Cleaning and disinfection programs against *Campylobacter jejuni* for broiler chickens: productive performance, microbiological assessment and characterization

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**ABSTRACT** Detailed cleaning and disinfection programs aims to reduce infection pressure from microorganisms from one flock to the next. However, studies evaluating the benefits to poultry performance, the sanitary status of the facilities, and the sanitary quality of the meat are rarely found. Thus, this study was designed to evaluate 2 cleaning and disinfecting programs regarding their influence on productive performance, elimination of *Campylobacter* spp. and characterization of *Campylobacter jejuni* strains when applied to broiler chickens’ facilities. Two subsequent flocks with 960 birds each were distributed into 32 pens containing 30 birds each. In the first flock, the whole flock was inoculated with a known strain of *Campylobacter jejuni* in order to contaminate the environment. In the second flock, performance and microbiological evaluations were done, characterizing an observational study between 2 cleaning and disinfection programs, regular and proposed. The regular program consisted of sweeping facilities, washing equipment and environment with water and neutral detergent. The proposed cleaning program consisted of dry and wet cleaning, application of 2 detergents (one acid and one basic) and 2 disinfectants (250 g/L glutaraldehyde and 185 g/L formaldehyde at 0.5% and 210 g/L para-chloro-meta-cresol at 4%). Total microorganism count in the environment and *Campylobacter* spp. identification were done for the microbiological assessment of the environment and carcasses. The positive samples were submitted to molecular identification of *Campylobacter* spp. and posterior genetic sequencing of the species identified as *Campylobacter jejuni*. The birds housed in the facilities and submitted to the proposed treatment had better performance when compared to the ones in the regular treatment, most likely because there was a smaller total microorganism count on the floor, walls, feeders and drinkers. The proposed program also resulted in a reduction of *Campylobacter* spp. on floors, drinkers and birds. Moreover, it was possible to identify 6 different *Campylobacter jejuni* strains in the facilities. The proposed treatment resulted in a positive influence on the birds’ performance and reduction of environment contamination for broiler chickens.

Key words: biosecurity, campylobacteriosis, disinfectant, health, poultry

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Received December 12, 2016.
Accepted May 10, 2017.

1The nucleotide sequence data reported in this paper have been submitted to GenBank (National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894 USA) nucleotide sequence database and have been assigned the accession numbers.

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2017 Poultry Science 96:3188–3198
http://dx.doi.org/10.3382/ps/pex153

**INTRODUCTION** Preventive practices that include cleaning and disinfection are fundamental steps for biosecurity programs and are indispensable for the maintenance of high productivity of poultry flocks. The aim of cleaning is the maximum removal of organic matter from facilities and equipment. Therefore,
detergents that reduce superficial tension, act in the emulsification of lipids, have dissolving powers on mineral residues and peptizing action on protein residues are utilized. Detergents can be alkaline, acid, or neutral (UGA, 2005). Alkaline detergents have high dissolving power on organic residues; acid detergents have high dissolving power on mineral residues and some organic ones; and the neutral detergents are indicated for delicate surfaces and with weakly adhered residues. The maximum efficacy of disinfection procedures is only possible on surfaces with appropriate removal of organic matter (Ward et al., 2006).

Another fundamental step is disinfection, which aims to reduce infection pressure from microorganism destruction as well as the transmission of pathogens from one flock to the next (UGA, 2005; Tokach et al., 2012). There are a great variety of active ingredients utilized as disinfectants in poultry production such as formaldehyde and glutaraldehyde, which are bactericides, spiroids, and fungicides. Their activity is due to the alkyla-
tion of sulfidryl, hydroxyl, carboxyl, and amino groups of microorganisms, altering their DNA, RNA, and protein synthesis. There is also peracetic acid acting as a bactericide by attacking the lipid membrane, DNA, and other cell components through toxic free radicals that a disinfectant produces (Dvorak, 2008). However, cresols have bactericidal and viricidal action on the protoplasm of bacterial cells, causing denaturation and protein precipitation (Spinosa et al., 2006).

