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Residual 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

Aya Nasser Ibrahim, Hanan Saad Khalefa* and Sherief Tawfik Mubarak
Department of Veterinary Hygiene and Management, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt.

THIS study demonstrated that some bacteria could form biofilms and detect microbial residues. Two broiler housing facilities at Giza Governorate were investigated for microbiological status during production cycle days 7, 21, and 31, and after disinfection. 27 water samples were taken: 18 using the sponge stick method from water lines, 3 from major water sources, and 6 from the cooling pad water. Additionally, 25 dust samples were collected from fans and house floors. The samples were analyzed for aerobic bacteria, coliforms, pseudomonas, fungi, and yeast. After disinfection, 18 swab samples were taken from water lines, floors, and fans to assess residual microbial counts and biofilm. Generally, the counts of microorganisms were higher at the entrance of water lines compared to the end. Total colony counts (TCC) were 342 and 23.99 CFU per 10^6/20 cm2, total coliform counts (TCFC) were 36 and 0.97 CFU per 10^6/20 cm2, pseudomonas counts were 257.50 and 12.61 CFU per 10^6/20 cm2, and fungal counts (TFC) were 10.65 and 1.97 CFU per 10^5/20 cm2, respectively. Additionally, the highest number of colonies was discovered at 31 days (3,375 and 2,145 CFU per 10^6 g from the floors and fans, respectively). After disinfection, a variety of bacteria were found; the predominant bacteria were identified using VITEK 2, and they included *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Proteus mirabilis.* Forty-five percent of the isolates *that created a moderate biofilm were P. aeruginosa and K. pneumoniae,* which could pose a risk to animal health in subsequent production cycles.

Keywords: Broiler farm, Microbial contamination, Dust, Drinking water system, Biofilm.

Introduction

The house environment, feed, and drinking water are the main sources of infection in broilers [1]. In poultry farms, the quality of drinking water and drinking lines plays a significant role in health and performance [2] because of the possibility of bacterial contamination from multiple sources [3]. Chlorine compounds are usually used in production cycles to sanitize drinking water sources. Various acids are sometimes used in water lines; however, this does not mean all pathogens could be eliminated. Conditions in drinking lines such as temperature, low flow rates, and adequate nutrients enhance the growth of microorganisms and the subsequent formation and attachment of biofilms [4]. Several studies have suggested that microbes form biofilms in poultry water systems [5,6]. Despite clean water supplies, biofilm formation can still occur [7]. Pathogens often appear in biofilms [8], which remains a challenge for the next flock of birds. Microbes' ability to form biofilms depends on other factors,

Corresponding author: Hanan Saad Khalefa, Email: hanansaad04@gmail.com, Tel.: 01125702638
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including growth conditions, contact surfaces, and strain types [9]. Recently, poultry house air quality has become a major concern, particularly for poultry health. Bacteria contaminate the air, equipment, and surfaces [10]. Poultry house dust contains viable and nonviable components such as microorganisms (bioaerosols) and feathers, bedding materials, and feces, which cause allergies; therefore, reducing dust will reduce airborne microorganism contamination [11]. Effective hygiene practices start with cleaning and disinfecting poultry houses. Although residual bacteria can be found after cleaning and disinfection, there is scanty information about them. There is also no explanation for why some bacteria remain after disinfection whereas others are eliminated [12]. Possibly, isolates developed resistance to disinfection compounds through repeated exposure [13]. Moreover, some bacteria have intrinsic resistance to some disinfectant compounds, a phenomenon often due to their cells' impermeability [14]. Additionally, bacteria can produce biofilms to protect themselves against disinfectants and induce tolerance [15]. After improper cleaning, organic debris (e.g., feces and feathers) can also form a physical barrier to protect microorganisms [16]. This bacteria's long-term survival in food, water, soil, and porous and nonporous surfaces plays a critical role in transmitting bacterial infections within and between farms and flocks [17]. It is difficult to find accurate information regarding microbial residuals or biofilms forming on broiler chicken environmental surfaces. Thus, the present study aimed to determine airborne and waterborne infections by collecting samples from the drinking system. It also collected samples from cooling pads, and dust from fans and floors. This was done throughout the grow-out period. It also identified the microbial residuals involved. The subsequent in vitro biofilm model system identified and evaluated the dominant bacteria. This residual contamination still affects subsequent production cycles since it was present during cleaning operations, and cleaning or disinfection cannot remove it.

Material and Methods

Study area

This research was conducted in Giza, Egypt, near the Egypt-Alexandria Desert Road, on two commercial closed-house, tunnel-ventilated, deep litter system poultry broiler farms from March to November 2020. The size of each house was 2,000 m^2 (20 × 100 m) with a stocking density of 25 kg/m^2, temperatures of 31°C to 32°C on the first 7 days, 27°C to 28°C until the 21st day, and then 27.5°C to 28.5°C until the end of the production period, and relative humidity of 60%–70%. The farm drinking water system was a nipple with a cup drinking system and a pan feeding system. Water and dust samples were collected from the two houses throughout the growing period and after cleaning and terminal disinfection.

Sampling

Water samples

Water lines: Twelve samples were collected from three points on the water lines of the drinking water systems in each house (water entry and the two ends of the lines). A sample was taken on days 7, 21, and 31, and 24 h after terminal cleaning and disinfection of the water lines. A sponge stick method was used to collect the samples by swapping water lines with a 5 × 2 × 2 cm sponge pre-moisturized with 10 mL of saline (during the growing cycle) and 10 mL of neutralizing broth after disinfection to neutralize the used disinfectant. Approximately 20 cm^2 of the internal water line surface was swapped, according to Maes [18], with minor modifications.

Water sources (main tanks): three Samples were collected in sterile vials for microbiological analysis, from the main water tank of the farm and water tanks in each of the two houses before being treated with chlorine compounds.

Cooling pad water: This was collected in 100-mL sterile vials.

Dust samples

Fans: We collected pooled dust from five fans with wire mesh covers and fan blades with a brush and spatula (approximately 50–150 g). Samples were then put in sterile plastic bags throughout different sampling times; after disinfection, we swapped three fan blades from individual fans. The swap area measured 4 x 5 cm^2. Each swab was taken in 10 mL of Dey-Engley neutralizing broth, according to Maes [18].

