Isolation and Identification of Biofilm-Forming Capability Bacteria in Raw Milk Collected from Individual Dairy Farms

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ABSTRACT

Biofilm formation is recognized as a hazard compromising product safety and raising the possibility of foodborne illness. Thus, it is regarded globally as an emerging public health risk. Various mechanisms contribute to the attachment process and growth of microbial colonies on surfaces in contact with milk during the dynamic process of biofilm formation. This study aims to determine whether bacterial strains are potent biofilm formers by assessing their capacity to form biofilms from raw milk samples collected from cow milk farms in the Egyptian province of Alexandria. A total of 29 samples were chosen randomly from the cow milk samples. Each of the single colonies had morphological characteristics, and their capacity to build biofilm was examined. Various bacterial strains that produce biofilms were identified as *Bacillus spp.*, *Escherichia coli*, *Salmonella spp.*, *Staphylococcus spp.*, *Listeria monocytogenes*, *Pseudomonas spp.*, *Shigella sp.*, *and Enterobacter spp.* Also, study the impact of using sodium hydroxide at different concentrations: T1 1%, T2 1.5%, T3 2%, and T4 2.5% for steel and rubber plates. These treatments were applied for 15, 30, 45, and 60 minutes at a temperature range of 70-80°C and used in the Clean-In-Place (CIP) simulation model. The results indicated that there was an increase in the bacterial counts in all treatments compared with the control sample. Also, results showed that a higher bacterial count was noticed in rubber plate treatments compared to steel plate samples. The higher the concentration of sodium hydroxide and the longer the duration, the lower the bacterial growth and total bacterial count.

Keywords: Biofilm Formation; Biofilm identification; Clean in place simulation.

INTRODUCTION

Naturally, biofilm formation is a significant part of microorganisms' cycle life. Biofilm production is often seen as a grave danger to health and safety. Biofilm formation is observed mostly inside the dairy industry, which poses a serious risk to product quality and safety and may result in foodborne illness. Because of this, the development of these biofilms is seen as a global public health emergency. The development process is dynamic and involves multiple steps, including attachment to contaminated surfaces, growth phases, and microbial colonization on touched surfaces. One of the main food sectors around the world, the dairy sector produces a vast variety of milk products that are perishable and semi-perishable. Microbiological guidelines are a crucial necessity to ensure the quality and safety of these goods (Mogha *et al*., 2014). Microbial attachment is impacted by several factors including hydrodynamics, substratum influences, and different cell surface properties (Donlan, 2002). Because they affect early cell adhesion, In the food processing sector, the physical characteristics of solid surfaces are essential for the production of biofilms. The solid surface's essential surface tension determines the bacterial adhesion. Bacterial adherence is enhanced by wet surfaces and high free energy. Hydrophilic surfaces see a higher number of cell attachments than hydrophobic ones. Furthermore, It was found that the hydrophilic portion of the border between hydrophilia and hydrophobia on the stainless steel's surface was the site of bacterial colonization (Bos *et al*., 2000). The rate at which bacteria attach to surfaces is also significantly influenced by surface conditioning. Depending on the concentration of milk present, a coating of organic compounds, like milk proteins or even Exopolysaccharides (EPS) produced by bacteria that, depending on the milk concentration, can either promote or prevent bacterial adhesion.

The physical and chemical characteristics of bacterial cell surfaces are important for active attachment. The hydrophobic characteristics of the bacterial cell surface are attributed to lipopolysaccharide (LPS), flagella, and fimbriae. Reducing the repellent power of contact between two surfaces, a hydrophobic surface is important. Cell appendages called fibrae have hydrophobic amino acid residues. They enhance the hydrophobicity of bacterial cell surfaces and facilitate adhesion even more. Fimbriae's primary job is to get through the first barrier of electrostatic repulsion that separates the cell from the substratum. On the surface of cells, flagella are a dynamic appendage that facilitates mobility. Therefore, rather than serving as adsorbents or

DOI: 10.21608/asejaiqjsae.2025.432799
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Received May 15, 2025, Accepted, June 12, 2025.

adhesives, the purpose of flagella is to transport bacteria to specific attachment sites (Karagulyan *et al.*, 2022).

