Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2022 / v.24 / n.3 / 001-008

http://dx.doi.org/10.1590/1806-9061-2021-1505

Original Article

Direct Detection and Quantification of Bacterial Pathogens from Broiler Cecal Samples in the Slaughter Line by Real-Time PCR

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■Keywords

Campylobacter, Clostridium perfringens, food safety, poultry, Salmonella.

Submitted: 25/October/2021
Approved: 20/February/2022

ABSTRACT

Chicken meat is an important source of foodborne pathogens, including *Salmonella*, *Campylobacter,* and *Clostridium perfringens*. These bacteria can occur in the intestinal microbiota of broilers and contaminate chicken carcasses in industrial meat processing. This study aimed to develop and evaluate a procedure based on real-time PCRs for the direct detection and quantification of these three bacteria in broilers' ceca collected in poultry slaughter houses and demonstrate the occurrence of these important foodborne pathogens in Brazilian poultry production flocks. Cecal contents were collected from 45 different broiler flocks in three different slaughterhouses in the state of Paraná, Brazil, totaling 45 samples (in pools of 10 different ceca/chickens per broiler flock). Then, these samples were tested for the detection and quantification of *Salmonella*, *Campylobacter,* and *Clostridium perfringens* by real-time PCRs. The results demonstrated the occurrence of three (6.7%) positive pools for *Salmonella*, 20 (44.4%) for *Campylobacter,* and 32 (71.1%) for *C. perfringens*. Mean bacterial concentrations in the positive samples were 4.3$\log_{10}$ cells/g for *Salmonella*, 6.4 $\log_{10}$ cells/g for *Campylobacter,* and 5.5 $\log_{10}$ cells/g for *C. perfringens*. In conclusion, *Salmonella*, *Campylobacter,* and *C. perfringens* could be detected and quantified directly from the broilers cecal contents collected in the slaughter line. This procedure will be certainly useful to more quickly detect these foodborne pathogens and prevent their occurrence in chicken meat and other poultry food products.

INTRODUCTION

Foodborne diseases can result in more than two million deaths per year worldwide. Chicken meat is a possible carrier of foodborne pathogens that cause human infectious diseases (Saif, 2008; FAO-WHO, 2009; WHO, 2020). The most common bacterial pathogens are *Salmonella* and thermotolerant *Campylobacter* (*C. jejuni*, *C. coli,* and *C. lari*), accounting together for 94.1% of total foodborne outbreaks (EFSA, 2019). *Clostridium perfringens* is also a concerning bacterial pathogen due to the production of toxins that can cause food poisoning, mainly in children, elderly, and immunosuppressed people (Van Immerseel *et al.*, 2004; Lindström *et al.*, 2011; CDC, 2017).

Reduction of the risk of foodborne diseases infection can be pursued with measures of biosecurity and pathogen control throughout the food chain (Gölz *et al.*, 2014; Rivera *et al.*, 2018). The total absence or the occurrence of low levels of pathogenic bacteria in foods is the main goal to minimize human risk. This is also imperative in broilers flocks processed to produce chicken meat in slaughterhouses (Brazil, 2011; EFSA, 2011; Hermans *et al.*, 2011). Besides broiler infection

in any step of the poultry production chain, cross-contamination by pathogenic bacteria can also occur in food processing at the slaughterhouse. Evisceration in the slaughter line is considered the point of highest risk for carcass contamination by enteric pathogens. Therefore, animal and environmental samplings in all slaughterhouse steps (mainly in the evisceration) have to be collected to further perform laboratory analyses aimed at the detection of specific pathogens (Allaart *et al.*, 2013; Rajan *et al.*, 2017). Additionally, several different biological samples can be used for pathogen detection: fecal drop, feces, drag swabs, etc. It is noteworthy that a previous study demonstrated that cecal drops reflect chickens' cecal microbiome (including pathogenic bacteria) better than any other animal sample and should be preferentially used to estimate the contamination risk in slaughterhouses and foods (Pauwels *et al.*, 2015).

