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Molecular Identification and Disinfectant Susceptibility of Biofilm-Forming Bacteria in Meat and Poultry Processing Establishments

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Abstract: Microbial biofilms in food processing plants present a significant challenge since they are resilient to cleaning and disinfection processes, considering as a persistent source of cross-contamination that affects food safety. This study investigated the presence of biofilm-forming bacteria in poultry processing environments and evaluated various disinfection protocols. Forty-four bacterial isolates were collected from equipment surfaces, processing areas, and workers' hands across three establishments (one processing plant and two slaughterhouses). All isolates demonstrated biofilm-forming capabilities (9% weak, 59% moderate, 32% strong), with slaughterhouse B exhibiting the highest proportion (36%) of strong biofilm producers. Standalone disinfectants (0.7% peroxyacetic acid and 150 ppm sodium hypochlorite) showed limited efficacy when tested against 21 moderate to strong biofilm-forming isolates. The combination of 3% commercial alkaline detergent with sodium hypochlorite demonstrated superior efficacy, eliminating biofilm formation in 47.6% of isolates. However, eight isolates (38%) persisted despite all treatments; they were identified through 16S rRNA sequencing as members of the *Bacillus*, *Listeria*, and *Alcaligenes* genera. These findings underscore the need for enhanced sanitation strategies in meat and poultry processing plants, especially against the identified bacteria, to ensure microbiological quality and protect public health.

1 Introduction

Bacterial flora and biofilm formation in poultry production present significant challenges across the production chain. Abd-Elall et al (2023a), Abd-Elall et al (2023b) revealed extensive bacterial contamination in poultry farms, particularly on antibiotic-resistant strains forming resilient biofilms on various surfaces. Their findings demonstrated that environmental conditions in poultry farms, including temperature and humidity, significantly influence biofilm development. Edris et al (2023) documented the prevalence of pathogenic bacteria in poultry facilities, highlighting the role of biofilms in persistent contamination cycles. Sayed et al (2021) demonstrated that equipment surfaces, particularly in processing areas, harbor complex bacterial communities resistant to conventional sanitization. Ismail et al (2019) contributed to this understanding by investigating the relationship between bacterial biofilm formation and antimicrobial resistance patterns in poultry processing environments, revealing concerning levels

of multi-drug-resistant organisms persisting within biofilm matrices. Regarding biofilm formation in poultry processing environments, the complexity and resilience of these bacterial communities present significant challenges for food safety management. The process begins with bacterial attachment to processing equipment surfaces, leading to sophisticated colonization mechanisms. According to Dayamoy (2022), these biofilms demonstrate remarkable adaptive capabilities, with multiple bacterial species coexisting in structured communities. The historical perspective provided by Carpentier and Cerf (1993) laid the groundwork for understanding biofilm formation mechanisms, while recent studies by Møretrø and Langsrud (2017) have expanded our knowledge of bacterial communication within these communities. Various studies (Reuter et al 2010, Afshin and Saeid 2011, Rothrock et al 2016, Ahmed et al 2021, Vanaki et al 2022) have identified diverse bacterial populations in processing environments, including pathogenic species like *Escherichia coli, Listeria monocytogenes, Campylobacter jejuni, Bacillus* spp., *Acinetobacter* spp., and *Pseudomonas* spp. The significance of this bacterial diversity is underscored by Kalia et al (2023), who documented up to 1000-fold increased antimicrobial resistance in biofilm-associated bacteria compared to their planktonic counterparts.

Dayamoy (2022), demonstrated the relationship and complex interactions between surface materials and biofilm development, influencing bacterial colonization across various surfaces. Römling and Balsalobre (2012) further elaborated on the sophisticated micro-ecosystems within biofilms, highlighting their collaborative behaviors and adaptive stress responses that enable survival under challenging processing conditions.

The molecular identification of bacteria using 16S rRNA gene sequencing has revolutionized our understanding of microbial diversity in food processing environments. This approach targets the highly conserved 16S ribosomal RNA gene, which contains both conserved regions for universal primer design and variable regions that provide species-specific sequence information. Recent research by Telli et al (2024) demonstrated how microbial diversity increases after chilling and storage stages due to the redistribution of microorganisms following the physical effects of slaughtering. Their study identified common genera in chicken carcasses during slaughter stages, including *Micrococcus*, *Acinetobacter*, *Enterococcus*, *Escherichia-Shigella*, *Psychrobacter*, *Streptococcus*, *Lactococcus,* and *Ligilactobacillus*, while environmental samples showed the highest relative abundance of *Bacillus*, *Anoxybacillus*, *Acinetobacter,* and *Psychrobacter*.

In general, there is critical importance to understanding and addressing biofilm formation in poultry processing environments to maintain food safety and quality standards.

Therefore, this study aimed to investigate the biofilm-forming capabilities of bacterial isolates obtained from various meat and poultry processing environments, including equipment surfaces, processing lines, and environmental samples. Additionally, the study sought to evaluate the efficacy of detergent-disinfectant combination protocols and to molecularly identify the bacteria resistant to cleaning and disinfection.

