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Biofilm production by *Escherichia coli* in poultry water drinkers*

Produção de biofilmes por *Escherichia coli* em bebedouros avícolas

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Abstract

This study evaluated the microbiological quality of the water of 33 poultry sheds seeking to identify the presence of biofilm-producing *Escherichia coli.* The swab samples of the surface of the drinkers were analyzed by the inhibition test to detect the most effective disinfectant for removing these bacterial communities. The multiple-tube and mesophilic counts techniques were used to analyze the water samples. The swabs were plated on EMB agar followed by Rugai medium to identify *E. coli*. The biofilm was detected by the optical density readings and the Congo red agar method. Overall, 15 strains of *Escherichia coli* were detected in the swabs of the drinkers, of which 8 were biofilm producers. Regarding the water quality, 15 (45.45%) of the 33 evaluated water samples had bacterial growth while only two (13.33%) produced gas. The test with disinfectants showed that chlorine and chlorhexidine were not effective to control the *E. coli* biofilm production. Therefore, a strict control is essential to ensure the safety of the water supplied to the animals in broiler sheds and to eliminate the biofilm-forming *E. coli* that have shown to be potentially resistant to the sanitizers commonly used in the cleaning processes.

Keywords: water, poultry, microbial communities, disinfection.

Resumo

Objetivou-se avaliar a qualidade microbiológica da água de 33 galpões avícolas, identificar a presença de *Escherichia coli* produtoras de biofilmes em amostras de suabes de superfície dos bebedouros e analisar por teste de inibição o desinfetante mais eficiente para remoção dessas comunidades bacterianas. Foi utilizada a técnica dos tubos múltiplos e contagem de mesófilas para as amostras de água. Os suabes foram semeados em Ágar EMB, e posteriormente em meio Rugai para identificação de *E. coli*. Para análise de produção de biofilme foi feita a leitura da Densidade Óptica e o método do Ágar Vermelho Congo. Foram identificadas 15 cepas de *E. coli* nos suabes de bebedouros, sendo que 8 foram produtoras de biofilme. Quanto ao padrão de qualidade da água, observou-se que das 33 amostras de água avaliadas, 15 (45,45%) tiveram crescimento bacteriano, sendo que somente duas (13,33%) produziram gás. Para o teste com desinfetantes, o cloro e o clorexidine não se mostraram eficientes no controle da produção de biofilmes de *E. coli.* Dessa forma, torna-se essencial o controle rigoroso nos galpões de frangos de corte para garantir a inocuidade da água fornecida aos animais e a eliminação de *E. coli* potencialmente formadoras de biofilmes resistentes aos sanitizantes mais comumente usados nos processos de limpeza.

Palavras-chave: água, avicultura, comunidades microbianas, desinfecção.

Introduction

The microbial growth as biofilm in the distribution network pipelines, equipment, and utensils used for animal production results in poor quality water. These networks provide an environment adequate for growth and maintenance of several microorganisms, including many pathogens (Chaves et al., 2007; Togashi et al., 2008). Because the biofilms adhere to places with the ideal survival conditions, planktonic cells and biofilm aggregates might be released and transported to colonize new areas, making it difficult to control these microorganisms.

Escherichia coli is a bacterium commonly found in poultry farms that adopt the intensive rearing system. This bacterium causes colibacillosis, which can lead to respiratory, enteric infections, pericarditis, perihepatitis, sepsis and necrotizing dermatitis (cellulitis) (Dziva; Stevens, 2008). Appropriate cleaning and disinfection can help to prevent this disease and reduce the presence of microorganisms in the poultry farm environment.

The intake of poor quality water can interfere with the biological indices and the spread of diseases because all animals have access to the same water drinker. This spread of diseases can cause serious economic losses, and carry pathogenic agents of diseases that are of public health interest (Togashi et al., 2008). Thus, performing the microbiological analysis of water drinkers and determining the biofilm-forming capacity of the *E. coli* strains present in the chicken farms is a matter of public health, so that producers and veterinarians can take the necessary preventive steps and control actions.

