Antimicrobial activity of poultry hatch baskets containing copper inserts

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Primary Audience: Researchers, Veterinarians, Plant Managers, Quality Assurance Personnel

SUMMARY

Commercial poultry hatcheries provide ideal conditions for the multiplication and spread of microorganisms. Formaldehyde is widely used as a disinfectant; however, it is harmful to human health and can cause abnormal morphology in chicks. An alternative microbiological control is the use of copper, a metallic antimicrobial agent for contact surfaces. The antimicrobial efficacy of copper surfaces has been established in healthcare environments. However, its use in the poultry chain is still limited. This study aimed to compare the antimicrobial activity of common polypropylene hatch baskets with hatch baskets composed of copper (polypropylene hatch baskets covered by copper hatch baskets; polypropylene hatch baskets with solid copper plates on the bottom; polypropylene hatch baskets covered by copper hatch baskets and with solid copper plates on the bottom). To simulate a hatching environment with high contamination, the eggs and hatching cabinet were not fumigated. Microbiological analysis of the hatching cabinet environmental, surface of hatch basket, and fluff were performed. The results indicated that the absence of bottom holes resulted in a higher volume of organic matter that interfered with the copper's antimicrobial activity. The presence of copper in the hatch baskets did not decrease microbial contamination under the conditions evaluated, confirming that the metal should only be used as a complement to standard hygiene and not as a substitute for surface disinfectants. Further analysis will evaluate the antimicrobial activity of hatch baskets composed only of copper and the ability of nanoparticles to remove the biofilms formed by bacteria isolated from the poultry environment.

Key words: copper surfaces, poultry hatcheries, microbial control, formaldehyde

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DESCRIPTION OF PROBLEM

Microbial contamination in a few hatching eggs is easily disseminated by air movement and thus can contaminate other chicks throughout the same hatcher room (Buhr et al., 2013; Warren et al., 2016). Commercial poultry hatcheries have ideal temperature, nutrient, and humidity conditions for the maintenance and multiplication of microorganisms (Graham et al., 2018). Common microorganisms such as *Escherichia coli, Staphylococcus, Streptococcus, Pseudomonas, Salmonella*, and *Aspergillus fumigatus* can adversely affect the eggs' hatchability, affect chick quality, and even result in embryonic deaths (Gehan, 2009). Contamination is especially high in hatch baskets, which must be thoroughly cleansed to prevent them from becoming a source of pathogen spread. Washing and sanitizing chicken transport cages reduces, but does not completely eliminate, bacterial contamination on the flooring surface (Northcutt and Berrang, 2006). Therefore, an effective sanitation program is essential for a poultry hatchery's successful operation (Graham et al., 2018). Formaldehyde is a chemical feedstock for numerous industrial processes and is widely used as a disinfectant and biocide in Brazilian hatcheries because it is cheap, easily available, noncorrosive, and kills most microorganisms (Salthammer et al., 2010). However, formaldehyde is a mucosal irritant and as a fumigant, has a persistent noxious odor, making venting its vapors difficult (Salthammer et al., 2010; Graham et al., 2018). In addition, due to its classification as a human carcinogen, it has been banned in several countries. This classification was based on studies of the relationship between formaldehyde exposure and nasopharyngeal cancer and leukemia (Salthammer et al., 2010; Swenberg et al., 2013). In the hatchery, exposing chicks to formaldehyde gas during pipping has been shown to cause ciliostasis and abnormal morphology (Hayretdag and Kolankaya, 2008). Other moderately effective disinfectants such as quaternary ammonium compounds, peroxides, glutaraldehyde, and phenolics are also currently utilized in the poultry industry (Gehan, 2009). Thus far, efforts to replace the use of formaldehyde have found large microbial populations in hatching cabinets despite the application of alternative sanitation procedures (Wright et al., 1995; Graham et al., 2018).

