



Genetic diversity, antimicrobial resistance and virulence profile of *Salmonella* isolated from the peanut supply chain



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ABSTRACT

Thirty-Eight *Salmonella* isolates recovered from different stages of the peanut supply chain in three Brazilian States (São Paulo, Minas Gerais and Bahia) were subtyped by pulsed-field gel electrophoresis (PFGE) and characterized by phenotypic and genotypic tests for antimicrobial resistance and virulence genes. The isolates were distributed into seven PFGE pulsotypes. All the isolates were resistant to sulfonamide. However, only one isolate from a production site in Minas Gerais had resistance to two types of antimicrobials (sulfonamide and ampicillin). Furthermore, the isolates had intermediary resistance to kanamycin (16/38), streptomycin (14/38) and ceftazidime (12/38). Four isolates had the antimicrobial resistance gene related to phenicols (*floR*) and 37 related to aminoglycosides (*strA*). The *bla_{shv}* gene related to β -lactams was detected in isolates recovered from all the production regions. Six virulence genes (*invA*, *sefA*, *sivH*, *mgtC*, *ssaQ* and *agfA*) were observed in all isolates. The *sopE* gene was detected in 24 isolates, *avrA* in 12. The *gtgB*, *ipfA* and *rck* genes were not detected. The results showed that the pulsotype 1 was restricted to Minas Gerais whereas the pulsotype 7 was present in São Paulo and Bahia. In addition, most of the isolates were not multidrug resistant.

1. Introduction

The peanut supply chain is divided into primary production, secondary processing, manufacturing of confectionary products, distribution, consumption and disposal. The primary production comprises pre and post harvesting steps: pulling out, sun drying and threshing (US EPA, 1995). The secondary processing is made up of artificial drying, shelling, sorting, blanching and roasting (Prusak et al., 2014). The manufacturing steps are determined by the nature of the final product and include blending, grinding, crushing, covering, heating (up to 100 °C), cooling and filling/packing (Prusak et al., 2014).

Microbial contamination of peanuts can occur at all stages of the production chain (Nascimento et al., 2018). Calhoun et al. (2013) recovered *Salmonella* from 2.33% of raw shelled peanut samples collected by the U.S. Department of Agriculture, Food Safety and Inspection Service (FSIS), with a contamination level ranging from < 0.03 to 2.4 MPN/g. In Brazil, the pathogen was detected in 2.2% of the peanuts sampled throughout the supply chain, with counts between 0.004 and 0.092 MPN/g (Nascimento et al., 2018). One of the latest outbreak involving peanut products occurred in 2012 in the U.S.A and affected

42 people in 20 states (CDC, 2013). In 2014 another outbreak occurred involving peanut butter and affected 6 people in 5 states (CDC, 2014).

The stress condition provided by the low water activity can trigger alteration of gene expression and protein synthesis as a way of defense. It enables the microorganisms to stand harsh conditions resulting in more virulent strains (Doménech et al., 2015; Esbelin et al., 2018; Podolak and Black, 2017). Nevertheless, most of available data on the characterization of *Salmonella* have focused on isolates from human and animal sources (Gad et al., 2018; Maka et al., 2014; Sallam et al., 2014). To understand the profile of strains recovered from low water activity food, which usually has a low infectious dose (0.04 MPN/g) (Weber et al., 2005), it is essential to have a suitable therapeutic and predictive choice. The aim of this study was to determine the PFGE profiles and the antimicrobial resistance and virulence genetic profiles of *Salmonella* isolates obtained at different steps of the peanut supply chain. Results can be relevant for proper risk assessment and management decisions.

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Table 1

Description of the *Salmonella* isolates used in this study, which were previously recovered from different stages of the peanut supply chain in Brazil (Nascimento et al., 2018).

