Benha Veterinary Medical Journal 47 (2024) 82-87

Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

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Original Paper

Isolation and biofilm characterization of *Klebsiella* spp. and pathogenic *E. coli* in raw milk and unpasteurized dairy products in Egypt; antibiogram of *Klebsiella* spp.

Hagar Nazmy*[1], Dina A.B. Awad [2], Enas A. Soliman[1]

[1] *Department of Bacteriology, Immunology and Mycology, Faculty of Veterinary Medicine, Benha University, Egypt*

[2] *Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Benha University, Egypt.*

ARTICLE INFO

Keywords

Klebsiella spp,

Escherichia coli,

Soft cheese

Cream

Public health

Received 18/09/2024
Accepted 05/11/2024
Available On-Line 31/12/2024

ABSTRACT

There has been growing interest in traditional popular dairy products in Egypt, especially in rural areas like white, acid-coagulated soft cheeses (Karish cheese) and soured cream. Still, they are highly susceptible to microbial contamination, including *Enterobacteriaceae*, which poses a health risk to consumers. This study aimed to isolate and identify *Klebsiella* spp. and E. coli from raw milk, karish and soured cream, assess their antibiotic sensitivity, and investigate their biofilm-forming capabilities. About 154 dairy samples, including raw buffalo and cow milk, karish cheese, and soured cream, were collected from various locations in Egypt. Both *Klebsiella* spp. and *E. coli* using spread plate methods on MacConkey and EMB agar were isolated and identified by morphological analysis via Gram staining and biochemical tests, followed by molecular identification through PCR targeting the gapA and pehX genes. Antibiotic sensitivity was assessed using the disc diffusion method and biofilm production was evaluated using crystal violet staining and Congo red agar plating. The results revealed that *Klebsiella* species were positive in 3 samples (1.94%), including *K. oxytoca* and *K. ozaenae*, and 19 isolates identified as pathogenic *E. coli*, indicating a prevalence of 12.33%. Antibiotic sensitivity testing showed high resistance in *Klebsiella* isolates to several antibiotics, including ceftazidime and amoxycillin/clavulanic acid. Biofilm production was evaluated, with most isolates showing weak to moderate biofilm formation, indicating varying degrees of adherence and pathogenicity. This study highlights the public health risks of antibiotic-resistant *Klebsiella* spp. and *E. coli* in raw milk and unpasteurized dairy products in Egypt.

1. INTRODUCTION

The degree of hygiene in the manufacturing process and the microbiological quality of the food are both indicated by the *Enterobacteriaceae* family of Gram-negative bacteria. Moreover, eating food contaminated with *Enterobacteriaceae* exposes people to microbiological hazards (Mladenović et al., 2021).

Dairy products contain proteins and carbohydrates, which are essential for the growth of microbes and make them perishable and highly subject to microbial infection. Bacterial contamination of milk and milk products is one of the biggest problems facing the dairy industry, especially in developing countries where traditional processing methods are still in use. Bacteria from primary sources (milk from mastitis cows, for example) or secondary sources (poor hygiene during production chains) can infect milk and milk products. Microorganism-contaminated milk spoils quickly, and the pathogens might infect consumers and cause illnesses (Gwandu et al., 2018).

Systematic analysis showed the overall prevalence of *Klebsiella* spp., the genus of important zoonotic pathogens belonging to the family *Enterobacteriaceae*, in the global dairy herds and the risk of cross-species transmission between human and dairy cows with mastitis worldwide. A meta-analysis of 55 manuscripts revealed the presence of *Klebsiella* spp, in 2,478 milk samples out of 79,852 milk samples (31.03%). The global pooled prevalence estimate was 7.95%. Milk samples from the 2013–2020 monitoring period had a higher ($p < 0.05$) percentage of *Klebsiella*-positive samples (12.16%). than that of 2007–2012 (3.85%), indicating that bovine mastitis caused by *Klebsiella* might become more common. Due to the potential hazard of cross-species transmission between humans and cows, the prevalence of mastitic milk-derived *Klebsiella*. and its high multiple-drug resistance rate needs to be monitored, especially in developing countries with high population densities (Song et al., 2023a).

