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EVALUATION OF IN-VITRO EFFICACY OF THREE TYPES OF DISINFECTANTS AGAINST BIOFILM FORMATION ON DIFFERENT POULTRY CONTACT SURFACES

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ABSTRACT

Biofilms pose a significant challenge to treating poultry diseases and managing poultry flocks. This study assessed the effect of three disinfectants frequently used in the poultry sector on 14-day mature mono- and mixed-species biofilms of *Staphylococcus aureus* (*S. aureus)* and *Salmonella typhimurium* (*S. typhimurium)* on galvanized steel (GS) and plastic (PL) surfaces. The surface type influenced cellular density in mono-species and mixed-species biofilms (p <0.05). A significant positive correlation was observed between the average log in our verified data and the biofilm density measurements made with crystal violet on various surface coupons (r = 0.94, r = 0.91; r = 0.94, r = 0.81) for *S. aureus* and *S. typhimurium*, respectively. Our findings indicated that Oxy Clean® is the most effective disinfection on PL surfaces, showing a 5-log reduction at low concentration (0.5%) and short contact time (10 minutes) when applied with *S. typhimurium* or mixed strain biofilm. On GS surfaces, the biofilm of the mono-species *S. aureus* exhibits total resistance to all disinfectants tested, except for Oxy Clean®, which is effective after an extended contact period of 20 minutes, resulting in the complete elimination of the biofilm. Conversely, *S. typhimurium* exhibits a high sensitivity to all disinfectants employed. The only disinfectants capable of effectively penetrating the mixed biofilm are Oxy Clean® and VirKon S®, provided they are used for 20 minutes at a concentration of 1%. Compared to mono-species biofilms, both bacteria exhibited greater resistance to disinfectants in mixed-species biofilms.

Keywords: Biofilm, Mixed-Species, Crystal Violet, Disinfectant, Oxy Clean®

INTRODUCTION

Biofilm formation facilitates microorganisms' survival in their natural environment (Ali et al., 2025; Hou *et al.,* 2012). Prevalent microorganisms responsible for biofilm formation in poultry farms and food sectors include *Salmonella spp., Staphylococcus spp., Listeria monocytogenes, E. coli, Klebsiella pneumoniae*, and *Campylobacter jejuni*. According to Wladyka *et al.* (2011), *S. aureus* is a dangerous bacterium that can cause several lesions in chickens and result in significant financial losses for the poultry sector. Furthermore, Nasrin *et al.* (2007) identify *Staph. aureus* from poultry environments, including litter, water, and feed. Researchers have found *S. aureus* in various settings and believe it is an extremely adaptable organism, capable of modifying its

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mRNA transcription levels in response to environmental changes (Rode *et al.,* 2007; Begley and Hill, 2015).

Salmonella species can stick to surfaces and form biofilms, which can become long-lasting environmental reservoirs. This may show a direct link between contamination in the environment and contamination in food processing (Musa *et al.,* 2024). Salmonella's principal survival strategy is biofilm development, as demonstrated by an extensive study (Merino *et al.,* 2019). Salmonella has aggregated fimbriae and lipopolysaccharides that allow it to stick to and form biofilm on a variety of surfaces, including rubber, plastic, and metal. Direct or indirect interaction with the environment can transmit infection. It can proliferate under low water activity (between 8 and 45 °C), with an ideal temperature of 37 °C (Hanes, 2003).

Pathogens have evolved many methods for survival in this environment, such as surface adhesion and biofilm development (Steenackers *et al.,* 2012; Elshafiee *et al.,* 2022). Biofilms are complex communities that form when microorganisms find surfaces and stick to abiotic or biotic substrates, forming an extracellular matrix mostly made up of exopolysaccharides (Karygianni *et al.,* 2020; Ibrahim *et al.,* 2023). They are crucial for foodborne infections' survival and constitute a major risk factor in their dissemination across the food chain (Abebe *et al.,* 2020; Samy *et al.,* 2022). Researchers have devised diverse techniques to inhibit biofilm formation during the initial phases. Numerous factors influence the bacterial adhesion process to a surface, including bacterial and surface characteristics (Karygianni *et al.,* 2020; Laban *et al.,* 2025).

In places with many kinds of bacteria, the interactions between these microorganisms may change the makeup and function of biofilms (Ibrahim *et al.,* 2023). Compared to planktonic cells, they may shield bacteria from physical, environmental, and antibiotic stressors and affect disinfection protocols' effectiveness (Varga *et al.,* 2008). Researchers have documented that efficient cleaning techniques and chemical disinfectants can inhibit microbial growth on surfaces and remove biofilms (Khalefa *et al.,* 2025; Simoes *et al.,* 2010). According to Simoes *et al.* (2010), the most used disinfectants in agricultural and food processing settings include hydrogen peroxide, potassium mono-persulfate (Virkon), sodium hypochlorite, quaternary ammonium compounds (QACs), and a disinfectant based on glutaraldehyde. Other studies have demonstrated that Salmonella biofilms are far more resistant than planktonic cells to commercial disinfectants, such as chlorine and quaternary ammonium (Nguyen and Yuk., 2013; Abd-Elall *et al.,* 2023).

Considering all the above, the goal of this work was to investigate the potential impact of bacterial interactions within and between species on the capacity of *S. Typhimurium* and *Staph. aureus* to form mixed-culture multi-strain biofilms on biotic and abiotic substrates under dry conditions over 14 days, and on the subsequent resistance of their sessile cells to chemical disinfection.

MATERIALS AND METHODS

1. Biofilm production by *S. aureus/S. typhimurium*

Bacterial species: The bacterial strains used were *S. typhimurium* and *S. aureus*, isolated from the poultry farm's environment. The strains were identified based on morphology and biochemical characteristics and subsequently confirmed using serological testing for *Salmonella* spp. and the staphylococci test kit (BioMérieux, France). Before use, the bacteria were cultured in tryptic soy broth (TSB) for 24 hours at 37°C to achieve a final concentration of 10^8 CFU/mL (Iniguez-Moreno *et al.,* 2018).

Used coupons: Coupons made of galvanized iron and plastic, each measuring 2x2 cm, were utilized to construct biofilm. Before executing the experiment, the coupons were

sanitized using the procedure outlined by Marques *et al.* (2007). The surfaces were soaked in pure acetone for 1 hour to eliminate dirt and grease, followed by immersion in diluted neutral detergent for 1 hour, rinsing with sterile distilled water, cleaning with 70% ethanol, drying for 2 hours at 60°C, and sterilizing in an autoclave at 121°C for 15 minutes before use.

Formation of mono- and mixed-species biofilms: Sterilized coupons were organized in groups within sterile glass Petri dishes. Coupons designated for mono-species biofilms were inoculated with 50 µL of cultures incubated at 37 °C for 24 hours for the respective strain. Coupons intended for mixed-species biofilms received 25 µL of each bacterial suspension. Subsequently, the surfaces were allowed to dry and were incubated at 37 °C for 14 days, as reported in a prior study by Laban *et al.* (2025). From day 1 to day 14, attached cells received nutrition and hydration once a day via a sterile saline solution enhanced with 20% BHI broth. Each coupon was administered a daily 80 µL of the feeding and hydration solution (Christine *et al.,* 2023; Laban *et al.,* 2025).

