FOODBORNE PATHOGENS AND DISEASE
Volume XX, Number XX, 2021

DOI: 10.1089/fpd.2021.0010

Prevalence and Antimicrobial Resistance of *Salmonella* spp. in Aquacultured Nile Tilapia (*Oreochromis niloticus*) Commercialized in Federal District, Brazil

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Abstract

This study aimed to assess *Salmonella* spp. prevalence in aquaculture Nile tilapia commercialized in the Federal District, Brazil, and determine the antimicrobial resistance profile of the isolates. Fifty-seven *Salmonella* spp. strains were isolated from 101 samples of fresh tilapia fillets collected in the Federal District, Brazil. These isolates were subjected to antimicrobial susceptibility testing by the Kirby–Bauer disk diffusion method and analyzed for the presence of *blaCTX*, *tetB*, *sul2*, and *floR* resistance genes. The *Salmonella* spp. prevalence in fresh tilapia fillets was 45.5%; that is, 46 of 101 samples were positive for the *InvA* gene. The antimicrobial resistance profile showed high resistance rates for amoxicillin/clavulanic acid (87.7%), tetracycline (82.5%), sulfonamide (57.9%), and chloramphenicol (26.3%). Additionally, 56.1% of *Salmonella* spp. isolates were multidrug-resistant (MDR) isolates. The beta-lactam-resistant gene *blaCTX* was identified in 66.7% of isolates, the tetracycline resistance gene *tetA* in 54.4%, and the chloramphenicol resistance gene *floR* in 50.9%, while the sulfonamide resistance gene *sul2* was present in 49.1%. The results revealed that tilapia fillets were highly contaminated with MDR *Salmonella*. These *Salmonella* spp. strains carried multiple antimicrobial resistance genes, which might facilitate their dissemination to consumers along the production chain. Hence, there is an evident need to control *Salmonella* in fish production systems to ensure public health.

Keywords: tilapia fillets, freshwater fish, resistance genes, multidrug-resistant *Salmonella*

Introduction

Tilapia is the second most farmed freshwater fish worldwide, after carps (FAO, 2018). Today, all commercially important tilapia outside of Africa belong to the genus *Oreochromis*, and more than 90% of these farmed fish are Nile tilapia (*Oreochromis niloticus*) (Wang and Lu, 2015). In Brazil, strong consumer demand and continuous investments in the tilapia farming sector have resulted in continued production growth, with 311.500 tons produced in 2018, corresponding to 60% of the Brazilian aquaculture fish production (IBGE, 2018). Tilapia is considered one of the most accepted fish in the consumer market due to its attractive characteristics, such as white and tasty fillets (Barroso *et al.*, 2019).

In recent years, tilapia fillet consumption has increased significantly in Brazil. Simultaneously, this food's safety, particularly related to microbiological contamination, has become a source of concern. *Salmonella* spp. is not part of the healthy fish microbiota, and its presence indicates fecal contamination either from polluted water or cross-contamination during the production chain (Wang and Lu, 2015; Fernandes *et al.*, 2018). *Salmonella* spp. is one of the most widespread, zoonotic foodborne pathogens worldwide (Heredia and García, 2018; Draeger *et al.*, 2019). In Brazil, *Salmonella* spp. is the second most prevalent foodborne pathogen, while *Escherichia coli* is the first (Draeger *et al.*, 2019). The European Union reports salmonellosis as the second most prevalent zoonosis, after campylobacteriosis (Pepe *et al.*, 2009; EFSA and ECDC, 2021). Therefore, the presence of *Salmonella* spp. in tilapia may represent a reason for an increasing number of enteric disease outbreaks (Heredia and García, 2018). Nevertheless, the prevalence of the gastrointestinal pathogen *Salmonella* in fish in Brazil is seldom reported in the literature (Fernandes *et al.*, 2018).

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The expanding antimicrobial resistance in foodborne pathogens, such as *Salmonella* spp., has caused a significant increase in public health safety concerns. The rise in resistant strains has been attributed to the inappropriate use of antimicrobial agents in human and veterinary medicine and their usage as growth promoters in animal production. This acquired antimicrobial resistance can be further transmitted to the human population through the food chain. Considering that multiresistant strains of *Salmonella* spp. have been isolated from different animal-origin foods worldwide, expanding resistance poses a serious threat to public health (Yang *et al.*, 2015; Nair *et al.*, 2018).

