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Application of MALDI-TOF Mass Spectrometry to Identify Lactic Acid Bacteria Isolated from Artisanal Dairy Products in Algeria

M. Bouchibane, Y. Zabouri, A. Cheriguene, A. Kaced, F. Chougrani, D. Ait Saada

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ABSTRACT

Background: Artisanal dairy products are considered an affordable food source and are highly valued by Algerian consumers.

Methods: In this study during the period from 2019 to 2020, phenotypic identification methods and protein analysis by MALDI-TOF MS were performed to identify lactic acid bacteria isolated from 19 sample and five types of artisanal dairy products collected in different Algerian regions (East, West, North, South).

Result: A number of 250 strains were isolated, only those that showed the characteristics of lactic acid bacteria were identified by analysing their proteins, The results showed that the strains belong to different species of LAB namely: *Lactobacillus fermentum* (31.03%), *Enterococcus faecium* (24.14%), *Lactobacillus plantarum* (21.55%), *Enterococcus faecalis* (17.24%), *Lactococcus lactis ssp lactis* (4.31%), *Lactobacillus paracasei* and *Lactobacillus paraplantarum* (0.86%). Identification by MALDI-TOF MS was similar to that provided by phenotypic Characterization. This new technique that has been used is considered as an effective tool for rapid identification of food-associated lactic acid strains and has also been successfully extended to dairy isolates.

Key words: Artisanal dairy products, Lactic acid bacteria, Maldi-Tof MS, Phenotypic identification.

INTRODUCTION

The traditionally fermented dairy products have a great importance in Algeria especially for people living in rural areas who consider these products very relevant for their daily diet. The difficulty of preserving milk in its fresh form leads to its transformation into different artisanal dairy products. Example of these products : J'ben, Smen, L'ben, Zebda and Raib are the most famous and consumed by the Algerian population. Lactic acid bacteria (LAB) are Gram positive microorganisms that represent a phylogenetically very heterogeneous group of bacteria with GRAS status. (Abhyankar *et al*., 2022). Several species of LAB were isolated from Algerian artisanal dairy products. Generally their identification is performed using phenotypic tests, nevertheless these tests have a low power distinction between isolates allowing an identification up to the genus level, moreover, they are not very accurate (Herbel *et al*., 2013). For this reason, genotypic techniques have been used to identify LAB associated with dairy products such as polymerase chain reaction (PCR) and amplification of ribosomal DNA 16S. (Benamara *et al*., 2016); (Dahou *et al*., 2021), (Patil *et al*., 2015), restriction enzyme analysis with pulsed field gel electrophoresis (REA-PFGE) (Domingos-lopes *et al*., 2017). Which allow identification that can achieve to the subspecies level with good precision and have good discriminatory power, but they are expensive, time consuming and difficult to apply in routine analyses (Doan *et al*., 2012). The identification of isolates by using an accurate and rapid technique helps to easily and quickly select strains that could be useful to improve the process of dairy products manufacturing (Seng *et al*., 2009).

Department of Agronomy, Abdelhamid Ibn Badis University, Mostaganem-2700, Algeria.

Corresponding Author: M. Bouchibane, Food Technology and Nutrition Laboratory, Mostaganem University, Algeria.
Email: malika.bouchibane.etu@univ-mosta.dz

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MALDI-TOF MS (Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry) is a molecular proteomic technique widely utilised to rapidly identify bacteria. This technique has been used recently as a research tool to determine the genera and species of LAB associated with foods. (Soro-Yo *et al*., 2014). For example *Lactobacillus curvatus, Lactobacillus diolivorans, Lactococcus lactis* and *Leuconostoc mesenteroide*, *Bifidobacterium animalis* and *Streptococcus thermophilus* have been accurately identified in yoghurts and probiotic foods (Angelakis *et al*., 2011; Tanigawa *et al*., 2010). While phenotypic and genotypic methods have been applied to identify LAB isolated from Algerian artisanal fermented milk products, little detailed information on their proteomic identification using MALDI-TOF MS is available (Meghoufel *et al*., 2017); (Arezki *et al*., 2019). This work aimed to isolate and to identify LAB from artisanal dairy products collected from different regions in Algeria and to confirm the use of the MALDI-TOF Mass Spectrometry technique as a method for the proteomic identification.