An alternative microbiological control for these structures is the use of surfaces made of copper, which the US Environmental Protection Agency registered in 2008 as the first metallic antimicrobial agent for contact surfaces (Depner et al., 2015). The antimicrobial efficacy of copper surfaces has been established for a variety of bacteria and fungi, including in healthcare environments (Noyce et al., 2006; Elguindi et al., 2011a; Depner et al., 2016; Vincent et al., 2016; Parra et al., 2018). Current worldwide concern about increasing antimicrobial resistance and growing concerns for the environment and excessive disinfectant use has led researchers to reconsider the use of alternative antimicrobial agents such as trace elements, e.g., copper (Elguindi et al., 2011a; Vincent et al., 2016). Although copper has been a part of the Earth for millions of years, microbial tolerance to copper is extremely rare. This is explained by copper's multisite kill mechanism and its mostly nonspecific damage mechanisms (Borkow and Gabbay, 2009). Some experimental studies have demonstrated in situ that surfaces containing at least 55 to 70% copper eliminate many seeded pathogenic microorganisms (Vincent et al., 2016). In addition, the incorporation of copper nanoparticles in polymeric matrices has produced excellent results in inhibiting the growth of a broad spectrum of microorganisms (Tamayo et al., 2016).

Copper is an essential nutrient for many organisms, including microbes, and enzyme-associated copper is a requirement for aerobic metabolism. However, excess copper accumulation or the intracellular release of free copper leads to severe toxicity (Quaranta et al., 2011). Copper toxicity is related to its tendency to alternate its oxidation state between cuprous (Cu^{1+}) and cupric (Cu^{2+}) ions (Depner et al., 2015). Under aerobic conditions, copper readily catalyzes reactions that result in the production of hydroxyl radicals. These radicals damage biomolecules such as proteins and lipids and may be involved in damage to nucleic acids (Quaranta et al., 2011; Souli et al., 2013). The genome and plasmid analyses of bacterial cells recovered from metal surfaces have indicated

substantial DNA degradation after exposure to copper. However, several authors claim the DNA damage is secondary, caused by cell death (Warnes et al., 2010; Souli et al., 2013). The mechanism by which microbial death occurs on copper contact surfaces is called "contact killing" (Depner et al., 2015). Thus, the antibacterial activity of copper relies on close contact between the bacteria and the surface releasing the ionic copper, which can be influenced by different factors such as temperature, humidity, wet or dry application, copper concentration, type of contact, bacterial species, and the oxidization state of the ions (Vincent et al., 2016; Parra et al., 2018). In this study, we aimed to evaluate the antimicrobial activity of hatch baskets composed of 99.9% copper.

MATERIALS AND METHODS

Animal Use Approval

All experimental procedures described were approved by the Ethics Committee on Animal Use at the Federal University of Santa Maria (**UFSM**) under protocol number 130/2014.

Local Description

The experiment was conducted in a commercial poultry hatchery located in the state Rio Grande do Sul (Southern Brazil). The hatchery produces a monthly average of 1,300,000 d-old chicks; the establishment has 16 conventional incubators with multistage operation and 8 hatching cabinets, 4 on each side of the room. The machines (model 576, Petersime, Içara, Brazil) operate with incubator trays having a capacity for 150 eggs.

To evaluate the copper antimicrobial activity in a hatching environment with high contamination, some of the hatchery cleaning and disinfection program practices were modified. First, the eggs used in the experiment were not fumigated with formaldehyde prior to their allocation in the egg holding room. The hatching cabinet used was not fumigated in the 3 previous hatchings or during the tests. Due to the volatility of formaldehyde, the other cabinets in the hatching room were also not fumigated during the experiment. The other practices in the cleaning and disinfection program of the hatchery were left unmodified, including the standard hygiene protocols for the hatch baskets in the experiment.

Experimental Design

For this experiment, a total of 5 hatchings were evaluated. Floor eggs from broiler breeder flocks between 56 and 66 wk old were identified. For each hatching evaluated, 4 basket compositions (treatments) were tested (Figure 1): 1) polypropylene hatch basket; 2) polypropylene hatch basket covered by a 99.9% Cu (Cu11000) hatch basket; 3) polypropylene hatch basket containing a 99.9% Cu (Cu11000) solid bottom plate; 4) polypropylene hatch basket covered by a 99.9% Cu (Cu11000) hatch basket and containing a 99.9% Cu (Cu11000) solid bottom plate. All hatch baskets were the same dimensions (580 mm × 755 mm × 83 mm) and were identified with different colored seals.

