



Short communication: Identification of subclinical cow mastitis pathogens in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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ABSTRACT

Subclinical mastitis is a common and easily disseminated disease in dairy herds. Its routine diagnosis via bacterial culture and biochemical identification is a difficult and time-consuming process. In this work, we show that matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) allows bacterial identification with high confidence and speed (1 d for bacterial growth and analysis). With the use of MALDI-TOF MS, 33 bacterial culture isolates from milk of different dairy cows from several farms were analyzed, and the results were compared with those obtained by classical biochemical methods. This proof-of-concept case demonstrates the reliability of MALDI-TOF MS bacterial identification, and its increased selectivity as illustrated by the additional identification of coagulase-negative *Staphylococcus* species and mixed bacterial cultures. Matrix-assisted laser desorption-ionization mass spectrometry considerably accelerates the diagnosis of mastitis pathogens, especially in cases of subclinical mastitis. More immediate and efficient animal management strategies for mastitis and milk quality control in the dairy industry can therefore be applied.

Key words: bacteria identification, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), subclinical mastitis diagnosis

Subclinical mastitis, an udder infection without clinical signs that is commonly encountered in cows, is a major problem in the dairy industry. The disease af-

fects both milk quality and yield, and may lead to rapid transmission of the infection to healthy cows in the herd. Microbiological methods routinely used to diagnose subclinical mastitis are based on changes in milk composition caused by bacterial growth and enzymatic and immunological reactions in the infected dairy cows (Peeler et al., 2003; Santos et al., 2004).

Many microorganisms have been identified as agents of mastitis infection. In Germany, for instance, 5 bacterial species (*Staphylococcus aureus*, *Corynebacterium* spp., *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli*) have been shown responsible for the majority (approximately 80%) of cases (Barkema et al., 1998). In Brazil, a recent survey showed that the 3 major pathogens causing mastitis are *Corynebacterium* spp., *Staphylococcus* spp., and *Streptococcus* spp. (Souto et al., 2010).

Routine microbiological methods applied to microorganism identification have remained nearly unchanged over the last century. These methods still employ classical approaches based on sample streaking, colony growth using various culture media, and morphological and biochemical characterization of the isolated bacterial species. On average, routine milk microbiological procedures take from 5 to 8 d to be completed and require the use of various biochemical tests and the evaluation of experts (NMC, 1999).

Recently, bacterial identification at the molecular level has been investigated using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Fenselau and Demirev, 2001). Many microorganisms have been identified by MALDI-TOF MS via characteristic “chemical signatures” in a high-throughput fashion using straightforward extraction and sample work-up protocols. After initial bacterial growth that requires 1 d on average, and using crude bacterial extracts or lysate supernatants of whole cells, bacterial determination has been performed by MALDI-TOF MS in a few minutes (Holland et al., 1996,

Received July 9, 2010.

Accepted August 31, 2010.

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Mellmann et al., 2008). This technique has therefore revolutionized the field of bacterial screening by providing a much faster, more reliable, and highly sensitive technique for bacteria identification. These features are particularly attractive for bacterial isolates screening in contaminated milk at subclinical levels.

Matrix-assisted laser desorption/ionization is a “soft” ionization technique that allows for the detection of intact molecular species from pure compounds or complex mixtures. Matrix-assisted laser desorption/ionization time-of-flight MS of bacteria identifies protein “fingerprints,” a group of mainly ribosomal proteins unique to each bacterial species (Ieven et al., 1995; Claydon et al., 1996; Keys et al., 2004; Carbone et al., 2007; Barbuddhe et al., 2008). Matrix-assisted laser desorption/ionization time-of-flight MS bacterial identification has also shown high interlaboratory reproducibility (Mellmann et al., 2009), and is rapidly being incorporated into human clinical microbiological routines. This technique requires little MS expertise, the use of simple instruments, and relatively fast operator training. When compared with DNA analysis-based technologies, MALDI-TOF MS requires smaller amounts of biological material and involves simpler sample preparation protocols with no initial assessment, such as Gram staining, (Fenselau and Demirev, 2001; Iliina et al., 2009; Mellmann et al., 2009; Iliina et al., 2010).

