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Prevalence of *Pseudomonas aeruginosa* in Milk and Some Dairy Products with Reduction Trials by Some Natural Preservatives

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Abstract

Contamination of milk and dairy products with spoilage and pathogenic microorganisms is a common problem worldwide. Therefore, this study was conducted on 200 samples (milk, Kareish cheese, Damietta cheese, and plain yoghurt, 50 of each) collected from Zagazig City, Sharkia, Egypt to be examined bacteriologically to isolate and identify the multi-drug resistant *Pseudomonas aeruginosa* as well as some reduction trials on cold stored soft cheese using some natural compounds including the essential oil (EO) of Clove (0.01%, 0.1%) and *Nigella sativa* (NS 0.5%, 1%), in addition to Nisin (10 ppm 12.5 ppm). The obtained results revealed the *Pseudomonas aeruginosa* prevalence in the examined milk and dairy product samples was 45(22.5%); 24(48%) from raw milk samples, 8(16%) from yoghurt, 9(18%) from soft cheese and 4(8%) from kareish cheese. The isolated *Pseudomonas aeruginosa* harbored some antibiotic-resistant genes including $bla_{TEM,}$ bla_{SHV}, *ermB*, and *Mcr1* genes, while bla_{OXA-1} failed to be detected, so it was resistant to different types of antimicrobial agents. The multiple antibiotic resistance index (MAR) of the isolated strains was 0.500. Clove (0.01%, 0.1%) and *Nigella sativa* (NS 0.5%, 1%), in addition to Nisin (10 ppm, 12.5 ppm) had an antibacterial effect against *Pseudomonas aeruginosa* compared with control samples and acted as good preservatives that extended the storage period and shelf life of soft cheese up to thirty days.

KEYWORDS
Clove, Contamination, Nisin, *Pseudomonas aeruginosa*, Resistance

INTRODUCTION

In dairy plants, milk and dairy products contamination by food poisoning organisms during different stages of processing and storage is a significant concern (Fox *et al*., 2009). Consumption of contaminated milk products results in more than 250 different illnesses (Abrar *et al*., 2020). *Pseudomonas* species particularly *Pseudomonas aeruginosa* have been allocated among the causative agents of such illnesses. This microorganism is an opportunistic Gram-negative with high veterinary and medical importance (Ebrahimpour *et al*., 2018). *Pseudomonas aeruginosa* is a widespread pathogen and can colonize and infect various animals (Haenni *et al*., 2015). In dairy cows, it can cause mastitis (Rasooli *et al*., 2018), while, in humans, it can cause serious health effects (Emami *et al*., 2015). High virulence of *Pseudomonas aeruginosa* is related to various cell-associated toxins including exoenzyme S, exotoxin A and secreted toxins such as exoenzyme T and exoenzyme Y (Mesquita *et al*., 2013). Inadequate biosecurity and inadequate hygiene are the principal causes of high risk of infection. Thus, it is necessary to use antibiotics as growth promotors, prophylaxis, and for therapeutic purposes (Van Boeckel *et al*., 2015), but improper use of antibiotics leads to the emergence of resistant pathogenic *Pseudomonas aeruginosa* between animals and humans. To cope with the wide spread of antibiotic resistance, it is very important to search for new methods. These alternative methods include using antibacterial plants and essential oils (EOs) in treatment (Fisher and Phillips, 2009). Using natural antimicrobials as preservatives is desirable to consumers due to the increased demand for clean-label milk. One of these natural preservatives is nisin. The antimicrobial effect of nisin is attributed to its ability to interfere with the biosynthesis of the cell wall, through binding to the cell wall precursor lipid II (Ahmad *et al*., 2017). Clove (*Syzygium aromaticum*) is one of the most important phytochemicals with various medical applications and it is categorized as a safe food additive by the FDA (FDA, 2011). *Nigella sativa* L. (NS) is an annual herb that belongs to the family Ranunculaceae; it is called the black cumin and is largely used in folk medicine (Zuridah *et al*., 2008). Recently NS oil and extracts have been proved to exert immunomodulatory, antimicrobial, and anticancer activity (Priani *et al*., 2020). Therefore, the present study was conducted to evaluate the prevalence of multidrug-resistant *Pseudomonas aeruginosa* in milk and some dairy products with reduction trials using some natural essential oils including clove, *Nigella sativa*, and nisin.