Floors: Dust was collected using a brush and spatula from 20 chosen points in the house, including the entrance, adjacent walls, and next to doors away from the bedding materials, and placed in sterile bags, according to Macher [19]. The samples were identified and marked (name, area of collection, date, age of flock) and transported in an icebox at 4°C to the laboratory for further microbiological examination.

Sample preparation

Water samples from water sources and lines were vortexed for 2 min to ensure homogenization, and one dilution from tenfold serial dilution was examined, according to Maes [18]. We prepared dust samples collected from fans and floors according to Macher [19] by mixing the samples thoroughly and then adding 0.2 g of weighted dust sample to 500 mL of sterile peptone water (0.75 g of peptone, 0.25 g of NaCl, and 0.05 g of Tween 80 per 500 mL of distilled water), followed by adding glass beads and vortexing for 2 min.

Microbiological examination

The spread method was applied to four different types of microbiological media by adding 0.1 mL of selected dilutions to plates containing the media. ISO [20]. Assessed the total colony count (TCC) on nutrient agar at 37°C for 24 hours. *Pseudomonas* spp. Were counted on pseudomonas-based agar with CFC selective supplement at 30°C for 48 h [21]**,** coliform culture was counted on MacConkey agar at 37°C for 24 h [22]**,** and yeast and mold were counted on Sabouraud's dextrose agar with chloramphenicol supplement [23]. As reported by Messer et al. [24]**,** countable plates having 30–300 colony-forming units (CFUs) were assessed as follows:

- Water lines, water sources, and cooling pads (during the growing cycle) as CFU/mL
- Dust samples were calculated using the following equation: CFU/g

$$\frac{(\text{Plate count})\times(\text{total volume of solution})}{(\text{Dilution factor})\times(\text{plated volume})\times(\text{dust mass})} = \frac{(CFU)(500\ ML)}{(10^{-X})(0.1)(0.2)} = \frac{CFU}{g}$$

CFU/20 cm^2 for equipment such as water lines, fans, and floor swaps were measured (Macher, 2001).

Detection of microbial contamination following disinfection

Isolated strains were subjected to gram's stain, colonial morphology, oxidase, catalase, indole, methyl red, Voges–Proskauer, citrate, urea, and triple sugar iron tests. Biochemical testing identified 20 strains using Bergey's Manual [25] and confirmed with the VITEK 2 (bioMérieux) [26].

Determination of the biofilm-forming ability of the isolates

After cleaning and disinfection, high levels of microorganisms on surfaces have been identified as indicating a biofilm [27]. According to Hassan et al. [28], *Staphylococcus epidermidis*, a positive biofilm producer, was used as a control positive, and sterile broth was used as a control negative. Biofilm formation was detected by the following methods:

Tissue culture plate

According to Christensen et al**.** [29], the quantitative test is considered the golden standard for detecting biofilms. A random selection of 20 isolated species (n = 20) was tested for the ability to form biofilms using 96-well microtiter plates made from polystyrene [30]**.** After first being isolated from fresh agar plates, test organisms were inoculated into 10 mL of trypticase broth with 1% glucose. The broth was incubated at 37°C for 24 h. Subsequently, the culture was diluted in a fresh medium at 1:100. Then, 200 µL of the diluted culture was added to the individual wells of 96-well tissue culture-treated polystyrene plates. Control species were incubated, diluted, and applied to tissue culture plates as well. Negative controls were inoculated with sterile broth. A 24-h incubation period was conducted at 37°C. Afterward, each well's contents were gently tapped out. We rinsed the wells four times with 0.2 mL of phosphate-buffered saline (pH 7.2). This method eliminated bacteria floating on the surface. Biofilms adhered to the wells were fixed with 2% sodium acetate and stained with crystal violet (0.1%), and the excess stain was rinsed with

TABLE 1. Biofilm production according to the ODc

OD value	Biofilm production
OD strain ≤ ODc	Biofilms not produced
ODc < OD strain ≤ (2 × ODc)	Produces poor biofilms
(2 × ODc) < OD strain ≤ (4 × ODc)	Produces moderate biofilms
(4 × ODc) < OD strain	Produces strong biofilms

ODc, optical density cutoff = negative control OD (average of OD) + 3× standard deviation of the negative control.

deionized water, followed by drying.

The following categories of biofilm were assigned according to the 590-nm absorbance values of crystal violet-stained samples based on the study results by Stepanović et al. [31]. The relative optical density cutoff was defined as three standard deviations above the absorption value of the negative control (Table 1).

Tube method

The tube method is a qualitative technique for detecting biofilms defined by [32]. Loops of test species were inoculated in 10 mL of trypticase soy broth containing 1% glucose. For 24 h, tubes were incubated at 37 °C. Decanted tubes were washed in buffered saline (pH 7.3) and dried after incubation. We stained the tubes with crystal violet (0.1%) and then used deionized water to remove any remaining stain. Tubes were dried upside down. Tube system scoring was conducted based on the performance of the control strains. A transparent film on its wall and bottom indicated a biofilm had formed inside the tube. It was rated as follows: 1, weak/none; 2, moderate, or 3, high/strong. The experiment was repeated three times.

Statistical analysis

Using SPSS for Windows (version 17.0) and Microsoft Excel for Windows 2010, a *t*-test was performed to determine the significance of variation between variables [33].

Results

Bacterial and fungal counts on water lines and dust throughout the growing period

In this study, all samples from the two broiler houses were collected and analyzed to detect microbial counts at different ages (days 7, 21, and 31) during the grow-out period. Table 2 shows the microbial counts of water lines and water sources. Generally, the counts at the entrance of water lines are higher than at the ends. At the entrance and ends of the pipelines, the mean total colony count (TCCs) was 342 and 23.99 CFU × 10^6/20 cm^2, respectively; similarly, the total coliform counts (TCFCs) were 36 and 0.97 CFU × 10^6/20 cm^2, the total pseudomonas counts were 257.50 and 12.61 CFU × 10^6/20 cm^2, and the total fungal counts (TFCs) were 10.65 and 1.97 CFU × 10^5/20 cm^2. Our observations also suggest that the microbial counts increased with age until day 21 and decreased after that. There was a statistically significant difference between the ages of water lines at the entry points in the

TABLE 2. Bacterial, fungal, and yeast loads of water lines and water sources from two broiler houses during the grow-out period at different ages.

