk, or 25 g of cheese or yogurt were added to 225 mL of buffered peptone water (Promega, Madison, Wisconsin, USA) in aseptic bags under sterile conditions, and the mixture was thoroughly agitated with a stomacher to form the first suspension for pre-enrichment [9]. This initial suspension was incubated at 37 °C for 18 hours. Next, 0.1 mL of the suspension was added to Rappaport Vassiliadis Soya broth (RVS) in 10 mL sterilized test tubes (Oxoid, Basingstoke, UK), and the test tubes were incubated for 24 hours at 41.5 °C. The culture was used to inoculate *Salmonella Shigella* (SS) agar (Oxoid, Basingstoke, UK) and MacConkey (MAC) agar (Oxoid, Basingstoke, UK) using a sterile loop, and then incubated for 24 hours at 37 °C. The colonies were transferred to nutritional agar for additional isolation and confirmatory biochemical assays, including L-lysine decarboxylation medium (LDC), urea agar, sulfide-indole-motility (SIM), and triple sugar/iron agar (TSI agar). The colonies were kept at 5 °C on nutrient agar slants, at – 80 °C in a broth glycerol medium, and on semisolid nutrient agar.

In order to isolate and identify *E. coli* sp., 10 mL of raw milk, or 10 g of cheese or yogurt were taken, added to 90 mL MRD, and placed in aseptic bags under sterile conditions. The initial suspension was prepared by thoroughly mixing the bags with a stomacher. MAC and eosin methylene blue (EMB) agar (Oxoid, Basingstoke, UK) were inoculated using the pour plate method, and the plates were then incubated at 37 °C for 18–24 hours. Subculturing was carried out by streaking colonies on MacConkey agar in order to get pure colonies for confirmation by biochemical assays such as motility indole ornithine media (MIO), TSI agar, lysine media, Simmon's citrate, and urea. *E. coli* colonies exhibited a shiny green color on EMB agar. The colonies were stored at 5 °C on nutrient agar slants in semisolid nutrient agar, and at – 80 °C in a broth glycerol medium.

Furthermore, 225 mL of half Fraser broth (Oxoid, Basingstoke, UK) was mixed with 25 mL of raw milk, or 25 g of cheese or yogurt to enrich the samples and isolate *Listeria monocytogenes*. The mixture was incubated at 30 °C for 24 hours. 0.1 mL of Half Fraser broth culture was added to a 10 mL complete Fraser broth test tube (Oxoid, Basingstoke, UK) containing secondary enrichment media. The test tube was then incubated for 24 hours at 37 °C. Half and complete Fraser broth cultures were applied to the surface of the chromogenic *Listeria* agar (Oxoid, Basingstoke, UK), and then incubated for 48 hours at 37 °C. *Listeria monocytogenes* was confirmed utilizing L-rhamnose, D-xylose, beta-hemolysis, and microscopic analysis [10].

Antimicrobial susceptibility testing

Antimicrobial susceptibilities were tested using the Kirby-Bauer disk diffusion method. The antibiotic test panel contained the following: trimethoprim-sulfamethoxazole (1.25/23.75 µg), ampicillin (10 µg), ceftazidime (30 µg), vancomycin (30 µg), colistin (10 mcg), tetracycline (30 µg), erythromycin (15 µg), and streptomycin (15 µg) (Oxoid, Basingstoke, UK). The isolates were categorized as susceptible, intermediate, or resistant; in line with the National Committee for Clinical Laboratory Standards recommendations.

Whole genome sequencing

Four randomly selected samples were used for whole genome sequencing to determine the resistance genes and virulence factors for the isolated *Staphylococcus* species. The PureLink Microbiome DNA Purification Kit (Invitrogen, Carlsbad, California, USA) was used following the manufacturer’s instructions. Lysis buffer (S1), lysis enhancer (S2), and beads were used for mechanical lysis of the cells. The quantity and purity of the DNA were assessed using Qubit (Carlsbad, California, USA) and Nanodrop (Waltham, Massachusetts, USA). Purified DNA was stored for a maximum of 1 week at 4 °C, or for a long period at – 20 °C. The DNA library was set up using a Rapid Barcoding Kit from Oxford Nanopore Technologies (Oxford, UK). The kit contained 12 distinct barcode tags, and the transposase located in its center attached barcoded tags to the cleft ends of template molecules while simultaneously cleaving them. Rapid sequencing adaptors were affixed to the tagged ends after the barcoded samples were pooled. Short library fragments were extracted from the library DNA using solid phase reversible immobilization (SPRI) beads, and the libraries were examined with a Qubit fluorometer (Carlsbad, California, USA). The flow cell was loaded after priming.