MATERIALS AND METHODS

Samples collection and preparation

Two hundred samples (raw milk, Kareish cheese, Damietta

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cheese, and yoghurt, 50 of each) were collected from healthy animals and supermarkets in Zagazig City, Sharkia, Egypt, and transferred in a cold insulated box to be examined bacteriologically at the Food Control Department, Faculty of Veterinary Medicine, Zagazig University. About 25 (g, ml) of the samples were mixed with 225 ml peptone 0.1% and homogenized, then incubated at 37°C/24 h.

Isolation and identification of Pseudomonas aeruginosa

Bacteria were inoculated in a broth called soybean casein (Oxoid, England) then isolation was done on the cetrimide agar (Oxoid, England) (Brown and Lowbury, 1965). Gram staining and growth at 42°C were carried out for preliminary identification. Pyomelanin, pyocyanin, pyoverdine, and pyorubin production was tested through *Pseudomonas* isolation agar culturing (Alpha Biosciences, Baltimore, MD, USA) (MacFadden, 2000). Molecular screening of antimicrobial resistance genes was conducted by QIAamp Genomic DNA Purification mini kit (51304). Oligonucleotide primers used in PCR were shown in Table 1.

Antimicrobial susceptibility testing of Pseudomonas aeruginosa

It was carried out using agar disk diffusion methods on Mueller-Hinton agar (Srivani, 2011). Results interpretation was carried out according to the Clinical and Laboratory Standards Institute (2015). As calculated by Singh *et al*. (2010), the multiple antibiotics resistance index (MAR) equals the resistant antibiotics divided by the total number of tested antibiotics.

Reduction trials using clove, Nigella sativa, and nisin

Commercial nisin (Nisaplin, Danisco Brasil Ltda, Pirapozinho, Brazil) in concentrations of 10 ppm and 12.5 ppm was added to milk during cheese manufacturing (Hurst, 1981). Clove and NS oil were extracted from seeds using Clevenger's apparatus according to Lamaty *et al*. (1987). Clove and NS EO were analyzed by Gas chromatography (GC) according to Adams (1995).

Soft cheese preparation and inoculation

Soft cheese was prepared according to the guidelines described by Youssef *et al*. (2016). *Pseudomonas aeruginosa* was added to milk as well as clove EO (01%, 1%), or NS EO (0.5%, 1%) and nisin (10 ppm, 12.5 ppm) separately. Control cheese samples were free from clove EO, NS EO, and nisin. Samples were collected at 0, 1, 3, 7, 15, 21, and 30 days and compared with the control. A tenfold serial dilution was performed to count *Pseudomonas aeruginosa* according to APHA (2004).

Statistical analysis

Bacterial counts were converted to $\log_{10}$ CFU/g and presented as means±standard error (S.E) then analyzed by SPSS and One-Way Analysis of Variance (ANOVA) at a 95% level of confidence to determine the significant differences among the different samples by using the Tukey test considering $p<0.05$ as significant.

RESULTS

Prevalence of Pseudomonas aeruginosa

Results illustrated in Table 2 declared that the total prevalence of *Pseudomonas aeruginosa* in the samples was 45(22.5%); it was isolated from 24(48%) raw milk samples, 8(16%) yoghurt, 9(18%) soft cheese and 4(8%) of kareish cheese. As shown in Figure 1, the isolated *Pseudomonas aeruginosa* harbored some antibiotic-resistant genes including bla_{TEM}, $bla_{SHV,}$ *ermB*, and *Mcr1* genes, while bla_{OXA-1} failed to be detected. The isolated strains of *Pseudomonas aeruginosa* were 100% sensitive to Piperacillin-tazobactam, Imipenem, and Meropenem meanwhile,
these strains were 100% resistant to Lincomycin, Clindamycin, and Amoxycillin-Claviolinic acid (Table 3). The antimicrobial resistance profile of *Pseudomonas aeruginosa* revealed that the average MAR index was 0.500.

Table 1. Oligonucleotide primers sequences of *Pseudomonas aeruginosa*.