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This study conducted microbiological analysis of the water wells and drinkers of chicken farms to identify biofilm-forming *E. coli* strains in the drinkers and to determine the effectiveness of the disinfection procedures adopted by the chicken farms in São Paulo state.

Material and methods

The potability analysis of 33 water samples was performed to assess the total coliforms, thermophilic and mesophilic bacteria and to determine whether these samples complied the sanitary microbiological standards for human consumption required by the RDC No 12 (BRASIL, 2001).

The surface swabs of the water troughs were analyzed for *E. coli* using eosin methylene blue (EMB - Merck) agar test and biochemical tests in Rugai medium (Newprov) (Araujo et al., 2008). Subsequently, these bacteria were evaluated for their biofilm-forming capacity followed by the inhibition test of sessile structures in chlorhexidine and chlorine.

The samples were collected from three chicken farms located in Santa Rita do Passa Quatro and five in Mococa, Sao Paulo, between May and July 2014. The water samples were collected from 10 nipple-type drinkers in each of the 33 chicken sheds evaluated and were preserved in sterile bags following the aseptic methods proposed by Silva et al. (2005).

The *E. coli* presence was determined in swab samples collected in the drinkers of the 33 sheds. Ten drinkers were randomly rubbed with sterile swabs, totaling 10 swabs per shed. The swabs were placed in tubes with Stuart medium transport (Silva et al., 2005). The bottles with the water samples and swabs were placed in isothermal boxes containing ice cubes.

The total and fecal coliform counts were performed using the multiple-tube method to determine the most probable number (MPN) of the target microorganisms in the sample, using the Sodium Lauryl Sulfate Broth (CLS) (Oxoid). The samples were incubated at 35 ± 1°C for 48 hours, and those with bacterial growth and gas production were cultured in tubes containing Brilliant Green Lactose Bile Broth (CLBVB) (Oxoid) at 35°C for 48 hours, and in selective detection of *E. coli* (EC Broth) at 45°C for 48 hours (Silva et al., 2006). The final score was determined using the Most Probable Number table proposed by the Brazilian Association of Technical Standards (ABNT) (1991).

The mesophilic bacteria test was conducted in Plate Count Agar (PCA) (Oxoid). Three serial dilutions were made per sample, and 1-mL aliquot was inoculated on sterile Petri dishes for further plating using the pour-plate technique, in duplicate. Subsequently, the inverted plates were incubated at 35°C for 48 hours, followed by the colony count. The result was given by the number of colonies multiplied by the reciprocal of the dilution plates with counts between 25 and 250 colonies, in colony-forming unit/mL (CFU/mL) (Osowsky; Gamba, 2001).

The results were compared to the standards set by the RDC 12/2001 of the National Health Surveillance Agency (ANVISA) (Brazil, 2001).

The surface swabs of the drinkers were transferred to plates containing eosin methylene blue (EBM- Merck) agar and incubated at 35°C for 24 hours. The black colonies formed, suspected of *E. coli,* with or without the metallic green sheen, were identified by biochemistry in Rugai medium (Newprov) (Araújo et al., 2008).

For phenotypic identification of the *E. coli* biofilm-producer strains, the bacteria were grown in brain heart infusion broth (BHI) (Himedia) at 37ºC for 24 hours, seeded in congo red agar (CRA) [0.8 g Congo red dye (Vetec) and 50 g of sucrose (Vetec) to 1 L brain heart infusion agar (Himedia)] (Corbett et al., 1987; Freeman et al., 1989). To this end, the Congo red agar plates were inoculated and incubated at 37°C for 24 hours, followed by incubation at room temperature for 48 hours. The production of rough black colonies distinguished the non-producing biofilm strains, which formed smooth red colonies.

This test was conducted with samples that expressed the phenotype in CRA to determine the biomass producing strains and quantify the absorbance before and after contact with chlorine and chlorhexidine.

