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Biofilm production by Pseudomonas species isolated from bulk tank milk and some milk products.

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ABSTRACT

Greater resistance to environmental aggressors, including resistance to antibiotics and other disinfectants is a characteristic of the Pseudomonas phenotype that forms biofilms. Consequently, the current study's goal was to isolate Pseudomonas spp. from two hundred samples of milk and milk products and determine their ability to form biofilm. Pseudomonas species were recovered from bulk tank milk, kareish cheese, yoghurt, and ice cream with the incidence of 36 %, 26%, 22% and 16 %, respectively. For biofilm formation, *Ps.* aeruginosa strain has the ability to produce biofilm as 9 (33.4%) of the isolated strains were strong biofilm producers, 5 (18.5%) were moderate, 5 (18.5%) were weak, while 8 (29.6%) were non biofilm producers. Also, 6 (46.3%) of the isolated *P. fluorescence* stains were strong biofilm former while, 2 (15.4%) were moderate and 3 (23.1%) were weak, but 5 (15.4%) were unable to form biofilm. Furthermore, 2 (40%) of *Ps. putida* strains had a strong ability for biofilm formation, 1 (20%) were moderate, 1 (20%) weak and 1 (20%) was non-biofilm producer. Moreover, 2 (40%) of *Ps. diminuta* strains had a strong ability for biofilm formation, 1 (20%) were moderate and 2 (40%) were weak biofilm former. It was concluded that some of Pseudomonas species that isolated from milk and milk products have the ability to form biofilm.

1. INTRODUCTION

A microbial cell will naturally build a biofilm on a solid surface in order to compete effectively with other cells for nutrients and space, to withstand any unfavorable environmental circumstances, and to boost the virulence of pathogens (Ya-Wen et al., 2015).

By creating a three-dimensional biofilm scaffold out of an extracellular polymeric substance (EPS), microbes adhere to surfaces. Solid surfaces and a physiologically active matrix of cells and extracellular substances are joined to form biofilms. The EPS serves as a metaphorical "house" for the bacteria in biofilms, providing it with shelter (Trevor et al., 2008).

Biofilm formation is a very rapid complex process that involves several physical, chemical, and biological factors (Flemmingand and Wingender, 2010). The ability of bacteria to form biofilms on surfaces is influenced by several factors, including cell surface properties, surface properties, environmental factors, EPS, polysaccharides, and virulence factors (Cho et al., 2022 ; Sherry et al., 2021). The Cell surface properties as the hydrophobicity, flagellation, and motility of bacterial cells can influence bacterial adhesion to surfaces (Matthew et al., 2020).

Surface properties such as roughness and hydrophobicity can also affect bacterial adhesion (Yuanet al., 2017). Environmental factors such as nutrient levels, temperature, pH, and ionic strength can influence biofilm formation (Zhao et al., 2017).

Extracellular polymeric substances are produced by bacteria and form a protective matrix around the biofilm, contributing to its stability and resistance to antimicrobial agents, Various polysaccharides, such as alginate, pel (cationic polymer composed of 1,4 linked N-acetylglucosamine and N-acetyl galactosamine), and psl (a neutral polysaccharide consisting of a penta saccharide repeat containing glucose, mannose, and rhamnose), determine the stability of biofilm structure as in *Pseudomonas aeruginosa* (Alotaibi and Bukhari, 2021). Some bacterial virulence factors, such as surface proteins, can play important roles in biofilm formation and pathogenesis (Hwang and Michael 2012; Xingjian et al., 2021).

A biofilm develops in stages, starting with a loose bacterial attachment to a surface and moving toward a firm adhesion. During the final phase of adhesion, the bacterial cell wall is deformed, which brings the cytoplasmic bacterial molecules closer to the surface, increasing the adherence of the bacteria to it. Structured channels in the biofilm allow implanted microorganisms and the environment to exchange food and byproducts, which encourages bacterial colonization, growth, and maturity (Kecheng et al., 2022). Bacteria leave the matured biofilm after it has reached maturity and move to another biofilm community to establish a new one (Hall-Stoodley et al., 2004).

Since viruses can be directly transmitted through contact in the environment of food preparation, biofilm formation poses a concern to food safety. Pathogens can also develop

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biofilms on food contact surfaces after transmission (Pinto et al., 2019).

