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Hygienic Studies on Biofilms in Drinking Water Systems in Poultry Farms: Isolation, Molecular Identification, and Antibiotic Sensitivity

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Abstract | In poultry production, drinking water must be free from pathogens that pose a risk for infection. Biofilms are significant contributors to water contamination with pathogens, and aid in the genetic exchange among bacterial populations that cause antibiotic resistance. The study included four-layer chicken houses receiving the same water source: A growing pullet house with iron water pipes and three production layer houses with polyvinyl chloride (PVC) water pipes. Biofilm samples were collected, during February 2020, by swabbing the inner surfaces of drinking water pipes. The heterotrophic bacterial counts were determined. Afterwards, colonies were purified and molecularly identified using the 16S rRNA by PCR test. A total of 31 antimicrobials were used for antibiotic sensitivity testing of the bacterial isolates. In the PVC pipes, more bacterial densities were found than in the iron pipes (2×10^{19} and 2×10^{12} colony forming units/ml, respectively). *Pseudomonas*, *Enterococcus*, *Staphylococcus*, and *Sphingopyxis* were identified from iron pipes, while *Acinetobacter*, *Pseudomonas*, and *Bacillus* were confirmed from PVC pipes. Multidrug resistance to at least three antibiotic groups was identified in 67% of the isolates. *Staphylococcus*, *Enterococci*, *Sphingopyxis*, *Bacillus*, and *Acinetobacter* were found to be originated from water sources highly contaminated with antibiotics overuse. While all *Pseudomonas* strains originated from water environments free from antibiotics contamination. In terms of bacterial density and antibiotic resistance patterns, biofilms possess a significant role in harboring and disseminating pathogenic strains leading to production problems in poultry. So, programs for the prevention and control of biofilm buildup in poultry drinking systems are required.

Keywords | Layer chicken; Iron and PVC water pipes; biofilm; *Enterococcus spp.; Staphylococcus saprophyticus.*

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INTRODUCTION

Improving the quality of drinking water by reducing biofilms is becoming increasingly important in poultry production. Biofilms consisted of aggregates of bacterial cells impeded in an extracellular matrix of their metabolites and pose regulatory cell–cell interaction networks (Lianou et al., 2020). *Pseudomonas*, *Acinetobacter*, *Sphingomonas*, and *Klebsiella* are pathogenic bacteria for poultry and were isolated from the biofilm of the drinking water systems of some poultry farms (Maes et al., 2019). *Sphingopyxis terrae* and *Pseudomonas aeruginosa* were found to be potent biofilm producers (Labella et al., 2021).

Several water systems are prone to biofilm formation. Bacterial cells adhere to surfaces of pipes and form biofilms rather than being found free in the water. A biofilm may act as a shelter against harsh environmental conditions for

pathogens inside a drinking water system (Szewzyk et al. 2000). Biofilm is commonly formed in nutrient-poor water pipes and is hard to be removed. The formation of the biofilm in the drinking water systems is influenced by the type of pipe surface, types of bacteria in water, available nutrients, temperature, and the system hydraulics (Lehtola et al., 2004).

Water is an essential nutrient for poultry. Biofilm could induce blockage of the water system and, subsequently, impair the adequacy of water flow as well as the flock performance (Fairchild and Ritz, 2009). The resistance of organisms in biofilm increases toward disinfection and medication, so the biofilm becomes a repository for the continuous dissemination of bacteria within the water flow to form biofilms in other parts of the pipelines and spreading pathogenic bacteria along the drinking line. In many cases, the aesthetic properties of drinking water are negatively affected by biofilm organisms. When biofilms are formed on surfaces of iron pipes, corrosion of iron pipes can occur, which lead to metal particles being detached into drinking water (Camper et al., 1998; September et al., 2007; Hoiby et al., 2010).

In the past few decades, extensive use of antibiotics for medication and promoting growth in veterinary sector has caused an upsurge in the emergence of multidrug-resistant (MDR) bacteria (Page and Gautier, 2012; Boeckel et al., 2015). Horizontal transfer of antibiotic resistance genes (ARGs) between antibiotic-resistant bacteria (ARBs) and environmental bacteria was found to be facilitated in water environments (Martinez, 2012; Labella et al., 2021). Resistance genes could be maintained and spread by those environmental resistant bacteria (Taylor et al., 2011). Thus, veterinary and public health risks may arise from acquiring ARGs through consuming drinking water obtained from such environments (Wellington et al., 2013). The implication of biofilm formation and the occurrence of multiple antibiotic-resistant bacteria have become a critical problem for veterinary and public health (Oliveira et al., 2010).