MATERIALS AND METHODS

19 samples from five types of Algerian artisanal dairy products made from raw milk, J'ben (*n*=7), Smen (n=5), L'ben (n=2), Raib (n=1) and Zebda (n=4) were studied in this work. They were collected from regions Khenchela and Tizi Ouzou in the East, Oran, El Bayadh and Naama in the West, Blida, Medea and Ain Defla in the North-West, Djelfa, Msila and Bechar in the South-West during the period from 2019 to 2020. The samples were analyzed by the method of plating dilutions poured on agar. For this purpose, 10 g and 1 ml of aliquots of each sample were aseptically collected, transferred to vials containing 90 ml of TSE solution and to tubes containing 9 ml of TSE. A series of decimal dilutions were performed for each sample. Then a volume of 0.1 ml was taken from the 10^{-5}, 10^{-6} and 10^{-7} dilutions and spreaded on the surface of M17 (Pronadisa, Madrid, Spain). (Terzaghi and Sandine, 1975) and MRS agar plates (Pronadisa, Madrid, Spain). (De Man Rogosa, 1960). Plates were incubated at 37°C and 45°C from 48 h to 72 h under aerobic and anaerobic conditions. After growth, Gram staining, catalase and oxidase tests were carried out. Isolates which resulted Gram-positive, catalase-negative and oxidase-negative were purified and then stored in MRS and M17 broths containing (30%) glycerol at -20°C (Samelis *et al*., 1994).

For the selected isolates of LAB, phenotypic identification was carried out at the Research Laboratory « Food Technology and Nutrition » of Abdelhamid Ibn Badis University. Was performed by physiological and biochemical tests according to the methods and criteria of Mathara *et al*., (2004), Carr *et al*., (2002) and López-Dìaz *et al*., (2000). The following tests were performed–: The ability of isolates to grow at different temperatures was examined on MRS and M17 liquid media at 15°C for 7 days and at 45°C and 37°C for 48 h. The ability to grow in the presence of 4% and 6.5% NaCl and at different pH (9.6 and 4) was observed after incubation at 30°C for 2-5 days. The culture on Sherman's milk test was performed on 0.1% and 0.3% methylene blue skimmed milk. Gas production from glucose was determined in MRS and M17 broths, containing Durham bell. The arginine dihydrolase assay was carried out by inoculating the isolates on MRS broth containing bromocresol purple and 0.3% (w/v) arginine. Hydrolysis of esculin was also tested by inoculating the strains tested on esculin agar medium by central pricking. Moreover, the strains were identified on the basis of their carbohydrates fermentation profiles, the latters were tested on the MRS and M17 broth with 1% of each sugar tested, containing (0.04 g/l) bromocresol purple as a pH indicator.

For the identification by the molecular technique MALDI-TOF MS, From overnight cultures, 0.1 ml was spread on M17 and MRS agar media. The obtained colonies were spread with the tip of a sterile toothpick on a steel MALDI target plate and then each colony was covered with a drop of the matrix solution (saturated solution of cyano-4-hydroxy-cinnamic acid at 50% acetonitrile - 2.5% trifluoroacetic acid) and allowed to dry. After the calibration of the MALDI-TOF spectrometer (Bruker Daltonics, Bremen, Germany) by the kit containing a protein extract enriched by *E. coli DH5 alpha* with two proteins 'RNase A and myoglobin' (Ferreira *et al.,* 2011), the plate was placed in the apparatus for the identification. It was performed using Biotyper software (Bruker Daltonics, version 3.0) by comparing the peaks of the generated mass spectra with the existing reference spectra in the database version provided by the manufacturer (BioTyper DB- 5989 MALDI-TOF MS reference profiles). Each experiment was repeated 02 time. This technique was done at the Technical and Scientific Research Center in Physical and Chemical Analysis, Tipaza, Algeria.

RESULTS AND DISCUSSION

Phenotypic identification

The results showed the presence of three genera: *Lactobacillus, Enterococcus* and *Lactococcus.* The cells of strains had different shapes : cocci and rod.

63 rod-shaped were divided into four groups

Group 1

25 isolates (17 homofermentative and 8 heterofermentative), arginine dihydrolase positive (ADH) strains, growing at 15°C, 37°C and 45°C, at pH 4 and with 4% NaCl, but not at pH 9.6 and 6.5% NaCl, they did not ferment Arabinose and Xylose.

