UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS DE RIBEIRÃO PRETO

Analyses of the genomes, transcriptomes and phenotypic characterization of *Salmonella* Typhimurium strains isolated from humans, food and swine in Brazil

Análises dos genomas, transcriptomas e caracterização fenotípica de linhagens de *Salmonella* Typhimurium isoladas de humanos, alimentos e suínos no Brasil

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Concentration area: Bioagents and Biotechnology Applied to Pharmacy

Candidate: Amanda Aparecida Seribelli
Supervisor: Profª Drª Juliana Pfrimer Falcão

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"I dedicate this work to my dear and
dedicated parents, my brother and sister
who are essential in my journey.
I love you...beyond life"

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"There are, in effect, two things, to know
and to believe one knows; to know is
science; to believe one knows is ignorance"
Hippocrates

RESUMO

SERIBELLI, A. A. **Análises dos genomas, transcriptomas e caracterização fenotípica de linhagens de *Salmonella* Typhimurium isoladas de humanos, alimentos e suínos no Brasil**. 2021. 176f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2021.

Salmonella enterica subsp. *enterica* sorovariedade Typhimurium (*S.* Typhimurium) é uma das principais causas de gastroenterite em vários países ao redor do mundo. Ademais, *S.* Typhimurium *sequence type* (ST) 313 é emergente e tem causado doença invasiva principalmente na África Subsaariana e sua presença foi recentemente descrita no Brasil. Entretanto, no país há poucos estudos que elucidaram possíveis diferenças na diversidade genotípica, expressão gênica e virulência de linhagens de *S.* Typhimurium isoladas de diferentes fontes e pertencentes a importantes STs. Na presente tese, seis importantes questões foram abordadas: (1) Qual é a diversidade de *single nucleotide polymorphism* (SNP) e de genes de resistência entre linhagens de *S.* Typhimurium isoladas de humanos e alimentos? (2) As linhagens de *S.* Typhimurium ST313 isoladas de humanos e alimentos são geneticamente distintas entre si e em comparação a outros STs? (3) Como linhagens de *S.* Typhimurium isoladas de humanos e alimentos se comportam diante de testes fenotípicos relacionados à virulência? (4) Qual é a diversidade genética de linhagens de *S.* Typhimurium isoladas de suínos? (5) Quais são as diferenças genômicas das linhagens de *S.* Typhimurium isoladas de humanos, alimentos e suínos? (6) Como a linhagem de *S.* Typhimurium ST313 se comporta em um modelo animal clássico e quais são as suas diferenças de expressão gênica em comparação a linhagens do ST19? Foi evidenciado que o resultado filogenético baseado em SNP agrupou as linhagens de *S.* Typhimurium em dois grandes grupos, sugerindo a existência de um subtipo prevalente, provavelmente mais adaptado para as linhagens isoladas de humanos e com alguma diversidade de subtipos para as linhagens de alimentos. A variedade e prevalência de genes de resistência encontrados nessas linhagens de *S.* Typhimurium reforçaram o potencial perigo destas para humanos sob tratamento e o risco da sua presença em alimentos no Brasil. Os genomas de linhagens de *S.* Typhimurium ST313 do Brasil mostraram grande semelhança entre si, cujas informações podem eventualmente ajudar no desenvolvimento de vacinas e antimicrobianos. A análise do pangenoma mostrou que os genomas de *S.* Typhimurium estudados apresentavam um pangenoma aberto, mas especificamente tendendo a se tornar fechado para as linhagens de *S.* Typhimurium ST313. A capacidade das *S.* Typhimurium estudadas invadirem as células epiteliais Caco-2 foi variável e não está relacionada com a fonte ou o ano de isolamento. Contudo, linhagens de *S.* Typhimurium isoladas de humanos mostraram maiores taxas de sobrevivência em macrófagos humanos U937 e apresentaram maior proporção de isolados com perfil virulento em larvas de *G. mellonella* quando comparadas com as linhagens isoladas de alimentos, sugerindo que essa diferença pode estar relacionada à maior frequência de isolados humanos que continham genes plasmidiais, tais como *operon spvABCDR*, *operon pefABCD*, *rck* e *mig-5*. O cgMLST e BLAST Atlas foram mais eficientes na discriminação das linhagens isoladas de suínos estudadas em comparação com o wgMLST. O potencial patogênico dessas linhagens de suínos foi corroborado pela presença de importantes *Salmonella pathogenicity islands* (SPIs) relacionadas à patogênese de *S.* Typhimurium. As análises filogenéticas agruparam a maioria dos isolados de *S.* Typhimurium de origens diversas em um único grupo, sugerindo a presença de um subtipo prevalente que contaminou com sucesso fontes humanas, alimentares e animais há 30 anos no Brasil. A análise de agrupamentos de proteínas ortólogas revelou genes únicos nas linhagens de *S.* Typhimurium estudadas, principalmente relacionados ao metabolismo bacteriano e que podem ser importantes em sua patogenicidade. Isolados de *S.* Typhimurium

de suínos apresentaram maior diversidade de STs e profagos em comparação com as linhagens de *S*. Typhimurium isoladas de humanos e alimentos. O potencial patogênico das linhagens de *S*. Typhimurium foi corroborado pela presença de profagos exclusivos dessa sorovariedade envolvidos em sua virulência. O elevado número de genes de resistência relacionados às bombas de efluxo é preocupante e pode levar a falhas terapêuticas quando houver necessidade de tratamento. *S*. Typhimurium STm30 (ST313) isolada de fezes de humano no Brasil demonstrou maior expressão de genes relacionados à patogênese a 37°C, além de melhor colonização e invasão no cólon murino, devido aos maiores níveis de expressão de genes de virulência e as citocinas pró-inflamatórias também foram mais expressas nesse órgão, sugerindo maior dano tecidual em comparação com as linhagens de *S*. Typhimurium SL1344 (ST19) e *S*. Typhimurium STm11 (ST19) isoladas de fezes humanas no Brasil. Finalmente, os resultados obtidos contribuíram para uma melhor caracterização da virulência e da diversidade genotípica desse importante enteropatógeno mundial.

Palavras-chave: *Salmonella* Typhimurium, sequenciamento do genoma completo, testes fenotípicos relacionados à virulência, transcriptoma.

ABSTRACT

SERIBELLI, A. A**. Analyses of the genomes, transcriptomes and phenotypic characterization of *Salmonella* Typhimurium strains isolated from humans, food and swine in Brazil.** 2021. 176f. Thesis (Doctorate). School of Pharmaceutical Sciences of Ribeirão Preto - University of São Paulo, Ribeirão Preto, 2021.

Salmonella enterica subsp. *enterica* serovar Typhimurium (*S*. Typhimurium) has been an important cause of gastroenteritis in various countries worldwide. In addition, *S*. Typhimurium sequence type (ST) 313 has been emerging as a cause of invasive disease mainly in sub-Saharan Africa and its presence was recently described in Brazil. However, in Brazil there are few studies that have elucidated possible differences in the genotypic diversity, gene expression and virulence of *S*. Typhimurium strains isolated from different sources and belonging to important STs. In the present thesis, six important questions were addressed: (1) What is the diversity of single nucleotide polymorphism (SNP) and resistance genes among *S*. Typhimurium strains isolated from humans and food? (2) Are genetically distinct the *S*. Typhimurium ST313 strains isolated from humans and food among each other and in comparison to other STs? (3) How do *S*. Typhimurium strains isolated from humans and food behave in phenotypic tests related to virulence? (4) What is the genetic diversity of *S*. Typhimurium strains isolated from swine? (5) What are the genomic differences of *S*. Typhimurium strains isolated from humans, food and swine? (6) How do the *S*. Typhimurium ST313 strain behave in a classic animal model and what are their differences in gene expression in comparison to ST19 strains? Phylogenetic results placed the *S*. Typhimurium strains into two major clades suggesting the existence of a prevalent subtype, likely more adapted, among strains isolated from humans, with some diversity in subtypes in isolates from food. The variety and prevalence of resistant genes found in these *Salmonella* Typhimurium strains reinforced their potential hazard for humans under treatment and the risk of its presence in foods in Brazil. The ST313 genomes from Brazil showed a high similarity among them which information might eventually help in the development of vaccines and antibiotics. The pangenome analysis showed that the *S*. Typhimurium genomes studied presented an open pangenome, but specifically tending to become closed for the ST313 strains. The ability of the studied *S*. Typhimurium to invade Caco-2 epithelial cells was strain dependent and was not related to the source or the year of isolation. However, *S*. Typhimurium strains isolated from humans showed greater survival rates in U937 human macrophages and presented higher proportion of isolates with a virulent related profile in *G. mellonella* larvae in comparison to strains isolated from food suggesting that this difference may be related to the higher frequency of human isolates which contained plasmidial genes, such as *spvABCDR* operon, *pefABCD* operon, *rck* and *mig-5*. The cgMLST and BLAST Atlas were more efficient at discriminating the swine isolates studied in comparison to wgMLST. The pathogenic potential of the swine strains studied was corroborated by the presence of important *Salmonella* pathogenicity islands (SPIs) related to the pathogenesis of *S*. Typhimurium. Phylogenetic analyses grouped the majority of the *S*. Typhimurium strains of diverse origins into a single cluster suggesting that there was one prevalent subtype that has successful contaminated human, food and animal sources for 30 years in Brazil. The orthologous protein clusters analysis revealed unique genes in the *S*. Typhimurium studied mainly related to bacterial metabolism and that may be important in their pathogenicity. *S*. Typhimurium isolates from swine showed greater diversity of STs and prophages in comparison to *S*. Typhimurium strains isolated from humans and foods. The pathogenic potential of *S*. Typhimurium strains was corroborated by the presence of exclusive prophages of this serovar involved in its virulence. The high number of resistance genes related to efflux pumps is worrying and may

lead to therapeutic failures when treatment is needed. *S*. Typhimurium STm30 (ST313) isolated from human feces in Brazil demonstrated greater expression of genes related to pathogenesis at 37°C, besides better colonization and invasion in the murine colon due to higher levels of expression of virulence genes and pro-inflammatory cytokines were also more expressed in this organ, suggesting greater tissue damage in comparison to *S*. Typhimurium SL1344 (ST19) and *S*. Typhimurium STm11 (ST19) isolated from human feces in Brazil. Finally, the results obtained contributed for a better characterization of the virulence and genotypic diversity of this important enteropathogen worldwide.

Keywords: *Salmonella* Typhimurium, whole genome sequencing, phenotypic tests related to virulence, transcriptome.

SUMMARY

1 - INTRODUCTION

1 - INTRODUCTION

1.1 *Salmonella* genus

The *Salmonella* genus was identified by an American scientist named Dr. Daniel E. Salmon in 1885, since then numerous advances in the understanding of this bacterium and salmonellosis disease have been revealed, including the identification of the two main *Salmonella* pathogenicity islands (SPIs) denominated SPI-1 and SPI-2 which are essential for *Salmonella* invasion and survival in the host cells (PETERSEN and MILLER, 2019).

Salmonella genus belongs to the *Enterobacteriaceae* family and consists of at least 2500 serovars, which approximately 80-90 of these serovars have been of considerable importance to the health of humans and animals. Besides, it is a Gram-negative bacilli, non-spore-forming and strains are usually not capable to ferment lactose, but some *Salmonella* strains can ferment this carbohydrate due to the acquisition of plasmids (lac+) (FERREIRA and CAMPOS, 2008; CARVALHO et al., 2016). Furthermore, this bacterium has the ability to ferment arabinose, maltose, mannitol, mannose, rhamnose, sorbitol, trehalose, xylose and dulcitol, perform decarboxylation of the amino acids lysine and ornithine, reduction of nitrate to nitrite and utilization of citrate as the sole carbon source (BRAZIL, 2011).

In addition, the classification and identification nomenclature of *Salmonella* are complex and different researchers use distinct systems to reference this genus. Currently, the most accepted approach has been that the genus is divided into two major species: *Salmonella enterica* and *Salmonella bongori* (ISSENHUTH-JEANJEAN et al., 2014). According to the Wang and collaborators (2019), *S. bongori* has been successful in infecting cold-blooded hosts, but there are some reports of this bacterium infecting warm-blooded animals (WANG et al., 2019a).

Salmonella enterica is divided into six subspecies with thousands of serovars: *S. enterica* subspecies *enterica*, *S. enterica* subspecies *salamae*, *S. enterica* subspecies *arizonae*, *S. enterica* subspecies *diarizonae*, *S. enterica* subspecies *houtenae* and *S. enterica* subspecies *indica* (BRENNER et al., 2000; ISSENHUTH-JEANJEAN et al., 2014).

Salmonella serovars have been usually identified using the White-Kauffmann-Le Minor (WKL) scheme since 1930 and this identification is based on the serology of the O (somatic), H (flagellar) and capsular (Vi – may not be present) antigens (BRENNER et al., 2000; FERREIRA and CAMPOS, 2008; NATARO et al., 2011; YOSHIDA et al., 2016).

Moreover, *Salmonella enterica* subspecies *enterica* can also be divided into two major groups: typhoidal and non-typhoidal, which have been associated with distinct diseases

(NATARO et al., 2011). Typhoid and paratyphoid fever are caused by the *S*. Typhi and *S*. Paratyphi serovars, respectively and the infection initially develops in the intestinal mucosa progressing to a systemic disease (FERREIRA and CAMPOS, 2008). The multiplication of the bacteria occurs in the spleen and liver of the host, causing bacteremia and the development of high fever, headache, poor appetite, abnormal heart rhythm, increase in spleen volume, diarrhea, among others (FERREIRA and CAMPOS, 2008).

Non-typhoidal *Salmonella* (NTS) has been an important cause of gastroenteritis worldwide (HOHMANN, 2001; EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL (ECDC), 2014; SERIBELLI et al., 2020; CENTERS FOR DISEASE FOR CONTROL AND PREVENTION (CDC), 2021). According to Majowicz and collaborators (2010), it was estimated that globally occur 93.8 million cases and approximately 155,000 deaths due to NTS every year in which 80.3 million cases have been transmitted by the ingestion of contaminated foods.

In Brazil, *Salmonella* has been one of the most common causes of foodborne outbreaks during the last decade and animal products have been the main vehicle for the transmission of this disease (BRAZIL, 2010; BRAZIL, 2019).

Salmonella transmission occurs mainly due to the ingestion of contaminated water and/or food, such as eggs, meat, poultry, milk and vegetables (WORLD HEALTH ORGANIZATION (WHO), 2018). Furthermore, there are other possibilities of contamination including person-to-person contact via fecal-oral route and contact with pets since these animals often do not show signs or symptoms of salmonellosis (WHO, 2018). It is important to mention that *Salmonella* is resistant to unfavorable environmental factors and it can proliferate in temperatures ranging from 35°C to 43°C with the extremes tolerated of 5°C and 46°C, as well as growth in pH 7.0 and 7.5 with the tolerated extremes of 3.8 and 9.5 (BRAZIL, 2011).

In addition, gastroenteritis caused by *Salmonella* is usually a self-limited disease which the symptoms disappear after four to seven days and there is no need of treatment with antimicrobials (CDC, 2019a). The main symptoms resulting from salmonellosis are diarrhea, fever and stomach cramps that have been predominantly caused by the *S*. Enteritidis and *S*. Typhimurium serovars in many countries worldwide (SCHULTE and HENSE, 2016; ARYA et al., 2017). However, systemic infections can occur in children and immunocompromised patients (SCHULTE and HENSE, 2016; ARYA et al., 2017). Specifically, it is known that children under 5 years old, infants, adults over 65 years and people who have other coexisting

diseases or use drugs that act by lowering the immune defense are more likely to develop severe disease due to *Salmonella* (CDC, 2019a).

Furthermore, a recent literature review indicated that nine cases of more serious complications from NTS infection in pregnant women were reported between 1966 and 2018 (MOLLO et al., 2019). The main complications were sepsis, spontaneous abortions and fatal outcomes, emphasizing that even in a small proportion of cases this bacterium can cause severe conditions in pregnant women (MOLLO et al., 2019).

It is important to mention that many NTS serovars have been described as host generalist, such as *S*. Typhimurium and *S*. Enteritidis, which are capable of infecting animals and humans. On the other hand, there are serovars host adapted, such as *S*. Gallinarum in poultry, *S*. Dublin in cattle and *S*. Choleraesuis in pigs (ARYA et al., 2017; WHO, 2018; CDC, 2021).

According to Fernandes and collaborators (2006), *S*. Typhimurium was the second most prevalent serovar in São Paulo State between 1996 and 2003. Similarly, in Pará State, *S*. Typhimurium was also the second most isolated serovar from outbreaks and sporadic cases of diarrhea during 2010 to 2013 (ASSIS et al., 2017). In addition, in Rio de Janeiro State, 129 isolates of *Salmonella* were identified between 2009 and 2013 with *S*. Typhimurium being the most frequent (48.8%) serovar among the isolates (PRIBUL et al., 2017).

Moreover, among the thousands NTS serovars it is estimated that globally the *S*. Typhimurium and *S*. Enteritidis serovars represent 50% of all isolates reported from humans with salmonellosis (ARYA et al., 2017). Specifically, *S*. Typhimurium has been the second most isolated serovar of salmonellosis cases between 2013-2014 in important countries including Canada, United States and European Union (EU) (ARYA et al., 2017).

Currently, the world has been under the threat of antimicrobial resistant pathogens affecting public health, food development and the environment (WHO, 2020). According to the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) (2020), the indiscriminate use of antimicrobials in humans and animals selects resistant bacteria, making treatment difficult when necessary. Increasing numbers of diseases caused by antimicrobial resistant bacteria, such as tuberculosis, gonorrhea and salmonellosis has been observed (WHO, 2020).

In the EU, the latest data in 2017/2018 indicated that there was an increase in the number of *Salmonella* multidrug resistant (MDR) isolates (resistant to three or more antimicrobials classes) from humans, animals and food (ECDC, 2020). *Salmonella* strains

isolated from humans resistant to ciprofloxacin had a significant increase, being this drug an important antibiotic used for the treatment of salmonellosis when necessary (ECDC, 2020).

In the United States, drug resistant NTS has been a serious threat to public health and causes more than 212,000 infections and 70 deaths every year (CDC, 2019b). Furthermore, in 2017, ciprofloxacin resistant NTS caused approximately 89,200 infections in this country, remembering that usually antibiotics such as ciprofloxacin, azithromycin and ceftriaxone may be needed for treatment of this disease (CDC, 2019b).

It is important to emphasize that the antimicrobial resistance in *S*. Typhimurium strains has also increased in the last year in many countries (WANG et al., 2019b). Data from CDC and NARMS between 1996 and 2016 showed that it was possible to observe that the most frequently reported resistance in this serovar isolated from food chain was ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (WANG et al., 2019b). In addition, the presence of ceftriaxone resistant *S*. Typhimurium strains was also reported in animals, meat and humans, causing concern for being a treatment option in severe cases as mentioned before (WANG et al., 2019b).

Therefore, this problem is so serious that there is an estimate that infections caused by antimicrobial resistant pathogens if the control measures fail will kill approximately 10 million people in all the world in 2050, thus over coming other diseases, such as cancer, cholera, diabetes, diarrhoeal disease, measles and tetanus (O' NEILL, 2014).

1.2 *Salmonella* Typhimurium

As mentioned earlier *S*. Typhimurium is a generalist serovar and can infect humans, cattle, pigs, sheep, horses, rodents, turtles, chickens, turkeys, ducks, pigeons and birds (RABSCH et al., 2002; WORK et al., 2019). The detection of *S*. Typhimurium in poultry and pigs is difficult due to the different outcomes of the bacteria in these animals, including asymptomatic cases, diarrhea or more severe cases causing deaths (BEST et al., 2007; ÖSTERBERG; LEWERIN; WALLGREN, 2010; DAR et al., 2017). Usually, such animals show no symptoms and can transmit the bacteria to humans through consumption and handling of their contaminated meat (BEST et al., 2007; ÖSTERBERG; LEWERIN; WALLGREN, 2010; DAR et al., 2017).

S. Typhimurium has been isolated mainly from humans and animals, including poultry and pigs (EVANGELOPOULOU et al., 2015; PORTER et al., 2019). According to Porter and collaborators (2019), *S*. Typhimurium was the main serovar isolated from pigs in Northern

Ireland between 1997 and 2016. Furthermore, this serovar has been the causative agent of several outbreaks associated with consumption of pork in the EU in recent years demonstrating its importance in pig's production (CAMPOS et al., 2019).

In Brazil, it is also possible to observe that *S*. Typhimurium has been isolated from the intestinal content of pigs, carcasses and surrounding pig environment, which can represent risk of contamination in all meat production processes (ALMEIDA et al., 2016a; PAIM et al., 2019). According to the Brazilian Association of Animal Protein (ABPA), Brazil has been ranked as the fourth largest producer of pig meat, surpassed only by China, EU and the United States, producing 3,983 thousand tons of pig meat and exporting 750 thousand tons in 2019 (ABPA, 2020).

Control and prevention measures have been of great importance in relation to *S*. Typhimurium contamination in pigs (RODRÍGUEZ and SUÁREZ, 2014). This contamination can occur during the different stages of pork production, including transport, carcass processing and meat handling and storage, generating damage for producers and consumers (RODRÍGUEZ and SUÁREZ, 2014; EVANGELOPOULOU et al., 2015). In addition, research should be encouraged mainly in pork producing and exporting countries such as Brazil due to the emergence of this pathogen related to the political, economic, and public health sectors (RODRÍGUEZ and SUÁREZ, 2014; EVANGELOPOULOU et al., 2015).

It is known that Invasive Non-typhoidal *Salmonella* (iNTS) has been a serious public health problem frequently reported in the African continent and mainly related to other diseases, such as acquired human immunodeficiency virus infection (HIV), malaria, malnutrition, cachexia, and sickle cell anemia (SINGLETARY et al., 2016; GILCHRIST and MACLENNAN, 2019).

According to Ao et al. (2015), it was estimated that occur annually 3.4 million cases and more than 680 thousand deaths due to iNTS worldwide, which the African continent has been the most affected with 227 cases per 100,000 populations. Unfortunately, in Ghana the estimated incidence was 2,520 cases of iNTS per 100,000 population between 2007 to 2009 in children under 5 years old (UCHE; MACLENNAN; SAUL, 2017).

It is important to emphasize that iNTS has been the most isolated agent in Africa, which has a mortality rate around 18 to 25% (REDDY; SHAW; CRUMP, 2010; FEASEY et al., 2012; GILCHRIST and MACLENNAN, 2019). Therefore, bloodstream infections have been frequently reported with high mortality rates, indicating the need for preventive and control measures mainly in this continent (REDDY; SHAW; CRUMP, 2010).

Specifically, a summary of 22 studies resulting from bloodstream infections in Africa between 1984-2006 indicated that in North Africa the prevalence was of the *S*. Typhi serovar in adults (REDDY; SHAW; CRUMP, 2010). During this period, in West and Central Africa, NTS was the predominant agent from bloodstream infections in children (REDDY; SHAW; CRUMP, 2010). In East Africa, NTS was the second most prevalent agent from bloodstream infections in children and adults (REDDY; SHAW; CRUMP, 2010). Finally, in Southern Africa, NTS was the most isolated agent from bloodstream infections in children and adults (REDDY; SHAW; CRUMP, 2010).

Among NTS serovars that have been successful in causing invasive disease in young age and adults with other underlying health conditions it should be highlighted the *S*. Enteritidis, *S*. Typhimurium and *S*. Dublin serovars (BALASUBRAMANIAN et al., 2019). According to Reddy and collaborators (2010), *S*. Typhimurium was the most isolated serovar (65.2%) in the African continent from bloodstream infections in children and adults. Specifically, there is an epidemic invasive disease mainly in sub-Saharan Africa caused by *S*. Typhimurium belonging to the sequence type (ST) 313 (BALASUBRAMANIAN et al., 2019).

The ST is obtained by the Multilocus sequence typing (MLST) technique and *S*. Typhimurium ST313 was characterized as being genetically distinct from *S*. Typhimurium ST19 which has been reported as the most frequent ST in strains of this serovar (FEASEY et al., 2012; FEASEY et al., 2014; KARIUKI and ONSARE, 2015). Clinical and epidemiological data indicated that *S*. Typhimurium ST313 has been closely linked to invasive systemic disease, such as bacteremia, septicemia and meningitis in Africa (RAMACHANDRAN et al., 2015). On the other hand, cases of gastroenteritis have been predominantly caused by *S*. Typhimurium ST19 in different parts of the globe (RAMACHANDRAN et al., 2015).

It is known that *S*. Typhimurium ST313 and *S*. Typhimurium ST19 present differences in their behavior during infection; but there are few studies that tried to elucidate such differences mainly *in vivo* (OKORO et al., 2015; YANG et al., 2015a; RAMACHANDRAN et al., 2015; RAMACHANDRAN et al., 2017). In addition, the presence of *S*. Typhimurium ST313 strains have been recently described in Brazil (ALMEIDA et al., 2017a; PANZENHAGEN et al., 2018). According to Almeida and collaborators (2017a), the ST19 was the most frequently reported in 88 *S*. Typhimurium strains isolated from humans and food in Brazil and the ST313 was the second most reported.

According to Pulford and collaborators (2020), *S*. Typhimurium ST313 strains may be successful in causing invasive disease in Africa due to resistance to antimicrobials and loss-of-function in genes that are not needed for systemic infection. Furthermore, these authors found a variant of ST313 denominated L3 that emerged in Malawi in 2016, which was pan susceptible and clonally related to ST313 strains isolated predominantly from cases of gastroenteritis found in the UK and Brazil (ALMEIDA et al., 2017a; PULFORD et al., 2020).

1.3 Virulence

The infection by *S*. Typhimurium strains begins in the fecal oral route through ingestion of contaminated water and/or food (GILCHRIST; MACLENNAN; HILL, 2015). *S*. Typhimurium has the ability to invade, survive and replicate within host cells due to the Type III Secretion Systems (T3SS-1 and T3SS-2) which are encoded by genes located in *Salmonella* pathogenicity islands 1 and 2 (SPIs) denominated SPI-1 and SPI-2, respectively (KIMBROUGH and MILLER, 2002; HURLEY et al., 2014; SANTOS; FERRARI; CONTE-JUNIOR, 2019).

Overall, the *sipA*, *sipB*, *sipC*, *sipD*, *sopE*, *sopE2*, *sopB*, *sopD* and *sopA* genes are SPI-1 effectors and decisive for the entry of *S*. Typhimurium in the host cells (IBARRA and STEELE-MORTIMER, 2009; HEIJDEN and FINLAY, 2012; HURLEY et al., 2014). Specifically, the T3SS-1 is basically formed by a needle complex, export apparatus and translocon, which is known as molecular syringe, because its activation triggers signaling cascades that manipulate the host cells and generate disarrangements (IBARRA and STEELE-MORTIMER, 2009; JONG et al., 2012; SANTOS; FERRARI; CONTE-JUNIOR, 2019).

In addition, *S*. Typhimurium can alternatively be taken to the intestinal submucosa without invading due to internalization by dendritic cells or M cell mediated transcytosis (SANTOS; FERRARI; CONTE-JUNIOR, 2019; WEMYSS and PEARSON, 2019). The induction and regulation of the T3SS-1 occurs in environments with changes in the osmolarity, density, pH and aeration, the *hilA* gene is the main transcriptional activator of the SPI-1 effectors (HEIJDEN and FINLAY, 2012).

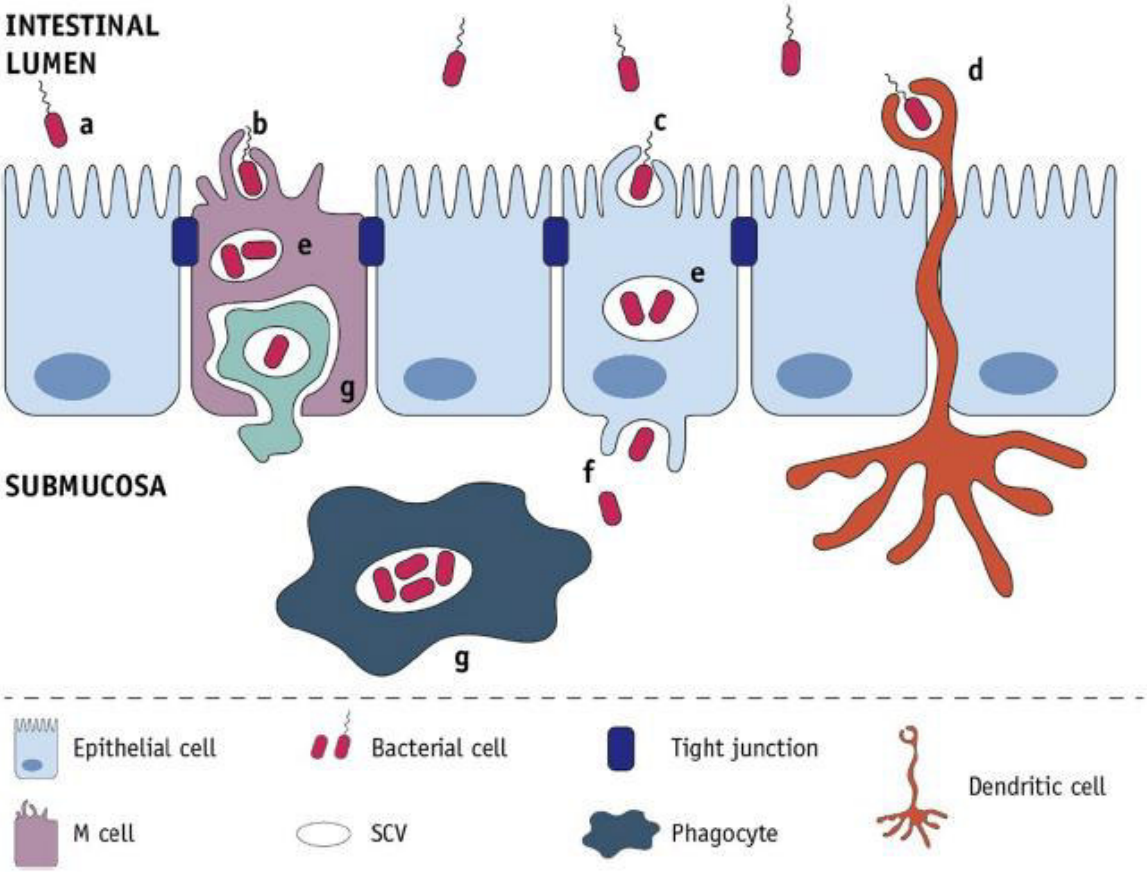
Once *S*. Typhimurium is already internalized, the SPI-1 effectors induce development of a vacuole denominated *Salmonella*-containing vacuole (SCV) and become downregulated (WEMYSS and PEARSON, 2019). While, the SPI-2 effectors become upregulated (T3SS-2), such as the *pipB2*, *sopD2*, *sifA*, *sspH2*, *steC*, *sseF*, *sseG*, *sseI* and *sseJ* genes that are mainly responsible by the SCV maturation and intracellular survival of *S*. Typhimurium inside of

intestinal epithelial cells or phagocytic cells as in local intestinal macrophages (HEIJDEN and FINLAY, 2012; HURLEY et al., 2014; WEMYSS and PEARSON, 2019).

It is important to mention that after *S*. Typhimurium have crossed the epithelial barrier, local macrophages engulf the bacteria trying to control the infection, but *S*. Typhimurium is capable to escape of these cells, because the expression of SPI-2 effectors (T3SS-2) prevent the fusion of the SCV with the lysosome, causing apoptosis of the local macrophages (HEIJDEN and FINLAY, 2012; HURLEY et al., 2014; WEMYSS and PEARSON, 2019). The induction and regulation of the T3SS-2 occurs after the production of the SCV and the SsrB transcriptional regulator is capable to connect and activate all SPI-2 effectors (HEIJDEN and FINLAY, 2012).

Salmonellosis can be summarized in some topics, including adhesion and invasion to intestinal epithelial cells (T3SS-1), survival and proliferation in host cells, such as intestinal epithelial cells and macrophages (SCV – T3SS-2), recruitment of more phagocytic cells to the infection site and intestinal homeostasis imbalance, triggering fluid and electrolyte loss through diarrhea (Fig. 1) (GILCHRIST; MACLENNAN; HILL, 2015; SANTOS; FERRARI; CONTE-JUNIOR, 2019).

Fig. 1 – Pathogenesis of *Salmonella* Typhimurium



Source: Santos; Ferrari; Conte-Junior, Current Microbiology, 2019.
a *Salmonella* adheres to the intestinal epithelial and M cells using many of adhesion factors present on its cell surface. **b, c** Effector proteins are released into enterocyte causing changes on its cytoskeleton and forming structures in its surface known as ruffles. **d** Alternatively, the bacterial cells can be directly taken by dendritic cell from the submucosa. **e** Once inside cytoplasm, *Salmonella* cells are located into SCV (*Salmonella*-containing Vacuoles), where it multiplies. **f** The SCV transcytose to the basolateral membrane and release to the submucosa. **g** Bacteria is internalized within phagocytes and then located again into SCV; this figure was based on the one illustrated in the article Sansonetti, Gut, 2002.

It is known that the invasive disease mainly caused in children by *S*. Typhimurium ST313 in sub-Saharan Africa presents in addition to the pathogenesis mentioned above, systemic dissemination through the bloodstream and establishment of new infectious sites culminating in cough, dyspnea, convulsions, tachycardia, tachypnea, respiratory distress, hepatomegaly, and splenomegaly (MACLENNAN et al., 2017; GILCHRIST and MACLENNAN, 2019).

According to Gilchrist and Maclennan (2019), it is not clear why the *S*. Typhimurium ST313 strains are being more invasive; but there are some phenotypes that may contribute to these cases, such as degradation of the complement system on the bacterial surface, reduction

in colitis and recruitment of neutrophils, resistance to death serum and attenuated inflammatory response in macrophages (GILCHRIST and MACLENNAN, 2019).

The virulence plasmid (pSLT) has been reported in *S*. Typhimurium and carries important genes for the pathogenesis of this serovar. Specifically, in this plasmid there is a highly conserved region denominated *spv* (*Salmonella* plasmid virulence) which encodes four structural genes *spvA*, *spvB*, *spvC* and *spvD* and the regulatory gene s*pvR* (GUINEY et al., 1995; GILCHRIST and MACLENNAN, 2019).

In addition, the *spvB* and *spvC* genes are T3SS-2 effectors, participating in the processes of host macrophages apoptosis and decreasing the inflammatory response, respectively (IBARRA and STEELE-MORTIMER, 2009; HEIJDEN and FINLAY, 2012).

Bacteriophages or phages are virus specialized in infecting bacteria and can perform the lytic and/or lysogenic cycles in the host cells (KROPINSKI, 2009; SWITT et al., 2015). In the lytic cycle, the virus "hijacks" the host cell machinery, giving rise to thousands of descending virus and leading the cell to death. On the other hand, in the lysogenic cycle, the virus integrates its DNA with bacterial DNA denominated prophage (SWITT et al., 2015). Prophages can be related to several functions in bacterial cells, such as virulence, metabolism, signaling, evolution and ecology (WAHL; BATTESTI; ANSALDI, 2019).

The Gifsy prophages carry genes that favor the virulence of *S*. Typhimurium in the host cells (KLUMPP and FUCHS, 2007; NGOI; YAP; THONG, 2018). Specifically, the Gifsy-1 and Gifsy-2 prophages encode genes involved in the intracellular survival of *Salmonella* in the host cells (FIGUEROA-BOSSI and BOSSI, 1999; WAHL; BATTESTI; ANSALDI, 2019).

It is important to mention that the Gifsy prophages are found only in *S*. Typhimurium, as well as the Fels-1 and Fels-2 prophages (NGOI; YAP; THONG, 2018). According to Brüssow, Canchaya and Hardt (2004), the Fels-1 and Fels-2 prophages encode genes related to adherence and survival of *S*. Typhimurium to host cells (WAHL; BATTESTI; ANSALDI, 2019).

Furthermore, it was described that the *S*. Typhimurium LT2 reference strain presented the Gifsy-1, Gifsy-2, Fels-1 and Fels-2 prophages and these prophages have been described in *S*. Typhimurium isolated in other countries including Australia, Europe and China (GARCÍA et al., 2013; PANG et al., 2013; YANG et al., 2015b; NGOI; YAP; THONG, 2018).

1.4 Immune response

Innate immunity is the first line of defense against pathogens, which is capable to induce a series of cellular and inflammatory responses that try to block the infection (BROZ; OHLSON; MONACK, 2012). It is known that *S*. Typhimurium causes severe intestinal inflammation due to its invasion into host cells (BROZ; OHLSON; MONACK, 2012). This bacterium is adapted to survive and multiply in this inflammatory environment, as well as uses this adaptation to benefit from other microorganisms found in the intestinal microbiota (BROZ; OHLSON; MONACK, 2012).

In the moment that *S*. Typhimurium reaches its target organ pattern recognition receptors (Toll-like) that detect the presence of the invading pathogen extracellularly and stimulate the release of pro-inflammatory cytokines, such as IL-23 (BROZ; OHLSON; MONACK, 2012; HURLEY et al., 2014). During the course of the infection, *S*. Typhimurium invades host cells promoting the release of different cytokines, including the interleukins IL-1β, IL-18, IL-6, interferons (IFN-γ) and tumor necrosis factor (TNF-α), which in general increase the systemic inflammatory response (Fig. 2) (BROZ; OHLSON; MONACK, 2012; HURLEY et al., 2014).

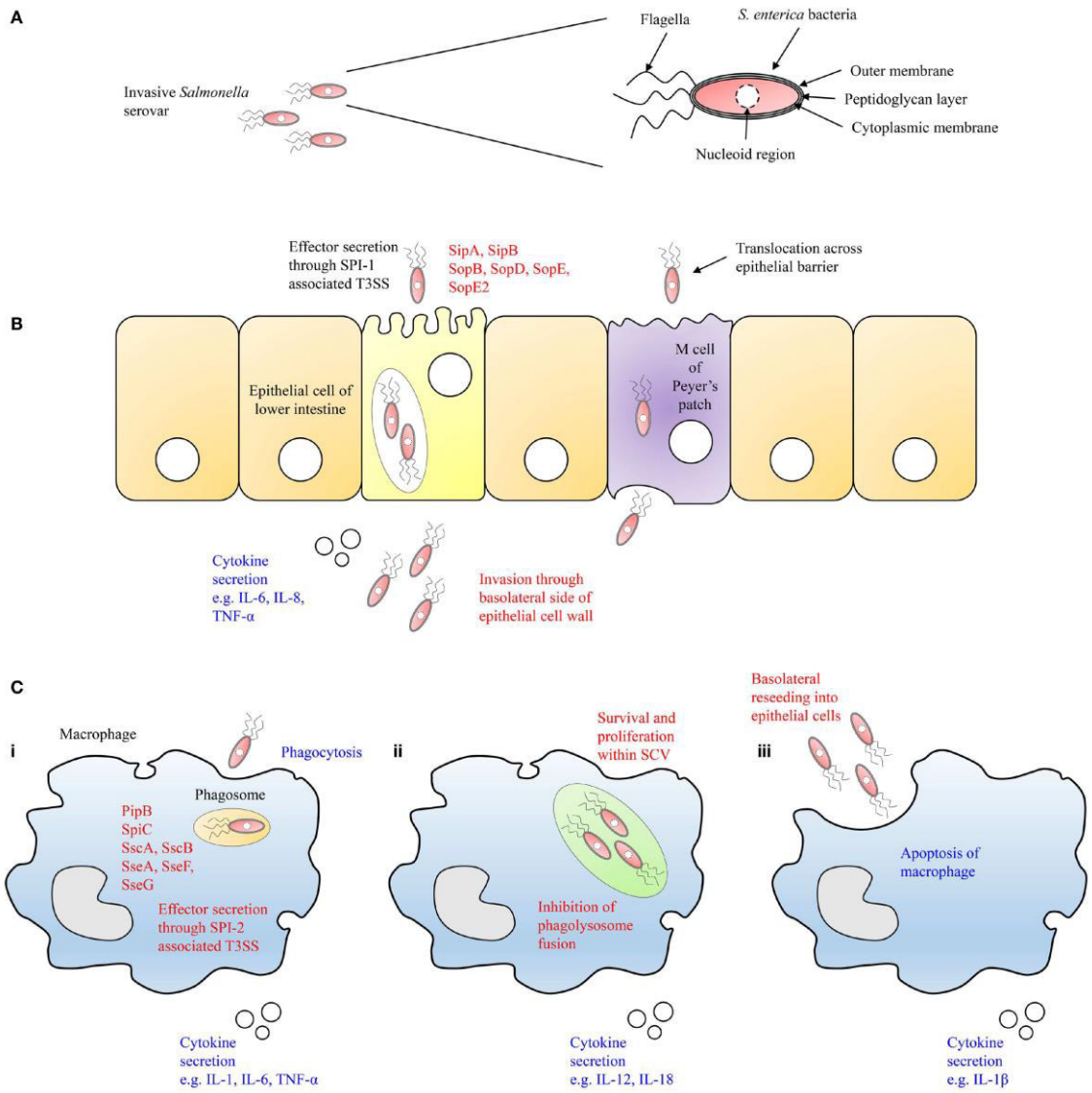
In addition, the interleukin IL-23 promotes the release of interleukins IL-17 and IL-22 that contribute to healing, secretion of antimicrobial molecules and induction of pro-inflammatory mediators, with consequent recruitment of neutrophils (BROZ; OHLSON; MONACK, 2012; VALERI and RAFFATELLU, 2016). Specifically, the interleukin IL-17 induces other pro-inflammatory cytokines and IL-22 is capable to induce tissue protection (BROZ; OHLSON; MONACK, 2012; VALERI and RAFFATELLU, 2016).

The greater recruitment of neutrophils to the infection site aims to eliminate *S*. Typhimurium strains that are still extracellularly and this cellular and inflammatory flow culminate in damage and disarrangement of the intestinal epithelium, losing the epithelial cell barrier with consequent elimination of fluids through diarrhea (BROZ; OHLSON; MONACK, 2012).

