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Investigation of *Listeria monocytogenes*, *Salmonella enterica* and *Yersinia enterocolitica* in pig carcasses in Southern Brazil[1]

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ABSTRACT.- Kich J.D., Souza A.I.A., Montes J., Meneguzzi M., Costa E.F., Coldebella A., Corbellini L.G. & Cardoso M. 2020. **Investigation of *Listeria monocytogenes*, *Salmonella enterica* and *Yersinia enterocolitica* in pig carcasses in Southern Brazil.** *Pesquisa Veterinária Brasileira 40(10):781-790*. Embrapa Suínos e Aves, BR-153 Km 110, Distrito de Tamanduá, Concórdia, SC 89715-899, Brazil. E-mail: jalusa.kich@embrapa.br

The intensification of pig production and advances in the sanitary control of herds profoundly changed the profile of risk attributed to pork consumption. In the actual scenario, most microorganisms related to macroscopic lesions observed in the *post mortem* inspection are not transmitted by food, while foodborne bacteria of importance to consumer health do not cause macroscopic lesions. In Brazil, the "*Ministério da Agricultura, Pecuária e Abastecimento*" requested a scientific opinion on the prioritizing of pathogens potentially transmitted by unprocessed pork. After conducting a qualitative risk assessment, only *Salmonella enterica* was classified as of high risk to consumers. The present study was part of the validation step of the risk assessment and aimed to investigate the frequency of *S. enterica*, *Yersinia enterocolitica* and *Listeria monocytogenes* and hygienic-sanitary indicators in pig carcasses of pigs rose under intensive production and slaughtered under the Federal Inspection System in three slaughterhouses located in Southern Brazil. Additionally, the antimicrobial resistance profile of the isolated pathogens was also investigated. A total of 378 carcasses were sampled by superficial sponges before the chilling step in three slaughterhouses. Samples were investigated for the presence of the three aforementioned pathogens and subjected to enumeration of Colony Formation Units (log CFU.cm^{-1}) of total aerobic mesophiles (TAM) and Enterobacteriaceae. *Salmonella* strains were tested by disc diffusion test for resistance to eleven antimicrobials. There were significantly statistical differences ($p<0.0001$) on the median counts of both indicators between the slaughterhouses. The median of TAM was very close for Slaughterhouses A and B: 1.573 log CFU.cm^{-1} and 1.6014 log CFU.cm^{-1}, respectively. While in Slaughterhouse C, a higher TAM median was detected (2.216 log CFU.cm^{-1}). A similar profile was observed regarding to Enterobacteriaceae, and medians were calculated as follow: -0.426 log CFU.cm^{-1} in Slaughterhouse A; 0.2163 log CFU.cm^{-1} in B; and 0.633 log CFU.cm^{-1} in C. Regarding the pathogens investigated, *L. monocytogenes* was not detected and only one carcass from Slaughterhouse C was positive for *Y. enterocolitica*. Thus, the results suggest a very low prevalence of *L. monocytogenes* and *Y. enterocolitica* in the sampled population. A total of 65 (17.2%) carcasses were positive for S. *enterica*, with a difference in frequencies between slaughterhouses and slaughter days. The prevalence of *Salmonella* positive carcasses was higher in the Slaughterhouse C (25.4%; CI 95% 19-32%) in comparison with A (9.5%; CI

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95% 9-14%) and B (18.3%; CI 95% 12-24%). There was no significantly statistical association between Enterobacteriaceae counts and *Salmonella* isolation on carcass surface (p=0.69). The slaughtering day, nested within the slaughterhouse, explains 31.3% of *Salmonella* prevalence variability. *S.* Typhimurium (38.1%) was the most prevalent, followed by *S.* Infantis (30.1%). Among the 61 *Salmonella* strains tested for resistance to antimicrobials, 18 (31.6%) were full-susceptible. No strain displayed resistance to azithromycin, ceftazidime, cefotaxime and meropenem. The highest resistance frequency was displayed to tetracycline (54.1%), followed by ampicillin (50.82%), nalidixic acid (42.62%) and chloramphenicol (42.62). Multi-resistance was detected in 52.54% of the, strains. In conclusion, *S. enterica* is more prevalent in pre-chill pig carcasses than *Y. enterocolitica* and *L. monocytogenes* and thus should be prioritized in monitoring and control programs at slaughter. *Salmonella* serovars varied among slaughterhouses and present significant differences in their resistance to antimicrobials. Slaughterhouses that present higher medians of TAM or Enterobacteriaceae in a monitoring period may have higher *S. enterica* prevalences as well. However, there is a high variation of *S. enterica* prevalence among slaughter days, which cannot be always related to the hygienic indicators counts observed on a given day.

INDEX TERMS: *Listeria monocytogenes*, *Salmonella enterica*, *Yersinia enterocolitica*, pig carcasses, pork, pigs, antimicrobial resistance, Brazil.