2. Biofilm determination:

Crystal violet staining: To detect the formation of biofilms, plastic and galvanized iron coupons were cleaned three times with distilled water to eliminate planktonic bacteria. The samples were then allowed to air dry and stained with 0.1% (w/v) crystal violet at 28°C for 20 minutes, according to Tang *et al.* (2012).

Biofilm quantification by cell enumeration: Following a 14-day experiment conducted in fully aseptic conditions, additional coupons were extracted from the glass Petri dishes using sterile forceps. Two mL of sterile saline was pipetted to remove any loosely attached cells during the rinse process (Kostaki *et al.,* 2012). The biofilm-attached cells on each coupon were removed from the coupon surface by thoroughly swabbing with a pair of cotton swabs previously soaked in a sterile saline solution. The cell suspension was serially diluted, and 0.1 mL of each dilution was plated onto pre-prepared sterile tryptic soy agar (TSA) plates for mono-species biofilms, and TSA with lactose (10 g/L) and phenol red (0.025 g/L) for mixed-species biofilms to quantify the biofilm bacteria. The inoculation plates were incubated at 37 °C for 24 hours. The colonies of *Staph. aureus* exhibited a yellow hue due to lactose fermentation, but the colonies of *Salmonella* spp. were devoid of color (Iniguez-Moreno *et al.,* 2018). After incubation, counts were ascertained, logarithmic values were computed and expressed as CFU /Coupon in logarithmic form.

3. Disinfectants applied to PL and GI surfaces have long-term biofilm development.

After the 14th day of biofilm formation, 10 ml of phosphate-buffered saline PBS (pH=7 ± 0.2) was carefully added to each coupon twice to remove any unattached cells. Subsequently, three disinfectants, as detailed in Table 1, were evaluated for their efficacy in eradicating established biofilm by submerging each coupon in 5 ml of disinfectant at concentrations ranging from 0.5% to 1% for two distinct contact durations (10 and 20 minutes) at room temperature.

Each coupon was placed into 1.5 mL of neutralizer solution following the exposure period. Following a 5-minute neutralization period, the surviving cells were quantified through sonication and plate counting, as previously outlined (Mariscal *et al.,* 2009) by enumerating viable cells after each disinfection treatment and comparing resistance among strains under varying biofilm growth conditions (i.e., mono- and mixed-species). All disinfectants were freshly diluted in sterile distilled water. Before application, all chemicals were verified for sterility. The experiment was repeated three times.

RESULTS

The results mean values after the 14-day incubation period were presented in Table 2. Demonstrated that log_{10} counts in both plastic and galvanized steel exhibited significant differences (P<0.05) throughout the experiment, with counts in plastic exceeding those in galvanized steel. This finding suggested that the materials used in poultry processing plants, PL and GS, were different in how well they stick to polystyrene, which was often used to measure biofilm development in the lab. It is advised to exercise caution when comparing or extrapolating results for other materials in light of these findings. Regarding bacterial species, cell densities attained were 4.22 log10 CFU/coupon for *Staph. aureus* in mono-species on day 9, thereafter declining to 3.81±0.06 log10 CFU/coupon, whereas *S. typhimurium* reached 3.08 log10 CFU/coupon before decreasing to 1.86 ±0.06 log10 CFU/coupon. There were more bacteria on the plastic surface than on the other surface. The difference between the densities of single-species and dual-species biofilm was 9.47 log10 CFU/coupon.

Table 1: Disinfectants used in experiment

Disinfectants	Composition	Concentration	Neutralizer
A: VirKon S®	potassium peroxy mono-sulfate	0.5%, 1%	General neutralizer: - 3% polysorbate 80 - 0.3% lecithin - 0.1% Histidine - 0.5% sodium thiosulphate, - 3% Saponin - 1% sodium Laureth-Sulphate) (Anonymous, 2002)
B: Super Quat ®	Quaternary Ammonium Compounds	0.5%, 1%	
C: Oxy clean®	Peracetic acid + hydrogen peroxide	0.5%, 1%	

Table 2: The biofilm development (log CFU/coupon) produced by *Staph. aureus* and *S. typhimurium* strains under mono- or dual-species conditions on PL and GS coupons

	Staph. aureus		*S. typhimurium*		dual-species	
Time	PL	GS	PL	GS	PL	GS
Day 2	2.85±.05^{c}	2.61±.04^{c}	3.17±.02^{c}	2.10±.06^{b}	3.05±.03^{d}	1.69±.01^{d}
Day 5	7.12±.03^{b}	4.52±.04^{a}	5.24±.02^{b}	2.26±.03^{b}	7.39±.08^{c}	4.07±.07^{b}
Day 8	7.79±.01^{b}	4.95±.03^{a}	5.32±.01^{b}	2.92±.03^{a}	8.43±.02^{b}	3.57±.06^{b}
Day 11	8.43±.01^{a}	4.22±.03^{b}	6.17±.01^{a}	3.09±.05^{a}	7.71±.03^{c}	4.51±.04^{a}
Day 14	8.20±.02^{a}	3.81±.03^{b}	5.50±.02^{b}	1.86±.03^{c}	9.47±0.03^{a}	3.10±.06^{c}
P value	0.000	0.000	0.000	0.000	0.000	0.000

SE: standard error PL: plastic GI: galvanized steel
A: Before exposure to disinfectant B: After exposure to disinfectant
$^{a, b,c,d,}$ means in the same column indicate significant difference (P <0.05)

The Crystal Violet method was employed to measure the biofilm biomass developed on coupons, as illustrated in **Fig. (1)**. The bulk of the biofilm extracellular matrix was affected by the interplay between the incubation conditions and the surface material (P< 0.001). The absorbance of *S. typhimurium* and *Staph. aureus* in mono- and dual-species conditions on plastic coupons was twice that observed on galvanized steel surface coupons at 570 nm **(Fig. 2).**

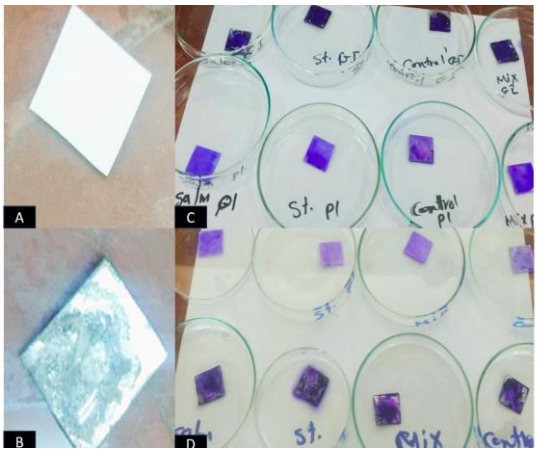

Fig. 1: The coupons were evaluated for biofilm growth and stained with 0.01% crystal violet. 1-A) Plastic coupon before biofilm development. 1-B) Galvanized steel coupon before biofilm development. 1-C) After six days of biofilm development, *Staph. aureus* and *S. typhimurium* form biofilms on plastic and galvanized steel coupons, either as mono-species or dual-species. 1-D) Plastic and galvanized steel coupons following biofilm formation by *Staph. aureus* and *S. typhimurium*, either as mono-species or dual-species, during 10 days of biofilm development.

