UNIVERSIDADE ESTADUAL PAULISTA – UNESP

FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS (FCAV)

CÂMPUS DE JABOTICABAL

CARACTERIZAÇÃO GENÉTICA DE SOROVARES DE *Salmonella* spp. ISOLADOS NA AVICULTURA POR WHOLE GENOME SEQUENCING

Valdinete Pereira Benevides

Bióloga

2024

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Valdinete Pereira Benevides

Orientador: Prof. Dr. Angelo Berchieri Junior

Coorientador: Dr. Mauro de Mesquita Souza Saraiva

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para obtenção do título de Doutora em Microbiologia Agropecuária

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DADOS CURRICULARES DA AUTORA

Valdinete Pereira Benevides – Nascida em Riacho de Santana, Bahia, em 11 de dezembro de 1995, filha de Janete Maria Pereira e Valdivino Alves Benevides. Em 2013, ingressou em Biologia no Centro Universitário da Fundação Educacional de Barretos (UNIFEB), obtendo o título de Bióloga em 2016. Durante a graduação foi bolsista de iniciação científica tendo desenvolvido projeto de pesquisa na área de Microbiologia, sob orientação da Prof. Dra. Patrícia Amoroso de Andrade e bolsista de iniciação a docência. Em agosto de 2017, ingressou no Mestrado na Universidade Estadual Paulista Júlio de Mesquita Filho, UNESP/FCAV, Câmpus de Jaboticabal pelo Programa de Pós-Graduação em Microbiologia Agropecuária como bolsista da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (N° Proceso: 2017/25743-7), sob orientação do Prof. Dr. Angelo Berchieri Junior e coorientação da Dra. Marcela da Silva Rubio. Em 2019, ingressou no curso de Doutorado do Programa de Pós-graduação em Microbiologia Agropecuária sob orientação do Prof. Dr. Angelo Berchieri Junior e coorientação do Dr. Mauro de Mesquita Souza Saraiva na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista "Júlio de Mesquita Filho" – Câmpus Jaboticabal, com bolsa de estudo da CAPES e FAPESP (N° Processo: 2020/07018-6), com período sanduíche na University of Copenhagen, Department of Veterinary and Animal Sciences, Dinamarca, sob supervisão do Prof. Dr. John Ermedahl Olsen (N° Processo BEPE/FAPESP: 2021/10285-9).

“*Great things are not done by impulse, but by a series of small things brought together.*”

Vincent Van Gogh

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CARACTERIZAÇÃO GENÉTICA DE SOROVARES DE *Salmonella* spp. ISOLADOS NA AVICULTURA POR WHOLE GENOME SEQUENCING

Resumo - As bactérias do gênero *Salmonella* são patógenos que podem infectar animais e seres humanos. Alguns sorovares de *Salmonella enterica* podem colonizar o trato gastrointestinal de aves de produção e se disseminarem pelo organismo sem provocar o aparecimento de sinais clínicos. Entretanto, a presença dessas bactérias, pode significar risco a saúde pública, tornando os alimentos de origem avícola potenciais fontes de infecção para os seres humanos. Pouco se sabe sobre os fatores associados à diversidade genética que podem contribuir para a adaptação e persistência de *Salmonella* spp. a um ou vários hospedeiros. Além disso, os métodos tradicionais de sorotipificação e identificação de marcadores genéticos no gênero *Salmonella* é laborioso e pode transcorrer por vários dias. Dessa forma, o presente estudo foi dividida em três manuscritos (capítulos 2-4). No capítulo 2 apresentamos características genômicas de seis estirpes brasileiras de *Salmonella* Mbandaka ST413 isoladas de granjas de postura comercial. Os genes de resistência mais frequentes foram *aac(6')-Iaa*, *sul1*, *qacE*, *bla*$_{OXA-129}$, *tet(B)* e *aadA1*, além de mutação no gene *parC* associada à resistência a quinolonas. Quatro dos seis genomas continham um plasmídeo IncHI2A de 112.960 pb, carreando genes de resistência a tetraciclina (tetACDR) e mercúrio (*mer*). A maioria das genomas também possuía a ilha genômica de *Salmonella* 1 (SGI1) e sete ilhas de patogenicidade (SPIs) relacionadas à virulência (SPIs 1-5, 9, and C63PI). A análise filogenética utilizando os seis novos genomas sequenciados em nosso estudo e mais 481 genomas do banco de dados de acesso público, mostrou que os isolados de origem Brasileira formaram clados bem definidos, relacionados a estirpes de humanos e alimentos, indicando sua importância epidemiológica e potencial para surtos alimentares. Esses resultados destacam que *S.* Mbandaka ST413 nesse estudo são resistentes a multiplos antimicrobianos e carregam genes de virulência conservados, características que favorecem sua disseminação regional e global. No capítulo 3 realizamos uma análise genômica comparativa de quatro sorovares (*S.* Schwarzengrund, *S.* Senftenberg, *S.* Saintpaul, *S.* Braenderup) de 301 genomas de *Salmonella enterica* de humanos e

poultry, coletados do Enterobase, além de incluir dez novos genomas sequenciados de isolados de amostras fecais de galinhas poedeiras no Brasil entre 2016 e 2017. Utilizando análise de bioinformática foram preditos tipos de *Multilocus Sequence Typing* (MLST), *replicons* plasmidiais, genes de resistência antimicrobiana, além da predição dos fatores de virulência típicos implicados nos mecanismos de virulência de *Salmonella enterica* e alguns especificos que podem estar relacionados a adaptação ao hospedeiro. Identificamos 52 genes de resistência antimicrobiana, com 48% (25/52) compartilhados entre isolados de aves e humanos, 21,1% (11/52) exclusivos de aves e 30,7% (16/52) exclusivos de humanos. Mutações cromossômicas associadas aos genes *gyrA* e *parC* também foram previstas. Em todos os genomas foram identificados SPIs, algumas variando conforme o hospedeiro e sorovar (SPI 1–5, SPI 8–14, CS54 e C63PI). To the best of our knowledge, this is the first work to report *Salmonella* Braenderup carrying the SPI-10. SGI-1 foi detectada em alguns isolados de *S*. Schwarzengrund de isolados de aves e a ilha CS54 foi observada exclusivamente em genomas dos sorovares *S*. Saintpaul e *S*. Braenderup. Detectamos uma diversidade de replicons de plasmídeos, sendo o sorovar Saintpaul o menos diverso. Analisamos 271 genes de virulência, com 161 comuns a todos os sorovares e os demais distribuídos de forma distinta entre os sorovares analisados. Nosso estudo destaca o potencial de sorovares de *Salmonella enterica* negligenciados na avicultura que ameaçam a saúde pública devido à seu potencial de virulência e resistência a múltiplos moléculas antimicrobianas e de desisnfetantes. No capítulo 4, foi realizada uma predição de caracteríticas genômicas de *Salmonella* Heidelberg, um importante patógeno de origem alimentar em produtos avícolas resistentes à diferentes antimicrobianos, que pode causar infecções graves em humanos. Nesse estudo, analisamos 317 genomas de *S*. Heidelberg isoladas de aves do Brasil e Estados Unidos, incluindo 314 do banco de dados Enterobase e três novos genomas brasileiros sequenciados. Os principais genes de resistência encontrados foram *aac(6')-Iaa*, *fosA7*, *sul2*, *tet(A)* e bla_{CMY-2}. Foram detectadas mutações nos genes *gyrA* e *parC*, relacionados à resistência a quinolonas. Os replicons de plasmídeos mais comuns foram ColpVC, IncC, IncI1-I(Gamma) e IncX1. O estudo destaca a diversidade genômica de SH relacionados as maiores regiões de produtoras de frango do mundo e a necessidade de melhorar a vigilância para prevenir surtos por este patógeno. De

forma geral, foi demonstrado em nossos estudos a diversidade genômica desses sorovares de *Salmonella enterica* e sua capacidade de colonizar e se estabelecer em diversos nichos, incluindo humanos, animais e o ambiente. Essa plasticidade genética e potencial evolutivo representam preocupações de segurança alimentar e de saúde pública.

Palavras-chave: genes de virulência, resistência antimicrobiana, salmonelas paratíficas, WGS

GENETIC CHARACTERIZATION OF *Salmonella* spp. SEROTYPES ISOLATED FROM POULTRY BY WHOLE GENOME SEQUENCING.

Abstract – Bacteria of the genus *Salmonella* are pathogens that can infect animals and humans. Some serovars of *Salmonella enterica* can be installed in poultry, in the digestive tract, and be disseminated without causing the appearance of clinical signs. However, the presence of these bacteria can pose a risk to public health, making foods of poultry origin potential sources of infection for humans. Little is known about the factors associated with genetic diversity that may be contributing to the adaptation and persistence of *Salmonella* spp. to one or more hosts. Furthermore, traditional methods of serotyping and identification of genetic markers in the *Salmonella* genus are laborious and can take several days. This study was divided into three manuscripts (chapters 2-4). In Chapter 2, we present genomic characteristics of six Brazilian strains of *Salmonella* Mbandaka ST413 isolated from commercial layer farms. The most frequent resistance genes were *aac(6')-Iaa*, *sul1*, *qacE*, $bla_{\text{OXA-129}}$, *tet(B)*, and *aadA1*, along with a mutation in the *parC* gene associated with quinolone resistance. Four of six genomes contained a 112,960 bp IncHI2A plasmid carrying tetracycline resistance genes (*tet*ACDR) and mercury resistance genes (*mer*). Most genomes also harbored the *Salmonella* genomic island 1 (SGI1) and seven *Salmonella* pathogenicity islands (SPIs) related to virulence (SPIs 1-5, 9, and C63PI). Phylogenetic analysis using the six newly sequenced genomes in our study and an additional 481 genomes from the public database showed that the Brazilian isolates formed well-defined clades, related to strains from humans and food, indicating their epidemiological importance and potential for foodborne outbreaks. These results highlight that *S*. Mbandaka ST413 in this study are resistant to multiple antimicrobials and carry conserved virulence genes, characteristics that favor their regional and global dissemination. In Chapter 3, we conducted a comparative genomic analysis of four serovars (*S*. Schwarzengrund, S. Senftenberg, *S*. Saintpaul, *S*. Braenderup) from 301 *Salmonella enterica* genomes from humans and poultry, collected from Enterobase. Additionally, we included ten newly sequenced genomes from isolates obtained from fecal samples of laying hens in Brazil between 2016 and 2017. Using bioinformatics analysis, we predicted Multilocus Sequence Typing (MLST) types, plasmid replicons, antimicrobial resistance genes, as well as typical virulence factors implicated in the virulence mechanisms of

Salmonella enterica and some specific factors that may be related to host adaptation. We identified 52 antimicrobial resistance genes, with 48% (25/52) shared between avian and human isolates, 21.1% (11/52) exclusive to avian isolates, and 30.7% (16/52) exclusive to human isolates. Chromosomal mutations associated with the *gyrA* and *parC* genes were also predicted. SPIs were identified in all genomes, with some variations depending on the host and serovar (SPI 1–5, SPI 8–14, CS54, and C63PI). To the best of our knowledge, this is the first study reporting *Salmonella* Braenderup carrying SPI-10. SGI-1 was detected in some isolates of *S*. Schwarzengrund from avian isolates, and the CS54 island was exclusively observed in genomes of the serovars *S*. Saintpaul and *S*. Braenderup. We detected a diversity of plasmid replicons, with the Saintpaul serovar being the least diverse. We analyzed 271 virulence genes, with 161 common to all serovars and the remainder distributed differently among the serovars analyzed. Our study highlights the potential of neglected *Salmonella enterica* serovars in poultry farming that pose a public health threat due to their virulence potential and resistance to multiple antimicrobial and disinfectant molecules. In Chapter 4, we conducted a prediction of genomic characteristics of *Salmonella* Heidelberg, an important foodborne pathogen in poultry products resistant to different antimicrobials, which can cause severe infections in humans. In this study, we analyzed 317 genomes of *S*. Heidelberg isolated from poultry in Brazil and the United States, including 314 from the Enterobase database and three newly sequenced Brazilian genomes. The main resistance genes found were *aac(6')-Iaa*, *fosA7*, *sul2*, *tet(A)*, and *bla*$_{CMY-2}$. Mutations in the *gyrA* and *parC* genes related to quinolone resistance were detected. The most common plasmid replicons were ColpVC, IncC, IncI1-I(Gamma), and IncX1. The study highlights the genomic diversity of *S*. Heidelberg related to the largest chicken-producing regions in the world and the need to improve surveillance to prevent outbreaks by this pathogen. Overall, our studies demonstrated the genomic diversity of these *Salmonella enterica* serovars and their ability to colonize and establish in various niches, including humans, animals, and the environment. This genetic plasticity and evolutionary potential represent concerns for food safety and public health.

Keywords: virulence genes, antimicrobial resistance, paratyphoidal salmonellosis, WGS

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº A28695F

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**A28695F**
Usuário:	**UNESP**
CPF/CNPJ:	**48.031.918/0001-24**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica

X

Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica
Salmonella enterica

Título da Atividade: **Caracterização genética de sorovares de Salmonella spp. isoladas na avicultura por Whole Genome Sequencing**

Equipe

Valdinete Pereira Benevides	**FCAV/UNESP**
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Angelo Berchieri Junior	**FCAV/UNESP**

Parceiras no Exterior

University of Copenhagen

Envios de Amostra

Espécie:	**Salmonella enterica**
Tipo do Patrimônio Genético:	-
Forma do Patrimônio Genético:	**Garrafa/frasco com meio de cultivo**
Instituição Destinatária:	**University of Copenhagen**
Sede da Instituição Destinatária:	**DEPARTMENT OF VETERINARY AND ANIMAL SCIENCES, Faculty of Health and Medical Scie**
Espécie:	**Salmonella enterica**
Tipo do Patrimônio Genético:	-
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Sede da Instituição Destinatária:	**DEPARTMENT OF VETERINARY AND ANIMAL SCIENCES, Faculty of Health and Medical Scie**
Espécie:	**Salmonella enterica**
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CAPÍTULO 1 – Considerações gerais

1. Características e nomenclatura do gênero *Salmonella*

As bactérias do gênero *Salmonella*, pertencentes à família *Enterobacteriaceae*, são bastonetes Gram-negativos, não formadores de esporos, aeróbios ou anaeróbios facultativos. Produzem sulfeto de hidrogênio (H_2S) e fermentam a glicose, mas não são capazes de fermentar lactose e sacarose. Quase a totalidade dos exemplares expressam flagelos e são móveis, com exceção de *Salmonella enterica* subespécie *enterica* sorovar Gallinarum biovares Gallinarum e Pullorum (Grimont et al., 2000).

Até o presente momento foram tipificados mais de 2.659 sorovares de *Salmonella* spp., dos quais cerca de 100 já foram isolados tanto em seres humanos quanto em animais(Issenhuth-Jeanjean et al., 2014). O gênero é dividido em duas espécies: *S*. bongori e *S*. enterica, sendo que a última abriga mais de 99% dos sorovares conhecidos. Por sua vez, *Salmonella enterica* é subdividida em seis subespécies (subesp.): *S*. *enterica* subesp. *enterica*, *S*. *enterica* subesp. *salamae*, *S*. *enterica* subesp. *arizonae*, *S*. *enterica* subesp. *diarizonae*, *S*. *enterica* subesp. *houtenae*, *S*. *enterica* subesp. *indica*. Dentre estas, a subespécie *enterica* alberga aproximadamente 60% dos sorovares, a maioria dos quais causadores de infecções em inúmeros hospedeiros, entre humanos e animais (Issenhuth-Jeanjean et al., 2014).

A complexidade para identificar os exemplares desse gênero é uma outra característica de *Salmonella* spp. As provas bioquímicas definem o gênero, enquanto que as provas antigênicas com base nos antígenos somáticos (O) e flagelares (H) determinam os sorovares (Grimont et al., 2000). Além disso, o antígeno de virulência ou capsular (Vi) pode ser identificado em sorovares específicos, como *S*. Typhi, *S*. Paratyphi C e *S*. Dublin (Grimont e Weill, 2007).

2. Salmonelose em avicultura e saúde pública

Em aves, podem ocorrer três enfermidades resultantes de infecções por *Salmonella* spp. O tifo aviário e a pulorose, causados respectivamente pelos biovares

hospedeiro-específicos *S*. Gallinarum e *S*. Pullorum, ambos pertencentes ao sorovar Gallinarum; e o paratifo aviário, que corresponde à infecção por qualquer outro sorovar (Poppe, 2000). As salmonelas paratíficas não são específicas de aves e podem infectar diversos hospedeiros, tais como seres humanos e outros animais, o que dificulta o sucesso dos programas de controle e prevenção na produção avícola. Assim, a presença de salmonelas paratíficas em aves de exploração comercial pode se constituir em um risco para saúde pública (Freitas Neto et al., 2020), mesmo que estes animais não demonstrem sinais clínicos. Por se tratar de uma fonte proteica de qualidade, os produtos avícolas são cada vez mais consumidos por brasileiros de todas as classes sociais (ABPA, 2023), o que torna essenciais o monitoramento e o controle de salmonelas paratíficas não só para o sucesso da indústria avícola como para saúde da população.

De acordo com o "*European Food Safety Authority*" (EFSA), no ano de 2016 foram reportados 67.418 casos de salmoneloses humanas, com maior prevalência para os sorovares *S.* Enteritidis (48,5%); *S.* Typhimurium (13,4%); *S.* Typhimurium monofásica 1.4.[5].12:i:- (8,4%); *S.* Infantis (2,4%); *S.* Newport (1,1%); *S.* Derby, *S.* Kentucky e *S.* Stanley (0,8%); *S.* Virchow e *S.* Saintpaul (0,7%); *S.* Agona, *S.* Paratyphi B var. Java e *S.* Braenderup (0,6%); e *S.* Panama (0,5%) (EFSA, 2017a). No Brasil, os dados epidemiológicos de surtos em seres humanos são imprecisos. Segundo relatório da Secretaria de Vigilância em Saúde (SVS), entre os anos de 2007 e 2018, foram isoladas 225 estirpes de *Salmonella* spp. provenientes de infecções de origem alimentar associadas ao consumo de produtos avícolas, tendo sido reportado apenas o sorovar Enteritidis (4,4%) (SVS, 2019).

Dados sobre a prevalência de *Salmonella* spp. em casca, albúmen e gema de ovos brancos e marrons provenientes do estado de Goiás, demonstrou ampla variabilidade de sorovares, como *S.* Agona (18,2%), *S. enterica* subsp. *enterica* O: 4,5 (18,2%), *S.* Schwarzengrund (18,2%), *S.* Cerro (13,6%), *S.* Anatum (13,6%), *S.* Enteritidis (9,1%), *S.* Joanesburgo (4,5%) e *S.* Corvallis (4,5%) (Moraes et al., 2016). Em estudo semelhante, Freitas Neto et al. (2014) avaliaram ovos para consumo provenientes da região de Jaboticabal-SP, os quais obtiveram 1,47% de amostras positivas para *Salmonella* spp., sendo identificados os sorovares *S*. Mbandaka, *S*. *enterica* subsp. *enterica* 6,7: z10: - e *S*. Braenderup.

Apesar da comprovada importância para o setor avícola, bem como à saúde pública, pesquisas que abordem alguns sorovares específicos, como *S*. Mbandaka, *S*. Saintpaul, *S*. Senftenberg e *S*. Braenderup são relativamente limitadas. No entanto, estes tem sido sorovares cada vez mais observados em vários países devido seu potencial zoonótico e ampla disseminação (EFSA, 2017a; Carvajal-Restrepo et al., 2017). No Brasil, o monitoramento das salmonelose em granjas avícolas exige apenas a identificação dos sorovares *S*. Gallinarum, *S*. Pullorum, *S*. Enteritidis, *S*. Typhimurium e *Salmonella* Typhimurium monofásica (1,4[5],12:-:1,2 e 1,4[5],12:i:-) (Brasil, 1995; Brasil, 2016a). No entanto, a presença de outros sorovares em animais destinados ao consumo humano podem promover insegurança do consumidor e perdas econômicas ao país devido aos custos associados a produção e comercialização de proteína animal, investigações de surtos e tratamentos médicos, principalmente quando as bactérias são resistentes aos antimicrobianos (Fouladkhah et a., 2019).

3. Caracterização genômica por *Whole Genome Sequencing* (WGS)

Os métodos clássicos de identificação bacteriana, como provas bioquímicas e moleculares como a Reação em Cadeia da Polimerase (*Polymerase Chain Reaction* **-** PCR), são os mais utilizados para detecção de espécies e determinantes genéticos em patógenos bacterianos. Entretanto, o recente avanço das técnicas de sequenciamento, como o WGS teve grande impacto no estudo da biologia molecular de patógenos zoonóticos, incluindo os associados a enfermidades veiculadas por alimento (Rantsiou et al., 2018).

Com o advento e recém popularização do WGS, alto rendimento em termos de quantidade de dados gerados, velocidade para realização da técnica e maior custo-benefício, tornaram essa tecnologia viável como ferramenta para caracterização de patógenos (Boolchandani et al., 2019). A rápida identificação de genes específicos de virulência, patogenicidade, além dos genes relacionados à resistência antimicrobiana com alta precisão e acurácia, é uma das grandes vantagens dessa tecnologia (Boolchandani et al., 2019). Além disso, a ferramenta permite que os dados gerados possam ser comparados com as análises de outros genomas previamente

depositados em bancos públicos de referência (Deng et al., 2016), facilitando as análises epidemiológicas.

4. Resistência antimicrobiana em *Salmonella* spp.

O uso inadequado de antimicrobianos, seja na produção animal ou em saúde pública, acarreta em uma pressão seletiva sobre a microbiota do indivíduo, favorecendo a emergência de estirpes resistentes (Saraiva et al., 2018). A pressão seletiva também tem beneficiado o estabelecimento de estirpes patogênicas, o que reflete no aumento de casos de infecções graves causadas por bactérias multirresistentes em seres humanos (Boolchandani et al., 2019).

Em estudo anterior foi demonstrada a persistência de 22 sorovares de *Salmonella* spp. multirresistentes em granjas de aves de postura de ovos para consumo no Brasil (Benevides et al., 2020), com potencial patogênico aos seres humanos. Sorovares de *Salmonella* spp. resistente aos antimicrobianos, entre os quais *S.* Enteritidis, *S.* Agona, *S.* Derby, *S.* Schwarzengrund, *S.* Mbandaka, *S.* Saintpaul, *S.* Kentucky e *S*. Typhimurium, são comumente encontradas em aves e seus produtos, e estão relacionadas a infecções em humanos (Antunes et al., 2016; Du et al., 2017; Shah et al., 2017; Reis et al., 2018; Benevides et al., 2020). Além disso, estudo do nosso grupo de pesquisa corrobora a hipótese de que o fenótipo de resistência de *Salmonella* spp. aos múltiplos princípios ativos é um entrave ao setor avícola (Souza et al., 2020) pelo difícil controle e ampla disseminação.