2 Materials and Methods

2.1 Isolation and purification of poultry-related bacteria

Forty swabs were collected from three meat and poultry processing establishments in Egypt. The first was a meat and poultry-product plant (A) located in the 10th of Ramadan industrial zone, Al-Sharkia governorate. The second and third were slaughterhouses (B) and (C) located in Al-Qalyubia and Al-Ismailia governorates, respectively. Sterilized cotton swabs were collected from different surfaces and workers' hands in the establishments studied. Swabs were aseptically transferred under cooling to the Food Microbiology Laboratory at the Department of Food Science, Faculty of Agriculture, Ain Shams University. Swabs were directly suspended in 10 mL Maximum Recovery Diluent (MRD), and 100 µL of this suspension was spread on the surface of nutrient agar plates and incubated at 37°C for 24 h (Humphrey et al 1995). Separate colonies were selected and streaked onto nutrient agar plates several times with the aid of light microscopic examination during purification until pure isolates were obtained.

2.2 Preliminary Identification

The morphological characteristics of bacterial colonies and cells were recorded for preliminary identification. In addition, Gram staining, motility, and catalase activity were carried out.

2.3 Quantification of biofilm formation on polystyrene

The commonly used microtiter-plates method for determining bacterial adhesion to plastic surfaces was applied in the present study according to Stepanovic et al (2000). Briefly, the wells of sterile 96-well polystyrene microtiter plates (Minitek, USA) were filled with 230 µL of tryptone soy broth (TSB). A quantity of 20 µL of each culture was added to each well. The negative control wells contained TSB only. The plates were incubated aerobically for 48h at 37°C. The content of the microtiter plates was poured off, and the wells were washed three times with 300 µL of P-buffer. The remaining attached bacteria were fixed with 250 µL of methanol per well. After 15 min, microtiter plates were emptied and air dried. The microtiter plates were stained with 250 µL per well of 1% crystal violet used for Gram staining (Merck, Germany) for 5 min. The excess stain was rinsed off by placing the microtiter plates under tap water. After the microtiter plates were Air dried, the dye bound to the adherent cells was extracted with 250 µL of a mixture (80% ethanol: 20% acetone) per well. The absorbance of each well was measured at 630 nm using an ELISA reader (Mastoor et al 2022). Based on the absorbance (A_{630}) produced by bacterial films, strains were classified into four categories (Christensen et al 1985, Stepanovic et al 2000). Briefly, the cut-off absorbance (Ac) was the mean absorbance of the negative control. Strains were classified as follows: A = Ac = no biofilm producer (0); Ac < A = (2 x Ac) = weak biofilm producer (+); (2 x Ac) < A = (4 x Ac) = moderate biofilm producer (++); (4 x Ac) < A = strong biofilm producer (+++). All tests were carried out in triplicate, and the results were averaged.

2.4 *In vitro* antibiofilm effect of commercial disinfectants

Two disinfectants commonly used in poultry establishments in Egypt were selected based on their mode of action to assess their effectiveness in eradicating established biofilm. These include peroxyacetic acid at a concentration of 0.7% with a 15 min contact time and sodium hypochlorite at 150 ppm for a 15 min contact duration. These disinfectants were tested using the previously mentioned microtiter plate method, with modifications to the washing step followed by incubation of the inoculated strains at 37°C for 48 h. In the phosphate buffer washing step, the process involved washing once with 300 µL of P-buffer, followed by washing with the disinfectant at the specified concentration for a contact period of 15 min. Afterward, the contents of the microtiter plate were discarded, and the walls were rinsed once with 300 µL of P-buffer. The attached bacteria in each well were then fixed with 250 µL of methanol for 15 min, after which the microtiter plates were emptied and air-dried. The experiment continued with the previously described process (Stepanovic et al 2000).

2.5 *In vitro* antibiofilm effect of detergent-disinfectant treatments

A proportion of 300 µL (3% of a commercial alkaline detergent consisting of sodium hydroxide, potassium hydroxide, and surfactant) was applied for 15 min to evaluate the impact of incorporating a cleaning step before applying the previously mentioned disinfectants. The experiment then continued with the same previously described process (Stepanovic et al 2000).

2.6 Partial sequencing of the 16S rRNA gene

Isolates that proved resistant to biofilm removal using the above methods (Parts 2.4 and 2.5) were identified by estimating the partial sequence of 16S rRNA gene**.** Genomic DNA was extracted and purified, and the 16S rRNA gene was amplified using universal primers 518F/800R (Weisburg et al 1991). The PCR reaction, optimized for approximately 10 ng of template DNA, involved initial denaturation, followed by 30 cycles of denaturation, annealing, and extension. The resulting PCR products were purified, sequenced using the BigDye terminator method, and compared to the GenBank database via BLAST (Elhariry et al 2012). The obtained 16S rDNA sequences were then deposited in GenBank, and accession numbers were assigned.

2.7 Statistical analysis

All tests were performed in three replicates, and the arithmetic means ± standard deviation were obtained using Microsoft Excel analysis sheet Mahato (2023).

3 Results and discussion

3.1 Isolation of poultry-related bacteria

The bacterial isolates from a meat and poultry product processing plant (A) were obtained from various surfaces, including deli meat slicers, conveyor belts, marinated chicken handling carts, and workers' hands. **Table 1** indicates the presence of both Gram-positive and Gram-negative bacteria, with most isolates being

Table 1. Some morphological and biochemical characteristics of bacterial isolates from the meat and poultry-product plant (A)