RESUMO.- [**Pesquisa de *Listeria monocytogenes*, *Salmonella enterica* and *Yersinia enterocolitica* em carcaças de suínos no sul do Brasil.**] A intensificação da produção de suínos e os avanços no controle sanitário dos rebanhos alterou de forma importante o perfil de risco do consumo de carne suína. No cenário atual, a maioria dos microrganismos causadores de lesões macroscópicas detectáveis na inspeção *post mortem* não são transmissíveis por alimentos, enquanto bactérias de importância como causadoras de doenças transmitidas por alimentos não causam lesões macroscópicas. No Brasil, o Ministério da Agricultura, Pecuária e Abastecimento solicitou uma opinião científica sobre a priorização de patógenos potencialmente transmitidos pela carne suína in natura. Após conduzir uma avaliação de risco qualitativa, apenas *Salmonella enterica* foi classificada como de alto risco para o consumidor. O presente estudo foi parte da etapa de validação da avaliação de risco e objetivou: investigar a frequência de *S. enterica*, *Yersinia enterocolitica* e *Listeria. monocytogenes*; e enumerar indicadores higiênico-sanitários em carcaças de suínos abatidos sob inspeção federal em frigoríficos dedicados ao abate de suínos sob sistema intensivo de criação no sul do Brasil. Além disso, o perfil de resistência a antimicrobianos dos patógenos isolados foi investigado. A superfície de um total de 378 carcaças foi amostrada por esponjas, na etapa de pré-resfriamento em três matadouros frigoríficos (A, B, C). As amostras foram investigadas quanto à presença dos três patógenos acima mencionados e quanto à enumeração de Unidades Formadoras de Colônia (log UFC.cm^{-1}) de mesófilos aeróbios totais (MAT) e Enterobacteriaceae. As cepas isoladas de *Salmonella* foram testadas quanto à resistência a onze antimicrobianos pela técnica de disco difusão. As medianas de contagem de ambos os indicadores apresentaram diferença significativa (p<0,0001) entre matadouros-frigoríficos. A mediana de MAT foi bastante próxima para A e B (1,573 log UFC.cm^{-1} e 1,6014 log UFC.cm^{-1}, respectivamente), enquanto em C uma mediana de MAT mais elevada foi determinada (2,216 log CFU.cm^{-1}). Um perfil semelhante foi observado em relação a Enterobacteriaceae, sendo as medianas calculadas para A, B e C, respectivamente: -0,426 log CFU.cm^{-1}; 0,2163 log UFC.cm^{-1}; e 0,633 log UFC.cm^{-1}. Em relação aos patógenos investigados, *L. monocytogenes* não foi detectada e apenas uma carcaça, do Matadouro C, foi positiva para *Y. enterocolitica.* Portanto, os resultados sugerem uma prevalência muito baixa desses patógenos na população amostrada. Em um total de 65 (17,2%) carcaças houve isolamento de *S. enterica*, com diferença nas frequências observadas entre matadouros e dias de abate. A prevalência de carcaças positivas para *S. enterica* foi maior no Matadouro C (25,4%; IC95% 19-32%) em comparação com A (9,5%; IC95% 9-14%) e B (18,3%; IC95% 12-24%). Não houve associação estatística entre o número de Enterobacteriaceae e o isolamento de *S. enterica* na superfície das carcaças (p=0,69). O dia de abate agrupado por frigorífico explica 31,3% da variação na prevalência de *Salmonella.* O sorovar mais frequente de *S. enterica* foi Typhimurium (38,1%) seguido de *S.* Infantis (30,1%). Entre as 61 cepas de *S. enterica* testadas quanto à resistência a antimicrobianos, 18 (31,6%) foram totalmente suscetíveis aos antimicrobianos testados. Nenhuma cepa apresentou resistência a azitromicina, ceftazidima, cefotaxima e meropenem. As maiores frequências de resistência foram demonstradas contra tetraciclina (54,1%), ampicilina (50,8%), ácido nalidíxico (42,62%) e cloranfenicol (42,62%). Em 52,54% das cepas foi detectada multi-resistência. Em conclusão, *S. enterica* é mais prevalente em carcaças suínas no pré-resfriamento do que *Y. enterocolitica* e *L. monocytogenes*. Portanto, *S. enterica* deve ser priorizada em programas de monitoramento e controle ao abate. Os sorovares de *Salmonella* variam entre matadouros e apresentam diferenças significativas na resistência a antimicrobianos. Matadouros de suínos que apresentam medianas de MAT e Enterobacteriaceae num período de monitoramento podem apresentar também prevalências mais de altas de presença de *S. enterica*. Entretanto, há uma alta variabilidade na frequência de S. *enterica* entre dias de abate, e nem sempre há relação entre essa frequência e a contagem de indicadores higiênico-sanitários determinados num determinado dia.

TERMOS DE INDEXAÇÃO: Carne suína, carcaças de suínos, *Salmonella enterica*, *Listeria monocytogenes*, *Yersinia enterocolitica*, resistência a antimicrobianos, suínos, Brasil.