Com o propósito de preservar a eficácia dos antimicrobianos, especialmente daqueles utilizados em saúde pública, assim como em reduzir os casos de infecções em seres humanos causadas por bactérias multiresistentes, o conhecimento do perfil de resistência aos fármacos e a utilização de métodos alternativos em produção animal estão sendo realizados em diversos países (Brasil, 2016b; EFSA, 2017b; FDA, 2018).

No entanto, os resultados ainda são incipientes, mesmo após a proibição do uso de alguns antimicrobianos em produção animal. No Brasil, Reis et al. (2018) demonstraram que pacientes hospitalizados com infecções por estirpes de *Salmonella enterica* subespécie *enterica* apresentaram resistência ao ácido nalidíxico (59,2%), a tetraciclina (37,6%), a ampicilina (35,1%), a nitrofurantoína (34,2%), a estreptomicina

(32,6%), a gentamicina (19,1%), a ciprofloxacina (15,6%), ao cloranfenicol (15,2%), ao trimetoprim/sulfametoxazol (11,8%), a cefoxitina (2,8%) e a ceftazidima (1,4%). Os autores ainda relataram a identificação de *S.* Typhimurium, *S* Infantis*. S.* Enteritidis, *S*. Newport, *S.* Panamá, *S.* Agona, *S.* Rissen, *S*. Braenderup, *S.* Derby, *S*. Heidelberg, *S.* Saintpaul, *S.* Oranienburg e *S*. Mbandaka resistentes a pelo menos um dos antimicrobianos testados.

A resistência a drogas de estirpes de *S*. Mbandaka, especialmente contra ciprofloxacina, estreptomicina e sulfonamidas, foi relatada ao longo dos últimos anos na produção avícola de diferentes países (Hoszowski et al., 2016; Velasquez et al., 2018; Li et al., 2020), frequentemente associada a infecções em seres humanos (Hoszowski et al., 2016). Já em relação a *S.* Schwarzengrund oriundos de carne de frango, a emergência de estirpes multirresistente têm aumentado a cada ano, tornando-se um dos sorovares paratíficos de grande importância em saúde pública (Mori et al., 2018; Duc et al., 2020). Em contraste, a identificação de determinantes de resistência antimicrobiana em *S.* Senftenberg isolados de aves e seres humanos têm sido relatada como de baixa frequência (Kay et al., 2015; Shah et al., 2017).

Em recente relato de estirpe de *S.* Saintpaul isolada de frango, foi identificado um plasmídio (pSGB23) que abrigava 11 genes que codificam a resistência aos antimicrobianos e aos compostos de amônia quaternária, além de carrear sistemas conjugativos e tipos de *replicons*, os quais podem contribuir na transferência destes genes para outros patógenos e a disseminação destes a outros hospedeiros (Ding et al., 2018).

Além de favorecer a prevalência de bactérias resistentes aos antimicrobianos, o uso *off-label* dessas drogas pode induzir o aumento da tolerância contra outros princípios ativos cujas características químicas possam ser semelhantes ou não, capacidade esta denominada de resistência cruzada (Lázár et al., 2014). Dessa forma, a resistência aos antimicrobianos de crítica importância à saúde humana (WHO, 2019) tem sido correlacionada à resistência cruzada. Como exemplo, o uso da ampicilina na produção animal pode estar diretamente relacionado à ocorrência de resistência às cefalosporinas de terceira e quarta gerações, fluoroquinolonas e gentamicina, por influência de fatores genéticos (Jensen et al., 2018), cenário em que se limita a possibilidade de tratamento com essas drogas. O principal desses fatores é a

ocorrência de mutações que podem, eventualmente, alterar a sensibilidade simultânea contra diferentes antimicrobianos (Lázár et al., 2014; Toprak et al., 2012).

O arcabouço genético bacteriano é quem, de fato, determina o potencial de resistência aos antimicrobianos. Essa resistência pode ser mediada por *single nucleotide polymorphism*s (SNPs), aquisição de ilhas genômicas adquiridas por transferência horizontal (transdução, conjugação ou transformação), além da super expressão ou duplicação de genes (Boolchandani et al., 2019). A resistência aos antimicrobianos em *Salmonella* spp. também tem sido mediada por genes cromossomais (Boyd et al., 2000; Shahada et al., 2011; Haley et al., 2019). *Salmonella* spp. que possuem essas ilhas genômicas em seu material genético podem carrear genes que contribuem na virulência e na disseminação da estirpe (Sahu et al., 2013). Contudo, os fatores que culminam na transferência gênica envolvendo *Salmonella* spp. entre os isolados da produção animal e saúde pública ainda não estão completamente elucidados.

5. Patogenicidade e virulência de *Salmonella* spp.

O sistema imunológico de um hospedeiro saudável é capaz de identificar e eliminar, de forma eficiente, os patógenos por meio da resposta imune inata e adquirida. Entretanto, *Salmonella* spp. pode escapar do sistema imunológico usando estratégias complexas que contribuem para a multiplicação e sobrevivência no interior da célula do hospedeiro (Levine et al., 2012).

Os genes envolvidos com a patogenicidade de salmonelas estão inseridos em regiões especificas do genoma bacteriano que abriga um conjunto de genes correlatos. Essas regiões são denominadas de Ilhas de Patogenicidade de *Salmonella* (*Salmonella Pathogenicity Island* - SPI). Dentre as SPIs identificadas, as de principal importância para a sobrevivência de sorovares paratíficos de aves estão localizadas no cromossomo e são enumeradas de uma a cinco: SPI-1, SPI-2, SPI-3, SPI-4 e SPI-5 (Coburn et al., 2007). As duas primeiras ilhas codificam aparatos denominados como Sistema de Secreção do Tipo III da SPI-1 (T3SS-1) ou da SPI-2 (T3SS-2), cujas funções se resumem em transportar proteínas efetoras da célula bacteriana para a célula hospedeira que, por sua vez, induzem alterações celulares como na estrutura do citoesqueleto, transporte da membrana e expressão de citocinas. Estas

modificações possibilitam a invasão e persistência da *Salmonella* spp. no hospedeiro (Coburn et al., 2007; Haraga et al., 2008). No geral, a ação dao T3SS-1 ocorre na fase de contato da bactéria com a célula eucariótica, enquanto que o T3SS-2 transporta proteínas efetoras para o Vacúolo Contendo *Salmonella* (*Salmonella*-containing Vacuole - SCV) com capacidade de ser ativado dentro desse fagossomo modificado (Hansen-Wester e Hensel, 2001).

Por outro lado, a capacidade dos integrantes do gênero *Salmonella* em causar enfermidades (invasão e sobrevivência) está relacionada aos fatores de virulência que são codificados por genes que se encontram em locais específicos, como em elementos genéticos móveis (*transposons*, plasmídios ou bacteriófagos) e nas SPIs (Santos et al., 2018). No entanto, análises genômicas comparativas demonstraram que os perfis dos genes de virulência apresentam variações entre *Salmonella* spp. isoladas de produtos avícolas e de seres humanos, no qual podem interferir no grau de virulência da bactéria e na capacidade de adaptação a diversos hospedeiros (Jacobsen et al., 2011; Gharieb et al., 2015; Kim et al., 2019).

A forma como *Salmonella* spp. se adapta às condições dentro do hospedeiro depende da virulência da estirpe (Wang et al., 2020). Normalmente, as infecções acarretadas por salmonelas paratíficas são autolimitantes, no qual não progridem além da lâmina própria, no entanto alguns sorovares expressam um conjunto de genes de virulência que contribuem para a invasão da mucosa intestinal e proliferação em fagócitos (Bumann e Schothorst, 2017; Wotzka et al., 2017). Contudo, investigações no genoma ainda não foram totalmente exploradas e a caracterização dos diferentes genes de virulência envolvidos nas SPIs de *S. enterica* ainda não são completamente elucidados.

As SPI-2, 3, 5–8, 10–13 e 16 secretam efetores de virulência envolvidos em auxiliar o patógeno a tolerar o ambiente ácido, desempenhando papel central na multiplicação dentro da célula hospedeira e para o escape imunológico. Aparentemente, os sorovares de *Salmonella* spp. contam com um *pool* de efetores relevantes, indicando a importância desses para a virulência em hospedeiros distintos, incluindo, PipA, PipB, PipB2, SifA, SipA, SipB, SipC, SipD, SopB, SopD, SpiC, SptP, SseF, SseG, SseL, SteA e SteD (Galán 1996; Hautefort et al., 2007; Johnson et al., 2018). Vale destacar que a adaptação relacionada ao hospedeiro em *Salmonella* spp

pode ter influenciado na presença ou ausência de alguns efetores em sorovares que afetam maior gama de hospedeiros e sorovares adaptados (Johnson et al., 2018).

5.1. Diversidade genética e outros fatores de virulência relacionados com a especificidade e adaptação ao hospedeiro

Alguns elementos favorecem significativamente para a ocorrência da diversidade genética entre os sorovares de *Salmonella* spp., como as SPIs, plasmídeos, profagos funcionais, restos de fagos (Parkhill et al., 2001; Boyd and Brüssow, 2002), além da aquisição de elementos genéticos por transferência horizontal de genes ou por inativação funcional de genes (Dagan et al., 2006; Boolchandani et al., 2019). Com isso, inúmeros genes influenciam na virulência, especificidade e adaptação dos sorovares de *Salmonella* spp. em relação aos seus hospedeiros (Sabbagh et al., 2010).

Em estudo sobre interação patógeno-hospedeiro usando sequenciamento genômico de *Salmonella enterica* sorovar Typhi (ST), restrito a humanos, e *Salmonella enterica* sorovar Typhimurium (STM), que afeta uma ampla gama de hospedeiro, foi demonstrado que aproximadamente 90% dos genes desses sorovares são idênticos (Mclelland et al., 2001). Os 10% de diferença incluem 479 genes exclusivos de STM e 600 genes exclusivos de ST, nos quais estão relacionados ao potencial patogênico (Figura 1) (Parkhill et al., 2001; Johnson et al., 2018). Essas regiões conservadas em ambos os sorovares sugerem a adaptação do patógeno para a colonização intestinal, sobrevivência e disseminação. Já os agrupamentos de genes específicos podem contribuir para a adaptação aos nichos intracelulares e à patogenicidade. Além disso, a alta proporção de pseudogenes em ST tem sido associada a um estilo de vida exclusivo ao hospedeiro (Parkhill et al., 2001; Jong et al., 2012).

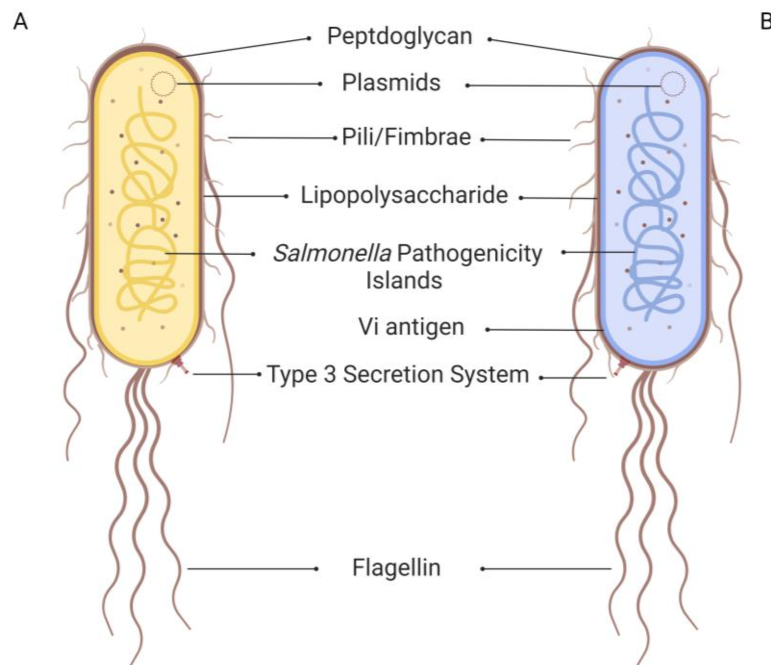

Figura 1. Fatores de virulência de *S*. Typhimurium (STM) (A) e *S*. Typhi (ST) (B). Um dos principais fatores de virulência entre ST e sorovares não-tifóides é a presença do antígeno Vi (cápsula polissacarídica). Fonte: Adaptado de Jong et al., 2012. Criado com BioRender.com.

A diversidade genética e a patogênese molecular das outras salmonelas paratíficas ainda é pouco compreendida em relação a STM. Mecanismos particulares de especificidade/adaptação (aquisição e perda de genes) são capazes de tornar sorovares virulentos para uma espécie animal em particular, independentemente do grau de patogenicidade (menos virulentos ou avirulentos) que exibe para um hospedeiro animal diferente (Evangelopoulou et al., 2013), como *S.* Dublin e *S.* Choleraesuis, que persiste em populações de bovinos e suínos, respectivamente (Lee et al., 2015; Mohammed et al., 2017). No entanto, existem outros fatores que contribuem na especificidade, como a dose infecciosa do patógeno, a espécie animal infectada, a idade e resposta imune do hospedeiro (Evangelopoulou et al., 2013).

Desse forma, a cadeia avícola, ao fornecer fontes proteicas de qualidade a baixo custo, pode contribuir para a transmissão de bactérias zoonóticas, como do genêro *Salmonella*. No entanto, pouco se sabe sobre as características genótipo-fenótipo que

influenciam a adaptação ao hospedeiro e a capacidade de causar doenças em diferentes hospedeiros relacionadas a alguns sorovares, como os aqui estudados (*S.* Schwarzengrund, *S.* Mbandaka, *S.* Braenderup, *S.* Saintpaul and *S.* Senftenberg). Conhecer os padrões genéticos de resistência, virulência e patogenicidade é crucial para entender as diferenças entre isolados de humanos e aves, visando prevenir enfermidades. Entretanto, faltam informações sobre o por que de alguns sorovares colonizarem o intestino de aves sem causar sintomas, atuando como reservatórios de doenças zoonóticas. Este projeto buscou elucidar os fatores genéticos que podem estar envolvidos na adaptação dos sorovares de salmonelas paratíficas em aves comerciais, focando na saúde pública.

6. Objetivos

6.1. Objetivo geral

Realizar análise genômica comparativa de sorovares de salmonelas paratíficas em aves, relacionadas à adaptação do hospedeiro, perfis de resistência antimicrobiana, virulência e patogenicidade associado a genomas de isolados de seres humanos e aves de produção.

6.2 Objetivos específicos

- Sequenciar o genoma de salmonelas paratíficas previamente isoladas em granjas de aves para postura de ovos para consumo mantidos na bacterioteca do Laboratório de Ornitopatologia da FCAV/Unesp - Jaboticabal;
- Comparar geneticamente os isolados com sorovares de salmonelas paratíficas isoladas de produtos avícolas e de seres humanos obtidas em banco de dados públicos de referência;
- Identificar variações genéticas que podem afetar a adaptação do hospedeiro e/ou aptidão de sorovares de *Salmonella* spp. isolados em granjas avícolas e de seres humanos, obtidas em banco de dados públicos de referência;

- Realizar a comparação genotípica de *Salmonella* spp. provenientes de granjas de aves para postura de ovos para consumo mantidos na bacterioteca do Laboratório de Ornitopatologia da FCAV/Unesp - Jaboticabal com os depositados em bancos de dados de referência de isolados de seres humanos;
- Predizer como os genes de resistência antimicrobiana, virulência e patogenicidade são adquiridos/propagados entre os sorovares de *Salmonella* spp.

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CAPÍTULO 2 – Genomic Features and Phylogenetic Analysis of Antimicrobial-Resistant *Salmonella* Mbandaka ST413 Strains[1]

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Abstract: In recent years, *Salmonella enterica* subsp. *enterica* serovar Mbandaka (*S.* Mbandaka) has been increasingly isolated from laying hens and shell eggs around the world. Moreover, this serovar has been identified as the causative agent of several salmonellosis outbreaks in humans. Surprisingly, little is known about the characteristics of this emerging serovar, and therefore, we investigated antimicrobial resistance, virulence, and prophage genes of six selected Brazilian strains of *Salmonella* Mbandaka using Whole Genome Sequencing (WGS). Multi-locus sequence typing revealed that the tested strains belong to Sequence Type 413 (ST413), which has been linked to recent multi-country salmonellosis outbreaks in Europe. A total of nine resistance genes were detected, and the most frequent ones were *aac(6')-Iaa*, *sul1*, *qacE*, $bla_{OXA\text{-}129}$, *tet(B)*, and *aadA1*. A point mutation in ParC at the 57th position (threonine → serine) associated with quinolone resistance was present in all investigated genomes. A 112,960 bp IncHI2A plasmid was mapped in 4/6 strains. This plasmid harboured tetracycline (*tet*ACDR) and mercury (*mer*) resistance genes, genes contributing to conjugative transfer, and genes involved in plasmid maintenance. Most strains (four/six) carried *Salmonella* genomic island 1 (SGI1). All *S.* Mbandaka genomes carried seven pathogenicity islands (SPIs) involved in intracellular survival and virulence: SPIs 1-5, 9, and C63PI. The virulence genes *csgC*, *fimY*, *tcfA*, *sscA*, (two/six), and *ssaS* (one/six) were absent in some of the genomes; conversely, *fimA*, *prgH*, and *mgtC* were present in all of them. Five *Salmonella* bacteriophage sequences (with homology to *Escherichia* phage phiV10, *Enterobacteria* phage Fels-2, *Enterobacteria* phage HK542, *Enterobacteria* phage ST64T, *Salmonella* phage SW9) were identified, with protein counts between 31 and 54, genome lengths of 24.7 bp and 47.7 bp, and average GC content of 51.25%. In the phylogenetic analysis, the genomes of strains isolated from poultry in Brazil clustered into well-supported clades with a heterogeneous distribution, primarily associated with strains isolated from humans and food. The phylogenetic relationship of Brazilian *S.* Mbandaka suggests the presence of strains with high epidemiological significance and the potential to be linked to foodborne outbreaks. Overall, our results show that isolated strains of *S.* Mbandaka are multidrug-resistant and encode a rather conserved virulence machinery, which is an epidemiological hallmark of *Salmonella* strains that have successfully disseminated both regionally and globally.

Keywords: antimicrobial resistance; bacteriophage; One Health; pathogenesis; salmonellosis; virulence factors

1. Introduction

Foodborne diseases caused by *Salmonella* spp. have been frequently associated with poultry products [1]. According to the European Food Safety Authority (EFSA), 65,208 cases of salmonellosis in humans were reported in 2022 [2]. More than 2.650 serovars of this pathogen have already been identified, of which about a hundred have been isolated from both animals and humans [3], wherein in humans, the serovars *S*. Enteritidis (54.6%), *S*. Typhimurium (12.1%), *S*. Typhimurium single-phase 1,4,[5],12:i:- (10.4%), and *S*. Infantis (2.3%) the main isolates [2].

The epidemiological importance of *Salmonella* serovars is related to their geographically widespread distribution and their ability to infect multiple hosts [4]. For instance, day-old chicks can arrive at poultry farms already infected with several *Salmonella* serovars [5]. Moreover, vectors including rodents play a central role in the maintenance and dissemination of *Salmonella* within poultry flocks [5].

Salmonella Mbandaka (*S*. Mbandaka) is a bacterial pathogen with diversity in its host range that includes bovines, poultry, and humans [2,6,7]. However, despite the identification of *S*. Mbandaka in other sources, including animal feed [8], the prediction of the Sequence Type (ST) is not always investigated in studies. Recently, the dissemination of *S*. Mbandaka ST413 in poultry farms has been reported [6] and in humans [7]. This clone was involved in a multi-country outbreak linked to the consumption of poultry meat in EU/EEA, Israel, and the UK, which resulted in 196 human cases, 19 hospitalizations, five septicaemic infections, and one death [7]. Likewise, this serovar was also related to multistate outbreaks in the United States [9]. Another emergent concern to public health is the multidrug-resistant (MDR) profiles exhibited by *S.* Mbandaka isolates from poultry farms in different countries, which involves tetracycline, fluoroquinolones, aminoglycosides, sulphonamides, and third-generation cephalosporins [10,11].

This combination of virulent and MDR phenotypes has been associated with invasive infections caused by *S*. Mbandaka, worsening the severity of clinical signs, and leading to higher mortality rates in humans [7,12]. Despite the emergent epidemiological importance of *S.* Mbandaka, there is little information on the genomic features that allow this serovar to persist in poultry production facilities and use poultry as asymptomatic carriers to disseminate. Thus, we carried out an in-depth genomic characterization of *S.* Mbandaka isolated from the caecal content of laying hens in São Paulo State, Brazil, to analyse the evolutionary, epidemiological, and adaptive potential of this serovar in poultry and humans.

2. Materials and Methods

2.1. Sample Collection

Six strains of *S*. Mbandaka were selected from a collection of 29 isolates using the antimicrobial resistance phenotypic profile observed in a previous study by our group as selection criteria, spanning the period between 2016 and 2017 [13]. In this study, a pool of caecal content samples was collected in 151 commercial layer farms located in the Midwest region of the São Paulo State, Brazil. In total, 2008 samples of 300 g each were collected in sterile flasks and refrigerated (4–8 °C) until analyses of isolation and identification as described previously [13]. The selected six strains were isolated between 2016 (1092/18, 1095/18, 1096/18, and 1097/18) and 2017 (1124/18 and 1158/18) from the caecal content from live laying hens and a laying quail (the isolate 1092/18) from commercial egg-laying farms.

2.2. Whole Genome Sequencing (WGS)

The genomic DNA of the strains was extracted using the Maxwell RSC Cultured Cells DNA kit (Promega, Madison, WI, USA). After extraction, DNA integrity was evaluated by electrophoresis in 1% agarose gel, quantified in a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA), and the concentration analyses using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Scoresby, Australia). A DNA library was prepared using the Flex DNA Library Preparation Kit (Illumina, San Diego, CA, USA) following the instructions from the supplier, followed

by paired-end sequencing performed in Illumina MiSeq (Anicon, Germany; NGS-MiSeq, University of Copenhagen, Denmark) using a Nextera XT v3 kit (2 × 300 bp insert size).