Isolate Code	Sample collection area	Gram stain	Catalase	Motility	Sporul	Cell morphology	Colony characteristics			
							Color	Sparkle	Edge	Highness
OH000	Deli meat slicer	+	+	-	-	Short rods	Creamy	Glossy	Entire margins	Flat
OH001	Marinated chicken handling cart	+	+	+	-	Short rods	Creamy	Glossy	Irregular	Slight raised
OH002	Conveyor belt surface (after wet batter process)	-	+	-	-	Short rods	Creamy	Glossy	Irregular	Slight raised
OH003		+	+	-	-	Short rods	Creamy	Glossy	Entire margins	Raised
OH004	Conveyor belt surface (after wet batter process)	+	+	-	-	Long rods	Creamy	Glossy	Entire margins	Flat
OH005		-	-	+	-	Cocci	Creamy	Glossy	Irregular	Slight raised
OH006	Worker's hand in the area after wet batter process. (Direct contact with no gloves)	+	+	+	+	Rods	Opaque to white	Rough - wrinkled	Irregular	Raised
OH007	Top surface of the conveyor after the blast freezing process	-	+	+	-	Short rods	White	Glossy	Entire margins	Flat
OH008	Automatic filling and weighing machine	+	+	+	-	Short rods	Creamy	Curly	Irregular	Flat
OH009	Cooling conveyor after the product comes out of the oven	+	+	+	+	Rods	White	Matte	Entire margins	Flat
OH010	Surface of the final conveyor belt before the filling and weighing process	+	+	+	-	Short rods	White	Rough	Entire margins	Raised
OH011		-	+	+	-	Short rods	Creamy	Glossy	Entire margins	Raised
OH012	Manual filling the table surface	-	+	-	-	Short rods	White	Rough	Irregular	Raised
OH013	Worker's hand in the area after preparation for frying	+	+	+	-	Short rods	White	Glossy	Entire margins	Raised
OH014	Worker's hand in the area after wet batter process	+	+	+	-	Short rods	Creamy	Glossy	Irregular	Raised
OH015		+	+	+	+	Long rods	White	Glossy	Entire margins	Raised
OH016		-	+	+	-	Short rods	White	Rough	Entire margins	Raised

catalase-positive and exhibiting varied motility and sporulation characteristics. The presence of bacterial isolates on conveyor belts, particularly after the wet batter process, is significant, as these surfaces come into direct contact with semi-processed poultry products, increasing the risk of contamination. Additionally, bacterial isolates detected on workers' hands highlight the importance of personal hygiene in food safety. In general, rod-shaped bacteria were the most common among the isolated bacteria, accounting for 94% of the total.

Poultry-slaughterhouse (B) focuses on the poultry processing environment, including additional contamination sources, such as feather removal machines, slaughtering area walls, and degutting equipment (**Table 2**). The isolates from these facilities are more diverse in terms of morphology and colony characteristics, with a high prevalence of rod-shaped bacteria (91%). Feather removal machines and degutting equipment are critical contamination points, as they handle raw poultry carcasses, making them a primary source of bacterial introduction into the production line. Bacterial isolates on liver-handling surfaces are also noteworthy, as poultry liver is highly susceptible to microbial contamination due to its nutrient-rich environment. The detection of bacterial isolates in these high-risk areas underscores the need for stringent cleaning and disinfection measures to reduce microbial persistence and prevent contamination from spreading throughout the facility.

The isolates from the poultry slaughterhouse (C) were obtained from different locations, such as preparation hangers, breast portioning machines, and conveyor belts after chilling (**Table 3**). Identifying bacterial isolates on breast-cutting machines and filleting knives is of particular concern, as these tools are used in direct contact with meat products. Additionally, the presence of bacterial isolates in the cold-water bath used for rinsing chicken before chilling suggests that waterborne contamination is a potential issue. Since chilling is a critical step in reducing microbial load, the persistence of bacterial isolates in this stage indicates that additional safety measures, such as water treatment and frequent equipment sanitation, are necessary to prevent bacterial survival and growth.

Across all three establishments, bacterial contamination was identified in multiple processing areas, indicating the widespread presence of microbial hazards in poultry production. The differences in bacterial distribution among facilities highlight the variation in contamination sources and risks. The establishment (A) showed contamination in handling and processing areas, establishment B exhibited bacterial presence in slaughter-related equipment, and establishment C revealed contamination in the early processing and chilling stages. The presence of bacterial isolates on workers' hands in multiple locations emphasizes the role of personnel in cross-contamination, reinforcing the importance of proper hygiene practices. Overall, these findings highlight the need for continuous microbial monitoring, effective sanitation protocols, and strict adherence to food safety regulations to ensure the microbiological quality of poultry products. These results provide new insights into the sources of bacterial contamination in poultry processing environments, emphasizing the importance of equipment surfaces and personnel hygiene in maintaining food safety standards in line with previous local and regional studies (Ahmed et al 2021, Abd-Elall et al 2023b, Edris et al 2023, Yhia and Elniema 2024).

3.2 The biofilm-forming ability of isolated bacteria

The biofilm-forming ability of the bacterial isolates obtained from different poultry processing establishments was evaluated based on the absorbance at 630 nm (**Fig 1 to 3**). Data presented in these figures indicated that none of these isolates were classified as non-biofilm producers. The purified isolates were categorized into weak (+), moderate (++), and strong (+++) producers. The results revealed that most of the isolates exhibited biofilm-forming ability, with a considerable proportion classified as moderate and strong biofilm producers (**Fig 1**). The presence of strong biofilm formers in poultry-processing environments poses a potential risk for bacterial persistence and contamination, as biofilms protect cleaning and disinfection measures (Galié et al 2018).