Production environments with high contamination levels have a direct influence on increased mortality, and/or indirect influence on uniformity and decreased broiler performance (Ristow, 2008; Renaudeau, 2009). Thus, cleaning and disinfection can have a positive influence on the increase in birds’ productive performance, mainly in environments where there is a sanitary challenge (Burbarelli et al., 2015). Besides guaranteeing high productivity, these practices are fundamental to ensure the quality of poultry products, making them appropriate for human consumption. The occurrence of diseases in humans, transmitted by poultry products, can be related to the birds’ contamination during their life in production facilities, and Campylobacter is one of the causative agents of disease (Shane et al., 1986; Stern, 1992; Smith et al., 2008). The contamination of chickens, and consequently of carcasses, is a reason to pay attention to the poultry productive chain (Evans and Sayers, 2000). In the field, one of the goals is the decrease of pathogen coloniza-
tion in the birds’ intestinal tract since the horizontal transmission of the pathogen is more efficient (Newell and Fearnley, 2003).

However, studies simultaneously evaluating efficiency of cleaning and disinfection protocols, benefits on poultry performance, sanitary status of the facilities and sanitary quality of the meat are rarely found. Thus, the objective of this study was to evaluate 2 cleaning and disinfection programs regarding their effectiveness on broilers’ productive performance and on the elimination and characterization of Campylobacter jejuni strains in environments that had been previously contaminated with Campylobacter jejuni.

**MATERIAL AND METHODS**

**Birds, Installations, and Experimental Scheme**

The experimental protocol was approved by the ethics committee for animal experimentation of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo, protocol 3025/2013.

A total of 1,920 day-old male Cobb 500 broiler chicks were divided into 2 subsequent flocks with 960 birds each. In both flocks, the birds were distributed into 32 pens containing 30 birds each. This work was an observational study study between 2 cleaning and disinfection programs: a Regular treatment and a Proposed one.

The floor was covered with new rice hulls litter and provided with a tubular feeder and bell drinkers. The housing density was 10 birds per m², with average initial weight of 45.5 g ± 0.763 g. The interval between the 2 flocks was 8 d.

The poultry house had average area of 500 m² (con-
sidering structure, ceiling, curtains, internal and exter-
nal parts, paving, flooring, and walls) which was utilized for the analysis and calculation of the cleaning and dis-
fection program. The poultry house had an internal room that divided it into 2 halves, guaranteeing the isolation of each experimental group.

The diets were formulated with corn and soybean bran according to Rostagno et al. (2011) and provided ad libitum. There was no addition of growth-promoting antibiotics in pre-mixtures. The chicks received chlo-
rinated drinking water at a concentration of 2.0 ppm.

The first-raised broiler group house experiment was designed to create a sanitary challenge by inoculating the birds with Campylobacter jejuni. Performance and microbiological evaluations were done in the second flock.

**Sanitary Challenge**

In the first housing, on d 11, the chicks were in-
oculated with standard Campylobacter jejuni (ATCC 33560) strains through an oral probe to deposit 1 mL of inoculum consisting of liquid BHI culture medium and 10⁵ UFC/mL of Campylobacter jejuni, considered a high dose by Chaveerach et al. (2004).

**Cleaning and Disinfection Programs**

Litter and all the equipment were removed from the poultry house to carry out the treatments. In both pro-
grams, approximately 0.4 L of diluted solution per m² was used for detergents as well as for disinfectants. The
water utilized in the treatments had chlorination at 2.0 ppm. The Regular treatment was applied in 16 pens and consisted of sweeping facilities, washing equipment (feeder, drinkers, buckets, boots) with neutral detergent, wetting and washing the environment (floors, walls, ceiling, curtains) with water and neutral detergent, and subsequently drying the environment and facilities. The Proposed treatment was also applied in 16 pens and started with cleaning and disinfecting the water supply system: washing the water reservoir and next applying 100 g/kg peracetic acid and 80 g/kg Benzyl-(C12-C16) chloro-alkyl dimethyl ammonium at 0.5%. The product was added to the water reservoir that was posteriorly drained by the water supply system, providing contact with the whole supply system, and kept like that for 12 h, and then drained through the triggers of the bell drinkers. Dry cleaning was done by removing bedding and sweeping the facilities followed by wet cleaning of the house and posterior with pressurized water. All equipment utilized in the poultry house (feeders, drinkers, buckets, boots, trays and other utensils) were washed under pressurized water. Next, alkaline and acid detergents in solution at 4% were applied on all internal and external surfaces (ceiling, walls, flooring, curtains), objects and equipment, followed by rinsing under pressurized water. Disinfectants were only applied after the environment and the equipment were partially dry, without water accumulation. The first utilized disinfectant consisted of 250 g/L glutaraldehyde and 185 g/L formaldehyde at 0.5% and was applied on all surfaces and equipment. The second one, composed of 210 g/L para-chloro-meta-cresol at 4%, was applied only on the floor and walls up to 0.5 m of height. A knapsack sprayer was utilized for both applications.

