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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

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ABSTRACT

Preservation of green palm leaves by silage preserves them as green feed in addition to the added value of probiotics and prebiotics. In this work, the characterization of fresh palm tree leaves was performed. The isolation of local lactic acid bacteria allowed selection of 4 isolates for the silage of the palm tree leaves. Dairy feed was used to enrich the leaves silage with the necessary nutrients. *Lactobacillus paracasei* and *Pediococcus acidilactici* were found to be dominating their respectively inoculated silages. The high occurrence of LAB found in the spontaneous silages of the palm tree leaves mixed with dairy feed is a good indication that ensiling palm tree leaves can support the growth of LAB and produce good-quality silage. However, a combination of the process by following the population dynamics using MALDI-TOF MS allowed the selection of the appropriate LAB strain, which is a new approach for application of silage.

1. Introduction

The severe environmental conditions generate challenges for the production of feed for livestock. Many semiarid and arid regions are mostly dominated by date palm trees. Of approximately 105 million palm trees in the world, 62 million are located in the Middle East and North African region (Nasser et al., 2016). Palm trees belonging to *Phoenix dactylifera* L. is one of the most valuable fruit trees in the Asian region, mainly the Middle East, due to their capacity to endure harsh arid environmental conditions. Palm trees shed their leaves every fall. A single palm tree generates roughly 20 kg of green leaves and therefore large quantities of leaves are accumulated in the agricultural field after yearly harvesting. Palm tree leaves contain high fibers and lignocellulose, i.e., cellulose (37–49 %) and lignin (28–36 %) contents (Nasser et al., 2016; Mahdi et al., 2021). The moisture in palm tree leaves is low, ranging from 5 to 20 %, and contains crude protein contents ranging from 5 to 8 % (Jonoobi et al., 2019). The lignocellulosic composition mainly cellulose and hemicellulose could be favorable to feed ruminants, if well preserved. Palm tree co-products (leaves and pits) are a good source of sugar and in certain situations; they may be adjusted with non-commercialized dates. Indeed, green (not dry) date palm leaves have been used as a source of feed for the ruminants in these regions (Aziz, 2020). Khattab and Abd El Tawab, 2018) reported that the nutritional value of palm tree leaves for feeding livestock is insufficient, although Echegaray et al. (2021) presumed that date palm leaves could be used as a source of feed for livestock. Palm tree leaves mixed with palm dates are a good source of bioactive compounds and are linked to ethnomedicine to improve human health. Mahrous et al. (2021) showed that feeding growing lambs with a ration of green leaves of *Phoenix dactylifera L* contributed in their productive performance. However, the issue with these products is that they dry rapidly, due their low moisture. They gradually lose their potential to feed animals, by precipitation of the valuable nutrients (Jonoobi et al., 2019). Their preservation under controlled moisture and temperature was always favorable to growth of fungi, especially the mycotoxigenic ones (Aziz, 2020). The consequence is that the leaves are generally dried and serve mainly for traditional uses or burned in rural regions. Almi et al. (2015) reported their potential utilization as wood for composite reinforcement. Searching for a technological innovative process to preserve and valorize palm tree leaves is necessary. Mirmehdi et al. (2014) showed that date palm leaves may be used as filler of linear low-density polyethylene. However, Khattab and Abd El Tawab (2018) showed that their co-silage by mixing with other feed could enhance their digestibility. Indeed, the silage of palm tree co-products such as leaves, pits, and other non-

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commercialized dates can represent an interesting substitute for the products that are imported such as wheat bran in animal feed. Ensiling palm tree leaves as a source of feed for the ruminants can be beneficial economically and agriculturally. If silage is properly performed, it is a source of prebiotics (lactic acid and fibers) and probiotics (lactic acid bacteria, LAB) (Pato et al., 2021).

Practice of silage involves the anaerobic growth of lactic acid bacteria (LAB) for performing lactic acid fermentation, which increases the nutritional value of the products. Commonly used crops to prepare silage are corn, wheat, and alfalfa with other substitutes also being used such as grasses, sorghum, and legumes, with corn and alfalfa comprising one-third of the silages in the US (Grant and Adesogan, 2018). Silage is mostly dominated by LAB, converting carbohydrates to organic acids, generally acetic acid, and lactic acid, which decreases the pH and inhibits the growth of other microorganisms, while maintaining the nutritional value of the feed for a longer period in harsh environmental conditions (Grant and Adesogan, 2018). Facultative homofermentative LAB such as *Lactobacillus plantarum* (*L. plantarum*) and *Pediococcus acidilactici (P. acidilactici*) are usually used for inoculating silages for their rapid growth and instant release of lactic acid and lowering of the pH of the silage. However, silages inoculated with homofermentative LAB tend to have lower aerobic stability in comparison to those prepared with heterofermentative LAB such as L. *buchneri* (Calasso et al., 2020). The aerobic stability of silage is determined by observing the increase of its pH at aerobic conditions. Silages, which tend to increase their pH quickly when placed in aerobic conditions, have lower aerobic stability (Liu et al., 2019). Aerobic instability of the silage tends to reduce the nutritional value of the silage and enhance the growth of undesirable microorganisms, which can pose a threat to livestock. Yeast has been known to assimilate lactic acid when exposed to air. They are redoubtable in silage. Chen et al. (2019) reported using weak organic acids with high anti-fungal properties such as propionic acid to improve aerobic stability, but it has also been reported that propionic acid reduces fermentation efficiency. Using homofermentative LAB with propionic acid has been reported to overcome the shortcoming of one another (Chen et al., 2019). These considerations make the silage of date palm tree leaves difficult to perform unless a rigorous approach based on the selection of the appropriate LAB strains is employed. Following the drop of acidity, increase of acidity and detection of fungi and yeast is not enough to perform silage with low nutrient containing agricultural products. Identification and differentiation of LABs and co-existing microorganisms are essential in silage preparation to analyze the outcomes of the decrease in pH and increase in acidity due to the release of lactic acid and other acids by LAB. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been more advantageous than other molecular techniques in analyzing and interpreting the data for the rapid identification of bacteria. The process of identification is rapid, cost-effective, and requires less sample size which has made MALDI-TOF MS more acceptable to microbiologists (Ashfaq et al., 2022; Carvalho et al., 2017). MALDI-TOF MS has proven to be reliable based on numerous studies and different uses not only in microbial identification but also in the detection of pathogens in food and water, identification of pathogens in blood, and epidemiological studies due to its ability to characterize strains at sub-species levels. MALDI-TOF MS is also used for the identification of antibiotic mechanisms and allergies through specific biochemical markers (Wieser et al., 2011; Dec et al., 2014; Ashfaq et al., 2019).