In this work, we tested the applicability of MALDI-TOF MS for bacterial screening of subclinical mastitis in cows. Clinical samples of 33 bacterial strains isolated from samples of milk were collected from dairy cows on various farms in São Paulo State, Brazil. For comparison, these bacterial strains were also submitted to classical routine biochemical identification testing.

To isolate bacteria from milk samples, 10 μ L of sampled milk was streaked onto blood agar plates and incubated at 37°C for 24 h to allow for bacterial growth. The biochemical identification of the species was performed by means of catalase production, coagulase test, spore staining, and (for some samples) Gram staining.

To prepare the samples for MALDI-TOF MS, the bacterial strains isolated from milk were thawed and cultured for 24 h in a brain heart infusion broth. The bacterial culture was centrifuged, inactivated in 75% ethanol, and submitted to bacterial extraction, which was performed as detailed in the literature (Barbuddhe et al., 2008; Lartigue et al., 2009; Iliina et al., 2010). By this protocol, the microtube containing isolated bacteria in 75% ethanol solution was centrifuged (all centrifugation steps were at 13,000 $\times g$ for 2 min), and the supernatant was removed by carefully pouring it from the microtube. A second centrifugation step was performed and the remaining liquid was carefully re-

moved with a pipette tip. Bacterial pellets were allowed to air dry at room temperature for 5 to 10 min. A solution of 70% formic acid was added to lyse bacterial cells and to release the inner-cell proteins, predominantly the ribosomal proteins that produce diagnostic ions in MALDI fingerprinting (Ryzhov and Fenselau, 2001). The 70% formic acid solution was added proportionally to the size of pellet to completely dissolve it. Subsequently, 100% acetonitrile was added to each sample in volumes equal to the 70% formic acid solution added, thus producing a bacterial extract in a 1:1 ratio of 70% formic acid and acetonitrile. A final centrifugation step was performed to separate bacterial cell debris from the supernatant containing the inner-cell proteins used for the MALDI-TOF MS identification.

To prepare the MALDI target plates, 1 μ L of bacterial extract was placed onto a steel target plate (MSP 96 polished-steel target; Bruker Daltonik, Bremen, Germany) and allowed to air dry. The dried supernatant, overlaid with 1 μ L of matrix solution, consisted of α -cyano-4-hydroxy-cinnamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid. The MALDI-TOF MS was performed in a Bruker Microflex LT MALDI-TOF mass spectrometer operated in the linear mode and equipped with a 337-nm nitrogen laser using FlexControl 3.3 software (Bruker Daltonik). The mass spectra were collected within the mass range of 2,000 to 20,000 m/z . Two hundred forty laser shots were accumulated to generate each spectrum. Spectra were analyzed with MALDI Biotyper 2.0 software (Bruker Daltonik) at default settings. For each sample, the automatic analysis generated a peak list, which was used to match a reference library by the integrated pattern-matching algorithm. The result was given by means of a log score with a maximum value of 3.0. Score values higher than 1.7 were considered reliable for genus identification, and scores higher than 2.0 were considered probable for species identification. In this study, only scores higher than 2.0 were considered. In general, the MALDI Biotyper pattern-matching algorithm considers the matches of the unknown sample spectrum against the reference database and the reverse matches of the main spectrum with the unknown spectrum; it also compares the relative intensities of unknown and database spectra (Lartigue et al., 2009).

Table 1 summarizes the results of biochemical and MALDI-TOF MS characterization of the 33 samples studied. The isolated bacteria were identified by classical microbiological routine protocols as *Staph. aureus* ($n = 13$), *Strep. agalactiae* ($n = 10$) or CNS ($n = 10$). Samples displaying divergent results for each identification technique used were sent for 16S rRNA gene sequencing for a more reliable identification. Figure 1 presents a comparison of the analysis workflow of

Table 1. Biochemical identification and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) identification of bacterial strains isolated from different milk samples from cows presenting subclinical mastitis