Multidrug-resistant bacteria are prevalent in poultry, poultry products, carcasses, litter, and fecal matter. Many studies reported the evolution of antibiotic-resistant bacteria in the poultry production sector, such as *Escherichia coli* (Tadesse et al., 2012), *Campylobacter* (Richter et al., 2015), and *Staphylococcus* (Bortolaia et al., 2016). A study on 337 strains of *Salmonella Pullorum* from China revealed that resistance to cefamandole, trimethoprim, and co-trimoxazole was higher for biofilm-forming bacteria when compared to the non-biofilm ones (Gong et al., 2013). A positive correlation was observed between antibiotic resistance and the biofilm-producing capability of the bacteria (Zhang et al., 2017). Vancomycin-resistant *Enterococcus faecium* was isolated from farm animals and surface water (Iversen et al., 2002), as well as, from several human hosts (Novais et al., 2006). Biofilm lining the drinking water system has mixtures of bacteria, nutrients, and antimicrobial agents (Hirsch et al., 1999), which could help resistant bacteria and gene transfer within the bacterial population. Bacterial biofilms showed resistance to several types of antibiotics either by mutations or the acquisition of foreign DNA (Hoiby et al. 2010). The control of biofilms will, therefore, improve bird health and minimize antibiotic treatments (Linden, 2014).

The current study aimed to isolate and identify biofilm bacteria found in the drinking water systems of layer chicken farms. Moreover, the study examined the effectiveness of different antibiotics in combating isolated bacteria inside poultry production facilities as an integral part of overall prevention and control measures.

MATERIALS AND METHODS

Bird houses

The present study was conducted at a shaver chicken layer farm in Ayyat (Giza), Egypt, in February 2020. The farm included one growing pullet house (15 weeks age; 7500 birds/house) and three egg-producing layer houses (69 weeks age; 30,000 birds/house). Pullets were raised in the growing house for 15 weeks before being transferred to the production houses. Growing pullet house was equipped with iron water pipelines with a diameter of one inch and bell drinkers. All three production houses were equipped with Polyvinyl chloride (PVC) water pipelines (1-inch diameter) and automatic drinking systems with drinking nipples. Houses were all closed with fully controlled environments. No treatment or sanitization was performed on underground water before it was used for birds. The average daily water consumption rates were 1300 and 7000 liters/house for growing and production houses, respectively. Drinking water was supplemented with vitamin E and selenium 4-weeks before sampling. The performance of both the growing and production houses was suboptimal throughout the production cycle (Tables 1- 2).

Sampling of biofilms from water pipelines

A total of eight biofilm samples were collected from the water systems of the four-layer chicken houses. Two samples were collected from the iron pipelines of the growing house (Biofilm A), and six samples were collected from the PVC pipelines of the three production houses (Biofilm B). Sterile cotton swabs, moistened with sterile normal saline, were used to scrape biofilm from the inner surface of the pipelines. Swabs were collected in sterile conical flasks and transferred to the laboratory in an icebox within four hours before being examined (Baird et al., 2017; Lin et al., 2015).

Table 1: Mean body weights of growing pullets compared with target body weights (g)

Age (weeks)	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th
Body weights (g)															
Target	60	120	190	275	360	450	540	630	720	810	900	1000	1095	1180	1265
Actual	56	110	179	254	337	421	499	588	668	755	839	928	1020	1095	1178
Change % [a]	-6.7	-8.3	-5.8	-7.6	-6.4	-6.4	-7.6	-6.7	-7.2	-6.8	-6.8	-7.2	-6.8	-7.2	-6.9

[a] Percentage of change between the actual and target body weights.

Table 2: Weekly egg production % in the producing layer houses compared with target egg production%