Gene	Sequence	Amplified product	Reference
bla_{TEM}	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	Colom *et al*. (2003)
bla_{SHV}	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392 bp	
Bla_{OXA-1}	ATATCTCTACTGTTGCATCTCC AAACCCTTCAAACCATCC	619 bp	
Mcr1	CGGT CAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	308 bp	Newton-Foot *et al.* (2017)
ermB	GAAAAAGTACTCAACCAAATA AATTTAAGTACCGTTACT	639 bp	Nguyen *et al*. (2009)

Table 2. Prevalence of *Pseudomonas aeruginosa* in milk and dairy products.

Samples	No. of samples	No. of positive	% of positive
Raw milk	50	24	48
Yoghurt	50	8	16
Soft Cheese	50	9	18
Kareish Cheese	50	4	8
Total	200	45	22.5

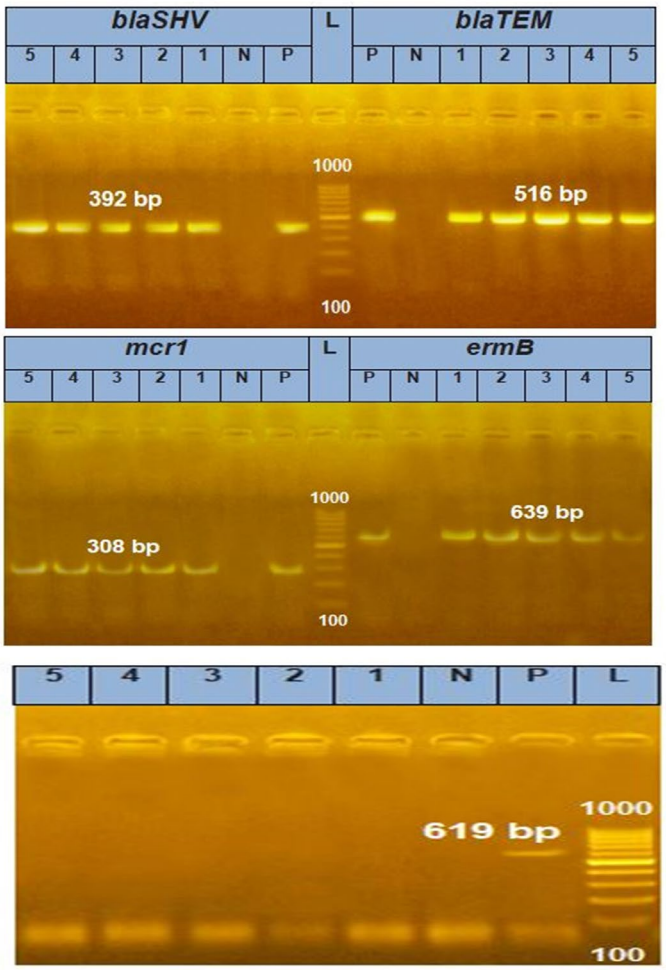

Figure 1. Agarose gel electrophoresis of PCR of *Pseudomonas aeruginosa* bla_{TEM} gene (516 bp), bla_{SHV} gene (392 bp), Bla_{OXA-1} gene (619 bp), Mcr1 gene (308 bp), ermB (639 bp).

Analysis of clove and NS essential oils by Gas chromatography illustrated various components responsible for the antibacterial activities of these EOs (Tables 4, 5). Data recorded in Table 6, evaluated the antibacterial effect of clove, NS, and nisin against *Pseudomonas aeruginosa*. At the beginning of the experiment, the mean count of *Pseudomonas aeruginosa* (log_{10} CFU/g) in control samples was 6.40±0.02, while it was 6.37±0.06 and 6.34±0.08 after treatment by clove 0.01% and 0.1%; 6.38±0.08 and 6.34±0.08 after treatment by NS 0.5% and 1%; and 6.39±0.04 and 6.36±0.03 after treatment by nisin 10 ppm and 12.5 ppm. After three days of the experiment, a significant difference was found between control and treated samples by nisin 12.5 ppm ($p<0.05$). Meanwhile, after seven days of the experiment, significant differences were detected between control and treated samples by clove, NS 1%, and nisin 12.5 ppm ($p<0.05$). After fifteen days of the experiment, significant differences between control and treated samples by clove, NS, and nisin at different concentrations after twenty-one days of treatment ($p<0.05$). After thirty days of treatment, the mean counts of *Pseudomonas aeruginosa* were 5.51±0.13, 5.50±0.01, 6.02±0.03, 5.67±0.06, 6.07±0.06, and 5.38±0.09 log_{10} CFU/g in clove 0.01%, clove 0.1%, NS 0.5%, NS 1%, nisin 10 ppm, and nisin 12.5 ppm treated samples, respectively compared with 6.72±0.01 log_{10} CFU/g in control untreated samples.