According to McMillan *et al.* (2019), *Salmonella* spp. can carry genes encoding resistance to several classes of antimicrobials, including β-lactams, tetracyclines, sulfonamides, and phenicols. The authors reported that a diverse group of plasmids present in strains of *Salmonella enterica* isolated from food animals, carrying antimicrobial resistance genes, is responsible for its phenotypic resistance. The antimicrobial resistance transfer ability between bacteria on mobile genetic elements can cause the rapid establishment of multidrug resistance in bacteria from animals, thus creating a foodborne risk to human health.

Few studies in Brazil have focused on *Salmonella* prevalence in tilapia and on its antimicrobial resistance characterization. Thus, this study evaluated *Salmonella* spp. prevalence in fresh tilapia fillet samples collected in the Federal District, Brazil, and determined the isolates' antimicrobial susceptibility and the presence of *blaCTX*, *tetB*, *sul2*, and *floR* resistance genes.

Materials and Methods

Sample collection, Salmonella *isolation, and biochemical confirmation*

From March 2018 to March 2020, 101 samples of fresh tilapia fillets were collected from different supermarkets in the Federal District, Brazil. These fish came from several Brazilian states (Paraná, São Paulo, Rondônia, Santa Catarina, Minas Gerais, and Federal District). Samples were stored in an icebox and immediately transported to the laboratory and analyzed within 2 h.

Salmonella strains were isolated as described in the Technical Guide for Laboratory Detection of *Salmonella* spp. (Brasil, Ministério da Saúde, 2011). For *Salmonella* spp. detection, in triplicate, 25 g of each sample was transferred into 225 mL of buffered peptone water (BPW; Kasvi, Brazil), homogenized, and incubated at 37°C for 18–20 h. Then, 1.0 mL each of the pre-enrichment BPW aliquots was transferred into 10 mL of tetrathionate broth (Himedia) and selenite cystine broth (Acumedia), respectively, and incubated at 37°C for 24 h. A loopful (10 μL) of enriched broth was streaked onto the xylose lysine deoxycholate (XLD) agar (Himedia) and Salmonella Shigella (SS) agar (Himedia) and incubated at 37°C for 24 h. Presumptive *Salmonella* colonies in XLD and SS agars were confirmed biochemically using triple sugar iron (TSI) agar (Himedia) and lysine iron agar (LIA; Himedia) slants. These slants were incubated at 37°C for 24 h. The presumptive *Salmonella* isolates, tested positive on TSI and LIA biochemical testing, were confirmed by amplifying a targeted *Salmonella*-specific invasive (*invA*) gene by polymerase chain reaction (PCR; Table 1).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed following the standard Kirby–Bauer disk diffusion method as per Brazilian Committee on Antimicrobial Susceptibility Testing (BRCAST, 2019) guidelines. The bacterial inoculum was obtained from a microbial growth suspension in Mueller-Hinton broth with turbidity equivalent to 0.5 McFarland standard (1.0×108 CFU/mL), adjusted to an optical density between 0.08 and 0.10 at 625 nm using a spectrophotometer (BRCAST, 2019). All the antimicrobial disks were purchased from Newprov (Brazil). The analyzed antimicrobial agents' concentrations and abbreviations are as described: amoxicillin/clavulanic acid (AMC, 20/10 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), gentamicin (GEN, 10 μg), chloramphenicol (CLO, 30 μg), tetracycline (TET, 30 μg), imipenem (IMP, 10 μg), sulfonamide (SUL, 300 μg), and ciprofloxacin (CIP, 5 μg). The isolates were classified as susceptible (S), intermediate (I), or resistant (R), according to the CLSI guidelines for *Enterobacteriaceae* (CLSI, 2020). *Salmonella* isolates resistant to three or more antimicrobials were defined as multidrug-resistant (MDR) isolates.

Salmonella *molecular confirmation and antimicrobial resistance gene detection by PCR*

The InvA gene PCR amplification confirmed the biochemically presumptive *Salmonella* isolates. The confirmed strains ($n = 57$) were then screened, by PCR, for the presence of antimicrobial resistance genes: *blaCTX* for beta-lactams, *tetB* for tetracycline, *sul2* for sulfonamide, and *floR* for chloramphenicol.