Fig 2 depicts the biofilm-forming ability of bacterial isolates collected from a poultry slaughterhouse (B). This figure allows for a comparison of biofilm formation potential among isolates from a different environment (slaughterhouse B) within the poultry production chain. Notably, a large proportion of the isolates were moderate to strong biofilm producers, which is consistent with previous studies highlighting the ability of bacteria to adapt and form biofilms in food-processing environments (Kumar and Anand 1998). The presence of strong biofilm producers in meat and poultry-processing plants may lead to persistent microbial contamination and increased resistance to conventional cleaning procedures.

Table 2. Some morphological and biochemical characteristics of bacterial isolates from the Poultry-slaughterhouse (B)

Isolate Code	Sample collection area	Gram stain	Catalase	Motility	Sporulation	Cell morphology	Colony characteristics			
							Color	Sparkle	Edge	Highness
OH017	Feather removing machine number 1	+	+	+	+	Rods	Creamy	Dry	Irregular	Flat
OH018	Slaughtering area wall	+	+	-	-	Cocci	Creamy	Glossy	Entire margins	Raised
OH019	Conveyor belt for chicken cages. (Alive)	+	+	+	-	Long rods	White	Glossy	Entire margins	Raised
OH020	Feather removing machine number 2	+	+	+	+	Rods	Opaque to white	Glossy	Irregular	Raised
OH021		+	+	+	+	Long rods	Creamy	Glossy	Entire margins	Slight raised
OH022		+	+	+	-	Long rods	White	Glossy	Entire margins	Raised
OH023	Plastic container for liver	-	+	-	-	Short rods	Creamy	Glossy	Irregular	Slight raised
OH024	Degutting area floor	+	-	+	-	Short rods	White	Glossy	Irregular	Slight raised
OH025	Feather removing machine number 1	-	+	+	-	Short rods	Creamy	Rough	Irregular	Flat
OH026	Liver handling line	+	+	+	+	Long rods	Creamy	Glossy	Entire margins	Raised
OH027	Degutting machine	-	+	-	-	Short rods	Creamy	Glossy	Entire margins	Raised

Table 3. Some morphological and biochemical characteristics of bacterial isolates from the poultry-slaughterhouse (C)

Isolate Code	Sample collection area	Gram stain	Catalase	Motility	Sporulation	Cell morphology	Colony characteristics			
							Color	Sparkle	Edge	Highness
OH028	Liver handling line	-	+	+	-	Short rods	Creamy	Glossy	Entire margins	Slight raised
OH029		+	+	+	-	Short rods	Creamy	Glossy	Entire margins	Flat
OH030	Preparation hangers	+	+	+	-	Short rods	White	Glossy	Irregular	Slight raised
OH031		+	+	+	-	Short rods	White	Glossy	Entire margins	Flat
OH032	Degutting Machine	+	+	+	-	Short rods	Creamy	Glossy	Irregular	Slight raised
OH033		+	+	-	-	Short rods	Creamy	Glossy	Irregular	Raised
OH034	Head removing machine	+	+	+	-	Short rods	White	Glossy	Irregular	Flat
OH035	Wall of preparation area	+	+	+	+	Long rods	Creamy	Curly	Irregular	Flat
OH036		+	+	+	+	Rods	Creamy	Rough	Irregular	Flat
OH037	Wing straightening machine to assist in the separation process	+	+	+	-	Short rods	Creamy	Glossy	Entire margins	Raised
OH038		+	+	+	-	Short rods	White	Rough	Irregular	Raised
OH039	Breast portioning machine	+	+	+	-	Short rods	Creamy	Glossy	Irregular	Raised
OH040	Conveyor belt after the Chiller	+	+	-	-	Short rods	Creamy	Dry	Irregular	Flat
OH041		+	+	+	-	Short rods	Creamy	Glossy	Irregular	Raised
OH042	Filleting knife used after the chiller	+	+	+	-	Short rods	Creamy	Glossy	Irregular	Slight raised
OH043	Cold water bath to rinse chicken before entering the chiller	+	+	+	-	Short rods	Creamy	Glossy	Irregular	Slight raised