After finishing the treatments, new bedding was distributed in the pens, the equipment was relocated, and the second group of day-old chicks was distributed in the rearing pens.

**Productive Performance**

The birds were weighed on d 1, 7, 21, 35, and 42 of the experiment for performance analysis. The measured response variables in each pen were: body weight gain (BWG), feed intake (FI), feed: gain ratio (F:G), viability (VB), and productive efficiency index (PEI).

**Microbiological Evaluation**

Surface swabs of the floor, wall, drinkers, and feeders were done for total count and evaluation of *Campylobacter* spp. presence from surfaces of 2 cm × 5 cm totaling a 10 cm² area. Samplings of 200 mL of the birds’ drinking water from the bell drinker tap of 5 pens in each treatment were collected. Besides the mentioned points, swabs of 5 birds’ cloaca per program were swabbed for the evaluation of *Campylobacter* spp. presence. The swabs were stored in tubes containing peptone water at 0.1% to maintain colony viability. The total microorganism count was done 24 h before the cleaning and disinfection procedures and 48 h after them. Plate count agar (PCA) was utilized in previously solidified plates with sowing on the surface as described by Evancho et al. (2001).

The evaluation regarding the presence of *Campylobacter* spp. was done in the first house before and after inoculation. In the second flock, the evaluation was done when the birds were 2, 11, and 42 d old. Moreover, harvesting was done right before slaughter, after feathering and after chiller. In each point, 10 carcasses of each experimental group were used.

Direct isolation of *Campylobacter* spp. was done with inoculation of 0.1 mL of peptone water solution at 0.1% in petri dishes containing mCCDA (CM739, Oxoid, Hampshire, England) culture medium with selective supplement (SR155, Oxoid Hampshire, England) where inoculation of 0.1 mL of solution obtained from samples was done. Then they were incubated in an environment whose microaerophilic atmosphere was modified with 5% of O₂, 10% CO₂ and 85% N₂, at 42°C for 48 h in jars for special atmospheres (Probac do Brasil, São Paulo, Brazil). From the positive samples, 1 to 3 possible *Campylobacter* spp. colonies were randomly selected and submitted to Gram staining to differentiate S bacilli from spiral ones. Oxidase and catalase assays were also carried out. The colonies with compatible characteristics to *Campylobacter* spp. morphology were collected for posterior PCR assay.

**Molecular Identification**

PCR testing was performed to distinguish *Campylobacter* species in positive samples from the microbiological culture. Five typical colonies from the positive samples of *Campylobacter* spp. were harvested from the same sampling point. The colonies were submitted to bacterial DNA extraction through an adapted thermal shock technique by Fang and Hedin (2003).

Multiplex PCR technique was utilized, based on Klena et al. (2004). Primer pairs of *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari*, and *Campylobacter upsaliensis* were utilized for the amplification of DNA fragments found in each of the cited species. The specific primers presented lpxA as the target gene. For *Campylobacter coli*, lpxAColi and lpxARKK2m primers were used; for *Campylobacter jejuni*, lpxARs and lpxARKK2m; for *Campylobacter lari*, lpxALari and lpxARKK2m; and for *Campylobacter upsaliensis*, lpxAUPS and lpxARKK2m.

