DOI: 10.56511/JIPBS.2021.v8.i03.pp24-30

Journal of Innovations in Pharmaceutical and Biological Sciences (JIPBS)
ISSN: 2349-2759
Available online at www.jipbs.com

Research article

Evaluation of protein mass Fingerprint-MS of clinical isolates of *Candida* spp. by MALDI-TOF MS

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Received on: 12/04/2021, Revised on: 07/05/2021, Accepted on: 08/05/2021, Published on: 01/07/2021.

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Keywords: Candida, Fingerprint, Fungal, Identification and MALDI-TOF.

Vol. 8 (3): 24-30, Jul-Sep, 2021.

Abstract

Invasive fungal infections have high levels of mortality, especially in immunocompromised patients. Clinical isolates of *Candida* spp. are responsible for more than 50% of the researched examples. Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) offers fast and accurate identification of the microorganism, optimizing the beginning of antifungal therapy. In recent years, studies are being carried out on the applicability of MALDI-TOF in fungal identification. However, few data were found on fingerprint-MS in different species of *Candida*. The present study aims to evaluate a Protein Mass Fingerprint (PMF) of clinical isolates of *Candida* spp identified by MALDI-TOF MS. For this purpose, it was performed a multivariate analysis of the spectra. There was a consistent spectrum profile among *Candida* species between 3 and 15 KDa. The most intense peaks being between 3 and 8 KDa. It was noted the existence of characteristic signs between species. *C. albicans* shows two intense signs, the first at 4.3 and 4.4 KDa and the other at 5.1 and 5.2 KDa. The others species show an intense signal in 4.4 - 4.5 KDa. *C. lusitaniae* shows intense signals between 6.0 - 6.1 and 6.6 - 6.7 KDa, C. *guilhermondii* between 6.0 - 6.1 and 6.1 –6.2 KDa. *C. glabrata* an intense signal in 5.5 - 5.6 KDa and a less intense signal in 11.0-11.1 Kda. Thus, it is possible to outline a spectral profile of *Candida* spp. The characterization of the spectral profile can help in the critical evaluation of fingerprint-MS, assisting the analyst in the decision-making process and in the expansion of reference library.

Introduction

Invasive fungal infections have high mortality rates, especially in immunocompromised patients [1, 2]. The main agents causing opportunistic fungal infections are *Candida* spp., *Aspergillus* spp., *Fusarium* spp. and *Cryptococcus* spp [3, 4]. Clinical isolates of *Candida spp.* are responsible for 40-90% of these infections, depending on the group of patients under study [1, 5].

The fast and accurate identification of the microorganism optimizes patient care and therapy [6], benefiting their prognosis [1]. Rapid identification can be done through commercially available methods based on the biochemical characteristics of the strains such as chromogen medium,

biochemical and enzymatic panels and automated identification and fungal characterization systems. The inherent disadvantages to these techniques are the limited database and little sensitivity [3, 6, 7].

The alternative to biochemical and molecular identification is mass spectrometry through matrix-assisted laser desorption ionization and detection in a flight time type analyzer (MALDI-TOF MS) for the routine identification of microorganisms in clinical centers in several parts of the world [3, 6, 8]. This technique provides microbial identification by comparing the spectrum profiles of its cytoplasmic proteins [3, 8]. This technique has some limitations, such as the difficulty in the determination of small or mucoid colonies, in the identification of closely related species and in mixed colonies [9].

In recent years, studies are being developed about the applicability of MALDI-TOF in fungal identification [10, 11, 12]. Most studies address performance and optimization of MALDI-TOF MS for the identification of clinical yeast isolates [13], expansion of the reference library, determination of antifungal susceptibility [14] and biofilm production [15] through MALDI-TOF-MS.

MALDI-TOF MS profiles for different *Candida* species are still restricted Databases about it are not freely accessible. The identification of the spectrum profile can help in the critical evaluation of protein mass fingerprint assisting the analyst in the decision-making processes and in the expansion of reference library. Little data is found about the profile of the spectra to the different species of Candida. The present study aims to determine the protein mass fingerprint of clinical isolates of five species of Candida *(C. albicans, C. tropicalis, C. lusitaniae, C. guilhermondii and C. glabrata)* by MALDI-TOF MS.