Finally, the immune response triggers a series of cellular and humoral inductions that try to control salmonellosis, among the different cytokines important for these processes it is highlighted the interleukins IL-12 and IL-10 (SALAZAR et al., 2017; ELSNER and SHLOMCHIK, 2019). The interleukin IL-12 is produced mainly by dendritic cells and phagocytes, which plays a key role in stimulating the Th1 response (cell-mediated immune response) (ELSNER and SHLOMCHIK, 2019). According to Salazar and collaborators

(2017), the interleukin IL-10 is classified as anti-inflammatory which is extremely important to limit the inflammation that occurs during the infectious process and prevent tissue damage, inhibiting other interleukins including IL-12 and IFN-γ.

Fig. 2 – Schematic illustration of the infection of epithelial cells of the lower intestine and macrophages by *Salmonella*



Source: Hurley and collaborators, Frontiers in Immunology, 2014.
(A) The complex membrane structure of *Salmonella* allows it to survive until reaching the epithelial cell wall of the host in the lower intestine. (B) *Salmonella* then translocates across M cells of Peyer's patches or actively invade epithelial cells by the secretion of effector proteins through the SPI-1 encoded T3SS-1. (C) (i) After crossing the epithelial barrier, *Salmonella* is engulfed by proximal macrophages that will secrete effector proteins into the cytosol of the cell via the SPI-2 encoded T3SS-2 and prevent fusion of the phagosome with

the lysosome. (ii) Within the SCV, *Salmonella* will proliferate resulting in cytokines secretion by the macrophage. (iii) Finally, the macrophage will undergo apoptosis, and *Salmonella* will escape the cell basolaterally reinvading epithelial cells or other phagocytic cells of the host innate immune system.

1.5 Whole genome sequencing (WGS) and RNA-seq

Over the past 56 years, researchers around the world have witnessed the advent of new-generation sequencing (NGS) for genetic material of prokaryotic and eukaryotic cells (HEATHER and CHAIN, 2016; ILLUMINA, 2021). Specifically, there are currently numerous possibilities for microbial whole genome sequencing (WGS) and RNA-seq in order to better understand its pathogenicity, virulence and epidemiology (ILLUMINA, 2021).

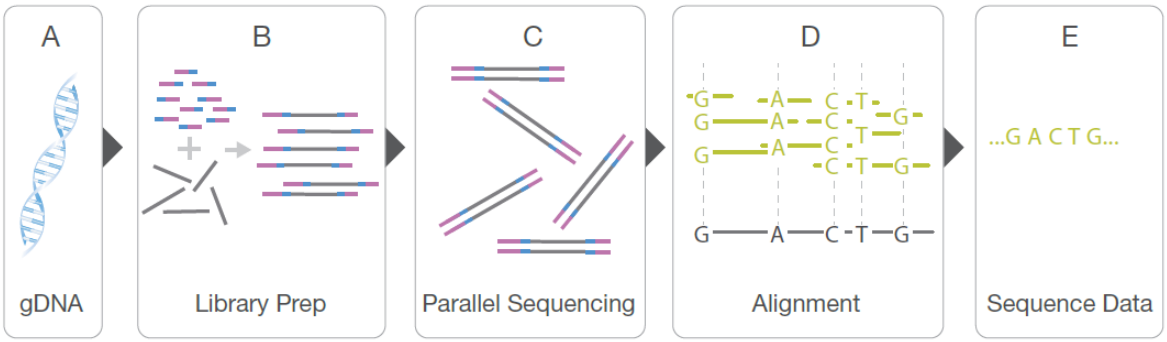
The first step towards the currently available technology was taken in 1965 by Holley and collaborators who sequenced the alanine tRNA from the yeast *Saccharomyces cerevisiae*. At the same time, Sanger and collaborators developed the sequencing of radiolabeled DNA fragments (HOLLEY et al., 1965; SANGER; BROWNLEE; BARRELL, 1965; HEATHER and CHAIN, 2016).

Currently, the WGS of bacterial strains generates several data that can provide important information for molecular epidemiology, such as genetic similarity among strains from different sources, years and countries, besides the technique allows the detection of virulence and resistance genes and prediction of prophages regions (SHIVANI et al., 2015; CDC, 2016). According to Centers for Disease Control and Prevention (2016), the WGS has been an essential tool for faster diagnoses and extremely useful for the solution or prevention of foodborne epidemics, facilitating work in the areas of health and epidemiological surveillance (CDC, 2016).

In addition, it is important to emphasize that these thousands of data can be analyzed by professionals trained in the area of bioinformatics, since specific softwares must be used for different analyzes, including important concepts for understanding the process of genetic material sequencing (Fig. 3) (FERREIRA and BORGES NETO, 2003, ILLUMINA, 2021). It worth mentioning that this technology allows us to differentiate extremely genetically related strains, as well as provides a refined analysis that differentiates strains at the level of single nucleotide polymorphism (SNPs) (FERREIRA and BORGES NETO, 2003; SHIVANI et al., 2015). Furthermore, the WGS is becoming more affordable because the costs are getting lower and facilities are spread around the world allowing partnership between universities and laboratories (SHIVANI et al., 2015; HEATHER and CHAIN, 2016; ILLUMINA, 2021).

Transcriptional analyses through RNAseq of *Salmonella* in different conditions have been useful for understanding the pathogenicity of this important foodborne pathogen (GRUZDEV et al., 2012). It is important to mention that transcriptome studies have provided relevant information on the pathogenicity and virulence of different bacteria, since through this technology it is possible to identify genes involved in different bacterial biological processes, also providing insights about the functions of genes that have not been characterized and indicating possible changes in the proteome and metabolome (CROUCHER et al., 2009; LI et al., 2017; OSHOTA et al., 2017).

Fig. 3 – Workflow of Next-Generation Sequencing (NGS)



Source: Illumina, 2021.
(A) Extracted genomic DNA. (B) Sample preparation fragments genomic DNA and adds adapters to generate a library. (C) DNA fragments within the library are each sequenced in parallel. (D) Individual sequence reads are reassembled by aligning to a reference genome. (E) The whole genome sequencing is derived from the consensus of aligned reads.

Finally, in Brazil, there are few studies that have characterized possible differences in the genomes, gene expression and virulence of *S*. Typhimurium strains isolated from different sources, belonging to distinct STs and geographic regions in this country. Therefore, studies using WGS analyses, invasion and survival assays, transcriptome and *in vivo* tests of *S*. Typhimurium strains isolated from humans, food and swine in Brazil are of great importance and should contribute for a better understanding of the genotypic diversity, gene expression and virulence of this important global enteropathogen.

2 - CORRELATION OF THE CHAPTERS

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In Brazil, there are few studies that have characterized possible differences in the genomes, gene expression and virulence of *S*. Typhimurium strains isolated from humans, food and swine. Therefore, six important questions were addressed in this thesis: (1) What is the diversity of single nucleotide polymorphism (SNP) and resistance genes among *S*. Typhimurium strains isolated from humans and food? (2) *S*. Typhimurium ST313 strains isolated from humans and food are genetically distinct from ST19 strains? (3) How do *S*. Typhimurium strains isolated from humans and food behave in phenotypic tests related to virulence? (4) What is the genetic diversity of *S*. Typhimurium strains isolated from swine? (5) What are the genomic differences of *S*. Typhimurium strains isolated from humans, food and swine? (6) How do a representative *S*. Typhimurium ST313 strain behave in a classic animal model and what are its differences in genes expressions in comparison to ST19 strains?

The Chapter 1 is an initial exploration of 90 *S*. Typhimurium genomes of strains isolated from humans and foods between 1983 and 2013 from different geographic regions in Brazil. We performed a phylogenetic tree based on single nucleotide polymorphism (SNP) analysis and search for antimicrobial resistance genes *in silico*, since the monitoring of resistant strains is essential and according to World Health Organization (WHO) (2019), among the Global Priority Pathogens List, *Salmonella* has been described as high in the priority category, corroborating with the importance of such analyses as described previously in this thesis (ASOKAN et al., 2019). This article was published in 2018 in the PLosOne and an interview was granted to FAPESP Agency, the first authorship is shared between Amanda Ap. Seribelli and Dr. Fernanda de Almeida who performed the whole genome sequencing at the Food and Drug Administration (FDA), College Park, under the supervision of Dr. Marc W. Allard.

The Chapter 2 is a comparison among 40 *S*. Typhimurium strains isolated from humans and various foods that were selected based in the phylogenetic tree (Chapter 1) containing different characteristics, such as important STs like ST313 and ST19, year of isolation and distinct subgroups in the tree. The ST313 genomes from Brazil showed a high similarity among them by 16S rRNA sequences, the Gegenees software, whole genome multilocus sequence typing (wgMLST), and average nucleotide identity (ANI) which information might eventually help in the development of vaccines and antibiotics. However, the ST313 genomes were usually clustered more distantly to other STs of strains isolated in

Brazil and in other parts of the world. Furthermore, the pangenome analysis showed that the *S.* Typhimurium genomes studied presented an open pangenome, but specifically tending to become closed for the ST313 strains. This article was published in 2019 in the Brazilian Journal of Microbiology and it is important to mention that *S*. Typhimurium strains typed as ST313 have been associated with invasive and feverish disease in countries on the African continent, on the other hand, ST19 strains have been related to gastroenteritis in humans worldwide (FEASEY et al., 2012; KARIUKI and ONSARE, 2015). The presence of *S*. Typhimurium ST313 strains in Brazil has been recently described by our research group (ALMEIDA et al., 2017a).

In addition, the Chapter 3 was performed with the majority of the 40 strains of *S*. Typhimurium isolated from humans and food described in Chapter 2. In this article, we selected 20 *S*. Typhimurium strains isolated from humans and 20 *S*. Typhimurium strains isolated from foods based on different groups of the phylogenetic tree described in Chapter 1. This article was published in PLosOne in 2020 and aimed to do a phenotypic and genotypic characterization of *Salmonella* Typhimurium isolated from humans and foods in Brazil regarding their invasion and survival in human epithelial cells (Caco-2) and macrophages (U937). Their virulence potential was determined using the *Galleria mellonella* larvae model combined with the analysis of virulence genes by whole genome sequencing (WGS).

Although, Brazil has been one of the largest pork meat exporters worldwide, there are few studies that characterized epidemiologically *S*. Typhimurium strains isolated from swine in this country. The Chapter 4 emerged from a new partnership with FDA for whole genome sequencing 26 *S*. Typhimurium strains isolated from swine that had not been explored before. Therefore, this article is a study on the genetic similarities of these strains isolated from swine among themselves and with 50 strains isolated from other sources and countries available on GenBank. Characterizations were made using different phylogenetic strategies such as whole genome multi locus sequence typing (wgMLST) and core genome multi locus sequence typing (cgMLST). In addition, it was compared the genomic content between the 26 swine strains studied to a reference *S*. Typhimurium LT2 by BLAST Atlas and it was verified the frequencies of *Salmonella* pathogenicity islands (SPIs). This study was submitted for publication in the Infection, Genetics and Evolution - Journal – Elsevier.

As a result of Chapter 4, in Chapter 5 we wanted to explore and compare all Brazilian strains belonging to our research group, totaling 117 *S*. Typhimurium genomes isolated from humans, food and swine in different regions. Comparative genomic techniques were carried

out for this purpose, such as phylogenetic analysis; orthologous protein clusters analysis; Multi-locus sequence typing (MLST); prophages and screening of resistance genes related to efflux pumps. It is important to mention that this work was submitted for publication in the BMC microbiology – Part of Springer Nature.

Finally, the Chapter 6 is an attempt to elucidate possible differences in the virulence of one *S*. Typhimurium ST313 strain and two *S*. Typhimurium ST19 strains that were chosen due to its similar ability to invade Caco-2 cells (Chapter 3). The aims of this work were to perform the RNA-seq *in vitro* under growth in Luria Bertani (LB) at 37°C comparing the *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains. In addition, it was aimed to evaluate the colonization and expression of virulence genes and cytokines in the murine colon after infection by such strains which in turn could explain possible differences in the pathogenesis of the invasive ST313. It is important to mention that this work was submitted for publication in the Microbiological Research – Journal – Elsevier.

3 - OBJECTIVES

3 - OBJECTIVES

- To uncover the genotypic diversity of *Salmonella* Typhimurium strains isolated from humans and food, between 1983 and 2013, from different geographic regions in Brazil and to verify the presence of antimicrobial resistance genes, as well as, the occurrence of mutations points in the *gyrA, gyrB, parC* and *parE* genes by WGS.

- To assess the genetic relationship of many *S.* Typhimurium genomes comparing strains of ST313 isolated from humans and food in Brazil among themselves, with other STs isolated in this country and in other parts of the globe by 16S rRNA sequences, the Gegenees software, whole genome multi locus sequence typing (wgMLST), pangenome and average nucleotide identity (ANI) for the genomes of ST313.

- To evaluate *S*. Typhimurium isolates from humans and foods in Brazil and their ability to invade Caco-2 epithelial cells, the ability to survive in U937 human macrophages, and to assess its virulence in the *Galleria mellonella* infection model, and lastly to characterize the repertoire of virulence genes present through WGS.

- To analyze the phylogenetic relationships of *S.* Typhimurium genomes from swine isolated in Brazil among themselves and with other genomes isolated from several sources and countries using different phylogenetic strategies such as wgMLST and cgMLST, besides to compare the genetic content between the genomes of the swine strains studied with the reference *S.* Typhimurium LT2 by BLAST Atlas and to perform the search of *Salmonella* pathogenicity islands (SPIs).

- To compare genetically 117 *S.* Typhimurium isolates from humans, foods and swine in Brazil during 30 years using different genomics strategies, such as phylogenetic trees, orthologous clusters analysis, MLST, prophages and resistance genes related to efflux pump occurrences.

- To perform the RNA-seq *in vitro* under growth in Luria Bertani (LB) at 37°C comparing the *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains. In addition, it was aimed to evaluate the colonization and expression of virulence genes and cytokines in the murine colon after infection by such strains which in turn could explain possible differences in the pathogenesis of the invasive ST313.

CHAPTER 1

Phylogenetic and antimicrobial resistance gene analysis of *Salmonella* Typhimurium strains isolated in Brazil by whole genome sequencing

Running title: Whole genome sequencing of *Salmonella* Typhimurium in Brazil

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ABSTRACT

Whole genome sequencing (WGS) has been used as a powerful technology for molecular epidemiology, surveillance, identification of species and serotype, identification of the sources of outbreaks, among other purposes. In Brazil, there is relatively few epidemiological data on *Salmonella*. In this study, 90 *Salmonella* Typhimurium strains had their genome sequenced to uncover the diversity of *Salmonella* Typhimurium isolated from humans and food, between 1983 and 2013, from different geographic regions in Brazil based on single nucleotide polymorphism (SNP) analysis. A total of 39 resistance genes were identified, such as aminoglycoside, tetracycline, sulfonamide, trimethoprim, beta-lactam, fluoroquinolone, phenicol and macrolide, as well as the occurrence of point mutations in some of the genes such as *gyrA, gyrB, parC* and *parE*. A total of 65 (72.2%) out of 90 *S.* Typhimurium strains studied were phenotypically resistant to sulfonamides, 44 (48.9%) strains were streptomycin resistant, 27 (30%) strains were resistant to tetracycline, 21 (23.3%) strains were gentamicin resistant, and seven (7.8%) strains were resistant to ceftriaxone. In the *gyrA* gene, it was observed the following amino acid substitutions: Asp(87)→Gly, Asp(87)→Asn, Ser(83)→Phe, Ser(83)→Tyr. Phylogenetic results placed the 90 *S.* Typhimurium strains into two major clades suggesting the existence of a prevalent subtype, likely more adapted, among strains isolated from humans, with some diversity in subtypes in foods. The variety and prevalence of resistant genes found in these *Salmonella* Typhimurium strains reinforces their potential hazard for humans and the risk in foods in Brazil.

INTRODUCTION

Foodborne diseases have a major impact on the economy and public health worldwide. Non-typhoidal *Salmonella* (NTS) is one of the most common causes of bacterial foodborne illnesses (MAJOWICZ et al., 2010; SCALLAN et al., 2011). It is estimated that NTS cause about 93.8 million annual cases of gastroenteritis and 155 thousand deaths per year worldwide (MAJOWICZ et al., 2010).

Among the *Salmonella enterica* serovars, *Salmonella* Typhimurium (*S.* Typhimurium) is among the most frequent ones isolated worldwide (HENDRIKSEN et al., 2011). From 2001 to 2007, this serovar was the most prevalent in the United States, Canada, Australia and New Zealand. In the same period, *S.* Typhimurium appeared as the second most prevalent serovar in Africa, Asia, Europe and Latin America, surpassed only by *S.* Enteritidis (HENDRIKSEN et al., 2011).

In Brazil, there are relatively little epidemiological data on *Salmonella* (TAUNAY et al., 1996; TAVECHIO et al., 1996; TAVECHIO et al., 2002; FERNANDES et al., 2006). However, it is known that in the State of São Paulo, *S.* Typhimurium was the most commonly isolated serovar from human sources and the third most common from non-human sources before the 1990's (TAUNAY et al., 1996). After this period, *S.* Typhimurium declined becoming the third most commonly isolated serovar from human and non-human sources in the period of 1991-1995in São Paulo State in Brazil, with *S.* Enteritidis being the most isolated serovar in both sources and, *S.* I 4, [5], 12:i:- and *S.* Havana the second most isolated serovar in human and non-human sources, respectively (TAVECHIO et al., 1996). Between 1996 and 2000, the isolation of *S.* Typhimurium declined even more from non-human sources (TAVECHIO et al., 2002). However, between 1996 and 2003, this serovar was ranked as the second most commonly isolated serovar from human sources (FERNANDES et al., 2006).

Epidemiological studies have been crucial to verify the relationship among pathogenic strains isolated from different sources, to elucidate contamination routes and to differentiate strains isolated from outbreaks and sporadic cases. Investigative capabilities have been greatly enhanced with the development and increasing feasibility of WGS as a molecular epidemiological tool (ALLARD et al., 2012; CAO et al., 2013; HOFFMANN et al., 2014). Over the last few years there has been a substantial reduction in the costs of WGS making this technology economically viable as a routine tool for molecular epidemiology. WGS has also been used for detection of antibiotic resistance determinant (MCDERMOTT et al., 2016; (WANG et al., 2017).

The use of antimicrobials is not recommended in cases of noninvasive *Salmonella* infection (HOHMANN, 2001; FÀBREGA and VILA, 2013). However, in some cases, the antibiotic therapy might be necessary. The drug of choice for the treatment of *Salmonella* infections is typically ciprofloxacin due to its broad spectrum antimicrobial activity (FÀBREGA and VILA, 2013).

The extensive use of antimicrobials has led to increasing numbers of non-typhoidal *Salmonella* strains that are resistant to quinolones and exhibited reduced susceptibility to fluoroquinolones (ANGULO et al., 2000; CHOI et al., 2005; CAMPIONI et al., 2017). This reduced susceptibility can lead to treatment failures in some cases (MØLBAK et al., 1999; HELMS et al., 2002). Quinolone resistances is usually mediated by mutations in the quinolone resistance determining regions (QRDRs) of the *gyrA*, *gyrB*, *parC*, and *parE* genes that code for bacterial DNA gyrase leading to changes in the binding site of the antimicrobial

to the enzyme (YOSHIDA et al., 1991; SÁENZ et al., 2003; CAMPIONI et al., 2017). Also, quinolone resistance may be due to the acquisition of plasmid-mediated quinolone resistance (PMQR) genes (HOPKINS; DAY; THRELFALL, 2008; CASAS et al., 2016; CUMMINGS et al., 2017), such as the *qnr* genes that encodes a group of pentapeptide proteins that bind to DNA gyrase and prevent the action of quinolones, *qepA* gene, an quinolone efflux pump, *aac(6')-Ib-cr* gene that encodes to the aminoglycoside acetiltranferase that can reduce susceptibility to ciprofloxacin and *oqxAB* genes, a multidrug resistance efflux pump (JABOBY; STRAHILEVITZ; HOOPER, 2014).

In previous studies of our group, we typed *S.* Typhimurium strains isolated from humans and food between 1983 and 2013 in Brazil by Pulsed-field gel electrophoresis (PFGE), multiple-locus variable number of tandem repeats analysis (MLVA), enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), Multilocus sequence typing (MLST) and CRISPR-multi-locus virulence sequence typing (CRISPR-MVLST). Moreover, the frequency of 12 virulence markers was assessed by PCR and the resistance profile against 12 antimicrobials was verified (ALMEIDA et al., 2015; ALMEIDA et al., 2017a; ALMEIDA et al., 2017b).

In this present work, WGS is used to uncover the diversity of *Salmonella* Typhimurium isolated from humans and food, between 1983 and 2013, from different geographic regions in Brazil. Additionally, WGS is used to verify the presence of antimicrobial resistance genes, as well as, the occurrence of mutations points in the *gyrA, gyrB, parC* and *parE* genes.

MATERIALS AND METHODS

Bacterial Strains

A total of 90 *S.* Typhimurium strains were sequenced including: 42 strains from human clinical material such as diarrheic feces (n=40), blood (n=1) and brain abscess (n=1) between 1983 and 2010; and 48 strains from food such as chicken (n=8), poultry (n=3), swine (n=11), meats (n=23), vegetables (n=2) and unknown (n=1). Samples were collected between 1999 and 2013 from seven States of Brazil including: São Paulo; Santa Catarina; Paraná; Mato Grosso do Sul; Rio Grande do Sul; Goiás; and Bahia (Table 1). Strains were provided by Adolf Lutz Institute of Ribeirao Preto and Oswaldo Cruz Foundation (FIOCRUZ).

DNA extraction and quantification

The genomic DNA extraction methods followed Campioni and Falcão (2014). The quality of the DNAs were checked using NanoDrop 1000 (Thermo Scientific, Rockford, IL), and the concentrations were determined using Qubit double-stranded DNA BR assay kit and Qubit fluorometer (Life Technologies, Grand Island, NY) according to each manufacturer's instructions.

Genome Sequencing, Assembly, and Annotation

All isolates were prepared using the Nextera Sample Preparation Kit (Illumina, San Diego, CA) and then sequenced on Illumina NextSeq (Illumina) for 2 x 151 cycles. *De novo* assemblies were generated from all raw sequence data. The Illumina reads were assembled with SPAdes 3.0 with the following parameters: only contigs of length ≥500 bp were included; mismatch (MM) 3.28; the genome fraction was 96.157; and number of mis-assemblies (MA) was 2 (NURK et al., 2013). The contigs for each isolate (draft genome) were annotated using NCBI's Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (KLIMKE et al., 2009). The draft genome sequences of *S.* Typhimurium strains are publicly available in GenBank, with accession numbers listed in Table S1. The presence of resistance genes, as well as points mutation in the QRDR of the *gyrA, gyrB, parC*, and *parE* genes, were determined using ResFinder (Center for Genomic Epidemiology, https://cge.cbs.dtu.dk/services/ResFinder/) with settings of threshold of 90%, and minimum length of 60% (ZANKARI et al., 2012).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the 90 *S.* Typhimurium strains were tested by the disc diffusion method of the Clinical and Laboratory Standards Institute (CLSI, 2015). The majority of these results were previously published in Almeida et al., (2015) for 12 antimicrobials including: cefotaxime; cefoxitin; ceftazidime; aztreonam; cefepime; amoxicillin-clavulanic acid; ampicillin; nalidixic acid; levofloxacin; trimethoprim-sulfamethoxazole; chloramphenicol; and ciprofloxacin (Oxoid). However, five additional antimicrobials were tested in this study including: gentamicin; streptomycin; tetracycline; sulfonamides; and ceftriaxone. Additionally, the minimum inhibitory concentrations (MIC) of fluoroquinolones in the nalidixic acid resistant and susceptible strains were evaluated using

Etest® following the Clinical and Laboratory Standards Institute (CLSI) guidelines. Strains with MIC ≤ 0.06 µg/mL were considered sensitive and ≥ 1 µg/mL resistant.

Phylogenetic Analysis

In addition to the 90 *S*. Typhimurium strains sequenced in this study, four additional *S.* Typhimurium strains (the sequencing reads were downloaded from NCBI with run accessions of SRR1060710, SRR1963606, SRR6325339, and ERR1556230 for strain DT104, LT2, 14028s, and SL1344, respectively) were added into the phylogenetic analysis for diversity purpose. The genomic analysis was performed using the CFSAN SNP Pipeline that generated the SNP matrix, which was then used to infer the maximum likelihood tree using GARLI (DAVIS et al., 2015) with 200 maximum likelihood replicates and 1000 bootstrap iterations. Three samples were included as outgroups including: *Salmonella enterica* serovar Saintpaul CFSAN000611; *Salmonella enterica* serovar Saintpaul CFSAN000614; and *Salmonella enterica* serovar Heidelberg CFSAN000443 (TIMME et al., 2013). The SNP matrix included 59,130 and 11,176 SNPs, with or without the three outgroups sample, respectively.

RESULTS

***In silico* antimicrobial resistance gene analysis**

A total of 39 antimicrobial resistance genes were identified (Table 1) and are described in detail below according to the different antimicrobial classes.

Aminoglycoside resistance genes. Thirteen distinct aminoglycoside resistance genes were detected including: the most common gene *aadA1* in 23 (25.6%) isolates (19 humans, 4 foods)*; aph(6)-Id* in 20 (22.2%) isolates (7 humans, 13 foods); *aph(3')-Ia* in 11 (12.2%) isolates (10 humans, 1 foods); *ant(2")-Ia* in 7 (7.8%) isolates from humans; *aacA4* in 5 (5.6%) isolates from humans; and *aph(3")-Ib* in 5 (5.6%) isolates (1 humans, 4 foods); *aph(4)-Ia* in 3 (3.3%) isolates from foods; *aac(3)-IVa* in 3 (3.3%) isolates from foods; and lastly both *aac(3)-IId* and *aadA15* in 1 (1.1%) food isolate each.

Tetracycline resistance genes. Five distinct tetracycline resistance genes were detected including: the most common *tet(B)* gene in 19 (21.1%) isolates (3 humans, 16 foods); *tet(A)* in 8 (8.9%) food isolate; *tet(C)*in 7 (7.8%) human isolates; *tet(M)* in 3 (3.3%) food isolates; and *tet(D)* in 1 (1.1%) food isolate.

Sulfonamide and trimethoprim resistance. Only two sulfonamide resistance genes were detected including: *sul1* in 19 (21.1%) strains (12 humans 7 foods); and *sul2* in 9 (10%)

strains (2 humans 7 foods). The 4 trimethoprim resistance genes detected included: the most common *dfrA1* in 24 (26.7%) isolates (22 human, 2 foods); *dfrA12* in 4 (4.4%) isolates; *dfrA8* in 2 (2.2%) foods; and *dfrA25* in 1 (1.1%) food isolate.

Beta-lactam resistance genes. Seven distinct beta-lactam resistance genes were detected including: $bla_{\text{TEM-1B}}$ in 16 (17.8%) strains (6 human, 10 foods); $bla_{\text{OXA-4}}$ in 7 (7.8%) human isolates; $bla_{\text{OXA-17}}$ in 5 (5.6%) human isolates; $bla_{\text{TEM-1A}}$in 2 (2.2%) food isolates; and $bla_{\text{CTX-M-8}}$ in 2 (2.2%) food isolates; $bla_{\text{TEM-187}}$ in 1 (1.1%) human isolate; and $bla_{\text{CTX-M-2}}$ in 1 (1.1%) food isolate.

Fluoroquinolone resistance genes. Five fluoroquinolone resistance genes were detected including: *aac(6')Ib-cr* in 5 (5.6%) human isolates; *oqxA* in 4 (4.4%) food isolates; *oqxB* in 4 (4.4%) food isolates; and *qnrB2* and *qnrB88* each in one (1.1%) food isolate.

Phenicol resistance genes. Two phenicol genes were detected including: *catA1* in 14 (15.6%) human isolates; and *floR* in 5 (5.6%) food isolates.

Macrolide resistance genes. Only one macrolide resistant gene (*mphA)* was detected in one food isolate.

Antimicrobial susceptibility testing

A total of 65 (72.2%) out of 90 *S.* Typhimurium strains studied were resistant to sulfonamides, 44 (48.9%) strains were streptomycin resistant, 27 (30%) strains were resistant to tetracycline, 21 (23.3%) strains were gentamicin resistant, and 7 (7.8%) strains were resistant to ceftriaxone. In our previously published paper (ALMEIDA et al., 2015), 34 strains were resistant to nalidixic acid (Nal^R). In this study we evaluated the reduced susceptibility to fluoroquinolones of 34 strains Nal^R and 12 strains susceptible to nalidixic acid (Nal^S). All the 12Nal^S strains and 21Nal^R strains studied were sensitive to ciprofloxacin (MIC ≤ 0.06 µg/ml), whereas 11Nal^R strains presented intermediate resistance to this drug (MIC 0.12 - 0.5 µg/ml) and two Nal^R strains were resistant to ciprofloxacin. All the antimicrobial susceptibility test results were presented in Table 1.

Table 1 - Phenotypic and genotypic resistance profiles of the 90 *Salmonella* Typhimurium strains studied isolated from humans and food in various States between 1983 and 2013 in Brazil

CFSAN nº	Isolate name	Source	State	Year of isolation	Phenotypic Resistance Profile	Genotypic Resistance Profile (Identity %)						
						Aminoglycoside	Tetracycline	Sulphonamide	Trimethoprim	Beta-lactam	Fluoroquinolone	Phenicol
CFSAN033848	STm01	Human feces	SP	1983	AMP-NA-SXT-STR	*aadA1* (100), *aph(3')-Ia* (99.57)	*tet(C)* (99.92)	—	*dfrA1* (100)	—	—	—
CFSAN033849	STm02	Human feces	SP	1983	AMC-AMP-NA-SXT-C-GEN-STR-SUL	*ant(2")-Ia* (99.06), *aadA1* (100)	—	*sul1* (100)	*dfrA1*(100)	$bla_{\text{OXA-4 (100)}}$	—	*catA1* (99.85)
CFSAN033850	STm03	Human feces	SP	1983	AMP-NA-SXT-C-GEN-STR-SUL	*aadA1* (100), *aph(3')-Ia* (99.39), *ant(2")-Ia* (99.06)	*tet(C)* (99.92)	*sul1* (100)	*dfrA1*(100)	$bla_{\text{OXA-4 (100)}}$	—	*catA1* (99.85)
CFSAN033851	STm04	Human feces	SP	1983	AMP-NA-SXT-C-GEN-STR-SUL	*aadA1* (100), *aph(3')-Ia* (99.39),*ant(2")-Ia* (99.06)	—	*sul1* (100)	*dfrA1*(100)	$bla_{\text{OXA-4 (100)}}$	—	*catA1* (99.85)
CFSAN033852	STm05	Human feces	SP	1983	AMP-NA-SXT-C-GEN-STR-SUL	*aadA1* (100), *aph(3')-Ia* (99.39),*ant(2")-Ia* (99.06)	*tet(C)* (99.92)	*sul1* (100)	*dfrA1* (100)	$bla_{\text{OXA-4 (100)}}$, $bla_{\text{TEM-187 (98.82)}}$	—	*catA1* (99.85)
CFSAN033853	STm06	Human feces	SP	1983	—	—	—	—	—	—	—	—
CFSAN033854	STm07	Human feces	SP	1983	AMP-NA-SXT-C-STR	*aph(3')-Ia* (99.25), *aadA1* (100)	—	—	*dfrA1*(100)	$bla_{\text{TEM-1B (100)}}$	—	*catA1* (99.85)
CFSAN033856	STm09	Human feces	SP	1984	AMP-NA-SXT-C-GEN-SUL	*aadA1* (100), *ant(2")-Ia* (99.06)	—	*sul1* (100)	*dfrA1*(100)	$bla_{\text{OXA-4 (100)}}$, $bla_{\text{TEM-1B (100)}}$	—	*catA1* (99.85)
CFSAN033857	STm10	Human feces	SP	1984	NA-SXT-SUL	—	—	—	*dfrA1* (100)	—	—	—
CFSAN033858	STm11	Human feces	SP	1984	AMP-NA-SXT-C-GEN-STR-SUL	*aadA1* (100), *ant(2")-Ia* (99.06)	—	*sul1* (100)	*dfrA1* (100)	$bla_{\text{OXA-4 (100)}}$	—	*catA1* (99.85)
CFSAN033859	STm12	Human feces	SP	1984	NA-GEN-STR-SUL	*aacA4* (99.64), *aadA1* (100), *aph(3')-Ia* (99.39)	—	*sul1* (100)	*dfrA1* (100)	$bla_{\text{OXA-17 (100)}}$	*aac(6')Ib-cr* (99.28)	*catA1* (99.85)
CFSAN033860	STm13	Human feces	SP	1984	AMP-NA-SXT-C-GEN-SUL	*aadA1 (100)*, *ant(2")-Ia* (99.06)	—	*sul1* (100)	*dfrA1* (100)	$bla_{\text{OXA-4 (100)}}$	—	*catA1* (99.85)
CFSAN033861	STm14	Human feces	SP	1984	AMP-NA-SXT	*aph(3')-Ia* (99.39)	*tet(C)* (99.92)	—	*dfrA1* (100)	$bla_{\text{TEM-1B (100)}}$	—	—

Continue

Continuation

CFSAN nº	Isolate name	Source	State	Year of isolation	Phenotypic Resistance Profile	Genotypic Resistance Profile (Identity %)						
						Aminoglycoside	Tetracycline	Sulphonamide	Trimethoprim	Beta-lactam	Fluoroquinolone	Phenicol
CFSAN033862	STm15	Human feces	SP	1985	SUL	—	—	—	—	—	—	—
CFSAN033863	STm16	Human feces	SP	1985	NA-SXT	*aadA1* (100)	—	—	*dfrA1* (100)	—	—	—
CFSAN033864	STm17	Human feces	SP	1985	—	—	—	—	—	—	—	—
CFSAN033865	STm18	Human feces	SP	1985	—	—	—	—	—	—	—	—
CFSAN033866	STm19	Human feces	SP	1986	AMP-NA-SXT-C-GEN-STR-SUL-CRO	*aacA4* (99.64), *aadA1* (100)	—	*sul1* (100)	*dfrA1* (100)	$bla_{\text{OXA-17 (100)}}$	*aac(6')Ib-cr* (99.28)	*catA1* (99.85)
CFSAN033867	STm20	Human feces	SP	1986	NA-SXT-C-TET-STR	*aadA1* (100), *aph(3')-Ia* (99.39)	*tet(C)* (99.92)	—	*dfrA1* (100)	—	—	*catA1* (99.85)
CFSAN033868	STm21	Human feces	SP	1986	NA-SXT-STR	*aadA1* (100), *aph(3')-Ia* (99.37)	—	—	*dfrA1* (100)	—	—	—
CFSAN033869	STm22	Human feces	SP	1986	AMC-AMP-NA-SXT-C-GEN-STR-SUL	*aadA1* (100), *aacA4* (99.64)	—	*sul1* (100)	*dfrA1* (100)	$bla_{\text{OXA-17 (100)}}$	*aac(6')Ib-cr* (99.28)	*catA1* (99.85)
CFSAN033870	STm23	Human feces	SP	1986	TET-STR	*strA* (100), *aph(6)-Id* (100)	*tet(B)* (100)	—	—	—	—	—
CFSAN033871	STm24	Human feces	SP	1986	AMP-NA-SXT-C-GEN-STR-SUL	*aadA1* (100), *aacA4* (99.64)	*tet(C)* (99.92)	*sul1* (100)	*dfrA1* (100)	$bla_{\text{OXA-17 (100)}}$	*aac(6')Ib-cr* (99.28)	*catA1* (99.85)
CFSAN033872	STm25	Human feces	SP	1986	AMP-NA	*aadA1* (100)	—	—	*dfrA1* (100)	$bla_{\text{TEM-1B (100)}}$	—	—
CFSAN033873	STm26	Human feces	SP	1986	NA-STR	*aadA1* (100), *aph(3')-Ia* (99.47)	—	—	*dfrA1* (100)	—	—	—
CFSAN033874	STm27	Human feces	SP	1986	AMP-NA-SXT-C-GEN-STR-SUL	*aadA1* (100), *aacA4* (99.64)	*tet(C)* (99.92)	*sul1* (100)	*dfrA1* (100)	$bla_{\text{OXA-17 (100)}}$	*aac(6')Ib-cr* (99.28)	*catA1* (99.85)
CFSAN033875	STm28	Human feces	SP	1988	SUL	—	—	—	—	—	—	—
CFSAN033876	STm29	Human feces	SP	1989	AMP-STR-SUL	*aph(6)-Id* (100), *aph(3")-Ib* (100)	—	*sul2* (100)	—	$bla_{\text{TEM-1B (100)}}$	—	—
CFSAN033877	STm30	Human feces	SP	1990	SUL	—	—	—	—	—	—	—

Continue

Continuation

CFSAN nº	Isolate name	Source	State	Year of isolation	Phenotypic Resistance Profile	Genotypic Resistance Profile (Identity %)						
						Aminoglycoside	Tetracycline	Sulphonamide	Trimethoprim	Beta-lactam	Fluoroquinolone	Phenicol
CFSAN033878	STm31	Human feces	SP	1991	SUL	—	—	—	—	—	—	—
CFSAN033879	STm32	Human feces	SP	1992	SUL	—	—	—	—	—	—	—
CFSAN033880	STm33	Human feces	SP	1992	—	—	—	—	—	—	—	—
CFSAN033881	STm34	Human feces	SP	1993	—	—	—	—	—	—	—	—
CFSAN033882	STm35	Human feces	SP	1995	SUL	—	—	—	—	—	—	—
CFSAN033883	STm36	Cold chicken	SP	1995	STR	—	—	—	—	—	—	—
CFSAN033884	STm37	Raw pork sausage	SP	1996	SUL	—	—	—	—	—	—	—
CFSAN033885	STm38	Human feces	SP	1997	SUL	—	—	—	—	—	—	—
CFSAN033886	STm39	Human feces	SP	1998	STR	—	—	—	—	—	—	—
CFSAN033887	STm40	Lettuce	SP	1998	STR-SUL	—	—	—	—	—	—	—
CFSAN033888	STm41	Raw kafta	SP	1998	TET-STR-SUL	*strA* (100), *aph(6)-Id* (100)	*tet(B)* (100)	—	—	—	—	—
CFSAN033889	STm42	Human feces	SP	1999	TET-STR	*strA* (100), *aph(6)-Id* (100)	*tet(B)* (100)	—	—	—	—	—
CFSAN033890	STm43	Human feces	SP	2000	TET-STR	*strA* (100), *aph(6)-Id* (100)	*tet(B)* (100)	—	—	—	—	—
CFSAN033891	STm44	Blood	SP	2000	SUL	—	—	—	—	—	—	—
CFSAN033892	STm45	Raw pork sausage	SP	2000	TET-STR-SUL	*strA* (100), *aph(6)-Id* (100)	*tet(B)* (100)	—	—	—	—	—
CFSAN033893	STm46	Raw tuscan sausage	SP	2002	STR	*strA* (100), *aph(6)-Id* (100)	—	—	—	—	—	—
CFSAN033894	STm47	Human feces	SP	2003	SUL	—	—	—	—	—	—	—
CFSAN033895	STm48	Brain abscess	SP	2005	AMP-SXT-STR-SUL	—	—	*sul2* (100)	*dfrA1* (100)	$bla_{\text{TEM-1B (100)}}$	—	—

Continue

Continuation

CFSAN nº	Isolate name	Source	State	Year of isolation	Phenotypic Resistance Profile	Genotypic Resistance Profile (Identity %)						
						Aminoglycoside	Tetracycline	Sulphonamide	Trimethoprim	Beta-lactam	Fluoroquinolone	Phenicol
CFSAN033896	STm49	Human feces	SP	2010	NA	—	—	—	—	—	—	—
CFSAN033897	702/99	Final product	SC	1999	—	—	—	—	—	—	—	—
CFSAN033898	12288/06	Swine	SC	2006	AMP-TET-STR-SUL	*strA* (100), *aph(6)-Id* (100)	*tet(B)* (100)	—	—	$bla_{\text{TEM-1B (100)}}$	—	—
CFSAN033899	12278/06	Swine	SC	2006	NA-TET-STR-SUL	*strA* (100), *aph(6)-Id* (100)	*tet(B)* (100)	—	—	—	—	—
CFSAN033900	12290/06	Swine	SC	2006	TET-STR-SUL	*aph(3")-Ib* (100), *aph(6)-Id* (100)	*tet(B)* (100)	*sul2* (100)	—	—	*oqxA (99.40)*, *oqxB* (98.86)	—
CFSAN033901	12268/06	Swine	SC	2006	AMP-NA-STR-SUL	*strA* (100), *aph(6)-Id* (100)	*tet(B)* (100)	—	—	$bla_{\text{TEM-1B (100)}}$	*oqxA (99.40)*, *oqxB* (98.83)	—
CFSAN033902	12381/06	Swine	SC	2006	TET-STR-SUL	*aph(6)-Id* (100), *aph(3")-Ib* (100)	*tet(B)* (100)	*sul2* (100)	—	—	—	—
CFSAN033903	5936/06	Cold chicken	SC	2006	STR-SUL	—	—	—	—	—	—	—
CFSAN033904	5937/06	Cold chicken	SC	2006	SUL	—	—	—	—	—	—	—
CFSAN033905	5934/06	Swine	SC	2006	NA-TET-GEN-STR-SUL	*strA* (100), *aph(4)-Ia* (100),*aph(6)-Id* (100), *aac(3)-IVa* (99.87)	*tet(B)* (100)	—	—	—	*oqxA (99.40)*, *oqxB* (98.83)	—
CFSAN033906	5961/06	Swine	SC	2006	TET-GEN-STR-SUL	*aadA1* (99.87)	*tet(B)* (100)	*sul1* (99.89)	—	—	—	—
CFSAN033907	5962/06	Swine	SC	2006	TET-STR-SUL	*aadA1* (99.87)	*tet(B)* (100)	*sul1* (99.89)	—	—	—	—
CFSAN033908	5929/06	Poultry	SC	2006	TET-SUL	—	—	—	—	—	—	—
CFSAN033909	13609/06	Poultry	SC	2006	—	—	—	—	—	—	—	—
CFSAN033910	3848/08	Food	SC	2008	SUL	—	—	—	—	—	—	—
CFSAN033911	16238/09	Ready-to-eat dish	MS	2009	AMP-NA-SXT-C-TET-GEN-STR-SUL	*aac(3)-IIa* (100), *strA* (100), *aph(6)-Id* (100), *aadA1* (99.75)	*tet(A)* (100)	*sul1* (100)	*dfrA1* (100)	$bla_{\text{TEM-1B (100)}}$	—	*floR* (98.19)
CFSAN033912	16239/09	Ready-to-eat dish	MS	2009	AMP-NA-TET-SUL-CRO	—	*tet(A)* (99.92), *tet(M)* (96.15)	—	—	$bla_{\text{TEM-1B (100)}}$	—	—