Another species of the *Enterobacteriaceae* family, *Escherichia coli*, is frequently found in the large intestine of warm-blooded animals like humans. Fecal contamination during the milking process, along with poor hygiene practices, is the main cause of *E. coli* in raw milk and milk products. As such, *E. coli* is often used as a reliable indicator of direct or indirect fecal contamination in addition to the possibility of enteric pathogens in raw milk and raw dairy products (Kornacki and Johnson, 2001). In addition to this, preserving the highest levels of cleanliness throughout the production process and monitoring the raw materials' microbiological quality is essential for both the safety of consumers and the quality of products (Mladenović, et al., 2021). So, the aim of the current study was the isolation and identification of *klebsiella* spp and *E. coli* from raw cow or buffalo milk and different dairy products (acid-coagulated soft cheese (karish and soured cream), as well as the determination of antimicrobial sensitivity to isolated strains against the

* Correspondence to: hagarnazmy6666@gmail.com

different antibiotics using the disc diffusion method and the investigation of isolates ability to form biofilm.

2. MATERIALS AND METHODS

2.1. Ethical Approval:

Under the Ethical Approval number BUFVTM17-11-23, the protocols utilized in this study were carried out according with the guidelines for animal use and care approved by the Faculty of Veterinary Medicine's ethical Approval Committee at Benha University in Egypt.

2.2 .Samples collection

Between January and April of 2024, randomly 154 dairy samples of raw buffalo and cow milk (33), karish cheese (92), and soured cream (29) were collected from various locations in Egypt, such as Menoufia, Qalyubia, and Cairo governorates. Dairy samples were collected separately in a sterile bottle under hygienic measures, and they were sent directly in an ice box to the food hygiene and control laboratory in the Faculty of Veterinary Medicine at Benha University for bacteriological examination

2.3 .Isolation of E. coli and Klebsiella species

Escherichia coli and *Klebsiella* was isolated from raw milk and dairy products using the ISO (2007) guidelines. Approximately 1 ml of milk/1 g of collected dairy product (homogenized, 5g: 45 ml diluent) was suspended in 9 ml of modified Tryptone Soya Broth (HiMedia Laboratories LLC, 507 School House Rd., USA). Prepared Samples are vortexed and incubated, overnight at 41°C. After selective enrichment. 50 µl of isolate was streaked on Eosin Methylene Blue (EMB) agar (HiMedia Laboratories LLC, 507 School House Rd., USA) for formation of metallic sheen. The same enrichments are streaked on MacConkey agar (HiMedia Laboratories LLC, 507 School House Rd., USA) plates and incubated at 37 °C for 24h. Characteristic red/pink mucoid colonies were selected.

2.4 .Morphological identification

Gram stain was used to stain a single colony. For all suspicious colonies, the isolate was examined using bright-field microscopy for stain and morphological features, according to Smith and Hussey.(2005)

2.5 .Biochemical tests

The bacterial isolates were identified by using the following biochemical tests: Motility test (Cheesbrough, 2000), Oxidase test (Shields and Catheart, 2013), Triple Sugar Iron test (Acharya, 2013), Simmon's Citrate Agar test (Murray et al., 2007), Indole test (MacFaddin 2000), Urease tests (Winn et al., 2006).

2.6 .Confirmatory identification of Klebsiella isolates using polymerase chain reaction (PCR)

2.6.1 .DNA extraction

The DNA extraction from bacterial cultures (3) was carried out. using the GF1 Bacterial DNA extraction kit (GF-BA-100, Vivantis Co., Malaysia) following manufacturer's instructions.