For DNA extraction, the biochemically identified *Salmonella* isolates were cultivated overnight in Mueller-Hinton broth and their DNA was extracted by employing the NucleoSpin Food® kit (Macherey-Nagel, Düren, Germany), as per the manufacturer's instructions. The DNA concentrations were then determined using a NanoDrop 2000 (Thermo Scientific, Pittsburgh), and DNA integrity was confirmed by agarose gel electrophoresis.

Table 1 shows the primer sequences and PCR conditions used to amplify the virulence gene *InvA* and the resistance genes, *blaCTX*, *tetB*, *sul2*, and *floR*. All PCRs were performed in a 25-μL final volume reaction mixture containing 2.5 μL of PCR buffer; 0.7 μL of MgCl2; 1.5 μL of dNTP (2.5 mM); 0.5 μL of Taq DNA polymerase; 1.5 μL of each primer, forward and reverse; and 18.3 μL of Milli-Q water. These thermal cycling reactions were conducted with Techne TC-512 thermal cycler (Bibby Scientific, Inc.), and each PCR run included negative and reagent controls. The reagent control consisted of all PCR components except for the DNA template. The amplified DNA was separated by electrophoresis at 100 V for 50 min in 1.5% (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light. A 100 bp DNA ladder was used as a molecular weight marker.

Results

Salmonella *spp. prevalence in fresh tilapia fillets*

Salmonella spp. prevalence was 45.5%, that is, 46 of 101 samples of fresh tilapia fillets analyzed presented this bacterium confirmed by *invA* gene detection.

Table 1. Primer Sequences and Polymerase Chain Reaction Conditions Used to Amplify the Virulence Gene *InvA* and the Resistance Genes, *blaCTX*, *tetB*, *sul2*, and *floR*

Target gene	*Primer sequence (5′→ 3′)*	*Product size (bp)*	*PCR amplification conditions*	*Reference*
invA	CATTGGTGATGGTCTTGTCG CTCGCCTTTGCTGGTTTTAG	298	Denaturation for 2 min at 95°C, followed by 35 cycles for 1 min at 95°C, annealing for 1 min at 60°C, and a final extension for 1 min at 72°C	Cruz *et al.* (2019)
blaCTX	CGATGTGCAGTACCAGTAA AGTGACCAGAATCAGCGG	585	Denaturation for 5 min at 94°C, followed by 30 cycles for 30 s at 94°C, annealing for 30 s at 55°C, 50 s at 72°C, and a final extension for 7 min at 72°C	Li *et al.* (2013)
tetB	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	659	Denaturation for 5 min at 94°C, followed by 34 cycles for 25 s at 94°C, annealing for 30 s at 55°C, 50 s at 72°C, and a final extension for 7 min at 72°C	Zishiri *et al.* (2016)
sul2	GCGCTCAAGGCAGATGGCATT GCGTTTGATACCGGCACCCGT	285	Denaturation for 10 min at 95°C, followed by 35 cycles for 45 s at 94°C, annealing for 50 s at 55°C, 50 s at 72°C, and a final extension for 10 min at 72°C	Zhu *et al.* (2017)
floR	CACGTTGAGCCTCTATAT ATGCAGAAGTAGAACGCG	868	Denaturation for 5 min at 94°C, followed by 30 cycles for 30 s at 94°C, annealing for 30 s, 1 min at 72°C, and a final extension for 5 min at 72°C	Thai *et al.* (2012)

PCR, polymerase chain reaction.

Antimicrobial resistance profile of Salmonella *isolates*

Figure 1 shows the antimicrobial resistance profile of 57 *Salmonella* isolates. Overall, only 5.3% (3/57) of isolates were susceptible to all tested antimicrobials and 38.6% (22/57) were resistant to one or two antimicrobials, while 56.1% (32/57) presented multidrug resistance (Table 2). High resistance rates were observed for amoxicillin/clavulanic acid (87.7%, 50/57), tetracycline (82.5%, 47/57), sulfonamide (57.9%, 33/57), and chloramphenicol (26.3%, 15/57), whereas lower resistance rates were found for ciprofloxacin (1.8%), imipenem (1.8%), ceftazidime (3.5%), and cefotaxime (3.5%). Notably, all the isolates were susceptible to gentamicin.