Figure (3) showed a strong positive relationship between the average log in our checked data and measurements of biofilm density and the amount of crystal violet found on different surface coupons. The regression for single-species *Staph. aureus* biofilm formation on plastic and galvanized steel surfaces is strong (r = 0.94, r = 0.91). The regression for *S. typhimurium* biofilm formation on both surfaces (PL and GS) is strong (r = 0.94, r = 0.81). Only plastic surfaces exhibited a strong connection (r = 0.94) for dual- species.

The effect of three disinfectants commonly used in the poultry industry on mature mono- and dual-species biofilm of *Staph. aureus* and *S. typhimurium* were evaluated on different surfaces (Tables 3, 4, and Figs. 4 and 5). Most studies focused only on mono-species models, where dual-species biofilm was not often studied. So, this study compared the effect of three disinfectants on these two types of biofilm on different surfaces. The effectiveness of used disinfectants was evaluated on mono-species biofilm of *Staph. aureus* and *S. typhimurium* beside dual species biofilm of them (Tables 3 and 4). The different concentrations of disinfectants (0.5–1%) applied to the biofilm formed on plastic.

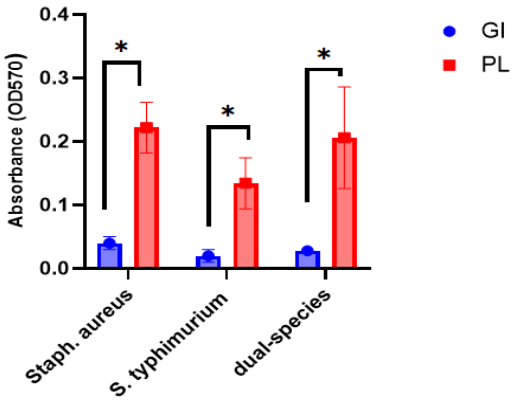

Fig. 2: Mono species and dual-species crystal violet quantification of *Staph. aureus* and *S. typhimurium* biofilm on plastic (PL) and galvanized steel (GS) coupons, mean values of 570 nm OD represented in the columns.
*Indicate significant difference (P<0.05)

The results indicated that VirKon S® was unable to achieve a 5-log reduction for either mono or dual biofilm on PL surfaces, with the exception of *S. typhimurium*, which successfully achieved a complete reduction in the count of formed biofilm. Moreover, even at higher concentrations and contact times (1% and 20 min), Super Quat® (0.5–1%) was unable to completely eradicate the biofilm that either *Staph*. or *Salmonella* had formed on the PL surface. However, it only demonstrated a positive effect at higher concentrations and contact times (1% and 20 min), achieving a 5-log reduction in the biofilm formed by both species. The results showed that Oxy Clean® is the most effective disinfectant for PL surfaces, achieving a 5-log reduction even at low concentrations (0.5%) and short contact times (10 min) when applied to *S. typhimurium* biofilm or dual strains of *Staph. aureus* and *S. typhimurium* biofilm. However, the single strain *Staph. aureus* biofilm exhibited some tolerance and recovered from the surface after disinfectant exposure.

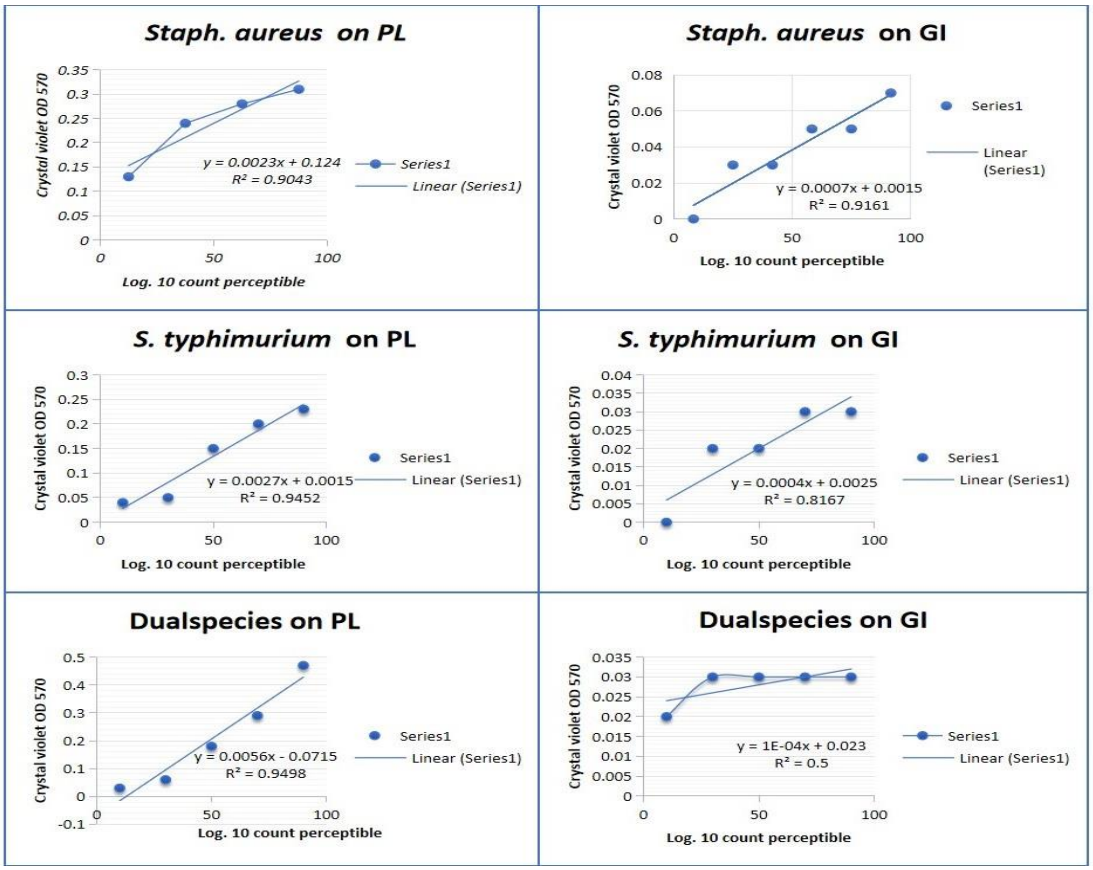

Fig. 3: The correlation between the $\log_{10}$ count perceptible and the absorbance of crystal violet on various coupons (plastic and galvanized steel) surfaces inoculated with *S. typhimurium* and *Staph. aureus* strains, whether mono-species or dual-species.

Table 3: The efficacy of different disinfectants on mono-species and dual-species biofilms on PL surfaces.

