Received: 16 July 2022, Accepted: 21 August 2022

Original Research

Journal of Advanced Veterinary Research
(2022) Volume 12, Issue 4, 415-421

Virulence Genes of Multi-drug Resistance *Pseudomonas* species Isolated from Milk and Some Dairy Products

Heba Gamal[1*], Mohamed El-Diasty[2], Amany Dapgh[2], Mohammed El-Sherbini[1], Amira El-Baz[1], Adel Abdelkhalek[1,3]

[1]Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt.
[2]Agricultural Research Center (ARC), Animal Health Research Institute- Mansoura provincial Lab (AHRI-Mansoura) P.O. Box 264-Giza, Cairo 12618, Egypt.
[3]School of Veterinary Medicine, Badr University in Cairo (BUC) P.O. Box 11829.

***Correspondence**
Heba Gamal
Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt.
E-mail address: hebagamalali7@gmail.com

Abstract

Pseudomonas species is one of the psychotropic bacteria that can survive in low-tempered milk and dairy products besides producing heat-resistant spoilage enzymes. In this study, one hundred and fifty samples of milk and some dairy products were analyzed. The overall prevalence of *Pseudomonas* spp. was 44.66% (0% pasteurized milk, 16% butter, 20% pasteurized cream, 48.5% Talaga cheese, 50% bulk milk tank, 66.6% raw market milk, and 70% in raw cream). From 67 positive samples, eighty-three isolates were confirmed biochemically as *Pseudomonas* spp. The most prominent species were *P. aeruginosa*, then *P. fluorescence*, *P. Fragi*, *P. psychrophile*, *P. proteolytica*, *P. alcaligens*, *P. lundensis*, and *P. brenneri* by a percent of 38.5%, 37.5%, 10.8%, 6%, 2.4%, 2.4%, 1.2%, and 1.2%, respectively. Fourteen antibiotic discs were selected to measure the antimicrobial susceptibility of 59 isolates of *Pseudomonas* spp. The higher antimicrobial resistance was against Ampicillin (100%) followed by Colistin (98%), while the antibiotic sensitivity was higher against Imipenem (96.6%) then Meropenem (91.5%). The average MAR index of isolated *Pseudomonas* spp. was 0.462. Ten isolates of antimicrobial resistance serotypes of *P. aeruginosa* were O11: E, O8: C, O5: B, O4: F, and O2: B. Molecular identification of *P. aeruginosa*, *P. fluorescence*, and *P. Fragi* was carried out using polymerase chain reaction (PCR) to determine their virulence genes (*LasB*, *ExoS*, *pilB* for *P. aeruginosa*, *aprX* for *P. fluorescence* and *carA* gene for *P. Fragi*). High levels of antimicrobial-resistant (AMR) *Pseudomonas* spp. threaten public health and cause global concern. The economic and public health impacts were discussed.

KEYWORDS
AMR, Dairy products, milk, PCR, *Pseudomonas* spp.

INTRODUCTION

Pseudomonas spp. is the most prominent bacterium detected in cold raw milk worldwide that produces heat-resistant spoilage enzymes (Jay *et al*., 2005; Machado *et al*., 2017). They are isolated with high concentrations from milk following cold reports or immediately after milking and are inhabitants of plants, soil, and surface water (Dogan and Boor, 2003).

In the coming three decades, it is expected that the Egyptian population to grow by 65%. Hence, between 2015 and 2050, the predicted changes in demand for livestock products are remarkable, as milk consumption increases by almost 300 percent (FAO, 2017). Liquid milk has resembled 34.8 % of the marketable milk, of which unhealthy is 54 % and supplied through the informal sector 70% where no awareness about the hygienic condition during milking and transportation of milk. The health impact of the direct sale of informal milk will be dangerous for human health (ECES, 2020; ILO, 2020).

Pseudomonas spp. have a public health hazard as it is an opportunistic pathogen that can affect any part of the body (Bhargava, 2020). Endotoxin is present in all strains and is a prominent virulence factor in bacteremia and septic shock (Baron, 1996). Public health was threatened by microbial multidrug resistance (MDR) which resulted from antibiotic abuse and antibiotic gene transfer between bacteria (Quintieri *et al*., 2019). Antimicrobial resistance (AMR) bacteria cause untreated diseases in 700,000 people every year globally, which is expected to cause the deaths of 10 million people every year by 2050 (Strathdee *et al*., 2020).

However, in the animal, there were economic losses due to intramammary infections that lead to mastitis. Its virulence is conducted in several antibiotic resistance as ceftriaxone, enrofloxacin, and levofloxacin (Ahmed *et al*., 2014; Ameen *et al*., 2019).

In terms of quality, psychotropic bacteria are considered the leading reason for spoilage that causes huge financial losses for the food sector (Samaržija *et al*., 2012). They cause apparent spoiling characteristics (discolorations, structural loss, rheology changes) as well as non-visual defects (protein breakdown, off-odors, and off-flavors), which severely decrease the quality and shelf-life of dairy products (Quintieri *et al*., 2021).

One of the virulent genes of *P. fluorescens* is *aprX* which causes proteolysis of milk and dairy products due to the degradation of extracellular proteins (Marchand *et al*., 2009a). Also, from *P. aeruginosa* virulence genes were the Pili gene (*pilB*) which is important for biofilms formation, initiation of colonization, and

ISSN: 2090-6277/2090-6269/

adherence of the bacteria. The Exoenzyme S gene (*ExoS* gene) is cytotoxic (Fadhil *et al*., 2016). The elastase B gene (*LasB*) has an elastolytic activity and destroys the structural proteins of the cell (Benie *et al*., 2017). In addition, *P. Fragi* participates in food spoilage through the carbamoyl phosphate synthase gene (*carA* gene) one of its virulence genes (Ercolini *et al*., 2007).