Samples, n	Biochemical identification	MALDI-TOF MS
11	<i>Staphylococcus aureus</i>	<i>Staph. aureus</i>
1	<i>Staph. aureus</i>	<i>Staph. haemolyticus</i> ¹
1	<i>Staph. aureus</i>	<i>Enterococcus faecalis</i> / <i>Staph. aureus</i> ²
10	<i>Streptococcus agalactiae</i>	<i>Strep. agalactiae</i>
1	CNS	<i>Staph. simulans</i> ¹
2	CNS	<i>Staph. aureus</i> ¹
5	CNS	<i>Staph. epidermidis</i> ¹
1	CNS	<i>Staph. chromogenes</i> ¹
1	CNS	<i>Staph. haemolyticus</i> ¹

¹The 16S ribosomal RNA sequencing result matched the MALDI-TOF MS identification.

²Algorithm-detected mixed bacterial culture.

routine microorganism biochemical identification and MALDI-TOF MS.

The MALDI-TOF MS and biochemical identifications were performed for all 13 *Staph. aureus* samples. Each sample was cultured twice and similar results were observed. Eleven out of 13 bacterial isolates presented the same results through both identification methods. All *Strep. agalactiae* samples yielded the same results for both biochemical and MALDI-TOF MS identification procedures. Superposed spectra of *Strep. agalactiae* (Figure 2, in red, from the Biotyper database) present the same ions observed in the investigated samples (Figure 2, in light blue). From the data shown in Figure 2, it is clear that MALDI-TOF MS (based on conserved protein profiles) provides characteristic protein profiles that allow for identification of different species from the same bacterial genus (i.e., *Strep. agalactiae* and *Strep. dysgalactiae*). The MALDI Biotyper Database comprises data from strain collections (e.g., American Type Culture Collection, ATCC) and sequenced bacterial strains, and has been optimized for *Strep. agalactiae* identification (Lartigue et al., 2009).

To confirm some identification results provided by MALDI-TOF MS as compared with the classical microbiology results (Table 1), the 16S rRNA gene of certain samples was sequenced (see Table 1). One sample was identified as *Staph. aureus* by the biochemical procedures previously mentioned, whereas the samples identified by MALDI-TOF MS were identified as a *Staph. haemolyticus* (compare with Figure 3, for *Staph. aureus* and *Staph. haemolyticus*, respectively). The 16S rRNA gene sequencing confirmed the MALDI-TOF MS identification as *Staph. haemolyticus*. Matrix-assisted laser desorption/ionization-MS is also able to identify mixed bacterial cultures, as exemplified by 1 of the samples identified as *Staph. aureus* by biochemical testing. The MALDI-TOF MS displayed a protein signature corresponding to a mixture of *Enterococcus faecalis* and

Staph. aureus (Table 1). This mixed bacterial culture may explain the positive coagulase reaction of this isolate.

Many coagulase-negative *Staphylococcus* species are classified as coagulase-negative and are commonly involved in mastitis infections. Routinely, CNS species are not further determined by biochemical procedures, but further identification was achieved by MALDI-TOF MS (Figure 4; Dubois et al., 2010). The coagulase-negative *Staph. aureus* was not expected, although *Staph. aureus* strains displaying negative reaction in the coagulase tube test do exist (Mlynarczyk et al., 1998). Similarly, the presence of coagulase-negative *Staph. aureus* has been reported in 1 out of 26 *Staph. chromogenes* (a coagulase-negative *Staphylococcus*) isolated from 44 subclinical mastitis isolates from dairy herds in Belgium. The 1 isolate identified as a *Staph. aureus* strain by molecular characterization via transfer RNA intergenic spacer PCR failed to be identified microbiologically as it showed no positive coagulase reaction (Catry et al., 2003). Note that characteristic MALDI-TOF MS of CNS were obtained from coagulase-negative species, such as *Staph. simulans*, *Staph. epidermidis*, and *Staph. chromogenes*. This information, easily obtained by MALDI-TOF MS, is important for a better characterization of pathogens causing mastitis in cows and increases the prospective applications of this technique in dairy science.

Four ATCC strains were also cultured and submitted to MALDI Biotyper identification: *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Staph. aureus* ATCC 29213, and *Escherichia coli* ATCC 25922. Because these ATCC strains also were present in the MALDI Biotyper 2.0 database, these strains could be identified unequivocally (data not shown).