Disin-fectant	CONC.	Time exposure (min)	Mean Log_{10} CFU/coupon on plastic								
			Staph. aureus			*S. Typhimurium*			dual-species		
			A	B	Log reduction %	A	B	Log reduction %	A	B	Log reduction %
VirKon S®	0.5 %	10	8.28	6.143[a]	2.25	5.51	0.00[e]	100	9.39	6.10[a]	٣,٢٤
		20		5.31[d]	3.12		0.00[e]	100		4.41[d]	٥,٠١
	1%	10		4.60[e]	3.73		0.00[e]	100		5.57[b]	٣,٨٣
		20		3.67[g]	4.68		0.00[e]	100		5.17[c]	٤,٢٣
Super quat®	0.5 %	10		5.93[ab]	2.23		3.28[c]	٢,٢٩		5.14[c]	٤,٣
		20		5.63[b]	2.71		3.10[d]	٢,٤٢		3.92[e]	٥,٥٣
	1%	10		4.86[e]	3.56		4.37[a]	١,٢٢		3.90[e]	٥,٥٣
		20		4.31[ef]	4.11		4.07[b]	١,٥0		3.27[f]	٦,١٣
Oxy clean®	0.5 %	10		5.51 [cd]	2.80		0.00[e]	100		0.00[g]	100
		20		4.16[f]	4.14		0.00[e]	100		0.00[g]	100
	1%	10		5.21[cd]	3.13		0.00[e]	100		0.00[g]	100
		20		2.28 [h]	5.80		0.00[e]	100		0.00[g]	100
SEM			0.00	0.18		0.00	0.30		0.01	0.39	
*P value			1.000	.000		1.000	0.000		1.000	0.000	

SE: standard error PL: plastic GI: galvanized steel
A: Before exposure to disinfectant B: After exposure to disinfectant

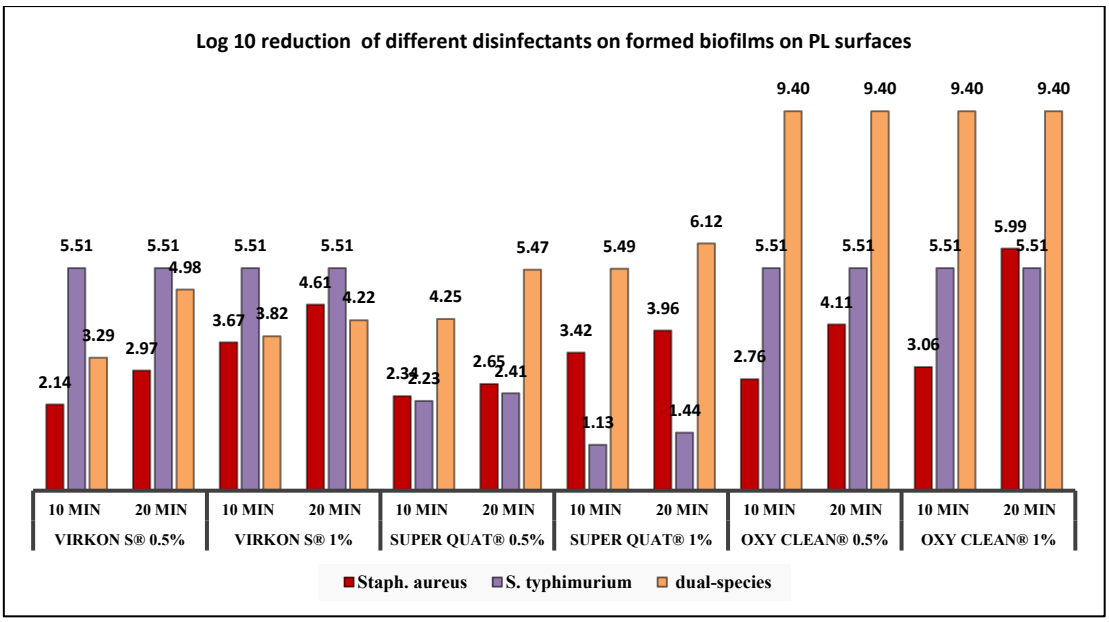

Fig. 4: The bars show the average log count of biofilm made by *Staph. aureus* and *S. typhimurium*, as well as the results of dual species after being disinfected for 10 and 20 minutes at different concentrations (0.5% and 1%). We used three disinfectants (VirKon S®), Super Quat® (10 ppm), and Oxy Clean® (10 ppm) after allowing biofilm to develop on plastic coupons for 14 days.

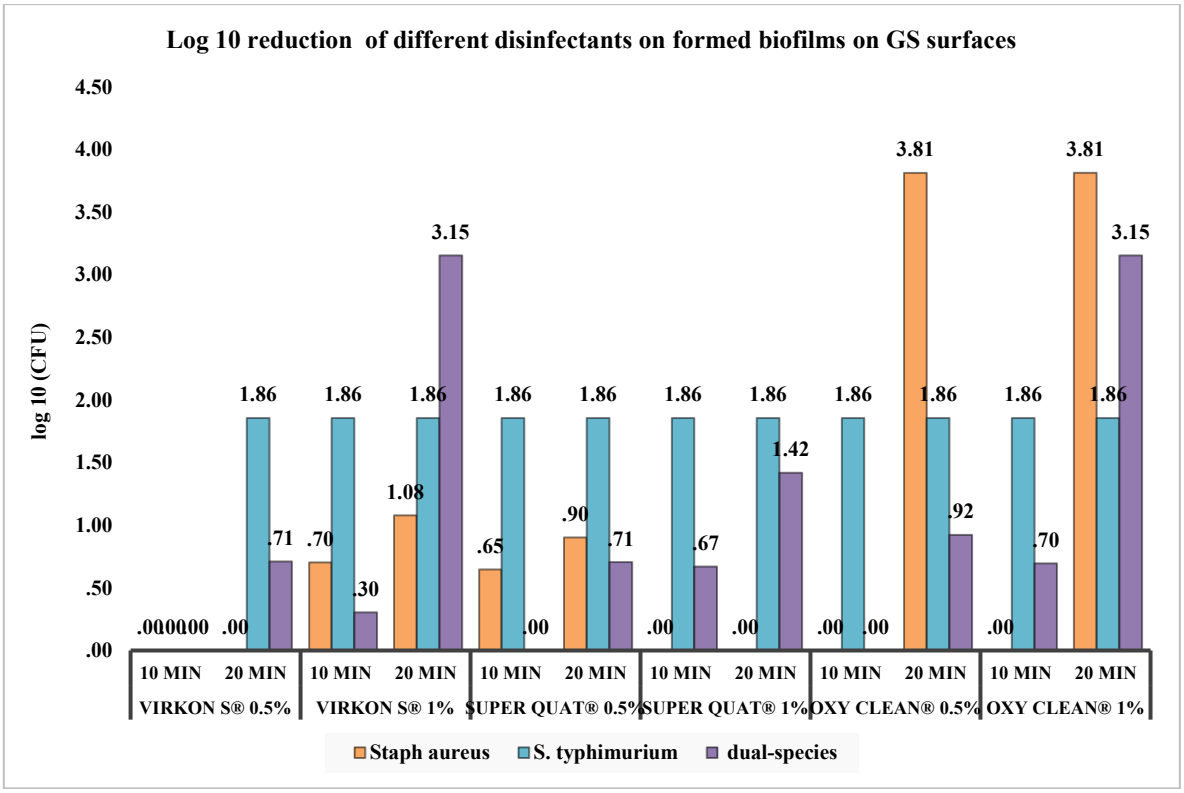

Fig. 5: The bars show the average log count of biofilms made by *Staph. aureus* and *S. typhimurium*, as well as the results of mixed species after being disinfected for 10 and 20 minutes at different concentrations (0.5% and 1%). We used three disinfectants (VirKon S®), Super Quat® (10 ppm), and Oxy Clean® (10 ppm) after allowing biofilms to develop on galvanized steel coupons for 14 days.