Antibiotic therapies are beneficial in treating a variety of infectious disorders. However, this method is ineffective in situations when bacterial biofilms are the main problem. Multiple mechanisms are thought to contribute to biofilms' resistance. The biofilm's several layers are not entirely penetrated by the antibacterial agent. Its diffusion is hampered by polymeric components in the biofilm matrix, which indicates that they never accumulate enough antibiotics. The biofilm's cells, at least some of which are nutrient-deficient, must transition into a phase of slow growth. Many antimicrobial drugs do not affect slow-growing or non-growing cells, and many of them can survive. In the biofilm, bacteria exchange resistance genes with one another (Ciftçi et al., 2005).

Therefore, the purpose of this investigation was to find out whether the isolates of the Pseudomonas species obtained from bulk tank milk and some dairy products have the ability to form biofilms.

2. MATERIAL AND METHODS

2.1. Collection of samples

A total of 200 random samples of bulk tank milk, Kareish cheese, yoghurt, and ice cream (50 of each) were gathered from various milk collecting centers and supermarkets in Menofia governorate. Random sampled (500ml) was maintained separately in a plastic bag before being swiftly and completely aseptically transported to the lab in an insulated ice box, where it was permitted to defrost in a refrigerator (2–5 °C). All obtained samples were as quickly as possible analyzed bacteriologically for *Pseudomonas* species isolation.

2.2. Preparation of samples

Under strict aseptic conditions, 10 ml/gm samples were transferred into a sterile jar containing 90 ml of sterile 0.1% peptone water. At room temperature (20 °C), the contents were homogenized using Fisher Scientific™ 850 Homogenizer for 2.5 minutes before being let to stand for 5 minutes .

2.3. Isolation and identification of pseudomonas species

Two separate petri dishes with Pseudomonas agar base (NutriSelect® Plus- Sigma-Aldrich P2102) supplemented with glycerol were uniformly dispersed with 0.1 ml of each sample homogenate. Purified and sub-cultured onto nutrient agar slopes, the suspicious colonies (blue-green or brown pigmentation, or fluorescence) were incubated at 37°C for 24 hours. The purified colonies were subjected to further morphological or microbiologic identification according to Krieg and Holt (1984).

2.3.1 Morphological examination

Microscopical examination and motility test were done according to APHA (1992) and McFadden (1976), respectively.

2.3.2 Biochemical identification

The purified Pseudomonas colonies were identified biochemically following Cruickshank et al. (1975) and Quinn et al. (2002). Moreover, pigment formation on nutrient agar (Collins and Lyne 1984) was done as the suspected colonies were inoculated on nutrient agar plates and incubated at 20-25 °C for 24 hours. The color of the media was observed and recorded.

2.4 Biofilm formation using crystal violet quantitative ELISA.

Each Pseudomonas spp. isolate was grown in trypticase soy broth (TSB; Himedia, India) for a whole night at 37 °C. Then, sterile 96-well polystyrene microtiter plates containing 195 µL of TSB were filled with 5 µL of cell suspension (Weinstein et al., 1985) Each test included 100 mL of uninoculated TSB in negative control wells. At 37 °C, the cells were cultured for 24 hours. Three gentle washings with 200 µl of phosphate-buffered saline (PBS) were performed on the wells. The wells were reverse-dried. Then, 125 µL of 0.1% crystal violet (Oxoid, UK) was used to stain the biofilm mass. The wells were gently cleansed three times with 200 µL of distilled water before being dried upside down. Wells were dried for 1 hour at 60 °C before the stain was solubilized in 200 µL of 30% acetic acid. The optical density of the wells was measured at 570 nm using a micro-ELISA auto reader (Sinothinker Microplate reader sk 202, China). The study was repeated three times in duplicate for each strain. An optical density of 0.240 was used to distinguish between species that generated biofilm and those that did not (Salih and AL-Ani 2013). Bacteria that formed weak biofilms had values greater than 0.120 but less than 0.240. When those strains' reading values were less than 0.120, it was determined that they did not form biofilms.

All the isolates were classified based on the adherence capabilities into the following categories: non-biofilm producers (OD ≤ ODc), weak biofilm producers (ODc< OD ≤ 2xODc), moderate biofilm producers (2ODc < OD ≤ 4xODc), and strong biofilm producers (4xODc < OD) (Stepanovic´ et al., 2007; Hamad et al., 2019).

3. RESULTS

It was evident from table (1) that the Pseudomonas species were recovered from bulk tank milk, Kareish cheese, Yoghurt, and Ice cream with an incidence of 36 %, 26%, 22%, and 16 %, respectively.