	Egg production%				Egg production%		
Age (weeks)	Target	Actual	Change %[a]	Age (weeks)	Target	Actual	Change %
17	0	0	0	44	91	78	-14.3
18	0	0	0	45	91	78	-14.3
19	12	4	-66.7	46	91	78	-14.3
20	32	12	-62.5	47	91	77	-15.4
21	62	25	-59.7	48	90	77	-14.4
22	85	54	-36.5	49	90	77	-14.4
23	93	79	-15.1	50	90	77	-14.4
24	94	83	-11.7	51	89	75	-15.7
25	95	85	-10.5	52	89	75	-15.7
26	96	86	-10.4	53	88	75	-14.8
27	96	85	-11.5	54	88	75	-14.8
28	96	84	-12.5	55	87	74	-14.9
29	96	84	-12.5	56	87	74	-14.9
30	95	84	-11.6	57	86	73	-15.1
31	95	84	-11.6	58	85	72	-15.3
32	95	85	-10.5	59	85	72	-15.3
33	94	84	-10.6	60	84	71	-15.5
34	94	84	-10.6	61	84	72	-14.3
35	94	83	-11.7	62	83	70	-15.7
36	94	84	-10.6	63	83	70	-15.7
37	93	82	-11.8	64	82	70	-14.6
38	93	82	-11.8	65	82	70	-14.6
39	93	82	-11.8	66	81	70	-13.6
40	92	81	-12.0	67	81	70	-13.6
41	92	82	-10.9	68	80	70	-12.5
42	92	81	-12.0	69	80	71	-11.3
43	92	81	-12.0	-	-	-	-

[a] Percentage of change between actual and target weekly egg production %.

Heterotrophic count and isolation of biofilm bacteria

The heterotrophic bacterial count of biofilm samples was done following the standard protocols of the American Public Health Association's (Baird et al., 2017). Briefly, 1-ml from the collected samples were serially diluted in 9 ml sterile saline solution and then 0.1 ml from each dilution were spread on plate count agar plates and incubated for 24 hours at 37°C to allow bacterial growth.

For bacterial isolation, microbial enrichment was performed by inoculating 1ml from each biofilm sample into a nutrient broth medium with the following ingredients (g/l): 3, Beef Extract; 5, Peptone and 5, NaCl, and then incubated at 37°C for 24 hours with shaking at 150 rpm (Gehring et al., 2012). Bacterial isolation and purification were done by streaking on nutrient agar plates (Sanders, 2012). Suspected colonies were picked up and transferred to sterile nutrient agar plates to check purity. Then purified

colonies were preserved in slant tubes at 4°C and in Cryotubes containing 50% glycerol in cell bank at -85°C.

Molecular identification and sequencing of biofilm bacteria

All DNA of bacterial isolates were extracted using Quick-DNA™ Fungal/Bacterial Microprep Kit (Zymo research #D6007) according to the manufacturer's protocol. For molecular identification of bacterial isolates, ribosomal 16S rRNA genes were amplified using the universal bacterial primers NVZ-1 (forward; 5′-GCGGATCCGCGGC-CGCTGCAGAGTTTGATCCTGGCTCAG-3′) and NVZ-2 (reverse; 5′-GGCTCGAGCGGCCGCCCGG-GTTACCTTGTTACGACTT-3′) (Lopez et al., 2006). The protocol for 16S rRNA genes amplification and sequencing were performed by Sigma Scientific Services Co. (http://sigmaeg-co.com/) (Tables 3 – 4). PCR products clean up were performed using GeneJET™ PCR Purification Kit (Thermo K0701), according to the manufacturer's instructions.

Table 3: Components of the PCR reaction

Ingredients	Quantities
Maxima® Hot Start PCR Master Mix (2X)	25µl
ITS1 Forward primer	1µl (20µM)
ITS4 primer	1µl(20µM)
Template DNA	5µl
Water, nuclease-free	18µl
Total volume	50µl

Table 4: The recommended PCR thermal cycling conditions:

Steps	Temperature (°C)	Time	Number of cycles
Initial denaturation / enzyme activation	95	10 min	1
Denaturation	95	30 s	35
Annealing	57	1min	
Extension	72	1 min30s	
Final Extension	72	10 min	1

The amplified 16S rRNA fragments of bacterial isolates were sequenced at GATC Company using ABI 3730xl DNA sequencer by using forward and reverse primers. The NCBI BLAST website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to get the most closely similar 16S rRNA gene sequences.

Antibiotic sensitivity of biofilm bacterial isolates

The antibiotic sensitivity of the biofilm bacterial isolates was assessed using a total of 31 antimicrobials representing 14 antibiotic groups: Aminoglycosides, Carbapenem, Cephalosporins, Piperacillin-Tazobactam, Fluoroquinolones, Monopactam, Penicillins, Sulfa, Nitrofurantoin, Macrolides, Lincosamides, Glyco-peptides, Tetracycline, and Anti-TB & Leprosy. Disc diffusion method was conducted using antibiotic-loaded discs (Sigma Aldrich, Egypt) (Bonev et al., 2008). Results were interpreted following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020).