The main objective of the work is the valorization of palm leaves (*Phoenix dactylifera L.*) produced seasonally in large quantities in Qatar and in the region, by silage, to preserve them as feed for animals that are originally accustomed to their consumption. Indeed, Mahrous et al. (2021) showed that palm leaves can be beneficial for growing lambs. The novelty of our work is to propose an alternative to preserve the nutritional value of palm leaves by silage as a process for protecting them from desiccation, especially their fibers serving as prebiotics and for their enrichment with LABs serving as probiotics, in addition to many others interests. In addition, the local LABs are adapted to the local harsh conditions and compete to dominate the silage. This requested the combination of a modern molecular technique of MALDI-TO MS to follow the population dynamics and ensure the conditions with allow dominance of the adequate LAB populations, ensuring the good quality of the silage. For the first time in literature, silage processes were analyzed, followed, and screened by coupling the microbial process to MALDI-TOF MS. The quality of the silage in terms of stability of the LABs and their dominance is a key parameter of a silage of high quality.

2. Material and Methods

2.1. Palm tree leaves sampling

The samples of palm tree leaves (*Phoenix dactylifera L.*) were collected from 5 farms in Qatar, (Al-Waab, Umm Salal, Umm Birka, Mazzraty (NGA), and Qatar University). One entire brush from each of the 5 palm trees was used and the green leaves of each were cut into small pieces (1 to 1.5 cm^2). All the small pieces of each sample were homogenously mixed in a container and preserved anaerobically in dark at 4 °C until use. The analyses were performed in triplicates and the results are presented as the average of three determinations using three samples.

2.2. Determination of the dry matter and the ash content of palm tree leaves

The dry matter was determined by desiccating the determined weight (around 5 g) of the sample of palm tree leaves at 103 °C for 2 d (constant weight achieved). The dry matter of the samples was incinerated at 550 °C for 5 h, to determine the ash content. The same calculation is valid for both dry matter and ash content using the AOAC methods (Tobaruela et al., 2018; Ahmad et al., 2020).

2.3. Determination of the pH and the acidity before and after silage

The pH of the cut palm tree leaves was determined by mixing 2 g of the sample in 50 ml of distilled water and homogenizing it for 1 h (Tobaruela et al., 2018). The mixture was centrifuged at 2000 $\times g$ for 15 min and the supernatant was used for pH measurement. For the determination of acidity, the solution was titrated with sodium hydroxide solution (0.1 N) using phenolphthalein as a color indicator (Tobaruela et al., 2018). The acidity was estimated as the equivalent of acidity per kg of fresh silage.

2.4. Determination of the protein content

The standard Total Kjeldahl Nitrogen method was used to determine the protein content using 1 g of sample in a total of 10 ml reactive medium (Goulding et al., 2020). The total protein conversion factor used is 6.25 (Tobaruela et al., 2018). The calculation reported the content per kg of leaves or fresh silage (Tobaruela et al., 2018).

2.5. Determination of the fibers content (Hemicellulose, Cellulose, and Lignin)

A sample of 2 g of cut leaves was dried at 103 °C for 2 days and then hydrolyzed with 2.5 ml of sulfuric acid solution (12 M) for 1 h at 30 °C. Then, 27.5 ml of water was added to the hydrolysate and the mixture was incubated at 100 °C for 1 h, cooled in a desiccator, and weighed to determine the fiber content (Tobaruela et al., 2018). For the determination of the water-soluble carbohydrates, 2 g of sample of the leaves were dried at 103 °C for 2 days and the dried matter was crushed into fine particles and refluxed for 2 h with 300 ml of distilled water at 100 °C. The mixture was filtered and dried at 103 °C for 2 d. The dry

weight of the residue was determined to evaluate the water-soluble carbohydrates. The dried filtered residue was then refluxed for 2 h with 300 ml of 0.5 M H_2SO_4 at 100 °C. The mixture was filtered and dried and the dried filtered residue was weighed to determine the hemicellulose content. The dried residue was further treated with 20 ml of 72 % (*v*/v) of 0.5 M H_2SO_4 at 25 °C for 4 h and then diluted with 300 ml of 0.5 M H_2SO_4 and refluxed for 4 h at 100 °C. The dried filtered residue after filtration was used to determine the cellulose and lignin content (Pagarra et al., 2018). The calculation reported the content per kg of leaves or fresh silage (Tobaruela et al., 2018).

