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Dry Biofilm Formation, Mono-and Dual-attachment, on Plastic and Galvanized Surfaces by *Salmonella typhimurium* and *Staphylococcus aureus* Isolated from Poultry House

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ABSTRACT

Biofilm significantly contributes to disease transmission on poultry farms by increasing microbial resistance to antimicrobials and disinfectants. Three Salmonella spp. and three *Staphylococcus aureus* (*S. aureus*) species from poultry farm environments were tested for biofilm formation using a tissue culture plate assay. All strains formed biofilms with varying densities. It was found that *S. typhimurium* strains were positive for the gcpA, csgD, and adrA genes, while S. aureus isolates were positive for the icaD, eno, and fnbA genes. The biofilm formation by mono- and dual-bacteria on plastic (PL) and galvanized steel (GS) coupons was tested in a lab setting similar to a chicken farm. The surface type impacted biofilm density; the S. aureus count on PL was 8.11±0.03 log10 CFU/coupon, and the *S. typhimurium* count was 1.54±0.02 log10 CFU/coupon. On GS, S. aureus count was 4.22 log10 CFU/coupon on day 9, then decreased to 3.81±0.06 log10 CFU/coupon, and *S. typhimurium* reached 3.08 log10 CFU/coupon before decreasing to 1.86±0.06 log10 CFU/coupon. The generated biofilms were identified using SEM. In conclusion, the bacteria formed more mono- and dual-species biofilm on PL than on GS under dry conditions. Species, serotypes, surfaces, and environment influence biofilm formation.

Key words: Dry biofilm, *S. typhimurium*, *S. aureus*, Galvanized Steel and Poultry house

INTRODUCTION

Poultry farming is a highly efficient animal husbandry method that offers a consistent protein source, especially in developing nations (Vaarst et al. 2015). *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus* spp. are well-established microbes that form biofilms on chicken farms (Mondal 2022). Despite disinfection efforts in the poultry house environment, numerous visible bacteria remained and could form biofilms. A study conducted by Ibrahim et al. (2023) revealed that 45% of bacteria from chicken homes formed a moderate biofilm, potentially endangering the animals' health during subsequent production cycles.

Salmonella enterica and *S. aureus* are harmful bacteria that cause foodborne illnesses due to their capacity to stick and dissociate from biofilm on various surfaces (Ibrahim et al. 2022; Samy et al. 2022). Bacterial biofilm, which forms on surfaces like feed troughs, walls, and pipes, is believed to enhance the resilience and growth of microbes like *Salmonella* (Maffei et al. 2017). Many bacteria commonly form biofilm, but the specificity depends on the species, isolate's serotype, and origin (Lamas et al. 2016). Previous research has investigated the development of biofilms under various temperatures and surface conditions (Cho et al. 2022; Obe et al. 2022). Investigating the impact of surface material on mixed biofilm growth is critical, as it affects biofilm matrix density and cell adhesion. Certain surfaces can promote biofilms' formation, thereby elevating contamination risk by releasing viable bacteria (Thames et al. 2023). Understanding the development capacity of microbial biofilms on different surfaces is crucial for comprehending adhesion mechanisms and preventing bacterial colonization. The World Health Organization has recognized *Salmonella* enterica serotype *typhimurium* as a significant global foodborne pathogen

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(WHO 2015). *Salmonella* is prevalent in poultry, and Porwollik et al. (2004) reported that serovars *typhimurium* and *Newport* are responsible for 41.8% of animal veterinary illnesses. Various vectors, including rodents, wild animals, insects and even people, can transmit *Salmonella* infection in chicken flocks (Khalefa et al. 2021; Du et al. 2023). According to Merino et al. (2019), *salmonella* uses biofilm to survive and acquire new genes that improve reproduction and survival.

S. aureus exhibits remarkable adaptability and the ability to adjust its mRNA transcription levels in response to various environmental conditions, as demonstrated by Balasubramanian et al. (2017). Over time, bacteria attach to hydrophilic or hydrophobic surfaces and develop disinfectant, antimicrobial, and antibiotic resistance, according to Iñiguez-Moreno et al. (2017). They damage farmland and infect humans, animals, and birds. Microorganisms' biofilm development capacity can be assessed using various techniques such as roll plates, tubes, microtiter assays, PCR assays, mass spectrometry, and biological tests. This study aims to investigate how common materials' surfaces, like galvanized steel and plastic, influence the formation of biofilm by mono- or dual-species *S. typhimurium* and *S. aureus* in dry conditions. The evaluation study, which lasted 12 days, used methods such as direct plating and scanning electron microscopy to detect and quantify the produced biofilms.