Material and methods

Preparation of cultures

In this study were used 12 clinical isolates of *Candida albicans*, eight of *Candida glabrata*, 10 of *Candida lusitaniae*, 10 of *Candida tropicalis* and one of *C. guilhermondii*. The microorganisms came from patients using dental prostheses (Approval by the ethics committee No. 2.236.863, CEP-UFRGS) provided by the Biomicolab Laboratory (Federal University of Rio Grande do Sul's Mycology Laboratory). *Candida* species were isolated and identified with conventional methods using CHROMagar TM Candida Difco® culture medium and only pure colonies were found.

The stock cultures were kept in freezing medium Soy Tripticaseine Broth (TSB; Himedia®) plus 10% glycerol at -18 °C. To reactivate the strains, 10 µL of the stock culture was inoculated into tubes containing Sabouraud dextrose Agar with chloramphenicol (Kasvi®) and incubated at 30 °C for 48 h.

Analysis by MALDI-TOF MS

Clinical isolates of *Candida* species samples were extracted according to the method described by Sauer and collaborators [16]. The spectra were automatically recorded using a MALDI-TOF mass spectrometer (Bruker Daltonics) in the partnership with the UFRGS Biosciences Institute. The spectra were acquired in doubles using the MBT_AutoX method, in a positive linear mode, with a mass range of 2,000 to 20,000 Daltons. Before spectra acquisition, the equipment was calibrated using 1 µL of calibrator (Bruker BTS, Bacterial Test Standard). Acquisition parameters were: accumulating 200 laser shots; nitrogen laser λ : 337 nm; frequency: 60 Hz; ion source 1 voltage: 20 kV; ion source 2 voltage: 18.2 kV; lens voltage: 5.90 kV; minimum laser power required for sample ionization: 30 - 40% maximum power.

The mass spectra obtained were exported to the MALDI Biotyper™ 4.0 Microflex software (Bruker Daltonics). Identifications using BioTyper™ are based on the pattern-matching algorithm, that is, in presence or absence of each peak in the spectrum, which compared the raw spectra (unknown isolate) with the reference main spectral pattern in the database (Bruker library).

The identification of clinical isolates of *Candida* species was performed by log (score) values, which ranged from 0 to 3, used as recommended by the manufacturer: a score ≥2 identifies at species level and a score from 1.7 - 1.9 identifies at genus level.

The analysis of the fingerprint mass spectra of clinical isolates of *Candida* species with a score greater than 2 (precision at the species level) was performed through Principal Component Analysis (PCA).

Principal Component Analysis (PCA)

PCA is a multivariate method that has been used to visualize trends in a data matrix, by reducing the dimensionality of the data set. [17]. In this case spectral peaks of ribosomal protein and *Candida* species. The visualization of the PCs shows trends and explained the differences and similarities between species of *Candida*. The Chemostat® v.2 software was used to do the PCA, without pre-processing the data.

Results and discussion

MALDI-TOF has been used for the routine identification of microorganisms in clinical centers in various parts of the world [3, 6, 8]. It compares the mass spectral fingerprints of predominantly ribosomal proteins belonging to a database, assigning classifications of genus and/or species according to their similarity [16].

Candida species identified with conventional methods were confirmed by MALDI-TOF. All organisms analyzed had spectral scores of ≥2.0, (Table 1) identifying at species level, as recommended by the manufacturer. These isolates consisted of 12 *Candida albicans*, eight *Candida glabrata*, 10

Candida lusitaniae, 10 *Candida tropicalis* and one *C. guilhermondii*.

Analysis of MALDI-TOF MS fingerprints from different clinical isolates of *Candida* showed a consistent spectral profile between species (Figure 1). Most ribosomal protein signals are between 3 and 15 kDa [18]. The signals with the highest intensity are between 3 and 8 kDa, being those with the lowest intensity between 8 and 15 kDa. Similar results have been reported in previous studies, where most of the signs were found are between 3 and 15 kDa. [3, 7].