The polypropylene hatch basket (Treatment 1) was used as a control. The copper hatch basket (Treatment 2) is a prototype that fits inside a conventional polypropylene hatch basket and is composed of 99.9% Cu. As the antimicrobial action of copper occurs through close contact between the bacteria and surface releasing ionic copper, solid copper bottoms (Treatments 3 and 4) were also tested.

The tests were always performed on the right side of the hatching cabinet. The hatch baskets from each treatment were organized in the same column and were tested in 3 different positions (upper, intermediate and lower levels) in the trolleys. A clockwise rotation of the columns for each treatment was established at each hatching to avoid the influence of treatment position in the trolleys. Thus, the first position of each treatment was only repeated at the fifth hatching. The standard temperatures for the incubators and hatching cabinets were 99.5°F and 98.8°F, respectively. Humidity in a wet bulb thermometer was maintained at 84°F for both machines.

Microbiological Analysis

Surface Swab. The level of microbial contamination on the hatch baskets was evaluated at 4 time points: 0 h (initial contamination after

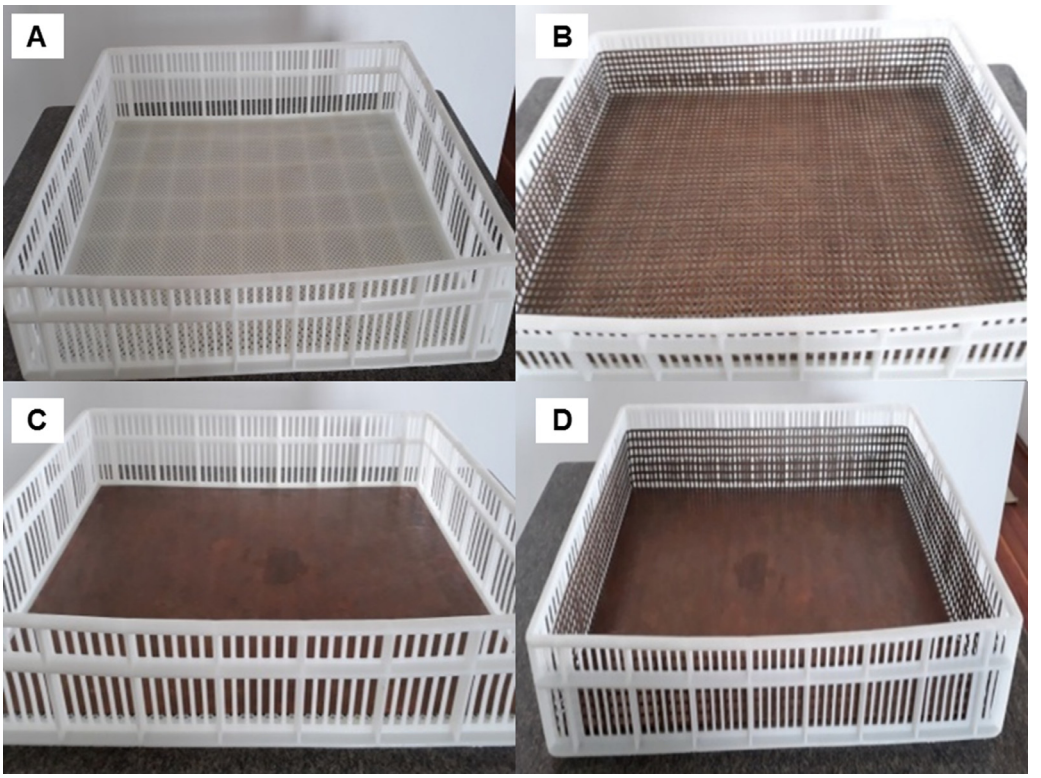

Figure 1. Hatch basket compositions used in the experiment: (A) polypropylene hatch basket (Treatment 1/control); (B) polypropylene hatch basket covered by a prototype 99.9% Cu (Cu11000) hatch basket (Treatment 2); (C) polypropylene hatch basket containing a solid 99.9% Cu (Cu11000) plate on the bottom (Treatment 3); (D) polypropylene hatch basket covered by a prototype 99.9% Cu (Cu11000) hatch basket and containing a 99.9% Cu (Cu11000) plate on the bottom (Treatment 4).

disinfection and transfer of the eggs to the trays); 24 h after the first sampling; 48 h (time that chicks are removed from the hatching cabinet); 7 d (average basket storage period after hatching and before sanitation for the next hatching). The bottom of each hatch basket was sampled with a swab soaked in 0.9% sterile saline over a 25 cm^2 area delimited by a sterile metal model. The samples were stored in sterile tubes and refrigerated until laboratory analysis. The enumeration of total molds and yeasts, mesophilic microorganisms, *Enterobacteria*, and *Escherichia coli* colonies were performed according to the guidelines of Normative Instruction 62 of the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA; Brazil, 2003). The results are expressed as colony forming units per square centimeter (cfu/cm^2).

