Sociedade Brasileira de Infectologia

The Brazilian Journal of
INFECTIOUS DISEASES

www.elsevier.com/locate/bjid

Original Article

Characterization of the virulence, *agr* typing and antimicrobial resistance profile of *Staphylococcus aureus* strains isolated from food handlers in Brazil

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ARTICLE INFO

Article history:
Received 17 April 2022
Accepted 5 August 2022
Available online 26 August 2022

Keywords:
Staphylococcus
Food poisoning
Toxins
MRSA
Food handlers

ABSTRACT

Staphylococcus aureus is one of the main pathogens associated with foodborne outbreaks in Brazil and food handlers can carry toxigenic and resistant *S. aureus* strains. The aims of this study were to verify the frequency of virulence genes, to identify the *agr* groups and to determine the antimicrobial resistance profile of *S. aureus* strains isolated from food handlers of pilot kitchens located in São Paulo, Brazil. A total of 74 strains of the *Staphylococcus* genus were detected and 50% were identified as of the species *S. aureus*. The enterotoxin genes detection, *tst* and *luk-PV* detection, *agr* typing, *mecA* detection, *ccr* complex detection and SCC*mec* typing were performed using PCR. The antimicrobial resistance testing was performed by the disk diffusion method. The enterotoxin genes were identified in 36 *S. aureus*, including *sea* (83.8%). The *tst* gene was detected in 18.92% of the strains and the *luk-PV* was detected in only one isolate. *Agr* typing classified 58.3% of the strains as type I. Seven (18.92%) strains were classified as MRSA and the *ccr2* complex was detected in six of these isolates. The SCC*mec* typing characterized strains as type II, III, IV and V. Moreover, there were also a greater number of resistant strains to penicillin (83.78%) and clarithromycin (67.57%). In conclusion, the study revealed a significant prevalence of *S. aureus*, and the presence of different virulence genes and a worrying resistance profile in *S. aureus* strains isolated from food handlers in this country.

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https://doi.org/10.1016/j.bjid.2022.102698

Introduction

Foodborne diseases are an important public health problem, and it is estimated that 600 million people get sick annually due to the consumption of contaminated food around the world.[1] In general, foods can be contaminated by several microorganisms, including bacteria which are one of the main causes (66%) of foodborne diseases.[2,3] The transmission of these pathogens usually occurs due to poor food handling and low sanitation practices during processing.[1,4] In Brazil, *Staphylococcus aureus* is one of the most frequent pathogens associated with foodborne outbreaks.[5]

S. aureus is an important opportunistic pathogen that colonizes the nasal cavity, oropharynx and skin of approximately 30% of the world population.[6] This microorganism invades host tissues and is responsible for causing various diseases.[7] To establish and maintain infection, *S. aureus* has been able to encode several virulence factors during their growth, including superantigenic toxins, such as classical Staphylococcal Enterotoxins (SEA, SEB, SEC and SED) and Toxic Shock Syndrome Toxin-1 (TSST-1).[7] Another important toxin group are leukotoxins, such as Panton-Valentine Leukocidin (PVL).[8]

The expression of these virulence factors is regulated and controlled by genetic regulatory systems, such as the accessory gene regulator (*agr*) system.[9] The *agr* system is a group with quorum sensing activity and controls expression of different genes involved in tissue colonization and invasion.[9,10]

Until this moment, there are four *agr* polymorphism types due to mutation and/or insertion of fragments, resulting in variations of the *agrC* and *agrD* genes, denominated *agr*I, *agr*II, *agr*III and *agr*IV.[9,10] It is important to emphasize that studies have also reported that different *agr* groups are indirectly linked to disease severity profiles due to relationship between *agr* group and the genetic background of the strain, although the reasons for this association are not yet clear.[9,11,12]

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is one of the main causes of infections in hospital and community settings and has been isolated from food and food handlers in different countries, including in Brazil.[5,13,14] In addition, MRSA strains can cause serious foodborne outbreaks due to the presence of numerous toxins in their genome, thus being a potential risk to public health.[5,15]

S. aureus may cause severe diseases and has been difficult to treat humans with this infection worldwide.[5] Food handlers can be a potential reservoir and transmission vehicle of virulent and antimicrobial-resistant *S. aureus* strains, including MRSA.[5]

Therefore, the aims of this study were to verify the frequency of virulence genes, to identify the *agr* groups, and to determine the antimicrobial resistance profile of *Staphylococcus aureus* strains isolated from nasal cavities and underside of nail of the food handlers of three pilot kitchens located in the West region of São Paulo State, Brazil.