The 16S rRNA gene polymorphism is widely accepted as the “gold standard” for bacteria identification with ambiguous biochemical profiles (Relman et al., 1990,

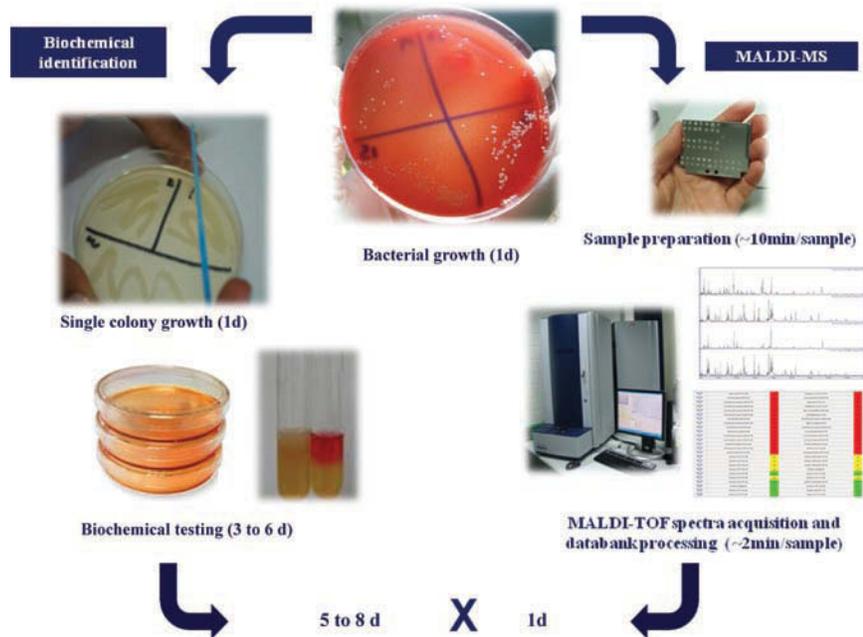


Figure 1. Schematics comparing the workflow of classical routine microorganism biochemical identification and the workflow normally used in matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) for clinical isolates identification. After initial growth of the microorganism (for 24 h after sample streaking), microorganism identification is performed in a few minutes by MALDI-TOF MS compared with 5 to 7 d for the classical biochemical methodologies. High-throughput MALDI-TOF MS can also be performed for 96 or 384 samples on each target plate.