MATERIALS AND METHODS

Ethical approval

The Research Ethical Committee of Cairo University's Faculty of Veterinary Medicine, Egypt, authorized the study's protocol and techniques (Vet CU 25122023841; approval date: 25-12-2023).

Bacterial isolation

Three strains of *S. aureus* and three *Salmonella* spp. strains were recovered from poultry houses' environmental samples. A specific Salmonella-Shigella (SS) agar medium (Oxoid) was used to separate the strains of *Salmonella*. In contrast, Mannitol Salt Agar (Oxoid) was used to separate and identify the *S. aureus* strains. Subsequently, isolates have been identified according to morphological and biochemical features, as Cruikshank et al. (1974) reported. They were then verified by serological testing for *Salmonella* spp. and the staphylococci test kit (BioMérieux, France).

Biofilm quantification by tissue culture plate assay

The biofilm-forming potential of all isolates was quantitatively investigated using the Tissue Culture Plate (TCP) method (Stepanović et al. 2004). The optical density (OD) was quantified using a micro-ELISA auto-reader, and the strains were classified as non-adherent, weakly adherent, moderately adherent, and strongly adherent. There are three replicates for each strain.

Molecular identification of biofilm genes

Bacterial DNA was extracted from original cultures using the QIAamp DNA kit (Qiagen, German) following the manufacturer's instructions. The primer sequences and PCR settings used to identify the biofilm genes of *Salmonella* and *S. aureus* are provided in Table 1. The *invA* gene, peculiar to *Salmonella* as a species was chosen based on earlier verification (Khalefa et al. 2021). The identification of the *S. aureus* strains was verified using 23S rDNA, as described by Straub et al. (1999). The PCR was performed using a thermal cycler (Applied Biosystems 2720), and the resulting products of PCR were observed on a 1.5% agarose gel.

Biofilm growth by *S. aureus* and *S. typhimurium* upon tested surfaces

Bacterial strains

Based on their strong biofilm formation assays obtained through the tissue culture plate method and identification of biofilm genes, two isolates (*S. typhimurium* and *S. aureus* were selected for biofilm formation on plastic and galvanized steel surfaces. Bacterial cultures were created by incubating each purified strain in sterile Tryptic Soy broth for 18–20h. The bacterial count of the acquired cultures was adjusted to 0.5 McFarland (10^8 CFU/mL).

Surface materials preparation

This study examined two surface materials: plastic (PL) and galvanized steel (GS) in the form of coupons in dimensions ($2\times2\times1cm^3$). Both surface materials were chosen based on their intended functions in the chicken house. Before the experiment, we prepared the coupons according to Laban and Hamoud's (2019) protocol. In conclusion, coupons were washed with 70% ethanol before being used, dried for two hours at 60°C, and then autoclaved for 15min at 121°C.

Table 1: Biofilm Primers sequences used in our study (target genes, and amplicon sizes)

Target gene	Oligonucleotide sequence (5´- 3´)	Final extension	Amplified segment (bp)	References
adrA	ATGTTCCCAAAAATAATGAA	72˚C	1113	Bhowmick et al. (2011)
	TCATGCCGCCACTTCGGTGC	12min		
gcpA	CTATTTCTTTTCCCGCTCCT	72˚C	1713	
	GTGCCGCACGAAACACTGTT	12min		
csgD	TTACCGCCTGAGATTATCGT	72˚C	651	
	ATGTTTAATGAAGTCCATAG	7min		
icaD	AAA CGTAAGAGAGGTGG	72˚C	381	Ciftci et al. (2009)
	GGCAATATGATCAAGATA	10min		
fnbA	CATAAATTGGGAGCAGCATCA	72˚C	127	Vancraeynest et al. (2004)
	ATCAGCAGCTGAATTCCCATT	7min		
eno	ACGTGCAGCAGCTGACT	72˚C	205	Tristan et al. (2003)
	CAACAGCATYCTTCAGTACCTTC	7min		

Development of mono- and dual-species biofilms under dry conditions

This experiment was conducted under dry conditions, simulated as poultry house's environment at 28°C with 50–55% relative humidity, according to the Christine et al. (2023) method. Eight sterile coupons for each species were in groups inside sterile petri dishes. To test the production of mono- and dual-species biofilms, coupons were inoculated with bacterial culture suspensions. For 12 days, the coupons were dried and kept at 28°C. To keep the biofilm cells nourished and hydrated, a sterile saline solution enhanced with 20% Brain Heart Infusion broth was utilized daily.

