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# Matrix-assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) for subgingival bacteriome identification in a group of treated periodontitis patients: a case series

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## Abstract

*Periodontitis is a chronic multifactorial polymicrobial infection, characterized by profound modifications of the composition and proportion of the subgingival microbiota. Microbiological laboratory tests are sometimes used in periodontal diagnosis and monitoring of treatment, but both conventional cultivation methods and molecular techniques have some major drawbacks. Therefore, other performant bacterial identification methods must be considered. The aim of the current study was to use Matrix-assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALTI-TOF MS) analysis in association with bacterial culture method to evaluate the modifications of the subgingival bacterial composition in periodontitis patients, before and after cause-related subgingival therapy. Subgingival plaque samples were collected from periodontal pockets before and after subgingival mechanical instrumentation and adjunctive local antimicrobial applications and were cultured in aerobic and anaerobic*

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*conditions. Microbial colonies were further assessed using MALDI-TOF-MS. A total of 36 bacterial strains were isolated from a group of 16 patients. All species from the orange complex were identified by MALDI-TOF MS. A marked reduction of detection frequency was observed in most bacterial strains, including the orange complex after cause-related periodontal treatment. The results of this study indicate that MALDI-TOF MS could be considered an accurate method for oral microbial identification and the cause-related periodontal treatment is useful for reducing the microbial burden.*

***Keywords**: periodontitis, MALDI-TOF MS, bacteriome*

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## Introduction

Periodontitis - a chronic multifactorial polymicrobial infection - is one of the most important causes of tooth loss and has raised concern for its potential to trigger or aggravate various systemic diseases (1,2).

Roughly 20-30 predominant species from 1,000 microbial species of the oral microbiome can be isolated from each sampling site (mucosa or dental plaque) (3). Therefore, periodontitis is associated with profound modifications of the composition and proportion of the subgingival bacterial fingerprint (4), mostly represented by Gram-negative bacterial species such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Prevotella intermedia,* and *Fusobacterium nucleatum* (5,6). However, other bacteria are currently being evaluated for their potential link to periodontitis (7).

Cause-related periodontitis treatment firstly aims to control subgingival biofilm in order to reduce the excessive bacterial load and the proportion of periodontopathogens through supragingival and subgingival mechanical instrumentation, eventually associated with locally applied adjunctive antimicrobials (8).

In periodontology, molecular techniques have been validated against conventional cultivation methods and could successfully replace them as gold standard in laboratory microbiological diagnosis of progressive periodontitis (9). However, nucleic acid-based analysis as Real Time Polymerase Chain Reaction (RT-PCR) is associated with a major drawback as the requirement to select the appropriate targets in advance (10,11). Matrix-assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) has emerged as a rapid and highly sensitive identification method for microbes in clinical laboratory. MALDI-TOF MS relies on a so-called "soft ionization" technique, using a laser to gently ionize target molecules of the sample, typically mixed with a matrix (analytes) and generating a gas-phase containing the spectrum of components. Ionized proteins from the bacterial colonies are subjected to mass spectrometry analysis to provide information on the specimen composition based on a spectrum of mass-to-charge (m/z) ratio calculations (10). The latter are electrodynamic measurements of the rapidity with which the charged ions travel from bacterial colonies through the time of flight (TOF) tube to reach a detector. Generated spectra are compared to the spectra of reference microorganisms collected in a database (10).

The aim of this study is to use MALDI-TOF MS identification method in association with bacterial culture to show the subgingival bacterial composition in periodontitis patients, before and after cause-related subgingival therapy.

## Materials and methods

### *Study design and population*

This study was carried out in the Periodontology Department of Iuliu Haţieganu University, Cluj-Napoca, Romania. A group of periodontitis

patients were treated by supragingival and subgingival mechanical instrumentation and adjunctive local antimicrobial applications. From each patient, subgingival plaque samples were harvested from five periodontal pockets before and after periodontal treatment. The samples were cultured in aerobic and anaerobic conditions and microbial colonies were further identified using MALDI-TOF-MS. Data analysis reported the microbiota of the two measured points in time.

