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Faculdade de Agronomia Eliseu Maciel
Departamento de Ciência e Tecnologia Agroindustrial
Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos

TESE

Diversidade genética e determinantes de virulência e de resistência a antimicrobianos e sanitizantes em isolados de *Salmonella* spp. provenientes de carcaças bovinas e produtos cárneos

Letícia Klein Scheik

Pelotas, 2023

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Orientador: Prof. Dr. Wladimir Padilha da Silva
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Tese aprovada, como requisito parcial para obtenção do grau de Doutora em Ciência a Tecnologia de Alimentos, Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas.

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“São as nossas escolhas, mais do que as nossas capacidades,
que mostram quem realmente somos.”

J. K. Rowling

Resumo

SCHEIK, Letícia Klein. **Diversidade genética e determinantes de virulência e de resistência a antimicrobianos e sanitizantes em isolados de *Salmonella* spp. provenientes de carcaças bovinas e produtos cárneos.** Orientador: Wladimir Padilha da Silva. 2023. 160 f. Tese (Doutorado em Ciência e Tecnologia de Alimentos) – Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, 2023.

Salmonella spp., a bactéria causadora da salmonelose, causa milhares de surtos dessa Doença Transmitida por Alimentos por ano no mundo. O tratamento de casos severos da infecção pode dar-se com o uso de antimicrobianos, aos quais a *Salmonella* pode adquirir resistência, bem como podem tornar-se tolerantes aos sanitizantes frequentemente aplicados na indústria de alimentos, fazendo com que a bactéria seja uma fonte persistente de contaminação no ambiente de produção. O objetivo dos manuscritos 1 e 2 deste estudo foi caracterizar 55 isolados de *Salmonella* spp. provenientes de carcaças bovinas e produtos cárneos da cidade de Pelotas, quanto aos seus perfis fenotípicos e genotípicos de virulência e de resistência a antimicrobianos e a sanitizantes, bem como avaliar a diversidade genética entre os isolados. Todos os isolados apresentaram pelo menos um dos genes de virulência avaliados no estudo. A resistência à tetraciclina e à ampicilina foram as mais prevalentes, e o perfil fenotípico de multirresistência a antimicrobianos foi observado em mais da metade dos isolados (56,4%), dos quais 32,2% pertenciam ao sorovar Typhimurium, e 96,8% desses isolados multirresistentes são provenientes de produtos cárneos. Onze genes de resistência a antimicrobianos foram detectados em 31 isolados de *Salmonella* spp., entre os quais os genes *tet(A)* e bla_{TEM} foram os mais prevalentes. Plasmídeos portadores de genes de resistência antimicrobiana foram encontrados em 41,9% dos isolados multirresistentes, sendo o gene *tet(A)* o mais detectado nos plasmídeos. A tolerância aos sanitizantes foi avaliada pela concentração inibitória mínima, apresentando valores de até 128 $\mu g.mL^{-1}$ para o cloreto de benzalcônio e 32 $\mu g.mL^{-1}$ para a clorexidina, sendo os isolados menos tolerantes a esses compostos do que ao ácido peracético e ao

hipoclorito de sódio, que apresentaram concentração inibitória mínima de 2048 $\mu g.mL^{-1}$. Além disso, quase 90% dos isolados que se mostraram tolerantes ao cloreto de benzalcônio foram isolados de produtos cárneos. O gene *qacEΔ1*, que foi o único gene de resistência a sanitizantes detectado nos isolados, foi correlacionado com o gene *int1*, sugerindo que existe uma relação entre a tolerância aos sanitizantes e a resistência antimicrobiana nesses isolados. Foi observada diversidade genética entre os isolados de *Salmonella*, especialmente nos sorovares Typhimurium, Anatum, Heidelberg e Derby. Clonalidade foi observada apenas no sorovar Senftenberg de carcaças bovinas. Já o objetivo do manuscrito 3 deste estudo foi investigar a ocorrência e a diversidade genética de *Salmonella enterica* subsp. *enterica* em linguiças comercializadas no sul do Brasil, bem como avaliar a presença de genes de virulência e determinar os perfis fenotípicos e genotípicos de resistência a antimicrobianos e sanitizantes. A prevalência de *Salmonella* nas amostras de linguiça analisadas foi de 5,5%. Os sorovares predominantes foram *S*. Infantis e *S*. Rissen. A análise por Eletroforese em Gel de Campo Pulsado (PFGE) revelou nove perfis distintos, e alguns deles foram reincidentes no mesmo estabelecimento em datas diferentes. Perfil de multirresistência a antimicrobianos foi observado em 21,4% dos isolados, e as resistências mais frequentes foram à ampicilina, sulfonamida, trimetoprima/sulfametoxazol e trimetoprima. Somente *S*. Schwarzengrund apresentou os genes *tet(B), strA, strB* e *sul2*. O cloreto de benzalcônio e a clorexidina foram mais efetivos que o ácido peracético e o hipoclorito de sódio, apresentando menores valores de concentração inibitória mínima. Os resultados observados neste estudo destacam a importância das boas práticas de fabricação em abatedouros e ambientes de processamento de alimentos, tanto em relação à contaminação cruzada quanto ao uso indevido dos compostos sanitizantes, a fim de reduzir a contaminação com *Salmonella* multirresistentes a antimicrobianos e tolerantes a sanitizantes em carnes e produtos cárneos. Também, seis sorovares de *Salmonella* foram encontrados em linguiças, demonstrando um risco potencial de salmonelose associado ao consumo deste alimento na região sul do Brasil.

Palavras-chave: Alimentos. Boas Práticas de Fabricação. Compostos desinfetantes. Genes de virulência. Multirresistência. PFGE.

Abstract

SCHEIK, Letícia Klein. **Genetic diversity and determinants of virulence and resistance to antimicrobials and sanitizers in *Salmonella* spp. isolates from bovine carcasses and meat products.** Advisor: Wladimir Padilha da Silva. 2023. 160 f. Thesis (Doctorate in Food Science and Technology) – Faculty of Agronomy Eliseu Maciel, Federal University of Pelotas, Pelotas, 2023.

Salmonella spp., bacterium that causes salmonellosis, is responsible for thousands of outbreaks of this Foodborne Disease each year worldwide. The treatment of severe cases of the infection can occur with the use of antimicrobials, to which *Salmonella* can acquire resistance, as well as they can become tolerant to sanitizers often applied in the food industry, making the bacteria a persistent source contamination in the production environment. The aim of manuscripts 1 and 2 of this study was to characterize 55 *Salmonella* spp. from beef and meat products from the city of Pelotas, regarding their phenotypic and genotypic profiles of virulence and resistance to antimicrobials and sanitizers, as well as to evaluate the genetic diversity among the isolates. All isolates presented at least one of the virulence genes evaluated in the study. Resistance to tetracycline and ampicillin were the most prevalent, and the phenotypic profile of multidrug resistance to antimicrobials was observed in more than half of the isolates (56.4%), of which 32.2% remained with serovar Typhimurium, and 96.8% of these multiresistant isolates were from meat products. Eleven antimicrobial resistance genes were detected in 31 *Salmonella* spp. isolates, among which the *tet(A)* and *blaTEM* genes were the most prevalent. Plasmids carrying antimicrobial resistance genes were found in 41.9% of the multiresistant isolates, with the *tet(A)* gene being the most detected in the plasmids. Tolerance to sanitizers was evaluated by the minimum inhibitory concentration, showing values of up to 128 $\mu g.mL^{-1}$ for benzalkonium chloride and 32 $\mu g.mL^{-1}$ for chlorhexidine, with the isolates being less tolerant to these compounds than to peracetic acid and sodium hypochlorite, which presented a minimum inhibitory concentration of 2048 µg.mL-1. Furthermore, nearly 90% of the isolates found to be benzalkonium chloride tolerant were isolated from meat products. The *qacEΔ1* gene,

which was the only sanitizer resistance gene detected in the isolates, is linked with the *int1* gene, suggesting that there is a relationship between sanitizer tolerance and antimicrobial resistance in these isolates. Genetic diversity was observed among *Salmonella* isolates, especially in serovars Typhimurium, Anatum, Heidelberg and Derby. Clonality was observed only in serovar Senftenberg from bovine carcasses. The objective of manuscript 3 of this study was to investigate the occurrence and genetic diversity of *Salmonella enterica* subsp. *enterica* in sausages sold in southern Brazil, as well as to evaluate the presence of virulence genes and determine the phenotypic and genotypic profiles of resistance to antimicrobials and sanitizers. The prevalence of *Salmonella* in the analyzed sausage samples was 5.5%. The predominant serovars were *S*. Infantis and *S*. Rissen. Analysis by Pulsed Field Gel Electrophoresis (PFGE) revealed nine different profiles, and some of them were repeat offenders in the same establishment on different dates. A profile of multidrug resistance to antimicrobials was observed in 21.4% of the isolates, and the most frequent resistances were to ampicillin, sulfonamide, trimethoprim/sulfamethoxazole and trimethoprim. Only *S*. Schwarzengrund presented the *tet(B), strA, strB* and *sul2* genes. Benzalkonium chloride and chlorhexidine were more effective than peracetic acid and sodium hypochlorite, with lower minimum inhibitory concentration values. The results observed in this study highlight the importance of good manufacturing practices in slaughterhouses and food processing environments, both in relation to cross-contamination and the misuse of sanitizing compounds, in order to reduce contamination with *Salmonella* that are multirresistant to antimicrobials and tolerant to sanitizers in meat and meat products. Also, six *Salmonella* serovars were found in sausages, demonstrating a potential risk of salmonellosis associated with the consumption of this food in southern Brazil.

Key-words: Food. Good Manufacturing Practices. Disinfectant compounds. Virulence genes. Multidrug-resistance. PFGE.

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Lista de Abreviaturas e Siglas

AMC	Amoxicilina+ácido clavulânico/amoxicillin+clavulanate
AMP	Ampicilina/ampicillin
AP	Ácido Peracético
ATCC	American Type Culture Collection
BKC	Benzalkonium chloride
BPF	Boas Práticas de Fabricação
^{o}C	graus Celsius
CB	Cloreto de Benzalcônio
CDC	Centers for Disease Control and Prevention
CFL	Cefalotina/cephalothin
CFU	Colony-forming unit
CHL	Cloranfenicol/chloramphenicol
CIM	Concentração Inibitória Mínima
CIP	Ciprofloxacina/ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CLX	Clorexidina/chlorhexidine
CQA	Compostos Quaternários de Amônio
CSB	Cell suspension buffer
CTX	Cefotaxima/cefotaxime
DNA	Deoxyribonucleic acid
DTHA	Doença de Transmissão Hídrica e Alimentar
ECDC	European Centre for Disease Prevention and Control

EDTA	Ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
EUA	Estados Unidos da América
FDA	Food and Drug Administration
Fig	Figure
g	gramas/grams
GEN	Gentamicina/gentamicina
GMP	Good Manufacturing Practices
h	horas/hours
HS	Hipoclorito de Sódio
IMP	Imipenem
IN	Instrução Normativa
Kb	Kilobase
LB	Luria Bertani
LPS	Lipopolissacarídeo
MDR	Multidrug-resistance
mg.L	miligramas por litro
MIC	Minimum inhibitory concentration
min	minutes
µg.mL	microgramas por mililitro
µL	microlitros
NAL	Ácido nalidíxico/nalidixic acid
NARMS	National Antimicrobial Resistance Monitoring System
ng	nanogramas

OMS	Organização Mundial da Saúde
PAC	Peracetic acid
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
QAC	Quaternary ammonium compounds
QRDR	Quinolone resistance determining regions
RDC	Regime Diferenciado de Contratações
s	seconds
SH	Sodium hypochlorite
SS	Saline solution
SST3	Sistema de Secreção Tipo 3
STR	Estreptomicina/streptomycin
SUL	Sulfonamida/sulfonamide
SUT	Sulfametoxazol+trimetoprima/sulfamethoxazole/trimethoprim
TET	Tetraciclina/tetracycline
TOB	Tobramicina/tobramycin
TSA	Tryptic soy agar
TRI	Trimetoprima/trimethoprim
U	Units
UE	União Europeia
UPW	Ultrapure Water
USA	United States of America
V	volts
W	wats

WHO	World Health Organization
w/p	weight/volume

Sumário

1 Introdução

Segundo a Organização Mundial da Saúde (OMS, 2018), *Salmonella* spp. está entre os quatro agentes etiológicos que mais causam doenças diarreicas no mundo, e o terceiro micro-organismo mais envolvido em surtos de Doenças de Transmissão Hídrica e Alimentar (DTHA) no Brasil (BRASIL, 2021). É uma bactéria capaz de colonizar o trato intestinal de mamíferos, répteis e aves (HOELZER; SWITT; WIEDMANN, 2011; BARILLI et al., 2018), sendo o consumo das carnes de aves, bovinos e suínos contaminadas com o patógeno, bem como de seus alimentos derivados com cocção insuficiente, considerado a principal via de transmissão de *Salmonella* spp. (BARILLI et al., 2018).

A salmonelose caracteriza-se, geralmente, como uma doença autolimitante, com os sintomas cessando entre cinco e sete dias após o início da infecção, porém, indivíduos pertencentes aos grupos de risco tendem a desenvolver uma infecção mais severa (CDC, 2022). Para desenvolver a patogênese, *Salmonella* spp. possui uma série de genes (por exemplo, o gene *pefA* que codifica para a fímbria Pef), que são necessários para estabelecer o processo de infecção no hospedeiro. Os fatores de virulência de *Salmonella* spp. estão diretamente relacionados com esses genes de virulência (VALDEZ; FERREIRA; FINLAY, 2009), sendo assim, a presença destes genes em sorovares de *Salmonella* indica uma maior probabilidade de desenvolvimento da doença nos humanos.

Além de causar a salmonelose, *Salmonella* spp. pode adquirir resistência aos antimicrobianos frequentemente aplicados no tratamento de casos de gastroenterite severa em humanos, bem como pode também apresentar resistência aos antimicrobianos utilizados em animais de produção (THRELFALL, 2002; NAIR; VENKITANARAYANAN; JOHNY, 2018), aumentando a taxa de multirresistência a antimicrobianos em uma população bacteriana. Dessa forma, a resistência bacteriana a antimicrobianos é considerada um problema de saúde pública, já que o tratamento das doenças utilizando antimicrobianos aos quais as bactérias possuem resistência pode tornar-se ineficaz. A resistência pode ser intrínseca, quando a bactéria possui o gene que confere a resistência em seu cromossomo, ou ainda pode ser adquirida, através da transferência de genes de resistência entre bactérias resistentes e bactérias suscetíveis, a partir de elementos genéticos móveis contendo

genes de resistência, como os plasmídeos e *integrons* (VON WINTERSDORFF et al., 2016).

A tolerância a sanitizantes também é considerada um problema, principalmente para a indústria de alimentos. Bactérias tolerantes a sanitizantes persistem na planta de processamento de alimentos, transformando-se em uma fonte contínua de contaminação (MØRETRØ & LANGSRUD, 2017). Além disso, os mecanismos de resistência a sanitizantes podem estar relacionados com os mecanismos de resistência a antimicrobianos (WU et al., 2015) através da localizaçãodos seus genes de resistência - por exemplo, o gene *qacEΔ1,* que confere resistência aos compostos quaternários de amônio, localiza-se no *integron* de classe 1, que também carreia o gene *sul1* que confere resistência às sulfonamidas. Sendo assim, é relevante pesquisar os perfis fenotípicos e genotípicos de resistência a antimicrobianos e a sanitizantes em isolados de *Salmonella* spp. provenientes de carcaças bovinas e de produtos cárneos, com o intuito de auxilar a entender a atual situação do quadro de resistência a antimicrobianos e da tolerância a sanitizantes em diferentes sorovares de *Salmonella* spp. da região sul do Rio Grande do Sul.

2 Revisão da Literatura

2.1 Características do gênero *Salmonella* spp.

O gênero *Salmonella* pertence a família Enterobacteriaceae*,* que apresentam forma de bastonete (ou bacilos) de tamanho que varia entre 0,2-1,5 x 2-5 μm. São Gram-negativas, anaeróbias facultativas, não esporuladas e móveis por flagelos peritríquios, com exceção dos sorovares *S*. Gallinarum e *S*. Pullorum (FÀBREGA & VILA, 2013; GRIMONT & WEILL, 2007; JAJERE, 2019). Este gênero bacteriano é composto por duas espécies, *Salmonella bongori* e *Salmonella enterica,* sendo esta última dividida em outras seis subespécies: *S. enterica* subesp. *enterica, S. enterica* subesp. *salamae, S. enterica* subesp. *arizonae, S. enterica* subesp*.diarizonae, S. enterica* subesp. *indica* e *S. enterica* subesp. *houtenae* (LAMAS et al., 2018).

Além de suas espécies e subespécies, *Salmonella* ainda apresenta mais de 2600 sorovares, sendo a maioria destes, especificamente 1531 sorovares, pertencentes à *S. enterica* subespécie *enterica* (FÀBREGA & VILA, 2013; LAMAS et al., 2018). A classificação de *Salmonella* em sorovares ocorre a partir de um sistema chamado Esquema de Kauffman & White, o qual classifica este gênero baseado em três determinantes antigênicos da bactéria: os seus antígenos somáticos (O), flagelares (H) e capsulares (K) (BRENNER et al., 2000; JAJERE, 2019). O antígeno somático (O) refere-se aos antígenos localizados na membrana celular externa da bactéria, e que formam o lipopolissacarídeo (LPS), de membrana, sendo que um mesmo sorovar de *Salmonella* pode expressar um ou mais antígenos O. Já os antígenos flagelares (H1 e H2) referem-se aos antígenos localizados nos flagelos da bactéria, e que estão envolvidos na ativação da resposta imune do hospedeiro no processo de infecção. A maioria dos sorovares de *Salmonella* possuem dois tipos de proteínas flagelares (H1 e H2). Os antígenos H1 são ligados à resposta imunológica e podem ser expressos por alguns sorovares; já os antígenos H2 são não-específicos e podem ser encontrados na maioria dos sorovares (MCQUISTON et al., 2008). Os antígenos de superfície, também chamados de capsulares (K), são localizados na superfície da cápsula bacteriana e são mais raros. Por exemplo, o antígeno de virulência (Vi), que é um subtipo de antígeno K, é somente encontrado nos sorovares Typhi, Paratyphi C e Dublin (JAJERE, 2019). Todos esses antígenos

são representados em uma fórmula antigênica, sendo que cada fórmula equivale a um sorovar específico.

2.2 Salmonelose

Embora todas as espécies e subespécies de *Salmonella* já tenham sido reportadas como causadora de doença, a maioria das infecções nos humanos são causadas por *S*. *enterica* subesp. *enterica* (LAMAS et al., 2018). Essa subespécie inclui sorovares chamados tifóides e não-tifóides. Os sorovares tifóides são *S.* Typhi e *S.* Paratyphi A, B e C, e infectam exclusivamente humanos, causando febre tifoide e paratifoide, respectivamente, que são infecções muito severas que podem levar à falência múltipla de órgãos, bacteremia e sepse (GAL-MOR, BOYLE & GRASSL, 2014). Os sorovares não-tifóides, como *S.* Typhimurium e *S*. Enteritidis, por exemplo, infectam humanos e animais e estão entre os agentes que mais causam gastroenterite, tendo diarreia como principal sintoma (CDC, 2023).

A manifestação clínica da salmonelose inclui quadros entéricos agudos ou crônicos, sendo seus sintomas a diarreia (que pode apresentar sangue ou não), febre e dores abdominais, e alguns indivíduos podem sentir náuseas, apresentar vômitos e dores de cabeça. Ainda há possibilidade de complicações (principalmente em indivíduos do grupo de risco, como idosos, crianças e indivíduos com o sistema imune debilitado): infecções extraintestinais, infecções septicêmicas, osteomielite, artrite e hepatite estão entre as complicações mais reportadas pós-infecção por *Salmonella* (BRASIL, 2011).

A dose infectante de *Salmonella* spp. em humanos pode variar de 10^5 a 10^8 células, mas em pacientes imunocomprometidos já foram observadas doses ≤ 10^3 para alguns sorovares envolvidos em surtos de DTHA (BRASIL, 2011). Além de variar de acordo com o sorovar e com as características de saúde do hospedeiro, a dose infectante pode variar de acordo com a via de transmissão de *Salmonella*, ou seja, com o tipo de alimento contaminado que está sendo consumido (GHARPURE et al. 2021). Por exemplo, em matrizes alimentares com teor de gordura elevado x baixa atividade de água (como a porção de gordura das carnes, toucinho, e produtos fermentados, como por exemplo, os salames), essa combinação de fatores pode proteger a célula de *Salmonella* spp*.* contra as condições ácidas do estômago do

hospedeiro, aumentando a probabilidade da bactéria, mesmo em concentrações mais baixas, de multiplicar-se ao chegar no epitélio intestinal e causar a doença (AVILES et al., 2013).

2.2.1 Dados epidemiológicos

O *Centers for Disease Control and Prevention* (CDC) estima que *Salmonella* spp. cause cerca de 1,35 milhão de infecções, 26.500 hospitalizações e 420 mortes anuais nos Estados Unidos da América (CDC, 2022), sendo considerada entre as quatro principais causas de doenças diarreicas no mundo (OMS, 2018). No Brasil, *Salmonella* spp. foi o terceiro principal agente etiológico identificado em surtos de DTHA entre os anos de 2012 e 2021, e foi responsável por 11,2% dos surtos notificados nesse período (BRASIL, 2022_a), devendo o número real ser significativamente maior, devido à alta taxa de subnotificação de casos no país.