2.6. Bacterial cultures

The isolation of the LAB was performed using the De Man, Rogosa and Sharpe medium (abbreviated MRS) and Luria-Bertani medium (LB). MRS medium was used since it is designed for the growth of *Lactobacilli*. The sodium acetate in the medium and the low pH prevent the growth of competing microorganisms. MRS medium is composed of (g/l): peptone proteose 10; meat extract 8; yeast extract 4; D (+) -glucose 20; sodium acetate 5; triammonium citrate 2; magnesium sulfate 0.2; manganese sulfate 0.05; dipotassium phosphate 2 and polysorbate 80 (Tween) 1with pH adjusted to 5.7 before sterilization. LB medium is composed of (g/l): tryptone, 10; NaCl 10 and yeast extract 5. LB agar and MRS agar media were obtained by adding 15 g/l agar before sterilization by autoclaving. Autoclaving was performed at 121 °C for 20 min.

2.7. Isolation of LAB and their identification by MALDI–TOF MS

The isolation of the LAB was performed from fresh palm leaves, dairy feed, and spontaneous silages prepared with palm leaves and a mixture of palm leaves and dairy feed. MRS was used as a selective medium, which was inoculated with 1 g of sample. Each enrichment culture was diluted 10,000 times and plated on MRS agar. The representative colonies were separated, and the corresponding bacterial strains were purified and identified by MALDI–TOF MS. For this purpose, selected colonies were sub-cultured for 24 h in LB agar. Then, a single colony was transferred to the MALDI Biotarget plate and was air-dried. Then, 1 µl of the α-Cyano-4-hydroxycinnamic acid (HCCA) matrix was overlaid and allowed to dry. The MALDI Biotarget plate was then loaded into the MALDI-TOF MS instrument (Bruker Daltonics / Germany) and Biotyper RTC 3 software was initiated to start the identification procedure. The bacterial identification results and their MALDI scores were interpreted as per the manufacturer guidelines and as reported in the literature (Alsayegh et al., 2021). The Principal component analysis (PCA) was necessary to reduce the dimensions of the set of data, maintaining the original information, and determining the similarities between the protein profiles.

2.8. Preparation of the inoculum for silage

The cultures of the isolated strains were prepared in 50 ml sterile tubes containing 15 ml of liquid MRS. An overnight single colony from the MRS agar of the selected strains was vortexed in the 1.5 ml MRS for 5 min. The optical density (OD) of the culture was measured at 600 nm. This inoculum suspension was used to inoculate the corresponding culture with an initial OD of 0.15.

2.9. Determination of viable cells in silage by Colony Forming Units (CFU)

A sample of 1 g of silage was suspended in 10 ml MRS and vortexed for 5 min. The CFU was determined by plating 100 µl of serial dilutions of the cultures on MRS agar. The dilution corresponding to a number of colonies between 30 and 100 was considered. Then the CFUs were calculated for each ml of the corresponding culture and reported to g of silage.

2.10. Silage procedure

For the preparation of the silages, the cutted palm tree leaves were mixed with different feed ratios ranging from 0 % feed to 50 % (*w*/w), at constant moisture corresponding to the addition of 100 ml of water per 550 ml silage at the time zero of inoculation. Silages were prepared in 500 ml glass bottles with a total volume of 550 ml, all filled with well-piled silage. The controls of the experiments were performed as silages without inoculation, while the studied silages were inoculated with LABs using an initial approximate OD of 0.15 (Table 1).

The mixture of palm tree leaves and feed was homogenized with moisture and the corresponding inoculum for the preparation of silage and then filled in the glass bottles to a point that almost all air is removed to create anaerobic conditions. The bottles were tightly sealed and incubated at 37 °C in the dark. The pH, acidity, and LAB growth were monitored on a weekly basis until the silage reached a stable state. The acidity was estimated as the equivalent of acidity per kilogram of fresh silage and the LAB growth was determined by taking the logarithm value of the CFU.

3. Result and discussion

3.1. Physical and chemical characterization of fresh palm tree leaves of Qatar

To evaluate the composition of the leaves date palm (*Phoenix dactylifera L.*) for further silage, the major physical and chemical parameters were evaluated (Table 2). The analyses were performed in triplicates and the results are presented as the average values.

Phoenix dactylifera L. leaves from Qatar contain high dry matter content of 63.95 % and low ash content of 4.34 %. Aziz (2020) also reported a dry matter content of 62.94 % in date palm tree leaves in Egypt. However, in literature, it was reported that the ash contents fluctuated between 2.6 % and 10.5 % (Almi et al., 2015; Khattab and Abd El Tawab, 2018; Mirmehdi et al., 2014). The crude proteins content is of 8.72 %, similar to that reported with date palm trees in Iran (Jonoobi et al., 2019). The palm tree leaves of Qatar are rich in total fibers (85.5 %). Their inorganic content is 4.34 %, determined by the ash content. Their organic content in dry matter is 59.61 %. However, cellulose, hemicellulose, lignin, and proteins represent 52.34 % of the leaves. Mirmehdi et al. (2014) reported similar results with palm leaves from Iran in terms of hemicellulose and lignin of 12.8 % and 32.3 %, respectively with a higher concentration of cellulose (40.21 %). Similarly, Nasser et al. (2016) reported higher cellulose composition (47.14 %) in date palm tree leaves from Saudi Arabia, with hemicellulose and lignin contents of 16.13 % and 36.73 %, respectively, being close to those of Qatari palm trees. AL-Oqla and Sapuan (2014) reported low cellulose and lignin contents of 20 % and 23 %, respectively, and high hemicellulose content of 55 % with a low ash content of 1.1 % in Malaysian palm tree leaves. These variations in the composition of the fiber contents could be mainly due to the variety of the plants, their age, and the environmental conditions (Al-Oqla, 2020). While palm leaves are interesting as a feed due their high fiber content, the low crude proteins and consequently the low digestibility may be corrected by mixing them with other protein-rich feeds. Lignin, representing the

Table 1
Composition of the silages.

Silage code	Feed (% w/w)	Cut date palm leaves
1	0	100
2	10	90
3	20	80
4	30	70
5	40	60
6	50	50

Table 2
Characterization of palm tree leaves.