The amplifications were carried out in 25 μL of solution containing 1.25 μL of reaction buffer 5× Colorless GoTaq Flexi Buffer, 2 μL of MgCl₂, 0.5 μL of dNTP, 1 μL of each primer (lpxAColi, lpxAJjej, lpxALari, lpxAUPS), 3 μL of lpxARKK2m primer, 0.25 U of GoTaq
DNA Polymerase (Promega, Madison, WI, USA), 11 μL of nuclease-free water, and 3 μL of DNA.

For the amplifications, a thermocycler was utilized, programmed for an initial denaturation cycle at 94°C for 2 min., followed by 40 cycles with denaturation at 94°C, 1 min; annealing at 52°C, 1 min; extension at 72°C, 1 min., and final extension at 72°C, 5 min. The PCR products were analyzed by electrophoresis in agarose gel at 3%, stained with SybrGold (Invitrogen, Karlsruhe, Germany) (0.1 μL/mL) and visualized in a UV trans-illuminator (BioAgency, São Paulo, Brazil). The product sizes were determined by comparing electrophoretic migration standard of a 100-pb molecular size marker (GE Healthcare, USA).

Nuclease-free water was utilized as negative control whereas a strain of Campylobacter jejuni (ATCC 33560), the same utilized for environment contamination, was used as positive control.

The samples with electrophoretic migration standard compatible to the positive control were sent to genomic sequencing.

Genomic Sequencing, Nucleotide Sequence Alignment and Phylogenetic Analysis

DNA fragments were extracted from agarose gel for samples with band size compatible to Campylobacter jejuni, and QIAquick Gel Extraction Kit (Qiagen, USA) was utilized after PCR re-amplification following the manufacturer’s recommendation.

The sequencing reactions were done utilizing BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Life Technologies, USA) containing AmpliTaq DNA Polymerase, according to the manufacturer’s specifications, and reactions for sense and anti-sense primers were carried out using an automated sequencer, 3730 DNA Analyzer (Applied Biosystems, Life Technologies, USA).

The search for consensus sequences generated by the program CAP 3 Contig (Huang and Madan, 1999) and edited by BioEdit 7.0.9 (Hall, 1999) was done by BLAST program version 2.0 (Altschul et al., 1997). The editing and multiple alignment of obtained nucleotide sequences as well as others deposited in GeneBank (Table 1) were done by ClustalW program version 1.4 (Thompson et al., 1997), implemented in BioEdit Sequence Alignment Editor version 7.0.9 (Hall, 1999), utilizing default parameters. Distance matrices, given in percentages of similarity/identity, between the nucleotide sequences were calculated through MatGAT program, version 2.0 (Campanella et al., 2003), using global alignment algorithm.

Phylogenetic reconstructions for sequences of 213 nucleotides, related to lpxA gene were done through maximum likelihood algorithm and Jukes and Cantor (JC) substitution model with nodal bootstrap support for 1000 pseudo-replicates, utilizing MEGA 5.0 program (Tamura et al., 2013). For the reconstructions, other sequences of Campylobacter spp. deposited in GenBank were used as shown in Table 1.

Statistical Analysis

Data were analyzed by Statistical Analysis System (SAS Institute, 2012). Normality of studentized residual was verified by Shapiro-Wilk’s Test PROC UNIVARIATE (SAS Institute, 2012) and the variances compared by Levene’s test. The data that did not meet these requirements were submitted to logarithmic transformation. The original or transformed data were submitted to analysis of variance utilizing PROC MIXED (SAS Institute, 2012). The occurrence frequency of Campylobacter was analyzed by Chi-square test through PROC FREQ (SAS Institute, 2012). The utilized level of significance was 5% of probability.

RESULTS

There was no significant effect of treatments in the initial periods (1 to 7, 1 to 21, and 1 to 35 d). However, during the total housing period (1 to 42 d) there was a significant effect of the Proposed treatment, where the birds exhibited greater BWG, FI, F:G, and PEI (Table 2).