Even though these bacteria are rendered inactive following pasteurization and sterilization of milk, they produce a large number of thermo-tolerant lipolytic and proteolytic enzymes during the storage of raw milk reducing both the shelf life and quality of processed milk (Wiedmann *et al*., 2000).

So, this research was done to determine the prevalence, virulence genes, and antimicrobial resistance pattern of *Pseudomonas* spp. in 150 samples of refrigerated market milk (raw and pasteurized), farm bulk milk tank (BMT), Talaga cheese, butter, and cream (raw and pasteurized) in Dakahlia, Egypt.

MATERIALS AND METHODS

The methods for sample collection were recommended by American Public Health Association (APHA, 1992). One hundred and fifty samples were collected in the period between April till July 2021(30 market raw milk, 20 BMT of dairy farms, 35 Talaga cheese, 10 pasteurized milk, 20 raw cream, 10 pasteurized cream, and 25 butter) from different supermarkets, dairy shops, and dairy farms in Dakahlia, Egypt. The collected samples are kept in an insulated icebox (4±1°C) to be transport to the laboratory of food hygiene and control department, faculty of veterinary medicine, Mansoura University for examination.

Preparation of samples

The samples were prepared following APHA (2004), 25ml of milk and 25 g of cheese (homogenized for 3 min) were obtained, while 25 g of butter and cream (melted in a water bath at 40ºC) were thoroughly mixed and added to 225 ml of buffered peptone water.

Isolation and identification of Pseudomonas spp.

An amount of 0.1 ml of suitable dilution of each sample was spread on *Pseudomonas* selective agar base media (HIMEDIA) supplemented with glycerol and CFC supplements (Cetrimide, Fusidic Acid, and Cefaloridin) and incubated aerobically at 25±1°C for 48 h. Plates showing white, circular, moist, 1.2 mm in diameter, with or without pigment production, and convex surfaces of the suspected *Pseudomonas* colonies were counted. Suspected *Pseudomonas* colonies (4-5) were purified for biochemical identification.

Confirmation of isolates was achieved microscopically (ISO, 2013) and biochemically according to Garrity and Holt (2001).

P. aeruginosa isolates were identified serologically according to Glupczynski *et al*. (2010) using four polyvalent and sixteen monovalent antisera (Bio-Rad, France). While the detection of *P. aeruginosa* groups was dependent on the International Antigen Typing Scheme (IATS) following Legakis *et al*. (1982).

Molecular detection of virulence genes: for some isolates of *P. aeruginosa* (elastase B (*LasB*), exoenzyme S (*ExoS*), and pili (*PilB*)), *P. fluorescens* (alkaline metalloprotease (*aprX*) and *P. Fragi* (carbamoyl phosphate synthase gene (carrA)) were done using primers as mentioned in Table 1, where DNA extraction was performed following Sambrook (1989).

Amplification of virulence genes of *P. aeruginosa*, *P. fluorescens*, and *P. Fragi* was carried out following Machado *et al*. (2013); Benie *et al*. (2017) and Hilario *et al*. (2004), respectively.

Antibiotic Resistance of Pseudomonas species (Antibiogramme)

Antimicrobial susceptibility for *Pseudomonas* spp. was examined by the single diffusion technique following Luczkiewicz *et al*. (2015). The antimicrobial discs and their concentrations and the inhibition zone diameters are demonstrated in Table 2.

Determination of Multiple antibiotic resistance (MAR) index

MAR index = Number of resistance (Intermediate isolates were considered to be MAR index sensitive) / Total Number of tested antibiotics.

Statistical Analysis

Results were analyzed as numbers and percentages in addition to MAR (Multiple Antibiotic Resistance) indexes for each isolate and the average is calculated through SPSS (Statistical Package for Social Science) software version 16.

RESULTS

Prevalence and count of Pseudomonas spp. in milk and some dairy products

The total prevalence of *Pseudomonas* spp. was 44.66% in all examined samples. Raw cream recorded the highest prevalence of 70%, while the raw market milk, bulk milk tanks from different dairy farms, Talaga cheese, pasteurized cream, and butter recorded 66.6%, 50%, 48.5%, 20%, and 16% respectively, however, the

Table 1. Primer sequences for virulence factors of *Pseudomonas* species:

Target gene	Oligonucleotide sequence (5′ → 3′)	size (bp)	References
LasB (F)	5′ GGAATGAACGAGGCGTTCTC ′3	300	Mehri *et al*. (2013).
LasB (R)	5′ GGTCCAGTAGTAGCGGTTGG ′3		
ExoS (F)	5′ CTTGAAGGGACTCGACAAGG ′3	504	Strateva *et al*. (2008).
ExoS (R)	5′ TTCAGGTCCGCGTAGTGAAT ′3		
pilB (F)	5′ ATGAACGACAGCATCCAACT ′3	826	
pilB (R)	5′ GGGTGTTGACGCGAAAGTCGAT ′3		
aprX (F)	5′ TAYGGBTTCAAYTCCAAYAC ′3	194	Bach *et al*. (2001).
aprX (R)	5′ VGCGATSGAMACRTTRCC ′3		
carX (F)	5′ AAAGTCGTCAGCACCGAAGCC ′3	370	Ercolini *et al*. (2007).
carX (R)	5′ CGTCAGCACCGAAAAAGCC ′3		

pasteurized milk recorded 0%. The Maximum count of *Pseudomonas* species was 2.8×10^7 CFU/g in Raw cream. While the minimum count was 3×10^3 CFU/g in pasteurized cream (Table 3).