2.3. Downstream Bioinformatic Analyses

The quality of raw reads was analysed using FastQC 0.11.9 [14]. Low-quality reads and adapters were removed using Trimmomatic 0.39 [15], before assembling genomes using Unicycler [16]. The resulting assembled sequences were automatically annotated with RAST 2.0 [17]. Utilizing bioinformatics tools available from Center for Genomic Epidemiology (CGE), including SeqSero 1.2 [18], and MLST 2.1 [19,20], the assembled sequences underwent in silico analysis. This analysis aimed to confirm the serotype and determine the Multilocus Sequence Typing (MLST) sequence type (ST) for *Salmonella enterica*, respectively.

The identification of *Salmonella* pathogenicity islands (SPIs) was performed using SPIFinder 2.0 [21] on April 6th, 2023. The results were compared by nucleotide alignment with the BLAST Ring Image Generator (BRIG) 0.95, in which *Salmonella* Typhimurium LT2 (NC_003197.2) was used as a reference strain [22]. Using Blast atlas analysis, conducted at the Gview server [23], we predicted the presence of SGI1 in the study genomes. For that purpose, we used the reference sequence of *Salmonella* Typhimurium genomic island I (AF261825.2) and ran the analysis with default BLAST parameters. This approach included only those query genome files that were found to harbour SGI1, as indicated by prior results generated by SPIFinder. The output file obtained was visualized using GView's tools, facilitating the investigation of both conserved and variable regions [23].

We also conducted a detailed analysis of the SPI 2-3 regions of *S.* Mbandaka. This investigation was prompted by observed variations within these islands, to elucidate the genetic nuances and potential associated functional implications. To accomplish this, sequences of SPI 2-3 regions from the six *S.* Mbandaka strains were initially collected, alongside the reference sequence of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 (NC_003197.2). Base-to-base alignment was performed using Seaview 5.0.4 [24], and posteriorly, multiple sequence alignment was carried out using the Clustal Omega tool within Seaview software to

reveal the genetic variations and similarities. The aligned regions were curated using Geneious [25].

The VFanalyzer tool available in the Virulence Factors Database [26] was utilized to detect putative virulence factors in the investigated *Salmonella* genomes, and *Salmonella* Typhimurium LT2 (chromosome: NC_003197) was used as the reference genome; this analysis was conducted on 6 April 2023. These data on the presence or absence of virulence determinants were used to generate a heatmap using Morpheus (https://software.broadinstitute.org/morpheus, accessed on 24 August 2023). Additionally, the CGE web server was used on 5 April 2023 to identify the presence of antimicrobial resistance genes using ResFinder 4.1 [27], whereas the detection of plasmid replicons was conducted with PlasmidFinder 2.0 [28] and manually curated using Geneious [25]. For this last analysis, the *S*. Mbandaka 1095/18 genome was aligned with the R478 plasmid (BX664015) using the Map to Reference tool with the default setting to build the contig [25]. Incongruent sequences were removed before circularizing the mobile genome.

The six assembled genomes were analysed by Phage Search Tool Enhanced Release (PHASTER) to identify the presence of prophages [29,30]. Only prophages identified as “intact” (score > 90) were considered for the results. The genome alignment tool [31] was used to assess linkages and correlations based on nucleotide identity between phage sequences.

To elucidate phylogenetic relationships amongst *S*. Mbandaka isolates, whole-genome phylogenies were reconstructed using *Escherichia coli* (U00096.2), *Shigella flexneri* (AE014073.1), and *Salmonella* Typhimurium LT2 (AE006468.2) as outgroups. In addition to the six genomes that were sequenced as part of this research, 475 assembled sequences of *S*. Mbandaka were gathered from the Enterobase database [32,33], spanning various isolation sources over the past five years (2019 to 2023). The genomes were selected on 15 August 2023, in the database according to the standards established by the authors, using the following filters: *S.* Mbandaka, ST413, and coverage ≥ 90. Moreover, the genomes were recovered already assembled (files in “.fasta” format). Input genomes were annotated with Prokka 1.14.5 [34] using default parameters. The Roary v3.12.0 [35] pipeline with default parameters generated the

matrix from the MAFFT alignment program [36]. The phylogenetic tree was constructed using the Maximum Likelihood method in IQ-TREE2 2.2.2.6 [37] with clade support estimates calculated using ultrafast bootstrap (UFBoot) of 1000 pseudoreplicates [38]. TN+F+R10 was applied as a best-of-fit model according to the BIC with ModelFinder [39]. To edit the phylogenetic tree, the online tool iTOL—Interactive Tree of Life was used [40].

2.4. Data Availability and Accession Numbers

The assembled genomes of six *Salmonella enterica* subsp. *enterica* serovar Mbandaka strains were deposited at the NCBI Sequence Read Archive (SRA) website, under Project PRJNA1015686. The complete genome data of 1092/18, 1095/18, 1096/18, 1097/18, 1124/18, and 1158/18 were deposited in GenBank with Accession No. SAMN37152295, SAMN37152294, SAMN37152296, SAMN37152297, SAMN37152298, and SAMN37152299, respectively.

3. Results

3.1. Genome Assembly, Genome Annotation, and MLST

Characteristics and quality parameters of the draft genome assemblies are shown in **Table 1**. Genomes had GC content between 51.8 and 52.2% and genome sizes between 4,738,760 and 4,934,713 bp, which are within the typical range of *S. enterica* (*Salmonella* Typhimurium LT2, NC_003197.2). MLST analysis showed that all strains belonged to the sequence type 413 (ST413).

Table 1. Quality features of draft genome assemblies of the *S.* Mbandaka strains from the caecal content of the laying quail (1092/18) and laying hens (1095/18, 1096/18, 1097/18, 1124/18, and 1158/18) in this study.

	Sample ID					
Assembly attributes	**1092/18**	**1095/18**	**1096/18**	**1097/18**	**1124/18**	**1158/18**
Strain ID	SAMN37152295	SAMN37152292	SAMN37152296	SAMN37152297	SAMN37152298	SAMN37152299
Genome size (bp)	4,854,182	4,929,569	4,929,238	4,738,760	4,934,713	4,916,385
Contigs number	41	36	37	28	51	52
% GC	52.2	51.9	51.9	52.2	51.9	51.8
L_{50}	5	6	7	6	7	8
N_{50} (bp)	322596	296604	277541	286548	187348	160426
Average depth of coverage	108	75	45	27	52	36
CDSs	4,956	4,936	4,977	4,753	5,010	4,993
rRNA	11	7	8	9	10	8
tRNA	80	78	77	75	79	76

N50: represents the sequence length of the shortest contig at which 50% of the total assembly length is reached; L50: represents the minimal count of contigs needed so that their total length equals half of the genome size; CDSs: Coding Sequences; rRNA: Ribosomal Ribonucleic Acid; tRNA: Transfer Ribonucleic Acid.

3.2. Antimicrobial Resistance Determinants

All isolates harboured the gene *aac(6')-Iaa*, which encodes resistance to amikacin and tobramycin, while the gene *aadA1*, responsible for conferring resistance to spectinomycin, was found in four out of six strains. The genes *sul1* (sulfonamides), *dfrA21*, and *dfrA25* (trimethoprim) were also found in five/six, four/six, and one/six of strains, respectively. *tetA* (one/six) and *tetB* (four/six) encoding tetracycline resistance encoding genes were detected in some strains, while the *bla*$_{OXA-129}$ gene related to resistance to β-lactams was detected in four/six of the genomes. Additionally, our results also disclosed the widespread distribution of the *qacE* gene (five/six) in these *S.* Mbandaka strains, which contributes to the resistance to quaternary ammonium compounds (QAC). Moreover, point mutation in ParC [T57S], which confers resistance to quinolones, was identified in all the strains (**Table 2**).

Table 2. Genotypic and phenotypic characterization of the antimicrobial resistance profiles of *S.* Mbandaka isolated from a quail and laying hen farms in Brazil.

Sample ID	AMR phenotypes*	Resistance gene							
		aac(6')-Iaa	*aadA1/ dfrA21*	*sul1*	*dfrA25*	*tet(A)*	*tet(B)*	*bla*$_{OXA-129}$	*qacE*
1092/18	StrSulSxtTetOxiCip	+	-	+	+	+	-	-	+
1095/18	StrSulSxtTetOxiAmoAmpAmcCip	+	+	+	-	-	+	+	+
1096/18	StrSulSxtTetOxiAmoAmpAmcCip	+	+	+	-	-	+	+	+
1097/18	StrSulCip	+	-	-	-	-	-	-	-
1124/18	StrSulSxtTetOxiAmoAmpAmc	+	+	+	-	-	+	+	+
1158/18	StrSulSxtTetOxiAmoAmpAmc	+	+	+	-	-	+	+	+
Total		6	4	5	1	1	4	4	5
%		100	66,7	83,3	16,7	16,7	66,7	66,7	83,3

*: Previous study of the phenotypic resistance profile of *S.* Mbandaka isolates carried out by Benevides et al., 2020 [13]; Str: streptomycin; Sul: sulfonamide; Sxt: trimethoprim/sulfamethoxazole; Tet: tetracycline; Oxi: oxytetracycline; Cip: ciprofloxacin; Amo: amoxicillin; Amp: ampicillin; Amc: amoxicillin/clavulanic acid.

3.3. Plasmid Replicons and Homology to Published Plasmid Sequences

Most strains (five/six) of *S.* Mbandaka harboured plasmid replicons, except the strain 1097/18. IncHI2A plasmid replicons were detected in four genomes (1095/18, 1096/18, 1124/18, and 1158/18), whereas a replicon of IncN type was identified in a single strain (1092/18) (**Table 3**). The detection of those replicons identified in known plasmids (R478 and R46) suggested the presence of a similar sequence to these plasmids in the genomes under study, which was further confirmed by aligning the reference sequence of plasmid R478 (BX664015) to genome 1095/18. The p109518 plasmid was assembled using R478 as a reference genome aligning this to the sequence of 1095/18. While the IncN replicon plasmid (R46 homologous plasmid) was predicted in the 1092/18 genome, attempts to circularize it were unsuccessful. Only a limited region of 6,823 bp was identified, containing the *ccgEIII*, *ardR*, *ardB*, *mucA*, *mucB*, *mpr*, *ardK*, and *repA* genes.

Table 3. General features and accession numbers of the predicted homologous plasmids with PlasmidFinder of *S.* Mbandaka isolated from the caecal content of commercial egg-laying flocks in Brazil.

Sample ID	Incompatibility group	Size (bp)	ORFs	NCBI Accession	Homologous plasmid
1095/18, 1096/18, 1097/18, 1124/18, and 1158/18	IncHI2A	274,762	295	BX664015	R478
1092/18	IncN	50,969	64	AY046276	R46

The results are based on raw reads with an identity threshold of 95% and a minimum coverage of 60% [28].

The manual assembly of the putative R478-like plasmid generated a high-quality circular sequence with only one contig of 112,960 bp (named p109518) (**Figure 1**). The annotation of p109518 predicted 103 Open Reading Frames (ORFs), 79 Coding Sequences (CDSs), and 44.3% G+C content. The p109518 plasmid harbours 36 genes of known function, of which two are involved in replication initiation (rep), that is, genes that encode the core IncHI2 plasmid determinants such as repHIA, position 1…875 (length: 875) and repHI2, position 8463…99551 (length: 1089). The plasmid contained nine genes predicted to be involved in conjugation, including six trh genes (ABELOV) and three htd (OTV) genes. The dam gene encoding for a DNA adenine methylase was also detected. Other genes associated with adaptive advantages for the bacteria included tet-genes (ACDR), which encode tetracycline resistance. A genetic region related to mercury resistance was also detected, including the mer operon (ACDEPT).

Figure 1. The predicted structure of the 112,960 bp p109518 plasmid from *S.* Mbandaka (isolate 1095/18) shows backbone and accessory module regions. Genes with a predicted function are denoted by green arrows, hypothetical proteins in yellow, and ORFs in orange. The innermost blue circle presents a GC content of 44.3%.

3.4. Analysis of SPIs and Virulence Determinants

All the sequenced isolates carried seven SPIs: C63PI, SPI 1 to 5, and SPI 9. We also detected the presence of SGI1 containing an antibiotic-resistance gene cluster in 1095/18, 1092/18 1096/18, and 1158/18 sequences (**Figure 2**). After the local alignment of SPIs 1-5, a deletion close to the 5′ region of SPI 3 (~2000 bp) was the main difference observed between the study isolates and the

reference *Salmonella* Typhimurium LT2 strain. The SPI 2 region, before 70 kbp in **Figure 3**, was shown to have variations in four of the *S.* Mbandaka genomes (except in genomes 1092/18 and 1097/18) when compared with the reference genome. Conversely, we found conserved full SPIs 1, 4, and 5 in all the sequenced isolates (**Figure 3**).

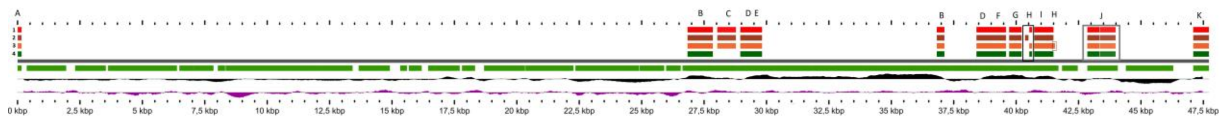

Figure 2. Linear BLAST atlas of SGI1 in *S.* Mbandaka strains 1158/18 (1), 1096/18 (2), 1095/18 (3), and 1092/18 (4). The backbone is represented in grey, the reference sequence (AF261825.2) is represented in a light shade of green, GC content is depicted in black, and GC skew is displayed in purple. A: *thdf* (product: tRNA-5-carboxymethylaminomethyl-2-thiouridine (34) synthesis protein MnmE), B: *intI1* (product: integron integrase Inti1), C: *aadA2* (product: aminoglycoside 3″-nucleotidyltransferase), D: *qacEdelta1* (product: small multidrug resistance (SMR) efflux transporter => QacE delta 1, quaternary ammonium compounds), E: *sul1delta* (product: dihydropteroate synthase type-2—sulphonamide resistance protein), F: *sul1* (product: dihydropteroate synthase type-2—sulphonamide resistance protein), G: product: similar to puromycin N-acetyltransferase, H: product: hypothetical protein, I: *tnpA* (product: transposase), J: *int2* (product: phage integrase) K: *yidY* (product: multidrug efflux pump MdtL (of MFS type)). The figure was built using the Blast Atlas tool (Gview server).

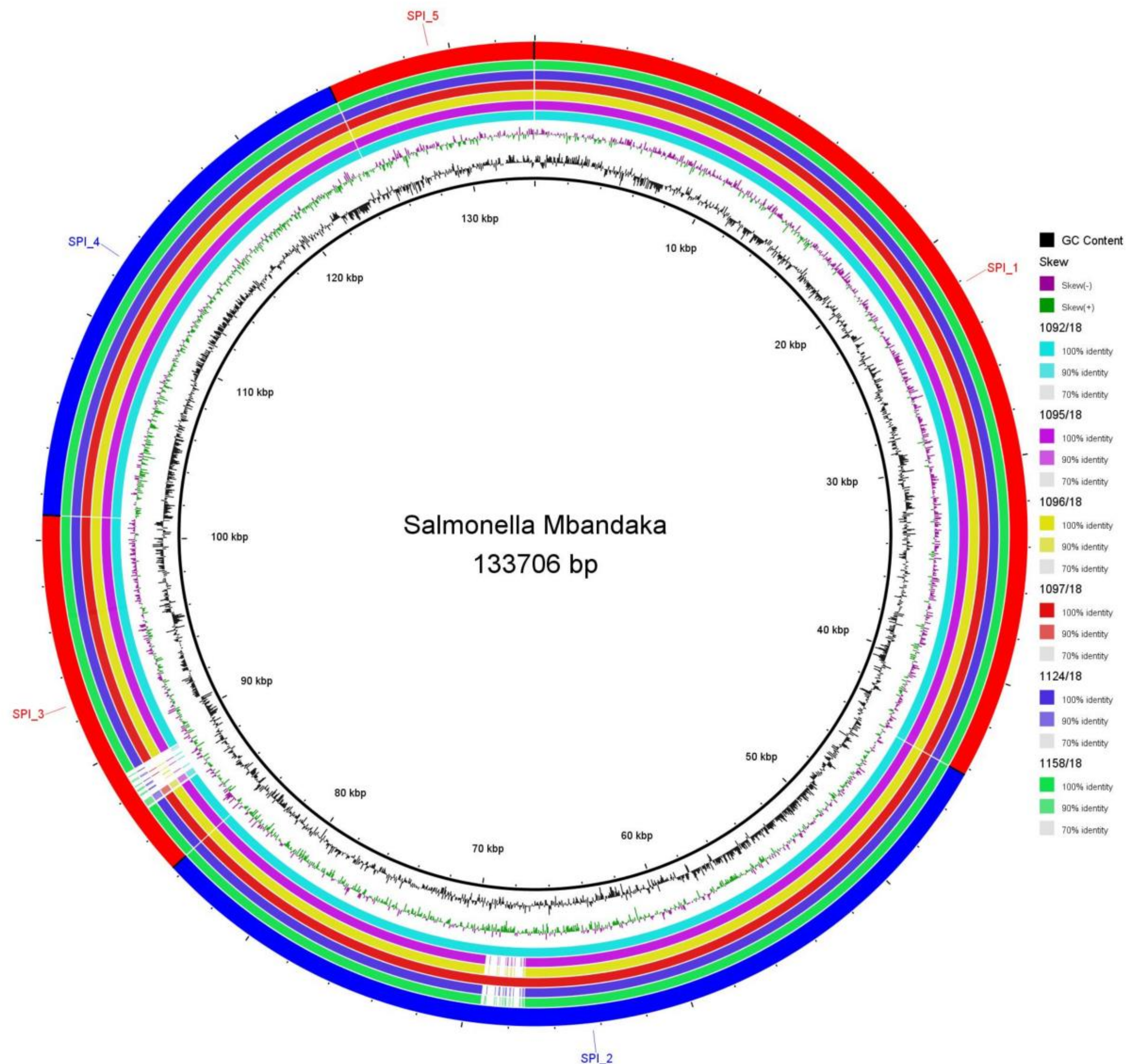

Figure 3. BLAST ring image of the SPIs detected in six *S.* Mbandaka isolates from laying hens and quail in São Paulo State, Brazil. Colour intensities represent the percentage of identity (>90%) with the reference strain *Salmonella* Typhimurium LT2, while blank areas indicate no identity with the reference. The figure is shown in order from inside to outside, starting from the isolates in the right column.

Variations related to deletions within SPI 2 were detected in some genomes (1096/18, 1124/18, and 1158/18); in the 1095/18 genome, the variation occurred in a region encoding a hypothetical protein of unknown function (**Figure 4**). It is worth

mentioning that the 1092/18 and 1097/18 genomes were identical to those found in the reference sequence. In SPI 3, the *rhuM* gene and the DUF4942 domain-containing protein were replaced by hypothetical proteins of 726 bp and 207 bp, respectively (except for genome 1158) (**Figure 4**).

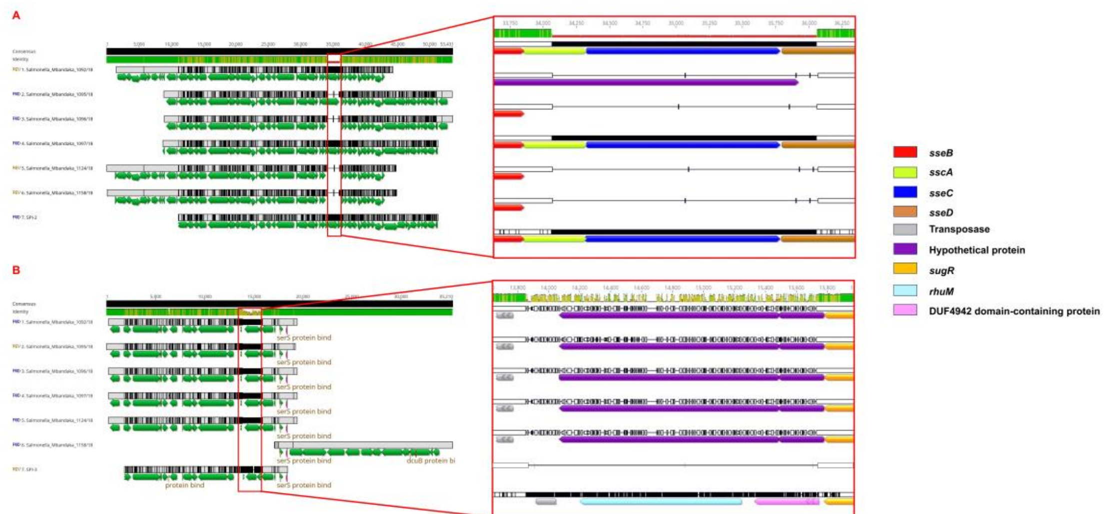

Figure 4. Comparative genomic analysis of SPIs 2 and 3. (**A**) Alignment of the SPI 2 locus and (**B**) alignment of the SPI 3 locus in *Salmonella enterica* serovar Typhimurium LT2 to six *S.* Mbandaka sequences (1092/18, 1095/18, 1096/18, 1097/18, 1124/18, and 1158/18).

The genomes under study carried multiple virulence genes, which were distributed into eight virulence factor (VF) classes. These included fimbrial and nonfimbrial adherence determinants, macrophage inducible genes, magnesium uptake, secretion systems, stress adaptation, and other adherence determinants. Some sequences of *S.* Mbandaka showed variations in relation to the presence of the fimbrial adherence determinants. For instance, the genes *csgC* (1092/18, 1097/18, 1124/18, and 1158/18), *fimY* (1095/18, 1092/18, 1097/18, and 1158/18), *tcfA* (1095/18, 1092/18, 1097/18, and 1124/18) and *stcB* (1095/18) were absent in some of the studied genomes (**Figure S1**). Conversely, genes involved in the macrophage inducible system (*mig-14*), magnesium uptake (*mgtCB*), and a two-component regulatory

system (*phoPQ*) were detected in all the analysed genomes. Furthermore, the analysis highlighted the absence of *sseA*, a gene associated with the secretion system class in the *Salmonella* genus.

Several genes encoded in SPI-2's type three secretion system (T3SS) were detected in all sequences. However, some absences were also observed for the *ssaI*, *ssaM*, *ssaS*, and *sscA* genes. The *sodCI* gene, which is involved in stress adaptation, was predicted in all genomes of *S.* Mbandaka. Likewise, the *fae* (CDEHI), *ste* (ABCDEF), and *stk* (ABCDEFG) operons related to bacterial adherence and fimbria were detected in all analysed genomes. Furthermore, three genes that are implicated in nonfimbrial adherence in *Salmonella* serotypes (*shdA* = 3/6; *ratB* = 5/6; *misL* = 6/6) were detected (**Figure S1**).