For all biofilm isolates, the Multiple Antibiotic Resistance Index (MARI) was estimated. A value above 0.2 indicates that the bacteria came from a potentially contaminated water source where antibiotics are commonly applied. Rates smaller than or equal to 0.2 refer to bacteria from water environments where antibiotic use is rare or nonexistent (Titilawo et al., 2015; Labella et al., 2021).

MARI= the number of antibiotics the bacterial isolate was resistant to ÷ the total number of antibiotics the isolate was tested against.

RESULTS

Bird performance

The growing pullet house displayed lower performance than what should be in the correspondent ages. Throughout the growing period, the average body weights were suboptimal. The differences from optimal weights ranged from -5.8% to -8.3% (Table 1). Furthermore, the production houses showed suboptimal egg production % throughout the production cycle. The change % from the optimal egg production levels ranged from -66.7% to -10.4% (Table 2).

Biofilms heterotrophic bacterial count and isolates

In the iron water pipes (sample A), the biofilm samples were rusty and brownish in color. Counting heterotrophic bacteria revealed a density of 2×10^{12} CFU/ml. Based on sequencing, the following isolates were identified: *Staphylococcus saprophyticus* (MW192643), *Enterococcus faecalis* (MW192866), *Enterococcus casseliflavus* (MW192868), *Pseudomonas aeruginosa* (MW192780), and *Sphingopyxis terrae* (MW192898) (Table 5).

In the PVC water pipes (sample B), the biofilm samples were blackish in color. Counting heterotrophic bacteria revealed a density of 2×10^{19} CFU/ml. Based on sequencing, the following isolates were identified: two isolates of *Pseudomonas aeruginosa* (MW193120 and MW035344), *Bacillus luti* (MW193092), and *Acinetobacter kookii* (MW193079) (Table 6).

Table 5: Heterotrophic bacterial count and isolates of the iron water pipe biofilm (A).

Biofilm isolates	Iron water pipe biofilm (A)	Accession no.*
Heterotrophic bacterial count (CFU/ ml)	2×10^{12}	-
Staphylococcus saprophyticus	1	MW192643
Enterococcus faecalis	1	MW192866
Enterococcus casseliflavus	1	MW192868
Pseudomonas aeruginosa	1	MW192780
Sphingopyxis terrae	1	MW192898

CFU: Colony-forming unit; * Gene bank accession number (identification based on 16S rRNA).

Table 6: Heterotrophic bacterial count and isolates of the PVC water pipe biofilm (B).

Biofilm isolates	PVC water pipe biofilm (B)	Accession no.*
Heterotrophic bacterial count (CFU/ ml)	2×10^{19}	-
Bacillus luti	1	MW193092
Pseudomonas aeruginosa	2	MW193120, MW035344
Acinetobacter kookii	1	MW193079

CFU: Colony-forming unit; * Gene bank accession number (identification based on 16S rRNA).

Antibiotic sensitivity of biofilm isolates

Table (7) displays bacterial isolates' sensitivity to antibiotics. In the iron water pipes (sample A), *P. aeruginosa* showed susceptibility to all antibiotics tested, while *Staph. saprophyticus*, *E. faecalis*, *E. casseliflavus*, and *S. terrae* showed varied resistance patterns. The *Staphylococcus saprophyticus* strain was resistant to cefpodoxime, penicillin, erythromycin, clindamycin, and doxycycline. Both *E. faecalis* and *E. casseliflavus* showed resistance to erythromycin and doxycycline. Additionally, we noticed rifampin resistance in *E. faecalis*, and gentamicin resistance in *E. casseliflavus*. *Sphingopyxis terrae* showed resistance to amikacin, doripenem, ceftriaxone, cefpodoxime, cefotaxime, cefepime, nalidixic acid, aztreonam, trimethoprim-sulfamethoxazole, and doxycycline.

In biofilm isolates of PVC water pipes (sample B), one *P. aeruginosa* isolate (MW193120) was sensitive to all tested antibiotics, while the other three isolates (*P. aeruginosa*-MW035344, *Bacillus luti*, and *Acinetobacter kookii*) displayed different levels of resistance. The *B. luti* strain was resistant to ceftazidime, cefpodoxime, cefepime, penicillin, and rifampin. The *P. aeruginosa* strain (MW035344) was only resistant to norfloxacin. The *Acinetobacter kookii* strain was resistant to ceftriaxone, cefepime, and trimethoprim-sulfamethoxazole (Table 7).

