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Reliability of MALDI-TOF Mass Spectrometry to identify oral isolates of *Streptococcus salivarius* and *Lactobacillus* spp.

François Jussiaux [a], Cécile Miot-Sertier [b], Darrène Nguyen-Lopez [c], Cécile Badet [a,b,c], Johan Samot [a,b,c]

[a] Univ. Bordeaux, UFR des Sciences Odontologiques, Bordeaux, France

[b] Univ. Bordeaux, ISVV, EA 4577, Unité de recherche Œnologie, USC 1366 INRAE, F-33140 Villenave d'Ornon, France.

[c] Centre hospitalier universitaire de Bordeaux, pôle de médecine et chirurgie bucco-dentaire, Bordeaux, France

Running title: MALDI-TOF for identification of oral isolates

Cécile Badet ORCID: 0000-0003-3518-8608

Johan Samot ORCID: 0000-0002-6087-2375

Corresponding author: Johan SAMOT

johan.samot@u-bordeaux.fr

Abstract

Objective: The aim of this study is to evaluate the performance of MALDI-TOF mass spectrometry in identifying bacteria isolated in the oral cavity known to be of probiotic interest.

Design: We evaluated Bruker MALDI Biotyper for the identification of 92 clinical oral isolates of probiotic interest (31 *Streptococcus salivarius* and 61 *Lactobacillus* spp.) by comparing direct colony method with on-plate formic acid extraction. Isolates were previously identified by use of biochemical methods and molecular biology.

Results: Using the manufacturer's suggested genus and species level cutoff scores, the direct colony method identified 42 (45.7%) isolates at the genus level and 35 (38%) at the species level while the on-plate extraction method correctly identified 90 (97.8%) isolates at the genus level and 82 (89.1%) at the species level. The difference between the two methods was statistically significant at the genus and species levels ($P \leq 0.0001$). After dividing the isolates into two subgroups, the analysis was repeated. The direct colony method identified correctly all isolates of *Streptococcus salivarius* at the species level. In contrast, the direct colony method allowed the identification of only 11 (18%) lactobacilli at the genus level and 4 (6.6%) at the species level. The on-plate extraction method was statistically ($P \leq 0.0001$) more efficient since 59 (96.7%) lactobacilli were identified at the genus level and 51 (83.6%) at the species level.

Conclusions: MALDI Biotyper can efficiently identify *Streptococcus salivarius* regardless of the preparative method but on-plate extraction is superior to direct colony method for the identification of lactobacilli.

Keywords: Bacteria; Probiotics; Mass spectrometry; MALDI-TOF; Oral cavity

Introduction

The identification of clinical isolates from polymicrobial flora is a lengthy procedure that usually involves several techniques such as GRAM staining, biochemical methods and molecular biology. In recent decades, identification by mass spectrometry has provided new solutions. Indeed, since the first descriptions of bacterial identification by mass spectrometry in the 1970s, the performance of these methods have constantly evolved to allow, through the constitution of increasingly precise spectral databases of proteins, a more sensitive identification of microorganisms (Anhalt & Fenselau, 1975; Lay, 2001; Meuzelaar & Kistemaker, 1973; Simmonds, 1970). Currently, using a soft ionization principle, Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry (MS) has become a gold standard for microbial identification in clinical microbiology laboratories (Sauer & Kliem, 2010; Schubert & Kostrzewa, 2017; van Belkum et al., 2017). The four present commercial MALDI-TOF systems (MALDI Biotyper, Saramis, Andromas and Vitek MS) can identify a wide range of microorganisms through the analysis of unique "fingerprints" of abundant proteins from whole cells or cell extracts (Angeletti, 2017; Khot et al., 2012). MALDI-TOF mass spectrometry has been described as a fast, cost-effective and accurate method for the identification of bacteria (Seng et al., 2009) and can therefore be particularly useful for the study of oral ones. The human oral cavity is a complex ecosystem in which more than 700 species of bacteria live together in a fragile balance. Modern lifestyles, age, nutritional status and general state of health are all factors that can cause disturbances of the oral microbiome leading to dysbiosis, which can have harmful consequences on oral health (candidiasis, caries, periodontitis...). Because of the interplay between oral pathologies and numerous systemic disorders, it is essential to avoid the development and progression of oral diseases, especially infectious ones (Dorfer et al., 2017). With the limited progress in the discovery of new antibiotics and the increase in emerging