In the total microorganism count, counts were similar before on the floor, wall, drinkers, feeders, and water (Table 3). After the procedures, there was a difference between the treatments, showing that the smallest counts were found in water, feeders, walls, and floor

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**Table 1. Utilized nucleotide sequences of Campylobacter spp. for phylogenetic reconstruction with genotype, name, origin and respective access numbers in GenBank.**

| Species                  | Isolate      | Origin          | Genbank Access Number |
|--------------------------|--------------|-----------------|-----------------------|
| Campylobacter jejuni     | NCTC 11168   | United Kingdom  | AL111168              |
|                          | RM 3668      | California      | AYS31515              |
|                          | F 38011      | Arizona         | AYS31520              |
|                          | KLC2851      | N. Zeland       | AYS31522              |
|                          | RM 3664      | California      | AYS31519              |
| Campylobacter coli       | RM 1878      |                 | AYS31493              |
|                          | RM 1858      |                 | AYS31495              |
|                          | RM 1865      |                 | AYS31494              |
|                          | RM 1857      |                 | AYS31496              |
|                          | WA 27        | N. Zeland       | AYS31510              |
|                          | RM 3232      |                 | AYS31504              |
|                          | RM 1896      | USA             | AYS31492              |
| Campylobacter Lari       | RM 3659      | United Kingdom  | AYS31477              |
|                          | RM 2825      | Canada          | AYS31479              |
|                          | RM 2824      | United Kingdom  | AYS99884              |
|                          | RM 2819      | Canada          | AYS31485              |
|                          | RM 2822      |                 | AYS31482              |
|                          | RM 2100      | USA             | AYS31474              |
|                          | RM 2823      | Canada          | AYS31481              |
|                          | RM 1890      |                 | AYS31476              |
| Campylobacter Upsaliensis| RM 3195      | South Africa    | AYS31473              |
|                          | RM 2093      |                 | AYS98987              |
|                          | RM 2089      |                 | AYS31472              |
|                          | RM 1488      | Canada          | AYS31471              |
| Helicobacter hepaticus   | ATCC 51449   | USA             | DN202995              |
with the Proposed program. The drinkers from different treatments were not different (Table 3).

Table 4 shows the occurrence frequencies of *Campylobacter* spp. There was no difference in the sampled points before and after inoculation. After cleaning and disinfection, there was a smaller occurrence of *Campylobacter* spp. in drinkers and floors with the Proposed program. The birds housed in the facilities after the Proposed treatment also had less *Campylobacter* spp. verified by cloaca swabs 7 d after housing. There were no differences in the occurrence of *Campylobacter* spp. at 42 d old and at slaughter time.

| Sampling point | Common | Proposed | SEM | Probability |
|----------------|--------|----------|-----|-------------|
| Water          | 4.10   | 4.20     | 0.01 | 0.99        |
| Drinker        | 6.40   | 6.50     | 0.01 | 0.99        |
| Feeder         | 4.60   | 4.70     | 0.01 | 0.99        |
| Wall           | 4.50   | 4.60     | 0.01 | 0.99        |
| Floor          | 4.40   | 4.50     | 0.01 | 0.99        |

Table 3. Total microorganism count before and after cleaning and disinfection procedures.

| Sampling point | Common | Proposed | SEM | Probability |
|----------------|--------|----------|-----|-------------|
| Water          | 4.10   | 4.20     | 0.01 | 0.99        |
| Drinker        | 6.40   | 6.50     | 0.01 | 0.99        |
| Feeder         | 4.60   | 4.70     | 0.01 | 0.99        |
| Wall           | 4.50   | 4.60     | 0.01 | 0.99        |
| Floor          | 4.40   | 4.50     | 0.01 | 0.99        |

After the contamination frequency analysis, the positive samples were submitted to PCR and genomic sequencing, totaling 54 samples. The sequencing results confirmed *Campylobacter jejuni* for approximately 43% (23/54) of the samples. Besides the samples identified as *Campylobacter jejuni*, other enterobacteria were found after sequencing, such as *Enterobacter cloacae*, *Enterobacter asburiae*, and *E. coli*.

Table 5 presents the percentages of similarity (under the diagonal) and identity (over the diagonal) of nucleotide sequences found among the selected and sequenced samples and the reference sequences for *Campylobacter* deposited in GenBank (Table 3). The high similarity level among the samples obtained in this study can be observed, varying from 99.1 to 100%. Moreover, all the samples have a high similarity level of 98.6% with the standard ATCC 33560 strain. The same occurs with the identity among the samples, 99.1 to 100% among samples and 98.6% with standard strain.

