Current Research in Microbial Sciences 6 (2024) 100235

Contents lists available at ScienceDirect

Current Research in Microbial Sciences

journal homepage: www.sciencedirect.com/journal/current-research-in-microbial-sciences

Insights into population adaptation and biodiversity of lactic acid bacteria in challenged date palm leaves silaging, using MALDI–TOF MS

Muhammad Zaid Jawaid, Mohammad Yousaf Ashfaq, Mohammad Al-Ghouti, Nabil Zouari [1,*]

Environmental Sciences Program, Department of Biological and Environmental Sciences, College of Arts and Sciences, Qatar University, P.O.B 2713, Doha, Qatar

ARTICLE INFO

Keywords:
Lactic acid bacteria
Silages
Palm tree leaves
MALDI–TOF MS
Dendrogram
PCA

ABSTRACT

The study focused on isolating indigenous Qatari lactic acid bacteria (LAB) from various challenged date palm tree leaf silages to construct a comprehensive strain collection, useful to study the diversity of these strains following their adaptation to the uncommon silage. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI–TOF MS) was employed for strain identification and differentiation. The diversity of LAB populations and strains was assessed through principal component analysis (PCA) and dendrogram analyses. A total of 88 LAB isolates were obtained from silages of fresh palm leaves, silage of mixed leaves and dairy feed, along with fresh palm tree leaves, and dairy feed, adapted to local harsh environments. These isolates were categorized according to the new classification of 2020, belonging to genera of *Pediococcus, Lactiplantibacillus plantarum, Lacticaseibacillus paracasei, Companilactobacillus farciminis, Limosilactobacillus oris, Limosilactobacillus vaginalis, Lactiplantibacillus pentosus* and *Lactobacillus johnsonii. Pediococcus* was the most prevalent genus, falling mostly within the species *Pediococcus lolii*. MALDI–TOF MS protein profiles, PCA, and dendrogram analyses successfully grouped the LAB isolates into five distinctive clusters based on the protein's similarities. The high diversity of the indigenous LAB in spontaneous palm leaf silages demonstrated their adaptation and mutualistic interactions, forming robust consortia that ensure the quality of the silage. The straightforward, quick, and accurate identification of LAB in this silage using MALDI–TOF MS presents a valuable approach for formulating LAB consortia for silaging harsh agricultural by-products.

1. Introduction

The role of Lactic acid bacteria~~'s~~ (LAB) ~~role~~ in preserving the natural balance among various species of microorganisms that inhabit the gastrointestinal and urogenital tracts along with the oral cavity of humans and animals is essential. Moreover, due to their ~~use~~ function as probiotics, they have great significance in food microbiology (Dec et al., 2014). Besides the role of LAB to help preservation of feed-stock and silage and inhibition of the proliferation of the harmful microorganisms, they exhibit benefits on the animals and their products (Du et al., 2023; Guo et al., 2020). Indeed, LAB in feed, enhance the feed digestibility and the nutrients quality, leading to higher concentrations of readily available nutrients to the animals (Wang et al., 2022). In addition, it was clearly shown that feeding the ruminants with LAB enriched feed, enhanced the quality and production of milk in dairy cows and improved growth and yield of meat-producing animals (El Jeni et al., 2023). Enriching feed with LAB, through silage processes, also contributed in reducing the risk of digestive disorders while promoting the overall animal health (Vieco-Saiz et al., 2019).

The quality of the silage depends on the accurate and quick identification of the growing LAB, to monitor the outcomes of the fermentation process, like beneficial LAB and corresponding drop of pH due to the production of volatile fatty acids by LAB. The LAB converts carbohydrates into organic acids during the ensilage process and dominate the medium by competition and adaptation routes. The lowering of the pH, hinders the growth of the undesirable microorganisms, leading to improving the quality and the nutritional content of the feed in challenging environmental circumstances (Grant and Adesogan, 2018). Indeed, the identification of LAB in silages is essential to control the fermentation process, due such high diversity. In 2020, Zheng et al. revised the taxonomy of LAB by analyzing their genomes alongside signature genes, their conserved amino acid identities, and considering other physiological and ecological factors (Zheng et al., 2020). Therefore, the genus *Lactobacillus* was reclassified into 25 genera comprising

* Corresponding author.
E-mail address: Nabil.Zouari@qu.edu.qa (N. Zouari).
[1] Senior

https://doi.org/10.1016/j.crmicr.2024.100235

Available online 15 April 2024

the genus *Lactobacillus*, including the *Lactobacillus delbrueckii, Paralactobacillus,* and 23 novel genera. In this study, this newly adopted taxonomy was used. However, precise, and rapid identification and differentiation of the LAB is a difficult task at the species level. Improving the process of microbial identification is in continuous demand in all sectors of science, which is precise, less time consuming and cost effective. Techniques like PCR (Polymerase Chain Reaction) and DNA sequencing target specific genetic regions, providing highly specific identification of bacteria. In addition, they may differentiate between closely related bacterial species that may have similar phenotypic characteristics. However, they are related to the highly conserved DNA regions characterizing the genus, species and sub-species, not the hole genome. Their accuracy relies on the availability and quality of reference databases, which may be incomplete or outdated for certain bacterial species. They can be expensive and require specialized equipment and expertise for sample preparation, amplification, and analysis (Aldi et al., 2019). However, Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a technique utilized for the identification of bacteria species, sub-species, and genus of bacterial isolates, based on the conserved proteins content and it generates a complete proteins profile for the strain, which may be compared to a database or among strains, analyzed at the same time (Nacef et al., 2017). The classification of bacteria through MALDI-TOF analysis relies on the mass spectra of the ribosomal proteins, assumed as the characteristics of each bacterial species (Seuylemezian et al., 2018). In comparison to other techniques used for microbial identification, MALDI–TOF MS is proving to be quicker, economical, and reliable, in numerous health and environmental sectors. MALDI-TOF MS requires a low sample size, without any pre-preparation in comparison to other PCR-based identification. The use of MALDI–TOF was reported by Karaduman et al. (2017) for LAB identification from non-commercial yogurts. The reliability of the findings was demonstrated, despite the use of the previous taxonomy. More interestingly, the protein profiles generated by MALDI-TOF for each strain, in parallel to score for identification, is multivariate data that can be statistically analyzed to distinguish between the strains., Indeed, MALDI-TOF analysis can be coupled to the principal component analysis (PCA), which is also a multivariate analysis tool ~~and~~ that can be used to eliminate redundancy in the data collection. PCA depicts the spectrum in 2D and 3D dimensions displaying the potential variation among the strains and groups them in clusters based on their high similarities. The differences between the strains can be investigated by using their proteins profiles to create a dendrogram. The dendrogram displays the strains with high proteins similarities in groups that can be classified as relatable strains. Both dendrogram and PCA can graphically depict the differences as well as the similarities (Alsaegh et al., 2021). Both are performant appropriate for the biodiversity studies. Indeed, the MALDI-TOF MS method coupled with PCA was shown reliable in the study of the diversity of the complex, dynamic, and adapted microbial populations in extreme environments, like sabkhas, mangrove sediments, and others (Abdelsamad et al., 2022; Alsaegh et al., 2021; Oualha et al., 2019).