Isolate	Sample type	Supply chain step	Brazilian State	Production/sampling site
1	In-shell	Retail	Bahia	BA 1
2	peanuts			
3	In-shell	Threshing	Minas Gerais	MG 1
4	peanuts			
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26	In-shell	Artificial drying	São Paulo	SP 1
27	peanuts			
28	In-shell	Threshing	São Paulo	SP 2
29	peanuts			
30	In-shell	Pulling out	São Paulo	SP 2
31	peanuts			
32				
33				
34				
35				
36	In-shell	Threshing	Minas Gerais	MG 1
37	peanuts			
38				

2. Material and methods

2.1. *Salmonella* isolates

This study was conducted with 38 *Salmonella* isolates recovered from different points of the peanut supply chain (Table 1), as described in the previous study (Nascimento et al., 2018). *Salmonella* isolates were given a serial designation from 1 to 38. The isolates were stored in a biofreezer (-80°C) at the Faculty of Food Engineering at the University of Campinas (UNICAMP). Each isolate was cultivated in tryptic soy broth (TSB, Difco, Becton Dickinson, Sparks, MD, USA) followed by tryptic soy agar (Difco) at 37°C for 18–24 h and maintained on slants at 4°C for use.

2.2. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed according to the protocol developed by the Centers for Disease Control and Prevention (<http://www.cdc.gov/pulsenet/protocols.htm>). Briefly, agarose embedded DNA was digested with the enzyme *Xba*I (Thermo Fisher Scientific, CA, USA) overnight in a water bath at 37°C . Fragments of digested DNA were separated by electrophoresis in $0.5\times$ Tris-borate-EDTA buffer at 14°C for 18 h using a CHEF-mapper system (Bio-Rad, Hercules, CA, USA). PFGE results were analyzed using BioNumerics software version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity among PFGE

patterns was assessed using the unweighted-pair group method with arithmetic averages, with 2.0% band position tolerances. Dice coefficients had 1.5% optimization values. PFGE patterns that were 80% similar were considered to be in the same genetic cluster; similarity coefficients were obtained by calculating dice coefficients.

2.3. Antimicrobial resistance

2.3.1. Phenotypic antimicrobial tests

Antimicrobial susceptibility of the 39 *Salmonella* isolates was evaluated by the disk diffusion test on Mueller-Hinton agar according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2016). Twelve antimicrobials were tested: ampicillin (AMP, 10 mg), cefotaxime (CTX, 30 mg), ceftazidime (CAZ, 30 mg), chloramphenicol (CHL, 30 mg), ciprofloxacin (CIP, 5 mg), gentamicin (GEN, 10 mg), kanamycin (KAN, 30 mg), nalidixic acid (NAL, 30 mg), streptomycin (STR, 10 mg), sulfonamide (SUL, 300 mg), tetracycline (TET, 30 mg), and trimethoprim (TMP, 5 mg) (Cefar Diagnóstica Ltda., Brazil). *Escherichia coli* ATCC 25922 was used as a reference strain for quality control purposes. The analysis was carried out in triplicate.

2.3.2. Genotypic antimicrobial tests

The genomic DNA of the *Salmonella* isolates was extracted using PureLink[®] Genomic DNA Mini Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Genes with reported contributions to antimicrobial resistance were tested in this study (Table 2). PCR assays were performed in 25 μL reaction mixtures containing $1\times$ PCR buffer, 50 μM deoxynucleoside triphosphates, 5 U of *Taq* polymerase (Thermo Fisher Scientific) and 2 μL (~ 10 ng) of template DNA. Amplification was performed using primer concentrations and conditions previously published with minor modifications. A negative control without template DNA was included in all experiments. Aliquots (5 μL) of amplification products were subjected to electrophoresis in a 1.5% (w/v) agarose gel stained with SYBR safe (Thermo Fisher Scientific), and visualized by UV light illumination.

2.4. Virulence genes

The obtained DNA from isolates was subjected to several PCR assays targeting virulence related genes, as detailed in Table 2.

3. Results and discussion

This study evaluated 38 *Salmonella* isolates recovered in a previous investigation (Nascimento et al., 2018), which analyzed 414 samples of peanuts and peanut-based products collected from primary production, secondary processing, manufacturing and retail in three Brazilian States (São Paulo, Minas Gerais and Bahia). Other studies also characterized a low number of isolates recovered from food, such as poultry and ready-to-eat products (Gad et al., 2018; Yang et al., 2016).