Frequency distribution of Pseudomonas species detected in milk and some dairy products

Culturally, one hundred more characterized isolates were identified as suspected *Pseudomonas* spp. Biochemically, eighty-three isolates were identified as *Pseudomonas* species (*P. aeruginosa* 38.5%, *P. fluorescence* 37.3%, *P. Fragi* 10.8%, *P. psychrophile* 6%, *P. proteolytica* 2.4%, *P. alcaligens* 2.4%, *P. lundensis* 1.2% and *P. brenneri* 1.2%). The most prevalent type was *P. aeruginosa*, followed by *P. fluorescence* (Table 4). There were 17 competitor bacterial isolates isolated from *Pseudomonas* selective agar media (*Klebsiella* spp. 47% (8/17), *Proteus* spp. 35% (6/17), *Serratia* spp. 11.7% (2/17), and *Citrobacter* spp. 5.8% (1/17))

P. aeruginosa was found in 34% (11/32) of the Talaga cheese samples. While *P. fluorescence* showed the highest incidence in retail market milk at 35% (11/31). *P. Fragi* showed the most prevalence in cheese, at 44% (4/9). *P. psychrophila* was detected more in raw cream and retail market milk with the same percentage of 40% (2/5). *P. alcaligens* was detected only in retail market milk. However, *P. proteolytica* was found in both BMT and retail market milk with the same values. Lastly, *P. lundensis* and *P. brenneri* were found only in butter and pasteurized milk, respectively (Table 4).

Table 2. Concentration of antimicrobial discs and their effect on bacterial isolates.

Antimicrobial agent	Sensitivity disc content (ug)	Resistant (mm)	Intermediate (mm)	Susceptible(mm)
Amikacin (AK)	30	12 or less	13-15	16 or more
Azithromycin (AZ)	15	18 or less	19-21	22 or more
Ipipenem (IPM)	10	18 or less	19-21	22 or more
Ceftazidime (CE)	30	14 or less	15-18	19 or more
Gentamicin (G)	10	12 or less	13-14	15 or more
Colistin (C)	25	10 or less	15-Nov	16 or more
Ampicillin (AM)	10	13 or less	14-17	18 or more
Piperacillin (P)	30	14 or less	15-17	18 or more
Cefepime (FEP)	30	18 or less	19-24	24 or more
Aztreonam (AT)	30	17 or less	18-22	23 or more
Ciprofloxacin (CP)	5	15 or less	15-19	20 or more
Amoxicillin (AMX)	30	14 or less	15-18	19 or more
Meropenem (M)	10	9 or less	12-Oct	13 or more
Tobramycin (T)	10	13 or less	14-22	23 or more

Table 3. *Pseudomonas* spp. prevalence and count (CFU/ml) in tested samples.

Type of samples	No. of examined samples	No. of positive samples	%	Minimum	Maximum	Mean ± SE
Raw cream	20	14	70	$2x10^5$	$4.40x10^7$	$4.11x10^6\pm3.07x10^6$
Talaga cheese	35	17	48.57	6.6×10^3	1×10^7	$2.14x10^6\pm6.40x10^5$
Market raw milk	30	20	66.66	2×10^4	6×10^5	$1.68 \times10^5\pm3.57x10^4$
Bulk milk Tank	20	10	50	8×10^3	2.30×10^7	$3.70 \times10^6\pm2.32x10^6$
Butter	25	4	16	1.20×10^6	2.80×10^7	$9.95 \times10^6\pm6.19 \times10^6$
Pasteurized cream	10	2	20	3×10^3	1.6×10^4	$9.500 \times10^3\pm6.50 \times10^3$
Pasteurized milk	10	Zero	Zero	-	-	-
Total	150	67	44.66			

Table 4. Frequency distributions of *Pseudomonas* species obtained from milk and some dairy products

Isolates	Sample types							
	Butter	Farm BMT	Raw cream	Talaga cheese	Market milk	Pasteurized cream	No	%
P. aeruginosa	1	5	5	11	9	1	32	38.5
P. fluorescence	1	4	6	7	11	2	31	37.3
P. Fragi	1	1	1	4	2		9	10.8
P. psychrophila		1	2		2		5	6
P. proteolytica		1			1		2	2.4
P. alcaligens					2		2	2.4
P. lundensis	1						1	1.2
P. brenneri						1	1	1.2
Total	4	12	14	22	27	4	83	100

Antimicrobial susceptibility of Pseudomonas species

Fourteen antibiotics were selected to detect the AMR in only 59 isolates of *Pseudomonas* spp., which were chosen from the previously identified 83 isolates. The higher antimicrobial resistance was against Ampicillin (100%) followed by Colistin (98%), Amoxicillin (93%), Azithromycin (79%), Gentamicin (66%), Amikacin (55%), Ceftazidime (44%), Tobramycin (33%), Piperacillin (23%), Aztreonam (20%), Ciprofloxacin (18%), Cefepime (8.5%), Meropenem (3.4%), and Imipenem (1.7%) (Table 5). The average MAR index of isolated *Pseudomonas* species was 0.462.

Serotyping of Pseudomonas aeruginosa

Ten isolates of antimicrobial resistance *P. aeruginosa* were serotyped and classified into various serotypes (O2, O4, O5, O8, and O11) and groups (B, C, E, and F). The serotypes O11E, O5B, and O8C were found in cheese samples, while O2B was detected in raw cream, O11E and O4F were found in bulk milk tanks, and O2B, O11E, and O4F were detected in raw market milk.

Molecular identification

Multiplex PCR method was used to identify virulence factors (*LasB* (300 bp), *ExoS* (504 bp), and *pilB* (826 bp)) in ten isolates of antimicrobial resistance *P. aeruginosa*. The incidence of *LasB* (Elastase B gene), *ExoS* (Exoenzyme S gene), and *pilB* (*Pili* gene) were 10 (100%), 8 (80%), and 4 (40%), respectively.