2.6.2 .DNA amplification

Amplification was done to identify *K. pneumoniae* subsp. *ozaenae* and *K. oxytoca* using primers specific for gapA and pehX genes, respectively (Table 1). PCR reactions were performed by using SimpliAmp™ Thermal Cycler (Cat. No. A24811, Applied Biosystems, USA) in final volume of 25 µl reaction having 12.5 µl of 2× EasyTaq® PCR SuperMix (Cat. AS111-01/11, Trans Co., China), 0.5 µl (10 µM) of each primer and 1µl of the target DNA, PCR cycling conditions are shown in table (2). The PCR products were separated by electrophoresis on 1.5% agarose gel then photographed and analyzes using InGenius 3 gel documentation system (Syngene, UK). The used primers and cycling conditions were listed in table (1and 2).

Table (1) PCR Primers used in this study

Gene	Sequence (5´-3´)	Amplicon Size (bp)	Reference
gapA	F-GTGATGGGCGTTAATGAGAG R-AAGCATTGTTCACCTCTTCG	391bp	Aziz and Lafta (2022)
pehX	F-GATACG GAG TATGCC TTTACG GTG R-TAG CCTTTATCA AGCGGATAC TGG	344bp	Kovtunovych *et al.* (2003)

Table (2) Cycling conditions for the detection of gene in this study

Gene	Initial denaturation	denaturation	Annealing	Extension	Final extension	Cycles
GapA	95˚C 2min	95˚C 30sec	56˚C 30sec	72˚C 45sec	72˚C 7min	35
PehX	95˚C 2min	95˚C 30sec	55˚C 30sec	72˚C 40sec	72˚C 7min	35

2.7. Antibiotic sensitivity assay

According to the guidelines of CSLI (Clinical and Laboratory Standards Institute (CLSI, 2019), the antibiotic sensitivity was identified using the disc diffusion method. The plates filled with sterile Mueller Hinton Agar (MHA) medium (Oxoid Limited, Thermo Fisher Scientific Inc Company). On MHA plates, the bacterial suspension was distributed, 19 antimicrobial discs (penicillin 1 µg Bio analysa - streptomycin 10 µg Bio analysa – erythromycin 15 µg bioanalysa – Norfloxacin 10 µg bio analysa - cefepime 30 µg hi media – amoxicillin / clavulanic acid 30 µg hi media – tetracycline 30 µg hi media – gentamicin 10 µg bio maxima - Clostine 10 mcg TM media – cefadroxil 30 µg Bio analysa - ampicillin / sulbactam 20 µg bio maxima - azithromycin 15 µg bio maxima - cefoxitin 30 µg bio maxima - ceftriaxone 30 µg bio maxima - doxycycline 30 µg bio maxima - , cephradine 30 mcg Bio analysa - cefaclor 30 µg - oxacillin 1 µg bio maxima - ceftazidime 30 µg bio maxima , They put on the surface of agar then incubated at 37 °C overnight. the inhibition zone surrounding the disc measured for each antibiotic (Magiorakos *et al*, 2012), multidrug resistance (MDR) is the inability to respond to more than one drug in three or more antimicrobial families.

2.8. Phenotypic assay of biofilm formation ability

2.8.1. Qualitative Congo red agar plating

The Congo red agar method (CRA) used to detect the production of biofilms. Agar (10 gm/L), sucrose (50 gm/L), brain heart infusion broth (37 gm/L), and Congo red indicator (Oxoid, UK) (8 gm/L) were combined to create the CRA medium. The Congo red stain was autoclaved at

121°C for 15 minutes apart from other ingredients of the medium, and then it combined at 55 °C. Test organisms were subsequently spread to CRA plates, and then aerobically incubated for 24 hours at 37 °C. Red or pink crystalline colonies showed no signs of biofilm formation, but black colonies with dry crystalline consistency showed biofilm generation (Jain and Agarwal, 2009).