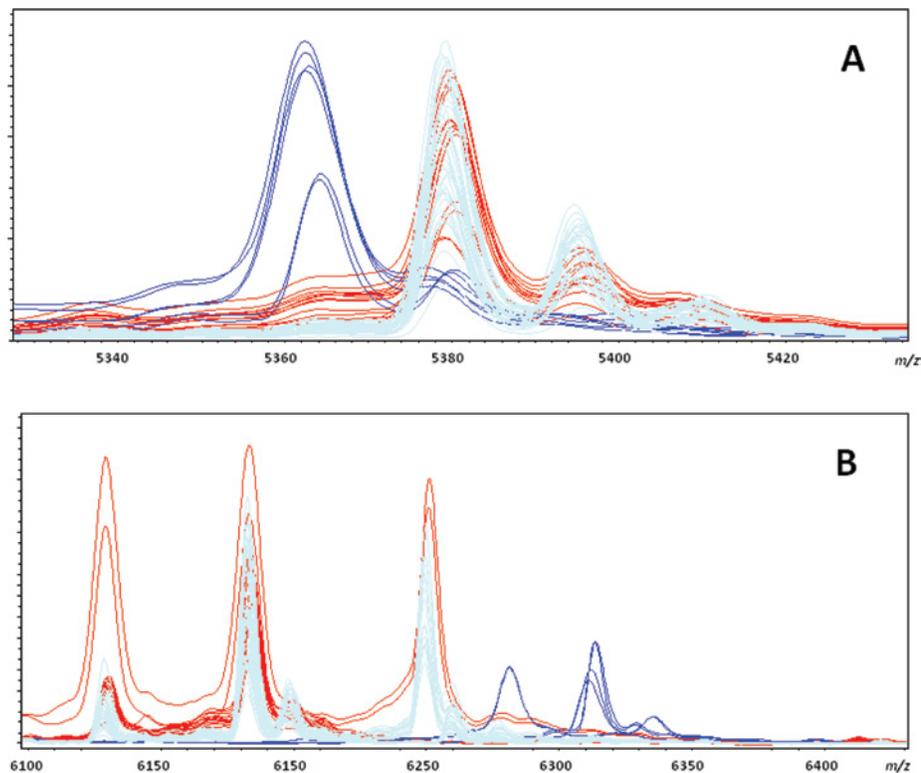


Figure 2. Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry at the ranges of (A) 5,300 to 5,450 m/z and (B) 6,100 to 6,500 m/z from reference spectra of *Streptococcus agalactiae* (red lines), reference spectra of *Streptococcus dysgalactiae* (dark blue lines), and the spectra obtained *Strep. agalactiae* samples (light blue lines). Note that the 10 *Strep. agalactiae* samples are clearly matched with the *Strep. agalactiae* from the reference spectra.

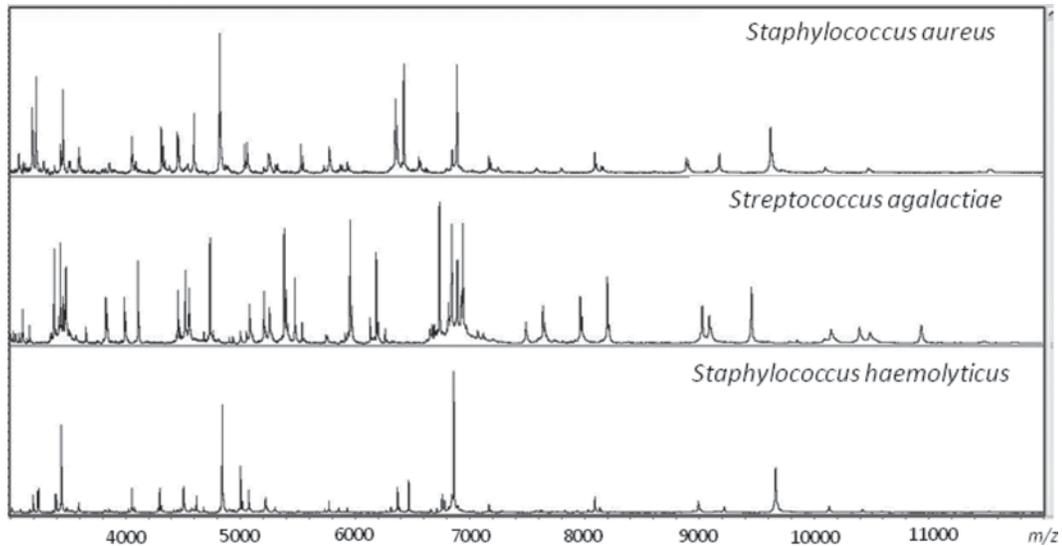


Figure 3. Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry of 3 bacterial isolates. Note the characteristic protein profiles obtained for *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Staphylococcus haemolyticus*.

1992). It is highly conserved within species and among species of the same genus; therefore, this method usually provides more accurate and faster results compared with conventional biochemical protocols (Ferroni et al., 2002; Becker et al., 2004; Cloud et al., 2004).

Some unexpected results presented in this work are related to the different methods of bacterial strain identification used in the classical biochemical procedures (colony morphology, staining, and enzymatic reactions) compared with the MALDI-TOF MS approach, which