The study was conducted in accordance with the protocol approved by the Ethical Board of the University (80/1.02.2018) and the Emergency Clinical Cluj County Hospital (10539/B/4.05.2018) and with European and Declaration of Helsinki regulations. All patients were informed on the research protocol and details, and they were asked to provide a signed informed consent.

The study group comprised 16 patients aged between 29-62 years with non-treated moderate and severe periodontitis, with a number of at least 10 present teeth and at least five periodontal pockets ≥ 5 mm. The exclusion criteria applied were as follows: mild periodontal destructions, previous periodontal treatment, antibiotic treatment in past 6 months, or immunosuppressant therapy. Data were collected between June 2018 and February 2020.

***Periodontal evaluation and treatment***

Full-mouth periodontal examinations were performed using standard methodology and equipment. For the diagnosis of periodontitis and the severity involvement, the 2012 CDC/AAP case definition was used based on clinical attachment loss (CAL) and probing depth (PD) measurements.

Each patient received supragingival and subgingival instrumentation associated with subgingival antimicrobial applications of a sulphonic/sulphuric acid-based product (HYBENX, Epien Medical, Minnesota, USA), and oral hygiene instructions. Patients were instructed to avoid antibiotic therapy for 6 weeks after treatment.

***Sample collection and cultivation***

The subgingival plaque samples were collected from the five deepest pockets before and at 6 weeks after treatment, using ISO 70 sterile paper cones (Marked Paper Cones 070/Green Series 2, Dr. Mayer Life & Health) inserted into the pockets for 60 seconds, placed in a single 13 ml sterile tube with Schaedler Broth+vitamin K3 (bioMerieux, Chemin del´Orme, France), and immediately transported at the Microbiology Laboratory of the University Hospital for Infectious Diseases, Cluj Napoca, Romania.

A volume of 100 µL from the samples was cultured: two Petri plates with Columbia agar with 5% sheep blood (bioMerieux Chemin de l´Orme-France) and one Petri plate with Chocolate agar PolyViteX (bioMerieux Chemin de l´Orme-France). One Columbia agar plate and Chocolate agar plate were incubated for 48 hours in an incubator 37ºC with 5% CO 2 (Sanyo Incubator, Osaka, Japan). Another Columbia agar plate was cultured for 7 days at 37ºC under anaerobic conditions using AnaeroGen Compact (Thermo-Scientific Oxoid Ltd, Wadw Road, Basingstoke, Hants RG24 8PW). During this period, in order to purify different bacterial strains based on colony morphology passage cultivation processes were repeated to obtain single colonies.

***MALDI-TOF MS***

MALDI-TOF MS Microflex LT/SH MALDI Biotyper (Bruker Daltonics GmbH & Co. KG Bremen Germany) was used to identify cultured bacterial strains. All organisms in all different morphotypes were identified. An isolated single colony was transferred with a toothpick onto a reusable barcoded polished steel MALDI target plate with 96 places covered with 1 µl matrix solution (50% acetonitrile, 1% α-cyano-4-hydroxycinnamic acid, and 2.5% trifluoro-

acetic acid). The assembly was allowed to dry at room temperature.

Acquisition of the spectral data has been completed, using a Microflex LT/SH MALDI Biotyper 3.1 database ® (MBT) (Bruker Daltonics GmbH & Co. KG Bremen Germany). The identification cut-off values higher than 2 and 1.7 were used for species and genus identification, respectively.

***Statistical analysis***

Our interpretation of the microbiological results took into consideration all the bacterial species isolated from all patients and provided by MALDI-TOF MS. The identified species were classified according to bacterial taxonomy.

The first part of the statistical analysis assessed the number of species present in each individual, before and after therapy. Depending on the presence or absence of a certain species, each patient was assigned a 1 / 0 code. In particular, the evolution of the orange complex bacteria (*Prevotella intermedia, Parvimonas micra, Campylobacter rectus, Fusobacterium nucleatum*) was recorded for our group of patients: the presence or the absence of the orange complex was assessed for each patient and all presences (code 1) were summed for the entire group, before and after treatment.