Table 4 illustrated the effectiveness of disinfectants following their application on the GS surface, demonstrating the growth of a biofilm layer containing either mono species (*Staph. aureus* - *S. typhimurium*) or a combination of both. The biofilm of the mono species *Staph. aureus* demonstrated complete resistance to all used disinfectants, except for Oxy Clean®, which is effective when used for a long contact time (20 min) and can completely remove the biofilm layer. On the other hand, Fig. 5 shows that *S. typhimurium* was more sensitive to all used disinfectants, even at low concentrations and short contact times. While *S. typhimurium* turned into a tolerant species when it grew with a resistant one (*Staph. aureus*) in the dual-species biofilm layer, the only disinfectants able to overcome this mix of biofilm were Oxy Clean® and VirKon S® when used for a long contact time (20 min. with 1% concentration).

Table 4: The efficacy of different disinfectants on mono-species and dual-species biofilms on GS surfaces.

Disinfectant	CONC.	Time exposure (min)	Mean Log_{10} CFU/coupon on galvanized steel								
			Staph. aureus			*S. Typhimurium*			dual-species		
			A	B	Log reduction %	A	B	Log reduction %	A	B	Log eduction %
VirKon S®	0.5%	10	3.8	3.61^{a}	0.18	1.86	1.52^{a}	0.35	3.15	2.66^{a}	0.59
		20		3.73^{a}	0.05		0.00^{b}	100		2.44^{ab}	0.7
	1%	10		3.11^{cd}	0.60		0.00^{b}	100		2.85^{a}	0.3
		20		2.73^{e}	1.07		0.00^{b}	100		0.00^{C}	100
Super quat®	0.5%	10		3.17^{c}	0.70		0.00^{b}	100		2.83^{a}	0.32
		20		2.91^{de}	0.91		0.00^{b}	100		2.45^{ab}	0.7
	1%	10		3.32^{bc}	0.52		0.00^{b}	100		2.48^{ab}	0.67
		20		3.73^{a}	0.08		0.00^{b}	100		1.73^{b}	1.42
Oxy clean®	0.5%	10		3.52^{ab}	0.27		0.00^{b}	100		2.81^{a}	0.34
		20		0.00^{f}	100		0.00^{b}	100		2.23^{ab}	0.92
	1%	10		3.72^{a}	0.07		0.00^{b}	100		2.46^{ab}	0.69
		20		0.00^{f}	100		0.00^{b}	100		0.00^{C}	100
SEM			0.00	0.22		0.00	0.07		0.04	0.16	
*P value			1.000	.000		1.000	0.000		1.000	0.000	

$^{a, b,c,d,}$ means in same column indicate significant difference (P <0.05)

DISCUSSION

A limited study has investigated the development of biofilm by mono-species or dual-species on various surfaces, such as plastic and galvanized steel, as well as the survival of bacteria in dry conditions. The results of our study showed that it was hard to form biofilm by *S. typhimurium* on a galvanized steel surface. This finding corresponds with recent studies that have shown *S. typhimurium* capacity to withstand adverse conditions, such as low temperatures, salt stress, starvation, and other challenging conditions. Morishige *et al.* (2017) and Highmore *et al.* (2018) have documented that *S. typhimurium* can enter a viable but non-culturable (VBNC) stage. As previously stated, various factors may have influenced the lack of inadequacy of cell attachments for the specific type of coupon used in this study. The new methods that use SEM images clearly show a well-developed biofilm in both cases, which supports what Nahar *et al.* (2021) and Laban *et al.* (2025) already found. Both investigations reveal multiple distinct layers of cells, accompanied by an abundance of extracellular components. According to

Wibisono *et al.* (2020), *Staphylococcus* and *Salmonella* are the two most prominent foodborne pathogens.

This study revealed that crystal violet (CV) was appropriate for application on PL surfaces. However, GS surfaces were unsuitable due to high retention, leading to results beyond detectable limits (Figs. 1&2). The plastic coupons exhibited greater retention of crystal violet than galvanized steel, but both were measurable. Despite the elevated OD570, there was a markedly reduced number of connected cells. Although a direct comparison of the crystal violet assay results to the log count of biofilm-attached cells was impossible, we determined that the counts of the examined bacteria, *Salmonella* spp., and *Staph. aureus*, on galvanized steel and plastic surfaces, whether mono-species or dual-species, exhibited a significant positive correlation (Figure 3). The regression for single-species *Staph. aureus* biofilm formation on plastic and galvanized steel surfaces is robust (r = 0.94, r = 0.91). The regression for *S. typhimurium* biofilm formation on both surfaces (PL and GS) is strong (r = 0.94, r = 0.81). Only plastic surfaces exhibited a robust connection (r=0.94) for dual-species. Nevertheless, while several methods can assess biofilm growth on various surfaces, crystal violet assays are more cost-effective and efficient, requiring minimal equipment. By combining elements of various methods, such as the use of biofilm reactor coupons, with the crystal violet test method, we can create a more streamlined approach that more accurately demonstrates biofilm formation on processing surfaces (Thames *et al.,* 2023).

The significant difference (P<0.001) between the two surfaces was reported, irrespective of whether the biofilm consisted of two distinct species or a single species (Table 2). In contrast to the plastic surface coupons, the average logarithm (log_{10}) of biofilm cells adhered to the galvanized steel (GS) surface exhibited a significant decline. Various surface properties, including hydrophobicity, coating, and roughness, may affect cell adhesion. De Oliveira *et al.* (2014) stated that hydrophobic surfaces exhibit a heightened tendency for biofilm formation and bacterial cell attachment, in contrast to hydrophilic materials, such as steel. Nonetheless, ambient factors considerably influence the physicochemical properties of surfaces, rendering laboratory studies on microbial adherence to various materials largely efficacious. Myszka and Czaczyk (2011) declared that most surfaces facilitate bacterial adhesion by coating organic and inorganic materials. Stress circumstances, environmental context, and surface type can influence biofilm formation (Kostaki *et al.,* 2012). Surface roughness enhances the surface area available for microbial adhesion. Surface constraints encourage reversible and irreversible adherence, protecting adherent bacteria from external factors and aiding initial colonization (Vancraeynest *et al.,* 2004). Diverse surfaces can facilitate biofilm development by various food borne pathogens; the characteristics of these surfaces influence both initial bacterial adhesion and subsequent growth and dissemination. A study by Iñiguez-Moreno *et al.* (2018) demonstrated that bacteria of the same species may establish biofilms on both hydrophilic and hydrophobic surfaces. Nevertheless, plastic harbored a higher quantity of multispecies biofilms compared to GS. This is because GS is a hydrophilic substance containing metallic ions that impede bacterial adherence.

The efficacy of disinfectants against biofilm may fluctuate based on the features of the disinfectant, the kind and nature of the surface, the bacteria present in the biofilm, the age of the biofilm, and additional factors such as temperature, organic load, and contact duration (Khalefa *et al.,* 2025). An effective biofilmcide must eradicate 99.99% of the biofilm following its contact period with biofilm-producing bacteria. This requires the appropriate selection of a suitable disinfectant at the recommended concentration and for the precise duration of contact. Therefore, we selected three commercial disinfectants commonly used in poultry farm

disinfection processes to test their effectiveness against *Staph. aureus* and *S. typhimurium in vitro*, aiming to eliminate the established biofilm layer on PL and GS surfaces.