Parameters	Average of triplicates
pH	6.35 ± 0.04
Equivalent acidity/kg of fresh leaves	0.025 ± 0.002
Water-soluble carbohydrates (%)	28.34 ± 0.05
Dry Matter (%)	63.95 ± 0.2
Ash Content (%)	4.34 ± 0.4
Fiber (%)	85.5 ± 0.5
Crude Protein (%)	8.72 ± 0.02
Cellulose (%)	22.07 ± 0.03
Hemicellulose (%)	11.47 ± 0.04
Lignin (%)	39.6 ± 0.03

highest fiber content in the palm tree leaves, is the cause of their low digestibility and low nutritional value if used to feed ruminants without adjustment of the composition (Kholif et al., 2022; Abd El et al., 2016). However, Khattab and Abd El Tawab (2018) suggested that the nutritional value and thus the digestibility of the palm tree leaves could be enhanced with addition of fibrolytic enzymes, or nutrients, or by mixing with other feed sources for increasing the ruminal fermentation. These alternatives add value to the palm tree leaves as a good source of bioactive compounds, which may be linked to ethnomedicinal properties to improve animal health (Mirmehdi et al., 2014; Kholif et al., 2017).

3.2. Selection of LAB for silage

Following the isolation of LAB and their identification by MALDI-TOF MS from ensiled leaves and ensiled mixed leaves/feed, it was noticed that one strain was dominating each of the silages since it was solely present in the serial dilution of 10^4 obtained from 1 g of each silage. The isolation was performed at aerobic and anaerobic conditions and the total number of colonies obtained at the dilution of 10^4 is provided in Table 3. All the isolated strains corresponding to the colonies of each condition were identified by MALD-TOF MS with the corresponding score (Table 3). Since the scores are higher than 2.000 and below 2.300, the isolates are identified at the genus level with a probable species-level identification (Alsayegh et al., 2021). The protein profiles of the isolates, generated by MALD-TOF MS, were compared and analyzed by PCA. The intra- and interspecific similarity between the isolated strains from each sample was established through the analysis of the similarity of their respective protein profiles. The results showed that all the isolates obtained from each silage with the dilution of 10^4 have almost 100 % similar protein profiles. One strain from each silage was used in further experiments (Table 3). The strain L. *plantarum* SMZ103 was isolated at anaerobic conditions from the mixed silage and L. *plantarum* SMZ46 from the silage of leaves at aerobic conditions. The strain *P. acidilactici* SMZ41 was isolated from the mixed silage at aerobic conditions, while L. *paracasei* SMZ20 at anaerobic conditions from the silage of leaves.

3.3. Evolution of pH in the silages

Each of the silages mentioned in Table 1 was inoculated with one of the selected strains, mentioned in Table 3. The control, for each of them, was performed at the same conditions and composition but without inoculation. Fig. 1 shows the evolution of the pH in each of the silages. The control silages showed that in the absence of any inoculated strain, the increase in the feed/leaves ratio accelerated the drop of the pH as expected in the silage process (Yang et al., 2019). The pH of all the silages performed with 100 % leaves showed the least drop in pH compared to silages performed with high percentages of feed. The highest drop in pH, from 6.17 down to 5.06, was observed with the inoculant 3 (*L. plantarum* SMZ46) and the least, from 6.18 down to 5.56, with the inoculant 1 (*L. plantarum* SMZ103). The maximal drop in pH was observed in the 50 % ratio silages with the highest drop (from 6.21 down to 3.85) observed with inoculant 3 and the least (from 6.31 down to 4.2) with inoculant 2 (*L. paracasei* SMZ20). These results clearly showed that the endogenous acid-forming microorganisms initially present in the feed and the leaves were performant to acidify the silage and thus ensure stability and prevention from pathogenic microorganisms. In addition, with the 10 %, 20 %, and 30 % feed-silages, the highest drop was observed with the inoculant 4 (*P. acidilactici* SMZ41) from 6.18, 6.13, and 6.11 down to 4.15, 4.12, and 4.09, respectively, reaching stability after the week 4 of incubation. The highest drop of pH (from 6.23 down to 3.98) in the 40 % feed silages was observed with inoculant 3, with stability reached after 4 weeks of incubation. These results clearly show that the pH in the silages inoculated with LAB tends to drop faster than that in controls performed without inoculation, due to higher initial LAB activities.

In addition, the results of Fig. 1 show that *P. acidilactici* SMZ41 initiate the acidification of the silage at a faster rate than the other LAB, which confirm the observations of Yang et al. (2019) using *P. acidilactici* in silage processes. *P. acidilactici* drops the pH rapidly in silages, making the conditions more favorable for other LABs.

Interestingly, even without any inoculation with LAB, the pH dropped down to 4.42 in the 10 % feed/leaves silages, showing that silages of palm trees leave may be performed with 10 % feed and 90 % leaves.

3.4. Evolution of acidity in the silages

All the silages showed that with the increase in feed/leaves ratios, the acidity tends to increase compared to that in the silages performed without feed (Fig. 2). The acidity of all the silages performed with 100 % leaves remained steady at 0.025 equivalent Acidity/kg, during incubation. For the control and inoculant 1 silage performed with L. *plantarum* SMZ103, the highest acidity of 0.225 equivalent Acidity/kg, was obtained in the silage containing 50 % feed/leaves, reaching stability after 3 weeks of incubation. The highest acidity of 0.3 equivalent Acidity/kg was observed with the inoculant 3 (*L. plantarum* SMZ46) in the silage performed with a 50 % feed/leaves ratio, stabilized after 3 weeks of incubation. The acidity of the silages prepared with a 10 % feed/leaves ratio was of 0.075 equivalent Acidity/kg for the control, the inoculant 2 (*L. paracasei* SMZ20) and the inoculant 3 (*L. plantarum* SMZ46) while it was of 0.1 equivalent Acidity/kg with the inoculant 1 (*L. plantarum* SMZ103) and the inoculant 4 (*P. acidilactici* SMZ41). The highest acidity observed in the 20 % feed/leaves ratio was with the inoculant 4 (*P. acidilactici* SMZ41) increasing from 0.05 to 0.175 equivalent Acidity/kg. The initial acidity of the prepared silages with 30 % of feed was 0.05 equivalent Acidity/kg, with the highest acidity (0.2 equivalent Acidity/kg) reached in the control, inoculant 3, and inoculant 4 and stabilized after 3 weeks of incubation. With the initial acidity of all the silages

Table 3
Selected lactic acid bacteria for silage inoculation.