Further on, we established an algorithm to calculate the microbiota evolution index (favourable if samples resulted negative to the presence of bacteria/ unfavourable if positivity for certain bacteria was recorded). Thus, for each patient, a total of favourable and unfavourable codes was calculated, and an individual evolution index was generated. The evolution index of the group was eventually calculated based on an age-adjusted cut-off value of 46 years (average age) and on an evolution index value of 5.

Correlation between age and score index was assessed using the Pearson methodology. Differences between percentages were evaluated by $\chi^2$ test. The p-values <0.05 were considered significant.

Statistical analysis was performed using the MedCalc® **Statistical Software version 19.7.1 (**MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021).

## Results

The study group consisted of 9 males and 7 females, with a mean age of 46 years (minimum = 29, maximum = 62). A total of 36 microbial species from 9 families were identified. All the species of the orange complex were identified, but none of the species belonging to the red complex (*Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola*) could be cultured. Distribution per species and per families of the study group, before and after treatment, is presented in figure 1. In most bacteria, initial detection exceeded post-treatment rates.

Figure 2 shows the evolution index of the study group microbiota across age groups. A decreasing trend of the evolution index is recorded: increased patient age is associated with a lower evolution index score, which translates into less persistent post-therapeutic species (r = 0.35).

The total sum of positive detections (code 1) of every species of the orange complex per patient was used to generate the evolution of the orange complex in the study group following the therapy (Table 1). There was a decrease in the detection of members of this community in the study group (p = 0.02) after treatment.

## Discussions

Within this group of periodontitis patients, a total of 36 bacterial strains were identified by MALDI-TOF MS. A general reduction of detection frequency of most bacterial strains including those belonging to the orange complex was observed, which is found to be in agreement with the gener-