INTRODUCTION

Meat inspection aims to protect the consumer's health by detecting and preventing hazards, which may be transmitted by meat (FAO 2000). The identification of meat unsuitable for human consumption has traditionally been performed by *post mortem* inspection of carcasses and viscera, which are subjected to visual examination, palpation and incisions for detecting lesions. The intensification of pig production and advances in the sanitary control of herds, however, profoundly changed the profile of risk attributed to pork consumption. While in the past parasitic diseases were the most important hazards transmitted by pork, nowadays parasites are very well controlled in the animal production phase with rare evidence of lesions. In the actual scenario, most microorganisms related to macroscopic lesions observed in the *post mortem* inspection are not transmitted by food; most of them cause animal diseases or are related to occupational exposure (EFSA 2011). Data collected from 2012 to 2014 in all Brazilian slaughterhouses under the Federal Inspection System demonstrated that cysticercosis, which had been an important zoonosis in the past, was the cause of only 9.2 condemnations per million pigs slaughtered in this period. Moreover, 74.5% of the detections occurred in only one slaughterhouse, indicating an epidemiological profile found in a restricted geographical area (Kich et al. 2019).

Foodborne bacteria of importance to consumer health, in turn, do not cause lesions and thus are not detectable by the technics adopted in the *post mortem* inspection (EFSA 2011). In addition, traditional inspection system practices, such as palpation and incision, can lead to the transfer of these bacteria between carcasses, the environment and employees (Buncic et al. 2014). As long as supported by scientific evidences, several countries have changed some traditional inspection practices, and adopted a risk-based meat inspection (EFSA 2011). In Brazil, the "*Ministério da Agricultura*, *Pecuária e Abastecimento*" (MAPA) requested from "Embrapa Suínos e Aves" a scientific opinion on this topic; in order to achieve this goal a multidisciplinary scientific project was conducted. The study aimed ultimately at generating scientific evidences to support decisions to be taken by the risk manager regarding changes in the meat inspection system. The first step of the project was a qualitative risk assessment for the prioritization of biological hazards transmissible to humans by pork (Costa et al. 2020), in which only *Salmonella enterica* was classified as of high risk to consumers. Other bacteria, such as *Yersinia enterocolitica* and *Listeria monocytogenes*, which are reported as possible causes of foodborne diseases transmitted by pork, were not prioritized in the risk assessment. However, *Y. enterocolitica* is often carried in slaughtered pig tonsils and can contaminate carcasses during processing (Drummond et al. 2012), while ready-to-eat pork products may be the vehicle of *L. monocytogenes* in foodborne cases (WHO 2018a). The presence of bacterial hazards in carcasses in turn is greatly influenced by the quality of the process and self-control programs implemented at slaughterhouses (Pearce et al. 2004, Arguello et al. 2013). The process hygiene has been monitored by the enumeration of hygienic-sanitary indicators, such as mesophilic aerobes and Enterobacteriaceae, since these bacteria can be eliminated by proper hygiene and sanitation procedures during slaughtering (Ghafir et al. 2008). Therefore, the investigation of these three pathogens and two hygienic-sanitary indicators (mesophilic aerobes and Enterobacteriaceae) was kept in the present study, which will be a part of the validation step that follows the qualitative risk assessment.

In addition to the risk posed by their presence in pork, pathogenic bacteria carrying antimicrobial resistance genes have been a growing concern to consumer's health. The hazard of multi-resistant strains selection by the antimicrobial use in animals has been stressed worldwide (WHO 2018b). Resistance to antimicrobials used for treatment of human diseases has been reported in foodborne pathogens, such as *S. enterica* isolated from swine (Lopes et al. 2015, McDermott et al. 2016, Cameron-Veas et al. 2018, Wang et al. 2019), highlighting the importance of monitoring the resistance of this pathogen. In this sense, gathering information of antimicrobial resistance profile of bacteria isolated from animals and animal products belongs to the goals of the antimicrobial resistance monitoring and control program launched in Brazil (Brasil 2018).

Therefore, the present study aimed to investigate the frequency of *S. enterica*, *Y. enterocolitica* and *L. monocytogenes* and hygienic-sanitary indicators in pig carcasses slaughtered under the Federal Inspection System in three slaughterhouses in Southern Brazil. This investigation was one of the steps for the development of the scientific opinion on the adoption of a risk-based inspection of pork in the scope of the Federal Meat Inspection System. Additionally, the antimicrobial resistance profile of the isolated pathogens was also investigated.

MATERIALS AND METHODS

Study design. The sample size calculation considered the minimum number of carcass samples needed to determine the prevalence of bacterial pathogens listed as important in pig carcasses. The parameters used to calculate a simple random sample were: infinite population, 95% confidence level, 6% absolute precision and 50% prevalence. A previous study (Corbellini et al. 2016) showed an important effect of the day of sampling on the variation of *Salmonella* prevalence in pig carcasses. The design effect (Deff) of sampling day was estimated in 1.42 in this study (Corbellini et al. 2016). Thus, this inflation factor was applied to correct the number of samples, resulting in a total of 378 carcasses to be analyzed. The Brazilian swine production system includes producers integrated into large agro-industries, producers who participate in a cooperative system and independent farmers who deliver finished animals to slaughterhouses. In order to achieve representativeness of the sample, the total number of carcasses was divided equally (n=126) among three slaughterhouses (A, B, C), which represent the aforementioned three modes of swine production. Slaughterhouse A was part of a cooperative system; approximately 4,600 pigs were slaughtered daily. In Slaughterhouse B, a similar number of pigs (4,200/day) were slaughtered, which were delivered by farmers engaged in an integration system with the company. The Slaughterhouse C received pigs from independent farmers, and slaughtered 900 pigs daily. In general, the moderate variation between clusters (sampling days) implies that the addition of sample units on a given day will not add much to the sample's representativeness, being more important to distribute the sample units in as many days as possible. Thus, six weekly sampling events were carried out in each of the slaughterhouses, with 21 carcasses sampled per event. Sampling was always conducted in the morning shift, with the first carcass sampled being that corresponding to the first slaughtered animal. The other samples were collected systematically in an interval of 35 carcasses.