The incidence of (alkaline protease X) *aprX* (194 bp) as a virulent gene for the characterization was found in the four examined isolates of *P. fluorescens*, While the (carbamoyl phosphate synthase gene) *carA* gene (370 bp) was found in the two examined isolates of *Pseudomonas fragi*.

DISCUSSION

Pseudomonas species pose a great danger to human health and animals, resulting in economic losses. They can be transmitted to customers through fresh dairy products, in particular, due to unhygienic practices during manufacturing and handling (Quintieri *et al*., 2019). This danger lies in cheeses as they are ready-to-eat food products (Vrdoljak *et al*., 2016), and other dairy products that are made from raw milk, such as raw cream (Friesland Campina Institute, 2015).

The prevalence of *Pseudomonas* species varied depending on the type of sample according to the results shown in Table 3. Higher results in cheese samples were shown by Hammad (2015) who detected *Pseudomonas* species in 70 samples of examined Domiattie cheese with a percent of 87.5 %. On the other hand, a lower result was reported by Arslan *et al*. (2011) who examined 140 homemade white cheese samples with 22.9% (32) of *Pseudomonas* spp. The results of butter samples were matched with (Al-Ashmawy and El-Dyasety, 2008). who detected 20% of *Pseudomonas* spp. from 25 samples of table butter unlike Meshref (2010) who was not detected *Ps. aeruginosa* in any butter samples, which may be due to the effect of high acidity and salt content. While in milk samples, Dogan and Boor (2003). detected *Pseudomonas* isolates in raw and pasteurized milk from 4 dairy processing plants. While Meng *et al*. (2017) and Condé *et al*. (2022). detected them in refrigerated bulked raw milk.

In comparison with the *Pseudomonas* count in Table 3, El-kholy *et al*. (2008). detected 72% *Pseudomonas* species in 25 samples of Talaga cheese with a count of $7.6\times10^4\pm5.2\times10^4$. However, Eleboudy *et al*. (2015) examined 50 samples of Damietta cheese with a 68% prevalence of *Pseudomonas* spp. with a mean count of $9.02x10^4\pm2.87x10^4$. When the count of bacteria reaches more than (10^6 CFU/mL), the bacteria produced hydrolytic enzymes in their stationary phase of growth and can be introduced into milk without bacterial dissociation from biofilms (Teh *et al*., 2014). Flavor problems in processed milk usually appear when the bacterial population of the milk exceeds 10^7 CFU/ml (Dogan and Boor, 2003).

At the start of storage, raw milk may be contaminated with larger amounts of bacteria, up to 10^7 CFU/mL depending on the hygienic conditions (Machado *et al*., 2015). On a farm level, the bulk milk tank is contaminated through external surface contamination (Murphy and Boor, 2000). The main sources of milk contamination are air, feed, water, feces, grass, and soil (Vacheyrou *et al*., 2011). In addition to the milking system and milking hygienic practices have a role (Monsallier *et al*., 2012). In contrast, there is no existence of *Pseudomonas* spp. in pasteurized milk because of the thermal effect of pasteurization (Wiedmann *et al*., 2000).

It is scientifically proven that protease activity and production in *Pseudomonas* species are induced by milk fat (Decimo *et al*., 2017; Zhang *et al*., 2020). This is achieved in full-fat cream and butter, which have a fat content of about 35% and 80%, respectively, (Friesland Campina Institute, 2015). However, various factors may harm the incidence of these bacteria in butter samples, such as the effect of high acidity and salt content (Meshref, 2010). Gram-negative bacteria found in pasteurized cream indicate

Table 5. Antimicrobial susceptibility of *Pseudomonas* species (n=59).

	Sensitive		Intermediate		Resistant	
Antimicrobial agent	NO	%	NO	%	NO	%
Ampicillin (AM)	-	-	-	-	59	100
Colistin (C)	-	-	1	1.7	58	98.3
Amoxicillin (AMX)	1	1.7	3	5.1	55	93.2
Azithromycin (AZ)	8	13.6	4	6.8	47	79.7
Gentamicin (G)	14	23.7	6	10.2	39	66.1
Amikacin (AK)	25	42.4	1	1.7	33	55.9
Ceftazidime (CE)	29	49.2	4	6.8	26	44.1
Tobramycin (T)	37	62.7	2	3.4	20	33.9
Piperacillin(P)	40	67.8	5	8.5	14	23.7
Aztreonam (AT)	44	74.6	3	5.1	12	20.3
Ciprofloxacin (CP)	46	78	2	3.4	11	18.6
Cefepime (FEP)	52	88.1	2	3.4	5	8.5
Meropenem (M)	54	91.5	3	5.1	2	3.4
Imipenem (IPM)	57	96.6	1	1.7	1	1.7

contamination after pasteurization or inadequate pasteurization (Dogan and Boor, 2003).

Cheeses are cold-stored dairy products that spoil due to an incidence of *Pseudomonas* spp. The variations in bacterial incidence are attributed to hygienic practices during the preparation of dairy products (Sospedra *et al*., 2009). Pseudomonads were discovered to be implicated in the proteolysis of spoiled cheeses as well as fresh cheese discoloration, particularly blue discoloration, which is a global issue (Del Olmo *et al*., 2018).

There are noticeable variations of *Pseudomonas* spp. isolated from milk and its dairy products as shown in Table 4. There are 14 species identified from 143 isolates from 87 samples of BMT (Meng *et al*., 2017). Another variable incidence was 8% *P. aeruginosa*, and 8% Ps. Alcaligenes, 6% Ps. Fluorescence (El-kholy *et al*., 2008). *Pseudomonas* spp. are implicated in the proteolysis of milk, leading to spoilage such as milk creaming, sediment formation, gelation, and bitterness. While rancidity, off-flavors, soapy strawberry flavor, and bitterness in milk are due to lipolysis (De Jonghe *et al*., 2011).