The two most relevant regions for the identification of most *Candida spp.* species were between 5 to 7 KDa, and the 11 KDa region specifically for the identification of *C. glabrata and C. bracarensis*, as we can see in figure 1. The data corroborate the work of Santos and collaborators [19]. It was also observed the presence of signs characteristic of species such as 4,3 to 4,4 and 5,1 to 5,2 KDa for *C. albicans*, 6,0 to 6,1 KDa for *C. lusitaniae* and 11,0 to 11,1 KDa for *C. glabrata*. Most of these peaks are specific peaks, and some of them are overlapping peaks (Table 2), for example peaks in the range 4,4 to 4,5 KDa are common in C. *tropicalis*, *C. guilhermondii,* C *glabrata,* and *C. lusitaniae*, but they are specific peaks of *C. albicans*.

Although other authors discuss the presence of signs capable of identifying resistance profile [7, 14], and biofilm production capacity [15] through MALDI-TOF MS, data about this is not reported. MALDI-TOF MS has recognized importance in the diagnosis of fungal infections and has great potential for use in scientific research regarding fungal resistance and biofilm production. However, the subject is still little explored. A survey in the Scielo and Pubmed databases resulted in 244 articles, of which the vast majority address the applicability of MALDI-TOF in fungal identification. Only five articles relate to the identification of *Candida spp.* by MALDI-TOF MS and your fingerprint-MS profile.

The principal components analysis (PCA) allows the compilation of data from protein mass fingerprints, showing the most relevant information for each *Candida* species studied [17]. The profile of fingerprints-MS for each species can be seen through the scatterplots PC1 *vs* PC2 and PC3 *vs* PC4. In the first, the separation of *C. glabrata, C. lusitaniae* and *C. albicans*, is observed, which relates to the presence of peaks in the ranges 11,0 to 11,1, 6,6 to 6,7 and 4,3 to 4,4 KDa (Figure 2). In the dispersion graph PC2 *vs* PC3 the separation of *C. guilhermondii* and *C. tropicalis* can be seen. They are co-related to the peaks in the range 3,0 to 3,1, 6.1 to 6.2 and 6.9 to 7.0 KDa (Figure 3).

Table 1. MALDI-TOF spectral scores of clinical isolates of Candida.

Species of *Candida*	Spectral scores
Candida albicans	2.186 - 2.382
Candida tropicalis	2.102 - 2.510
Candida lusitaniae	2.214 - 2.345
Candida guilhermondii	2.014
Candida glabrata	2.309 - 2.412

Table 2: Profile of the protein mass fingerprint of species of *Candida* (*C. albicans*, *C. tropicalis*, *C. lusitaniae*, *C. guilhermondii* e *C. Glabrata*).

C. albicans m/z	*C. tropicalis* m/z	*C. guilhermondii* m/z	*C. glabrata* m/z	*C. lusitaniae* m/z
3.0 - 3.1	3.0 - 3.1			
4.3 - 4.4				
	4.4 - 4.5	4.4-4.5	4.4-4.5	4.4-4.5
5.1 - 5.2				
			5.5 - 5.6	
6.0 - 6.1		6.0 - 6.1		6.0 - 6.1
6.1 - 6.2		6.1 - 6.2		
6.2 -6.3	6.2 - 6.3		6.2 - 6.3	
				6.6 - 6.7
6.9 - 7.0	6.9 - 7.0		6.9 - 7.0	
			11.0 - 11.1	