In this experiment, Oxyclean®, a mixture of hydrogen peroxide and peracetic acid, performed optimally as a germ killer against *S. typhimurium* at concentrations of 0.5% and 1%. This disinfectant eliminated the mono *S. typhimurium* biofilm layer on both surfaces in just 10 minutes, and it only had a destructive effect on the dual- biofilm on the PL surface, but no effect observed on the GS surface. This disinfectant attains complete biofilm reduction with statistical significance (*P* value ≤ 0.05). According to De Carvalho (2007), the product with H_2O_2 and peracetic acid works well because H_2O_2 can make free radicals that damage the biofilm matrix. Our findings align with those of Abd-Elall *et al.* (2023), who employed H_2O_2 at concentrations of 2% and 5% for 120 minutes to eradicate *Salmonella* biofilm, achieving a full reduction of 100%. However, our results were different from those of Marin *et al.* (2009), who showed that a 1% concentration of H_2O_2 was only slightly effective against *Salmonella* biofilm.

Oxyclean® (0.5–1%) was applied to *Staph. aureus* biofilm on both PL and GS surfaces for 10 minutes. The results showed no significant reduction and the bacteria were resistant to the disinfectant. However, when when extended the contact time to 20 minutes, we observed sensitivity on the GS surface, while resistance remained on the PL surface. The plastic surface is hydrophobic, while the galvanized surface is hydrophilic. This means that the aqueous disinfectant has a harder time sticking to the plastic surface because of the surface tension of the solution. Our results agree with those of Abd-Elall *et al.* (2023), who found that 5% H_2O_2 dropped the 7 days old biofilm of *Staph. Aureus* by 100% after 120 minutes of contact. Köse and Yapar (2017) indicated that 5% H_2O_2 can eradicate just 70% and 80.3% of one-day-old *Staph. aureus* biofilm after 1 and 60 minutes of contact time, respectively.

At a concentration of 0.5 to 1%, Virkon S® completely eliminate the 14-day-old mature biofilm made by *S. typhimurium*. There were significant differences ($P \leq 0.05$) between the surfaces that were treated either GS or PL. The results obtained were similar to those of Rodrigues *et al.* (2011), who documented that low concentrations of sodium hypochlorite were highly effective in eradicating 1-day-old *S. enterica* biofilm. On the other hand, Abd-Elall *et al.* (2023) found that Virkon S® was extremely effective against *Salmonella* biofilm, but it was not quite efficient to eliminate it at 5% concentration for 120 min, and treatment achieved judgement edge 96.1 (90%) for biofilm reduction.

The present study found that neither Virkon S® nor Super Quat® were effectively eliminated the biofilm generated by *Staph. aureus* at concentrations of 0.5% to 1%, even with extended exposure time on both surfaces. Iiguez-Moreno (2018) found that applying sodium hypochlorite at concentrations of 100 and 200 ppm was significantly reduced the *Staph. aureus* biofilm on both surface types. Rossoni and Gaylarde (2000) determined that sodium hypochlorite concentrations of 100 and 200 ppm did not significantly impact *Staph. aureus* cell adhesion to stainless steel after 10 minutes contact with the disinfectant at room temperature. The variance among these outcomes may originated from the disinfectant concentration, biofilm age, initial bacterial count, and surface type. *Staph. aureus* may be able to handle disinfectants like quaternary ammonium compounds (QACs) and chlorine compounds because of a mechanism in planktonic cells called QAC efflux. This mechanism is what makes planktonic cells resistant to QACs and cationic biocides (Cervinkova *et al.,* 2012). Furthermore, the influence of the three-dimensional architecture of *Staph. aureus* biofilms on QAC resistance are minimal, but the physiological alterations in the biofilm cells are more significantly associated with

them. Bridier *et al.* (2011) explained that the positive charge and hydrophobic properties of QAC explain why the EPS layer of the biofilm takes longer to break through. Mature biofilms exhibit increased resistance owing to several causes, including elevated concentrations of extracellular polymeric substances (EPS). The EPS stops disinfectants from getting into the biofilm, which means that antimicrobials and biofilm components might interact. As a result, microorganisms in biofilms are less likely to be killed by disinfectants because they are growing slowly.

According to this study, According to this study, mature 14 days biofilms made by *Staph. aureus* and *S. typhimurium* were more resistant to disinfectants than biofilms made by only one species. These findings were consistent with the majority of studies that showed multispecies biofilms to be resistant to disinfectants, as highlighted by Gkana *et al.* (2017) and Bridier *et al.* (2012). Regrettably, the mechanisms of resistance remain unclear. Nevertheless, certain potential mechanisms involve chemical interactions among microbes, which result in a more viscous matrix being formed (Burmølle *et al.,* 2006). There appears to be an interaction among the examined strains that positively influences their resistance. In contrast, Iñiguez-Moreno (2018) found that biofilms made up of both *Staph. aureus* and *Salmonella* spp. were more sensitive to disinfectants like peracetic acid, QACs, and sodium hypochlorite than biofilms made up of only one species.

CONCLUSION

In conclusion, the resistance of microorganisms such as *Staph. aureus* and *S. typhimurium* to bactericidal chemicals in biofilms is influenced by various parameters, including surface type, initial bacterial load, biofilm maturity, and interspecies interactions. This research looks at how the surface affects the number of adhered planktonic cells and the density of cells in mono-species biofilms. The galvanized surface exhibited less bacterial strain attachment, whereas the plastic surface demonstrated a higher biofilm count. Both microorganisms displayed greater resilience to disinfectants in dual-species biofilms compared to mono-species biofilms, although their sensitivity varied in degree. Different types of chemical disinfectants, like chlorine compounds, peroxides, and QACs, reduced biofilm in different ways, depending on the concentration and length of contact. In particular, an oxidizing disinfectant (a mix of peracetic acid and hydrogen peroxide) can eliminate biofilms very well, especially when used in large amounts and for long periods. Bacterial strains tolerated quaternary ammonium compounds (QACs), the least effective agents. Nonetheless, the bacteria present in the biofilm and the substrate upon which the biofilm formed largely determined the disinfectant's efficacy.

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REFERENCES

Abd-El-all, A.M.; El-Bana, M.H.; Gamal, N. and Megahed, A. (2023): Biofilm Production Capacity Exerted by some Bacterial Pathogens Recovered from Poultry Farms in Egypt with a Trial of Control Using Chemical Disinfectants. Journal of Advanced Veterinary Research. 13(6), 1136-1141.

Abebe, E.; Gugsa, G. and Ahmed, M. (2020): Review on major food-borne zoonotic bacterial pathogens. Journal of tropical medicine. 1, 4674235.

ALI, A. M., NASR, S. A., & KHALEFA, H. S. (2025). in vitro evaluation of organic acids'biofilmicidal and antimicrobial efficacy against salmonella kentucky and pseudomonas aeruginosa strains isolated from broiler farms'drinking water. *Assiut Veterinary Medical Journal,* 71(185), 93-107.

Anonymous. (2002): ASTM E 1054-02. Standard test method for evaluation of inactivators of antimicrobial agents.