With these potentials, MALDI-TOF MS can be used to replace the biochemical and molecular biology-based techniques, for the identification and categorization of bacteria (Seuylemezian et al., 2018). MALDI–TOF MS being robust and requiring a small sample size has made it more appealing to microbiologists. Its application has shown its reliability in many studies such as pathogen identification in blood, food, epidemiological studies, and water (Ashfaq et al., 2022; Singhal et al., 2015; Wieser et al., 2011).

The use of MALDI-TOF to identify LAB and follow their occurrence and abundance in silage for a better ensiling process, ensuring the conservation of feedstock becomes of high utility, especially for improving the nutritional quality of unconventional agricultural by-products, suffering from non-reproducibility and non-useful bacterial growth (Lorenzo et al., 2018). Since ensilage is a natural process under the influence of varieties of factors, the population dynamics and the growth of the dominating LAB are random, if highly adapted LAB are not introduced (Sharma et al., 2020). In addition, it is not always easy to act on contamination in the field, the harsh climate conditions, the impact of previous crops, the work carried out on the ground, the composition which would vary with season among others. Indeed, although LAB are already found naturally on the leaves and stems of fodder, they may change under the effect of these factors (Gómez-Camacho et al., 2021). It is always speculated and supposed that they should adapt to all the random factors, employing their potential of competition, antagonism, and adaption (Gómez-Camacho et al., 2021). Palm leaves are complex feedstocks, which should undergo a similar future during spontaneous silage. It becomes now necessary to demonstrate the potentials of LAB in silage processes of complex biomass to establish a strategy of selection of inoculated LAB to silage. The objective of this research is to study the diversity, population adaptation and interactions of LAB in fresh and fermented palm leaves, for a future orientation of their silage processing. The main hypothesis is that the naturally existent LAB, would adapt in the challenging silages based on palm tree leaves, and develop mutualistic interactions. MALDI-TOF MS and PCA are used to explain the adaptation and biodiversity in consortia formed based on mutualistic interactions. This study would help in the formulation of LAB consortia, appropriate to perform silages based on uncommon agricultural by-products such as date palm tree leaves, growing in harsh environmental conditions (Qatar).

2. Material and methods

2.1. Date palm leaves, feed, and silage for the isolation of LAB

To isolate the LABs from the Qatari environment, 5 different sources were used (1) fresh palm leaves; (2) cow dairy feed; (3) spontaneous silage of only palm tree leaves; (4) spontaneous silage of only cow dairy feed, and (5) spontaneous silage of mixed palm leaves with dairy cow feed (50% w/w). The composition of the palm tree leaves was provided by Jawaid et al. (2023). The cow dairy feed was kindly provided by a local cow milk and meat-producing company. The composition, provided by the producer, contains (% dry matter): 30, soybean meal; 20, maize; 18 carob pods; 17 wheat bran; 10 sunflower meal; supplemented with calcium phosphate, calcium carbonate, vitamin and trace mineral premix (personal communication). The spontaneous silages were incubated at 38 °C, which is the average temperature in Qatar. Palm tree leaves were cut into 1 cm^2 pieces and mixed, if any, with the cow dairy feed at a 50 % ratio (w/w). Ensiling was performed in 1000 ml well-piled silages (leaves pieces or a mixture of leaves and dairy feed, or dairy feed only) mixed with 200 ml water before being filled in the 1000 ml glass bottles. The bottles were hermetically closed prior to incubation at 38 °C for 28 d

2.2. Isolation of lactic acid bacteria

The LAB isolation process was done through enrichment cultures with an MRS liquid medium at an acidic pH of 5.7. One gram of each sample was separately put in 15 ml liquid MRS, in a 50 ml sterile tube and then incubated in a shaker at 200 rpm at 38 °C for a period of 48 h. After the incubation, 100 μl was sampled and plated on a solid MRS medium for the isolation of all the separate colonies, at aerobic conditions for the fast growth of LAB. A serial dilution was performed if necessary to reduce the quantity of colonies on the plate. Purification of the isolates from the colonies was performed after 5 successive plating with the use of a single separate colony in each. After the 5 purification runs, 1 colony was isolated, coded, and used for the preservation of vegetative cells in Luria-Bertani medium containing 30% glycerol (v/v) at -70 °C, until further use.

The growth at the aerobic conditions was evaluated by incubation of the plates in the incubator, while the growth at the anaerobic conditions was evaluated by incubation of the plates in a Jar in which the oxygen

was depleted by burning a candle, then the jar was introduced in the incubator. Indeed, the plates were incubated for 16 h only, and the size of the colonies is was an indicator of the preference of the strain for aerobic or anaerobic conditions.

2.3. Determination of the pH of the solid samples

By combining 2 g of each sample with 50 ml of distilled water and homogenizing it for 1 h, the sample's pH was ascertained. The supernatant from the centrifuge mixture (15 min at 2000xg) was used to measure the pH (Tobaruela et al., 2018).

2.4. Sample preparation for MALDI–TOF MS analysis

Ethanol and formic acid, in equal quantities, were used for the protein extraction. After being suspended in 300 μL of sterile water, a single colony was then resuspended in 900 μL of pure ethanol. 1 mL of formic acid (70 %) and 1 mL of acetone (100 %), after centrifuging at 10,000 rpm for 5 min, were added to the pellet. 1 μL of the supernatant was added to the biotarget 48 sample spot following centrifugation. 1 μL of alfa-cyano-4-hydroxycinnamic acid (HCCA) matrix in ultrapure water containing 50 % acetonitrile and 2.5 % trifluoroacetic acid was added to the supernatant for protein crystallization. Following that, the equipment created mass fingerprints, generating protein profiles and MALDI–TOF MS scores for all samples. The machine used in this work was MALDI Biotyper (Bruker Daltonics, Leipzig, Germany) associated to a RTC 3 software and database.

2.5. Mass spectra acquisition

The protein profiles were created as mass spectra by Bruker Flex Control program with acceleration and a source voltage set at 20 kV and 18.7 kV, respectively. For each spectrum, 240 laser shots were acquired from different spots of the sample in 10-shot spots. For each strain, two sample spots were used and three profiles were obtained for each spot. Hence, a total of 6 profiles were obtained for each strain which was subsequently analyzed using Flex Analysis software and Biotyper RTC 3 software.

2.6. Identification of the bacterial isolates by MALDI-TOF MS

Utilizing MALDI-TOF MS, isolated strains were identified. To distinguish between the strains the bacterial isolates were run through a reference dataset for identification, hence, ribosomal proteins (proteins highly sustained across species, sub-species, and genera) were utilized. The outcomes are supplied as scores based on similarities between the mass spectra of the various strains in the database.