Sendo um patógeno de grande importância em alimentos, a pesquisa de *Salmonella* spp. é obrigatória na maioria dos países. A União Europeia (UE) estabeleceu uma abordagem integrada para controlar *Salmonella* spp. nos países membros. Essa abordagem envolveu diversas autoridades do governo dos Estados-Membros da UE, da Comissão Europeia, do Parlamento Europeu, da Autoridade Europeia para a Segurança Alimentar (EFSA) e do Centro Europeu de Prevenção e Controle de Doenças (ECDC), aplicando legislações que cobrem todas as vias de exposição do patógeno na cadeia de produção de alimentos (EFSA, 2021; EHUWA, JAISWAL & JAISWAL, 2021). Dentre estas regulamentações europeias, fica estabelecido que as indústrias devem apresentar um laudo microbiológico comprovando a ausência da *Salmonella* spp. em diversos alimentos, como carne e produtos de carne e de frango, laticínios e produtos prontos para o consumo (EFSA, 2021). No Brasil, a RDC n^{o} 724/2022, através da IN 161, da Agência Nacional de Vigilância Sanitária (ANVISA), preconiza a ausência de *Salmonella* spp. em 25 g de alimento sólido ou 25 mL de alimento líquido para diversas classes de alimentos (BRASIL, 2022_b). Em 2018, a OMS publicou recomendações para o controle de *Salmonella* spp. e outros patógenos de importância em alimentos. Essas medidas visam fortalecer os padrões de segurança dos alimentos, orientando desde manipuladores até consumidores de alimentos sobre as melhores práticas a serem

adotadas na prevenção da contaminação por *Salmonella* spp. em toda a cadeia de produção de alimentos (OMS, 2018).

2.2.2 Vias de transmissão de *Salmonella* spp.

Salmonella spp. é uma bactéria que pode estar presente no ambiente, altamente adaptável, apresentando uma distribuição ubíqua, tanto em reservatórios geográficos como biológicos. O patógeno pode persistir fora dos animais em ambientes naturais, incluindo águas superficiais doces e marinhas, solo e poeira, além de vegetais (BELL et al., 2015), podendo ser fonte de contaminação em alimentos das mais diversas origens. Entretanto, por tratar-se de uma enterobactéria, e estar naturalmente presente no trato intestinal de animais, principalmente de mamíferos, répteis e aves (BARILLI et al., 2018, BROWN et al., 2021), alimentos de origem animal são os mais relacionados com casos de salmonelose humana (LI et al., 2018).

Alguns sorovares são adaptados aos animais, como *S*. Gallinarum e *S*. Pullorum em aves, podendo desencadear o tifo aviário e a pulorose, respectivamente, que causam sintomas e levam a sepse desses animais (BARROW, FREITAS & NETO, 2011). Entretanto, as aves são assíntomáticas à maioria dos outros sorovares de *Salmonella* spp*.,* e uma vez presente no seu trato intestinal, a bactéria pode contaminar a carne desses animais após o seu abate. Sendo assim, a transmissão de *Salmonella* spp. ao homem pode ocorrer pelo contato com animais infectados e suas fezes, mas principalmente pelo consumo de alimentos contaminados com a bactéria, e as carnes de aves, suínos e bovinos, bem como ovos, são os alimentos mais frequentemente relacionados com a transmissão de *Salmonella* spp. aos humanos (GUT et al., 2018).

Na Europa, os alimentos fora dos padrões para *Salmonella* spp. são geralmente de origem cárnea. Uma das fontes mais relevantes de casos de salmonelose humana na UE é a carne suína, onde *Salmonella* spp. é comumente detectada em vários pontos da cadeia de produção (BONARDI, 2017; CAMPOS et al., 2019; ECDC, 2018). No Brasil, que é o quarto maior produtor mundial de suínos, o consumo de carne suína contaminada com *Salmonella* spp. é considerado uma das principais vias de transmissão deste patógeno, sendo responsável por até 25% dos

casos de salmonelose reportados em humanos no país (ABPA, 2018; KICH et al., 2011; VIOTT et al., 2013).

A região sul do Brasil é a maior produtora de suínos do país, e aproximadamente 70% da carne suína produzida é consumida na forma de produtos cárneos, como as linguiças (MÜRMANN, DOS SANTOS & CARDOSO, 2009). As linguiças de carne suína, e também mistas com carne de frango, são amplamente consumidas na região sul do Brasil, e também são fontes potenciais de transmissão de *Salmonella* spp. aos humanos, tanto por serem produzidas com matérias-primas com alta probabilidade de estarem contaminadas com a bactéria (carne, gordura), como por serem alimentos altamente manipulados, podendo ser contaminados através de colaboradores e também por contaminação cruzada durante o seu processamento (MAMBER et al., 2018). Além disso, a contaminação causada pelo consumo desses alimentos produzidos na região pode criar proporções maiores, já que esses produtos são comercializados em diversas partes do país e também são exportados para outros países.

2.3 Virulência de *Salmonella* spp.

O quadro de salmonelose e outras complicações podem agravar de acordo com o grau de virulência da estirpe de *Salmonella* spp. (CHENG; EADE & WIEDMANN, 2019). A virulência das estirpes de *Salmonella* spp. é multifatorial, incluindo fatores como a motilidade pelos flagelos peritríquios, habilidade de aderir, invadir e multiplicar-se nas células epiteliais, resistência à ação do sistema complemento, entre outros, e esta virulência é mediada através de genes da bactéria que codificam para proteínas envolvidas nesses fatores de virulência. Os principais genes de virulência estão presentes em Ilhas de Patogenicidade de *Salmonella* (SPI). Até o momento, 23 Ilhas SPI já foram descritas, algumas mais elucidadas e conhecidas que outras (DESAI et al., 2013; HAYWARD; JANSEN; WOODWARD, 2013; PAVON et al., 2022), sendo que algumas SPI são conservadas entre sorovares de *Salmonella*. Na SPI-1, a mais estudada até o momento, encontram-se genes como o *hilA* (ativador transcricional que regula o processo de invasão celular), *invA* e *sipA* (codificam para proteínas de adesão e invasão em células epiteliais) e genes que

codificam para sistemas de secreção tipo 3 (SST3) (ALMEIDA et al., 2016; BARILLI et al., 2018).

Em alguns sorovares, a virulência ainda pode ser mediada por um plasmídeo de virulência de *Salmonella*, como o pSPV. Neste plasmídeo há uma região chamada *operon spv,* de 8 kb, que contém os genes *spvRABCD,* relacionados com a sobrevivência e multiplicação da bactéria em macrófagos (RYCHLIK; GREGOROVA & HRADECKA, 2006; GUINEY & FIERER, 2011). A adesão da bactéria à superfície celular do hospedeiro também é essencial para a patogênese da doença, e é mediada através de fímbrias. As fímbrias PEF e SEF14 desempenham importantes papéis na patogênese de *Salmonella* spp. A fímbria PEF é codificada por genes do *operon pef* localizado em um plasmídeo de 90 kb. (RYCHLIK; GREGOROVA; HRADECKA, 2006), e adere-se às vilosidades do intestino, causando acúmulo de fluidos (BÄUMLER et al., 1997). Já a fímbria SEF14 é assim chamada porque foi primeiramente identificada em *S*. Enteritidis, e contribui para a adesão em células epiteliais do hospedeiro (QUAN et al., 2019). As suas subunidades são codificadas por genes do *operon sef,* dentre estes o gene *sefA,* localizados na SPI-10 (MORGAN, 2007).

2.4 Resistência e multirresistência a antimicrobianos

Para alguns casos específicos de infecção por *Salmonella* spp. recomenda-se o tratamento da doença com o uso de antimicrobianos, principalmente em indivíduos que apresentam sintomas mais severos (diarreia intensa, febre alta e persistente ou outras condições que requerem hospitalização), imunocomprometidos (pacientes soropositivos, transplantados, em tratamento oncológico e também indivíduos com mais de 50 anos com problemas cardíacos), além de idosos acima de 65 anos (CDC, 2022). Apesar disso, o uso de antimicrobianos não é recomendado para a maioria dos casos de salmonelose, primeiramente porque em indivíduos saudáveis que apresentam a infecção, o tratamento com essas drogas não diminui o tempo da doença, e nem mesmo abranda os sintomas. Além disso, podem ocorrer outros efeitos adversos, como a possibilidade do indivíduo se tornar portados assintomático prolongado da bactéria e de alteração do seu microbioma. Entretanto, o grande problema envolvendo o uso desnecessário de antimicrobianos é a contribuição para

o aumento da resistência bacteriana aos antimicrobianos nas últimas décadas (CDC, 2022; PULINGAM et al., 2022).

A resistência aos antimicrobianos é definida como a habilidade dos micro-organismos de resistirem à ação destes agentes, e isso ocorre quando um antimicrobiano perde sua capacidade de inibir a multiplicação das bactérias através de mecanismos de resistência desenvolvidos por elas (BECEIRO et al., 2013; NADEEM et al., 2020). A resistência bacteriana a antimicrobianos, que ocorre principalmente por causa do uso indiscriminado dessas substâncias, tanto na terapêutica humana e animal, como de forma profilática na produção animal, vêm aumentando consideravelmente nas últimas décadas (POPOFF; LE MINOR, 2015). Esta questão tornou-se uma das principais ameaças mundiais à saúde pública da atualidade, e a OMS estimou que até o ano de 2050 podem ocorrer cerca de 10 milhões de mortes devido ao aumento da resistência aos antimicrobianos (DE KRAKER; STEWARDSON & HARBARTH, 2016).

As estirpes bacterianas podem tornar-se multirresistentes a antimicrobianos, o que consensualmente é definido como a resistência a três ou mais classes de antimicrobianos (MAGIORAKOS et al., 2012; SWEENEY et al., 2019). A multirresistência a antimicrobianos em *Salmonella* spp. vem sendo cada ano mais reportada em estudos do mundo inteiro (LI et al., 2020; GODÍNEZ-OVIEDO et al., 2020). Apesar deste fato, e também do frequente relato de multirresistência observada em diferentes sorovares envolvidos em surtos (HASSENA et al., 2019; MCDERMOTT; ZHAO & TATE, 2018), poucos países apresentam programas de vigilância para monitorar as taxas de resistência em *Salmonella* spp. na produção de alimentos (MCDERMOTT; ZHAO; TATE, 2018). Nos EUA, o Sistema Nacional de Monitoramento da Resistência Antimicrobiana para Bactérias Entéricas, chamado de NARMS, foi criado em 1996, e tem como principal objetivo rastrear as mudanças nos perfis de suscetibilidade aos antimicrobianos de enterobactérias isoladas em surtos, e também de carnes no varejo e em rações para animais (CDC, 2023). Este sistema é de grande importância para a saúde pública daquele país, pois fornece informações sobre o aumento da resistência bacteriana, bem como as formas pelas quais as resistências aos diferentes antimicrobianos se disseminam e como as infecções causadas por bactérias resistentes diferem das infecções causadas por

estirpes suscetíveis. Sistemas semelhantes existem na UE, Canadá, alguns países da América Latina, Ásia e África, sendo a maioria desses programas focada no monitoramento da suscetibilidade à antimicrobianos de isolados clínicos humanos (MCDERMOTT, ZHAO & TATE, 2018), portanto a maioria dos dados que se tem sobre a situação atual de resistência em *Salmonella* envolvida em surtos alimentares são obtidos através dos estudos realizados com isolados de cada região dos diferentes países.

2.4.1 Uso de antimicrobianos no tratamento de salmonelose

Quando um indivíduo é acometido por uma gastroenterite, o recomendado é que seja diagnosticado qual é o agente etiológico causador da doença, para então realizar um teste de suscetibilidade a antimicrobianos, e proceder com a melhor escolha de tratamento a ser seguida para aquele paciente (CDC, 2019). Entretanto, sabe-se que na prática nem sempre esta recomendação é levada em consideração, por diferentes motivos, como por exemplo, a urgência no tratamento do paciente. No passado, recomendava-se o tratamento de casos de salmonelose em humanos com antimicrobianos como ampicilina, cloranfenicol e sulfametoxazol-trimetoprima. Com isso, em meados dos anos 1980 e início de 1990, começou a disseminação de estirpes de *Salmonella* spp*.* com o perfil penta-resistente ACSSuT (caracterizado pela resistência a ampicilina, cloranfenicol, estreptomicina, sulfametoxazol e tetraciclina) (PARRY, 2003; WANG et al., 2017). O perfil ACSSuT entre isolados de *S*. Typhimurium provenientes de bovinos sofreu uma considerável queda, passando de 67% de ocorrência em 2009 para 7% no ano de 2014 (MCDERMOTT, ZHAO & TATE, 2018). Apesar desta queda na frequência de isolados ACSSuT resistentes na década passada, estudos recentes ainda observam a presença deste perfil de resistência em isolados de *Salmonella* spp. provenientes de amostras de carnes, produtos cárneos e seus ambientes de processamento (MION et al., 2016; NGUYEN THI et al., 2020).

Devido ao aumento da resistência a estes antimicrobianos em estirpes de *Salmonella* spp. nas últimas décadas, antimicrobianos das classes das quinolonas, macrolídeos e cefalosporinas de terceira geração passaram a ser amplamente utilizados (BARILLI et al., 2018). Os antimicrobianos orais de primeira linha mais

comumente administrados atualmente em casos de salmonelose são as fluoroquinolonas, como a ciprofloxacina, para os adultos, e a azitromicina para crianças. Já as cefalosporinas de terceira geração, como a ceftriaxona e a cefotaxima, têm tornado-se alternativas de tratamento da infecção nos últimos tempos (CDC, 2019) Entretanto, a emergência da resistência a esses antimicrobianos de importância clínica também vem sendo reportada ultimamente em isolados de *Salmonella* spp.*,* fato que requer atenção dos órgãos de saúde e deve ser monitorado (CLOTHIER et al., 2018).

Estudos recentes apontam a preocupação com a resistência e a multirresistência a antimicrobianos em sorovares de *Salmonella* spp. provenientes de amostras de carnes e de produtos cárneos. No estudo de PLAWINSKA-CZARNAK et al. (2022), que investigou perfis de multirresistência em isolados de *Salmonella* spp. de produtos de carne *in natura*, um total de 53,84% dos isolados foram classificados como multirresistentes, sendo resistentes a seis classes de antimicrobianos: penicilinas, cefalosporinas, aminoglicosídeos, fluoroquinolonas, sulfonamidas e tetraciclinas. Alguns dos isolados apresentaram resistência às cefalosporinas de terceira geração, que são utilizadas no tratamento de salmonelose em humanos. Além disso, todos os isolados foram resistentes à gentamicina, que é um dos principais antimicrobianos utilizados no tratamento de infecções urinárias em humanos, e foram resistentes à estreptomicina, usada para tratar tuberculose e infecção por *Burkholderia* spp. (PLAWINSKA-CZARNAK et al., 2022). Portanto, essas resistências representam um problema, já que limitam as opções de tratamento para diversas doenças.

2.4.2 Uso de antimicrobianos na terapêutica animal

A crescente tendência de estirpes de *Salmonella* spp. resistentes e multirresistentes a antimicrobianos ocorre também devido ao uso frequente de algumas dessas drogas em sistemas de criação de animais, com o objetivo de controlar, tratar e até mesmo prevenir zoonoses em bovinos, suínos, aves e ovinos (CHUANCHUEN et al., 2010; DOMÉNECH et al., 2015; FEIYANG et al., 2021; VILELA et al., 2019). Estudos das últimas duas décadas demonstram que *Salmonella* spp. isoladas de alimentos, principalmente de origem animal, tornaram-

se altamente resistentes a classes de antimicrobianos que normalmente são usados como terapia de primeira linha de doenças infecciosas em animais (HU et al., 2020). Praticamente um quarto dos isolados (24,64%) apresentaram resistência às tetraciclinas no estudo de PLAWINSKA-CZARNAK et al. (2022), na Polônia, apesar do fato de que, desde 2006, a UE impôs a proibição da utilização não-terapêutica de antimicrobianos, como as tetraciclinas, na produção animal. No Brasil, o Ministério da Agricultura, Pecuária e Abastecimento (MAPA) proibiu, desde 1998, o uso de alguns antimicrobianos, como a tetraciclina e as quinolonas, como aditivos alimentares para rações animais, devido à resistência antimicrobiana. Desde então, o uso destas drogas sofreu uma queda significativa, no entanto, o acesso a esses agentes proibidos ainda é uma realidade do país, e seu uso ilegal ainda ocorre (BRASIL, 1998; MARON, SMITH & NACHMAN, 2013; VILELA et al., 2019), o que se reflete nos perfis de suscetibilidade de isolados no Brasil. LOPES et al. (2015) obtiveram 225 isolados de *Salmonella* spp. a partir de amostras de ração animal, suínos e carcaças suínas de abatedouros da região sul do Brasil, entre os anos de 2008 e 2011, e a taxa de resistência à tetraciclina nesses isolados foi de 54,5%. Mais recentemente, DOS SANTOS BERSOT et al. (2019) encontraram uma taxa de resistência à tetraciclina de 83,8%, em 210 isolados de *Salmonella* spp. provenientes da cadeia de produção suína, também na região sul do país.

Como citado anteriormente, as fluoroquinolonas e as cefalosporinas de terceira geração são atualmente as principais opções de tratamento para casos severos de salmonelose em humanos, e antimicrobianos dessas mesmas classes são empregados na medicina veterinária para o tratamento de doenças em animais de corte. Embora as fluoroquinolonas tenham sido banidas para uso em aves (FDA, 2017), a enrofloxacina ainda é aplicada para tratamento de doenças respiratórias em bovinos e suínos (FDA, 2021). Portanto, o uso frequente destes importantes antimicrobianos, tanto no tratamento de doenças em humanos como em animais, tornaram-se um problema de saúde pública global, uma vez que as opções terapêuticas disponíveis estão se esgotando em consequência do aumento da resistência bacteriana a essas drogas (JAJERE et al., 2019).

2.4.3 Determinantes genéticos de resistência

A resistência bacteriana aos antimicrobianos é causada por proteínas da bactéria que atuam como impedientes da ação bactericida desses agentes, e essas proteínas são codificadas por genes de resistência, que a bactéria pode apresentar intrinsicamente em seu cromossomo, mas também pode adquirir essas resistências através de mutações que ocorrem em seus genes alvo, ou ainda através de elementos genéticos móveis transferidos de outras bactérias, como os plasmídeos (MUNITA & ARIAS, 2016; REYGAERT, 2018).

A ação das tetraciclinas na bactéria ocorre através da sua ligação à subunidade ribossomal bacteriana 30S, inibindo assim a síntese de proteínas, afetando a multiplicação celular. Os principais mecanismos de resistência bacteriana a essa classe antimicrobiana são mediados principalmente por bombas de efluxo. Apesar da grande variedade de genes de resistência *tet* que existem, os que codificam para bombas de efluxo são os mais fortemente relacionados com sorovares de *Salmonella enterica,* principalmente os genes *tet(A)* e *tet(B)* (PEZZELLA et al., 2004; VILELA et al., 2019).

O principal mecanismo bacteriano de resistência a agentes da classe das quinolonas está relacionado a mutações pontuais específicas que ocorrem nos genes *gyrA, gyrB, parC* e *parE,* que codificam para a DNA girase a topoisomerase IV, proteínas-alvo desses antimicrobianos. Estas mutações levam a substituições de aminoácidos dessas proteínas, que mudam sua conformação e resultam em diferentes níveis de resistência na bactéria, tanto às quinolonas quanto para fluoroquinolonas (ALDRED, KERNS & OSHEROFF, 2014; VILELA et al., 2019). Apesar de geralmente a resistência às fluoroquinolonas ocorrer por mutações nesses genes, essa resistência também pode ser mediada por genes presentes em plasmídeos. Na resistência a quinolonas mediada por plasmídeo (RQMP), os genes *qnr* codificam proteínas que bloqueiam a ação da ciprofloxacina na DNA girase e na topoisomerase IV. Outro gene plasmidial, o *aac(6')-Ib-cr*, codifica para uma aminoglicosídeo acetiltransferase que confere suscetibilidade reduzida à ciprofloxacina, enquanto os genes *qepA* e *oqxAB* codificam para bombas de efluxo, que expulsam o composto ativo das fluoroquinolonas para fora da célula (ALDRED, KERNS & OSHEROFF, 2014).

Os genes de resistência presentes em elementos genéticos móveis podem disseminar-se em uma população bacteriana, e essa aquisição de material genético que confere resistência antimicrobiana é possível a partir de vias horizontais de transferência genética, que pode ocorrer por transformação, transdução e/ou conjugação (REYGAERT, 2018). Plasmídeos conjugativos podem transferir diversos genes de resistência entre diferentes espécies bacterianas, e a presença de vários genes de resistência facilita a sobrevivência bacteriana no hospedeiro (ROBERTS & SCHWARZ, 2016). As enzimas responsáveis pela resistência aos antimicrobianos da classe dos β-lactâmicos em *Salmonella* spp. são principalmente as β-lactamases, que são codificadas por genes como o bla_{TEM}, geralmente localizados em plasmídeos (REYGAERT, 2018). Ainda, de acordo com PAVELQUESI et al. (2021), os genes *sul1* e *sul2* são os genes de resistência a sulfonamidas mais frequentemente presentes em sorovares de *Salmonella* spp., e também são frequentemente associados a plasmídeos e transposons, destacando o potencial de transferência desses genes para outras bactérias, disseminando essas resistências.

2.5 Tolerância a sanitizantes e principais sanitizantes aplicados na indústria de alimentos

Sanitizantes são substâncias que contém compostos ativos capazes de reduzirem a população bacteriana de um ambiente em números significativos (EPA, 2018). A etapa de sanitização dentro da indústria de alimentos faz parte das Boas Práticas de Fabricação (BPF), e é considerada essencial para garantir a segurança e a qualidade dos alimentos (ALONSO et al., 2022). Entretanto, o uso frequente desses compostos em superfícies de ambientes de processamento de alimentos pode conduzir à tolerância bacteriana a sanitizantes, que assim como a resistência a antimicrobianos, ocorre principalmente a partir do seu uso indevido, como subdoses (BRIDIER et al., 2011). Não existem valores de corte definidos para classificar isolados de *Salmonella* spp. como tolerantes ou suscetíveis aos compostos sanitizantes. Entretanto, existe uma recomendação da OMS em relação aos limites para o uso da maioria das classes de biocidas utilizados na sanitização de superfícies.

Os compostos quaternários de amônio (CQA) são sanitizantes frequentemente aplicados no ambiente industrial, devido às suas características de não serem corrosivos, não causar irritabilidade em quem o manipula, sua baixa toxicidade e sua elevada eficácia (BORE et al., 2007). Isolados de *Salmonella* spp. provenientes de frango e ovos avaliados por LONG et al. (2016) foram testados contra o cloreto de benzalcônio (CB), o CQA mais comumente utilizado na etapa de sanitização das indústrias alimentícias, e apresentaram baixa susceptibilidade ao composto, com valores de Concentração Inibitória Mínima (CIM) variando entre 32 e 256 $mg.L^{-1}$, sendo que a OMS recomenda o uso de até 500 $mg.L^{-1}$ deste composto para sanitização de superfícies (OMS, 2020). Mais recentemente, HAUBERT et al. (2022) avaliaram a tolerância ao CB em isolados de *Salmonella* spp. provenientes de ovos, e observaram tolerância dos isolados ao composto, que apresentaram CIM nos valores de 64 e 128 $mg.L^{-1}$.