The “in vitro” biofilm-producing capacity was determined following the methodology proposed by Cucarella et al. (2001), with minor modifications as described below. The *Escherichia coli* ATCC 25922 was used as positive control. Polystyrene flat bottom microplates with 96 wells were used, with all strains cultivated in triplicate, diluted to 1: 200 in BHI containing 0.25% glucose (Synth) and incubated for 24 hours at 37°C with stirring. Then, the wells were washed 3 times with 200µL of sterile saline (0.85% NaCl - Dynamics).

Subsequently, the first treatment consisted of adding 200µL chlorine (5ppm) followed by incubation at 37°C for 12 hours so that the disinfectant could act. The second treatment consisted of adding 200µL 2% chlorhexidine, rubbing the swabs on the microplate wells to simulate the cleaning of the chicken farm that is carried out with this product, and then incubated for 10 minutes. The solutions were prepared and left in contact with the samples, according to the label or suppliers’ instructions. After incubation, the microplates were washed three times with 200µL sterile saline (0.85% NaCl - Dynamics). Last, in the third treatment, the microplate was washed with water only, without the disinfectant, and incubated to evaluate the O.D. for each tested strain.

After washing, the microplates were incubated in an oven at 60°C and allowed to dry for about 30 minutes. Then, 200µL 1% crystal violet (Synth) was added for five minutes, followed by washing with distilled water and drying. Subsequently, 200µL 33% acetic acid (Isofar) was added and the readings were performed in the ELISA reader at 570 nm. Wells containing non-inoculated BHI broth and glucose were used as controls. The biofilm-producing strains were considered the samples with absorbance greater than 0.1 (Mack et al., 2000). The products were tested in triplicate, with three repetitions for the same sample.

The experiment followed a completely randomized design with three treatments and eight repetitions. A preliminary analysis of the data was performed to check the basic assumptions for the analysis of variance (ANOVA). Thus, the normality of the mathematical model residues was checked by the Anderson-Darling test and the variance homoscedasticity by the Levene test, at 5%. As the p-value of the Anderson-Darling test was lower than 5% (p-value = 0.013), the data were log (X) transformed to obtain new p-values (AD = 0.08 and Levene = 0.16).

The ANOVA checked for significant differences between the chlorine and chlorhexidine treatments and the control, followed by Tukey test when means were significantly different.

All procedures were performed by the SISVAR software (Ferreira, 2011) at 5% significance.

Results and discussion

Of the 33 water samples collected from the drinkers in the chicken sheds, only two had gas formation according to the results of the MPN technique, indicating the presence of microorganisms. These were transplanted to the tubes containing lactose brilliant green bile broth and selective EC broth, which also resulted in gas formation. Although the two samples formed gas, after seeding on EMB, no *E. coli* was detected in either sample. However, the detection of mesophilic bacteria showed that 15 of 33 evaluated samples displayed growth, of this, six were identified as biofilm-producing E. coli. This result suggests that there is no connection between the gas formation in water samples (detection of bacteria in water), and the presence of biofilm-forming bacteria (Table 1).

Moreover, 15 of the 33 swabs collected in the drinkers had *E. coli* as shown by the EMB and confirmed in Rugai medium. However, no relationship was observed between the *E. coli* bacteria present in the positive swabs and water samples that had gas formation. Of the 15 samples that presented bacterial growth, eight were identified as biofilm-producing *E. coli* by the phenotypic tests (Table 1).

Table 1: Water analysis results and swab surfaces of positive drinking fountains for total coliforms (CLS, CLBVB, EC Broth) by the MPN technique, presence of mesophilic bacteria (PCA), identification of *E. coli* (EMB) and ability to form biofilms (CRA)

Sample	CLS	CLBVB	EC Broth	PCA	EMB	CRA
Water	2	2	2	-	-	-
Water	-	-	-	15	6	6
Swab	-	-	-	-	15	8
Total	33	33	33	33	33	66

*CLS: sodium lauryl sulfate broth, CLBVB: lactose brilliant green bile broth, EC Broth: selective detection of *E. coli,* PCA: plate count agar, EMB: eosin methylene blue, CRA: congo red agar, -: unrealized test.