Counting of biofilm cells

This experiment involved enumerating biofilm formed on various days (1st, 3rd, 7th, 9th, and 12th days) to confirm the formation and survival of biofilm on surfaces. Using sterile forceps, coupons were randomly selected from different groups and quietly rinsed by water, then the biofilm cells were removed from coupon surfaces using cotton swabs wetted in sterile saline solution. Glass tubes with five milliliters of sterile saline within held the swabs. The tubes were agitated in a vortex for a duration of 30s to extract and uniformly disperse the bacterial cells present in the biofilm. The cell suspensions from the original samples were diluted in a series and then transferred to modified tryptic soy Agar plates for the formation of biofilm consisting of a single species or two species. The plates were placed in a controlled environment and kept at a temperature of 37°C for 24 hours. The number of living cells was measured and reported as the logarithm of colony-forming units per coupon.

Detection of biofilm formation by scanning electron microscopy

SEM was utilized to verify cellular adhesion and track the growth of extracellular matrix in biofilms. The scanning was performed at the Applied Centre for Entomonematodes, Cairo University, using a JEOL GM 5200 microscope and gold-palladium sputter coating (Shamseldean and Platzer 1989)

Statistical analysis

Using the SPSS program, we performed statistical analyses (SPSS PASW statistic 18). Using analysis of variance (ANOVA), we investigated the effects of different surface materials, time, mono and dual-species of bacteria, and bacterium species on biofilm formation. A Tukey test was conducted with a significance level of $P<0.05$.

RESULTS

The study identified *Salmonella* spp. on S-S agar media, and serology identified the isolates as *Salmonella enterica subspecies diarizionea*, *S. enteritidis*, and *S. typhimurium* Biochemical analysis identified the staphylococci as Gramme-positive cocci, revealing oxidase-negative, catalase-positive, and coagulase-positive staphylococci.

The TCP method detected biofilm development in all *Salmonella* and *S. aureus* isolates, with inter-group variations statistically significant among *Salmonella* spp. As shown in Fig. 1, *S. typhimurium* had the highest mean OD of 0.88±.012, while no significant difference was found among *Staphylococcus* strains. Two *Salmonella* strains (66.6%) showed moderate adherence, while only *S. typhimurium* showed strong adherence. All three *S. aureus* isolates (100%) showed moderate adherence.

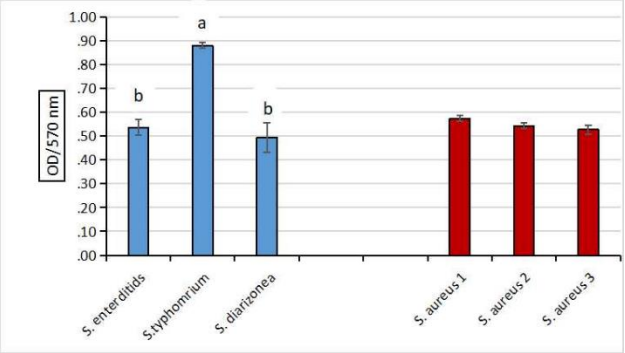

Fig. 1: Using the TCP approach, the optical density at 570nm was measured to determine the biomass of biofilms that were 24 hours old. The figure displays the average±SE of three separate experiments. Different letters on bars between same bacterial species isolates indicate significant difference ($P<0.05$).

PCR analysis in Table 2 revealed all *Salmonella* strains had positive *csgD* genes, with only two strains (*S. enteritidis* and *S. typhimurium*) testing positive for the *adrA* and *gcpA* genes. All *S. aureus* isolates, however, included the *icaD* and *eno* genes.

Table 2: The variation in biofilm gene patterns among specific isolates of *Salmonella* spp. and *S. aureus*

Isolate		No. of isolates	Biomarker biofilm genes
Salmonella	*S.enteritidis*	1	*adrA, csgD , gcpA*
	S. typhimurium	1	*adrA, csgD , gcpA*
	S. diarizionea	1	*csgD*
S.aureus	*S. aureus* 1	1	*icaD, eno , fnbA*
	S. aureus 2	1	*icaD, eno , fnbA*
	S. aureus 3	1	*icaD, eno*

The cellular density of mono- and dual-species biofilms was shown to be influenced by the surface type, as illustrated in Fig. 2-4. *S. aureus* exhibited a higher preference for plastic surfaces. Still, the biofilm of *S. typhimurium* was less inclined to form on GS surfaces. The density of *S. aureus* remained consistent on both surfaces, although the density of *S. typhimurium* decreased. The study also discovered that the density of dual-species biofilms remained consistent. The study indicates that the type of surface can substantially impact the development of biofilms.