Foodborne bacteria are usually detected in laboratories by bacterial isolation in different culture media, followed by additional biochemical and serological characterization of bacterial colonies (Lee *et al.*, 2015). Although these traditional microbiological methods are considered the gold standard, they are laborious and require several steps and reagents. These time-consuming analytical processes can take days to obtain a final result, which reduces the effectiveness in controlling the contamination of poultry flocks. Additionally, some fastidious bacteria such as *Campylobacter* are difficult to isolate in culture media, requiring other analytical procedures (Schnider *et al.*, 2010; Maurischat *et al.*, 2015; Nagpal *et al.*, 2015; Ricke *et al.*, 2019). These methods are also usually not quantitative, making the effective estimation of the contamination risk of positive foods for any bacteria impossible (Navidshad *et al.*, 2012; Ricke *et al.*, 2019).

Thus, DNA-based methods like real-time polymerase chain reaction (qPCR) and loop-mediated isothermal amplification (LAMP) have been increasingly developed and used to detect and quantify foodborne pathogens in the poultry production chain (Souza *et al.*, 2019; Waldman *et al.,* 2020). Other important advantage of DNA-based methods is the possibility of performing qualitative and quantitative analysis of food pathogens in biological samples simply with prior enrichment or even directly from biological samples (Albini *et al.*, 2008; Rodgers *et al.*, 2012; Park *et al.*, 2014; Ricke *et al.*, 2019).

The present study aimed to develop and evaluate a procedure based on real-time PCRs for the direct detection and quantification of the foodborne pathogens *Salmonella*, *Campylobacter,* and *C. perfringens* in broilers´ ceca collected in poultry slaughterhouses.

MATERIAL AND METHODS

Samples

A total of 450 griller broilers with 30 days of production (weight of birds ~ 1.4 kg) from 45 flocks in 39 different farms located in the state of Paraná, Brazil, were selected by convenience (10 broilers per flock) in three different slaughterhouses between 2017 and 2019. The cecum of each broiler was collected aseptically in the slaughter line after mechanical evisceration, placed in 45 sterile plastic bags (pools of 10 ceca per flock), and maintained at 4°C for a maximum of 24 h until laboratorial processing (Stern *et al.*, 2005; Rasschaert *et al.*, 2007).

Samples pre-processing

The 45 pools of ceca collected in slaughterhouses were prepared in the laboratory before analytical processing. First, cecal contents were released from the pools manually and homogenized. Then approximately 50 mg of each sample was collected with a sterile spatula and mixed with 1,250 µL of lysis solution (NewGene Prep, Simbios Biotecnologia, Rio Grande do Sul, Brazil). It was then incubated at 60°C for 10 min and centrifuged for 1 min at 9,410 x *g*. Tubes containing pre-processed samples were separated for DNA extraction, which was performed immediately after.

DNA extraction

DNA extraction of cecal content samples was performed using a commercial kit for total nucleic acid purification (NewGene Prep and Preamp, Simbios Biotecnologia). This procedure was performed according to the manufacturer´s instructions with some modifications. Initially, 100 µL of supernatant from the pre-processed sample prepared in the previous step was transferred to another tube (1.5 mL) containing 400 µL of cell lysis solution. After homogenization, 20 µL of silica suspension was added into the tube, which was incubated at room temperature for 10 min and shaken by inversion every 2 min. Then each sample was centrifuged for 1 min at 9,410 x g and the pellet was washed once with 150 µL of wash solution A, once with wash solution B, and once with wash solution C from the extraction kit. All tubes were placed in a thermoblock at 60°C to remove water. Then, 50 µL of elution solution was added and each sample tube

was centrifuged for 3 min at 9,410 x g. The liquid with soluble DNA was removed to another tube and stored at -20°C until DNA amplification.