Sample collection and analysis. Carcasses were sampled at the pre-chill step using individual sterile abrasive sponges (NASCO®) rubbed in an area of 100cm^2 from each: loin, jowl, ham and belly (Brasil 2007). The four sponges were pooled in sterile plastic bags and constituted the carcass sample, which was kept refrigerated until processing. To each carcass sample, 100mL of 1% Buffered Peptone Water (BPW 1%) were added; the suspension was then homogenized and used for the enumeration of hygienic indicators: total aerobic mesophiles (TAM) and Enterobacteriaceae; and detection of pathogens: *Salmonella enterica*, *Yersinia enterocolitica* and *Listeria monocytogenes*.

The enumeration of TAM and Enterobacteriaceae was performed in duplicate, in Petrifilm™ Aerobic Count Plates (3M Company) and Petrifilm™ Enterobacteriaceae Count Plates (3M Company), respectively, following the manufacturer's recommendations. After incubation at 37ºC for 48h, typical colonies were counted and the result was multiplied by 0.25 to achieve the number of colony forming units (CFU) per cm^2 of carcass surface.

S. enterica was investigated following the ISO 6579:2002 Amendment 1:2007 protocol (ISO 2007), using Xylose-Lysine-Deoxycholate agar (Merck) and Brilliant Green Phenol Red Lactose Sucrose agar (Merck) for the selective differential isolation step. Typical colonies were confirmed by their biochemical profile and agglutination with somatic polyvalent serum (Probac). The isolates confirmed as *S. enterica* were sent for serotyping at the "Fundação Instituto Oswaldo Cruz" (Fiocruz).

The detection of *Y. enterocolitica* was performed according to the methodology of the American Public Health Association (Weagant & Feng 2001). Briefly, 1mL of the sample suspension was added to 9mL of Peptone Sorbitol Bile broth (PSB, Himedia) and incubated for 10 days at 10°C. After this period, 10µL of the culture was transferred to 100µL of either 0.5% potassium hydroxide (KOH) or NaCl 0.5% solutions. After homogenization for five seconds, an aliquot was immediately transferred to Cefsulodin-Irgasan-Novobiocin agar (Fluka) and to MacConkey agar (Oxoid) plates, and incubated at 30°C for 24h. Typical colonies were subjected to identification by Matrix-assisted Laser Desorption Ionization Time-of-flight (MALDI-TOFF).

L. monocytogenes was investigated according to the IN62 (Brasil 2003) protocol. An aliquot of 1mL of the sample suspension was added to 9mL of Enrichment Broth for Listeria (UVM). After incubation at 30±1°C for 24h, an aliquot (100µL) was transferred to 9.9mL of Fraser broth (Merck) and incubated at 30±1°C for 24h. Afterwards, aliquots were transferred to Tryptose agar with Nalidixic Acid, Palcam agar (Oxoid) and Chromocult© Listeria Selective Agar acc. to Agosti and Otttaviani (ALOA, Merck). After incubation at 30°C±1°C for 48h, typical colonies were identified by biochemical tests and CAMP test (Silva et al. 2010).

Antimicrobial susceptibility testing. *S. enterica* isolates were tested for antimicrobial susceptibility against eleven different antimicrobials. The agar disc diffusion method was performed and evaluated according to the specifications of the Clinical and Laboratory Standards Institute (CLSI) documents VET01-S3 and M100-S26 (CLSI 2015, 2016). The following discs (Oxoid) were used: ampicillin (10µg); azithromycin (15µg); cefotaxime (30µg); ceftazidime (30µg); chloramphenicol (30µg); ciprofloxacin (5µg); gentamicin (10µg), meropenem (10µg); nalidixic acid (30µg); tetracycline (30µg); trimethoprim (5µg). *Escherichia coli* ATCC© 25922 was used as a reference strain for quality control purposes. Antimicrobial multi-resistance was defined as resistance to three or more classes of antimicrobials (CLSI 2016).

Data analysis. The proportions of slaughterhouses in the quantiles of log CFU of mesophilic and Enterobacteriaceae counts, box-plot with the distribution of these indicators and Kruskal-Wallis test to assess if there are differences in the median counts of these indicators between slaughterhouses were made with the function *Desc* of the package *DescTools* (Signorell 2020) in R environment. The prevalence of *Salmonella* in each slaughterhouse was estimated using the package *survey* (Lumley 2020) in R environment.