O digluconato de clorexidina, ou simplesmente clorexidina (CLX), é um biocida amplamente utilizado na área da saúde, por exemplo, em antissépticos para a pele e sabonetes antimicrobianos, sendo recomendado seu uso em concentrações entre 1200 $mg.L^{-1}$ a 2000 $mg.L^{-1}$ (OMS, 2020). Estudos já testaram a ação antibacteriana deste composto em isolados de *Salmonella* spp*.* de origem alimentar. HUMAYOUN et al. (2018) avaliaram 88 isolados de *Salmonella* spp. provenientes de animais (bovinos, suínos, frangos, perus, cavalos, cães e gatos) e de carnes provenientes do varejo, e identificaram tolerância à clorexidina, sendo a maioria provenientes de bovinos.

O ácido peracético (AP) também é considerado um sanitizante de elevada eficácia, sendo muito utilizado em ambientes de processamento de alimentos e bebidas (LUUKKONEN & PEHKONEN, 2017). Já os sanitizantes das classes de compostos clorados, como o hipoclorito de sódio (HS), também são uma escolha frequente da indústria de alimentos para a sanitização, pois não são onerosos e possuem elevada ação antimicrobiana, tanto para serem utilizados em solução com água para higienização por imersão, como para serem usados em superfícies de contato de alimentos (FAO/WHO, 2008). Ambos compostos foram testados em *Salmonella* spp. isoladas de animais, carne e também de ovos nos estudos de HUMAYOUN et al. (2018) e HAUBERT et al. (2022), e em ambos, os isolados

mostraram-se tolerantes ao AP e ao HS, sendo observados altos valores de CIM, sendo estes valores maiores do que o recomendado pela OMS, que é a concentração de 1000 e 5000 $mg.L^{-1}$ para o HS e para o AP, respectivamente (OMS, 2020).

2.5.1 Mecanismos de tolerância a sanitizantes e correlação com a resistência a antimicrobianos

A tolerância a sanitizantes é, geralmente, classificada como intrínseca ou adquirida, assim como ocorre com a resistência aos antimicrobianos. A tolerância adquirida refere-se a modificações genéticas que podem ocorrer ao acaso ou ainda após a exposição contínua a um determinado composto. Essas modificações genéticas são o que determinam os mecanismos de tolerância bacteriana aos sanitizantes, e podem envolver alterações na permeabilidade da membrana ou o uso de bombas de efluxo e a atividade de algumas enzimas, que expelem o composto para fora da célula. O mecanismo das bombas de efluxo é o mais elucidado em relação à tolerância ocasionada pelo uso de diferentes classes de sanitizantes (ALONSO et al., 2022).

As bombas de efluxo mais elucidadas em *Salmonella* spp., frequentemente relacionadas com a tolerância aos CQA, são codificadas por genes do operon *qac,* que geralmente estão localizados em plasmídeos ou integrons (ZOU et al., 2014). Os genes *qacA, qacE, qacF* e *qacH* já foram identificados em *Salmonella* spp. (CHUANCHUEN, 2007; WU et al., 2015), entretanto, o gene *qacEΔ1* é o mais observado em isolados de diversos estudos (LONG et al., 2016; WU et al., 2015). Este gene está localizado na porção conservada 3’ dos *integrons* de classe 1, que também podem portar genes de resistência a antimicrobianos (CHAPMAN, 2003; ROMERO et al., 2017).

Sanitizantes e antimicrobianos apresentam distintos mecanismos de ação: uma vez que os sanitizantes possuem um espectro de atividade mais amplo, atuam em diversos locais-alvo na célula bacteriana; já os antimicrobianos agem em um alvo celular específico, variando de acordo com sua classe. Por este motivo, raramente uma mutação que ocorre em um local alvo de um antimicrobiano resultará também em uma tolerância a sanitizante (HUMAYOUN et al., 2018; WEBBER et al., 2013).

Entretanto, existe a possibilidade de ocorrer resistência cruzada entre esses dois agentes, justamente porque alguns determinantes de tolerância a sanitizantes e de resistência a antimicrobianos podem estar localizados em um mesmo elemento genético móvel, sendo essas resistências mediadas através da transferência desse material genético entre as bactérias (HUMAYOUN et al., 2018; MCCARTHY & LINDSAY, 2012). WU et al. (2015) observaram que a tolerância aos CQA pode estar relacionada com a presença de genes de resistência a antimicrobianos, o que pode justificar-se pelo fato de os genes *qac* frequentemente serem encontrados em *integrons* que também carreiam genes de resistência a alguns antimicrobianos, como o gene *sul1,* que confere resistência às sulfonamidas.

3 Objetivos

3.1 Objetivo geral

Caracterizar isolados de *Salmonella* spp. provenientes de carcaças bovinas e produtos cárneos de Pelotas, Rio Grande do Sul, quanto aos seus perfis fenotípicos e genotípicos de virulência e de resistência a antimicrobianos e a sanitizantes, e quanto a sua diversidade genética.

3.2 Objetivos específicos

Objetivo 1: Avaliar os determinantes de virulência em isolados de *Salmonella* spp. de carcaças bovinas e produtos cárneos, através da pesquisa de genes de virulência com a técnica de reação em cadeia da polimerase (PCR).

Objetivo 2: Verificar os perfis fenotípicos de resistência a antimicrobianos em isolados de *Salmonella* spp. de carcaças bovinas e produtos cárneos através de disco difusão em ágar e concentração inibitória mínima, bem como identificar os determinantes genéticos de resistência a antimicrobianos nos isolados, através da pesquisa de genes de resistência a antimicrobianos com a técnica de PCR.

Objetivo 3: Identificar os perfis fenotípicos de suscetibilidade a sanitizantes em isolados de *Salmonella* spp. de carcaças bovinas e produtos cárneos utilizando a técnica de concentração inibitória mínima, bem como avaliar os determinantes genéticos de resistência a sanitizantes nos isolados, através da pesquisa de genes de resistência a sanitizantes com a técnica de PCR.

Objetivo 4: Avaliar a diversidade genética entre os isolados de *Salmonella* spp. de carcaças bovinas e produtos cárneos através da técnica de gel de eletroforese em campo pulsado (PFGE).

4 Manuscrito 1 – A longitudinal study of the virulence and antimicrobial resistance rates in *Salmonella* spp. from bovine carcass and meat products in Southern Brazil

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A longitudinal study of virulence and antimicrobial resistance rates in *Salmonella* spp. from bovine carcasses and meat products in Southern Brazil

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Abstract

This study aimed to evaluate the genotypic virulence profile, the phenotypic and genotypic antimicrobial resistance profiles, and the genetic relatedness of *Salmonella* isolates from bovine carcasses and meat products. All isolates presented at least one of the virulence genes evaluated (*invA*, *spvC* and/or *pefA*); however, the *sefA* gene was not detected. Resistance to tetracycline (54.5%) and ampicillin (49.1%) were the most prevalent. Multidrug-resistance (MDR) phenotypic profile was observed in more than half of the isolates (56.4%), of which 32.2% belonged to serovar Typhimurium. Interestingly, 96.8% of MDR isolates are from meat products. Thirty-one isolates carried 11 resistance genes, among which *tet(A)* and bla_{TEM} genes were the most prevalent. Plasmids harboring antimicrobial resistance genes were found in 41.9% of MDR isolates, being the *tet(A)* gene the most detected in plasmids. PFGE showed a high variability in the genomic structure of *Salmonella* isolates, especially in serovars Typhimurium, Anatum, Heidelberg and Derby. Clonality was observed only in the serovar Senftenberg from bovine carcasses. *Salmonella* serovars with MDR profiles and genetic diversity, carrying a variety of resistance genes, including in plasmids, were more frequently among meat products. This data highlights the importance of good manufacturing practices in slaughterhouses and food processing environments, in order to reduce contamination in meat and meat products, which are highly manipulated, and spread of antimicrobial resistant *Salmonella* serovars.

Keywords: genetic diversity; multidrug-resistance; plasmids; resistance genes; salmonellosis

1 Introduction

Salmonellosis, the infection caused by non-typhoidal *Salmonella* serovars, affects about 1.35 million people only in the United States of America every year, resulting in approximately 26.500 hospitalizations and 420 deaths (CDC, 2021). Contaminated foods are the source of most of these illnesses, being meat, and broiler/pork meat products associated with a significant number of human cases (Chittick, Sulka, Tauxe and Fry, 2006; Yang et al., 2019). The severity of salmonellosis and the occurrence of other complications may worsen depending on the degree of virulence of the *Salmonella* strain (Cheng, Eade and Wiedmann, 2019). Several genes encode for virulence factors in *Salmonella* spp., including genes that encoding for adhesion and invasion proteins (*invA* gene), fimbriae (*pefA* and *sefA* genes) and virulence genes located in plasmids, as *spvC* gene, which can be transmitted for other bacteria.

Over time, while antimicrobials have been used to treat severe cases of salmonellosis (WHO, 2018), the emergence and spread of bacterial resistance mechanisms have contributed to the serious situation of antimicrobial resistance, further reducing the ability to treat the disease (Hu et al., 2020; WHO, 2018). Since resistance to older antimicrobials (e.g. ampicillin, chloramphenicol and folate pathway inhibitors, such as trimethoprim/sulfamethoxazole) has been reported for at least 30 years, the options for salmonellosis treatment include fluoroquinolones (such as ciprofloxacin) and third-generation cephalosporins (such as cefotaxime) (Parry and Threlfall, 2008; Chen, Wang, Su and Chiu, 2013). However, resistance to these antimicrobials is also increasing, being reported resistance to extended-spectrum cephalosporins (ESC) in *Salmonella* spp. since the late 1980's (Ranjbar et al., 2013), and have increased ever since, leading to the need to use of last-line antimicrobials (e.g. carbapenems) in the therapy of serious salmonellosis cases (Nordmann and Poirel, 2014; WHO, 2012).

It is important to emphasize that the increase in antimicrobial resistance is a global public health concern, and several resistant serovars of *Salmonella enterica* subsp. *enterica* have emerged in the last decades (WHO, 2018). According to CDC (2018), *S.* Typhimurium, one of the most commonly reported serovars related to foodborne human salmonellosis cases worldwide (Sun et al., 2020), was the most

associated with multidrug-resistance profiles, and a *S*. Typhimurium clone, typically resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole/sulfisoxazole and tetracycline (ACSSuT), was widely distributed.

Pulsed-field Gel Electrophoresis (PFGE), a widely used technique for bacterial molecular typing, uses restriction enzymes that cleave bacterial DNA, grouping the strains into clones based on the PFGE banding patterns, defined through the generated DNA fragments (Neoh et al, 2019; Kang, Meng and Pan, 2021). PFGE is still an important tool for molecular typing of bacterial foodborne pathogens, such as *Salmonella* spp. (Kang, Meng and Pan, 2021; Ribot et al., 2006), and the detection of PFGE patterns of *Salmonella* isolated from food can aid in the investigation of foodborne illness outbreaks (CDC, 2012).

In this context, this study aimed to evaluate the genotypic virulence profile, the phenotypic and genotypic antimicrobial resistance profiles, and the genetic relatedness of *Salmonella* isolates from bovine carcasses and meat products.

2 Material and Methods

2.1 Source of *Salmonella* spp. isolates

A total of 55 *Salmonella* spp. isolates was obtained from a longitudinal study conducted in Pelotas, Southern Brazil. A total of 200 bovine carcasses were sampled after bleeding, after skinning, after evisceration, and after pre-cooling washing at two bovine slaughterhouses, totalizing 15 isolates. To avoid duplicates, only one *Salmonella* isolate per positive sample was selected. Additionally, 40 *Salmonella* isolates from meat products obtained from 14 establishments of Pelotas and belonged from the collection culture of the Laboratório de Microbiologia de Alimentos of DCTA/UFPEL were evaluated (Table 1). All isolates were identified as *Salmonella enterica* as described by the International Organization for Standardization (ISO 6579-1:2017). Serological tests were performed at the National Reference Center, Fundação Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, by standard slide agglutination using commercially available antisera in order to characterize the serovar of the isolates.

2.2 Detection of virulence genes

Firstly, genomic DNA was extracted according to Ellington, Kistler, Livermore and Woodford (2007) with minor modifications, dissolving colonies of the *Salmonella* isolates in 200 µL of ultrapure water (UPW) and freezing at -20 ^{o}C for at least 1 h. The confirmation of *Salmonella* spp. isolates by the detection of *hilA* gene and the detection of virulence genes (*invA, spvC, sefA,* and *pefA*) were performed by PCR assays. The oligonucleotides used are shown in the Table S1 in the supplementary material, as well as the respective annealing temperatures of each primer. The reaction mixtures contained 12.5 µL of GoTaq® Green Master Mix 2x (Promega, USA), 1 µL of each primer at a concentration of 10 µmol, 2 µL of DNA (10 ng) and 8.5 µL of UPW (Promega, USA) to a total volume of 25 µL. The amplification was performed in a thermocycler MJ Research® PTC 100. *Salmonella* Enteritidis ATCC® 13076 and *S.* Typhimurium ATCC® 14028 were used as positive controls. After, the PCR products were submitted to electrophoresis on a 1.5% (w/v) agarose gel (Invitrogen™, USA) stained with GelRed® at 80 V for 70 min using a molecular weight marker of 1 Kb (Invitrogen™, USA). The amplified products were visualized in an UV transilluminator (Loccus Biotecnologia®, Brazil).

2.3 Antimicrobial susceptibility testing

The antimicrobial susceptibility of the *Salmonella* spp. isolates was evaluated by the agar disk diffusion method, according to the Clinical and Laboratory Standards Institute for the Enterobacteriaceae group (CLSI, 2018). The following antimicrobial disks were evaluated: ampicillin 10 µg (AMP), amoxicillin/clavulanic acid 30 µg (AMC), cefotaxime 30 µg (CTX), cephalothin 30 µg (CFL), gentamicin 10 µg (GEN), tobramycin 10 µg (TOB), streptomycin 10 µg (STR), imipenem 10 µg (IMP), nalidixic acid 30 µg (NAL), ciprofloxacin 5 µg (CIP), sulfonamide 300 µg (SUL), trimethoprim/sulfamethoxazole 1.25/23.75 µg (SUT), trimethoprim 5 µg (TRI), tetracycline 30 µg (TET), and chloramphenicol 30 µg (CHL) acquired from Laborclin (Laborclin Produtos para Laboratórios Ltda, Brazil). The isolates were classified as susceptible or resistant to the antimicrobial agents tested, being the isolates with intermediate resistance according classification of CLSI (2018), were considered resistant isolates. Isolates resistant to three or more antimicrobial classes were classified as multidrug-resistance (MDR) (Basak, Singh and Rajurkar, 2016).

The minimum inhibitory concentration (MIC) of antimicrobials ampicillin, ciprofloxacin, nalidix acid and tetracycline, also was evaluated, using the broth microdilution method (CLSI, 2017). Firstly, the inoculum was prepared at ~0.5 x 10^8 McFarland standard from a 24 h/37 ^oC growth on TSA in 0.85% (w/v) saline solution (SS) (Synth, Brazil). Concentrations of antimicrobials were prepared using a stock solution at 2048 $\mu g.mL^{-1}$, diluting until 0.125 $\mu g.mL^{-1}$ in Mueller-Hinton broth (Kasvi, Brazil). Microtiter plates were filled with the inoculum and the antimicrobial concentrations in a proportion of 1:9, with incubation of the microtiter plates at 37 ºC for 24 h. The MIC of antimicrobials was defined as the lowest concentration completely inhibited the bacterial growth, observed visually.

2.4 Detection of antimicrobial resistance genes

The isolates that presented antimicrobial resistance profile in the phenotypic test were evaluated for the presence of the respective antimicrobial resistance genes. The coding resistance genes to aminoglycosides (*aadA*, *aadB, aac(6')-Ib, strA,* and *strB*), β-lactams (bla_{TEM} and *blaZ*), class 1 integrase (*int1*), folate pathway inhibitors (*dfrA, dfrD, dfrG, sul1, sul2,* and *sul3*), phenicols (*catA1* and *floR*) and tetracyclins [*tet(A)* and *tet(B)*] were investigated by PCR assays using the oligonucleotides listed in Table S1 in the supplementary material. The cycling conditions of the PCR assays followed the recommendations of the referenced authors' studies (Table S1).

The presence of these resistance genes was also evaluated in the plasmid DNA of the MDR isolates. Firstly, the plasmid DNA was extracted and purified following the alkaline lysis protocol (Birnboim and Doly, 1979). The extracted plasmids were subjected to an electrophoresis on a 0.8% agarose gel (Kasvi®, Brazil) in a Tris/Acetate/EDTA buffer (TAE, Ludwig Biotec, Brazil) for 2 h at 70 V. To estimate plasmids sizes, the plasmids of *Escherichia coli* V517 (Macrina et al., 1978) were used as size standard. Finally, the resistance genes were investigated in the plasmids by PCR assays using the same oligonucleotides and cycling conditions cited above.

2.5 DNA sequencing

After the antimicrobial resistance phenotypic tests, the genes *gyrA* and *gyrB* (that codified for two subunits of DNA gyrase, GyrA and GyrB, respectively), and the genes *parC* and *parE* (that codified for two subunits of topoisomerase IV, ParC and ParE, respectively), were sequenced. Two isolates nalidixic acid and ciprofloxacin resistant were selected, given that resistances to these antimicrobials are associated with mutations in specific regions of these genes, called quinolone resistance-determining regions (QRDR), since proteins encoded for these genes are targets of quinolones and fluoroquinolones. The two selected isolates (*S*. Anatum S27 and *S*. Typhimurium S39) presented different MIC values for nalidixic acid (64 and >2048 $\mu g.mL^{-1}$, respectively) and for ciprofloxacin (4 and 8 $\mu g.mL^{-1}$, respectively).

For the identification of the mutations, the PCR products of the four genes from the two isolates were purified using the protocol of DNA precipitation with sodium acetate and ethanol, with modifications (Sambrook and Russel, 2001). Firstly, in 100 µL of PCR products, were added 10 µL of sodium acetate (3M, pH 5.2), and then 250 µL of cold absolute ethanol. The samples were incubated for 15 min at room temperature and then centrifuged for 20 min/18400 *g*/4 ^{o}C. Supernatant was discarded and 300 µL of cold ethanol 70% were added and the samples were centrifuged again for 10 min/18400 *g*/4^{o}C. After removing the supernatant, the pellet was dried for ~5 min/60 ^{o}C, and ressuspended in 50 µL of UPW DNases and RNases free. The purified products and their respective primers were sent to ACTGene Análises Moleculares (Brazil) and the DNA sequencing was performed by the Sanger method using the AB 3500 Genetic Analyzer (Applied Biosystems™, USA). The sequences were compared with references strains using the Basic Local Alignment Search Tool (BlastX).

2.6 Molecular typing

Pulsed-Field Gel Electrophoresis (PFGE) typing was performed using the standard CDC PulseNet protocol for *Salmonella* spp. (Ribot et al., 2006), with minor modifications, with the *Salmonella* isolates inoculated in 4 mL of Luria Bertani broth (LB) at 37 °C for 24 h. At day one, 1 mL of inoculated LB was centrifuged at 4000 rpm for 20 min, the supernatant was discarded and 500 µL of Cell Suspension Buffer (CSB) were added. The next steps (preparation of agarose plugs, cell lysis and

washing of agarose plugs) were prepared according with the CDC PulseNet protocol. Genomic DNA of *Salmonella* spp. isolates and *S.* Braenderup H9812 (reference marker) were digested with 20 U of *Xba*I (Invitrogen^{TM}, USA) restriction enzyme. Macrorestriction fragments were separated in a CHEF-DR II system apparatus (Bio-Rad Laboratories Inc., USA). The electrophoresis running conditions were: temperature at 14 °C, initial time of 2.2 s, final time of 63.8 s, run time of 19 h at 6 volts. After, the gel was stained with ethidium bromide (stock solution of 20 mg.mL^{-1}, Sigma, USA) for 20 min and destained in distilled water for 20 min twice. The restriction fragments were viewed in a photodocumentation L-Pix system (Loccus Biotecnologia®, Brazil). Band patterns were analyzed by BioNumerics 7.1 (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice coefficient, with a maximal position tolerance of 1.0% and optimization of 1.0% and the patterns were clustered using the unweighted pair group method with arithmetic averages (UPGMA).

3 Results and discussion

3.1 Detection of virulence genes

All 55 isolates harbored the *Salmonella* invasion genes *hilA* and *invA,* which are essential for bacteria invasion in epithelial cells, and are widely used to identify *Salmonella* at the genus level (Favier et al*.,* 2013; Galan and Curtiss, 1991; Tafida et al*.,* 2013). The *spvC* and *pefA* genes were detected in five (9.9%) and nine (16.4%) isolates, respectively, however the *sefA* gene, that codified for one of the subunitis of the fimbriae SEF14, was not detected. On the other hand, Haubert et al. (2018b) evaluated the same virulence genes used in this study in 26 *Salmonella* spp. isolates from food and food environments, mainly meat products, and all four genes were detected, being *invA* detected in 100% isolates, *spvC* in 15.38%, *pefA* in 19.23%, and *sefA* in 3.84% isolates. Weber et al. (2019) evaluated virulence genes in 126 *Salmonella* Heidelberg isolated from chicken carcasses and, as in the present study, none of the isolates carried the *sefA* gene. According these authors, fimbriae have tropism by distinct cell types in different hosts, and for this reason, it is possible that the absence of some fimbriae, such as SEF14, could affects the virulence of the bacteria, making the strains less virulent.