Environmental Contamination. Three Petri dishes containing plate count agar (**PCA**; Oxoid, Basingstoke, UK) and Sabouraud dextrose agar (**SDA**; Oxoid, Basingstoke, UK) for enumerating the total mesophilic microorganisms and molds and yeasts, respectively, were placed uncovered in the hatching cabinet environment 15 min before the chicks' removal. Plates were distributed in the front, middle, and back of the machine. After a 15-min exposure period, all plates were covered and refrigerated until laboratory analysis. The colony counts were performed according to the MAPA Normative Instruction 62 (Brazil, 2003) guidelines and are expressed in cfu. The same procedure was performed in a closed unfumigated hatching cabinet in the same room during the experiment.

Fluff Testing. At the end of the hatching period, fluff samples from each treatment (pool of the 3 hatch baskets) were collected and refrigerated until laboratory analysis. The total mold and yeast counts were performed according to MAPA Normative Instruction 62 (Brazil, 2003) guidelines, and the results are expressed as cfu/g.

Statistical Analysis

A design with 15 repetitions (hatch baskets) was used for each treatment in the 5 hatchings. Levene and Shapiro-Wilk tests were performed to evaluate the homogeneity of variance and normality of the parameters, respectively. The counts of microbiological microorganisms in surface swabs and environmental contamination did not show a normal distribution by the Shapiro-Wilk test analysis ($P < 0.05$), regardless of the treatment. On the other hand, the microbial counts in the fluff were normally distributed, according to the analysis by the Shapiro-Wilk test ($P > 0.05$). Based on these analyses, nonparametric Kruskal-Wallis and Mann-Whitney tests were used to compare the means for the total molds and yeasts, mesophilic microorganisms, *Enterobacteria*, and *E. coli* colonies obtained from the surface swabs and the environmental contamination collections. A one-way analysis of variance (**ANOVA**) was performed to examine the statistical significance of the other results. The microorganism counts within each treatment were analyzed as a function of time using a repeated-measure ANOVA. When a Mauchly test indicated that the sphericity hypothesis was violated ($P < 0.05$), the degrees of freedom were corrected using the Greenhouse-Geisser procedure. The Statistical Package for Social Sciences (**SPSS**), version 20.1, was used for the analyses, adopting a reference significance level of 5%.