Sixty-seven percent of the isolates showed resistance to at least three antibiotic groups (Table 7). Multidrug resistance was primarily observed in *Staph. saprophyticus*, *E. faecalis*, *E. casseliflavus*, and *S. terrae* isolated from iron pipes biofilms (sample A), and *Bacillus luti*, and *Acinetobacter kooki* isolated from PVC pipes biofilms (sample B).

The MARI index was used to trace the source of antibiotic resistance of the isolates. Using the Krumperman (1983) criteria, the isolated strains of *Staph. saprophyticus*, *E. faecalis*, *E. casseliflavus*, and *S. terrae* isolated from iron pipes, as well as *Bacillus luti*, and *Acinetobacter kookii* isolated from PVC pipes originated from highly contaminated water sources, involving frequent use of antibiotics. Nevertheless, all isolates of *P. aeruginosa* strains from iron and PVC pipes originated from water environments free from antibiotics contamination (Table 8).

DISCUSSION

Biofilms samples were collected from drinking water pipes of 4-layer houses to characterize the bacterial population and test their antimicrobial resistance. Physical examination of biofilm samples revealed that they were slimy. This finding is consistent with (Cunha et al., 2019), who stated that biofilm slime was a pseudo-capsule formed by bacteria, especially staphylococci. Moreover, the biofilm layer found on the inner surface of the PVC pipes was more abundant and blackish than the layer formed within the iron pipes. These findings agreed with what was stated by (Cerrato et al., 2006) that water in PVC pipes showed more manganese levels and black colour than detected in water flowed in iron pipes. According to Cerrato et al. (2006), the scale layer of the PVC pipe was composed of white and brown layers, with manganese making up approximately 6% of the brown layer. Furthermore, Lehtola et al. (2004) mentioned in their study that PVC pipe was used as a cost-efficient alternative to iron pipe. On the other hand, PVC could release phosphorous and biodegradable compounds that enhance biofilm formation and microbial regrowth.

In the current study, the PVC pipe showed a total microbial count higher than the iron pipe (Tables 5-6). The PVC pipes were constructed in the production layer houses of 69-weeks of age, while the iron pipes were in the growing pullet house of 15-weeks of age. Biofilms are highly hydrated structures that protect bacteria from desiccation and antibacterial agents. The formation of biofilm may be attributed to the time factor and the lack of concurrent water sanitization, as untreated groundwater was used in all layer houses. Drinking water system hydrodynamics

Table 7: Antibiotic sensitivity testing of bacterial strains isolated from biofilm samples of iron and PVC water pipes

			Iron pipe Biofilm (A)					PVC pipe Biofilm (B)			
Group	Scientific name	Disk Content	*Staph. saprophyticus*	*E. faecalis*	*E. caselifavus*	*P. aeruginosa* MW 192780	*S. terrae*	*B. luti*	*P. aeruginosa* MW1 93120	*P. aeruginosa* MW03 5344	*A. kookii*
Aminoglycosides	Amikacin	30µg	S	-	-	S	R	S	S	S	S
	Gentamicin	10µg	S	S	R	S	S	S	S	S	S
	Tobramycin	10µg	-	-	-	S	I	-	S	S	S
Carbapenem	Imipenem	10µg	S	-	-	S	S	S	S	S	S
	Ertapenem	10µg	-	-	-	-	S	-	-	-	-
	Doripenem	10µg	-	-	-	S	R	-	S	S	S
Cephalosporins	Cefuroxime	30µg	-	-	-	-	I	-	-	-	-
	Ceftriaxone	30µg	-	-	-	-	R	-	-	-	R
	Ceftazidime	30µg	S	-	-	-	I	R	S	S	I
	Cefpodoxime	10 µg	R	-	-	-	R	R	-	-	-
	Cefotaxime	30µg	-	-	-	-	R	-	-	-	-
	Cefepime	30µg	S	-	-	S	R	R	S	S	R
Combinations	Amoxicillin-Clavulanic acid	20/10 µg	-	-	-	-	S	-	-	-	-
	Piperacillin-Tazobactam	100/ 10 µg	-	-	-	S		-	-	-	-
Fluoroquinolones	Ciprofloxacin	5µg	S	S	S	S	I	S	S	S	S
	Ofloxacin	5µg	S	-	-	S	S	S	I	S	
	Nalidixic Acid	30µg	-	-	-	-	R	-	-	-	-
	Norfloxacin	10µg	I	S	-	S	S	S	S	R	-
Monopactam	Aztreonam	30µg	-	-	-	S	R	-	I	S	-
Penicillins	Ampicillin	10µg	-	S	S	-	S	-	-	-	-
	Piperacillin	100µg	-	-	-	S	S		S	S	S
	Penicillin	10 units	R	S	S	-	-	R	-	-	-
Sulfa	Trimethoprim-Sulfamethoxazole	1.25/ 23.75 µg/mg	S	-	-	-	R	S	-	-	R
Urinary Antiseptics	Nitrofurantoin	300µg	S	S	S	-	S	S	-	-	-
Macrolides	Erythromycin	15µg	R	R	R	-	-	I	-	-	-
Lincosamides	Clindamycin	2µg	R	-	-	-	-	S	-	-	-
Glyco-peptides	Linezolid	30µg	-	S	S	-	-	-	-	-	-
	Teicoplanin	30µg	-	S	S	-	-	-	-	-	-
	Vancomycin	30µg	-	S	S	-	-	-	-	-	-
Tetracycline	Doxycycline	30µg	R	R	R	-	R	S	-	-	S
Anti-TB & Leprosy	Rifampin	5µg	S	R	S	-	-	R	-	-	-