resistant pathogenic bacteria, it has become imperative to try new approaches such as biotherapy. The cornerstone of this biotherapy strategy is currently the use of beneficial microbes, commonly referred to as probiotics. According to little but increasing number of randomized double-blind clinical studies, beneficial bacteria may play a role in maintaining oral health through their interaction with the oral microbiome, thereby contributing to a healthy microbial equilibrium and preventing oral infectious conditions (Bustamante et al., 2019; Gruner et al., 2016; Nadelman et al., 2018). The persistence in the oral cavity of these beneficial bacteria is therefore essential and like other authors, we believe that an adequate strategy is to use microorganisms directly isolated from their natural oral habitat as oral probiotics (Samot et al., 2017; Samot et al., 2011; Strahinic et al., 2007; Wescombe et al., 2012). However, prior to any clinical use, the selection and evaluation of potential probiotic candidates requires a multi-step approach starting with precise strain identification (FAO/WHO, 2002). Our aim was to evaluate the performance of MALDI-TOF mass spectrometry in identifying bacteria isolated in the oral cavity known to be of probiotic interest. In this study, we evaluated the Bruker Maldi Biotyper for identification of clinically isolates *Streptococcus salivarius* and lactobacilli using or not on-plate formic acid preparation.

Materiels and Methods

Bacterial isolates

Thirty-one oral strains of *Streptococcus salivarius* were isolated among children before and after dental care under general anesthesia. The strains were identified using both biochemical (API20Strep, BioMerieux, Marcy l'Etoile, France) and PCR method. The primers used were Ssa442F (5'- AACGTTGACCTTACGCTAGC-3') and Ssa2712R (5'- GATTCTGTCAAAGAAGCC-3') targeting the dextranase gene of *Streptococcus salivarius* (Igarashi et al., 2001). Sixty-one lactobacilli strains, isolated from human salivary samples and previously identified, were used in this study (Román-Méndez et al., 2009; Samot et al., 2017). Sequencing of the 16S-rDNA fragment was performed for all strains using the primers FD1 et RD1 according to Weisburg et al.(Weisburg et al., 1991). *Streptococci* were cultured in Trypticase Soy broth (Oxoid, Dardilly, France), and *Lactobacilli* were cultured in Man, Rogosa and Sharpe Medium (Fischer Scientific, Illkirch, France) at 37°C. All strains were cryo-preserved at -80°C.

DNA extraction

DNA was isolated from each bacterial strain by using GenElute Bacterial Genomic DNA kit (Sigma Aldrich, St Quentin Fallavier, France) according to the manufacturer's supplied protocol.

Mass spectrometry identification

To obtain fresh bacteria, isolates were cultured overnight or until visible growth was observed on Trypticase Soy agar (Oxoid, Dardilly, France) for Streptococci or on Man, Rogosa and Sharpe Agar (Fischer Scientific, Illkirch, France) for Lactobacilli. A colony of each isolate, taken directly from the agar plate, was deposited on a polished steel target (Bruker Daltonics, Bremen, Germany) in a single spot and allowed to dry at room temperature. One microliter of

matrix solution (saturated solution of a cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile) was added to the sample and was then crystallized by air-drying at room temperature for 5 min. For the direct transfer-formic acid method, 1µl of 70% formic acid (Sigma-Aldrich) was added to the bacterial spot and allowed to air dry, before the matrix solution was added. The samples prepared by each method were applied to a MicroFlex LT mass spectrometer (Bruker Daltonik), and the results were analyzed using the database (containing 6,903 reference spectra of 2,641 microbial species at the time of the study) associated with MALDI Biotyper 3.1 software (Bruker Daltonik). Each measurement was performed only once for each culture and each preparation. Escherichia coli DH5α was used as a quality control as recommended by the manufacturer on each experiment.