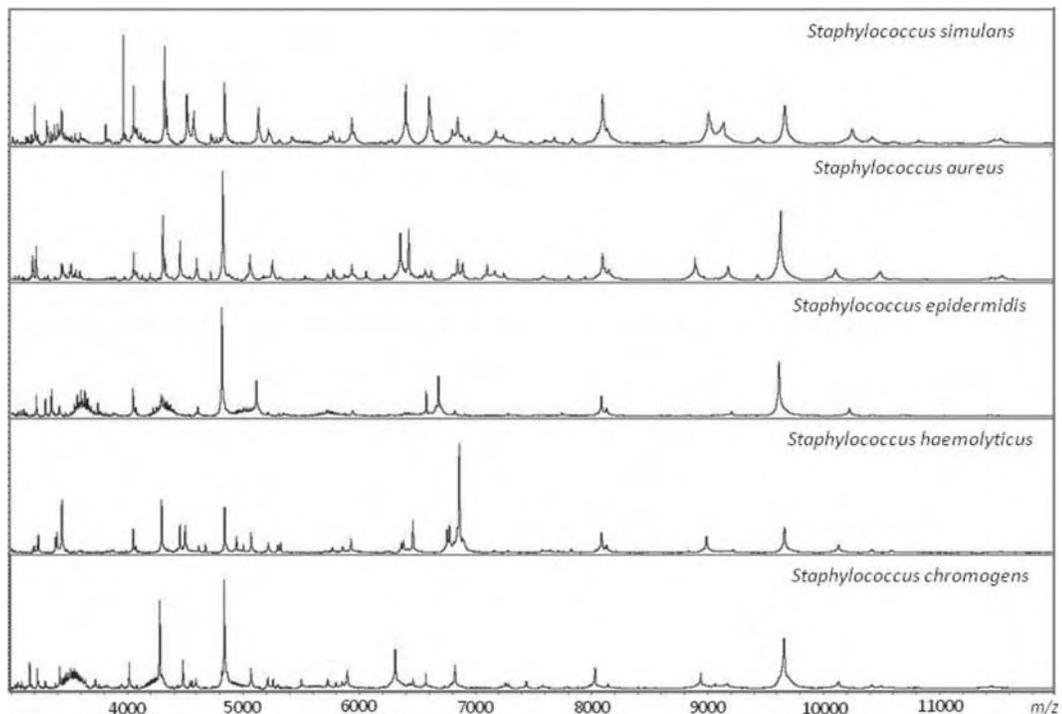


Figure 4. Matrix-assisted laser desorption/ionization (MALDI)–time-of-flight mass spectrometry CNS isolates. Note the identification of CNS isolates. These profiles have been readily related to a bacterial genus and species by data treatment with the MALDI Biotyper Database.

is based on chemical signatures provided by protein profiles. The classical biochemical procedures for bacteria identification are more susceptible to incorrect human evaluation or errors during sample work-up, as indicated by the identification of *Strep. uberis* isolated from cow mastitis samples (Odierno et al., 2006). Approaches at the molecular level have long been recognized as important tools in microbiology (Kolbert and Persing, 1999), and MALDI-TOF MS employing “molecular fingerprints” seems to provide a reliable tool for microorganism identification. This seems to open a new era in microbiology screening in which conventional phenotypic methods will shift to molecular methods capable of providing faster and more accurate responses and similar genome-related methods of taxonomic and phylogenetic analysis.

The results presented herein, supported by similar and extensive data for clinical human microbiology (Barbuddhe et al., 2008; Mellmann et al., 2008, 2009; Sauer et al., 2008; Ilina et al., 2009; Lartigue et al., 2009; Marklein et al., 2009; Nagy et al., 2009; Dubois et al., 2010; Ilina et al., 2010; Seibold et al. 2010), show that MALDI-TOF MS provides faster detection (1 d vs. 5–8 d) and therefore the possibility of an earlier treatment of subclinical and clinical mastitis with appropriate antibiotics. In the dairy industry, MALDI-TOF MS can also provide a faster, cheaper, and more reliable identification of microorganisms for a more comprehensive microbiological quality control of milk.

ACKNOWLEDGMENTS

We are grateful to Simone Stumpf and Thomas Wenzel (both of Bruker Daltonik GmbH, Bremen, Germany) for their collaboration in this work, to Carol H. Collins (University of Campinas, Campinas, SP, Brazil) and Beatrice Razzo (University of Pennsylvania, PA) for English review, and to the Brazilian National Council for Scientific and Technological Development (CNPq, Brasilia, DF, Brazil) and the São Paulo State Science Foundation (FAPESP, Sao Paulo, SP, Brazil) for funding.

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