The MinION Mk1CA (Oxford Nanopore, Oxford, UK**)** was sequenced in accordance with the manufacturer’s instructions. Standard settings were used during the 6-hour run. The quality of Fastq readings was tested using Fastqc [11]. Subsequently, the reads that were of low quality or insufficient length were removed using the NanoFilt tool (https://github.com/wdecoster/nanofilt). Moreover, adapters sequences present in the reads were eliminated using Porechop_ABI [12]. The filtered reads were then subjected to de novo (https://github.com/Nextomics/NextDenovo) assembly by canu flye, NECAT polishing by Medaka (https://github.com/nanoporetech/medaka), and annotation by prokka (https://github.com/tseemann/prokka). Next, the quality of the resulting assembly (i.e., the consensus sequence) was evaluated using Quast, and the identification of antimicrobial resistance genes and virulence factors was carried out using Amr finderplus and Abricate tools (ABRICATE software 2022, https://github.com/tseemann/abricate), utilizing the National Center for Biotechnology Information (NCBI) and Virulence Factor Database (VFDB) databases [13]. Finally, the taxonomic classification of the sample was performed using the Kraken database and the Kraken2 tool [14].

Statistical analysis

Statistical analysis was done using the Statistical Package for the Social Sciences (SPSS) software version 26 (IBM Corp, Armonk, NY, USA). Qualitative variables were recorded as frequencies and percentages.

Ethical considerations

The ethical board at the Suez Canal University approved this investigation (No. 201809M1). The study was carried out in compliance with all applicable regulations and guidelines.

Results

Bacterial identification and isolation

We identified 33.6% *Staphylococcus*, 0.3% *Salmonella*, and 3.6% *E. coli* in the 300 samples that we cultured. A total of 101 (33.6%) isolates exhibited strong effervescence with hydrogen peroxide on MSA agar. Among them, 74 fermented the mannitol in the MSA media, confirming that they were *Staphylococcus*. One isolate (0.3%) formed colorless colonies with black centers on SS media, but it did not ferment the lactose on MAC agar. There was only one *Salmonella* isolate and it was confirmed by H_2S production and SIM test. A total of 11 (3.6%) *E. coli* isolates were confirmed based on the development of pink colonies on MAC agar, production of a green metallic sheen on EMB

Table 1. Antimicrobial drug resistance patterns of bacterial isolates.

Antimicrobial agent	**Bacterial isolates**								
	Number of *Staphylococcus species* (%)			**Number of *E. coli species* (%)**			**Number of *Salmonella species* (%)**		
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Streptomycin	13 (12.8%)	19 (18.8%)	69 (68.4%)	4 (36.4%)	3 (27.2%)	4 (36.4%)	—	—	1 (100%)
Trimethoprim / Sulphamethoxazole	18 (17.8%)	13 (12.9%)	70 (69.3%)	—	3 (27.2%)	8 (72.8%)	—	—	1 (100%)
Erythromycin	46 (45.5%)	37 (36.7%)	18 (17.8%)	10 (91%)	1 (9%)	—	—	1 (100%)	—
Tetracycline	6 (5.9%)	17 (16.8%)	78 (77.3%)	1 (9%)	2 (18.1%)	8 (72.9%)	1 (100%)	—	—
Colistin	95 (94%)	2 (2%)	4 (4%)	2 (18.1%)	7 (63.6%)	2 (18.3%)	1 (100%)	—	—
Vancomycin	1 (1%)	22 (21.7%)	78 (77.3%)	11 (100%)	—	—	1 (100%)	—	—
Ceftazidime	93 (92%)	2 (2%)	6 (6%)	3 (27.2%)	3 (27.2%)	5 (45.6%)	—	—	1 (100%)
Ampicillin	90 (89%)	4 (4%)	7 (7%)	10 (91%)	1 (9%)	—	1 (100%)	—	—

R: resistant; I: immediate; S: susceptible.

agar, and production of turbid purple to a faded yellow-purple color on MIO media. *Listeria monocytogenes* was not detected in any of the samples examined.