Among the five isolates that carried the *spvC* gene, four (80%) belonged to the serovar Typhimurium, which also carried the *pefA* gene, and one isolate belonged to the serovar Derby. Among the nine isolates that harbored the *pefA* gene, seven (77.8%) were *S.* Typhimurium, while one was Derby and other was S. *enterica* subsp. *enterica* (Table 1). Elkenany et al. (2019) didn't find *pefA* gene in 36 *S.* Typhimurium isolates from broiler chickens and chicken carcasses in Egypt. Furthermore, Thung et al. (2018) found this gene in three of 23 isolates (13.04%) from retail beef in Malaysia, and two of them were *S.* Typhimurium. SpvC protein, encoded by the *spvC* gene, is an anti-inflammatory effector which plays an important role in the host's proinflammatory response (Fàbrega and Vila, 2013), while the plasmid encoded fimbriae (*pefA*) gene contributes to the adhesion of *Salmonella* to epithelial cells (Murugkar et al., 2003). In this way, the presence of these important virulence genes, mainly in the serovar Typhimurium, the more involved in foodborne human salmonellosis, is a relevant data found in this study.

Table 1 - Characteristics of *Salmonella* spp. isolates from bovine carcass and meat products in Southern Brazil.

Origin	ID	Sample	Date/ Abattoir or establishment	Serovar	PFGE profile (*XbaI*)	Virulence genes	Resistance Phenotype[a]	Resistance Genotype
Bovine Carcass	S3	After bleeding	Nov. 2010/ B	Derby	-	*invA*	STR-SUL-TET	*aadA, int1, sul1, tet(A)*
	S4	After bleeding	Jan. 2011/ A	*S. enterica* subsp. *enterica* (O:6,7)	-	*invA*	Susceptible	-
	S5	After bleeding	Jan. 2011/ A	Livingstone	-	*invA*	Susceptible	-
	S6	After bleeding	Jan. 2011/ A	*S. enterica* subsp. *enterica* (O:6,7)	-	*invA*	Susceptible	-
	S7	After bleeding	Jan. 2011/ A	Ohio	-	*invA*	STR	-

S11	After bleeding	Aug. 2011/ A	Senftenberg	P4	*invA*	Susceptible	-
S12	After bleeding	Aug. 2011/ A	Senftenberg	P1	*invA*	Susceptible	-
S13	After bleeding	Aug. 2011/ A	Senftenberg	P2	*invA*	Susceptible	-
S14	After bleeding	Aug. 2011/ A	Senftenberg	P5	*invA*	Susceptible	-
S15	After bleeding	Aug. 2011/ A	Senftenberg	P5	*invA*	Susceptible	-
S16	After bleeding	Aug. 2011/ A	Senftenberg	P3	*invA*	Susceptible	-
S17	After bleeding	Mar. 2012/ B	Senftenberg	P6	*invA*	Susceptible	-
S18	After bleeding	Mar. 2012/ B	Muenster	-	*invA*	Susceptible	-

	S19	After bleeding	Mar. 2012/ B	Anatum	-	*invA*	Susceptible	-
	S20	After bleeding	Mar. 2012/ B	Muenster	-	*invA*	Susceptible	-
Meat products	S1	Fresh sausage	Jan. 2010/ K	Derby	P36	*invA*	STR-SUL-TET	*aadA*, *int1*, *sul1*, *tet(A)*
	S2	White pudding	July. 2010/ K	Infantis	-	*invA*	Susceptible	-
	S8	Pork sausage	Oct. 2011/ K	Derby	P34	*invA*, *pefA*, *spvC*	STR-SUL-TET	*aadA*, *int1*, *sul1*, *tet(A)*
	S9	Poultry sausage	Nov. 2011/ L	Derby	P35	*invA*	NAL	-
	S10	Skinless bacon	Dec. 2011/ M	Typhimurium	P9	*invA*	AMP-CHL-GEN[b] -NAL-STR-SUL-SUT-TET-TRI	*aadA*, bla_{TEM}, *catA1*, *floR*, *int1*, *strA*, *strB*, *sul1*, *tet(A)*

S22	Beef	Feb. 2012/ B	Typhimurium	P7	*invA*, *pefA*, *spvC*	Susceptible	-
S23	Smoked mixed colonial sausage	Feb. 2018/ C	Anatum	P23	*invA*	CFL[b]	-
S24	Smoked mixed colonial sausage	Feb. 2018/ D	Anatum	P19	*invA*	CFL[b]	-
S25	Fresh mixed sausage	Mar. 2018/ C	Anatum	P26	*invA*	AMP-CFL-CHL-CIP[b]-NAL[b]-STR-SUL-TET	*aadA, bla*$_{TEM}$, *floR, int1, sul1, tet(A)*
S26	Fresh mixed sausage	Apr. 2018/ C	Anatum	P20	*invA*	AMP-CHL[b]-CIP[b]-NAL[b]-STR[b]-SUL-SUT-TET-TRI	*aadA, bla*$_{TEM}$, *floR, int1, strA, strB, sul1, tet(A)*
S27	Smoked dried sausage	Apr. 2018/ C	Anatum	P21	*invA*	AMP-CFL[b]-CHL-CIP[b]-GEN[b]-NAL[b]-STR[b]-SUL-SUT-TET-TRI	*aadA, bla*$_{TEM}$, *floR, strA, strB, sul1, tet(A)*

S28	Bacon	Apr. 2018/ C	Anatum	P25	*invA*	Susceptible	-
S29	Pork fat	Apr. 2018/ E	*S. enterica* subsp. *enterica* (O:4,5)	-	*invA*	AMP-CFL[b]-CHL-CIP[b]-NAL[b]-TET-TRI	*bla*$_{TEM}$, *tet(A)*, *tet(B)*
S30	Smoked mixed sausage	Apr. 2018/ E	Anatum	P27	*invA*	AMP-CHL-CIP[b]-NAL[b]-TET	*bla*$_{TEM}$, *tet(B)*
S31	Smoked dried mixed sausage	May. 2018/ E	Heidelberg	P29	*invA*	AMP-CHL-CIP[b]-CFL[b]-STR[b]-SUL	*aadA*, *bla*$_{TEM}$, *strA*, *strB*, *sul2*
S32	Fresh sausage	June. 2018/ N	Typhimurium	P13	*invA*	AMP-CHL-CIP[b]-CFL[b]-NAL-STR-SUL-TET	*floR*, *strA*, *strB*, *sul2*
S33	Fresh sausage	June. 2018/ F	Agona	-	*invA*	Susceptible	-
S34	Cooled pork	June. 2018/ O	Anatum	P28	*invA*	AMP-CHL-CIP[b]-NAL[b]-STR[b]-SUL-SUT-TET[b]-TRI	*bla*$_{TEM}$, *floR*, *int1*, *strA*, *strB*, *sul1*,

							tet(A)
S35	Fresh sausage	June. 2018/ O	Anatum	P24	*invA*	AMP-CFL[b]-CHL-NAL[b]-STR[b]-SUL-SUT-TET[b]-TRI	*bla*$_{TEM}$*, floR, int1, strA, strB, sul1, tet(A)*
S36	Fresh sausage	June. 2018/ H	Senftenberg	-	*invA*	AMP-SUL-SUT-TET-TRI	*bla*$_{TEM}$*, sul1, tet(A)*
S37	Pork	July. 2018/ F	Typhimurium	P8	*invA, spvC, pefA*	AMP-CHL-CFL[b]-GEN-NAL-SUL-TET	*bla*$_{TEM,}$ *floR, int1, sul1, sul2, tet(A), tet(B)*
S38	Vacuum packed rump trim	July. 2018/ B	Lexington	-	*invA*	AMP-SUL-TET	*bla*$_{TEM}$*, sul1, tet(A)*
S39	Pork sausage	July. 2018/ F	Typhimurium	P14	*invA*	AMP-CFL[b]-CIP[b]-NAL-STR[b]-SUL-SUT-TET-TRI	*bla*$_{TEM}$*, int1, strA, strB, sul1, tet(A)*
S40	Pork sausage	Aug. 2018/ F	Derby	P37	*invA*	AMP-CHL-CFL[b]-TET	*floR, tet(A)*
S41	Pork sausage	Aug. 2018/ F	Derby	P38	*invA*	AMP-CHL-SUL-STR-TET	*bla*$_{TEM}$*, floR, int1, sul1*

S42	Smoked mixed colonial sausage	Nov. 2018/ C	Heidelberg	P30	*invA*	AMP-CHL-TET	*bla*$_{TEM}$
S43	Pork fat	Nov. 2018/ C	Typhimurium	P12	*invA*	AMP-CFL^{b}-CHL-NAL-TET	*bla*$_{TEM}$, *floR, tet(A)*
S44	Smoked mixed colonial sausage	Nov. 2018/ C	Heidelberg	P31	*invA*	AMP-CHL-NAL-SUL-STR^{b}-TET	*bla*$_{TEM}$, *floR, strA, strB, sul1, sul2, tet(B)*
S45	Fresh mixed sausage	Nov. 2018/ J	Heidelberg	P32	*invA*	AMP-CHL-NAL-TET	*bla*$_{TEM}$, *floR, tet(A)*
S46	Fresh special pork sausage	Feb. 2019/ G	Heidelberg	P33	*invA*	AMP-CHL-TET	*bla*$_{TEM}$, *floR, tet(A)*

S47	Fresh pork sausage	Feb. 2019/ F	Anatum	P22	*invA*	AMP-CHL-CFL[b]-STR-SUL	*floR, strA, strB, sul2*
S48	Fresh pork sausage	Apr. 2019/ H	*S. enterica* subsp. *enterica**	-	*invA*	Susceptible	-
S49	Smoked mixed colonial sausage	June. 2019/ I	Typhimurium	**	*invA, pefA*	TET	*tet(A), tet(B)*
S50	Fresh mixed sausage	June. 2019/ J	*S. enterica* subsp. *enterica**	-	*invA, pefA*	AMP-CFL[b]-CHL-TET	*bla_{TEM}, floR, tet(A)*
S51	Colonial salami	June. 2019/ J	Typhimurium	P10	*invA, pefA*	AMP-CFL[b]-CHL[b]-NAL-SUL-TET	*floR, sul2, tet(B)*
S52	Smoked mixed sausage	June. 2019/ I	Typhimurium	P16	*invA, pefA*	CFL[b]	-
S53	Pork	Sept. 2019/ H	Typhimurium	P11	*invA, spvC, pefA*	AMP-CFL[b]-GEN[b]-NAL-SUL-TET	*int1, sul2, tet(B)*

S54	Smoked fresh mixed sausage	Oct. 2019/ I	Typhimurium	P17	*invA, spvC, pefA*	CFL[b]-NAL-TET	-
S55	Fresh sausage	Oct. 2019/ F	Typhimurium	P18	*invA*	AMP-CFL-CHL[b]-CTX-NAL-TET	*bla*$_{TEM}$, *tet(A)*
S56	Fresh sausage	Oct. 2019/ C	Typhimurium	P15	*invA*	AMP-CFL-CHL-STR[b]-SUL-TET	*bla*$_{TEM}$, *floR, int1,strB, sul1, sul2, tet(A)*

*flagellate structure not detectable

**isolate not digested with XbaI enzyme

[a]AMP: ampicillin, CTX: cefotaxime, CFL: cephalothin, GEN: gentamicin, STR: streptomycin, TET: tetracycline, CHL: chloramphenicol, NAL: nalidixic acid, CIP: ciprofloxacin, SUL: sulfonamide, SUT: trimethoprim/sulfamethoxazole, TRI: trimethoprim

[b]Intermediate resistance

3.2 Antimicrobial resistance profiling

Of the 55 *Salmonella* isolates included in this study, 37 (67.3%) showed resistance to at least one antimicrobial agent. The main phenotypic resistance was to tetracycline (54.5%), followed by ampicillin (49.1%), chloramphenicol (41.8%), and sulfonamides (38.2%), and lowest resistance rates were found to trimethoprim (14.5%), sulfamethoxazole (12.7%) and cefotaxime (1.8%). It is interesting that the vast majority of these resistances were found in isolates from meat products: among the 30 isolates resistant to tetracycline and to sulfonamides, 29 (96.6%) and 20 (95.2%) are from meat products, respectively, and among the 27 isolates ampicillin-resistant and the 23 isolates chloramphenicol-resistant, all (100%) are from meat products. Yang et al. (2019) evaluated *Salmonella* spp. from retail meat and meat products, and also observed higher resistance rates to tetracycline (65.6%) and ampicillin (45.4%). According to these authors, tetracycline and ampicillin are traditional antimicrobial agents, and tetracycline is still one of the most used antimicrobials in feed additives on farms in several countries, therefore, this uncontrolled use can be one of the causes of the spread of resistance to this antimicrobial among *Salmonella* isolates. Furthermore, the high susceptibility rates of *Salmonella* isolates from bovine carcasses and the high resistance rates in isolates of meat products, that are more manipulated foods, may suggest that the major source of the dissemination of resistance genes occurs in the food processing environments during the processing of meat products, which includes raw material from different origins, handlers that can carry resistant strains, equipment, surfaces, and devices. Biofilms, especially on equipment that comes into contact with products, can result in *Salmonella*-contaminated meat products that are resistant and even multi-resistant to antimicrobials (Siddique et al., 2021).

Isolates with intermediate resistances according the classification of CLSI (2018), were considered as resistant isolates: 17 (85%) of 20 isolates resistant to cephalotin; seven (36.8%) of 19 isolates resistant to nalidixic acid; eight (47%) of 17 isolates resistant to streptomycin; three of four isolates (75%) to gentamicin and all of nine isolates (100%) resistant to ciprofloxacin were intermediate resistant (Table 1). All of these intermediate resistances are from isolates of meat products. These data suggest that resistance rates of *Salmonella* spp. in meat products marketed in the

evaluated region will increase, since intermediate resistances can soon become resistances, with the continuous exposure of *Salmonella* isolates to these compounds over time. The development of resistance mechanisms in microbial populations is a normal evolutionary process, but it is accelerated due the selective pressure caused by the frequent exposure of microorganisms to antimicrobials (Christopher et al., 2021).

One isolate (1.8%) of *S*. Typhimurium (S55) presented phenotypic resistance to cefotaxime in the agar disc diffusion method, resistance that also was reported by Sasaki et al. (2021) in 2% of *Salmonella* isolated from chicken products. Despite the resistance to this antimicrobial being low, this data deserves attention, since cefotaxime is still one of the last alternatives for the treatment of severe cases of salmonellosis (Ghaly et al., 2021). The increase of resistance to clinically relevant antimicrobials used in salmonellosis treatment, such as third-generation cephalosporins and fluorquinolones (Yang et al., 2019), also have been observed in non-typhoidal *Salmonella* isolates in Cambodja and China (Nadimpalli et al., 2019; Zhang et al. 2019), representing an increasingly threat for global public health.

Six isolates (10.9%) presented phenotypic resistance to only one antimicrobial tested, and MDR profile was observed in 31 isolates (56.4%), being 30 (96.8%) from meat products, and 10 (32.2%) belonging to serovar Typhimurium, percentage almost three times higher than cited by CDC in a NARMS annual report (CDC, 2018). Although the increase in the rates of MDR *Salmonella* isolates in the last years, already in the early 1990s, *S*. Typhimurium was reported to be simultaneously resistant to five antimicrobial agents (ACSSuT) (Su et al., 2004).However, it is observed that currently the MDR profile of the isolates presents in addition to resistance to traditional antimicrobial, resistance to antimicrobial of last choice for the treatment of human salmonellosis, which tends to make the treatment of infection even more difficult (Luo, Yi, Yao, Zhu and Qin, 2018), as mentioned above to cefotaxime. Distinct MDR profiles were identified in this study, being detected eight and six different MDR profiles in the establishments C and F, respectively, and all of these MDR profiles belonged to isolates from meat products (Table 1). A high diversity of resistance genes among isolates in packaged products intended for retail,

enhances that the global problematic of MDR *Salmonella* spp. is also present in the Southern Brazil, where this study was carried out.

All isolates were susceptible to amoxicillin/clavulanic acid, tobramycin and imipenem; 18 isolates (32.7%) were susceptible to all antimicrobials evaluated, being seven (38.9%) *S.* Senftenberg; two (11.1%) S*. enterica* subsp. *enterica* (O:6,7), *S*. Muenster and *S*. Anatum; and one isolate (5.5%) of each of the following serovars: *S. enterica* subsp. *enterica**, *S*. Infantis, *S*. Livingstone, *S*. Typhimurium and *S*. Agona. Furthermore, 13 (72.2%) of them were from bovine carcasses.

Minimum inhibitory concentration (MIC) of tetracycline and ampicillin (the more prevalent in the isolates), as well as nalidixic acid and ciprofloxacin were quantified through broth microdilution method, evaluating concentrations between 1 and 2048 $\mu g.mL^{-1}$ (varying with the antimicrobial) in the isolates that showed resistances to these antimicrobial agents (Table 2). Of 30 isolates resistant to tetracycline in the disc diffusion method, 21 (70%) presented MIC 32 $\mu g.mL^{-1}$, seven (23.3%) presented MIC 64 $\mu g.mL^{-1}$, and two isolates (6.7%) presented MIC 128 $\mu g.mL^{-1}$. Considering the MIC breakpoints described by CLSI (2018) for Enterobacteriaceae, all these isolates are tetracycline resistant, since the breakpoint value to this antimicrobial is 16 $\mu g.mL^{-}$

Table 2 - Distribution of the minimum inhibitory concentration for the *Salmonella* spp. isolates that presented resistance to ampicillin, tetracycline, nalidixic acid or ciprofloxacin in the agar disk diffusion method.

Antimicrobial agent	**Ranges of antimicrobial agents and MIC (μg.mL^{-1}) of the *Salmonella* spp. isolates that have resistance profile in the ADD method**														**Total of tested isolates**
	>1	1	2	4	8	16	32	64	128	256	512	1024	2048	>2048	
Ampicilin	0	0	0	0	0	0	0	0	0	1	0	0	0	26	27
Ciprofloxacin	0	1	1	3	4	0	0	0	0	0	0	0	0	0	9
Nalidixic acid	0	0	0	0	0	0	2	3	3	0	0	0	0	11	19
Tetracycline	0	0	0	0	0	0	21	7	2	0	0	0	0	0	30

ADD: agar disk diffusion method

MIC: minimum inhibitory concentration

Regarding to ampicillin (CLSI 2018 MIC breakpoint 32 μg.mL^{-1}), one isolate (S41) presented MIC 256 μg.mL^{-1} and the other 26 were resistant even to the highest tested concentration (2048 μg.mL^{-1}). Although the resistance to ampicillin is very consolidated in *Salmonella* spp., the high MIC values obtained are of concern, since not even the highest tested concentration (2048 μg.mL^{-1}) of this antimicrobial was capable to inhibit the *Salmonella* isolates tested.

The resistance to nalidixic acid (CLSI 2018, MIC breakpoint 32 μg.mL^{-1}) was quantified in 19 isolates of which two showed MIC 32 μg.mL^{-1} (10.5%), three presented MIC 64 and three MIC 128 μg.mL^{-1} (15.8%). Of these eight resistant isolates, seven presented intermediate resistance in the disc diffusion method. Most isolates (11) resistant to nalidixic acid (57.9%), were resistant even to the highest tested concentration (2048 μg.mL^{-1}), and all of them presented resistance in the disc diffusion method, i.e, no one intermediate resistances. It's noteworthy that among these 11 isolates with highest MIC value, nine (81.8%) are *S.* Typhimurium.

Regarding the nine isolates that presented resistance to ciprofloxacin (all intermediate resistances according with CLSI 2018) in the disc diffusion method, only two presented MIC 1 and 2 μg.mL^{-1} (22.2%). Three isolates presented MIC 4 μg.mL^{-1} (33.3%), and most of the isolates (four, 44.4%) presented MIC 8 μg.mL^{-1}, being all of them considered resistant, since MIC breakpoint to this antimicrobial according to CLSI (2018) is 1 μg.mL^{-1} for *Salmonella* spp.. Fluoroquinolones are widely used to treat respiratory diseases in animals, so it is possible that the use of antimicrobial agents of this class, such as ciprofloxacin, may contribute to propagate an acquired resistance to this drug among *Salmonella* spp. isolates (McDermott, Zhao and Tate, 2018).

3.3 Genotypic resistance

Salmonella isolates with phenotypic resistance profile were evaluated for the presence of antimicrobial genes described in item 2.4. Of the 37 isolates that presented antimicrobial phenotypic resistance profile, 31 harbored antimicrobial resistance genes: *aadA* (8 isolates, 25.8%), *int1* (13 isolates, 41.9%), *strA* (10 isolates, 32.2%) and *strB* (11 isolates, 35.5%), that confers resistance to aminoglycosides; bla_{TEM} (22 isolates, 70.9%), that confers resistance to β-lactams;

sul1 (16 isolates, 51.6%) and *sul2* (8 isolates, 25.8%), that confers resistance to folate pathway inhibitors; *catA1* (1 isolate, 3.2%) and *floR* (18 isolates, 58.1%), that confers resistance to phenicols; *tet(A)* (22 isolates, 70.9%) and *tet(B)* (7 isolates, 22.6%), that confers resistance to tetracyclines (Table 1). It is observed that, in the same way that phenotypic resistance to tetracycline was the most prevalent among the isolates, *tet* genes were also the most detected, followed by bla_{TEM} gene, which may have been responsible for the high rate of the ampicillin resistance found in this study.

Zhu et al. (2017) also detected similar rates of tetracycline-resistant *Salmonella* spp. isolates from broiler chickens in China that harbored at least one *tet* gene (85.7%); however, the authors observed that the *tet(B)* gene was more prevalent (50%) than *tet(A)* gene (23.5%). According Haubert, Cruxen, Fiorentini and Da Silva (2018a), the presence of *tet* genes is a challenging problem due to the fact that they are usually encoded by mobile genetic elements, such as conjugative plasmids and/or transposons, highlighting a possible transfer of these resistance genes to other bacteria. Furthermore, among efflux pumps *tet* that have already been identified in *Salmonella* isolates, the *tet(A)* and *tet(B)* genes have been found the most frequently detected (Michael and Schwarz, 2016).

