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Original Article

Qualitative and Quantitative Analysis of *Salmonella* spp. in Broilers Technological Processing and Determination of a Performance Objective (PO) for Frozen Chicken Breast

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ABSTRACT

The present study investigated the frequency, level of contamination and serotyping of *Salmonella* strains isolated from broiler flocks in different processing sites and the fulfillment of a Performance Objective (PO) in frozen chicken breasts, as a risk assessment to measure the efficacy of prevention and control programs applied to reduce the risk of *Salmonella* spp. in raw poultry meat that contribute to reach food safety and public health goals. From 1,800 samples of cloacal swabs, carcasses before and after immersion chilling and frozen breasts derived from 20 broiler flocks slaughtered at two processing plants located in the mid-west and southern regions of Brazil, 278 samples were positive for *Salmonella* spp. by polymerase chain reaction (PCR) automated BAX System (DUPONT QUALICOM, USA), and 118 were enumerated by miniaturized most probable number technique. 122 *Salmonella* spp. strains were serotyped at the National Reference Laboratory of Cholera and Enteric Diseases of Oswaldo Cruz Institute Foundation (FIOCRUZ), showing a dominance of *Salmonella* Minnesota in every processing steps of the slaughterhouse located in the Brazilian mid-west region. Only 1 lot failed to reach the expected result for the Performance Objective (PO), using a maximum of 10% positivity acceptance for *Salmonella* spp. in frozen chicken breasts. Qualitative and quantitative results combined may be considered an effective tool to evaluate the effect of prevention and control programs for *Salmonella* spp. on the safety of the final product.

INTRODUCTION

Salmonella spp. appears as the most important foodborne pathogen in the whole world, widely distributed in nature (FAO/OMS, 2002). The most common non typhoid *Salmonella* reservoir is the intestinal tract of a wide range of domestic and wild animals and a variety of food matrices that can serve as a vehicle for transmission of *Salmonella* spp. to humans through fecal contamination. The transfer frequently occurs when theses microorganisms are introduced into food preparation areas, with subsequent proliferation in food items through improper storage temperature, inadequate cooking, and/or cross contamination, as well as through direct contact with infected animals and humans (EFSA, 2017).

Raw chicken products are an important part of international food trade and the poultry products are highly reported as a contamination source in cases of human salmonellosis with public health and economic implications (FAO/WHO, 2002; Mead *et al.* 2010).

CDC estimates *Salmonella* bacteria cause about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States every year. Food is the source for most of these illnesses (CDC, 2020). The annual cost associated with salmonellosis in the United States has

been estimated to be approximately $14,6 billion and health related economic cost of each foodborne illness is approximately $2.000, taking into account the quality of life calculations (Scharff, 2010).

In 2016, the USDA-FSIS reported that 3,7% broiler chicken carcasses were *Salmonella* positive, with *Salmonella* serotypes Kentucky (60,8%), Enteritidis (13,6%), Typhimurium (7,7%) Infantis (6,5%) and Heidelberg (3,4%) being responsible for approximately 92% of the serotypes detected. Despite the percentage of poultry has been decreasing since the implementation of the PR-HACCP rule, the human incidence of salmonellosis reported to the Center of Disease Control and Prevention (CDC) has not greatly changed over time (NACMCF, 2019).

Salmonellosis remains in European Union as the second most commonly reported gastrointestinal infection in humans after campylobacteriosis, and an important cause of foodborne diseases. The trend for salmonellosis in humans in member states has stabilized over the last five years after a long period of a declining trend. As in previous years, the three most commonly reported *Salmonella* serovars in 2018 were *S.* Enteritidis, *S*. Typhimurium and monophasic *S*. Typhimurium (1,4,[5],12:i:-), *S*. Infantis and *S*. Newport. The first three sorotypes represent 71.0% of the 79,698 confirmed human cases with known serovar in 2018. (EFSA, 2019 a,b). Broiler meat products was related in 2,4% foodborne outbreaks caused by *Salmonella* in member states. The EFSA annual report, reports the 7,15% occurrence of *Salmonella* spp. in fresh broiler meat samples in member states, considering the entire production chain and in broilers flocks. *Salmonella* was found in 3.5% of the broiler flocks compared with 3.3% in 2017. The number of flocks positive for *S.* Typhimurium increased in 2018 (N = 433) compared with 2017 (N = 363) (EFSA, 2019a,b).

Survey studies have shown that poultry consumption is one of the major causes of *Salmonella* infection in Korea, which was the third most common cause of foodborne diseases in humans reported between 2002 and 2017 (Jeong *et al.*,2018).

Alali *et al.*, 2012 related a prevalence of 31,5% of chilled and frozen whole chicken carcasses on retail in Russia from national poultry industry despite the official Russian Federation Central Research Institute of Epidemiology report that just 4% of domestic poultry meat and poultry products tested in Russia failed the country's microbiological standards for food.

The results of a meta-analysis *Salmonella* serovars worldwide published by Ferrari *et al.* (2019) identified that in poultry, *S.* Enteritidis is the most prevalent in Asia, Latin America, Europe and Africa, *S.* Sofia is the most prevalent in Oceania and *S.* Kentucky the most prevalent in North America. *S.* Typhimurium displays a cosmopolitan profile and is considered an example of a generalist serovar.