ASTM, Villanova, PA. Blazej A: Tensides, Bratislava: Alfa, 1977, pp: 389.

Begley, M. and Hill, C. (2015): Stress adaptation in foodborne pathogens. Annu. Rev. Food Sci. Technol. 6, 191–210.

Bridier, A.; Briandet, R. and Thomas, V. Dubois-Brissonnet, F. (2011): Resistance of bacterial biofilms to disinfectants: A review. Biofouling. 27, 1017–1032.

Bridier, A.; Sanchez-Vizuete, M.D.P.; Le Coq, D.; Aymerich, S.; Meylheuc, T.; Maillard, J.Y. and Briandet, R. (2012): Biofilms of a Bacillus subtilis hospital isolate protects Staphylococcus aureus from biocide action. PLOS ONE. ,7. e 44506.

Burmølle, M.; Webb, J.S.; Rao, D.; Hansen, L.H.; Sørensen, S.J. and Kjelleberg, S. (2006): Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Applied and environmental microbiology.72, 3916–3923.

Cervinkova, D.; Babak, V.; Marosevic, D.; Kubikova, I. and Jaglic, Z. (2013): The role of the qacA gene in mediating resistance to quaternary ammonium compounds. Microbial Drug Resistance. 19(3), 160-167.

Christine, E.; Olive, C.; Louisin, M.; Dramé, M. and Marion-Sanchez, K. (2023): A new spray-based method for the in-vitro development of dry-surface biofilms. Microbiology Open. 12(1), e1330.

De Carvalho, C.C. (2007): Biofilms: recent developments on an old battle. Recent Patents on Biotechnology.1, 49-57.

De Oliveira, D.C.V.; Fernandes Junior, A.; Kaneno, R., Silva, M.G.; Araujo Junior, J.P.; Silva, N.C.C. and Rall, V.L.M. (2014): Ability of Salmonella spp. to produce biofilm is dependent on temperature and surface material. Foodborne pathogens and diseases. 11(6), 478-483.

El Shafiee, E.A.; Khalefa, H.S.; Al-Atfeehy, N.M.; Amer, F.; Hamza, D.A. and Ahmed, Z.S. (2022): Biofilms and efflux pump regulatory gene (mexR) in multidrug-resistant Pseudomonas aeruginosa isolated from migratory birds in Egypt. Veterinary World.15(10), 2425.

Gkana, E.N.; Giaouris, E.D.; Doulgeraki, A.I.; Kathariou, S. and Nychas, G.J.E. (2017): Biofilm formation by Salmonella Typhimurium and Staphylococcus aureus on stainless steel under either mono or mixed-species multi-strain conditions and resistance of sessile communities to sub-lethal chemical disinfection. Food Control. 73, 838–846.

Hanes, D. (2003): Nontyphoid Salmonella. In International Handbook of Foodborne Pathogens; CRC Press: Boca Raton, FL, USA.157–170.

Highmore, C.J.; Warner, J.C.; Rothwell, S.D. Wilks, S.A. and Keevil, C.W. (2018): Viable-but-nonculturable Listeria monocytogenes and Salmonella enterica serovar Thompson induced by chlorine stress remain infectious. MBio. 9(2), 10-1128.

Hou, W.; Sun, X.; Wang, Z. and Zhang, Y. (2012): Biofilm-forming capacity of Staphylococcus epidermidis, Staphylococcus aureus, and Pseudomonas aeruginosa from ocular infections. Investing Ophthalmol. Vis. Sci. 53, 5624–5631.

Ibrahim, A.N.; Khalefa, H.S. and Mubarak, S.T. (2023): Resimixed contamination and biofilm formation by opportunistic pathogens Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa in poultry houses isolated from drinking water systems, fans, and floors. Egyptian Journal of Veterinary Sciences, 54, 1041-1057.

Iñiguez-Moreno, M.; Gutiérrez-Lomelí, M.; Guerrero-Medina, P.J. and Avila-Novoa, M.G. (2018): Biofilm formation by Staphylococcus aureus and Salmonella spp. under mono and mixed-species conditions and their sensitivity to cetrimonium bromide,

peracetic acid and sodium hypochlorite. Brazilian journal of microbiology. 49, 310-319.

Iniguez-Moreno, M.; Gutiérrez-Lomelí, M.; Guerrero-Medina, P.J. and Avila Novoa, M.G. 2018. Biofilm formation by Staphylococcus aureus and Salmonella spp. under mono and mixed-species conditions and their sensitivity to cetrimonium bromide, peracetic acid and sodium hypochlorite. Brazilian journal of microbiology. 4 9, 310–319.

Karygianni, L.; Ren, Z.; Koo, H. and Thurnheer, T. (2020): Biofilm matrixome: extracellular components in structured microbial communities *Trends in microbiology*.28, 668-681.

Khalefa, H.S.; Ahmed, Z.S.; Abdel-Kader, F.; Ismail, E.M. and Elshafiee, EA. (2021): Sequencing and phylogenetic analysis of the *stn* gene of Salmonella species isolated from different environmental sources at Lake Qarun protectorate: The role of migratory birds and public health importance. Veterinary World. 14(10), 2764.

Khalefa, H.S.; Arafa, A.A.; Hamza, D.; El-Razik, K.A.A. and Ahmed, Z. (2025): Emerging biofilm formation and disinfectant susceptibility of ESBL-producing Klebsiella pneumoniae. *Scientific Reports*, *15*(1), 1599.

Köse, H. and Yapar, N. (2017): The comparison of various disinfectants? efficacy on Staphylococcus aureus and Pseudomonas aeruginosa biofilm layers. Turkish Journal of Medical Sciences 47, 1287-1294.

Kostaki, M.; Chorianopoulos, N.; Braxou, E.; Nychas, G.J. and Giaouris, E. (2012): Differential biofilm formation and chemical disinfection resistance of sessile cells of Listeria monocytogenes strains under mono-species and mixed-species (with Salmonella enterica) conditions. Applied and environmental microbiology. 78(8), 2586-2595.

Laban, S.E.; Arafa, A.A.; Ibrahim, E.S.FEE. and Khalefa, H.S. (2025): Dry Biofilm Formation, Mono-and Mixed-attachment, on Plastic and Galvanized Surfaces by Salmonella typhimurium and Staphylococcus aureus Isolated from Poultry House. International Journal of Veterinary Science. 14(1), 25-31.

Marin, C.; Hernandiz, A. and Lainez, M. (2009): Biofilm development capacity of Salmonella strains isolated in poultry risk factors and their resistance against disinfectants. Poultry Science. 88, 424-431.

Mariscal, A.; Lopez-Gigosos, R.M.; Carnero-Varo, M. and Fernandez-Crehuet, J. (2009): Fluorescent assay based on resazurin for detection of activity of disinfectants against bacterial biofilm. Applied Microbiology and Biotechnology. 82, 773–783.

Marques, S.C.; Rezende, J.D.G.O.S.; Alves, L.A.D.F.; Silva, B.C.; Alves, E.; Abreu, L.R.D. and Piccoli, R.H. (2007): Formation of biofilms by Staphylo-coccus aureus on stainless steel and glass surfaces and its resistance to some selected chemical sanitizers. Brazilian Journal of Microbiology. 38, 538-543.