Table 1 The incidence of Pseudomonas species isolated from bulk tank milk and milk products (n= 50 of each)

Samples	No. of samples	No. of positive samples	% of positive samples
Bulk tank milk	50	18	%36
Kareish cheese	50	13	%26
Yoghurt	50	11	%22
Ice cream	50	8	%16
Total	200	50	%25

% was calculated according to the total number of samples

As seen in table (2) the incidence of *P. aeruginosa*, *P. fluorescence*, *P. putida*, and *P. diminuta* that were isolated from Bulk tank milk was 16%, 12%, 4%, and 4%, respectively. While the incidence of *P.* aeruginosa, *P.* fluorescence, *P. putida* and *P. diminuta* that were isolated from Kareish was 14%, 6%, 4%, and 2%, respectively. Furthermore, the incidence of *P. aeruginosa*, *P. fluorescence*, *P. putida*, and *P. diminuta* that were isolated from yoghurt was 12%, 6%, 0%, and 4%, respectively. The incidence of *P. aeruginosa*, *P. fluorescence*, *P. putida*, and *P. diminuta* that were isolated from ice cream was 12%, 2%, 2%, and 0%, respectively.

Table 2 The percent of identified pseudomonas species in milk and some dairy products

Pseudomonas strains	Bulk tank milk		Kareish cheese		Yoghurt		Ice cream	
	No	%	No	%	No	%	No	%
P. aeruginosa	8	16	7	14	6	12	6	12
P. fluorescence	6	12	3	6	3	6	1	2
P. putida	2	4	2	4	0	0	1	2
P. diminuta	2	4	1	2	2	4	-	-
Total	18	36	13	26	11	22	8	16

Fifteen Pseudomonas species isolates were evaluated for biofilm formation by using crystal violet staining method as seen in tables (3 and 4). *P. aeruginosa* has the ability for biofilm formation as 9 (33.4%) of the isolated strains were strong, 5 (18.5%) was moderate, 5 (18.5%) was weak, while 8 (29.6%) was non biofilm producer. Also, 6 (46.3%) of the isolated *P. fluorescence* were strong biofilm former while, 2 (15.4%) was moderate and 3 (23.1%) was weak, but 5 (15.4%) was a non-biofilm producer. Furthermore, 2 (40%) of *P. putida* was strong biofilm formation, 1(20%) was moderate, 1 (20%) weak and 1 (20%) was non biofilm producer. Moreover, 2 (40%) *P. diminuta* has a strong ability for biofilm formation, 1 (20%) was moderate and 2 (40%) was weak biofilm former.

Table 3 Biofilm forming ability of pseudomonas strains isolated from the examined samples.

Pseudomonas		Biofilm producer		Non biofilm producer	
Strains	Total	No	%	No	%
P. aeruginosa	27	19	70.4	8	29.6
P. fluorescence	13	11	84.6	2	15.4
P. putida	5	4	80.0	1	20.0
P. diminuta	5	5	100.0	0	0.0
Total	50	39	78	11	22

% is calculated in relation to no. of each isolated strain

Table 4 Degree of Biofilm forming ability of Pseudomonas strains isolated from the examined samples.

Pseudomonas		Strong		Moderate		Weak	
species	Total	No	%	No	%	No	%
P. aeruginosa	19	9	33.4	5	18.5	5	18.5
P. fluorescence	11	6	46.3	2	15.4	3	23.1
P. putida	4	2	40.0	1	20.0	1	20.0
P. diminuta	5	2	40.0	1	20.0	2	40.0
Total	39	19	48.7	9	23.07	11	28.2

% is calculated in relation to no. of each strain

4. DISCUSSION

Pseudomonas species pose a great danger to human health and animals, resulting in financial losses (Abd El-Ghany, 2021. Due to unclean manufacturing and handling procedures, they might be spread to consumers through fresh dairy products in particular (Quintieri et al., 2019).