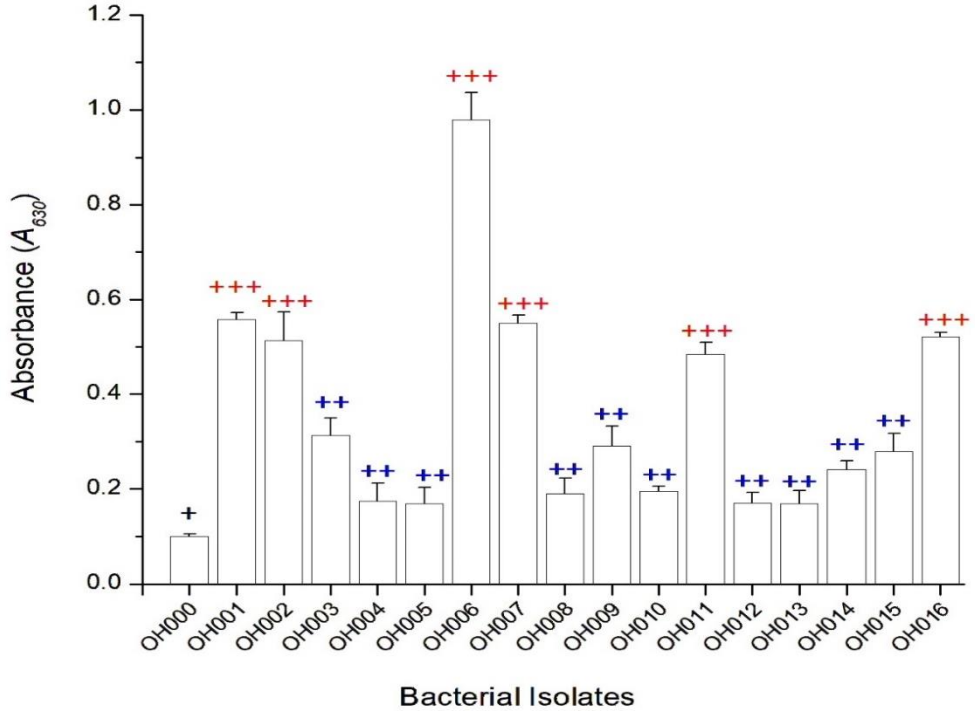

Fig 1. Biofilm-forming ability of different bacterial isolates obtained from the meat and poultry-product plant (A). The isolates were categorized as weak (+), moderate (++), and strong biofilm producers (+++). Absorbance was measured at 630 nm and presented as mean (*n=3*)±SD

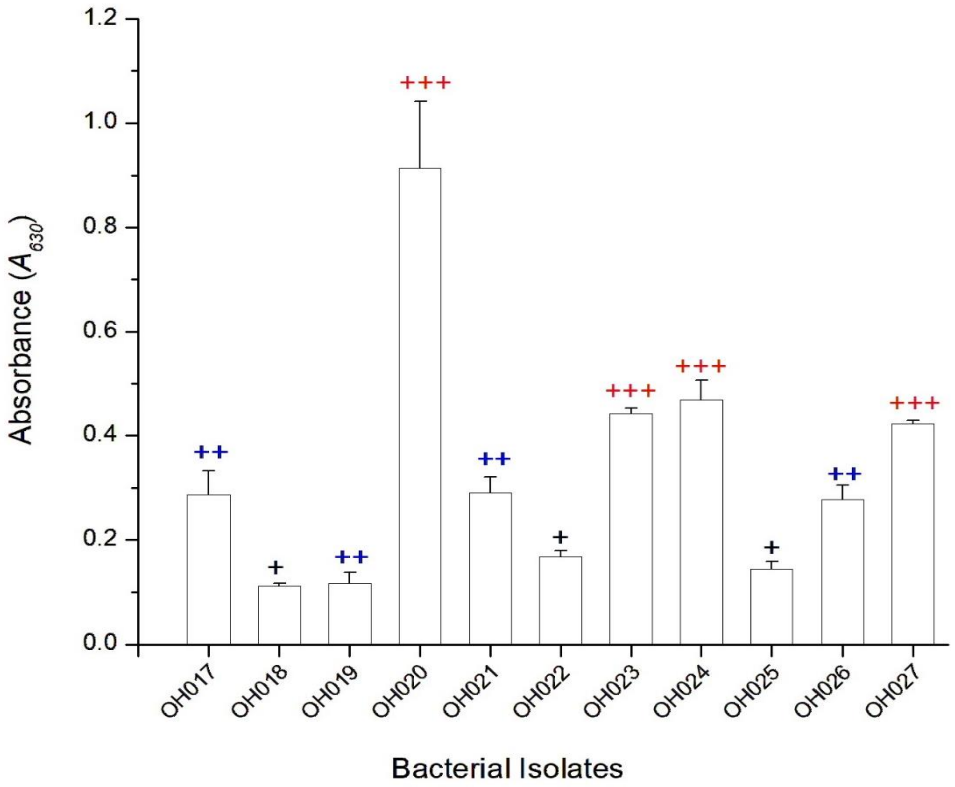

Fig 2. Biofilm-forming ability of different bacterial isolates obtained from the poultry slaughter-house (B). The isolates were categorized as weak (+), moderate (++), and strong biofilm producers (+++). Absorbance was measured at 630 nm and presented as mean (n=3)±SD.

Bacterial isolates from a poultry slaughterhouse (C) were also assessed for their biofilm-forming ability (**Fig 3**). The results indicated a predominance of moderate and strong biofilm producers, highlighting the ability of bacteria to establish resilient communities in slaughterhouse environments. Previous studies have shown that slaughterhouses provide favorable conditions for biofilm formation due to the presence of organic residues and moisture, which enhance bacterial adhesion and survival (Chmielewski and Frank 2003).

Table 4 presents the distribution of biofilm-forming bacterial isolates across different poultry establishments. Among the 44 isolates examined, none were classified as non-producers, indicating that all isolates possessed some degree of biofilm-forming ability. Weak biofilm producers were found in low numbers (9%), while most isolates (59%) were classified as moderate biofilm producers. Strong biofilm producers constituted 32% of the isolates, highlighting a large proportion of bacteria with robust biofilm-forming capacity.

The highest proportion of moderate biofilm producers was observed in poultry slaughterhouse C, where 75% of the isolates exhibited moderate biofilm-forming ability. This finding aligns with a previous study indicating that poultry slaughterhouses provide an ideal environment for bacterial colonization and biofilm development due to the presence of organic material and protein-rich residues (Carpentier and Cerf 1993). Poultry slaughterhouse (B) had the highest proportion of strong biofilm producers (36%), suggesting that certain processing conditions may favor the persistence of highly resilient bacterial populations.

The absence of non-producers suggests that the conditions in the poultry processing environment may favor the selection or survival of biofilm-forming bacteria. The presence of strong biofilm producers in poultry processing facilities is a significant concern for food safety. Biofilms can protect bacteria from disinfectants and antimicrobial agents, increasing the risk of contamination and transmission of foodborne pathogens (Bridier et al 2011, Pang et al 2023). Moreover, biofilms facilitate horizontal gene transfer, potentially enhancing antibiotic resistance among bacterial populations in food-processing environments (Gebreyohannes et al 2019). These findings underscore the need for effective sanitation protocols and biofilm-targeting control measures to mitigate microbial risks in poultry establishments.