Antimicrobial susceptibility test

All the bacterial isolates from milk samples were tested for antibiotic susceptibility. The overall resistance pattern of the isolated samples is summarized in Table 1. The results indicate higher resistance of *Staphylococcus* isolates to colistin, followed by ceftazidime, ampicillin, and erythromycin. All *E. coli* and *Salmonella* isolates were resistant to vancomycin, ampicillin, and erythromycin. Some isolates were resistant to more than 2 antibiotics, classifying them as multidrug-resistant (MDR) isolates (Table 2). Out of the 11 *E. coli* isolates, 6 were MDR; among them, 2 isolates were resistant to erythromycin, vancomycin, ampicillin, and ceftazidime; another 2 isolates were resistant to erythromycin, vancomycin, ampicillin, and streptomycin; and the remaining 2 isolates were resistant to erythromycin, vancomycin, and ampicillin. Out of the 101 *Staphylococcus* isolates, 19 were MDR. Of these, 13 isolates were resistant to erythromycin, colistin, and ampicillin; 3 were resistant to trimethoprim/sulfamethoxazole, erythromycin, colistin, and ampicillin; and 3 were resistant to colistin, ceftazidime, and ampicillin. The single *Salmonella* isolate was resistant to 4 antimicrobial agents from different classes: tetracycline, colistin, vancomycin, and ampicillin.

Whole genome sequencing

Four samples were chosen randomly for whole genome sequencing, based on the significant antibiotic resistance observed in the *Staphylococcus* species isolates. Each of these samples showed resistance to more than 2 antibiotics. The samples; identified with barcodes 13, 29, 122, and 153 were determined to be *Staphylococcus lentus*, *Staphylococcus sciuri*, *Staphylococcus lentus*, and *Staphylococcus aureus*, respectively. The sample sequences were uploaded to the NCBI database with accession numbers SAMN43026219 for *Staphylococcus lentus* (barcode 13), SAMN43026220 for *Staphylococcus sciuri* (barcode 29), SAMN43026221 for *Staphylococcus lentus* (barcode 122), and SAMN43026222 for *Staphylococcus aureus* (barcode 153). The virulence factors identified in the 4 samples are summarized in Tables 3 to 6. Numerous genes influencing the virulence factors were identified in the 4 samples. For example, various intercellular adhesion genes were identified. The polysaccharide intercellular adhesin (PIA) deacetylase enzyme encoded by the *IcaB* gene was identified in the *S. lentus* strain (barcode 13). In contrast, *S. sciuri* contained N-acetylglucosaminyl-transferase encoded by the *IcaA* gene, O-succinyltransferase encoded by the *IcaC* gene, and the *IcaB* gene. The *S. aureus* isolate contained the *IcaC* gene. These genes are known for encoding proteins that are able to form strong bonds with the host surfaces and effectively resist the immune system.

Table 2. Multi-drug-resistant (MDR) phenotype of isolates.

Isolated pathogens	**No of isolates with MDR pattern**	**Number of antibiotics**
***Staphylococcus* species (n = 101)**	13	3
	3	4
	3	3
***E. coli* species (n = 11)**	2	4
	2	4
	2	3
***Salmonella* species (n = 1)**	1	4

N: total number of isolated pathogens.

Table 3. Virulence factors of *Staphylococcus lentus* isolate (barcode 13).

Virulence class	Virulence factor	Related genes	*S. lentus*	
			contig_1	contig_3
Adherence	Intercellular adhesion	*icaB*		orf03380
Immune evasion	Capsule	Undetermined	orf01857; orf01864; orf01865; orf01866; orf01867; orf02894; orf03260	
	Polysaccharide capsule (*Bacillus*)		orf01856	
Intracellular survival	Lipoate protein ligase A1 (*Listeria*)	*lplA1*	orf01344	
Iron uptake	Periplasmic binding protein-dependent ABC transport systems (*Vibrio*)	*vctC*	orf01612	
Nutritional factor	Allantoin utilization (*Klebsiella*)		orf02848 orf02851 orf02853	
Phagosome arresting	Nucleoside diphosphate kinase (*Mycobacterium*)	*ndk*	orf00662	Nucleoside diphosphate kinase (*Mycobacterium*)
Serum resistance and immune evasion	Lipopolysaccharide, LPS (*Francisella*)	*wbtP*	orf01859	LPS (*Francisella*)
Surface protein anchoring	Lipoprotein diacylglyceryl transferase (*Listeria*)	*lgt*	orf01575	Lipoprotein diacylglyceryl transferase (*Listeria*)