Real-time quantitative PCRs (qPCRs)

Real-Time PCRs were carried out using commercial kits SALAmp, CPBAmp and CPERAmp (NewGene, Simbios Biotecnologia). All these kits are TaqMan® based-PCRs (including only one primers pair and one probe) with the following specific molecular targets: a) *inv*A gene for *Salmonella spp.* (Hoorfar *et al.*, 2000); b) 16S rRNA target for thermotolerant *Campylobacter* (*C. jejuni*, *C. coli* and *C. lari*) (Josefsen *et al.*, 2004); and c) *plc* gene for *C. perfringens* (Abildgaard *et al.*, 2010). Three gBlocks™ Gene Fragments (Integrated DNA Technologies, Iowa, USA) were synthesized with DNA sequences specific for all three targets and used as positive controls in the qPCRs. All assays were carried out on the 7300 Real-Time PCR equipment (Applied Biosystems, Massachusetts, USA) with the following cycling conditions: one initial denaturation cycle of 3 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Also, the three gBlocks™ were serially ten times diluted (initial concentrations of standard samples: 4,000,000 copies) and five dilutions with known loads (40 to 400,000 copies) of each gBlocks™ were used as standard samples for preparing standard curves for each bacterium in all runs of qPCR. CTs (cycle threshold) observed in all positive samples were compared with CTs of the standard curve to determine the bacterial load and to estimate the number of bacteria cells. All results were converted to $\log_{10}$ cells/gram of cecal content.

Data analysis

Analytical sensitivity of the assays (LOD, limit of detection) was calculated using the PROBIT procedure based on the methodology described by Waldman *et al.*, 2020. In summary, replicates of positive control samples of each pathogen with loads of specific bacteria DNAs (*Salmonella* 5,000,000 copies; *Campylobacter* 4,000,000 copies; and *C. perfringens* 3,000,000 copies) were serially ten times diluted (diluted 10^5 to 10^{-1} fold). The procedure was repeated on three different days by the same operator and equipment to evaluate reproducibility, totaling 9 replicates of each dilution. The number of cells of each dilution, the total number of repetitions, and number of positive repetitions were used to calculate the 50% and 95% cut-off values, which represented the LOD of the assays.

Assessments of possible statistical differences between qualitative variables were verified by Pearson's chi-square test or Fisher's exact test as appropriate. Distributions of quantitative variables were verified by the Kolmogorov-Smirnov test with Lilliefors correction and comparisons between groups were performed by Student's t-test and ANOVA. All analyses were bilateral with a pre-established significance level of 5% alpha error ($p<0.05$). Data were compiled and analyzed using SPSS® software (23.0 version, Chicago, IL Statistical Package for the Social Sciences).

RESULTS

Development of the pre-processing procedure and limit of detection of qPCRs assays

First, pre-processing conditions were evaluated by testing different volumes of samples and cell lysis solutions to avoid the occurrence of amplification inhibitors. Three DNA extractions protocols were developed: A, B, and C (Table 1). All the 45 samples collected in this study were submitted to these DNA extraction protocols and amplified with the specific qPCR for *Salmonella*. The final quantities of cecal content per qPCR reaction (mg/qPCR) for each protocol were 0.077, 0.0154, and 0.00308 mg/qPCR, respectively. The results demonstrated the occurrence of 1, 3, and 2 PCR positive samples for *Salmonella* in protocols A, B, and C, respectively. So, we assumed protocol B (0.0154 mg/qPCR) to be the most sensitive (Table 1).

Table 1 – Adjustment of the DNA extraction protocol to avoid amplification inhibitors.

DNA extraction protocol	Volume of pre-processed sample extracted (µL)	Volume of cell lysis solution added in the DNA extraction step (µL)	Final quantity of cecal content per qPCR reaction (mg/qPCR)	Number of *Salmonella* positive samples/total samples[2]
A[1]	500	0	0.077	1/45
B	100	400	0.0154	3/45
C	20	480	0.00308	2/45

qPCR= quantitative real-time PCR.

[1] Original protocol, as described by the manufacturer´s instructions purification (NewGene Prep and Preamp, Simbios Biotecnologia).

[2]Repeated three times.