3.3 Effect of standalone disinfectants on bacterial biofilm eradication from poultry environments

The efficacy of two common commercial disinfectants - peroxyacetic acid (PAA, 0.7%) and sodium hypochlorite (150 ppm) - against biofilm-forming bacteria isolated from poultry environments was evaluated. Twenty-one bacterial isolates demonstrating moderate to strong biofilm formation capacity were assessed using a quantitative microtiter plate method (**Table 5**).

Table 5 reveals that neither peroxyacetic acid (0.7%) nor sodium hypochlorite (150 ppm) alone demonstrated substantial efficacy against most bacterial biofilms when compared to the control. Of the 21 isolates tested, 18 (85.7%) maintained moderate (++) biofilm production capacity after peroxyacetic acid treatment, while 14 (66.7%) maintained moderate biofilm production after sodium hypochlorite treatment.

Notably, isolates OH006 and OH020 exhibited strong biofilm-forming capabilities (+++) that remained unaffected by either disinfectant. Conversely, isolate OH021 showed partial susceptibility, with reduced biofilm formation following peroxyacetic acid treatment (+) and complete eradication after sodium hypochlorite exposure (-).

Sodium hypochlorite demonstrated slightly superior efficacy compared to peroxyacetic acid, with four isolates (OH014, OH028, OH037, and OH041) showing reduced biofilm formation (+) after treatment. This aligns with the findings by Byun et al (2021), who reported that sodium hypochlorite exhibits enhanced penetration capability into bacterial biofilms compared to peracetic acid-based compounds.

3.4 Effect of Detergent-Disinfectant Combinations on Bacterial Biofilm Eradication from Poultry Environments

The data in **Table 6** demonstrate markedly improved efficacy when the commercial alkaline detergent (3%) was combined with either disinfectant. The combination of the alkaline detergent with sodium hypochlorite (150 ppm) showed superior efficacy, eliminating biofilm formation completely in 10 isolates (47.6%), compared to the alkaline detergent with peroxyacetic acid combination, which eliminated biofilm formation in only one isolate (4.8%). These findings corroborate the observations of Nguyen et al (2020), who demonstrated that the sequential application of detergents and disinfectants significantly enhances biofilm removal efficacy through the disruption of the extracellular polymeric substance (EPS) matrix by detergents, facilitating subsequent penetration of disinfectants.

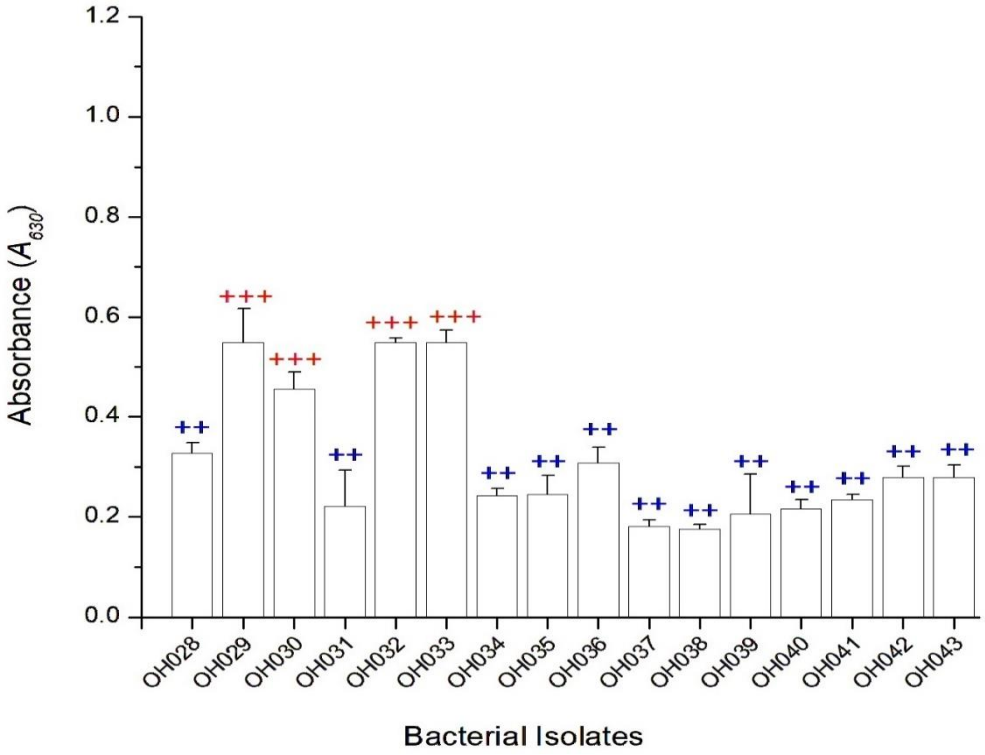

Fig 3. Biofilm-forming ability of different bacterial isolates obtained from the poultry slaughterhouse (C). The isolates were categorized as weak (+), moderate (++), and strong biofilm producers (+++). Absorbance was measured at 630 nm and presented as mean (n=3) ±SD.