In addition, the pathogenicity of the *S. aureus* isolate involved the production of toxins such as alpha hemolysin encoded by the *hly*/*hla* gene; and gamma hemolysin, encoded by the *hlgA*, *hlgB*, and *hlgC* genes. These genes are known to bind to the cell membrane and cause cell damage. In addition, the exotoxin genes *set16*, *set18*, *set19*, *set21*, *set23*, *set26*, and *set34*, were also identified. Lukotoxin D, encoded by the *lukD* gene, is known for evading the host immune response, and was also detected in the isolates. Type VII secretion system genes, including *esaA*, *esaB*, *esaC*, *essB*, *essC*, and *esxA*, were detected in the *S. aureus* isolate. The type VII secretion system specializes in secreting proteins through the cell membrane of bacteria.

Based on the results of the whole genome sequencing, it was determined that the 4 isolates carried different genes responsible for their resistance to the various antimicrobial agents (Table 7). All isolates had a high temperature requirement A factor, encoded by the *Htra* gene, which is a serine protease colistinase enzyme that triggers colistin resistance; and the glucose transport gene *glup*, which participates in the antimicrobial resistance mechanism. The *S. aureus* isolate contained a serine protease-like protein encoded with the *splc* gene, belonging to the family of serine protease colistinase enzymes, which is responsible for colistin resistance. A magnesium transporter gene from the *Cora* gene family was detected in all isolates, and it is known to alter the action of colistin by facilitating the entry of magnesium ions into bacteria. Additionally, efflux ABC transporters that are responsible for MDR, were detected in *S. lentus* isolates (barcode 13) and *S. sciuri*. These ABC transporters were encoded with the *LPTB* gene in *S. lentus*, and by *cydD* gene in *S. sciuri*. These genes are crucial in converting the destabilization effect of colistin by the production of capsular polysaccharides. Resistance genes *aadD*, *str*, *bleo*, *dfrK*, *fexA*, *inuA*, and *mecA* were also detected in the *S. sciuri* isolate. These genes confer resistance to aminoglycosides, streptomycin, bleomycin, trimethoprim, chloramphenicol, lincosamide, and penicillin; respectively. Only the *S. lentus* isolate had the *FusB* gene which is responsible for fusidic acid resistance.

Discussion

In this study, 300 raw milk samples were collected and *Staphylococcus* species were observed in 33% of the samples. This is in agreement with a study in Sharkia governorate [15]; and is not in agreement with another study conducted in Beni-Suef, El-Fayoum, and Giza, where the proportion of *Staphylococcus* species

Table 4. Virulence factors of *Staphylococcus sciuri* isolate (barcode 29).

Virulence class	Virulence factor	Related genes	b23
Adherence	Intercellular adhesion	*icaA*	orf02427
		icaB	orf02426
		icaC	orf02425
Enzyme	Serine V8 protease	*sspA*	orf01381
Immune evasion	Capsule	Undetermined	orf00723
	Polysaccharide capsule (*Bacillus*)	*manA*	orf01897
Antiphagocytosis	Capsule (*Enterococcus*)	*cdsA*	orf00959
Surface protein anchoring	Lipoprotein diacylglyceryl transferase (*Listeria*)	*lgt*	orf00404

Table 5. Virulence factors of *Staphylococcus aureus* isolate (barcode 153).