Group 2

36 heterofermentative, ADH positive, esculin-positive strains growing at pH 4 and 4% NaCl, at 37°C and 45°C, but not 15°C, at pH 9.6 and 6.5% NaCl. They did not ferment Sorbitol, Rhamnose and Xylose.

Group 3

01 homofermentative strain, ADH negative, esculin positive, grows at 15°C and 37°C, at pH 4 and with 4% and 6.5% NaCl, but not at 45°C, at pH 9.6. This strain did not ferment Mannitol, Sorbitol, Melibiose, Arabinose, Xylose and Ribose. Group 4 : 01 heterofermentative strain, ADH positive, esculin positive, grows at 37°C, pH 4 and 4% NaCl, but not at 15°C and 45°C, pH 9.6 and 6.5% NaCl. It did not ferment Arabinose and Xylose.

53 strains in the form of cocci were also divided into three groups

Group 1

05 homofermentative strains, ADH negative, esculin positive, growing at 0.1% and 0.3% methylene blue, at 15°C and 37°C, at pH 4 and with 4% NaCl, but not at 45°C, at pH 9.6 and with 6.5% NaCl, they did not ferment Inositol, Sorbitol, Rhamnose, Melibiose, Amygdaline, Xylose.

Groups 2 and 3 are similar in all tests, except in the sugar fermentation test where there is some difference. 48 homofermentative strains, growing at 0.1% methylene blue, not 0,3%, at 15°C, 37°C and 45°C, at pH 4 and 9.6 and with 4% and 6.5% NaCl, esculin positive, ADH positive, all strains are thermoresistance, group 2 : they not ferment Inositol,

Rhamnose, Saccharose, Melibiose, Amygdaline, Xylose and Ribose. Group 3: they not ferment Mannitol, Inositol, Sorbitol, Rhamnose, Saccharose, Melibiose, Amygdaline, Xylose and Ribose.

Molecular identification

Strains that were identified by MALDI-TOF MS generated mass spectra with peaks varying from one species to another. Fig 1 represent the spectra of some identified species. For each of them we have chosen an example of raw profile spectrum (F1, G1, H1, I1), the profiles of strains that were compared with those of the reference strains in the MALDI-TOF MS Biotyper database are represented as (F2, G2, H2, I2) in the form of stick spectra. The sticks of the upper part colored in green, yellow and red show the matching of the profiles of tested strains and the sticks of the lower part colored in blue refers to the profiles of the reference strains.

The results revealed a score close to and higher than 2.0. The score values found for each identified strain are detailed in the (Table 1). Among 116 strains were identified, 11 of them identified with log(score) ≥2.3, 65 with log(score) ≤2.3 and ≥2 and 40 strains with log(score) ≤2 and ≥1.7. The values found in our results are different and higher than those of the score values of the strains that were also isolated from some Algerian artisanal dairy products by Arezki *et al.,* (2019), who reported that among the twenty isolates identified as *Lactobacillus plantarum* only four