2.7. Studying the diversity of the strains through principal component analysis (PCA)

For the analysis of the strain protein profile, MALDI Biotyper software (v3.0) was used resulting in PCA analysis using default algorithms. Prior to analysis, the individual protein profiles were preprocessed i.e., the baseline was subtracted and smoothed. The original information of the data was maintained while reducing the dimensionality using PCA. The mass spectra generated were multivariate data with each signal depicting distinct molecular dimensions. For the differentiation, PCA clusters and dendrogram groups were created and classified based on protein profiles. Only proteins of m/z higher than 2000 were considered. The results are graphically illustrated in PCA and dendrogram showing species having high similarities aggregated and grouped together (Al-Kaabi et al., 2018).

3. Result and discussion

3.1. Isolation and identification of LAB

Enrichment cultures in liquid MRS media were used for LAB isolation from fresh palm leaves and performed silages, based on palm leaves. This enrichment approach was used to select bacteria in the samples, that could grow in the MRS medium, at a pH of 5.7, selective to LAB. Fresh palm leaves, dairy feed, a mixture of palm leaves and feed for spontaneous silages, and 50 % (w/w) amalgam of palm leaves and diary feed were used. Since the LABs are facultative bacteria and grow more quickly in aerobic environments, the isolation was carried out in these circumstances. The numbers of pure strains are given in Table 1. The pH of the spontaneous silage varied from 5.71 in leaves-silage to 3.67 with feed-silage, while it was 4.12 in the silage of 50 % mixed leaves and feed, depicting low growth of LAB in fresh leaves in comparison to silages, as expected. In total, 117 pure LAB isolates were isolated for identification with the highest number of isolates (83) obtained from the spontaneous mixed silages. The number of isolates obtained from fresh leaves, not ensiled feed, ensiled leaves, and ensiled feed was low.

The isolated strains' MALDI–TOF scores were determined, serving for their identification (Table 2). The score indicates how accurate the identification of the strains is performed. Indeed, the score varies from 0 to 3, and if the scores is below 1.700 the strains are not detected. A score of 1.700–2.000 shows that the strain identification is accurate at the genus level. A score more than 2.000 and less than 2.300 implies genus and probably species identification. A score greater than 2.300 shows a high likelihood of species-level identification (Abdel Samad et al., 2020). It is clear that the genus *Staphylococcus* ($n = 29$), represented all the strains isolated from the fresh palm tree leaves ($n = 15$), dairy feed ($n = 9$), and some of the isolated strains from the mixed silage ($n = 5$). LAB was only present in the silages. All were cultured in aerobic and anaerobic conditions. The identified strains showing higher growth at aerobic conditions were 58 and those showing higher growth at anaerobic conditions were 30. These later 30 strains were identified as *Lacticaseibacillus paracasei* ($n = 13$), *Limosilactobacillus oris* ($n = 5$), *Limosilactobacillus vaginalis* ($n = 7$), and *Lactiplantibacillus plantarum* ($n = 5$). The 58 strains that showed higher growth at aerobic conditions were identified as *Lactiplantibacillus plantarum* ($n = 7$), *Lactobacillus jhonsonii* ($n = 1$), *Lactiplantibacillus pentosus* ($n = 1$), *Companilactobacillus farciminis* ($n = 16$), and *Pediococcus lolii* ($n = 33$). Notably, *Pediococcus lolii* in particular is better adapted to the mixture of palm leaves and dairy feed, than *Companilactobacillus farciminis* and *Lactiplantibacillus plantarum*. They may be considered adapted to the silage composition and forming populations with mutualistic interactions for co-existence. In addition, the dependence on aeration of their growth is variable as shown by the colony size in plates, after 16 h incubation.

The identification was based on the score of each strain with reference to the database. The scores depict the importance of evaluating the identification accuracy of the LAB strains at genus and species levels, as well as differentiating between strains from the same species. Indeed, *Pediococcus lolii* is represented by 33 isolates, 6.2 % of them identified with scores higher than 2.3, while 56.2 % have scores ranging between 2.0–2.3 and 37.5% between 1.7–2.0. Twelve *Lactiplantibacillus plantarum* strains were isolated with 8.33 % of scores higher than 2.3, 41.6 %

Table 1
The number of isolated strains from different sources.

Source	pH, at the isolation time	Number of bacterial isolates at the aerobic conditions
Fresh palm leaves	6.34	15
Not ensiled dairy feed	7.12	9
Ensiled palm leaves	5.71	3
Ensiled feed	3.67	7
Ensiled 50% w/w (leaves/feed)	4.12	83

Table 2
Isolated strains identified by MALDI TOF MS. n: number of corresponding species.

Source	Identification	Strain code
Palm leaves	*Staphylococcus galliranum*	SMZ1, SMZ5, SMZ12, SMZ13, SMZ14, SMZ23, SMZ27, and SMZ28
	Staphylococcus epidermidis	SMZ6, SMZ10, SMZ11, and SMZ32
	Staphylococcus warneri	SMZ8, SMZ9 and SMZ36
Dairy feed	*Staphylococcus galliranum*	SMZ2, SMZ3, and SMZ4,
	Staphylococcus epidermidis	SMZ7, SMZ21, and SMZ22
	Staphylococcus capitis	SMZ29, SMZ30 and SMZ33
Palm leaves silage	*Lactiplantibacillus plantarum* ($n = 1$)	SMZ46
	Pediococcus lolii ($n = 2$)	SMZ41 and SMZ43
Feed silage	*Lactiplantibacillus plantarum* ($n = 2$)	SMZ104 and SMZ105
	Pediococcus lolii ($n = 5$)	SMZ107, SMZ108, SMZ109, SMZ110 and SMZ112
Mixed silage	*Lactiplantibacillus plantarum* ($n = 3$)	SMZ19, SMZ102, SMZ103, SMZ106, SMZ53, SMZ34, SMZ25, SMZ39, and SMZ35
	Lacticaseibacillus paracasei ($n = 10$)	SMZ15, SMZ115, SMZ119, SMZ117, SMZ116, SMZ118, SMZ120, SMZ121, SMZ20, SMZ24, SMZ50, SMZ78, SMZ79.
	Companilactobacillus farciminis ($n = 16$)	SMZ56, SMZ65, SMZ69, SMZ73, SMZ72, SMZ70, SMZ71, SMZ57, SMZ62, SMZ61, SMZ64, SMZ60, SMZ63, SMZ57, SMZ59, SMZ58 and SMZ122
	Limosilactobacillus oris ($n = 5$)	SMZ84, SMZ81, SMZ83, SMZ80 and SMZ82.
	Limosilactobacillus vaginalis ($n = 7$)	SMZ91, SMZ86, SMZ88, SMZ90, SMZ87, SMZ89, and SMZ85
	Lactiplantibacillus pentosus ($n = 1$)	SMZ74
	Lactobacillus johnsonii ($n = 1$)	SMZ47
	Pediococcus lolii ($n = 26$)	SMZ48, SMZ51, SMZ68, SMZ75, SMZ52, SMZ77, SMZ49, SMZ54, SMZ67, SMZ55, SMZ66, SMZ92, SMZ76, SMZ100, SMZ95, SMZ101, SMZ94, SMZ111, SMZ96, SMZ114, SMZ98 SMZ93, SMZ113, SMZ99, and SMZ98
	Staphylococcus warneri	SMZ16, SMZ17, and SMZ18
	Staphylococcus galliranum	SMZ26 and SMZ31