Virulence class	Virulence factor	Related genes	Sample code (b25)
Adherence	Autolysin	*atl*	orf01257
	Clumping factor A	*clfA*	orf01461
	Clumping factor B	*clfB*	orf01460
	Collagen adhesion	*cna*	orf02380
	Elastin binding protein	*ebp*	orf00721
	Fibrinogen binding protein	*efb*	orf01137
	Fibronectin binding proteins	*fnbA*	orf02606
		fnbB	orf02608
	Intercellular adhesion	*icaC*	orf02409
	Ser-Asp rich fibrinogen-binding proteins	*sdrC*	orf01739
		sdrD	orf01737
		sdrE	orf01736
	Staphylococcal protein A	*spa*	orf02262
Enzyme	Cysteine protease	*sspB*	orf01262
	Hyaluronate lyase	*hysA*	orf02934; orf02935
	Lipase	*geh*	orf02019
		lip	orf02406
	Serine V8 protease	*sspA*	orf01261
	Serine protease	*splA*	orf00339
		splB	orf00340
		splC	orf00341
		splD	orf00342
		splE	orf00343
		splF	orf00344
	Staphylocoagulase	*coa*	orf02127
	Staphylokinase	*sak*	orf00195
	Thermonuclease	*nuc*	orf00904
Immune evasion	*AdsA*	*adsA*	orf02321
	Capsule	*Undetermined*	orf02198; orf02199; orf02200; orf02202; orf02203; orf02204; orf02205; orf02206; orf02209; orf02210; orf02213; orf02214; orf02215
	SCIN	*scn*	orf00200
	Sbi	*sbi*	orf02699
Secretion system	Type VII secretion system (T7ss)	*esaA*	orf02065; orf02066
		esaB	orf02062
		esaG	orf02041; orf02044; orf02045; orf02046; orf02047; orf02048
		essB	orf02061
		essC	orf02060
		esxA	orf02067
Toxin	Alpha hemolysin	*hly/hla*	orf01132
	Exotoxin	*set16*	orf01925
		set18	orf01921
		set19	orf01920
		set21	orf01918
		set23	orf01915
		set26	orf01906
		set34	orf01919
	Gamma hemolysin	*hlgA*	orf00331; orf02697
		hlgB	orf02694
		hlgC	orf02695
	Leukotoxin D	*lukD*	orf00333

The most significant finding was colistin resistance in the majority of the isolates, and this was validated by whole genome sequencing. Serine protease, which is encoded by *htra* (*Dpgp*) genes, was found in all 4 isolate genomes. This enzyme is known to break down colistin through an enzymatic method that involves hydrolyzing the particular peptide link, DAB-DAB, by colistinase [24]. In addition to *htra*, other serine protease genes resistance across bacterial species in raw milk. The findings underscore a critical need for stringent regulation of antibiotic use, by minimizing the indiscriminate administration of antibiotics, especially in veterinary practices, to combat the rise of MDR bacterial strains.

In addition to strong antibiotic resistance mechanisms, our isolates had a varied set of virulence

Table 6. Virulence factors of *Staphylococcus lentus* isolate (barcode 122).

Virulence class	Virulence factor	Related genes	b24
Enzyme	Serine V8 protease	*sspA*	orf01685
Immune evasion	Capsule	Undetermined	orf00292; orf00293; orf00294; orf00295; orf00301; orf02185; orf02551
	Polysaccharide capsule (*Bacillus*)		orf00302
Intracellular survival	Lipoate protein ligase A1 (*Listeria*)	*lplA1*	orf00819
Iron uptake	Periplasmic binding protein-dependent ABC transport systems (*Vibrio*)	*vctC*	orf00544
Nutritional factor	Allantoin utilization (*Klebsiella*)		orf02596 orf02593 orf02591
Phagosome arresting	Nucleoside diphosphate kinase (*Mycobacterium*)	*ndk*	orf01480
Regulation	LisR/LisK (*Listeria*)	*lisR*	orf01209
Serum resistance and immune evasion	Lipopolysaccharide, LPS (*Francisella*)	*wbtP*	orf00299
Surface protein anchoring	Lipoprotein diacylglyceryl transferase (*Listeria*)	*lgt*	orf00583

genes, which helps understand bacterial pathogenicity. The *S. lentus*, *S. sciuri*, and *S. aureus* isolates contained *ica* adhesion genes, which are important for biofilm formation. Notably, the *SspA* gene, a proteolytic enzyme that modifies the host's immune response and cleaves surface proteins, was present in these species, suggesting its significance in tissue disintegration. Moreover, all isolates included genes for capsular polysaccharide formation, which protects bacteria from phagocytic uptake, with specific pathogenic genes such as *lplA1*, *vctc*, *ndk*, and *wbtb* detected in *S. lentus*, highlighting their importance in bacterial survival and virulence. *S. aureus* isolates were highly virulent, generating hemolysins and other exotoxins known to cause severe cytotoxicity, as well as several genes that aid in adhesion, immune evasion, and biofilm formation. This extensive discovery of both resistance determinants and virulence factors highlights the crucial need for strict antibiotic control measures to tackle the rising threat of these deadly infections in clinical and agricultural settings.