Materials and methods

Socioeconomic, sanitary, and health-related data collection

The study was approval was obtained at the local research ethics committee (CEP) as Protocol CAAE: 59635316.6.0000.5515.

The study was carried out with the participation of 41 food handlers from three Pilot Kitchens (PK) located in the West region of São Paulo State in Brazil. Each participant answered a standardized questionnaire, comprising 22 objective questions, containing information about the processing way and handling of food. In addition, the socioeconomic, sanitary, and health-related data of the participants were collected.

Isolates

Two bacterial samples were collected from each participant, one from the nasal cavities and one from the underside of nails. The specimens were collected using a sterile swab, moistened with saline solution (0.85%). The samples were stored at the Microbiology Laboratory located on Campus 1 of UNOESTE, Presidente Prudente - SP, in Brazil.

Phenotypic Staphylococcus identification

The isolates obtained from samples of nasal cavities and the underside of nails were plated on Baird-Parker medium and were subjected to Gram stain for observation of colony morphology. Subsequently, the catalase and coagulase tests were performed.

DNA extraction

Bacteria DNA was extracted by using the Illustra tissue and cells prep genomic mini spin kit (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions, following an adapted protocol described by Pereira et al.[16] The extracted DNA was stored in a freezer at -20°C.

Species identification and detection of virulence genes

The Polymerase Chain Reaction (PCR) was performed to confirm the *S. aureus* identification from the *sau* gene amplification. The PCR technique was also used to detect the *sea, seb, sec-1, sed,* tst, and *luk*-PV genes.

The *sea, seb, sec-1, sed,* and tst genes were amplified using a protocol described by Cunha & Calsolari[17] and Johnson et al.[18] PCR reactions for the detection of PVL genes (*lukS-PV* and *lukF-PV*) were performed as described by Lina et al.[19] The amplification was revealed by electrophoresis in a 1% agarose gel and stained with ethidium bromide. International reference toxigenic *S. aureus* strains were used as positive controls, including, ATCC 13565 (EEA), ATCC 14458 (EEB), ATCC 19095 (EEC) e ATCC 23235 (EED). For negative control, *S. xylosus* ATCC 29971 was used.

Determination of agr *group*

The *S. aureus* strains that carried one or more virulence genes analyzed were submitted to the *agr* group typing method by PCR. The amplification occurred from 1.5 μL of extraction DNA in a 13 μL reaction mixture, containing 1.3 U *Taq* DNA polymerase, 104 μmoL/L of Deoxyribonucleotide Triphosphates (dNTP), 10.4 mmoL/L of Tris-HCl, Ph 8.4, 0.39 mmoL/L $MgCl_2$ and 0.5 μL primers *agr1, agr2, agr3, agr4* and *pan* described by Gilot et al.,[20] where the *pan* was used together with the four *agr* primers.

The PCR products were submitted to electrophoresis in a 1% agarose gel and stained with ethidium bromide. The amplified products size was compared with the standards: *pan* and *agr1* with 441 bp, *pan* and *agr2* with 575 bp, *pan* and *agr3* with 323 bp, and *pan* and *agr4* with 659 bp. International reference strains were used as positive controls, including R137 (*agr*I), N315 (*agr*II) e *S. aureus* ATCC 25923 (*agr*III).