of scores between 2.0–2.3, and 50 % of scores between 1.7–2.0. Among the 13 isolates of *Lacticaseibacillus paracasei,* 38.46 % of the strains were identified with scores higher than 2.3, while 53.8 % were identified with scores between 2.0–2.3, and 7.6 % with scores between 1.7–2.0. All 16 isolates of *Companilactobacillus farciminis* were identified with scores between 1.7–2.0. The isolates of *Limosilactobacillus vaginalis* identified with scores between 2.0–2.3 represent 85.71 % while 14.28 % of the isolates were identified with scores between 1.7–2.0. Of the strains identified as *Limosilactobacillus oris*, 40 % had scores between 2.0–2.3, and 60 % of scores between 1.7–2.0. Single strains of *Lactobacillus johnsonii* were identified with a score between 2.0–2.3 and *Lactiplantibacillus pentosus* with scores between 1.7–2.0. The results are summarized in Fig. 1, which shows that *Lacticaseibacillus paracasei,* having the highest percentage of isolates with a score higher than 2.3 followed by *Lactiplantibacillus plantarum* and *Pediococcus lolii*. These results clearly show the appropriateness of MALDI-TOF methods for the accurate identification of LAB at the species level.

On the other hand, the results show that application MALDI-TOF MS to control LAB dynamic populations during the ensilage processes would be an alternative. Indeed, silage is prone to microbial spoilage, and LAB play a crucial role in its fermentation process. In this study, MALDI-TOF offered assessment of the LAB in silage of palm-trees leaves. To truly appreciate the remarkable quality of the obtained silages, it's essential to explore the significance of each LAB species in the final product. LAB are pivotal players in the ensiling process, exerting profound effects on silage fermentation, preservation, and nutritional value. Different LAB species exhibit varying metabolic activities and preferences. For instance, species like *Lactobacillus plantarum* are renowned for their rapid acidification capabilities, facilitating a swift drop in pH and ensuring efficient preservation. On the other hand, *Pediococcus pentosaceus* contributes to flavor development through its production of aromatic compounds, enhancing the palatability of the silage. The results demonstrate that the silage based on palm trees may be rich of the most interesting LAB, searched for the final quality. Indeed, the interest of each of the LAB species in the silage is reported. *Lactiplantibacillus plantarum* is a facultatively homofermentative lactic acid bacterium. Due to its vigorous lactic acid fermentation, it is frequently used (Corsetti and Valmorri, 2011). Another species from homofermentative LAB is *Lacticaseibacillus paracasei* known to hydrolyze starch into simple lactic acid, glucose, and carbohydrates (Gobbetti and Minervini, 2014). The survivability of this species in pH below 2.0 makes it highly resistant in the alimentary canal. *Lacticaseibacillus paracasei* in feed can improve organism performance by increasing protein and carbohydrate metabolism (Gobbetti and Minervini, 2014). *Companilactobacillus farciminis* is a probiotic that has been claimed to be utilized as a stress reliever because of the colonial lumen's spontaneous release of nitric acid (Ait-Belgnaoui et al., 2009). Stress-induced hypersensitivity is reported to be reduced in animals when *Companilactobacillus farciminis* is found in feed (Ait-Belgnaoui, 2006). *Lactobacillus johnsonii* has been reported to coexist with numerous other microbes in the intestines of numerous organisms (Pridmore et al., 2004). *Lactobacillus johnsonii* is "acidophilus complex" of the *Lactobacillus* genus, involved in probiotic activities, and helpful to the wellbeing of animal and human health. Pathogen inhibition and immune modulation are also effectively characterized by

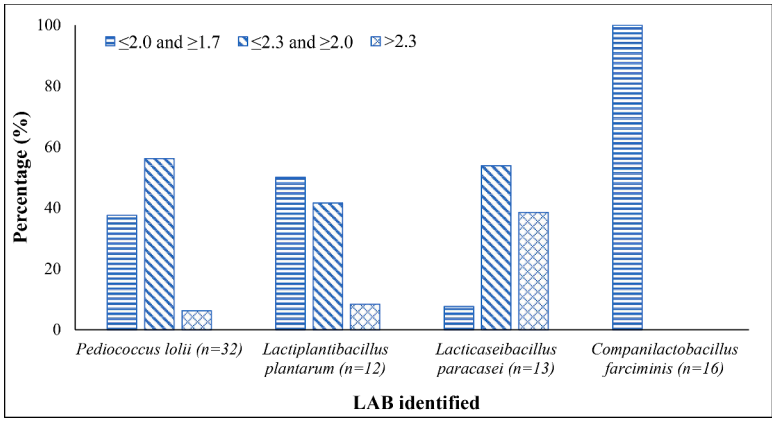

Fig. 1. Distribution of the isolated strains based on the MALDI-TOF MS.

Lactobacillus johnsonii (Pridmore et al., 2004). *Lactiplantibacillus pentosus* is a LAB that is facultatively homofermentative and a probiotic that is most commonly found in the gastrointestinal tract and demonstrates higher growth in the aforementioned conditions (Abriouel et al., 2017). *Lactiplantibacillus pentosus* can defend itself against pathogenic bacteria by fermenting prebiotics and lactose. *Lactiplantibacillus pentosus* is extremely reliant to its environmental habitat (Abriouel et al., 2017). These authors showed that Lactiplantibacillus pentosus strains were also isolated from diverse environmental niches like fermented products and food, and silage of corn. Interestingly, several strains of *Lactiplantibacillus pentosus* exhibit health-promoting properties (antiproliferative and immunomodulatory activities). Some of them are potential probiotic strains. *Limosilactobacillus vaginalis* is often detected in dairy animals' uteri. It contributes to the health of vaginal ecosystem by functioning as the first line of defense by releasing acids and antibacterial molecules (Gärtner et al., 2015). *Limosilactobacillus oris* is widely found in the oral cavity of animals and humans. *Limosilactobacillus oris* has a high survivability in harsh conditions with pH as low as 1.0, making its survival easy in gastrointestinal tract (Afrin et al., 2021). The latter authors observed *Limosilactobacillus oris* consistently reduced harmful microbes. *Pediococcus lolii* is a facultative homofermentative LAB usually employed as a silage inoculant. Due to its high similarities with other LABs, based on phenotypically and genotypically, it is often used in food products (Crow and Curry, 2002).