2.8.2. Crystal violet (CV) quantitative microtiter plate method

By isolating isolates of *E. coli* and *Klebsiella*, the microtiter plate method was used to measure the development of biofilms (Hamad, *et al*., 2019). Brain Heart Infusion (BHI) (Oxoid Limited, Thermo Fisher Scientific Inc Company) broth was used to culture each isolate for an entire night at 37 °C. Afterwards, 200 µL of cell suspension was injected into each of the sterile 96-well polystyrene microtiter plates, and 198 µL of BHI was added. 200 µL of sterile BHI was kept in negative control well. Afterwards, the microtiter plate was incubated for 24 hours at 37 °C. The wells were subjected to 3 mild washings with 200 µL of phosphate buffered saline. The wells were inverted and had dried up. On the biofilm mass, 125 µL of 0.1% CV (Oxoid, UK) stain was applied. The wells were dried upside-own after being carefully cleaned three times with 200 µL of distilled water. Lastly, the wells solved in 200 µL of 30% acetic acid to solubilize the pigment. Using a microplate reader, the biofilm mass optical density (OD) was determined at 595 nm. The OD cut-off (ODc) was defined as three standard deviations above the mean OD of the negative control. Based on their ability to attach, all of the isolates were divided into four categories: OD ≤ ODc, weak biofilm producers (ODc< OD ≤ 2× ODc), moderate biofilm producers (2ODc < OD ≤ 4× ODc), and strong biofilm producers (4× ODc < OD).

3. RESULST

3.1 Prescreening survey of *Enterobacteriaceae* (*E. coli* and *Klebsiella*) in unpasteurized dairy products using presumptive specific media

The recorded results from bacterial culture screening of 154 dairy samples represented by; raw milk (cow or buffalo origin) (33), karish cheese (92), soured cream (29), it was observed that, MacConkey agar media resulted in 69 isolates showing pink color. By their further purification on EMB agar, there were 41 isolates that had mucoid pink colonies with prevalence rate 26.62% characteristic for *klebsiella* spp and 19 green metallic colonies which is characteristic for pathogenic *E. coli* with prevalence of pathogenic *E. coli* in examined dairy samples 12.34% which is distributed as the following 14 out of them (15.2%) were isolated from Karish Cheese and 5 (17.2%) from Soured Cream.

3.2 Prevalence of isolated Enterobacteriaceae *(E. coli* and *Klebsiella*) in unpasteurized dairy products using biochemical tests

Application of biochemical tests on selected mucoid or sticky pink colonies (n=32) yielded identification of (3) *Klebsiella* isolates*;* two *K. oxytoca* and one *K. ozaenae.* The Prevalence of *Klebsiella* in the examined dairy samples was 1.94% (Table 3). The two isolates of *K. oxytoca* were found in Karish Cheese and soured cream while *K. pneumoniae subsp. ozaenae* was isolated from raw milk samples.

By performing Indole test, Lysin and Voges – Proskauer tests were the key tests that differentiate between *K. oxytoca* and *K. pneumoniae subsp. ozaenae.* The former was positive in Indole and Lysin tests, while *K. ozaenae* was positive in Voges – Proskauer test (Table 4).

Also, biochemical tests revealed identification of different bacterial species, including non-pathogenic *E. coli* (13 isolates), *Escherichia vulnaris* (3 isolates), *Escherichia agglomerans* (3 isolates), *Yersinia berocieri* (1 isolates), *Enterobacter diversusi* (1 isolates), Enterobacter sakazakii (1 isolates), *Enterobacter pyrins* (2 isolates), *citrobacter* (1 isolates), *vibrio* (2 isolate) and untyped (2 isolate).