The farm to form concept is essential to prevent and control *Salmonella* in raw poultry meat, however a key public health issue is the *Salmonella* contamination level on positive carcasses at the end of the processing operation (FAO, 2009). Linking the presence and numbers of a particular pathogen in specific food with the proportion of illnesses caused in a human population constitutes a further challenge but this information is needed to estimate the magnitude of risk and stablish clear goals for public health protection that can be communicated to both industry and the public. Sounds risk management requires allocation of resources that are proportional to the magnitude of the risk and feasibility and effectiveness of risk reduction measures (Mead *et al.*, 2010). The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) in United States, recommended that the agency and industry move toward risk-based disposition of finished raw product. This approach would be informed by *Salmonella* level and serotype or subtype, and diverted products would be subject to a validated lethality step or reprocessing (USDA, 2019). Evidence suggests that when *Salmonella* is present, the most probable number (MNP) is generally low, often no more than 100 cells per carcass (Mead *et al.,* 2010).

Infectious doses can be defined as the minimum number of live *Salmonella* bacteria that will take to cause illness. This is dependent on a number of factors, including host susceptibility and being taken by the host, the food matrix and virulence factors of the pathogen (McEntire *et al.* 2014). The ability of *Salmonella* species to cause human infection involves attachment and colonization of intestinal columnar epithelial cells and specialized microfold cells overlying Peyer's patches. In healthy humans, the infective dose for salmonellosis is estimated to be in the range of 10^4 to 10^6 cells or higher, but can be as low as 10^1 to 10^2 cells in highly susceptible individuals or if contained in a food with high fat matrix as cheese or chocolate (Cosby *et al.*, 2015). Teunis *et al.* (2012), evaluated non-typhoid *Salmonella* outbreaks to determine a dose-response model that could be utilized when the *Salmonella* dose or the number of exposed was unknown and found that as the dose increased, the probability of illness increase. Dose above 10^2 CFU had

probabilities of illness ranging from 0.05 to 1.0, where doses less than 10^2 CFU has probabilities of illness ranging from 0.01 to 0,56. A probit regression analysis conducted by Akil & Ahmad (2018) for a Qualitative Risk Assessment (QRA) model of human salmonellosis resulting from consumption of broiler chicken showed that the consumption of at least $1{,}46x10^4$ CFU/g for *Salmonella* Enteritidis or $6{,}4x10^3$ CFU/g for *Salmonella* Typhimurium is required to develop infection in 50% of the population.

The genus *Salmonella* exhibits great diversity with more than 2649 serotypes identified and at least 100 serotypes included in the specie *enterica* sub-specie *enterica* could be important in terms of both public and animal health, particularly those that are not restricted to a single species and affect humans and animals and cause foodborne diseases (Pulido-Landinez, 2019; Ferrari *et al.*, 2019).

Although different serotypes have been associated with salmonellosis, a limited number are responsible for most human infections. Worldwide data about *Salmonella* serotype prevalence in humans and in the diverse range of foodstuffs have contributed to establish an epidemiological link between salmonellosis and poultry products, with diverse serotypes overlapping between humans and poultry meat, otherwise, the shift in *Salmonella* serotypes related to poultry and poultry production has been associated with the spread of certain clones (Antunes, 2016).

Besides the implication in public health, *Salmonella* spp. is considered a pathogen of great economic impact and is frequently a target of sanitary barriers at the international meat trade, barriers that are enforced without the proper scientific basis and support (Mead *et al.*, 2010).

Given the relevance of this subject, the Codex Alimentarius Food Hygiene Commission (CCFH) published in 2011 the document CAC/GL 78-2011, which establishes the "Guidelines for the Control of *Campylobacter* and *Salmonella* in Chicken Meat", with prevention and control measures focused on good practices, hazards and risks (Codex Alimentarius, 2011) supported by a risk assessment conducted for FAO experts (FAO/WHO, 2009). As such document was being structured, a team of scientists and experts in *Salmonella* from chicken meat production chain and from several countries, and also under follow-up from FAO, published a scientific review concerning the applicability of a microbiological criterion for *Salmonella* spp. in raw chicken meat, concluding that the concept of zero tolerance is unfeasible (Mead *et al.*, 2010).

Considering the importance of the globalization in the international trade of food and in an attempt to address the differences between capability-driven levels of hazard control between countries, the World Trade Organization (WTO) has issued the Sanitary and Phytosanitary Measures (SPS) agreement and in conjunction with this Codex Alimentarius has developed the Risk Analysis framework to help countries link food control measures to public health. Within Risk Analysis, risk assessment is the scientific and technical component that can determine the risk in a population associated with a particular pathogen & food combination and can evaluate risk mitigation options (Membré *et al.*, 2007).

Codex Alimentarius and The International Commission on Microbiological Specifications for Foods (ICMSF), in turn, introduced new concepts that are intended to help in the process by translating risk management decisions on risking the population to measures that industry needs to implement in their daily operations as part of the prevention and control programs at the food production chain, looking for food safety and public health goals. One of these concepts is the Performance Objective (PO), which establishes a maximum frequency or concentration for some hazards in foods, to be reached in a specific step of the production chain, before the consumption. In the case of *Salmonella* spp. present in chicken meat, which is usually consumed after cooking but can effectively cause a cross-contamination during its handling and preparation, the ICMSF recommends that the industry defines as a PO that a percentage of carcasses may contain an established maximum limit of *Salmonella*, in order to reduce the probability of contamination of other foods (Membré *et al.,* 2007, Straver *et al.*, 2007; van der Fels-Klerx *et al.*, 2008; van Schothorst *et al.*, 2009; Tromp *et al.*, 2010).