3.2. Biodiversity of the isolated LAB strains

The PCA analysis using the protein profiles illustrated large biodiversity among the strains (Fig. 2). The three major components PC1, PC2, and PC3, vary by 28 %, 13 %, and 11 % among strains, for a total of 52 % diversity. The three main components generate five cluster groups of LAB isolates. Each cluster represents a species with high similarity, and the distance between clusters depicts variation at the group level. The distance within a group reveals differences at the strain level. Cluster 1 contains 13 *Companilactobacillus farciminis* isolates with a negative association with PC1 and a positive correlation with PC2 and PC3 (SMZ56-SMZ63, SMZ69, SMZ71-SMZ73, SMZ122). These 13 isolates are among the 16 *Companilactobacillus farciminis* isolates from mixed silage. Cluster 2 contains nine isolates of *Lacticaseibacillus paracasei* (SMZ115-SMZ117, SMZ20, SMZ119-SMZ121, SMZ50, SMZ79) and one isolate of *Companilactobacillus farciminis* (SMZ70), having positive association with PC1 and PC2 and negative with PC3. Cluster 3 contains a single strain of *Lacticaseibacillus paracasei* (SMZ78), three strains of *Lactiplantibacillus plantarum* (SMZ105, SMZ102, SMZ104), two strains of *Companilactobacillus farciminis* (SMZ65, SMZ64) and 32 *Pediococcus lolii* (SMZ41, SMZ75-SMZ77, SMZ92-SMZ101, SMZ107-SMZ114, SMZ49, SMZ66-SMZ68, SMZ48, SMZ51, SMZ52, SMZ43, SMZ54, SMZ55). A single strain of *Lactobacillus johnsonii* (SMZ47), nine strains of *Lactiplantibacillus plantarum* (SMZ19, SMZ103, SMZ25, SMZ106, SMZ37, SMZ53, SMZ34, SMZ46, SMZ39) and three strain of *Lacticaseibacillus paracasei* (SMZ24, SMZ15, SMZ118) make up Cluster 4 having negative association with all components. One strain of *Lactiplantibacillus pentosus* (SMZ74), each isolate of *Limosilactobacillus oris* (SMZ80-SMZ84), and *Limosilactobacillus vaginalis* (SMZ85-SMZ91) are found in Cluster 5 which has negative association with all the components. The differences between cluster 4 and cluster 5, having both negative correlations with all three components.

As can be seen, similar LAB species are clustered together in the same clusters. For example, Cluster 1 has *Companilactobacillus farciminis*, Cluster 2 contains *Lacticaseibacillus paracasei,* and Cluster 4 contains

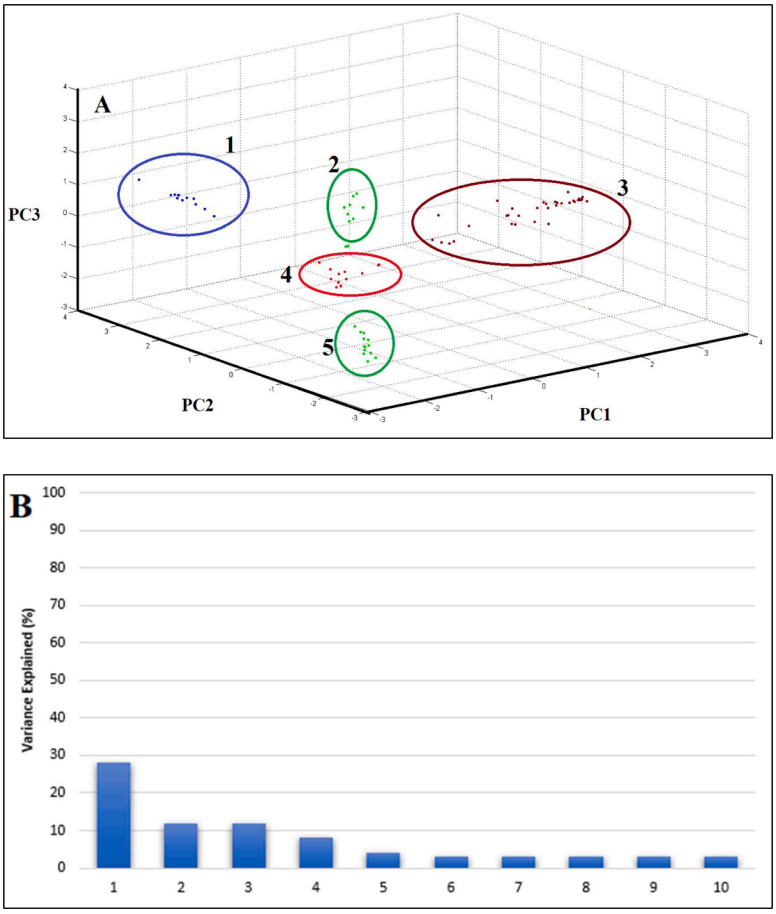

Fig. 2. PCA classification of LAB strains (A) and PCA's share of the variation explained (B).

Lactiplantibacillus plantarum., Cluster 3 was found to be the most diverse containing 4 four distinct LABs *Companilactobacillus farciminis, Lacticaseibacillus paracasei, Lactiplantibacillus plantarum,* and *Pediococcus lolii,* all showing highly comparable protein profiles. Three separate LAB species, exhibiting strong resemblance to one another make up Cluster 5, including *Lactiplantibacillus pentosus, Limosilactobacillus oris,* and *Limosilactobacillus vaginalis*.

The related dendrogram, which serves as a phylo-proteomic tree, was produced as a result of the PCA analysis (Fig. 3). Based on similarities, the isolated strains were divided into five groups. Group I contain *Pediococcus lolii* (SMZ68) and Thirteen *Companilactobacillus farciminis* (SMZ57, SMZ58, SMZ60-SMZ65, SMZ70-SMZ73, SMZ122) and Group II contains one *Pediococcus lolii* (SMZ75) and Twelve *Lacticaseibacillus paracasei* (SMZ15, SMZ50, SMZ24, SMZ79, SMZ20, SMZ115-SMZ121). Group III comprised One *Lactiplantibacillus pentosus* (SMZ74), five *Limosilactobacillus oris* (SMZ80-SMZ84), and seven *Limosilactobacillus vaginalis* (SMZ85-SMZ91). In group IV, there are Eight *Lactiplantibacillus plantarum* (SMZ19, SMZ106, SMZ46, SMZ25, SMZ53, SMZ34, SMZ39, SMZ37) as well as one strain each of *Pediococcus lolii* (SMZ108) and *Lactobacillus johnsonii* (SMZ47). Group V includes 2 *Pediococcus lolii* (SMZ109 and SMZ112) and *Lactiplantibacillus plantarum* (SMZ103). Group VI included 24 *Pediococcus lolii* (SMZ107, SMZ41, SMZ108, SMZ43, SMZ110-SMZ114, SMZ48, SMZ92, SMZ51, SMZ67, SMZ49, SMZ52, SMZ66, SMZ77, SMZ55, SMZ54, SMZ51, SMZ76, SMZ52, SMZ92-SMZ101, SMZ67) three *Companilactobacillus farciminis* (SMZ69, SMZ59, SMZ56), 3 *Lactiplantibacillus plantarum* (SMZ104, SMZ102, SMZ105) and *Lacticaseibacillus paracasei* (SMZ78).