The change in phenotype of planktonic cells into sessile forms is influenced by several environmental factors, such as pH, temperature, nutritional composition, and population features of bacteria. It was demonstrated that *L. monocytogenes* exhibited maximum adherence to surfaces made of stainless steel at 30 C and pH 7. Furthermore, Nutrient It has been demonstrated that the presence of carbohydrates stimulates the phosphate level at which biofilm development occurs, which is an optimal amount. Biofilm cannot form when there is little oxygen available because the bacteria cannot stick to the surface of the substrate (Anderson, 2012). Dairy producers are now very concerned about bacterial biofilms. Biofilms that grow on pipe interiors cause corrosion and blockage, particularly in designed systems. Because they are shielded by EPS from the sanitizer within the biofilm, biofilm on floors can improve hygiene challenges in places used for milk production (Geetha, 2011).

Food products may get contaminated by secondary sources due to microorganisms found in the food business, when milk and milk products stick to surfaces in dairy facilities that come into contact with food, biofilm can form and contaminate the milk and milk products (Flint *et al.,* 1997b and Sharma & Anand, 2002). Many bacterial genera, such as *Staphylococcus* and *Pseudomonas,* form multispecies bacterial biofilms that facilitate the adherence of other important pathogens, such as Listeria monocytogenes (Sasahara and Zottola, 1993). These microbes can survive on food processing equipment and exhibit heightened tolerance to environmental challenges when they are present in biofilms (Zottola & Sasahara, 1994 and Campanac *et al.,* 2002). The other significant dairy sector biofilm-forming genera are *Bacillus, Streptococcus, Listeria, Lactobacillus, Pseudomonas, Staphylococcus, Salmonella enterica* serovar Typhimurium and *Coronobacter sakazakii* etc. (Durango *et al*., 2004; Seifu *et al*., 2004 and Kandhai *et al*., 2010).

This investigation aims isolated and identify bacterial strains that could form biofilms in dairy farms using different ways such as microspic examined and study the features of bacterial growth on nutrient agar. Also, identification of bacterial isolates' homology sequences in GenBank using 16 sr RNA. Also, study effect of appling clean in place system by using different concentration of sodium hydroxide on biofilm bacterial growth.

MATERIAL AND METHODS

Nutrient Broth from TITAN BIOTECH LTD.BHIWADI-301019, Rajasthan, India.Nutrient Agar fromHiMedia Laboratories Pvt Ltd B/4-6, M.I.D.C, Nashik, India.Petri plates. All chemical was obtained from El-Nasr Company for chemicals, Egypt.

Isolation of bacteria, colony characteristics, and microscopic examination:

Twenty-nine swab samples were collected randomly from dairy milking units in some dairy farms in Behira and Alexandria Governorates, and from lines receiving raw milk in some dairy industry. Sterilized swabs were struck from these lines after full washing. Swab samples were kept in ice box until they arrived to the laboratory for examination. Peptone saline solution (0.85%) was used to serially dilute each sample. The enumerations were conducted in nutrient media. Plates were incubated under ideal temperature.

The isolation of bacteria was carried out according to Flint and Harley (1996); cultures were streaked on the media to obtain single colonies as described by Luong *et al.* (2003). The pure isolates were subcultured onto the nutrient agar plates and incubated at 37 °C for 48 hours before testing. Morphological characterization of the bacterial colonies was carried out depending on different features-their shape, size, color, margin, and elevation on the media. Cell morphologies of the isolates were observed using optical microscopes (Olympus BX51 Microscope 100x).

Identification of bacteria strain

Preidentification of isolates has been carried out by morphological characteristics, Gram stain, Catalase reaction and oxidase test (MacFaddin, 2000).

- **Examination of isolates for biofilm-forming capability**

DNA Extraction: DNA was extracted from the biological samples collected from dairy products using a standardized extraction protocol. Then, performing PCR using the 16S rRNA primer, the 16S rRNA gene region was amplified using polymerase chain reaction (PCR) with specific primers designed for the 16S rRNA gene. Isolating the DNA Fragment from the Gel and Purifying It: The amplified DNA fragment was separated from the agarose gel and purified to remove any contaminants. Conducting Sequencing: DNA sequencing was performed on the purified DNA fragments to determine their nucleotide sequences. Matching the Sequence with the Database: The obtained DNA sequences were compared with sequences in the database to identify the microbial isolates present in the dairy products (Weisburg *et al.,* 1991 and Antil *et al.,* 2023).