P. fluorescens was identified in 51 % of the 338 *Pseudomonas* isolates obtained from raw and pasteurized milk (Dogan and Boor, 2003). Moreover, the predominant bacteria of the genus *Pseudomonas* were more than 80% *P. fluorescens* from the examined refrigerated bulk raw milk (Condé *et al*., 2022). In addition, the predominant species in refrigerated raw cream was *P. fluorescens* (6/14) 43%. This proved that the *P. fluorescens* species group was the most predominant and variable in refrigerated milk and dairy products (Mulet *et al*., 2010). *P. fluorescence* occupies the first incidence with a percentage of 45% in Domiattie cheese (Hammad, 2015). In another study, the most prevalent types were *P. fluorescens* (35%), and *P. aeruginosa* (21.6%) (Eleboudy *et al*., 2015). While in white cheese, the predominant isolate was *P. alcaligenes* (5%), *P. aeruginosa* (1.4%), *P. fluorescens* (0.7%) (Arslan *et al*., 2011).

On another level, *Pseudomonas* species detected in butter were (10% *P. Fragi*, 6% *P. fluorescence*, and 2% *P. aeruginosa*) (Al-Ashmawy and El-Dyasety, 2008).

Enterobacteriaceae was identified as a competitor bacterium to *Pseudomonas* spp. Their existence in dairy products indicates an unhygienic condition (Sobeih *et al*., 2020). and causes a variety of severe infections in human health (Sheu *et al*., 2019).

Antimicrobial resistance bacteria pose a serious threat to human health. In this study, 59 isolates of *Pseudomonas* species isolates show resistance to fourteen antibiotics shown in Table 5. Antimicrobial resistance was widespread in the most prevalent bacterial infections including *P. aeruginosa* worldwide in 2018, according to the World Health Organization (WHO, 2018). In the Arab region, previous studies between (2010 – 2018) in most countries analyzed *P. aeruginosa* which produces β-lactamase resistant to 35 antibiotics (Nasser *et al*., 2020). In Egypt, fifty isolates of *P. aeruginosa* with a high incidence of MDR (El-Shouny *et al*., 2018).

In 2011, *Pseudomonas* spp. was susceptible 100% to Ceftazidime, Ciprofloxacin, Amikacin, Gentamicin, and Imipenem (Arslan *et al*., 2011). Furthermore, in 2015, Imipenem, Meropenem, Amikacin, Gentamycin, and Tobramycin sensitivity was 100 percent in all *P. aeruginosa* strains, followed by 69.23 percent for Ciprofloxacin and Moxifloxacin sensitivity (Eleboudy *et al*., 2015).

Several *Pseudomonas* spp. strains obtained from dairy products are resistant to the four structural types of beta-lactams (penicillins, cephalosporins, carbapenems, and monobactams) (Quintieri *et al*., 2019). Because of their resistance to all available antibiotics, *Pseudomonas* spp. was the leading cause of nosocomial infections (Lupo *et al*., 2018). *P. aeruginosa* was the most often detected pathogen (17%) causing healthcare-associated pneumonia (Nasrin *et al*., 2022).

Ten isolates of antimicrobial resistance *P. aeruginosa* were serotyped by an antibody-based agglutination technique in this study. In another study, *P. aeruginosa* serotypes O1:11 are the most common serotypes of *P. aeruginosa* among the 20 serotypes (Nasrin *et al*., 2022).

Molecular characterization of *P. aeruginosa* was detected by multiplex PCR. In the case of the *ExoS* gene lower result was detected by Younis *et al*. (2015) (17.14%) and (Banerjee *et al*., 2017). (36.8%). Also, Younis *et al*. (2015) showed lower results in the las B gene (14.7%).

Detection of *aprX* virulent gene for *P. fluorescens* was done in this study. As *aprX* is the most important proteolytic enzyme which causes milk and dairy products spoilage because the product of this gene degrades extracellular proteins (Marchand *et al*., 2009a; Marchand *et al*., 2009b). Also, this gene was detected by Hammad (2015) and Meng *et al*. (2017).

The virulent gene (*carA*) of *P. Fragi* was detected. Also, Ercolini *et al*. (2007) utilized the *carA* gene for confirmation of *P. Fragi*.

The economic cost of *Pseudomonas* spp. spoilage is around one-third of the edible sections of produced food that are lost or wasted globally. The dairy industry accounts for around 25–30% of the losses, depending on the product kinds (Quintieri *et al*., 2019).

Bacterial adaptation increased at low temperatures due to increases in cellulose production, biofilm biomass, motility, and pigment production (Rossi *et al*., 2018). These bacteria cause discoloration (blue color in cheese, grayish color in milk, and black color in butter), loss of structure, and off-flavors based on both the bacterial enzymatic activities and their numbers, with fatal implications for the shelf-life and quality of products (Quintieri *et al*., 2021).

As psychrotrophic species belonging to the *P. fluorescens* lineage cause great food spoilage potential identified as Specific Spoilage Organisms (SSO) (Andreani and Fasolato, 2017).

As a result, applying microbial contamination control measures in the bulking process should be highlighted because even under refrigeration, microbial contamination can occur (Condé *et al*., 2022).

CONCLUSION

The prevalence of *Pseudomonas* spp in raw milk and its dairy products indicates an unhygienic condition. Thus, these bacteria have a public health hazard due to their wide existence and multidrug resistance. So, milk should be properly pasteurized and proper hygienic measures, such as the implementation of HACCP, and applying GMPs during the dairy products' manufacture. Applying ISO 22000 in dairy shops will prevent spoilage of food and extend the dairy products' shelf-life. Personal hygiene education for food handlers is critical for food safety.