In terms of grouping of the LAB isolates the PCA and dendrogram results showed similar results. Based on the substantial similarities in terms of protein profiles, *Limosilactobacillus oris, Limosilactobacillus vaginalis,* and *Lactiplantibacillus pentosus* displayed similarities in both dendrogram and PCA. The isolated strains of *Companilactobacillus farciminis* have been classified into single groups in PCA and dendrogram, demonstrating high similarity. Similarly, *Lacticaseibacillus paracasei* isolates have also been clustered together, demonstrating a high degree of similarities amongst the isolates of this species. *Lactiplantibacillus plantarum* and *Lactobacillus johnsonii* share similarities in both PCA and dendrogram. Tiani et al. (2017) have reported that *Lactiplantibacillus plantarum* and *Lactobacillus johnsonii* share similar environmental conditions, as found in the intestinal tract, secrete antimicrobial substances and limit the growth of the pathogens. As shown in both PCA and dendrogram graphs, the isolated strains of *Pediococcus lolii* share similarities with other LABs, such as *Companilactobacillus farciminis, Lacticaseibacillus paracasei,* and *Lactiplantibacillus plantarum*. Phenotypically the LABs vary greatly with certain species resembling more than others (Crow and Curry, 2002). As previously described, *Pediococcus* and *Lactobacillus* both exhibit comparable growth traits and transformation patterns (Bosma et al., 2017). *Pediococcus lolii, Lactiplantibacillus plantarum,* and *Lacticaseibacillus paracasei* have been reported by Bosma et al. (2017) to share similarities with one another (similar to this research). *Pediococcus lolii* isolates have similarities to *Lactobacilli* species based on the findings of the identification. Both in PCA and dendrogram, the isolated SMZ68 of *Pediococcus lolii* was shown to be associated with *Companilactobacillus farciminis.* In both the dendrogram and PCA, the isolated SMZ75 was placed alongside *Lacticaseibacillus paracasei*. Similarly, *Lacticaseibacillus paracasei* was discovered, in both dendrogram and PCA, to be in the same group. Similar to PCA, in Cluster 3 high quantity of *Pediococcus lolii* was found in the dendrogram as well, with sharing similarity with same *Lactobacilli* species. In a study reported by Santos et al. (2019), LAB strains from the genera *Lactobacilli* and *Pediococcus* shared similarities with one another. Based on phylogenic connections, the results show that the genera *Lactobacilli* and *Pediococcus* are highly relatable to one another (Puntillo et al., 2020). Sade & Bjorkroth (2019) reveal comparative findings of the similarities of the

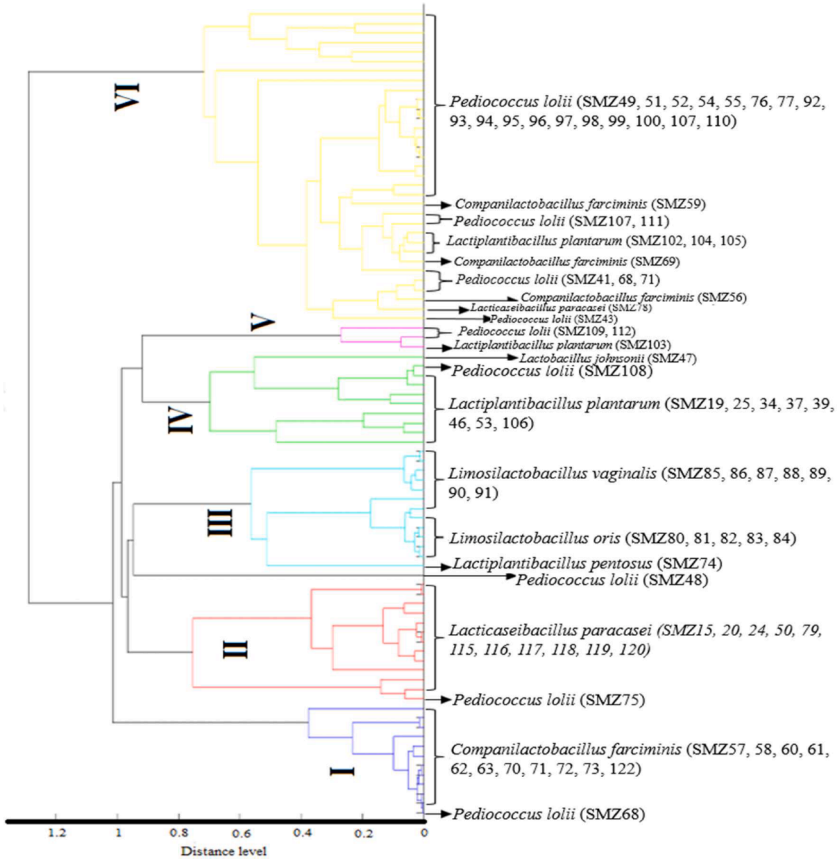

Fig. 3. Dendrogram showing the grouping of 87 strains of LABs determined by MALDI-TOF MS.

general *Lactobacilli* and *Pediococcus* due to their close phylogenetic relationship. It is demonstrated that *Lactobacillus* and *Pediococcus*, based on intergenic spacer region between 16S and 23S genes, share characteristics.

LAB is widely found in silages that include a significant number of different species of the genus *Lactobacilli*. Along with *Lactobacilli*, a large number of genera of *Pediococcus* species were found in spontaneous silage. From this study, preparing silages using palm leaves with diary feed can evidently encourage the growth of LABs and create quality silages. Interestingly, these LABs inhibit the growth of undesirable bacteria as was shown with *Staphylococcus* strains.

4. Conclusion

The study showed that the prospect of producing silage from palm tree leaves presents an intriguing opportunity, particularly in regions where palm cultivation is abundant. Palm leaves, rich in fibers and nutrients, offer potential as a valuable feed source for livestock. However, their utilization in silage production necessitates careful consideration of microbial dynamics, particularly regarding LAB. By implementing precise control measures, such as inoculating with selected LAB strains or adjusting ensiling conditions to favor LAB growth, it's possible to ensure optimal fermentation and preserve the nutritional integrity of the silage. This approach not only enhances the palatability and digestibility of the final product but also maximizes nutrient retention, offering a sustainable solution for enhancing feed availability and livestock productivity in palm-growing regions. Applying to LAB, MALDI–TOF MS has demonstrated to be a precise, quick, reliable, and economical technique. In this research, it allowed to demonstrate that a high diversity of LAB may be associated to ensilaging process of palm tree leave, with 88 different LAB strains able to occur in the silage. They belong to almost the major LAB species. However, similarities and differences were demonstrated, which may not easily allow to predict the evolution of the silage quality. Following our approach, based on MALDI-TOF MS, it would be possible to select the appropriate endogenous strains with the most representative strains within the determined LAB clusters to inoculate palm tree leaves for an oriented ensilage process and to ensure a final quality of the silages. Combining fast molecular techniques with bioprocesses of silage will have considerable practical significance.