Continue

Continuation

CFSAN nº	Isolate name	Source	State	Year of isolation	Phenotypic Resistance Profile	Genotypic Resistance Profile (Identity %)						
						Aminoglycoside	Tetracycline	Sulphonamide	Trimethoprim	Beta-lactam	Fluoroquinolone	Phenicol
CFSAN033913	16240/09	Ready-to-eat dish	MS	2009	AMP-NA-C-TET-STR-SUL-CRO	—	*tet(A)* (99.92), *tet(M)* (96.15)	—	—	$bla_{\text{TEM-1B (100)}}$	—	*floR* (98.11)
CFSAN033914	16202/09	Industrialized product	RS	2009	TET-SUL	—	—	—	—	—	—	—
CFSAN033915	16251/09	Industrialized product	GO	2009	AMP-SXT-C-TET-GEN-SUL	*strA* (100), *aph(4)-Ia* (100), *aac(3)-IVa* (99.87), *aph(6)-Id* (100)	*tet(A)* (100), *tet(B)* (100)	*sul1* (100), *sul2* (100)	*dfrA25* (100), *dfrA8* (100)	$bla_{\text{TEM-1A (100)}}$	*qnrB2* (100)	*floR* (98.19)
CFSAN033916	16273/09	Industrialized product	GO	2009	AMP-NA-TET-GEN-SUL	*aac(3)-IId* (99.88), *aadA2* (100), *aph(3")-Ib* (100), *aph(6)-Id* (100)	*tet(B)* (100)	*sul2* (100)	—	$bla_{\text{TEM-1B (100)}}$	—	—
CFSAN033917	17307/09	Industrialized product	-	2009	AMP-NA-SXT-TET-GEN-STR-SUL-CRO	*strA* (100), *aac(3)-IIa* (100), *aadA1* (100), *aph(6)-Id* (100)	*tet(A)* (100)	*sul1* (100)	*dfrA1* (100)	$bla_{\text{TEM-1B (100)}}$	*qnrB88* (100)	—
CFSAN033918	9461/10	In natura meat	SC	2010	SUL	—	—	—	—	—	—	—
CFSAN033919	9479/10	In natura meat	SC	2010	SUL	—	—	—	—	—	—	—
CFSAN033920	7032/10	Poultry	PR	2010	CTX-ATM-FEP-AMP-SXT-TET-STR-SUL-CRO	*strA* (100), *aadA2* (100), *aph(6)-Id* (100)	*tet(B)* (100), *tet(D)* (100)	*sul1* (100), *sul2* (100)	*dfrA12* (100)	$bla_{\text{CTX-M-2 (100)}}$	—	—
CFSAN033921	3057/10	Frozen chicken carcass	PR	2010	STR-SUL	—	—	—	—	—	—	—
CFSAN033922	6346/10	Chicken	SP	2010	SUL	—	—	—	—	—	—	—
CFSAN033923	5635/10	Unknown	RS	2010	NA	—	—	—	—	—	—	—
CFSAN033924	9109/10	Swine	PR	2010	SUL	—	—	—	—	—	—	—

Continue

Continuation

CFSAN nº	Isolate name	Source	State	Year of isolation	Phenotypic Resistance Profile	Genotypic Resistance Profile (Identity %)						
						Aminoglycoside	Tetracycline	Sulphonamide	Trimethoprim	Beta-lactam	Fluoroquinolone	Phenicol
CFSAN033925	426/10	Chicken	SC	2010	CTX-FEP-AMP-SUL-CRO	—	—	—	—	*bla*$_{\text{CTX-M-8 (100)}}$	—	—
CFSAN033926	447/10	Chicken	SC	2010	CTX-FEP-AMP-SUL-CRO	—	—	—	—	*bla*$_{\text{CTX-M-8 (100)}}$	—	—
CFSAN033927	2452/11	Frozen chicken carcass	SP	2011	TET-SUL	*aadA2* (100)	*tet(B)* (100)	—	*dfrA12* (100)	—	—	—
CFSAN033928	6709/11	Cold chicken	RS	2011	AMP-NA-SXT-C-TET-GEN-STR-SUL	*aph(6)-Id* (100), *aph(3")-Ib* (100)	*tet(B)* (100)	*sul2* (100)	*dfrA8* (100)	*bla*$_{\text{TEM-1A (100)}}$	*oqxA (99.40)*, *oqxB* (98.83)	*floR* (98.19)
CFSAN033929	948/12	Raw salad	BA	2012	SUL	—	—	—	—	—	—	—
CFSAN033930	1103/12	Swine (homemade salami)	RS	2012	SUL	—	—	—	—	—	—	—
CFSAN033931	1104/12	Swine (homemade salami)	RS	2012	—	—	—	—	—	—	—	—
CFSAN033932	3330/12	Roast beef	SC	2012	SUL	—	—	—	—	—	—	—
CFSAN033933	994/13	Final product sales (animal origin)	SP	2013	SUL	—	—	—	—	—	—	—
CFSAN033934	374/13	Final product sales (animal origin)	SC	2013	SUL	—	—	—	—	—	—	—

Continue

Conclusion

CFSAN nº	Isolate name	Source	State	Year of isolation	Phenotypic Resistance Profile	Genotypic Resistance Profile (Identity %)						
						Aminoglycoside	Tetracycline	Sulphonamide	Trimethoprim	Beta-lactam	Fluoroquinolone	Phenicol
CFSAN033935*	465/13	Final product sales (animal origin)	SP	2013	AMP-SXT-TET-GEN-STR-SUL	*aph(4)-Ia* (100), *aph(3')-Ia* (99.75), *aadA1* (99.87), *aph(3")-Ib* (100), *aac(3)-IVa* (99.87), *aadA15* (96.46), *aph(6)-Id* (100)	*tet(A)* (100), *tet(B)* (100)	*sul1* (100), *sul2* (100)	*dfrA12* (100)	$bla_{\text{TEM-1B (100)}}$	—	—
CFSAN033937	622/13	Final product sales (animal origin)	SC	2013	NA	—	—	—	—	—	—	—
CFSAN033938	583/13	Final product sales (animal origin)	SC	2013	AMP-TET-SUL	*aadA2* (100)	*tet(A)* (99.92), *tet(M)* (96.15)	—	*dfrA12* (100)	$bla_{\text{TEM-1B (99.88)}}$	—	—
CFSAN033939	623/13	Final product sales (animal origin)	SP	2013	AMP-NA-C-TET-STR	*aadA1* (100), *aph(3')-Ia* (99.57)	*tet(A)* (100)	—	—	$bla_{\text{TEM-1B (100)}}$	—	*floR* (98.19)

* This genome was the only one that presented the *mph(A)* (identity 100%) gene that confers resistance to macrolide.

Detection of mutations in the *gyrA, gyrB, parC* and *parE* genes and of the presence of *qnr*, *qepA, oqxAB* and *aac(6')-Ib-cr* genes

A total of 33 (36.7%) out of 90 strains studied presented mutation points in the *gyrA* gene, with all being resistant to nalidixic acid (Table 2). The non synonymous points of mutation in the *gyrA* gene included: aspartate/glycine, Asp(87)→Glyin 21 strains; aspartate/asparagine, Asp(87)→Asnin 7 strains; serine/tyrosine, Ser(83)→Tyr in 4 strains; and serine/phenylalanine, Ser(83)→Phe in one strain. None of the strains had more than one mutation point (Table 2). One strain (5934/06 isolated from swine) Nal^R did not show mutation in the *gyrA* gene. Seven (7.8%) strains presented synonymous nucleotide mutation, and these strains were Nal^S (data not shown) suggesting undiscovered mutations. Thirty-two (35.6%) strains presented synonymous nucleotide mutation in the *parC* gene and 10 of those strains was Nal^R with two strains resistant to ciprofloxacin (data not shown). No strains presented mutations in the *parE* gene.

The *qnrB88* gene was found in 1 (1.1%) Brazilian strain that previously had been reported both in *Klebsiella pneumoniae* (GenBank: KX118608) and under another gene (*qnrE1)* found in *Klebsiella pneumonia* (GenBank: KY781949). Additionally, one strain had the *qnrB2* gene present in *Salmonella* Bredeney (GenBank: FJ844401). The *oqxAB* gene was found in 4 (4.4%) strains. However, these genes diverged in having 6 mutations compared to the *oqxAB* of *Salmonella* Derby (GenBank: FN811184). The *aac(6')Ib-cr* gene was identified in 5 strains isolated from humans.

Table 2 - Quinolone resistance profiles of the 90 *Salmonella* Typhimurium strains studied isolated from humans and food in various States between 1983 and 2013 in Brazil

CFSAN nº	Isolate Name	CIP E-test	QRDRs mutations			
			gyrA mutation	*gyrB* mutation	*parC* mutation	*parE* mutation
CFSAN033848	STm01	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033849	STm02	Intermediate	Asp(87)→Gly	—	—	—
CFSAN033850	STm03	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033851	STm04	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033852	STm05	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033853	STm06	—	—	—	—	—
CFSAN033854	STm07	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033856	STm09	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033857	STm10	Intermediate	Asp(87)→Gly	—	—	—
CFSAN033858	STm11	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033859	STm12	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033860	STm13	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033861	STm14	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033862	STm15	—	—	—	—	—
CFSAN033863	STm16	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033864	STm17	—	—	—	—	—
CFSAN033865	STm18	—	—	—	—	—
CFSAN033866	STm19	—	Asp(87)→Gly	—	—	—
CFSAN033867	STm20	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033868	STm21	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033869	STm22	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033870	STm23	—	—	—	—	—

Continue

Continuation

CFSAN nº	Isolate Name	CIP E-test	QRDRs mutations			
			gyrA mutation	*gyrB* mutation	*parC* mutation	*parE* mutation
CFSAN033871	STm24	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033872	STm25	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033873	STm26	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033874	STm27	Susceptible	Asp(87)→Gly	—	—	—
CFSAN033875	STm28	Susceptible	—	—	—	—
CFSAN033876	STm29	Susceptible	—	—	—	—
CFSAN033877	STm30	—	—	—	—	—
CFSAN033878	STm31	Susceptible	—	—	—	—
CFSAN033879	STm32	—	—	—	—	—
CFSAN033880	STm33	—	—	—	—	—
CFSAN033881	STm34	Susceptible	—	—	—	—
CFSAN033882	STm35	Susceptible	—	—	—	—
CFSAN033883	STm36	Susceptible	—	—	—	—
CFSAN033884	STm37	Susceptible	—	—	—	—
CFSAN033885	STm38	—	—	—	—	—
CFSAN033886	STm39	—	—	—	—	—
CFSAN033887	STm40	Susceptible	—	—	—	—
CFSAN033888	STm41	—	—	—	—	—
CFSAN033889	STm42	—	—	—	—	—
CFSAN033890	STm43	—	—	—	—	—
CFSAN033891	STm44	Susceptible	—	—	—	—
CFSAN033892	STm45	Susceptible	—	—	—	—
CFSAN033893	STm46	Susceptible	—	—	—	—

Continue

Continuation

CFSAN nº	Isolate Name	CIP E-test	QRDRs mutations			
			gyrA mutation	*gyrB* mutation	*parC* mutation	*parE* mutation
CFSAN033894	STm47	Susceptible	—	—	—	—
CFSAN033895	STm48	—	—	—	—	—
CFSAN033896	STm49	Intermediate	Asp(87)→Asn	—	—	—
CFSAN033897	702/99	—	—	—	—	—
CFSAN033898	12288/06	—	—	—	—	—
CFSAN033899	12278/06	Susceptible	Asp(87)→Asn	—	—	—
CFSAN033900	12290/06	—	—	—	—	—
CFSAN033901	12268/06	Intermediate	Asp(87)→Asn	—	—	—
CFSAN033902	12381/06	—	—	—	—	—
CFSAN033903	5936/06	—	—	—	—	—
CFSAN033904	5937/06	—	—	—	—	—
CFSAN033905	5934/06	Susceptible	—	—	—	—
CFSAN033906	5961/06	—	—	—	—	—
CFSAN033907	5962/06	—	—	—	—	—
CFSAN033908	5929/06	—	—	—	—	—
CFSAN033909	13609/06	—	—	—	—	—
CFSAN033910	3848/08	—	—	—	—	—
CFSAN033911	16238/09	Resistant	Ser(83)→Tyr	—	—	—
CFSAN033912	16239/09	Intermediate	Asp(87)→Asn	—	—	—
CFSAN033913	16240/09	Intermediate	Asp(87)→Asn	—	—	—
CFSAN033914	16202/09	—	—	—	—	—
CFSAN033915	16251/09	—	—	—	—	—
CFSAN033916	16273/09	Intermediate	Ser(83)→Phe	—	—	—

Continue

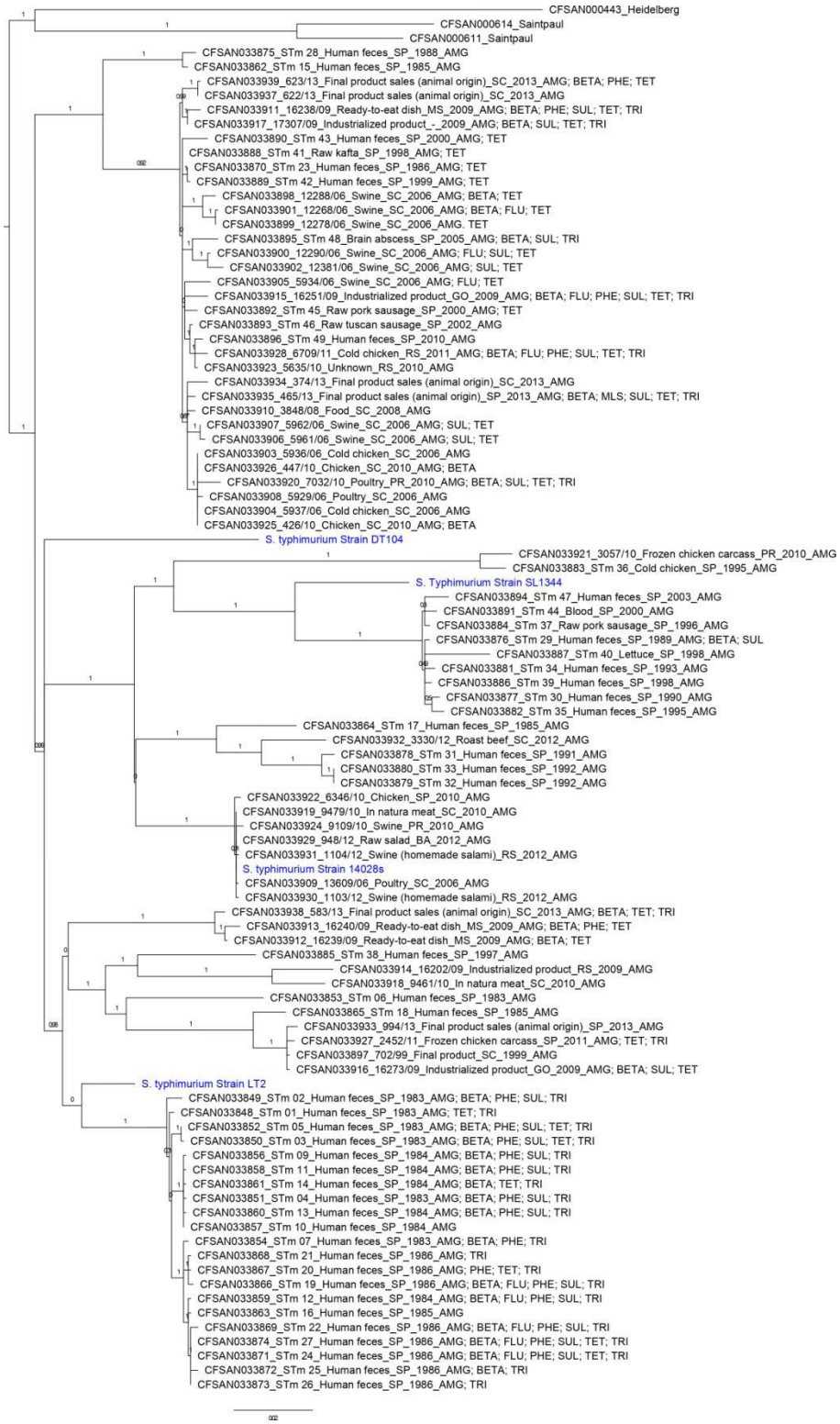
Conclusion

CFSAN nº	Isolate Name	CIP E-test	QRDRs mutations			
			gyrA mutation	*gyrB* mutation	*parC* mutation	*parE* mutation
CFSAN033917	17307/09	Resistant	Ser(83)→Tyr	—	—	—
CFSAN033918	9461/10	—	—	—	—	—
CFSAN033919	9479/10	—	—	—	—	—
CFSAN033920	7032/10	—	—	—	—	—
CFSAN033921	3057/10	—	—	—	—	—
CFSAN033922	6346/10	—	—	—	—	—
CFSAN033923	5635/10	Intermediate	Asp(87)→Asn	—	—	—
CFSAN033924	9109/10	—	—	—	—	—
CFSAN033925	426/10	—	—	—	—	—
CFSAN033926	447/10	—	—	—	—	—
CFSAN033927	2452/11	—	—	—	—	—
CFSAN033928	6709/11	Intermediate	Asp(87)→Asn	—	—	—
CFSAN033929	948/12	—	—	—	—	—
CFSAN033930	1103/12	—	—	—	—	—
CFSAN033931	1104/12	—	—	—	—	—
CFSAN033932	3330/12	—	—	—	—	—
CFSAN033933	994/13	—	—	—	—	—
CFSAN033934	374/13	—	—	—	—	—
CFSAN033935	465/13	—	—	—	—	—
CFSAN033937	622/13	Intermediate	Ser(83)→Tyr	—	—	—
CFSAN033938	583/13	—	—	—	—	—
CFSAN033939	623/13	Intermediate	Ser(83)→Tyr	—	—	—

Phylogenetic analysis

The 90 *S.* Typhimurium strains studied were distributed into 2 major clades (designated A and B, Figure 1). Clade A comprised 34 (37.8%) strains with 7 isolated from humans between 1985 and 2010, and 27 isolated from food between 1998 and 2013. Thirty-four strains located in Clade A were isolated from South, Southeast and Midwestern Regions in Brazil. Of the 34 strains in Clade A, 15 strains (14 foods, 1 human) were resistant to three or more antimicrobial classes being multidrug-resistant (MDR). Clade B comprised 56 (62.2%) strains with 35 isolated from humans between 1983 and 2003, and 21 strains isolated from food between 1995 and 2013. Fifty-six strains located in Clade B were from South, Southeast, Northeast and Midwestern Regions in Brazil. Of the 56 strains in Clade B, 23 strains (18 humans, 5 foods) were MDR. All reference genomes added were grouped in clade B (DT104, SL1344, 14028s and LT2).

Fig. 1 - Phylogenetic analysis based on SNPs of the 90 *Salmonella* Typhimurium strains of this study and four additional *S.* Typhimurium strains (the sequencing reads were downloaded from NCBI with run accessions of SRR1060710, SRR1963606, SRR6325339, and ERR1556230 for strain DT104, LT2, 14028s, and SL1344, respectively). The genomes of one *Salmonella* Heidelberg and two *Salmonella* Saintpaul were used as outgroup.



DISCUSSION

In this study 90 *S.* Typhimurium strains isolated from food and humans in Brazil were sequenced by next generation sequencing technology to evaluate their antimicrobial resistance gene profiles and phylogenetic diversity. This is the first study of *S.* Typhimurium strains isolated in Brazil that used WGS to access the genetic diversity and the molecular bases of antimicrobial resistance. In previous studies, the same strains were typed by PFGE, MLVA, ERIC-PCR, MLST and CRISPR-MVLST (ALMEIDA et al., 2015; ALMEIDA et al., 2017a; ALMEIDA et al., 2017b).

In this study, 47 (52.2%) strains presented phenotypic resistance to gentamicin and/or streptomycin. Streptomycin is not frequently used to treat *Salmonella enterica* infections; but, it has been commonly used as a growth promoter in food-producing animals and for this reason may serve as a marker for resistant strains moving through the food supply (MCDERMOTT et al., 2016).

Our results confirm McDermott et al's (2016), observations of discrepancies between phenotypic resistance and genotypic resistance of aminoglycoside resistant genes. We observed 35 isolates carrying streptomycin resistance genes, but these isolates were phenotypically susceptible to the drugs. It is unclear why the genes while present in the genomes were not expressed to provide phenotypic resistance. Presence of the known streptomycin resistance genes does not predict phenotypic resistance well for this class.

The tetracycline resistance genes were found in 32 (35.5%) strains. Interestingly, 2 strains that were phenotypically resistant to tetracycline did not present any known tetracycline resistance genes suggesting a possible alternative mode of resistance. In contrast, seven strains that presented tetracycline resistance genes were phenotypically susceptible. Of these seven, six strains had two tetracycline resistance genes and one strain had only one tetracycline resistance gene. Tetracycline has been used commonly as an antibiotic in swine husbandry (ALMEIDA et al., 2016a). Brazil is a major producer of pigs with 3.73 million tons of pork produced and exported in 2016 (VIOTT et al., 2013; ABPA, 2017). The *Salmonella* Typhimurium serovar usually does not cause severe disease in pigs and sometimes it is asymptomatic in these animals, which may be a serious public health problem, since it may be an important source of contamination of carcasses in slaughterhouses. In addition, the contamination by *S.* Typhimurium may not be detected while the pigs are on the farm, which may eventually lead to human contamination (KICH et al., 2011; ALMEIDA et al., 2016a).

Cefoxitin resistance has been used to indicate certain types of beta-lactamases production by *Salmonella* and *E. coli*. First and second-generation cephalosporin susceptibility results are not reported in clinical medicine for *Salmonella*, because the drugs may appear active *in vitro*, but are not therapeutically effective (CLSI, 2015). Regarding the beta-lactam resistance genes found in Brazil, the most common was $bla_{\text{TEM-1B}}$ gene presented in 16 (17.8%) isolates (6 humans, 10 foods). The $bla_{\text{TEM-1B}}$ gene has been associated with ampicillin resistance and 32 (35.6%) strains were phenotypically resistant to the ampicillin. The $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-2}}$ genes have been more closely associated to cephalosporin resistance and 7 strains were resistant to ceftriaxone (CRO), third generation cephalosporin, but only 3 strains presented a bla_{CTX} allele. The most common resistant gene was *aac(6')Ib-cr* found in 5(5.6%) human isolates followed by *oqxA* and *oqxB* found in 4(4.4%) food isolates. The *qnrB2* and *qnrB88* genes were found each in 1 (1.1%) food isolate.

Some of the discrepancies observed when a resistance gene is present but no phenotypic resistance in bacterial growth is observed, or when the phenotype is present but no known resistance gene is observed, is likely due to new unidentified resistance genes or mutations conferring resistance in undiscovered genes. Therefore, it is important to study any discrepancy as each represents new ways that bacteria are acquiring resistance as was reported for a new mechanisms discovered for *Campylobacter* gentamicin resistance (ZHAO et al., 2015). Pribul et al. (2017) evaluated the prevalence of PMRQ genes in129 isolates of non-typhoidal *Salmonella* from Brazil by PCR amplification. *Qnr* genes were found in 15 (11.6%) isolates (8 *qnrS*, 6 *qnrB*, and 1 *qnrD*), and the *aac(6')-Ib* gene was found in 23 (17.8%) isolates. Regarding mutation points in the QRDRs, *gyrA* mutation was the only one found among the strains studied. Thirty-three (36.7%) of nalidixic acid resistant strains presented mutations in the *gyrA* gene (22 human, 11 foods).

McDermott and colleagues (2016) used WGS technology to identify known antimicrobial resistance genes among 640 non-typhoidal *Salmonella* strains for 43 different serotypes and correlated these with susceptibility phenotypes to evaluate the utility of WGS for antimicrobial resistance surveillance. Overall, genotypic and phenotypic resistance correlated in 99.0% of the cases. They concluded that WGS is an effective tool for predicting antibiotic resistance in non-typhoidal *Salmonella* (MCDERMOTT et al., 2016). Regarding QRDR mutations and PMQR genes, 21 isolates had either QRDR mutations or PMQR genes, all of which were from human clinical cases. In contrast, in this study QRDR mutations were found in both human and food isolates.

Salmonella Typhimurium ST313 had been described only in sub-Saharan Africa, with high levels of antibiotic resistance associated with bloodstream infections and mortality rates of >25% (FEASEY et al., 2014; LEY et al., 2014). In 2017 nine strains were typed as ST313 in Brazil, with only 1 MDR, human strain (STm29 feces), presenting resistant to ampicillin, streptomycin and sulfonamide. Five Brazilian strains (STm30, STm35, STm37, STm47, STm44) were resistant just to sulfonamide with STm37) isolated from food. Other resistant strains included: STm40 isolated from food (streptomycin and sulfonamide); STm39 isolated from human feces (streptomycin); and STm34 isolated from human feces (pan susceptible) (ALMEIDA et al., 2017a).

Food isolates were distributed in Clades A and B in relatively similar numbers suggesting that there is more than one subtype in circulation, in foods in Brazil. Human's isolates were more prevalent in the Clade B suggesting the existence of a prevalent subtype. Genomic and phenotypic testing results suggest clinical isolated before the mid-1990s presented more antimicrobial resistance compared to later strains. The diversity and prevalence of resistant genes found in Brazilian *Salmonella* Typhimurium is an alert of their potential hazard for food safety and public health.

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CHAPTER 2

Phylogenetic analysis revealed that *Salmonella* Typhimurium ST313 isolated from humans and food in Brazil presented a high genomic similarity

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Keywords: *Salmonella* Typhimurium; ST313; phylogeny; pangenome.

ABSTRACT

Salmonella Typhimurium sequence type 313 (*S.* Typhimurium ST313) has caused invasive disease mainly in sub-Saharan Africa. In Brazil, ST313 strains have been recently described and there is a lack of studies that assessed by whole genome sequencing (WGS) the relationship of these strains. The aims of this work were to study the phylogenetic relationship of 70 *S.* Typhimurium genomes comparing strains of ST313 (n=9) isolated from humans and food in Brazil among themselves, with other STs isolated in this country (n=31) and in other parts of the globe (n=30) by 16S rRNA sequences, the Gegenees software, whole genome multi locus sequence typing (wgMLST) and average nucleotide identity (ANI) for the genomes of ST313. Additionally, pangenome analysis was performed to verify the heterogeneity of these genomes. The phylogenetic analyses showed that the ST313 genomes were very similar among themselves. However, the ST313 genomes were usually clustered more distantly to other STs of strains isolated in Brazil and in other parts of the world. By pangenome calculation, the core genome was 2,880 CDSs and 4,171 CDSs singletons for all the 70 *S.* Typhimurium genomes studied. Considering the 10 ST313 genomes analyzed the core genome was 4,112 CDSs and 76 CDSs singletons. In conclusion, the ST313 genomes from Brazil showed a high similarity among them which information might eventually help in the development of vaccines and antibiotics. The pangenome analysis showed that the *S.* Typhimurium genomes studied presented an open pangenome, but specifically tending to become close for the ST313 strains.

INTRODUCTION

Salmonella enterica subsp. *enterica* serovar Typhimurium can cause gastroenteritis and invasive disease in humans and other animals around the world (HENDRIKSEN et al., 2011; MOFFATT et al., 2016; ALMEIDA et al., 2017a). It is estimated that more than one million cases of salmonellosis, 23.000 hospitalizations and 450 deaths occur in the United States each year (CDC, 2018). According to the European Center for Disease Prevention and Control (ECDC, 2014), *Salmonella* is one of the leading bacteria found in food-borne infections in the European Union and *S.* Enteritidis and *S.* Typhimurium have been the most prevalent serovars (HENDRIKSEN et al., 2011).

In Brazil, *Salmonella enterica* has been reported as the main isolated pathogen in foodborne outbreaks and *S.* Typhimurium has been pointed as the first or second most

isolated serovar in the country, depending on the location (FERNANDES et al., 2006; ASSIS et al., 2017; PRIBUL et al., 2017).

S. Typhimurium invades host cells through the Type III Secretion System (T3SS) and genes which encode this system and the effectors proteins are located mainly in two regions on the chromosome denominated pathogenicity islands 1 and 2 (SPI-1 and SPI-2) that have the capacity to modulate a series of cellular functions related to the survival and replication of *S.* Typhimurium in host cells (GALÁN, 2001; SUN et al., 2016).

Clinical and epidemiological data indicate that *S.* Typhimurium sequence type (ST) ST313 has been frequently linked to invasive systemic disease, bacteremia, septicemia and meningitis in Mali and West Africa (FEASEY et al., 2012; FEASEY et al., 2014). It has been reported that *S.* Typhimurium ST313 can also cause systemic infections in children and adults with HIV (FEASEY et al., 2012; FEASEY et al., 2014; KARIUKI and ONSARE, 2015). On the other hand, cases of gastroenteritis have been mainly caused by *S.* Typhimurium ST19 worldwide (CARDEN et al., 2015; RAMACHANDRAN et al., 2015; SINGLETARY et al., 2016). According to Gilchrist and Maclennan (2019), *S.* Typhimurium ST313 strains is genetically distinct when compared to non-invasive *S.* Typhimurium ST19 strains, but it is not clear what these strains have of difference, some authors suggest the presence of plasmids, prophage-like elements and the presence of different genes, such as, *st313-td* (GUINEY et al., 1995; KINGSLEY et al., 2009; HERRERO-FRESNO et al., 2014).

By Multilocus Sequence Typing (MLST), nine *S.* Typhimurium ST313 strains isolated from humans and food between 1989 to 2003 have been recently described, for the first time, in Latin America and Hela cells invasion and intramacrophage survival assays were performed for those strains (ALMEIDA et al., 2017a). Comparative analyses using the complete genomes of *S.* Typhimurium ST313 and ST19 strains are of great importance and can help to elucidate the diversity and phylogenetic relations among the strains and can also improve epidemiologic data of this important global pathogen (PANZENHAGEN et al., 2018). Furthermore, there is a lack studies that characterized possible phylogenetic differences of *S.* Typhimurium strains isolated from diverse sources and distinct genetic subtypes in Brazil (ALMEIDA et al., 2017a; PANZENHAGEN et al., 2018).

Frequently, the 16S rRNA sequences analysis has been successfully used for taxonomic classification and in phylogenetic studies of different bacterial genus such as *Salmonella*, *Listeria*, *Escherichia coli,* among others, but in some cases, this approach cannot distinguish strains of the same species and is necessary to use other methods like some based

on whole genome sequences (SRINIVASAN et al., 2015; CEUPPENS et al., 2017; PETTENGILL and RAND, 2017).

Whole genome sequencing (WGS) has been proved to be a tool with a high discriminatory power capable to improve epidemiological and phylogenetic studies. Moreover, WGS has become financially more accessible in the last years, allowing the understanding of the genomic variability of some important foodborne pathogens such as *Salmonella* spp. (OKORO et al., 2012; HAWKEY et al., 2013; PHILLIPS et al., 2016).

The aims of this work were to study the phylogenetic relationship of 70 *S.* Typhimurium genomes comparing ST313 strains isolated from humans and food in Brazil among each other, with other STs isolated in this country and in other parts of the globe by using different phylogenetic strategies such as 16S rRNA sequences, the alignment of fragmented genomes for inference of phylogenetic distances using the Gegenees software (AGREN et al., 2012),whole genome multi locus sequence typing (wgMLST) and average nucleotide identity (ANI). Furthermore, it was aimed to verify the heterogeneity of these genomes by pangenome analysis to better understand their genotypic diversity.

Altogether, the results obtained in this work contributed for a better characterization of the *S.* Typhimurium strains studied regarding its genotypic diversity.

MATERIALS AND METHODS

Bacterial strains and genome sequencing

A total of 40 *S.* Typhimurium strains isolated from human diarrheic faeces and food in the São Paulo State in Brazil, between 1983 and 2013 were selected from the collections of the Adolfo Lutz Institute of Ribeirão Preto (IAL-RP) and Oswaldo Cruz Foundation (FIOCRUZ-RJ). The genomic DNA extraction of these 40 *S.* Typhimurium strains was performed according to Campioni and Falcão (2014). The concentration of the genomic DNA was detected in NanoDrop 1000 (Thermo Scientific). Libraries were prepared using 1 ng of genomic DNA with the Nextera XT DNA library preparation kit (Illumina, San Diego, CA) (ALMEIDA et al., 2016b).

The whole genome sequencing was performed using the NextSeq 500 desktop sequencer with the NextSeq 500/500 high-output version 2 kit (Illumina) for 2 x 151 cycles according to the manufacturer's instructions in the U.S. Food and Drug Administration (FDA), College Park, MD, USA (ALMEIDA et al., 2016b).

The genomes were assembled using the SPAdes software (BANKEVICH et al., 2012) and the quality of the assemblies was evaluated using QUAST software (GUREVICH et al., 2013). The contigs for each isolate (draft genomes) were annotated using NCBI's Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (KLIMKE et al., 2009). These draft genome sequences are available in GenBank database and their accession numbers are detailed in Table S1.

Beside these 40 genomes, 30 other ones of *Salmonella* Typhimurium isolated from different sources, geographical areas and sequence types (STs) were retrieved from GenBank database (Table S2). The genomes of all these strains were used in the phylogenetic and pangenome analyses, except in the Average Nucleotide Identity (ANI) analysis which was performed only for the ST313 strains.

Phylogeny

The genomes of all the 70 *S.* Typhimurium strains described in item 2.1 (Table S1 and S2) were used in the phylogenetic analyses.

For the phylogenetic analyses of 16S rRNA sequence, all sequences were retrieved from genomic annotation and aligned using the multiple sequence alignment CLUSTALW that is integrated in the MEGA6 software (TAMURA et al., 2013). The appropriate evolutionary model was defined, and the evolutionary history was inferred using the Maximum likelihood (ML) criterion, based on the Jukes-Cantor model and the rates among sites has invariant (I) with 1000 bootstrap replicates. *Escherichia coli* K12 (MG1655) was used to root the final tree.

The alignment of fragmented genomes for inference of phylogenetic distance was performed using the Gegenees software (AGREN et al., 2012). This software calculates the percentage of similarity among the genomes of all strains. The alignment method BLASTn was used with sequence fragmentation length of 200 bp and a step size of 100 bp. The heatmap resulting from this analysis was exported in the ".nexus" format for phylogenomic analysis using SplitsTree4 software (HUSON and KLOEPPER, 2005) with NeighborNet and equal angle methods.

The wgMLST analysis was performed using the module *Build_PGAdb* on the software PGAdb-builder (LIU; CHIOU; CHEN, 2016) for creating a *PGAdb* allelic profile. The wgMLST tree was constructed using the *Build_wgMLSTtree* module from uploaded genome contigs by *PGAdb* database. We used as input files the genomes contigs in the ".fasta" format.

The parameters used for PGAdb were alignment coverage and identity ≥ 90% (LIU; CHIOU; CHEN, 2016).

Average Nucleotide Identity (ANI)

The Average Nucleotide Identity (ANI) analysis was performed using the whole genome sequences of nine Brazilian strains and the reference ST313-lineage II from Africa designated D23580.

ANI is based on the mean values of identity or similarity between homologous regions that are shared by two genomes. ANI values of 95-96% are equivalent to a DNA-DNA hybridization index of 70% and can be used as a threshold for species delineation (KONSTANTINIDIS and TIEDJE, 2005; KIM et al., 2014).

Pangenome calculation

The genomes of all 70 *S.* Typhimurium strains were used in the pangenome analysis. Furthermore, the 10 genomes *S.* Typhimurium ST313 had the pangenome performed separately. Initially, the amino-acid sequences from all DNA coding sequences (CDSs) in all genomes were used in the OrthoMCL software (LI; STOECKERT; ROOS, 2003) for an all-vs.-all BLASTp analysis with an e-value of 1e−6. The CDSs observed in all strains were considered as the core genome, while the CDSs harbored by only one strain were considered as singletons and those presented in more than one genome, but not in all, were classified as shared genome.

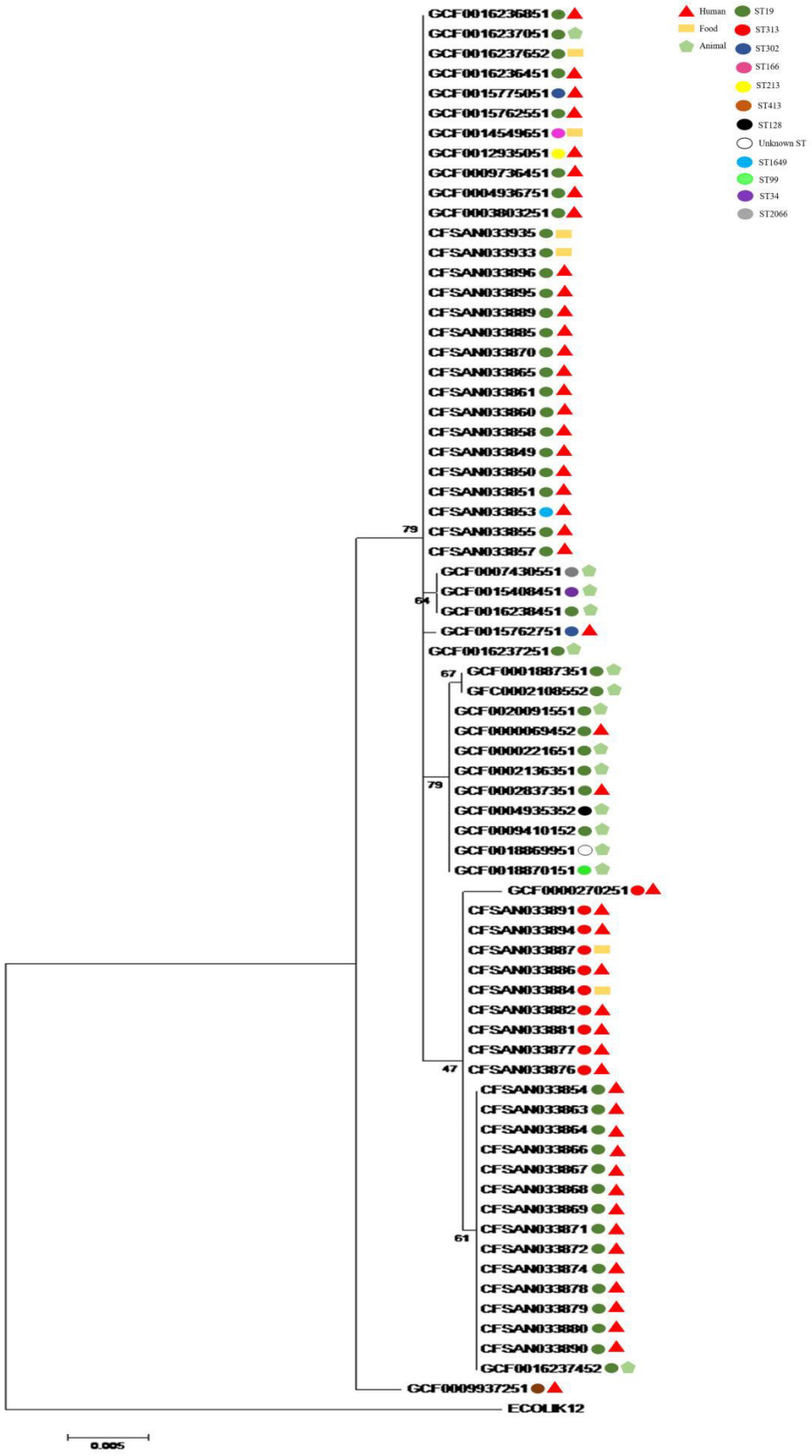
The pangenome development was calculated using the Heap's Law and the extrapolations of the curves of the core genome and singletons were calculated using the least-squares fit of the exponential regression decay of the mean values, as described by Benevides and collaborators (2017).

RESULTS

Phylogeny

The phylogenetic analysis using the 16S rRNA sequences showed that 69 out of 70 *S.* Typhimurium strains analyzed were grouped in a single large cluster regardless of the source of isolation (Figure 1). The dark green circle was designated for the ST19 *S.* Typhimurium strains that were widely distributed along different subclusters. In this analysis, we could observe the existence of many polytomies and some low bootstrap values. The 10 red circles were designated for the ST313 *S.* Typhimurium strains (CFSAN033876, CFSAN033877, CFSAN033881, CFSAN033882, CFSAN033884, CFSAN033886, CFSAN033887, CFSAN033891, CFSAN033894 and GCF0000270251). The nine ST313 sequences from Brazil were grouped closely among each other and with the reference ST313-lineage II from Africa D23580 (GCF0000270251) isolated in Malawi, Africa (Figure 1). All the others STs (ST1649, ST34, ST99, ST128, ST213, ST302, ST2066 and ST166) were grouped in this large cluster, except the ST413 represented by a brown circle that was not grouped.

Fig. 1 - Phylogenetic analysis based on 16S rRNA gene sequences of the 70 *Salmonella* Typhimurium strains studied. The bootstrap analysis was performed with 1000 replicates. Evolutionary analyses were conducted in MEGA6 (TAMURA et al., 2013)



The Gegenees software generated a distance matrix based on the similarity among all genomes that was plotted as a heatmap (Figure 2). In this matrix, the similarity varied between 100% and 79% among the 70 genomes. The nexus file exported from the Gegenees software was further used in the software SplitsTree4 to generate a phylogenetic tree. In this analysis, *S.* Typhimurium were grouped in two large clusters designated A and B (Figure 3). The cluster A comprised six ST19 *S.* Typhimurium genomes (dark green circles) isolated from humans in Brazil. The cluster B comprised 62 *S.* Typhimurium genomes and this cluster was subdivided into B1 and B2 subclusters. The subcluster B1 grouped ST19 (dark green circles), ST1649 (light blue circle) and all the nine ST313 (red circles) *S.* Typhimurium genomes isolated from humans and food in Brazil. The subcluster B2 grouped ST19 (dark green circles), ST34 (purple circle), ST128 (black circle), ST213 (yellow circle), ST302 (dark blue circles), ST99 (light green circle), unknown ST (white circle), ST2066 (grey circle) and ST313 (red circle) *S.* Typhimurium genomes isolated from humans, food and animals in Brazil and in other parts of the world (Table S2). The ST166 (pink circle GCF001454965) and ST413 (brown circle GCF000993725) *S.* Typhimurium genomes were not grouped in any of the two clusters.