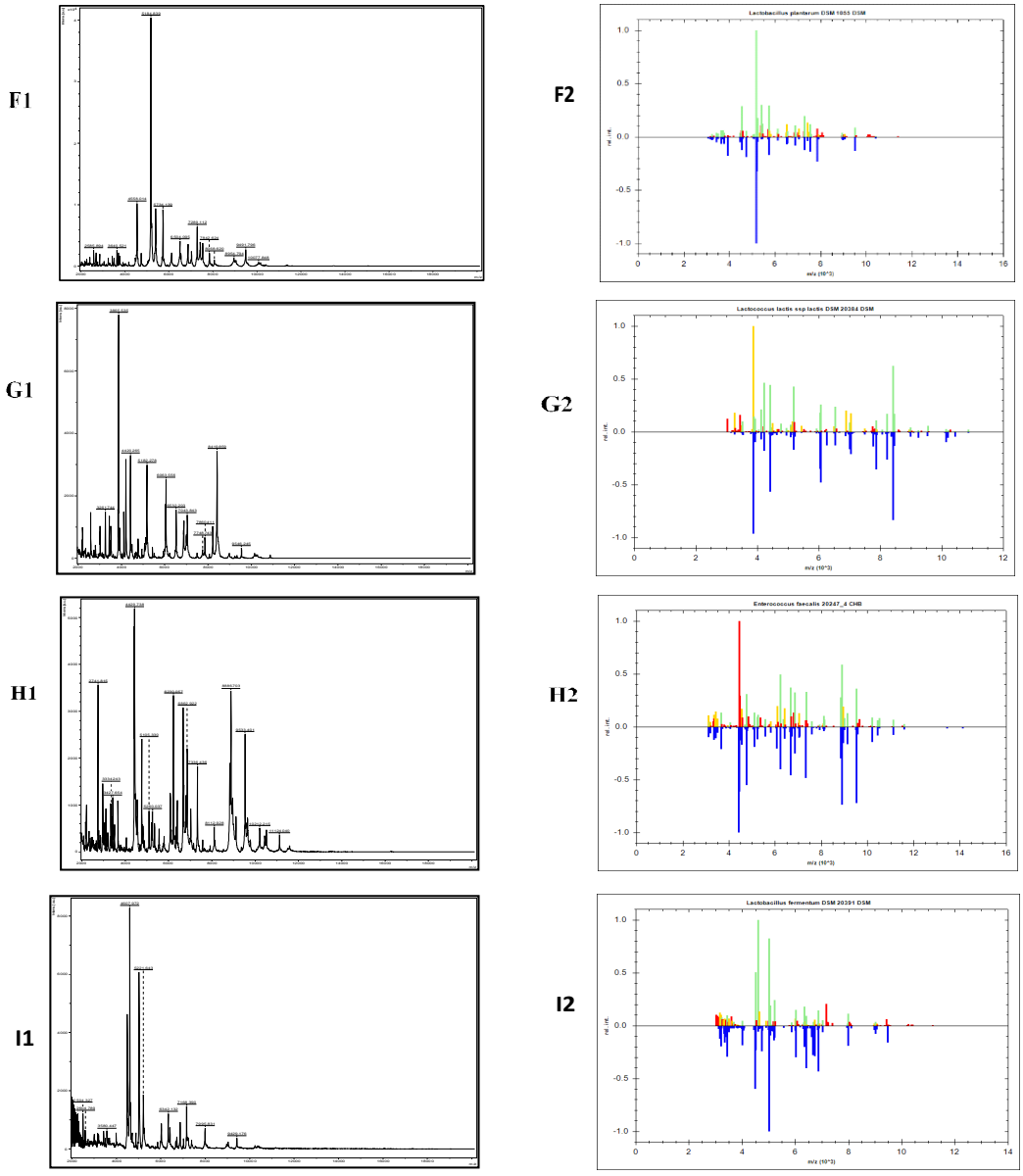

Fig 1: profile of species *Lactobacillus plantarum* (panels F1 and F2), *Lactococcus lactis ssp lactis* (panels: G1, G2), *Enterococcus faecalis* (panels: H1, H2), *Lactobacillus fermentum* (panels I1 and I2).

strains had scores higher than 2. On the other hand, the value of *Lactobacillus paracasei* species is higher than the value found in our strain. This difference is explained by generation of spectra with bruises containing weak peaks which indicates that the colonies tested are not pures colonies or their ribosomal and membrane proteins are low. According to Cherkaoui *et al.,* (2010), the determination of the score value is based on three factors : the number of peaks corresponding to the tested colony, the total number of peaks in the spectra of the reference strain and the tested strain, correlation of the intensity related to similar peaks.

The identification of our isolates by the MALDI-TOF MS confirmed that they belong to the lactic acid bacteria with different species and divided into 7 groups. The first group includes the species *Lactobacillus plantarum* which correspond to *Lactobacillus plantarum ssp plantarum* DSM 20174T DSM, *Lactobacillus plantarum* DSM 12028 DSM, *Lactobacillus plantarum* DSM 2601 DSM. The strains of the second group were identified as *Lactobacillus fermentum*, all strains correspond to the reference strain *Lactobacillus fermentum* DSM 20391 DSM. The third and the fourth groups include only one strain correspond to *Lactobacillus paraplantarum* DSM 10667T DSM and *Lactobacillus paracasei ssp paracasei* DSM 2649 DSM respectively. The strains of the fifth group refer to *Lactococcus lactis ssp lactis* DSM 20384 DSM, *Lactococcus lactis ssp lactis* DSM 20661 DSM. The sixth group contains strains identified as *Enterococcus faecalis* ATCC 7080 THL, *Enterococcus*

Table 1: Results of the score values of analyzed strains.