CRediT authorship contribution statement

N.Z. conceived the original idea. N.Z., M.A., and M.Y.A. planned the experiments and wrote the manuscript. M.Z.J. and partially MYA conducted the experiments. N.Z. took care of funding acquisition, supervision, and project administration.

Funding

The publication of this article was funded by the Qatar National Library.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors acknowledge all the staff of the Central Unit Laboratory of the College of Arts and Sciences at Qatar University, for providing support performing several chemical characterizations.

References

AbdelSamad, R., Al Disi, Z., Ashfaq, M., Zouari, N., 2020. The use of principle component analysis and MALDI-TOF MS for the differentiation of mineral forming *virgibacillus* and *Bacillus* species isolated from Sabkhas. RSC Adv. 10, 14606–14616. https://doi.org/10.1039/D0RA01229G.

Abdelsamad, R., Al Disi, Z., Abu-Dieyeh, N., AlGhouti, M., Zouari, N., 2022. Evidencing the role of carbonic anhydrase in the formation of carbonate minerals by bacterial strains isolated from extreme environments in Qatar. Heliyon 8 (10), e11151. https://doi.org/10.1016/j.heliyon.2022.e11151.

Abriouel, H., Pérez Montoro, B., Casimiro-Soriguer, C.S., Pérez Pulido, A.J., Knapp, C.W., Caballero Gómez, N., Castillo-Gutiérrez, S., Estudillo-Martínez, M.D., Gálvez, A., Benomar, N., 2017. Insight into potential probiotic markers predicted in *Lactobacillus pentosus* MP-10 genome sequence. Front. Microbiol. 8, 00891. https://doi.org/10.3389/fmicb.2017.00891, 2017.

Afrin, S., Akter, S., Begum, S., Hossain, M.N., 2021. The prospects of *Lactobacillus Oris* as a potential probiotic with cholesterol-reducing property from Mother's Milk. Front. Nutr. 8, 619506 https://doi.org/10.3389/fnut.2021.619506.

Ait-Belgnaoui, A., 2006. *Lactobacillus farciminis* treatment suppresses stress induced visceral hypersensitivity: a possible action through interaction with epithelial cell cytoskeleton contraction. Gut 55 (8), 1090–1094. https://doi.org/10.1136/gut.2005.084194.

Ait-Belgnaoui, A., Eutamene, H., Salvador-Cartier, C., Durand, H., Houdeau, E., Fioramonti, J., Bueno, L., Theodorou, V., 2009. P.35 Un traitement probiotique (*Lactobacillus farciminis*) atténue la réponse au stress de l'axe hypothalamo-hypophyso-surrénalien (HPA chez Le Rat. Gastroentérologie Clinique Et Biologique 33 (3). https://doi.org/10.1016/s0399-8320(09)72667-3.

Al Disi, Z., Zouari, N., Dittrich, M., Jaoua, S., Al Saad Al-Kuwari, H., Bontognali, T., 2019. Characterization of the extracellular polymeric substances (EPS) of *Virgibacillus strains* capable of mediating the formation of high Mg-calcite and protodolomite. Mar. Chem. 216, 103693 https://doi.org/10.1016/j.marchem.2019.103693, 2019.

Al-Kaabi, N., Al-Ghouti, M.A., Oualha, M., Mohammad, M.Y., Al-Naemi, A., Sølling, T.I., Al-Shamari, N., Zouari, N., 2018. A MALDI-TOF study of bio-remediation in highly weathered oil contaminated soils. J. Petrol. Sci. Eng. 168, 569–576. https://doi.org/10.1016/j.petrol.2018.05.013.

Alsayegh, S.Y., Disi, Z.A., Al-Ghouti, M.A., Zouari, N., 2021. Evaluation by MALDI-Tof MS and PCA of the diversity of biosurfactants and their producing bacteria, as adaption to weathered oil components. Biotechnol. Rep. 31, e00660. https://doi.org/10.1016/j.btre.2021.e00660.

Ashfaq, M.Y., Da'na, D.A., Al-Ghouti, M.A., 2022. Application of MALDI-TOF MS for identification of environmental bacteria: a review. J. Environ. Manage. 305 https://doi.org/10.1016/j.jenvman.2021.114359.

Bosma, E.F., Forster, J., Nielsen, A.T., 2017. *Lactobacilli* and *Pediococci* as versatile cell factories – evaluation of strain properties and genetic tools. Biotechnol. Adv. 35 (4), 419–442. https://doi.org/10.1016/j.biotechadv.2017.04.002.

Corsetti, A., Valmorri, S., 2011. Lactic acid bacteria *Lactobacillus* spp.: *lactobacillus plantarum*. Encyclop. Dairy Sci. 111–118. https://doi.org/10.1016/b978-0-12-374407-4.00263-6.

Crow, V., Curry, B., 2002. *Pediococcus* spp. Encyclop. Dairy Sci. 2245–2247. https://doi.org/10.1016/b0-12-227235-8/00762-8.

Dec, M., Urban-Chmiel, R., Gnat, S., Puchalski, A., Wernicki, A., 2014. Identification of lactobacillus strains of goose origin using MALDI-Tof Mass Spectrometry and 16S–23S rdna intergenic spacer PCR analysis. Res. Microbiol. 165 (3), 190–201. https://doi.org/10.1016/j.resmic.2014.02.003.

Du, Z., Yang, F., Fang, J., Yamasaki, S., Oya, T., Nguluve, D., Kumagai, H., Cai, Y., 2023. Silage preparation and sustainable livestock production of natural woody plant. Front. Plant Sci. 14 https://doi.org/10.3389/fpls.2023.1253178.

El Jeni, R., Villot, C., Koyum, O.Y., Osorio-Doblado, A., Baloyi, J.J., Lourenco, J.M., Steele, M., Callaway, T.R., 2023. Probiotic" approaches to improving dairy production: reassessing "magic foo-foo dust. J. Dairy Sci. https://doi.org/10.3168/jds.2023-23831.

Gärtner, M.A., Bondzio, A., Braun, N., Jung, M., Einspanier, R., Gabler, C., 2015. Detection and characterisation of *Lactobacillus* spp. in the bovine uterus and their influence on bovine endometrial epithelial cells in vitro. PLoS ONE 10 (3), 0119793. https://doi.org/10.1371/journal.pone.0119793.

Gobbetti, M., Minervini, F., 2014. Lactobacillus casei. Encyclop. Food Microbiol. 432–438. https://doi.org/10.1016/b978-0-12-384730-0.00180-4.

