ISSN: 2279-9761
Working paper of public health [Online]

# Identification of culturable vaginal *Lactobacillus* species by means of MALDI-TOF MS: a proof-of-concept study

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**Original article**

**Keywords (MeSH) Lactobacillus; Gardnerella vaginalis; Spectrometry, Mass, Matrix-Assisted Laser Desorption-Ionization; Statistics, Biological;**

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ABSTRACT

**Background:** the study of vaginal microbiota is mainly performed by molecular methods. Nevertheless, they are expensive and not easily available in clinical microbiology laboratories. MALDI-TOF MS is being increasingly adopted for fast and accurate identification of pathogens from clinical samples. Aim of this study was to evaluate MALDI-TOF MS for identification of culturable bacterial species directly from plates streaked with vaginal swabs from a population of patients in childbearing age.

**Methods:** We included women of childbearing age evaluated from October 2020 to October 2021. Identification was performed by means of Vitek® MS system.

**Results:** A total of 381 patients were included in the study. Mean age was 33.3 years (±7.7) and 218/381 (57.2%) were pregnant. The most frequent isolates were: *Lactobacillus crispatus* 85/381 (22.3%), *Lactobacillus acidophilus/gasseri* 77/381 (20.2%) and *Lactobacillus iners* 64/381 (16.8%), identified with a confidence value of 99.9%. *Gardnerella vaginalis* was identified in 74/381 (19.4%) patients.

**Conclusions:** In this Proof of Concept study we found that MALDI-TOF MS has the potential to be used to identify main *Lactobacillus* species directly from plates streaked with vaginal swabs.

**Obiettivi:** per lo studio del microbiota vaginale vengono utilizzati principalmente metodi molecolari, gold standard per la migliore comprensione di un ecosistema microbico, ma dai costi elevati e quindi non sempre disponibili nei laboratori di microbiologia clinica. Il MALDI-TOF MS è una metodica molto diffusa per l'identificazione rapida di agenti patogeni da campioni clinici. Scopo dello studio è stato di valutare il sistema Vitek® MS per l'identificazione di specie batteriche coltivabili, direttamente da piastre seminate da tamponi vaginali prelevati da pazienti in età fertile.

**Metodi:** abbiamo incluso donne in età fertile valutate da Ottobre 2020 ad Ottobre 2021.

**Risultati:** sono state incluse un totale di 381 pazienti. L'età media era di 33,3 anni (±7,7) e 218/381 (57,2%) erano in gravidanza. Gli isolati più frequenti sono stati: *Lactobacillus crispatus* 85/381 (22,3%), *Lactobacillus acidophilus/gasseri* 77/381 (20,2%) e *Lactobacillus iners* 64/381 (16,8%), identificati con un livello di confidenza del 99,9%. *Gardnerella vaginalis* è stata identificata in 74/381 (19,4%) pazienti.

**Conclusioni:** questo studio ha mostrato come MALDI-TOF MS possa essere utilizzato per identificare le principali specie di *Lactobacillus* direttamente da piastre seminate da tamponi vaginali.

# Introduction

Lactobacillus is a genus of Gram-positive, non-spore-forming bacilli (Carroll et al, 2019). Most species are facultative anaerobic, although some grow best under either anaerobic or microaerophilic conditions (Procop et al, 2017). The vagina is an organ colonized by various species of bacteria and during childbearing age the bacterial flora of a healthy vagina consists mainly of Lactobacillus species (Chee et al, 2020). Lactobacillus species exert a protective action on the vaginal environment by producing various substances such as lactic acid, hydrogen peroxide and others, competitively excluding other microorganisms causing disease (Kovachev, 2018). The main Lactobacillus species composing vaginal flora has been identified as Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners and Lactobacillus jensenii by means of molecular methods such as 16S rRNA gene analysis (Ravel et al, 2011; Zhou et al, 2007). Gardnerella vaginalis is a Gram-variable-staining, non-spore-forming coccobacillus, facultative anaerobic (Carroll et al, 2019), known as associated with Bacterial Vaginosis (BV) a vaginal dysbiosis characterized by disruption of normal microbial balance with loss of Lactobacillus dominance and increase of other species such as G. vaginalis, Prevotella spp., Mobiluncus spp., Atopobium vaginae, as well as other anaerobic organisms (Coudray et al, 2020; Ling et al, 2010). Nevertheless, G. vaginalis has also been found in vaginal samples of healthy women without BV (Janulaitiene et al, 2017).

Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) is a relatively new technology for rapid and accurate identification of bacteria, mycobacteria and certain fungal pathogens in clinical microbiology (Angeletti, 2017; Torres-Sangiao et al, 2021). The MALDI-TOF is based on an analytical technique in which chemical compounds in biological samples are ionized into charged molecules and ratio of their mass to charge (m/z) is evaluated (Fenselau and Demirev, 2001). The biological samples to be analyzed are spotted on a target plate and subsequently mixed with an organic compound called matrix. The mixture undergoes ionization by means of a laser beam and the generated ions separate from each other in relation to their m/z in a flight tube and then analyzed by a detector on the basis of the time-of-flight (TOF). The device then generates a spectrum composed by peaks corresponding to the relative abundance of the m/z of the ions detected and compares the spectrum with those present in its spectra database (Singhal et al, 2015).

Although other Authors already reported how MALDI-TOF MS is a reliable and fast tool to identify lactobacilli to the species level compared to 16S rDNA analysis from single colonies obtained from vaginal and oral samples (Anderson et al, 2014) to the best of our knowledge,

there is a lack of evidence of the use of MALDI-TOF MS for identification of lactobacilli directly from bacterial growth on agar plates inoculated with vaginal swabs. The aim of this study was therefore to evaluate the MALDI-TOF MS technology for identification of Lactobacillus species directly from plates streaked with vaginal swabs obtained from a population of patients in childbearing age.

# Materials and methods

## Study design

This is a cross-sectional study aimed to assess the prevalence of *Lactobacillus* species from vaginal swabs in a population of patients of childbearing age from October 2020 to October 2021. Demographical data were obtained from the Laboratory Information System. Inclusion criteria: all consecutive female outpatients aged 18-50, referred to the collection center of the SS. Antonio e Biagio e C. Arrigo Hospital of Alessandria for vaginal sampling and from whom a bacterial growth compatible with *Lactobacillus* spp. was obtained (Goldstein et al, 2015), evaluated only once during the period considered. Exclusion criteria: all patients younger than 18 or older than 50 years of age or from whose swabs no growth was obtained.

## Bacterial culture and identification

All vaginal swabs (ESwab® transport medium, Copan Italia, Brescia, Italy) were plated onto Columbia colistin-nalidixic acid agar (bioMérieux, Marcy l'Etoile, France) and incubated overnight at 35°C. Identification was performed by means of Vitek® MS MALDI-TOF system (bioMerieux, Marcy l'Etoile, France). Briefly, an amount of the microbial growth from the agar plate was spotted onto the MALDI-TOF target plate, immediately overlaid with 1 µl of matrix solution and air dried. The target plate was then placed into the MALDI-TOF MS device and underwent identification. The Vitek® MS system identifies the organism by means of an advanced spectrum classifier algorithm that calculates a confidence value as a percent probability that represents the similarity in terms of presence/absence of specific peaks between the generated spectrum and the database spectra (Dubois et al, 2012).

## Statistical analysis

Categorical variables were expressed as count and percentage, continuous variables as mean and standard deviation (±SD). Tests for association were performed by Yates's chi-square test and comparisons of mean values were performed using the two-sample unpaired t-test. Statistical significance was assumed if a null hypothesis could be rejected at a p value of ≤0.05. SPSS statistical package, release 17.0 (SPSS Inc, Chicago, IL, USA) was used for all statistical analyses.

## Ethical considerations

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Ethical approval was not needed because this is a secondary analysis of data part of standard care and those included in the database were deidentified before access. No personal information was stored in the study database. No patient intervention occurred with the obtained results.