By SEM picture analysis as in Fig. 5, the experiment showed that microorganisms rapidly colonized on PL and GS surfaces, with biofilm formation observed by single- and dual-species species.

DISCUSSION

A biofilm is a group of bacteria that attach to a surface using a matrix they manufacture themselves. Biofilms are more resistant to antimicrobials, biocides, and the host immune system than individual microbes floating freely in

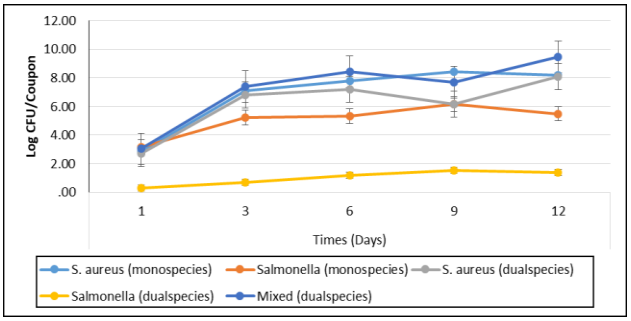

Fig. 2: Showing the average development of biofilm (log CFU/coupon) on plastic coupons by strains of *S. typhimurium* and *S. aureus*, either mono- or dual-species, under dry conditions after a 12-day incubation period at 28ºC.

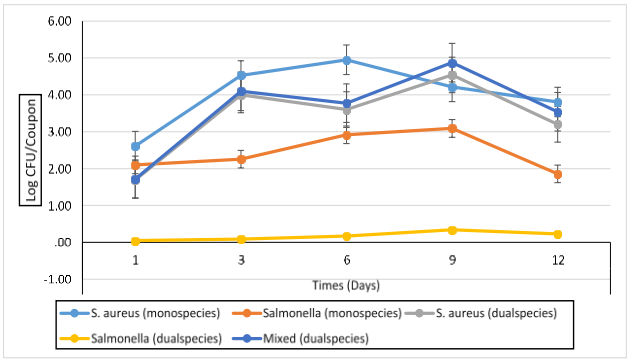

Fig. 3: Showing the average development of biofilm (log CFU/coupon) on galvanized steel coupons by strains of *S. typhimurium* and *S. aureus*, either mono- or dual-species, under dry conditions after a 12-day incubation period at 28ºC.

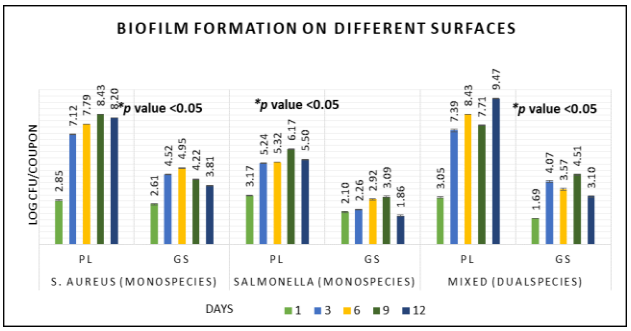

Fig. 4: Showing the development of biofilm (log CFU/coupon) that *S. typhimurium* and *S. aureus* strains under either mono- or dual-species conditions were able to produce on PL and GS coupons after 12 days of incubation at 28ºC.

a liquid (Steenackers et al. 2012). The study measured biofilm production of *S. aureus* and *Salmonella* spp. strains isolated from the environment of poultry houses using the TCP method. All strains produced biofilms, with *S. typhimurium* having the highest mean OD. Similarly, De Oliveira et al. (2014) discovered that the capacity to create biofilms differed among serotypes when temperature and nutritional conditions were kept constant. The capacity of a certain isolate to create a biofilm relies on numerous factors that differ based on the environmental conditions of a given ecological niche. This study observed that the three *S. aureus* strains could form a moderate biofilm; no statistically significant differences were found (P>0.05) between strains. The fact that these strains could form biofilm after taken from chicken houses shows that they