3.5. Prophage Regions in Genomes of S. Mbandaka

Overall, ten prophage sequences were identified in the genomes. The *Salmonella* phage SW9 prophage was uniquely detected in four (1095/18, 1096/18, 1124/18, and 1158/18) *S.* Mbandaka sequences. In contrast, the highest number of prophage sequences was detected in two other strains, which contained three prophage sequences per genome, including prevalent prophages such as *Escherichia* phage phiV10 (1092/18 and 1097/18), *Enterobacteria* phage Fels-2 (1092/18 and 1097/18), *Enterobacteria* phage HK542 (1092/18), and *Enterobacteria* phage ST64T (1097/18). We identified an extensive variation in genome phage sizes from *S.* Mbandaka sequences (24.7–47.4 kb) and % GC content (48.57–52.83%). More details about the predicted phages in *S.* Mbandaka genomes are described in **Table 4**. The genes found in all phage sequences involve proteins to prevent the degradation of viral genetic material, DNA packaging, phage structural proteins, lysis components, DNA recombination, regulation, and replication. No phage-borne virulence or antimicrobial-resistance genes were predicted in any of the six *S.* Mbandaka sequences (**Figure S2**).

Table 4. Genomic features of prophages detected in six *S.* Mbandaka genomes analysed from the caecal content of a commercial laying quail and hens, São Paulo, Brazil.

Genome	Region Length[a]	Total Proteins	Contig	Region Position[b]	Phage identity	Access Number	% GC
1092/18	46.4Kb	45	11	3-46484	*Escherichia* phage phiV10	NC_007804	49.99
	26.7Kb	34	15	66909-93641	*Enterobacteria* phage Fels-2	NC_010463	49.10
	24.7Kb	31	24	2-24741	*Enterobacteria* phage HK542	NC_019769	53.23
1095/18	36.3Kb	45	1	399882-436265	*Salmonella* phage SW9	NC_049459	52.82
1096/18	36.4Kb	45	2	185676-222104	*Salmonella* phage SW9	NC_049459	52.83
1097/18	47.7Kb	54	9	152566-200279	*Enterobacteria* phage ST64T	NC_004348	48.57
	46.4Kb	45	13	3-46484	*Escherichia* phage phiV10	NC_007804	49.99
	38Kb	46	18	42660-80693	*Enterobacteria* phage Fels-2	NC_010463	50.29
1124/18	36.3Kb	45	2	455491-491874	*Salmonella* phage SW9	NC_049459	52.82
1158/18	36.4Kb	45	1	138261-174689	*Salmonella* phage SW9	NC_049459	52.82

3.6. Phylogenetic Insights into S. Mbandaka

The pangenome analysis indicated that all the analysed *S.* Mbandaka ST413 genomes ($n = 481$) harboured a total set of 16,663 genes, consisting of 3983 core genes, 203 soft-core genes, 513 shell genes, and 11,964 cloud genes. The 3983 core and soft-core genes, which represent a conserved set of genes shared among the majority of all the analysed genomes, were utilized as the basis for conducting the phylogenetic analysis. The Maximum Likelihood tree (**Figure 5**) revealed six clades, with two well-defined clades (purple and red) that distinctly grouped the genomes according to their geographical origin.

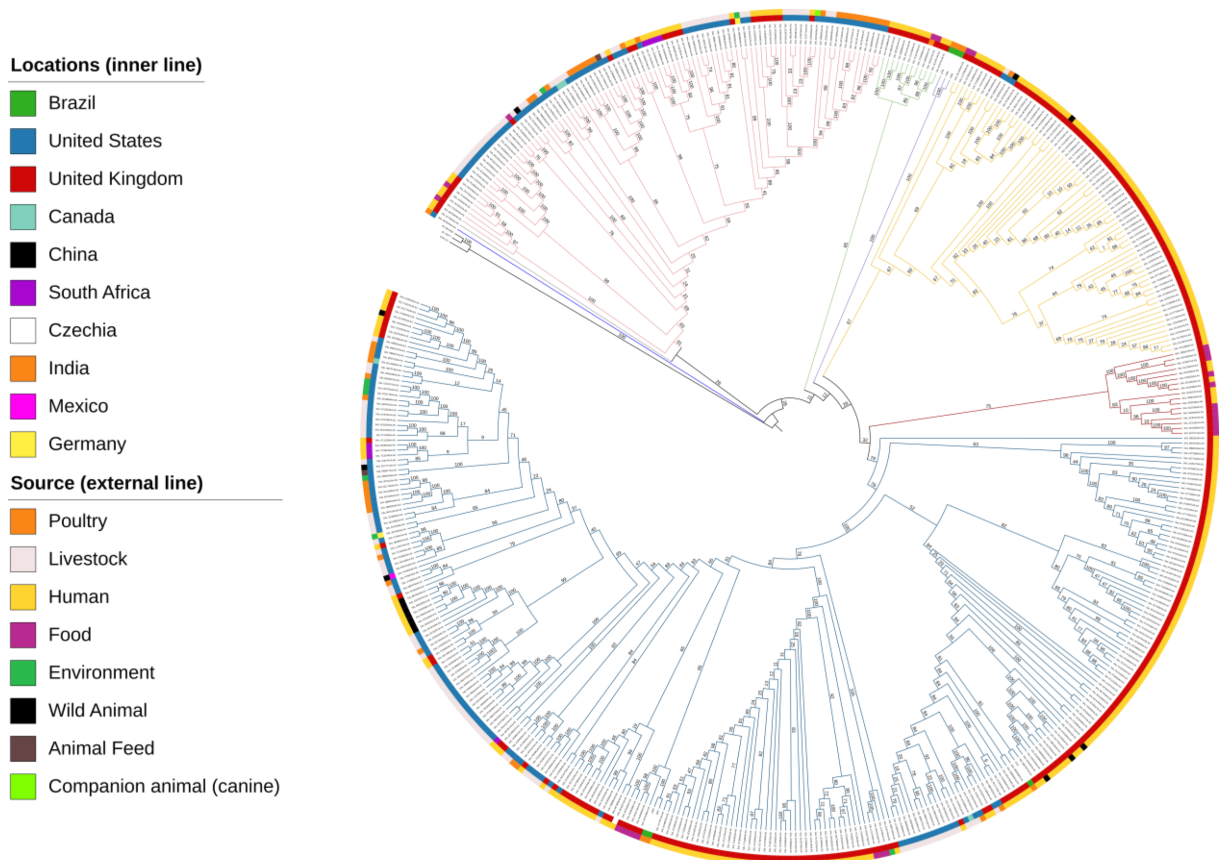

Figure 5. Maximum Likelihood phylogenetic tree of 481 ST413 *S.* Mbandaka strains recovered from poultry. *Escherichia coli* (U00096.2), *Shigella flexneri* (AE014073.1), and *Salmonella* Typhimurium LT2 (AE006468.2) were used as outgroups and to root the tree. The clade support is indicated above or next to each branch as bootstrap values, as calculated from 1000 pseudoreplicates. The colours in the inner ring of the tree represent the location of isolation and the colours in the outer ring indicate the source of isolation (see legend). The coloured branches represent the division of clades: clade 1 (light pink branch), clade 2 (green branch), clade 3 (purple branch), clade 4 (yellow branch), clade 5 (red branch), and clade 6 (blue branch).

The genomes SAL MC9044AA AS (United States—Poultry—2020) and SAL XC7954AA AS (United Kingdom—Human—2022) did not cluster within any defined clade on the phylogenetic tree; instead, strains appeared as standalone lineages. Clade 1 (light pink branch) contains strains from the United States, the United Kingdom, Canada, Germany, and South Africa isolated from different sources. In clade 2 (green branch), a total of 12 *S.* Mbandaka ST413 were grouped, with the majority consisting of 11 strains originating from the United Kingdom, including 91% (10/11)

human isolates, and 9% (1/11) isolates from food, and beyond a single strain isolated from food in India (**Figure 5**).

Clade 3 (purple branch) was well-defined and comprised three poultry strains isolated in Brazil, specifically, the isolates 1158/18, 1096/18, and 1095/18 from our study, indicating a close phylogenetic relationship between these strains, whereas clade 4 (yellow branch) encompassed isolates predominantly derived from the United Kingdom (95.9%), and a smaller portion from United States strains (4.1%). The *S.* Mbandaka ST413 strains from clade 5 (red branch) all originated from the United Kingdom, with 11 strains isolated from food sources (64.7%) and six from human hosts (35.3%), out of a total of 17 strains. Clade 6 (blue branch) represented the largest assemblage of isolates, encompassing Brazilian genomes of *S.* Mbandaka ST413 1124/18, 1092/18, and 1097/18, which exhibited a closer phylogenetic affinity to other strains isolated from poultry and food sources from the United Kingdom (**Figure 5**).

4. Discussion

Our findings present genomic features of the new six genomes of *S*. Mbandaka ST413 and evolutionary insight into the dynamics of this serovar with sequences from different regions of the world. This sequence type has been implicated in foodborne outbreaks in the EU/EEA, Israel, and the UK [7,41] and identified in cases of salmonellosis in Brazil [42]. The recent identification of *S*. Mbandaka ST413 in clinical cases highlights the importance of understanding its genetic characteristics. However, in Brazil, the lack of epidemiological data on this serovar in both humans and poultry led to an unknown scenario, making it difficult to prevent outbreaks and control strategies.

The phylogenetic tree suggests an epidemiological link in the spread of *S.* Mbandaka ST413, with sequences from Brazilian poultry closely related to those from food and human isolates in the United Kingdom and the United States. Non-typhoid serovar *Salmonella enterica* has been a burden to public health, especially for groups of immunocompromised individuals who can develop serious infections that result in death [43]. Thus, the fact that the sequence of *S.* Mbandaka 1124/18 isolated

from poultry in Brazil is closely linked to SALMB7937AA (a human isolate from the United Kingdom) raises an alert for the need for health surveillance since *S.* Mbandaka ST413 was associated with outbreaks in Europe, with cases of septicaemia and one person dying; the most likely the cause of this outbreak was the consumption of contaminated chicken meat [7]. In 2019, a study reported the case of a 69-year-old immunocompromised man who developed mitral valve endocarditis contracted by cereal consumption during an *S.* Mbandaka outbreak in the United States (2018). The authors report the difficulty of treating this infection for this patient due to the MDR feature of that strain [44]. The arrangement of a clade with sequences 1097/18 and 1092/18 with a sister group of food isolates derived from the United Kingdom reinforces the need for control and prevention measures for these pathogens in products of animal origin, which are one of the forms of transmission of infection to humans and are often associated with food outbreaks [7].

Several virulence genes are clustered within SPIs on the chromosome of strains of *S. enterica* and are involved in cell invasion, intracellular survival, and inflammation [45]. The presence of C63PI (six/six) and SPI 9 (six/six) was noticed herein in all *S.* Mbandaka genomes. Although SPIs 1-5 are common in *Salmonella* serovars [46], there are divergences regarding the presence and absence of genes in these islands [45,47]. Many of those mainly found in SPI 2 encode structural and effector proteins of a type III secretion system (T3SS) that transfer essential virulence effectors with important functions during cell infection [45]. Although *Salmonella* Typhi has specific virulence factors, many effectors are absent in this serovar and present in nontyphoidal *Salmonella* (such as *S.* Mbandaka) related to wide-range hosts [48]. Notably, four/six of the *S.* Mbandaka genomes analysed herein lack *sseE*, *sseC*, and *sseD* genes in SPI 2 (**Figure 3**). They compound the *sse* operon (ABCDEFG), in which the *sseC* and *sseD* genes are enrolled to the expression type III secretion system (T3SS) components, and *sseE* has been linked to the possible role of chaperone or helper function for SPI 2 dependent type III reinforcement [49,50]. This implies that the absence of those key genes in SPI 2 of *S.* Mbandaka can lead to the potential attenuation of virulence; however, this hypothesis needs validation through in vivo experiments, not addressed in this work.

The analysis of *S.* Mbandaka virulence factors in the present study also showed the absence of *sseA*. *In vivo* experimentation using *S.* Typhimurium with *sseA* mutation has shown that this gene is the chaperone for the translocon components *sseB* and *sseD*, leading to a high level of virulence attenuation and an intracellular replication defect, while a strain with a mutation in *sseE* is still capable of carrying out systemic *Salmonella* infection of the mouse, suggesting that *sseE* is not essential to this virulence system [49,50]. SPI 3 harbours the *mgtCB* virulence genes that are related to Mg^{2+} uptake when under restricted availability of this cation, such as within the macrophage environment [45,51]. While the *mgtCB* genes were predicted in *S.* Mbandaka isolates, other regions of SPI 3 were absent in all the genomes [47], possibly implying that these genes are not essential for *Salmonella* serovar infection. Although the expressions of virulence genes are not the focus of this work, we acknowledge that additional research into the variation in the virulence factors and the interaction of those genes will further our understanding of the role of the virulence mechanism in this serovar and contribute valuable insights into the broader landscape of *Salmonella* pathogenesis.

Additionally, for virulence factors, this study showed that *S.* Mbandaka ST413 strains carry plasmid-borne or chromosomal genes conferring resistance to several antimicrobials commonly used in both the poultry industry and human medicine, such as aminoglycosides (*aac(6') -Iaa*), sulfonamide (*sul2*), streptomycin (*aph(6)-Id*), tetracycline (*tetA* and *tetB*), and trimethoprim (*dfrA14*) [41]. The presence of the *aac(6')-Iaa* gene is in line with the wide distribution of aminoglycoside resistance genes among *Salmonella* strains of different serovars [52,53]. However, despite the prevalence of aminoglycoside resistance genes and their *in vitro* characteristics, it has been reported that this antimicrobial class exhibits clinical inefficacy against *Salmonella* spp. [54].

The plasmid replicon IncHI2A was detected in four *S*. Mbandaka genomes and harboured tetracycline resistance genes (*tet*ACDR). Our findings align with a broader context of antimicrobial resistance in *Salmonella* spp., where an average of 71.1% of tetracycline-resistant isolates and an average of 57.4% of sulphonamide-resistant strains, with the *tetA*, *tetB*, *sul1*, and *sul2* genes being the most prevalent [55]. This underscores the significance of conjugation events in gene dissemination since those

are often associated with plasmids [56]. Furthermore, the selective pressure exerted using these antimicrobials in agriculture further fosters the emergence of antibiotic resistance [57].

bla$_{\text{OXA-129}}$ is an Extended Spectrum β-lactamase (ESBL)-encoding gene that belongs to the class D beta-lactamase enzymes, and it is often carried by plasmids [58]. The presence of pathogens such as *S*. Mbandaka that harbour β-lactamase genes carried by mobile genetic elements is a burden that creates a multifaceted risk, which, allied to the potential inefficiency of antibiotic treatment, facilitates the spread of resistance among several bacterial species [59]. In contrast to previous findings in *Salmonella* spp. related to the poultry industry, the presence of this plasmid-borne gene in our isolates highlights the complexity of the antimicrobial resistance dynamics and the need for continued research on plasmids and other genetic elements that may be involved in its dissemination [60].

Another QAC resistance gene (*qacF*) was detected in multidrug-resistant isolates of *S. enterica* from chickens, which was carried in a plasmid pSGB23 along with 11 antimicrobial and disinfectant resistance genes [61]. This corrobora

tes with our study, where all five QAC-resistant sequences are multidrug-resistant strains. The dissemination of these genes can become a problem for public health because QAC is an essential biocide used to disinfect food processing facility environments and to prevent and control nosocomial infections in hospitals [62].

In *Salmonella* spp., the main mechanism of resistance to quinolones is associated with mutations in the *gyrA* and *parC* genes [63]. In the present study, all *S.* Mbandaka sequences had a single point mutation in ParC [T57S], a common mutation in *Salmonella* spp. serovars since the early 2000s [64]. *qnr* genes encoding plasmid-mediated resistance to quinolones (PMQR) in *S. enterica* isolated from animals have been reported [65] but were not detected in the *S.* Mbandaka genomes in the present study.

One of the most striking genomic features of the strains studied here is SGI1, which was present in most study genomes (four/six). This resistance island was first identified in a global epidemic of multidrug-resistant *Salmonella* Typhimurium DT104

[66]. A feature of this island is that it often contains various genes endowing their hosts with new traits, like antimicrobial resistance and virulence that enhance bacterial adaptation to the environment [67]. Beyond *Salmonella* serovars, the dissemination of the SGI1 extends to other bacteria that already circulate in humans and production animals (poultry and swine), suggesting that its spread is influenced by various genomic events such as insertions, deletions, and homologous recombination [68,69]. In a study encompassing 35 *Salmonella* serovars, variations in these moving elements were observed, in which the *dfrA12* and *aadA2* genes were the most prevalent [68]. Our study corroborates these findings, with the presence of the *intl* gene in all *S*. Mbandaka genomes accompanied by antimicrobial resistance genes (*sul1*, *aadA2*, and *qacE*), emphasizing the imperative for ongoing surveillance to comprehend and address the spread of genetic elements associated with the determinants of resistance.

While *S*. Mbandaka's multidrug-resistant profile has been acknowledged, little is understood about the genetic mechanisms behind this trait and the transferability of resistance [29]. Our study identified the IncHI2A plasmid replicon in four *S*. Mbandaka genomes as a key vehicle for horizontally transferring tetracycline (*tet*) and mercury (*mer*) resistance genes. The plasmid also carries genes facilitating transfer mechanisms (*trh* e htd). The IncHI2 plasmid is implicated in spreading antibiotic resistance genes in clinical and food isolates of *Salmonella*, such as β-lactamase ($bla_{OXA\text{-}1}$ and $bla_{TEM\text{-}1}$), and Plasmid-Mediated Quinolone Resistance (*qnrA* and *acc (6') -ib-cr*), with potential implications for clinical and public health [70].

Due to the extensive diversity within the *Salmonella* genus, identifying phages and interactions with the host cell is crucial, given their role in bacterial virulence, significant potential as biocontrol agents, and for use as therapeutics [71]. The *Enterobacteria* phage ST64T, previously identified in *S*. Typhimurium, integrates near tRNAs and was found to be associated with tRNA-Arg (anticodon CCT) in our study with *S.* Mbandaka, facilitating pathogenicity islands integration, which can affect gene expression and modulate virulence [72]. While temperate phages like SW9 and phiV10 are related to aid *Salmonella* adaptation during infection [73], further investigation is needed on phiV10 integration in the *Salmonella* genus, given its specific association with *E. coli* O157:H7 [74], which is associated with severe

intestinal infections [75]. Despite the diversity of phages described in this study, no genes encoding virulence or antibiotic resistance were predicted in the 10 phage sequences; however, our understanding of the ecology and diversity of *S.* Mbandaka phages is limited, and it is imperative to conduct additional tests to confirm the absence of these genes.

5. Conclusions

This study showed some *S*. Mbandaka ST413 strains circulating in egg-laying flocks in the Brazilian southeast harbour genomic features associated with strong antimicrobial resistance profiles, which is a hallmark of epidemiologically relevant strains. They also have conserved virulence machinery and are genetically close to strains involved in foodborne outbreaks and invasive salmonellosis cases around the world, granting the need for further research on the factors behind the emergence of this *Salmonella* serovar. This study expands our knowledge of *S*. Mbandaka ST413 and highlights the necessity for further investigations into the genetic and environmental determinants contributing to the evolution and dissemination of these serovars.

Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms12020312/s1, **Figure S1**: Heatmap comprising the presence (black cells) or absence (white cells) of 67 virulence genes among Brazilian *S.* Mbandaka isolates. The strains are clustered by their prediction of gene similarities. The strains were analysed with hierarchical clustering using Pearson's correlation coefficient. The virulence gene profiles that exhibited differentiation among the studied genomes were used to generate the heat map. *Salmonella* Typhimurium LT2 (chromosome: NC_003197) was used as the reference genome.; **Figure S2**: Genomic maps of *S.* Mbandaka prophages using multiple alignments of the genomic sequence [27]. The CDSs are shown in white, and the connected blocks indicate matching regions between the phages. The phage alignment sequence: *Enterobacteria* phage Fels-2 (1092/18 and

1097/18), *Escherichia* phage phiV10 (1092/18 and 1097/18), and *Salmonella* phage SW9 (1095/18, 1096/18, 1124/10, and 1158/18).

Author Contributions

Study conception and design, V.P.B., M.M.S.S., J.E.O. and A.B.J.; acquisition of data, analysis, and interpretation of data, V.P.B., M.M.S.S., C.F.N., S.R.S., V.F.O.M., H.C. and J.E.O.; drafting of this manuscript, V.P.B., M.M.S.S., E.J.D.-S., C.J.B.O. and J.E.O.; supervision and critical revision, M.M.S.S., E.J.D.-S., C.J.B.O., A.B.J., H.C. and J.E.O. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

All data needed to evaluate the results and conclusions can be found in online repositories and the associated Supplementary Material. Additional data related to this study can be requested from the authors.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CAPÍTULO 3 – Genomic profiling of neglected *Salmonella enterica* serovars from poultry and human[1]

[1] Este capítulo corresponde ao artigo científico que será submetido à revista Zoonoses and Public Health (IF = 2.4; Qualis CAPES A1)

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Abstract: Bacteria of the genus *Salmonella* are pathogens of zoonotic importance. Some serovars can establish themselves in poultry in the digestive tract and spread without causing any apparent clinical signs, which poses a risk to public health. However, the factors associated with some *Salmonella* serovars (*S.* Saintpaul, *S.* Braenderup, *S.* Senftenberg, and *S.* Schwarzengrund) genetic diversity remain unclear. In this context, we investigated genotypic profiles of antimicrobial resistance, plasmid replicons, and virulence factors in 301 *Salmonella enterica* genomes from humans and animals collected in a public database. Additionally, we included ten new *Salmonella enterica* genomes isolated from fecal samples of laying hens from Brazil

between 2016 and 2017. A large number of antimicrobial resistance genes has been detected across various *Salmonella* serovars; a limited number of unique resistance genes was predicted in *Salmonella* isolates from poultry hosts compared to those from human hosts. Specifically, among the 52 antimicrobial resistance genes identified, 48% (25/52) were shared between poultry and human isolates, while 21.1% (11/52) were exclusive to poultry isolates and 30.7% (16/52) were exclusive to human isolates. The chromosomal point mutations associated with the *gyrA* and *parC* genes were also predicted. All sequences from *Salmonella* spp. harbored at least one of the pathogenicity islands (SPI 1–5, SPI 8–14 CS54, and C63PI), varying according to host and serovar analyzed. For instance, SPI-1 to SPI3 and SPI-5 were identified in all strains of *Salmonella enterica*, regardless of the host. To the best of our knowledge, this is the first work to report *Salmonella* Braenderup carrying the SPI-10. SGI-1 was detected in a few isolates of *S*. Schwarzengrund from poultry and the CS54 island was solely noticed in genomes referring to the serovars *S*. Saintpaul and *S.* Braenderup. A high diversity of plasmid replicons was identified in *Salmonella enterica*; the serovar Saintpaul was the one with the lowest diversity. A total of 271 virulence genes were analyzed across 311 genomes, showing variation in their occurrence among serovars (ranging from 197 to 212). While 161 genes were common to all serovars, the remaining genes were exclusively identified within specific serovars, revealing a distinct distribution pattern within the *Salmonella enterica* population. Overall, our study brings to light the genetic potential of *Salmonella* serovars frequently neglected in poultry production, which threaten public health, particularly due to multidrug-resistant profiles against active principles used to treatment human infections.