ACKNOWLEDGMENTS

The authors would like to thank the Faculty of Veterinary Medicine at Mansoura University and the Animal Health Research Institute (AHRI) in Egypt for their assistance.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

REFERENCES

APHA (American Public Health Association), 1992. Standard Methods for the Examination of Dairy Products. 16th Ed. APHA. Washington: D. C., USA.

APHA (American Public Health Association), 2004. Standard method for the examination of dairy products. Michael H. and Frank J., eds, 17th edition., APHA Press.

Ahmed, M., Hameed, Malla, S., Kumar, R.S., 2014. Molecular characterization of *Pseudomonas* sp. isolated from milk samples by using RAPD-PCR. Eur. J. Exp. Biol. 4, 78-84.

Al-Ashmawy, M. A., El-Dyasety, A.S., 2008. Lipolytic and proteolytic activities of *Pseudomonas* spp. isolated from table butter. Mansoura Vet. Med. J. 10, 73-78.

Ameen, F., Reda, S.A., El-Shatoury, S.A., Riad, E.M., Enany, M.E., Alarfaj, A.A., 2019. Prevalence of antibiotic resistant mastitis pathogens

in dairy cows in Egypt and potential biological control agents produced from plant endophytic actinobacteria. Saudi J. Biol. Sci. 26, 1492-1498.

Andreani, N.A., Fasolato, L., 2017. *Pseudomonas* and Related Genera. In The microbiological quality of food (pp. 25-59). Woodhead Publishing.

Arslan, S., Eyi, A., Özdemir, F., 2011. Spoilage potentials and antimicrobial resistance of *Pseudomonas* spp. isolated from cheeses. J. Dairy Sci. 94, 5851-5856.

Bach, H.J., Hartmann, A., Schloter, M., Munch, J.C., 2001. PCR primers and functional probes for amplification and detection of bacterial genes for extracellular peptidases in single strains and in soil. J. Microbiol. Methods 44, 173-182.

Banerjee, S., Batabyal, K., Joardar, S.N., Isore, D.P., Dey, S., Samanta, I., Samanta, T.K., Murmu, S., 2017. Detection and characterization of pathogenic *Pseudomonas aeruginosa* from bovine subclinical mastitis in West Bengal, India. Vet. World 10, 738-742.

Baron, S., 1996. The book of Medical microbiology. Chapter 27 *Pseudomonas*. 4th edition. University of Texas Medical Branch of Galveston 1996.

Benie, C.K.D., Dadié, A., Guessennd, N., N'gbesso-Kouadio, N.A., Kouame, N.Z.D., N'golo, D. C., Solange, A., Etienne, D., Koffi, M.D., Mireille, D., 2017. Characterization of virulence potential of *Pseudomonas aeruginosa* isolated from bovine meat, fresh fish, and smoked fish. Eur. J. Microbiol. Immunol. 7, 55-64.

Bhargava, H.D., 2020. *Pseudomonas* Infection. Medscape Education. WebMD. https://emedicine.medscape.com/article/970904-overview

Condé, P.R., Pinto, C.L.D.O., Gandra, S.O., Campos, R.C.A.B., Silva, R.R.D., Costa, J.F.D., Scarlet, O. G., Renata, C.A.B.C., 2022. Identification of the contaminating psychrotrophic bacteria in refrigerated bulked raw milk and the assessment of their deteriorating potential. Semina:Cienc. Agrar. 43, 739-750.

De Jonghe, V., Coorevits, A., Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P., Marc, H., 2011. Influence of Storage Conditions on the Growth of *Pseudomonas* species in Refrigerated Raw Milk. Appl. Environ. Microbiol. APPL. 77, 460-470.

Decimo, M., Brasca, M., Ordóñez, J.A., Cabeza, M.C., 2017. Fatty acids released from cream by psychrotrophs isolated from bovine raw milk. Int. J. Dairy Technol. 70, 339-344.

Del Olmo, A., Calzada, J., Nuñez, M., 2018. The blue discoloration of fresh cheeses: A worldwide defect associated to specific contamination by *Pseudomonas fluorescens*. Food Control 86, 359-366.

Dogan, B., Boor, K.J., 2003. Genetic Diversity and Spoilage Potentials among *Pseudomonas* spp. Isolated from Fluid Milk Products and Dairy Processing Plants. Appl. Environ. Microbiol. APPL. 69, 130-138.

ECES (The Egyptian Center for Economic Studies), 2020. Views on the Crisis (Manufacturing Industries – Cont'd) Industries that Benefited from the Crisis Food Industries: Dairy Products. Report of The Egyptian Center for Economic Studies, Issue 17, 7-9-2020

El-kholy, A.M, Saadia, H.E., Hassan, G. M., Khadiga, A.H., 2008. Monitoring of psychrotrphic bacteria in milk and some dairy products with special reference to *Pseudomonas* species. Assiut Vet. Med. J. 54, 1-17.

Eleboudy, A., Amer, A., Nasief, M., Eltony, S., 2015. Occurrence and Behavior of *Pseudomonas* Organisms in White Soft Cheese. Alex. J. Vet. Sci. 44, 74.

El-Shouny, W.A., Ali, S.S., Sun, J., Samy, S.M., Ali, A., 2018. Drug resistance profile and molecular characterization of extended spectrum beta-lactamase (ESβL)-producing *Pseudomonas aeruginosa* isolated from burn wound infections. Essential oils and their potential for utilization. Microb. Pathog. 116, 301-312.

Ercolini, D., Russo, F., Blaiotta, G., Pepe, O., Mauriello, G., Villani, F., 2007. Simultaneous Detection of *Pseudomonas fragi* , *P. lundensis* , and P. putida from Meat by Use of a Multiplex PCR Assay Targeting the *carA* Gene. Appl. Environ. Microbiol. APPL. 73, 2354-2359.