An intercept-only multilevel logistic model that predicts the prevalence conditional to slaughterhouse and day of sampling was made in which the random effect was the day nested within slaughterhouse. The predicted conditional prevalence for each day of sampling within slaughterhouse was extracted from the model estimates. The model dimension has 18 subjects (i.e. the day of sampling, six in each selected slaughterhouse) with 21 observations per subject (21 carcasses in each day). The intra cluster correlation (ICC) was given by:

$$ICC = \frac{\sigma_0^2}{\sigma_0^2 + \frac{\pi^2}{3}}$$

Where σ_0^2 is the between-cluster variance (i.e., the variance between days nested within slaughterhouse) and $\left(\sigma_0^2 + \frac{\pi^2}{3}\right)$ is the total variance.

The same model structure was used to check the effect of Enterobacteriaceae counts (log CFU.cm^{-1}) on the *Salmonella* isolation, and the ICC was calculated using the same formula described above. This model estimates the average effect of Enterobacteriaceae (fixed-effect) as well as its effect conditional to the day of sampling and slaughterhouse, i.e., the model generates 18 estimates for each day of sampling nested within slaughterhouse (random-effects).

The multilevel logistic models were analyzed with SAS Studio using the procedure PROC GLIMMIX. A significance level of 0.05 was considered for all statistical tests.

The antimicrobial susceptibility testing was analyzed using Fisher's exact test to identify its association with serotype and slaughterhouse. The procedure PROC FREQ from SAS 9.3 (SAS 2012) was used to do this evaluation.

RESULTS

The distributions of log CFU of TAM and Enterobacteriaceae counts and the proportion of slaughterhouse in the quantiles of log counts are demonstrated in Figure 1 and 2. There were significantly statistical differences ($p<0.0001$) on the median counts of both indicators between the slaughterhouses. The median of TAM was very close for Slaughterhouses A and B: 1.573 log CFU.cm^{-1} and 1.6014 log CFU.cm^{-1}, respectively. While in Slaughterhouse C, a higher TAM median was detected (2.216 log CFU.cm^{-1}). A similar profile was observed regarding to Enterobacteriaceae, and medians were calculated as follow: -0.426 log CFU.cm^{-1} in Slaughterhouse A; 0.2163 log CFU.cm^{-1} in B; and 0.633 log CFU.cm^{-1} in C. For both hygienic indicators, in Slaughterhouse C the collected data were concentrated in the fourth and fifth quantiles, while in A and B there was a concentration in the first and second quantiles.

Regarding the pathogens investigated, *Listeria monocytogenes* was not detected and only one carcass from Slaughterhouse C was positive for *Yersinia enterocolitica*. A total of 65 (17.2%) carcasses were positive for *Salmonella enterica*,

with a difference in frequencies between slaughterhouses and slaughter days. The prevalence of *Salmonella* positive carcasses was higher in the Slaughterhouse C (25.4%; CI 95% 19-32%) in comparison with A (9.5%; CI 95% 9-14%) and B (18.3%; CI 95% 12-24%). Only in Slaughterhouse C was *S. enterica* detected in all slaughter days.

The *Salmonella* prevalence conditional to the day of sampling and slaughterhouse estimated by the model was 17.7% and the between-cluster variance estimates was 1.67. The resulted ICC was 33.6% [1.67/(1.67+9.9/3)], which means the between-cluster (day of slaughtering nested within the slaughterhouse) variability in the prevalence of *Salmonella* on the carcasses. Table 1 depicts the predicted and observed prevalence in each day of sampling and model estimates. It is possible to observe a marked variation of the prevalence among days.

There was no significantly statistical association between Enterobacteriaceae counts and *Salmonella* isolation on carcass surface (p=0.69, Table 2). The between-cluster variance estimates was 1.50, resulting in an ICC value of 31.3% [1.50/(1.50+9.9/3)], which demonstrate the between-cluster (day of sampling nested within slaughterhouse) variability in the effect of Enterobacteriaceae counts on the prevalence of *Salmonella*. There were three statistically significant days nested within slaughterhouses (random intercepts), which are demonstrated in Table 2. These results mean that in these days the probability of isolation of *Salmonella* was significantly different (higher) than the average probability of *Salmonella* isolation given the Enterobacteriaceae count.

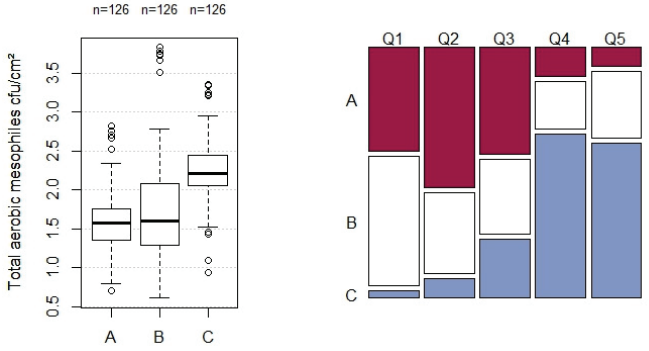

Fig.1. Box plot and quantile distribution (Q1-Q5) of total aerobic mesophiles counts on pig pre-chill carcasses in three slaughterhouses (A, B, C).