Merino, L.; Procura, F.; Trejo, F.M.; Bueno, D.J. and Golowczyc, M.A. (2019): Biofilm formation by Salmonella sp. in the poultry industry: Detection, control and eradication strategies. Food Research International. 119, 530-540.

Morishige, Y.; Koike, A.; Tamura-Ueyama, A. and Amano, F. (2017): Induction of viable but nonculturable Salmonella in exponentially grown cells by exposure to a low-humidity environment and their resuscitation by catalase. Journal of Food Protection. 80, 288-294.

Musa, L.; Toppi, V.; Stefanetti, V.; Spata, N.; Rapi, M.C.; Grilli, G. and Casagrande Proietti, P. (2024): High Biofilm-Forming Multidrug-Resistant *Salmonella Infantes* Strains from the Poultry Production Chain. Antibiotics. 13, 595.

Myszka, K. and Czaczyk, K. (2011): Bacterial biofilms on food contact surfaces-a review. Polish Journal of Food and Nutrition Sciences. 61(3), 173-180.

Nahar, S.; Ha, A.J.W.; Byun, K.H.; Hossain, M.I.; Mizan, M.F.R. and Ha, S.D. (2021): Efficacy of flavoenzyme against Salmonella Typhimurium, Escherichia coli, and Pseudomonas aeruginosa biofilms on food-contact surfaces. International Journal of Food Microbiology. 336, 108897.

Nasrin, M.S.; Islam, M.J.; Nazir, K.H.M.N. H.; Choudhury, K.A. and Rahman, M. T. (2007): Identification of bacteria and determination of their load in adult layer and its environment. J. Bangladesh Soc Agric Sci Technol. 4, 69-72.

Nguyen, H.D.N. and Yuk, H.G. (2013): Changes in resistance of Salmonella Typhimurium biofilms formed under various conditions to industrial sanitizers. Food Control. 29 (1), 236-240.

Rode, T.M.; Langsrud, S.; Holck, A. and Moretro, T. (2007): Different patterns of biofilm formation in Staphylococcus aureus under food-related stress conditions. International Journal of Food Microbiology. 116, 372–383.

Rodrigues, D.; Cerca, N.; Teixeira, P.; Oliveira, R.; Ceri, H. and Azeredo, J. (2011): Listeria monocytogenes and Salmonella enterica Enteritidis biofilms susceptibility to different disinfectants and stress-response and virulence gene expression of surviving cells. Microbial Drug Resistance. 17, 181-189.

Rossoni, E.M. and Gaylarde, C.C. (2000): Comparison of sodium hypochlorite and peracetic acid as sanitizing agents for stainless steel food processing surfaces using epi fluorescence microscopy. International Journal of Food Microbiology. 61, 81–85.

Samy, A.A.; Arafa, A.A.; Hedia, R.H. and Ibrahim, E.S. (2022): Multiple drug resistance Salmonella and antibiotic residues in Egyptian animal products. World's Veterinary Journal. 4, 363-373.

Simoes, M.; Simoes, L.C. and Vieira, M.J. (2010): A review of current and emergent biofilm control strategies. LWT Food Sci Technol. 43, 573–583.

Steenackers, H.; Hermans, K.; Vanderleyden, J. and De Keersmaecker, S.C. (2012): Salmonella biofilms: An overview on occurrence, structure, regulation and eradication. Food Research international 45, 502–531.

Tang, P.L.; Pui, C.F.; Wong, W.C.; Noorlis, A. and Son, R. (2012): Biofilm forming ability and time course study of growth of Salmonella typhi on fresh produce surfaces. International food research journal. 19(1), 71-76.

Thames, H.T.; Pokhrel, D.; Willis, E.; Rivers, O.; Dinh, T.; Zhang, L. and Sukumaran, A.T. (2023): Salmonella biofilm formation under fluidic shear stress on different surface materials. Foods. 12 (9), 1918.

Vancraeynest, D.; Hermans, K. and Haesebrouck, F. (2004): Genotypic and phenotypic screening of high and low virulence Staphylococcus aureus isolates from rabbits for biofilm formation and MSCRAMMs. Veterinary Microbiology. 103, 241-247.

Varga, J.J.; Therit, B. and Melville, S.B. (2008): Type IV pili and the CcpA protein are needed for maximal biofilm formation by the gram-positive anaerobic pathogen Clostridium perfringens. Infection and Immunity. 76, 4944 – 4951.

Wibisono, F.M.; Wibisono, F.J.; Effendi, M.H.; Plumeriastuti, H.; Hidayatullah, A.R.; Hartadi, E.B. and Sofiana, E.D. (2020): A review of salmonellosis on poultry farms: Public health importance. Systematic Review in Pharmacy. 11, 481–486.

Wladyka, B.; Dubin, G. and Adam, D. (2011): Activation mechanism of thiol protease precursor from broiler chicken specific Staphylococcus aureus strain CH-91. Vet. Microbiology. 147, 195-199.

تقييم فعالية ثلاثة أنواع من المطهرات في المختبر ضد تكوين الأغشية الحيوية على أسطح الدواجن المختلفة

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تشكل الأغشية الحيوية تحديا كبيرا امام علاج أمراض الدواجن. قيمت هذه الدراسة تأثير ثلاثة مطهرات تستخدم بشكل متكرر في قطاع الدواجن على الأغشية الحيوية الأحادية والمختلطة المتكونة لمدة ١٤ يوما من المكورات العنقودية الذهبية (*Staph. aureus*) والسالمونيلا التيفية (*S. typhimurium*) على أسطح الفولاذ المجلفن والبلاستيك ، حيث أثر نوع السطح على الكثافة الخلوية في كل من الأنواع الأحادية والأغشية الحيوية للأنواع المختلطة. لوحظ ارتباط إيجابي كبير بين قراءة الكم الكلي البكتيري وقياسات كثافة الأغشية الحيوية التي تم إجراؤها باستخدام البلوري البنفسجي على كوبونات سطحية مختلفة . كما تشير النتائج التي توصلنا إليها إلى أن Oxy Clean® هي طريقة التطهير الأكثر فعالية على أسطح البلاستيك، حيث تظهر انخفاضا من ٥ لوجات بتركيزات منخفضة (٠,٥٪) وأوقات تلامس قصيرة (١٠ دقائق) عند تطبيقها مع *S. typhimurium* أو الأغشية الحيوية السلالة المختلطة. بينما علي الاسطح المجلفنة ، يظهر الأغشية الحيوية للنوع الأحادي *Staph. aureus* مقاومة كاملة لجميع المطهرات التي تم اختبارها ، باستثناء Oxy Clean® ، والتي تكون فعالة بعد فترة تلامس طويلة مدتها ٢٠ دقيقة ، مما يؤدي إلى القضاء التام على الأغشية الحيوية. على العكس من ذلك ، تظهر *S. typhimurium* حساسية عالية لجميع المطهرات المستخدمة. المطهرات الوحيدة القادرة على اختراق الأغشية الحيوية المختلطة بشكل فعال هي Oxy Clean® و VirKon S® ، بشرط استخدامها لمدة ٢٠ دقيقة بتركيز ١٪. بالمقارنة مع الأغشية الحيوية أحادية النوع ، أظهرت كلتا البكتيريا مقاومة أكبر للمطهرات في الأغشية الحيوية المختلطة الأنواع.