Fadhil, L., Al-Marzoqi, A.H., Al Taee, Z.M., Shalan, A.A., 2016. Molecular and Phenotypic Study of Virulence Genes in a Pathogenic Strain of *Pseudomonas aeruginosa* isolated from various clinical origins by PCR: Profiles of genes and Toxins. Res. J. Pharm. Biol. Chem. Sci. 7, 590-598.

FAO (Food and Agriculture organization of the United Nations), 2017. FAO statistical yearbook. Africa Sustainable Livestock 2050: Country Brief Egypt. Technical meeting and regional launch, Addis Ababa, Ethiopia, 21:23 Feb. FAO Animal Reproduction and Health Report No. 12, Rome, Italy. https://www.fao.org/3/a-i7312e.pdf

Friesland Campina Institute for Dairy Nutrition and Health, 2015. Fat content and fat composition of dairy products. Dairy articles, milk. Published 23 December. Copy rights Friesland Campina Institute for dairy nutrition and health institute@frieslandcampina.com www.frieslandcampinainstitute.com.

Garrity, G.M., Holt J.G., 2001. The Road Map to the Manual. Bergey's Manual® of Systematic Bacteriology. Springer New York. 119-166.

Glupczynski, Y., Bogaerts, P., Deplano, A., Berhin, C., Huang, T.D., Van Eldere, J., Rodriguez-Villalobos, H., 2010. Detection and characterization of class A extended-spectrum- -lactamase-producing *Pseudomonas aeruginosa* isolates in Belgian hospitals. J. Antimicrob. Chemother. 65, 866-871.

Hammad, A.M., 2015. Spoilage potential of *Pseudomonas* spp. isolated from domiati cheese. Assiut. Vet. Med. J. 61, 18-23.

Hilario, E., Buckley, T. R., Young, J. M., 2004. Improved resolution on the phylogenetic relationships among *Pseudomonas* by the combined analysis of atpD, *carA*, recA and 16S rDNA. Antonie van Leeuwenhoek 86, 51-64.

ILO (International Labour Organization), 2020. Developing the Dairy Value Chain in Egypt's Delta Market System Analysis. Copyright © International Labour Organization 2020. I ILO Publishing (Rights and Licensing), International Labour Office, CH1211 Geneva 22, Switzerland, or by email: rights@ilo.org. www.ilo.org/publns.

ISO (International Standards Organization), 2013. Microbiology of food and animal feeding stuffs. ISO 10272: 2013 (E) International Standards Organization, Geneva, Switzerland.

Jay, J.M., Loessner, M.J., Golden, D.A., 2005. Taxonomy, role, and significance of microorganisms in foods. Modern Food Microbiol. 13-37.

Legakis, N.J., Aliferopoulou, M., Papavassiliou, J., Papapetropoulou, M., 1982. Serotypes of *Pseudomonas aeruginosa* in clinical specimens in relation to antibiotic susceptibility. J. Clin. Microbiol. 16, 458-463.

Luczkiewicz, A., Kotlarska, E., Artichowicz, W., Tarasewicz, K., Fudala-Ksiazek, S., 2015. Antimicrobial resistance of *Pseudomonas* spp. isolated from wastewater and wastewater-impacted marine coastal zone. Environ. Sci. Pollut. Res 22, 19823-19834.

Lupo, A., Haenni, M., Madec, J.Y., 2018. Antimicrobial Resistance in Acinetobacter spp. and *Pseudomonas* spp. Microbiol. Spectr. 6, 3-6.

Machado, S.G., Bazzolli, D. M.S., Vanetti, M.C.D., 2013. Development of a PCR method for detecting proteolytic psychrotrophic bacteria in raw milk. Int. Dairy J. 29, 8-14.

Machado, S.G., da Silva, F.L., Bazzolli, D.M.S., Heyndrickx, M., Costa, P.M.D.A., Vanetti, M. C. D., 2015. *Pseudomonas* spp. and *Serratia liquefaciens* as Predominant Spoilers in Cold Raw Milk. J. Food Sci. 80, 1842-1849.

Machado, S.G., Baglinière, F., Marchand, S., Van Coillie, E., Vanetti, M.C.D., De Block, J., Marc, H., 2017. The Biodiversity of the Microbiota Producing Heat-Resistant Enzymes Responsible for Spoilage in Processed Bovine Milk and Dairy Products. Front. Microbiol. 8, 302.

Marchand, S., Heylen, K., Messens, W., Coudijzer, K., De Vos, P., Dewettinck, K., Lieve, H., Jan, D., Marc, H., 2009a. Seasonal influence on heat-resistant proteolytic capacity of *Pseudomonas lundensis* and *Pseudomonas fragi*, predominant milk spoilers isolated from Belgian raw milk samples. Environ. Microbiol. 11, 467-482.

Marchand, S., Vandriesche, G., Coorevits, A., Coudijzer, K., De Jonghe, V., Dewettinck, K., Paul, D., Bart, D., Marc, H., ,Jan D., 2009b. Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. Int. J. Food Microbiol. 133, 68-77.

Mehri, I., Yousra, T., Imen, D., Asma, B. R., Abdennaceur, H., Ben, R.A., Gtari, M., 2013. Molecular identification and assessment of genetic diversity of fluorescent pseudomonads based on different polymerase chain reaction (PCR) methods. Afr. J. Microbiol. Res. 7, 2103-2113.

Meng, L., Zhang, Y., Liu, H., Zhao, S., Wang, J., Zheng, N., 2017. Characterization of *Pseudomonas* spp. and Associated Proteolytic Properties in Raw Milk Stored at Low Temperatures. Front. Microbiol. 8, 2158.

Meshref, A.M.S., 2010. Microbiological quality and safety of cooking butter in Beni-Suef governorate-Egypt. Afr. Health Sci. 10, 193–198.