Keywords: antimicrobial resistance; epidemiology; foodborne pathogen; plasmids; salmonellosis; virulence.

1. Introduction

Salmonella spp. is a high-priority pathogen in the antimicrobials resistance research field, since this foodborne agent is responsible for many deaths especially in emerging and least-developing countries (WHO, 2017). The bacteria was responsible for 65,208 cases of human salmonellosis in the European Union in 2022, which 38.9%

lead to hospitalized cases (ESFA, 2023). Nowadays, more than 2,650 serovars of *Salmonella enterica* (*S. enterica*) were identified and closely to a hundred of them can infect both human beings and animals (Issenhuth-Jeanjean et al., 2014). Infections by non-typhoid *Salmonella* in poultry may not trigger clinical signs and the poultry becomes an asymtopmatic carrier, hampering its diagnosis (Saraiva et al., 2022). Taking it into account, poultry are the main reservoirs of potential zoonotic salmonellae (Ruvalcaba-Gómez et al., 2022).

Throughout the years, an increase in the frequency of multidrug-resistant *Salmonella* has been noticed (Saraiva et al., 2022) and it put light on possible failures of critically important antimicrobial treatments against human salmonellosis infections (WHO, 2019). Furthermore, the concern extends to the transference of resistance genes to other pathogens (WHO, 2019). Worsenen the situation, some serovars are neglected, which compromises the diagnosis and treatment efficacies due to the fact that the resistance and virulence profiles vary among serovars and the host range (Kim et al, 2019; Benevides et al., 2020). In this scenario, the present study focuses on the genetic profiles of serovar genomes of *S*. Saintpaul, *S.* Senftenberg, *S*. Braenderup, and *S*. Schwarzengrund of poultry and human origins that are poorly enlightened.

2. Materials and methods

2.1. Bacterial strains

Ten strains of five serovars of *Salmonella enterica* (**Figure 1**) were isolated from cecal feces in commercial egg-laying farms in the State of São Paulo, Brazil, as reported by Benevides et al. (2020).

Figure 1. Phenotypic characterization of antimicrobial resistance profiles of *Salmonella enterica* serovars isolated from commercial egg-laying farms in Brazil. Hierarchical clustering was performed using Euclidean distance and the average linkage method. A previous study of the phenotypic resistance profile of *Salmonella enterica* isolates carried out by Benevides et al., 2020; Sul: Sulfonamide; Sxt: Trimethoprim/Sulfamethoxazole; Amc: Amoxicillin/Clavulanic acid; Amo: Amoxicillin; Amp: Ampicillin; Tet: Tetracycline; Oxi: Oxytetracycline; Enr: Enrofloxacin; Str: Streptomycin; Cip: Ciprofloxacin.

2.2. DNA Extraction and Whole-Genome Sequencing

The genomic DNA from the *Salmonella* spp. strains were extracted using the Maxwell RSC Cultured Cells DNA kit (Promega, Madison, USA). Then, DNA integrity was assessed through electrophoresis on a 1% agarose gel, quantified using a spectrophotometer (NanoDrop, Thermo Fisher Scientific, USA), and concentration analyses were conducted with the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Scoresby, Australia). Subsequently, a DNA library was prepared using the Flex DNA Library Preparation Kit (Illumina, San Diego, CA, United States) following the supplier's instructions. Paired-end sequencing was performed on Illumina MiSeq (Anicon, Germany; NGS-MiSeq, University of Copenhagen, Denmark) platform using a Nextera XT v3 kit (2 x 300 bp insert size).

2.3. Genomic analysis

Raw data were checked for quality using FastQC 0.11.9 (Andrews, 2010). The trimming of both adapter and low-quality reads was performed using Trimmomatic-0.39 (Bolger et al., 2014). Moreover, the assembly was carried out by means of Unicycler (Wick et al., 2017), and an automated annotation was done with Rapid Annotation using Subsystem Technology (RAST) (Aziz et al., 2008).

In addition to the new ten genomes sequenced in this research, assembled sequences of *Salmonella enterica* (*S*. Saintpaul, *S*. Senftenberg, *S*. Braenderup, and *S.* Schwarzengrund) were gathered from the Enterobase database (Alikhan et al., 2018; Achtman et al., 2020), comprising human and poultry isolates from 2016 to 2020. The genomes were harvested in August 2020 from the database according to the standards established by the authors, using the following filters: serovar (serovar name), source type (poultry or human), and coverage ≥ 90. Due to the large number of genomes available, a selection standard was established, in which up to ten genomes from each serovar/host were selected per year. Moreover, the genomes were recovered in ".fasta" format.

Utilizing bioinformatics tools available from Center for Genomic Epidemiology (CGE), including SeqSero 1.2 (Zhang et al., 2015), MLST 2.1 (Larsen et al., 2012; Achtman et al., 2012), PlasmidFinder 2.1 (Carattoli et al., 2014), SPIFinder 2.0 (Roer et al., 2016) and ResFinder 4.1 (Zankari et al., 2012), the assembled sequences were submitted to *in silico* analysis. It was performed to confirm the serotype, to determine the Multilocus Sequence Typing (MLST) for *Salmonella enterica*, to identify plasmid replicons, *Salmonella* Pathogenicity Islands (SPIs), and acquired antimicrobial resistance genes, respectively.

VFanalyzer tool available in the Virulence Factors Database (Chen et al., 2005) was used to detect putative virulence factors in the investigated *Salmonella* genomes. For this, *Salmonella* Typhimurium LT2 (chromosome: NC_003197) was used as the reference genome. The matrix of presence and absence of virulence factors identified with the VFDB tool was used for comparative analysis between *Salmonella enterica* genomes associated with samples of poultry and human origins. Genes were filtered based on their frequent presence, selecting those with higher occurrence in *Salmonella*

genomes isolated from poultry relative to *Salmonella* genomes of human origin. Then, the selected genes were subjected to a clustering analysis. The filtered data matrix was used to calculate the Euclidean distances between virulence factors using the “dist” function of the stats package (v.4.1.2), and then complete hierarchy clustering (full linkage) was performed using the "hclust" function in R (R Core Team, 2021). To visualize the results, a heat map was constructed using the “ggplot2” package (v3.4.4) (Wickham, 2016).

2.4. Phylogenetic analysis

Aiming to provide a better visual overview of the strains analyzed, an additional SNP analysis was performed in the web-based tool CSI Phylogeny 1.4 (Kaas et al., 2014). The 19 Brazilian strains of serovars of *Samonella* were The genome of *Salmonella* Typhimurium LT2 (AE006468.2) was used as reference for the alignment and the default parameters were applied. The phylogenetic tree was constructed using Maximum Likelihood (ML) method, implemented in RAxML-HPC2, with 1000 bootstrap replicates on the XSEDE workflow provided by the CIPRES Science Gateway v. 3.3 (Miller et al., 2010). In the advanced parameters, the "dna_gtrcat_" option was supplemented with the "GTRGAMMA" topic. Subsequently, the resulting phylogenetic tree was modified using the online tool iTOL – Interactive Tree of Life (Letunic et al., 2021). Accession numbers and related metadata of the *Salmonella enterica* serovars genomes are displayed in **Table S1.**

2.5. Data availability and accession numbers

The genomes of the ten *Salmonella enterica* strains were deposited into the NCBI (BioProject PRJNA1015686). The GenBank BioSample IDs for the individual strains are SAMN39832332 (1093/18), SAMN39832339 (1099/18), SAMN39832335 (1105/18), SAMN39832338 (1107/18), SAMN39832340 (1108/18), SAMN39832334 (1118/18), SAMN39832333 (1122/18), SAMN39832336 (1128/18), SAMN39832337 (1133/18), and SAMN39832341 (1164/18).

3. Results

3.1. Genomic Sequencing, Genomic Annotation and Multilocus Sequence Typing

Characteristics and quality parameters of the draft genome assemblies are shown in **Table 1**. Genomes had GC content between 51.7 and 52.2 %, and genome sizes ranged from 4,712,070 and 4,969,463 bp, which are within the typical range of *S*. *enterica* (*Salmonella* Typhimurium LT2, NC_003197.2).

Four distinct sequence type (ST) were recognized , corresponding to one for each which four sequenced serovars: one strain belonged to ST96 (*S*. Schwarzengrund), followed by two ST50 (*S*. Saintpaul) strains, three ST14 (*S*. Senftenberg) strains, and four ST22 (*S*. Braenderup) strains.

Table 1. Quality features of draft genome assemblies of the *Salmonella enterica* strains isolated from cecal feces samples of laying farms in Brazil.

	Sample ID									
Assembly Attributes*	1093/18	1099/18	1105/18	1107/18	1108/18	1118/18	1122/18	1128/18	1133/18	1164/18
Genome size (bp)	4,890,648	4,733,889	4,781,586	4,734,540	4,770,017	4,669,367	4,712,070	4,779,237	4,969,463	4,725,118
Contigs number	35	25	24	27	28	59	50	29	33	27
% GC	51.7	52.2	52.1	52.2	52.1	52.1	52.1	52.1	52.0	52.2
L_{50}	7	4	6	4	4	10	7	6	6	4
N_{50} (bp)	204,728	459,384	347,841	459,384	457,923	126,650	150,042	286,918	286,917	417,103
Average depth of coverage	90.53	50.45	29.42	79.00	57.83	57.32	40.99	52.74	39.7	28.79
CDSs	4,729	4,730	4,597	4,596	4,596	4,833	4,836	4,836	4,980	4,753
rRNA	9	12	10	22	22	22	22	22	22	22
tRNA	73	78	85	85	84	84	85	89	84	78

* N50: represents the sequence length of the shortest contig at which 50% of the total assembly length is reached; L50: represents the minimal count of contigs needed so that their total length equals half of the genome size; CDSs: Coding Sequences; rRNA: Ribosomal Ribonucleic Acid; tRNA: Transfer Ribonucleic Acid.

3.2. Selected sequences from genomic database

A total of 301 sequences were selected: *S.* Saintpaul (poultry=15/humans=43), *S.* Senftenberg (poultry=40/humans=35), *S.* Braenderup (poultry=46/humans=44), and *S.* Schwarzengrund (poultry=43/humans= 35). From them, 47.8% (n = 144) were isolated from poultry and 52.1% (n = 157) were isolated from humans. The assembled genomes were downloaded from the EnteroBase website. Details of each sequence used herein are provided in **Table S2**.

3.3. Antimicrobial resistance determinants

In the prediction of antimicrobial resistance genes acquired in serovars of *Salmonella enterica*, a substantial number of genes were identified in the 311 sequences analyzed. Of these genes, 48% (25/52) were common across all genomes, 21.1% (11/52) were unique to *Salmonella* isolates from poultry, and 30.7% (16/52) were unique to *Salmonella* isolates from humans (**Figure 2**).

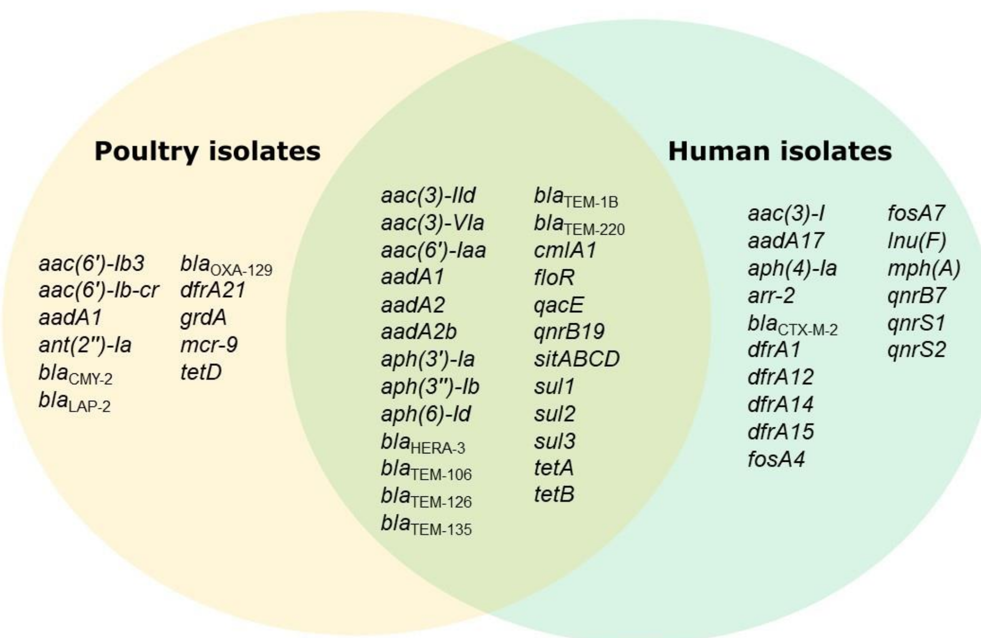

Figure 2. Venn diagram showing antimicrobial resistance genes identified in 311 genomes of *Salmonella enterica* (*S.* Saintpaul, *S.* Senftenberg, *S.* Braenderup, and *S.* Schwarzengrund) isolated from poultry (left/yellow) and humans (right /blue).

The genes shared in both isolates are shown at the intersection (middle/green) of the diagram.

At least one antimicrobial resistance gene was found in all serovars, regardless the source. A total of 10,6% (33/311) genomes contained resistance determinants against three or more classes and were considered as a potentially multidrug-resistant strain. The most prevalent gene that encodes beta-lactam resistance detected in *Salmonella* Schwarzengrund was *bla*$_{TEM-1B}$, with a frequency of 8.6% (3/38) in isolates from human sources. In contrast, other genes exhibited even lower prevalence rates. Specifically, in poultry isolates, the genes *bla*$_{HERA-3}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-129}$, and *bla*$_{LAP-2}$ were detected at a frequency of 2.3%. For human isolates, the genes *bla*$_{CTX-M-2}$, *bla*$_{TEM-106}$, *bla*$_{TEM-220}$, *bla*$_{TEM-126}$, and *bla*$_{TEM-135}$ were identified with a prevalence of 2.9%.

A more pronounced dissimilarity in gene diversity was noticed within sequences of *S.* Saintpaul isolated from humans. Four genes that confer resistance against β-lactams (*bla*$_{CMY-2}$, *bla*$_{TEM-106}$, *bla*$_{TEM-126}$, *bla*$_{TEM-135}$, *bla*$_{TEM-220}$) were exclusively detected in 11% of the analyzed poultry genomes. However, 17 genes were exclusive to human isolates, encoding resistance to fluoroquinolones (*qnrB19*, *qnrS1*), macrolides (*mph(A)*), phenicols (*floR*), aminoglycosides (*aph(3'')-Ib*, *aph(6)-Id*, *aadA17*, *aph(3')-Ia*), folate pathway antagonists (*sul2*, *sul3*, *dfrA14*, *dfrA15*), fosfomycin (*fosA4*, *fosA7*), β-lactams (*bla*$_{HERA-3}$), rifampicin (*arr-2*), and lincosamides (*lnu(F)*) (**Figure 3**).

In a comparative analysis of *S.* Senftenberg genomes, avian isolates exhibited the highest abundance of resistance genes. On the other hand, human-isolate *S.* Senftenberg strains only harbored the *aac(6')-Iaa* gene (100%) and the *qnrB19* gene (8.6%). Notably, *qnrB19* gene was absent in the genomic sequences of poultry source strains **(Figure 3)**.

S. Braenderup genomes of human origin exhibited high diversity of genes encoding resistance to fluoroquinolones, including *qnrB7* (6.8%), *qnrB19* (6.8%), *qnrS1* (2.3%), and *qnrS2* (2.3%). In 2.0 % of the genomes of S. Braenderup isolated from poultry, exclusive predictions were made for genes aac(3)-Via, aph(3'')-Ib, aph(6)-Id, sul1, qacE, and blaCMY-2 (Figure 2).

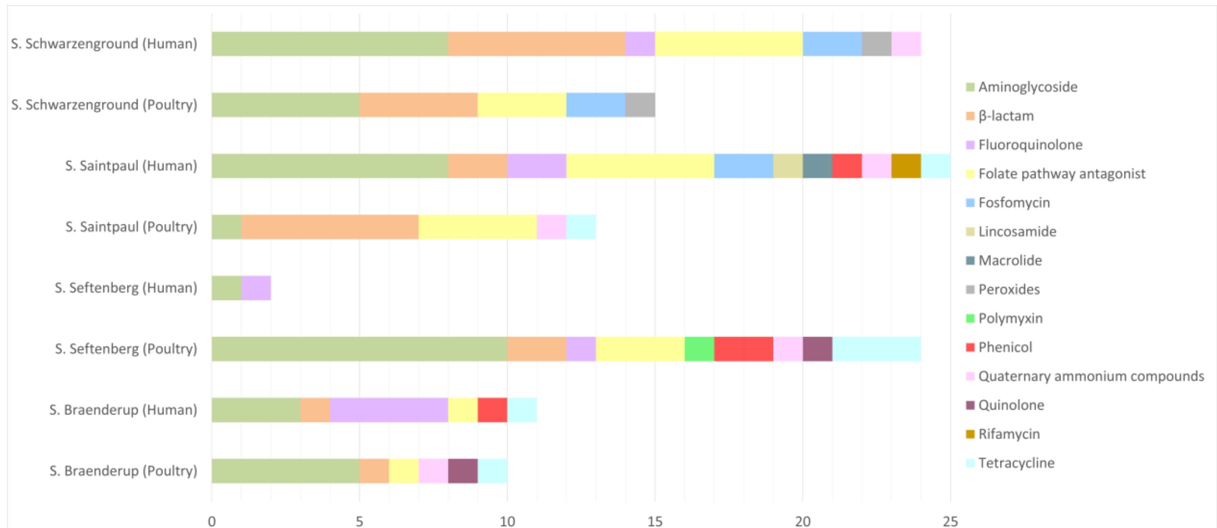

Figure 3. Diversity of resistance genes class predicted in *Salmonella* serovars isolated from humans beings and poultry.

Chromosomal point mutations associated with antimicrobial resistance were identified in the *gyrA* and *parC* genes, which confer resistance to quinolones, as detailed in **Table 2**.

Table 2. Percentage of known chromosomal point mutations detected in *Salmonella enterica* serovars isolated from humans and poultry.

Serovar/source	**Chromosomal point mutations**			
	parC	*gyrA*		
	T57S	S83F	D87N	S83Y
Schwarzengrund/Poultry	100%	-	-	-
Schwarzengrund/Human	100%	8,5%	2,8%	20%
Saintpaul/Poultry	-	-	-	-
Saintpaul/Human	11,6%	-	-	6,9%
Senftenberg/Poultry	100%	-	-	-
Senftenberg/Human	100%	-	-	-
Braenderup/Poultry	100%	-	-	-
Braenderup/Human	98%	-	4,5%	-

-: Unidentified mutation. Number of sequences analyzed: *S.* Schwarzengrund/Poultry (44); *S.* Schwarzengrund/Human (35); *S.* Saintpaul/Poultry (17); *S.* Saintpaul/Human (43); *S.*

Senftenberg/Poultry (43); *S*. Senftenberg/Human (35); *S*. Braenderup/Poultry (50); *S*. Braenderup/Human (44).

3.4. Salmonella pathogenicity islands

Table 3 shows the presence of pathogenicity islands among the 311 *Salmonella enterica* genomes analyzed. All sequences of *Salmonella* spp. harbored at least one of the pathogenicity islands (SPI 1–5, SPI 8–14 CS54 and C63PI).

SPI-4 was present in 100% of the analyzed genomes isolated from poultry, however, for human isolates, variation in the percentage of presence was identified (**Table 3**). SPI-8 was identified in the sequences of *S*. Schwarzengrund isolated from humans (11.4%) and *S.* Senftenberg (86%) isolated from humans and from poultry (84%).

The main differences were found in the presence of SPI-10, SPI-11, and SPI-12. Only 2.3% of *S*. Braenderup sequences from humans harbored SPI-10, while 6.8% carried SPI-12. The latter was also predicted in sequences from *S*. Braenderup (14%), and *S*. Saintpaul (24%). It is worth mentioning that in 5% and 16% of *S*. Saintpaul genomes from humans, SPI-11 and SPI-12 were detected, respectively.

The pathogenicity islands SPI-13 and SPI-14 were detected in the genomes of serovars Schwarzengrund, Saintpaul, and Braenderup, but were not identified in the genomes of *S*. Senftenberg. Furthermore, SPI-10-12 was not detected in all *S*. Senftenberg genomes.

The C63PI island was detected in variable percentages in all serovars, except serovar Senftenberg, in which it was present in 100% of the analyzed sequences. The CS54 island was identified in the genomes of *S*. Saintpaul isolated from humans and poultry, with an occurrence of 88%. It was also predicted in 88 % of *S*. Braenderup genomes from poultry and 81.8% in isolates from humans.

Table 3. Predicted percentages of Pathogenicity Islands in *Salmonella enterica* isolates from humans and poultry sources.