RESULTS AND DISCUSSION

Microbial contamination in hatcheries and eggs can seriously impact the viability and quality of chicks, as well as the overall growth performance of chickens (Chen et al., 2002; Graham et al., 2018). The progressive development of the poultry industry has led to increased hatchery sizes, many of them operating nonstop throughout the year. In addition, poor standards of hygiene in a hatchery facility can favor an explosion of pathogenic organisms, resulting in severe economic losses (Samberg and Meroz, 1995; Gehan, 2009; Buhr et al., 2013). Hatchery hygiene level is recognized as an important factor in healthy poultry production (Gehan, 2009), and the use of materials with copper, a metal with antimicrobial properties, could assist in this process (Vincent et al., 2016). In addition, the physical properties of copper include low corrosion, high thermal and electrical conductivity, and ease in malleability (Ameh and Sayes, 2019). However, copper alloys, which have more anticorrosion properties, may not perform well as bactericidal surfaces. Under these circumstances, maintaining higher corrosion rates without the application of corrosion inhibitors is imperative to achieve the maximum bactericidal effects (Elguindi et al., 2011b). Microbiological environmental monitoring is an essential element of hygiene control in the hatchery. Diverse monitoring methods can be used, including sampling the hatchery air, surfaces, and fluff, as was performed in our study (Chen et al., 2002; Warren et al., 2016). Despite being a low cost and an easy method to perform, the microbiological counts of air sampling may be below the real levels. Thus, surface swabbing and microbiological examination of fluff are more reliable methods for evaluating the hygienic status of a hatchery. Furthermore, fluff can be easily sent by mail to a distant laboratory for evaluation of the hygienic status (Gehan, 2009).

In this study, there were no significant differences ($P > 0.05$) in the mesophile, *Enterobacteria, E. coli*, or mold and yeast counts within each treatment among the 5 hatchings, regardless of sample type (data not shown). Thus, we consider it unlikely that individual hatching conditions influenced the results. For statistical analysis purposes, we considered the mean microorganism counts from the 4 differing sample collection time points or observation periods. The microbial counts from the surface swabs also showed no differences ($P > 0.05$) among the 5 sampling periods evaluated.

Table 1 summarizes the results of the microbial counts recovered from the hatch basket surfaces, which show that the hatch basket composition influenced the amount of contamination on the surface area. The microbial counts, in general, were lower ($P < 0.05$) for Treatment 2 than for Treatments 3 and 4, regardless of the microorganism. The exception was the lower concentration

Table 1. Microorganism counts recovered from the hatch basket surfaces, according to treatment.

Treatment	Mesophiles cfu/cm^2	*Enterobacteria* cfu/cm^2	*E. coli* cfu/cm^2	Molds and yeasts cfu/cm^2
T1	704.44 ± 265.63^{ab}	125.79 ± 72.26^{ab}	2.95 ± 1.84^{ab}	323.82 ± 157.11^{a}
T2	576.88 ± 255.09^{b}	118.09 ± 108.84^{b}	0.39 ± 0.26^{b}	168.23 ± 124.66^{b}
T3	1185.67 ± 524.60^{a}	225.37 ± 175.7^{a}	8.12 ± 6.50^{a}	779.79 ± 364.49^{a}
T4	1286.61 ± 448.65^{a}	228.46 ± 114.32^{a}	0.14 ± 0.10^{a}	264.86 ± 124.66^{a}

Data are expressed as the mean ± standard deviation and correspond to the mean of the 5 hatchings observed.
Abbreviation: cfu/cm^2, colony forming units per square centimeter.
^{ab}Different letters in the same column indicate there is a statistical difference ($P < 0.05$) in the microorganism count among treatments according to a Student-Newman-Keuls test (P-value < 0.05); n = 15/group.

of *E. coli* recovered from the surface of hatch baskets in Treatment 4 in relation to Treatment 2. The release of copper ions from a copper surface is a key element of the bacterial killing process. The intimate contact between the bacteria and copper causes significant cell membrane damage, which in turn makes the cells more susceptible to the released copper ions (Vincent et al., 2016). Thus, a lower microbial count was expected for Treatments 3 and 4. However, an important difference in the hatch baskets for Treatments 3 and 4 was probably responsible for their worse results. These hatch baskets had a solid plate of copper on the bottom rather than the holes present in the hatch baskets used in Treatment 2 (Figure 2). In addition to reducing air circulation among the hatch baskets, the lack of holes probably resulted in a higher accumulation of organic matter. This suggests that despite the greater contact between the surface and microorganisms in Treatments 3 and 4, the amount of accumulated organic matter was greater than the antimicrobial capacity of the copper. Rich culture media, as seen in this situation, can prolong survival on copper surfaces due to the copper ions' binding to organic molecules, decreasing the copper bioavailability and subsequent ion influx (Noyce et al., 2006; Elguindi et al., 2011b).