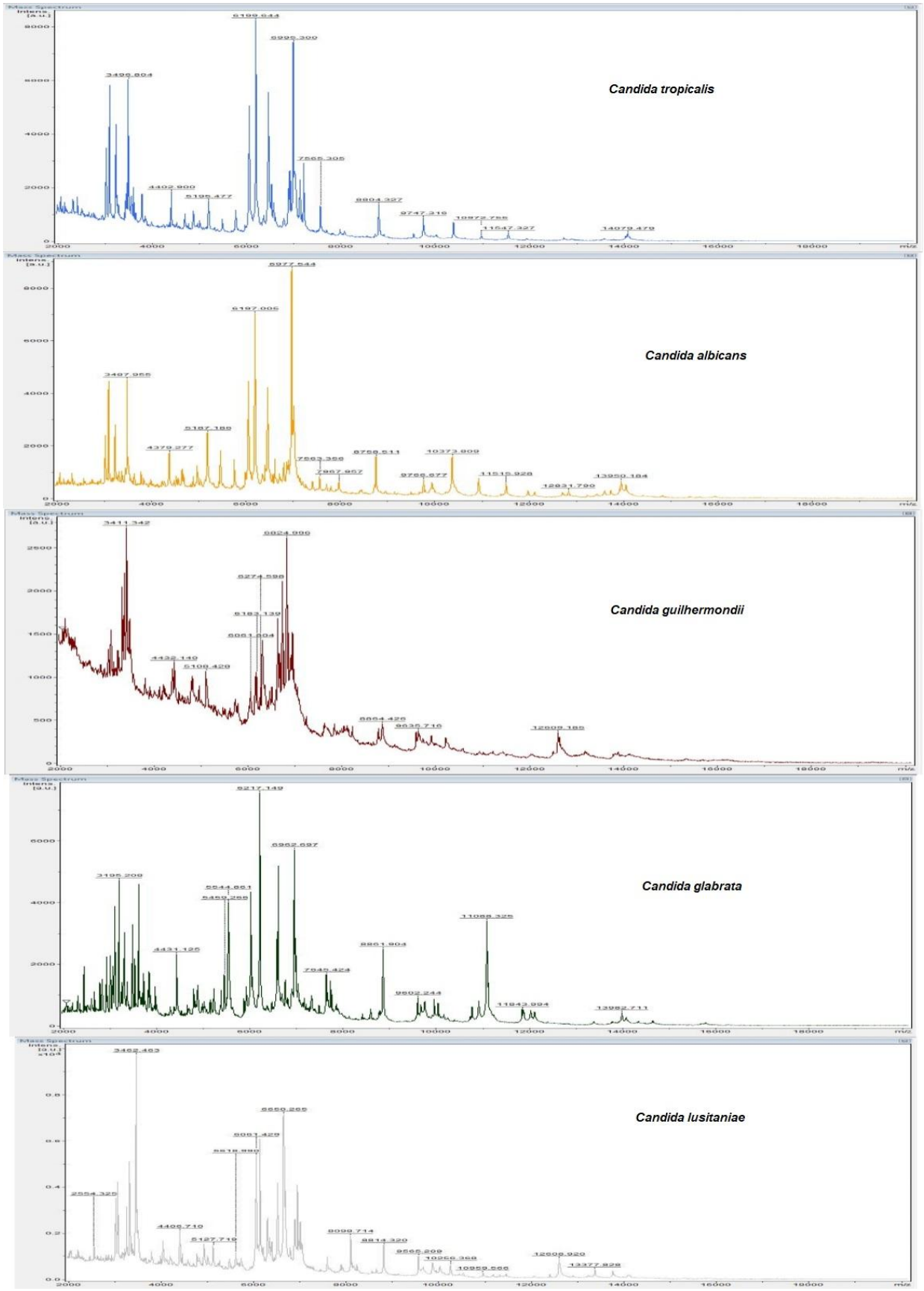
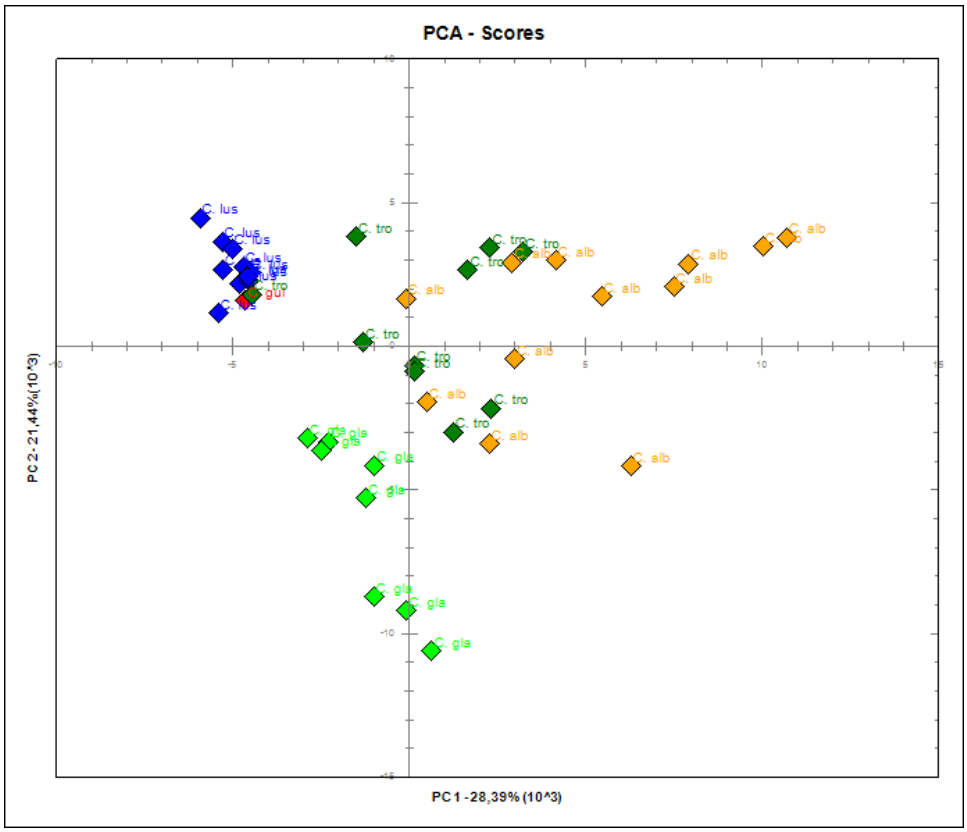