# Results

## Prevalence in the whole population

A total of 381 patients fulfilled the inclusion criteria and were included in the study. Mean age was 33.3 years (±7.7) and 218/381 (57.2%) were pregnant. The geographical origin of the patients is displayed in Figure 1, 294/381 (77.2%) were Italian. The bacterial species identified are summarized in Figure 2. All the isolates identified as single species had a confidence value of 99.9%, whereas the identification of two isolates from the same sample had a confidence value of 50% for each one. The most frequent isolates were: *Lactobacillus crispatus* 85/381 (22.3%), *Lactobacillus acidophilus/gasseri* 77/381 (20.2%) and *Lactobacillus iners* 64/381 (16.8%). *Gardnerella vaginalis* was identified, as single isolate or in association with lactobacilli, in 74/381 (19.4%) patients. The comparison of mean age values showed how foreign patients were significantly younger: 31.9 (±6.9) vs 33.8 (±7.8); p=0.035. Conversely, no significant differences were found between pregnant and not pregnant: 33.2 (±6.8) vs 33.5 (±8.8); p=0.696 nor between patients positive and negative for *G. vaginalis*: 32.8 (±7.4) vs 33.5 (±7.8); p=0.488.

## Prevalence and geographical origin

The prevalence of bacterial species found according to geographical origin is shown in Table 1. The most frequent *Lactobacillus* species identified in Italian patients was *L. crispatus* 67/294 (22.8%) whereas in patients from East Europe prevailed *L. iners* 13/52 (25%) and in patients from Africa, *L. acidophilus/gasseri* and *L. crispatus* were equally frequent 5/20 (25%). The comparison of proportions among Italian and foreign patients positive and negative for *G. vaginalis* showed no significant difference (chi-square = 0.015; p=0.902).

## Prevalence and pregnancy

In Table 2 is displayed the prevalence of bacterial species according to pregnancy. The comparison of proportions between pregnant and non-pregnant patients positive or negative for *G. vaginalis* showed no significant difference: chi-square = 0.048; p=0.826.

# Discussion

The main result of the study is the confirmation of the ability of MALDI-TOF MS in identifying *Lactobacillus* species directly from agar plates streaked with vaginal swabs. Indeed in 365/381 (95.8%) samples the identification confidence value was 99.9%. In the remaining 16/381 (4.2%) the device identified two bacteria, both with a score of 50%. The latter result could be nevertheless interpreted in the light of possible coexistence of two bacteria with comparable amount of viable cell counts. Indeed, in a recent study, Mörtelmaier et al. (2019) reported how the performance of MALDI-TOF MS with mixed culture relies on the species, the phylogenetic distance, and their ratio of viable cell counts per ml (Mörtelmaier et al, 2019). Moreover, we found that *G. vaginalis* was identified associated with *L. iners* in 12/74 (16.2%) samples and this result matches other reports in whom *L. iners* was found associated also with vaginal dysbiosis (Zheng et al, 2019) and in BV-positive and partial BV samples (Janulaitiene et al, 2017).

With regard to the species identified in this study, the main *Lactobacillus* species (278/381; 73%) identified as single isolates with confidence values of 99.9% (*L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*) match those reported in studies performed by pyrosequencing of barcoded 16S rRNA Genes (Ravel et al, 2011) and by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes (Zhou et al, 2007).

The specific differences in the prevalence of certain species in relation to geographical origin found in this study only partially match those found by other Authors (Ravel et al, 2011; Zhou et al, 2007). This is conceivable, since the identification methods were anyway different and in this study only culturable bacteria were evaluated.

The lack of a significant difference in the recovery rate of *G. vaginalis* in relation to geographical origin or pregnancy is in line with the notion that *G. vaginalis* is commonly found also in healthy pregnant women without BV (Machado et al, 2017).

This study has limitations. The main one is that only culturable microbial flora has been studied, while it is known that the use of culture-independent approaches based on the analysis of 16S rRNA gene sequences has revealed that several vaginal bacterial communities exist. Nevertheless, the molecular methods for studying vaginal microbiota are expensive and not easily available in clinical microbiology laboratories, on the other hand, MALDI-TOF MS is accurate, widely used and cheaper. Moreover, also by molecular methods, the main *Lactobacillus* species found by other Authors, such as *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* are the same found also by MALDI-TOF MS. Other limitations are the lack of clinical data and a gold standard molecular analysis to compare the results with. This will be the subject of future research.

# Conclusions

In this Proof of Concept study we found that MALDI-TOF MS has the potential to be used to identify main *Lactobacillus* species directly from plates streaked with vaginal swabs.