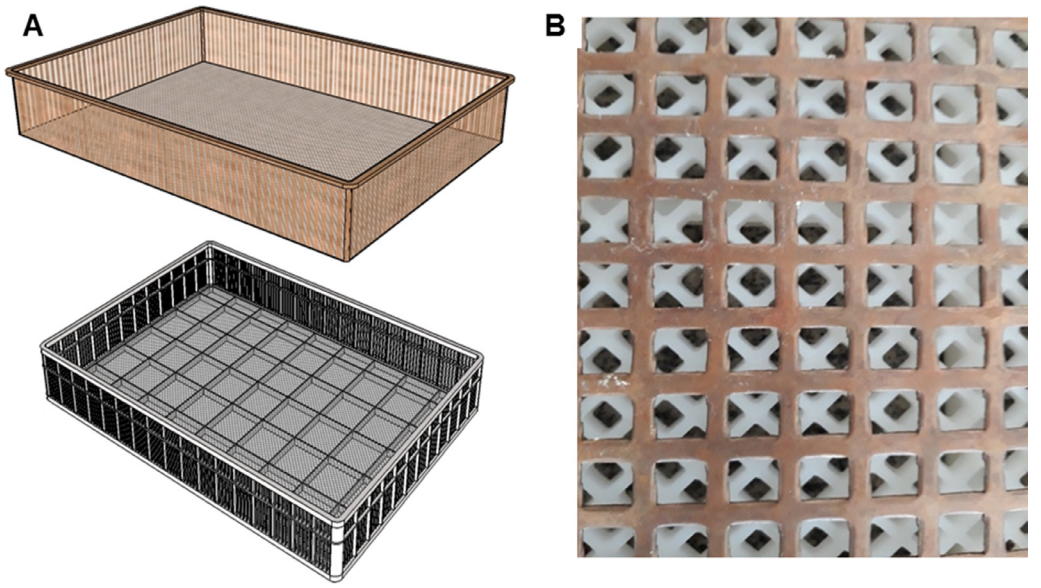

Figure 2. (A) Plug-in model of the copper hatch basket prototype inside the polypropylene hatch basket. (B) Detail of the bottom of the attached hatch baskets (Treatment 2), showing the overlapping of the holes and reduction in void space.

In contrast, there was no significant difference ($P > 0.05$) in the reduction of bacterial contamination between Treatments 1 (control) and 2. In this case, only the mold and yeast counts were lower ($P < 0.05$; Table 1). Copper and copper compounds have been shown to effectively kill a wide range of yeast and fungi such as *Aspergillus* sp. (Borkow and Gabbay, 2009). However, the hatch baskets in Treatment 2 had smaller gaps due to an overlap of the holes in the copper prototype and polypropylene hatch baskets (Figure 2). As a result, compared to the control group (Treatment 1), the polypropylene hatch baskets in Treatment 2 had a greater accumulation of organic material, which may have influenced the copper's antimicrobial activity.

Our group has previously shown the efficacy of antimicrobial copper against bacteria and fungi isolated from commercial poultry hatcheries in vitro. In that experiment, copper plates were immersed in standardized concentrations of the microorganisms and achieved promising results (Depner et al., 2016). Thus, the aim of this study was to evaluate the antimicrobial activity of copper in hatching cabinets with higher environmental contamination and with some modifications in the hatchery's sanitation program. It should be noted, however, the Environmental Protection Agency (**EPA**) emphasizes that copper and its alloys should only be used as a complement to hygiene control and not as a substitute for standard surface cleaning and disinfection practices (EPA, 2008).