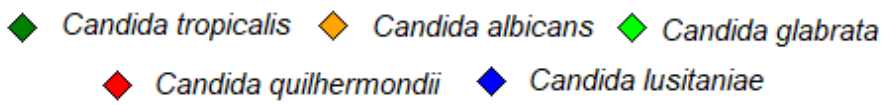
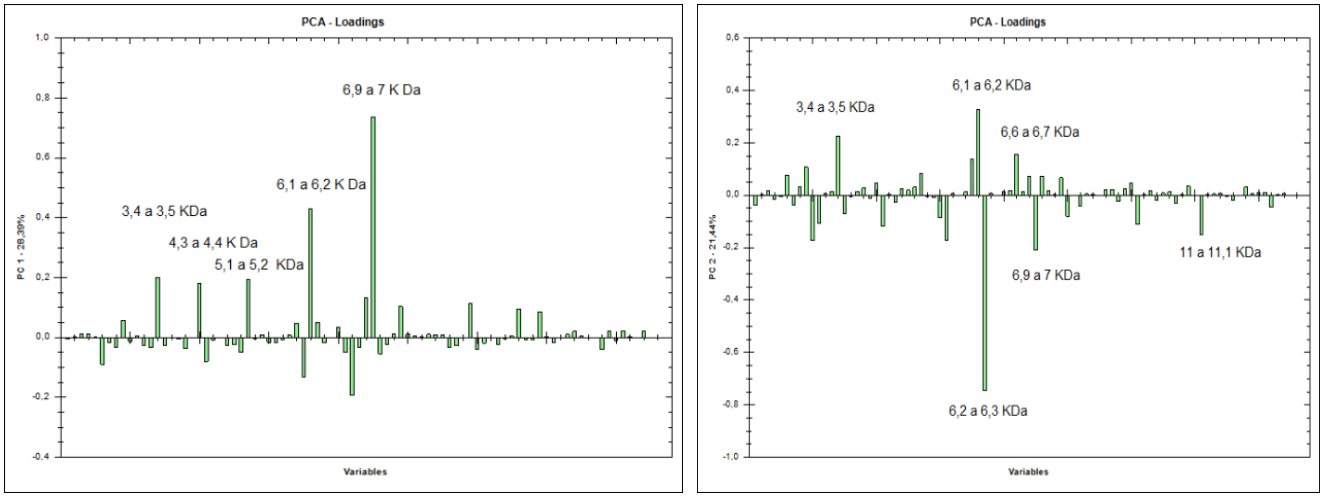