Sampling time	Broiler house	Total colony count (CFU × 10^6/20 cm^2)		Total coliform count (CFU × 10^6/20 cm^2)		Total pseudomonas count (CFU × 10^6/20 cm^2)		Total fungal count (CFU × 10^5/20 cm^2)		Total yeast count (CFU × 10^5/20 cm^2)	
		A	B	A	B	A	B	A	B	A	B
7 days	H_1	110	10.93	8.20	.02	57.5	8.7	40	1.3	0	2.81
	H_2	195	40.06	4.10	0.17	100	41.83	8.5	0.58	4.3	2.12
	Mean ± SD	152.50 ± 60.10 [a]	25.49± 15.13	6.15± 2.89 [a]	0.09±0.09	78.75± 49.05	25.26± 12..99	24.25± 22.27	0.9± 0.50	2.1± 1.04	2.46± 0.06
21 days	H_1	4900	199.50	2600	1.25	482	1.21	29	6	7	29.2
	H_2	8600	57	2000	0.64	7700	6.35	60	85.2	20	12
	Mean ± SD.	6750± 2616.29 [ab]	128.3 ± 71.9	2300± 424.26 [ab]*	0.94± 0.42*	4091± 3609.9	3.78± 3.63	44.5± 30.37	42.92± 37.8	13.5± 6.54	20.6± 8.67
31 days	H_1	172	6.48	34	1.75	85	3.97	4.7	1.45	13	7.7
	H_2	512	31.50	38	0.80	430	21.25	16.6	2.5	20	2.3
	Mean ± SD	342± 240.41 [b]	18.99± 12.57	36± 2.82 [b]*	1.27± 0.47*	257.50± 243.95	12.61± 12.21	10.65± 8.41	1.97± 0.74	16.56± 4.22	5± 3.81
Major source (tanks) (CFU × 10^4/20 cm^2)		2180		0		210		0		0	
H_1 (CFU × 10^4/20 cm^2)		30		0		0		0		0	
H_2 (CFU × 10^4/20 cm^2)		80		0		50		0		0	

A *indicates the entrance of the water line system expressed by CFU/20 cm².* **B** *indicates the average of two ends of the water line system expressed by CFU/20 cm²*

The means with the same letter within the same column are significantly different at P < 0.05

TCC and TCFC, as shown in Figure 1. There was also a statistically significant difference between the entrance of the waterline and the average of two water line ends in the TCFC at 21 days and TCFC at 31 days as shown in Figure 2. In the water sample taken from the source, 2,180 CFU × 10^4/20 cm^2 were found, followed by 210 CFU × 10^4/20 cm^2; but the coliform, fungal, and yeast counts were not isolated. Only the TCC of 30 CFU × 10^4/20 cm^2 was isolated from the tank of house 1, whereas from the tank of house 2, only the TCC of 80 CFU × 10^4/20 cm^2 and total pseudomonas count were isolated.

In Table (3) The results of measuring the distribution of airborne bacteria and fungi from dust collected from fans and floors showed that the highest count of colonies was found at the age of 31 days (3,375 and 2,145 CFU × 10^6/g from the floors and fans, respectively). In contrast, the TFCs were 16.43 and 12.06 CFU × 10^6/g from the floors and fans, respectively. Figure 3 shows the statistical difference between the flock ages in the TCC and TCFC in fan dust, and Fig. 4 shows the statistical difference between flock ages in the TCC and TCFC in floor dust. Table 4 shows the total bacterial count increased during

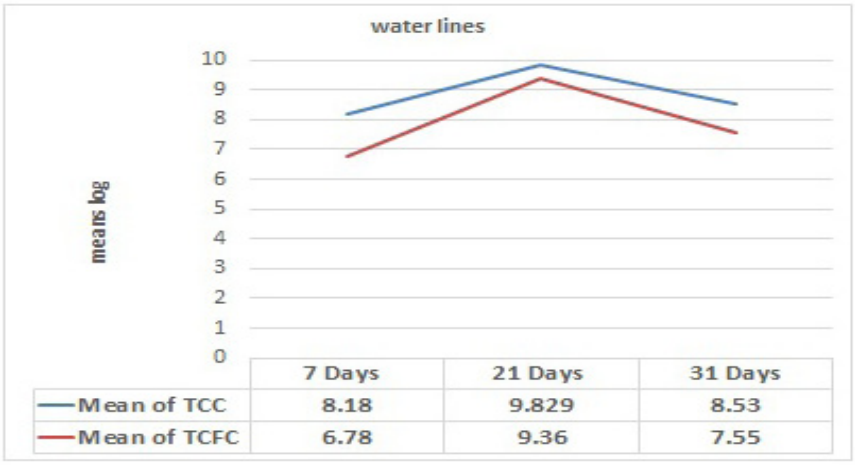

Fig. 1. The statistical difference between the ages at the entrance of water lines (A) by log10 of means in the total colony count (TCC) and total coliform count (TCFC).

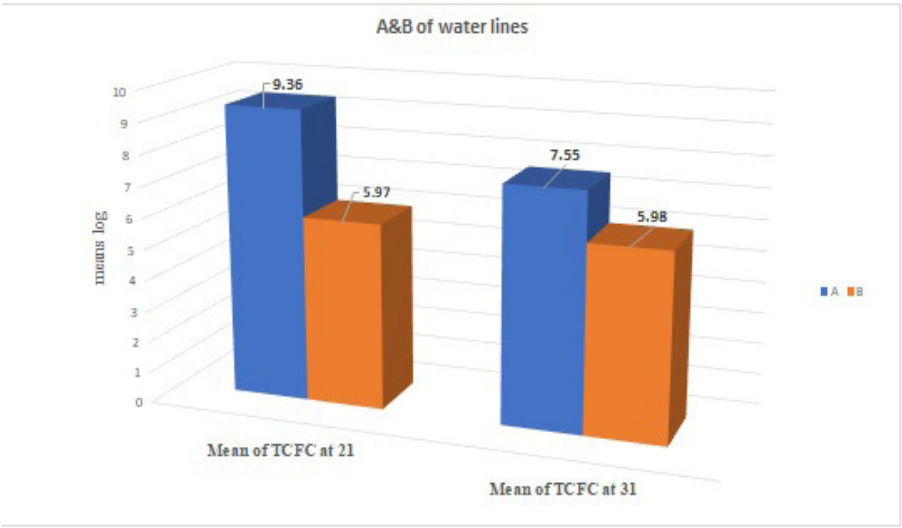

Fig. 2. The statistical difference between the entrance of the water line (A) and the average of two water line ends (B) in the TCFC at 21 days and TCFC at 31 days.

growth, reaching 1,732.5 CFU × 10^3/mL. Then, it decreased on day 21, reaching 560 CFU × 10^3/mL. The same pattern was observed in the cooling pads. The overall pseudomonas count at 31 days was 100.12 CFU × 10^3/mL, and the TFCs at 21 and 31 days were 6.06 and 0.150 CFU × 10^3/mL, respectively, whereas the TCFC was 20.5 CFU/mL on day 21 and was undetectable. Fig. 5 illustrates the difference between flock ages in the TCFCs and total yeast counts (TYCs) in the water from the cooling pads.