3.1. PFGE profiles

The isolates were grouped into seven PFGE pulsotypes (Fig. 1). Twenty-three out of 26 isolates recovered from four samples of threshing at the same production site located in Minas Gerais (MG 1) were grouped in cluster 7. The other three were non-typable by *Xba*I-mediated PFGE, even after the addition of thiourea to the electrophoresis buffer. In São Paulo State, two isolates recovered from two samples of artificial drying collected at a processing plant (SP 1) were displayed in two clusters, 2 and 4. In addition, eight isolates obtained from post-harvesting steps at the same production site (SP 2) were grouped in three clusters: six in cluster 5, one in cluster 6 and one in cluster 3. Two isolates recovered from the same sample of in-shell peanuts collected at a marketplace in Bahia (BA 1) were displayed in two different clusters, 1 and 3. Thus, only cluster 3 was detected in

Table 2
PCR primers used for amplification of antimicrobial resistance and virulence genes in *Salmonella* isolates.

	Target	Primer sequences (5'–3')	References	
Antimicrobial resistance genes	<i>bla_{pSE}</i>	fw: CGCTTCCGGTTAACAAGTAC rv: CTGGTTCATTTCAGATAGCG	Silva et al. (2012)	
	<i>floR</i>	fw: CTGAGGGTGTGCTCATCTAC rv: GCTCCGACAATGCTGACTAT	Chen et al. (2004)	
	<i>aadA1</i>	fw: GCGCTAAATGAAACCTTAAC rv: TCGCCTTTCACGTAGTGGAC	Guerra et al. (2002)	
	<i>strA</i>	fw: TGACTGGTTGCCTGTGAGAGG rv: CCAGTTGTCTTCGGCGTTAGCA	Kich et al. (2011)	
	<i>bla_{TEM}</i>	fw: ATGAGTATTC AACATTTCCG rv: GACAGTTACCAATGCTTAATCA	Haley et al. (2012)	
	<i>qnrS</i>	fw: CGACGTGCTAACTTGCCTGATA rv: TACCAGTGTCTCGAGAATCAG	Cavaco et al. (2009)	
	<i>bla_{shv}</i>	fw: ATGCGTTATATTCGCCTGTGTA rv: TTAGCGTTGCCAGTGTCTGATCAG	Nobrega et al. (2013)	
	Virulence genes	<i>sivH</i>	fw: CTGAGGGTGTGCTCATCTAC rv: GCTCCGACAATGCTGACTAT	Kingsley et al. (2003)
		<i>lpfA</i>	fw: CTTTCGCTGCTGAATCTGGT rv: CAGTGTTAACAGAAACCAGT	Bäumler and Heffron (1995)
		<i>agfA</i>	fw: ATAAAATTCCTGAAGACGAAA rv: GACAGTTACCAATGCTTAATCA	Borges et al. (2013)
<i>sefA</i>		fw: CGCTTCCCGTTAACAAGTAC rv: CTGGTTCATTTCAGATAGCG	Oliveira et al. (2002)	
<i>invA</i>		fw: GTGAAATTTATCGCCACGTTCCGGGCAA rv: TCATCGCACCGTCAAAGGAACC	Oliveira et al. (2002)	
<i>avrA</i>		fw: GTTATGGACGGAACGACATCGG rv: ATTCTGCTTCCCGCCGCC	Prager et al. (2003)	
<i>sopE</i>		fw: ACACACTTTCACCGAGGAAGCG rv: GGATGCCTTCTGATGTTGACTGG	Prager et al. (2003)	
<i>gtgB</i>		fw: TGCACGGGGAAAACACTACTTC rv: TGATGGGCTGAAACATCAAA	Capuano et al. (2013)	
<i>sspH1</i>		fw: TGCAGAAAAAGGGGAATACG rv: GCAGCCTGAAGGTCTGAAAC	Borriello et al. (2012)	
<i>rck</i>		fw: AACGGACGGAACACAGAGTC rv: TGTCTGACGAAAAGTGCATC	Capuano et al. (2013) Borriello et al. (2012)	
<i>mgtC</i>		fw: TGAATATCAATGCTCCAGTGAAT rv: ATTTACTGGCCGCTATGCTGTTG	Soto et al. (2006)	
<i>ssaQ</i>		fw: GAATAGCGAATGAAGAGCGTCC rv: CATCGTGTATCTCTGTGTCAGC	Soto et al. (2006)	