The identification of a larger number of strains of *E. coli* by the EMB swab samples that water concentration occurs because the sample rate, wherein the microorganisms on surfaces are more grouped with a higher probability of being identified when seeded on EMB different water samples in which microorganisms are dispersed. It was also observed that when the sample is sown in the PCA and then peaked for the EMB, the strain is pre enriched with great chances to grow in selective medium EMB as in this study.

Silva et al. (2006) used the multiple-tube technique to detect *E. coli* in EMB agar and reported coliforms at 45ºC and *E.coli* in several samples of cheese, sausage, vegetables and corn meal, while some samples with coliforms were negative for *E. coli*, similar to the results of this study. The multi-tube technique is considered a standard method, but it is subject to various interferences such as the presence of antagonistic bacteria and natural inhibitors of the selective medium, use of high incubation temperature, and competition for lactose between non-coliform bacteria. These factors can lead to underestimated detection or non-detection of *E. coli* (Evans et al., 1981; Koneman et al., 1997).

In this work, 53.40% (8/15) and 53.40% (8/15) of *E. coli* samples/strains identified on the swabs and in the mesophilic test, respectively, had the phenotypic traits for biofilm production. These results are lower than the 63.00% of the *Salmonella* strains identified as biofilm-producing on the surfaces of polystyrene and canvas mats in cutting areas of slaughterhouses (Vivian, 2014). Barros et al. (2014) identified 31 (64.58%) strains of biofilm-producing *E. coli* in the water of rivers nearby the Atlantic Ocean.

Eight biofilm-producing *E. coli* samples swabs were used to test how effectively chlorine and chlorhexidine are to destroy the biofilm by microplate assay, performed in triplicate with three replicates, as shown in Figure 1.

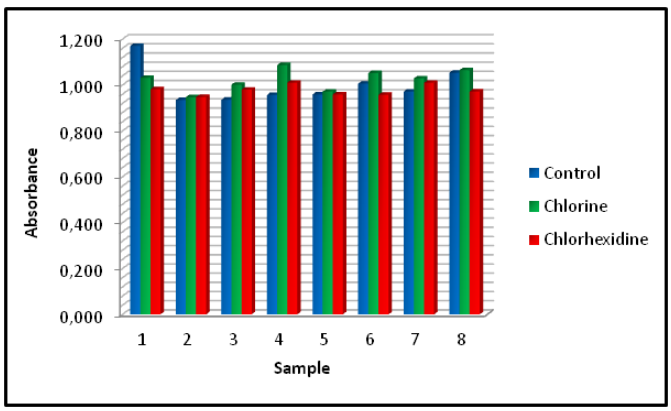

Figure 1: Phenotypic analysis of *Escherichia coli* biofilm to evaluate the effectiveness of chlorine and chlorhexidine treatments, and control.

The ANOVA showed no significant differences between treatments. Even though the absorbance of the samples showed that the chlorhexidine treatment reduced the biofilm, this difference was not significant.

Telles (2011) reported that removing the adhered microorganisms that form the biofilm requires either a mechanical force or chemical disruption of the adhesion force by applying enzymes, detergents, surfactants, disinfectants and/or heat. Nevertheless, this study did not reach satisfactory results regarding biofilm removal either with disinfectant or by applying mechanical force with the swabs.

Vivian (2014) used the stainless steel, a hydrophilic material, as a reference compared to plastic and aluminum materials, which are hydrophobic. This information is extremely important, as it has been previously shown that the microorganisms adhere in higher numbers to more hydrophobic materials (Donlan, 2002), helping to explain the occurrence of biofilms in the surveyed farms.