Microbial counts on water lines, fans, and floors after disinfection

The data analyzed in Table 5 represent the bacterial and fungal counts after infection. Generally, the colony, coliform, pseudomonas, fungal, and yeast counts decreased severely after the disinfection of water lines, while the fans and floors did not have any coliforms following disinfection. Table 6 shows the Cleaning and disinfection products (mostly based on alkaline base or glutaraldehyde and quaternary ammonium compound) were regularly applied in the drinking water, Floors, and fans in all farms during production.

Identification and formation of biofilm

After disinfection, twenty randomly selected isolates from the water sources, water lines, cooling pad waters, floors, and fans were tested for their biofilm-forming ability (Table 7). VITEK 2 and traditional methods were used to identify the isolates microbiologically. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Escherichia coli* were identified as the isolates. Moreover, 45% of all tested microorganisms (n = 20) formed moderate biofilms (Fig. 6a&6b), while the rest (55%) formed weak biofilms. Many moderate biofilms were isolated from the water sources and floors. *P. aeruginosa* and *K. pneumoniae* were the major producers of moderate biofilms (Fig. 7a, b, c, and d).

TABLE 3. Bacterial, fungal, and yeast loads of dust of the fans and floors taken from two broiler houses during the grow-out period at different ages.

Sampling time	Broiler house	Total colony count (CFU × 10^6/g)		Total coliform count (CFU × 10^6/g)		Total pseudomonas count (CFU × 10^6/g)		Total fungal count (CFU × 10^6/g)		Total yeast count (CFU × 10^4/g)	
		Fans	Floor	Fans	Floor	Fans	Floor	Fans	Floor	Fans	Floor
7 days	H_1	132.50	72.50	3.32	365	2.45	12.75	67.50	0.67	27.50	0.70
	H_2	112.50	1250	1.12	550	0.57	6	72.50	0.70	0	15
	Mean ± SD	122.50± 14.14 [a]	661.25± 832.61 [a]	2.22± 1.55	457.5 ± 130.815[a]	1.51 ± 1.32	9.37 ± 4.77	70 ± 3.53 [a]	0.68± 0.017	13.75± 19.44	7.85± 10.11
21 days	H_1	950	160	0	0	5	22.50	77.50	5	27.50	20
	H_2	1030	772.50	2.25	15	14.50	11.95	45	2	17.50	8
	Mean± SD	990± 56.56 [ab]	466.25 ± 433.10 [b]	1.12 ± 1.59	7.5 ± 6.6 [a]	9.7 ± 6.71	17.22 ± 7.45	61.25 ± 22.98 [b]	3.50± 2.12	22.50 ± 7.07	14 ± 8.48
31 days	H_1	2300	4000	0.32	297.5	5.75	12.50	1.62	3.2	4.07	12.50
	H_2	1990	2750	0.75	342.5	23	7.50	22.50	0.37	15	7.50
	Mean± SD	2145± 219.20 [b]	3375± 883.88 [ab]	0.53± 0.30	320± 31.8198	14.37± 12.19	10± 3.53	12.06 ± 14.76 [ab]	1.62± 1.31	9.53± 7.72	10± 3.53

The means with the same letter within the same column are significantly different at $P < 0.0$.

TABLE 4. Bacterial, fungal, and yeast loads of cooling pad water taken from two broiler houses during the grow-out period at different ages

Sampling time	Broiler house	Total colony count (CFU × 10^3/ mL)	Total coliform count (CFU × 10^3/ mL)	Total pseudomonas count (CFU × 10^3/ mL)	Total fungal count (CFU × 10^3/ mL)	Total yeast count (CFU × 10^3/ mL)
7 days	H_1	113	0.26	3.02	0.31	0.70
	H_2	5.40	0.09	1.67	0.10	0.50
	Mean ±SD	59.20± 53.08	0.17± 0.12 [a]	2.34 ± 0.95	0.20 ± 0.14	0.60 ± 0.14 [a]
21 days	H_1	565	17	300	12	0
	H_2	3200	24	1780	0.8	0
	Mean ±SD	1882.5± 1317.5	20.5± 4.94 [ab]	1040 ± 1046.51	6.4 ± 5.6	0[a]
31 days	H_1	900	0	190	0.14	0
	H_2	220	0	10.24	0.16	0.30
	Mean ± SD	560 ± 480.83	0[b]	100.12 ± 90.10	0.15 ± 0.01	0.15 ± 0.21

The mean with the same letter within the same columns is significantly different at $P < 0.05$.

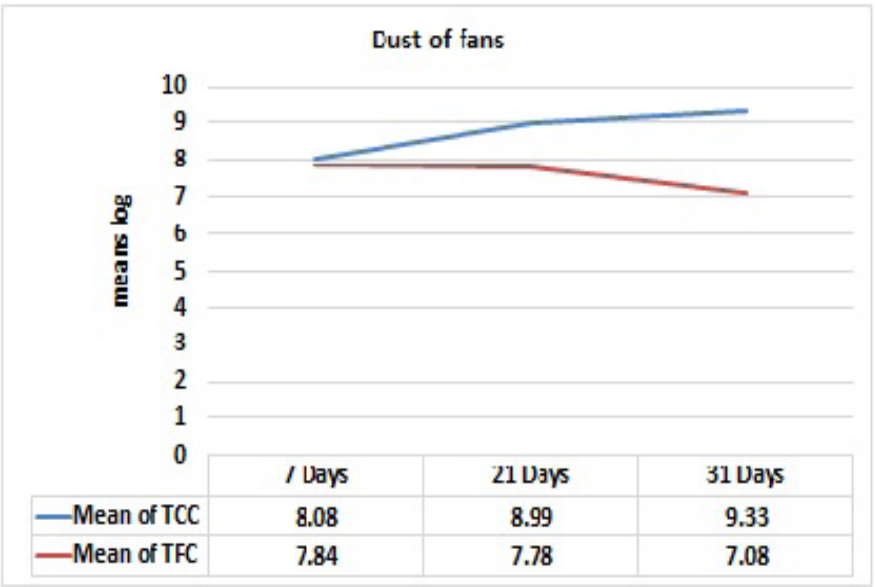

Fig. 3. The statistical difference between different ages of flock in (TCC)and Total fungal count (TFC) in fan dust.