Table 4. Number and percentage of biofilm-producing bacteria in different poultry establishments

Biofilm category	Meat and Poultry-product plant (A)		Poultry slaughterhouse (B)		Poultry slaughterhouse (C)		Total Isolates	
	No.	%	No.	%	N0.	%	No.	%
Non-producer	0	0	0	0	0	0	0	0
Weak producer	1	6	3	27	0	0	4	9
Moderate producer	10	59	4	36	12	75	26	59
Strong producer	6	35	4	36	4	25	14	32
Total	17		11		16		44	100

Particularly noteworthy was the persistent resistance exhibited by isolates OH006 and OH020, which maintained strong biofilm formation (+++) despite exposure to both combination treatments. Also, isolates OH009 and OH017 demonstrated moderate biofilm formation (++) even after exposure to either combination treatment. Moreover, four isolates (OH001, OH024, OH028, and OH032) still resist complete eradication by the tested cleaning and disinfecting protocols (**Table 6**). This finding indicated a strain-specific resistance pattern that warrants further investigation. The observed resistance patterns align with the growing concern regarding biofilm-mediated antimicrobial resistance in food production environments (Uruén et al 2021). The persistence of biofilm formation capacity in isolates OH006 and OH020 despite exposure to multiple disinfection protocols represents a significant challenge for biosecurity measures in poultry production settings.

The enhanced efficacy of detergent-disinfectant combinations, particularly with sodium hypochlorite, supports the findings of Maillard and Centeleghe (2023), who demonstrated that multi-step sanitation protocols targeting both the structural and cellular components of biofilms yield superior results. Alkaline detergent likely disrupts the biofilm matrix structure, allowing the subsequent disinfectant to access and eliminate bacterial cells more effectively.

Table 5. Effect of commercial disinfectants on eradicating biofilm* formed by bacteria isolates from poultry environment

Isolate Code	Control			Peroxyacetic acid 0.7%			Sodium hypochlorite (150 ppm)		
	Category	A_{630}	±SD	Category	A_{630}	±SD	Category	A_{630}	±SD
OH001	+++	0.458	0.014	++	0.223	0.100	++	0.222	0.020
OH003	++	0.313	0.037	++	0.167	0.071	++	0.307	0.002
OH006	+++	1.078	0.198	+++	0.508	0.261	+++	0.781	0.255
OH007	+++	0.455	0.017	++	0.244	0.09	++	0.258	0.011
OH009	++	0.227	0.063	++	0.211	0.103	++	0.206	0.019
OH011	+++	0.384	0.025	++	0.203	0.081	++	0.273	0.027
OH014	++	0.201	0.018	++	0.153	0.022	+	0.112	0.006
OH017	++	0.286	0.047	++	0.255	0.151	++	0.232	0.112
OH020	+++	0.914	0.127	+++	0.671	0.386	+++	0.607	0.171
OH021	++	0.221	0.029	+	0.112	0.037	-	0.072	0.004
OH023	+++	0.443	0.010	++	0.15	0.015	++	0.260	0.013
OH024	+++	0.468	0.038	++	0.209	0.015	++	0.181	0.013
OH028	++	0.327	0.021	++	0.179	0.053	+	0.108	0.036
OH032	+++	0.481	0.009	++	0.159	0.056	++	0.306	0.025
OH033	+++	0.487	0.025	++	0.217	0.125	++	0.211	0.009
OH034	++	0.242	0.014	++	0.208	0.054	++	0.213	0.019
OH037	++	0.180	0.013	++	0.112	0.057	+	0.118	0.009
OH039	++	0.205	0.080	++	0.176	0.09	++	0.19	0.012
OH041	++	0.234	0.011	++	0.182	0.036	+	0.126	0.023
OH042	++	0.278	0.023	++	0.207	0.018	++	0.186	0.012
OH043	++	0.278	0.026	++	0.178	0.036	++	0.185	0.023

*Biofilm was formed on tryptic soy broth (TSB) at 37°C for 48h. Peroxyacetic acid (0.7%) or sodium hypochlorite (150 ppm) was used for washing instead of phosphate buffer (control). The isolates were categorized as no (-), weak (+), moderate (++), and strong biofilm producers (+++) according to their absorbance at 630 nm (A_{630}). Values are presented as the mean of three replicates ($n=3$) ±SD.

Table 6. Effect of commercial detergent and disinfectants on eradicating biofilm* formed by bacteria isolates from poultry environment

Isolate Code	Control			Alkaline detergent 3% Peroxyacetic acid 0.7%			Alkaline detergent 3% Sodium hypochlorite 150 ppm		
	Category	A_{630}	±SD	Category	A_{630}	±SD	Category	A_{630}	±SD
OH001	+++	0.458	0.014	+	0.082	0.005	+	0.096	0.025
OH003	++	0.313	0.037	+	0.083	0.007	-	0.077	0.003
OH006	+++	1.078	0.198	+++	0.397	0.171	+++	0.39	0.037
OH007	+++	0.455	0.017	+	0.076	0.001	-	0.078	0.003
OH009	++	0.227	0.063	++	0.324	0.064	++	0.252	0.04
OH011	+++	0.384	0.025	+	0.080	0.004	-	0.076	0.002
OH014	++	0.201	0.018	+	0.076	0.002	-	0.079	0.004
OH017	++	0.286	0.047	++	0.281	0.034	++	0.186	0.023
OH020	+++	0.914	0.127	+++	0.5	0.157	+++	0.392	0.147
OH021	++	0.221	0.029	-	0.069	0.002	-	0.073	0.004
OH023	+++	0.443	0.010	+	0.083	0.002	-	0.078	0.002
OH024	+++	0.468	0.038	+	0.079	0.001	+	0.116	0.005
OH028	++	0.327	0.021	+	0.083	0.008	+	0.085	0.003
OH032	+++	0.481	0.009	+	0.078	0.002	+	0.091	0.001
OH033	+++	0.487	0.025	+	0.084	0.005	-	0.083	0.005
OH034	++	0.242	0.014	+	0.074	0.003	-	0.082	0.003
OH037	++	0.180	0.013	+	0.007	0.0	-	0.077	0.002
OH039	++	0.205	0.080	+	0.079	0.005	-	0.074	0.003
OH041	++	0.234	0.011	+	0.081	0.009	-	0.072	0.003
OH042	++	0.278	0.023	+	0.085	0.009	-	0.078	0.01
OH043	++	0.278	0.026	+	0.081	0.004	-	0.077	0.005