Table (3) Screening of pathogenic *E. coli* and *Klebsiella* spp in dairy products in all 154 samples

Type of examined sample	NO. of samples	*Klebsiella* species		Pathogenic *E. coli*		Non-Pathogenic *E. coli*	
		NO.	%	NO.	%	NO.	%
Raw Milk	33	1 *(K. ozaenae)*	3.03%	-	-	1	3.03%
Karish Cheese	92	1 *(K. oxytoca)*	1.08%	14	15.2%	9	9.7%
Soured Cream	29	1 *(K. oxytoca)*	3.44%	5	17.2	3	10.3
Total	154	3	1.94%	19	12.33%	13	23.03

Table (4) Screening of pathogenic *E. coli* and *Klebsiella* spp using biochemical characteristics of the isolated strains.

Biochemical tests	*K.pneumoniae subsp. ozaenae*	*Klebsiella oxytoca*
Oxidase test	-ve	-ve
Indole Test	-ve	+ve
Motility Test	-ve	-ve
Urease Test	+ve	+ve
Triple Sugar Iron Test	Yellow slant, yellow butt	Yellow slant, yellow butt
Simmon's Citrate Agar Test	+ve	+ve
Lysin	-ve	+ve
Voges – Proskauer	+ve	-ve

3.3 Confirmation of *Klebsiella* Spp. using PCR

Application of PCR for confirmation of 3 *Klebsiella* isolates revealed one as *K. pneumoniae subsp. ozaenae* (391bp) while the others were diagnosed as *K. oxytoca* (344bp) as shown in Figure (1).

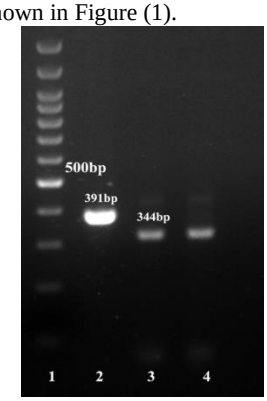

Fig (1) Results of PCR amplifications of the *gapA* (391bp) and *pehX* (344bp) genes from total bacterial DNA , Lane 1: 100 bp DNA Ladder; Lane 2: *K. pneumoniae* subsp. ozaenae; Lane 3: *K. oxytoca* and lane 4: *K. oxytoca*

3.4 Antibiogram of isolated *E. coli* and *Klebsiella spp*. from unpasteurized dairy products using disc assay

Antimicrobial susceptibility test for the *Klebsiella* isolates showed complete sensitivity to cefaclor, azithromycin, tetracycline and norfloxacin. Meanwhile complete resistance was seen to cefoxitin, ceftriaxone, cefadroxil, Ampicillin/ sulbactam, amoxycillin/ clavulanic, penicillin, oxacillin, –amoxicillin, clostine sulphate, streptomycin and erythromycin (Table 5). Interestingly, the isolates showed the characters of MDR.

Table (5) Antimicrobial susceptibility testing of *klebsiella* Isolates.

Antibiotic group	Antibiotic	Sensitive		Intermediate		Resistant	
		NO.	%	NO.	%	NO.	%
Cephalosporins	Ceftazidime	1/3	33.33 %	-	-	2/3	66.66 %
	Cefapime	1/2	50 %	1/2	50 %	-	-
	Cefoxitin	-	-	-	-	2/2	100 %
	Ceftriaxone	-	-	-	-	2/2	100 %
	Cefaclor	2/2	100%	-	-	-	-
	Cefadroxil	-	-	-	-	1/1	%100
	Cephradine	-	-	1/2	50 %	1/2	50 %
Penicillin	Ampicillin/sulbactam	-	-	-	-	2/2	100 %
	Amoxycillin /clavulanic acid	-	-	-	-	3/3	%100
	Pencillin	-	-	-	-	1/1	100 %
	Oxacillin	-	-	-	-	2/2	100 %
	Amoxicillin	-	-	-	-	2/2	100 %
	Clostine sulphate	-	-	-	-	3/3	100 %
Macrolides	Azithromycin	2/2	100%	-	-	-	-
Tetracycline	Tetracyclin	2/2	100 %	-	-	-	-
	Doxycycline	2/3	66.66 %	-	-	1/3	33.33 %
Florfenicol	Norfloxacin	3/3	100 %	-	-	-	-
Aminoglycoside	gentamicin	-	-	1/1	100 %	-	-
	Streptomycin	-	-	-	-	1/1	100%
	Erythromycin	-	-	-	-	1/1	100 %