Monsallier, F., Verdier-Metz, I., Agabriel, C., Martin B., Montel, M.C., 2012. Variability of microbial teat skin flora in relation to farming practices and individual dairy cow characteristics. Dairy Sci. Technol. 92, 265-278.

Mulet, M., Lalucat, J., García-Valdés, E., 2010. DNA sequence-based analysis of the *Pseudomonas* species. Environ. Microbiol. 12, 1513–1530

Murphy, S.C., Boor, K. J., 2000. Trouble-shooting sources and causes of high bacteria counts in raw milk. Dairy, Food and Environ. Sanitation 20, 606-611.

Nasrin, S., Hegerle, N., Sen, S., Nkeze, J., Sen, S., Permala-Booth, J., Myeongjin, C., James S., Milagritos, D.T., Kristie, J., Samba, O.S., Joshua, T.T., Vance, G.F., Karen, A.K., Annelie, B., Efthymia, P., Eirini, C.,

Yuichiro, S., Andrea, L.K., Sadia, S., Ashika, S.M., Olga, P., Jan, J.,Octavie, L., Raphael, S., Alan, S.C., Sharon, M.T., 2022. Distribution of serotypes and antibiotic resistance of invasive *Pseudomonas aeruginosa* in a multi-country collection. BMC Microbiol. 22, 1-12.

Nasser, M., Gayen, S., Kharat, A.S., 2020. Prevalence of β-lactamase and antibiotic-resistant *Pseudomonas aeruginosa* in the Arab region. J. Glob. Antimicrob. Resist. 22, 152-160.

Quintieri, L., Fanelli, F., Caputo, L., 2019. Antibiotic Resistant *Pseudomonas* Spp. Spoilers in Fresh Dairy Products: An Underestimated Risk and the Control Strategies. Foods 8, 372.

Quintieri, L., Caputo, L., Brasca, M., Fanelli, F., 2021. Recent Advances in the Mechanisms and Regulation of QS in Dairy Spoilage by *Pseudomonas* spp. Foods, 10, 3088.

Rossi, C., Serio, A., Chaves-López, C., Anniballi, F., Auricchio, B., Goffredo, E., Beniamino, T.C., Florigio, L., Silvia, F., Antonello, P., 2018. Biofilm formation, pigment production and motility in *Pseudomonas* spp. isolated from the dairy industry. Food Control 86, 241-248.

Samaržija, D., Zamberlin, Š., Pogačić, T., 2012. Psychrotrophic bacteria and their negative effects on milk and dairy products quality. Mljekarstvo: časopis za unaprjeđenje proizvodnje i prerade mlijeka 62, 77-95.

Sambrook, H.C., 1989. Molecular cloning: a laboratory manual. 2nd Edition, Cold Spring Harbor, NY.

Sheu, C.C., Chang, Y.T., Lin, S.Y., Chen, Y.H., Hsueh, P.R., 2019. Infections Caused by Carbapenem-Resistant *Enterobacteriaceae*: An Update on Therapeutic Options. Front. Microbiol. 10, 80.

Sobeih, A., Al-Hawary, I., Khalifa, E., Ebied, N., 2020. Prevalence oF Enterobacteriaceae in raw milk and some dairy products. Kafrelsheikh Vet. Med. J. 18, 9-13.

Sospedra, I., Rubert, J.V., Soler, C., Soriano, J.M., Mañes, J., 2009. Microbial Contamination of Milk and Dairy Products from Restaurants in Spain. Foodborne Pathog. Dis 6, 1269-1272.

Strateva, T., Ouzounova-Raykova, V., Markova, B., Todorova, A., Marteva-Proevska, Y., Mitov, I., 2008. Problematic clinical isolates of *Pseudomonas aeruginosa* from the university hospitals in Sofia, Bulgaria: current status of antimicrobial resistance and prevailing resistance mechanisms. J. Med. Microbiol. 56, 956-963.

Strathdee, S.A., Davies, S.C., Marcelin, J.R., 2020. Confronting antimicrobial resistance beyond the COVID-19 pandemic and the 2020 US election. The Lancet 396, 1050-1053.

Teh, K.H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., Lindsay, D., 2014. Biofilm—An unrecognized source of spoilage enzymes in dairy products?. Int. Dairy J. 34, 32-40.

Vacheyrou, M., Normand, A.C., Guyot, P., Cassagne, C., Piarroux R., Bouton, Y., 2011. Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. Int. J. Food Microbiol. 146, 253-262.

Vrdoljak, J., Dobranić, V., Filipović I., Zdolec, N., 2016. Microbiological Quality of Soft, Semi-Hard and Hard Cheeses During the Shelf-Life. Maced. Vet. Rev. 39, 59-64.

WHO (World Health Organization), 2018. Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2017-2018. License: CC BY-NC-SA 3.0 IGO. 19 July. Geneva. © World Health Organization. https://apps.who.int/iris/handle/10665/279656.

Wiedmann, M., Weilmeier, D., Dineen, S.S., Ralyea, R., Boor, K.J., 2000. Molecular and Phenotypic Characterization of *Pseudomonas* spp. Isolated from Milk. Appl. Environ. Microbiol. 66, 2085-2095.

Younis, G. Awad, A., Maghawry, M., Selim, F., 2015. Extracellular Enzymes and Toxins of *Pseudomonas aeruginosa* Strains Isolated from Clinically Diseased Egyptian Cows. Adv. Anim. Vet. 3, 522-526.

Zhang, D., Palmer, J., Teh, K.H., Calinisan, M.M.A., Flint, S., 2020. Milk fat influences proteolytic enzyme activity of dairy *Pseudomonas* species. Int. J. Food Microbiol. 320, 108543.