Salmonella contamination is usually expressed in terms of prevalence, but evidence from microbiological risk assessment indicates that levels of contamination can be even more important to public health, and efforts at any stage of production or processing that reduce the level of *Salmonella* on the end product will reduce risk. With the development of better means of enumerating *Salmonella* and methods that are internationally acceptable, this aspect should receive greater attention in the future, enabling more heavily contaminated items to be identified and suitable interventions developed. Data suggest that the probability of illness is increased as exposition to greater numbers of salmonella increases. The exact number of

Salmonella needed to cause illness is dependent on a number of factors and can vary, the challenge as to whether improvements in public health will result from more stringent performance standards or from efforts that decrease the load of *Salmonella* in ground poultry products or from both (Mead *et al.*, 2010; McEntire *et al.*, 2014).

The importance of enumeration *Salmonella* was emphasized in an expert report by the American Society and the National Advisory Committee on Microbiological Criteria for Foods, both report that the efficacy of pathogen reduction cannot be determined without enumeration, because control efforts could reduce *Salmonella* cells number and the risk for consumer exposition, even the prevalence is not changing in a risk assessment (Cox *et al.*, 2010).

Risk assessments studies have also revealed that the risk for human salmonellosis related to consumption of chicken meat contaminated with high number of *Salmonella* with a greater probability of transferring rate *Salmonella* cells to other surfaces than a carcass contaminated with only a few cells of *Salmonella* (Straver *et al.*, 2007, Uyttendale *et al.*, 2009; WHO, 2002).

Brazil is signatory of the Codex Alimentarius and, as so, follows the recommended international guidelines and principles. In 2019 the National Health Surveillance Agency (ANVISA) published a review on microbiological criteria for foods and the new standards should be in force as of December 2020. The criteria adopted to raw poultry meat is the absence of *Salmonella* Enteritidis and *Salmonella* Typhimurium (n=5, c=0). In Brazil it is mandatory hazard communication of a possible presence of *Salmonella* in chicken meat to the consumer, including, to this end, precautions related to meat handling, preparation and storage and prevention of cross-contamination in the label (Brasil, 2019).

The control of *Salmonella* spp. contamination in the chicken slaughter process is established in Brazil by a National Program of Pathogen Reduction (PNRP), ruled by Normative Instruction nº. 20/2016 (Brasil, 2016), which determines a tolerance baseline of 25% (2/8) of positivity in carcasses after pre-chilling in regular cycles of official monitoring for chicken and turkey slaughtering in establishments under Federal Inspection Service (SIF).

The annual report published in 2019 by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) reveal that the *Salmonella* spp. Prevalence reaches 12,71% (352/2.791 samples) in poschill carcasses. The result shows a descendent curve in reference to 2017 (Brasil, 2019).

The aim of this study was to investigate the presence, level of contamination and serological profile of *Salmonella* spp. in different steps of broiler processing as a database to evaluate the implementation of a PO in frozen chicken breast.

MATERIAL AND METHODS

The collection and processing of samples were performed in two broiler slaughterhouses belonging to the same company, under Federal Inspection Service, located in the mid-west (Plant A) and southern (Plant B) regions of Brazil between May 2012 and December 2013.

10 broiler flocks were assessed in each slaughterhouse: 5 flocks with a positive result and 5 with a negative result in the pre-slaughter monitoring for *Salmonella* spp.

The samples were collected from chickens at the reception platform, from carcasses before (after final washing shower) and after (at the hook for second handling) immersion chilling and from frozen chicken breasts without bone and skin after 30 days of storage at -18°C.

10 cloacal swabs (each swab was used to collect material from five chickens, with a total of 50 animals), 25 broiler carcasses before immersion chilling, 25 broiler carcasses after immersion chilling and 30 chicken breasts were collected in each of the 20 flocks assessed, with a total of 1,800 samples analyzed for detection, enumeration and isolation of *Salmonella* spp.

The number of samples to be sufficient to assess compliance of the lot of frozen chicken breast was established according to the sampling recommended to meet an acceptance Performance Objective (PO) with a confidence interval of 95%, considering a proportion of 15% contaminated carcasses and 10% frozen chicken breast tolerated (van Shothorst *et al.*, 2009).

The material collected was identified and packaged in isothermal boxes and then forwarded to process at the company's quality assurance laboratory. The preparation of cloacal swabs' samples was performed at the animal health laboratory. The chicken breasts were stored for 30 days in a separate location at the cold chamber at -18°C before they were sent to process at the laboratory.

The cloacal swabs were put into tubes containing 25mL of buffered peptone water (BPW) 1% and then homogenized. After transferring an aliquot of 7.5mL

for the mMPN test, the tubes were incubated at 37ºC for 18 to 24 hours. 25g of muscle and skin were collected from the carcasses and frozen breasts, which were previously stored in sterile and sealed plastic bags, and then weighed. The samples were homogenized in a stomacher-like homogenizer with 225mL of BPW 1% and an aliquot of 7.5mL was transferred for mMPN, with further incubation at 37°C for 18 to 24 hours. After a pre-enrichment, the cloacal swabs' samples passed through a new enrichment by adding 500µL of Brain-Heart Infusion broth (BHI) and incubated at 37°C before starting the A-PCR protocol. Such protocol included the extraction, replication and identification steps and lasted approximately three hours and 30 minutes. The results were obtained by comparing the fragments with positive control standard through the equipment software. Parameterized positive and negative controls were used in all the assays.