PROBIT analysis demonstrated a limit of 2.8 $\log_{10}$ cells/g of cecal content of *Salmonella*, 2.7 $\log_{10}$ cells/g of cecal content of thermotolerant *Campylobacter* and 2.6 $\log_{10}$ cells/g of cecal content of *C. perfringens* to obtain 50% positive results, and 3.8 $\log_{10}$ cells/g of cecal content of *Salmonella*, 3.7 $\log_{10}$ cells/g of cecal content of thermotolerant *Campylobacter* and 3.6 $\log_{10}$ cells/g of cecal content of *C. perfringens* to obtain 95% positive results for the qPCR, with a 95% confidence level (Table 2).

Table 2 – PROBIT analysis – Analytical sensitivity of the qPCR assays in cecal content samples.

Assay	Confidence level (%)	Sensitivity ($\log_{10}$ cells/g)
qPCR *Salmonella*	50%	2.8
	95%	3.8
qPCR *Campylobacter*	50%	2.7
	95%	3.7
qPCR *Clostridium perfringens*	50%	2.6
	95%	3.6

qPCR= quantitative real-time PCR.

Prevalence of foodborne pathogens in the slaughterhouses

The complete procedure described above, its mean qPCR for the three foodborne pathogens, was used in the analysis of the broiler flocks' cecal content pools collected in the slaughter line. *Salmonella* was detected in 3 (6.7%), thermotolerant *Campylobacter* in 20 (44.4%), and *C. perfringens* in 32 (71.1%) poultry flocks in the three Brazilian slaughterhouses. The detection frequencies were statistically different for the three bacterial pathogens (p<0.01).

All flocks were evaluated for single or multiple infections. Five flocks (11.1%) showed negative results for the three bacteria analyzed, 8 flocks (17.8%) were positive only for thermotolerant *Campylobacter*, 18 flocks (40.0%) were positive only for *C. perfringens*, 2 flocks (4.4%) tested positive for *Salmonella* and *C. perfringens*, 11 flocks (24.4 %) tested positive for thermotolerant *Campylobacter* and *C. perfringens*, and only one flock (2.2 %) tested positive for the three bacteria analyzed.

Bacterial loads were determined in all positive samples. Overall results ranged from 2.8 to 6.4 $\log_{10}$ cells/g of cecal content (mean 4.3± 1.9) to *Salmonella*, 3.8 to 10.0 $\log_{10}$ cells/g (mean 6.4± 1.7) to thermotolerant *Campylobacter* and 3.7 to 7.8 $\log_{10}$ cells/g (mean 5.5± 1.0) to *C. perfringens* (Figure 1).

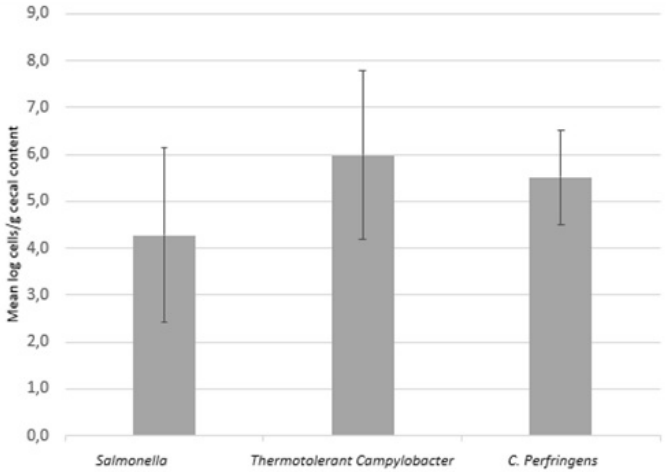

Figure 1 – Mean and standard deviation of bacterial concentrations found in cecal content samples from 45 broiler flocks from slaughterhouses in the state of Paraná, Brazil.

Flowchart diagram for detection and quantification of foodborne pathogens in cecal content

Based on these previous findings, an analytical procedure was defined, and a flowchart diagram was designed to summarize the detection and quantification of *Salmonella*, thermotolerant *Campylobacter,* and *C. perfringens* direct in cecal content samples by real-time PCR assays (Figure 2). It has been used in the routine analysis of the broilers samples in the slaughterhouse.