Mainly, 12 resistance patterns of *Salmonella* isolates were detected, as seen in Table 3. The dominant resistance pattern was SUL AMC TET (24.6%, 14/57), followed by AMC TET (21.1%, 12/57) and SUL AMC TET CLO (17.5%, 10/57).

Antimicrobial resistance genotypes of Salmonella *isolates*

Antimicrobial resistance genes were screened in all 57 *Salmonella* isolates and 66.7% (38/57) presented the *blaCTX* gene associated with resistance to beta-lactams, 54.4% (31/57) presented the *tetb* gene associated with resistance to tetracycline, 49.1% (28/57) presented the *sul2* gene associated with resistance to sulfonamide, and 50.9% (29/57) presented the *floR* gene associated with resistance to chloramphenicol (Table 4). Resistance genes were also detected in phenotypically susceptible and intermediate

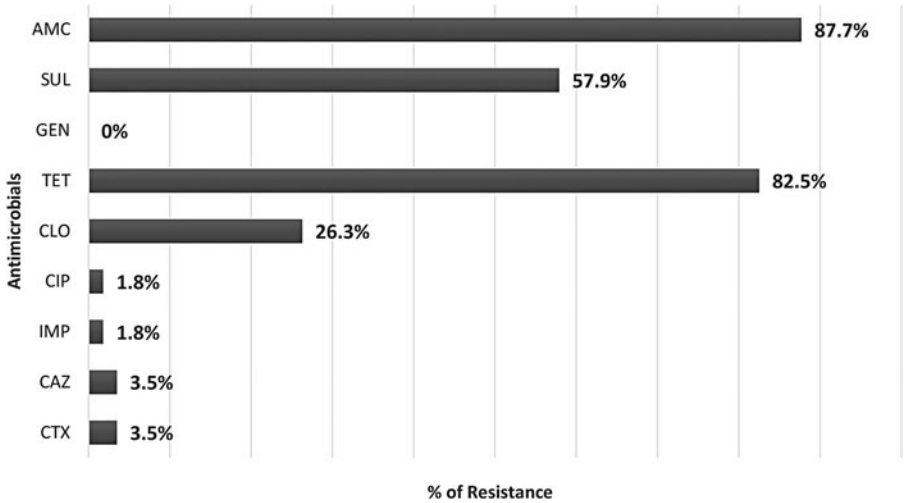

FIG. 1. Antimicrobial resistance profile of *Salmonella* isolates.

Table 2. Antimicrobial Resistance and Multidrug Resistance Profile of *Salmonella* Isolates

No. of antimicrobials with resistance	*Number and percentage of isolates*	
	n	%
0	3	5.3
1	5	8.8
2	17	29.8
3	19	33.3
4	11	19.3
5	2	3.5
Total	57	100
MDR (sum of 3, 4, and 5)	32	56.1

MDR, multidrug-resistant.

isolates (Table 4), *blaCTX* in 8.8% (4/38), *tetb* in 7.0% (4/31), and *sul2* in 15.8% (8/28), whereas *floR* was detected in 31.6% (18/29).

Table 5 displays the various resistance gene patterns found in each of the *Salmonella* isolates. All 57 *Salmonella* strains in this study, including those susceptible to all antimicrobials, had at least one of the resistance genes tested in their genome. The isolated *Salmonella* registered 15 distinct genotype resistance profiles, indicating considerable resistance gene pattern diversity.

Discussion

Salmonella spp. prevalence in fresh tilapia fillets commercialized in Federal District, Brazil, was 45.5%; that is, 46 samples were unfit for consumption according to the Brazilian legislation, which prohibits the *Salmonella* spp. presence in fish (Brasil, Agência Nacional Vigilância Sanitária, 2019). Other studies also reported high rates of tilapia contamination with *Salmonella*. Budiati *et al.* (2013) isolated *Salmonella* spp. from 43.8% (14/32) of the tilapia samples obtained from Malaysia. Elhadi (2014) reported a 64.9% *Salmonella* spp. prevalence in tilapia imported from Thailand and 28.0% prevalence in those imported from India. Seel *et al.* (2016) found that of 20 fresh tilapia samples collected in different Bangladesh markets, 75% (15/20) were positive for *Salmonella* spp. Lerma-Fierro *et al.* (2020) also detected *Salmonella* spp. in 41.7% (5/12) of fresh Nile tilapia fillets marketed in Tepic Nayarit city, Mexico.