When the samples (Table 6) were compared to other species of *Campylobacter*, there was smaller similarity and identity, which ranged from 69 to 86.9% for both. When compared to *Helicobacter hepaticus*, the values were 53.5% for similarity and 52.8% for identity.

Figure 1 illustrates the cladogram obtained in phylogenetic nucleotide reconstruction for *Campylobacter* sequences. The sequenced samples in this study were grouped into 2 subclades of *Campylobacter jejuni*. The
Identification Sampling point Accession n

BR212Cjej Bird 12 -initial environment KY321332
BR597 Cjej Bird 3 -After inoculation KY321333
BR1241Cjej Bird - 42 d Proposed Program KY321334
BR1236Cjej Bird 30–42 d Common Program KY321335
BR1241Cjej Bird - 42 d Proposed Program KY321334
BR597 Cjej -After inoculation KY321333
BR212Cjej Bird 12 –initial environment KY321332
BR1236Cjej Bird 30–42 d Common Program KY321335

Table 5. Comparison of similarity (under diagonal) and identity (over diagonal) percentages of nucleotide sequences of 331pb fragments of Campylobacter jejuni IpxA gene, among 6 samples detected, posteriorly sequenced, by multiplex PCR and sequences of other Campylobacter recovered in GenBank.

Table 6. Legends of sequenced samples utilized to build the phylogenetic tree.

| Similarity/Identity (%) | Isolated |
|------------------------|----------|
| BR597 Cjej             | – 100.0  |
| BR212Cjej              | 99.1     |
| BR1241Cjej             | 99.1     |
| BR1236Cjej             | 99.1     |
| BR1240Cjej             | 99.1     |
| BR1037Cjej             | 99.1     |
| RM3668Cjej             | 99.1     |
| NCTC11168Cjej          | 99.1     |
| KLC2851Cjej            | 99.1     |
| FD3011Cjej             | 99.5     |
| ATCC33560Cjej          | 98.6     |
| RM2825Clari            | 76.1     |
| RM1878Ccoli            | 86.4     |
| RM1915Cups             | 77.0     |
| ATCC531449Hhep         | 54.5     |

DISCUSSION

High bacterial populations are responsible for a decrease in broiler chickens’ performance (Payne et al., 2005). Thus, cleaning and disinfection practices have positive effects on broilers’ performance (Sharma, 2010) and on prevention of disease (Cozad and Jones, 2003; Newell et al., 2011); however, there are few studies that directly relate cleaning and disinfection to poultry’s performance characteristics. The positive effects presented in this study corroborate the ones by Burbarelli et al. (2015), who observed an improvement in broilers’ productive performance and reduction in microorganism count with a detailed cleaning and disinfection program for a flock with reutilized bedding. Bragg and Plumstead (2003) and Ka-Oud et al. (2008) also found beneficial effects of cleaning and disinfection such as greater final weight and lower mortality rate.

In poultry, the satisfactory performance expression is related to intestinal health (Mayorka et al., 2002); therefore, the microbiota balance is an important factor for productivity. Approximately 20% of ingested crude energy is spent on the maintenance of the intestinal epithelium. In addition, the reduction in nutrient absorption has negative influences on F:G, carcass yield, and production cost (Hoerr, 2001). Cleaning and disinfection practices are responsible for reduction of environment infection pressure (Sesti et al., 1998), favoring the intestinal microbiota balance, increasing nutrient absorption and consequently resulting in better expression of broiler chickens’ genetic potential.

The decrease in total microorganism count in equipment and facilities as a result of the Proposed treatment is in accordance to the studies by Luyckx et al. (2015), who observed a greater decrease in total microorganism count when a wet phase was included in the environment. This seems to be related to the easy organic matter removal of microorganisms with high-pressure washing (Grezzi, 2008). Organic matter removal seems to be fundamental to cleaning and disinfection programs because their residues are able to decrease the action of disinfectants (Stringfellow et al., 2009; Chima et al., 2012; Luyckx et al., 2017), resulting in greater microorganism counts even after disinfection.