The mecA *gene detection and determination of* SCCmec

The *mecA* gene detection was performed by conventional PCR reactions according to the protocol described by Murakami et al.[21] *S. aureus* strains ATCC 33591 (positive control) and ATCC 25923 (negative control) were included in all reactions. Staphylococcal Cassette Chromosome *mec* (SCC*mec*) was typed in MRSA strains. Reactions were performed by multiplex PCR, as described by Oliveira & De Lencastre.[22]

Antimicrobial resistance test

The antimicrobial resistance test was performed using the disk diffusion method described in the guidelines of the Clinical Laboratory Standards Institute (CLSI).[23] The choice of antimicrobials was based on *Staphylococcus* infection treatment. The antimicrobials tested were penicillin (10 μg), oxacillin (1 μg), clarithromycin (15 μg), erythromycin (15 μg), cefoxitin (30 μg), tetracycline (30 μg), levofloxacin (5 μg), amoxicillin (10 μg), clindamycin (2 μg), linezolid (10 μg), and vancomycin (30 μg).

Data analysis

Data analysis was done using software Bioestat 5.3. Chi-Square test was used for comparing the frequencies of the *sea, seb, sec-1, sed,* tst and *mecA* genes with the following variables: sex, age, hospitalization/surgery, antibiotic use and medicine use by assuming p-value ≤0.05 as significant difference.

Results

Socioeconomic, sanitary, and health-related data

Forty-one food handlers from three Pilot Kitchens (PK) participated in this study, including 16 from PK1, 11 from PK2, and 14 from PK3. Among the 41 participants, 35 (85.37%) were female and 6 (14.63%) male. Related data to the functions performed and the health of the participants, including hospitalization episodes, surgical procedures, and use of antimicrobials in the last 12 months, as well as use of other medications are shown in Table 1.

Table 1 – Questionnaire data answered by the food handlers of the three Pilot Kitchens.

	Number	Percentage (%)
Functions performed by food handlers		
Meal preparation	8	19.51
Meal preparation + material and food cleaning	14	34.15
Food transport	6	14.63
Nutritionists/administration	4	9.76
Environment and material cleaning	8	19.51
Transport, material cleaning + administration	1	2.44
Food handlers' health issues	**Number**	**Percentage (%)**
Hospitalization (last 12-months)	6	14.63
Surgical process (last 12-months)	3	7.32
Use of antimicrobials (last 12-months)	13	31.71
Use of medicines	25	60.98

Participants were also asked about the use of Personal Protective Equipment (PPE) and the drying hands habit during their workday. Of the 41 participants, 33 (80.49%) reported using PPE, such as aprons and gloves, and 8 (19.51%) reported not using them, and all reported using disposable paper towels and cloth towels for drying hands.

Identification of isolates

Eighty-two bacterial samples were collected from the underside of nails and nasal cavities of each participant in the pilot kitchens. Specifically, 32 in PK1, 22 in PK2 and 28 in PK3. Of all isolates, 74 (90.24%) were identified as *Staphylococcus* through Gram method and catalase and coagulase tests, and four samples were discarded due to contamination. In four samples there was no growth of the bacterial genus in question. The *sau* gene was detected in 37 (50%) isolates, confirming the *S. aureus* identification.

Detection of virulence genes

Of the 37 *S. aureus* isolates submitted to the PCR method to detect the enterotoxins genes, 31 (83.78%) were positive for the presence of the *sea* gene, 4 (10.81%) for the *seb* gene, 18 (48.65%) for the *sec-1* gene and 5 (13.51%) for the *sed* gene. Regarding the *tst* gene amplification, this occurred in 7 (18.92%) isolates. The *luk*-PV gene was detected in only one isolate.