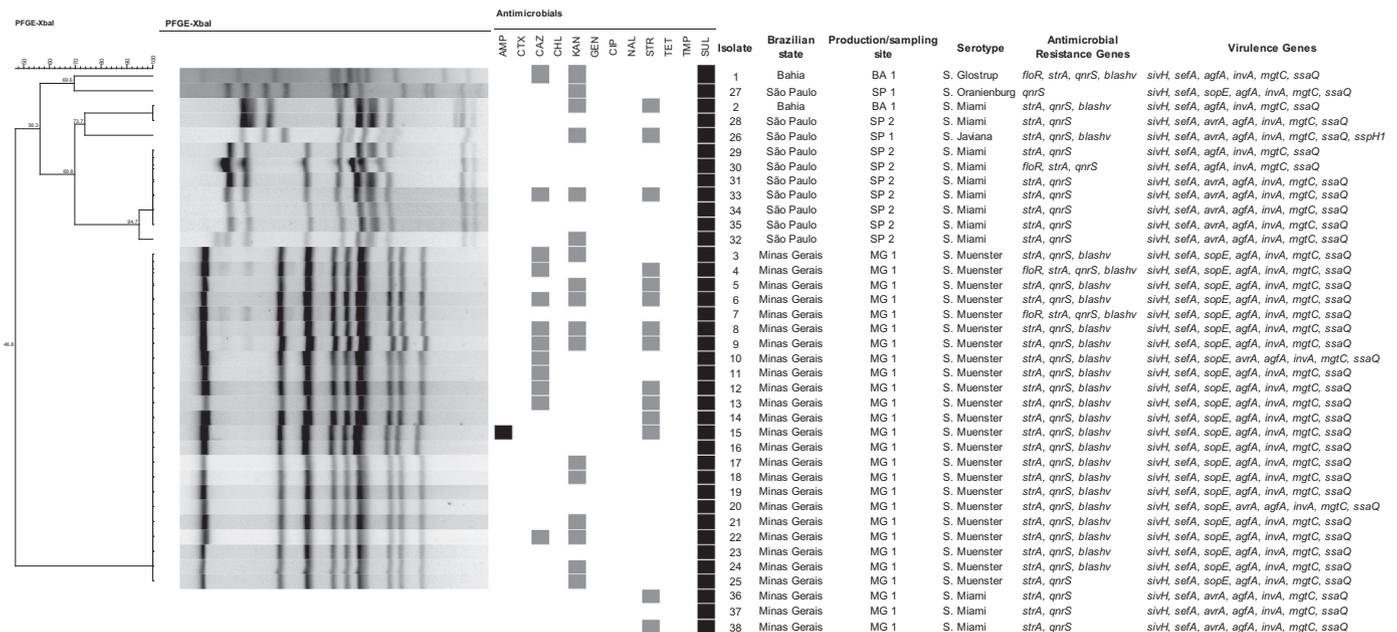


Fig. 1. Dendrogram of *Salmonella* isolates recovered from peanut supply chain showing antibiotic resistance profiles, Brazilian states, production/sampling sites, serotypes, presence of antimicrobial resistance genes and virulence genes. Black squares indicate resistance. Grey squares indicate intermediate resistance and white squares susceptibility. Abbreviations: AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic; STR, streptomycin; SUL, sulfonamide; TET, tetracycline; TMP, trimethoprim.

more than one Brazilian State.

3.2. Antimicrobial susceptibility

Resistance or decreased susceptibility to five antimicrobials was verified (Fig. 1). Only one isolate from the threshing step in Minas Gerais (15) was resistant to more than one antimicrobial: sulfonamide and ampicillin. Brar et al. (2016) obtained an index of 20% of multi-drug-resistance in *Salmonella* isolated from in-shell pecans. Resistance to sulfonamide was observed in 100% of the isolates. Elevated rates of resistance to sulfonamides were also reported in other countries in *Salmonella* obtained from human and animal sources (CDC, 2016; EFSA, 2015; Van et al., 2012). Intermediate resistance to kanamycin (42.1%), streptomycin (36.8%) and ceftazidime (31.6%) was detected. All isolates were susceptible to cefotaxime, chloramphenicol, tetracycline, ciprofloxacin, gentamicin, nalidixic acid and trimethoprim. The susceptibility to most of the antimicrobial agents tested could be related to the fact that the isolates were recovered from vegetable products. In agriculture, the antimicrobial agents are not as widely employed as in livestock (Frech et al., 2003; Huehn et al., 2010).