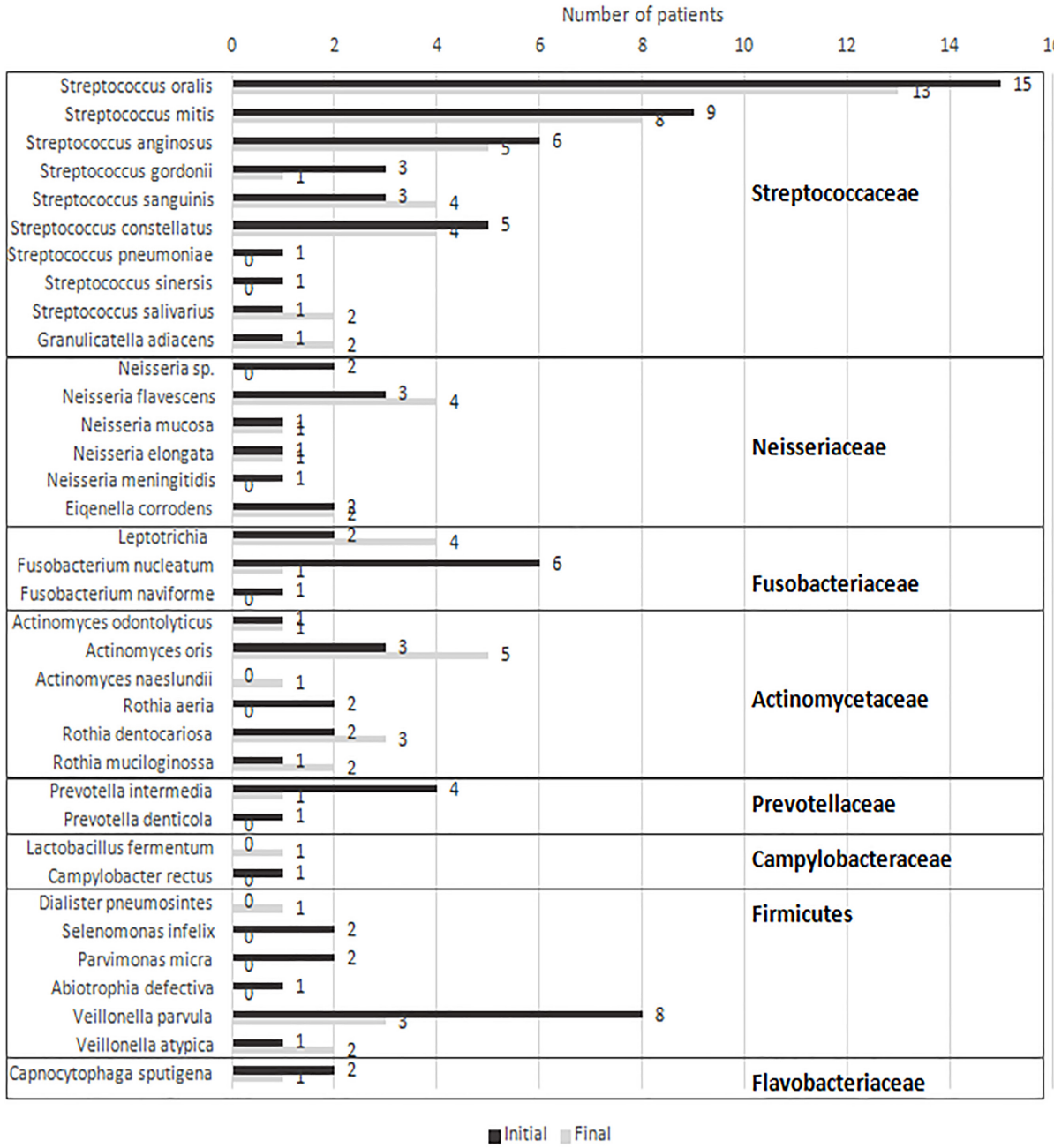


**Fig. 1. Distribution of the subgingival bacteria identified in the study group before and after treatment**

al findings of cause-related therapy in periodontitis, reporting a significant decrease of subgingival bacterial load and qualitative changes of microbiota (8). The orange complex precedes and allows the colonization of the red complex bacteria associated with clinical attachment loss (6).

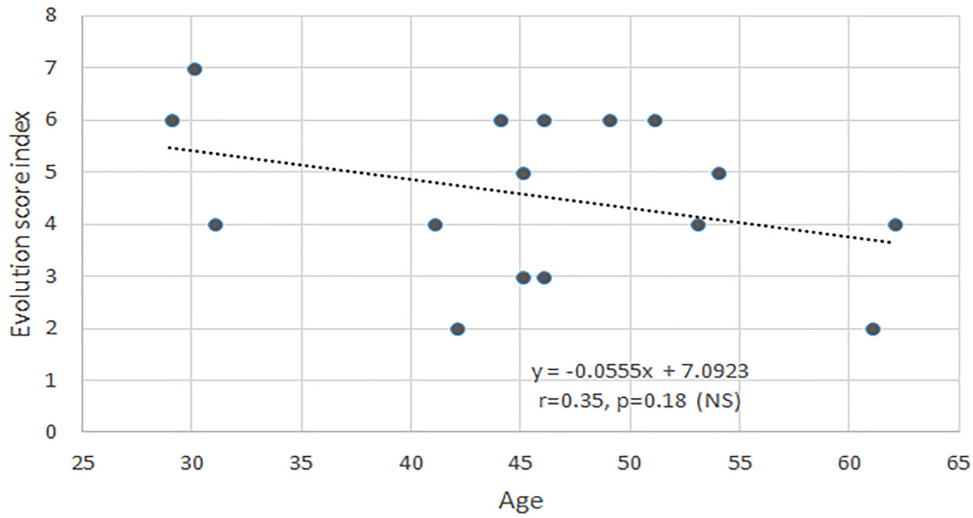


**Fig. 2. Evolution index trend of microbiota in relation with the age of patients**

**Table 1. The evolution of the orange complex in the study group**

| | Frequency of detection | | |
|---|---|---|---|
| **Bacterial complex** | **Before treatment** | **After treatment** | |
| Orange | 12 | 2 | p=0.02 |
| non Orange | 83 | 71 | |
| Total | 95 | 73 | |