Fig. 2 - Heatmap of the 70 *Salmonella* Typhimurium genomes analyzed. The numbers in the heatmap show the percentage of similarity among the genomes; the colors vary from red (low similarity) to green (high similarity)

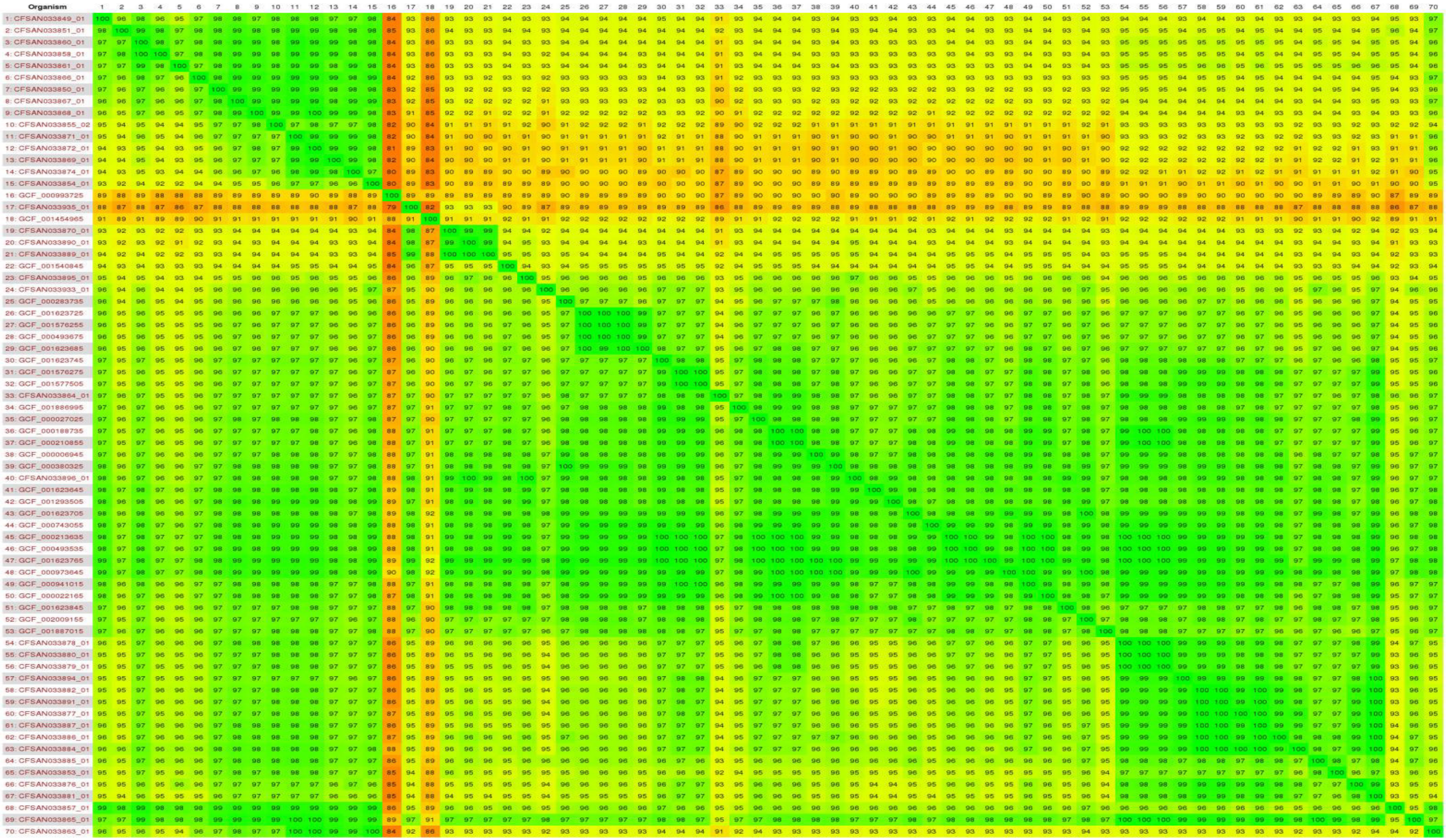
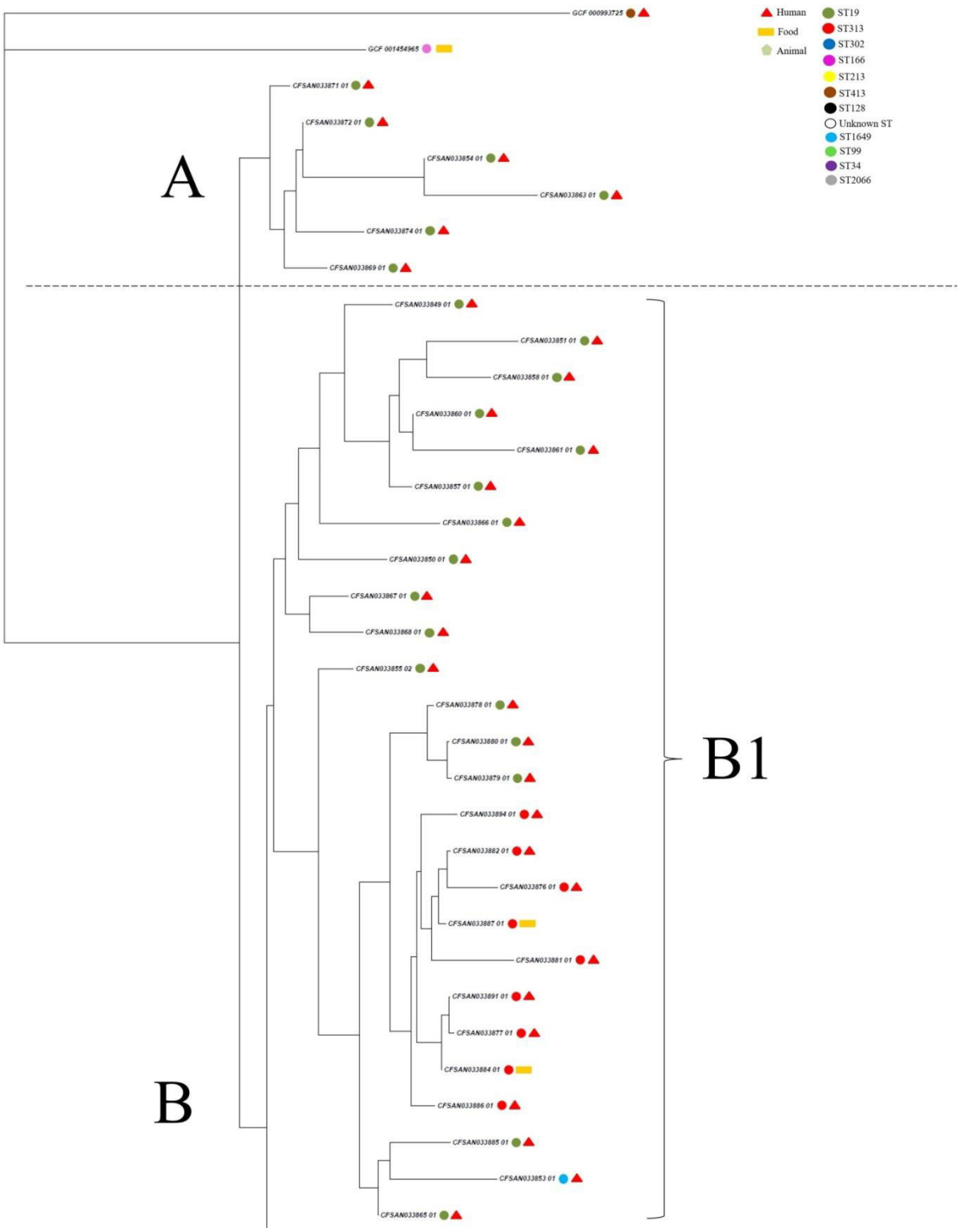
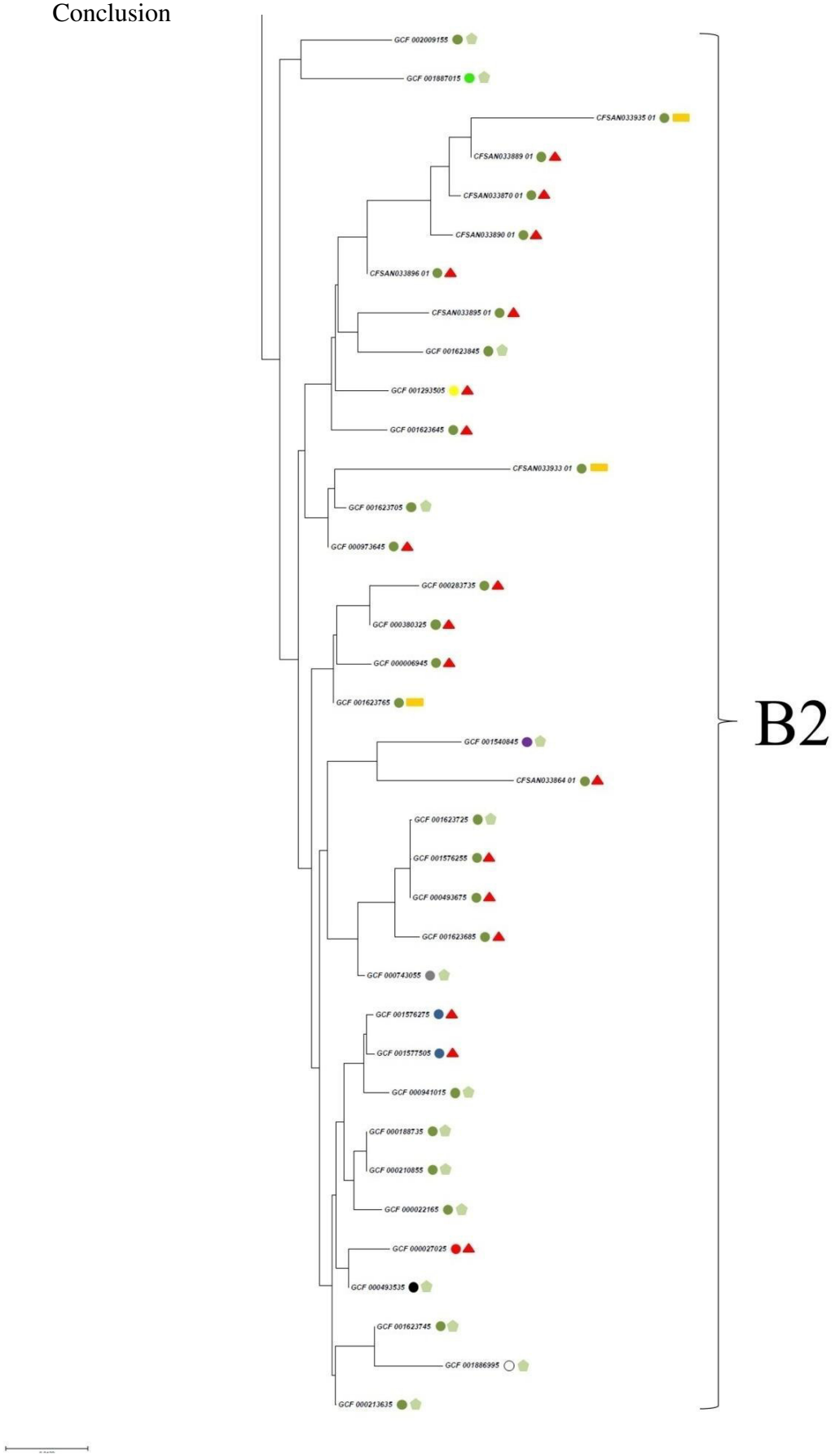


Fig. 3 - Phylogenetic analysis based in the genomes of the 70 *Salmonella* Typhimurium strains. The network was constructed using SplitsTree software (HUSON and KLOEPPER, 2005) with NeighborNet and equal angle methods, based on a distance matrix from Gegenees software (AGREN et al., 2012)



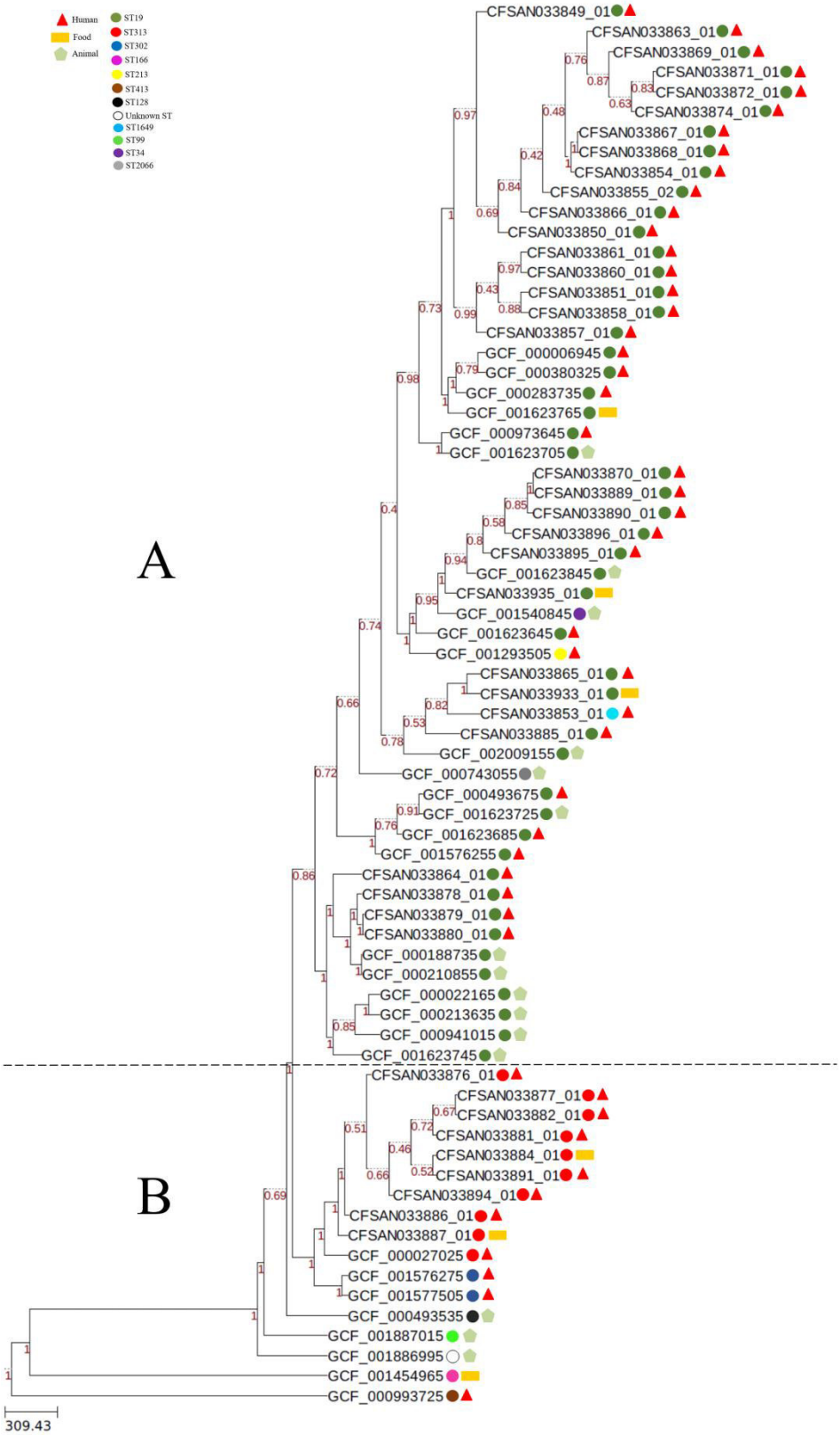
Continue

Conclusion


GCF 002009155
GCF 001887015
CFSAN033935 01
CFSAN033889 01
CFSAN033870 01
CFSAN033890 01
CFSAN033896 01
CFSAN033895 01
GCF 001623845
GCF 001293505
GCF 001623645
CFSAN033933 01
GCF 001623705
GCF 000973645
GCF 000283735
GCF 000380325
GCF 000006945
GCF 001623765
GCF 001540845
CFSAN033864 01
GCF 001623725
GCF 001576255
GCF 000493675
GCF 001623685
GCF 000743055
GCF 001576275
GCF 001577505
GCF 000941015
GCF 000188735
GCF 000210855
GCF 000022165
GCF 000027025
GCF 000493535
GCF 001623745
GCF 001886995
GCF 000213635
B2

The wgMLST analysis using a gene-by-gene approach showed two large clusters designated A and B (Figure 4). The cluster A comprised 53 *S.* Typhimurium genomes containing all the ST19 (dark green circles), ST34 (purple circle), ST213 (yellow circle), ST1649 (light blue circle) and ST2066 (grey circle) isolated from humans, food and animals in Brazil and in other parts of the world. The cluster B grouped 12 *S.* Typhimurium genomes including all the ST313 (red circles) isolated from humans and food in Brazil and Africa. Also, two ST302 (dark blue circles) *S.* Typhimurium genomes isolated from humans in Mexico. The ST166 (pink circle GCF001454965), ST413 (brown circle GCF000993725), ST128 (black circle GCF000493535), ST99 (light green circle GCF001887015) and unknown ST (white circle GCF001886995) *S.* Typhimurium genomes were not grouped in any of the two clusters.

Fig. 4 - Phylogenetic analysis with wgMLST profiles for 70 *Salmonella* Typhimurium genomes. The PGAdb profile from the genomes was used to construct a wgMLST tree using the Build_wgMLST tree module (LIU; CHIOU; CHEN, 2016). Bootstrap values are shown next to the nodes. The dendrogram was constructed with the UPGMA clustering algorithm



Average Nucleotide Identity (ANI)

Using an identity cut-off of 95%, this analysis revealed that the nine ST313 *S.* Typhimurium genomes isolated from humans and food were very similar among themselves and with the reference ST313-lineage II from Africa D23580 (Table S3).

Pangenome calculation

To take a global view of the strains and to further explore the genome diversity of this genus, the size of the pangenome was calculated (i.e. the total number of non-redundant CDSs). The orthology analysis showed that the pangenome contained a total of 9,883 CDSs. The core genome showed that 2,880 CDSs were shared by all genomes and 4,171 CDSs singletons (i.e. unique to a single genome) were found in the studied genomes (Figure 5A). Using the Heap's law and considering that $\alpha = 1-\gamma$, we inferred that the α value of the pangenome development was 0.722, indicating that the pangenome is open ($\alpha < 1$) but tending to become close ($\alpha \geq 1$). By examining the extrapolated curve of the core genome and singletons, we found that the size of the core genome tended to converge at ~ 960 genes and the singletons at ~782 (Figure 6).

A separate analysis of the 10 genomes *S.* Typhimurium ST313 revealed that the core genome contained 4,112 CDSs and 76 CDSs singletons (Figure 5B). Using the Heap's law and considering that $\alpha = 1-\gamma$, we inferred that the α value of the pangenome development was 0.970, indicating that the pangenome is open ($\alpha < 1$) but tending to become close ($\alpha \geq 1$) (Figure 6).

Fig. 5 - Diagram depicting the subsets of the *Salmonella* Typhimurium pangenome. The numbers represent the coding sequences belonging to each subset. Left chart (a): pangenome subsets from an analysis based on all 70 *Salmonella* Typhimurium genomes. Right chart (b): subset based on analysis of 10 genomes *Salmonella* Typhimurium ST313

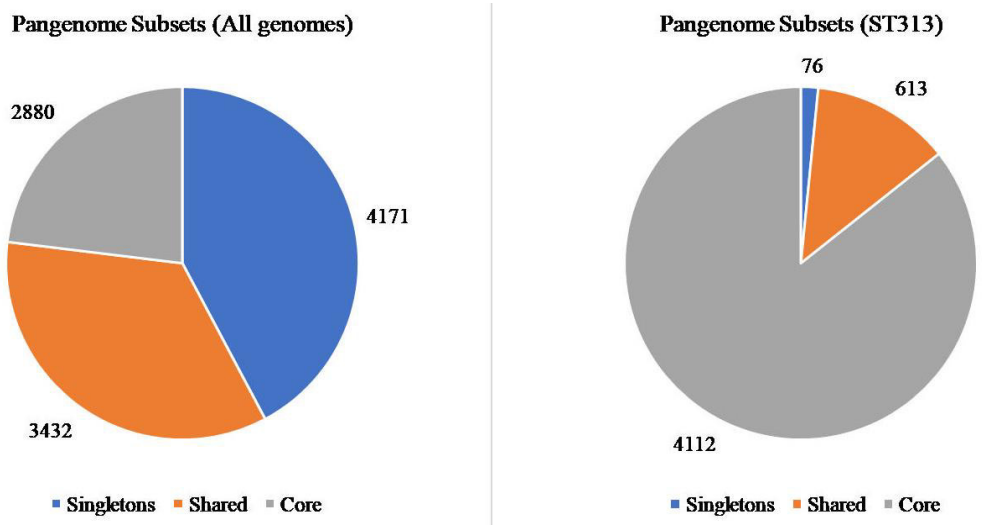
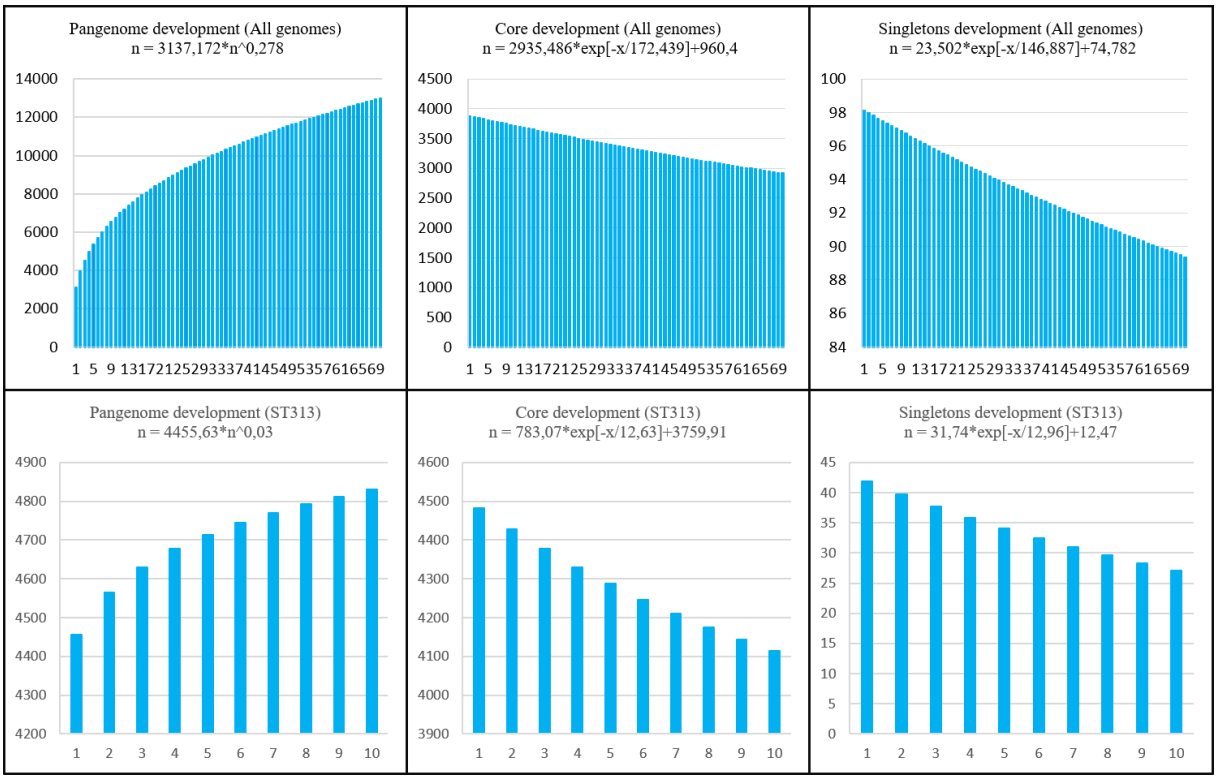


Fig. 6 - Development of the pangenome, core genome and singletons. Upper chart (a): pangenome, core genome, and singleton development based on permutations of all 70 *Salmonella* Typhimurium genomes. Lower chart (b): development based on permutations of 10 genomes *Salmonella* Typhimurium ST313



DISCUSSION

During the last decades, an epidemic of invasive infections of *S.* Typhimurium ST313 in Africa has been witnessed. Clinical observations and genomic studies suggested that such strains have been evolving concerning the known virulence patterns (SINGLETARY et al., 2016). In addition, the presence of *S.* Typhimurium ST313 strains has been recently described in Brazil, being the first time that the highly invasive ST313 was reported in another continent than Africa (ALMEIDA et al., 2017a; PANZENHAGEN et al., 2018).

The 16S rRNA sequences analysis has been the method of choice of many researchers to study phylogenetic relationships and the investigation of microbial diversity, but it is important to consider not only these sequences. Therefore, the 16S rRNA sequences analysis can be used together with whole genome to complement studies of genomic diversity within the same genus or species (QUAST et al., 2013; YILMAZ et al., 2014).

In the present work, the 16S rRNA gene sequencing was not able of accurately differentiating the *S.* Typhimurium strains analyzed, but it was important to confirm that all the strains studied are of the same serovarity. In addition, for the *Salmonella* genus, 16S rRNA gene sequencing has been widely used for its identification in diverse sources such as food, animals and humans (TRKOV and AVGUSTIN, 2003; JARVIS et al., 2015).

The similarity matrix obtained with Gegenees software and used into the SplitsTree4 software for a phylogenomic analysis showed the evolutionary relationship among the strains, highlighting that all the nine ST313 strains from Brazil isolated from humans and food stayed grouped in subcluster B1. However, the ST313 strain from Africa was clustered in subcluster B2. This unexpected cluster pattern may be explained because the parameters used in Gegenees software does not use only the probably homologous genes, but the fragmented alignment of whole genomes, including repetitive regions, genomic islands, duplicated genes and other elements that can create biases in this analysis. The analysis of this software can also be influenced by the different sizes in the genomes. To eliminate this bias, we used other methods.

The wgMLST tool was used to subtype the strains of this work. As opposed to conventional MLST analysis, which uses only a few housekeeping genes, the wgMLST approach takes advantage of a larger number of tracked loci, enabling higher resolution in intraspecies differentiation (MAIDEN et al., 2013). The constructed phylogenetic tree separated with accuracy the *S.* Typhimurium strains studied, showing that the 10 ST313 strains from Africa and Brazil were in a different cluster apart from all ST19 strains. The resolution of wgMLST resulting tree was better when compared to 16S rRNA sequences

based phylogenetic tree and Gegenees, because more conserved genes were considered allowing a better differentiation among the strains. It is important to mention that the different sources of isolation of the strains studied did not influence their grouping in any of the phylogenetic trees constructed.

In the present study, the ANI analysis showed a high similarity between the ST313 genomes isolated from humans and food in Brazil and Africa. ANI is based on the mean values of identity or similarity between homologous regions that are shared by two genomes. Furthermore, the ANI has been widely used to characterize and identify the genomic relationship of two or more strains, because it is a fast, easy and reproducible method (YI and CHUN, 2015; LEE et al., 2016; MAHATO et al., 2017). The ANI analysis has also been used for prokaryotic taxonomic classification studies and is considered the new gold standard for bacterial species determination (KONSTANTINIDIS and TIEDJE, 2005; SENTAUSA and FOURNIER, 2013).

The present work provided additional information about *S.* Typhimurium ST 313, ST19 and ST1649 strains that were previously molecularly typed by Pulsed-field gel electrophoresis (PFGE), Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), Multiple-locus variable-number tandem-repeat analysis (MLVA) and Clustered regularly interspaced short palindromic repeats-Multi-locus virulence sequence typing (CRISPR-MVLST) (ALMEIDA et al., 2015; ALMEIDA et al., 2016a; ALMEIDA et al., 2017b). Additionally, resistance genes were searched by the whole genome sequencing in these strains and genes that confer resistance to aminoglycoside, tetracycline, sulphonamide, trimethoprim, beta-lactam, fluoroquinolone and phenicol were found (ALMEIDA et al., 2018). In sub-Saharan Africa high levels of antibiotic resistance have been found in *S.* Typhimurium ST313 strains (KINGSLEY et al., 2009; FEASEY et al., 2014; KARIUKI and ONSARE, 2015). On the other hand, most of the *S.* Typhimurium ST313 strains isolated in Brazil showed sensitivity to different antimicrobials classes searched (ALMEIDA et al., 2015) and genes that confer resistance to aminoglycoside, sulphonamide and beta-lactam were found in only one strain (ALMEIDA et al., 2018). The other eight *S.* Typhimurium ST313 strains studied did not show any resistance genes (ALMEIDA et al., 2018).

Therefore, the ST313 genomes from Brazil presented a high similarity among themselves regardless of the source being from humans or food by 16S rRNA, wgMLST and ANI analyses, which was also observed using single nucleotide polymorphism (SNP) by (PANZENHAGEN et al., 2018).

The ST302 was genotipically similar to ST313 by wgMLST analysis in the present work (Figure 4), being that in accordance to Vinuesa and colleagues (2016) that reported ST302 strains to be closely related to ST313 human-invasive strains from Africa. The ST302 was first described in Mexico and isolated from humans. This ST was later described in two African strains being characterized as single locus variant (SLV) of ST19 that is the predominant ST among *S.* Typhimurium strains and is usually related to gastroenteritis worldwide (SILVA et al., 2016a; SILVA et al., 2016b).

Furthermore, others STs of *S.* Typhimurium with different characteristics retrieved from GenBank database were studied in this work and compared with ST313 strains of this study. The ST128 was clonally related to ST313 and was described as a cause of systemic disease in pigeons (ANDREWS-POLYMENIS et al., 2010). In contrast, in this work this close phylogenetic relationship was not observed between the ST128 and ST313. The ST213 and ST34 were related to resistance to multiple drugs, ST213 was also associated to invasive disease in humans and animals (WONG et al., 2013; CALVA et al., 2015). Finally, the ST166 was described in poultry and the ST99 was reported in wild birds and pigs (WU et al., 2010; PEREZ et al., 2012). All these STs above mentioned were not clonally related to the ST313 herein study by the different tools used. According to the published literature, this is the first article that brings this epidemiological information comparing the genomes of ST313 strains isolated in Brazil with different STs isolated in other countries.

The pangenome analysis showed that *S.* Typhimurium genomes studied presented an open pangenome because the number of orthologous genes increased when other genomes were added in the analysis. According to Alikhan and collaborators (2018), *Salmonella* is a recombinant bacterial genus characterized by an open pangenome. In addition, the two subsets showed α values close to 1, but is evident that when only the 10 genomes *S.* Typhimurium ST313 were analyzed in this study the α value was higher because these strains are very similar to each other, as it was observed in the other analyzes.

In conclusion, the ST313 genomes from Brazil showed a high similarity among them regardless of the source being from humans or food by all methods used which might eventually help in the development of vaccines and antibiotics. However, those ST313 genomes presented different similarities in comparison with other STs isolated in Brazil and from other parts of the world depending on the method performed. The pangenome analysis showed that the *S.* Typhimurium genomes studied presented an open pangenome in accordance to our results from the phylogenetic analyses. Altogether, the results obtained in this work contributed for a better characterization of the *S.* Typhimurium strains studied

regarding its genotypic diversity. Detailed studies of the ST313 genomes should be performed in order to try to elucidate differences among them.

Acknowledgements

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CHAPTER 3

Phenotypic and genotypic characterization of *Salmonella* Typhimurium isolates from humans and foods in Brazil

Running title: Invasion and survival of *S*. Typhimurium isolates

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Keywords: *Salmonella* Typhimurium, Caco-2 epithelial cells, U937 human macrophages, *Galleria mellonella*, virulence genes

ABSTRACT

Salmonella enterica subsp. *enterica* serovar Typhimurium (*S*. Typhimurium) causes gastroenteritis in many countries. However, in Brazil there are few studies that have conducted a virulence characterization of this serovar. The aim of this study was to evaluate the virulence potential of *S*. Typhimurium strains isolated in Brazil. Forty *S*. Typhimurium strains isolated from humans (n=20) and food (n=20) from Brazil were studied regarding their invasion and survival in human epithelial cells (Caco-2) and macrophages (U937). Their virulence potential was determined using the *Galleria mellonella* larvae model combined with the analysis of virulence genes by whole genome sequencing (WGS). A total of 67.5% of the *S*. Typhimurium studied (32.5% isolated from humans and 35% isolated from food) invaded Caco-2 epithelial cells at levels similar to or greater than the *S*. Typhimurium SL1344 prototype strain. In addition, 37.5% of the studied strains (25% isolated from humans and 12.5% isolated from food) survived in U937 human macrophages at levels similar to or greater than SL1344. *S*. Typhimurium strains isolated from humans (40%) and food (25%) showed high or intermediate virulence in *G. mellonella* larvae after seven days exposure. Approximately, 153 virulence genes of chromosomal and plasmidial origin were detected in the strains studied. In conclusion, the ability of the *S*. Typhimurium to invade Caco-2 epithelial cells was strain dependent and was not related to the source or the year of isolation. However, *S*. Typhimurium strains isolated from humans showed greater survival rates in U937 human macrophages, and presented higher proportion of isolates with a virulent profile in *G. mellonella* in comparison to strains isolated from food suggesting that this difference may be related to the higher frequency of human isolates which contained plasmid genes, such as *spvABCDR* operon, *pefABCD* operon, *rck* and *mig-5*.

INTRODUCTION

Salmonella Typhimurium has been an important cause of gastroenteritis in different parts of the globe (COLOMBE et al., 2019; GODÍNEZ-OVIEDO et al., 2020; WANG et al., 2020a). It is important to emphasize that the transmission of this bacterium is mainly due to the ingestion of contaminated food such as eggs, beef, poultry, swine, and vegetables (WORLD HEALTH ORGANIZATION (WHO), 2018). In addition, the transmission can occur person-to-person by fecal oral routes and, contaminated asymptomatic pets also can transmit to humans (WHO, 2018).

According to the Centers for Disease Control and Prevention (CDC) (2020), it was estimated that 1.35 million infections, 26,500 hospitalizations and 420 deaths occur in the

United States every year due to salmonellosis. In humans the symptoms are diarrhea, fever, and stomach cramps, with food being the main source of transmission of this disease. Therefore for prevention and control of *Salmonella* spp. food safety and hygenic handling practices are very important (CDC, 2020a).

In Brazil, *Salmonella* spp. has been one of the main bacterial genera isolated from foodborne outbreaks (BRAZIL, 2019). However, until now there are few published studies that have characterized the possible differences between Brazilian *S*. Typhimurium strains isolated from human and food sources. No studies have examined the invasiveness of these isolates to Caco-2 epithelial cells (human colon adenocarcinoma), their survival in U937 human macrophages, or described the repertoire of virulence genes present by whole genome sequencing (WGS).

Several genes are responsible for the virulence of *Salmonella* spp. in different hosts. The Type III Secretion System (T3SS) is an important virulence factor for the invasion and survival of this pathogen in epithelial and phagocytic cells at the beginning of the infection (JOENSEN et al., 2017; BHUNIA, 2018). The genes are located mainly in two regions of the chromosome denominated pathogenicity islands 1 and 2 (SPI-1 and SPI-2) encode by T3SS (JOENSEN et al., 2017; BHUNIA, 2018).

The virulence of *Salmonella* usually has been studied in mice, but there are some studies that used alternative infection models such as *Galleria mellonella* in which larvae are easily grown in large numbers and have components of the innate immune response similar to mammals, formed mainly by hemocytes and opsonins (TSAI; LOH; PROFT, 2016).

Whole genome sequencing is a powerful tool for assessing phylogenetic relationships, virulence, and antimicrobial resistance content, as well as providing information about the presence of plasmids, among other data in different bacterial genera (SHIVANI et al., 2015; BENEVIDES et al., 2017; ALMEIDA et al., 2018).

The aims of this study were to evaluate *S*. Typhimurium isolates from humans and foods in Brazil and their ability to invade Caco-2 epithelial cells, the ability to survive in U937 human macrophages, and to assess virulence in the *Galleria mellonella* infection model, and lastly to characterize the repertoire of virulence genes present through WGS.

MATERIALS AND METHODS

Bacterial strains

A total of 40 *S.* Typhimurium strains isolated from humans (n=20) and food (n=20) between 1983 to 2013 in Brazil were studied (Table 1). These isolates were selected from the

collections of the Adolfo Lutz Institute of Ribeirão Preto (IAL-RP) and of the Oswaldo Cruz Foundation from Rio de Janeiro (FIOCRUZ). *S.* Typhimurium SL1344 prototype strain was used as control in all experiments.

Table 1 - Characteristics of the 40 *Salmonella* Typhimurium strains sequenced and studied, isolated from different sources in Brazil between 1983 and 2013

Research Institute	Isolate name	CFSAN nº	GenBank nº	Source	Year of isolation	Sequence Type (ST)
IAL-RP	STm02	CFSAN033849	LVHB00000000	Human feces	1983	19
IAL-RP	STm06	CFSAN033853	LVGX00000000	Human feces	1983	1649
IAL-RP	STm11	CFSAN033858	LVGT00000000	Human feces	1984	19
IAL-RP	STm17	CFSAN033864	LVGO00000000	Human feces	1985	19
IAL-RP	STm23	CFSAN033870	LVGJ00000000	Human feces	1986	19
IAL-RP	STm27	CFSAN033874	LVGF00000000	Human feces	1986	19
IAL-RP	STm28	CFSAN033875	LUJE00000000	Human feces	1988	3343
IAL-RP	STm29	CFSAN033876	LVGE00000000	Human feces	1989	313
IAL-RP	STm30	CFSAN033877	LVGD00000000	Human feces	1990	313
IAL-RP	STm31	CFSAN033878	LUJD00000000	Human feces	1991	19
IAL-RP	STm33	CFSAN033880	LVGB00000000	Human feces	1992	19
IAL-RP	STm34	CFSAN033881	LVGA00000000	Human feces	1993	313
IAL-RP	STm35	CFSAN033882	LVFZ00000000	Human feces	1995	313
IAL-RP	STm36	CFSAN033883	LVFY00000000	Cold chicken	1995	19
IAL-RP	STm37	CFSAN033884	LVFX00000000	Raw pork sausage	1996	313
IAL-RP	STm38	CFSAN033885	LUJC00000000	Human feces	1997	19
IAL-RP	STm39	CFSAN033886	LUJB00000000	Human feces	1998	313
IAL-RP	STm40	CFSAN033887	LUJA00000000	Lettuce	1998	313
IAL-RP	STm41	CFSAN033888	LVFW00000000	Raw kafta	1998	19
IAL-RP	STm42	CFSAN033889	LUIZ00000000	Human feces	1999	19
IAL-RP	STm44	CFSAN033891	LVFU00000000	Blood	2000	313
IAL-RP	STm45	CFSAN033892	LUIY00000000	Raw pork sausage	2000	19
IAL-RP	STm46	CFSAN033893	LVFT00000000	Raw tuscan sausage	2002	19
IAL-RP	STm47	CFSAN033894	LUIX00000000	Human feces	2003	313
IAL-RP	STm48	CFSAN033895	LUIW00000000	Brain abscess	2005	19
IAL-RP	STm49	CFSAN033896	LVFS00000000	Human feces	2010	19
FIOCRUZ	702/99	CFSAN033897	LVFR00000000	Final product	1999	19
FIOCRUZ	12278/06	CFSAN033899	LUIU00000000	Swine	2006	19
FIOCRUZ	5937/06	CFSAN033904	LUIQ00000000	Cold chicken	2006	19
FIOCRUZ	13609/06	CFSAN033909	LUIM00000000	Poultry	2006	19
FIOCRUZ	3848/08	CFSAN033910	LUIL00000000	Food	2008	19
FIOCRUZ	16238/09	CFSAN033911	LUIK00000000	Ready-to-eat dish	2009	19
FIOCRUZ	16273/09	CFSAN033916	LVFN00000000	Industrialized product	2009	19
FIOCRUZ	6346/10	CFSAN033922	LUIC00000000	Chicken	2010	19
FIOCRUZ	9109/10	CFSAN033924	LVFK00000000	Swine	2010	19
FIOCRUZ	6709/11	CFSAN033928	LVFJ00000000	Cold chicken	2011	19
FIOCRUZ	948/12	CFSAN033929	LUHY00000000	Raw salad	2012	19
FIOCRUZ	3330/12	CFSAN033932	LUHW00000000	Roast beef	2012	19
FIOCRUZ	583/13	CFSAN033938	LUHR00000000	Final product sales (animal origin)	2013	19
FIOCRUZ	623/13	CFSAN033939	LVFH00000000	Final product sales (animal origin)	2013	1921

Whole genome sequencing

Whole genome sequencing of the 40 *S.* Typhimurium isolates (Table 1) was performed on the NextSeq platform (Illumina) at the U.S. Food and Drug Administration (FDA), College Park, MD, USA. The genomes were assembled using the software SPAdes (BANKEVICH et

al., 2012) and the quality of the assemblies were evaluated using the software QUAST (GUREVICH et al., 2013) as described in Almeida et al. (2018).

Invasion assay in Caco-2 epithelial cells and survival assay in U937 human macrophages

These assays were performed for all 40 *S*. Typhimurium isolates (Table 1) and for the SL1344 prototype strain according to (FINLAY; RUSCHKOWSKI; DEDHAR, 1991; FIERER et al., 1993; PFEIFER et al., 1999; MOREIRA; WEINSHENKER; SPERANDIO, 2010).