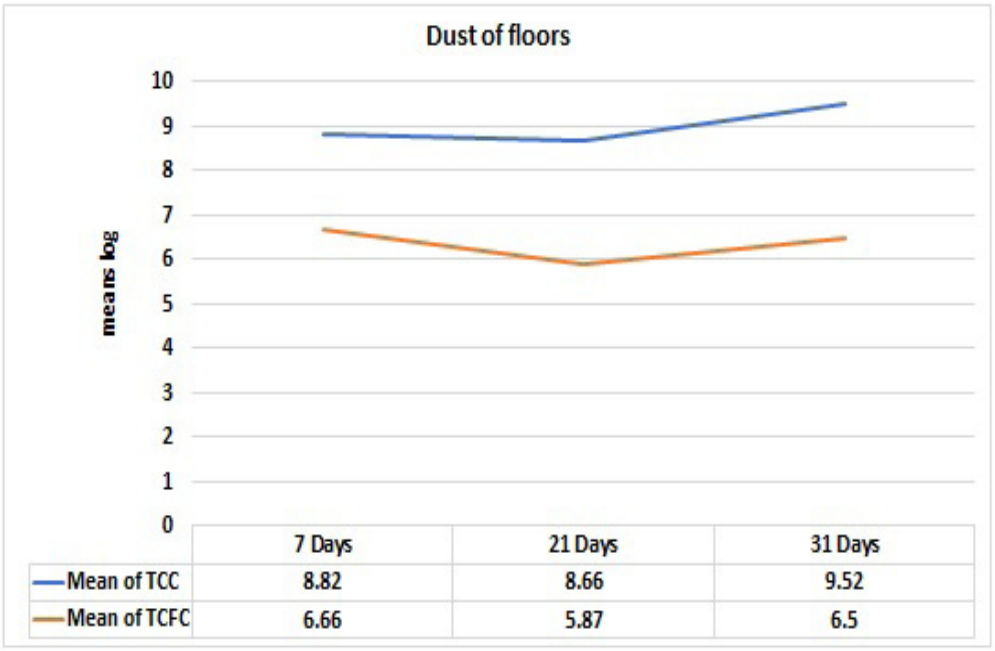

Fig. 4. The statistical difference between different ages of flock in (TCC) and (TCFC) in floor dust

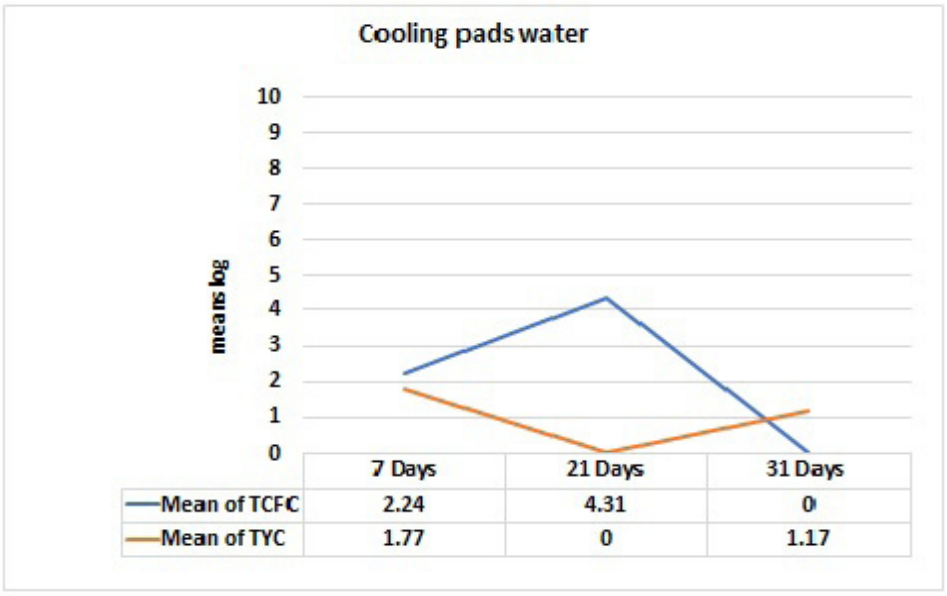

Fig. 5. The statistical difference between the ages of flock in the TCFC and total yeast count (TYC) in the water returned from cooling pads.

TABLE 5. Bacterial, fungal, and yeast loads of water lines, fans, and floors from two broiler houses after disinfection.

Broiler house	Total colony count (CFU × 10^4/20 cm^2)		Total coliform count (CFU × 10^4/20 cm^2)		Total pseudomonas count (CFU × 10^4/20 cm^2)		Total fungal count (CFU × 10^4/20 cm^2)		Total yeast count (CFU × 10^4/20 cm^2)	
Lines after disinfection (CFU × 10^4/20 cm^2)										
	A	B	A	B	A	B	A	B	A	B
H_1	39	2460	3.35	0.08	10	1548.5	81	60	0	159.5
H_2	0.03	205.92	0	9	0	250.95	0.43	2.08	0.11	53.36
Mean ± SD	19.51 ± 27.55	1332.96 ± 1125.87	1.675 ± 1.53	4.54 ± 6.31	5 ± 7.07	899.72 ± 649.50	40.71 ± 39.97	31.04 ± 29.95	0.05 ± 0.07	106.43 ± 50.71
Dust of fans and floor after disinfection (CFU × 10^2/20 cm^2 of swapping)										
	Fans	Floors	Fans	Floors	Fans	Floors	Fans	Floors	Fans	Floors
H_1	7	167	0	0	2	108	3	5	0	4
H_2	19	35	0	0	0	0	17	15	0	0
Mean ± SD	13 ± 8.48	101 ± 93.33	0	0	1 ± 98.9	54 ±56	10 ± 9.89	10 ± 7.07	0	2 ±1.99

Lines A indicate the entrance of the water line system expressed by CFU × 10^4/20 cm. Lines B indicate the average of the two ends of the water line system expressed by CFU × 10^4/20 cm.