Plasmids were detected in 77.4% (n=24) of the MDR isolates (n=31). In all MDR isolates with resistance profile to tetracycline, ampicillin, sulfonamides and chloramphenicol, the presence of the respective genes that confer these resistances was evaluated in the plasmid DNA. Plasmid-encoded *tet(A)* and *tet(B)* genes were detected in 34.8% (n=8) and 13% (n=3) of the MDR isolates with phenotypic tetracycline resistance, respectively. The bla_{TEM} gene was detected in the plasmid DNA of 15% (n=3) of MDR isolates resistant to ampicillin. Furthermore, plasmid-encoded *sul1* and *sul2* genes were detected in 35.7% (n=5) and 14.3% (n=2) of the MDR isolates with phenotypic sulfonamides resistance, respectively. Finally, the *floR* gene was detected in the plasmid DNA of 31.25% (n=5) of MDR isolates resistant to chloramphenicol.

Corroborating these results, Castro-Vargas, Herrera Sanchéz and Rondón-Barragán (2021) reported *S*. Heidelberg isolates from poultry farms in Colombia presenting *tet(B)*, *sul1* and *sul2* genes located in plasmids in the study. However,

tet(A) and *bla*$_{TEM}$ genes were not detected in plasmids of the same isolates. The presence of plasmids harboring antimicrobial resistance genes in *Salmonella* isolates, which is of concern, since these mobile genetic elements enable the transference of resistance determinants to other bacteria, spreading this characteristic in a bacterial population (Sin et al., 2020).

3.4 Mutations in quinolones resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *parC* and *parE*

No point mutations were detected in the QRDR of the *gyrA, gyrB, parC and parE* in the evaluated isolates S27 and S39, which were resistant to quinolones and fluoroquinolones. According to Munita and Arias (2016), resistance to this antimicrobial class in bacteria can occur due to three different mechanisms: through mutations in the genes *gyrA*, *gyrB*, *parC* and *parE*, that encode the target site of this antimicrobial agent (DNA gyrase and topoisomerase IV); by over-expression of efflux pumps that expel the drug from the cell; and through the protection of the DNA gyrase and topoisomerase IV by the quinolone resistance protein, called Qnr protein, codified by *qnr* genes, located in plasmid-mediated quinolone resistance (PMQR) (Cuypers et al., 2018). The fact that more than one mechanism may be involved resistance to these compounds may explain the absence of mutations on QRDR in the evaluated genes of isolates resistant to quinolones and fluoroquinolones observed in this study.

3.5 Genetic diversity

The genetic diversity of *Salmonella* spp. isolates was analysed using *Xba*I-macrorestriction. Were selected isolates belonging to the five prevalent serovars: isolates belonging to the serovars *S.* Typhimurium (n=12), *S.* Anatum (n=10), *S.* Heidelberg (n=5), and *S.* Derby (n=5), all from meat products. Seven isolates of *S*. Senftenberg (the more prevalent in bovine carcasses) also were included in this analysis (Fig. 1). The *S*. Seftenberg isolates from bovine carcasses were separated in six different *Xba*I-restriction profiles (P1 to P6). Only two isolates (S14 and S15) shared the same *Xba*I-restriction profile (P5), with 100% of similarity (Fig. 1.A). Furthermore, the isolates S12, S13 and S16 were classified as P1, P2 and P3, respectively, sharing more than 90% of similarity among them. It should be noted that

all seven *S*. Seftenberg isolates from bovine carcasses were susceptible to all tested antimicrobials (Table 1).

Salmonella Typhimurium from meat products showed a wide genetic diversity, given that among the 12 *S.* Typhimurium isolates, 12 different *Xba*I-restriction profiles (P7 to P18) were detected. Only the isolates S32 and S39, from sausages of different establishment, showed more than 70% of similarity among them (Fig. 1.B). The isolates are from many different sources, varying from salami and smoked sausages until bacon and cooled pork. Regarding antimicrobial resistance, only one isolate (S22) was susceptible to all tested antimicrobials; one isolate (S52) was resistant only to cephalothin, and ten isolates presented a MDR profile, including resistance to cefotaxime (S55). This data is of concern, since demonstrates that have a wide diversity of MDR *S*. Typhimurium profiles circulating in retail products in this region, representing a high risk to consumer's health. One isolate of *S*. Typhimurium (S49) was not digested by *Xba*I enzyme, and was not included in the dendrogram (Fig. 1.B). Han et al. (2020) used PFGE for molecular typing of *Salmonella* spp. and also failed to carry out cleavage by the *Xba*I enzyme of eight isolates. According to Soyer et al. (2010), digestion with *Xba*I may not be efficient enough in some isolates, requiring the use of additional enzymes, such as *Bln*I.

For the 10 *S*. Anatum isolates from meat products, 10 *Xba*I-restriction profiles (P19 to P28) were identified. The isolates S24 and S26, from smoked sausage and fresh sausage from different establishment, showed 83% of similarity, however, while the isolate S24 was resistant only to cephalothin, the isolate S26 was MDR and susceptible to cephalothin. Realpe-Quintero et al. (2018) also found *S*. Anatum isolates presenting low genetic similarity (5.33 to 35.88% of genetic similarity), and high variability in their antimicrobial resistance phenotypes, as observed in this study.

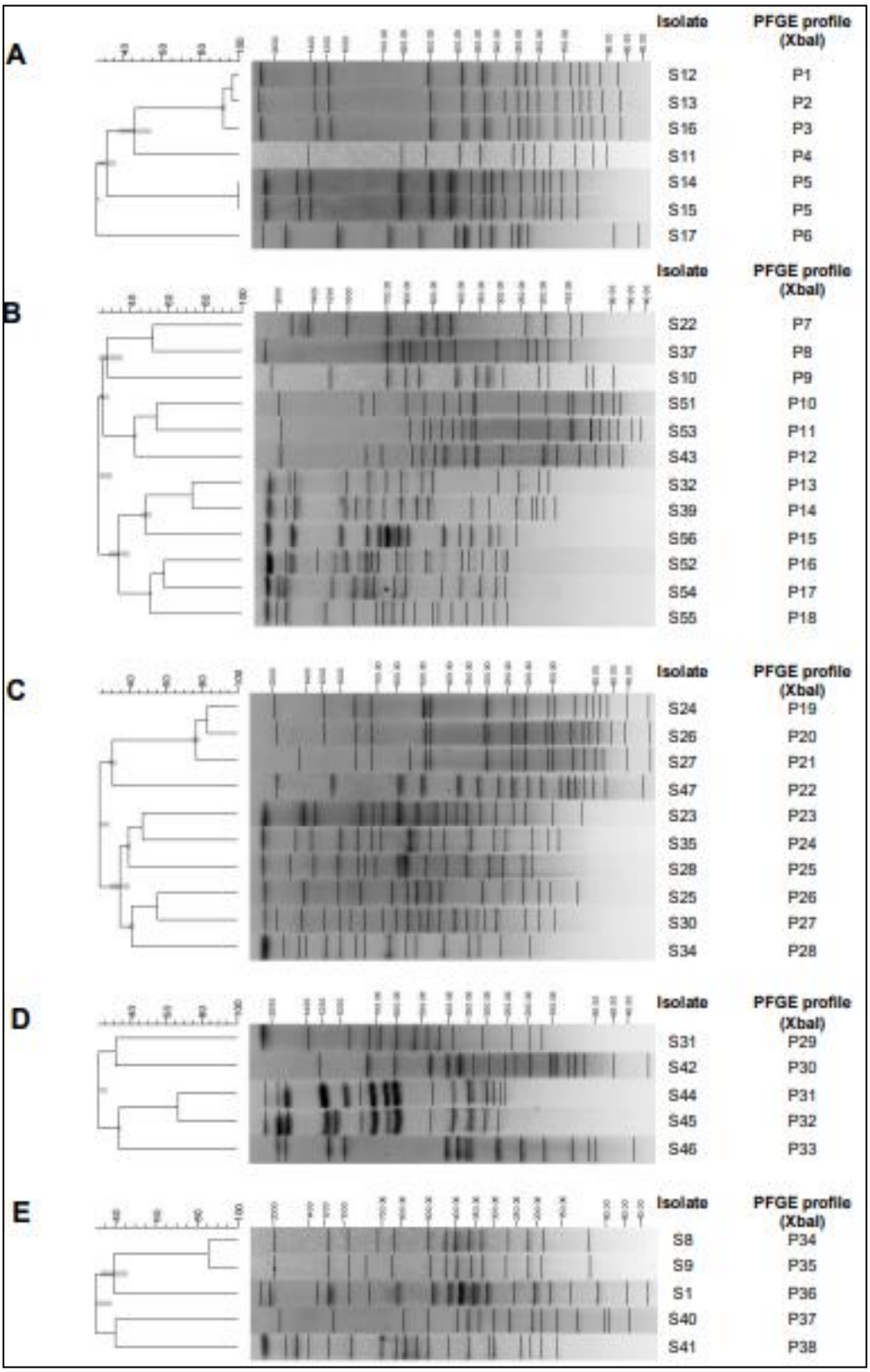

Figure 1 - Dendrogram of the genetic relationships among *Salmonella* isolates: (**A**) *S.* Senftenberg from bovine carcasses; (**B**) *S.* Typhimurium from meat products; (**C**) *S.* Anatum from meat products; (**D**) *S.* Heidelberg from meat products; (**E**) *S.* Derby from meat products; analysed by PFGE, using *Xba*I endonuclease, with Dice correlation coefficient , with maximal position tolerance of 1.5%. Using Molecular Weight Marker *Salmonella* Braenderup H9812 digested with *Xba*I endonuclease

Genetic diversity among *S*. Heidelberg isolates was also observed, being generated five *Xba*I-restriction profiles (P29 to P33) (Fig. 1.D). The isolates S44 and S45 showed PFGE profiles with more than 70% of similarity. All five *S*. Heidelberg isolates presented MDR profile, and were from sausages (three of them from smoked sausages). *Salmonella* Derby PFGE showed five *Xba*I-PFGE profiles (P34 to P38), with the isolates S8 and S9 being the most genetic related, with 85% of similarity (Fig. 1.E). The first isolate was from pork sausage and showed a MDR profile, while the isolate S9 was from poultry sausage, and was resistant only to nalidixic acid.

Realpe-Quintero et al. (2018) also studied the genetic profiles by PFGE of antimicrobial resistant *S*. Typhimurium from beef production chain, and also observed high genetic variability among the isolates, as well as verifying clonality among some isolates. The authors identified 28 pulsotypes grouped in two major clusters, with similarities varying between 67 and 100% in the cluster II, which included 21 isolates. Furthermore, Proietti et al. (2020), evaluating the PFGE profiles and the resistance patterns of *S.* Infantis of broiler meat production, reported that isolates sharing the same MDR profile not necessarily belonged to the same genetic profile, as well as was observed by the *S*. Derby isolates S1 and S8, and *S*. Heidelberg isolates S42 and S46, in this study, which concluded that was no relationship between the PFGE profiles and the resistance patterns of *S.* Infantis of broiler meat production.

4 Conclusions

In conclusion, it was observed the prevalence of *S*. Typhimurium in the meat sources evaluated, however, other ten serovars were also observed. *Salmonella* isolates harboring resistance genes in plasmids showed variability in their genomic structure evaluated by PFGE, as well as in their phenotypic and genotypic resistance profiles, including elevated MIC values for AMP and NAL. These findings support the requirement of surveillance programs in Brazil to better trace the dissemination of MDR *Salmonella* serovars in meat chain, and indicate the need of prudent use of antimicrobial agents in both human and veterinary medicine. The large number of *Salmonella* spp. resistant to antimicrobials, including MDR, in meat products sold in the evaluated region is a potential risk to human health, both in relation to salmonellosis itself, and in the difficulty of therapeutic options for the disease.

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Table S1 - Oligonucleotides used in this study.

Target genes	Sequence (5’ – 3’)	PCR conditions*	Annealing temperature (°C)	Amplicon size (bp)	Reference
hilA	Fw: GCGAGATTGTGAGTAAAAACACC Rv: CTGCCCGGAGATATAATAATCG	P1	63	413	Crâciunas, Keul, Flonta, & Cristea (2012)
invA	Fw: TTGTTACGGCTATTTTGACCA Rv: CTGACTGCTACCTTGCTGATG	P2	53	521	Swamy, Barnhart, Lee, & Dreesen (1996)
sefA	Fw: GCAGCGGTTACTATTGCAGC Rv: TGTGACAGGGACATTTAGCG	P2	53	330	Woodward , & Kirwan (1996)
pefA	Fw: TTCCATTATTGCACTGGGTG Rv: AAGCCACTGCGAAAGATGCC	P2	53	497	Haneda et al. (2001)
spvC	Fw: CGGAAATACCATCTACAAATA Rv: CCCAAACCCATACTTACTCTG	P2	53	669	Swamy, Barnhart, Lee, & Dreesen (1996)
blaZ	Fw: ACTTCAACACCTGCTGCTTTC Rv: TGACCACTTTTATCAGCAACC	P3	56	172	Martineau et al. (2000)
bla_{TEM}	Fw: ATGAGTATTCAACATTTCCG Rv: TTAATCAGTGAGGCACCTAT	P4	50	851	Grimm et al. (2004)
aadA	Fw: GTGGATGGCGGCCTGAAGCC Rv: ATTGCCCAGTCGGCAGCG	P5	60	526	Sandvang & Aarestrup (2000)

aadB	Fw: GGGCGCGTCATGGAGGAGTT Rv: TATCGCGACCTGAAAGCGGC	P5	58	328	Sandvang & Aarestrup (2000)
aac(6')-Ib	Fw: TTGCGATGCTCTATGAGTGGCTA Rv: CTCGAATGCCTGGCGTGTTT	P6	60	482	Park et al. (2006)
strA	Fw: TGACTGGTTGCCTGTCAGAGG Rv: CCAGTTGTCTTCGGCGTTAGCA	P7	64	645	Kehrenberg & Schwarz (2001)
strB	Fw: ATCGTCAAGGGATTGAAACC Rv: GGATCGTAGAACATATTGGC	P7	56	510	Kikuvi et al. (2007)
int1	Fw: CGGAATGGCCGAGCAGATC Rv: CAAGGTTCTGGACCAGTTGCG	P4	50	871	Sandvang, Diggle, & Platt (2002)
sul1	Fw: ATGGTGACGGTGTTCGGCATTCTG Rv: CTAGGCATGATCTAACCCTCGGTT	P8	64	840	Grape, Sunström, & Kronvall (2003)
sul2	Fw: GCGCTCAAGGCAGATGGCATT Rv: GCGTTTGATACCGGCACCCGT	P9	69	293	Kerrn, Klemmensen, Frimodt-Moller, & Espersen (2002)

sul3	Fw: GGGAGCCGCTTCCAGTAAT Rv: TCCGTGACACTGCAATCATTA	P10	57	500	Chuanchuen, Koowatananukul, & Khemtong (2008)
dfrA	Fw: CCTTGGCACTTACCAAATG Rv: CTGAAGATTCGACTTCCC	P11	52	350	Perreten et al. (2005)
dfrD	Fw: GGGCAGATTTGTTTAGTAAGG Rv: GTATCTCCTTCGAATTCATGATG	P11	52	785	Bertsch et al. (2013a)
dfrG	Fw: TTTCTTTGATTGCTGCGATG Rv: CCCTTTTTGGGCAAATACCT	P11	52	422	Bertsch et al. (2013b)
tetA	Fw: GTAATTCTGAGCACTGT Rv: CCTGGACAACATTGCTT	P12	43	953	Frech & Schwarz (2000)
tetB	Fw: ACGTTACTCGATGCCAT Rv: AGCACTTGTCTCCTGTT	P12	50	1169	Frech & Schwarz (2000)
catA1	Fw: GGCATTTCAGTCAGTTG Rv: CATTAAGCATTCTGCCG	P7	45	550	Kikuvi et al. (2007)
floR	Fw: AGGGTTGATTCGTCATGACCA Rv: CGGTTAGACGACTGGCGACT	P13	55	765	Kadlec, Kehrenberg, & Schwarz (2007)
gyrA	Fw: AGGGTTGATTCGTCATGACCA Rv: CGGTTAGACGACTGGCGACT	P4	53	313	Wajid et al. (2019)

gyrB	Fw: AGGGTTGATTCGTCATGACCA Rv: CGGTTAGACGACTGGCGACT	P4	53	181	Wajid et al. (2019)
parC	Fw: AGGGTTGATTCGTCATGACCA Rv: CGGTTAGACGACTGGCGACT	P4	53	270	Wajid et al. (2019)
pare	Fw: AGGGTTGATTCGTCATGACCA Rv: CGGTTAGACGACTGGCGACT	P4	53	240	Wajid et al. (2019)

Fw: forward primer; Rv: reverse primer

*PCR conditions: P1 (initial denaturation at 94 °C/4 min., followed by 30 cycles of denaturation at 94 °C/60 s, annealing according with the respective gene for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/10 min.); P2 (initial denaturation at 94 °C/2 min., followed by 35 cycles of denaturation at 94 °C/30 s, annealing according with the respective gene for 45 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P3 (initial denaturation at 95 °C/5 min., followed by 28 cycles of denaturation at 95 °C/30 s, annealing according with the respective gene for 30 s, and extension at 72 °C/45 s, ending with final extension of 72 °C/5 min.); P4 (initial denaturation at 95 °C/5 min., followed by 30 cycles of denaturation at 95 °C/60 s, annealing according with the respective gene for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P5 (initial denaturation at 94 °C/5 min., followed by 30 cycles of denaturation at 94 °C/45 s, annealing according with the respective gene for 45 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P6 (initial denaturation at 94 °C/4 min., followed by 34 cycles of denaturation at 94 °C/45 s, annealing according with the respective gene for 45 s, and extension at 72 °C/45 s, ending with final extension of 72 °C/10 min.); P7 (initial denaturation at 94 °C/2 min., followed by 30 cycles of denaturation at 94 °C/60 s, annealing according with the respective gene for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P8 (initial denaturation at 94 °C/5 min., followed by 30 cycles of denaturation at 94 °C/60 s, annealing according with the respective gene for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P9 (initial denaturation at 94 °C/5 min., followed by 30 cycles of denaturation at 94 °C/15 s, annealing according with the respective gene for 30 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P10 (initial denaturation at 94 °C/5 min., followed by 30 cycles of denaturation at 94 °C/45 s, annealing according with the respective gene for 3 min., and extension at 72 °C/5 min., ending with final extension of 72 °C/10 min.); P11 (initial denaturation at 95 °C/5 min., followed by 30 cycles of denaturation at 95 °C/60 s, annealing according

with the respective gene for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/5 min.); P12 (initial denaturation at 94 °C/4 min., followed by 34 cycles of denaturation at 94 °C/60 s, annealing according with the respective gene for 2 min., and extension at 72 °C/3 min., ending with final extension of 72 °C/7 min.); P13 (initial denaturation at 95 °C/2 min., followed by 25 cycles of denaturation at 95 °C/60 s, annealing according with the respective gene for 30 s, and extension at 72 °C/2,5 min., ending with final extension of 72 °C/7 min.).

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5 Manuscrito 2 – Tolerance to sanitizers in *Salmonella* spp. from bovine carcasses and meat products in Southern Brazil with different profiles of susceptibility and resistance to antimicrobials

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Tolerance to sanitizers in *Salmonella* spp. from bovine carcasses and meat products from Southern Brazil with different profiles of resistance to antimicrobials

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Abstract

The aim of this study was to evaluate *Salmonella* spp. from bovine carcasses and meat products showing different antimicrobial resistance profiles and relating it to their profile of susceptibility to sanitizers commonly used in slaughterhouses and food processing environments. Fifty five *Salmonella* spp. from bovine carcasses and meat products with different profiles of resistance to antimicrobials were evaluated for their tolerance to four sanitizers. Minimum inhibitory concentration (MIC) of benzalkonium chloride (BKC), chlorhexidine (CLX), peracetic acid (PAC) and sodium hypochlorite (SH) was determined, being 128, 32, 2048 and 2048 $\mu g.mL^{-1}$ the highest MIC values found, respectively, being the *Salmonella* spp. isolates less tolerant to BKC and CLX than to PAC and SH. *Salmonella* Typhimurium was the serovar that showed the highest MIC values for BKC. Besides, almost 90% of isolates that presented the highest values of MIC for BKC were from meat products. Although all isolates that showed higher MIC values for BKC were resistant to antimicrobials, and 80% of the less tolerant isolates to CLX were susceptible, there was no relationship between to tolerance to sanitizers and antimicrobial profile in the *Salmonella* isolates evaluated. The *qacEΔ1* gene was the only one detected, in 27.3% of isolates, which is linked with the *int1* gene, suggesting that there is a relationship between tolerance to sanitizers and antimicrobial resistance in these isolates. The data of present study highlights the importance of conscious use of sanitizers in the food industry, since *Salmonella* spp. isolates showed tolerance to sanitizers widely used in industry sanitation programs, increasing the chances of the pathogen remaining in the environment and causing salmonellosis infection in the final consumer.

Keywords: benzalkonium chloride; chlorinated compounds; efflux pumps; good manufacturing practices; salmonellosis

1 Introduction

Good manufacturing practices (GMP) are established by competent organizations of each country for help food industry to ensure the food safety, including appropriate personal hygienic practices, as well as maintenance of sanitary operations and sanitization of equipments and surfaces during food production process (FDA, 2020), in order to avoid the persistence of pathogenic bacteria, such as *Salmonella* spp., in the food processing plant. The presence of this bacterium in the final product can lead to salmonellosis in the consumer, in addition to causing financial loss to food producers, since it makes the product unfit for consumption, according to current Brazilian legislation (Brazil, 2022).

Sanitizers are biocides used by food industry in sanitization steps. There are different classes of sanitizers: chlorinated compounds, such as sodium hypochlorite (SH); oxidizing agents, such as peracetic acid (PAC); and surface active compounds, such as benzalkonium chloride (BKC), which represents the class of quaternary ammonium compounds (QAC). All these sanitizers are widely used in food industries (Kumar and Anand, 1998; Van Houdt and Michiels, 2005); while chlorhexidine (CLX) is more used in hospital environments (Zheng et al., 2022).