Initially, the Caco-2 epithelial cells were cultured in DMEM medium (Dulbecco's Modified Eagle Medium - Gibco - low glucose) supplemented with 10% fetal bovine serum (Life Technologies) and antibiotic in 5% CO_2 at 37°C. In addition, $1x10^5$ cells were added to each well of a 12-well microplate. The assay was performed after 12 days of incubation until the cells were polarized and differentiated.

The monocytes were cultured in suspension in RPMI medium (1640 - powder - Gibco) supplemented with 10% fetal bovine serum (Life Technologies) and antibiotic in 5% CO_2 at 37°C. In addition, $1x10^5$ cells were added to each well of a 24-well microplate. For the differentiation of monocytes into macrophages, 1µl of phorbol 12-myristate-13-acetate (PMA) (Sigma-Aldrich) was used in 50 mL of the RPMI medium (50 ng/mL) and maintained in 5% CO_2 at 37°C for 48h. All the *S*. Typhimurium strains were opsonized with 20% mouse serum (Sigma-Aldrich) at 37°C for 15 min after three washes with PBS (centrifuged at 12000 × rpm for 1 min).

The invasiveness in Caco-2 epithelial cells of these strains was performed after 90 minutes of bacteria-cell interaction with intraepithelial survival during 3 hours. In addition, the survival in U937 human macrophages was performed after 30 minutes of bacteria-cell interaction with intramacrophage survival during 3 hours and in both assays the (multiplicity of infection) MOI was 100:1.

Serial dilutions were performed and plated in LB agar medium plates with incubation during 18-24 hours at 37°C for later counting of colony forming units (CFU). The experiments were carried out in biological triplicate and in all plates there was a negative control with only cells in the wells.

Virulence analysis in *Galleria mellonella*

The analysis it was performed according to (Renwick et al., 2006) - adapted in *Galleria mellonella*. The larvae were maintained at 28°C in the dark in glass containers (30 cm height - 20 cm wide - 2 L capacity) with appropriate oxygen and access to food until reaching the sixth instar, whose weight is between 200 mg and 250 mg. After complete development, the larvae were deprived of food and separated into groups of 10 units in glass Petri dishes for each bacterial isolate and controls.

A Hamilton micro-syringe (model 7000.5KH of 10 µL) was used for artificial inoculation of *G. mellonella* larvae into the center of the last right pro-leg with 5 µL of *S.* Typhimurium (10^5 CFU/mL) for each of the40 isolates studied and for the positive control infected with *S.* Typhimurium strain ATCC14028. The negative control was inoculated with PBS. After inoculation, the larvae were incubated at 37°C, deprived of food and direct light. During the experimental period of 7 days the larvae were removed every 24 hours of the pre-pupae, in order to delay their metamorphosis with data recorded daily.

Virulence gene characterization

The virulence genes were identified for all isolates using the Virulence Factors Database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm). Bacteria *Salmonella* was assessed with BLAST using a threshold of ≥70% identity, and ≥70% coverage comparing with *S. enterica* subsp. *enterica* serovar Typhimurium str. LT2, 4857432 bp, NC_003197, and plasmid *S. enterica* subsp. *enterica* serovar Typhimurium str. LT2 pSLT, 93939 bp, NC_003277 (CHEN et al., 2005).

The presence of plasmids were determined using PlasmidFinder (Center for Genomic Epidemiology, https://cge.cbs.dtu.dk/services/PlasmidFinder/) with a threshold set for a minimum of 95% identity, and minimum coverage of 60% (CARATTOLI et al., 2014). BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm the location and percentages of identity, and coverage between the *S. enterica* subsp. *enterica* serovar Typhimurium str. LT2 pSLT, 93939 bp, NC_003277 reference sequences of the pSLT plasmid, and the *spvABCDR*, *pefABCD*, *rck* and *mig-5* genes of the positive and negative genomes for IncFIIs.

Statistical analyses

The comparisons between the means of the virulence tests were performed using Student's t-test for two means and two-way analysis of variance (ANOVA) with *post hoc*

Tukey test for more than two means in the Minitab® statistical software (version 18.1). The analysis of the virulence genes identification was performed using two proportion z-test in the Minitab® statistical software (version 18.1) comparing the difference in isolates proportion which contained genes of interest. The graphics and statistical analyses of the virulence assay in *Galleria mellonella* were performed using the Log-rank (Mantel-Cox) method, both in the Prism5 program for Windows of the GraphPad® software (version 5.01). For all analyses, the level of significance was $\alpha = 5\%$.

RESULTS

Caco-2 epithelial cells invasion assay

A total of 67.5% of the 40 *S*. Typhimurium isolates studied (32.5% isolated from humans and 35% isolated from food) invaded the epithelial cells at various levels compared to the SL1344 reference (Fig. 1). Considering all the isolates studied the invasion in Caco-2 cells ranged from 1×10^5 to 1×10^7 CFU/mL. From the statistical analysis of the Student's t-test, three subgroups were found comprising: isolates that invaded more (black) (35%), equal (grey) (32.5%) or less (white) (32.5%) than the *S*. Typhimurium SL1344 isolate. The bidirectional analysis of variance (ANOVA) categorized the 14 *S*. Typhimurium isolates that invaded more than the SL1344 reference in groups from A to G. In addition, ANOVA also categorized the 13 *S*. Typhimurium isolates that invaded less than the SL1344 reference in groups from A to C (Fig. 1).

Survival assay in U937 human macrophages

A total of 37.5% of the 40 *S*. Typhimurium isolates studied (25% isolated from humans and 12.5% isolated from food) survived in the human macrophages at various levels compared to the SL1344 reference (Fig. 2). All the isolates studied survived in U937 human macrophages and ranged from 1×10^6 to 1×10^7 CFU/mL. From the statistical analysis of Student's t-test, three subgroups were formed comprising: isolates that survived more (black) (27.5%), equal (grey) (10%) or less (white) (62.5%) than the *S*. Typhimurium SL1344 reference. The bidirectional analysis of variance (ANOVA) categorized the 11 *S*. Typhimurium isolates that survived more than the SL1344 reference in groups from A to E. In addition, ANOVA also categorized the 25 *S*. Typhimurium isolates that survived less than the SL134 reference in groups from A to K (Fig. 2).

Fig. 1 - Invasion assay in Caco-2 epithelial cells for the 40 *S*. Typhimurium strains isolated from humans and foods and for the *S*. Typhimurium SL1344 reference. The means followed by different letters differ statistically from each other at a level of 5% significance. The error bars represent standard deviation of biological triplicate.

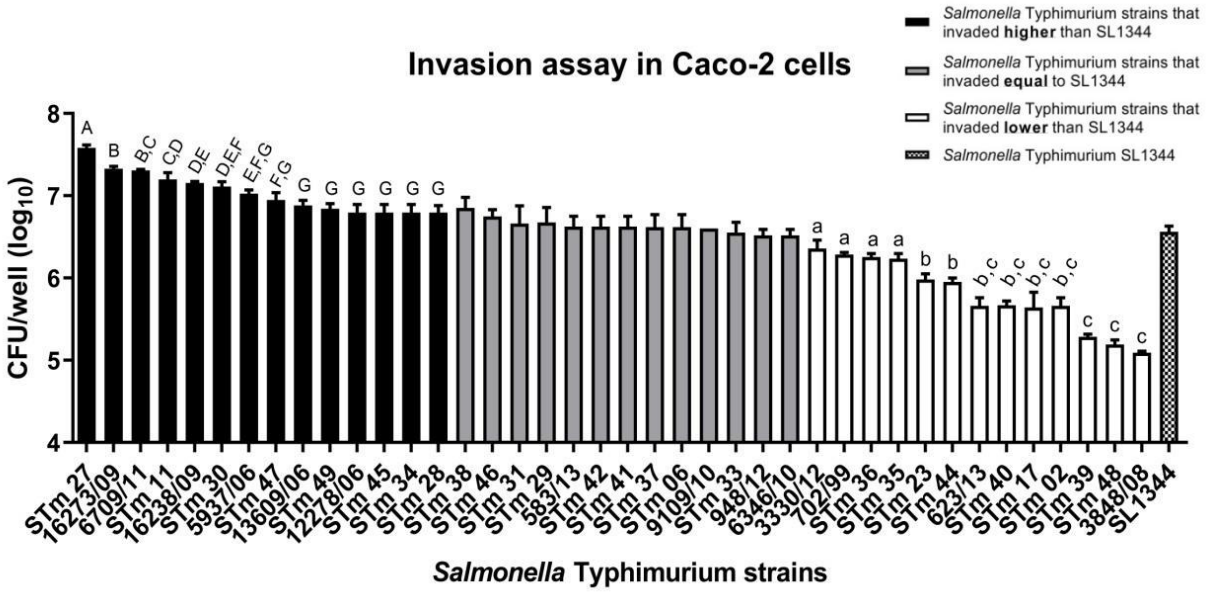
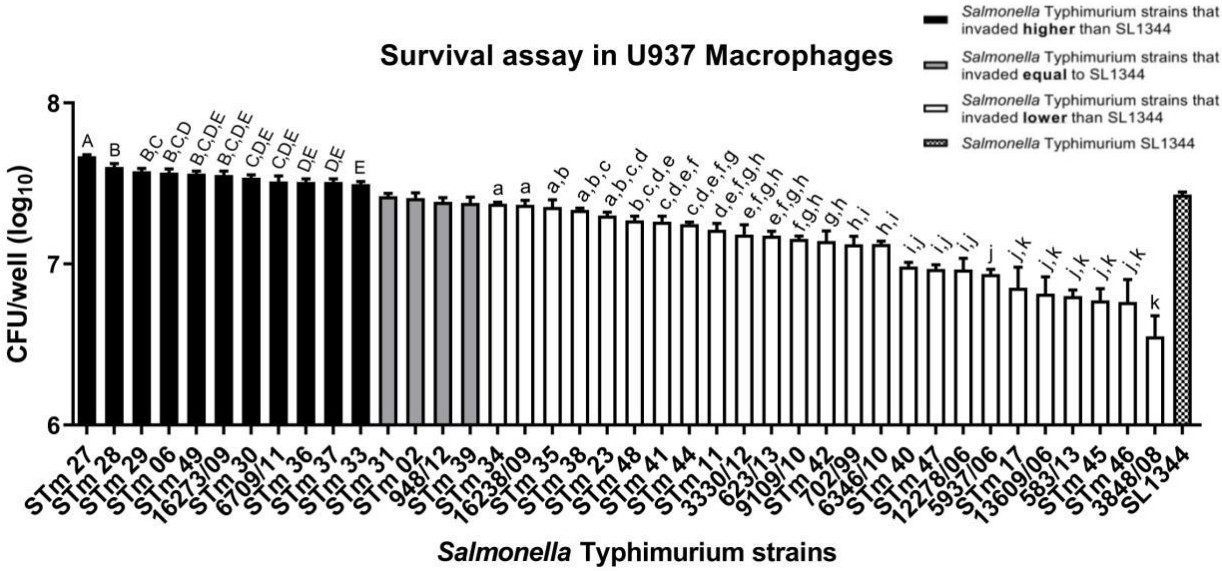


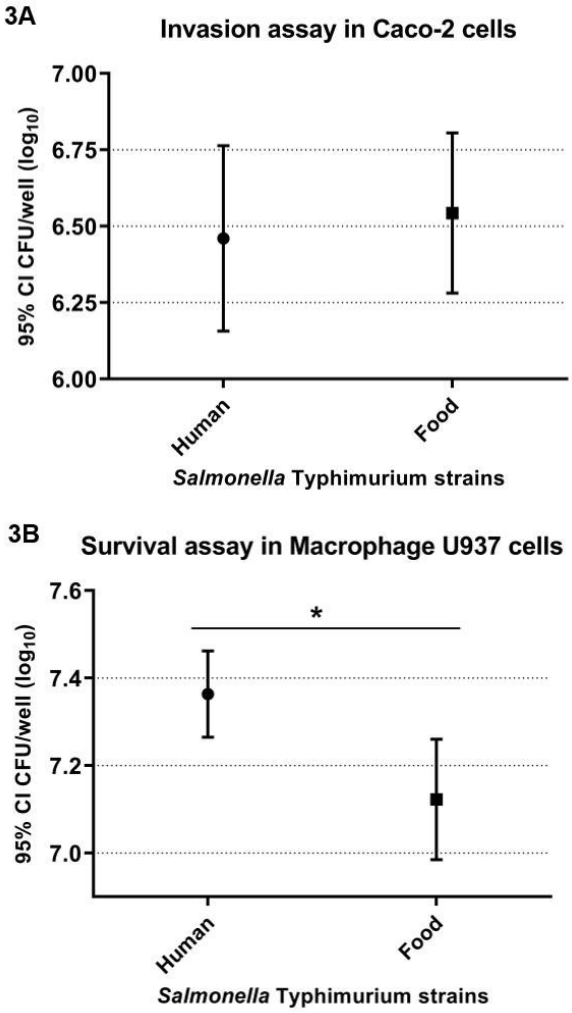
Fig. 2 - Survival assay in U937 human macrophages for the 40 *S*. Typhimurium strains isolated from humans and foods and for the *S*. Typhimurium SL1344 reference. The means followed by different letters differ statistically from each other at a level of 5% significance. The error bars represent standard deviation of biological triplicate.



Comparison between sources after invasion assay in Caco-2 epithelial cells and survival assay in U937 human macrophages

In both assays, *S*. Typhimurium isolates were classified with invasion and survival levels similar, greater or lower than the SL1344 reference with the mean of CFU/well (Log_{10}). Invasion in Caco-2 cells of 6.46 and 6.54 CFU/well (Log_{10}) were observed for isolates from humans and foods, respectively (Fig. 3A). Furthermore, the survival in U937 human macrophages was 7.36 and 7.12 CFU/well (Log_{10}) for humans and food isolates, respectively (Fig. 3B). Therefore, no evidence of statistically significant differences were found between isolates from humans and foods for invasion in Caco-2 cells. However, statistically significant differences were found between isolates from humans and foods for survival in U937 human macrophages.

Fig. 3 - Comparison among the means of CFU/well (log10) with 95% confidence interval (CI) of *Salmonella* Typhimurium strains isolated from humans and foods after the assays of Caco-2 epithelial cell invasion (A) and U937 human macrophages survival (B).



Virulence analysis in *Galleria mellonella*

The results of the *S.* Typhimurium studied isolates are presented separately in Fig. 4 and Fig. 5, respectively. In Fig. 4, the ATCC14028 control strain isolated from chickens showed high virulence (black circle) killing 100% of the larvae. For human isolates, four groups were formed after seven days of experiments. The STm48, STm29, STm34 and STm38 isolates killed 70-90% of the larvae, forming the group of virulent isolates (red shapes). The intermediate virulence group (orange shapes) killed between 30-50% of the larvae, comprised of STm39, STm33, STm31 and STm49 isolates. The STm35, STm42, STm06, STm23, ST28, STm30 and STm44 isolates formed the group of low virulence (green shapes) killing between 10-20% of the larvae. Finally, the avirulent group (white circle) comprised of STm02, STm11, STm17, STm27, STm47 isolates and the negative control did not kill any larvae (Fig. 4).

In Fig. 5, four groups were formed after seven days of experiment for isolates from food. The 583/13 isolate and the positive control ATCC14028 showed a high virulence killing 100% of the larvae (black circle). The 702/99, 9109/10, 16273/09 and 948/12 isolates killed between 50-60% of the larvae, forming a group of intermediate virulence (orange shapes). The 12278/06, 13609/06, 16238/09, 3330/12, 623/13, STm37, STm40, STm45, 6346/10 and 6709/11 isolates formed a group of low virulence (green shapes) killing 10-20% of the larvae. Finally, the avirulent group (white circle) comprised of isolates STm36, STm46, 5937/06, 3848/08 and the negative control did not kill any larvae. Thus, there were fewer virulent isolates identified from foods compared to humans (Fig. 4 and Fig. 5). Food isolate 583/13 was highly virulent and more so than any strain isolated from humans studied here in (Fig.5).

The STm41 isolate was not classified into either the intermediate or low virulence groups because its measurement fell between the two groups (Fig. 5).

Comparing the mortality of the isolates belonging to the same group (human and food) no significant difference was observed. On the other hand, significant difference was found among the different groups mentioned above, except for the groups of low virulence (green shapes) and avirulent (white circle). These evidences were found by comparing representatives of each group.

Fig. 4 - Survival percentages of *Galleria mellonella* larvae infected with 20 *Salmonella* Typhimurium isolates from humans in Brazil after seven days.

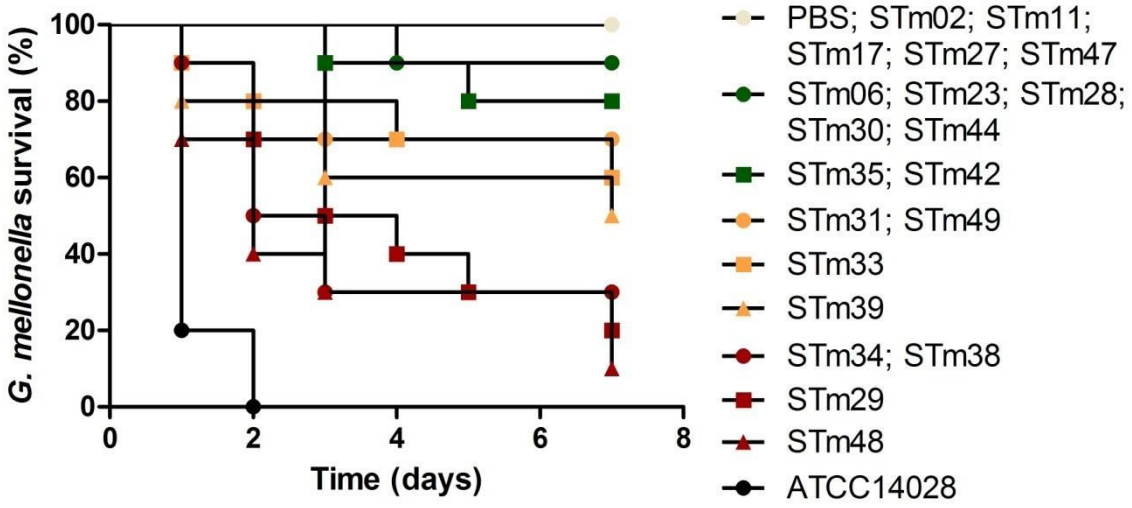
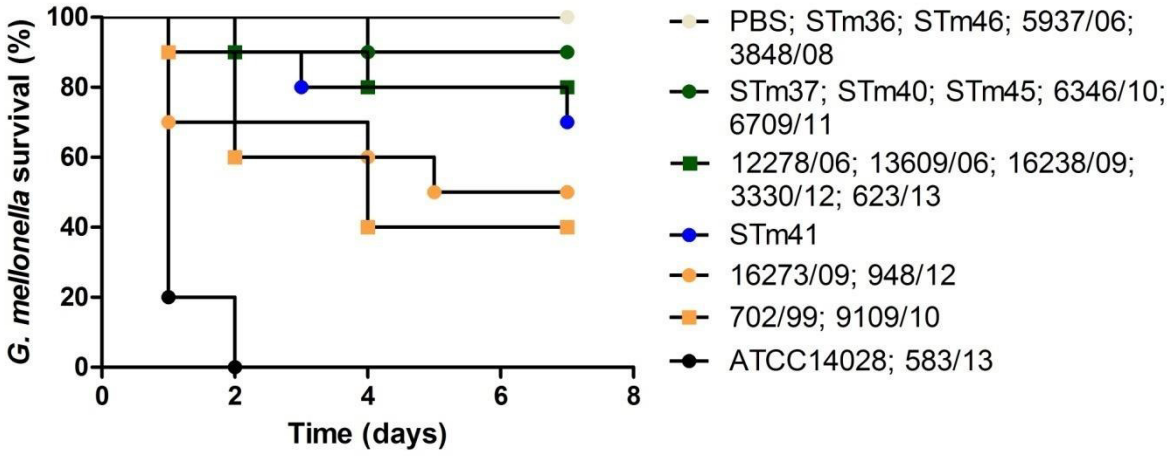


Fig. 5 - Survival percentages of *Galleria mellonella* larvae infected with 20 *Salmonella* Typhimurium isolates from foods in Brazil after seven days.



Genetic characterization focused on virulence genes

Genetic characterization focused on discovering virulence genes using the Virulence Factors Database (VFDB) which detected 153 genes related to invasion, survival, colonization, fimbriae and flagella production, among others for all isolates studied (Table 2).

The similarity rate varied between 87% and 100% of identity for all strains with coverage between 78% and 100% (Table 2). The *spvABCDR* operon, *pefABCD* operon, *rck* and *mig-5* genes were detected in 13 *S*. Typhimurium isolates from humans and 9 isolates from foods (Table 3). In order to confirm the location of genes, Plasmid Finder was used to document the presence of the IncFIIs plasmid incompatibility group, which belongs to the pSLT plasmid (Table 4). Independent, BLASTn analysis for all isolates confirmed the presence of the pSLT plasmid carrying the *spvABCDR* operon, *pefABCD* operon, *rck* and *mig-5* genes for 22 *S*. Typhimurium isolates with coverage of 100% and 99-100% of identity (Table 4). The other studied isolates did not present IncFIIs, pSLT plasmid, *spvABCDR* operon, *pefABCD* operon, *rck* and *mig-5* genes.

Table 2 - Proportion of the detection of virulence genes in *Salmonella* Typhimurium strains isolated from humans (n=20) and food (n=20) in Brazil

Gene	Proportion of isolates	Query cover (%)	Identity (%)	Gene	Proportion of isolates	Query cover (%)	Identity (%)	Gene	Proportion of isolates	Query cover (%)	Identity (%)	Gene	Proportion of isolates	Query cover (%)	Identity (%)
csgA	40/40	100	100	*invA*	40/40	100	100	*orf32*	40/40	100	100	*sseG*	40/40	100	100
csgB	40/40	100	100	*invB*	40/40	100	100	*orf48*	40/40	100	100	*sseJ*	40/40	100	100
csgC	40/40	100	100	*invC*	40/40	100	100	*orf70*	40/40	100	98-100	*sspH2*	39/40	100	87-100
csgE	40/40	100	99-100	*invE*	40/40	100	100	*orf242*	40/40	100	99-100	*ssrA*	40/40	100	99-100
csgF	40/40	100	100	*invF*	40/40	86	100	*orf245*	40/40	100	100	*ssrB*	40/40	100	100
csgG	40/40	100	99-100	*invG*	40/40	100	100	*orf319*	40/40	100	100	*ttrA*	39/40	100	99-100
fimA	40/40	100	100	*invH*	40/40	100	100	*orf408*	40/40	100	100	*ttrB*	40/40	100	100
fimC	40/40	97-100	100	*invI*	40/40	100	100	*pykF*	40/40	100	100	*ttrC*	40/40	100	99-100
fimD	40/40	100	100	*invJ*	40/40	100	100	*sifA*	40/40	100	100	*ttrR*	40/40	100	100
fimF	40/40	100	100	*orgB*	40/40	100	99-100	*spiC/ssaB*	40/40	100	99-100	*ttrS*	40/40	100	99-100
fimH	40/40	100	99-100	*prgH*	40/40	95	100	*ssaC*	40/40	100	100	**SPI-3**			
fimI	40/40	100	100	*prgI*	40/40	100	100	*ssaD*	38/40	100	99-100	*cigR*	39/40	90	99-100
fimW	40/40	100	100	*prgJ*	40/40	100	99-100	*ssaE*	40/40	100	100	*fidL*	40/40	96-100	100
fimY	40/40	100	100	*prgK*	40/40	100	99-100	*ssaG*	40/40	100	100	*marT*	40/40	100	100
fimZ	40/40	94-100	99-100	*sicA*	40/40	100	100	*ssaH*	40/40	78	100	*mgtB*	40/40	100	100
fur	40/40	100	100	*sicP*	40/40	99-100	10	*ssaI*	40/40	94	98-100	*mgtC*	40/40	100	100
lpfA	40/40	100	100	*sipA/sspA*	40/40	100	99-100	*ssaJ*	40/40	100	100	*misL*	40/40	100	100; 96
lpfB	40/40	100	99-100	*sipB/sspB*	40/40	100	99-100	*ssaK*	40/40	100	100	*slsA*	40/40	99-100	100
lpfC	39/40	100	99-100	*sipC/sspC*	40/40	100	100	*ssaL*	40/40	100	100	*sugR*	40/40	100	100
lpfD	40/40	100	100	*sipD*	40/40	100	100	*ssaM*	40/40	100	99-100	*rhuM*	40/40	100	100
lpfE	40/40	100	100	*sitA*	39/40	100	100	*ssaN*	40/40	100	99-100	*rmbA*	40/40	100	100
mig-14	40/40	96-100	100	*sitB*	39/40	100	100	*ssaO*	40/40	100	99-100	**SPI-4**			
phoP	40/40	100	99-100	*sitC*	39/40	100	100	*ssaP*	40/40	100	99-100	*siiE*	16/40	100	99-100
phoQ	40/40	100	99-100	*sitD*	39/40	100	100	*ssaQ*	40/40	100	100	*soxR*	40/40	100	100
ratB	40/40	98-100	99-100	*slrP*	40/40	100	99-100	*ssaR*	40/40	100	100	*soxS*	40/40	100	100
rpoS	40/40	96-100	100	*sopA*	40/40	100	99-100	*ssaS*	40/40	100	100	*ssb*	40/40	100	100
shdA	12/40	99	88-90	*sopB/sigD*	40/40	99-100	100	*ssaT*	40/40	100	100	*yjcB*	40/40	100	100
sinH	40/40	100	100	*sopD*	40/40	88	100	*ssaU*	40/40	100	99-100	*yjcC*	40/40	100	100
sodCI	40/40	100	100	*sopE2*	40/40	88-100	100	*ssaV*	40/40	100	100	**SPI-5**			
SPI-1				*spaO*	40/40	100	100	*sscA*	40/40	100	99-100	*copR*	40/40	100	100
avrA	38/40	90-100	95-100	*spaP*	40/40	100	99-100	*sscB*	40/40	100	99-100	*copS*	40/40	100	100
fhlA	39/40	93-100	99-100	*spaQ*	40/40	100	100	*sseA*	40/40	100	100	*orfX*	31/40	100	97
hilA	40/40	100	100	*spaR*	40/40	100	100	*sseB*	40/40	100	99-100	*pipA*	40/40	97-100	100
hilC	39/40	100	99-100	*spaS*	40/40	100	99-100	*sseC*	40/40	100	99-100	*pipB*	40/40	100	100
hilD	39/40	100	99-100	*sprB*	40/40	100	100	*sseD*	40/40	100	99-100	*pipC*	40/40	88-100	100
iagB	40/40	100	100	*sptP*	40/40	98-100	99-100	*sseE*	40/40	100	99-100	*pipD*	40/40	83-94	100
iacP	40/40	100	100	**SPI-2**				*sseF*	40/40	100	100				

Table 3 – Characteristics of the plasmid genes of 22 *Salmonella* Typhimurium isolates that presented these genes by Virulence factors database (VFDB)

Gene	Human			Food		
	Proportion of isolates	Query cover (%)	Identity (%)	Proportion of isolates	Query cover (%)	Identity (%)
spvA	13/20	100	100	9/20	100	100
spvB	13/20	100	100	9/20	100	100
spvC	13/20	100	100	9/20	100	100
spvD	13/20	100	99-100	9/20	100	100
spvR	13/20	100	100	9/20	100	100
pefA	13/20	100	100	9/20	100	100
pefB	13/20	100	100	9/20	100	100
pefC	13/20	100	99-100	9/20	100	99-100
pefD	13/20	100	100	9/20	100	100
rck	13/20	100	100	9/20	100	100
mig-5	13/20	100	100	9/20	100	100

Table 4 – PlasmidFinder and BLASTn of the 22 *Salmonella* Typhimurium isolates that presented the plasmid genes studied

Isolate name	CFSAN nº	IncF plasmid (Identity %)	pSLT (Query cover %) (Identity %)	*spvABCDR* (Query cover %) (Identity %)	*pefABCD* (Query cover %) (Identity %)	*mig-5* (Query cover %) (Identity %)
STm06	CFSAN033853	IncFIIs (100)	(99) (99.97)	(100) (100)	(100) (99.74)	(100) (99.70)
STm11	CFSAN033858	IncFIIs (100)	(99) (99.91)	(100) (100)	(100) (99.81)	(100) (99.70)
STm27	CFSAN033874	IncFIIs (100)	(99) (99.90)	(100) (100)	(100) (99.81)	(100) (99.70)
STm29	CFSAN033876	IncFIIs (100)	(99) (99.92)	(100) (100)	(100) (99.77)	(100) (99.70)
STm30	CFSAN033877	IncFIIs (100)	(99) (99.93)	(100) (100)	(100) (99.77)	(100) (99.70)
STm31	CFSAN033878	IncFIIs (100)	(99) (99.94)	(100) (99.94)	(100) (99.81)	(100) (99.70)
STm33	CFSAN033880	IncFIIs (100)	(99) (99.93)	(100) (99.94)	(100) (99.81)	(100) (99.70)
STm34	CFSAN033881	IncFIIs (100)	(99) (99.93)	(100) (100)	(100) (99.77)	(100) (99.70)
STm35	CFSAN033882	IncFIIs (100)	(99) (99.93)	(100) (100)	(100) (99.77)	(100) (99.70)
STm36	CFSAN033883	IncFIIs (100)	(99) (99.92)	(100) (99.66)	(100) (99.81)	(100) (99.70)
STm37	CFSAN033884	IncFIIs (100)	(99) (99.94)	(100) (100)	(100) (99.77)	(100) (99.70)
STm38	CFSAN033885	IncFIIs (100)	(99) (99.98)	(100) (100)	(100) (99.71)	(100) (99.70)
STm39	CFSAN033886	IncFIIs (100)	(99) (99.93)	(100) (100)	(100) (99.77)	(100) (99.70)
STm40	CFSAN033887	IncFIIs (100)	(99) (99.92)	(100) (99.94)	(100) (99.77)	(100) (99.70)
STm44	CFSAN033891	IncFIIs (100)	(99) (99.94)	(100) (100)	(100) (99.77)	(100) (99.70)
STm47	CFSAN033894	IncFIIs (100)	(99) (99.94)	(100) (100)	(100) (99.77)	(100) (99.70)
13609/06	CFSAN033909	IncFIIs (100)	(99) (99.96)	(100) (100)	(100) (99.74)	(100) (99.56)
16273/09	CFSAN033916	IncFIIs (100)	(99) (99.96)	(100) (100)	(100) (99.71)	(100) (99.74)
6346/10	CFSAN033922	IncFIIs (100)	(99) (99.90)	(100) (100)	(100) (99.77)	(100) (99.65)
9109/10	CFSAN033924	IncFIIs (100)	(99) (99.96)	(100) (100)	(100) (99.74)	(100) (99.70)
948/12	CFSAN033929	IncFIIs (100)	(99) (99.97)	(100) (100)	(100) (99.74)	(100) (99.65)
3330/12	CFSAN033932	IncFIIs (100)	(99) (99.95)	(100) (99.94)	(100) (99.74)	(100) (99.61)

The *rck* gene was detected for all isolates with query cover and identity of 100%.

DISCUSSION

In this study, 40 S. Typhimurium isolates from humans (n=20) and foods (n=20) in Brazil were compared after invasion assays in Caco-2 epithelial cells, survival assays in U937 human macrophages, *Galleria mellonella* assays and virulence gene analysis.

S. Typhimurium being considered a generalist serovar has been documented to infect several hosts including humans, cattle, pigs, sheep, horses, rodents, chickens, turkeys, ducks, pigeons, and birds (RABSCH et al., 2002; FERRARI et al., 2019). This serovar invades host cells through the Type III Secretion System (T3SS) where the genes are mainly located in pathogenicity island 1 and 2 (GALÁN, 2001; FIGUEIRA and HOLDEN, 2012; SUN et al., 2016).

Among the 40 *S*. Typhimurium isolates studied, from humans and foods many invaded the epithelial cells at similar or higher levels compared to the SL1344 reference control. Furthermore, analysis of variance (ANOVA) was performed for all *S*. Typhimurium isolates that differed from the SL1344 reference (Fig. 1). By this analysis three main groups were formed and the source and/or year of isolation did not correlate with the observed virulence profiles. Therefore, these results reinforce that the ability for *S*. Typhimurium to invade is probably isolate dependent and not related to the source or the year of isolation (Fig. 3A).

During the infection process, host neutrophils and macrophages try to control invasion by generating reactive oxygen species (ROS). The degranulation of these cells occurs in a process called respiratory burst (WESTERMAN et al., 2018).

S. Typhimurium has the ability to infect epithelial cells and macrophages in the small intestine, replicating in a niche called *Salmonella*-containing vacuole (SCV) and consequently triggering an inflammatory process culminating in gastroenteritis (WEMYSS and PEARSON, 2019). It is important to emphasize that in the present study, among the 40 *S*. Typhimurium strains studied, from humans and food survived at various levels compared to SL1344 control. In addition, analysis of variance (ANOVA) was performed for all *S*. Typhimurium strains that differed from the SL1344 control (Fig. 2). Three main groups were formed by this analysis and statistical significance was observed among the profiles, suggesting that *S*. Typhimurium isolates from humans survive more intramacrophage than isolates from food (Fig. 3B).

Among the possible causes of this difference we highlight a lesser dispersion of the survival assay data in U937 human macrophages and subtle differences in the genetic characterization for some important virulence genes.

The virulence assay in *Galleria mellonella* larvae divided the 40 *S.* Typhimurium isolates into four groups according to their virulence profiles (Fig. 4 and Fig. 5). It is

important to emphasize that there was a higher proportion (60%) of strains isolated from humans that demonstrated a virulent profile in comparison to strains isolated from foods. Therefore, this result may suggest that the *S.* Typhimurium studied isolated from humans were more virulent than strains isolated from food in Brazil according to the *G. mellonella* infection model.

G. mellonella larvae are easily grown in large numbers at low costs and produce innate immune response components very similar to humans composed by hemocytes and opsonins (TSAI; LOH; PROFT, 2016). A limitation of this infection model is that insects do not present the second line of defense characterized by an adaptive immune response formed by antibody-producing and memory cells (TSAI; LOH; PROFT, 2016).

It is important to mention that the melanization of insects such as *G. mellonella* occurs when it is infected by a pathogen, followed by melanin synthesis and deposition of this substance to encapsulate the infectious agents at the inoculation site (TANG, 2009; TSAI; LOH; PROFT, 2016). Therefore, this process is stimulated by the presence of bacteria and fungi, initiating a serine protease cascade responsible for the activation of phenoloxidase that catalyzes the formation of melanin ((TANG, 2009; TSAI; LOH; PROFT, 2016).

The genetic repertoire research focused in virulence genes by WGS revealed that the isolates regardless of the source were very similar. Moreover, several essential genes for the pathogenesis of salmonellosis were identified in this study with high identity and coverage reinforcing the pathogenic potential of these strains. It is important to mention that the ability of *S.* Typhimurium strains to invade and to survive in the host cells is closely linked to virulence genes present in the bacterium (SANTOS; FERRARI; CONTE-JUNIOR, 2019).

The *invG*, *invH*, *prgH*, *prgK*, *prgI*, *prgJ* and *iagB* genes are found in the SPI-1 of *Salmonella* spp. and are involved with the formation of the basal body of the T3SS [31]. The export apparatus of the T3SS are encoded by the *spaS*, *spaP*, *spaQ*, *spaR* and *invA* genes present in the SPI-1. Furthermore, the *spaO*, *invC*, *invI*, *orgB*, *invJ*, *invE*, *sipC*, *sipB* and *sipD* genes also are present in the SPI-1 and related to cytoplasmic ring, ATPase complex, regulation and translocation of the T3SS (DENG et al., 2017).

According to Deng and collaborators (2017), the *ssaC, ssaD, ssaJ, ssaG, ssaI, ssaU, ssaV, ssaR, ssaS, ssaT, ssaQ, ssaN, ssaK, ssaO, ssaP, ssaL, spiC, sseD, sseC and sseB* genes also encode proteins related to basal body, export apparatus, cytoplasmic ring, ATPase complex, regulation and translocation of the T3SS and are located in the SPI-2 de *Salmonella* spp (DENG et al., 2017).

All the genes mentioned above were found in the *S*. Typhimurium genomes of this study, suggesting that although these strains belong to collections from different years the essential genes for the injection of T3SS effectors proteins have been preserved (Table 2).

Moreover, genes present in the SPI-3, SPI4 and SPI-5 are related to virulence of *Salmonella* spp. However, more studies are needed in this area, due to the lack of theoretical and scientific information (SANTOS; FERRARI; CONTE-JUNIOR, 2019). For example, the *pip* (*pipA*, *pipB*, *pipC* and *pipD*) genes may be related to the rate of fluid secretion and inflammatory response during salmonellosis, suggesting that such genes are related to the bacterial enteropathogenicity, but the exact mechanism has not been yet fully elucidated (WOOD et al., 1998; SANTOS; FERRARI; CONTE-JUNIOR, 2019). Plasmids are known to be essential for the resistance and virulence of different bacteria, the IncF plasmid incompatibility group is heterogeneous and often described in enterobacteria (VILLA et al., 2010). The virulence plasmid (pSLT) which belongs to the IncFIIs plasmid incompatibility group has been reported in *S*. Typhimurium and carries important genes for the pathogenesis of this serovar (HILEY; GRAHAM; JENNISON, 2019). Among the content of the plasmid pSLT can highlight the following genes: *spvABCDR*, *pefABCD*, *rck* and *mig-5*.

The *spv* operon (*Salmonella* plasmid virulence) which is formed of five genes (*spvA, spvB, spvC, spvD* and *spvR*) has been associated with *Salmonella* spp. survival and multiplication in macrophages (RYCHLIK; GREGOROVA; HRADECKA, 2006). In addition, the *pef* fimbrial operon (plasmid encoded fimbriae) is responsible for the adhesion of *Salmonella* spp. to intestinal epithelium in infant mouse resulting in fluid accumulation and is consisted by four genes (*pefA*, *pefB*, *pefC* and *pefD*) (BAUMLER and HEFFRON, 1995; LEDEBOER et al., 2006).

It is important to emphasize that the *rck* (resistance to complement killing) and *mig-5* (macrophage inducible gene coding for putative carbonic anhydrase) plasmid genes have been associated to the resistance of *S*. Typhimurium strains to the host complement system which would cause bacterial cell disruption and related to neutralization of toxic compounds produced by macrophages after phagocytosis, respectively (RYCHLIK; GREGOROVA; HRADECKA, 2006).

Interestingly, plasmid genes (*spvABCDR*, *pefABCD, rck* and *mig-5*) were detected in the present work in equal numbers and its location was confirmed in the plasmid pSLT, being that the *S*. Typhimurium strains isolated from humans and food studied had all the plasmid genes mentioned previously or none (Table 3 and 4). According to Kuijpers and collaborators

(2019), the *spvABCDR*, *pefABCD*, *rck* and *mig-5* plasmids genes were detected only in the human cases associated with serovars Typhimurium and Enteritidis (KUIJPERS et al., 2018).

The predominant sequence type (ST) of *S*. Typhimurium from fecal samples has been ST19 worldwide. However, ST313 has caused a significant mortality rate in sub-Saharan Africa and has been described in recent years in Brazil (ALMEIDA et al., 2017a). In the present study, all the ST313 isolates presented the plasmid pSLT carrying the *spvABCDR* operon, *pefABCD* operon, *rck* and *mig-5* genes showing a high genomic similarity among each other regardless of the isolation source as previously described (SERIBELLI et al., 2019).

Considering that the *S*. Typhimurium strains isolated from humans survived more in U937 human macrophages than strains isolated from food in the present study, it is important to emphasize that a complex response like survival in human macrophages probably is not triggered for one or a few genes, highlighting specifically the importance of plasmidial gene groups. In addition, despite the high similarity of the genes present among the isolates studied, a difference was observed in the proportion of human isolates which contained the plasmid gene group *spvA, spvB, spvC, spvD*, *spvR*, *pefA*, *pefB*, *pefC*, *pefD*, *rck* and *mig-5* between *S*. Typhimurium strains isolated from humans and foods.

Therefore, a statistically significant difference was found (p-value = 0.001) suggesting that a higher proportion of human isolates which contained these genes may contribute to their greater survival in U937 human macrophages. It is important to mention that this plasmid gene group was the only one that showed the same pattern of presence/absence among the isolates. Furthermore, it was also the only group in which p-value differed statistically, calculated using both the normal approximation method and the Fisher's exact method, that latter tends to be more conservative, reinforcing the statistical significant difference.

Similarly, among 9 (69%) of the 13 *S*. Typhimurium isolates from humans and foods that had a virulent profile (high and intermediate virulence) in *G. mellonella*, the plasmid gene group was detected reinforcing their importance in the virulence of these isolates.

Finally, many other genes were detected in the *S*. Typhimurium genomes in the present study, specifically genes related to the fimbriae production, such as the *fim* (f*imA*, *fimC*, *fimD, fimF, fimH, fimI, fimW, fimY* and *fimZ*) genes, which encode structural subunits and fimbrial proteins. The *lpf* (*lpfA, lpfB, lpfC, lpfD* and *lpfE*) fimbrial genes have been described as important in the intestinal colonization in murine mucosa (BAUMLER and HEFFRON, 1995; LEDEBOER et al., 2006). Moreover, no relationship was found between

the isolates proportion that presented these researched chromosomal genes suggesting that the different profiles found in the invasion assay in Caco-2 epithelial cells and in the survival assay in U937 human macrophages can be linked to the expression of such genes.

In conclusion, a significant percentage of *S*. Typhimurium isolates from humans and foods showed high invasion in Caco-2 epithelial cells regardless of the source suggesting that the invasiveness to Caco-2 cells is probably isolated dependent and not related to the source or the year of isolation. However, *S*. Typhimurium isolates from humans showed greater survival rate in U937 human macrophages and higher proportion of isolates with a virulent profile in *Galleria mellonella* than isolates from foods suggesting that this difference may be related to the higher proportion of human isolates which contained plasmid genes, such as *spvABCDR*, *pefABCD*, *rck* and *mig-5*. Moreover, several virulence genes present in the pathogenicity islands 1, 2, 3, 4, 5 were detected in the *S*. Typhimurium isolates from humans and foods studied reinforcing the virulence of this important serovar independent of their clinical or non-human origin.