TABLE 6. Products used for cleaning and disinfection of poultry house surfaces (water tank, water lines, floors, fans, and cooling pads) with its applied concentration and use

	Disinfection location	Disinfection product	Active compound	Applied concentration and use
One day before the cycle	**Water lines**	**Bio scale**	**Water acidifier**	**Adjust the dilution to achieve a pH of between 3.7 and 4**
Disinfection	Water lines Water tanks Cooling pads	Bio VX	Stabilized blend of peroxygen compounds, surfactants, organic acids, and an inorganic buffer system	Defra General orders 1:100
Cleaning	Floors and fans	BioFoam	Blend of high-foaming surfactants, foam stabilizers, and alkaline builders	High-foaming pre-wash detergent. Cleaning a house size of 10,000 ft^2/1,000 m^2, dilute 3–6 L depending on the level of soiling in 600 L of clean water. Apply via a pressure washer fitted with a foaming lance.
Disinfection	Floors and fans	Bioshield	Liquid blend of glutaraldehyde and quaternary ammonium compound	Routine disinfection 1:100
Fogging solutions	Sanitizer and disinfectant per 4-m^2 surface	BioShield P	Liquid blend of glutaraldehyde and quaternary ammonium compound	Routine disinfection 1:100
		Bi-OO-Cyst	Powerful disinfectant, effective against endoparasites	Defra general orders 1:160

TABLE 7. Identification of strains and biofilm formation ability

Isolate number	Organism	Location	Biochemical tests	VITEK 2	Biofilm production
	Klebsiella pneumoniae	Water sources	✓	✓	Moderate
	Klebsiella pneumoniae		✓	✓	Moderate
	Klebsiella pneumoniae		✓	✓	Moderate
	Pseudomonas aeruginosa		✓	✓	Moderate
	Pseudomonas aeruginosa		✓	✓	Moderate
	Proteus mirabilis		✓	✓	Weak
	Proteus mirabilis		✓	✓	Weak
	Klebsiella pneumoniae	Water lines	✓	✓	Moderate
	Pseudomonas aeruginosa		✓	✓	Weak
	Pseudomonas aeruginosa		✓	✓	Weak
	Proteus mirabilis		✓	✓	Weak
	Escherichia coli		✓	✓	Weak
	Escherichia coli		✓	✓	Weak
	Pseudomonas aeruginosa	Floors	✓	✓	Moderate
	Pseudomonas aeruginosa		✓	✓	Moderate
	Proteus mirabilis		✓	✓	Weak
	Klebsiella pneumoniae	Cooling pads	✓	✓	Moderate
	Pseudomonas aeruginosa		✓	✓	Weak

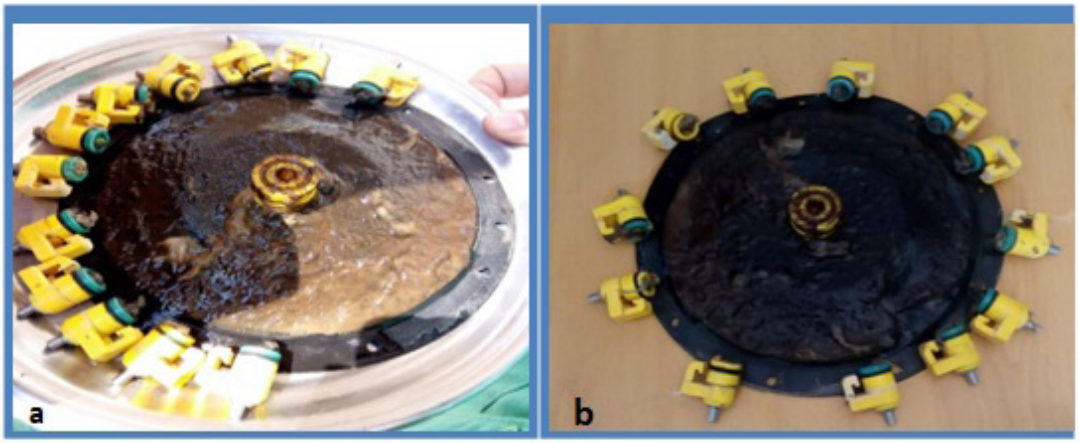

Fig. 6a, b. Biofilm attached to the entrance of the drinking water system; highly contaminated water pressure regulator.