Macari et al. (2012) stated that chlorine is the most recommended disinfectant due to good efficacy, low cost, convenience of use, and the fact that it is harmless to the birds when correctly used. In this work, the chlorine was chosen by the studied company as the primary disinfectant, 5ppm (parts per million). According to Palhares and Kunz (2011), the recommended dosage of chlorine in the drinker output varies from 1 to 3 ppm for chicks, and from 5 to 6 ppm for chicken older than 28 days, without decreasing

consumption. The recommended range would vary between 3 and 5 ppm on average. However, the results showed that the biofilm persisted even in the presence of chlorine in the water, in the test microplate, although this test is not to identify the bacteria that make up the biofilm are viable or not.

Among the disinfectants, chlorhexidine stands out especially due to low toxicity, broad spectrum, and the physical properties; it is odorless, colorless, and non-corrosive (Redu, 2014). For these reasons, it was chosen by the studied company to clean the poultry drinkers; however, the results were not satisfactory since the microorganisms were not completely removed. According to Redu (2014), this cleaning agent is more effective for Gram-positive bacteria and less effective for Gram-negative, which may explain the persistence of *E. coli* even after cleaning with chlorhexidine.

Several studies in dentistry have shown that chlorhexidine has antimicrobial activity against oral fungal biofilms such as *Candida* spp. in bovine dental enamel (Machado et al., 2010). The biofilm isolates composed of *Streptococcus mutans*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (Solmaz; Korachi, 2013) were significantly reduced *in vitro* by chlorhexidine for dosages varying from 3 to 12 mg/L. In this study, on the other hand, chlorhexidine used as a disinfectant of drinkers with *E. coli* biofilm did not show satisfactory results.

Ebrahimi et al. (2014) evaluated the efficacy of different chlorhexidine concentrations against planktonic cells and *in vitro* biofilm produced by *E. coli*, *Salmonella* spp., *Staphylococcus aureus* and *Streptococcus agalactiae*. These authors observed that the chlorhexidine concentration has to be two times higher against biofilm compared to the planktonic cells to reach similar results while the sub-inhibitory dose can stimulate the formation of biofilms.

The persistence of microorganisms in the water even in the presence of chlorine might be due to several reasons. Chlorine quantities were not evenly distributed in all points of the distribution network since low chlorine or practically zero concentrations were detected in the terminal areas that are very distant from the post-chlorination points, thus allowing bacterial growth. The chlorine deficiency in the water can also result from its reaction with the organic matter and/or corrosion products (Connell, 1996). These factors contribute to biofilm formation in the distribution network, equipment, and utensils in chicken farms, and in this study, the chlorine dosage used by the farms was evaluated and proven not sufficient to eliminate the *E. coli* biofilms.

Herczegh et al. (2013) conducted a study with *Streptococcus mutans*, *Lactobacillus acidophilus*, *Enterococcus faecalis*, *Veillonella alcalescens*, *Eikenella corrodens*, *Actinobacillus actinomycetemcomitans* and *Candida albicans* oral biofilm to test the effectiveness of sodium hypochlorite, chlorhexidine gluconate and chlorine dioxide against the biofilms. These authors observed that chlorine dioxide was better than other antiseptics against the biofilms produced by aerobic bacteria and fungi, but the antiseptics were not significantly different against the biofilms produced by anaerobic bacteria. These results are similar to this study because *Eikenella corrodens* and *Actinobacillus actinomycetemcomitans* are also Gram-negative bacteria, facultative anaerobic, and chlorhexidine and chlorine were not effective to destroy them in the form of biofilms.

Thus, it is essential to investigate the presence of biofilms with anaerobic Gram-negative in contact with food and water since the use of sanitizers in the tested dosages of 5 ppm of chlorine and 2% chlorhexidine might not be effective to destroy the biofilms and contribute to the spread of new biofilms, harming animal health.

Conclusions

Eight *E. coli* strains found on the surface of poultry drinkers, produced biofilms. The two disinfectants tested were not able to remove *E. coli* biofilms. The results suggest the need for great care regarding the cleaning procedures and disinfection of poultry equipment to prevent buildup of microorganisms and biofilms, and consequent animal production losses.

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