- **Extraction of DNA, PCR amplification, and 16S rRNA gene sequencing:**

The isolates that have biofilm-forming capability have been identified by 16S rRNA. DNA of the bacterial isolates was extracted by using the easy extraction kit BIO BASIC INC. (96-Well Plate Bacterial Genomic DNA Mini-Preps Kit). DNA concentration was conducted using Nanodrop and the determination of 1 % agarose gels stained with RedSafe. DNA stain was also used for the optical examination of DNA. The PCR amplification of the 16S rRNA gene was accomplished using a thermal cycler (Applied Biosystems 2720 ABI, Foster City, USA). For 16S rRNA gene amplification, forward primer (27F:5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer 1492R:5′-TACGGYTACCTTGTTACGACTT-3′ were used (Frank *et al.,* 2008). The PCR reaction mixture (25 µl) was composed of 50 ng of isolated DNA, 2 µl of primers mix (10 µM of each primer), 1.5 µl of (25 mM) $MgCl_2$, 5 µl of (5X) PCR buffer, 0.5 µl of (10 mM) dNTPs, 0.5 µl of (50 units/µl) GoTaq® Flexi DNA Polymerase, Promega, USA, Cat. No. M8297 and The volume was increased to 25. µl in the end by using nuclease-free water. The PCR amplification condition was initial denaturation for 3 min at 95°C, then 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 53°C, extension for 90 s at 72°C, followed by a final extension step for 7 min at 72 °C. The agarose gel (1 %) stained with RedSafeDNA stain was used to resolve PCR products. QIAquick PCR purification kit (QIAGEN Inc., USA) was used to purify PCR products following the manufacturer's protocol. Purified PCR products were sequenced with a forward 16S rRNA primer using Macrogene, Inc. (Seoul, South Korea). The partial 16S rDNA gene sequences of 16S rRNA were BLAST searched with the NCBI database (Altschul *et al.,* 1990).

Stimulate cleaning in place in the lab using different concentrations of sodium hydroxide at different times:

Stainless steel and/or rubber plates, along with various concentrations of sodium hydroxide, T1 1%, T2 1.5%, T3 2%, and T4 2.5% wt/wt for steel and rubber plates. These treatments were applied for durations of 15, 30, 45, and 60 minutes at a temperature range of 70-80 °C and were used in the Clean-In-Place (CIP) simulation model within dairy factories, as mentioned in Flint *et al.* (1997a). Previously mentioned strains were used to recontaminate sterilized steel and rubber plates. Each plate was treated with a different concentration of sodium hydroxide for varying durations. Total bacterial counts for each treatment were determined using nutrient agar media, and the results were compared to the control sample.

RESULTS AND DISCUSSION

Biofilms have been a significant issue for global healthcare professionals, industry, and researchers (Bakhtiari and Javadmakoei, 2017). Also, food pathogens are common and have been the main source of issues in the dairy sector. Thus, the threat posed by biofilm is transformed into an opportunity by the presence of dairy-originating bacteria (Guerrieri *et al.,* 2009; Furukawa, 2015 and Sadishkumar & Jeevaratnam, 2017).

Preidentification of bacteria cells using microscopic examination

In this study, a total of 29 samples were selected at random from cow milk farms in Alexandria and Behira Governorates. The characteristics of bacteria cells using microscopy appeared in rod-shaped and Gram-positive sizes ranging from 1 x 3-4 micrometers. Also, it appeared as straight or slightly curved slender bacilli with square ends singly or in short chains. The color was opaque white, clear white, and flat elevation. The strain's growth performance on nutrient agar is where it is evident that the colony that developed was medium-sized, dry, opaque, spherical, and gray-white in appearance. Also, a Gram-negative, rod-shaped, non-spore-forming, non-acid-fast, uniformly staining bacterium that belongs to the Enterobacteriaceae family is observed. It is one of the most prevalent pathogenic agents in avians (Barnes *et al.,* 2008).

Many bacteria have the ability to transition between their planktonic and biofilm forms, *E. coli* included. Bacteria need to form biofilms for a number of reasons. Firstly, the cells within biofilms are approximately 1000 times more resistant to water flow than those in planktonic forms (Jefferson, 2004). *E. coli* O157:H7 has demonstrated the capacity to adhere to, occupy, and create biofilms on a range of surfaces (Uhlich *et al.,* 2006).

The bacteria characteristics also were Gram-positive bacteria, long bacilli, and non-spore formers. *Lactobacillus* the ability of Rhamnosus to produce biofilms in vitro on an abiotic surface (polystyrene) is a trait that is heavily impacted by the circumstances present in the gastrointestinal environment and the culture medium utilized. Strong biofilm-forming abilities have also been demonstrated by *L. rhamnosus* strains isolated from dairy products, such as *L. rhamnosus* 183 (Lebeer *et al.,* 2007).