Source	# colonies in 10^4 dilutions	Growth conditions	Range of MALDI-TOF MS scores	Protein profile similarity of all colonies	Genus and species	Code of the used strain
Leaves	8	Anaerobic	2.01–2.11	100	*L. paracasei*	SMZ20
silage	19	Aerobic	2.09–2.22	99	*L. plantarum*	SMZ46
Mixed	17	Anaerobic	2.19–2.34	100	*L. plantarum*	SMZ103
silage	34	Aerobic	2.07–2.15	100	*P. acidilactici*	SMZ41

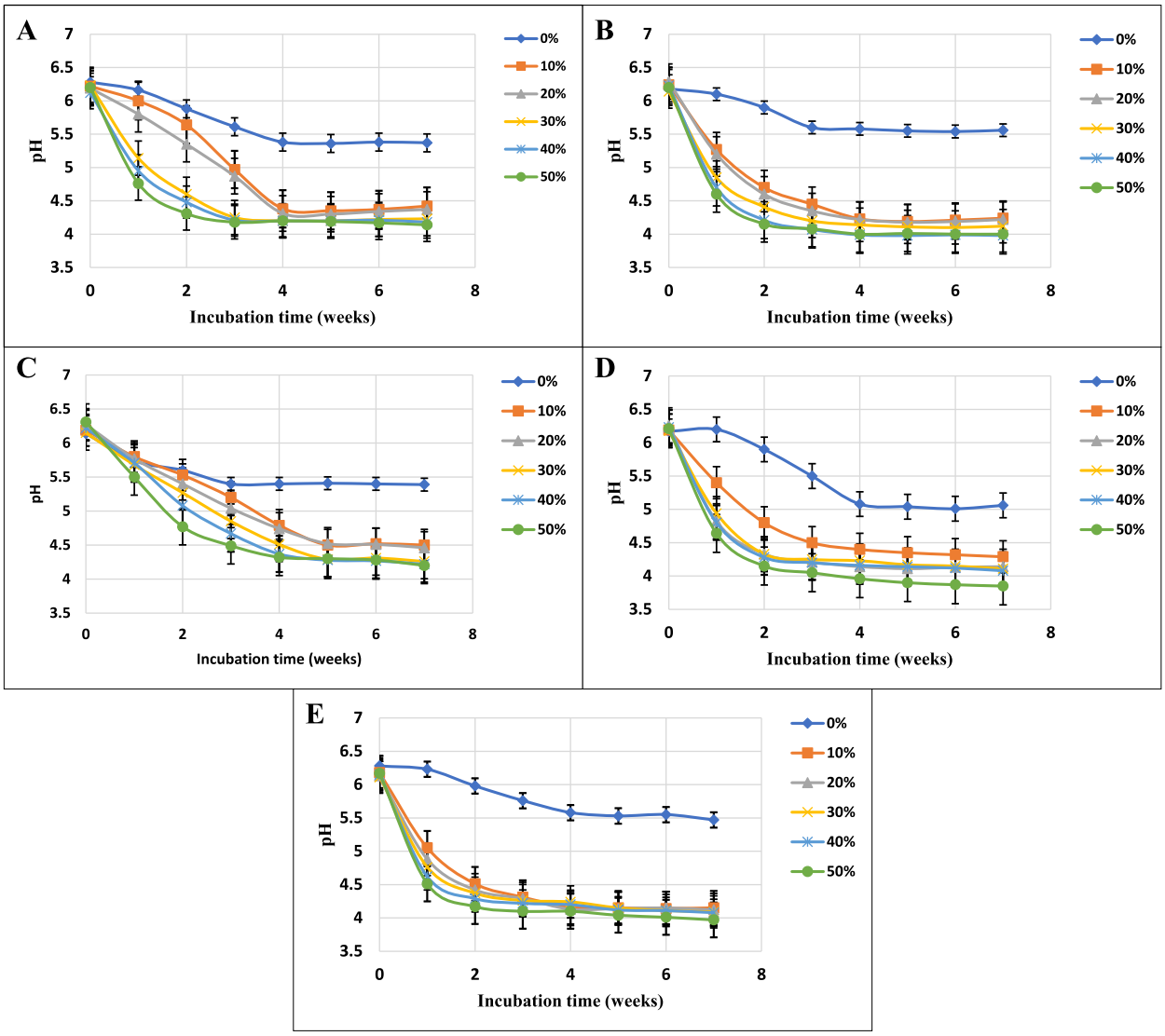

Fig. 1. pH in the silages during the incubation. Control Silage (A): Silages without inoculation, Inoculant 1 (B): *L. plantarum* SMZ103, Inoculant 2 (C): *L. paracasei* SMZ20, Inoculant 3 (D): *L. plantarum* SMZ46 and Inoculant 4 (E): *P. acidilactici* SMZ41. Silages were performed with different percentages of Feed: 0 % feed, 10 % feed, 20 % feed, 30 % feed, 40 % feed, and 50 % feed.

performed with 40 % of feed being 0.05 equivalent Acidity/kg, the highest acidity (0.225 equivalent Acidity/kg) was observed with the inoculant 1, inoculant 3, and inoculant 4, stabilized also after week 3 of incubation. The trend of the increase of acidity was the same for all the inoculated silages and the controls. The highest increase in acidity was observed with the inoculant 3 (*L. plantarum* SMZ103). The increase of the acidity by *P. acidilactici* SMZ46 was observed to be the fastest.