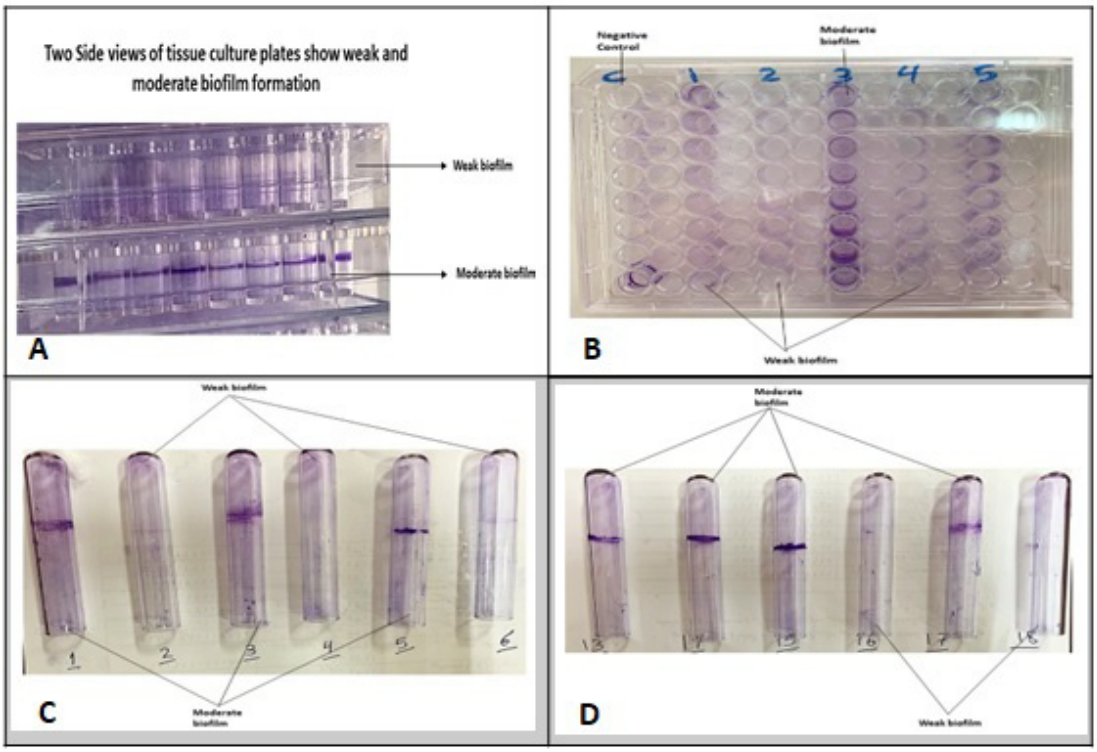

Fig. 7(a, b, c, and d). A variety of methods have been developed to cultivate and quantify biofilms, including microtiter plate tests (a and b) and tube tests (c and d)

Discussion

Bacterial and fungal counts on water lines and dust throughout the growing period.

As shown in Table 2, there was a significant difference ($P < 0.05$) between ages 7, 21, and 31 days related to the TCFC. The microbial load was very high at 21 days compared with those at other ages. This may be attributable to the addition of minerals, vitamins, and antibiotics to the water at 18, 19, and 20 days, which may be the matter of promoting microbiological aggregation and biofilm development [34]. Another reason may be from the flow rate of drinking water; it is lower at this age of growth, which was not present at 7 days (manually drinkers), and it was high at 31 days because their weight and age were high. There was a significant difference ($P < 0.05$) between the entry and two ends of the water lines associated with the TCFC (Fig. 1), and all pipes attached to the entrance had highly contaminated water pressure regulators (Fig. 6). These were the primary production sites for biofilm. The coliform counts were used to assess sanitation conditions, with high counts reflecting poor sanitation or post-processing contamination [35]. Figure 2 illustrates that the TCFC and TCC of water lines were higher at the entrances than at the ends. According to the study of Watkins [36] less than 1,000 CFU/mL of bacteria are acceptable in poultry drinking water. This demonstrates, once again, the importance of water sanitation and properly maintaining lines between flocks to eliminate much of the microbial problem in water systems. Different samples from the two-house farms demonstrated similar microbial growth patterns. In terms of microbial results, there was a significant difference between ages ($P < 0.05$). According to some authors [37,38] dominant bacteria entered water supply lines due to source contamination. As a result, chlorine is crucial to water tank treatment. Bacterial tests on water tanks showed that before addition of chlorine to the main water source, the TCC was 2,180 CFU/mL and the total Pseudomonas count was 210 CFU/mL. Nevertheless, there were no total counts of yeast, fungi, or coliforms (Table 2).

Farms and hatcheries have a wide range of bacteria in the air and on the surfaces of equipment and buildings [10]. The main means

disease-causing organisms are spread throughout poultry houses is by airborne dust. This is the main cause of infection next cycle. Our study's results showed a significant difference between ages 7, 21, and 31 days in the TCC due to the hygienic condition of the poultry house during the growing period ($P < 0.05$). As organic matter and dandruff increased with aging, it was not surprising that the TFC varied at different sampling times; this is consistent with [39]. Like this, the TCC and TCFC showed that the number of microorganisms in floor dust (Table 3) was significantly different ($P <0.05$) at the age of 31 days than at the age of 7 days due to the increase in dust on the floor. The results showed that the air concentration of microorganisms increased with bird growth and age. At the same time, it was low at the beginning and end of the fattening period, possibly due to limited bird activity. In their studies of the effects of fattening poultry age and season on the concentration of bioaerosols in poultry houses**,** [40-41] reached similar conclusions. Additionally, Sauter et al. [42] found that microflora concentrations in the air were related to bird density. Earlier studies have established that ammonia and dust productions affect litter type and stock density in broilers, according to [43]. There is no evidence that the microbial load on cooling pads expressed by returned water can affect house air quality. The TCFC values were significantly different between days 21 and 31 ($P < 0.05$), suggesting that this very high load on day 21 accumulated 3 days earlier. Cooling pads worked 3 days after the water was diluted by water action; however, the TYC increased on day 7, suggesting that the yeast had the chance to grow away from the water current. The turbidity of water was higher on day 7 compared with that in other ages and then decreased with aging.

In poultry farms, sanitation is properly implemented, which contributes to farm animal health. In confined animal facilities, environmental contamination directly affects the prevalence and severity of diseases**.** A disinfection program can significantly reduce the numbers of bacteria, total fungi, and isolates of common bacteria present in water lines, as shown in Table 5. Ward et al. [44] also demonstrated this. Due to their outer membranes, Gram-negative bacteria, such as Enterobacteriaceae, are generally more resistant to disinfectants than Gram-positive bacteria **[**45]. According to Aboelseoud et al.[46] many dominant genera discovered in water pipes were isolated from poultry environments during the grow-out cycle. After disinfection, bacteria may still exist due to residual moisture or extraneous material (like organic material), which could affect the disinfectant negatively or dilute it. This agrees with Luyckx et al., [12] findings. There were fewer counts recorded from the water lines' entrances than from their ends, primarily because the entrances of the pipes are where most of the flow occurs and where flushed acids are concentrated more than at the ends. A broad spectrum of disinfectants must be applied to the surface of objects to prevent their infection and colonization in poultry farms and their transmission to humans. In addition, following disinfection, the microbial counts of swaps taken from the fans and floors showed a significant reduction in the total bacterial count, total *Pseudomonas* count, and TFCs, where the TFCs on the fans and floors were also zero. Field trials with drag swabs, agar pour plates, and total counts of microorganisms should be used to determine whether the disinfectants are effective. Tamasi [47] stated that the in vitro method requires the use of representative strains. There is no evidence that other factors, such as mutations, physiological injuries, underdosing, or presence of organic matter, which may cause resistance to develop, influence these strains. Table 6 lists the disinfectant products. Products containing quaternary ammonium and/or glutaraldehyde are most widely used for disinfecting poultry houses [48]**.** However, using two or more active ingredients in commercial disinfectants is likely to increase its antibacterial activity. Additionally, residual disinfectants do not prevent microbial colonization and can lead to resistance [49-50]**,** resulting in a greater threat to water quality.