Altogether, the results obtained in this work contributed for a better characterization of *S*. Typhimurium isolates from humans and foods in Brazil over decades regarding its ability to invade Caco-2 epithelial cells, to survive in U937 human macrophages, virulence, and pathogenic potential.

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CHAPTER 4

Phylogenetic relationship and frequency of pathogenicity islands characterized by whole genome sequencing of *Salmonella* Typhimurium strains isolated from swine in Brazil

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KEYWORDS: Whole genome sequencing; swine; phylogenetic analyses; *Salmonella* pathogenicity islands

ABSTRACT

The aim of this work was to study the phylogenetic relationship of *S*. Typhimurium genomes isolated from swine in Brazil (n=26) and to compare them to other genomes isolated from humans, animals and food from different countries (n=50). Characterizations were made using different phylogenetic strategies such as wgMLST and cgMLST. In addition, it was compared the genomic content between the 26 swine strains to a reference *S*. Typhimurium LT2 by BLAST Atlas and it was verified the frequencies of *Salmonella* pathogenicity islands (SPIs). Using wgMLST the majority of the strains (69%) studied isolated from swine in Brazil were grouped into single cluster. The cgMLST grouped the strains into three main clusters. The BLAST Atlas analysis showed some different deletions in the strains studied in comparison to *S*. Typhimurium LT2. All 26 isolates studied contained SPI-1, SPI-2 and SPI-3 and 85% to 88% contained SPI-5, SPI-13 and SPI-14. In conclusion, molecular typing based on the wgMLST grouped the majority of the *S*. Typhimurium strains isolated from swine in Brazil in the same cluster suggesting that these isolates are genetically related. On the other hand, molecular typing based in the cgMLST and the comparison to *S*. Typhimurium LT2 by BLAST Atlas suggested greater genetic diversity among some *S*. Typhimurium isolated from swine in Brazil. Therefore, cgMLST and BLAST Atlas were more efficient at discriminating these isolates. The pathogenic potential of the strains studied was corroborated by the presence of important SPIs related to the pathogenesis of *S*. Typhimurium.

INTRODUCTION

Salmonellosis is a serious public health problem that has been caused by different serovars of *Salmonella* (CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC), 2020a). It has been estimated that 1.35 million infections, 26,500 hospitalizations and 420 deaths occur every year in the United States due to this disease (CDC, 2020a). In Brazil, it has been reported that *Salmonella* is one of the main causes of foodborne outbreaks annually (BRAZIL, 2019).

Salmonella enterica subsp. *enterica* serovar Typhimurium (*S*. Typhimurium) cause usually gastroenteritis in humans worldwide and this serovar is capable of infecting several hosts, and is defined as a non-specific host bacterium (WORLD HEALTH ORGANIZATION (WHO), 2018). Therefore, *S*. Typhimurium is transmitted easily between humans and animals, such as poultry, pigs and cattle (WHO, 2018).

Pork has been an important source of salmonellosis transmission mainly in countries related to swine production; consequently this mechanism of contamination has become a serious public health problem in different parts of the world due to the international trade of pigs (CAMPOS et al., 2019).

According to the Brazilian Association of Animal Protein (ABPA), Brazil is currently ranked as the fourth largest producer of pig meat, surpassed only by China, the European Union and the United States. In 2019, Brazil produced 3,983 thousand tons of pig meat and exported 750 thousand tons with the State of Santa Catarina producing 29.59% of the total production of pork in Brazil (ABPA, 2020). Therefore, studies that aim to better understand the epidemiological characterization of this important pathogen isolated from swine in Brazil are needed.

Until now there are no published studies in Brazil that have epidemiologically characterized *S*. Typhimurium strains isolated from swine by whole genome sequencing (WGS). Furthermore, those strains genomes have never been compared with strains isolated from other sources and different countries.

According to Liu and collaborators (2016), whole genome multi locus sequence typing (wgMLST) is a tool based on two steps, the construction of a pan-genome and allelic sequence generation for the subsequent construction of the genetic relatedness tree (LIU; CHIOU; CHEN, 2016). Core genome multi locus sequence typing (cgMLST) is based on the core genome and in the case of *Salmonella*, a soft core of 3,002 genes was selected as a subset from a whole genome MLST set (ALIKHAN et al., 2018).

The BLAST Atlas is based on the comparison of coding sequence (CDS) regions of the reference genome BLASTed against the CDS regions of the query genomes (PETKAU et al., 2010). The interpretation and visualization of the results is provided in a circular diagram with the blanks indicating areas where there were no BLAST hits between the reference and the query files (PETKAU et al., 2010).

Pathogenicity islands were present in some bacteria and normally carry genes essential for bacterial virulence, associated with adhesion, invasion and survival in the host (KARAOLIS, 2001). In addition, the genus of *Salmonella* has approximately 23 pathogenicity islands, with SPI-1 and SPI-2 being the two most studied and well characterized (WANG et al., 2020b).

The aims of this work were to study the phylogenetic relationships of *S.* Typhimurium genomes isolated from swine in Brazil among themselves and with other genomes isolated from several sources and countries using different phylogenetic strategies such as wgMLST

and cgMLST, besides to compare the genetic content between the genomes from swine in this country with the reference *S*. Typhimurium LT2 by BLAST Atlas and to perform the search of *Salmonella* pathogenicity islands (SPIs).

MATERIALS AND METHODS

Bacterial strains

A total of 26 *S.* Typhimurium strains isolated from swine in the Santa Catarina State located in the South Region of Brazil were sequenced (Table 1). Those strains were selected from the collection of the Brazilian Agricultural Research Corporation (EMBRAPA) between 2000 and 2012. Fifty other *Salmonella* Typhimurium genomes of strains isolated from humans, animals and food in different geographical areas were retrieved from GenBank database to perform wgMLST and cgMLST. Additional information on these genomes can be observed in Fig. 1 and Fig. 2.

Genome sequencing and assembly

The genomic DNA extraction was performed according to Campioni and Falcão (2014) and the concentration of the genomic DNA was detected in NanoDrop 1000 (Thermo Scientific).

The whole genome sequencing was performed in the NextSeq 500 desktop sequencer with the NextSeq 500/500 high-output version 2 kit (Illumina) for 2x151 in the laboratory of the U.S. Food and Drug Administration (FDA). Each strain genome was assembled *de novo* followed by multi-reference scaffolding. First, raw reads were processed by the a5 pipeline (August 2016 version, COIL; JOSPIN; DARLING, 2015), which started trimming the reads with Trimmomatic (BOLGER et al., 2014), performing quality filtering removing adaptor sequences, low quality bases (phred score quality < 28), and short reads (>35 bp). Following read error correction was performed by SGA (SIMPSON and DURBIN, 2012). The a5 uses IDBA-UD algorithm (PENG et al., 2012) for *de novo* assembling succeeded by SSPACE (BOETZER et al., 2011) scaffolding using the error-corrected paired reads. However, this pipeline was not enough to accomplish a closed genome due to the short read length. Thus, next we executed a reference-assisted scaffolding through MeDuSa 1.6 (BOSI et al., 2015), which uses information from a set of closely-related genomes to guide the scaffolds into the correct order and orientation, building a chromosome-size scaffold. Four different references were chosen based on the strains serovar, which included LT2, SL1344, ST4-74 and 14028S. These references are the main *S*. Typhimurium representatives with fully closed genomes

publicly deposited. The final scaffolds were passed through up to 20 interactions in Gapfiller 1.10 (NADALIN; VEZZI; POLICRITI, 2012), which used the error-corrected reads to determine the gaps between the contig sequences and improving the L_{50}contig count.

Phylogenetic analyses

The wgMLST analysis was performed using the module *Build_PGAdb* on the software PGAdb-builder and the tree was constructed using the *Build_wgMLSTtree* module. The parameters used for PGAdb were alignment coverage and identity ≥ 90% (LIU; CHIOU; CHEN, 2016). The cgMLST analysis from a set of reads was determined using the services of the center for genomic epidemiology for *Salmonella* (Enterobase) available at (https://cge.cbs.dtu.dk/services/cgMLSTFinder/) (ALIKHAN et al., 2018).

BLAST Atlas

After achieving the complete genomes a BLAST Atlas was created for each of the strains described herein compared to the LT2 *S*. Typhimurium main representative strain through GView (PETKAU et al., 2010).

***Salmonella* Pathogenicity Islands (SPIs)**

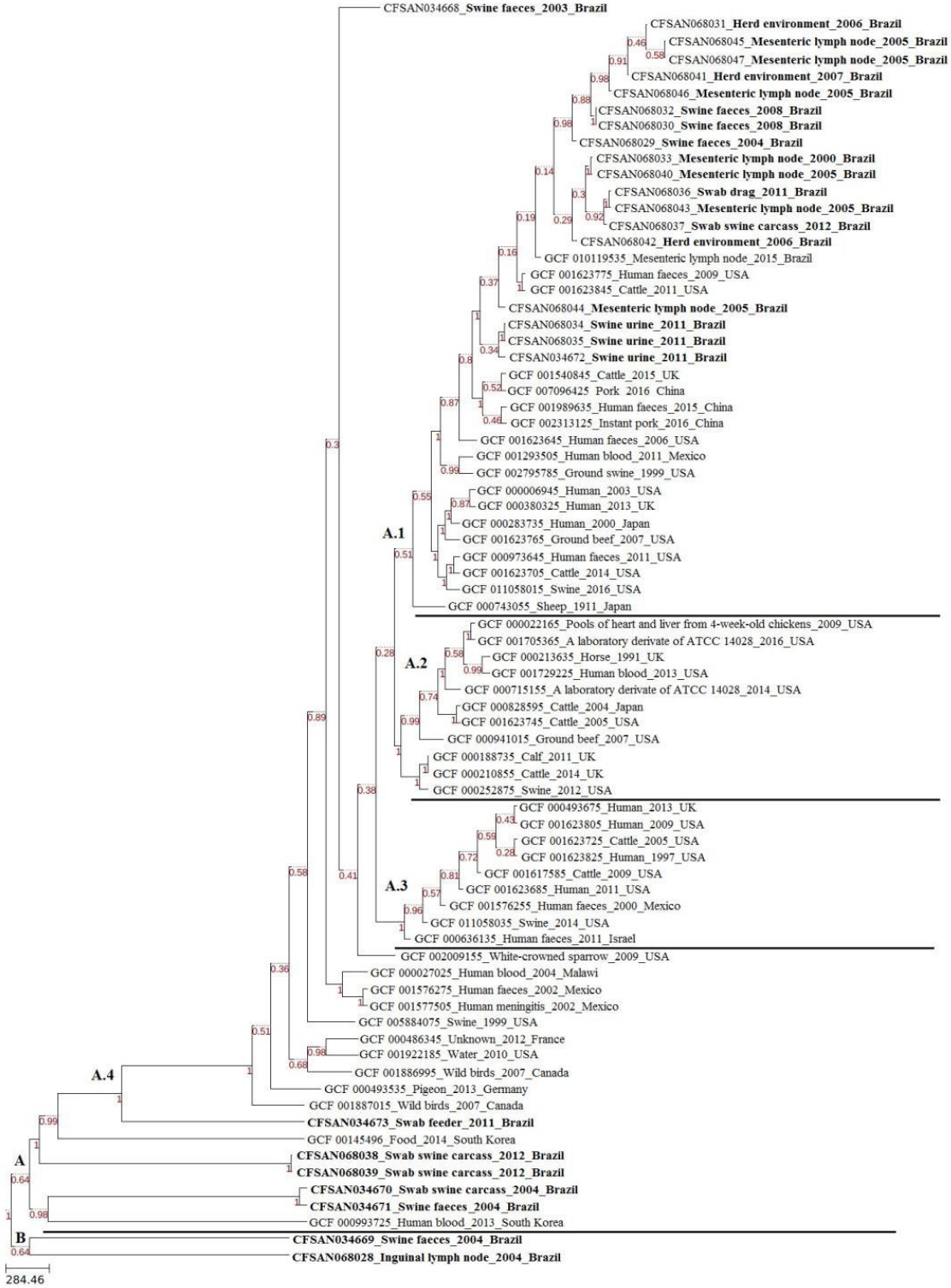
The SPIFinder was performed using the web services of the center for genomic epidemiology for *Salmonella* available at (https://cge.cbs.dtu.dk/services/SPIFinder/) with threshold of 95% and minimum length of 60% (ROER et al., 2016).

RESULTS

Phylogenetic analyses

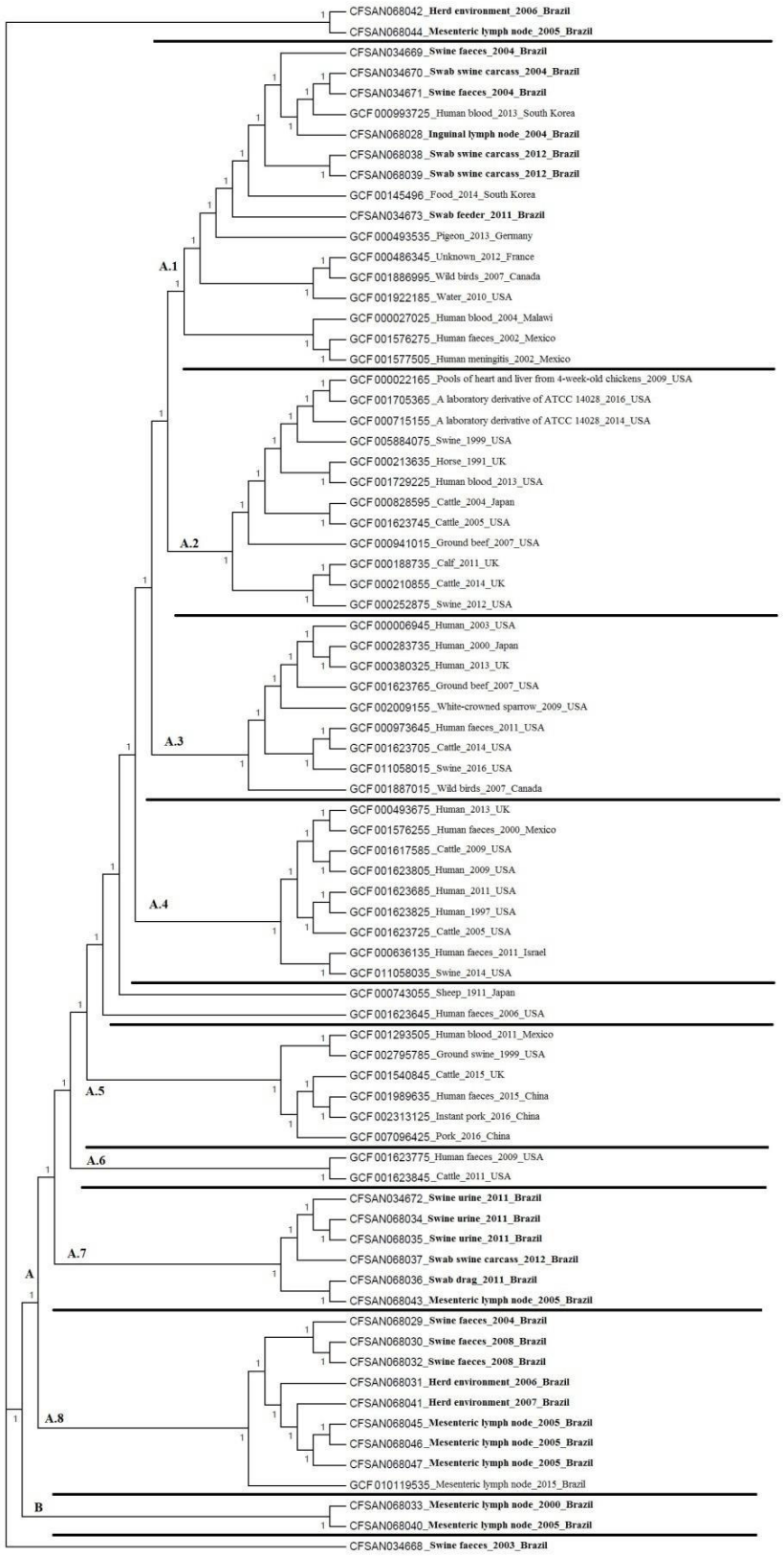
The PGAdb wgMLST grouped the 76 *S.* Typhimurium strains studied into two groups designated A and B, being group A subdivided in A.1, A.2, A.3 and A.4 (Fig. 1). Cluster A.1 comprised 36 genomes including 18 genomes (69%) isolated from swine in Brazil. Cluster A.2 comprised 11 genomes isolated from humans, animals and food in the United States (USA), United Kingdom (UK) and Japan. Cluster A.3 comprised 11 genomes isolated from humans and animals in Israel, Mexico, USA and UK. Cluster A.4 comprised 17 genomes including five genomes isolated from swine in Brazil. Finally, group B comprised two genomes isolated from swine in Brazil. The CFSAN034668 genome isolated from swine in Brazil was not grouped in any cluster.

Fig. 1 - Phylogenetic analysis with wgMLST profiles for 76 *Salmonella* Typhimurium genomes. The PGAdb profile from the genomes was used to construct a wgMLST tree using the *Build_wgMLSTtree* module. Bootstrap values are shown next to the nodes. The dendrogram was constructed with the UPGMA clustering algorithm.



The cgMLST grouped the 76 *S.* Typhimurium strains studied into two groups designated A and B, being group A subdivided in A.1, A.2, A.3, A.4, A.5, A.6, A.7 and A.8 (Fig. 2). Cluster A.1 comprised 16 genomes including seven genomes isolated from swine in Brazil. Clusters A.2, A.3, A.4, A.5 and A.6 comprised 12, 9, 9, 6 and 2 genomes isolated from humans, animals and foods in USA, UK, Japan, Canada, Israel, Mexico and China, respectively. Cluster A.7 comprised six genomes isolated from swine in Brazil. Cluster A.8 comprised nine genomes including eight genomes isolated from swine in Brazil. Finally, group B comprised two genomes isolated from swine in Brazil. The CFSAN034668, CFSAN068042 and CFSAN068044 genomes isolated from swine in Brazil were each not grouped in any cluster.

Fig. 2 - Phylogenetic analysis with cgMLST profiles for 76 *Salmonella* Typhimurium genomes a soft core of 3,002 genes was selected as a subset from a whole genome MLST set.



BLAST Atlas

The Fig. 3 represents the results of the BLAST Atlas between the reference genome and the 26 complete genomes of *S*. Typhimurium isolated from swine in Brazil. The blanks indicate areas present in the reference genome and absent in the query genomes.

***Salmonella* Pathogenicity Islands (SPIs)**

All the 26 isolates studied contained the SPI-1, SPI-2 and SPI-3. The SPI-4, SPI-5, SPI-8, SPI-9, SPI-13 and SPI-14 were detected in 16 (62%), 22 (85%), 2 (8%), 1 (4%), 23 (88%) and 23 (88%), genomes respectively. These data are summarized in Table 1.

Fig. 3 - BLAST Atlas showing the similarity between *Salmonella* Typhimurium LT2 and 26 genomes of *Salmonella* Typhimurium isolated from swine in the Santa Catarina State in Brazil. The blanks indicate areas where there were no BLAST hits between the reference and the query files and colored circles represent the isolates.

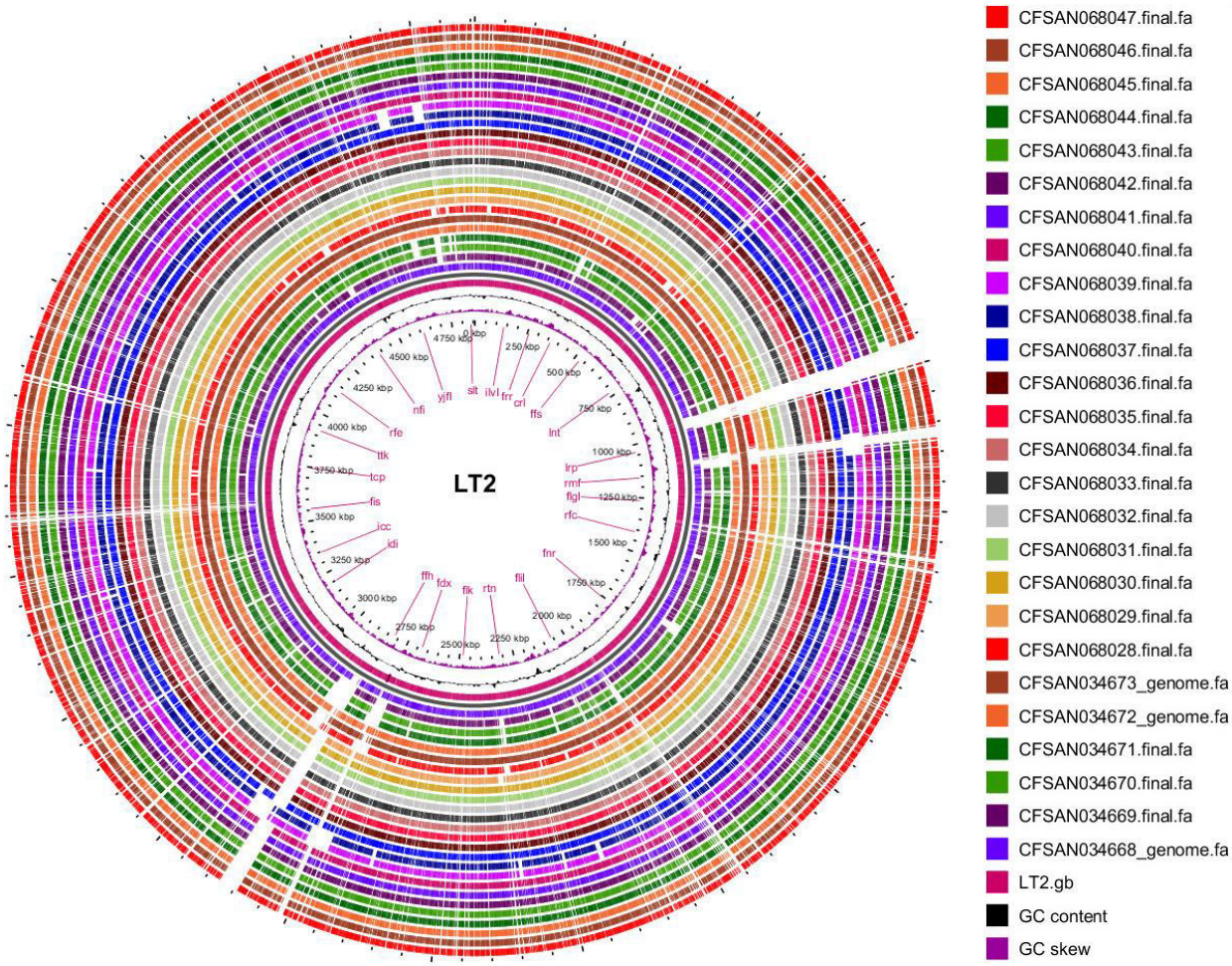


Table 1 – Characteristics of the 26 *S.* Typhimurium strains isolated from swine between 2000 and 2012 in Santa Catarina State in Brazil

CFSAN[a] no.	BioSample	Source	*Salmonella* pathogenicity islands SPIs (% identity)
CFSAN068028	SAMN08017374	Inguinal lymph node	SPI-1 (98.62); SPI-2 (98.52); SPI-3 (97.30); SPI-4 (98.08); SPI-13 (99.71); SPI-14 (99.80)
CFSAN068029	SAMN08016403	Swine feces	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.99); SPI-4 (98.94); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068030	SAMN08016388	Swine feces	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.95); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068031	SAMN07782279	Herd environment	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.95); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068032	SAMN08016037	Swine feces	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.95); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068033	SAMN08016458	Mesenteric lymph node	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.95); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068034	SAMN08017375	Swine urine	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.94); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068035	SAMN08015734	Swine urine	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068036	SAMN08016465	Swab drag	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.95); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068037	SAMN08016442	Swab carcass	SPI-1 (99.99); SPI-2; (99.99); SPI-3 (99.39); SPI-4 (98.95); SPI-5 (99.99); SPI-13 (100); SPI-14 (100)
CFSAN068038	SAMN08016431	Swab carcass	SPI-1 (99.35); SPI-2; (98.96); SPI-3 (98.28); SPI-5 (98.81); SPI-13 (100); SPI-14 (100)
CFSAN068039	SAMN08016411	Swab carcass	SPI-1 (99.35); SPI-2; (99.15); SPI-3 (99); SPI-5 (98.81); SPI-13 (100); SPI-14 (100)
CFSAN068040	SAMN08016390	Mesenteric lymph node	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.95); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068041	SAMN08016210	Herd environment	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068042	SAMN08017409	Herd environment	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.95); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068043	SAMN08016211	Mesenteric lymph node	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.95); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068044	SAMN08016474	Mesenteric lymph node	SPI-1 (100); SPI-2; (99.99); SPI-3 (98.41); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068045	SAMN08016038	Mesenteric lymph node	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068046	SAMN08015904	Mesenteric lymph node	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.94); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN068047	SAMN08016441	Mesenteric lymph node	SPI-1 (100); SPI-2; (99.99); SPI-3 (99.98); SPI-4 (98.95); SPI-5 (100); SPI-13 (100); SPI-14 (100)
CFSAN034668	SAMN03941172	Swine feces	SPI-1 (99.36); SPI-2; (99.86); SPI-3 (99.89); SPI-5 (99.29); SPI-13 (100); SPI-14 (100)
CFSAN034669	SAMN03941173	Swine feces	SPI-1 (99.01); SPI-2; (97.17); SPI-3 (98.57); SPI-4 (98.17)
CFSAN034670	SAMN03941174	Swab swine carcass	SPI-1 (99); SPI-2; (98.60); SPI-3 (98.02); SPI-8 (97.85)
CFSAN034671	SAMN03941175	Swine feces	SPI-1 (99); SPI-2; (98.60); SPI-3 (98.02); SPI-8 (97.85)
CFSAN034672	SAMN03941176	Swine urine	SPI-1 (100); SPI-2; (99.48); SPI-3 (99.47); SPI-4 (98.94); SPI-5 (99.56); SPI-13 (100); SPI-14 (99.80)
CFSAN034673	SAMN03941177	Swab feeder	SPI-1 (99.47); SPI-2; (99.29); SPI-3 (99.59); SPI-5 (99.36); SPI-9 (98.09); SPI-13 (100); SPI-14 (100)

DISCUSSION

The present study investigated the phylogenetic relationships of 76 *S.* Typhimurium genomes comparing strains isolated from swine in Brazil to each other and to other genomes isolated from different sources and geographical areas by wgMLST and cgMLST. Furthermore, genetic content among the genomes isolated was compared against *S.* Typhimurium LT2 by BLAST Atlas and it was characterized the SPIs.

The constructed phylogenetic tree by wgMLST grouped the majority of the strains (69%) studied isolated from swine in Brazil into the same cluster suggesting that these isolates are genetically related (Fig. 1). However, the constructed phylogenetic tree by cgMLST provided increased resolution differentiating more the *S.* Typhimurium strains studied in comparison to wgMLST, showing that the strains isolated from swine in Brazil are more genetically diverse among themselves due to the presence of three main clusters comprising those strains (Fig. 2). It is important to emphasize that the isolates in this study genetically are related very closely because coming from the same host source and region of the country.

According to Liu and collaborators (2016), the wgMLST technology proved to be as effective as a SNP-based approach to discern isolates of *S.* Typhimurium from epidemiologically related and non-related outbreaks. In the same way, the cgMLST tool has also been used to elucidate outbreaks involving patients, food and asymptomatic carriers caused by other *Salmonella* serovar showing it to be a good technology for epidemiological investigations (SIMON et al., 2018).

The main difference between the tools is that according to Alikhan and collaborators (2018), cgMLST focuses on the small differences between the set of 3,002 genes conserved genes for *Salmonella*. On the other hand, wgMLST has the characteristic of producing a clustering based on the allelic scheme of 3,609 genes (variable number) that are part of the core for all *S.* Typhimurium strains studied (LIU; CHIOU; CHEN, 2016).

It is important to mention that the present study provided additional information about *S.* Typhimurium strains isolated from swine in Brazil that were previously molecularly typed by Pulsed-field gel electrophoresis (PFGE), Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and Multiple-locus variable-number tandem-repeat analysis (MLVA). Additionally, virulence genes were searched by PCR and antimicrobial resistance profiles were assessed for those strains (ALMEIDA et al., 2016a).

According to Almeida and collaborators (2016a) the genotyping results showed a high similarity between some *S.* Typhimurium strains isolated from swine and humans in Brazil. In

addition, the pathogenic potential of these strains was documented by the presence of important virulence genes and multi-drug resistant strains were detected (ALMEIDA et al., 2016a).

The wgMLST and cgMLST demonstrated the similarity of some Brazilian strains with *S.* Typhimurium strains isolated from humans, animals and food of different parts of the globe, such as USA, Mexico, South Korea, among others suggesting that possibly in those cases the strains might have descended from a common ancestor.

In the present study, it was possible to observe several differences between the reference genome LT2 and the genomes isolated from swine in Brazil by BLAST Atlas. The blanks indicate deletions, with the largest spaces being regions of phages found in the reference strain and absent in the studied isolates. This result showed a greater diversity among the swine strains, because it was observed for some regions variable presences and deletions among themselves (Fig. 3).

In the present study, SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-8, SPI-9, SPI13 and SPI-14 were detected among the isolates from swine. All the isolates contained the SPI-1, SPI-2 and SPI-3 and the SPI-5, SPI-13 and SPI-14 were detected in 85%, 88% and 88%, respectively.

It is known that SPI-1 and SPI-2 contain several genes involved in the production of the Type III Secretion System (T3SS) essential for *Salmonella* invasion and replication in host cells (JOENSEN et al., 2017). *Salmonella* survival in human macrophages such as adaptation to low nutritional condition in the phagosome is mainly related to the genes present in SPI-3 (WANG et al., 2020b).

SPI-5, SPI-13 and SPI-14 were detected in high frequencies in the strains studied, with SPI-5 being responsible for encoding genes related to the fluid secretion and inflammatory response (WOOD et al., 1998; SANTOS; FERRARI; CONTE-JUNIOR, 2019). Moreover, SPI-13 and SPI-14 were detected in the same isolates and present genes involved in the internalization of *S*. Enteritidis in murine macrophages and a protein denominated LoiA (low oxygen induced factor A) which is expressed in low oxygen concentrations, activating the main regulator of SPI-1 (ESPINOZA et al., 2017; JIANG et al., 2019).

In general, the other SPI are involved in the processes of escape of defense cells, adhesion, invasion, survival in acidic environments, inflammatory responses and intestinal persistence; but more detailed studies on function in pathogenicity of *Salmonella* should be performed (WANG et al., 2020b).

S. Typhimurium has been a serovar of economic and political importance, since its contamination impacts the pork producing industry due to the financial losses resulting from expenses with diagnosis, treatment and consequently loss in the number of animals raised (RODRÍGUEZ and SUÁREZ, 2014). The contamination can occur during the different stages of pork production, including transportation, carcass processing and meat handling and storage, generating losses for producers and consumers (RODRÍGUEZ and SUÁREZ, 2014).

In conclusion, molecular typing based in the wgMLST grouped the majority *S*. Typhimurium strains isolated from swine in Brazil in the same subgroup suggesting that these isolates are genetically related. On the other hand, molecular typing based on the cgMLST and the comparison to *S*. Typhimurium LT2 by BLAST Atlas suggested greater genetic diversity among S. Typhimurium isolated from swine in Brazil. Therefore, cgMLST and BLAST Atlas were more efficient at discriminating these isolates. The pathogenic potential of the strains studied was corroborated by the presence of important SPIs related to the pathogenesis of this important pathogen.Altogether, this study provided relevant data on the epidemiological characterization of *S*. Typhimurium strains isolated from swine in Brazil by WGS technology that has been little explored in Brazil, currently one of the largest pork meat exporters in the world.

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CHAPTER 5

Insights about the epidemiology of *Salmonella* Typhimurium isolates from different sources in Brazil using comparative genomics

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ABSTRACT

Background: *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S*. Typhimurium) is an important zoonotic agent worldwide. In Brazil, there are few published studies that have characterized the possible differences of *S*. Typhimurium strains isolated from humans, foods and animal by whole genome sequencing (WGS). The aim of this work was to compare genetically 117 *S*. Typhimurium isolates from different sources from 30 years (1983 to 2013) in Brazil using different genomics strategies, including: phylogenetic analysis; orthologous protein clusters analysis; Multi-locus sequence typing (MLST); prophages and resistance genes screening related to efflux pumps. **Results:** The majority of the 117 *S*. Typhimurium strains studied were grouped into a single cluster (≅90%) by the genome core (cgMLST) and (≅77%) by single copy marker genes (ggTree). The different orthologous protein clusters found for some *S*. Typhimurium strains isolated from humans and food are involved in metabolic and regulatory processes. For 26 *S*. Typhimurium isolates from swine the sequence type (ST) 19 was the most common, the ST1921 was the second most prevalent and the STs 14, 64, 516 and 639 were also detected. The main prophages detected were: Gifsy-2 in 79 (67.5%) and Gifsy-1 in 63 (54%) *S*. Typhimurium isolates. All of the *S*. Typhimurium isolates contained the *acrA*, *acrB*, *macA*, *macB*, *mdtK*, *emrA*, *emrB*, *emrR* and *tolC* efflux pump genes. **Conclusions:** Phylogenetic analyses grouped the majority of the *S*. Typhimurium isolates into a single cluster suggesting that there is one prevalent subtype that has successful contaminated human, food and animal sources for 30 years in Brazil. The orthologous protein clusters analysis revealed unique genes in the *S*. Typhimurium studied mainly related to bacterial metabolism and that may be important in their pathogenicity. *S*. Typhimurium isolates from swine showed greater diversity of STs and prophages in comparison to *S*. Typhimurium strains isolated from humans and foods. The pathogenic potential of *S*. Typhimurium strains was corroborated by the presence of exclusive prophages of this serovar involved in their virulence. The high number of resistance genes related to efflux pumps is worrying and may lead to therapeutic failures when treatment is needed.

Keywords: *Salmonella* Typhimurium, phylogenetic trees, protein orthologous clusters, prophages, efflux pump.

BACKGROUND

Nontyphoidal *Salmonella* (NTS) strains have been an important enteric agent transmitted mainly by contaminated foods worldwide (WORLD HEALTH ORGANIZATION

(WHO), 2018). According to Kirk and collaborators (2015), it was estimated that 153 million infections and 56,969 deaths occurred around the globe due to salmonellosis in 2010. Moreover, data from the Centers for Disease Control and Prevention (CDC), estimated that 1.35 million infections, 26,500 hospitalizations and 420 deaths occur in the United States every year due to *Salmonella* (CDC, 2020a).

In Brazil, *Salmonella* has been the first or second most common foodborne pathogen isolated from outbreaks in recent years (BRAZIL, 2019). However, until now there are few published studies that have characterized the possible differences between Brazilian *S.* Typhimurium strains isolated from human, food and animal sources by whole genome sequencing (WGS).

S. Typhimurium is one of the main *Salmonella* generalist serovar, which has been isolated from pork in Europe, Oceania, Asia and North America, from poultry in North America and Oceania, from beef in Africa, Latin America and Europe, and from seafood in Europe (FERRARI et al., 2019). Therefore, this serovar has been transmitted from animals and humans in different parts of the world and is characterized as a zoonotic agent causing losses of million of dollars for the meat producing industry (BRAZIL, 2011; WHO, 2018).

According to the Centers for Disease Control and Prevention (CDC), *S.* Typhimurium can also infect domestic pets and recently was responsible for an outbreak linked to contact with small pet turtles that affected 35 people from nine states and generated 11 hospitalizations (CDC, 2020b).

WGS has been more accessible in the last few years and is used for molecular characterization studies (SHIVANI et al., 2015). Furthermore, different phylogenetic strategies can be performed after sequencing, such as construction of phylogenetic trees based on the core genome (cgMLST) and from single copy marker genes, comparison and analysis of orthologous protein clusters (OrthoVenn) and verification of the sequence type (ST) through Multilocus sequence typing (MLST) (WANG et al., 2015; ALIKHAN et al., 2018; WU, 2018). In addition, it has been possible to characterize the different prophages that contribute to *Salmonella* pathogenicity including identification of genes known to have functions such as virulence, metabolism and signaling (WAHL; BATTESTI; ANSALDI, 2019).

It is important to emphasize that the monitoring of resistant NTS strains has been of great importance due to its continued emergence worldwide (CDC, 2019b; JAJERE, 2019). According to Jajere (2019), multidrug resistant (MDR) *Salmonella* has been a serious public health problem because it may lead to treatment failure when the use of antimicrobial is

necessary (JAJERE, 2019). In the United States, it was estimated that 212,500 infections and 70 deaths occur due to drug resistant NTS every year (CDC, 2019b).

It is known that hundreds of genes can confer resistance to antibiotics in NTS and some were previously described for the *S*. Typhimurium strains isolated from humans and different foods in Brazil including genes related to resistance to aminoglycosides, tetracyclines, sulfonamides, trimethoprim, beta lactams, fluoroquinolones, phenicol and macrolides (ALMEIDA et al., 2018). However, antibiotic resistance is multifactorial and little is known about resistance genes related to efflux pump, which can be an important factor that confers resistance to some antibiotics, such as fluoroquinolones, beta lactams, macrolides and aminoglycosides (PIDDOCK, 2014; ALMEIDA et al., 2018).

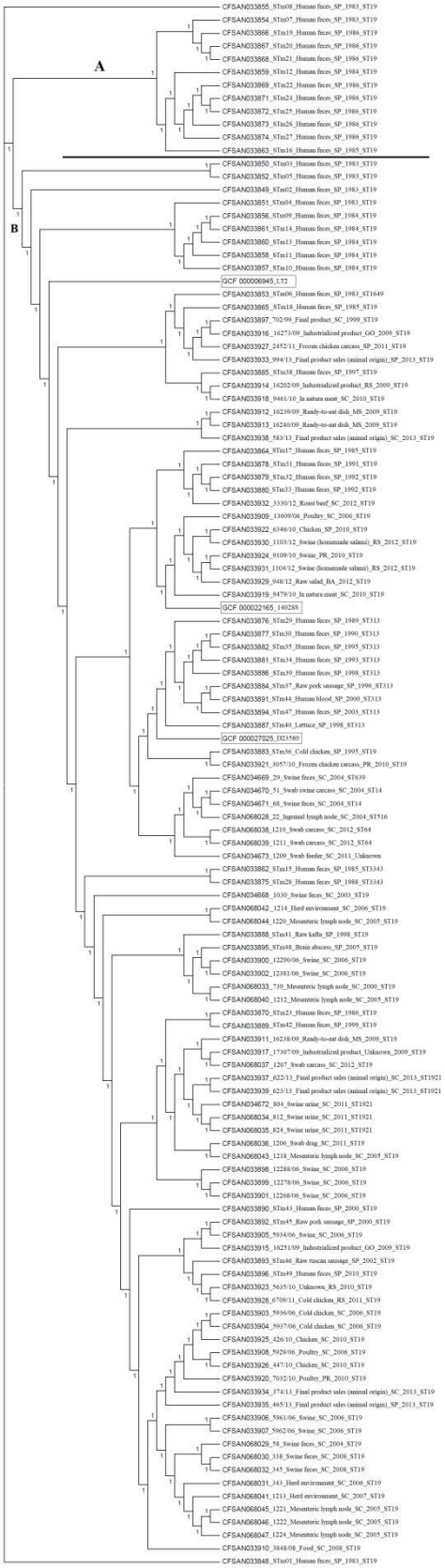
The aim of this work was to compare genetically *S.* Typhimurium isolates from humans, food and swines in Brazil during 30 years using different genomics strategies, such as phylogenetic trees, protein orthologous clusters analysis, MLST, prophages and resistance genes related to efflux pump.

RESULTS

cgMLST

The cgMLST grouped the 120 *S.* Typhimurium genomes studied in two main groups designated A and B (Fig. 1). Cluster A comprised 12 genomes of ST19 isolated from humans. Cluster B comprised a total of 108 genomes comprising strains isolated from humans, different foods and swines of ST19, ST1649, ST3343, ST1921 and ST313 in the case of strains isolated from humans and food, besides ST19, ST639, ST14, ST516, ST64 and ST1921 concerning strains isolated from swines. All three references were allocated in Cluster B. The CFSAN033848 and CFSAN033855 genomes isolated from humans were genetically distinct and did not grouped closely to any cluster.

Fig. 1 - Phylogenetic analysis with cgMLST profiles based on soft core of 3,002 genes selected for 117 *Salmonella* Typhimurium genomes isolated from humans (n=43), different foods (n=48) and swines (n=26) in Brazil.



Phylogenetic tree (ggTree) and orthologous protein clusters analysis

The phylogenetic tree grouped the 120 *S.* Typhimurium genomes studied in three groups designated A, B and C with cluster A subdivided in A.1 and A.2, cluster B subdivided in B.1 and B.2 (Fig. 2). Cluster A.1 comprised 84 genomes of ST19, ST1649, ST14, ST516, ST639, ST64, ST313, ST3343 and ST1921 isolated from humans, diverse foods and swines and the reference genomes. Cluster A.2 comprised nine genomes of ST19 isolated from humans, food and swines. Cluster B.1 comprised 20 genomes of ST19 from food and swines. Cluster B.2 comprised four genomes of ST19 isolated from human and food. Cluster C comprised three genomes of ST19 isolated from food and swine.

The orthologous protein clusters analysis was performed for the genomes that were more related to LT2, 14028S and D23580 references (Fig.2). The comparisons indicated the orthologous protein clusters presented in the genomes of the strains of this study and absent in the references. The different unique orthologous protein clusters found are involved in metabolic and regulatory processes showed in detail in Table 1.

MLST

Of the 26 *S.* Typhimurium strains isolated from swine studied, 16 (61.5%) belonged to the ST19, three (11.5%) to the ST1921, two (7.6%) to the ST14, two (7.6%) to the ST64, one (3.8%) to the ST516, one (3.8%) to the ST639 and one isolate did not match any known ST type.

Fig. 2 - Phylogenetic analysis based on a list of single copy marker genes for 117 *Salmonella* Typhimurium genomes isolated from humans (n=43), different foods (n=48) and swines (n=26) in Brazil.