Data analysis

According to the manufacturer's recommended score identification: a score of ≥2.0 indicated a species-level identification; ≥1.7 but <2.0, a genus-level identification and an isolate with a score <1.7 is considered to be unidentified. Discordant results between MALDI-TOF MS and molecular identification were categorized as misidentifications. The rates for different criteria (genus-level, species-level, unreliable identification, misidentifications) were calculated by the number of different criteria divided by the total number of isolates.

Statistical analysis

Comparisons of genus- or species-level identification using on-plate extraction versus direct colony methods were made by using McNemar's test of paired proportions. P values of less than 0.05 were considered statistically significant. All analyses were performed by using R 3.6.3 (R Core Team, 2020).

Results

Ninety-two isolates were included in this study. Molecular identifications were considered as the gold standard. Both the direct colony method and the on-plate extraction method were performed from a colony growing on one of the isolate preferred solid culture media. The "score" (ranging from 0.00-to-3.00) provided by the Biotyper database determined the level of identification for each isolate. For the direct colony method, results for each strain are shown in Table 1. The direct colony method identified 42 (45.7%) isolates at the genus level and 35 (38%) isolates at the species level. Using the on-plate extraction method, the Bruker Biotyper correctly identified 90 (97.8%) isolates at the genus level and 82 (89.1%) isolates at the species level (Table 2). The difference between the two methods was statistically significant at the genus and species levels ($P \leq 0.0001$). No isolate returned without identification after treatment with 70% formic acid (on-plate extraction) while with the direct colony method, 53.4% of the isolates remained unidentified. This positive evolution of the rate of unidentified isolates with the on-plate extraction method is to be modulated with the number of misidentified strains. Indeed, the number of misidentified strains increased from 7.6% (for the direct colony method) to 10.9% (for the on-plate extraction method). Surprisingly, some misidentifications were at the genus level (2 enterococci detected instead of 2 lactobacilli) with the on-plate extraction method while all misidentifications with the direct colony method were at the species level. The direct colony method was sufficiently conclusive to identify correctly all isolates of *Streptococcus salivarius* at the species level (Figure 1). The direct colony method allowed the identification of only 11 (18%) lactobacilli at the genus level and 4 (6.6%) at the species level. The on-plate extraction method was statistically ($P \leq 0.0001$) more efficient since 59 (96.7%) lactobacilli were identified at the genus level and 51 (83.6%) at the species level. Overall, MALDI-TOF mass spectrometry was less accurate for the identification of lactobacilli up to the precision of the species level.

Discussion

Lactobacilli as *Streptococcus salivarius* belong to the phylum Firmicutes, class Bacilli and order Lactobacillales. Bacteria belonging to this order are more commonly known as lactic acid bacteria (LAB). Lactic acid bacteria have a prominent place among bacteria of probiotic interest but because of their genetic closeness, it is sometimes complicated to identify them quickly and accurately (Argyri et al., 2013; Garcia et al., 2016). The purpose of our study was to estimate the value of MALDI-TOF mass spectrometry for the rapid identification of certain oral lactic acid bacteria. Our findings show that the method of sample preparation has a significant impact on the accuracy of strain identification. These results are consistent with other previous studies that have shown that a simple formic acid layer on the colony would be sufficient to increase the identification rate (Hsu & Burnham, 2014; Schulthess et al., 2013). Formic acid, by removing salt residues and some additives from the media, would allow obtaining clearer mass spectra, free of certain adducts (Lay, 2000; Walker et al., 1995). This aspect of the formic acid treatment is very interesting because it would make it possible to counterbalance the variations in the spectral fingerprint that a given strain may present depending on the agar culture medium on which it is grown. According to some previous studies, the complete extraction of proteins (a more laborious process) seems to offer only minimal gains and is above all not compatible with rapid routine identification methods (Barcelos et al., 2019; Schulthess et al., 2013).