Figure 1. Representative MALDI-TOF mass spectra of clinical isolates of five species of Candida. The m/z values are expressed in Da and the amplitudes are reported in a scale of intensity arbitrary units (a.u.).



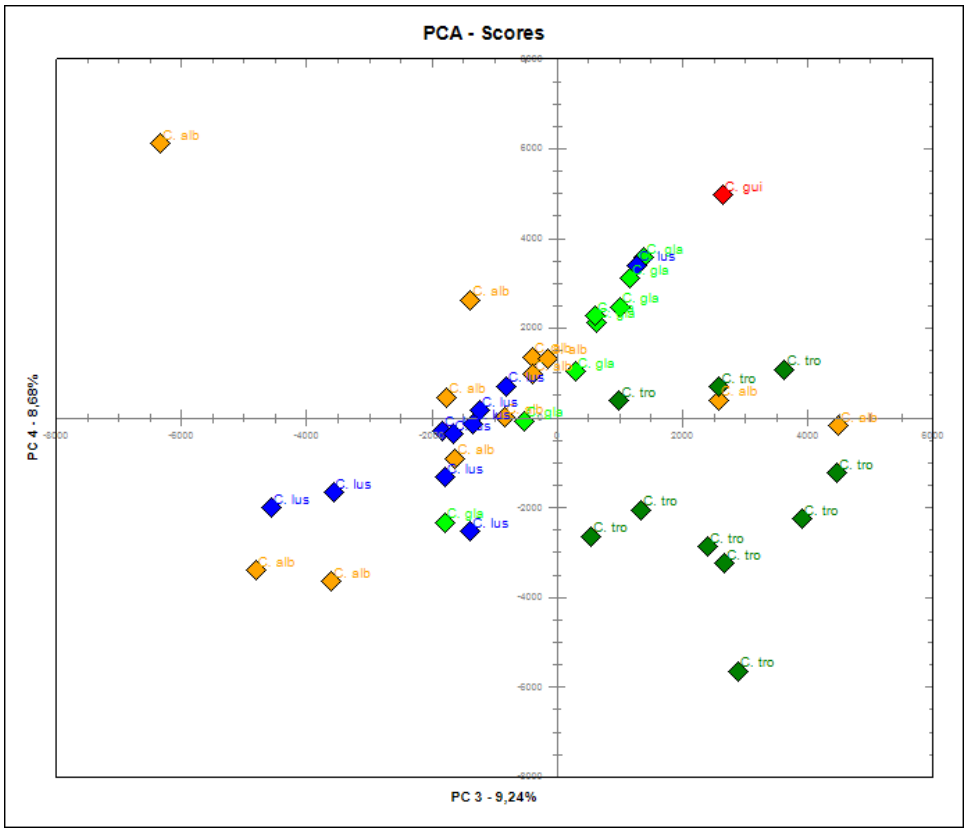
(A)