*Biofilm was formed on tryptic soy broth (TSB) at 37°C for 48h. Peroxyacetic acid (0.7%) or sodium hypochlorite (150 ppm) was used for washing instead of phosphate buffer (control). The isolates were categorized as no (-), weak (+), moderate (++), and strong biofilm producers (+++) according to their absorbance at 630 nm (A_{630}). Values are presented as the mean of three replicates ($n=3$) ±SD.

The variable susceptibility patterns observed among isolates suggest that standardized disinfection protocols may be insufficient for comprehensive biofilm management in poultry environments. This heterogeneity in resistance profiles necessitates tailored approaches based on the specific bacterial communities present, as emphasized by recent metagenomic studies (Caraballo Guzmán et al 2020).

3.5 Identifying the isolates resistant to the complete removal of the biofilm

Overall, the 48-hour biofilm formed by 8 of the 21 samples (38%) resisted complete removal by the cleaning and disinfection protocol used in the current study. These isolates were identified by determining the partial sequence of the 16S rRNA gene. The sequence was obtained, processed, and submitted to the NCBI GenBank, and the accession number for each strain was obtained for each of the eight isolates (**Table 7**). These strains were identified as four strains belonging to the genus *Bacillus*, three to the genus *Listeria*, and one to the genus *Alcaligenes*. The observation that 38% of isolates from meat and poultry processing environments formed biofilms resistant to standard cleaning and disinfection protocols highlights a significant challenge to food safety. Specifically, the identification of *Bacillus*, *Listeria*, and *Alcaligenes* species within these persistent biofilms raises concerns, as these genera are known for their ability to form robust biofilms and harbor virulence factors. *Bacillus* spp., for example, are frequently associated with spoilage and can produce heat-resistant spores, making them difficult to eradicate (Velusamy et al 2015, Haque et al 2022). Similarly, *Listeria monocytogenes*, a known pathogen, is notorious for its biofilm-forming capabilities and persistence in food processing environments, posing a risk of listeriosis outbreaks (Rodrigues et al 2010, Osaili et al 2021). The presence of *Alcaligenes*, while less frequently associated with foodborne illness, indicates a broader issue of bacterial resilience within these environments, as some species have been shown to develop resistance to disinfectants (Tong et al 2021) The resistance of these biofilms underscores the need for enhanced cleaning and disinfection strategies, potentially incorporating novel antimicrobial agents or physical methods, to ensure the effective removal of persistent microbial contaminants and mitigate risks in meat and poultry processing.

Table 7. Molecular identification of the most based on BLAST comparison to the GeneBank database

Isolate Code	Identified Bacteria	Strain	Accession No.	Closest Relative Accession No.	Sequence Identity (%)
OH001	*Listeria* sp.	OH001a	PV276811	NR_043518.1	97.42
OH006	*Bacillus* sp.	OH006a	PV276812	NR_165685.1 NR_137407.1 NR_042338.1	98.56
OH009	*Bacillus* sp.	OH009a	PV276813	NR_116187.1 NR_116188.1 NR_116186.1	98.54
OH017	*Bacillus* sp.	OH017a	PV276814	NR_112116.2	97.63
OH020	*Bacillus* sp.	OH020a	PV276815	NR_165685.1	96.65
OH024	*Listeria* sp.	OH024a	PV276816	NR_116805.1	97.26
OH028	*Alcaligenes* sp.	OH028a	PV276817	NR_113606.1	96.40
OH032	*Listeria* sp.	OH032a	PV276818	NR_043518.1	97.27

4 Conclusion

Bacterial biofilms present a significant food safety challenge in poultry processing environments, where 44 isolates exhibited biofilm-forming ability (59% moderate, 32% strong producers). Conventional disinfectants (0.7% peroxyacetic acid and 150 ppm sodium hypochlorite) showed limited efficacy, with 85.7% and 66.7% of isolates maintaining biofilm production after respective treatments.

The combination of 3% alkaline detergent with sodium hypochlorite demonstrated superior efficacy, completely eliminating biofilm formation in 47.6% of isolates. However, 38% of isolates exhibited persistent resistance to all tested protocols.

Molecular identification using 16S rRNA gene sequencing revealed that these resistant strains belonged primarily to the *Bacillus* and *Listeria* genera, with one *Alcaligenes* species also identified. This strain-specific resistance pattern represents a significant challenge for biosecurity in poultry production.

These findings emphasize the necessity for sophisticated, multi-step sanitation protocols targeting both structural and cellular components of the biofilms. The variable susceptibility patterns suggest standardized disinfection protocols may be insufficient, necessitating the development of novel anti-biofilm strategies tailored to these resilient bacterial communities to protect poultry product quality and public health.

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