Also, there was A striking characteristic of the bacteria in the Enterobacteriaceae family is Salmonella. The organisms are negative to Gram stain and oxidase test, and they are motile (due to the presence of peritrichous flagella), rod-shaped, non-spore producing, and facultative anaerobes (Lertworapreecha *et al.,*

2013). Salmonella typically produces hydrogen sulfide; to produce hydrogen and carbon dioxide, it breaks down D-glucose, while nitrates are reduced to nitrites (Pui *et al.*, 2011).

Staphylococci are cocci-shaped, Gram-positive bacteria that tend to be arranged in clusters and are described as being like grapes. The genus Staphylococcus are part of the normal skin flora of animals and humans (Becker *et al.*, 2014). Listeria are gram-positive, non-spore-forming rods and short cocci (Metwally and Ali, 2014). Pseudomonas spp. are aerobic, gram-negative bacteria that are rod-shaped and non-spore-forming (Macfaddin, 2004).

Identification of bacteria cells using the characteristic of growth on nutrient medium

The bacteria's development colonizes features on nutrient agar during incubation indicated that *Bacillus sp*. created round, creamy yellow colonies in a nutrient agar medium following a 48-hour incubation period.

Bacillus sp. is a typical germ and represents a common rod-shaped Gram-positive germ. When cultured on ordinary nutrient agar, the structure of this bacteria's circular colony has jagged edges and is rough, opaque, fuzzy white, or slightly yellow (Ming *et al.*, 2008 and Bai *et al.*, 2013). Also, the features of *Escherichia coli* growth on nutrient agar. It was demonstrated by the growth of round, smooth, white to grayish-white colonies (Hossain *et al.*, 2021). Also, the characteristics of growth *Salmonella spp*. The medium-sized 2-3 mm in diameter, wet, off-white, with entire margins and smooth, convex surfaces (Wang, 2022).

Staphylococcus spp. is hemolytic, salt-tolerant, coagulase- and catalase-positive, and when cultivated on nutrient agar, it forms enormous golden-yellow colonies (Gnanamani *et al.*, 2017). *The Listeria monocytogenes* growth characteristics on nutrient agar are small bacteria (~1 mm), creamy white, and dome-shaped. Smooth With a regular edge (Jamali *et al.*, 2013). *Pseudomonas* produced circular, mucoid, smooth colonies on nutrient agar. The colony surface is typically smooth and shiny. The edges of the colonies are often irregular and spreading. Which gives the colonies a distinctive color. Common pigments include pyocyanin (blue-green), pyoverdin (yellow-green), and fluorescein (greenish-yellow fluorescent) (Hossain *et al.*, 2013).

The biochemical characteristics of isolated strains

The biochemical characteristics are summarized in Table (1). Rethus showed that *Bacillus sp.* was oxidase negative, catalase positive, aerobic or anaerobic facultatively, and an endospore spore former (Al-Saraireh *et al.*, 2015). While *Escherichia coli* is facultatively anaerobic, catalase positive, oxidase negative, and does not form spores (Madigan *et al.*, 2018 and Talaro & Chess, 2018). Furthermore, results revealed that *Salmonella spp.* is aerobic, catalase-positive, oxidase-negative, and non-spore-forming. They are commonly associated with foodborne illnesses (Brenner *et al.*, 2000). *Staphylococcus spp.* are facultative anaerobes, catalase-positive, oxidase-positive, and non-spore-forming. Includes pathogenic strains like *Staphylococcus aureus* (Willey *et al.*, 2017). Additionally, *Listeria monocytogenes* is a facultative anaerobe, catalase-positive, oxidase-negative, and non-spore-forming (Swaminathan and Gerner-Smidt, 2007). *Pseudomonas spp.* ranges from aerobic to facultatively anaerobic, catalase-positive, oxidase-positive, and non-spore-forming known for environmental versatility and biofilm formation (Stover *et al.*, 2000 and Moore *et al.*, 2006).

Table 1. The biochemical characteristics of bacterial isolates

	Bacteria strains		Catalase	Oxidase	Spor former
D	*Bacillus spp.*	aerobic or facultatively anaerobic	+	-	Endospore former
E	*Escherichia coli*	facultative anaerobic	+	-	Non-spore former
F	*Salmonella spp.*	Aerobic	+	-	Non-spore former
B	*Staphylococcus spp.*	facultative anaerobic	+	+	Non-spore former
C	*Listeria monocytogenes*	A facultative anaerobic	+	-	Non-spore former
A	*Pseudomonas spp.*	aerobic–facultatively anaerobic	+	+	Non-spore former

Identification of bacterial isolates' homology sequences to in GenBank

Using the 16S rRNA gene sequence to study the phylogeny and taxonomy of bacteria is the most significant tool in identifying bacteria, by far the most common housekeeping genetic marker (Patel, 2001). Identification of isolates as strong biofilm formers by sequencing 16S rRNA. The sequences of the 16S rRNA gene of three isolates as strong biofilm formers were presented in Figures (2, 3, 4). Results of the homology search of the 16S rRNA gene sequence of the selected isolate in GenBank were presented in Table (2).