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Table 1. Prevalence of bacterial species according to geographical origin.

| Geographical origin | N | % |
|---|---|---|
| Italy | | |
| Lactobacillus crispatus | 67 | 22.8 |
| Lactobacillus acidophilus/gasseri | 57 | 19.4 |
| Gardnerella vaginalis | 47 | 16 |
| Lactobacillus iners | 46 | 15.6 |
| Lactobacillus jensenii | 43 | 14.6 |
| Lactobacillus casei/paracasei/rhamnosus | 12 | 4.1 |
| Gardnerella vaginalis + Lactobacillus iners | 8 | 2.7 |
| Others | 14 | 4.8 |
| Subtotal | 294 | 100 |
| East Europe | | |
| Lactobacillus iners | 13 | 25 |
| Lactobacillus acidophilus/gasseri | 12 | 23.1 |
| Lactobacillus crispatus | 8 | 15.4 |
| Lactobacillus jensenii | 7 | 13.5 |
| Gardnerella vaginalis | 6 | 11.5 |
| Gardnerella vaginalis + Lactobacillus iners | 2 | 3.8 |
| Others | 4 | 7.7 |
| Subtotal | 52 | 100 |
| Africa | | |
| Lactobacillus acidophilus/gasseri | 5 | 25 |
| Lactobacillus crispatus | 5 | 25 |
| Gardnerella vaginalis | 4 | 20 |
| Lactobacillus iners | 3 | 15 |
| Lactobacillus jensenii | 1 | 5 |
| Streptococcus mitis/oralis | 1 | 5 |
| Gardnerella vaginalis + Lactobacillus iners | 1 | 5 |
| Subtotal | 20 | 100 |
| South America | | |
| Lactobacillus iners | 2 | 20 |
| Lactobacillus acidophilus/gasseri | 2 | 20 |
| Lactobacillus crispatus | 2 | 20 |

| | | |
|---|---|---|
| Gardnerella vaginalis | 2 | 20 |
| Lactobacillus jensenii | 1 | 10 |
| Gardnerella vaginalis + Lactobacillus iners | 1 | 10 |
| Subtotal | 10 | 100 |
| Asia | | |
| Lactobacillus crispatus | 2 | 66.7 |
| Lactobacillus acidophilus/gasseri | 1 | 33.3 |
| Subtotal | 3 | 100 |
| Middle East | | |
| Lactobacillus casei/paracasei/rhamnosus | 1 | 50 |
| Lactobacillus crispatus | 1 | 50 |
| Subtotal | 2 | 100 |
| Total | 381 | 100 |

ISSN: 2279-9761
Working paper of public health [Online]

Table 2. Prevalence of bacterial species according to pregnancy.

| Bacterial species | Pregnant | % | Not pregnant | % | Total | % |
|---|---|---|---|---|---|---|
| Lactobacillus crispatus | 48 | 22 | 37 | 22.7 | 85 | 22.3 |
| Lactobacillus acidophilus/gasseri | 54 | 24.8 | 23 | 14.1 | 77 | 20.2 |
| Lactobacillus iners | 34 | 15.5 | 30 | 18.4 | 64 | 16.7 |
| Gardnerella vaginalis | 29 | 13.3 | 30 | 18.4 | 59 | 15.4 |
| Lactobacillus jensenii | 26 | 11.9 | 26 | 16 | 52 | 13.6 |
| Lactobacillus casei/paracasei/rhamnosus | 6 | 2.7 | 7 | 4.3 | 13 | 3.4 |
| Gardnerella vaginalis + Lactobacillus iners | 9 | 4.1 | 3 | 1.9 | 12 | 3.1 |
| Streptococcus mitis/oralis | 3 | 1.4 | 1 | 0.6 | 4 | 1 |
| Lactobacillus delbrueckii | 2 | 0.9 | 0 | 0 | 2 | 0.5 |
| Lactobacillus pentosus/plantarum/paraplantarum | 2 | 0.9 | 0 | 0 | 2 | 0.5 |
| Aerococcus urinae | 1 | 0.5 | 0 | 0 | 1 | 0.3 |
| Bifidobacterium spp | 0 | 0 | 1 | 0.6 | 1 | 0.3 |
| Lactobacillus fermentum | 0 | 0 | 1 | 0.6 | 1 | 0.3 |
| Lactobacillus reuteri | 1 | 0.5 | 0 | 0 | 1 | 0.3 |
| Lactobacillus salivarius | 0 | 0 | 1 | 0.6 | 1 | 0.3 |
| Streptococcus anginosus | 0 | 0 | 1 | 0.6 | 1 | 0.3 |
| Streptococcus vestibularis | 0 | 0 | 1 | 0.6 | 1 | 0.3 |
| Gardnerella vaginalis + Pediococcus acidilactici | 1 | 0.5 | 0 | 0 | 1 | 0.3 |