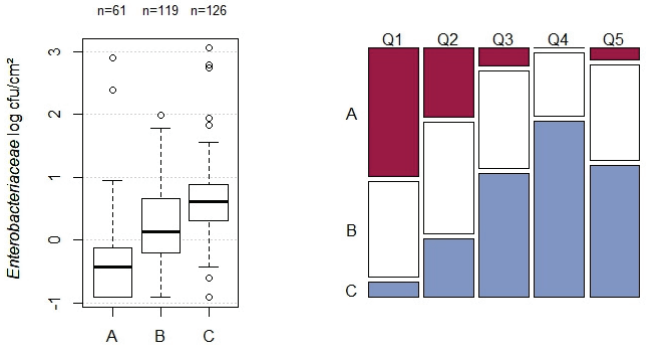

Fig.2. Box plot and quantile distribution (Q1-Q5) of Enterobacteriaceae (II) counts on pig pre-chill carcasses in three slaughterhouses (A, B, C).

Table 1. Observed *Salmonella* prevalence and predicted prevalence estimated by the model on pig pre-chill carcasses in each day of sampling conducted in three slaughterhouses in southern Brazil

Model effect	Slaughterhouse	Day of sampling	Model estimates			Observed data	
			Estimate	SE	Prevalence	No. *Salmonella* positive (n=21/day)	Prevalence
Fixed effect intercept	.	.	-1.89	0.35	-	-	-
Random effect intercept	A	1	-1.34	0.88	3.8%	0	0.0%
	A	2	-1.34	0.88	3.8%	0	0.0%
	A	3	-0.27	0.68	10.3%	2	9.5%
	A	4	-0.27	0.68	10.3%	2	9.5%
	A	5	-0.27	0.68	10.3%	2	9.5%
	A	6	0.85	0.56	26.1%	6	28.6%
	B	1	-1.34	0.88	3.8%	0	0.0%
	B	2	-1.34	0.88	3.8%	0	0.0%
	B	3	0.37	0.60	18.0%	4	19.0%
	B	4	-0.27	0.68	10.3%	2	9.5%
	B	5	0.37	0.60	18.0%	4	19.0%
	B	6	2.12	0.52	55.8%	13	61.9%
	C	1	2.12	0.52	55.8%	13	61.9%
	C	2	-0.72	0.76	6.8%	1	4.8%
	C	3	-0.27	0.68	10.3%	2	9.5%
	C	4	0.08	0.63	14.1%	3	14.3%
	C	5	1.78	0.52	47.3%	11	52.4%
	C	6	-0.27	0.68	10.3%	2	9.5%
Average prevalence					17.7%		17.7%

SE = Standard error.

Among the 63 isolates of *S. enterica* subjected to serotyping, *S.* Typhimurium (38.1%) was the most prevalent, followed by *S.* Infantis (30.1%). The profile of serovars found varied between slaughterhouses, with *S.* Typhimurium being significantly more prevalent (P<0.0001) in Slaughterhouses A and B than in C (Table 3). In Slaughterhouse C, *S.* Infantis was the most prevalent serovar.

Among the 61 *Salmonella* strains tested for resistance to antimicrobials, 18 (31.6%) were full-susceptible (Table 4). No strain displayed resistance to azithromycin, ceftazidime, cefotaxime and meropenem. The highest resistance frequency was displayed to tetracycline (54.1%), followed by ampicillin (50.82%), nalidixic acid (42.62%) and chloramphenicol (42.62). Multi-resistance was detected in 52.54% of the strains; in most cases (62.5%) the MDR profile included tetracycline, ampicillin and chloramphenicol. The association between the serovars with the resistance frequency demonstrated that *S.* Typhimurium presented significantly more resistance (P<0.05) to almost all antimicrobials than the other serovars. Therefore, the greater antimicrobial resistance frequencies were observed in slaughterhouses, in which *S*. Typhimurium was isolated.

DISCUSSION

Human salmonellosis is mainly attributed to food transmission (Scallan et al. 2011), reinforcing the need for control in food animals and their products. The high frequency of *Salmonella enterica* in pre-chill carcasses evidenced here supports the prioritizing of this hazard among those transmitted to human by unprocessed pork, which was pointed out in the risk assessment conducted by Costa et al. (2020). On the contrary and also corroborating the risk assessment results, the other two investigated bacterial hazards proved to have a very low frequency.

Table 2. Association between Enterobacteriaceae counts and *Salmonella* isolation on pig pre-chill carcasses in three slaughterhouses in southern Brazil

Model structure			Model results for Enterobacteriaceae count			Observed data			
Effects	Subjects[a]		Solutions			*Salmonella*	Enterobacteriaceae		
	Abattoir	Day of sample	Estimate	SE	*p*-value	Prevalence	Mean	Min	Max
Enterobacteriaceae count	All	All	0.10	0.25	0.69	17.7%[b]	0.28[b]	-0.9[b]	3.06[b]
Random intercepts	B	6	1.86	0.52	0.0004	61.9%	0.47	-0.90	1.13
	C	1	1.96	0.51	0.0002	61.9%	0.25	-0.90	1.94
	C	5	1.57	0.53	0.0032	52.4%	0.79	-0.43	1.54

[a] There are 18 subjects (sampling event), only three were statistically demonstrated; [b] prevalence, mean, and min-max of all data points in three slaughterhouses; SE = standard error.