3.5. Evolution of LAB growth in silages

Results shown in Fig. 3, illustrate the trends of evolution of growth of the LAB in silages, as CFU counts. The trend is similar to that of the acidity evolution of Fig. 2, with a notable increase in growth with the increase of the feed/leaves ratio in the silage. This is expected since the growth of the LAB is normally increased with the readily assimilated nutrients provided by the feed. For the silages performed with and without inoculation, growth was observed until week 3 of incubation after which the silages showed stability in terms of viable LAB counts. The highest LAB growth of 5.9 logs (CFU/g), in silages performed with 100 % leaves, was with the inoculant 4 (*P. acidilactici* SMZ41). For the silages performed with 50 % feed, the highest CFU of 7.7 logs (CFU/g) was also observed with the inoculant 4 (*P. acidilactici* SMZ41), with an initial CFU of 4 logs (CFU/g). In the 10 % feed/silages, the highest CFU of 6.47 logs (CFU/g) was recorded with the inoculant 3 (*L. plantarum* SMZ46). The highest CFUs of silages performed with 20 % and 30 % feed were obtained with the inoculant 2 (*L. paracasei* SMZ20), increasing from 2.7 to 6.8 logs (CFU/g) and 3.07 to 7.4 logs (CFU/g) respectively. In the silages prepared with 40 % feed, the highest LAB count of 7.45 logs (CFU/g) was obtained with the inoculant 2 (*L. paracasei* SMZ20), increased from 3.3 logs (CFU/g).

3.6. LAB population dynamics during palm tree ensilaging

Based on the previous results, inoculating the silages with selected LAB as well as their incubation for a period of 7 weeks are necessary conditions to reach the stability of pH, acidity, and CFU in palm tree leaves silages. The study of the evolution of the CFU in the silages provided enough information on the capability of the palm tree leaves and the supplemented feed to support the growth of LAB that can form colonies when plated on MRS agar. These CFUs are expected to be of LAB growing in the silage. However, the evolution and the balance between the LAB species existing in the silage during incubation, cannot be