The bacterial tolerance to sanitizers occurs mainly due the frequent use of these compounds on surfaces of food processing environments (Bridier, Briandet, Thomas and Dubois-Brissonnet, 2011). The resistance to sanitizers is considered a major problem for the food industry, since antimicrobial-resistant bacteria can persist in food processing plants, contaminating the final product and increasing the chances of *Salmonella* reaching the final consumer and causing illness (Møretrø and Langsrud, 2017). The mechanisms of tolerance to sanitizers in *Salmonella* spp. are not well understood. It is know that sanitizers have a large spectrum of activity, acting on multiple target sites, causing a series of changes which affect membrane integrity and bacteria cell morphology (Humayoun et al., 2018). Thus, antimicrobial resistance, which occurs as a result of changes at specific target sites in the bacterial cell, will rarely result in resistance to sanitizers. Sanitizers and antimicrobials have different mechanisms of action; however, there is the possibility of cross-resistance between these compounds. Genes encoding sanitizers tolerance can be frequently found in horizontally transferred mobile genetic elements, such as integrons, that also

carry antimicrobial resistance genes, causing a spread of resistance genes among bacteria (Antunes, Machado and Peixe, 2006; McCarthy and Lindsay, 2012). A relationship has already been observed between the presence of the integrase codified by the gene *int1* in and the presence of *qacEΔ1* gene in *Salmonella* spp. isolates with QAC resistance (Siriken, Türk, Yildirim, Durupinar and Erol, 2015; Zou et al., 2014). In the same way, the resistance to QAC was related to the presence of antimicrobial resistance genes in *Salmonella* spp. isolates (Wu et al., 2015).

Therefore, this study aimed to evaluate *Salmonella* spp. from bovine carcasses and meat products showing different antimicrobial resistance profiles, relating it to their profile of tolerance to sanitizers commonly used in slaughterhouses and food processing environments.

2 Material and Methods

2.1 *Salmonella* spp. isolates

A total of 55 *Salmonella* spp. isolates from bovine carcasses and meat products, previously characterized, were evaluated in this study (Table S1). These isolates were provided by the culture collection of the Laboratório de Microbiologia de Alimentos (DCTA-FAEM-UFPel), located in Pelotas city, Southern Brazil.

2.2 Sanitizers tolerance testing

The susceptibility of the *Salmonella* isolates to sanitizers compounds was evaluated by performing the minimum inhibitory concentration (MIC), using the broth microdilution method according to Wu et al. (2015). Four sanitizers compounds were used: benzalkonium chloride (BKC) (Sigma-Aldrich, UK), chlorhexidine digluconate (CLX) (Sigma-Aldrich, UK), peracetic acid (PAC) (Proc9, Brazil) and sodium hypochlorite (SH) (Dinâmica Química Contemporânea, Brazil). Firstly, the inoculum was prepared at ~0.5 x 10^8 McFarland standard with 37 ^oC for 24h growth on TSA and 0.85% (w/v) saline solution (SS) (Synth, Brazil). The concentrations of sanitizers were prepared using a stock solution at 2048 $\mu g.mL^{-1}$, diluting until 1 $\mu g.mL^{-1}$ in Mueller-Hinton broth (Kasvi, Brazil). Microtiter plates were filled with the inoculum and the sanitizers concentrations in a proportion of 1:9 (20/180 µL), with incubation of the microtiter plates at 37 ºC for 24 h. The MIC of sanitizers was defined as the

lowest concentration that completely inhibited the bacterial growth, evaluated visuality.

2.3 Detection of genes related to sanitizers tolerance

The isolates also were evaluated for the presence of genes related to sanitizers tolerance. Genomic DNA was extracted according to Ellington, Kistler, Livermore and Woodford (2007), with minor modifications, dissolving colonies of *Salmonella* isolates in 200 µL of ultrapure water (UPW) and freezing at -20 ^{o}C for at least 1 h. The genes that encode sanitizer tolerance to QAC (*qacA*, *qacEΔ1* and *smr*) and efflux pumps (*mepA*, *norA* and *norB*) were investigated by PCR assays using the oligonucleotides listed in Table 1. The cycling conditions of the PCR assays were the following for QAC genes: initial denaturation at 95 °C/5 min, followed by 30 cycles of denaturation at 95 °C/60 s, annealing according with the respective gene (Table 1) for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min. Regarding the efflux pumps genes, the PCR conditions were: initial denaturation at 94 °C/4 min, followed by 35 cycles of denaturation at 94 °C/30 s, annealing according with the respective gene (Table 1) for 55 s, and extension at 72 °C/55 s, ending with final extension of 72 °C/5 min. The PCR products were submitted to an electrophoretic run in an agarose gel 1.5% (Ludwig Biotecnologia©, Brazil), stained with GelRed® at 80 V for 70 min using a molecular weight marker of 1 Kb (Invitrogen™, USA). The amplified products were visualized in an UV transilluminator (Loccus Biotecnologia®, Brazil).

3 Results and discussion

3.1 Minimum inhibitory concentration to sanitizers

Minimum inhibitory concentration (MIC) of the four tested sanitizers was quantified (Table 2). Regarding to BKC, among 55 *Salmonella* isolates, 46 (83.6%) presented MIC 32 µg.mL^{-1}, seven (12.7%) presented MIC 64 µg.mL^{-1}, and two isolates (3.6%) presented MIC 128 µg.mL^{-1}. It is noteworthy that among the nine isolates that showed the highest MIC values (64 and 128 µg.mL^{-1}) for BKC, six (66.6%) belonged to the serovar Typhimurium (Table S1). Long et al. (2016) also found similar results evaluating the susceptibility to disinfectant of different

Salmonella serovars isolated from chicken and egg production chains. Although other *Salmonella* serovars are increasingly involved in foodborne salmonellosis outbreaks, serovar Typhimurium is still the most involved non-typhoidal *Salmonella* serovar in most parts of the world (WHO, 2018), and is often associated with the consumption of contaminated meat, such as poultry, pork and beef (EFSA, 2015). Furthermore, among the nine isolates with the highest MIC values (64 and 128 $\mu g.mL^{-1}$) for BKC, eight (88.9%) were from meat products, suggesting that this sanitizer may be being used in underdoses or the sanitation program is ineffective in the environments where these meat products were produced. Misuse can select *Salmonella* spp. with tolerance to these compounds, facilitating their persistence in the food production environment. Humayoun et al. (2018) evaluated 88 multidrug-resistant (MDR) *Salmonella* spp. isolates from animals (cattle, swine, chickens, turkeys, horses, dogs and cats) and retail meat, and observed that 95% of isolates presented MIC 40 $\mu g.mL^{-1}$ for BKC, similar to the found in most of the isolates in this study.

Table 1 - Oligonucleotides used in this study

Target genes	Sequence (5’ – 3’)	Annealing temperature (°C)	Amplicon size (bp)	Reference
qacA	Fw: ACTACTGATATGATGACATCA Rv: AGTTATATCAAGTGATTTGGG	53	1512	Mereghetti, Quentin, Mee, & Audurier (2000)
qacEΔ1	Fw: ATCGCAATAGTTGGCGAAGT Rv: CAAGCTTTTGCCCATGAAGC	53	225	Paulsen et al. (1993)
smr	Fw: ATAGCCATAAGTACTGAAGTT Rv: ACCGAAAATGTTTAACGAAAC	53	330	Mereghetti, Quentin, Mee, & Audurier (2000)
mepA	Fw: ATGTTGCTGCTGCTCTGTTC Rv: TCAACTGTCAAACGATCACG	61	718	Hassanzadeh et al. (2017)
norA	Fw: TTCACCAAGCCATCAAAAAG Rv: CTTGCCTTTCTCCAGCAATA	60	620	Hassanzadeh et al. (2017)
norB	Fw: AGCGCGTTGTCTATCTTTCC Rv: GCAGGTGGTCTTGCTGATAA	62	213	Hassanzadeh et al. (2017)

Fw: forward primer; Rv: reverse primer

Table 2 - Distribution of the minimum inhibitory concentration of sanitizers compounds for the *Salmonella* spp. isolates from bovine carcasses and meat products.

Sanitizers	**MIC (µg.mL^{-1}) of the *Salmonella* spp. isolates for sanitizers compounds**									**Total of tested isolates**
	8	16	32	64	128	256	512	1024	2048	
Benzalkonium chloride	0	0	46	7	2	0	0	0	0	55
Chlorhexidine	10	34	11	0	0	0	0	0	0	55
Peracetic Acid	0	0	0	0	0	0	0	36	19	55
Sodium hypochlorite	0	0	0	0	0	0	0	30	25	55

MIC: minimum inhibitory concentration

For CLX, most isolates (n = 34, 61.8%) presented MIC 16 μg.mL^{-1}, while 10 (18.2%) and 11 (20%) isolates presented MIC 8 and 32 μg.mL^{-1}, respectively, being all 11 isolates with MIC 32 μg.mL^{-1} from meat products (Table S1). Humayoun et al. (2018) evaluated *Salmonella* spp. from animals and retail meat and observed that the isolates were more susceptible to this compound, with MIC 3 μg.mL^{-1} for most isolates (29%), followed by MIC 12 μg.mL^{-1} in 25% of isolates. Although chlorhexidine is less used in the food industry in comparison with BKC, PAC and SH, tolerance of *Salmonella* isolates to this compound is an important result, since this sanitizer is widely used in patient care and other settings, such as consumer products and households (Kampf, 2019), and can also contribute to the spread of sanitizers tolerant *Salmonella* in the environment.

Visible bacterial growth was observed in the highest concentrations tested of PAC and SH. For PAC, 36 isolates (65.4%) presented MIC 1024 μg.mL^{-1}, while other 19 isolates (34.5%) presented MIC 2048 μg.mL^{-1} (Table 1). Similar results were observed to SH, which inhibited 30 isolates (54.5%) with a concentration of 1024 μg.mL^{-1}, and 25 isolates (45.5%) in a concentration of 2048 μg.mL^{-1}.

Although there are no cut-off values to define bacterial tolerance to sanitizers compounds, WHO recommends limits for the use of this biocides for sanitizing surfaces, which are 0.1% for SH and 0.5% for PAC, equivalent to 1000 and 5000 μg.mL^{-1}, respectively (WHO, 2020). According to this recommendation, 45.5% (n=25 with MIC 2048 μg.mL^{-1}) of *Salmonella* spp. isolates were tolerant to twice the concentration recommended for SH. For PAC, all 55 isolates presented MIC values below the limit established by WHO. Both PAC and SH are widely used in different sanitization steps in the food processing line (Lee and Huang, 2019). Humayoun et al. (2018) found that 90% of the *Salmonella* isolates from animals and retail meat had MIC values between 880 and1760 μg.mL^{-1} for PAC, and almost half of the isolates (43%) had MIC of 6304 μg.mL^{-1} for SH, a result significantly higher than found in this study, therefore, they were more tolerant to SH than *Salmonella* spp. isolates in the present study. Regarding the source of the isolates, there was no relationship between the source of the isolates and their tolerance to PAC and SH.

Among nine isolates showing higher MIC values for BKC (64 and 128 μg.mL^{-1}), five (55.5%) presented MDR profile, and the other four isolates (44.4%) presented

resistance to only one antimicrobial (streptomycin, tetracycline, cephalothin or nalidixic acid) (Table S1). Furthermore, among the 10 isolates with the lowest MIC values for CLX (8 μg.mL^{-1}), eight (80%) were susceptible to all antimicrobials tested (Table S1). Regarding the 11 isolates with the highest MIC value for this biguanide (32 μg.mL^{-1}), seven (63.6%) were MDR, three (27.3%) presented resistance to cephalothin or nalidixic acid, and only one isolate (9.1%) was susceptible to antimicrobials. These results suggest that the tolerance to BKC and CLX can be related with the antimicrobial profile of the isolates, since some isolates with the lowest MIC values for BKC and CLX were generally susceptible to all antimicrobials tested, and the highest MIC values for these compounds were mostly observed in isolates with an MDR profile. However, not all *Salmonella* MDR isolates showed high MIC values, as well as not all antimicrobial susceptible isolates showed lower tolerance to sanitizers. Chuanchuen, Pathanasophon, Khemtong, Wannaprasat, and Padungtod (2008) also evaluated the relationship between *Salmonella* isolates from poultry and swine with MDR profile and their tolerance to sanitizers, and verified that some *Salmonella* isolates with high MIC values to BKC and CLX showed antimicrobials resistance, however, the MDR isolates did not have higher MIC values for BKC or CLX than those with non-MDR profile.

No relationship was observed between PAC and SH and the antimicrobial profile of the isolates (Table S1). Humayoun et al. (2018) also did not found any clear relationship between tolerance to sanitizers and antimicrobial resistance in MDR *Salmonella* spp. isolates, highlighting that further studies are needed to clarify this relationship.

3.2 Genotypic resistance to sanitizers

The presence of genes encoding resistance to QAC (*qacA*, *qacEΔ1* and *smr*) and efflux pumps (*mepA*, *norA* and *norB*) was investigated. Only the *qacEΔ1* gene was detected in 27.3% (n = 15) of the isolates (Table S1). Among this 15 isolates, only two presented high MIC values for BKC (both 64 μg.mL^{-1}), being one from bovine carcass and one from meat product. Five (33.3%) and four (26.6%) of the isolates that carried the *qacEΔ1* gene belonged to serovars Anatum and Derby, respectively. Long et al. (2016), evaluating *Salmonella* spp. isolates from poultry and

eggs, also found prevalence of the *qacEΔ1* gene in *S*. Derby. These same authors also observed that *qacEΔ1* gene was the most prevalent among the QAC genes evaluated, being present in 26.7% of the isolates, similar rate to found in present study.

It was observed that 93.3% (n = 14) of isolates that carried *qacEΔ1* gene had an MDR profile, and one isolate was resistant to only one antimicrobial (isolate S7, resistant to streptomycin). The *qac* genes are frequently found in mobile genetic elements, usually encoded in integrons (Zou et al., 2014), such as class 1 integron, which is encoded by the *int1* gene. Corroborating this data, 73.3% (n = 11) of *Salmonella* isolates carrying *qacEΔ1* gene also carried the *int1* gene. According Chapman (2003), the *qacEΔ1* gene is located in the conserved 3' portion of class 1 integrons, which also codes for genes that may confer antimicrobial resistance, suggesting the relatedness between antimicrobial resistance and tolerance to QAC, such as BKC, in isolates that carry the genes *int1* and *qacEΔ1*.

4 Conclusions

Salmonella spp. from bovine carcasses and meat products isolated in southern Brazil were less tolerant to BKC and CLX than to PAC and SH, with *S*. Typhimurium showing the highest MIC values to BKC. The occurrence of *Salmonella* isolates from meat products with greater tolerance to BkC suggests the misuse of this sanitizer in these food industries. Eleven *Salmonella* spp. isolates carried the *qacEΔ1* gene which is related with the *int1* gene, suggesting that there is a relationship regarding sanitizers tolerance genetic mechanism between tolerance to sanitizers and antimicrobial resistance in these isolates.

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Table S1 **-** Characteristics of *Salmonella* spp. isolates from bovine carcass and meat products in Southern Brazil.

Origin	ID	Sample	Serovar	Antimicrobial Resistance Profile[a]	Antimicrobial and Sanitizers Resistance Genotype
Bovine Carcass	S3	After bleeding	Derby	STR-SUL-TET	*aadA, int1, sul1, tet(A), qacEΔ1*
	S4	After bleeding	*S. enterica* subsp. *enterica* (O:6,7)	Susceptible	-
	S5	After bleeding	Livingstone	Susceptible	-
	S6	After bleeding	*S. enterica* subsp. *enterica* (O:6,7)	Susceptible	-
	S7	After bleeding	Ohio	STR	*qacEΔ1*
	S11	After bleeding	Senftenberg	Susceptible	-
	S12	After bleeding	Senftenberg	Susceptible	-
	S13	After bleeding	Senftenberg	Susceptible	-
	S14	After bleeding	Senftenberg	Susceptible	-
	S15	After bleeding	Senftenberg	Susceptible	-
	S16	After bleeding	Senftenberg	Susceptible	-

	S17	After bleeding	Senftenberg	Susceptible	-
	S18	After bleeding	Muenster	Susceptible	-
	S19	After bleeding	Anatum	Susceptible	-
	S20	After bleeding	Muenster	Susceptible	-
Meat products	S1	Fresh sausage	Derby	STR-SUL-TET	*aadA, int1, sul1, tet(A), qacEΔ1*
	S2	White pudding	Infantis	Susceptible	-
	S8	Pork sausage	Derby	STR-SUL-TET	*aadA, int1, sul1, tet(A), qacEΔ1*
	S9	Poultry sausage	Derby	NAL	-
	S10	Skinless bacon	Typhimurium	AMP-CHL-GEN[b] -NAL-STR-SUL-SUT-TET-TRI	*aadA, bla_{TEM}, catA1, floR, int1, strA, strB, sul1, tet(A), qacEΔ1*
	S22	Beef	Typhimurium	Susceptible	-
	S23	Smoked mixed colonial sausage	Anatum	CFL[b]	-
	S24	Smoked mixed colonial sausage	Anatum	CFL[b]	-
	S25	Fresh mixed sausage	Anatum	AMP-CFL-CHL-CIP[b]-NAL[b]-STR-SUL-TET	*aadA, bla_{TEM}, floR, int1, sul1, tet(A), qacEΔ1*

S26	Fresh mixed sausage	Anatum	AMP-CHL[b]-CIP[b]-NAL[b]-STR[b]-SUL-SUT-TET-TRI	*aadA, bla*$_{TEM}$*, floR, int1, strA, strB, sul1, tet(A), qacEΔ1*
S27	Smoked dried sausage	Anatum	AMP-CFL[b]-CHL-CIP[b]-GEN[b]-NAL[b]-STR[b]-SUL-SUT-TET-TRI	*aadA, bla*$_{TEM}$*, floR, strA, strB, sul1, tet(A), qacEΔ1*
S28	Bacon	Anatum	Susceptible	-
S29	Pork fat	*S. enterica* subsp. *enterica* (O:4,5)	AMP-CFL[b]-CHL-CIP[b]-NAL[b]-TET-TRI	*bla*$_{TEM}$*, tet(A), tet(B)*
S30	Smoked mixed sausage	Anatum	AMP-CHL-CIP[b]-NAL[b]-TET	*bla*$_{TEM}$*, tet(B)*
S31	Smoked dried mixed sausage	Heidelberg	AMP-CHL-CIP[b]-CFL[b]-STR[b]-SUL	*aadA, bla*$_{TEM}$*, strA, strB, sul2*
S32	Fresh sausage	Typhimurium	AMP-CHL-CIP[b]-CFL[b]-NAL-STR-SUL-TET	*floR, strA, strB, sul2*
S33	Fresh sausage	Agona	Susceptible	-
S34	Cooled pork	Anatum	AMP-CHL-CIP[b]-NAL[b]-STR[b]-SUL-SUT-TET[b]-TRI	*bla*$_{TEM}$*, floR, int1, strA, strB, sul1, tet(A), qacEΔ1*
S35	Fresh sausage	Anatum	AMP-CFL[b]-CHL-NAL[b]-STR[b]-SUL-SUT-TET[b]-TRI	*bla*$_{TEM}$*, floR, int1, strA, strB, sul1, tet(A), qacEΔ1*
S36	Fresh	Senftenberg	AMP-SUL-SUT-	*bla*$_{TEM}$*, sul1, tet(A),*

	sausage		TET-TRI	*qacEΔ1*
S37	Pork	Typhimurium	AMP-CHL-CFL[b]-GEN-NAL-SUL-TET	*bla*$_{TEM,}$ *floR, int1, sul1, sul2, tet(A), tet(B)*
S38	Vacuum packed rump trim	Lexington	AMP-SUL-TET	*bla*$_{TEM}$*, sul1, tet(A), qacEΔ1*
S39	Pork sausage	Typhimurium	AMP-CFL[b]-CIP[b]-NAL-STR[b]-SUL-SUT-TET-TRI	*bla*$_{TEM}$*, int1, strA, strB, sul1, tet(A), qacEΔ1*
S40	Pork sausage	Derby	AMP-CHL-CFL[b]-TET	*floR, tet(A)*
S41	Pork sausage	Derby	AMP-CHL-SUL-STR-TET	*bla*$_{TEM}$*, floR, int1, sul1, qacEΔ1*
S42	Smoked mixed colonial sausage	Heidelberg	AMP-CHL-TET	*bla*$_{TEM}$
S43	Pork fat	Typhimurium	AMP-CFL[b]-CHL-NAL-TET	*bla*$_{TEM}$*, floR, tet(A)*
S44	Smoked mixed colonial sausage	Heidelberg	AMP-CHL-NAL-SUL-STR[b]-TET	*bla*$_{TEM}$*, floR, strA, strB, sul1, sul2, tet(B)*
S45	Fresh mixed sausage	Heidelberg	AMP-CHL-NAL-TET	*bla*$_{TEM}$*, floR, tet(A)*
S46	Fresh special pork	Heidelberg	AMP-CHL-TET	*bla*$_{TEM}$*, floR, tet(A)*

	sausage			
S47	Fresh pork sausage	Anatum	AMP-CHL-CFL[b]-STR-SUL	*floR, strA, strB, sul2*
S48	Fresh pork sausage	*S. enterica* subsp. *enterica**	Susceptible	-
S49	Smoked mixed colonial sausage	Typhimurium	TET	*tet(A), tet(B)*
S50	Fresh mixed sausage	*S. enterica* subsp. *enterica**	AMP-CFL[b]-CHL-TET	*bla*$_{TEM}$*, floR, tet(A)*
S51	Colonial salami	Typhimurium	AMP-CFL[b]-CHL[b]-NAL-SUL-TET	*floR, sul2, tet(B)*
S52	Smoked mixed sausage	Typhimurium	CFL[b]	-
S53	Pork	Typhimurium	AMP-CFL[b]-GEN[b]-NAL-SUL-TET	*int1, sul2, tet(B)*
S54	Smoked fresh mixed sausage	Typhimurium	CFL[b]-NAL-TET	-
S55	Fresh sausage	Typhimurium	AMP-CFL-CHL[b]-CTX-NAL-TET	*bla*$_{TEM}$, *tet(A)*
S56	Fresh sausage	Typhimurium	AMP-CFL-CHL-STR[b]-SUL-TET	*bla*$_{TEM}$*, floR, int1,strB, sul1, sul2, tet(A), qacEΔ1*

6 Manuscrito 3 – Occurrence, genetic diversity and resistance profiles of *Salmonella enterica* from Brazilian sausages collected at production facilities

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Occurrence, genetic diversity and resistance profiles of *Salmonella enterica* from Brazilian sausages collected at production facilities

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Abstract

This study aimed to investigate the occurrence and the genetic diversity of *Salmonella enterica* subsp. *enterica* in sausages from Southern Brazil, as well as evaluating virulence genes and determine the phenotypic and genotypic basis of antimicrobial and sanitizer resistance. *Salmonella* was detected in sausage samples with an overall prevalence of 5.5%. The prevalent serovars were *S*. Infantis and *S*. Rissen. Pulsed-field Gel Electrophoresis (PFGE) analysis yielded nine distinct PFGE profiles, and some of them were recurrently recovered in the same establishment on different dates. Among tested isolates, 28.5% showed resistance to at least one antimicrobial agent and multidrug-resistance (MDR) profile was observed in 21.4%. Resistance occurred most frequently to ampicillin, sulfonamide, trimethoprim/sulfamethoxazole and trimethoprim. Regarding the genotypic antimicrobial resistance profile, *S.* Schwarzengrund carried *tet(*B*)*, *strA*, *strB*, and *sul2* genes. Benzalkonium chloride and chlorhexidine were more effective than peracetic acid and sodium hypochlorite, showing lower minimum inhibitory concentration values. Six *Salmonella* serovars were found demonstrating a potential risk of salmonellosis associated to the consumption of this food. *Salmonella* carrying virulence genes, MDR profile, and tolerance to sanitizers is a public health concern and a challenge for the food industry, suggesting that new strategies should be developed to control this pathogen.