Our results also show that the performance of Bruker MALDI Biotyper also depends on the species we are trying to identify. The strains of *Streptococccus salivarius* were all correctly identified even with the direct colony method. This excellent identification rate of *S. salivarius* was also reported by the review by Fan et al (Fan et al., 2017). In contrast, even with the on-plate formic acid extraction method, the MALDI biotyper had more difficulty in identifying lactobacilli species accurately, even leading to misidentification at the genus level

(*Enterococcus*) for two poorly described species belonging to the *L. buchneri* phylogroup (*L. farraginis* and *L. parafarriginis*). Another strain (*L. kefiri*) belonging to this phylogroup was also misidentified (*L. fermentum*). In view of the small number of representatives of certain lactobacilli species in this study, no general statistical data on the identification of lactobacilli by Bruker MALDI biotyper can be derived. Nevertheless, it can be observed that the MALDI biotyper tends to slightly generate a poor identification of lactobacilli belonging to the same phylogenetic group. This is particularly the case here for lactobacilli of the *L. casei* group (*L. casei*, *L. paracasei*, *L. rhamnosus*, *L. zeae*) which accounts for more than a third (4/10) of misidentifications. This discordance in the identification of casei group lactobacilli by MALDI-TOF MS has already been documented, and has been explained as probably related to the fact that the four bacteria mentioned above cannot be distinguished by classical phenotypic tests (Garcia et al., 2016). This tendency to discriminate less well between species of the same phylogenetic group seems to be confirmed by the misidentification of *L. vaginalis* as *L. fermentum* (*L. reuteri* group). In this study, however, two results nuance this hypothesis; the above-mentioned misidentification of *L. kefiri* in *L. fermentum* and one of the four *Lactobacillus gasseri* (*L. delbrueckii* group) was misidentified as *L. rhamnosus* (*L. casei* group). The lower performance in the identification of lactobacilli can be explained by the complexity of this genus, which is regularly subject to taxonomic updates (Huang et al., 2020; Salvetti et al., 2018; Zheng et al., 2020). To allow better identification of lactobacilli, an improvement in the quality and richness of information of the protein spectra in Bruker Biotyper database seems essential. An alternative solution for microbiology laboratories remains the possibility of developing in-house databases, which can have the advantage of taking into account variations in the bacterial proteome depending on the culture medium used, and can even be adapted to identify antibiotic resistances (Bove et al., 2012; Huang et al., 2018; Oviano & Bou, 2019).

Conclusion

In summary, our data illustrate that the Bruker MALDI Biotyper can efficiently identify *Streptococcus salivarius* regardless of the preparative method but that on-plate formic acid extraction is superior to direct colony testing for the identification of lactobacilli. The addition of protein spectra to the database to reflect the diversity of closely related species may further improve identification.

Declaration of competing interest

The authors declare no conflict of interest.

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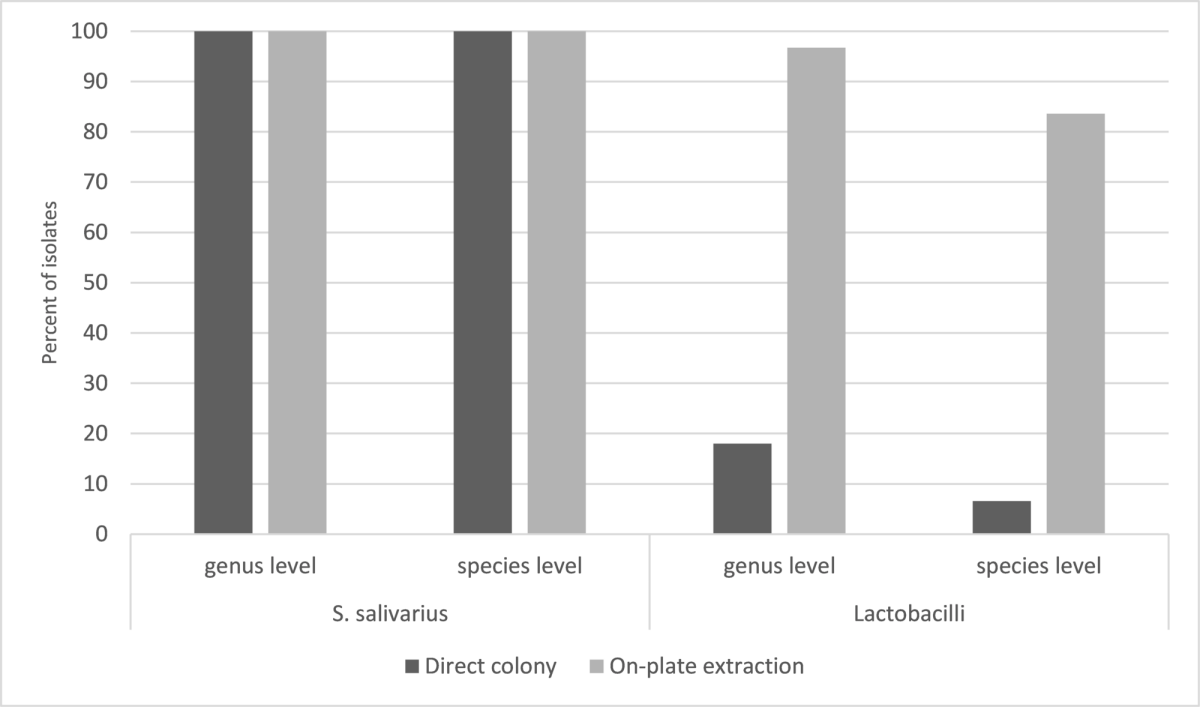
Figure legend