DISCUSSION

Pseudomonas aeruginosa is one of the most common psychrotrophic bacteria related to milk and dairy products spoilage which can grow at refrigeration temperature. It can metabolize milk fat and protein resulting in putrefaction, and fermentation. In the present study, raw milk samples were the most contaminated samples by *Pseudomonas aeruginosa*, followed by soft cheese, yoghurt, and kareish cheese. A nearly similar result was reported by Atia *et al*. (2022), while these results were higher than those reported by El-Leboudy *et al* (2015) and Bhunia (2008) and were lower than Arslan and Özdemir (2011) and AbdelAziz *et al*. (2022). *Pseudomonas aeruginosa* can cause various infections with difficult treatment as a result of resistance to various antibiotics. Extended-spectrum ß-lactamases (ESBLs) production is the principal cause of ß-lactam resistance (Peymani *et al*., 2017); TEM and SHV are major groups of ESBLs (Bradford, 2001). In this study, the achieved results were in line with Shahcheraghi *et al*. (2009), Polotto *et al*. (2012), and Peymani *et al*. (2017) who found that *Pseudomonas aeruginosa* carried bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{SHV-1}, and bla_{SHV-12} genes. The colistin resistance which is encoded by the mcr-1 gene is of great concern (Liu *et al*., 2016). In line with

Table 3. Antimicrobial susceptibility of *Pseudomonas aeruginosa* (n.=22).

Antimicrobial agent	Antimicrobial group	Sensitive		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Piperacillin-tazobactam	β-Lactams	22	100	0	0	0	0
Imipenem	Antipseudomonal carbapenem	22	100	0	0	0	0
Meropenem	Antipseudomonal carbapenem	22	100	0	0	0	0
Ciprofloxacin	Fluroquinolone	20	90.91	2	9.09	0	0
Amikacin	Aminoglycosides	17	77.27	4	18.18	1	4.55
Chloramphenicol	Phenols	17	77.27	1	4.55	4	18.18
Trimethoprim-sulfamethoxazole	Folate pathway inhibitors	16	72.73	0	0	6	27.27
Ceftazidime	Cephalosporins	14	63.64	1	4.55	7	31.82
Tetracycline	Tetracyclines	11	50	2	9.09	9	40.91
Colistin	Polypeptides	1	4.55	1	4.55	20	90.91
Erythromycin	Macrolides	0	0	1	4.55	21	95.45
Oxacillin	β-Lactams	0	0	1	4.55	21	95.45
Vancomycin	Glycopeptides	0	0	0	0	22	100
Lincomycin	Glycosamides	0	0	0	0	22	100
Clindamycin	Glycosamides	0	0	0	0	22	100
Amoxycillin-Clavulanic acid	β-Lactams	0	0	0	0	22	100
MAR	0.5						

Table 4. Gas chromatography analysis of Clove.

Components	Area sum %	Area
Eucalyptol	0.3	2236137.38
Terpinen-4-ol	0.23	1695813.34
Methyl salicylate	0.82	6048042.33
Chavicol	0.31	2251523.74
Eugenol	78.73	580201404.7
Copaene	0.31	2285140.44
E-Methyl cinnamate	0.32	2355924.84
β-Caryophyllene	6.03	44469041.53
α-Humulene	0.88	6476166.62
Eugenol acetate	11.52	84901889.57
Caryophyllene oxide	0.54	3985306.78

Table 5. Gas chromatography analysis of *Nigella sativa*.

Components	Area %	g/100g
Myristic acid (C14:0)	0.056	0.055
Palmitic acid (C16:0)	7.95	7.791
Stearic acid (C18:0)	1.298	1.272
Elaidic acid (C18:1n9t)	19.41	19.022
Linoleic acid (C18:2n6t)	63.039	61.778
Linolenic acid (C18:3n3)	3.79	3.714
γ- Linolenic acid (C18:3n6)	2.074	2.033
Arachidic acid (C20:0)	2.36	2.313

Table 6. Effect of Clove, *Nigella sativa* and Nisin on *Pseudomonas aeruginosa* count (Mean±SE).