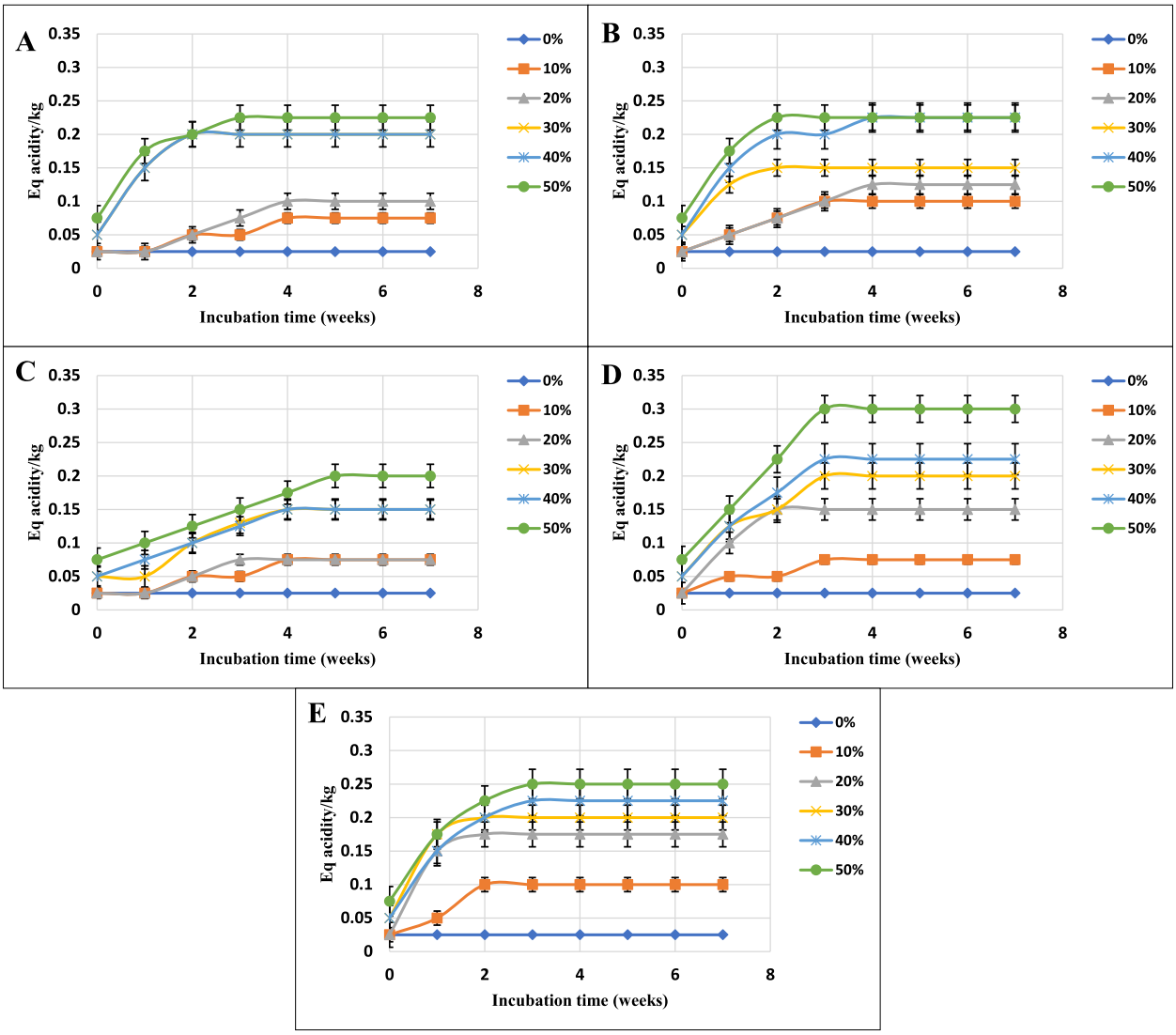

Fig. 2. Acidity of the silages: Control Silage (A), Inoculant 1 (B): *L. plantarum* SMZ103, Inoculant 2 (C): *L. paracasei* SMZ20, Inoculant 3 (D): *L. plantarum* SMZ46 and Inoculant 4 (E): *P. acidilactici* SMZ41. Silages were performed with different percentages of Feed: 0 % feed, 10 % feed, 20 % feed, 30 % feed, 40 % feed, and 50 % feed.

evaluated only by CFU determination because the colonies in the MRS agar are highly similar. In addition, it is necessary to know if the inoculated strains were able to dominate the endogenous ones or the latter would compete and dominate the silage. To study the population dynamics of the LAB growing in the silages, their identification was performed using MALDI-TOF MS. In addition, since 10 % of feed/silages showed interesting evolution of the three parameters (pH, acidity, and CFU) for a good silage quality and stability, it was selected for this study. It also allowed the study of the interaction between the LAB in a low feed/leaf's ratio. However, in order to demonstrate the potential applicability of the 10 % feed/silages, a comparison with the richest silage (50 % feed/leaves ratio) was performed to evaluate the possibility of the dominance of the inoculated LAB strain in a rich medium. At least 30 colonies obtained with serial dilutions on MRS agar were used at each condition, for their identification and the study of their distribution as species. Indeed, 100 µl of serial dilutions of the liquid MRS harvested after suspending 1 g silage were plated on MRS agar. The plates with 50 to 100 colonies were used. 30 colonies were selected randomly from each plate. All the results are shown in Fig. 4. The dominant species in 10 % feed-silage performed with the inoculant 1 (*L. plantarum* SMZ103) are shown in Fig. 4-a. The total number of colonies was 53 and only 30 colonies were randomly identified. *P. acidilactici* was found to be represented by 30 % of the colonies and L. *pentosus* by only 10 %, while 20 % of the colonies were of the yeast *Candida* sp. The results show that there are no dominant species but rather a mixture of LAB (80 %) and *Candida* sp. (20 %) was growing in the silage. However, none of the colonies was of L. *plantarum*. This means that the inoculated strain L. *plantarum* SMZ103 disappeared or became a minority, not detectable at the used serial dilution. The endogenous LAB (*P. acidilactici*) dominated the inoculated LAB strain SMZ103 of *L. plantarum*. Regarding the 10 % feed-silage inoculated with L. *paracasei* SMZ20, 30 colonies were randomly selected for identification, out of the 54 conies growing on MRS agar. The results of Fig. 4-b show that L. *paracasei* was represented by 63 % of the used colonies. *P. acidilactici* represented 27 % of the colonies and *Candida* sp. 10 %. The results show that the inoculated strain L. *paracasei* SMZ20 was dominating the silage and the endogenous *P. acidilactici* was also competing to dominate the silage. Both represented 100 % of the LAB colonies and 90 % of the total colonies. The 10 % feed-silage performed with the inoculant 3 (*L. plantarum* SMZ46) provided a total colony number of 58, and 30 random colonies were selected for the identification (Fig. 4-c). After identification, 56 % of colonies were of L. *farciminis*, 27 % of *P. acidilactici,* and 17 % of *Candida*