SPI	Serovars (%)							
	SSc_P	SSc_H	SSa_P	SSa_H	SSe_P	SSe_H	SB_P	SB_H
C63PI	97,7	88,6	12	5	100	100	100	97,7
CS54	-	-	88	88	-	-	88	81,8
SGI-1	2,3	-	-	-	-	-	-	-
SPI-1	100	100	100	100	100	100	100	100
SPI-2	100	100	100	100	100	100	100	100
SPI-3	100	100	100	100	100	100	100	100
SPI-4	100	74,3	100	88	100	100	100	90,9
SPI-5	100	100	100	100	100	100	100	100
SPI-8	-	11,4	-	-	84	86	-	-
SPI-9	100	100	100	100	100	100	100	100
SPI-10	-	-	-	-	-	-	-	2,3
SPI-11	-	-	-	5	-	-	-	-
SPI-12	-	-	24	16	-	-	14	6,8
SPI-13	100	88,6	100	100	-	-	100	100
SPI-14	100	88,6	100	100	-	-	100	100

- : Unidentified SPI. SSch_P: *S.* Schwarzengrund/Poultry (n= 44); SSch_H: *S.* Schwarzengrund/Human (n= 35); SSa_P: *S.* Saintpaul/Poultry (n= 17); SSa_H: *S.* Saintpaul/Human (n= 43); SSe_P: *S.* Senftenberg/Poultry (n= 43); SSe_H: *S.* Senftenberg/Human (n= 35); SB_P: *S.* Braenderup/Poultry (n= 50); SB_H: *S.* Braenderup/Human (n= 44).

3.5. Plasmid replicons

A high diversity of the plasmid replicons was identified in the *Salmonella enterica* sequences analyzed by PlasmidFinder, as shown in **Figure 4**. The Col(pHAD28) replicon was found to be the most common among all serovars isolated from humans. The serovar with the lowest diversity was *S.* Saintpaul, mainly from poultry isolates, in which only the replicons of plasmids IncI1-I(Alpha) (52.9%), IncX4 (5.9%), Col(pHAD28) (5.9 %), and IncX1 (11.8 %) were detected.

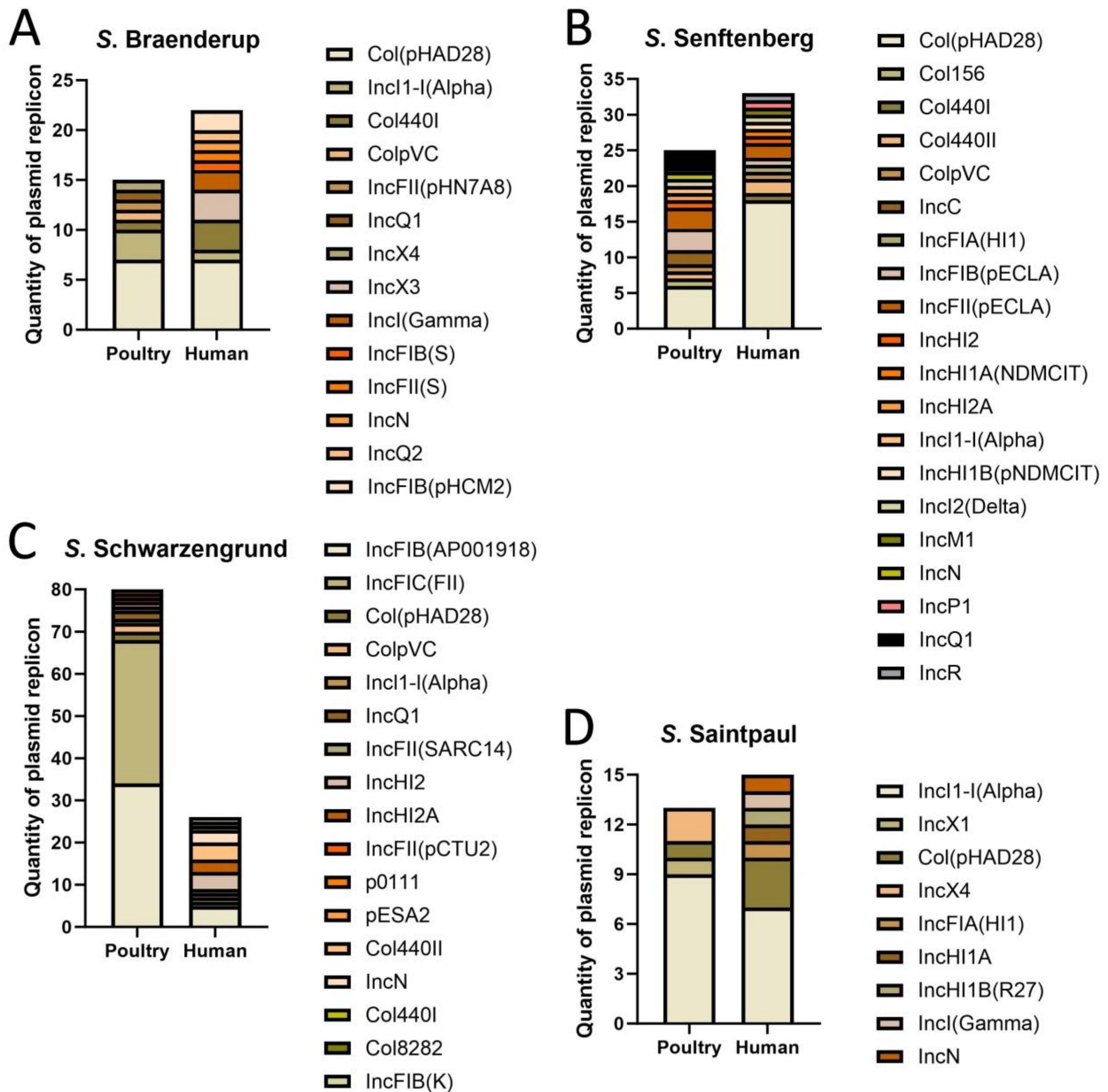

Figure 4. The diversity of plasmid replicons identified in the genomes of *Salmonella* isolates of humans and poultry origins varies according to the serovar. The letters correspond to different serovars analyzed: A: *S.* Braenderup; B: *S.* Senftenberg; C: *S.* Schwarzengrund; and D: *S.* Saintpaul.

3.6. Virulence factors

From the VFDB outcome, a total of 271 virulence genes were analyzed among the 311 *Salmonella enterica* genomes. The number of virulence genes varied among individual serovars, ranging from 197 to 212 genes. Among these 271 virulence genes,

161 were shared between all the tested serovars, whereas the remaining genes were exclusively detected in specific serovars or among them (**Figure 5**). A proportion of 8.1% (22/271) of these distinctive genes was observed in serovar *S.* Schwarzengrund, followed by 4.0% (11/271) in *S.* Senftenberg, 2.9% (8/271) in *S.* Saintpaul, and 2.2% (6/271) in *S.* Braenderup (**Figure 5**).

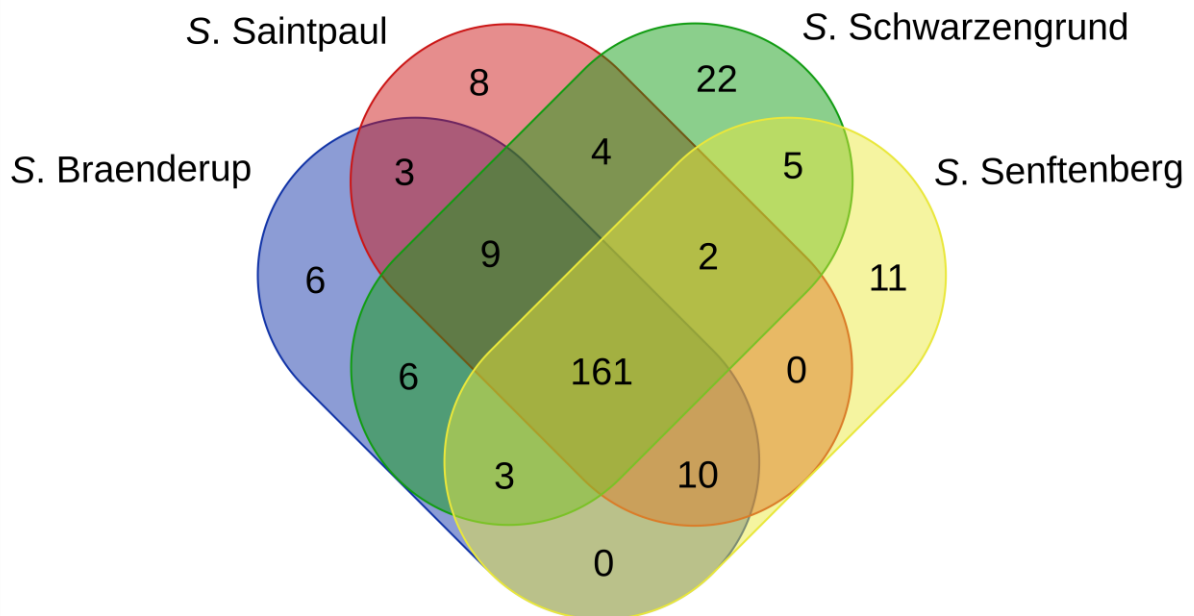

Figure 5. Venn diagram demonstrating the overlap of virulence genes of *Salmonella* serovars used in the present study. Figure was created using Bioinformatics & Evolutionary Genomics tool (https://bioinformatics.psb.ugent.be/webtools/Venn/).

The results also showed that these genes are related to enzymes, iron uptake, secretion system, and others. For example, *cdiB*, *eno*, *trbE*, *virB*, *pilL*, *pilN*, *traG*, *cdiA*, *sopD2*, *agn43*, and *faeJ* were solely noticed in the genomes of *S.* Senftenberg. On the other hand, *tsh*, *iucC*, *sitB*, *aec24*, *pltB*, *iutA*, *iucA*, *aec18*, *aec23*, *aec26*, *aec16*, *aec28*, *sitC*, *aec25*, *sitD*, *aec17*, *aec27/clpV*, *iucB*, *aec22*, *sitA*, *aec19*, and *aec29* was

observed only in *S.* Schwarzengrund. Moreover, *icsP*/*sopA*, *afaC*, *pilS*, *gogB*, *ireA*, *espO1-1*, *afaB*, and *basG* were present in *S.* Saintpaul isolates. Finally, *spvB*, *sefC*, *rck*, O-antigen (*Yersinia*), *mig-5*, and *pefA* could be only noted in *S.* Braenderup strains (**Figure 6**). In addition to features specific to the serovars, the host contributed to some differences in the profile of virulence genes, such as the presence of the iron uptake class, which in turns was only noticed in the genomes of *S*. Schwarzengrund from poultry (**Figura 6**).

The virulence profiles predicted within the sequenced Brazilian genomes of the Schwarzengrund, Saintpaul, Senftenberg, and Braenderup serovars show similarities alongside slight variations in the presence or absence of certain genes (**Figure 6**). Furthermore, most of these genes are in SPI-2 and SPI-1, which are involved in encoding structural proteins and secreted effectors of the Type III Secretion System (T3SS).

Figure 6. Heat map of the virulence profile based on the presence and absence of 47 genes using the VFDB database. The genes showed a higher occurrence in *Salmonella* genomes from poultry compared to those from humans. originating from humans. The dendrogram was generated by hierarchical clustering using Euclidean distance and complete linkage method. A total of 311 genomes of *Salmonella enterica* were analyzed concerning virulence genes, using both the plasmid (pSLT NC_003277) and the chromosome (NC_003197) as references. The bars at the top of the heat map indicate the classes of virulence factors, while the bars on the right side of the graph indicate the characteristics of the genomes, such as the source of isolation (human or poultry) and the *Salmonella* serovars (Schwarzengrund, Saintpaul, Senftenberg, and Braenderup).

3.7. Phylogenetic Relationships of Salmonella enterica isolated from Brazil

In the phylogenetic tree based on SNPs (**Figure 7**), the separation of serovars with the formation of exclusive clades is evidenced, according to the unique genetic characteristics of each serovar. In addition to very strong bootstrap support for both clades. One of the features shown in the phylogenetic analyses is the high similarity of *S.* Enteritidis str. P125109 genome with *S.* Braenderup strains compared with *S.* Typhimurium LT2.

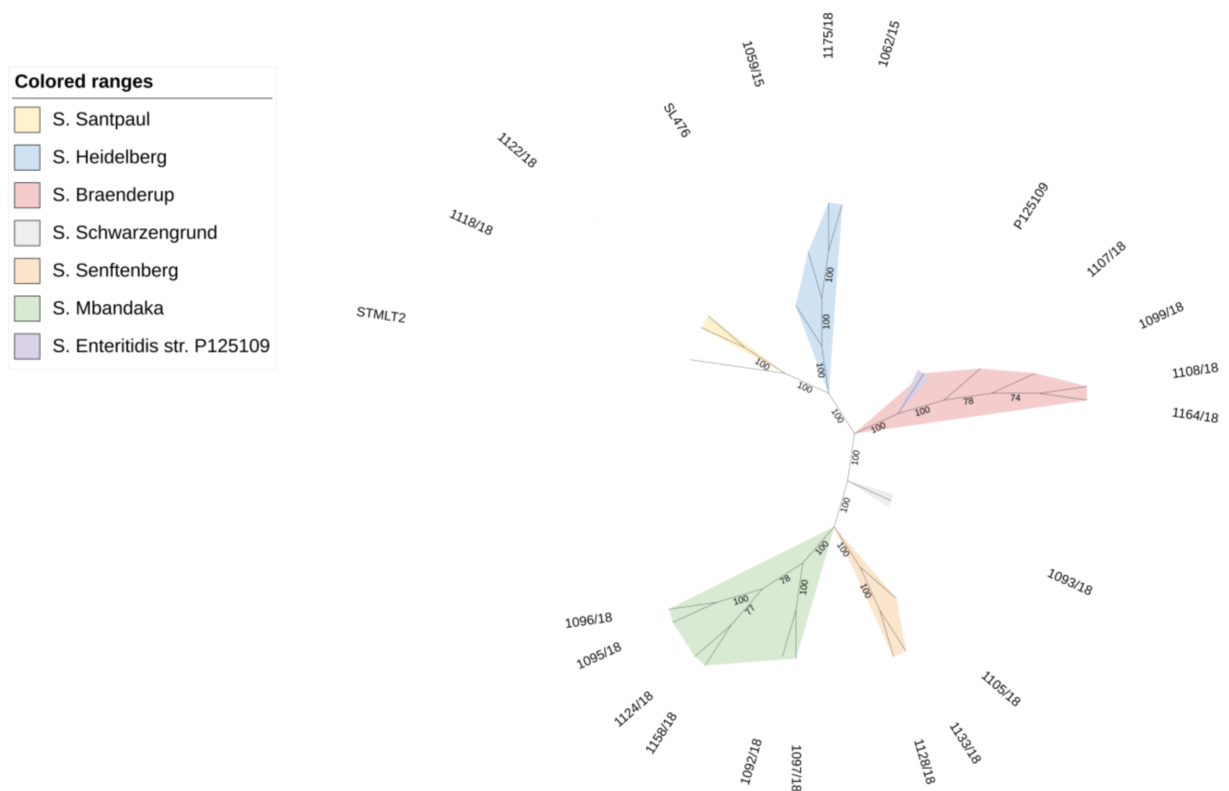

Figure 7. Maximum likelihood (ML) midpoint-rooted tree based on SNP analysis of 21 strains of *Salmonella enterica* serovars. Genomes were aligned using CSI phylogeny v1.4 provided by CGE (Rolf et al., 2014), comparing 19 *Salmonella enterica* genomes of strains isolated in Brazil with two additional representative genomes of *Salmonella* Heidelberg (NC_011083.1) and *Salmonella* Enteritidis (NC_011294.1). *Salmonella* Typhimurium LT2 (AE006468.2) was used as an outgroup and to root the tree. The phylogenetic tree was inference using ML, run on RAxML-HPC2 with bootstrapping with 1000 pseudoreplicates on Workflow XSEDE pertaining to the CIPRES Science

web server Gateway v. 3.3 (Miller et al., 2010). The resulting phylogenetic tree was edited using the online tool iTOL – Interactive Tree of Life (Letunic et al., 2021).

4. Discussion

Bacteria from the Enterobacteriaceae family are well adapted to the intestinal environment due to the acquisition/mutation of genes acquired in the colonization process, which incorporated several functions into their physiological framework, such as resistance to antimicrobials, important for their survival (Szmolka and Nagy, 2013). Thus, these resistance genes acquired by commensal strains can be lately transferred to pathogenic strains, such as *Salmonella* spp. (Perdens et al., 2013).

Our findings elucidate a diversity of antimicrobial resistance determinants in *Salmonella* serovars originating from human hosts compared to those isolated from avian sources. This observation suggests that poultry production serves as a notable pathway for the emergence and dissemination of these genes. Moreover, our results highlight the necessity of curtailing the administration of antimicrobials solely for therapeutic purposes, sparingly for prophylaxis, and never as growth promoters in animal production (Paterson 2006). Additionally, the indiscriminate and excessive application of antimicrobial agents across diverse sectors including agriculture, livestock farming, and human medical practice reinforces the importance of a “One Health” approach, as advocated by the World Health Organization (WHO, 2014).

At least one gene that confers resistance to aminoglycosides was present in all analyzed sequences, mainly the *aac(6')-Iaa* gene. It corroborates Cao et al. (2018) findings. Bacterial resistance to aminoglycosides may arise through enzymatic inactivation by acetyltransferases (*aac*), nucleotidyltransferases/adenylyltransferases (*ant*), and phosphotransferases (*aph*) (CLSI, 2023). At least one gene that confers resistance to aminoglycosides was present in all analyzed sequences, mainly the *aac(6')-Iaa* gene, supporting Cao et al. (2018) findings. Our data also showed a diverse array of aminoglycoside resistance genes in the genomes, including *aadA1*, *aph(6)-Id*, *aph(3'')-Ib*, and *aac(3)-IId*. Nonetheless, despite the potential activity of aminoglycosides against *Salmonella in vitro*, their clinical effectiveness against this genus remains limited (CLSI, 2023). Variants in the aad, aac, and aph gene groups can confer resistance to gentamicin, tobramycin, kanamycin, neomycin, and

streptomycin were reported in a recent study with *Salmonella enterica* isolated from poultry subproducts (eggs and meat) (Hu et al., 2020).

The data on the predicted resistance genes in *S*. Schwarzengrund genomes analyzed indicate resistance to beta-lactam antibiotics, despite the rate being lower than those reported in isolates of *Salmonella enterica* from poultry (Souza et al., 2020; and Sabry et al., 2020), this result showed the potential of this sorovar as an Extended-spectrum β-lactamase (ESBL) producers that circulate in poultry and humans. Even though low rates of ESBL-producing bacteria were observed in this study, isolates positive for genes such as *bla*$_{TEM-1B}$ (penicillin and early cephalosporin), *bla*$_{OXA-129}$ (oxacillin and methicillin), and *bla*$_{CTX-M-2}$ (third-generation cephalosporins) were associated with multidrug resistance. Therefore, the efficacy of these antibiotics against diseases caused by these bacteria is reduced (Hussain et al., 2021).

The presence of genes that confer resistance to folate pathway antagonists (*dfrA* and *sul*) in *Salmonella* spp. originating from food products, such as chicken, beef, pork, eggs, and vegetables, is predominantly carried by various types of plasmids, most of which are classified as conjugative, enabling horizontal gene transfer (Maka et al., 2015). The isolates under study, which harbored the *floR* gene conferring resistance to both florfenicol and chloramphenicol, also carried the *tetA* gene responsible for tetracycline resistance. This genetic composition is characteristic of multiresistant *Salmonella* strains (Brunelle et al., 2015).

The *qacE* gene, which encodes resistance to quaternary ammonium compounds commonly used for the disinfection of food processing facilities, has also been detected in poultry isolates. Several factors may be involved in the acquisition of this gene, including mobile genetic elements, such as pSGB23 plasmids. This element carries more than 11 antimicrobial and disinfectant resistance genes detected in *S*. Senftenberg isolates from chicken (Ding et al., 2018). Thus, this scenario increasingly difficult the treatment of infections in humans.

Quinolone resistance in *Salmonella* spp. is mainly mediated by mutations in the *gyrA* and *parC* genes (Misra et al., 2016). In the present study, all sequences of *S.* Senftenberg (humans and poultry) and *S.* Schwarzengrund (poultry) solely had mutations in the *parC* (T57S). Reports since the early 2000s indicate that these mutations are common in *Salmonella* spp. (Eaves et al., 2004; Qian et al., 2020).

However, mutations in the codons 83 and 87 of *gyrA* in *Salmonella* spp. are less common and are usually associated with a reduced susceptibility to quinolones (Lunguya et al., 2013; Eguale et al., 2017). In addition to chromosomal mutations, resistance to ciprofloxacin in *Salmonella enterica* can also be mediated by plasmid-encoded mechanisms (Martínez-Martínez et al., 1998). Our study predicted the presence of *qnr* genes in isolates of *S*. Schwarzengrund (*qnrB19*=11.4%), *S.* Saintpaul (*qnrB19*=7%; *qnrS1*=4.7%), *S*. Senftenberg (*qnrB19*=8.6%), and *S*. Braenderup (*qnrB7* and *qnrB19*= 6.8%; *qnrS1* and *qnrS2*=2.3%) obtained from humans. However, in the genomes of *S*. Senftenberg isolated from poultry, the *aac(6')-Ib-cr* (7%) gene was detected. Plasmid-Mediated Quinolone Resistance (PMQR) genes, such as *qnr*, *aac(6')-Ib-cr*, *qepA*, and *oqxAB*, have been identified in *Salmonella enterica* serovars with resistance and reduced susceptibility to ciprofloxacin in Brazil (Casas et al., 2016) and other countries in Latin America (Vieira et al., 2019), demonstrating the ease of propagation of these determinants through horizontal transfer as the main route (Ruiz et al., 2012).

The acquisition of antimicrobial resistance genes is typically mediated by plasmids (Hopkins et al., 2006; Carattoli, 2009). This study revealed that multiple plasmid replicons are disseminated in *Salmonella* serovars isolated from humans and poultry, which may harbor resistance genes to several classes of antimicrobials, including β-lactams, tetracyclines, aminoglycosides, and fluoroquinolones (Jain et al., 2018; McMillan et al., 2019). It is noteworthy that although the pathogen can harbor various mobile genetic elements, these plasmids are unlikely to belong to the same incompatibility group (Inc) in the same isolate (Carattoli, 2009). In addition to antimicrobial resistance determinants, plasmids can also harbor resistance genes that protect the pathogen against quaternary ammonium compounds, mainly the *qacE* gene (Hoffmann et al., 2017), which was also found in the genomes analyzed in this study.

The widespread distribution of plasmids in *Salmonella enterica* isolates from humans and poultry highlights the urgence of newly investigations to elucidate the virulence potential of the zoonotic pathogen. Furthermore, due to the common transfer of antimicrobial resistance genes through plasmid exchange, *Salmonella* genus demonstrates the presence of several virulence genes clustered in SPIs. These SPIs

play crucial roles at cell invasion, intracellular survival, and induction of inflammation by *Salmonella* spp. (Coburn et al., 2007).