Figure 1. Percent identification of *Streptococcus salivarius* and lactobacilli to the genus and species levels by the Bruker MALDI Biotyper system.


100
90
80
70
60
50
40
30
20
10
0
Percent of isolates
genus level
species level
genus level
species level
S. salivarius
Lactobacilli
Direct colony
On-plate extraction

Oral isolates tested	Total number of isolates	Number of isolates			
		correctly identified to each level		Not identified	Misidentified (misidentification)
		genus	species		
Streptococcus salivarius	31	31	31	0	0
Lactobacillus rhamnosus	47	3	2	44	1 (*Lactobacillus gasseri*)
Lactobacillus casei	4	4	0	0	4 (2 *Lactobacillus rhamnosus*, 2 *Lactobacillus paracasei*)
Lactobacillus gasseri	4	1	1	3	0
Lactobacillus farraginis	1	0	0	1	0
Lactobacillus fermentum	1	1	1	0	0
Lactobacillus kefiri	1	1	0	0	1 (*Lactobacillus fermentum*)
Lactobacillus parafarraginis	1	0	0	1	0
Lactobacillus plantarum	1	0	0	1	0
Lactobacillus vaginalis	1	1	0	0	1 (*Lactobacillus fermentum*)
Total	92	42	35	50	7 (all at the species level)
% identification		45,7	38	54,3	7,6

Table 1. MALDI Biotyper identification of isolates of *Streptococcus salivarius* and *Lactobacillus* spp. to the genus and species levels by use of direct colony method

Oral isolates tested	Total number of isolates	Number of isolates			
		correctly identified to each level		Not identified	Misidentified (misidentification)
		genus	species		
Streptococcus salivarius	31	31	31	0	0
Lactobacillus rhamnosus	47	47	46	0	1 (*Lactobacillus zeae*)
Lactobacillus casei	4	4	0	0	4 (2 *Lactobacillus rhamnosus*, 2 *Lactobacillus paracasei*)
Lactobacillus gasseri	4	4	3	0	1 (*Lactobacillus rhamnosus*)
Lactobacillus farraginis	1	0	0	0	1 (*Enterococcus faecium*)
Lactobacillus fermentum	1	1	1	0	0
Lactobacillus kefiri	1	1	0	0	1 (*Lactobacillus fermentum*)
Lactobacillus parafarraginis	1	0	0	0	1 (*Enterococcus faecium*)
Lactobacillus plantarum	1	1	1	0	0
Lactobacillus vaginalis	1	1	0	0	1 (*Lactobacillus fermentum*)
Total	92	90	82	0	10 (8 at the species level, 2 at the genus level)
% identification		97,8	89,1	0	10,9

Table 2. MALDI Biotyper identification of isolates of *Streptococcus salivarius* and *Lactobacillus* spp. to the genus and species levels by use of on-plate formic acid extraction