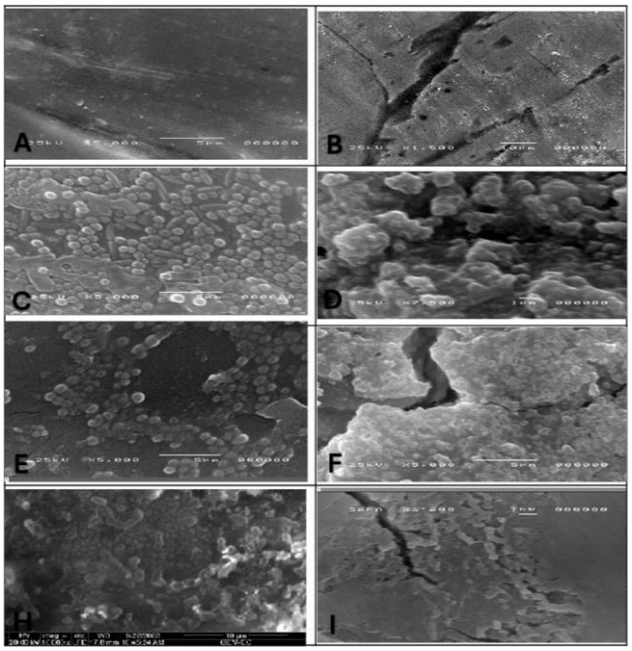

Fig. 5: SEM images of biofilm enhancement on PL and GS coupons by *S. aureus* and *S. typhimurium* isolate under either single- or mixed-species conditions, following incubation at 28ºC after 12 days (A=control plastic surface before biofilm formation; B=control galvanized steel surface before biofilm formation; C=dual-species on PL; D=dual-species on GS; E=*S. aureus* biofilm on PL; F=*S. aureus* biofilm on GS; H=*S. typhimurium* biofilm on PL; and I=*S. typhimurium* biofilm on GS).

may be able to adapt and live in the environment without a host (Lamas et al. 2016). Though, cleaning and disinfection techniques are essential in poultry houses to prevent the development of *Salmonella* and *S. aureus* on plastics inside the farm environment.

All *Salmonella* strains tested positive *csgD* genes; however, only two strains (*S. enteritidis* and *S. typhimurium*) tested positive for *adrA* and *gcpA* genes (Table 2). These findings match with those of De Oliveira et al. (2014) in that they discovered the genes *csgD* and *adrA* in all *Salmonella* strains they examined. In this stud, *S. typhimurium* strain tested was positive for the three genes analyses, like the results of Lamas et al. (2016). Genetic analysis that, even in the presence of low nutritional concentrations, is in the process of cellulose production and biofilm formation. although, *Salmonella diarizionea* is negative for *gcpA* and *adrA*, it is still able to produce a moderate biofilm. Therefore, it is not possible to conclude that a particular strain is capable of biofilm formation based solely on molecular analysis without considering environmental factors (Monds and O'Toole 2009). Staphylococci genes, *icaA* and *icaD*, play a role in creating and maintaining biofilms. These genes produce polysaccharide intercellular adhesion (*PIA*), forming a biofilm around bacteria cells (Rohde et al. 2009). Microbes possess proteins with strong adhesive properties, which interact with host proteins such as laminin-binding protein (*eno*), and fibronectin-binding proteins A and B (*fnbA* and *fnbB*) (Seo et al., 2008). The invasion and adhesion of bacteria are contingent upon the presence of the *fnbA* gene, which may be associated with their capacity to generate biofilms. The *fnbA* gene was identified in 66% of the strains. The genes *icaD* and *eno* were present in all isolates.

The previous study examined how bacterial interactions affect the development of single-species biofilms by *S. typhimurium* and *S. aureus* on different surfaces in chicken houses. While several studies have investigated the formation of biofilms by these two species individually, only a few have explored the kinetics of biofilm formation when both species are present. The functionality of bacterial cells relies on the interplay of the cells themselves, the surface they attach to, and the ambient circumstances in their vicinity (Giaouris et al. 2015). Our work investigates the production of biofilms in laboratory circumstances by mono- and dual-species *S. typhimurium* and *S. aureus* on plastic and galvanized steel surfaces. These surfaces are often seen in poultry farms, where *the* strains were originally obtained.