Strains	Score values	Source
Lactobacillus plantarum		
JBS24, JBS32, JBS5, JBS4, JBS8, JBS6C1,	2.155, 2.18, 2.129, 2.029, 2.016, 2.124,	J'ben
JBS23, JBS19, JBS11, JBS22, JBS20, JAS3C3,	1.858, 1.995, 2.139, 1.963, 2.093, 2.117,	
JAS1, JAS3C4, JAS16, JAS17, JAS11, JAS14.	2.152, 2.137, 2.213, 2.012,	
	1.774, 2.186	
LMS1, LMS2, LMS3, LMS4	2.232, 1.815, 2.161, 1.802	L'ben
ZMS1	2.166	Zebda
SVS1, SVS9	2.04, 1.072	Smen
Lactobacillus fermentum		
JOS1, JOS4, JBES1, JBES8, JBES4, JKS8,	2.03, 1.8802, 2.129,1.998, 2.059, 2.119,	J'ben
JKS1, JKS10, JKS9, JKS5, JKS7, JBS1,	1.933, 1.96, 1.859, 1.705, 1.9, 1.806,	
JNS2, JNS5, JNS9, JNS14	2.023, 1.707, 1.946, 1.905	
LMS8, LMS5, LMS7, LAS1,	1.831, 1.9555, 1.929, 1.74,	
LAS2, LAS7, LAS8, LAS6	1.862, 1.869, 2.024, 1.791	L'ben
SDS3, SMS6	2.232, 1.754	Smen
ZMS7, ZMS9, ZMS6, ZMS4	2.031, 2.096, 1.913, 1.72,	Zebda
ZMS2, ZMS1, ZMS3, ZTS1, ZKS3	1.898, 1.965, 1.742, 2.119, 2.183	
RC1	1.9555	Raib
Lactobacillus paraplantarum		
JAS15	1.857	J'ben
Lactobacillus paracasei ssp paracasei		
JKS13	1.71	J'ben
Lactococcus lactis ssp lactis		
E9S2, E7, E9S1, E10S1, E10S2	2,359, 2,313, 2,298, 2,292, 2,267	J'ben
Enterococcus faecalis		
JBS13, JBS5, JBS14, JBS30,JBS9, JAS18,	2.171, 2.298, 2.052, 2.141, 2.087, 2.125,	J'ben
JAS33, JAS30, JAS15, JAS2, JAS35, JAS20,	2.224, 2.181, 2.117, 2.059, 2.277, 2.322,	
JAS11, JAS16C3, JAS4, JAS34,	2.156, 1.784, 1.921, 2.223,	
JAS37, JAS39, JBES6	1.868, 2.175, 2.166	
SCS1	2.052	Smen
Enterococcus faecium		
JBS29, JBS26, JBS8, JBS36, JBS10, JAS8,	2.336, 2.369, 2.462, 2.256, 2.223, 2.318,	J'ben
JAS12, JAS16, JAS14, JAS24,JAS30, JAS42,	2.276, 2.24, 2.289, 2.276, 2.13, 2.143,	
JAS28, JAS25, JAS9, JAS37, JAS40, JAS41,	2.361, 2.091, 2.212, 2.384, 1.813, 2.218,	
JBAS4, JBAS6, JOS3, JOS6	2.113, 2.439, 2.222, 2. 085,	
SVS1, SCS4, SCS5, SMS2, SMS4	2.215, 2.431, 1.928, 2.262, 2.185	Smen
ZAS5	1.754	Zebda