The results showed that 16SrRNA gene sequences have been helpful in genus-level phylogenetic analyses; their use in closely related species groupings, like *Bacillus*, hindered the resolution of strain and species relationships. For taxonomy categorization, it has been shown that it has a 92.65% resemblance to *Bacillus pumilus*, as illustrated in Figure (3). Also, the isolated sample showed different similarity levels (92.66-92.88%) when compared with reference strain sequences in GenBank, whereas these results indicated the isolate belonged to species *Enterobacter sp*. and *Enterobacter hormaechei.* The results showed the phylogenetic relatedness of *Enterobacter spp.* to each other and other related genera constructed. The sequence alignment of 16SrDNA genes of isolated *Enterobacter* spp. and some other related species was performed as represented in Figure (2).

Also, the nucleotide sequence of the 16S rRNA gene from a *Shigella sp.* isolate is depicted in Figure (4). The sequence plays a crucial role in identifying and classifying bacterial species due to its highly conserved nature across different organisms. The isolated sample showed similarity levels (93.13%) with *Shigella sp*. CH-38 when compared with reference strain sequences in GenBank.

TGGAGTGCGGCAGCTACACATGCAGTCGACGGTAACAGGAAGCAGCTTGC
TGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCT
GATGGAGGGGGATAACTACTGGAAACGGTAACTAATACCGCATAACGTCG
CAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAG
ATGGGATTAGCTAGTACGTGGGGTAACGGCTCACCTAGGCCACGATCCCT
AGCTGGTCTGAAAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT
GATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGTTTGTAAAGTACT
TTCAGCGGGGAGGAAGGGGTTTACGCTAATAACCTTCTCATTTCACGTTT
CCCCCTCATTAACGTACGGATACCTCCGTGCCTTTTCCATCAGTAATACG
GGTGGTGCAAGCGTTAACCTCAATTACTGGGCGGTCGCTCACCCCTCCTT
GTTTGTCCTTGCCCTATTTTCAATCCCCTGCCTCCTCCTTTGGTCCTCTG
TTCTGGGTTGTTGATCGTGAGTCCTGTTTGCGGCCTCTAATGCCTTTTTT
CTGTCTCTTTGGCTTTTGGC

Fig. 1. The sequence of the 16S rRNA gene *of Enterobacter sp.* isolate

TTGCCAGTGGCGGGTGCTATACATGCAGTCGAGCGGACAGAAGGGAGCTT
GCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCC
TGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCC
TTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGGTGGCACTTACAG
AGGGACCCGCGGCGTATTAGCTACTTGGAGATGGAACTGCTCCCCATGGC
GACGATGCGTATTTTACCTGAGCGGGTGAGCGGCCACACTGGGTCTGGAA
CTCGGTCCAGACCCTAACGTAGGTTTGTTAGGGAAGCTGCTATTACGGCT
ATCTGATTACTTCATTCTCTTCCTGTTTTAGCTGGAGCTATCACTATCTT
ATTAACTGACCGAAACTTAAATACATCTTTCTTTGATCCAATCCGAGGAG
GAGATTCACTTTTATACCAGCACTTATTCTGATTCTTTGGACACCCAGAA
CTTTATATTTTATTTTACCCGGATTTGAAATAATTTCTCATATGATTACT