* mcg: Micrograms.

Table 8: Multiple Antibiotic Resistance Index (MARI) calculated for the bacterial strains isolated from biofilms of iron and PVC water pipes:

Biofilm source	Isolated bacteria	Accession no.	a/b[1]	MARI
Iron pipe Biofilm (A)	*Staphylococcus saprophyticus*	MW192643	5/16	0.31
	Enterococcus faecalis	MW192866	3/12	0.25
	Enterococcus casseliflavus	MW192868	3/11	0.27
	Pseudomonas aeruginosa	MW192780	0/13	0.00
	Sphingopyxis terrae	MW192898	10/23	0.43
PVC pipe Biofilm (B)	*Bacillus luti*	MW193092	5/16	0.31
	Pseudomonas aeruginosa	MW193120	0/12	0.00
	Pseudomonas aeruginosa	MW035344	1/12	0.08
	Acinetobacter kookii	MW193079	3/12	0.25

[1] a: the number of antibiotics the bacterial isolate was resistant to; b: the total number of antibiotics the isolate was tested against. MARI: Multiple Antibiotic Resistance Index (MARI= a/b).

(flow rate, velocity, turbulence, and shear stress) vary from growing to production houses. Hydrodynamics affect the exchange of trace nutrients, disinfectants, oxygen, heat, and microorganisms inside the pipe system (Fish et al., 2016). Douterelo et al. (2013) stated that the biofilm community will vary according to water system hydrodynamics. Cowle et al., 2020 stated that lower water flows assisted the attachment and propagation of biofilm bacterial biomass. While higher water flows weakened the biofilm attachment and hindered the development of biofilm.

Nine bacterial isolates were detected in water pipes biofilms, five isolates from iron pipes (*Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Enterococcus casseliflavus*, *Pseudomonas aeruginosa*, and *Sphingopyxis terrae*) and four isolates from PVC pipes (two *Pseudomonas aeruginosa* isolates, *Bacillus luti*, and *Acinetobacter kookii*). Drinking water can transmit various pathogenic bacteria that affect layer hen performance (do Amaral, 2004). Groundwater was considered a crucial problem in poultry production because it is vulnerable to contamination from sewage water and other possible sources (Cloete et al., 2003). A previous study detected indicative bacteria of fecal pollution in three different types of water samples: creek, drain, and artesian well, confirming the contamination of underground and superficial water resources (do Amaral et al., 1994).

Prior studies revealed that staphylococci and enterococci are common microbiota in the intestinal tract of chickens but are also opportunistic pathogens that cause diseases in poultry (Rosenstein and Gotz, 2012; Nowakiewicz et al., 2017; Syed et al., 2020). In previous research, staphylococci were isolated from eggs of layer chickens which were contaminated from the environment or the faeces of the birds (Fahim et al., 2021). Recently, *Staphylococcus saprophyticus* was identified as an emerging foodborne uropathogenic bacterium developing resistance to antibiotics (Sommers et al., 2017). *Staphylococcus* spp. has been known to have the biofilm-forming ability due to its microbial surface components recognizing adhesive matrix molecules (MSCRAMM) and biofilm formation genes that help in better host colonization (Culotti and Packman, 2015; Maes et al., 2019). Enterococci are resistant to unfavorable environmental conditions and have a considerable impact on soil and water contamination. Furthermore, enterococci can acquire antimicrobial resistance and virulence determinants (Nowakiewicz et al., 2017). All enterococci strains can form a biofilm, as reported by Woźniak-Biel, et al. (2019). As recommended by the European Council directive (ECD), that no enterococci should be spotted in drinking waters (ECD, 1998).