Keywords: PFGE; meat products; multidrug-resistance; antimicrobials; sanitizers.

1 Introduction

Nontyphoidal *Salmonella* is considered one of the most common causes of foodborne disease worldwide. In the United States of America (USA), the Centers for Disease Control and Prevention estimates that *Salmonella* cause about 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths every year (CDC 2022). The transmission of *Salmonella* to humans normally occurs by ingestion of contaminated food, including chicken, beef, pork, eggs, fruits, vegetables, and even processed foods (CDC 2022). *Salmonella* can cause salmonellosis, which is usually characterized by acute onset of fever, abdominal pain, diarrhea, nausea and sometimes vomiting (WHO 2018). Usually, human salmonellosis is self-limiting and does not require treatment unless it is severe, invasive, or occurs in the elderly, children, or people with underlying comorbidities (McDermott et al. 2018).

Most of the genes required for *Salmonella* pathogenicity are clustered in genomic islands known as *Salmonella* pathogenicity islands (SPIs). Until now, 23 SPIs have been described (Pavon et al. 2022). The *invA* gene, which encodes for the putative inner membrane InvA, an essential protein for the invasion of epithelial cells, is located in SPI-1, the most elucidated and studied among the SPIs (Pavon et al. 2022). Furthermore, some virulence genes can be located on plasmids, such as the *spvC* gene in the *spv* operon, which encodes for a protein associated with the systemic spread of *Salmonella* infection (Guiney and Fierer 2011).

In severe cases of salmonellosis, the use of antimicrobial therapy becomes necessary. In this way, *Salmonella* resistant to antimicrobial agents is a global public health concern, as it can make it difficult to treat the disease (WHO 2018). In addition, *Salmonella* resistant to sanitizers widely used in food industries and on livestock farms is another challenge, as it can facilitate the selection of strains that exhibit acquired resistance to sanitizers and can carry genes that encode cross-resistance to antimicrobials (Long et al. 2016; Tong et al. 2021).

In Southern Brazil, pork and chicken sausages are widely consumed and the presence of *Salmonella* in these products has already been reported. Meat products such as sausages have high water activity and rich nutritional composition, which favor the microbial growth. Moreover, sausages are exposed to more handling than meat, increasing the risk of contamination by foodborne pathogens in food

processing plants. Thus, the aims of this study were to investigate the occurrence and the genetic diversity of *Salmonella* in sausages from Southern Brazil, as well as evaluating virulence genes and determine the phenotypic and genotypic basis of antimicrobial and sanitizer resistance.

2 Material and Methods

2.1 Sampling

A total of 252 sausage samples collected at production facilities from 24 establishments submitted to sanitary inspection from Pelotas, Southern Brazil, were evaluated between the years 2010 and 2013. The samples included mixed sausage, pork sausage and pork sausage with cheese, chicken and chicken sausage with cheese, fresh Calabrese sausage, fresh pork sausage, fresh mixed sausage, smoked sausage, withe pudding, and black pudding in original packaging (Table 1).

2.2 Isolation of *Salmonella*

The detection of *Salmonella* was performed according to the method described by the International Organization for Standardization (ISO-6579-1:2017), with modifications. After pre-enrichment, samples were selectively enriched in Rappaport Vassiliadis broth (Acumedia®, Brazil) and Tetrationate broth (Acumedia®, Brazil) with incubation at 42 ºC and 37 ºC, respectively, for 24 h. Cultures were then streaked onto Hektoen Enteric agar (Himedia®, India) and Xylose Lysine Deoxycholate agar (Kasvi, Brazil) and incubated at 37 ºC for 24 h. The suspect colonies were subjected to biochemical tests, and the phenotypic confirmation of the *Salmonella* isolates was performed using serology tests with polyvalent somatic and flagellar anti-*Salmonella* sera (Probac, Brazil). The isolates were stored in Brain Heart Infusion broth (Kasvi, Brazil) with 20.0% of glycerol at -80 ^{o}C, until the beginning of the experiments.

2.3 Serotyping

Serological tests were performed at the National Reference Center, Fundação Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, by standard slide agglutination using commercially available antisera in order to characterize the *Salmonella* serovars.

2.4 Molecular typing

Pulsed-Field Gel Electrophoresis (PFGE) typing was performed using the standard CDC PulseNet protocol for *Salmonella* (Ribot et al. 2006), with minor modifications. Genomic DNA of *Salmonella* isolates and *S.* Braenderup H9812 (reference marker) were digested with 20 U of *Xba*I (Invitrogen™, USA) restriction enzyme. Macrorestriction fragments were separated in a CHEF-DR II system apparatus (Bio-Rad Laboratories Inc., USA). The gel was stained with ethidium bromide (stock solution of 20 $mg.mL^{-1}$, Sigma, USA) for 20 min and destained in distilled water, also for 20 min. The restriction fragments were viewed in a UV trans-illumination L-Pix system (Loccus Biotecnologia®, Brazil). Band patterns were analyzed with BioNumerics 7.1 (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice coefficient, with a maximal position tolerance of 1.0% and optimization of 1.0%, and the patterns were clustered using the unweighted pair group method with arithmetic averages (UPGMA).

2.5 Molecular confirmation and detection of virulence genes

The genomic DNA was extracted according to Ellington et al. (2007) with minor modifications. DNA template was prepared by emulsifying five colonies in 200 µLof ultrapure water and freezing at -20 ^{o}C for at least 1h. The confirmation of *Salmonella* isolates by the detection of *hilA* gene and the detection of virulence genes (*invA*, *spvC*, *sefA* and *pefA*) was performed by PCR assays. For this, 12.5 µL of GoTaq® Green Master Mix 2x (Promega, USA), 1 µL of each primer at a concentration of 10 µmol, 2 µL of DNA (10 ng), and 8.5 µL of ultra-pure water (Promega, USA) were mixed in a final volume of 25 µL. The reactions were amplified in a thermocycler MJ Research® PTC 100. The primer sequences, references, and PCR conditions used are listed in Table S1 in the supplementary material. As positive controls, *S.* Enteritidis ATCC® 13076 and *S.* Typhimurium ATCC® 14028 were used in the reactions. After that, the PCR products were subjected to electrophoresis at 80 V for 70 min on a 1.5% (w/v) agarose gel (Invitrogen™, USA) in a 0.5 Tris/Acetate/EDTA buffer using a molecular weight marker of 1 Kb (Invitrogen™, USA). The amplified products were visualized in a UV transilluminator (Loccus Biotecnologia®, Brazil).

2.6 Antimicrobial susceptibility testing

Salmonella isolates were tested for antimicrobial susceptibility against 15 distinct antimicrobial agents. The agar disk diffusion was performed and evaluated according to the specifications of the Clinical and Laboratory Standards Institute for the Enterobacteriaceae group (CLSI 2018). The following discs were used: ampicillin 10 µg, amoxicillin/clavulanic acid 20/10 µg, cefotaxime 30 µg, cephalothin 30 µg, gentamicin 10 µg, tobramycin 10 µg, streptomycin 10 µg, imipenem 10 µg, nalidixic acid 30 µg, ciprofloxacin 5 µg, sulfonamide 300 µg, trimethoprim/sulfamethoxazole 1.25/23.75 µg, trimethoprim 5 µg, tetracycline 30 µg, and chloramphenicol 30 µg, acquired from Laborclin (Laborclin Produtos para Laboratórios Ltda, Brazil). *Escherichia coli* ATCC® 25922 was used as reference strain for quality control purposes. Multidrug-resistance (MDR) was defined as resistant to three or more classes of antimicrobials (CLSI 2018).

2.7 Sanitizer susceptibility testing

The MIC for benzalkonium chloride (BKC, Sigma-Aldrich, Irvine, UK), chlorhexidine (CLX, Sigma-Aldrich, UK), peracetic acid (PAC, Proc9 Indústria, Brazil) and sodium hypochlorite (SH, Dinâmica Química Contemporânea Ltda, Brazil) was determined according to Wu et al. (2015). Firstly, *Salmonella* isolates were cultivated on Tryptone Soy agar (Oxoid, UK) at 37 ºC for 24 h. After incubation, the bacterial suspensions were prepared in 0.85% (w/v) saline solution (Synth, Brazil) using 0.5 McFarland standard (10^8 CFU.mL^{-1}). The microtiter plates (CralPlast, Brazil) were filled with the bacterial suspensions and Mueller-Hinton broth (Oxoid, UK) in a proportion of 1:9, using variable concentrations of the compounds (ranging from 0.25 to 128 mg.L^{-1} for BKC and CLX; and 64 to 1024 mg.L^{-1} for PAC and SH) with incubation at 37 ºC for 24 h. The MICs were defined as the lowest concentration that prevented visible growth of the isolates.

2.8 Detection of antimicrobial and sanitizer resistance genes

The isolates that showed phenotypic resistance to antimicrobials and/or sanitizers were evaluated for the presence of resistance genes. The resistance genes encoding for aminoglycosides [*aadA*, *aadB*, *aac(6')-Ib*, *strA* and *strB*], β-

lactams (*blaTEM* and *blaZ*), class 1 integrase (*int1*), folate pathway inhibitors (*dfrA*, *dfrD*, *dfrG*, *sul1*, *sul2* and *sul3*), phenicols (*catA1* and *floR*) and tetracyclines [*tet(*A*)* and *tet(*B*)*], and genes encoding for efflux pumps (*norA*, *norB*, *mepA*, *qacA*, *qacH*, *qacE*Δ*1* and *smr*) were investigated by PCR. For this, 12.5 µL of GoTaq® Green Master Mix 2x (Promega, USA), 1 µL of each primer at a concentration of 10 µmol, 2 µL of DNA (10 ng), and 8.5 µL of ultra-pure water (Promega, USA) were mixed in a final volume of 25 µL. The primer sequences, references, and PCR conditions used are listed in Table S2 in the supplementary material. The cycling conditions of the PCR assays followed the recommendations of the referenced author's studies.

3 Results and discussion

3.1 Occurrence and genetic diversity of *Salmonella* isolates

Salmonella was detected in four out of 24 establishments producing pork, chicken, and mixed sausages, with an overall prevalence of 5.5% (14/252) (Table 1). A similar result was observed by Pala et al. (2019) in Italy, that found *Salmonella* spp. in four out 56 food products collected from eight establishments (meat cutting laboratories and sausage factories), showing a prevalence of 7.1%. *Salmonella* was isolated from pork bacon and fresh pork sausages. In the USA, a lower occurrence of *Salmonella* (0.05%) in ready-to-eat meat and poultry products was reported, among 91,038 samples collected from 5,807 establishments between 2005 and 2012. Three product types (sausage products, pork barbecue, and head cheese) accounted for 62.0% of all positive samples (Mamber et al. 2018). In Brazil, *Salmonella* was detected in 24.4% of 336 samples of fresh pork sausage from supermarkets and butcher shops in Porto Alegre (Mürmann et al. 2009). In the present study, sausages were evaluated because they are widely consumed in southern Brazil, which was the studied area. Furthermore, sausages are exposed to more extensive handling than meat, presenting a higher risk of *Salmonella* contamination in food processing plants. The raw materials (meat and fat) are the primary source of contamination of sausages by *Salmonella* (Piras et al. 2019). In addition, contamination can occur during processing, when the lethality step is not adequate to eliminate *Salmonella;* from ingredients after primary lethality treatment; from facility staff; and from environmental vectors, such as vertebrates and insects (Mamber et al. 2018).

Table 1 - Types of sausage samples collected from 24 establishments (A-X) submitted to sanitary inspection from Pelotas, Southern Brazil, and number of positive samples for *Salmonella enterica* subsp*. enterica*.

Establishments	**Sausage types**											**Total**	**Positive samples**
	Mixed	**Pork**	**Pork with cheese**	**Chicken with cheese**	**Fresh Calabrese**	**Fresh pork**	**Fresh mixed**	**Smoked**	**Chicken**	**White pudding**	**Black pudding**		
A	9	7	1	0	3	0	0	2	4	6	1	33	3
B	5	21	3	4	1	1	0	1	2	1	2	41	4
C	17	35	0	17	0	0	0	17	32	0	0	118	0
D	1	0	0	0	0	0	0	0	1	0	0	2	0
E	0	0	0	0	0	1	1	0	0	0	0	2	0
F	0	0	0	0	1	1	0	0	0	0	0	2	0
G	1	0	0	0	1	0	0	0	0	0	0	2	0
H	1	0	0	0	5	0	0	0	0	0	0	6	2
I	0	2	0	0	1	1	0	0	0	0	0	4	0
J	1	1	0	0	0	2	1	2	0	0	0	7	5
K	1	0	0	0	0	0	0	1	1	0	0	3	0

L	1	2	0	0	0	0	0	1	0	0	0	4	0
M	0	0	0	0	0	0	1	1	0	0	0	2	0
N	0	0	0	0	0	0	0	2	0	0	0	2	0
O	0	0	0	0	0	0	2	2	0	0	0	4	0
P	0	2	0	0	0	0	0	0	0	0	0	2	0
Q	1	0	0	0	0	0	0	1	0	0	0	2	0
R	0	0	0	0	0	0	0	1	0	0	0	1	0
S	0	0	0	0	0	1	1	1	0	0	0	3	0
T	0	0	0	0	0	1	1	1	0	0	0	3	0
U	0	0	0	0	0	1	1	1	0	0	0	3	0
V	0	0	0	0	0	0	1	1	0	0	0	2	0
W	0	0	0	0	0	0	1	1	0	0	0	2	0
X	0	0	0	0	0	0	1	1	0	0	0	2	0
Total	38	70	4	21	12	9	11	37	40	7	3	252	14

Six different *Salmonella* serovars were identified in this study: *S*. Infantis (n=4, establishment J), *S*. Rissen (n=4, 3 from establishment B and 1 from establishment J), *S*. Give (n=2, establishment H), *S*. Typhimurium (n=2, establishment A), *S*. Derby (n=1, establishment B) and *S*. Schwarzengrund (n=1, establishment A) (Table 2). The serovars *S.* Infantis and *S.* Rissen were the most common found. In fact, *S.* Infantis and *S*. Typhimurium were the most prevalent serovars among *Salmonella* isolates from ready-to-eat meat and poultry products from USA (Mamber et al. 2018). Moreover, *S*. Rissen is among the five most commonly reported serovar in meat products in China (Yang et al. 2019).

Salmonella isolates were characterized by *Xba*I-macrorestriction, resulting in a total of nine PFGE profiles (Fig. 1, Table 2), which were correlated with the serovars. In previous study, a correlation between PFGE types and *Salmonella* serovars was also observed (Piras et al. 2019). On the other hand, Bridier et al. (2019) showed that five *Salmonella* serovars were distributed in 15 PFGE patterns, with isolates belonging to the same serovar being grouped into different pulsotypes. PFGE profiles were specific for each facility with no overlapping between different processing plants. In the PFGE profile 1 (P1), the isolates were clustered with 100% of similarity, belonging to the serovar *S.* Rissen, all from establishment B. It is noteworthy that these isolates were obtained from different samples (mixed sausage, chicken sausage with cheese, and pork sausage with cheese) and on different dates (June and September, 2011). Isolates clustered in the PFGE profile 4 (P4) showed 100% of similarity, belonging to the serovar *S.* Infantis, and all of them were from establishment J. As observed in P1, the isolates are from different samples (fresh mixed sausage, fresh sausage, and smoked sausage) and collected on different dates (September and October, 2013). The results of this study corroborate the scientific literature, which demonstrate that *Salmonella* isolates can persist for several months in the environment of animal production and food industries, and can contaminate the final product. Bridier et al. (2019) also found persistent *Salmonella* isolates in a pig slaughterhouse in France. They evaluated six different steps in the slaughter line, on four sampling dates, and observed the same PFGE pattern among *Salmonella* isolates obtained from different steps, and even at different sampling event.

Table 2 - Characteristics of *Salmonella* isolates from sausages collected at production facilities in Southern Brazil.

Isolate	**Source**	**Date/ Establishment**	**Serovar**	**PFGE profile (*Xba*I)**	**Virulence genes**	**Resistance phenotype[a]**	**Resistance genotype**
S1	Mixed sausage	March, 2010/A	Typhimurium	P7	*invA, spvC, pefA*	AMP-CHL-GEN-NAL-STR-SUL-SUT-TRI	-
S2	Mixed sausage	July, 2010/A	Typhimurium	P8	*invA, spvC, pefA*	AMP-NAL-SUT-TRI	-
S3	Pork sausage	March, 2011/A	Schwarzengrund	P6	*invA, spvC*	AMP-CHL-STR-SUL-SUT-TET-TRI	*tet*(B), *sul2, strA, strB*
S4	Mixed sausage	June, 2011/B	Rissen	P1	*invA*	Susceptible	-
S5	Fresh calabrese sausage	June, 2011/H	Give	P9	*invA*	Susceptible	-
S6	Chicken sausage with cheese	September, 2011/B	Rissen	P1	*invA*	Susceptible	-
S7	Pork sausage with cheese	September, 2011/B	Rissen	P1	*invA*	Susceptible	-

S8	Mixed sausage	February, 2012/H	Give	P5	*invA*	SUL	-
S9	Pork sausage with parmesan cheese	May, 2012/B	Derby	P2	*invA, sefA*	Susceptible	-
S10	Pork sausage	August, 2013/J	Rissen	P3	*invA*	Susceptible	-
S11	Fresh mixed sausage	September, 2013/J	Infantis	P4	*invA*	Susceptible	-
S12	Fresh sausage	October, 2013/J	Infantis	P4	*invA*	Susceptible	-
S13	Smoked sausage	October, 2013/J	Infantis	P4	*invA*	Susceptible	-
S14	Fresh sausage	October, 2013/J	Infantis	P4	*invA*	Susceptible	-

[a]AMP: ampicillin, CHL: chloramphenicol, GEN: gentamicin, NAL: nalidixic acid, STR: streptomycin, SUL: sulfonamide, SUT: trimethoprim/sulfamethoxazole, TET: tetracycline, TRI: trimethoprim.

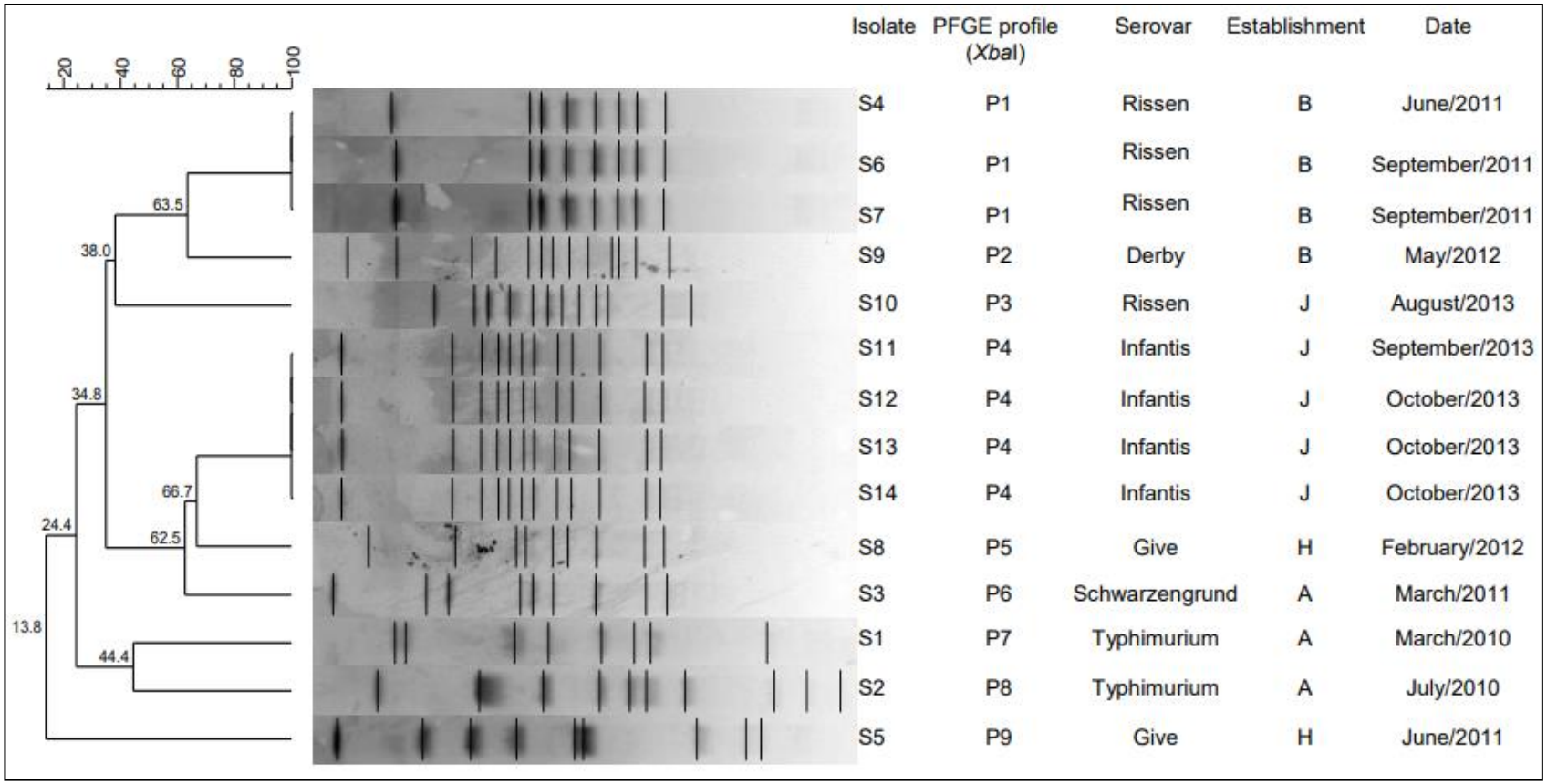

Figure 1 - Dendrogram of the genetic relationships among 14 *Salmonella enterica* isolates analysed by PFGE, using *Xba*I endonuclease, with Dice correlation coefficient, with maximal position tolerance of 1.5%. Using Molecular Weight Marker *Salmonella* Braenderup H9812 digested with *Xba*I endonuclease

3.2 Virulence genes

All isolates were confirmed as belonging to the genus *Salmonella*, through the amplification of the *hilA* gene (413 bp). The isolates also harbored the *Salmonella* invasion gene *invA,* commonly located in the chromosome*,* which is involved in the internalization in epithelial cells (Pavon et al. 2022). The *spvC* gene was detected in three (21.4%) isolates. This gene is part of *spvRABCD*, a plasmid-encoded operon of *Salmonella* that contributes to the systemic phase of the infection (Guiney and Fierer 2011). Regarding fimbrial genes, *pefA* (plasmid encoded fimbriae) and *sefA* (SE-based fimbriae) were detected only in two (14.3%) and one (7.1%) isolate, respectively (Table 2). The two isolates that carried the *pefA* gene (responsible for encoding Pef fimbria, involved in adhesion to the intestinal epithelium) belonged to the serovar *S.* Typhimurium. The *sefA* gene, which is involved in the encoding of structural subunits of the fimbriae SEF14 (Gong et al. 2014), was found in a single *S*. Derby isolate. The virulence genes detected in the isolates are related to the ability of *Salmonella* to increase its growth rate within the host during infection. Haubert et al. (2018b) evaluated the presence of the same virulence genes in *Salmonella* isolates from food and food environments, mainly meat products, and all of these genes were detected, with *invA* present in 100% of isolates, *pefA* and *spvC* in 19.2% and 15.4% of isolates, respectively, while *sefA* was found in just 3.8% of the isolates.