Fig. 2. The sequence of the 16S rRNA gene of,*Bacilluspumilus* isolate

GTTTTGTTTTTGGGATGCGGCAGCTACCATGCAAGTCGACGGTAACAGGA
AGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGG
GAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCG
CATAATGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTAGCCATCGG
ATGTGGCCTGATGGGATTAGCAATGAGGTGCTGTAAAGGCTCACACCCGC
CACTATCCCTAGCTGGGCTGAGTCGACTGCTACTGCCCTTGGATTTCTTG
CCCGGGCCTGATCCTTCCTGACGCGTCCGTTGGGACCCTGCCATGTGGCT
TTTAGCTTTCGTTTGATCTATGATCTGTCATATTTGTCCCTTACCGTTCT
GTACACTATCCGGTGGCTCTGTTTCCGGGCATATCTCGGCCAAACGCGCC
GCTTGCCTTTTCTCCTAGGTTCCCGGCGTCCTCCCGTGTTTCTCTACCTC
CTTCTACTCTCTGAGCTTCAGGCTTTCTCCTTTTATTCCTGCTGCTATCT
CCTTCCTCCTCCTTCCGCTTCGGTTCTTCCCCTTTTAGCACCCTCTGTTG
CTTTCTGTCCTAGGCTGCCTGCTTCGTATTCCTGTCCTCTCCCTCCTTCC
TGAGCTTGGTTTGTTTTCCTAAGCATTCCTTCGTGTGCTTGTCCGTGTTT
GTCTTTTCTTTCACTTGGTTTGCTTTTTTTGCTTCACGATTCTTTTCGGT
TTGTTGGTCCTTTTC

Fig. 3. The sequence of the 16S rRNA gene of the *Shigella sp* .isolate

Table 2. Bacterial isolates' homology sequences o in GenBank

Bacterial isolates	**Scientific Name**	Percentage Identity (%)	Accession number
1	*Enterobacter sp.*	92.80%	MK209686.1
	Enterobacte rhormaechei	92.66%	
2	*Bacillus pumilus*	92.65%	JX315323.1
3	*Shigella sp*. CH-38	93.13%	KR148988.1

The effect of applying a clean-in-place system by using different concentrations of sodium hydroxide at different durations

Data in Tables (2 and 3) revealed the effect of applying four different sodium hydroxide concentrations: 1%, 1.5%, 2%, and 2.5% for steel and rubber plates. These treatments were applied for durations of 15, 30, 45, and 60 minutes at a temperature range of 70-80°C on the viability of biofilm bacteria strains A: *Pseudomonas spp.; B: Staphylococcus spp.; C: Listeria monocytogenes; D: Bacillus spp.; E: Staphylococcus spp.; F: Salmonella spp.* these bacterial species had previously been isolated from the milk farm and have been generally reported to foul dairy manufacturing plants. The results indicated that there was an increase in the bacterial counts in all treatments compared with the control sample. Also, results showed that a higher bacterial count was noticed in rubber plate treatments compared to steel plate samples. This may be due to the variability of resistance strains, which depends on the treatment applied with sodium hydroxide concentration and varying time durations. (The higher the concentration of sodium hydroxide and the longer duration resulted in lower bacterial growth and total bacterial count). The most common and aggressive caustic cleaner is sodium hydroxide (NaOH), which is typically used in 1–5 % wt/wt concentrations for plate-type and tubular heat exchangers, and other heavily soiled surfaces, and 1–2 %wt/wt for general use (Flint *et al.,* 1997a). The primary role of the caustic (alkali) wash step is the removal of proteins and carbohydrates (Chisti, 1999). Increasing the effectiveness of the caustic step may reduce the amount of nitric acid required and the need to use a sanitizer. To enhance cleaning effectiveness, caustic blends and caustic additives have been developed that contain surfactants, emulsifying agents, chelating compounds, and complexing agents. The results also illustrated that the bacterial count obtained from rubber plates was higher than from steel plates. This was more pronounced in the C: (*Listeria monocytogenes*), which had a total count of 0.7 log cfu/ml in rubber plates, while the count of bacteria in steel plates of the same sample was not detected.

Alkali detergents which have a pH higher than 7 are commonly used in the dairy industry because they saponify fat and convert the fat to soap, and can be removed with water. These usually consist of sodium hydroxide (caustic soda) (Dairy Practice Council, 1993). Milk stones in pipelines are removed by hot alkali (caustic soda) assisted by wetting agents that break up the protein into water-soluble units. Typically, 0.5 – 2 % wt/wt caustic soda has been used at temperatures of up to 85°C. For highly fouled surfaces. Caustic soda is a very strong alkali and is a commonly used material in formulating detergents for use in CIP and other mechanical means of cleaning. It exhibits excellent removal of proteinaceous soils and fatty oils by saponification (Tamime, 2008). The most common and

aggressive alkali cleaner is sodium hydroxide (NaOH). It is typically used in concentrations between 0.15 and 1.0 % at temperatures in the range of 70–80 °C for 10 to 30 min. However, for heavily soiled surfaces, especially those containing burnt-on protein, such as found in plate-type and tubular heat exchangers, concentrations up to 5 % are used (Bremer and Seale, 2010). The effectiveness of cleaning of detergents improves as temperature is increased (Reinemann *et al.,* 2003), and lower temperatures can be associated with more variability in farm milk TBC (Bava *et al.,* 2009). The presence of these species in biofilms may be due to of the heat treatment that the milk undergoes.

