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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Doctoral Thesis presented to the Graduation Program of Biosciences and Biotechnology of the School of Pharmaceutical Sciences of Ribeirão Preto/USP for the degree of Doctor in Sciences

Concentration area: Bioagents and Biotechnology Applied to Pharmacy

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

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1. *Salmonella* Typhimurium 2. Whole genome sequencing 3. Phenotypic tests related to virulence 4. Transcriptome

# 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.

# *1 - INTRODUCTION*

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## 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).

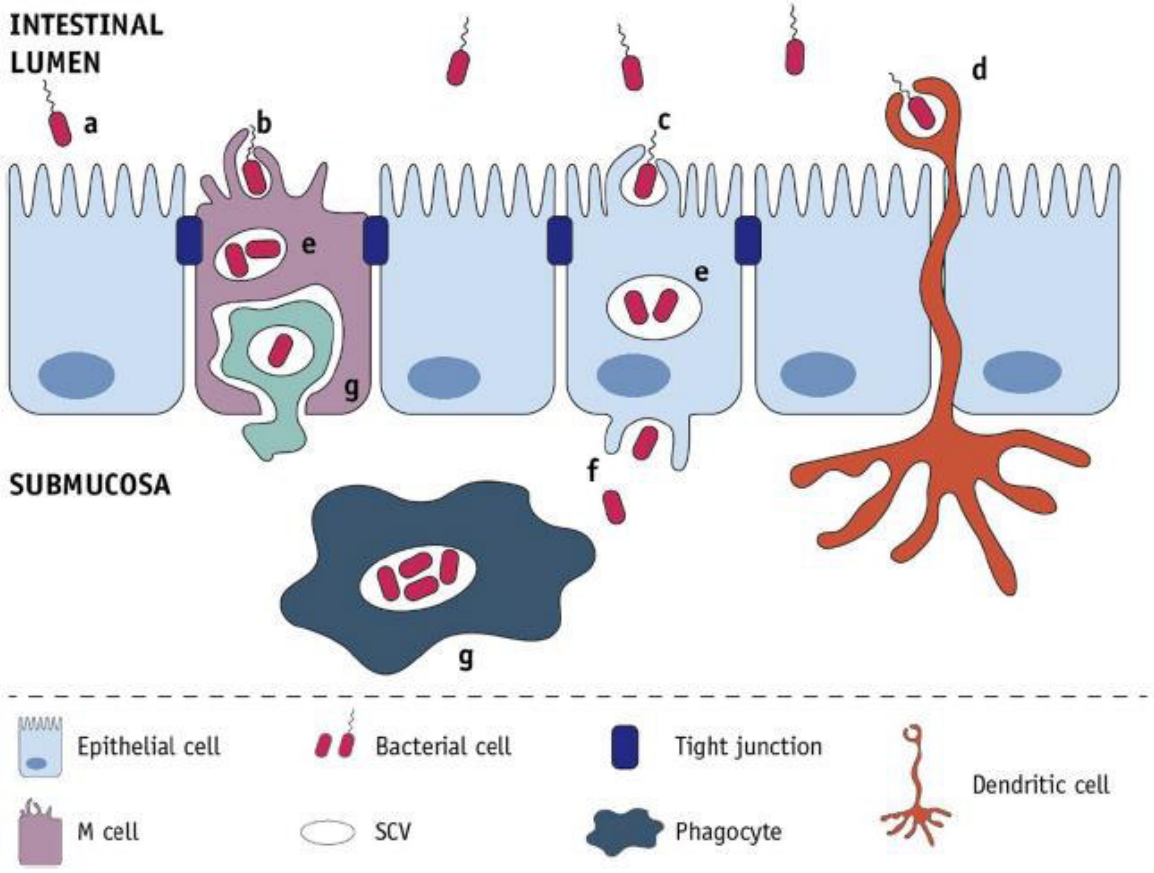
In addition, *S*. Typhimurium can alternatively be taken to the intestinal submucosa without invading due to internalization by dendritic cells or M cell mediated transcytosis (SANTOS; FERRARI; CONTE-JUNIOR, 2019; WEMYSS and PEARSON, 2019). The induction and regulation of the T3SS-1 occurs in environments 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).