Fewer positive samples of Campylobacter spp. were observed from drinkers, floor, and birds after the Proposed program, corroborating Van de Gissen et al. (1998) and Newell and Fearnley (2003) regarding the reduction capacity and elimination of Campylobacter spp. from the environment; however, that contradicts Bouwknecht et al. (2004), who did not find any effect of this type of treatment on facilities for broiler chickens.

Although it did not differ between the analyzed groups, the occurrence of Campylobacter spp. in the birds’ drinking water even after the Proposed program deserves attention. The parts of the water provision system of broiler chickens’ houses were a location of biofilm formation due to constant water contact (Araijo et al., 2011). Bacteria such as Campylobacter frequently are associated with biofilm (Shi and Szu, 2009). Because it is a gram-negative bacterium, Campylobacter is more
resistant to the action of disinfectants (Dahl et al., 1989), through a complex enzymatic system of resistance to oxidative stress, and among the enzymes of this system are superoxide dismutase, catalase and cytochrome C peroxidase (Atack and Kelly, 2009). The disinfectants utilized in the water system disinfection were peracetic acid and benzalkonium chloride, the former is an oxidant agent and the latter a quaternary ammonium compound.

Besides the bacterial resistance to the active ingredient we used, biofilm represents an additional resistance to bacteria (Chapman, 2003), because it is able to form a protection through its compounds, making oxidant compounds be inactivated even before getting in contact with the microorganisms (Chen and Stewart, 1996). Efficiency reduction of peracetic acid, an oxidant agent, was also observed by Trachoo and Frank (2002) against Campylobacter in the presence of biofilms. The
same authors also observed that quaternary ammonium compounds such as benzalkonium chloride have their efficiency affected as well. These factors can be related to re-colonization of facilities by *Campylobacter* after the Proposed treatment when the birds are 42 d old.

The absence of *Campylobacter* spp. in the other samples right after the Proposed treatment of cleaning and disinfection may be related to low resistance of *Campylobacter* spp. to glutaraldehyde and formaldehyde as found by Wang et al. (1983) and Gutiérrez-Martín et al. (2011).

At 42 d old, there was no difference between the contamination frequency of *Campylobacter* for both treatments, which can be related to the high dissemination capacity of these bacteria, as Knudsen, et al. (2006) observed in their studies. Contamination by *Campylobacter* was found in samples from the environment, considered negative for *Campylobacter* spp. as also reported by Van de Gissen et al. (1998). When investigating the contamination origin of those samples, the same authors found the bacteria in insects and staff shoes, suggesting that even the facilities that were previously free from *Campylobacter* spp. can be contaminated during the birds’ stay due to external sources.

Overall, the absence of sanitizing procedures can be considered a *Campylobacter* spp. contamination risk factor for broiler chickens (Evans and Sayers, 2000; Newell and Fearnley, 2003; Bouwknegt et al., 2004; Chon et al., 2011) observed low sensitivity and selectivity to this medium, mainly when the sample microflora was abundant. Bolton et al. (1996) found 13% of contamination of this medium when evaluating samples of human feces.

The utilized multiplex PCR was based on the methodology proposed by Klena et al. (2004), which uses lpxA gene in species differentiation of *Campylobacter* spp. (*C. jejuni, C. coli, C. lari,* and *C. upsaliensis*) that codifies Lpxa enzyme, the initial step of lipid A production, an essential molecule of LPS system found in bacteria of *Campylobacter* genus. This gene, found in several gram-negative bacteria (Weckesser and Mayer, 1988), was identified in *Neisseria meningitidis* (Odegaard et al., 1997), *Pseudomonas aeruginosa* (Dotson et al., 1998), *Enterobacter asburiae* (Osei Sekyere et al., 2016), *Enterobacter cloacae* (Meggann et al., 2015) and *Escherichia fergusonii* (Touchon et al., 2009).