Table 1 – Unique orthologous protein clusters in some selected *S*. Typhimurium strains in comparison reference genomes

Groups	Biological processes (protein orthologous clusters)
LT2 – STm07, STm12, STm16, STm19, STm20, STm21, STm22, STm24, STm25, STm26 and STm27 **(Comparison 1)**	Transposition (DNA-mediated), Transposition, Viral procapsid maturation, Virion attachment to host cell, Viral genome integration into host DNA, Trehalose transport, DNA replication, Viral capsid assembly, DNA binding, Histidine catabolic process to glutamate and formate
LT2 – STm01 and STm08 **(Comparison 2)**	Transposition (DNA-mediated), Transposition, Viral genome integration into host DNA, Virion attachment to host cell, DNA replication, Trehalose transport, DNA replication initiation, Viral procapsid maturation, Formate oxidation, DNA binding
LT2 – STm04, STm09, STm10, STm11, STm13 and STm14 **(Comparison 3)**	Transposition (DNA-mediated), Transposition, Viral genome integration into host DNA, Trehalose transport, Histidine catabolic process to glutamate and formate
LT2 – STm02, STm03 and STm05 **(Comparison 4)**	Transposition (DNA-mediated), Transposition, Viral procapsid maturation, Viral genome integration into host DNA, Trehalose transport, DNA replication, Response to mercury ion, Mercury ion transmembrane transporter activity, Formate oxidation, DNA restriction-modification system
14028S – 13609/06, 6346/10, 9109/10, 9479/10, 948/12, 1103/12 and 1104/12 **(Comparison 5)**	Transposition (DNA-mediated), Transposition, Formate oxidation, Trehalose transport, Cell adhesion, DNA binding
D23580 – STm29, STm30, STm34, STm35, STm37, STm39, STm40, STm44 and STm47 **(Comparison 6)**	Transposition (DNA-mediated), Transposition, Formate oxidation, Trehalose transport, Lyase activity, Viral tail assembly, Cell adhesion
D23580 – STm29, STm30, STm34, STm35, STm36, STm37, STm39, STm40, STm44, STm47 and 3057/10 **(Comparison 7)**	Transposition (DNA-mediated), Formate oxidation, Trehalose transport, Lyase activity, Cell adhesion, Metal ion binding

Prophages detection

The Gifsy-2 prophage was detected in 79 (67.5%) *S*. Typhimurium isolates, Gifsy-1 in 63 (54%), Salmon 118970_sal3 in 46 (39%) and Haemop - HP1 in 21 (18%). Two dozen other prophages were also detected in the genomes studied and are described in detail in Table 2.

Table 2 – Proportion of intact prophages detected in the 117 *Salmonella* Typhimurium studied in Brazil

Prophages	Humans (n=43) (%)	Foods (n=48) (%)	Swine (n=26) (%)
Aeromo_phiO18P	Not detected	48/01 (2.1)	26/01 (3.8)
Burkho_BcepMu	Not detected	48/01 (2.1)	Not detected
Edward_GF_2	43/02 (4.6)	48/06 (12.5)	Not detected
Entero_186	43/01 (2.3)	48/01 (2.1)	26/01 (3.8)
Entero_BP_4795	43/01 (2.3)	Not detected	Not detected
Entero_fiAA91_ss	Not detected	Not detected	26/06 (23.1)
Entero_mEp235	Not detected	Not detected	26/01 (3.8)
Entero_N15	43/03 (7)	Not detected	Not detected
Entero_P22	43/03 (7)	Not detected	Not detected
Entero_Tyrion	43/01 (2.3)	Not detected	Not detected
Entero_UAB_Phi20	43/05 (11.6)	Not detected	Not detected
Escher_RCS47	Not detected	Not detected	26/02 (7.7)
Gifsy_1	43/10 (23)	48/34 (71)	26/19 (73)
Gifsy_2	43/37 (86)	48/33 (68.7)	26/09 (34.6)
Haemop_HP1	43/18 (41.9)	Not detected	26/03 (11.5)
Salmon_118970_sal3	43/25 (58.1)	48/15 (31.2)	26/06 (23.1)
Salmon_118970_sal4	43/01 (2.3)	Not detected	Not detected
Salmon_epsilon34	Not detected	Not detected	26/01 (3.8)
Salmon_Fels_1	Not detected	48/01 (2.1)	Not detected
Salmon_Fels_2	43/04 (9.3)	48/01 (2.1)	26/04 (15.4)
Salmon_RE_2010	43/07 (16.3)	48/02 (4.2)	26/01 (3.8)
Salmon_SEN34	Not detected	Not detected	26/01 (3.8)
Salmon_SP_004	43/01 (2.3)	48/04 (8.3)	26/01 (3.8)
Salmon_SPN1S	43/17 (39.5)	Not detected	Not detected
Salmon_SPN3UB	Not detected	Not detected	26/02 (7.7)
Salmon_SPN9CC	Not detected	48/03 (6.2)	Not detected
Salmon_SSU5	Not detected	48/02 (4.2)	26/01 (3.8)
Salmon_ST64T	43/01 (2.3)	48/02 (4.2)	Not detected
Shigel_Sfll	Not detected	48/01 (2.1)	26/01 (3.8)

Efflux pump

The *acrA*, *acrB*, *macA*, *macB*, *mdtK*, *emrA*, *emrB*, *emrR* and *tolC* genes were detected in 117 (100%) *S*. Typhimurium isolates. The *mdsA* and *mdsB* genes were detected in 91 (100%) *S*. Typhimurium isolates from humans and different foods, but in only 18 (69.2%) *S*. Typhimurium isolates from swines. The *mdfA* gene was detected in 26 (100%) isolates from swines, 39 (81.2%) isolates from food and 18 (42%) isolates from humans. Finally, the *cmlA1* gene was detected only in isolates from swine 05 (19.2%). The percentage of query cover and identity for all genes ranged between 72-100 and 87-100, respectively (Table 3).

Table 3 – Frequencies of resistance genes related to efflux pumps in the 117 *Salmonella* Typhimurium studied

Genes	Humans (n=43) (Query cover %) (Identity %)	Food (n=48) (Query cover %) (Identity %)	Swine (n=26) (Query cover %) (Identity %)
acrA	43/43 (100) (91.69)	48/48 (100) (91.69)	26/26 (100) (91.69)
acrB	43/43 (100) (94.66)	48/48 (100) (94.66)	26/26 (100) (94.66)
macA	43/43 (100) (88.65-88.84)	48/48 (100) (88.65-88.84)	26/26 (100) (88.39-88.84)
macB	43/43 (100) (88.60-88.70)	48/48 (100) (88.70)	26/26 (100) (88.29-88.70)
mdtK	43/43 (100) (99.79)	48/48 (100) (99.79)	26/26 (100) (99.16-99.79)
emrA	43/43 (100) (99.92-100)	48/48 (100) (100)	26/26 (72-100) (98.40-100)
emrB	43/43 (100) (95.7)	48/48 (100) (95.7)	26/26 (99.80-100) (95.7)
emrR	43/43 (100) (93.14)	48/48 (100) (93.14)	26/26 (100) (93.14)
tolC	43/43 (99-100) (100)	48/48 (100) (100)	26/26 (100) (98.78-100)
mdsA	43/43 (100) (99.75-100)	48/48 (100) (100)	26/18 (100) (100)
mdsB	43/43 (100) (100)	48/48 (100) (100)	26/18 (100) (100)
mdfA	43/18 (100) (87.93)	48/39 (100) (87.93)	26/26 (100) (87.93)
cmlA1	Not detected	Not detected	26/05 (100) (99.76)

DISCUSSION

In this study, 117 *S*. Typhimurium isolates from humans (n=43), food (n=48) and swines (n=26) in Brazil were compared using genomic analyses, such as phylogenetic neighbor joining, orthologous protein clusters detection, MLST analysis, and blast identification of prophages and resistance genes related to efflux pump.

The constructed neighbor joining trees grouped the 117 *S*. Typhimurium isolates into two and three groups, respectively. The majority of the 117 *S*. Typhimurium strains studied were grouped in a single cluster (≅90%) by the genome core (cgMLST) and (≅77%) by single copy marker genes (ggTree) suggesting that there is one prevalent subtype that has been successful in contaminating human, food and animal sources for 30 years in Brazil (Fig. 1 and Fig. 2). It is important to mention that the present study provided additional information about *S.* Typhimurium strains isolated from humans, food and swines in Brazil because such strains have never been studied together.

According to Jensen (2001), homologous genes can be divided into orthologous and paralogs. Orthologous genes are originated from a common ancestor during speciation events and kept the same function; on the other hand, paralogs genes are originated from duplication events and do not kept the same function (XU et al., 2019).

Therefore, the OrthoVenn2 is a web server capable to annotate and compare orthologous protein clusters from the whole genome among different species (XU et al., 2019). In the present study, *S*. Typhimurium genomes more related to LT2, 14028S and D23580 references were compared and had their unique protein orthologous clusters revealed (Fig. 2). All *S*. Typhimurium isolates used for LT2 – comparison 1, 2, 3 and 4 were composed by a subcluster containing only strains of ST19 isolated from humans in the São Paulo State before the 1990s. The *S*. Typhimurium isolates used for 14028S – comparison 5 was formed by a subcluster containing only strains of ST19 isolated from food in the Rio Grande do Sul, Santa Catarina and Bahia States between 2006-2012. The *S*. Typhimurium isolates used for D23580 – comparisons 6 and 7 were formed by a subcluster containing strains of ST313 and ST19 isolated from humans and food in the São Paulo and Paraná States between 1995-2010.

Moreover, the different orthologous protein clusters found are involved in metabolic and regulatory processes, such as transposition, DNA replication, cell adhesion, formate oxidation, trehalose transport, lyase activity and response to mercury ion. These results show that despite being of the same serovar there are unique orthologous protein clusters in the strains studied in comparison to the reference strains and that may be important for their pathogenicity and were kept in these *S*. Typhimurium strains during natural selection and adaptation (Table 1).

In this study, MLST was performed only for swine isolates, because the STs for humans and food isolates were previously described in (ALMEIDA et al., 2017a). Of the 26 *S*. Typhimurium strains isolated from swine studied, 16 (61.5%) belonged to the ST19, three (11.5%) to the ST1921, two (7.6%) to the ST14, two (7.6%) to the ST64, one (3.8%) to the ST516, one (3.8%) to the ST639 and one did not have its ST detected.

According to Almeida and collaborators (2017a), the ST19 was the most common ST found, the ST313 was the second most prevalent. The STs 1649, 3343 and 1921 were also detected among the *S*. Typhimurium strains isolated from humans and different foods in Brazil.

S. Typhimurium isolates from swine showed greater diversity in the seven housekeeping genes studied despite having a lower number of strains (n=26) in comparison to the number of *S*. Typhimurium strains isolated from humans (n=43) and food (n=48). In

strains from swine the ST19 was the most common, the ST1921 was the second most prevalent and the STs 14, 64, 516 and 639 were also detected.

Accessing the Enterobase for *Salmonella*, it was observed that 29,572 samples isolated from human, reptile, ovine, swine, poultry, food and bovine in France, Mexico, China, Germany, Scotland, Portugal, Qatar, Korea, Ireland, United States (US), United Kingdom (UK) and Denmark deposited belonged to the ST19. For the ST313 3,049 samples isolated predominantly from human in Kenya, Ethiopia, Zimbabwe, Malawi, Mali and Nigeria were deposited (ALIKHAN et al., 2018).

Moreover, the STs 1649, 3343 and 1921 were found for 16, 4 and 7 strains respectively, isolated from humans, livestocks, food and swines in Venezuela, Ireland, US, UK, Colombia, Ecuador, Vietnam and Brazil (ALIKHAN et al., 2018). Finally, the ST516, ST64, ST639 and ST14 were linked to 370, 3,850, 237 and 2,149 strains respectively, isolated from humans, poultry, food, aquatic animals, reptiles and the environment in the US, Mexico, Senegal, Germany, Portugal, Qatar, Canada, UK, India, Ghana, Thailand, Malaysia, Malta, Vietnam, Pakistan, Greece, France, Germany, China, Denmark, Scotland, Norway and South Korea (ALIKHAN et al., 2018).

In the present study, the Gifsy-2 prophage was detected in 79 (67.5%) *S*. Typhimurium isolates, Gifsy-1 in 63 (54%), Salmon 118970_sal3 in 46 (39%) and Haemop - HP1 in 21 (18%). Specifically, Gifsy-1 prophage was detected in 10 (23%) *S*. Typhimurium strains isolated from humans, 34 (71%) strains isolated from different foods and in 19 (73%) strains isolated from swine (Table 3). Gifsy-2 prophage was detected in 37 (86%) *S*. Typhimurium strains isolated from humans, 33 (68.7%) strains isolated from foods and 9 (34.6%) strains isolated from swine (Table 2).

It is important to be mentioned that Gifsy prophages carry genes that are related to virulence of *S*. Typhimurium in the host (KLUMPP and FUCHS, 2007; NGOI; YAP; THONG, 2018). The Gifsy-1 prophage encodes three genes involved in the intracellular survival of *Salmonella* spp. in the host, denominated *gogB* (leucine-rich repeat protein), *sarA* (anti-inflammatory response activator) and p*agK2*. In the same way, the Gifsy-2 prophage encodes a superoxide dismutase (*sodC1*) that contributes to the survival of *Salmonella* spp. destroying the toxic radicals of the host macrophages (FIGUEROA-BOSSI and BOSSI, 1999; WAHL; BATTESTI; ANSALDI, 2019). It is important to emphasize that Gifsy prophages have been found only in *S*. Typhimurium strains, as well as the Fels-1 and Fels-2 prophages (NGOI; YAP; THONG, 2018). In the present study, Fels prophages were detected in four *S*.

Typhimurium strains isolated from humans, two strains isolated from food and four strains isolated from swine (Table 2).

According to Brussow and colleagues (2004), the Fels-1 prophage encodes the *sodCIII* and *nanH* genes related to the production of superoxide dismutase and neuraminidase in *S*. Typhimurium, respectively (BRÜSSOW; CANCHAYA; HARDT, 2004). Furthermore, the Fels-2 prophage carries genes that are apparently related to regulation and adhesion of *S*. Typhimurium to host cells (WAHL; BATTESTI; ANSALDI, 2019).

The Gifsy and Fels prophages have already been described in *S*. Typhimurium isolated in various parts of the world, such as Australia, Europe, China, among others (GARCÍA et al., 2013; PANG et al., 2013; YANG et al., 2015b). It is important to emphasize that other prophages were also found in the *S*. Typhimurium strains studied including Salmon 118970_sal3 and Haemop - HP1 (Table 2). Moreover, two dozen other prophages were detected in the *S*. Typhimurium strains studied, but there is less information about them related to pathogenicity and/or virulence of this serovar (Table 2).

In addition, *S*. Typhimurium isolates from swine showed 6 (23.1%) unique prophages despite having a lower number of strains analysed (n=26) in comparison to *S*. Typhimurium strains isolated humans (n=43) and food (n=48) that presented 7 (16.3%) and 3 (6.25%) unique prophages, respectively, suggesting the greater diversity in these mobile genetic element for *S*. Typhimurium strains isolated from swine in Brazil (Table 2).

Resistance to multiple drugs in bacteria has been a serious public health problem worldwide (ASOKAN et al., 2019). It is known that there are four main mechanisms that can cause this resistance, such as target alteration, drug inactivation, decreased permeability and drug expulsion through the production of efflux pump (SUN; DENG; YAN, 2014).

In the present study, the *acrA*, *acrB*, *macA*, *macB*, *mdtK*, *emrA*, *emrB*, *emrR, tolC*, *mdsA*, *mdsB*, *mdfA* and *cmlA1* genes were detected among the *S*. Typhimurium strains isolated from humans, food and swines. All of the isolates contained the *acrA*, *acrB*, *macA*, *macB*, *mdtK*, *emrA*, *emrB*, *emrR* and *tolC* genes (Table 3). Other genes related to production efflux pump, such as *oqxAB* and *floR* were previously reported in (ALMEIDA et al., 2018).

The AcrAB efflux system has been described as responsible for the intrinsic resistance to many antibiotics that can be used in medical practice for the treatment of *S*. Typhimurium, such as fluoroquinolones and beta-lactams (PIDDOCK, 2014). According to the World Health Organization (WHO) (2017), *Salmonella* spp. was described as a high priority category pathogen in fluoroquinolones resistance of the Global Priority Pathogens List (ASOKAN et al., 2019).

The *macA* and *macB* genes encode proteins that characterize an efflux pump related to macrolides resistance (MIRYALA and RAMAIAH, 2018; Universal Protein Resource (UniProt), 2020). According to the Universal Protein Resource (UniProt) (2020), the *mdtK*, *emrA*, *emrB*, *emrR*, *mdsA*, *mdsB*, *mdfA* and *cmlA1* genes encode mainly proteins involved in multidrug efflux transporter and confers resistance to different antibiotics, such as aminoglycosides, tetracyclines, novobiocin, nalidixic acid, chloramphenicol and norfloxacin (HORIYAMA; YAMAGUCHI; NISHINO, 2010). Furthermore, the *tolC* gene has been described as important for the formation of some multidrug efflux systems (AcrAB, MacAB, EmrAB and MdsAB) in *S*. Typhimurium (HORIYAMA; YAMAGUCHI; NISHINO, 2010).

CONCLUSIONS

The phylogenetic trees grouped the majority of the *S*. Typhimurium isolates into a single cluster suggesting that there is one prevalent subtype that has been successful in contaminating human, food and animal sources for 30 years in Brazil. The orthologous protein clusters analysis revealed unique genes in the strains studied mainly related to bacterial metabolism that may be important in their pathogenicity. *S*. Typhimurium isolates from swine showed greater diversity of STs and prophages in comparison to *S*. Typhimurium strains isolated from humans and food. The pathogenic potential of *S*. Typhimurium strains was corroborated by the presence of exclusive prophages of this serovar involved in their virulence. The high number of resistance genes related to efflux pump is worrying and may cause therapeutic failures when treatment is needed. Altogether, this study provided relevant data on the genomic characterization of *S*. Typhimurium strains isolated from different sources in Brazil using WGS.

METHODS

Bacterial strains

A total of 117 *S*. Typhimurium strains isolated from humans (43), food (48) and swines (26) between 1983 to 2013 in Brazil were studied (Table 4). These strains were selected from the collections of the Adolfo Lutz Institute of Ribeirão Preto (IAL-RP), of the Oswaldo Cruz Foundation from Rio de Janeiro (FIOCRUZ-RJ) and of the Brazilian Agricultural Research Corporation (EMBRAPA).

Table 4 - Characteristics of the 117 *Salmonella* Typhimurium strains studied isolated from different sources in Brazil

CFSAN nº	Isolate name	Source	State	Year of isolation	Sequence Type (ST)
CFSAN033848	STm01	Human feces	SP	1983	19
CFSAN033849	STm02	Human feces	SP	1983	19
CFSAN033850	STm03	Human feces	SP	1983	19
CFSAN033851	STm04	Human feces	SP	1983	19
CFSAN033852	STm05	Human feces	SP	1983	19
CFSAN033853	STm06	Human feces	SP	1983	1649
CFSAN033854	STm07	Human feces	SP	1983	19
CFSAN033855	STm08	Human feces	SP	1983	19
CFSAN033856	STm09	Human feces	SP	1984	19
CFSAN033857	STm10	Human feces	SP	1984	19
CFSAN033858	STm11	Human feces	SP	1984	19
CFSAN033859	STm12	Human feces	SP	1984	19
CFSAN033860	STm13	Human feces	SP	1984	19
CFSAN033861	STm14	Human feces	SP	1984	19
CFSAN033862	STm15	Human feces	SP	1985	3343
CFSAN033863	STm16	Human feces	SP	1985	19
CFSAN033864	STm17	Human feces	SP	1985	19
CFSAN033865	STm18	Human feces	SP	1985	19
CFSAN033866	STm19	Human feces	SP	1986	19
CFSAN033867	STm20	Human feces	SP	1986	19
CFSAN033868	STm21	Human feces	SP	1986	19
CFSAN033869	STm22	Human feces	SP	1986	19
CFSAN033870	STm23	Human feces	SP	1986	19
CFSAN033871	STm24	Human feces	SP	1986	19
CFSAN033872	STm25	Human feces	SP	1986	19
CFSAN033873	STm26	Human feces	SP	1986	19
CFSAN033874	STm27	Human feces	SP	1986	19
CFSAN033875	STm28	Human feces	SP	1988	3343
CFSAN033876	STm29	Human feces	SP	1989	313
CFSAN033877	STm30	Human feces	SP	1990	313
CFSAN033878	STm31	Human feces	SP	1991	19
CFSAN033879	STm32	Human feces	SP	1992	19
CFSAN033880	STm33	Human feces	SP	1992	19
CFSAN033881	STm34	Human feces	SP	1993	313
CFSAN033882	STm35	Human feces	SP	1995	313
CFSAN033883	STm36	Cold chicken	SP	1995	19
CFSAN033884	STm37	Raw pork sausage	SP	1996	313
CFSAN033885	STm38	Human feces	SP	1997	19
CFSAN033886	STm39	Human feces	SP	1998	313
CFSAN033887	STm40	Lettuce	SP	1998	313

Continue

Continuation

CFSAN nº	Isolate name	Source	State	Year of isolation	Sequence Type (ST)
CFSAN033888	STm41	Raw kafta	SP	1998	19
CFSAN033889	STm42	Human feces	SP	1999	19
CFSAN033890	STm43	Human feces	SP	2000	19
CFSAN033891	STm44	Blood	SP	2000	313
CFSAN033892	STm45	Raw pork sausage	SP	2000	19
CFSAN033893	STm46	Raw tuscan sausage	SP	2002	19
CFSAN033894	STm47	Human feces	SP	2003	313
CFSAN033895	STm48	Brain abscess	SP	2005	19
CFSAN033896	STm49	Human feces	SP	2010	19
CFSAN033897	702/99	Final product	SC	1999	19
CFSAN033898	12288/06	Swine	SC	2006	19
CFSAN033899	12278/06	Swine	SC	2006	19
CFSAN033900	12290/06	Swine	SC	2006	19
CFSAN033901	12268/06	Swine	SC	2006	19
CFSAN033902	12381/06	Swine	SC	2006	19
CFSAN033903	5936/06	Cold chicken	SC	2006	19
CFSAN033904	5937/06	Cold chicken	SC	2006	19
CFSAN033905	5934/06	Swine	SC	2006	19
CFSAN033906	5961/06	Swine	SC	2006	19
CFSAN033907	5962/06	Swine	SC	2006	19
CFSAN033908	5929/06	Poultry	SC	2006	19
CFSAN033909	13609/06	Poultry	SC	2006	19
CFSAN033910	3848/08	Food	SC	2008	19
CFSAN033911	16238/09	Ready-to-eat dish	MS	2009	19
CFSAN033912	16239/09	Ready-to-eat dish	MS	2009	19
CFSAN033913	16240/09	Ready-to-eat dish	MS	2009	19
CFSAN033914	16202/09	Industrialized product	RS	2009	19
CFSAN033915	16251/09	Industrialized product	GO	2009	19
CFSAN033916	16273/09	Industrialized product	GO	2009	19
CFSAN033917	17307/09	Industrialized product	-	2009	19
CFSAN033918	9461/10	In natura meat	SC	2010	19
CFSAN033919	9479/10	In natura meat	SC	2010	19
CFSAN033920	7032/10	Poultry	PR	2010	19
CFSAN033921	3057/10	Frozen chicken carcass	PR	2010	19
CFSAN033922	6346/10	Chicken	SP	2010	19
CFSAN033923	5635/10	Unknown	RS	2010	19
CFSAN033924	9109/10	Swine	PR	2010	19
CFSAN033925	426/10	Chicken	SC	2010	19
CFSAN033926	447/10	Chicken	SC	2010	19
CFSAN033927	2452/11	Frozen chicken carcass	SP	2011	19
CFSAN033928	6709/11	Cold chicken	RS	2011	19
CFSAN033929	948/12	Raw salad	BA	2012	19

Continue

Conclusion

CFSAN nº	Isolate name	Source	State	Year of isolation	Sequence Type (ST)
CFSAN033930	1103/12	Swine (homemade salami)	RS	2012	19
CFSAN033931	1104/12	Swine (homemade salami)	RS	2012	19
CFSAN033932	3330/12	Roast beef	SC	2012	19
CFSAN033933	994/13	Final product sales (animal origin)	SP	2013	19
CFSAN033934	374/13	Final product sales (animal origin)	SC	2013	19
CFSAN033935	465/13	Final product sales (animal origin)	SP	2013	19
CFSAN033937	622/13	Final product sales (animal origin)	SC	2013	1921
CFSAN033938	583/13	Final product sales (animal origin)	SC	2013	19
CFSAN033939	623/13	Final product sales (animal origin)	SC	2013	1921
CFSAN068033	739	Mesenteric lymph node	SC	2000	ST19
CFSAN034668	1030	Swine feces	SC	2003	ST19
CFSAN068028	22	Inguinal lymph node	SC	2004	ST516
CFSAN034669	29	Swine feces	SC	2004	ST639
CFSAN034670	51	Swab swine carcass	SC	2004	ST14
CFSAN034671	68	Swine feces	SC	2004	ST14
CFSAN068029	58	Swine faeces	SC	2004	ST19
CFSAN068040	1212	Mesenteric lymph node	SC	2005	ST19
CFSAN068043	1218	Mesenteric lymph node	SC	2005	ST19
CFSAN068044	1220	Mesenteric lymph node	SC	2005	ST19
CFSAN068045	1221	Mesenteric lymph node	SC	2005	ST19
CFSAN068046	1222	Mesenteric lymph node	SC	2005	ST19
CFSAN068047	1224	Mesenteric lymph node	SC	2005	ST19
CFSAN068031	343	Herd environment	SC	2006	ST19
CFSAN068042	1214	Herd environment	SC	2006	ST19
CFSAN068041	1213	Herd environment	SC	2007	ST19
CFSAN068030	338	Swine faeces	SC	2008	ST19
CFSAN068032	345	Swine faeces	SC	2008	ST19
CFSAN068034	812	Swine urine	SC	2011	ST1921
CFSAN068035	824	Swine urine	SC	2011	ST1921
CFSAN068036	1206	Swab drag	SC	2011	ST19
CFSAN034672	804	Swine urine	SC	2011	ST1921
CFSAN034673	1209	Swab feeder	SC	2011	Unknown
CFSAN068037	1207	Swab carcass	SC	2012	ST19
CFSAN068038	1210	Swab carcass	SC	2012	ST64
CFSAN068039	1211	Swab carcass	SC	2012	ST64

Whole genome sequencing

The whole genome sequencing of the 117 *S.* Typhimurium strains was performed on the NextSeq platform (Illumina) at the U.S. Food and Drug Administration (FDA), College Park, Maryland, USA. The genomes were assembled using the software SPAdes (BANKEVICH et al., 2012) and the quality of the assemblies were evaluated using the software QUAST (GUREVICH et al., 2013) as described in Almeida et al., 2018.

cgMLST

The cgMLSTFinder 1.1 analysis from a set of reads was determined for all 117 *S.* Typhimurium genomes and three different references of this serovar were chosen, which included LT2, 14028S and D23580 using the services of the center for genomic epidemiology for *Salmonella* (Enterobase) available at https://cge.cbs.dtu.dk/services/cgMLSTFinder/ (ALIKHAN et al., 2018).

Phylogenetic tree (ggTree) and orthologous protein clusters analysis

Three different references of *S.* Typhimurium serovar were chosen, which included LT2 (GCF_000006945), 14028S (GCF_000022165) and D23580 (GCF_900538085), all with fully closed deposited genomes. To evaluate the evolutionary distance between the sequenced genomes and the three reference strains, a neighbor-joining tree was built with the ezTree algorithm (WU, 2018) and ggTree R package (YU et al., 2017; YU et al., 2018a) (Fig. 2). Additional characterization of the orthologous protein clusters for some of the key *S.* Typhimurium strains were performed. The phylogroups selected included: Comparison 1 - LT2 with 11 genomes (CFSAN033873, CFSAN033871, CFSAN033874, CFSAN033859, CFSAN033869, CFSAN033872, CFSAN033863, CFSAN033854, CFSAN033867, CFSAN033866 and CFSAN033868); Comparison 2 - LT2 with two genomes (CFSAN033855 and CFSAN033848); Comparison 3 - LT2 with six genomes (CFSAN033856, CFSAN033860, CFSAN033861, CFSAN033857, CFSAN033858 and CFSAN033851); Comparison 4 - LT2 with three genomes (CFSAN033852, CFSAN033849 and CFSAN033850); Comparison 5 - 14028S with seven genomes (CFSAN033930, CFSAN033931, CFSAN033919, CFSAN033924, CFSAN033922, CFSAN033929 and CFSAN033909); Comparison 6 - D23580 with nine genomes (CFSAN033882, CFSAN033877, CFSAN033887, CFSAN033886, CFSAN033876, CFSAN033881, CFSAN033894, CFSAN033891 and CFSAN033884); Comparison 7 - D23580 with 11 genomes (CFSAN033882, CFSAN033877, CFSAN033887, CFSAN033886, CFSAN033876,

CFSAN033881, CFSAN033894, CFSAN033891, CFSAN033884, CFSAN033921 and CFSAN033883) via OrthoVenn2 (XU et al., 2019) in order to determine unique features and metabolic pathways defining each group.

Multi-locus sequence typing

MLST was performed for all the 26 isolates *S*. Typhimurium from swine using the MLST 2.0 of the Center for Genomic Epidemiology for *Salmonella enterica* available in https://cge.cbs.dtu.dk/services/MLST/ (Larsen et al., 2012). The STs of the *S*. Typhimurium isolates from humans and different foods were previously described in Almeida et al., 2017a.

Prophages detection

The genomes of all 117 *S*. Typhimurium strains were used to search the prophages by PHAge Search Tool Enhanced Release (PHASTER) that is an online platform for the rapid identification and annotation of prophages sequences in bacterial genomes and plasmids available in http://phaster.ca/ (ARNDT et al., 2016).

Efflux pump

The genomes of all 117 *S*. Typhimurium strains were used to search resistance genes related to efflux pump. Resistance gene identifier (RGI) is part of the Comprehensive Antibiotic Resistance Database (CARD) and was performed with high quality/coverage (includes contigs > 20,000 bp and excludes prediction of partial genes) available in https://card.mcmaster.ca/analyze/rgi (MCARTHUR et al., 2013).

LIST OF ABBREVIATIONS

NTS: Nontyphoidal *Salmonella*

CDC: Centers for Disease Control and Prevention

WGS: Whole genome sequencing

cgMLST: Core genome multilocus sequence typing

ST: Sequence type

MLST: Multilocus sequence typing

MDR: Multidrug resistant

DNA: Deoxyribonucleic acid

WHO: World Health Organization

UniProt: Universal Protein Resource

FDA: Food and Drug Administration

PHASTER: PHAge Search Tool Enhanced Release

RGI: Resistance gene identifier

CARD: Comprehensive Antibiotic Resistance Database

IAL-RP: Adolfo Lutz Institute of Ribeirão Preto

FIOCRUZ-RJ: Oswaldo Cruz Foundation from Rio de Janeiro

EMBRAPA: Brazilian Agricultural Research Corporation

DECLARATIONS

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data from 117 *S*. Typhimurium genomes were deposited in the GenBank (NCBI) under the identification numbers: LVHC00000000, LVHB00000000, LVHA00000000, LVGZ00000000, LVGY00000000, LVGX00000000, LVGW00000000, MABI00000000, LVGV00000000, LVGU00000000, LVGT00000000, LUJG00000000, LVGS00000000, LVGR00000000, LVGQ00000000, LVGP00000000, LVGO00000000, LVGN00000000, LVGM00000000, LVGL00000000, LUJF00000000, LVGK00000000, LVGJ00000000, LVGI00000000, LVGH00000000, LVGG00000000, LVGF00000000, LUJE00000000, LVGE00000000, LVGD00000000, LUJD00000000, LVGC00000000, LVGB00000000, LVGA00000000, LVFZ00000000, LVFY00000000, LVFX00000000, LUJC00000000, LUJB00000000, LUJA00000000, LVFW00000000, LUIZ00000000, LVFV00000000, LVFU00000000, LUIY00000000, LVFT00000000, LUIX00000000, LUIW00000000, LVFS00000000, LVFR00000000, LUIV00000000, LUIU00000000, LUIT00000000, LVFQ00000000, LUIS00000000, LUIR00000000, LUIQ00000000, LUIP00000000, LUIO00000000, LVFP00000000, LUIN00000000, LUIM00000000, LUIL00000000, LUIK00000000, LVFO00000000, LUIJ00000000, LUII00000000, LUIH00000000, LVFN00000000, LUIG00000000, LUIF00000000, LUIE00000000, LVFM00000000, LUID00000000, LUIC00000000, LVFL00000000, LVFK00000000, LUIB00000000, LUIA00000000, LUHZ00000000, LVFJ00000000, LUHY00000000, LUHX00000000,

LVFI00000000, LUHW00000000, LUHV00000000, LUHU00000000, LUHT00000000, LUHS00000000, LUHR00000000, LVFH00000000, SRR8291813, SRR8291805, SRR8291802, SRR8291817, SRR8291806, SRR8291814, PHJE00000000, PHJD00000000, PHJC00000000, PIJC00000000, PHJB00000000, PHJA00000000, PHIZ00000000, PHIY00000000, PHIX00000000, PHIW00000000, PHIV00000000, PHIU00000000, PHIT00000000, PHIS00000000, PHIR00000000, PHIQ00000000, PHIP00000000, PHIO00000000, PHIN00000000, PHIM00000000, released under the project PRJNA186035 (https://www.ncbi.nlm.nih.gov/bioproject/186035).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AAS participated in the design of the work, performed to methods, analysed and interpreted all the data and wrote the original manuscript. PS performed to methods. MFC analysed and interpreted the data. FA performed the whole genome sequencing of the *S*. Typhimurium strains isolated from humans and foods. MRF performed the whole genome sequencing of the *S*. Typhimurium strains isolated from swine. MICM collected *S*. Typhimurium strains isolated from humans and foods. DPR collected *S*. Typhimurium strains isolated from foods. JDK collected *S*. Typhimurium strains isolated from swine. LJB performed to methods. SCS performed to methods. MWA was whole genome sequencing supervisor. JPF participated in the design of the work and supervisor. All authors read and approved the final manuscript.

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CHAPTER 6

Salmonella Typhimurium ST313 isolated in Brazil revealed to be more invasive and inflammatory in murine colon compared to ST19 strains

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KEYWORDS: *Salmonella* Typhimurium ST313; Invasion; Inflammation; Cytokines; RNA-seq

ABSTRACT

Salmonella Typhimurium ST313 has caused an epidemic of invasive disease mainly in sub-Saharan Africa. However, in Brazil this ST was recently described and there is a few studies that tried to elucidate the virulence of ST313 strains. The aims of this study were to perform the RNA-seq *in vitro* under growth in Luria Bertani (LB) at 37°C and compare *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains. In addition, it was aimed to evaluate the colonization and expression of virulence genes and cytokines in the murine colon. The transcriptome and *in vivo* experiment showed a greater virulence and inflammation profile of *S*. Typhimurium STm30 (ST313) in comparison to SL1344 (ST19) and STm11 (ST19). The *hilA*, *sopD2*, *pipB* and *ssaS* virulence genes, besides other genes found or effectors of SPI-1 and SPI-2 and the IL-1β, IFN-γ, TNF-α, IL-6, IL-17, IL-22 and IL-12 cytokines presented high levels of expression during the infection in C57BL/6J mice by the ST313 strain. In conclusion, *S*. Typhimurium STm30 (ST313) isolated from human feces in Brazil demonstrated greater expression of genes related to pathogenesis at 37°C, besides better colonization and invasion in the murine colon due to higher levels of expression of virulence genes. In addition, pro-inflammatory cytokines were also more expressed in this organ, suggesting greater tissue damage in comparison to *S*. Typhimurium SL1344 (ST19) and *S*. Typhimurium STm11 (ST19) isolated from human feces in Brazil.

INTRODUCTION

Invasive Non-typhoidal *Salmonella* (iNTS) includes important serovars responsible for causing bacteremia and meningitis, such as *S*. Typhimurium belonging to sequence type (ST) 313 mainly described and isolated in sub-Saharan Africa (AO et al., 2015; GILCHRIST and MACLENNAN, 2019). In Brazil, *S*. Typhimurium ST313 strains isolated from blood, human feces and food have been recently reported (ALMEIDA et al., 2017).

It is important to mention that *S*. Typhimurium ST313 has been associated to systemic infections in infants, older adults and people with immunosuppressive conditions including HIV, hemoglobinopathies and malignant neoplasm (CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC), 2019a). On the other hand, cases of gastroenteritis have been mainly caused by *S*. Typhimurium ST19, which has been reported as the predominant ST in strains of this serovar in various parts of the globe (FEASEY et al., 2014; RAMACHANDRAN et al., 2015; RAMACHANDRAN et al., 2017).

According to Ao and collaborators (2015), it was estimated that 3.4 million infections occur for iNTS every year worldwide and the African continent is the most affected with an incidence of 227 cases per 100,000 population, totaling approximately 1.9 million cases annually. The mortality rate of this disease is high, representing approximately 20% of the total number of cases or 681,316 deaths (AO et al., 2015).

S. Typhimurium invades host cells through the Type III Secretion System (T3SS) that has the ability to modulate a series of cellular functions related to their survival and replication in humans which is encoded by genes present in the pathogenicity islands 1 and 2 (SPI-1 and SPI-2) (SUN et al., 2016; SANTOS et al., 2019). Specifically, the proteins encoded by SPI-1 are necessary for an initial interaction and invasion of *S*. Typhimurium in host epithelial cells found in the large intestine (HURLEY et al., 2014).

Furthermore, important proteins encoded by SPI-2 are expressed only when *S*. Typhimurium is internalized by the host local macrophages, favoring the escape of this bacterium by inhibiting the fusion of the phagosome with the lysosome resulting in their survival and proliferation inside vacuolar compartment denominated *Salmonella* containing vacuole (SCV) (HURLEY et al., 2014; LAROCK; CHAUDHARY; MILLER, 2015; SUN et al., 2016).

During the course of salmonellosis, pro-inflammatory cytokines that act by promoting a systemic inflammatory process are released, such as interleukins (IL-1β and IL-6), interferons (IFN-γ), and tumor necrosis factor (TNF-α) (HURLEY et al., 2014). In addition, there are two interleukins that contribute to healing, secretion of antimicrobial molecules and induction of pro-inflammatory mediators, with consequent recruitment of neutrophils. These interleukins are denominated IL-17 and IL-22, which IL-17 induces other pro-inflammatory cytokines and IL-22 is able to induce tissue protection (VALERI and RAFFATELLU, 2016).

The interleukin IL-12 is produced mainly by dendritic cells and phagocytes, which plays a key role in stimulating the Th1 response (cell-mediated immune response) (ELSNER and SHLOMCHIK, 2019). According to Salazar and collaborators (2017), the interleukin IL-10 is classified as anti-inflammatory which is extremely important to limit the inflammation that occurs during the infectious process and prevent tissue damage, inhibiting other interleukins including IL-12 and IFN-γ.

It is known that *S*. Typhimurium ST313 and *S*. Typhimurium ST19 present differences in their behavior during infection; but there are few studies that tried to elucidate such differences *in vivo* (OKORO et al., 2015; YANG et al., 2015a; RAMACHANDRAN et al., 2015; RAMACHANDRAN et al., 2017).

Previous studies performed by our research group evaluated the invasiveness of these strains *in vitro* isolated from human and non-human sources in HeLa and Caco-2 and survival in J774 murine and U937 human macrophages, besides the investigation of the virulence of these strains in the alternative *Galleria mellonella* infection model was performed (ALMEIDA et al., 2017; SERIBELLI et al., 2020).

S. Typhimurium strains of ST313 and ST19 studied showed high levels of invasion in HeLa and Caco-2 cells and survival in J774 and U937 macrophages, as well as virulence in *G. mellonella* similar to SL1344 and ATCC14028 levels, which are considered references of virulent strains of this serovar (ALMEIDA et al., 2017; SERIBELLI et al., 2020). Therefore, the differential and importance of this study are linked to the need to explore *in vivo* assays and gene expression of *S*. Typhimurium ST313 and ST19 strains isolated in Brazil.

The aims of this work were to perform the RNA-seq *in vitro* under growth in Luria Bertani (LB) at 37°C comparing the *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains. In addition, it was aimed to evaluate the colonization and expression of virulence genes and cytokines in the murine colon after infection by such strains which in turn could explain possible differences in the pathogenesis of the invasive ST313.

MATERIALS AND METHODS

Bacterial strains

Three strains were chosen due to its similar ability to invade Caco-2 cells (Seribelli et al., 2020) including one reference strain and two strains isolated from human feces in Brazil. Specifically, these strains were: *S.* Typhimurium STm11 (ST19) isolated in 1984, *S.* Typhimurium STm30 (ST313) isolated in 1990, both belonged to the culture collection of the Adolfo Lutz Institute of Ribeirão Preto (IAL-RP) and *S*. Typhimurium SL1344 prototype (ST19).

RNA-seq

The three *S*. Typhimurium strains chosen were grown overnight in 5 mL of Luria Bertani (LB) broth (Difco) at 37ºC. Then, 1 mL of the culture was added to 7 mL of LB broth (Difco) and incubated under shaking at 37°C for approximately 3 hours until reaching an O.D.600 = 0.8.

The RNAs were extracted with PureLink™ RNA Mini Kit (Thermo Fisher Scientific). The Qubit RNA BR (Broad-Range) Assay Kit (Invitrogen™) and Qubit™ dsDNA BR Assay

Kit (Invitrogen™), besides Bioanalyzer RNA 6000 Nano Kit (Agilent Technologies) and Bioanalyzer DNA 1000 kit (Agilent Technologies) were used for the quantification and quality check of the samples.

Ribosomal RNAs were depleted with RiboMinus™ Transcriptome Isolation Kit, bacteria (Invitrogen™) and were concentrated with RiboMinus™ Concentration Module (Invitrogen™). The sequencing was performed using NextSeq 550 series (Illumina®) and the strains were prepared according to the manufacturer's instructions. The experiment was performed in biological triplicate.