Antimicrobial resistance genes were not often detected in the isolates, which is in agreement with the low rates of multi-drug resistance observed in the phenotypic profile (Fig. 1). The only gene observed in all isolates was *qnrS* related to fluoroquinolones. Four isolates showed the antimicrobial resistance gene related to phenicols (*floR*): one from Bahia (retail, BA 1), one from São Paulo (pulling out, SP 2) and two from Minas Gerais (threshing, MG 1). One-third of the isolates did not carry the *bla_{shv}* gene; most belonged to *S. Miami*. The presence of the plasmid-mediated quinolone resistance (*qnrS*) and resistance to extended spectrum betalactamases (*bla_{shv}*) could not predict the antimicrobial resistance phenotype profiles shown by the isolates. This could be related to the large variety of the existing antibiotic resistance genes (> 70), structured as gene cassettes and coding for particular resistance patterns (Huang et al., 2013; Rowe-Magnus and Mazel, 2002). The isolate 27 was the only one lacking the aminoglycoside related gene (*strA*). According to Yau et al. (2010), the *strA* gene seems to be carried on the plasmid in many bacterial species. It can explain the high rate of this genetic profile in our *Salmonella* isolates. The *bla_{tem}* and the *bla_{pse}* genes related to β -lactams and the *aadA1* gene linked to aminoglycosides were not detected.

3.3. Virulence genes

The detection rate for virulence genes was higher than for antimicrobial resistance genes (Fig. 1). The high incidence rate of virulence genes and the variability of some virulence factors observed are similar to the findings reported for *Salmonella* strains isolated from humans, animals and foods, including ready-to-eat products (Capuano et al., 2013; Drahovska et al., 2007; Kuang et al., 2015; Yang et al., 2016). All isolates carried the *invA*, *sefA*, *sivH*, *mgtC*, *ssaQ* and *agfA* genes related to fimbriae production and invasion capacity. The high frequency rate of the *invA* gene observed in several studies, suggests that it is a conserved gene among the *Salmonella* genus (Dahshan et al., 2010; Oliveira et al., 2002; Oliveira et al., 2003; Salehi et al., 2005). The presence of the *agfA* gene at different steps of the peanut supply chain points out a concern to public health and food safety areas, since this gene is related to adhesion in the infection process and in biofilm formation (Borges et al., 2013; Yoo et al., 2013). The *sopE* was identified in 23 isolates recovered from post-harvesting in one production site in Minas Gerais (MG 1) and in one isolate from the artificial drying in São Paulo. The *avrA* was detected in 12 isolates, seven from two production sites in São Paulo (SP 1 and SP 2) and five from a production site in Minas Gerais (MG 1). The *sspH1* gene was only detected in one isolate recovered from artificial drying at a processing plant located in São Paulo (SP 1). This gene is related to the presence of prophages and consequently more virulent strains. The *gtgB*, *ipfA* and *rck* genes were not detected.

Although few studies have reported the presence of *sefA* and *sivH* genes, the occurrence of these virulence factors in all isolates were also found by Borges et al. (2013). The *mgtC* and *ssaQ* genes incorporated in *Salmonella* pathogenicity islands (SPIs) were frequently distributed with no variations among our isolates and as reported in other studies (Beutlich et al., 2011; Huehn et al., 2010; Yang et al., 2016).

4. Conclusion

In summary, this study brings up new information on PFGE patterns, virulence gene content and antimicrobial resistance in *Salmonella* isolated from the peanut supply chain. The results can enable us to understand better the characteristics of strains isolated from low-moisture food and allow for establishing control strategies and risk management in this food category.

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