For enumeration by mMPN, the plates were previously prepared by adding 2mL of BPW in wells of columns 2, 3 and 4. 2.5mL and the homogenized samples were inoculated in rows A, B and C of the first column. After that, a three-fold aliquot of 500µL each was transferred in 3 sequential dilutions in columns 2, 3 and 4 using a multichannel automatic pipette. The plates were incubated after adding 2mL in all the wells at 37°C for 16 to 20 hours. The selective enrichment was performed in similar plates adding 2mL of Semi-Solid Rappaport Vassiliadis medium (RVSS) with novobiocin in each well. Before the inoculation, the plates with the pre-enriched samples were kept in a centripetal movement for 3 to 5 minutes and, with a multichannel pipette, 20µL were transferred to the surface of each corresponding well at the plate with RVSS. The plates were then incubated at 41.5°C with readings at 24 and after 48 hours. The plates reading was based on bacterial growth and migration, with the development of a white or light blue halo caused by the reaction of the substrate and change of color of the medium. The positive samples were plated in selective mediums Rambach and XLT4 for isolation of typical colonies and confirmation by biochemical and serological tests.

The calculation of most probable number was obtained using the software *MPN Calculator* (Cariale, no date), where the parameters of four series of tubes/wells were fixed and the volumes of the samples inoculated were 2mL; 0.5mL; 0.1mL and 0.02mL, which, in function of the primary dilution, represent 0.20g; 0.04g; 0.008g and 0.0016g of the tested matrix. The software also establishes minimum and maximum limits within a confidence interval of 95%. The results of quantification analysis were divided into five groups according to the contamination level found, in accordance with the parameters used by Petton *et al.* (2004). The categories were: below the enumeration limit (<1 cell/g), low contamination (<10 cells/g), average (10-100 cells/g), high (>100 cells/g) and very high (>710 cells/g).

Strains of samples that were positive in the mMPN method and isolated from the 10 flocks that were positive at the pre-slaughter monitoring, were forwarded for antigenic characterization conducted at the National Reference Laboratory of Cholera and Enteric Diseases of the Oswaldo Cruz Institute Foundation (FIOCRUZ, Rio de Janeiro, Brazil) by means of rapid slide agglutination tests, conforming the White-Kauffman scheme for *Salmonella* using somatic and flagellar antisera.

Biostat 5.3 software was used for statistical calculation. To compare flocks knowingly positive and negative with the results found in all the process steps combined, the parametric method of G-test of Independence was applied. To evaluate the steps separately and according to the flocks' origin at the pre-slaughter and to perform the qualitative and quantitative analysis, the 2 Binomial proportions tests were used. The Kappa test was chosen to assess the agreement between the results of the different analytical methods A-PCR and mMPN.

The approval certificate number from the Ethics Commission on Animal Use (CEUA) of Fluminense Federal University regarding this study is 696.

RESULTS AND DISCUSSION

From the 1,800 assays performed from samples collected in different broiler processing steps, 278 (15,4%) were positive to *Salmonella* spp. and just 118 (6.5%) growth on MSRV plates to be enumerated by mMPN. The detailed results are show in table 1.

The comparison of results of the analytical methods A-PCR and mMPN showed a weak or null agreement in all analyzed processing steps: cloacal swabs (0.5575), carcasses before (0.5610) and after immersion chilling (0.577) and frozen chicken breasts (0.5067). Differences between the sensibility of those methods should be considered to explain the absence of correlation of results by different analytical methods. The automated PCR system BAX SYSTEM (DUPONT QUALICOM, USA) has an analytical basis similar to a conventional *polymerase chain reaction*

Table 1 – Frequency of *Salmonella* spp. detection and enumeration in contaminated samples in different broiler processing sites evaluated respectively by automated polymerase chain reaction (A-PCR) and miniaturized Most Probable Number (mMPN) techniques in chicken slaughterhouses under Federal Inspection, Brazil.

drag swabs	cloacal swabs		carcasses before chill		carcasses after chill		frozen chicken breast	
	A-PCR	mMPN	A-PCR	mMPN	A-PCR	mMPN	A-PCR	mMPN
negative	24% (24/100)	14% (14/100)	21.6% (54/250)	15.6% (39/250)	6.8% (17/250)	1.2% (3/250)	2.3% (7/300)	1.3% (4/300)
positive	29% (29/100)	17% (17/100)	31.2% (78/250)	15.2% (38/250)	25.6% (64/250)	1.2% (3/250)	1.6% (5/300)	0% (0/300)
total	27% (53/200)	15.5% (31/200)	26.4% (132/500)	14% (77/500)	16.2% (81/500)	1.2% (6/500)	2% (12/600)	0.6% (4/600)

*A-PCR: Automated polymerase chain reaction; **mMPN: Miniaturized Most Probable Number.