3.5 Biofilm formation of isolated Enterobacteriaceae (*E. coli* and *Klebsiella*) in unpasteurized dairy products using qualitative and quantitative method

Quantitative microtiter plate method was applied on 22 isolates; 3 *Klebsiella* isolates and 19 pathogenic *E. coli.* Two *Klebsiella* spp. (*Klebsiella oxytoca* and K. *pneumoniae subsp. ozaenae*) formed weak biofilm and one isolate produced intermediate biofilm. Ten *E. coli* isolates showed weak biofilm (45.45%), 5 intermediate biofilms (22.72 %), 2 strong biofilm (9.09%) and 2 isolates did not produce biofilm (9.09%), (Table 6 and Fig 2).

Through application of qualitative Congo red method on 22 isolates; on 3 *Klebsiella* isolates show weak to moderate black color biofilm formation. *E. coli* isolates show 3 weak biofilms (15.7%), 9 intermediate biofilm (40.9%), 2 strong biofilm (9.09%) and 5 no biofilm (22.72%) as illustrated in figure (3).

Table (6) Interpretation of microtiter plate method and Congo Red Agar method

optical Density Cut off (ODc)	microtiter plate method					Congo Red Agar method			
	Type	*Klebsiella spp.*		*E. coli*		*Klebsiella* spp.		*E. coli*	
		No.	%	No.	%	No.	%	No.	%
No biofilm	OD<Odc	-	-	2	9.09%	2	7.4%	5	22.72%
weakly adherent	ODc<OD<2×Odc	2	7.4%	10	45.45%	-	-	3	15.7%
moderately adherent	2×ODc<OD<4×ODc	1	3.7%	5	22.72%	1	3.7%	9	40.9%
Strong Biofilm (strongly adherent)	4×ODc<OD	-	-	2	9.09%	-	-	2	9.09%

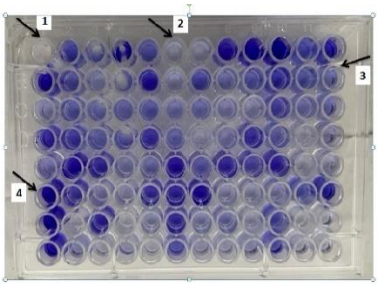

Fig. (2) Microtiter plate method showing different biofilm intensities;(number1): Non biofilm producer; (number 2): weak biofilm producer; (number 3): moderate biofilm producer;(number 4): strong biofilm producer.

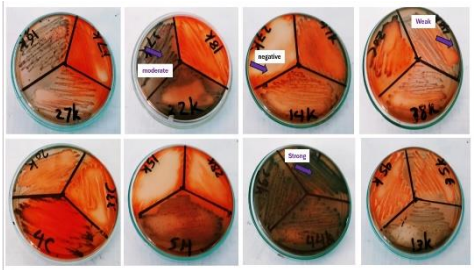

Fig. (3) Screening of biofilm by Congo Red Agar method. the result showing different degree of biofilm formation which represented in black color biofilm formation with black background (weak, moderate, and strong) while the samples showing white color with orange background represent negative result.

4. DISCUSSION

The isolation and identification of *E. coli* and Klebsiella spp. from raw milk and unpasteurized dairy products raise serious public health concerns, particularly in developing countries where microbial contamination is a result of old processing techniques and inadequate hygiene standards (Gwandu et al., 2018). The study's findings support worldwide statistics on the frequency of these bacteria in dairy products and present new concerns regarding food safety, antibiotic resistance, and zoonotic transmission.