Determination of agr group

The 36 *S. aureus* strains that carried one or more analyzed virulence genes were submitted to the *agr* group typing method. After the analysis, amplification of *agr*I was observed

Table 2 – Characteristics of the 37 *S. aureus* isolates from food handlers of Pilot Kitchens.

	sau	*sea*	*seb*	*sec-1*	*sed*	*tst*	*luk-PV*	*agrI*	*agr II*	*agr III*	*agr IV*	*mecA*	*ccr*	*mec complex*	SCC*mec*
N1	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
N3	+	-	-	-	-	+	-	+	-	+	-	-	-	-	-
N5	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
N6	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
N7	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
N8	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-
N9	+	+	+	+	-	-	-	+	-	+	-	-	-	-	-
N13	+	+	-	+	-	+	-	+	-	+	-	-	-	-	-
N14	+	+	-	-	-	+	-	+	-	+	-	-	-	-	-
N15	+	+	-	-	-	-	+	+	-	+	-	-	-	-	-
N16	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-
N22	+	+	+	+	+	-	-	+	-	-	-	+	*ccr2*		NT
N24	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-
N25	+	+	+	+	+	-	-	-	-	-	-	+	*ccr2*	A	II
N32	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-
N34	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-
N36	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-
N37	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-
N38	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-
N39	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
N40	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-
N41	+	+	-	+	-	-	-	-	-	-	-	+	*ccrC*	C	V
U2	+	+	-	-	-	+	-	-	-	+	-	-	-	-	-
U4	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
U5	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
U6	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
U9	+	+	+	-	-	+	-	+	-	-	-	-	-	-	-
U10	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
U11	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-
U14	+	+	-	-	-	+	-	+	-	+	-	-	-	-	-
U20	+	-	-	-	-	-	-	-	-	-	-	+	*ccr2*	B	IV
U22	+	+	-	+	+	-	-	-	-	-	-	+	*ccr2/ccr3*		NT
U27	+	+	-	+	+	-	-	-	-	-	-	+	*ccrC/ccr2*	B	III
U28	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
U33	+	+	-	+	-	-	-	-	+	-	-	+	*ccr2*		NT
U35	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
U41	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-

U, Underside of nails; N, Nasal cavities; NT, Not typed.

in 21 (58.33%) isolates, *agr*II in 4 (11.11%) isolates, and *agr*III in 15 (41.67%) isolates. In none of the samples there was the amplification of *agr*IV. In 11 (35.56%) samples, there was no detection of any of the four polymorphisms studied.mecA gene detection and determination SCC*mec*

Regarding the presence of the *mec*A gene, there was amplification in seven of the 37 isolates, therefore 18.92% of *S. aureus* were classified as MRSA and were submitted to the analysis of the *ccr* gene complex. The *ccr2* was detected in 6 (85.71%) isolates, and *ccr3* and *ccrC* in 2 (28.57%) different isolates. In 1 (14.28%) isolate only *ccrC* was detected. SCC*mec* typing revealed the presence of one isolate type II, one type III, one type IV, and one type V. Three (42.86%) strains were characterized as Not Typed (NT).

The data about virulence genes, *agr* groups, *mec*A detection and SCC*mec* typing are summarized in Table 2.

The frequency of toxins genes and the *mec*A gene and the variables sex, age, hospitalization/surgery, antibiotic use, and use of another medication are shown in detail in Table 3 (*p*-values).

Antimicrobial resistance test

The antimicrobial resistance test showed that 31 (83.78%) *S. aureus* isolates were resistant to penicillin, 22 (59.46%) to oxacillin, 24 (64.86%) to clarithromycin, and 25 (67.57%) to erythromycin. Seven (18.92%) strains were resistant to tetracycline, 3 (8.11%) strains to cefoxitin, 3 (8.11%) to levofloxacin, 2 (4.41%) to amoxicillin, 1 (2.70%) to clindamycin and none of the isolates were resistant to linezolid and vancomycin.

Discussion

The present study investigated the presence of important toxins genes, identified the *agr* groups, determined the antimicrobial resistance profile against 11 antimicrobials and carried out *mec*A detection and SCC*mec* typing in *Staphylococcus aureus* isolated from nasal cavities and underside of nail

Table 3 – Associations between frequency of toxins genes, *mecA* and questionnaire data (*p*-values).