(PCR), based on the detection of a fragment of genetic material whose sequencing is exclusive of a specific target organism. Such fragment is replicated, creating millions of copies, in the case it is present in the sample. In a few hours, it delivers a clear result, and for *Salmonella* spp., the results present a >98% sensibility and a >97% specificity. The *Salmonella* spp. enumeration by miniaturized Most Probable Number (mMPN) was recognized as the reference method in 2012 when part 2 of ISO 9579:2002 was published. In mMPN, sterile plastic plates with 24 wells of 2.5mL capacity each and 3x4 format are used, instead of conventional tubes. For each sample, a plate is used in a three-fold assay, being the volume of primary dilution tested lower than the detection volume of conventional method ISO6579:2002; consequently, the sensitivity is lower. The mMPN sensitivity limit is approximately 1 cfu/g, but it may vary according to the serovar and matrix, while the conventional methods' sensitivity limit is approximately 1 cfu/25g (0.04 cfu/g) (ISO, 2012). Considering that the A-PCR sensitivity is equivalent to the conventional cultivation method and that, as the general rule, the contamination levels are very low (Cox *et al.*, 2011), the performance difference between methods was expected.

No significant difference was observed between the results of flocks with a positive and negative origin for *Salmonella* spp. in qualitative analysis (p=0.4754) nor in quantitative analysis (p=0.1918), when considered all the processing steps evaluated in combination.

One of the flocks tested there was a status reversion in one flock with negative origin and negative status at the platform and before immersion chilling, that presented a high positivity frequency after the immersion chilling in the qualitative results. Such result profile was not replicated in the quantitative analysis, indicating possible low contamination, typical of cross-contamination in the chiller, as mentioned by Straver *et al.* (2007). The results found are similar to those of Burfoot *et al.* (2010), highlighting that several factors from the pre-slaughter monitoring and during the processing interfere in the frequency of *Salmonella* spp. isolation throughout the production chain, and it may affect the status reversion or the contamination level.

NACMCF (2019) related that several preslaughter strategies to reduce the burden of *Salmonella* in flocks before slaughter have been effective and demonstrating a correlation between flock status of *Salmonella* and pre and postchill contamination. However, correlation between preslaughter status and finish product contamination with *Salmonella* is not certain in commercial settings.

Analyzing the results of the qualitative and quantitative analysis of *Salmonella* spp. positivity in the processing steps separately, a difference with a significant reduction between frequencies in carcasses before and after immersion chilling was observed (p=<0.0001). However, when evaluating the results between carcasses before and after immersion chilling (p<0.0001) and between carcasses after immersion chilling and frozen chicken breast (p=0.0053), a difference with a significant reduction in *Salmonella* spp. frequency was observed.

In broiler flocks with a positive origin, only a significant reduction in *Salmonella* spp. frequency was found between carcasses after immersion chilling and frozen chicken breasts (p<0.0001) in the quantitative analysis. In the quantitative results, a difference with a significant reduction in *Salmonella* spp. frequency was only observed between carcasses before and after immersion chilling (p<0.0001).

Such information confirm the findings of a study performed by Straver *et al.* (2007), which highlights a reduction effect in the frequency and level of contamination during immersion pre-chilling, and reinforces that even a controlled process has a limitation of the contamination reduction potential and

therefore, emphasizes the importance of an efficient control in the previous processing steps, Hardie *et al.* (2019).

The immersion system to chill carcasses can introduce cross contamination by direct contact between carcasses and contaminated water, however at the same time the efficacy of control process with controlling flow rate, flow direction, low organic materials and temperature bellow 4°C will inhibit *Salmonella* growth and mitigate the risk to cross contamination and reduce the level of contamination (FAO, 2009; Codex Alimentarius,2011; NACMCF, 2019). Furthermore, chlorine is effective at reducing *Salmonella* spp. from carcasses in immersion chiller system, reducing the concentration below the limit of detection of the method used (Hardie *et al.*, 2019). A combination of these factors can be related with this result of mitigating the risk of *Salmonella* contamination in this site of processing.

A difference with a significant reduction between carcasses after immersion chilling and frozen breasts was also observed ($p<0.0001$). The effect of reduction of viable cells beyond the action of cold is expected, in case the control and prevention measures implemented are efficient and effective to avoid the cross contamination. The skin removal represents a relevant factor to reduce the risk of contamination (Straver *et al.,*2007). In a transmission model to estimate PO for *Salmonella* in the broiler supply chain designed by van der Fels-Klerx *et al.*, 2008, improved hygiene measures at slaughtering may also reduce contamination and increase reduction of contaminated flocks during the removal of the breast skin considering the *Salmonella* prevalence of 2,5% in the end processing.

Rimet *et al.* (2019) used bioluminescence, imaging, culture and immunohistochemistry to localize *Salmonella* isolates in the skin, skeletal muscle and bone of chicken and turkeys in order to reveal the contribution of these sites in the contamination of ground poultry meat. Their findings indicate that fecal *Salmonella* shedding results in contamination of chicken skin and the chicken skin contained low numbers of salmonella but at high prevalence significantly contributing to contamination of ground chicken.

Meanwhile, it is relevant to consider the influence of risk factor within an establishment that vary day by day during poultry processing, such as incoming flock prevalence, cleanliness, establishment personnel, ability of the bacterial present to persist in the environment as a biofilm for e.g.