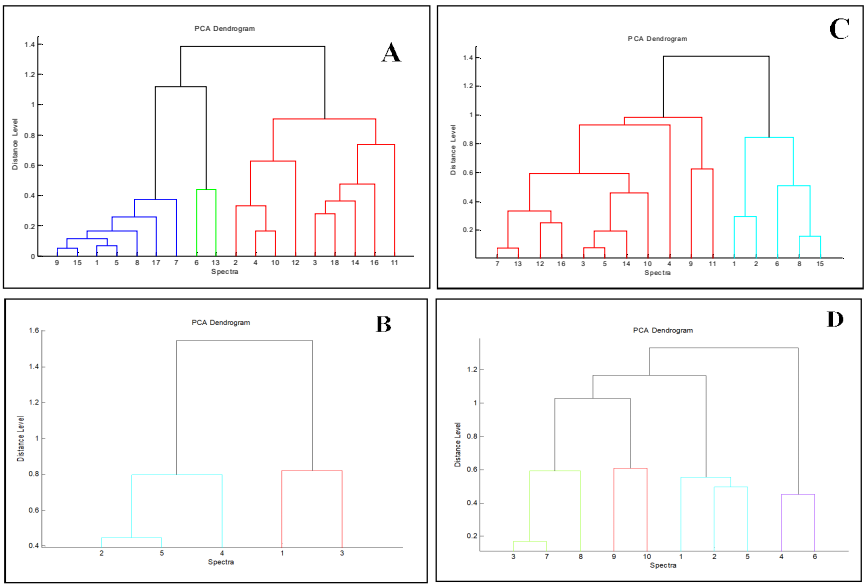

Fig 2: Dendrogram generated by MALDI-TOF MS of *Lactobacillus plantarum* (A), *Lactococcus lactis ssp lactis* (B), *Enterococcus faecalis* (C) and *Lactobacillus fermentum* (D).

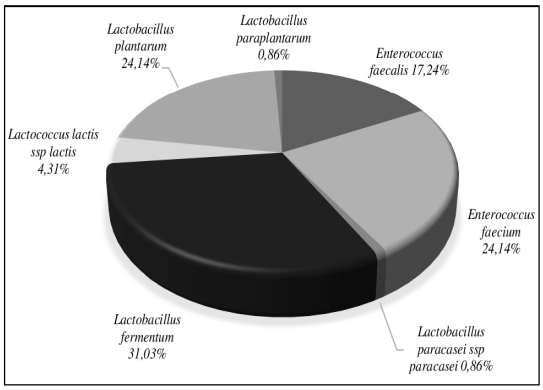

Fig 3: Distribution of the species identified from artisanal dairy products of Algeria.

faecalis DSM 20409 DSM, *Enterococcus faecalis* 20247_4 CHB, Whereas those in the seventh group belong to the reference strains *Enterococcus faecium* 20218_1 CHB, *Enterococcus faecium* 11037 CHB, *Enterococcus faecium* DSM 2146 DSM.

Representative strain with scores greater than 2 were grouped in the dendrograms shown in Fig 2. Then the dendrogram was performed by the standard Biotyper PCA (principal component analysis) method. The dendrogram provides the similarity relationships between strains belonging to the same species and obtained from different products, illustrated by different colours, the colours show the difference between the phylogenetic characteristics according to their protein component, strains belonging to the same colour are close and have the same characteristics. For example, the spectra of isolates belonging to the species *Lactobacillus plantarum* with the numbers (2, 4, 10 and 12) colored red were closer and have identical phylogenetic characteristics to those of the other isolates in the dendrogram colored green and blue. With the separating branches connected a distance level of 0.9.

The data from the proteomic analyses of the general distribution of species showed that *Lactobacillus fermentum* represents the dominant strains with a percentage of 31.04%, followed by *Enterococcus faecium* (24. 14%), *Lactobacillus plantarum* (21.55%) and *Enterococcus faecalis* (17.24%), with the subspecies *Lactococcus lactis ssp lactis* that represent 4.31% and finally the two subspecies *Lactobacillus paracasei ssp paracasei* and *Lactobacillus paraplantarum* with a low percentage of 0.86% (Fig 3).

Both species *Lactobacillus fermentum* and *Lactobacillus plantarum* were present in the five products. However *paracasei ssp paracasei, Lactobacillus paraplantarum* and *Lactococcus lactis ssp lactis* were found only in one type of product named “J’ben”. Interestingly, it was noted that all strains isolated from the samples of “L’ben and Raib” belong only to the genus. The strains isolated from the samples Zebda showed the existance of the genus *Enterococcus* with the dominance of *Lactobacillus.* However, the results of Smen samples highlighted the presence of two genera *Lactobacillus* and *Enterococcus,* in which *Enterococcus* is quantitatively more important than *Lactobacillus* (Fig 4).