3.3 Phenotypic and genotypic antimicrobial resistance profile

Of the 14 *Salmonella* isolates, four (28.5%) showed resistance to at least one antimicrobial agent. The most frequently resistances were to ampicillin (21.4%), sulfonamides (21.4%), trimethoprim/sulfamethoxazole (21.4%), trimethoprim (21.4%), chloramphenicol (14.3%), streptomycin (14.3%), nalidixic acid (14.3%), gentamicin (7.1%) and tetracycline (7.1%) (Table 2). All isolates were susceptible to amoxicillin/clavulanic acid, cefotaxime, cephalothin, tobramycin, imipenem, and ciprofloxacin. Among resistant isolates, one (7.1%) presented phenotypic resistance only against sulfonamides, whereas a MDR profile was observed in three isolates (21.4%) from pork sausage and mixed sausages (prepared with pork and poultry). They belong to the serovars *S*. Schwarzengrund and *S.* Typhimurium, the latter one

of the most resistant serovars to the main antimicrobials used in the treatment of salmonellosis (Bridier et al. 2019).

It is important to emphasize that the *Salmonella* isolates evaluated in the present study were obtained between 2010 and 2013 and can be used to compare changes in resistance patterns over time. In 2017, Ed-dra et al. evaluated *Salmonella* isolates from sausage samples in Morocco and MDR profile was found in 44.1% (15/34) of the isolates. All *Salmonella* isolates were resistant to ampicillin, 76.5% to streptomycin, 20.6% to sulfonamides, 17.7% to tetracycline, and 11.8% to ofloxacin, demonstrating higher resistance rates than those found in the isolates in this study (2010-2013). Zhou et al. (2018) evaluated antimicrobial resistance among *Salmonella* obtained from retail chicken products in Shangai (China) and found an MDR profile in 20.6% (30/146) of the isolates. Furthermore, more than half of the isolates (50.7%) were resistant to ampicillin, 49.3% to sulfisoxazole, 17.1% to tetracycline, and 15.8% to doxycycline. In 2022, a study with *Salmonella* from slaughterhouses and traditional pork dry sausage, in Italy, showed that all isolates were resistant to aminoglycosides and gentamicin, 86.1% to tetracycline, 55.5% to ampicillin, 25.0% to trimethoprim, and 5.5% to chloramphenicol, while MDR profile was found in 55.6% (20/36) of the isolates (Lauteri et al. 2022). It is observed that resistance continues to be more frequent to antimicrobials such as ampicillin, streptomycin, sulfonamides, and tetracyclines, however, rates of antimicrobial resistance and MDR have increased over the years.

Salmonella isolates with phenotypic resistance profile were evaluated for the presence of genes conferring resistance to the antimicrobials and sanitizers listed in item 2.8. Among four isolates with antimicrobial phenotypic resistance profile, just *S*. Schwarzengrund (isolate S3) carried antimicrobial resistance genes. This MDR isolate harbored the *tet*(B)*, strA, strB* and *sul2* genes*,* which confer resistance to tetracyclines, aminoglycosides and sulfonamides, respectively*.* In *S.* Typhimurium (isolates S1 and S2) and *S*. Given (isolate S8) no resistance genes were detected, suggesting that other mechanisms or genes not evaluated in this study are involved in the resistance profile.

One of the most commonly antimicrobial used as feed additives in livestock farming of several countries is tetracycline (Yang et al. 2019), which is also the major

resistance phenotype found in *Salmonella* isolates from pork products, especially sausages. The presence of *tet* genes, encoding tetracyclines resistance, is a challenging problem due to the fact that they are usually encoded in mobile genetic elements, such as conjugative plasmids and/or conjugative transposons, favoring a possible transfer of resistance genes to other bacteria (Haubert et al. 2018a). Among the *tet* genes encoding efflux pumps that have already been identified in *Salmonella* isolates, the *tet*(A) and *tet*(B) genes are the most frequently detected (Lopes et al. 2016). A study conducted in a pig production chain in Southern Brazil observed the predominant tetracycline-resistant gene *tet*(A) in 67.0% of the *Salmonella* isolates from lairage, the intestinal content of pigs and carcasses, followed by the *tet*(B) gene, identified in 33.0% of the isolates (Lopes et al. 2015).

The *strA* and *strB* genes encode for aminoglycoside-3"-phosphotransferase (APH(3")-Ib) and aminoglycoside-6-phosphotransferase (APH(6)-Id) proteins, respectively, and are responsible for resistance to streptomycin (Lopes et al. 2016). Haubert et al. (2018b) also found these genes in 7.7% of *Salmonella* isolated from food and food environments. On the other hand, the *sul2* gene is involved in resistance to antimicrobials of the sulfonamides class, and associated with a small mobile genetic element called CR2, but is also linked with the streptomycin-resistance genes, *strA* and *strB* (Yau et al. 2010).

3.4 Phenotypic and genotypic sanitizer tolerance profile

Regarding sanitizer tolerance, 11 isolates (78.6%) presented MIC 32 $mg.L^{-1}$ and three isolates (21.4%) presented MIC 64 $mg.L^{-1}$ for BKC. For CLX, seven isolates (50.0%) presented MIC 16 $mg.L^{-1}$, six isolates (42.9%) presented MIC 32 $mg.L^{-1}$, and one isolate (7.1%) presented MIC 64 $mg.L^{-1}$. In relation to PAC and SH, all 14 isolates (100%) presented MIC 1024 $mg.L^{-1}$ for both compounds (Table 3). These results demonstrate that the sanitizers BKC and CLX were more effective than PAC and SH for the control of *Salmonella*.

Table 3 - Distribution of the MIC of sanitizers compounds for the *Salmonella* spp. isolates from sausages collected at production facilities in Southern Brazil.

Sanitizers	**Number of *Salmonella* isolates with MIC (mg.L^{-1}) equal to:**							**Total of tested isolates**
	16	**32**	**64**	**128**	**256**	**512**	**1024**	
Benzalkonium chloride	0	11	3	0	0	0	0	14
Chlorhexidine	7	6	1	0	0	0	0	14
Peracetic Acid	0	0	0	0	0	0	14	14
Sodium hypochlorite	0	0	0	0	0	0	14	14

There are no cut-off values to establish bacterial tolerance profile for sanitizer compounds; however, *Salmonella* isolates continued to grow even at the maximum concentration of PAC and SH tested (> 1024 $mg.L^{-1}$). These results are very interesting, because these compounds are widely applied in different sanitation programs used in the food and beverage processing chain (Lee and Huang 2019). Humayoun et al. (2018) evaluated 88 MDR *Salmonella* isolates from animals (cattle, pigs, chickens, turkeys, horses, dogs and cats) and beef, and observed that 90.0% showed MIC values ranging from 880 to 1760 $mg.L^{-1}$ for PAC, and almost half of the isolates (43.0%) showed MIC values of 6304 $mg.L^{-1}$ for SH, values significantly higher than those found in this study.

None of the sanitizer resistance genes evaluated were found in the 14 *Salmonella* isolates. Fuentes et al. (2014) also evaluated the same sanitizer resistance genes, and did not find these genes in any Gram-negative bacteria evaluated, including *Salmonella* isolates, corroborating the results of this study. This fact suggests that other mechanisms that do not involve the evaluated genes, such as multidrug efflux pumps, may be involved in the resistance phenotype for sanitizer compounds tested in this study (Fuentes et al. 2014).

4 Conclusions

Different *Salmonella* serovars were found in sausage samples industrialized in Southern Brazil, indicating a potential risk of salmonellosis associated with the consumption of this meat product. PFGE profiles were specific for each facility and some clones were recurrently recovered on different dates. *Salmonella* carrying virulence genes, MDR profile, and tolerance to sanitizers is a public health concern and a challenge for the food industry, suggesting that new strategies should be developed to control this pathogen.

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Table S1 - Oligonucleotides of virulence genes used in this study.

Target genes	Sequence (5' – 3')	PCR conditions*	Amplicon size (bp)	Reference
hilA	Fw: GCGAGATTGTGAGTAAAAACACC Rv: CTGCCCGGAGATATAATAATCG	P1	413	Crâciunas, Keul, Flonta and Cristea (2012)
invA	Fw: TTGTTACGGCTATTTTGACCA Rv: CTGACTGCTACCTTGCTGATG	P2	521	Swamy, Barnhart, Lee and Dreesen (1996)
sefA	Fw: GCAGCGGTTACTATTGCAGC Rv: TGTGACAGGGACATTTAGCG	P2	330	Woodward and Kirwan (1996)
pefA	Fw: TTCCATTATTGCACTGGGTG Rv: AAGCCACTGCGAAAGATGCC	P2	497	Haneda et al. (2001)
spvC	Fw: CGGAAATACCATCTACAA ATA Rv: CCCAAACCCATACTTACTCTG	P2	669	Swamy, Barnhart, Lee and Dreesen (1996)

Fw: forward primer; Rv: reverse primer

*PCR conditions: P1 (initial denaturation at 94 °C/4 min., followed by 30 cycles of denaturation at 94 °C/60 s, annealing according with the respective gene for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/10 min.); P2 (initial denaturation at 94 °C/2 min., followed by 35 cycles of denaturation at 94 °C/30 s, annealing according with the respective gene for 45 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.).

Table S2 **-** Oligonucleotides of antimicrobial and sanitizers resistance genes used in this study

Target genes	Sequence (5' – 3')	PCR conditions*	Annealing temperature	Amplicon size (bp)	Reference
blaZ	Fw: ACTTCAACACCTGCTGCTTTC Rv: TGACCACTTTTATCAGCAACC	P1	56	172	Martineau et al. (2000)
bla_{TEM}	Fw: ATGAGTATTCAACATTTCCG Rv: TTAATCAGTGAGGCACCTAT	P2	50	851	Grimm et al. (2004)
aadA	Fw: GTGGATGGCGGCCTGAAGCC Rv: ATTGCCCAGTCGGCAGCG	P3	60	526	Sandvang and Aarestrup (2000)
aadB	Fw: GGGCGCGTCATGGAGGAGTT Rv: TATCGCGACCTGAAAGCGGC	P3	58	328	Sandvang and Aarestrup (2000)
aac(6')-Ib	Fw: TTGCGATGCTCTATGAGTGGCTA Rv: CTCGAATGCCTGGCGTGTTT	P4	60	482	Park et al. (2006)
strA	Fw: TGACTGGTTGCCTGTCAGAGG Rv: CCAGTTGTCTTCGGCGTTAGCA	P5	64	645	Kehrenberg and Schwarz (2001)
strB	Fw: ATCGTCAAGGGATTGAAACC Rv: CGATCGTAGAAGATATTGCG	P5	56	510	Kikuvi et al. (2007)
int1	Fw: CGGAATGGCCGAGCAGATC Rv: CAAGGTTCTGGACCAGTTGCG	P2	50	871	Sandvang, Diggle and Platt (2002)
sul1	Fw: ATGGTGACGGTGTTCGGCATTCTG Rv:	P6	64	840	Grape, Sunström and Kronvall (2003)

sul2	Fw: GCGCTCAAGGCAGATGGCATT Rv: GCGTTTGATACCGGCACCCGT		69	293	Kerrn, Klemmensen, Frimodt-Moller and Espersen (2002)
sul3	Fw: GGGAGCCGCTTCCAGTAAT Rv: TCCGTGACACTGCAATCATTA	P8	57	500	Chuanchuen, Koowatananukul and Khemtong (2008)
dfrA	Fw: CCTTGGCACTTACCAAATG Rv: CTGAAGATTCGACTTCCC	P9	52	350	Perreten et al. (2005)
dfrD	Fw: GGGCAGATTTGTTTAGTAAGG Rv: GTATCTCCTTCGAATTCATGATG	P9	52	785	Bertsch et al. (2013a)
dfrG	Fw: TTTCTTTGATTGCTGCGATG Rv: CCCTTTTTGGGCAAATACCT	P9	52	422	Bertsch et al. (2013b)
tetA	Fw: GTAATTCTGAGCACTGT Rv: CCTGGACAACATTGCTT	P10	43	953	Frech and Schwarz (2000)
tetB	Fw: ACGTTACTCGATGCCAT Rv: AGCACTTGTCTCCTGTT	P10	50	1169	Frech and Schwarz (2000)
catA1	Fw: GGCATTTCAGTCAGTTG Rv: GATTAAGCATTCTGCCC	P5	45	551	Kikuvi et al. (2007)
floR	Fw: AGGGTTGATTCGTCATGACCA Rv: CGGTTAGACGACTGGCGACT	P11	55	1291	Kadlec, Kehrenberg and Schwarz (2007)
mepA	Fw: ATGTTGCTGCTGCTCTGTTC Rv: TCAACTGTCAAACGATCACG	P12	61	718	Hassanzadeh et al. (2017)
norA	Fw: TTCACCAAGCCATCAAAAAG Rv: CTTGCCTTTCTCCAGCAATA	P12	60	620	Hassanzadeh et al. (2017)

norB	Fw: AGCGCGTTGTCTATCTTTCC Rv: GCAGGTGGTCTTGCTGATAA	P12	62	213	Hassanzadeh et al. (2017)
qacA	Fw: ACTACTGATATGATGACATCA Rv: AGTTATATCAAGTGATTTGGG	P13	53	1512	Meregheti, Quentin, Mee and Adurier (2000)
qacC/D smr	Fw: ATAGCCATAAGTACTGAAGTT Rv: ACCGAAAATGTTTAACGAAAC	P13	53	291	Meregheti, Quentin, Mee and Adurier (2000)
qacH	Fw: ATAGTCAGTGAAGTAATAG Rv: AGTCTGATCATCGCAATGT	P13	53	550	Taheri et al. (2016)
qacEΔ 1	Fw: ATCGCAATAGTTGGCGAAGT Rv: CAAGCTTTTGCCCATGAAGC	P13	53	225	Paulsen et al. (1993)

Fw: forward primer; Rv: reverse primer

*PCR conditions: P1 (initial denaturation at 95 °C/5 min., followed by 28 cycles of denaturation at 95 °C/30 s, annealing according with the respective gene for 30 s, and extension at 72 °C/45 s, ending with final extension of 72 °C/5 min.); P2 (initial denaturation at 95 °C/5 min., followed by 30 cycles of denaturation at 95 °C/60 s, annealing according with the respective gene for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P3 (initial denaturation at 94 °C/5 min., followed by 30 cycles of denaturation at 94 °C/45 s, annealing according with the respective gene for 45 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P4 (initial denaturation at 94 °C/4 min., followed by 34 cycles of denaturation at 94 °C/45 s, annealing according with the respective gene for 45 s, and extension at 72 °C/45 s, ending with final extension of 72 °C/10 min.); P5 (initial denaturation at 94 °C/2 min., followed by 30 cycles of denaturation at 94 °C/60 s, annealing according with the respective gene for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P6 (initial denaturation at 94 °C/5 min., followed by 30 cycles of denaturation at 94 °C/60 s, annealing according with the respective gene for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P7 (initial denaturation at 94 °C/5 min., followed by 30 cycles of denaturation at 94 °C/15 s, annealing according with the respective gene for 30 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.); P8 (initial denaturation at 94 °C/5 min., followed by 30 cycles of denaturation at 94 °C/45 s, annealing according with the respective gene for 3 min., and extension at 72 °C/5 min., ending with final extension of 72 °C/10 min.); P9 (initial denaturation at 95 °C/5 min., followed by 30 cycles of denaturation at 95 °C/60 s, annealing according with the respective gene for 60 s, and extension

at 72 °C/60 s, ending with final extension of 72 °C/5 min.); P10 (initial denaturation at 94 °C/4 min., followed by 34 cycles of denaturation at 94 °C/60 s, annealing according with the respective gene for 2 min., and extension at 72 °C/3 min., ending with final extension of 72 °C/7 min.); P11 (initial denaturation at 95 °C/2 min., followed by 25 cycles of denaturation at 95 °C/60 s, annealing according with the respective gene for 30 s, and extension at 72 °C/2,5 min., ending with final extension of 72 °C/7 min.); P12 (initial denaturation at 94 °C/4 min., followed by 35 cycles of denaturation at 94 °C/30 s, annealing according with the respective gene for 55 s, and extension at 72 °C/55 s, ending with final extension of 72 °C/5 min); P13 (initial denaturation at 95 °C/5 min., followed by 30 cycles of denaturation at 95 °C/60 s, annealing according with the respective gene for 60 s, and extension at 72 °C/60 s, ending with final extension of 72 °C/7 min.).

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7 Considerações finais

Os resultados obtidos no presente estudo confirmam a presença de *Salmonella* spp. resistentes e multirresistentes a antimicrobianos na região de Pelotas, principalmente provenientes de produtos cárneos, devendo-se destacar a importância das boas práticas de fabricação na indústria de alimentos, a fim de evitar a contaminação com *Salmonella* spp. nesses produtos altamente manipulados. Além disso, grande parte dos isolados apresentaram genes que conferem resistência aos antimicrobianos testados, e em alguns isolados, esses genes estão presentes inclusive em plasmídeos, sendo necessárias pesquisas futuras para avaliar a capacidade de transferência horizontal desses genes de resistência para outras espécies bacterianas. Os isolados de *Salmonella* spp. também apresentaram tolerância a compostos sanitizantes, sendo menos suscetíveis ao ácido peracético e ao hipoclorito de sódio do que ao cloreto de benzalcônio e a clorexidina, e, de acordo com os dados obtidos na literatura, a presença do gene *qacEΔ1* associado ao gene *int1* pode indicar uma correlação entre a tolerância aos sanitizantes e a resistência a antimicrobianos nos isolados. Ainda, foi observada uma elevada variabilidade genética entre os isolados avaliados, sugerindo uma disseminação de *Salmonella* com diversos perfis genéticos na região.

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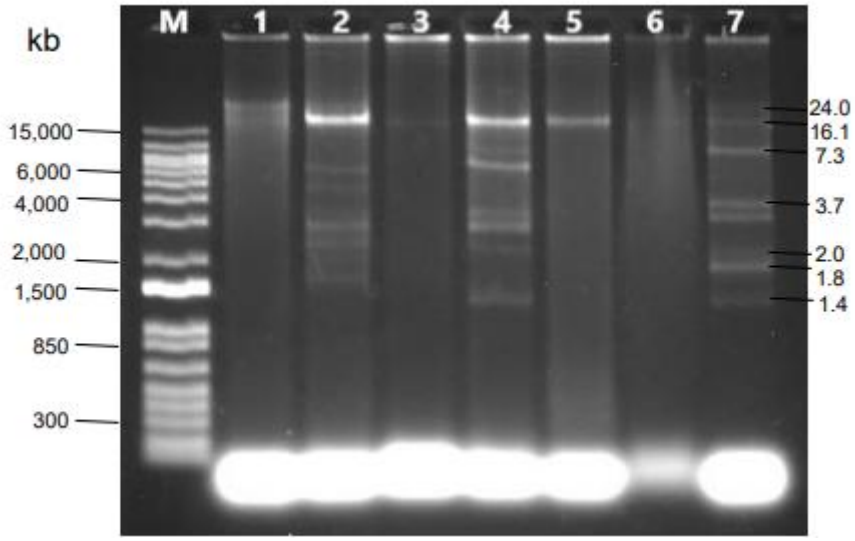
APÊNDICE



Gel de agarose 0,8% após corrida de eletroforese para visualização do DNA plasmidial de isolados de *Salmonella* spp. multirresistentes a antimicrobianos. Canaleta M: marcador molecular 1kb. Canaletas de 1 a 6: isolados S8, S29, S37, S39, S53 e S55. Canaleta 7: plasmídeos de Escherichia coli V517 (padrão para peso molecular de plasmídeos).