Biofilm formation enables bacteria to adhere to both biotic and abiotic surfaces, providing protection against biocides—including disinfectants and antibiotics—and allowing them to evade the immune responses of animal hosts. Compared to planktonic (free-floating) cells, biofilm-associated bacteria are significantly more resistant, making them a major concern in the dairy industry. These bacteria can cause clinical infections in livestock, such as mastitis, and are capable of colonizing milking equipment and processing surfaces, ultimately compromising the quality and safety of dairy products. A wide range of bacterial species commonly isolated from dairy environments possess biofilm-forming abilities. Notable examples include *Staphylococcus aureus* and other *staphylococci* associated with intramammary infections, as well as *Bacillus spp*., *Listeria* monocytogenes, and *Pseudomonas spp*., which contribute to product spoilage and foodborne illnesses. The economic impact of biofilm contamination in dairy production is considerable, prompting continuous efforts to develop effective antibiofilm strategies. In recent years, there has been growing interest in natural biocides, which may enhance the efficacy of traditional disinfectants or antibiotics while reducing environmental impact (Goetz *et al.,* 2024).

Table 2. The effect of sodium hydroxide (NaOH) at different concentrations and exposure durations on the growth (log cfu/ml) of biofilm-forming bacteria isolated from milk on rubber plates

Treatment	Time	Biofilm samples							
		A	B	C	D	E	F	Min,	Max.
C	15 min	2.50	2.77	2.57	2.55	2.49	2.54	2.49	2.77
	30 min	2.55	2.76	2.57	2.49	2.44	2.51	2.44	2.76
	45 min	2.31	2.73	2.59	2.49	2.41	2.48	2.41	2.73
	60 min	2.49	2.71	2.48	2.46	2.38	2.46	2.38	2.71
T1	15 min	2.42	2.51	2.39	1.88	1.79	2.26	1.79	2.51
	30 min	2.35	2.50	2.32	1.84	1.81	2.17	1.81	2.50
	45 min	2.17	2.16	2.13	1.50	1.54	1.95	1.50	2.17
	60 min	2.02	1.80	1.83	ND	ND	1.67	ND	2.02
T2	15 min	2.27	2.50	2.30	1.30	1.35	2.08	1.30	2.50
	30 min	2.08	2.47	2.22	ND	ND	1.88	ND	2.47
	45 min	1.99	2.26	2.00	ND	ND	1.76	ND	2.26
	60 min	1.54	1.47	1.70	ND	ND	1.46	ND	1.70
T3	15 min	1.95	2.21	2.28	ND	ND	1.83	ND	2.28
	30 min	1.91	1.95	2.19	ND	ND	1.65	ND	2.19
	45 min	1.89	ND	1.94	ND	ND	1.23	ND	1.94
	60 min	1.30	ND	1.30	ND	ND	0.78	ND	1.30
T4	15 min	ND	ND	0.70	ND	ND	ND	ND	0.70
	30 min	ND	ND	ND	ND	ND	ND	ND	ND
	45 min	ND	ND	ND	ND	ND	ND	ND	ND
	60 min	ND	ND	ND	ND	ND	ND	ND	ND

Data: C: without NaOH; T1:NaoH 1.0; T2: NaoH 1.5; T3: NaoH 2.0; T4: NaoH 2.5. A: *Pseudomonas spp.; B: Staphylococcus spp.; C: Listeria monocytogenes; D: Bacillus spp.; E: Staphylococcus spp.; F: Salmonella spp.*

Table 3. The effect of sodium hydroxide (NaOH) at different concentrations and exposure durations on the growth (log cfu/ml)of biofilm-forming bacteria isolated from milk on steel plates