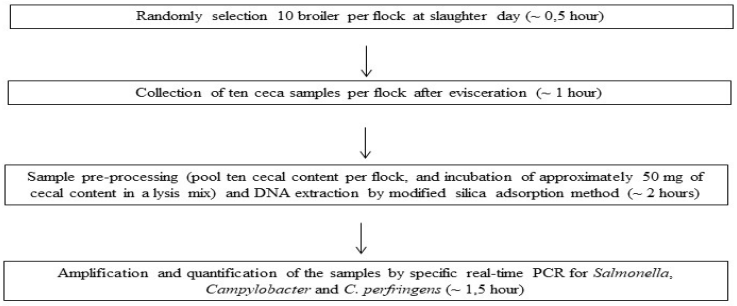

Figure 2 – Flowchart diagram for the detection and quantification of *Salmonella enterica*, thermotolerant *Campylobacter*, and *Clostridium perfringens* in cecal content samples by Real-time PCR assays.

DISCUSSION

To increase food security it is necessary to reduce the occurrence of foodborne pathogens. For poultry products, it is also important to control bacterial contamination levels in all production chain processes, from farm to slaughterhouses, distribution, and storage (Brazil, 2011, EFSA, 2011; Back, 2014). Complete elimination of pathogens in chicken meat and other poultry products is an extremely difficult task, as they can become persistent in food processing environments (Rivera *et al.*, 2018), but some measures can be taken

to reduce the risk of food contamination, such as the rapid identification of infected broiler flocks to avoid sending contaminated chicken products to the market (Josefsen *et al.*, 2004; Park *et al.*, 2014).

Risk of chicken carcasses contamination by pathogenic bacteria could be assessed by evaluating the intestinal microbiota content, including the detection and quantitation of *Salmonella, Campylobacter,* and *C. perfringens*. Furthermore, pathogen tracking is more efficient when broilers are analyzed immediately after evisceration in the slaughter line, thus monitoring intestinal poultry microbiota and providing safer food products to human consumption (Seliwiorstow *et al.*, 2015; Yeh *et al.*, 2019). Traditionally, feces and fecal material have been the samples collected in poultry farms as well as in slaughterhouses, respectively. However, cecal drop and/or cecal content are better samples to evaluate the broilers' intestinal microbiota because birds' ceca retain the microbial content longer than the small and large intestines, including high concentrations of bacteria, fungi, and other microorganisms (Clench, 1999; Pauwels *et al.*, 2015; Stanley *et al.*, 2015; Ijaz *et al.*, 2018). Moreover, feces and fecal material are not good estimators of the bacterial community of the cecal content (Pauwels *et al.*, 2015; Seliwiorstow *et al.*, 2015; Stanley *et al.*, 2015). For these reasons, cecum was already known as a spot for bacterial isolation in poultry (Pauwels *et al.*, 2015).

The present study evaluated the application of a procedure to detect and quantify three important foodborne pathogens (*Salmonella, Campylobacter,* and *C. perfringens*) in broiler's cecal samples by real-time PCR. First, PROBIT assays were carried out to determine the LOD of the qPCRs in cecal samples and the results demonstrated that between 100 and 1000 bacterial cells/g could be detected as previously reported (Waldman *et al.*, 2020). In addition, all these three bacterial pathogens were detected in more than one sample, demonstrating the good performance of the complete procedure. Previous studies have already demonstrated that PCR methods can be used to detect foodborne pathogens in chicken and other poultry samples (Alonso *et al.,* 2011; Park *et al.,* 2014). However, they have been carried out only after bacterial pre-enrichment and/or isolation (Borges *et al.,* 2019; Souza *et al.,* 2019; Borges *et al.*, 2020). The present study demonstrated that *Salmonella, Campylobacter,* and *C. perfringens* can be detected directly from broilers' cecal samples obtained in slaughter houses. With the removal of the pre-enrichment step in BPW, the whole procedure could be reduced in more than 12 hours (Park *et al.*, 2014; Ricke *et al.*, 2019). Additionally, quantitative data could be used to estimate the risk of contamination of chicken meat.