The prevalence of pseudomonas species isolated from bulk tank milk and milk products in table (1) is nearly similar to results reported by Abou EL-Roos et al. (2013); Delphine et al. (2008) and Laura and Mauro (2007), while, higher than results have been recorded by Atia et al. (2022). The prevalence of Pseudomonas spp. varied depending on the sample type, with *P. aeruginosa* being the most prevalent strain, followed by *P. fluorescence*, *P. putida*, and *P. diminuta*. While *P. aeruginosa*, *P. fluorescence*, and *P. Putida* could be isolated from ice cream by 10%, 4%, and 2%, respectively (Table 2). Abdel hameed, A. (2019) isolated *P. aeruginosa*, and *P. fluorescence* in lower incidence from raw milk by 11.6% and 3.3%, respectively. Amin et al. (2015) found a higher incidence of *P. fluorescence* and *P. putida* isolated from raw milk, at 35.4% and 18.2%, respectively. Moreover, Atia et al. (2022) reported that *P.* aeruginos*a* was isolated from raw milk, kareish cheese, yoghurt, and ice cream in the incidence of 20 %, 16 %, 8%, and 8 %, respectively in the examined samples, while *P.* fluorescence was found in 28, 20, 12 and 8%, respectively.

An important step in the establishment of Pseudomonas spp. in dairy processing plants is the ability of these bacteria to adhere to solid surfaces, which is followed by the development of a well-organized bacterial biofilm community (CHIRKENA et al. 2019). It is also widely known that the change from planktonic to biofilm formation is a complicated process that occurs in response to modifications in environmental conditions (O' Toole et al., 2000).

One of the most frequent causes of Pseudomonas treatment failure is biofilm development. According to Watnick and Kolter (2000) the exopolysaccharide (EPS) in the biofilm structure is believed to be essential to the bacterium's ability to live.

The majority of *Pseudomonas* spp. strains isolated from milk and dairy products, with some variances linked to strain diversification, were found to be able to generate biofilm in microtiter plate wells.

Most isolated *P. aeruginosa* had the ability for biofilm formation as (33.4%) of the isolated strains was strong while, (18.5%) was moderate and weak, while (29.6%) was a non-biofilm producer (Tables 3&4). These results were lower to Aziz, et al. (2022) as 22 (62.8%) of *P. aeruginosa* that were isolated from milk was a strong biofilm producer while 13 (37.1%) was a non-biofilm producer. Research on the production of biofilms has accelerated due to the rise in the frequency of biofilm infections. With the aid of evolving technology, numerous in vitro and in vivo techniques based on biofilm infection in experimental animals are utilized nowadays to detect biofilm formation. Chiara et al. (2016) recorded 57/64 *Ps. fluorescens* strains isolated from milk and milk products formed biofilm. Additionally, Pseudomonas spp. strains isolated from milk, dairy products, and dairy plants were examined by Chiara et al., (2018) for their capacity to build biofilm on polystyrene surfaces and engage in various forms of motility. Out of 72 *Pseudomonas* spp. isolates, molecular analysis showed that *P. fluorescens* (50 isolates) was the most prevalent species, followed by *P. putida* (9), *P. koreensis* (4), *P. brenneri* (4), *P. aeruginosa* (2), *P. granadensis* (2), and *P. veronii* (1). These findings demonstrated that the Pseudomonas strains had more biofilm cells than the pathogens. According to a study by Lauer and de Souza (2019), *Pseudomonas fluorescens*, which was isolated from chilled raw buffalo milk, produced biofilms as the strains produced varied amounts of exopolysaccharide, biofilm, and proteolytic activity. Savaşan and Sezener (2022) determined biofilm formation in 9 (37.5%) of isolates. This result proved that the formation of biofilm was high in raw milk contaminated with *Ps. aeruginosa* strains. Also, Abd el Aziz (2017) revealed that 65.3% of raw milk samples were non-biofilm formers by Pseudomonas sp. while 24%, were weak biofilm formers, 9.3%, were moderate biofilm formers, 1.3% were strong biofilm formers. The biofilm production from cheese was 53.5% considered moderate biofilm former and 46.1% was considered high biofilm production (El-Hamshary et al., 2021).

Evaluation of the dangers posed by psychrotrophic biofilm formation to stop product spoiling at an early stage, Pseudomonas is crucial (Minghuiet al., 2023). The most likely places to find heat-sensitive *Pseudomonas* and *Listeria* species are in the pipelines and silos that hold milk before pasteurization (Sophie et al., 2012).

Overall, the capability of Pseudomonas species to develop biofilms varies depending on the strain and the conditions in which they are grown. Biofilm formation by Pseudomonas species in milk and dairy products is an important issue for food safety and quality.

5. CONCLUSIONS

The ability of the vast majority of Pseudomonas strains isolated from milk and dairy products to develop biofilm identified the potential public health danger for Pseudomonas species in dairy manufacturing.

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