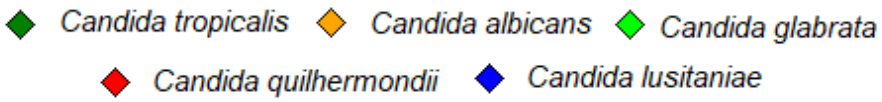


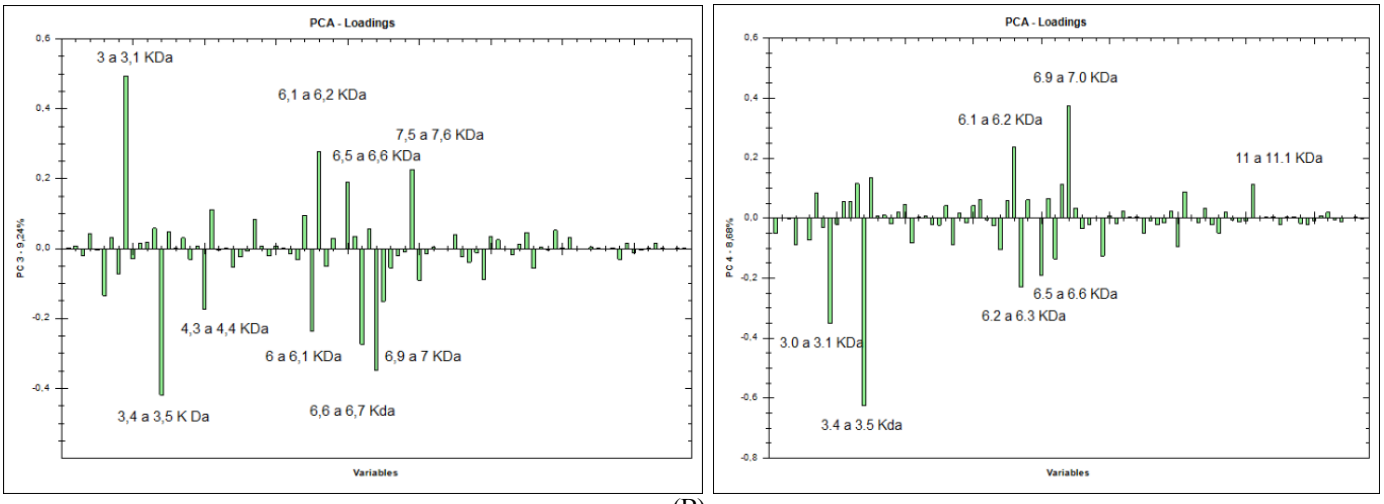
(B)

Figure 2. Scatter Plot PC1 vs. PC2 of fingerprints - MS of *Candida* spp.; (b) loadings. Graphic subtitle: *C. albicans* (C alb), *C. tropicalis* (C. tro), *C. lusitaniae* (C.lus), *C. guilhermondii* (C. gui) e *C. glabrata* (C. gla).



(A)





(B)

Figure 3. Scatter Plot PC3 vs. PC4 of fingerprints - MS of *Candida* spp.; (b) loadings. Graphic subtitle: *C. albicans* (C alb), *C. tropicalis* (C. tro), *C. lusitaniae* (C.lus), *C. guilhermondii* (C. gui) e *C. glabrata* (C. gla).

Conclusion

MALDI TOF-MS proved that it is a rapid and accurate identification method for *Candida* species when compared to traditional methods to identify fungi. There was a consistent spectrum profile among *Candida* species between 3 and 15 KDa. The most intense peaks being between 3 and 8 KDa. *C. albicans* shows two intense signs, the first at 4.3 and 4.4 KDa and the other at 5.1 and 5.2 KDa. The others species show an intense signal in 4.4 to 4.5 KDa. *C. lusitaniae* shows intense signals between 6.0 to 6.1 and 6.6 to 6.7 KDa, C. *guilhermondii* between 6.0 to 6.1 and 6.1 to 6.2 KDa. *C. glabrata* an intense signal in 5.5 to 5.6 KDa and a less intense signal in 11.0 to 11.1 KDa. The identification can help in the critical evaluation of protein mass fingerprint, assisting the analyst in decision-making processes and in the expansion of the reference library.

Acknowledgments

To the Federal University of Rio Grande do Sul – UFRGS, the Foundation of State Research of Rio Grande do SUL - FAPERGS, the National Council for Scientific and the Technological Development Organization - CNPq for financial support. The authors declare that they have no conflicts of interest in the authorship and publication of this article.

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