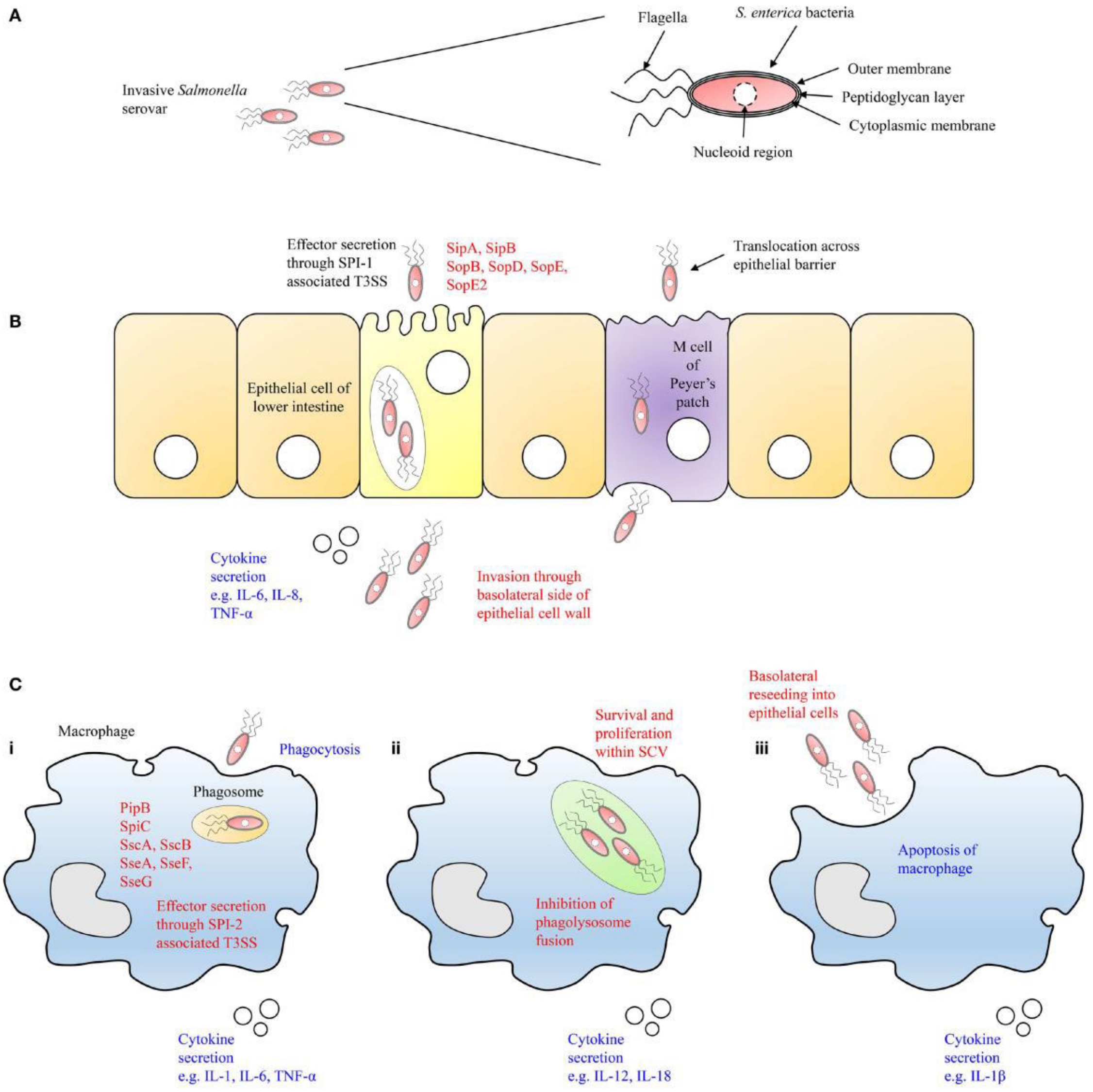
In addition, the interleukin IL-23 promotes the release of interleukins IL-17 and IL-22 that contribute to healing, secretion of antimicrobial molecules and induction of pro-inflammatory mediators, with consequent recruitment of neutrophils (BROZ; OHLSON; MONACK, 2012; VALERI and RAFFATELLU, 2016). Specifically,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

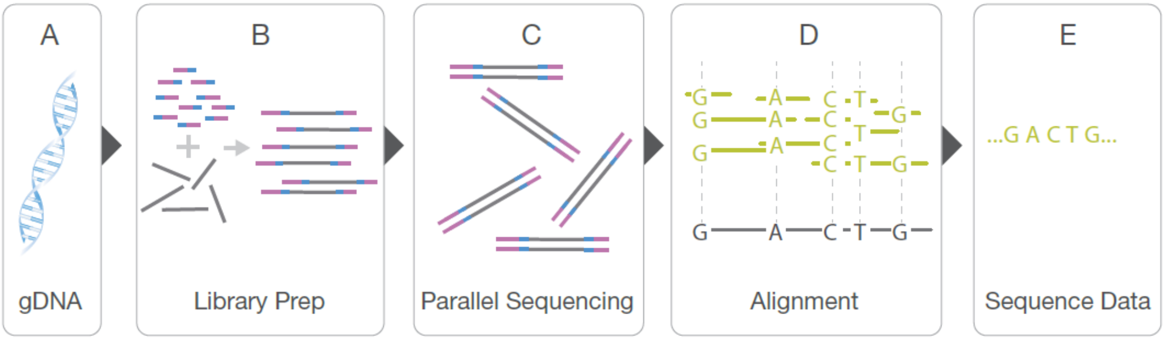
The first step towards the currently available technology was taken in 1965 by Holley and collaborators who sequenced the alanine tRNA from the yeast *Saccharomyces cerevisiae*. At the same time, Sanger and collaborators developed the sequencing of radiolabeled DNA fragments (HOLLEY et al., 1965; SANGER;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

# 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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