In summary, our results indicate that raw milk and its products contain a wide variety of bacteria; and that raw milk is an obvious source of MDR bacteria. When we isolated three different types of bacteria; *Salmonella*, *E. coli* and *Staphylococcus* sp.; we observed that *Staphylococcus* sp. were the most prevalent and also showed significant resistance to colistin. Therefore, it was necessary to analyze its genetic content by conducting whole genome sequencing procedures on 4 *Staphylococci* samples to identify the genes that are responsible for their resistance and increase their virulence. We identified the genes responsible for the bacteria's resistance to antibiotics, as well as other genes produced by the bacteria to increase their pathogenicity. This means that the bacteria present in milk may pose a serious danger to humans, if they consume milk and its products in raw form. This can be addressed by enhancing the hygienic environment during milking. Antimicrobial-resistant *Staphylococci* should be prevented and controlled from spreading by carefully evaluating and monitoring the appropriate use of antibiotics.

Conclusions

This study found MDR bacteria in unpasteurized milk and dairy products, raising serious concerns about their safety and possible harm to public health. The

Table 7. Antimicrobial resistance genes identified in the isolates.

Staphylococcus lentus **(13)**	***Staphylococcus sciuri*** **(29)**	***Staphylococcus lentus*** **(122)**	***Staphylococcus aureus*** **(153)**
Methicillin (*mecA*)	Tetracycline *Tet*(*L*)	Macrolides (*Erm*)	Beta lactam antibiotics (*BlaI*)
Macrolides (*erm(13)*)	Tetracycline *Tet*(*M*)	Macrolides (*Mph*)	Tetracycline (*Tet*)
Macrolides (*Msr(A)*)	Aminoglycosides (*Aad*)	Lincosamide / pleuromutilin / streptogramin *Sal*(*B*)	Penicillin (*Blaz*)
Macrolides (*Mph*)	Macrolides (*MsrA*)		Lincosamide (*Inu(A)*)
Lincosamide / pleuromutilin / streptogramin *(Sal(B))*	Streptomycin (*Str*)		
Fusidic acid (*FusB*)	Methicillin (*MecA*)		
	Trimethoprim (*Dfrk*)		
	Bleomycin (*Bleo*)		
	Chloramphenicol (*FexA*)		
	Macrolides (*Erm*)		
	Lincosamide (*InuA*)		
	Lincosamide and Streptogramin A (*SalA*)		
	Macrolides (*Mph*)		

emergence of such resistant strains highlights the critical need for strict regulations to limit the overuse of antimicrobial treatments in dairy herd management. This situation underscores the need for novel natural antibacterial medicines. Furthermore, prudent use of older antibiotics such as colistin is critical, given the rise in colistin resistance as a result of its growing use in veterinary settings. It is critical that clinicians stay watchful about the potential development of colistin resistance among MDR bacteria, which can occur through mutations or adaptive mechanisms.

Authors' contributions

All authors contributed fully to the manuscript.

Corresponding author

Dr. Shymaa Enany, PhD
Department of Microbiology and Immunology, Faculty of Pharmacy,
Suez Canal University, Ismailia, New University - Km 4.5, Egypt.
Tel: 01025801366
Fax: (+202) 22621217, (+2064) 3205208
Email: shymaa21@yahoo.com

Conflict of interests

No conflict of interests is declared.