	Genes detected in strains from the m underside of nails					
	sea	*seb*	*sec-1*	*sed*	*tst*	*mecA*
Questionnaire data						
Sex (female or male)	0.45	0.68	0.76	0.10	0.25	0.42
Age (younger or older than 30-years old)	0.77	0.09	0.63	0.36	0.77	0.93
Hospitalization/surgery (last year)	0.02[a]	0.60	0.29	0.45	0.33	0.24
Antibiotic use (last year)	1.00	0.46	1.00	0.28	0.17	0.10
Medicine use (last year)	1.00	0.46	0.26	0.03[a]	1.00	0.00[a]
	Genes detected in strains from nasal cavities					
	sea	*seb*	*sec-1*	*sed*	*tst*	*mecA*
Questionnaire data						
Sex (female and male)	0.11	0.56	0.89	0.12	0.00[a]	0.55
Age (younger or older than 30-years old)	0.80	0.80	0.48	0.80	0.26	0.29
Hospitalization/surgery (last year)	0.01[a]	0.68	0.26	0.68	0.00[a]	0.68
Antibiotic use (last year)	0.64	0.31	0.46	0.31	0.90	0.64
Medicine use (last year)	0.16	0.16	0.00[a]	0.16	0.13	0.16

[a] $p \leq 0.05$ – Significant values.

of the food handlers of three pilot kitchens located in the West region of São Paulo State, Brazil.

Good food handling practices are responsible for ensuring the hygienic-sanitary quality of foods during processing.[4] Specifically, from the information obtained by the questionnaires answered, regarding knowledge about the use of PPE, all employees demonstrated to be aware about this topic. In addition, all admitted to use paper and cloth towels that were in common use for drying hands. It is known that food handlers who use cloth towels have increased bacterial colonies counts from hand samples, suggesting that these towels are capable of disseminating *S. aureus* in a food handling environment.[24]

In this study, it was observed that *S. aureus* was isolated in half of the samples of nasal cavities and underside of nails from food handlers. According to Castro et al.,[15] Ahmed,[25] and Saber et al.[26] the presence of *S. aureus* in the hands and/or noses of food handlers was of 30%, 25%, and 30%, respectively.

The pathogenic potential of *S. aureus* strains was investigated by the presence of toxins genes, such as classical enterotoxins (SEs). Specifically, SEs are characterized as superantigens, which are proteins that can trigger severe and systemic manifestations, including fever, vomiting, diarrhea, abdominal cramps, and sweating.[7]

Of all 37 *S. aureus* isolates, 35 (94.6%) had at least one of the classical enterotoxins genes. The *sea* gene was detected in 83.78% of *S. aureus*. This gene is commonly associated with contaminated food and is considered the enterotoxin gene most prevalent and leading cause of staphylococcal food poisoning worldwide.[15,25,27-30]

The *seb* and *sec-1* genes were detected in 10.81% and 48.65% of *S. aureus* strains studied, respectively. In contrast, other studies with food handlers demonstrated that the *seb* gene was more frequent than *sec* in Iran and Sudan.[25,30] Finally, the *sed* gene was detected in 13.51% in the present study and this gene has been the least described among the other classical SEs genes.[25,30,31]

It is important to mention that the TSST-1 is also described as a superantigen. Unlike enterotoxins, TSST-1 has not yet been associated with food consumption, but if contaminated hands with TSST-1 producer strains touch foods, cross-contamination can occur, which can lead to Toxic Shock Syndrome (TSS) in humans.[32,33] In the present study, the *tst* gene was found in 7 (18.92%) *S. aureus* isolates. The presence of toxin-producing *S. aureus* has been frequently reported in healthy individuals and, generally, these *S. aureus* strains have high prevalence rates of enterotoxin-producing genes and a lower detection of TSST-1 and Panton-Valentine Leukocidin (PVL) genes.[15,31,33,34]

PVL is a cytolysin encoded by the *lukF-PV* and *lukS-PV* genes, which participate in the pores formation in the leukocyte membrane, leading to cell lysis predisposing skin and soft tissue infections and necrotizing pneumonia.[33,35] Aung et al.[33] observed that 12.5% of *S. aureus* strains presented the *luk*-PV genes, while the TSST-1 gene was the least prevalent among these isolates from food handlers.