Table 3. Distribution of *Salmonella enterica* serovars isolated from pig carcasses in three southern Brazilian slaughterhouses

Serovar	Slaughterhouses			Total (%)
	A	B	C	
Typhimurium	8	16	-	24 (38.1%)
Infantis	-	-	19	19 (30.1%)
Mbandaka	-	-	7	7 (11.1%)
Panama	3	1	-	4 (6.35%)
Hadar	-	-	4	4 (6.35%)
London	1	1	-	2 (3.2%)
Saintpaul	-	-	1	1 (1.6%)
S. O:4,5:-:1,2	-	-	1	1 (1.6%)
Rugose morphotype	-	-	1	1 (1.6%)
TOTAL	12	18	33	63

Table 4. Percentage distribution of antimicrobial resistance to different antimicrobials in *Salmonella enterica* serovars isolated from pig carcasses in three slaughterhouses of southern Brazil

Antimicrobials	*S.* Infantis (n=17)	*S.* Typhimurium (n=24)	Other serovars (n=20)	Fisher's probability (P)	Total (n=61)
Ampicillin	47.1[a]	83.3[b]	15.0[a]	<0.0001	54.4
Ciprofloxacin	0[a]	4.2[b]	0[a]	0.0065	1.75
Chloramphenicol	35.3[a]	75.0[b]	10.0[a]	<0.0001	45.6
Gentamicin	0[a]	54.2[b]	15.0[a]	<0.0001	28.1
Nalidixic acid	17.6[a]	83.3[b]	15.0[a]	0.0010	45.6
Tetracycline	35.3[a]	83.3[b]	35.0[a]	0.0010	57.9
Trimethoprim	35.3	12.5	20.0	0.2557	22.8
Full susceptible	41.2[a]	4.2[b]	55.0[a]	0.0003	31.2
Multi-drug resistant	41.2[b]	83.3[a]	25.0[b]	0.0003	52.5

[a,b] = Different letters in a same line differ significantly by Fisher's exact test (P≤0.05).

Regarding *Listeria monocytogenes* isolation, despite none carcass has being positive, the study design used here allow us only to conclude that the prevalence in carcasses is low, i.e. below the detection power. Although frequencies above 30% have already been reported in pig carcasses in Brazil (Ferronato et al. 2012, Pissetti et al. 2012), in both studies, the high frequency of isolation was concentrated in just one slaughterhouse. The high adaptation of *L. monocytogenes* to moist and low temperature, which characterize the slaughterhouse environment, corroborate with the hypothesis that high frequencies in pork may be associated with poor hygiene and biofilm formation on surfaces in contact with the carcasses. Moreover, *L. monocytogenes* outbreaks are more often vehiculated by ready-to-eat products than by unprocessed pork, which is usually subjected to heat treatment before consumption (Valk et al. 2001, Awofisayo-Okuyelu et al. 2016). Therefore, monitoring programs of *L. monocytogenes* targeted to processed pork products are usually the strategy adopted by the sanitary authorities (Brasil 2009, EFSA 2018) followed by a thorough investigation of the production process in cases of detection of contaminated products.

Although *Yersinia enterocolitica* has been recognized as cause of foodborne disease cases in humans, particularly associated with eating raw or undercooked pork in European countries (Huovinen et al. 2010, Rosner et al. 2011), only one carcass resulted positive in this study. Pigs have shown to be a major reservoir of pathogenic *Y. enterocolitica*, particularly strains of bioserotype 4/O:3 (Fredriksson-Ahomaa et al. 2007). A similar scenario was reported in Brazil, and *Y. enterocolitica* 4/O:3 was found in 30% of tongues and tonsils from pigs at slaughter, while none of 192 samples of pork resulted positive (Paixão et al. 2012). These results corroborate to the low priority of investigation of this pathogen in a monitoring program of pig carcasses in Brazil. Similarly, *Y. enterocolitica* was not classified as of high risk in the scientific opinion on biological hazards transmitted by pork published in the European Union (EFSA 2011). The high frequency reported in tongue and tonsils highlights the hazard represented by the tissues associated with the head regarding the cross contamination during the pluck set removal (Borch et al. 1996). Moreover, edible tissues in the head area, such as tongue and cheek meat, are often commercialized or included in processed products. In this sense, post-fabrication interventions to be applied in the trimmings may be considered and further evaluated in future studies.