In the present study the genus *Lactobacillus* is quantitatively more important than the genera *Enterococcus* and *Lactococcus*. Meghoufel *et al*., (2017) reporte the dominance of the genus *Enterococcus* followed by the two genera *Leuconostoc* and *Lactobacillus* in traditional J’ben. Furthermore, the identification of lactic strains isolated from some dairy products by the same technique by Arezki *et al.,* (2019) showed the presence of the genus *Lactobacillus*. To the best of our kcnowledge there is no much studies about the isolation and identification of LAB from five types « J’ben

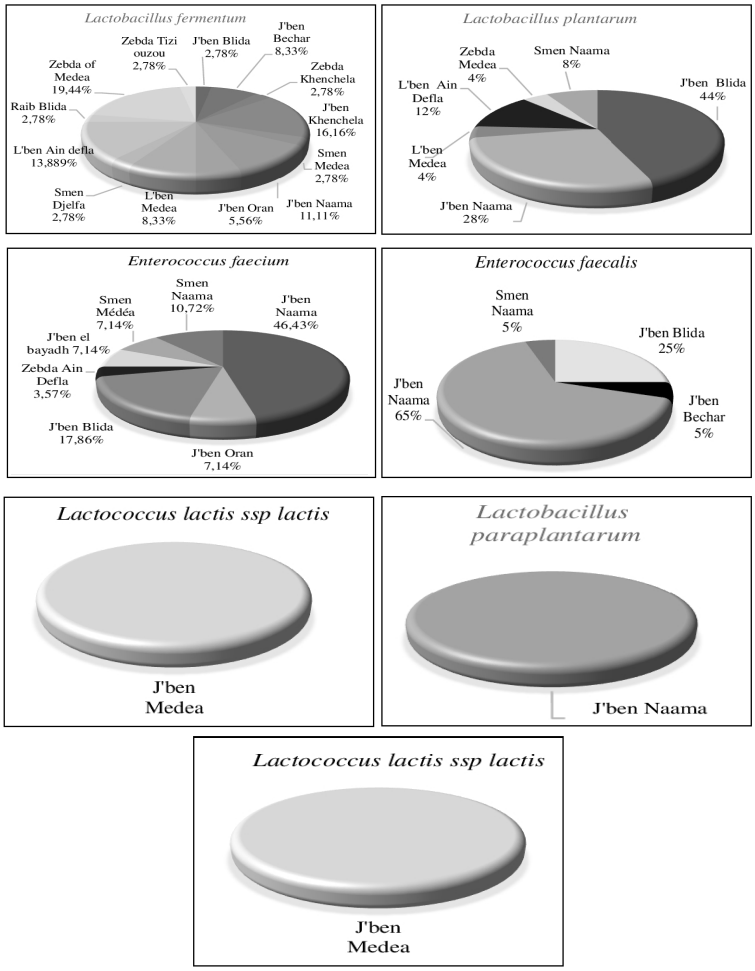

Fig 4: The distribution of each species in the five products analyzed with the regions.

Smen, l'ben, Raib and Zebda » of Algerian artisanal dairy products collected from several regions by the MALDI-TOF technique. This technique was used to identify LAB from a Spanish artisanal cheese, showing the dominance of the genus *Lactobacillus* which encompasses several species and subspecies compared to the genera *Lactococcus* and *Leuconostoc* (Sánchez-Juanes *et al.,* 2020). The identification the strains of lactic acid bacteria by the same technique isolated by Gantzias *et al.,* (2020) and by Nacef *et al.,* (2017) showed the predominance of the genus *Lactobacillus* with the presence of seven species with both genus *Enterococcus* and *Leuconostoc.* This last one goes along with our results in terms of the two species *Lactobacillus paracasei* ssp *paracasei*, *Lactobacillus plantarum* found in the sample "J'ben". However, Kanak and Yilmaz, (2019) have found that species of the genus *Enterococcus* were predominant in turkey artisanal cheese.

CONCLUSION

The present work aimed to determine the profil of lactic acid flora from five artisanal dairy products that exist in different regions of Algeria. The results showed that all isolates tested by MALDI-TOF MS were identified as lactic acid bacteria and confirmed that is a reliable technique comparable to phenotypic tests and has advantages over other genetic identification techniques in terms of rapidity and low cost. This work also confirm that dairy products can be a valuable source of lactic acid bacteria.

Conflict of interest: None.

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