The identification of *Enterobacter cloacae,* *Enterobacter asburiae,* and *E. coli* can be related to the presence of lpxA gene in these bacteria and there is also the possibility of genetic information exchange between the environmental microbiota bacteria through plasmids, transposons and gene insertion sequences. Fouts et al. (2005) when studying the genome of some *Campylobacter* strains, found plasmids involved in the transfer and secretion of virulence factors, sequences of chromosome and plasmid DNA insertion.

As the utilized gene, lpxA, belongs to LPS virulence factor of *Campylobacter,* there is the probability of lateral genetic transfer occurrence, which makes it possible that the primers utilized in multiplex PCR reaction, specific for *Campylobacter* species (*jejuni, coli, lari,* and *upsaliensis*), have interacted with similar sequences, but with other bacteria.

Moffat et al. (2011) when studying *Acinetobacter baumannii,* a bacterium that possess lpxA gene, found a gene insertion element of this same gene, showing that bacteria that have it can perform lateral genetic transfers. In this same study, the studied insertion sequence was related to resistance to antibiotics against *Acinetobacter baumannii,* which means a serious public health problem.

In *Campylobacter,* LPS composition, codified by several genes, including lpxA, is also related to resistance to antibiotics (Van Mourik et al., 2010). Thus, it is important to point out that as reported for *A. baumannii* (Potron et al., 2015), *Campylobacter* can have an important role in the dissemination of genes resistant to antibiotic against other gram-negative microorganisms when they may transfer genes laterally.

Evaluating the phylogenetic tree and the similarity and identity table, it is possible to verify that the sample strains are very similar among themselves, are very close to one another in cladogram for lpxA gene, and have high similarity and identity indices. When comparing the samples to the standard strains, there was a slight distancing in the cladogram and lower similarity and specificity indices, showing that it is small despite the differences among these strains.
The high level of similarity and specificity found in this study are in accordance with Lucien (2012) when evaluating the tuf gene of these bacteria, with similarity and identity varying from 98 and 99% of Campylobacter jejuni samples with standard ATCC 33560 strain.

In the present study, it was possible to observe 4 distinct clades for the phylogenetic reconstruction of Campylobacter spp, one for each studied species of Campylobacter, Campylobacter jejuni, Campylobacter coli, Campylobacter lari, and Campylobacter upsaliensis, similarly to Kärenlampi et al. (2004), Klena et al. (2004), Hill et al. (2006) and Lucien (2012) when studying groEL gene, lpxA gene, tuf gene, and cpn60 gene, respectively.

Our results showed that the strains of Campylobacter jejuni circulating the assessed experimental environment are very similar to the ones that could be found in different countries and types of samples, making it possible to observe that even with a great diversity of Campylobacter jejuni strains, they have genetic similarity among themselves, making the utilized methodology reliable for their identification and phylogenetic reconstruction.

Although the recovery of standard ATCC 33560 strains in the samples was expected, no sample could be identified after sequencing. This result can be related to a reduced adaptation to the environment and competition with the other Campylobacter jejuni, inhibiting a possible growth of standard ATCC 33560 strain. Cawthraw et al. (1996) reported the fragility of bacteria from in vitro cultures, since the laboratory conditions in which they are submitted differ from the in vivo environment, reducing their resistance, adhesion factors, motility, and virulence (Ringoir and Korolik, 2003). There is also the possibility of genomic rearrangement occurrence, insertions, deletions, or mutations of Campylobacter jejuni DNA as described by Wassenaar et al. (1998), Hämynen et al. (1999), and De Boer et al. (2002), resulting in modifications of these same factors.

CONCLUSION

The Proposed program shows greater efficiency in the total environmental microorganism count reduction and the capacity to eliminate Campylobacter from the floor and drinkers of facilities. With the adoption of the Proposed treatment, it is possible to obtain better performance of the birds. Both programs do not influence the occurrence frequency of Campylobacter in the facilities and birds at 42 d old and at slaughter time. It was possible to identify 6 different strains of Campylobacter jejuni, which occupy the same phylogenetic clade, and become effectively differentiated with high values of nodal support associated with other species of Campylobacter to which they were compared.

ACKNOWLEDGMENTS

The authors are grateful to FAPESP for providing the graduate scholarship (2013/02457-8) to carry out this study.

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