Additionally, it was possible to estimate the prevalence of these bacterial pathogens in the evaluated chicken broilers flocks. *Salmonella* was detected in 6.7% of the ceca from the sampled flocks. Some previous studies in Brazil described a wide range of *Salmonella* frequencies in chicken samples collected at different stages of slaughter: 48.9% in samples obtained during the slaughtering process in the Rio Grande do Sul state (Borges *et al.*, 2019); 9.33% of slaughterhouse samples (whole carcasses, cuts, viscera, and chiller water) in Mato Grosso do Sul state (Boni *et al.*, 2011); 0.2% of cloacal swabs collected from broilers chicken flocks in the metropolitan region of Fortaleza (Bezerra *et al.*, 2016); 3.7% of chicken carcasses from a slaughterhouse in the state of Mato Grosso (Cunha-Neto *et al.*, 2018); and 1.66% of cloacal swabs and 26.66% of chicken carcasses in the state of Rio de Janeiro (Baptista *et al.*, 2018). Moreover, 37.5% of raw chicken legs sold at retail in the state of São Paulo were positive for *Salmonella* (Ristori *et al.*, 2017). Bacterial loads were estimated in all positive samples and the results ranged from 2.8 $\log_{10}$ to 6.4 $\log_{10,}$ with a mean of 4.3 $\log_{10}$ bacteria/g of cecal content. This result could be compared to another Brazilian study that presented *Salmonella* loads ranging from 1.16 to 3.64 $\log_{10}$ CFU/mL in chicken carcasses (Borges *et al.*, 2019).

Campylobacter was observed in 44.4% of the broiler flocks analyzed here. Previous studies have also demonstrated the high frequency of *Campylobacter* in cecal samples from broiler flocks, ranging from 50% to 73% of positive flocks (Rasschaert *et al.*, 2007; Reich *et al.*, 2008; Boer *et al.*, 2015; Seliwiorstow *et al.*, 2015). Unfortunately, there are no studies in Brazil regarding cecal content samples, making more detailed comparisons impossible. However, a previous study with cecal drops demonstrated a high frequency (81.8%) of *Campylobacter* (Kuana *et al.*, 2008). Some Brazilian studies tested other types of samples (water, cloacal swabs, carcasses, organs, among others) and reported a wide range of bacterial frequencies: 69.4% (Borges *et al.*, 2020); 71.3% (Franchin *et al.*, 2007); 4.9% (Cortez *et al.*, 2006); 16.8% (Hungaro *et al.*, 2015); and 8.73% (Castro Chaves *et al.*, 2010). It is noteworthy that all these studies were performed in different farms and also different Brazilian geographic regions. At the retail, two studies conducted at

Southern Brazil found *Campylobacter* in 17% of poultry meat (Silva *et al.*, 2016) and 91.7% of poultry meat products (Würfel *et al.*, 2018), while a study conducted at São Paulo state detected this bacterium in 7.7% of raw chicken (Lopes *et al.*, 2018).

Previous studies have also shown that *Campylobacter* spreading is frequently fast in poultry flocks, with most birds from the same flock hosting this bacterium (Berndtson *et al.*, 1996; Rudi *et al.*, 2004). Also, a direct correlation between *Campylobacter* concentrations in broiler intestinal content and carcass is known and when a previously negative poultry flock is slaughtered after a positive flock, the more recent processed chickens become positive by cross-contamination (Rosenquist *et al.*, 2006; Reich *et al.*, 2008). These findings reinforce the importance of monitoring and decreasing *Campylobacter* intestinal concentrations in broilers at the flock level to reduce the transmission risk of this microorganism to humans through food consumption (Rudi *et al.*, 2004; Rosenquist *et al.*, 2006; Franchin *et al.*, 2007; Reich *et al.*, 2008).