Similar to our study, Zishiri *et al.* (2016) used the *invA* gene to confirm *Salmonella* spp. isolated from commercial chickens in South Africa and Brazil. The invasion A gene (*invA*) is a well-known virulence gene in *Salmonella*, which is responsible for host invasion. This gene located in *Salmonella* pathogenicity island 1 (SPI-1) is conserved in all *Salmonella* serovars. Consequently, researchers use it as a marker to detect *Salmonella* isolated from different sources (Zishiri *et al.*, 2016).

This study demonstrated a high incidence of resistance to amoxicillin/clavulanic acid (87.7%), tetracycline (82.5%), and sulfonamide (57.9%) in *Salmonella* isolates. These results were comparable with findings of Elhadi (2014), in which the highest antimicrobial resistance was for tetracycline (90%) and amoxicillin/clavulanic acid (45%) in *Salmonella* spp. isolated from freshwater fish imported by the eastern province of Saudi Arabia. Zhang *et al.* (2015) reported high resistance to sulfonamides (56.5%) and tetracycline (34.1%) in *Salmonella* serovars isolated from retail aquaculture products in China. The resistance to chloramphenicol (26.3%) observed in our study was similar to findings of Budiati *et al.* (2013), in which *Salmonella* isolates were resistant to chloramphenicol (37.2%) and tetracycline (67.4%). Furthermore, MDR *Salmonella* spp. was often found in our study (56.1%). Yang *et al.* (2015) and Zhang

Table 3. Antimicrobial Resistance Patterns of *Salmonella* Isolates

Resistant phenotypes		n	%
1	CAZ AMC CTX TET CLO	1	1.8
2	SUL CAZ AMC CTX TET	1	1.8
3	SUL AMC TET CLO	10	17.5
4	SUL IMP AMC TET	1	1.8
5	AMC TET CLO	4	7.0
6	SUL AMC TET	14	24.6
7	SUL CIP AMC	1	1.8
8	AMC TET	12	21.1
9	SUL AMC	2	3.5
10	SUL TET	3	5.3
11	AMC	4	7.0
12	SUL	1	1.8

AMC, amoxicillin/clavulanic acid; CAZ, ceftazidime; CIP, ciprofloxacin; CLO, chloramphenicol; CTX, cefotaxime; GEN, gentamicin; IMP, imipenem; SUL, sulfonamide; TET, tetracycline.

Table 4. Presence of Antimicrobial Resistance Genes Screened from 57 *Salmonella* Isolates

Resistance genes	*Antimicrobial resistance profile*			
	R n (%)	*S* n (%)	*I* n (%)	*Total* n (%)
blaCTX	33 (57.9)	1 (1.8)	3 (7.0)	38 (66.7)
tetb	27 (47.4)	2 (3.5)	2 (3.5)	31 (54.4)
sul2	19 (33.3)	6 (10.5)	3 (5.3)	28 (49.1)
floR	11 (19.3)	14 (24.6)	4 (7.0)	29 (50.9)

Table 5. Antimicrobial Resistance Genotype Patterns of *Salmonella* Isolates

	Resistance genes	n	%
1	*blaCTX, tetB, sul2, floR*	5	8.8
2	*blaCTX, sul2, floR*	5	8.8
3	*blaCTX, tetB, floR*	4	7.0
4	*blaCTX, tetB, sul2*	2	3.5
5	*tetB, sul2, floR*	4	7.0
6	*blaCTX, floR*	7	12.3
7	*blaCTX, sul2*	3	5.3
8	*blaCTX, tetB*	5	8.8
9	*tetB, floR*	1	1.8
10	*tetB, sul2*	5	8.8
11	*sul2, floR*	1	1.8
12	*blaCTX*	6	10.5
13	*Tetb*	4	7.0
14	*sul2*	3	5.3
15	*floR*	2	3.5
Total		57	100

et al. (2015) reported 34.0% and 43.3%, respectively, of MDR *Salmonella* isolated from aquatic food products from retail markets in China. This increased antimicrobial resistance of *Salmonella* may limit therapeutic options for treating salmonellosis in humans and animals (Elhadi, 2014; Heredia and García, 2018).