In total, 126 *Salmonella* spp. strain were identified from the slaughterhouse A (mid-west Brazil) and the serotype most frequent isolated was *S.* Minnesota (88,09%), present in all steps tested. 3 samples were not serotyped, as they did not present growth (2.38%). In slaughterhouse B (southern Brazil), only 6 strains were identified, and the single strain found in the slaughterhouse was *S.* Anatum. The distribution of serotypes of *Salmonella* strains isolated in the processing sites is summarized in table.2.

Strains of different serovars were isolated in the same flock 3 times in plant A (mid-west Brazil), always in carcasses before immersion chilling. The serotypes found were *S.* Minnesota, *S.* Newport and rough S. *enterica* subsp. *enterica*. These findings reproduce the review brought by Cox *et al.* (2011) concerning mixed colonization of flocks in nature, even with a prevalence of a dominant serotype or clone.

The dominance and spread of *S.* Minnesota observed in our results had been described by Voss-Rech *et al.* (2015) in drag swabs received between 2009 and 2010 from commercial broiler farms from the state of Mato Grosso do Sul. The dominance by geographical location has been reported by Cox, *et al.* (2011).

In the same study, Voss-Rech *et al.* reported the identification of *S.* Anatum in broiler farms in the states of Santa Catarina and Parana in the south of Brazil in low prevalence and absence of antibiotic resistance profile. However, their study didn't isolate *S.* Newport strains like our findings. The result of Brazilian National *Salmonella* surveillance program in broiler carcass related the prevalence of *S.* Minnesota as the second serotype most frequent isolated between 2009-2010 (Freitas, 2011), and the internal data revealed the dominance since 2010 in this company. Different serotypes were related by Cunha-Neto *et al.* (2018) in samples of chilled chicken carcass isolated in a slaughterhouse in the state of Mato Grosso between 2014 and 2015. In this study, the most frequently serotypes isolated were *S.* Infantis (34,4%, 11/31), *S.* Abony (25,8%, 8/31), *S.* Agona (12,9%, 4/31), *S.*Schuwarzengrund (9,7%, 3/31), *S.* Anatum and *Salmonella* enterica O:4,5 (6,5%, 2/31) and *Salmonella* enterica O:6,7 (3,2%, 1/31). The lapse time of 3 years should be considered.

Salmonella serovars come and go, often introduced or becoming prevalent through changes in husbandry or other practices (Barrow *et al.,* 2012). New serotypes can emerge because changes in the farm ecology, new technologies to do a more precise identification, by transfer of mobile genetic elements (Pullido-Landinez, 2019; Mastrorilli *et al.*, 2018), as the occurrence of type

II toxin-antitoxin systems promoting adaptability and persistence of the most prevalent *Salmonella* serovars circulating (Di Cesare *et al.,* 2016).

Although understanding the exact mechanisms of their persistence and spread in poultry are still largely unknown, recent studies focusing on emergent poultry associated Salmonella strains unveiled specific features that could provide a significant advantage both in the environment and in the host (Foley *et al*, 2011). Also genomic study of several predominant Salmonella serotypes from Canadian boiler chickens showed the presence of multiple features related with pathogenicity (e.g. genes encoding adhesins, flagellar proteins, iron acquisition systems, type II secretion system) and stress tolerance (e.g. metal and antiseptic tolerance genes, better acid-stress response) (Dhanani *et al.*, 2015). These features can play a role in the successful spread of emergent and virulent serotypes or clones that could contribute in a short time to replacing other *Salmonella* (Antunes, 2016).

It is relevant to consider that *S.* Minnesota is rarely responsible for human salmonellosis outbreaks worldwide (Ferrari *et al.,* 2019, EFSA, 2018). However, the detection of resistance genes have been related in strains of *Salmonella* Minnesota isolates from Brazilian poultry farms and raw poultry meat (Voss-Rech *et al.,* 2015; Rodrigues *et al.*, 2017, Campos *et al.*, 2018).

The single *S.* Anatum strain isolated in slaughterhouse A were not considered relevant in terms of epidemiology trend.

Table 2 – Serological identification of *Salmonella* strains isolated from broiler flocks evaluated in pre-harvest and in different sites of processing in chicken slaughterhouses under Federal Inspection, Brazil.

No. cell/mMPN	cloacal swabs		carcasses before chill		carcasses after chill		frozen chicken breast	
	positive flocks	negative flocks	positive flocks	negative flocks	positive flocks	negative flocks	positive flocks	negative flocks
<1 CFU/g*	83	86	213	211	247	247	300	296
<10 CFU/g		5	7	6		3		2
10-100 CFU/g	2	2	5	7	2			2
>100 CFU/g		5	3	6				
>710 CFU/g	16	2	22	20	1			

*Plant A, located at the mid-west region of Brazil; Plant B, located at the southern region of Brazil.

Just 118 (6.5%) samples growth on MSRV plates to be enumerated by mMPN. The results below the limit of the sensitive of the method were considered <1 CFU/g. Cloacal swabs and carcasses before chill reveal very high levels of *Salmonella* contamination, representing 50,84% of samples enumerated. One single sample of carcass after chill appear with a very high level of contamination. In the other hand, carcasses after chill shows low frequency of enumeration and low or average level of contamination. Just 0,6% (4/600) of frozen chicken breast were enumerated (low and average level of contamination). Table 3. Distribution of results of enumerated samples considering the range of levels of *Salmonella* contamination.