Pseudomonas aeruginosa, *Sphingopyxis terrae, Bacillus* and *Acinetobacter* bacteria are widely spread in environments and can be easily detected in soil, freshwater, and could endure intense environmental conditions. *Pseudomonas aeruginosa* and *Sphingopyxis terrae* are strong biofilm producers could co-exist and interact with a wide range of bacteria (del Mar Cendra and Torrents, 2021; Labella et al., 2021; Sharma et al., 2020). *Pseudomonas* has biofilm-forming capabilities, besides the ability to support the attachment of other pathogenic bacteria like *Campylobacter*. In the current study, *Pseudomonas* isolates represented one-third the numbers of isolates, and these results are in agreement with those of (Maes et al., 2019) who regarded *Pseudomonas* as the most abundant isolates in drinking water systems of broiler houses. *Sphingopyxis* is a gram-negative bacterium that was isolated from the biofilm of iron pipe, and this agrees with the results of (Lee et al., 2010). *Sphingopyxis* possesses the ability of biofilm formation as a secondary

colonizer (Douterelo et al., 2014). Sphingomonas was reported to be especially abundant in biofilms developed at low water flows, which agreed with our findings (Cowle et al., 2020).

Bacillus and *Acinetobacter* bacteria has high capacity to adhere to surfaces and forms biofilm (Ebrahimi et al., 2021; Rajitha et al., 2021). Acinetobacter is gram-negative bacteria exhibiting a strong biofilm-forming ability (Maes et al., 2019). Acinetobacter was previously isolated from diseased chickens (Liu et al., 2016); besides it possesses public health importance, as it was isolated from wild animals and humans, therefore it needs further investigation (Wilharm et al., 2018; Wareth et al., 2019). Gram-positive *Bacillus* spp. was isolated from the biofilm of the PVC pipe (Maes et al., 2019). *Bacillus* can induce corrosion in the iron pipe which represents economic loss and the need to change the pipe (Makris et al., 2014). *Bacillus cereus* is a common contaminant of poultry feed and lead to severe diarrhea and malnutrition. Bacillus was isolated from hemorrhagic lung infected chicken (Zuo et al., 2020).

Results of the antibiotic sensitivity revealed *Staphylococcus* resistance to penicillin, doxycycline, cefpodoxime, clindamycin, and erythromycin antibiotics. Additionally, *Staphylococcus* showed intermediate resistance to norfloxacin. These results agreed with (Bakheet et al., 2018), who proved the resistance of 90% of *Staphylococcus* isolates to penicillin. Also, Onaolapo et al. (2017) reported *Staphylococcus* resistance to doxycycline. Intermediate resistance of staphylococcal isolate to norfloxacin agreed with Farghaly et al. (2015), who reported intermediate resistance to norfloxacin in 7.1% of poults staphylococcal isolates. In Egypt, tetracycline and erythromycin are used frequently by veterinarians to treat staphylococcal infections, as well as other bacterial infections. Hence, these traditional antibiotics may not remain able to control staphylococcal infections, as previously reported in Belgium (Nemati et al., 2008).

Two *Enterococcus* isolates were identified in the biofilm of the iron pipe, *Enterococcus faecalis* and *Enterococcus casseliflavus*. Both enterococci strains showed resistance to doxycycline and sensitivity to vancomycin, and this agreed with (Stępień-Pyśniak et al., 2016). Enterococci showed sensitivity to teicoplanin, ciprofloxacin, and resistance to erythromycin. These results agree with findings reported by (Van den Bogaard et al., 2002). *Enterococcus* showed sensitivity toward ampicillin and nitrofurantoin. On contrary, da Costa et al. (2007) observed ampicillin and nitrofurantoin resistance in 36.2% and 1.2% of enterococci isolates from broiler feed; respectively.