SPI-10 and SPI-11 harbor genes that contribute to macrophage survival in humans (Gunn et al., 1995; Faucher et al., 2008). In the present study, SPI-11 was detected only in *S*. Saintpaul sequences from humans. Nevertheless, the mechanisms of survival within macrophages related to this SPI remain unclear (Wang et al., 2020). Previous findings indicated that SPI-10 was limited to specific serovars, such as Typhi, Paratyphi A, Dublin, Enteritidis, Gallinarum (Townsend et al., 2001), Typhimurium, and Washington (Saroj et al., 2008). To the best of our knowledge, this is the first work to report *Salmonella* Braenderup carrying the SPI-10 genes. This expands the understanding of the distribution of SPI-10 and suggests a broader genomic diversity among *Salmonella* serovars.

In contrast to other serovars, *S*. Senftenberg sequences lacked SPI 10-14, indicating a substantial gap in our understanding of these specific elements. It is noteworthy that comprehensive information regarding these serovars is still limited. Furthermore, SPI-13 has a major role in enhancing the virulence and metabolic adaptability of *Salmonella* Enteritidis in mammalian hosts (Elder et al., 2018). In a mice-model infection, the knockout of SPI-13 from *S.* Enteritidis genome attenuated the pathogen due to a lesser ability of colonization and diminished intestinal inflammation, followed by a reduced systemic invasion and intracellular survival (Elder et al., 2016). The genes associated with SPI-14 play a central role in the activation of SPI-1 genes, operating not through direct interactions with host cellular processes but through a regulator encoded within SPI-14 (STM14_1008), named LoiA (low oxygen-induced factor A), acting as the virulence determinant (Jiang et al., 2017).

The adaptation of *Salmonella* spp. to several organisms has led to the development of resistance mechanisms to overcome the host's physical barriers, alongside the inhibition and evasion of subsequent immune response activation by SPIs (Wang et al., 2020). Nevertheless, there is a lack of knowledge about the interaction between *Salmonella* serovars and several hosts, besides the genomic impact of this adaptation within each host species.

We demonstrated differences in the distributions of virulence genes among *Salmonella* serovars, which may represent a variation in pathogenicity, survival

advantages, and host adaptation (Tanner and Kingsley, 2018). These variations could be attributed to genes encoded within SPI-1 and SPI-2. These genes play crucial roles in invasion, survival, and replication within phagocytes and epithelial cells. Moreover, they regulate the course of infection, encompassing both systemic spread and intracellular pathogenesis (Wang et al., 2020). The *sit*ABCD operon, involved in the primary iron transport system, plays a crucial role in the overall virulence of *S.* Typhimurium (Janakiraman and James, 2002). These genes, located in SPI-1, appear to be restricted to certain serovars and hosts, being detected only in *S.* Schwarzengrund genomes isolated from poultry in our study.

5. Conclusion

The findings exposed herein contributes to epidemiological insights about the spread of *Salmonella* spp. and antimicrobial resistance genes within the poultry food chain. The variation observed among these serovars bring into lights their genetic flexibility and genomic features that may be helpful to our understanding about the host-pathogen interactions. Moreover, it highlights the importance of further in-depth studies to elucidate the virulence and adaptation mechanisms within the *Salmonella* genus.

Supplementary Materials

Table S1. Metadata for the 21 *Salmonella enterica* genomes used to perform phylogenetic analysis.

Table S2. Comprehensive overview of sequences of targeted serovars present in the genomic database by prescribed selection criteria.

Author Contributions

Study conception and design, V.P.B, M.M.S.S., J.E.O., and A.B.J.; acquisition of data, analysis, and interpretation of data, V.P.B., M.M.S.S., A.M.A., H.C., and J.E.O.; drafting of the manuscript, V.P.B., M.M.S.S., V.A.F., and M.I.G.F.; supervision and critical revision, M.M.S.S., A.B.J., H.C., and J.E.O. All authors read and approved the final manuscript.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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CAPÍTULO 4 – *Salmonella* Heidelberg isolates from poultry in Brazil and United States share a large number of resistance and virulence determinants [1]

[1] Este capítulo corresponde ao artigo científico que será submetido à revista Transboundary and Emerging Diseases (IF = 4.3; QUALIS CAPES A1).

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Abstract: *Salmonella enterica* subps. *enterica* serovar Heidelberg (SH) is one of the most isolated serovars in poultry. It can cause severe infections in humans due to the prevalence of resistance to multiple drugs, representing a risk to public health. We retrieved 314 genomes of SH from the Enterobase database, which included isolates from Brazil and the United States. In addition, three extra genomes from Brazil were sequenced, totalizing 317 of analyzed genomes. Regarding the both countries, the main predicted resistance determinant genes were: *aac(6')-Iaa*, *fosA7*, *sul2*, *tet(A)*, and bla_{CMY-2}, which confer resistance to aminoglycosides, fosfomycin, sulfonamides, tetracyclines, and β-lactams, respectively. Mutations in *gyrA* (S83F only in US genomes and S83Y and D87N in Brazilian genomes) and *parC* (T57S) genes, which confer quinolone-resistance. The plasmid replicons most identified in both countries were ColpVC, IncC, IncI1-I(Gamma), and IncX1. Overall, this study enriches the public genome databases with three newly sequenced genomes from Brazilian poultry production and brings into light varied genomic profiles of SH circulating in the worldwide poultry production regions. Furthermore, this study highlights the need for improved surveillance measures to protect both human and animal populations from potential outbreaks.

Keywords: Antimicrobial resistance; pathogenicity; virulence; whole-genome sequencing.

1. Introduction

The United States and Brazil stand out as the world's largest poultry producers. Nowadays, Brazil leads the panel of the greatest chicken meat exporter and is under constant expansion [1]. In this scenario of globalized poultry production, demands related to animal health and food safety are increasingly urgent. Bacteria of the *Salmonella* genus can colonize the intestinal tract of chickens and cross borders through the poultry production system, being a potential foodborne risk to public health [2]. *Salmonella* Heidelberg (SH) is one of the most isolated serovars in poultry production systems in Brazil [3] and is often linked to a higher risk of invasive disease in humans [4].

The emergence of the virulence potential of this serovar has been attributed to factors such as host susceptibility, virulence factors, and other adaptive elements [5]. Additionally, the occurrence of multidrug-resistant *Salmonella* Heidelberg, including resistance to the cephalosporins class, has been reported over the years, leading to antibiotic therapy failures [6, 7]. Understanding the genotypic characteristics of *Salmonella* Heidelberg contributes to the development of control measurements to minimize the risk of contamination, ensuring a safer product for consumers [8]. Therefore, we compared the genomes of *Salmonella* Heidelberg isolated from poultry in Brazil and the United States to elucidate genetic features related to virulence potential and antimicrobial resistance that may contribute to the spread of this serovar to represent a health risk.

2. Material and methods

2.1. Bacterial genomes

Salmonella Heidelberg genomes available in the Enterobase database [9–11], were selected in the database on August 4th, 2023, according to the standards filters established by the authors, as follows: *Salmonella* Heidelberg, poultry, coverage ≥ 90, Brazil/United States, related to the years from 2016 to 2023. Moreover, the assembled genome files were retrieved in ".fasta" format.

Due to the low number of genomes of Brazilian isolates, we sequenced three more isolates of *Salmonella* Heidelberg (1059/15, 1062/15, and 1175/18), which were previously obtained from poultry products sourced from Brazil by Souza et al. [7], constituting a novel aspect of our study.

2.2. Genome Sequencing and Data Processing

The genomic DNA from the three new *Salmonella* Heidelberg strains was extracted using the Maxwell RSC Cultured Cells DNA kit (Promega, Madison, USA). Following extraction, DNA integrity was evaluated through electrophoresis on a 1% agarose gel, quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and concentration analyses were conducted with the Qubit dsDNA

HS Assay kit (Thermo Fisher Scientific, Scoresby, Australia). Subsequently, a DNA library was prepared using the Flex DNA Library Preparation Kit (Illumina, San Diego, CA, United States) following the supplier's instructions. Paired-end sequencing was then conducted on an Illumina MiSeq platform (Anicon, Germany; NGS-MiSeq, University of Copenhagen, Denmark) using a Nextera XT v3 kit with a 2 x 300 bp insert size.

The quality assessment of raw data was conducted through FastQC 0.11.9 [12]. The Trimmomatic-0.39 [13] was employed for adapter trimming and the exclusion of low-quality reads. The assembly process was executed using Unicycler [14], and automated annotation was performed using Rapid Annotation using Subsystem Technology (RAST) [15].

2.3. Genomic features analysis

The Center for Genomic Epidemiology (CGE) webserver was used to identify acquired genes and detect chromosomal mutations mediating antimicrobial resistance – ResFinder 4.1 [16, 17], and identify plasmids – PlasmidFinder 2.1 [18]. Moreover, *Salmonella* Pathogenicity Islands (SPIs) identification was performed using SPIFinder 2.0 [19]. For all CGE tools, the default settings were used, with a minimum percentage of nucleotide identification (% ID) of 95% and minimum coverage of the target sequence of 60%, except for investigations of resistance genes in which the threshold for %ID it was 90% and minimum coverage 60%.

2.4. Phylogenetic Relationships

To elucidate phylogenetic relationships amongst *Salmonella* Heidelberg isolates, whole-genome phylogenies were reconstructed using *Salmonella* Typhimurium LT2 (AE006468.2) as an outgroup. In addition to the three genomes that were sequenced as part of this research, the additional set of 314 assembled sequences of *S*. Heidelberg from the Enterobase database was incorporated into this analysis. Genomic annotations were performed using Prokka 1.14.5 [20] with default parameters. The Roary v3.12.0 [21] pipeline, employing default settings, generated a matrix from alignments produced by the MAFFT program [22].

The construction of the phylogenetic tree utilized the Maximum Likelihood method in IQ-TREE2 software [23], with clade support estimates determined by ultrafast bootstrap (UFBoot) of 1,000 pseudoreplicates [24]. The best-fit model, TIM+F+I+G4, was selected based on the Bayesian Information Criterion (BIC) using ModelFinder [25]. The resulting phylogenetic tree was edited using the online tool iTOL – Interactive Tree of Life [26].

2.5. Genomic Data Deposition

The assembled genomes of three *Salmonella enterica* subsp. *enterica* serovar Heidelberg were deposited at the NCBI Sequence Read Archive (SRA) website, under Project PRJNA1015686. The complete genome data of 1059/15, 1062/15, and 1175/18 have been deposited in GenBank with BioSample SAMN38927491, SAMN38927540, and SAMN38927546, respectively.

3. Result

3.1. Selection of sequences in genomic databases

The amount of data deposited and available in the Enterobase related to *Salmonella* Heidelberg genomes from Brazil is substantially low compared to the United States. A total of 314 genomes of *S.* Heidelberg were collected in the Enterobase database, distributed between Brazil (n=31) and the United States (n=283). Furthermore, no *S*. Heidelberg genomes from Brazil were uploaded into the Enterobase database over the years 2019 and 2021-2023. Detailed information on each isolate can be found in **Table S1.**

3.2. Quality of genomic data

Characteristics and quality parameters of the draft genome assemblies are shown in **Table 1**. Genomes had GC content of 52.0 % and sizes ranging from 4,871,559 to 5,097,746 bp, which are within the typical range of *Salmonella enterica* (*Salmonella* Typhimurium LT2, NC_003197.2).

Table 1. Quality features of draft genome assemblies of the *S.* Heidelberg strains from poultry in the study.

Assembly Attributes*	1059/15	1062/15	1175/18
Genome size (bp)	4,871,559	4,874,905	5,097,746
Contigs Number	28	26	38
% GC	52.0	52.0	52.0
L_{50}	4	4	4
N_{50} (bp)	412,096	412,152	355,562
Average depth of Coverage	56.5	88.45	58.07
CDSs	4,529	4,830	4,936
rRNA	22	16	22
tRNA	86	81	86

* N50: represents the sequence length of the shortest contig at which 50% of the total assembly length is reached; L50: represents the minimal count of contigs needed so that their total length equals half of the genome size; CDSs: Coding Sequences; rRNA: Ribosomal Ribonucleic Acid; tRNA: Transfer Ribonucleic Acid.

3.3. Antimicrobial resistance determinants

In the context of predicting antimicrobial resistance genes, the analysis of *Salmonella* Heidelberg genomes from Brazil and the United States reveals a large number of shared resistance genes (n=13). Nevertheless, there is a variability in antimicrobial resistance determinants within the genomes from the United States (n=16), possibly stemming from the comprehensive sampling of genomes from this region (**Figure 1**).

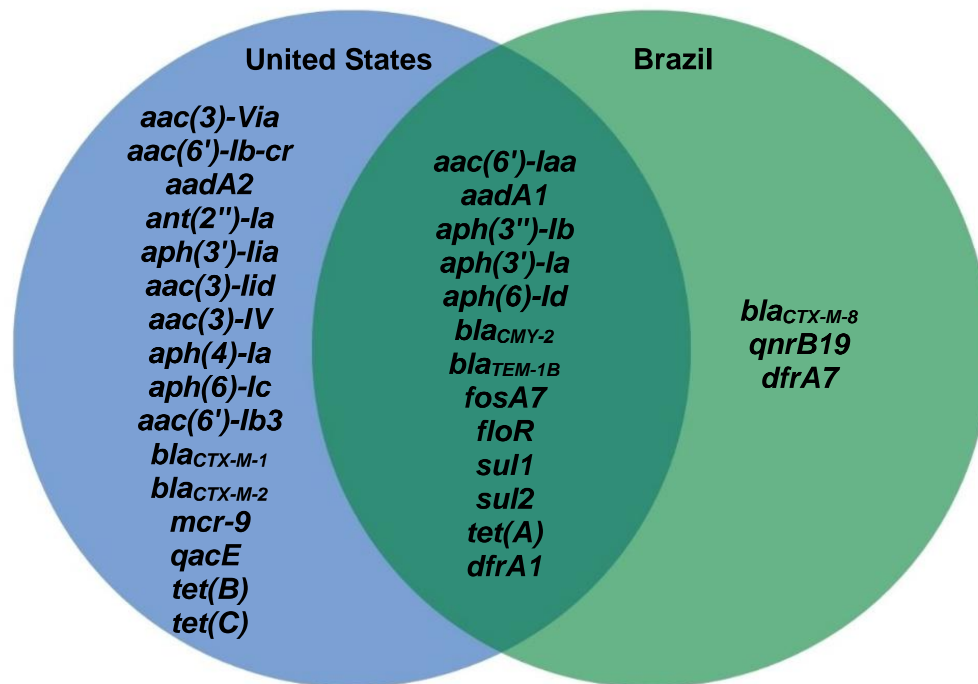


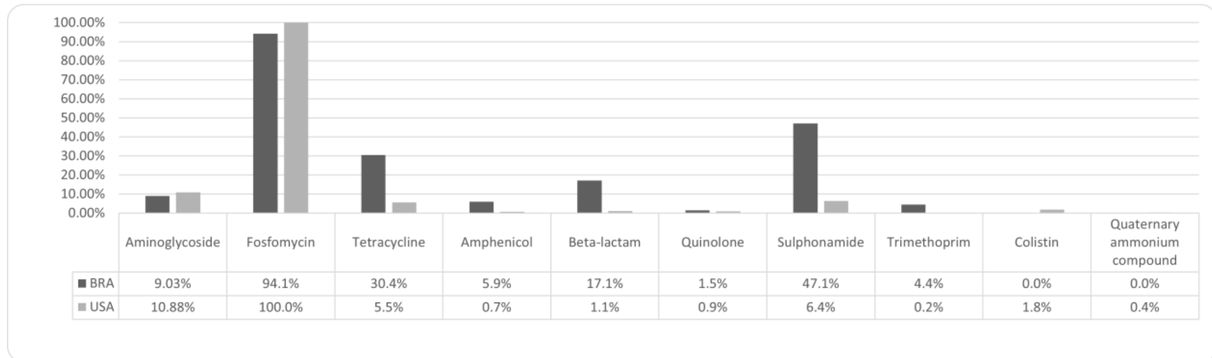
Figure 1. Venn Diagram demonstrating antimicrobial resistance genes identified in 317 genomes of *S.* Heidelberg isolated from poultry products in Brazil (right/green) and the United States (left/blue). The genes shared by both country isolates are shown at the intersection of the diagram (middle).

The genomic analysis of *S*. Heidelberg from both countries showed that the *aac(6')-Iaa* gene, associated with aminoglycoside resistance is present in all 317 genomes studied. Although other aminoglycoside resistance genes were predicted, the greatest diversity was observed in genomes from the United States. The predicted genes in the genomes of both countries were *aadA*, *aph(3")-Ib*, *aph(3')-Ia*, and *aph(6)-Id* (**Table S1**).

A variety of genes associated with tetracycline or sulfonamide resistance, such as *tet(ABC)*, *sul1,* and *sul2* were detected in both Brazilian and US genomes, also some genes exhibited higher prevalence rates. The *tet(A)* gene was predicted in 11.7% (33/283) of the US genomes, whereas the *sul2* gene was predicted in 2.1% (6/283) of the US genomes, while in Brazilian genomes those genes were found in 91.2% (31/34).

The *bla*$_{CMY-2}$ and *fosA7* genes, which encode resistance to β-lactams and fosfomycin respectively, showed an increase in frequency over the years in Brazilian

isolates. We observed that 1,8% (5/283) of the US isolates carried the *mcr-9* gene, which encodes colistin resistance (**Figure 2**).



	Aminoglycoside	Fosfomycin	Tetracycline	Amphenicol	Beta-lactam	Quinolone	Sulphonamide	Trimethoprim	Colistin	Quaternary ammonium compound
BRA	9.03%	94.1%	30.4%	5.9%	17.1%	1.5%	47.1%	4.4%	0.0%	0.0%
USA	10.88%	100.0%	5.5%	0.7%	1.1%	0.9%	6.4%	0.2%	1.8%	0.4%

Figure 2. This figure illustrates the distribution of antimicrobial resistance genes predicted in 317 genomes of *S.* Heidelberg isolated from poultry products in two distinct regions.

This study revealed that all genomes of *S*. Heidelberg exhibited a mutation at position 57 of ParC protein in which threonine (T) was substituted by serine (S). The difference observed between the US and Brazilian genomes was related to mutations in the *gyrA* gene, in which 15.2% (43/283) of the genomes from the US exhibited a missense mutation at position 83 of GyrA protein in which serine (S) was substituted by tyrosine (Y). Furthermore, 88.2% (30/34) of the genomes from Brazil harbored a missense mutation at position 83 of GyrA protein in which serine (S) was substituted by phenylalanine (F). The last and the least frequent form of mutation was at position 87 of GyrA protein in which aspartic acid (D) was substituted by asparagine (N) (D87N), which was detected in a single Brazilian genome.

3.4. Distributions of SPIs and plasmid replicons

All *S.* Heidelberg genomes showed at least one pathogenicity island (SPI 1–5, SPI 9, SPI 12, SPI 13, SPI 14, C63PI, and CS54), in which SPI-12 (7.1%) was found to be exclusive to US genomes (**Figure 3**). The SPI-13 was found in 100% of the analyzed genomes highlighting the preservation of this genomic segment within this serovar (**Figure 3**).

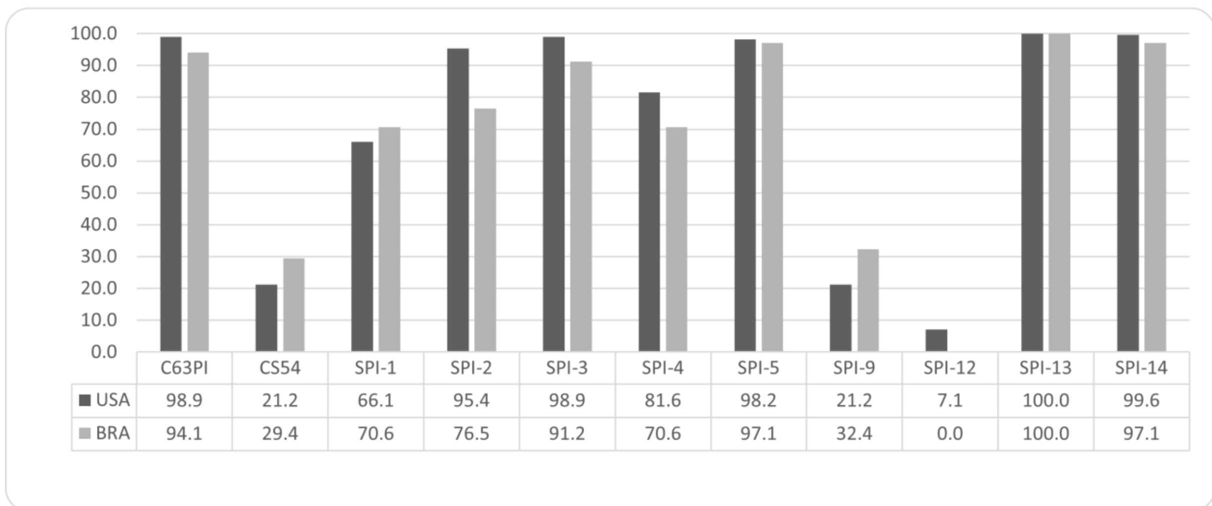


Figure 3. The percentage distribution of *Salmonella* Pathogenicity Islands (SPIs) in the genomes of 317 *S.* Heidelberg isolates from poultry products in Brazil and the USA.

Most of the Brazilian genomes (97.1%) harbored 2 to 3 plasmid replicons such as ColpVC, IncC, and IncX1 being the most frequent, demonstrating a predictive accuracy of 91.2% (**Table S1**). In addition, some genomes exhibited a presence of exclusive replicon plasmids, such as Col(pHAD28), Col156, IncFII (SARC14), p0111, IncHI2, IncHI2A, and IncY (**Table S1**). While a strain isolated from Brazil (Accession Number: SRR7250125) was devoid of plasmid replicons, others harbored plasmid replicons such as IncI1-I(Gamma) (38.2%), IncI1-I(Alpha) (14.7%), and IncQ1 (5,9%).

In 56.1% of US isolates, no plasmid replicon was predicted. The exclusive replicon identified in the US genomes was Col8282 (0.4%), detected in a single genome. On the other hand, despite the smaller sample size of genomes analyzed from Brazil, a greater diversity of plasmid replicon types was observed in comparison to those from the US.