Time (days)	Control	Clove		Nigella Sativa		Nisin	
		0.01%	0.10%	0.50%	1%	10 ppm	12.5 ppm
Zero	6.40±0.02	6.37±0.06	6.34±0.08	6.38±0.08	6.34±0.08	6.39±0.04	6.36±0.03
1	6.40±0.02	6.28±0.10	6.25±0.12	6.33±0.07	6.32±0.09	6.32±0.02	6.22±0.07
3	6.44 ± 0.02^{a}	6.18 ± 0.07^{ab}	6.10 ± 0.12^{ab}	6.24 ± 0.04^{ab}	6.15 ± 0.03^{ab}	6.27 ± 0.02^{ab}	5.92 ± 013^{b}
7	6.48 ± 0.02^{a}	5.93 ± 0.17^{b}	5.84 ± 0.21^{b}	6.20 ± 0.03^{ab}	5.95 ± 0.05^{b}	6.24 ± 0.02^{ab}	5.74 ± 0.08^{b}
15	6.58 ± 0.002^{a}	5.81 ± 0.14^{bc}	5.79 ± 0.14^{bc}	6.08 ± 0.04^{bc}	5.83 ± 0.07^{bc}	6.21 ± 0.01^{ab}	5.68 ± 0.08^{c}
21	6.67 ± 0.01^{a}	5.62 ± 0.10^{d}	5.57 ± 0.11^{d}	6.04 ± 0.02^{bc}	5.74 ± 0.07^{cd}	6.17 ± 0.02^{b}	5.61 ± 0.10^{d}
30	6.72 ± 0.01^{a}	5.51 ± 0.13^{c}	5.50 ± 0.01^{c}	6.02 ± 0.03^{b}	5.67 ± 0.06^{d}	6.07 ± 0.06^{b}	5.38 ± 0.09^{d}

the obtained result, Hameed *et al.* (2019) also detected the mcr-1 gene. Macrolides resistance is mediated by methylases which are encoded by erm (A, B, C) genes (He *et al.*, 2016). Regarding the antimicrobial sensitivity test, the isolates lacked the susceptibility to many of the tested antimicrobials; these results agreed with Peymani *et al.* (2017), while it differed from what had been reported by Dapgh *et al.* (2019) who reported different susceptibility to Chloramphenicol, Amikacin, Erythromycin, Tetracycline, Trimethoprim-sulfamethoxazole. Virulence factors responsible for *Pseudomonas aeruginosa* pathogenicity include antibiotic-resistant genes. Therefore, the isolated *Pseudomonas aeruginosa* strains were MAR, this result agreed with Rawat and Nair (2010). The MAR *Pseudomonas aeruginosa* has also been reported by Al-Orphaly *et al.* (2021) and Murray *et al.* (2022).

In Middle Eastern, soft cheese is preferred by consumers, it contains many nutrients (proteins, fat, carbohydrates, and vitamins) in addition to water. However, with this nutritive value, different microbiological and physicochemical alterations can affect it (Charfi *et al.*, 2021). Therefore, natural compounds are very important to reduce contamination and control the growth of spoilage bacteria in milk and dairy products. Antibacterial activities of natural compounds attribute to the high content of phenolic compounds, in addition to sesquiterpenes, terpenoids, and diterpenes (Tajkarimi *et al.*, 2010). In the present study, clove EO exhibited antibacterial activity against *Pseudomonas aeruginosa* compared with the control group, this result agreed with Charfi *et al.* (2021). The EO of NS had an inhibitory effect against *Pseudomonas aeruginosa*; other studies also reported similar results (Hassanien *et al.* 2014; Georgescu *et al.*, 2019; Puvača *et al.*, 2020). Nisin had a significant antibacterial effect ($p< 0.05$); this result agreed with Ibrahim and Sobeih (2005) who reported that nisin prolonged the shelf life of salted cheese to 20 days at refrigeration temperature.

CONCLUSION

Multi-drug-resistant *Pseudomonas aeruginosa* was isolated from milk and dairy products due to a lack of hygiene. Natural compounds including the essential oils of clove and *Nigella sativa* as well as nisin have an antibacterial effect and act as good preservatives to prolong the shelf life of soft cheese by up to thirty days.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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