References

1. Lando AM, Bazaco MC, Parker CC, Ferguson M (2022) Characteristics of US consumers reporting past year intake of raw (unpasteurized) milk: results from the 2016 food safety survey and 2019 food safety and nutrition survey. J Food Prot 85: 1036–1043. doi: 10.4315/JFP-21-407.
2. Ntuli V, Sibanda T, Elegbeleye JA, Mugadza DT, Seifu E, Buys EM (2023). Dairy production: microbial safety of raw milk and processed milk products. In Present Knowledge in Food Safety. Elsevier. 439-454. doi: 10.1016/B978-0-12-819470-6.00076-7.
3. Elmonir W, Abo-Remela E, Sobeih A (2018) Public health risks of *Escherichia coli* and *Staphylococcus aureus* in raw bovine milk sold in informal markets in Egypt. J Infect Dev Ctries 12: 533–541. doi: 10.3855/jidc.9509.
4. Girma A, Tamir D (2022) Prevalence of bovine mastitis and its associated risk factors among dairy cows in Ethiopia during 2005-2022: a systematic review and meta-analysis. Vet Med Int 2022: 1–19. doi: 10.1155/2022/7775197.
5. Faour-Klingbeil D, Todd ECD (2019). Prevention and control of foodborne diseases in Middle-East North African countries: review of national control systems. Int J Environ Res Public Health 17: 70. doi: 10.3390/ijerph17010070.
6. Pol M, Ruegg PL (2007) Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. J Dairy Sci 90: 249–261. doi: 10.3168/jds.S0022-0302(07)72626-7.
7. Hammad AM, Hoffmann M, Gonzalez-Escalona N, Abbas NH, Yao K, Koenig S, Allue-Guaradia A, Eppinger M (2019) Genomic features of colistin resistant *Escherichia coli* ST69 strain harboring *mcr-1* on IncHI2 plasmid from raw milk cheese in Egypt. Infect Genet Evol 73: 126–131. doi: 10.1016/j.meegid.2019.04.021.
8. Lee D-H, Cha J-H, Kim D-W, Lee K, Kim Y-S, Oh H-Y, Cho Y-H, Cha C-J (2022) Colistin-degrading proteases confer collective resistance to microbial communities during polymicrobial infections. Microbiome 10: 129. doi: 10.1186/s40168-022-01315-x.
9. Gebeyehu A, Taye M, Abebe R (2022) Isolation, molecular detection and antimicrobial susceptibility profile of *Salmonella* from raw cow milk collected from dairy farms and households in southern Ethiopia. BMC Microbiol 22: 84. doi: 10.1186/s12866-022-02504-2.
10. Lianou DT, Skoulakis A, Michael CK, Katsarou EI, Chatzopoulos DC, Solomakos N, Tsilipounidaki K, Florou Z, Cripps PJ, Katsafadou AI, Vasileiou NGC, Dimoveli KS, Bourganou MV, Liagka DV, Papatsiros VG, Kontou PI, Mavrogianni VS, Caroprese M, Petinaki E, Fthenakis GC (2022) Isolation of *Listeria ivanovii* from bulk-tank milk of sheep and goat farms-from clinical work to bioinformatics studies: prevalence, association with milk quality, antibiotic susceptibility, predictors, whole genome sequence and phylogenetic relationships. Biology 11: 871. doi: 10.3390/biology11060871.
11. De Sena Brandine G, Smith AD (2021) Falco: high-speed FastQC emulation for quality control of sequencing data. F1000Research 8: 1874. doi: 10.12688/f1000research.21142.2.
12. Bonenfant Q, Noé L, Touzet H (2023) Porechop_ABI: discovering unknown adapters in Oxford Nanopore Technology sequencing reads for downstream trimming. Bioinform Adv 3: vbac085. doi: 10.1093/bioadv/vbac085.
13. Feldgarden M, Brover V, Fedorov B, Haft DH, Prasad AB, Klimke W (2022) Curation of the AMRFinderPlus databases: applications, functionality and impact. Microb Genom 8: 1–10. doi: 10.1099/mgen.0.000832.
14. Wood DE, Lu J, Langmead B (2019) Improved metagenomic analysis with Kraken 2. Genome Biol 20: 257. doi: 10.1186/s13059-019-1891-0.
15. Abou-Khadra SH, El-Shorbagy IM, El-Azzouny MM (2020). Rapid detection of enterotoxigenic *Staphylococcus aureus* isolated from raw cow milk in Sharkia Governorate, Egypt. Advances in Animal and Veterinary Sciences 8: 11–17. doi: 10.17582/journal.aavs/2020/8.s1.11.17.
16. Abed AH, Menshawy AMS, Zeinhom MMA, Hossain D, Khalifa E, Wareth G, Awad MF (2021) Subclinical mastitis in selected bovine dairy herds in north upper Egypt: assessment of prevalence, causative bacterial pathogens, antimicrobial resistance and virulence-associated genes. Microorganisms 9: 1175. doi: 10.3390/microorganisms9061175.
17. Tartor YH, Abd El-Aziz NK, Gharieb RMA, El Damaty HM, Enany S, Soliman EA, Abdellatif SS, Attia ASA, Bahnass MM, El-shazly YA, Elbediwi M, Ramadan H (2021) Whole-genome sequencing of Gram-negative bacteria isolated from bovine mastitis and raw milk: the first emergence of colistin *mcr-10* and fosfomycin *fosA5* resistance genes in *Klebsiella pneumoniae* in Middle East. Front Microbiol 12: 770813. doi: 10.3389/fmicb.2021.770813.
18. Elafify M, Darwish WS, Al-Ashmawy M, Elsherbini M, Koseki S, Kawamura S, Abdelkhalek A (2019) Prevalence of *Salmonella* spp. in Egyptian dairy products: molecular, antimicrobial profiles and a reduction trial using D-tryptophan. Journal of Consumer Protection and Food Safety 14: 399–407. doi: 10.1007/s00003-019-01248-y.