In our study group, deep pockets contained *Fusobacterium nucleatum,* as well as, *Prevotella intermedia, Eikenella corrodens, Streptococcus constellatus*, but a low frequency of detection was recorded. These results are in agreement with other studies although they identified these strains more constantly (9). Even if *Fusobacterium nucleatum* abundantly colonizes the oral cavity of both healthy and diseased individuals, the relatively low frequency of detection of this opportunistic commensal in the present study may be due to difficulty of cultivation. This bacterium is known to be easily missed in routine culture performed in hospital laboratories (12). The prevalence of *Fusobacterium nucleatum* increases with the severity of the periodontal status, being closely linked to the progression of inflammation and pocket depth (13).

Some members of *Firmicutes* domain were identified by the present study mostly isolated after periodontitis treatment. Some members of this family were involved in the development of periodontitis (7), but others were well-recognized commensals, which could explain our findings.

Our results indicate that MALDI-TOF MS is a highly accurate method recommended for microbial identification. One of the advantages of using MALDI-TOF MS is that it allows the exploration and analysis of new microbial strains that were previously unknown for certain diseases. More particularly, the periodontopathogenic role of strains such as *Campylobacter rectus*, *Eikenella corrodens,* and *Sreptococcus constellatus*, isolated in the present study, is less documented (9). Notwithstanding its advantages, MALDI-TOF MS depends on culture approach-

es which are time-consuming and particularly labour-intensive, as is the case with anaerobic strains, which are difficult to maintain during sample collection and transportation (9). This could be a possible explanation for the fact that culture methods were shown to be less reliable than commercial molecular kits in detecting *Porphyromonas gingivalis* positive samples (9). However, results of different studies reporting data on oral microbiome identification using MALDI-TOF MS seem rather heterogenous (11). In clinical practice for periodontal purposes, MALDI-TOF MS remains a highly efficient perspective, although the available multiplex-PCR microbial tests such as DMDxA test (Omnigene, Cambridge, MA, USA) or micro-DentA kit (Hain Diagnostika Ltd., Nehren, Germany) offer a reasonable approach, in spite of their ability to only address a limited number of bacteria. In this context, a possible limitation of the present study would be the fact that it does not provide a validation of MALDI-TOF MS against a commercial multiplex-PCR approach. Our laboratory was not authorized to access such tests, therefore, the perspective of referring the samples to another laboratory having this privilege would have affected the research approach. Another limitation of this study is the lack of microbiota quantification.

Further MALDI-TOF MS - based microbial identification should be performed to provide relevant information on subgingival microbiota changes consecutive to various periodontal treatments.

## Conclusions

Using sample cultures and MALDI-TOF MS identification, this study showed the subgingival microbiota after cause-related periodontal treatment. MALDI-TOF MS could be an accurate method for bacterial identification provided that the culture approaches manage to highlight the entire bacterial spectrum of the sample, but more studies with a higher number of patients are required.

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## Authors' contributions

Conceptualization: IL, MF, AR, AS; Methodology: MF, AR, EP, AS; Validation: IL, MF, AR, DMP, AS; Formal analysis: IL, AS; Investigation: IL, MF, AR, ICM, EP, AS; Resources: IL, AC, ICM, EP; AS; Data Curation: IL, AC, ICM, AR; Writing – original draft preparation: IL, AR, AS; Writing – review and editing: IL, AR, DMP, AS; Visualization: IL, AC, ICM, DMP; Supervision: MF, AR, AS; Project administration: IL, MF, AR, EP, AS; Funding acquisition: IL, AR. All authors have read and agreed to the published version of the manuscript.

## Conflicts of interest

The authors have no conflicts of interest to declare.

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