The distinction between high and low levels and the establishment of a threshold level for process control is certainly debatable, but it is certain that it can be very helpful to investigate the factors that resulted in high loads, enabling the implementation of more effective mitigations and comparing levels with the processing involved to allow the industry to be better understood, which interventions works and when (McEntire *et al.,* 2014).

There is already evidence from microbiological risk assessment studies that levels of contamination can be even more important to public health than prevalence as they are directly related to the likelihood that the ingested dose exceeds the minimum infectious dose needed for disease development. There is a need to test new performance standards based on prevalence and enumeration level rather than just on absence or presence alone (Sampedro *et al.* 2018).

Recent studies have been published using the *Salmonella* enumeration to evaluate the risk assessment even as the impact of risk factor and interventions along the processing chain under the final product.

In Canada, the study conducted between 2012-2013 Hardie *et al.* (2019) evaluated the prevalence and enumeration of broiler carcasses postchill and parts at the end of the processing from 38 federally registered slaughterhouses. They considered the variables of processing and risk factors impact to evaluate the results of prevalence and concentration of *Salmonella* prior to and immediately after each processing intervention would inform processor on the best practices for reduction of *Salmonella* on broiler chicken products. The selected enumeration rate of lower limit of <0.03 MPN/mL and upper limit >11 MPN/mL and the mean concentration of these samples was -0,96 $\log^1$ MPN/mL but complete results were not available to compare with our results.

Table 3 – Samples enumerated for *Salmonella* spp. by mMPN and range of contamination level in different processing sites and according the result of *Salmonella* spp. detection in drag swabs of broiler flocks before slaughter in chicken slaughterhouses under Federal Inspection, Brazil.

No. cell/mMPN	cloacal swabs		carcasses before chill		carcasses after chill		frozen chicken breast	
	positive flocks	negative flocks	positive flocks	negative flocks	positive flocks	negative flocks	positive flocks	negative flocks
<1 CFU/g*	83	86	213	211	247	247	300	296
<10 CFU/g		5	7	6		3		2
10-100 CFU/g	2	2	5	7	2			2
>100 CFU/g		5	3	6				
>710 CFU/g	16	2	22	20	1			

*the limit of the sensibility of the method is 1 CFU/g. mMPN: miniaturized Most Probable Number; *sensitivity limit of enumeration method (1CFU/g); 1CFU/25g = 0,004CFU/g.

Waghamare *et al.* (2019) used the MPN miniaturized to evaluate the contamination in critical stages of poultry processing in different sets of practices in automated, semi-automated and retails shops in India, observing higher concentration of *Salmonella* at defeathering and evisceration stages.

Jeong *et al.* 2018 reproduced in South Korea the quantitative microbial risk assessment (QMRA) for *Salmonella* and whole chickens to determine the relationship between the most probable number (MPN) of *Salmonella* cells and the illness probability, and how this relationship is affected by each stage in the retail to the table. Their findings reveal that the prevalence of *Salmonella* in chicken has a predominant impact on the likelihood of illnesses and suggests that the efforts to decrease the contamination level should preceed to the importance of prevention of *Salmonella* contamination at stages of rearing and slaughtering, and also additional research on concentration of *Salmonella* on chicken for e.g. are needed.

In United States, Peng *et al.*(2016) found 13.7%,19.7% and 25% *Salmonella* prevalence in turkey drumstick skin, thigh skin and wing skin and the *Salmonella* enumeration by traditional MPN 1.18, 1.29 and 1.45 log MPN respectively, in natural contaminated samples of turkey ground collected postchill to evaluate skin of turkey parts and concluded that the high prevalence associated with the skin of turkey parts could be a potential source for ground turkey contamination.

Santos *et al.,* 2014 used the miniaturized MPN in 6 sites in triplicate in a total of 108 samples along the processing line in 3 Brazilian broiler slaughterhouses between 2013-2014. Cloacal swabs, sponge samples from transport cages before and after sanitation, carcasses before and after chiller and frozen carcasses after 24 hours were analyzed and revealed 5,5% prevalence of *Salmonella* but none of the samples were performed for enumeration.

Smadi & Sargent's, 2012 study on raw chicken breast in retail in Canada in the Quantitative Risk Assessment of human salmonellosis, revealed a prevalence of 30% and 82% on chicken breast samples had concentration below the MPN detectable limit (0,3 MPN/g). The input settings for the cumulative distribution for *Salmonella* set the minimum value as 0 MPN/g to 44.5 MPN/g. *Salmonella* were transferred through the model, considering the transfer rate and the number of *Salmonella* estimated on raw chicken breast. Their conclusion revealed that reduction of concentration of *Salmonella* on chicken breast retail and washing utensils and hands after handling raw chicken breast and proper cooking at consumers' homes can result in a predicted probability of illness.

Straver *et al.* (2007) examined chicken breast filets at the retail level in Netherland to perform a quantitative risk assessment and found that 8,6% samples tested *Salmonella* positive with MPN value of 10 to >1.000 CFU/fillet (corresponding a range of 0.05 to 5.5 CFU/g). In total, 0,8% of the samples yielded *Salmonella* MPN counts greater than 1.000CFU/filet and the highest contamination level observed was 3.81log MPN/filet. They concluded that fillet with high numbers of Salmonella determine a large fraction of the risk of salmonellosis, it's important to consider not only the prevalence, but also the number of *Salmonella* present. Our results for frozen chicken breast have some correlation with their findings, although we failed to proceed to convert the base of numbers.