Gómez-Camacho, C.E., Alborch, K.P., Bockisch, A., Neubauer, P., Junne, S., Ruggeri, B., 2021. Monitoring the physiological state in the dark fermentation of maize/grass silage using flow cytometry and electrooptic polarizability measurements. Bioenergy Res. 14, 910–923. https://doi.org/10.1007/s12155-020-10184-x.

Grant, R.J., Adesogan, A.T., 2018. Journal of dairy science silage special issue: introduction. J. Dairy Sci. 101 (5), 3935–3936. https://doi.org/10.3168/jds.2018-14630.

Guo, G.C.Q., Shen, C., Liu, Q., Zhang, S., Shao, T., Wang, C., Wang, Y., Xu, Q., Huo, W., 2020. The effect of lactic acid bacteria inoculums on in vitro rumen fermentation, methane production, ruminal cellulolytic bacteria populations and cellulase

activities of corn stover silage. J. Integr. Agric. 19, 838–847. https://doi.org/10.1016/S2095-3119(19)62707-3.

Jawaid, M.Z., Ashfaq, M.Y., Al-Ghouti, M., Zouari, N., 2023. Valorization of Palm tree (*Phoenix dactylifera* L.) leaves from harsh weather climate by silage using endogenous Lactic Acid Bacteria, and application of MALDI-TOF MS for study of populations dynamics. Bioresour. Technol. Rep. 22 (2023), 101408 https://doi.org/10.1016/j.biteb.2023.101408.

Karaduman, A., Ozaslan, M., Kilic, I.H., Bayil-Oguzkan, S., Kurt, B.S., Erdogan, N., 2017. Identification by using MALDI-TOF mass spectrometry of lactic acid bacteria isolated from non-commercial yogurts in southern Anatolia, Turkey. Int. Microbiol. 20 (1), 25–30. https://doi.org/10.2436/20.1501.01.282.

Lorenzo, J.M., Munekata, P.E., Dominguez, R., Pateiro, M., Saraiva, J.A., Franco, D., 2018. Main Groups of Microorganisms of Relevance for Food Safety and Stability. Innov. Technol. Food Preserv. 53–107. https://doi.org/10.1016/B978-0-12-811031-7.00003-0.

Nacef, M., Chevalier, M., Chollet, S., Drider, D., Flahaut, C., 2017. MALDI-TOF mass spectrometry for the identification of lactic acid bacteria isolated from a French cheese: the maroilles. Int. J. Food Microbiol. 247, 2–8. https://doi.org/10.1016/j.ijfoodmicro.2016.07.005.

Oualha, M., Al-Kaabi, N., Al-Ghouti, M., Zouari, N., 2019. Identification and overcome of limitations of weathered oil hydrocarbons bioremediation by an adapted Bacillus sorensis strain. J. Environ. Manage. 250, 109455 https://doi.org/10.1016/j.jenvman.2019.109455.

Pridmore, R.D., Berger, B., Desiere, F., Vilanova, D., Barretto, C., Pittet, A.-C., Zwahlen, M.-C., Rouvet, M., Altermann, E., Barrangou, R., Mollet, B., Mercenier, A., Klaenhammer, T., Arigoni, F., Schell, M.A., 2004. The genome sequence of the probiotic intestinal bacterium lactobacillus johnsonii NCC 533. Proc. Natl Acad. Sci. 101 (8), 2512–2517. https://doi.org/10.1073/pnas.0307327101.

Puntillo, M., Gaggiotti, M., Oteiza, J.M., Binetti, A., Massera, A., Vinderola, G., 2020. Potential of lactic acid bacteria isolated from different forages as silage inoculants for improving fermentation quality and aerobic stability. Front. Microbiol. 11 https://doi.org/10.3389/fmicb.2020.586716.

Santos, A.de, Ávila, C.L., Soares, C., Carvalho, B.F., Schwan, R.F., Lima, N., 2019. Lactic acid bacteria diversity in corn silage produced in Minas Gerais (Brazil). Ann. Microbiol. 69 (13), 1445–1459. https://doi.org/10.1007/s13213-019-01528-w.

Seuylemezian, A., Aronson, H.S., Tan, J., Lin, M., Schubert, W., Vaishampayan, P., 2018. Development of a custom MALDI-TOF MS database for species-level identification of bacterial isolates collected from spacecraft and associated surfaces. Front. Microbiol. 9 https://doi.org/10.3389/fmicb.2018.00780.

Sharma, R., Garg, P., Kumar, P., Bhatia, S.K., Kulshrestha, S., 2020. Microbial fermentation and its role in quality improvement of fermented foods. Fermentation 6 (4), 106. https://doi.org/10.3390/fermentation6040106, 2020.

Singhal, N., Kumar, M., Kanaujia, P.K., Virdi, J.S., 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front. Microbiol. 6 https://doi.org/10.3389/fmicb.2015.00791.

Säde, E., Björkroth, J., 2019. Introduction to the genera Pediococcus, Leuconostoc, Weissella, and carnobacterium. Lactic Acid Bacteria 65–85. https://doi.org/10.1201/9780429057465-6.

Tiani, K.A., Yeung, T.W., McClements, D.J., Sela, D.A., 2017. Extending viability of *Lactobacillus plantarum* and *Lactobacillus johnsonii* by microencapsulation in alginate microgels. Int. J. Food Sci. Nutr. 69 (2), 155–164. https://doi.org/10.1080/09637486.2017.1343285.

Tobaruela, E.de, Santos, A.de, Almeida-Muradian, L.B., Araujo, E.da, Lajolo, F.M., Menezes, E.W., 2018. Application of dietary fiber method AOAC 2011.25 in fruit and comparison with AOAC 991.43 method. Food Chem. 238, 87–93. https://doi.org/10.1016/j.foodchem.2016.12.068.

Vieco-Saiz, N., Belguesmia, Y., Raspoet, R., Auclair, E., Gancel, F., Kempf, I., Drider, D., 2019. Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. Front. Microbiol. 10 https://doi.org/10.3389/fmicb.2019.00057.

Wang, Y., Xia, K., Wang, X.N., Lin, X., Liu, J., Li, Y.J., Liu, X.L., Zhao, W.J., Zhang, Y.G., Guo, J.H., 2022. Improvement of feed intake, digestibility, plasma metabolites, and lactation performance of dairy cows fed mixed silage of sugar beet pulp and rice straw inoculated with lactic acid bacteria. J. Dairy Sci. 105 (10), 269–280. https://doi.org/10.3168/jds.2021-20494.

Wieser, A., Schneider, L., Jung, J., Schubert, S., 2011. MALDI-TOF MS in Microbiological Diagnostics—Identification of microorganisms and Beyond (Mini Review). Appl. Microbiol. Biotechnol. 93 (3), 965–974. https://doi.org/10.1007/s00253-011-3783-4.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'Toole, P., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S., 2020. A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. Int. J. Syst. Evol. Microbiol. 70 (4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107.