The frequency of *S. enterica* positive carcasses corroborates the prevalence in previous studies conducted in Brazil, which varied from 9.8 to 24% (Kich et al. 2011, Silva et al. 2012, Corbellini et al. 2016, Brasileiro et al. 2017). As previously reported (Corbellini et al. 2016), there is a high variation in the frequencies among slaughterhouses and slaughter days. The model constructed considering these variations estimates prevalence values between 3.8% and 55.8%, with an average prevalence similar to the observed. The day of slaughter nested within the slaughterhouse represents 31.3% of the whole variation in the *Salmonella* prevalence, indicating that several factors in a slaughter day may influence the number of positive carcasses. The *Salmonella* prevalence variation may be associated to failures in the slaughter process, which should be monitored by the profile of hygienic indicators over a time period. Actually, in our study, the median of TAM and Enterobacteriaceae was significantly higher in Slaughterhouse C, which presented also the highest *Salmonella* prevalence in the pre-chill carcasses. Therefore, data of monitoring of hygienic indicators may be of outmost importance in auto-control quality programs as well as for official auditing of slaughterhouses. Deviations in the hygienic indicators should be interpreted as an enhancement of the hazard of carcass contamination with pathogens, which may be present in the slaughtered pig or in the environment. However, there is no strict correlation between the hygienic indicator counts in a given carcass or in a given slaughter day with the isolation of *Salmonella* in pre-chill carcasses. Although, in a few cases the *Salmonella* prevalence was associated with higher Enterobacteriaceae counts in our study (Table 2), in general this association was not supported by the statistical analysis. This fact highlights that, even in slaughterhouses with a controlled hygienic process, *Salmonella* can present a higher prevalence in pre-chill carcasses in some days. Therefore, the monitoring programs of *Salmonella* in carcasses cannot be discontinued in auto-control programs and official audits, even in slaughterhouses with a good hygienic process. Another important aspect to be considered is the delivery of slaughter batches with high number of *Salmonella* carrier pigs or the presence of high shedders among the slaughtered pigs, since these factors have been pointed out as leading to enhancement of carcass contamination probability (Duggan et al. 2010, Silva et al. 2012, Kerouanton et al. 2019, Paim et al. 2019). In this case, other approaches targeted to the pre-harvest stage are needed, in order to diminish the pressure in the slaughtering process represented by a high number of *Salmonella* carrier pigs.

The characterization of *S. enterica* isolated in the three slaughterhouses demonstrated a significant difference among the serovars found. While *S.* Typhimurium predominated in Slaughterhouses A and B, in C this serovar was not found and most strains were identified as *S.* Infantis. The prevalence of *Salmonella* serovars often varies among production systems and over time (Denis et al. 2013, Colello et al. 2018). This fact reflects the multiple sources of *Salmonella,* which includes, associated or not, carrier animals, contaminated feed and environment (Funk & Gebreyes 2004, Kich et al. 2005). Still, *S.* Typhimurium is often reported as the most prevalent serovar in pig and pork (Kich et al. 2011, Silva et al. 2012, Campos et al. 2019, Paim et al. 2019). Moreover, this serovar is also frequently reported in human cases and outbreaks associated with pork consumption (Campos et al. 2019). While *S*. Infantis does not figure among the most prevalent serovars identified in swine, human cases present frequently serious symptoms indicating that this serovar may be highly virulent (Almeida et al. 2013). Therefore, the serovars identified in our study highlights the need of *Salmonella* control for the consumer's health.

An additional growing concern in public health has been the antimicrobial resistance presented by pathogenic or non-pathogenic bacteria colonizing food animals. The hazard of transmission of these resistant bacteria to human has been considered as a factor that may contribute to failures of antibiotic treatments in human patients. Foodborne pathogens are of special relevance, since antimicrobial resistance represents an additional hazard for patients with low immunity, which will need antibiotic therapy. In this sense, a marked difference

was observed in the resistance level of *S*. Typhimurium compared to the other serovars. Considering the first choice for antibiotic treatment of salmonellosis in humans, resistance to ciprofloxacin was displayed by 4.2% of the *S*. Typhimurium strains, while all strains of other serovars were susceptible to this drug. Moreover, 83.3% of the *S*. Typhimurium strains were resistant to nalidixic acid, which is often the first step for full resistance to fluorquinolones (Oteo et al. 2000). These results corroborate the recommendation of phasing out the use of antimicrobials highly important to human medicine in animal production (WHO 2018b). The resistance profile in general demonstrated that *S*. Typhimurium was significantly more resistant to most tested antimicrobials and presented more MDR strains in comparison to the other serovars. *S*. Typhimurium is reported as a serovar which often presents multiresistance and, therefore, besides its pathogenic potential may also carry a plethora of resistance genes (Almeida et al. 2018, McMillan et al. 2019). In recent years, genes codifying extended spectrum beta-lactamases (ESBL) or carbapenemases as well as resistance to macrolides have been emerged as a particular concern (WHO 2018b). In this regard, all tested strains were susceptible to these antimicrobial groups in agreement with other reports of very low frequency or absence of resistance to these drugs in *Salmonella* originated from the Brazilian swine production chain (Bersot et al. 2019, Monte et al. 2019, Viana et al. 2019). On the contrary, antimicrobials such as ampicillin, tetracycline and nalidixic acid have been often reported among the group of highest levels of resistance in food borne pathogens (Lopes et al. 2015, Almeida et al. 2018, Bersot et al. 2019, Monte et al. 2019, Viana et al. 2019). Those drugs belong to antimicrobial classes broadly used in pig production in Brazil (Dutra 2017), which justify the high level of resistance found.

CONCLUSIONS

Salmonella enterica is more prevalent in pre-chill pig carcasses than *Yersinia enterocolitica* and *Listeria monocytogenes* and thus should be prioritized in monitoring and control programs at slaughter.

Salmonella serovars varied among slaughterhouses and present significant differences in their resistance to antimicrobials.

Slaughterhouses that present higher medians of total aerobic mesophiles or Enterobacteriaceae in a monitoring period may have higher *S. enterica* prevalences as well. However, there is a high variation of *S. enterica* prevalence among slaughter days, which cannot be always related to the hygienic indicators counts observed on a given day.

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