One *Pseudomonas* isolate was found in the biofilm of iron pipe and two isolates were detected in the biofilm of the PVC pipe. *Pseudomonas* isolates were tested for antibiotic sensitivity, and they displayed sensitivity to most of the tested antibiotics. However, one showed resistance to norfloxacin and intermediate sensitivity to ofloxacin and aztreonam. These results are not agreeable with Kebede (2010) and Isichei-Ukah et al. (2018). Variation in antibiotic sensitivity results might be due to the misuse of antibiotics in the field, and the physicochemical properties of the cell wall, besides the antibiotic inhibiting enzymes (Koncicki et al., 1988).

One *Acinetobacter* isolate was identified from the biofilm of the PVC pipe. *Acinetobacter* displayed resistance to cefepime, ceftriaxone, and trimethoprim-sulfamethoxazole. While it showed intermediate resistance to ceftazidime and susceptibility to other tested antibiotics. These results showed differences from what was reported by (Kittinger et al., 2018). They reported the highest resistance to cefotaxime and low resistance to cefepime. Results agreed with Van Looveren et al. (2004) and Labella et al. (2021) who mentioned that the majority of *Acinetobacter* strains exhibited resistance toward cephalosporins.

The *Sphingopyxis terrae* isolate from iron pipes was resistant to 66.7% of cephalosporins and intermediately resistant to the remaining 33.3% (Table 7). *Sphingopyxis* was commonly resistant to cephalosporins, according to Labella et al. (2021). In addition, it showed resistance to nalidixic acid and trimethoprim-sulfamethoxazole. These results agreed with Vaz-Moreira et al. (2011) who reported fluoroquinolone and sulfonamide resistances as the second most widespread in Sphingomonadaceae, following beta-lactam resistance. *Bacillus luti* isolated from the PVC biofilm exhibited resistance to cephalosporins, penicillin, and intermediate resistance to erythromycin. These results agreed with Labella et al. (2021) who reported *Bacillus* resistance to ceftazidime, cefipime, and erythromycin.

All isolates except *Pseudomonas* (6 out of 9 - 66.7%) exhibited multiple resistance to antibiotics belonging to three or more groups. The percentage represented two times the prevalence (37.2%) reported by Labella et al. (2021). In addition, they found a significant relationship between the multiple antibiotic resistance index (MARI) and the capacity of bacteria to produce biofilm. In the current study, MARI highlighted contamination of the water environment with bacteria originated from antibiotic-rich sources. Hence, most of these bacteria were suggested to be recycled from the poultry farm wastewater and then leaked to underground water. Therefore, control measures should be targeted to the well, including the appropriate design and location of the well besides maintenance to protect the well from any contamination. Proper cleaning and disinfection of the drinking water system between flocks and regular water testing and treatment are crucial to protect

bird flocks. Farm's wastewater should be carefully handled to decrease soil contamination and subsequently the well water, especially shallow wells (Akinbile et al., 2012).

CONCLUSION

Multiple factors contributed to the formation of biofilm inside water pipelines used in layer chicken farms. Various surface materials have an impact on biofilm formation, composition, and bacterial population. Polyvinyl chloride (PVC) pipes enhanced the build-up of biofilm when compared to iron pipes. Additionally, water flow, water sanitization routine, and duration of production affected the propagation of bacteria and the development of biofilms inside the water systems. Various bacteria could grow and multiply within biofilm formed inside drinking systems, some of which represented a veterinary health concern and could affect the performance, production, and health of birds, like *Pseudomonas aeruginosa* and *Enterococcus* spp. The bacterial population identified from biofilms inside iron and PVC water pipes in the poultry farm differed from each other. Many of the isolated bacteria from the biofilms represented potential hazards to poultry health and performance. The biofilm environment increases pathogenic bacteria's antimicrobial resistance. Interestingly, the antibiotic resistance profile of these bacteria varied from one isolate to another. Doxycycline, trimethoprim, and erythromycin, which were frequently used in commercial poultry farms, showed inefficacy against multiple tested isolates as *Enterococcus* spp. and *Staphylococcus* spp. that have risk impacts on poultry health. Hence, control programs of monitoring, testing, cleaning, and disinfection are a must for combating the biofilm build-up within drinking systems. The responsible use of antibiotics became essential beside the long-term policies to ban the use of antibiotics in food-producing animals and poultry. Otherwise, consumers of poultry eggs and meat, as well as public health are at risk.

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CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

AUTHORS CONTRIBUTION

Hossam Aboelseoud: collected the samples, carried out the laboratory analyses, and wrote the initial manuscript draft. Elsayed M. Badawy, Gehan Z. Moustafa, and Elshaimaa Ismael: designed and supervised the study and revised the manuscript.

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