In this study, *agr* typing in the *S. aureus* strains showed that *agr*I type was the most prevalent (58.33%). This polymorphism can be involved in food poisoning due to enterotoxins production.[36] Similarly to our results, different authors demonstrated that *agr*I was the most common polymorphism found in strains isolated from food handlers or food products.[37-40]

The *agr*II group was found in 11.11% of the *S. aureus* isolates. This low frequency has been observed in other studies with food handlers or food products and more associate to clinical isolates.[27,39-41] The *agr*III group, that may be associated with toxic shock syndrome, was detected in 41.67% of the *S. aureus* strains. In contrast, other authors found a lower frequency of the *agr*III group in *S. aureus* isolated from food handlers and food products (3.1%, 12.84%, 23.5%, and 19.2%) compared to the present study.[27,36,37,41,42]

Finally, the polymorphism *agr*IV is associated with the production of exfoliative toxins.[36] This polymorphism has rarely been described and was not detected in the present

study as well as in other studies carried out in different parts of the world.[27,37,38,42]

Regarding antimicrobial resistance, 91.89% of the *S. aureus* isolates were resistant to at least one of the antibiotics tested, including resistance to penicillin (83.78%), erythromycin (67.57%) and clarithromycin (64.86%). Furthermore, a total of 18.92% of the isolates were identified as (MRSA).

Methicillin resistance and β-lactams is associated with the *mecA* gene, which is transported through a mobile genetic element denominated SCC*mec*. SCC*mec* encompasses the *mec* complex, regulatory genes, ccrAB or/and ccrC, plus other accessory genes encoding for a new specific Penicillin-Binding Protein (PBP2a).[5,43,44]

MRSA has been recognized as an important nosocomial pathogen and listed by World Health Organization as one of the high-priority antibiotics-resistant pathogens. Furthermore, in 2019 MRSA caused more than 100,000 deaths worldwide.[45,46] This bacterium have been isolated from food in different countries, including Brazil, and is also associated with the occurrence of foodborne diseases.[5,14,45,47]

In Brazil, Rodrigues et al.[47] isolated *Staphylococcus* spp. from cheese processing plants, including food handlers, and the *mecA* gene was present in six strains (6%). On the other hand, still in Brazil, Ferreira et al.,[48] demonstrated that 28.6% of the food handlers of public hospitals had MRSA strains in hands or nostrils. This incidence of MRSA isolated from food and food handlers in the country emphasize the need for better food handling practices, preventing these strains from being transmitted to the community.[5]

The present study also performed SCC*mec* typing. Specifically, the SCC*mec* type IV is more associated with healthy individuals' colonization, while the types I–III are related to nosocomial infections.[45] This difference was not observed in this data, since the types II, III, IV, and V were detected in *S. aureus* isolates from healthy food handlers. In addition, three samples were classified as non-typeable, which can be attributed to the diversity of the elements that make up the SCC*mec*.[49]

Finally, the data obtained in the present study also indicated that in isolates from the underside of the nails, there was association between the *sea* gene with hospitalization/surgical process, and the *sed* and the *mecA* gene with the use of medicines. In isolates from the nasal cavities, there was association between the *sec*-1 gene with the use of medicines and the *tst* gene with sex and hospitalization/surgical process. There is a scarcity of data literature about these associations, suggesting the need for further investigation of the relationship of these genes with the related variables.

Conclusions

In conclusion, the study revealed a significant prevalence of *S. aureus* colonizing the nasal cavities and lower nails of food handlers in the São Paulo State in Brazil, as well as the pathogenic potential was corroborated by the presence of important virulence genes and the prevalence of MRSA strains, reinforcing the threat for humans. Thus, biosecurity measures should be prioritized in this environment due to the risk of food contamination.

Conflicts of interest

The authors declare no have conflicts of interest.

Acknowledgments

We thank the São Paulo State Research Foundation for Scientific and Technological Development (FAPESP; Grants 2018/12652-6 and 2018/00056-0) for supporting research development and the Universidade do Oeste Paulista (UNOESTE) for structure and financial support.

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