Several factors probably influenced the observed results. In addition to the excess organic matter that accumulated in the bottom of the hatch baskets, another factor that probably influenced the higher counts was the lack of the minimum contact time. Initial microbial sampling occurred immediately after contact, i.e., there was not enough time for microbe inactivation. Previous studies have shown that it takes up to 3 h for copper ions to be released (Santo et al., 2012). This is not viable in hatcheries due to the chicks' movements in the hatch baskets, which alter the positions of the organic matter (fluff, eggshell, blood, and meconium) in the baskets. According to Wilks et al. (2006), bacteria exposed to metallic copper surfaces do not enter the physiological state classified as viable but not cultivable; generally, the bacteria are instead completely inactivated.

Table 2. Microorganism counts recovered from the environment, according to hatching cabinet.

Hatching cabinet	Mesophiles cfu	Molds and yeasts cfu
Test	215.42 ± 24.18^{a}	67.42 ± 21.36^{a}
Control	135.25 ± 35.08^{a}	56.83 ± 20.5^{a}

Data are expressed as the mean ± standard deviation and correspond to the mean of the 5 hatchings observed.
Abbreviation: cfu, colony forming units.
[a]Different letters in the same column indicate there is a statistical difference ($P < 0.05$) in the microorganism count among between test and control hatch cabinets according to Mann-Whitney test (P-value < 0.05); n = 15/group.

Table 2 shows the results of the mesophile, mold and yeast counts in the hatching cabinet environment. There were no significant differences ($P > 0.05$) between the counts inside the hatching cabinets of the experimental and control machines. Both machines showed high contamination, associated with not fumigating the environment and eggs during the experiment. The ability of copper to kill bacteria on contact is suppressed if bacterial-metal contact is prevented (Mathews et al., 2013; Vincent et al., 2016), as it was in this case, by the accumulation of organic material.

Table 3 shows the results of the mold and yeast counts recovered from the fluff. There were no significant differences ($P > 0.05$) among the treatments, despite the lower mold and yeast count means observed in Treatment 2. The fluff samples were collected from the

Table 3. Mold and yeast counts recovered from the fluff, according to treatment.

Treatment	Molds and yeasts (cfu/g x 10^3)
T1	40.4 ± 24.8^{a}
T2	36.9 ± 18.5^{a}
T3	51.5 ± 25.8^{a}
T4	87.9 ± 48.7^{a}

Data are expressed as the mean ± standard deviation and correspond to the mean of the 5 hatchings observed.
Abbreviation: cfu/g, colony forming units per gram.
[a]Different letters on the same column indicate that there is statistical difference ($P < 0.05$) in microorganism count among treatments, according to ANOVA (P-value > 0.05). n = 5/group.

hatching cabinet after hatching and from the chick processing room. Chick fluff is the most representative sample of the immediate hatching atmosphere (Warren et al., 2016) and is useful for evaluating the sanitary conditions of a hatchery as a means to prevent the spread of harmful bacteria and fungi (Samberg and Meroz, 1995; Chen et al., 2002; Gehan, 2009; Warren et al., 2016). Although the results reveal the magnitude of the mold and yeast contamination in the environment, they do not indicate how the mold and yeast reach the hatching cabinet or where they multiply. This information can only be determined by periodically surveying the microbial populations on the surfaces that can harbor microorganisms in a hatchery (Gehan, 2009). Our future studies will evaluate the antimicrobial activity of hatch baskets composed only of copper and the ability of nanoparticles to remove the biofilms formed by bacteria isolated from the poultry environment.

CONCLUSIONS AND APPLICATIONS

1. The copper did not decrease microbial contamination under the conditions evaluated, emphasizing that the metal should only be used as a complement to standard hygiene and not as a substitute for the common disinfectants used during standard surface cleaning and disinfection in hatcheries.
2. Regardless of the amount of copper surface contact area available, an accumulation of organic matter impairs the antimicrobial action of the copper.

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DISCLOSURES

The authors declare that there is no conflict of interest regarding the publication of this article.

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