The assay performed by Petton *et al.* (2004) used a sampling of 180 replicates of turkey meat artificially contaminated. However, the results converged regarding the contamination reduction throughout the processing like the trend we observed in our study.

A single lot of frozen chicken breast reached 16% of samples with a *Salmonella* positive detection and were not able to comply the Performance Objective (PO) formulated as "*not more than 10% of frozen chicken breast in a lot may test positive for Salmonella*

spp. and the consumer's acceptable level for safety is set at 95% probability" (table 4).

Table 4 – Sampling plans derived for *Salmonella* in poultry carcasses intended to test compliance with different POs.

Proportion of contaminated carcasses tolerated (PO)%	Number of samples (n) required to reject lots with 95% probability (c=0)	Proportion of contaminated carcasses accepted with 95% probability (%)
15	19	0.27
10	29	0.18
5	59	0.09
1	298	0.02

*Van Schothorst *et al.*, 2009.

The enumeration of 4 samples show levels of contamination up to the sensitivity of mMPN method (>1 CFU/g), 2 of them presenting a low contamination level (<10 CFU/g) and 2 an average contamination level (10-100 CFU/g). The drag swab of this broiler flock was negative to *Salmonella* spp. in the pre-harvest monitoring, but 80% of cloacal swabs at the platform and 64% of carcasses before immersion chilling were positive. Besides that, 48% of carcasses before chill revealed a very high level of contamination (>710 cells/g). The detailed and completed results data were tabulated in table 5.

Table 5 – Frequency of detection of *Salmonella* spp. by automated polymerase chain reaction (A-PCR) and frequency of samples enumerated by miniaturized most probable number technique (mMPN) in chicken slaughterhouses under Federal Inspection, Brazil.

Drag swabs	Plant	cloacal swabs		carcasses before chill		carcasses after chill		frozen chicken breast	
		a-PCR	mMPN	a-PCR	mMPN	a-PCR	mMPN	a-PCR	mMPN
negative	A	0/10	0/10	0/25	0/25	0/25	0/25	0/30	0/30
		9/10	3/10	24/25	13/25	17/25	3/25	2/30	0/30
		8/10	8/10	20/25	16/25	0/25	0/25	5/30	4/30
		1/10	1/10	0/25	0/25	0/25	0/25	0/30	0/30
		2/10	2/10	10/25	10/25	0/25	0/25	0/30	0/30
	B	0/10	0/10	0/25	0/25	0/25	0/25	0/30	0/30
		2/10	0/10	0/25	0/25	0/25	0/25	0/30	0/30
		0/10	0/10	0/25	0/25	0/25	0/25	0/30	0/30
		0/10	0/10	0/25	0/25	0/25	0/25	0/30	0/30
		2/10	0/10	0/25	0/25	0/25	0/25	0/30	0/30
positive	A	4/10	0/10	13/25	1/25	10/25	1/25	1/30	0/30
		10/10	9/10	9/25	8/25	1/25	1/25	2/30	0/30
		0/10	0/10	12/25	7/25	12/25	0/25	0/30	0/30
		10/10	7/10	24/25	20/25	18/25	1/25	0/30	0/30
		0/10	0/10	1/25	1/25	0/25	0/25	2/30	0/30
	B	1/10	0/10	0/25	0/25	0/25	0/25	0/30	0/30
		0/10	0/10	0/25	0/25	0/25	0/25	0/30	0/30
		0/10	0/10	1/25	1/25	0/25	0/25	0/30	0/30
		4/10	1/10	18/25	0/25	23/25	0/25	0/30	0/30
		0/10	0/10	0/25	0/25	0/25	0/25	0/30	0/30

A-PCR: Automated polymerase chain reaction; mMPN: miniaturized Most Probable Number; *A - Slaughter plant located at the mid-west region of Brazil; B - Slaughter plant located at the southern region of Brazil.

The move towards a risk-based management approach is a major step in advancing a science-base food safety system by clearly linking food safety requirements and criteria to the public health problems they are designed to address, as establishing targets earlier in the food chain, such as POs, and allowing credible limits to be established and verified by processors to indicate control (Mead *et al.*, 2010).

Some mathematical models have been published in the last years to estimate Performance Objectives (PO) for *Salmonella* in the broiler supply chain (Oscar,2013; Tromp *et al.*, 2010; van Schothorst *et al.,* 2009; van der Fels-Klerx *et al.*, 2008; Membré *et al.*, 2007). However, we didn't find any register or paper relating a practical application of PO in a raw poultry product using natural contaminated samples under industrial scale published until now.

CONCLUSION

The serological evaluation of isolated strains confirms the endemic profile of *Salmonella* Minnesota in the slaughter plant located at the middle-west region, with distribution throughout all the processing steps. The identification of the serotype circulating is one important element for hazard characterization.

The combination of analysis of prevalence and concentration of *Salmonella* and the application of a PO in raw poultry meat products is feasible and can be an important risk assessment tool along the processing chain to set best practices for risk mitigation, evaluate the effect of risk factor and support the decisions about the impact of food safety for public health.

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