Among the 87.7% (50/57) isolates of *Salmonella* resistant to amoxicillin/clavulanic acid, 57.9% (33/57) presented the resistance gene *blaCTX*. Studies have exposed a wide range of beta-lactamase genes occurring in *Salmonella*. Moreover, among *Enterobacteriaceae*, including *Salmonella*, the *blaCTX* gene is the most widespread beta-lactamase-resistant gene and is mainly associated with a diverse set of transmissible plasmids (Zhang *et al.*, 2019).

In this study, 47.4% (27/57) of the 82.5% (47/57) isolates of *Salmonella* phenotypically resistant to tetracycline presented the resistance gene *tetB*. According to Zishiri *et al.* (2016), *tetA* and *tetB* are the most common genes found in tetracycline-resistant *Salmonella* isolates. These genes are responsible for encoding efflux pumps associated with plasmids, transposons, or both and are often conjugative, highlighting the potential transference of these genes to other bacteria, environment, animals, and humans (Mąka and Popowska, 2016).

The resistance gene *sul2* was present in 33.3% (19/57) of the 57.9% (33/57) isolates of *Salmonella* phenotypically resistant to sulfonamide. The two most frequently found genes among sulfonamide-resistant isolates are *sul1* and *sul2*, both encode forms of dihydropteroate synthase whose product has a low affinity for sulfonamides. The *sul1* gene is regularly linked to other resistance genes in class 1 integrons, while *the sul2* gene is usually associated with small multicopy plasmids or large, transmissible multiresistance plasmids (Mąka *et al.*, 2015).

Among the 26.3% (15/57) of *Salmonella* isolates phenotypically resistant to chloramphenicol, 19.3% (11/57) had the resistance gene *floR*. This gene has been identified mainly in Gram-negative bacteria and is responsible for encoding drug efflux pumps. It has been identified on both chromosomes and plasmids, often associated with mobile genetic elements and genomic islands (Lu *et al.*, 2018).

Our study also detected resistant genes in phenotypically susceptible and intermediate isolates. Curiously, the *floR* gene was more prevalent in phenotypically susceptible and intermediate isolates than in resistant isolates. According to Adesiji *et al.* (2014), some antimicrobial-resistant genes are silent in bacteria *in vitro* and can spread to other bacteria or turn on *in vivo*, especially under the selective pressure of antibiotic use.

The extensive use of antimicrobials in animal production systems for controlling bacterial infections and growth promotion has contributed to the development of drug-resistant bacteria. The fecal excretion of antibiotic-resistant pathogens such as *Salmonella* from livestock and poultry causes water system contamination. Aquaculture isolates have shown similar resistance patterns to the isolates recovered from terrestrial agriculture, indicating that the water source contamination comes from farmland (Nair *et al.*, 2018). According to Wang and Lu (2015), integrated fish farming involves raising fish alongside livestock, and aquatic environment and product investigations revealed an increase of pathogens, such as *Salmonella*, in the water and intestine of tilapia in these cases.

Conclusions

This study revealed a high *Salmonella* spp. prevalence in fresh tilapia fillets collected in the Federal District, Brazil. Furthermore, these *Salmonella* isolates frequently exhibited multidrug resistance patterns, and antimicrobial resistance genes, *blaCTX*, *tetB*, *sul2*, and *floR*, were also prevalent among them. This high *Salmonella* spp. occurrence in fresh tilapia fillets indicates the need for programs to monitor microbiological safety in tilapia production systems and rational use of antimicrobials in aquaculture production to reduce the risk of developing and spreading antimicrobial resistance.

Disclosure Statement

No competing financial interests exist.

Funding Information

This research was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES - Finance Code 001), by Fundação de Apoio à Pesquisa do Distrito Federal (FAPDF - Edital 3/2018), and by UnB (Edital DPG 0004/2021).

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