Fig. (2) Demonstrates the biofilm formation on plastic coupons. The dual-species biofilm had the highest log count (7.21±1.10), followed by *S. aureus* mono-species (6.88±1.03) and *S. typhimurium* (5.08±0.50). *Salmonella* produced fewer biofilms in dual species than *S. aureus*, with comparable outcomes when measuring biofilm, as reported by Obe et al. (2022). *Salmonella* spp. can influence the biofilm formation of other bacteria, with researchers observing both synergistic and antagonistic interactions in miscellaneous-culture biofilm of *S. aureus* and other bacteria. This fits with what Gkana et al. (2017) found when they study the biofilm formation of *S. typhimurium* and *S. aureus* in both mono-and dual biofilms situations and found that in dual-species biofilms, both microorganisms had a lot fewer dead cells ($P<0.05$) than in single biofilms.

On galvanized steel coupons, we discovered that the mono-species *S. aureus* had a considerable rise in biofilm production, followed by the dual-species Staph. On Day 12, our experiment was concluded, and *S. typhimurium* had the lowest count (2.44±0.24 CFU/coupon) because of its slow growth in fully established biofilms. In a study by Knowles et al. (2005), the authors evaluated the biofilm production by *S. typhimurium* and *Staph. aureus* on stainless steel (SS) during a period of 12 days at a temperature of 25°C. The results indicated that *S. aureus* constituted the majority of the biofilm, accounting for roughly 99% (7-8 log CFU/section), whereas *Salmonella* was present in smaller amounts (6 log CFU/section). Alternatively, the research conducted by Utgikar et al. (2003) illustrates that the development of a biofilm consisting of several species on galvanized steel is influenced by various factors such as a medium lacking in nutrients, prolonged periods of drying, and the presence of water and nutrients, despite the detrimental impact of zinc on microorganisms.

The considerable significance ($P<0.05$) between the two surfaces regardless of whether the biofilms were composed of two different species or a single species, is illustrated in Fig. 4. Unlike the PL coupons, the average logarithm of biofilm cells attached to the GS surface showed a considerable drop. Various surface characteristics, such as hydrophobicity, coating, and roughness, might influence cell adhesion. De Oliveira et al. (2014) found that hydrophobic surfaces tend to promote biofilm formation and bacterial cell adhesion more than hydrophilic materials like steel. According to Myszka and Czaczyk (2011), most surfaces promote bacteria attachment through a layer of organic and inorganic substances. A study by Iñiguez-Moreno et al. (2017) revealed that bacteria belonging to the same species can form biofilms on surfaces that are both hydrophilic and hydrophobic. Nonetheless, PL contained a greater number of multispecies biofilms than GS. This is since GS is a hydrophilic material containing metallic ions that inhibit bacterial adhesion.

SEM images show the bacterial species' ability to maintain extracellular structure in all cases and on different surfaces. The intricate structure of biofilm matrices on GS was not easily observable in dual-species biofilms, aligning with recent research showing *S. typhimurium*'s ability to survive in challenging environments. Morishige et al. (2017) reported that *S. typhimurium* can enter state known as viable but non-culturable (VBNC), and Conventional methods based on traditional culture are not capable of detecting VBNC cells.

Conclusion

The ability of *Salmonella* and *S. aureus* strains to create biofilms on different densities of tissue culture medium (TCP) was investigated in this work. *S. typhimurium* was positive for every biofilm gene, and all three strains of *Salmonella* tested positive for *csgD* genes. Whereas *S. aureus* species displayed an *fnbA* gene that was reliant on adhesion and invasion. The dual-species biofilm on plastic coupons had the highest log count when the strains were evaluated for biofilm development on surfaces made of galvanized steel and plastic. On the other hand, little biofilm was found on galvanized steel surfaces, and it was difficult to see the complex arrangement of biofilm matrices created by *S. typhimurium* in a dual-species biofilm. Additional research is required to comprehend the fundamental reasons behind the inadequate adhesion and biofilm growth on GS surfaces.

Author's contribution

Samah E. Laban & Hanan S Khalefa designed the plan of work, performed in vitro testing for biofilm formation on plastic and GS surfaces, Amany A. Arafa shared in bacterial isolation and identification, performed PCR and drafting the manuscript. Eman S. Ibrahim and Eman Fathy shared in molecular identification and detection of biofilm genes. Hanan S Khalefa & Samah E. Laban writing and drafting the manuscript. Amany A. Arafa and Eman S. Ibrahim drafting the manuscript. All authors read and approved the final manuscript.

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