19. Saleh E, El-Lawendy H, Khedr E, Ali E (2022). Molecular detection of some virulence genes in multidrug resistant *Salmonella* species isolated from chicken meat products and raw milk. Damanhour Journal of Veterinary Sciences 7: 24–27. doi: 10.21608/djvs.2022.234975.
20. Lee S, Cappato L, Guimarães J, Balthazar C, Rocha R, Franco L, Cruz A, Corassin C, De Oliveira C (2019) *Listeria monocytogenes* in milk: occurrence and recent advances in methods for inactivation. Beverages 5: 1–14. doi: 10.3390/beverages5010014.
21. Ahmed W, Neubauer H, Tomaso H, El Hofy FI, Monecke S, Abd El-Tawab AA, Hotzel H (2021). Characterization of Enterococci- and ESBL-producing *Escherichia coli* isolated from milk of bovides with mastitis in Egypt. Pathogens 10: 97. doi: 10.3390/pathogens10020097.
22. Hammad AM, Eltahan A, Hassan HA, Abbas NH, Hussien H, Shimamoto T (2022). Loads of coliforms and fecal coliforms and characterization of thermotolerant *Escherichia coli* in fresh raw milk cheese. Foods 11: 332. doi: 10.3390/foods11030332.
23. Ombarak RA, Hinenoya A, Awasthi SP, Iguchi A, Shima A, Elbagory A-R M, Yamasaki S (2016). Prevalence and pathogenic potential of *Escherichia coli* isolates from raw milk and raw milk cheese in Egypt. Int J Food Microbiol 221: 69–76. doi: 10.1016/j.ijfoodmicro.2016.01.009.
24. Ito-Kagawa M, Koyama Y (1980) Selective cleavage of a peptide antibiotic, colistin by colistinase. J Antibiot (Tokyo) 33: 1551–1555. doi: 10.7164/antibiotics.33.1551.
25. Trachsel E, Redder P, Linder P, Armitano J (2019) Genetic screens reveal novel major and minor players in magnesium homeostasis of *Staphylococcus aureus*. PLoS Genet 15: e1008336. doi: 10.1371/journal.pgen.1008336.
26. Ding Y, Hao J, Xiao W, Ye C, Xiao X, Jian C, Tang M, Li G, Liu J, Zeng Z (2023) Role of efflux pumps, their inhibitors, and regulators in colistin resistance. Front Microbiol 14: 1207441. doi: 10.3389/fmicb.2023.1207441.
27. Nikaido H, Takatsuka Y (2009) Mechanisms of RND multidrug efflux pumps. Biochim Biophys Acta 1794: 769–781. doi: 10.1016/j.bbapap.2008.10.004.
28. Ahmed W, Neubauer H, Tomaso H, El Hofy FI, Monecke S, Abdeltawab AA, Hotzel H (2020) Characterization of *Staphylococci* and *Streptococci* isolated from milk of bovides with mastitis in Egypt. Pathogens 9: 381. doi: 10.3390/pathogens9050381.