A biofilm is a community of specialized microbial cells in close association with survival and is permanently attached to hydrophobic surfaces [51-52]**.** After disinfection of water sources, water lines, cooling pad waters, floors, and fans, 20 isolates were collected at random for biofilm determination. *K. pneumoniae*, *P. aeruginosa*, *P. mirabilis*, and *E. coli* were identified as the isolates. All four have biofilm-forming capabilities but at different degrees. Table 7 shows that 45% of all microorganisms tested produced moderate biofilm, while 55% produced weak biofilm, with most of the moderate biofilm isolated from the water sources and floors. Biofilm can be observed in water lines and drinker systems and has been discovered to create microbial populations that thrive and sustain health problems

not easily addressed. The formation of biofilm on pipe surfaces can occur even in networks where disinfectants are used. Morvay et al. [53] found that biofilm formation could reach values as high as 10^7cells/cm^2 on various plumbing materials in chlorinated drinking water systems after only 30 days. Furthermore, biofilms can clog water pipes and filters, restricting water flow, which can sometimes result in poor flock performance [54-56]. In Table 7, the bacterial isolates with the capability of forming a biofilm on each surface are summarized by genus. *P. aeruginosa* and *K. pneumoniae* were among the most prominent producers of biofilm. An analysis of drinking water distribution systems reported by Rożej et al.[57] found abundant *Stenotrophomonas maltophilia* and *P. aeruginosa*. Several studies have indicated that the prevalence of these bacteria was due to their ability to settle and multiply on plastic pipes. Further, the presence of these two species is in water supply networks used in human infrastructure, including homes, schools, and hospitals **[**58**].** Additionally, several studies have reported high mortality rates in broiler chicks caused by *P. aeruginosa* infection [59]. It is significant to consider the limitations of the used assay to evaluate biofilm-forming potential. The CFU densities differ between organisms regardless of positive control, and the optical density is used to normalize the cultures [3]. Various methods have been developed to cultivate and quantify biofilms, including tube tests, microtiter plate tests, radiolabeling, microscopy, and Congo red agar plate tests [60]. The microtiter plate method remains one of the most used assays for investigating biofilm, and several modifications have been designed to measure and cultivate bacterial biofilms in vitro (Fig. 7).

Conclusion

Waterline biofilm is hardly removed completely by acid flushing. Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Proteus mirabilis were the most prevalent bacteria found after disinfection, which were identified using VITEK 2. The most frequent bacteria found on floors and water sources were *P. aeruginosa* and *K. pneumoniae,* which made up 45% of the isolates that produced a moderate biofilm. *Therefore, pe*riodically changing the water lines is preferable, with continuous water sanitation in water tanks and cooling pads by chlorine components because of its significant microbial load. Dust from floors and fans affects air quality; thus, constant sweeping and cleaning of fans and floors are necessary to avoid its spread.

Conflicts of interest

All Authors declare that there is no conflict of interest.

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No funding was received for conducting this study.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Aya Nasser Ibrahim], [Hanan Saad Khalefa] and [Sherief Tawfik Mubarak]. The first [Aya Nasser Ibrahim], collect the samples and made practical part; draft of the manuscript was written by [Hanan Saad]; while all over supervision for the paper done by [Sherief Tawfik Mubarak], and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."

Statements & Declarations

Ethics approval: All standard national and international ethical guidelines dealing with environmental sampling were fully adopted and approved by the Ethics Committee of the Faculty of Veterinary Medicine, Cairo University**.**

Availability of data and material: The data is available within the article.

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I confirm that instruction for authors of this journal has been read carefully and all points are compiled for the whole manuscript.

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التلوث المتبقي وتكوين البيوفيلم في مزارع الدواجن بسبب أنظمة مياه الشرب والمراوح والأرضيات

اية ناصر ابراهيم ، حنان سعد خليفه و شريف توفيق مبارك
قسم الصحة والرعاية البيطرية - كليه طب بيطري - جامعة القاهرة - القاهرة - مصر.

يعد تنظيف وتطهير مزارع الدواجن أمرا أساسيا للإدارة الجيدة للمزرعة والوقاية من الأمراض.هناك دراسات قليلة تناولت التلوث المتبقي على أسطح مزارع التسمين ، ومنصات التبريد ، وغبار المروحة قبل وبعد التطهير. في هذه الدراسة تم بحث الحالة الميكروبيولوجية للأسطح المختلفة في مزرعتين تسمين في محافظة الجيزة أثناء وبعد دورة التربية. تم تحليل إجمالي عدد المستعمرات والقولونيات والزائفة والفطريات والخميرة. في الأيام 7 و 21 و 31 من فترة النمو ، تم جمع 27 عينة من مصادر المياه الرئيسية وخطوط المياه ومياه منصات التبريد وكذلك تم تجميع 25 عينه من الغبار من أسطح المروحة وأرضيات المنازل. بعد أربع وعشرين ساعة من تطهير المنزل ، تم جمع 18 عينة مسحة من خطوط المياه والأرضيات والمراوح لتقييم عدد الميكروبات المتبقية ووجود الأغشية الحيوية. أظهرت النتائج أن عدد الميكروبات زاد في عينات الماء والغبار مع نمو القطيع. بشكل عام، كان عدد الميكروبات أعلى عند المدخل منه في نهاية خطوط المياه ، وكانت تلك الموجودة في الطوابق أعلى من تلك الموجودة على المراوح. ولكن بعد التطهير انخفض عدد الميكروبات بشكل ملحوظ. في هذه الدراسة تم عزل بعض البكتيريا وتحديدها لتحديد البقايا الميكروبية والقدرة على تكوين الأغشية الحيوية. تم جمع عشرين عزلة بكتيرية تنتمي إلى أربعة أنواع بكتيرية (Klebsiella pneumoniae و Pseudomonas aeruginosa و Proteus mirabilis و Escherichia coli) من الماء والغبار ومياه التبريد. خمسة وأربعون في المائة من العزلات كانت منتجة بشكل معتدل للأغشية الحيوية. العزلات هي في الغالب مسببات الأمراض المزمنة. لذلك ، يمكن أن تشكل خطرا على صحة الحيوان في دورات الإنتاج اللاحقة.

الكلمات الدالة: مزرعة االتسمين ، التلوث الميكروبي ، الغبار ، نظام مياه الشرب ، الأغشية الحيوية.