3.5. Phylogenetic Relationship

The pangenome analysis indicated that all the analyzed *S.* Heidelberg genomes (n = 481) harbored a total set of 13,653 genes, consisting of 3,575 core genes, 625

soft-core genes, 433 shell genes, and 9,020 cloud genes. The 3,575 core and soft-core genes, representing a conserved set of genes shared among most of all the analyzed genomes, were utilized as the basis for conducting the phylogenetic analysis. The clades generated by the phylogenetic tree were associated with the geographic source of the genomes. All these sequences were clustered in seven clades (A-G) (**Figure 4**).

Overall, the majority of *S*. Heidelberg genomes recovered from poultry in the United States were grouped into five well-supported clades, as evidenced by high bootstrap values (Clades B, C, D, E, and F). Clade A, encompassing *S*. Heidelberg sequences from Brazil, did not show clustering correlated with genomes from the United States. In contrast, Clade G comprised 105 genomes, of which 31 (32.5%) were isolated from poultry in Brazil. Notably, this clade is more heterogeneous and could be subdivided into smaller subgroups, each containing a few strains from Brazil and the United States. However, all genomes not included in the other clades were assigned to Cluster G, for better visualization.

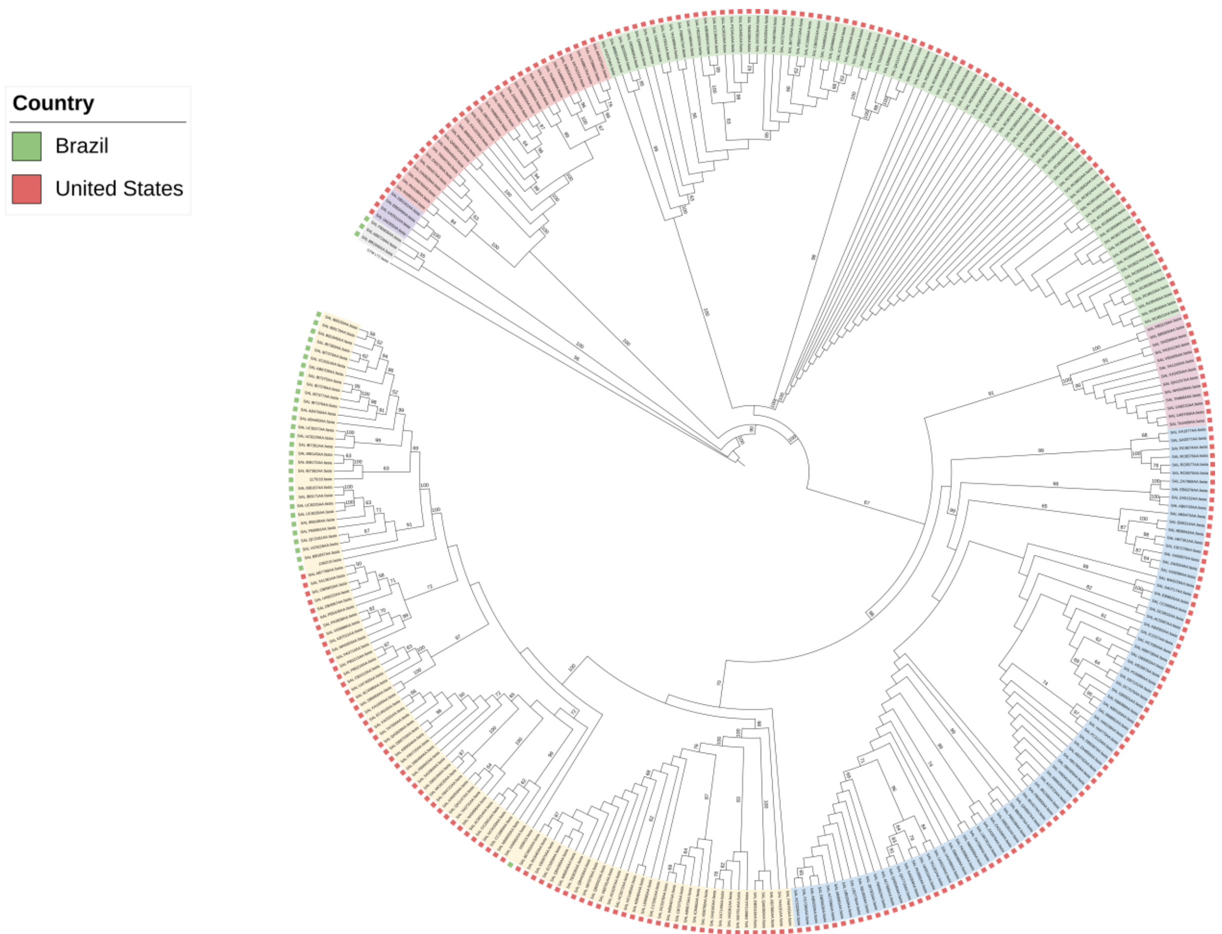


Figure 4. Maximum Likelihood phylogenetic tree of 317 *S.* Heidelberg strains recovered from poultry. *Salmonella* Typhimurium LT2 (AE006468.2) was used as an outgroup and to root the tree. The clade support is indicated above or next to each branch as bootstrap values calculated from 1000 pseudoreplicates. The colors in the inner ring of the tree represent the location of isolation (see legend). The colored branches represent the division of clades: clade A (grey branch), clade B (purple branch), clade C (red branch), clade D (green branch), clade E (pink branch), clade F (blue branch), and clade G (yellow branch). The tree has been annotated and visualized using iTOL [26].

4. Discussion

Salmonella Heidelberg has been isolated since 1962 in Brazil [27], whereas it is circulating in the United States since 1954 [28]. Recently, this serovar has been detected in chicken farms and has been quickly spreading within the poultry industry [7]. According to the Centers for Disease Control and Prevention (CDC), *Salmonella*

spp. is the major cause of 1.2 million infections, 23,000 hospitalizations, and 450 deaths in North America per year, being *S.* Heidelberg among the most frequent isolate serovars with poultry products identified as the primary source of the infection [29]. In Brazil, 13163 cases of foodborne disease were reported from 2000-2018, leading to 195 deaths. *Salmonella* spp. is the most common agent, but there is an estimation of underreporting cases [30, 31], which reflects directly on the amount of available data.

As one of the main emerging serovars in both countries, there is a concern related to the multidrug resistance detected in this pathogen [7, 32]. Herein, the analyzed genomes from both countries share a large number of resistance determinants, providing advantages to this serovar, enabling them to resist to antimicrobials used in infection treatment [33].

The *aac(6')-Iaa* gene was present in all analyzed genomes, which is responsible for encoding resistance such as gentamicin, tobramycin, and amikacin [34]. However, this gene is not often expressed in *Salmonella enterica*, has no clinical significance [35, 36]. On the other hand, the genomes of *Salmonella* spp. from poultry production carried variants of the *aad*, *aac,* and *aph* gene groups, which can confer resistance to gentamicin, kanamycin, tobramycin, streptomycin, and neomycin [37]. For instance, *aadA1*, *aac(3)-IV*, *aph(3') -Ia*, *aph(3'')-Ib,* and *aph(4)-Ia* showed gene variation more frequently in the US sequences. This antimicrobial class is related to the prevention and treatment of respiratory and enteric infections in poultry [38].

The *fosA7* gene is responsible for encoding resistance to fosfomycin and has been identified with high prevalence in *S.* Heidelberg isolates from broiler chickens [39], including in the present study and other studies involving fecal isolates from Brazilian poultry [5, 40, 41]. A public health concern is the frequent use of fosfomycin for the treatment of urinary infections and as an alternative agent in human medicine [42]. In addition, the transfer via plasmid of the *fosA7* gene has been associated with a high level of resistance to fosfomycin in transconjugant bacteria, highlighting the potential power of dissemination of this gene [39].

The data showed that *bla*$_{CMY-2}$ was predominant in genomes from Brazil (76.5%). The Extended-Spectrum beta-Lactamase (ESBL) production in *S.* Heidelberg is well-reported in Brazil [7, 34, 43], mainly due to the location of this gene in different plasmids (IncA, IncC, and IncI1) [41, 43, 44]. It is noteworthy that, exogenous

Enterobacteria can transfer *bla*$_{CMY-2}$ encoding IncI1 plasmids to the human microbiota, contributing to the dissemination of CMY-2 beta-lactamases [45] and occasional therapeutic failure, related to clavulanic acid, cephamycins, third-generation cephalosporins, and aztreonam [46].

This scenario shows that despite the application of the National Action Plan for the Prevention and Control of Antimicrobial Resistance [47] in Brazil, the use of those molecules in animal production has been related to the emergence of multidrug-resistant strains, putting light on the importance of preserving therapeutic options for the treatment of severe infection in human [48].

Regarding the *tet(A)* and *sul-2* genes, which encode resistance to tetracyclines and sulfonamides, respectively, Brazil presented a higher frequency when compared to the USA. Both genes were co-detected in a study with *S*. Heidelberg isolates from poultry flocks in Brazil [5, 49-51]. Despite the ban on their use as performance-enhancing zootechnical additives or as food preservatives for animals in Brazil [52], the intensive use of the molecules must have resulted in the development of this resistance.

Plasmid-mediated and transmissible colistin resistance (*mcr*) has raised public health concerns [53]. In our study, the *mcr-9* gene was predicted in five (1.8%) US genomes but was not detected in Brazilian genomes. Although it was first reported in *Salmonella* Typhimurium isolated from a human patient in Washington State in 2010 [54], the reports of enterobacteria harboring the *mcr-9* gene in livestock in the US have been low [55]. In 2020, the *mcr-9* gene was predicted in *Salmonella* Typhimurium ST19 isolated from swine in Brazil [56]. However, due to the scarcity of genomic data, the prediction of colistin-resistance genes in farm animals in Brazil is partially evaluated [55].

Most serovars of *Salmonella enterica* carrying the *mcr-9* gene do not exhibit resistance to colistin [57-60]. However, additional factors or induction/derepression conditions [58] can be involved in the expression of the *mcr-9* gene, leading to increased MIC levels. For example, exposure to subinhibitory concentrations, encoded by the *qseC* and *qseB* genes mediate a two-component regulatory system related to inducible colistin expression [61]. Colistin has been used as a crucial antimicrobial to treat serious human infections caused by multidrug-resistant bacteria [54, 62]. Thus,

efforts to reduce colistin usage in animals under the One Health approach have been applied in several countries [63]. The US government prohibited the use of colistin in food-producing animals [55] and Brazil banned its usage as a food additive in livestock in 2016 [64]. Colistin has been used as a crucial antimicrobial to treat serious human infections caused by multidrug-resistant bacteria [54, 62]. Thus, efforts to reduce colistin usage in animals under the One Health approach have been applied in several countries [63]. The US government prohibited the use of colistin in food-producing animals [55] and Brazil banned its usage as a food additive in livestock since 2016 [64].

In the present study, the point mutation at position 57 of ParC protein in which threonine (T) was substituted by serine (S) was identified in practically all genomes from both countries over the last five years. In contrast, the point mutation at position 83 of GyrA protein in which serine (S) was substituted by phenylalanine (F) was identified only in genomes from Brazil. Quinolone resistance in *Salmonella* spp. is primarily mediated through mutations in these two genes [65], and a combination of these mutations lead to resistance against fluoroquinolones (ciprofloxacin resistance) [66–68].

The main mechanism of resistance genes dissemination is the horizontal gene transferance [69], mainly through bacterial conjugation with conjugative plasmids [70]. Among all the genomes analyzed, the plasmid replicons with the highest frequency for both countries were ColpVC (US = 49.12% (139/283) and Brazil = 91.18% (31/34)) and IncX1 (USA = 69.96% (198/283) and Brazil = 91.18% (31/34)), which were also the most identified in other studies with *Salmonella enterica* isolated from poultry and swine production chains [40, 71].

The IncC plasmid replicon was present in 91.18% (31/34) of isolates from Brazil, which may be related to genes that encode ESBL [41], such as the bla_{CMY-2} gene that was also found in 76.5% of the Brazilian sequences (26/34). The evolution of this plasmid has been associated with the wide host range, and acquisition and dissemination of accessory DNA [72].

In addition to plasmid exchange, several virulence genes are clustered in SPIs on the chromosome and play a role in inflammation, cell invasion, and survival of *Salmonella enterica* [73]. Often *Salmonella* spp. isolates carry SPI 1-5, each

contributing to ensure that *Salmonella* can invade host cells, evade the immune system, and create a suitable intracellular environment for mutiplication [74, 75]. The SPI-13 was present in all analyzed genomes from both countries. It remarks the significance of this genomic region in the virulence of *Salmonella enterica*, particularly in the transcription of proteins related to bacterial metabolism [76]. SPI-14 is another genomic island that was frequently observed among *Salmonella* serovars lacking of host specificity and playing a role in the pathogenicity mechanisms, which is implicated in the modulation of bacterial invasion and the activation of SPI-1 genes [77].

SPI-9 encodes Type 1 Secretion Systems (T1SS) and contributes to the adherence to epithelial cells, with increased expression under conditions of high osmolarity and low pH, thereby facilitating intestinal colonization [78]. Further investigations about SPI-9 in *S.* Heidelberg may elucidate its interplay with host eukaryotic cells, given that this island has been studied in a few serovars, such as *S*. Enteritidis [79] and *S*. Typhi [78], revealing differences, such as biofilm formation, and possible adaptation to the host without provoke clinical signs [7, 32, 68].

Our phylogeny likely supports these findings linking to imported poultry meat/food, and indicates a relationship sample origin, suggesting that the evolution of *S.* Heidelberg may be influenced by local factors such as agricultural practices, food production systems, and sanitary regulations [80]. The presence of subgroups within Clade G, including samples from both Brazil and the United States, suggests possible events of international dissemination and/or coexistence of genetic lineages in different regions, thus the significant vehicle of salmonellosis in humans [4]. On the other hand, Clade A, composed of Brazilian samples (SAL_FB6656AA, SAL_AB6724AA, and SAL_BB1846AA), appears to have a distinct genetic origin, with the presence of exclusive antimicrobial resistance genes ($bla_{CTX-M-8}$, and *qnrB19*), not clustering with US samples. Brazilian poultry meat contaminated with multidrug-resistant *S*. Heidelberg may play an important role in the introduction and persistence of these isolates in other countries [81], given the importance of Brazil as the largest exporter of poultry meat in the world [82].

5. Conclusion

Salmonella Heildelberg from Brazil and US share a large number of resistance genes, suggesting that this genetic material was possibly transferred from different geographic locations at some point. In addition, this serovar represents a public health threat since it might increase the risk of transmission of multi-resistance strains to humans and contaminated poultry products can play a role in these cases. Finally, our study provides genomic features of *S*. Heidelberg isolated from poultry from two of the largest chicken meat-producing countries and provides a background to prevent the spread of resistance genes.

Supplementary Materials

Table S1: Metadata for the 317 *Salmonella* Heidelberg genomes retrieved from the Enterobase database to perform genomic analysis in this study.

Author Contributions

Study conception and design, V.P.B, M.M.S.S., J.E.O., and A.B.J.; acquisition of data, analysis, and interpretation of data, V.P.B., M.M.S.S., I.C.C, S.R.S, V.F.O.M, A.M.A., H.C., and J.E.O.; drafting of the manuscript, V.P.B., M.M.S.S., I.C.C., S.R.S, and J.E.O.; supervision and critical revision, M.M.S.S., P.R.G, A.B.J., H.C., and J.E.O. All authors read and approved the final manuscript.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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CAPÍTULO 5 – Considerações Finais

Neste estudo, as características genômicas dos sorovares analisados fornecem a base para a compreensão do potencial de virulência e resistência antimicrobiana, bem como dezenove novos genomas de *S*. Mbadaka, *S*. Senftenberg, *S*. Saintpaul, *S*. Schwarzengrund, *S*. Braenderup, e *S*. Heidelberg isolados da produção avícola brasileira. Além disso, ressaltamos a escassez de genomas de *Salmonella* spp. no Brasil em comparação com outras nações, destacando uma lacuna na amostragem genômica na região. Portanto, é relevante a integração de WGS em pesquisas com o gênero *Salmonella*. O impacto do WGS e o barateamento da técnica têm propiciado melhores resultados em análises epidemiológicas, permitindo uma compreensão mais abrangente da dinâmica de disseminação e evolução de sorovares de *Salmonella enterica*, além de contribuir para a vigilância e o monitoramento de doenças transmitidas por alimentos.

A partir dos resultados obtidos nos capítulos 2, 3 e 4 deste estudo, fica evidente a complexidade e a importância epidemiológica dos sorovares de *Salmonella* enterica analisados, especialmente no contexto da produção avícola e da segurança alimentar.

Nesses capítulos, foram detectados diversos genes de resistência antimicrobiana, tais como *aac(6')-Iaa*, *sul1*, *sul2*, *bla*$_{OXA-129}$, *bla*$_{CMY-2}$, *tet(A)*, *tet(B) aadA1*, *fosA7*, e *qacE*. Esses genes conferem resistência a uma ampla variedade de antimicrobianos, incluindo aminoglicosídeos, sulfonamidas, tetraciclinas e beta-lactâmicos, o que pode ocasionar falha nos tratamentos de infeções bacterianas.

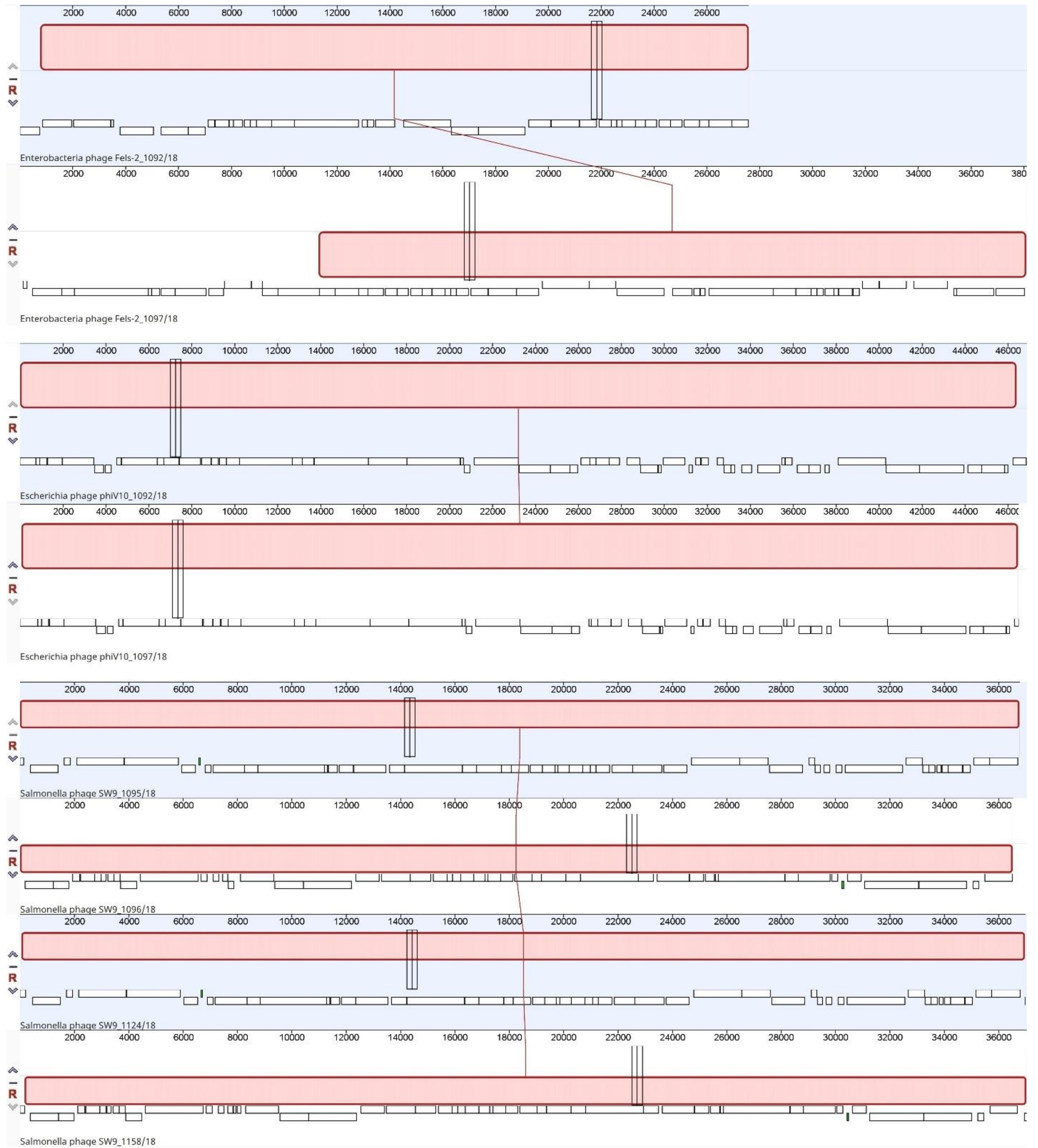
Nesse contexto, os diversos perfis genômicos preditos entre os sorovares, juntamente com os fatores específicos e sua plasticidade genômica, demonstram a capacidade genômica desses sorovares em colonizar uma ampla gama de nichos, abrangendo desde seres humanos e animais até o ambiente. Essas descobertas têm o potencial de enriquecer estudos sobre a relação *Salmonella*-hospedeiros, contribuindo para a otimização das estratégias de prevenção e para o tratamento da salmonelose em um futuro próximo.

Portanto, aqui está uma importante contribuição com informações genômicas de um dos maiores patógenos causadores de problema à saúde pública em todo o mundo. Entretanto, o monitoramento contínuo de estirpes de *Salmonella enterica* deve ser contínuo e realizado com técnicas de alta precisão, sempre com intuito de mitigar a disseminação da bacteria nos animais, humanos e ambiente.

APÊNDICES

Apêndice A: Figura Suplementar 1 apresentada no Capítulo 2.

Apêndice B: Figura Suplementar 2 apresentada no Capítulo 2.



Apêndice C: Tabelas Suplementares 1 e 2 apresentadas no Capítulo 3 estão disponíveis no Zenodo, um repositório de uso geral de acesso aberto mantido pelo OpenAIRE e CERN. O acesso aos apêndices deste capítulo pode ser encontrado no seguinte link permanente:

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Apêndice D: Tabelas Suplementares 1 apresentadas no Capítulo 4 estão disponíveis no Zenodo, um repositório de uso geral de acesso aberto mantido pelo OpenAIRE e CERN. O acesso aos apêndices deste capítulo pode ser encontrado no seguinte link permanente:

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