ISSN: 2279-9761
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| | | | | | | |
|---|---|---|---|---|---|---|
| Gardnerella vaginalis + Lactobacillus jensenii | 1 | 0.5 | 0 | 0 | 1 | 0.3 |
| Lactobacillus iners + Lactobacillus crispatus | 0 | 0 | 1 | 0.6 | 1 | 0.3 |
| Gardnerella vaginalis + Lactobacillus crispatus | 1 | 0.5 | 0 | 0 | 1 | 0.3 |
| Total | 218 | 100 | 163 | 100 | 381 | 100 |

1 Figure 1. Geographical origin of the patients (n=381)

ISSN: 2279-9761
Working paper of public health [Online]

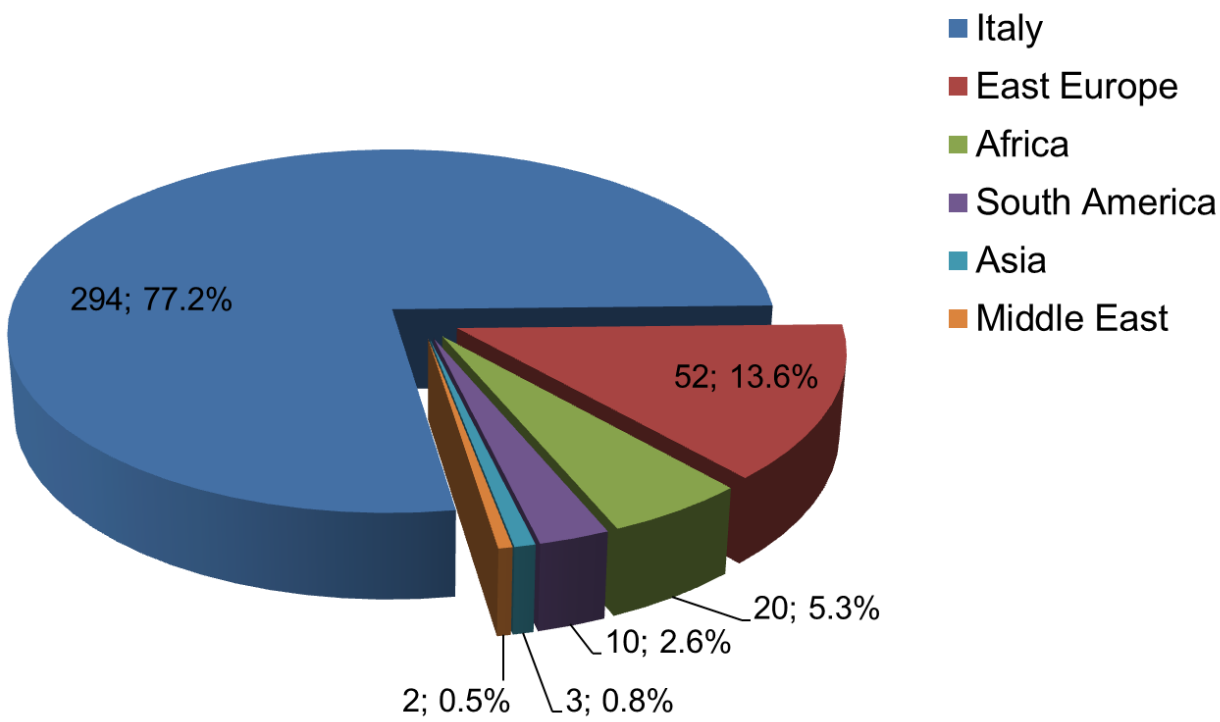


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Figure 2. Prevalence of bacterial species in the whole sample (n=381).

ISSN: 2279-9761
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