In this study, bacterial loads of *Campylobacter* ranged from 3.8 $\log_{10}$ to 10 $\log_{10}$, with a mean of 6.4 $\log_{10}$ bacteria/g of cecal content. According to a previous study (Seliwiorstow *et al.*, 2015), highly contaminated broiler flocks (> 7.5 $\log_{10}$ CFU/g) should be excluded to reduce the potential risk of campylobacteriosis transmission to humans. The present study found five broiler flocks (11.1%) with loads > 7.5 $\log_{10}$ bacteria/g of cecal content, i.e., presenting a high transmission risk of campylobacteriosis through poultry product consumption. Another study quantified *Campylobacter* in other different types of samples collected during slaughter in Brazil, with bacterial loads ranging from 0 to 2.1 log CFU/mL (Borges *et al.*, 2020). In worldwide studies that analyzed cecal samples, bacterial concentrations ranged from 6.1 $\log_{10}$ to 11.1 $\log_{10}$, with means ranging from 6.9 $\log_{10}$ to 8.5 $\log_{10}$ CFU/g (Rudi *et al.*, 2004; Reich *et al.*, 2008; Seliwiorstow *et al.*, 2015; Vinueza-Burgos *et al.*, 2018).

C. perfringens was found in 71.1% of broiler flocks analyzed in this study. A study conducted in Swedish slaughterhouses found *C. perfringens* in 18% of broiler carcasses after chiller (Lindblad *et al.*, 2016). To our knowledge, this is the first Brazilian study to evaluate the occurrence of *C. perfringens* in poultry slaughterhouses. At the retail level, when analyzing chicken liver sold in USA markets, Cooper *et al.* (2013) detected *C. perfringens* in 69.6% of samples. *C. perfringens* loads ranged from 3.7 to 7.8 $\log_{10}$, with a mean of 5.5 $\log_{10}$ $\log_{10}$ bacteria/g of cecal content. *C. perfringens* is a major cause of human food poisoning. Bacteria survive in the animal body when food is contaminated with very high concentrations (> 6 $\log_{10}$ cells/g of food) because viable bacterial cells can form spores in vivo (Shrestha *et al.*, 2018). The present study found 10 broiler flocks (22.2%) with loads ≥ 6.1 $\log_{10}$ bacteria/g of cecal content, demonstrating concern in regards to contamination of chicken meat.

Brazil is an important poultry-producing country and exports chicken meat to different countries of the world (ABPA, 2020). The results reported in this study are concerning, as they show that three important bacterial pathogens are present in chickens from broiler flocks in commercial slaughterhouses and could contaminate poultry food products. It is noteworthy that dissemination of these pathogens in Brazilian poultry is widely variable depending on the region, poultry establishment, poultry flock, and type of sample analyzed (Cortez *et al.*, 2006; Bezerra *et al.*, 2016; Würfel *et al.*, 2018; Borges *et al.*, 2019). Specific hygiene and biosecurity measures to control these foodborne pathogens throughout the poultry production chain are extremely necessary to reduce economic losses and minimize the risk of exposure of consumers to these bacteria.

In conclusion, a procedure for detection and quantitation of the main poultry bacterial pathogens in cecal content samples was developed and used in the routine analysis of three slaughterhouses to assess the risk for bacterial contamination in chicken meat. The complete procedure, from collection of samples to the analysis of the results in the laboratory, takes less than one day (approximately 5 h if the industry has a molecular biology facility). So it is possible to predict the risk of food infection, take sanitary measures before the food reaches the end of the production chain, and provide safe food for consumers. This procedure can also be useful to detect the occurrence and to determine the prevalence of these pathogens in both broiler flocks and slaughter houses from different poultry-producing regions in Brazil.

ACKNOWLEDGMENTS

This study was supported by Simbios Biotecnologia. MNS and JMW were supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) [Finance Code 001]; and VRL was supported by the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) [Process number 311010/2017-2].

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