The overall incidence of *Klebsiella* spp. (1.94%) and pathogenic *E. coli* (12.33%) in the examined dairy products is consistent with global findings that underscore the presence of these pathogens in milk and dairy products (Song et al., 2023 b). The lower prevalence incidence of *Klebsiella* spp. compared to *E. coli* could be attributed to the specific niches that these bacteria occupy. While *E. coli* is a natural inhabitant of the gastrointestinal tract of humans and animals, its presence in milk is mainly the result of fecal contamination during the milking process (Kornacki and Johnson, 2001). On the other hand, Klebsiella spp. can be more commonly associated with mastitis in cows, which explains its lower prevalence in dairy products as compared to E. coli (Song et al., 2023 b).

In terms of biofilm formation, *Klebsiella oxytoca* and *K. pneumoniae* subsp. ozaenae produced weak biofilms, while one isolate showed intermediate biofilm production. This suggests that there is diversity in the ability to form biofilms among Klebsiella species, potentially due to genetic and environmental factors influencing this behavior. For *E. coli*, 45.45% of the isolates showed weak biofilm production, while 22.72% showed intermediate production. The low number of strong biofilm producers (9.09%) suggests that these strains may be less common or that strong biofilm formation is not a common trait among

the studied species. Given that *E. coli* is known to cause gastrointestinal infections, understanding its biofilm potential is critical for food safety.

Antimicrobial resistance is the capacity of bacterium to live and multiply in the presence of antibiotic doses that were previously believed to be effective against them. AMR has become a significant worldwide concern. Since AMR has extremely high levels in both human and veterinary medicine in most regions of the world, it has emerged as a major rising threat for food security and public health in the current period (Chuanchuen. et al., 2014). Although global livestock production has been growing quickly and depends on the use of antibiotics (AMU), overuse of these medications may cause bacteria to become resistant to them (Phu et al., 2016). The possibility that milk and milk products include bacterial genes resistant to antibiotics is also causing worry for the food industry and public health (Cenci-Goga et al., 2004).

Antimicrobial Resistance and Public Health Implications: One of the key findings in this study was the high resistance observed among Klebsiella isolates to several antibiotics that belong to 3 different antimicrobial groups: aminoglycosides, cephalosporins, and beta-lactam antibiotics, indicating multi-drug resistance as defined before (Magiorakos et al., 2012). This multidrug resistance (MDR) is particularly alarming, as *Klebsiella* species are known to acquire resistance genes via horizontal gene transfer, leading to treatment failures in both human and veterinary medicine (Grosjean et al., 2024). MDR pathogens pose a severe risk to public health, especially in developing countries where access to advanced healthcare and antibiotics is limited. The resistance patterns observed in this study highlight the urgent need for continuous monitoring and the implementation of antibiotic stewardship programs to mitigate the spread of MDR bacteria in the food chain.

The ability of both *Klebsiella* spp. and *E. coli* to form biofilms enhances their pathogenicity and resistance to environmental stress and antibiotics. This study demonstrated that a significant proportion of the isolated *Klebsiella* strains were biofilm producers, with varying degrees of biofilm intensity (Hamad et al., 2019). Biofilm formation is a key virulence factor as it facilitates bacterial survival in hostile environments, such as during cleaning and disinfection processes in dairy plants. The presence of biofilm-forming *Klebsiella* in dairy products suggests potential difficulties in eliminating these bacteria through conventional sanitation methods, increasing the risk of persistent contamination in dairy production chains.

5. CONCLUSIONS

The study's findings draw attention to the dangers that consuming raw milk and other unpasteurized dairy products poses to the general public's health. The presence of biofilm-forming *E. coli* and Klebsiella species, which increase their virulence against antimicrobial agents and are resistant to antibiotics, highlights the need for stringent regulations and better hygiene practices in the dairy industry to protect Egypt's traditional dairy products.

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