Treatment	Time	Biofilm samples							
		A	B	C	D	E	F	Min	Mix
Control	15 min	2.52	2.70	2.54	2.48	2.45	2.48	2.45	2.70
	30 min	2.47	2.75	2.56	2.36	2.33	2.43	2.33	2.75
	45 min	2..46	2.63	2.51	2.32	2.30	2.49	2.30	2.63
	60 min	2.44	2.55	2.49	2.33	2.31	2.48	2.31	2.55
T1	15 min	2.30	2.41	2.11	1.70	1.68	1.82	1.68	2.40
	30 min	2.10	2.44	1.99	1.54	1.52	1.72	1.52	2.44
	45 min	1.86	2.30	1.48	1.18	1.06	1.08	1.06	2.30
	60 min	1.65	1.70	ND	ND	ND	0.90	ND	1.70
T2	15 min	1.85	2.50	2.03	0.50	0.50	1.65	0.50	2.50
	30 min	1.30	2.34	1.90	ND	ND	0.60	ND	2.34
	45 min	1.15	1.96	ND	ND	ND	ND	ND	1.96
	60 min	ND	1.50	ND	ND	ND	ND	ND	1.50
T3	15 min	1.60	2.06	1.99	ND	ND	ND	ND	2.06
	30 min	1.00	1.99	1.79	ND	ND	ND	ND	1.99
	45 min	0.90	1.70	ND	ND	ND	ND	ND	1.70
	60 min	ND	0.90	ND	ND	ND	ND	ND	0.90
T4	15 min	ND	ND	ND	ND	ND	ND	ND	ND
	30 min	ND	ND	ND	ND	ND	ND	ND	ND
	45 min	ND	ND	ND	ND	ND	ND	ND	ND
	60 min	ND	ND	ND	ND	ND	ND	ND	ND

Data: C: without NaOH;T1:NaoH 1.0; T2: NaoH 1.5; T3: NaoH 2.0; T4: NaoH 2.5; ND: not detected. A: *Pseudomonas spp.; B: Staphylococcus spp.; C: Listeria monocytogenes; D: Bacillus spp.; E: Staphylococcus spp.; F: Salmonella spp.*

CONCLUSION

Biofilm formation in the dairy industry is always noted as a threat that affects the product safety and thereby results in foodborne illness. In the current study, six bacterial isolates were found isolated from dairy samples. All isolates were diagnosed based on colony morphology and biochemical characteristics. Further study of selected bacterial isolates of three strains is performed by 16S rDNA gene sequencing to determine their species.

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الملخص العربي

عزل وتحديد البكتيريا القادرة على تكوين الأغشية الحيوية في الحليب الخام المجمع من مزارع الألبان الفردية

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يُعتبر تكوّن الأغشية الحيوية خطرًا يُهدد سلامة المنتج ويزيد من احتمالية الإصابة بالأمراض المنقولة بالغذاء خاصة فى مجال الألبان. وبالتالي، يُنظر إليه عالميًا على أنه خطر ناشئ على الصحة العامة. تساهم آليات مختلفة في عملية التصاق المستعمرات الميكروبية وتطورها ونموها على الأسطح الملامسة للحليب أثناء العملية الديناميكية لتكوّن الأغشية الحيوية. تهدف الدراسة إلى تحديد ما إذا كانت السلالات البكتيرية مُكوّنة للأغشية الحيوية من خلال تقييم قدرتها على تكوين أغشية حيوية من عينات الحليب الخام التي جُمعت من مزارع حليب الأبقار في محافظة الإسكندرية. تم اختيار ٢٩ عينة عشوائيًا من عينات حليب الأبقار. تميزت كل مستعمرة بخصائص مورفولوجية، وتم فحص قدرتها على تكوين الأغشية الحيوية. تم تحديد سلالات بكتيرية مختلفة تُنتج الأغشية الحيوية مثل:

Bacillus spp., Escherichia coli, Salmonella spp., Staphylococcus spp., Listeria monocytogenes, Pseudomonas spp., Shigella sp., and Enterobacter spp.

كذلك، تم دراسة تأثير استخدام هيدروكسيد الصوديوم بتركيزات مختلفة، كما يلي: T1 1%، T2 1.5%، T3 2%، وT4 2.5%، على صفائح من الاستانلس ستيل ورقائق المطاط. طُبِّقَت هذه المعاملات لفترات ١٥، ٣٠، ٤٥، و٦٠ دقيقة عند درجات حرارة تتراوح بين ٧٠ و٨٠ درجة مئوية، واستُخدمت في نموذج محاكاة التنظيف في الموقع بنظام (CIP). أشارت النتائج إلى زيادة في أعداد البكتيريا في جميع المعالجات مقارنةً بعينة الكنترول . كما أظهرت النتائج ارتفاعًا في أعداد البكتيريا في معاملات رقائق المطاط مقارنةً بعينات صفائح الاستانلس ستيل. كلما ارتفع تركيز هيدروكسيد الصوديوم وطالت مدته، انخفض نمو البكتيريا والعدد الإجمالي للبكتيريا.

الكلمات المفتاحية: تكوين الأغشية الحيوية، التعرف على الأغشية الحيوية، محاكاة التنظيف في الموقع.