Mapping of RNA-seq libraries and differential gene expression analysis

Initially raw reads served as input for Trimmomatic (BOLGER et al., 2014), which performed quality filtering removing Illumina adaptor sequences, low quality bases (phred score quality > 20) and reads shorter than 35 bp. Trimming was followed by read error correction by SGA *k*-mer-based algorithm (SIMPSON AND DURBIN, 2012). The pre-processed reads were compared to the raw reads through FastQC analysis (ANDREWS, 2010) to evaluate the trimming and quality control performed previously.

Next the reads were mapped against the *Salmonella enterica* serovar Typhimurium SL1344 reference genome (KROGER et al., 2012) Genbank access code GCA_000210855.2, using Bowtie2 software (LANGMEAD AND SALZBERG, 2012) following previously described optimization (BARUZZO et al., 2017).

The gene differential expression calling was achieved initially by counting the number of reads in each transcript through HTSeq (ANDERS; PYL; HUBER, 2015). Finally, the count data were direct to differential analysis with DESeq2 R package (LOVE; HUBER; ANDERS, 2014).

Gene ontology

The genes that showed adjusted p value < 0.1 and log fold change > 2 or < -2 for each comparison were subjected to gene ontology analysis using the PANTHER database (MI et al., 2019). A False Discovery Rate (FDR) p value < 0.05 was used as threshold to select significant pathways.

Mice

The animals used in this study were obtained from CEMIB/UNICAMP and the experiments were performed according to the Animal Ethics Committee

(CEUA/FCF/CAr.05/2020). We used six C57BL/6J UNIB female mice per group, weighing between 17g and 20g of seven to nine week-old. In order to deplete partially the intestinal microbiota, the mice were pretreated orally with 1g/Kg of streptomycin 24 hours before infection. Mice were infected with $1x10^8$ of *S*. Typhimurium SL1344 (ST19), *S.* Typhimurium STm11 (ST19) and *S.* Typhimurium STm30 (ST313) via oral gavage and monitored for 2 days. Feces were collected on days 1 and 2 after infection to recover Colony Forming Units (CFU). For gene expression analysis via qRT-PCR and tissue colonization, the animals were euthanized on day 2 after infection and the colon was collected for RNA extraction and CFU count. All experiments were performed as previously described by Barthel et al., 2003.

qRT-PCR

RNA extraction was performed from colon collected on day 2 after mouse infections. In order to disrupt cells and inactivate nucleases, it was used 1 mL of TRIzol (Life Technologies) per 100 mg of samples. RNA was isolated using the Ribo Pure bacterial isolation kit (Ambion) according to the manufacturer's instructions. qRT-PCR was performed with primers to detect virulence genes and cytokines (Table 1). The experiments were carried out with Master Mix SYBR®, Multiscribe® Reverse Transcriptase, RNAse inhibitor (Thermo Fisher Scientific) and 100 ng of RNA in a one-step reaction using the QuantStudio3 (Thermo Fisher Scientific). All data were normalized with endogenous control, *rpoA* (RNA polymerase subunit A) for virulence genes and *Actb* (Beta-actin) for host genes. Results were analyzed by comparative critical threshold ($\Delta\Delta CT$) method, as previously described in Walters and Sperandio, 2006.

Table 1. Oligonucleotides used in this study

Primer	Forward sequence	Reverse sequence	Reference or source
rpoA	GCGCTCATCTTCTTCCGAAT	CGCGGTCGTGGTTATGTG	Moreira; Weinshenker; Sperandio, 2010
pipB	GGTGGAGTAAGAAGAAGCAA	AGTTTTCCAATTACCTCCCG	This study
ssaS	TTTTACGTCTATGCCGGTAG	CTGTATTTGAGTCAAGGCCT	This study
hilA	CATGGATCAATTACGCCCCG	AGCGGGTTGGTGTTCTATCA	This study
sopD2	AGAAGAAGCGCTATACATGG	ATAATACCTCCAGCACCTCT	This study
Il1β	ATGCCTTCCCCAGGGCATGT	CTGAGCGACCTGTCTTGGCCG	Yu et al., 2018b
Il6	TTCCATCCAGTTGCCTTCTTG	AGGTCTGTTGGGAGTGGTATC	McGuire et al., 2016
Ifnγ	GCTTTGCAGCTCTTCCTCAT	GTCACCATCCTTTTGCCAGT	Liu et al., 2018
Tnfα	GCCTCTTCTCATTCCTGCTTG	CTGATGAGAGGGAGGCCATT	Yamakawa et al., 2011
Il10	CCCTTTGCTATGGTGTCCTTTC	GATCTCCCTGGTTTCTCTTCCC	Darling et al., 2017
Il12p40	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG	Zhou et al., 2015
Il17a	GCTCCAGAAGGCCCTCAGA	AGCTTTCCCTCCGCATTGA	Behnsen et al., 2014
Il22	GGCCAGCCTTGCAGATAACA	GCTGATGTGACAGGAGCTGA	Behnsen et al., 2014
Actb	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	Behnsen et al., 2014

Statistical analysis

All statistical analyses were done using the GraphPad Prism v7.04 (GraphPad Software, San Diego, CA) by one-way analysis of variance (ANOVA) and Dunnett's multiple comparisons post-test. Data were considered significant when $p < 0.05$.

RESULTS

Transcriptome analysis

The transcriptome was performed for the *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains after growth in LB at 37°C. Overall, 303 genes were differentially expressed (153 upregulated and 150 downregulated) in STm30 (ST313) compared to SL1344 (ST19). Specifically, *S*. Typhimurium STm30 (ST313) presented upregulated genes linked to various biological processes including pathogenesis, propanediol degradation pathway, ethanolamine utilization, bacteriophage, electron acceptors, aminoacid metabolism, sugar metabolism and peptidoglycan synthesis (Fig. 1A). Moreover, the STm30 (ST313) strain presented downregulated genes linked to various biological processes including bacteriophage, anaerobic metabolism, motility/chemotaxis, sugar metabolism, nucleotide/amino acid metabolism, glycerol catabolic process, TCA cycle/glycolysis, transport and pathogenesis (Fig. 1B). The Fig. 2A indicates some of the main upregulated genes related to pathogenesis, such as genes found in SPI-1 and SPI-2.

In another analysis, comparing *S*. Typhimurium STm30 (ST313) and STm11 (ST19,) 867 genes were differentially expressed (493 upregulated and 374 downregulated). Similarly, *S*. Typhimurium STm30 (ST313) presented upregulated genes linked to various biological processes including pathogenesis, anaerobic metabolism, bacteriophage, ribosome assembly, propanediol utilization, ethanolamine utilization and cobalamin biosynthesis (Fig. 1C). Furthermore, the STm11 (ST313) strain presented downregulated genes linked to various biological processes including motility/chemotaxis, transport, bacteriophage, anaerobic metabolism, transcriptional control and DNA replication and repair (Fig. 1D). The Fig. 2B indicates some of the main upregulated genes related to pathogenesis, such as genes found in SPI-1 and SPI-2.

The most marked differences among the *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) were upregulated genes related to pathogenesis in ST313. Specifically, the heatmaps presented in Fig. 3 illustrate the

correlation between the transcriptomes of such strains and the differential expression of the genes of SPI-1, SPI-2, SPI-1 and SPI-2 effectors, SPI-3 and SPI-4.

Fig. 1 – Transcriptome analyses of *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains after *in vitro* growth in Luria Bertani (LB) at 37°C. A) STm30xSL1344 - 153 upregulated genes, B) STm30xSL1344 - 150 downregulated genes, C) STm30xSTm11 – 493 upregulated genes, D) STm30xSTm11 – 374 downregulated genes.

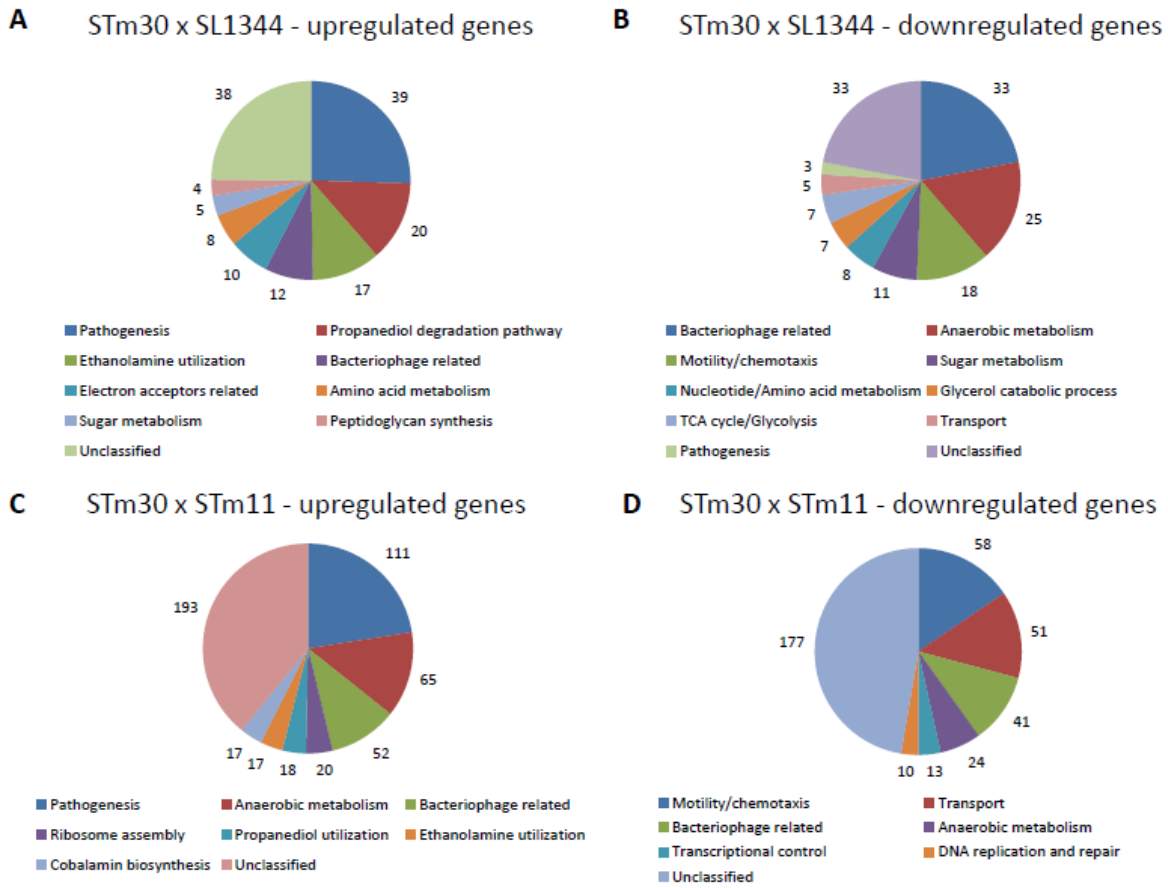


Fig. 2 – Volcano plot of *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains after *in vitro* growth in Luria Bertani (LB) at 37°C. A) STm30xSL1344 - upregulated genes related to SPI-1 and SPI-2 pathogenicity islands. B) STm30xSTm11 - upregulated genes related to SPI-1 and SPI-2 pathogenicity islands.

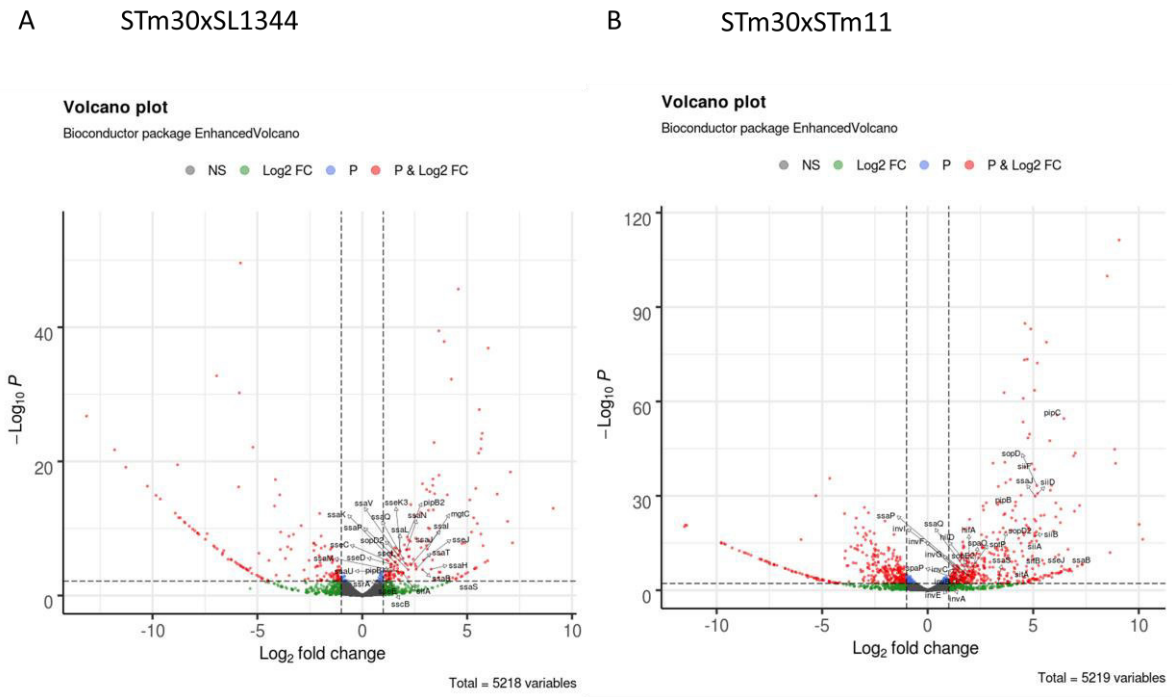
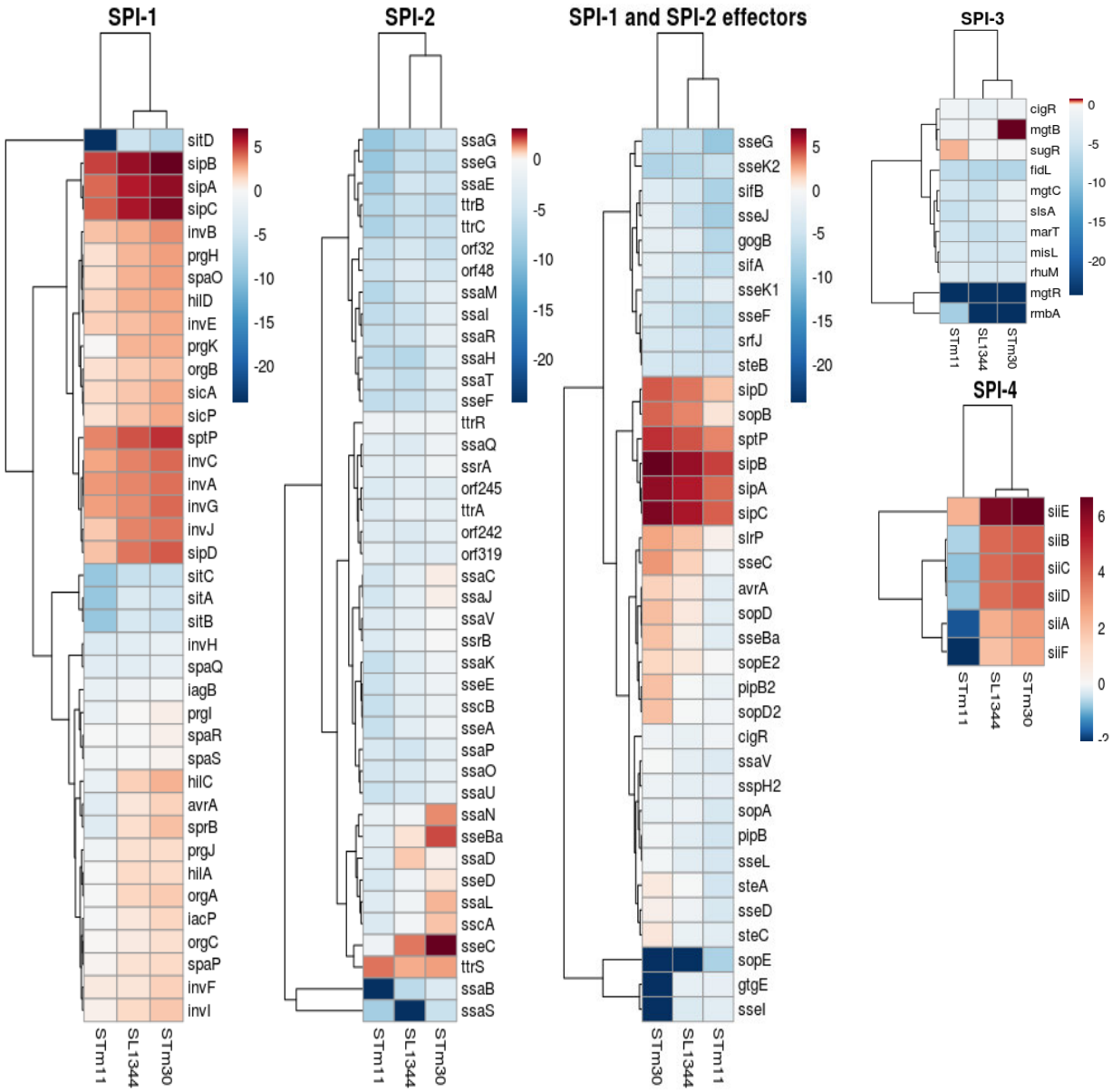


Fig. 3 – Heatmaps comparing the *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains after *in vitro* growth in Luria Bertani (LB) at 37°C of genes related to SPI-1, SPI-2, SPI-3 and SPI-4.

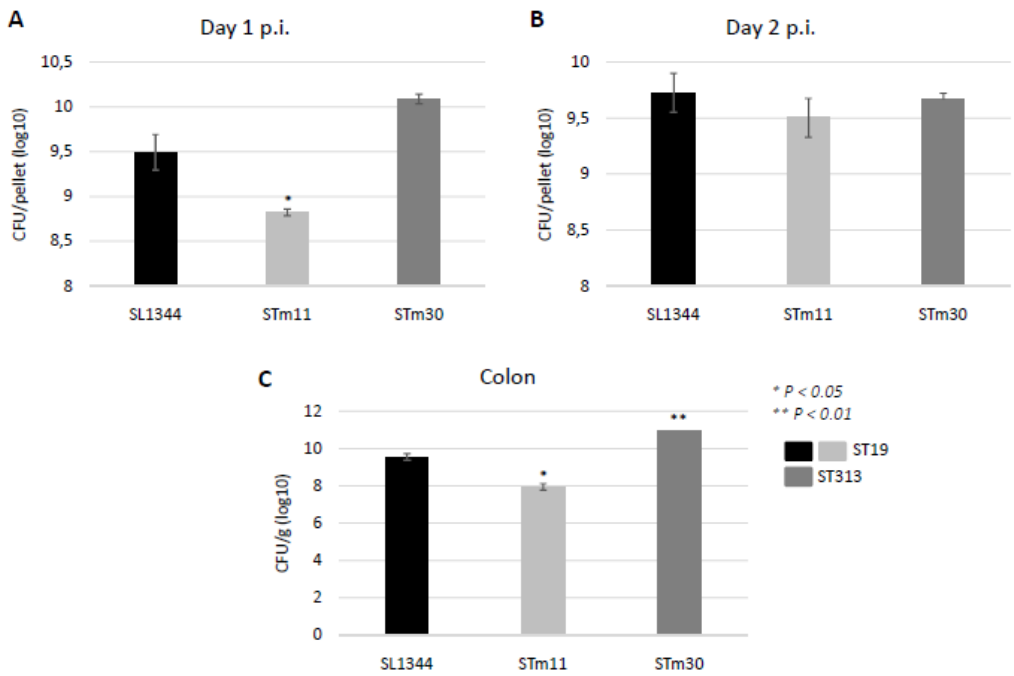


Colitis model infection with *S*. Typhimurium

In order to analyze the number of bacteria present in the feces during the infection period by *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains, fecal pellets were collected on days 1 and 2 p.i. On day 1 p.i. the STm11 (ST19) strain showed a difference of 0.7 orders of magnitude lower and there was a significant difference in comparison to *S*. Typhimurium SL1344 (ST19). The STm30 (ST313) strain had no significant difference in comparison to SL1344 (ST19) (Fig. 4A). However, on day 2 p.i. all strains showed a similar colonization profile (Fig. 4B).

S. Typhimurium strains has the ability to invade intestinal epithelial cells predominantly causing diarrhea, fever and stomach cramps (CDC, 2019a). Therefore, we evaluated the number of bacteria present in colon collected on day 2 p.i. Specifically, in the large intestine, its main infection site, when compared to SL1344 (ST19), the STm11 (ST19) strain presented a lower colonization with a difference of 1.6 orders of magnitude, while the STm30 (ST313) strain showed a notable intestinal cells invasion efficiency with 1.4 orders higher of CFU recovered (Fig. 4C).

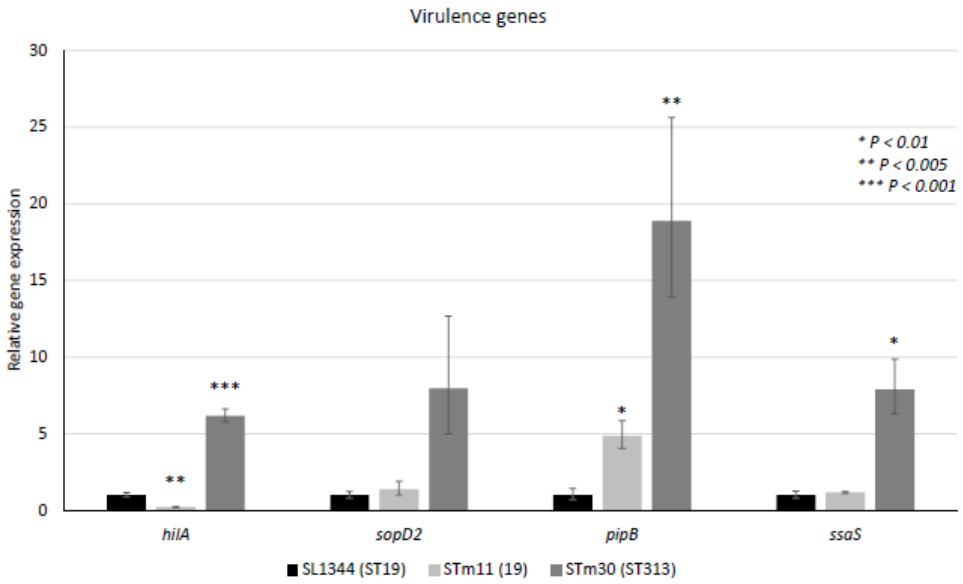
Fig. 4 – *S*. Typhimurium infection in C57BL/6J mice. Colony Forming Units (CFU) of SL1344 (ST19), STm11 (ST19) and STm30 (ST313) strains were recovered from feces collected on days 1 and 2 p.i. (A and B) and from intestines collected on day 2 p.i. (C). Statistical significance for each replicate was assessed by ANOVA. Standard deviation is indicated by error bars (*p<0.05, **p<0.01).



To measure the expression of virulence genes and cytokines via qRT-PCR, RNAs were extracted from colon of animals infected with the *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains on day 2 p. i. It is important to emphasize that the SL1344 (ST19) strain was used as a comparison standard for all the results described below.

Specifically, to investigate the mechanism of the STm30 (ST313) strain associated with the better colonic colonization (Fig. 4C) it was performed the gene expression measurement of some virulence genes (Fig. 5). In the colon environment on day 2 p.i. the STm30 (ST313) strain presented a higher expression of epithelial cells invasion and intracellular replication genes compared with both ST19 strains (Fig. 5). The *hilA* gene is described as trascriptional activator of SPI-1 (THIJS et al., 2007) and it was 5-fold over expressed in the STm30 (ST313) strain (Fig. 5). Similarly, the STm30 (ST313) strain had higher levels of expression for the SPI-2 effectors *sopD2* and *pipB*, both related to *Salmonella* inducing filaments (Sifs), an important structure to intracellular fitness and replication (KNODLER et al., 2003; JIANG et al., 2004), *pipB* was more than 15-fold increased (Fig. 5). The *ssaS* gene encodes a T3SS apparatus protein (MCCLELLAND et al., 2001) and it was more than 7-fold over expressed in the STm30 (ST313) (Fig. 5).

Fig. 5 – Gut inflammation during infection with *S*. Typhimurium strains. q-RT-PCR was performed with RNA collected from intestines of mice infected with SL1344 (ST19), STm11 (ST19) and STm30 (ST313) strains on day 2 p.i. The *hilA*, *sopD2*, *pipB* and *ssaS* genes expressions were analyzed. Statistical significance for each replicate was assessed by ANOVA. Standard deviation is indicated by error bars (**p*< 0.01, ***p*< 0.005, ****p*< 0.001).



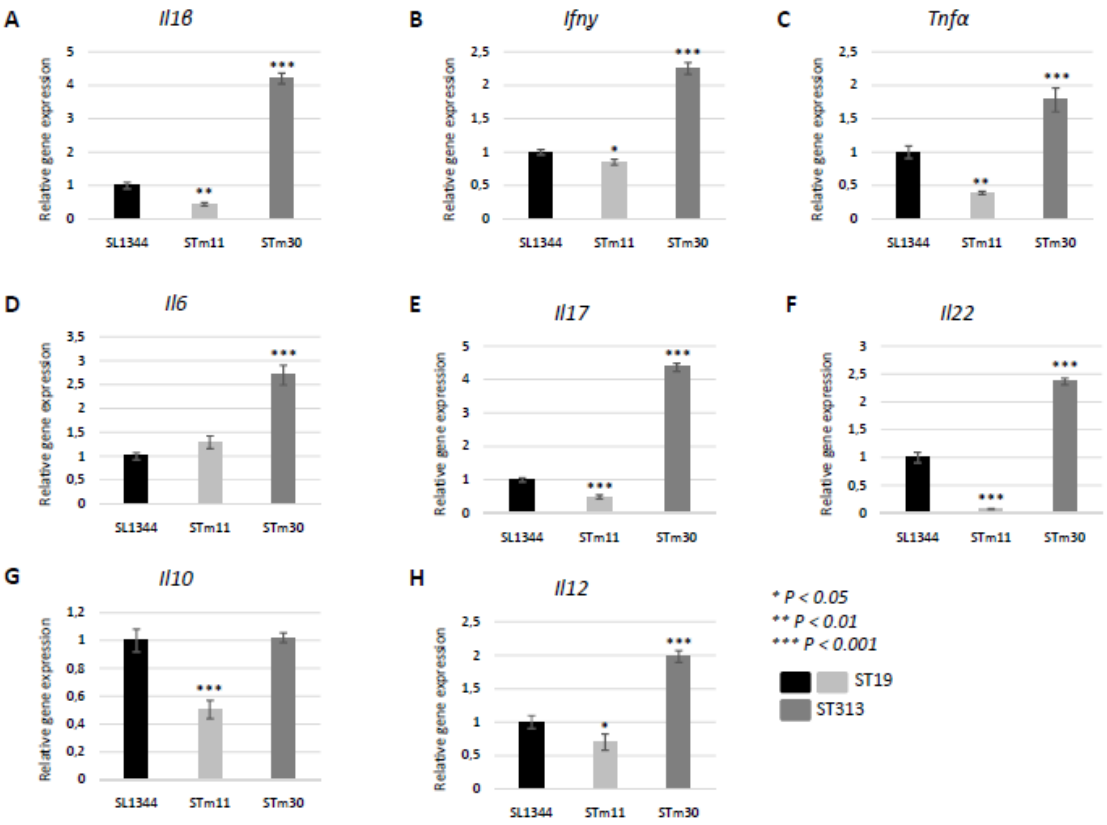
The IL-1β, IFN-γ, TNF-α and IL-6 cytokines are important mediators of systemic inflammation (HURLEY et al., 2014). For IL-1β, the STm11 (ST19) strain showed 2.3-fold decrease and the STm30 (ST313) strain an increase of 4.2-fold (Fig. 6A). For IFN-y, a decrease of 1.2-fold was observed for STm11 (ST19) and a 2.3-fold increase for STm30 (ST313) (Fig. 6B). For TNF-α, a decrease of 2.6-fold was observed for STm11 (ST19) and a 1.8-fold increase for STm30 (ST313) (Fig. 6C). For IL-6, only the STm30 (ST313) strain presented a significant difference with a 2.7-fold increase (Fig. 6D).

Moreover, the interleukin IL-17 induces the production of other pro-inflammatory cytokines (VALERI and RAFFATELLU, 2016) and here it was observed a 2.1-fold decrease for the STm11 (ST19) strain and a 4.4-fold increase for the STm30 (ST313) strain (Fig. 6E). On the other hand, the interleukin IL-22 is able to induce tissue protection (VALERI and RAFFATELLU, 2016) and its expression was 13.4-fold lower in STm11 (ST19) and a 2.4-fold higher in STm30 (ST313) (Fig. 6F).

Another interleukin able of inducing repair and consequent limitation of inflammation is IL-10 (SALAZAR et al., 2017), which showed a two-fold reduction for the STm11 (ST19) strain, while the STm30 (ST313) strain showed no significant difference (Fig. 6G).

Finally, the interleukin IL-12 is important for cellular response (ELSNER and SHLOMCHIK, 2019) and here it showed a 1.4-fold decrease for the STm11 (ST19) strain and a 2-fold increase for the STm30 (ST313) strain (Fig. 6H).

Fig. 6 – Gut inflammation during infection with *S*. Typhimurium strains. q-RT-PCR was performed with RNA collected from intestines of mice infected with SL1344 (ST19), STm11 (ST19) and STm30 (ST313) strains on day 2 p.i. The IL-1β, IFN-γ, TNF-α, IL-6, IL-17, IL-22, IL-10 and IL-12 cytokines expressions (A-H) were analyzed. Statistical significance for each replicate was assessed by ANOVA. Standard deviation is indicated by error bars (**p*< 0.05, ***p*< 0.01, ****p*< 0.001).



DISCUSSION

In this study, *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains were compared after performing the transcriptome with growth LB at 37°C and evaluation of colonization and expression of virulence and cytokines genes in the colon using the C57BL/6J infection model.

It is important to mention that in Africa *S*. Typhimurium ST313 strains has been a serious public health problem and during the last decades, an epidemic of invasive infections has been evidenced with mortality rates of 25% (FEASEY et al., 2012; GILCHRIST and MACLENNAN, 2019).

A recent article of our research group showed data on invasion in Caco-2 epithelial cells and survival in U937 human macrophages of *S*. Typhimurium strains of different STs including nine ST313 and 28 ST19 strains isolated from humans and food (SERIBELLI et al.,

2020). *S*. Typhimurium strains of ST313 and ST19 showed mean of CFU/well (log_{10}) of 6.37 and 6.57, respectively for invasion in Caco-2 cells and with the mean of CFU/well (log_{10}) of 7.33 and 7.2, respectively for survival in U937 human macrophages (data not shown). Therefore, no evidence of statistically significant difference was found between ST313 and ST19 strains suggesting that the high virulence described for these strains was not observed under the *in vitro* assays performed.

This article initially checked the transcriptome of *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains. In general, the majority of the upregulated genes were related to the pathogenesis of this important pathogen, mainly present in SPI-1, SPI-2, SPI-1 and SPI-2 effectors, SPI-3 and SPI-4 (Fig. 1, Fig. 2 and Fig. 3).

It is known that *S*. Typhimurium has the capacity to invade epithelial cells of the intestine, during this process the T3SS is fundamental and some SPI-1 effectors genes are involved including *sipA*, *sipB*, *sopB*, *sopD* and *sopE2* (HURLEY et al., 2014; ARYA et al., 2017). Interestingly, these genes were more expressed in the STm11 (ST313) strain compared to SL1344 and STm11 (ST19) (Fig. 3).

In addition, *S*. Typhimurium crosses the epithelial barrier and is phagocyted by host local macrophages, activating genes present in SPI-2 and its effectors, such as *pipB*, *sscA*, *sscB*, *sseA*, *sseF* and *sseG*, secreting proteins that prevent the fusion of the phagosome with the lysosome (HURLEY et al., 2014). Similarly to the results described above, these genes were more expressed in the STm11 (ST313) strain compared to SL1344 and STm11 (ST19) (Fig. 3).

The genes present in SPI-3 are important for the adaptation of *S*. Typhimurium in environments with low nutritional conditions, such as inside macrophages and low concentrations of magnesium, the expression of the *mgtB* and *mgtC* genes are necessary for adaptation in these environments (WANG et al., 2020b). The *mgtB* and *mgtC* genes were more expressed in the STm11 (ST313) strain compared to SL1344 and STm11 (ST19) (Fig. 3).

Finally, SPI-4 is formed by the *siiA*, *siiB*, *siiC*, *siiD*, *siiE* and *siiF* genes responsible for the production of the Type I Secretion System (T1SS), favoring the adhesion and internalization of *S*. Typhimurium in host epithelial cells (WANG et al., 2020b). All of these genes were also more expressed in the STm11 (ST313) strain compared to SL1344 and STm11 (ST19) (Fig. 3).

After the *in vivo* experiment it was possible to observe that the number of CFU recovered from feces on day 1 p.i. was lower in the STm11 (ST19) strain in comparison to SL1344 (ST19) and between the STm30 (ST313) strain and the SL1344 (ST19) no significant difference was detected (Fig. 4A). Furthermore, the number of CFU recovered from feces on day 2 p.i. was similar among the strains studied (Fig. 4B). Also, on day 2 p.i. the animals were euthanized, and the colon collected, the recovered CFU was higher in the STm30 (ST313) strain compared to SL1344 (ST19) (Fig. 4C). For the STm11 (ST19) strain the recovered CFU was lower in comparison to SL1344 (ST19) (Fig. 4C).

Moreover, the expression of four virulence genes was detected in the murine colon including *hilA*, *sopD2*, *pipB* and *ssaS*. As well as, for what was observed for the virulence genes described in the transcriptome, these genes were also more expressed in the STm30 (ST313) strain in comparison to SL1344 (ST19) (Fig. 5). On the other hand, the *hilA* gene was less expressed in the STm11 (ST19) strain compared to SL1344 (ST19), for the *sopD2* and *ssaS* genes there was no significant difference and for the *pipB* gene there is a greater expression in relation to SL1344 (ST19) (Fig. 5).

Therefore, these data indicate that *S*. Typhimurium STm30 (ST313) was more efficient for colonizing and invading the large intestine of C57BL/6J due to increased expression of virulence genes, such as *hilA*, *sopD2*, *pipB* and *ssaS* that are found or effectors of SPI-1 and SPI-2.

In this study, the expression of eight cytokines (IL-1β, IFN-γ, TNF-α, IL-6, IL-17, IL-22, IL-12 and IL-10) was investigated in the murine colon after infection by the *S*. Typhimurium SL1344 (ST19), *S*. Typhimurium STm11 (ST19) and *S*. Typhimurium STm30 (ST313) strains (Fig. 6). According to Hurley and collaborators (2014), the cytokines IL-1β, IL-6, IFN-γ and TNF-α are released after *S*. Typhimurium strains invade and survive to intestinal epithelial cells and macrophage apoptosis. Another interleukin that favors inflammation is IL-17, inducing the release of inflammatory mediators; however IL-22 induces tissue repair and protection (VALERI and RAFFATELLU, 2016).

The control of inflammation is due to the production of anti-inflammatory cytokines and interleukin IL-10 is the main representative of this class, inhibiting the proliferation of Th1 cells and the synthesis of inflammatory cytokines, such as IFN-γ and IL-12 (SALAZAR et al., 2017).

S. Typhimurium has the ability to survive and proliferate intra macrophage due to the formation of SCV and this stimulates the release of some cytokines, such as IL-12 which acts

favoring the response mediated by phagocytic cells against intracellular pathogens (Th1) (HURLEY et al., 2014).

The IL-1β, IFN-γ, TNF-α, IL-6, IL-17, IL-22 and IL-12 cytokines were more expressed in the STm30 (ST313) strain compared to SL1344 (ST19), suggesting a greater inflammatory process in the murine colon by the first strain mentioned (Fig. 6). The expression of the interleukin IL-10 was similar between the STm30 (ST313) strain and the SL1344 (ST19) (Fig. 6G). In contrast, the cytokines IL-1β, IFN-γ, TNF-α, IL-17, IL-22, IL-10 and IL-12 were less expressed in the STm11 (ST19) strain compared to SL1344 (ST19) (Fig. 6). The expression of the interleukin IL-6 was similar between the STm11 (ST19) strain and the SL1344 (ST19) (Fig. 6D).

Therefore, this study showed a more virulent profile of *S*. Typhimurium STm30 (ST313) in comparison to *S*. Typhimurium SL1344 (ST19) and *S*. Typhimurium STm11 (ST19) due to better colonization in the murine colon, greater expression of virulence genes and inflammatory cytokines.

It is known that local inflammation of the intestinal tract provides advantages for Non-typhoidal *Salmonella* (NTS), increasing bacterial transmission and proliferation (HAPFELMEIER et al., 2004; LAROCK; CHAUDHARY; MILLER, 2015). For example, SPI-1 and SPI-2 effectors, such as *sipA*, *sipC*, *sopA*, *sopB*, *sifA*, *sseF*, *sseG*, *sopD2* and *pipB2* contribute to intestinal inflammation, release of pro-inflammatory cytokines, destruction of intestinal cells which in turn, inhibit the migration and antigen presentation of dendritic cells and T cell proliferation (MCLAUGHLIN et al., 2014; LAROCK; CHAUDHARY; MILLER, 2015).

Studies conducted during three to five days after infection in BALB/c mice showed that the D23580 (ST313) strain isolated in Africa was able to colonize more quickly the spleen, mesenteric lymph nodes and gallbladder compared to SL1344 (ST19) (YANG et al., 2015a). Moreover, BALB/c mice infected with *S*. Typhimurium ST313 showed significantly higher bacteremia compared to animals infected with *S*. Typhimurium ST19 (RAMACHANDRAN et al., 2015).

In contrast, Okoro and collaborators (2015), investigated cecal inflammation in C57BL/6 mice after 48h of infection among ST1313 (A130 and D23580) and ST19 (SL1344) strains and no significant difference was found. Ramachandran and collaborators (2017), studied cecal inflammation in BALB/c mice after four days of infection with *S*. Typhimurium ST313 and ST19 strains and no difference was significant. These authors also investigated the serum and some organs, such as colon and liver of rhesus macaques infected with *S*.

Typhimurium ST313 and *S*. Typhimurium ST19 strains and no significant difference was detected in the expression of IL-6 and IFN-γ cytokines or in the organs studied (RAMACHANDRAN et al., 2017).

In conclusion, *S*. Typhimurium STm30 (ST313) isolated from human feces in Brazil demonstrated greater expression of genes related to pathogenesis after growth in LB at 37°C, besides better colonization and invasion in the murine colon due to higher levels of expression of virulence genes. In addition, pro-inflammatory cytokines were also more expressed in this organ, suggesting greater tissue damage in comparison to *S*. Typhimurium SL1344 (ST19) and *S*. Typhimurium STm11 (ST19) isolated from human feces in Brazil.

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Data Accessibility

Raw data is available at NCBI BioProject repository under the identification number PRJNA686200. Direct URL to data: (Not available yet - the SRA files are being processed).

Conflict of interest

The authors declare no conflict of interest.

4 – CONCLUSIONS

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- Phylogenetic results placed the studied *S*. Typhimurium strains from humans and foods into two major clades suggesting the existence of a prevalent subtype, likely more adapted, among strains isolated from humans, with some diversity in subtypes in strains from foods;
- The variety and prevalence of resistant genes found in the *Salmonella* Typhimurium isolated from humans and foods studied reinforced their potential hazard for humans under treatment and the risk of its presence in foods in Brazil;
- The ST313 genomes from analysed Brazil showed a high similarity among them which information might eventually help in the development of vaccines and antimicrobials;
- The pangenome analysis showed that the selected S. Typhimurium genomes presented an open pangenome, but specifically tending to become closed for the ST313 strains studied;
- The ability of the studied *S*. Typhimurium isolated from humans and foods to invade Caco-2 epithelial cells was strain dependent and was not related to the source or year of isolation;
- *S*. Typhimurium strains isolated from humans showed greater survival rates in U937 human macrophages, and presented higher proportion of isolates with a virulent profile in *G. mellonella* in comparison to strains isolated from food suggesting that this difference may be related to the higher frequency of human isolates which contained plasmidial genes, such as *spvABCDR* operon, *pefABCD* operon, *rck* and *mig-5*;
- The cgMLST and BLAST Atlas were more efficient at discriminating *S*. Typhimurium strains isolated from swine in Brazil in comparison to wgMLST, suggesting greater genetic diversity among these isolates;
- The pathogenic potential of the strains isolated from swine studied was corroborated by the presence of important SPIs related to the pathogenesis of *S*. Typhimurium;
- Phylogenetic analyses grouped the majority of the *S*. Typhimurium strains from diverse origins into a single cluster suggesting that there was one prevalent subtype that has successful contaminated human, food and animal sources for 30 years in Brazil;
- The orthologous protein clusters analysis revealed unique genes in the *S*. Typhimurium of diverse origins studied mainly related to bacterial metabolism and that may be important in their pathogenicity;
- *S*. Typhimurium isolates from swine showed greater diversity of STs and prophages in comparison to *S*. Typhimurium strains isolated from humans and foods;
- The pathogenic potential of *S*. Typhimurium strains of diverse origins was corroborated by the presence of exclusive prophages of this serovar involved in its virulence;

- The high number of resistance genes related to efflux pumps found in the studied *S.* Typhimurium of diverse origins is worrying and may lead to therapeutic failure when treatment is needed;
- *S*. Typhimurium STm30 (ST313) isolated from human feces in Brazil demonstrated greater expression of genes related to pathogenesis at 37°C, besides better colonization and invasion in the murine colon due to higher levels of expression of virulence genes in comparison to ST19 strains;
- Pro-inflammatory cytokines were also more expressed in murine colon by STm30 (ST313) strain, suggesting greater tissue damage in comparison to *S*. Typhimurium SL1344 (ST19) and *S*. Typhimurium STm11 (ST19), all isolated from human feces in Brazil;
- Finally, the results obtained contributed to a better characterization of virulence and genotypic diversity of this important enteropathogen worldwide.

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