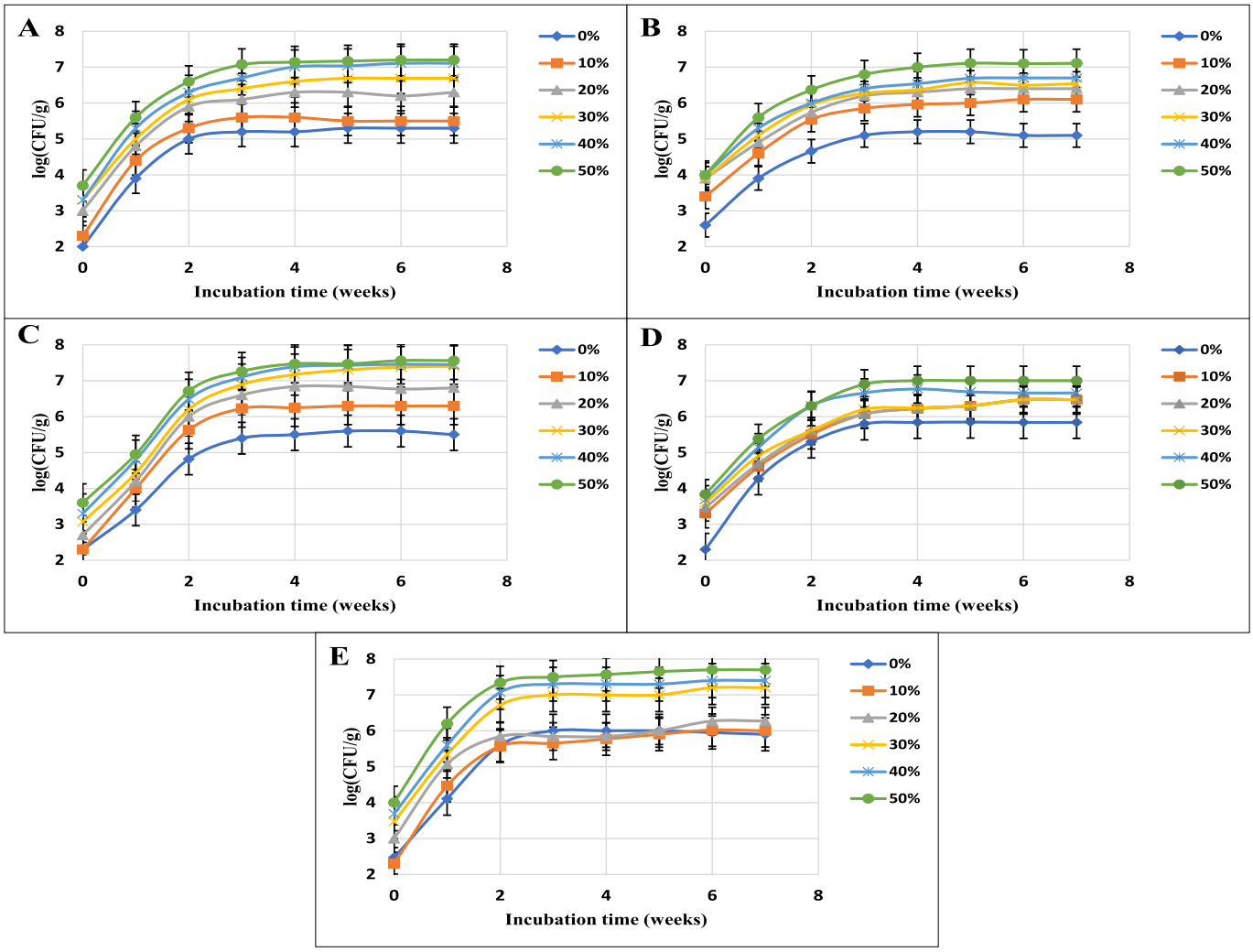

Fig. 3. Growth of the LAB in silages over incubation time, expressed as log (CFU/g). Control Silage (A), Inoculant 1 (B): *L. plantarum* SMZ103, Inoculant 2 (C): *L. paracasei* SMZ20, Inoculant 3 (D): *L. plantarum* SMZ46 and Inoculant 4 (E): *P. acidilactici* SMZ41. Silages were performed with different percentages of Feed: 0 % feed, 10 % feed, 20 % feed, 30 % feed, 40 % feed, and 50 % feed.

sp. This result clearly shows that the silage is dominated by the endogenous strains of L. *farciminis* and *P. acidilactici*. Using the 10 % feed-silage performed with the inoculant 4 (*P. acidilactici* SMZ41), there were 63 colonies on the plate and 30 random colonies were identified. The results of Fig. 4-d clearly show that *P. acidilactici* dominated 75 % of the colonies, while *Candida* sp. was represented with 25 %. None of the other endogenous LAB was detected at the dilution employed in the plating.

The same procedure was employed for the 50 % feed-silages. Using the inoculant 1 (*L. plantarum* SMZ103), the total plate count was 103 colonies. Among them, 45 colonies were randomly selected, and the corresponding LABs were identified (Fig. 5-a). *L. plantarum* was represented by 51 % of the colonies, while *P. acidilactici* by 22 % and *Candida* sp. by 27 %. The results show that L. *plantarum* was almost the dominating species in 50 % of feed/leaf silage. Using the inoculant 2 (*L. paracasei* SMZ20), there were 134 colonies on the plate and 50 of them were randomly selected for identification (Fig. 5-b). The results indicate that L. *paracasei* corresponded to 38 % of the colonies and *P. acidilactici* to 62 % without any colony corresponding to yeast. This shows that the studied silage was dominated by LAB, especially *P. acidilactici*. The 50 % feed-silage performed with the inoculant 3 (*L. plantarum* SMZ46) had a plate count of 108 colonies, of which 60 were randomly selected for identification (Fig. 5-c). 20 % of the colonies were of *P. acidilactici* and 77 % of *Candida* sp. with 1 colony of *Vegococcus fluralis* and 1 of *Staphylococcus warneri*. This indicates that the silage was dominated by the yeast *Candida* sp. and other bacteria than LAB, which is not appropriate for producing a silage of high quality. Using the inoculant 4 (*P. acidilactici* SMZ41), there were 183 colonies on the plate and 74 colonies were identified (Fig. 5-d). The results indicate that *P. acidilactici* was represented with 72 % of the identified colonies, *Candida* sp. with 21 %, *Staphylococcus epidermidis* with 3 colonies, and *Staphylococcus capitis* with 2 colonies. This silage containing bacteria belonging to staphylococcus is not appropriate for animal feeding.

The results clearly show that L. *plantarum* does not dominate the silage containing a low concentration of feed (10 % feed-silages). Growth of other LABs such as L. *pentosus, L. oris*, *L. vaginalis, and* L. *farciminis* was noticed, indicating that the inoculated L. *plantarum* was dominated by the endogenous LAB. However, *L. plantarum* was observed to represent almost 50 % of the colonies in silages containing 50 % feed/leaves. *L. paracasei* dominated the silages containing 10 % and 50 % feed. In the silage performed with 50 % feed, *P. acidilactici* was also represented in the LAB populations but with much lower than L. *paracasei*. This result is novel, since Alhaag et al. (2019) demonstrated that, if present in a silage, *P. acidilactici* is always the most dominant species even if the silage is inoculated with other LAB, because *P. acidilactici* is known to dominate the silage in the initial stages of fermentation until the pH drops below 4. At such low pH in the silage, the other LAB start to grow. In the silages performed with palm tree leaves, the pH of the

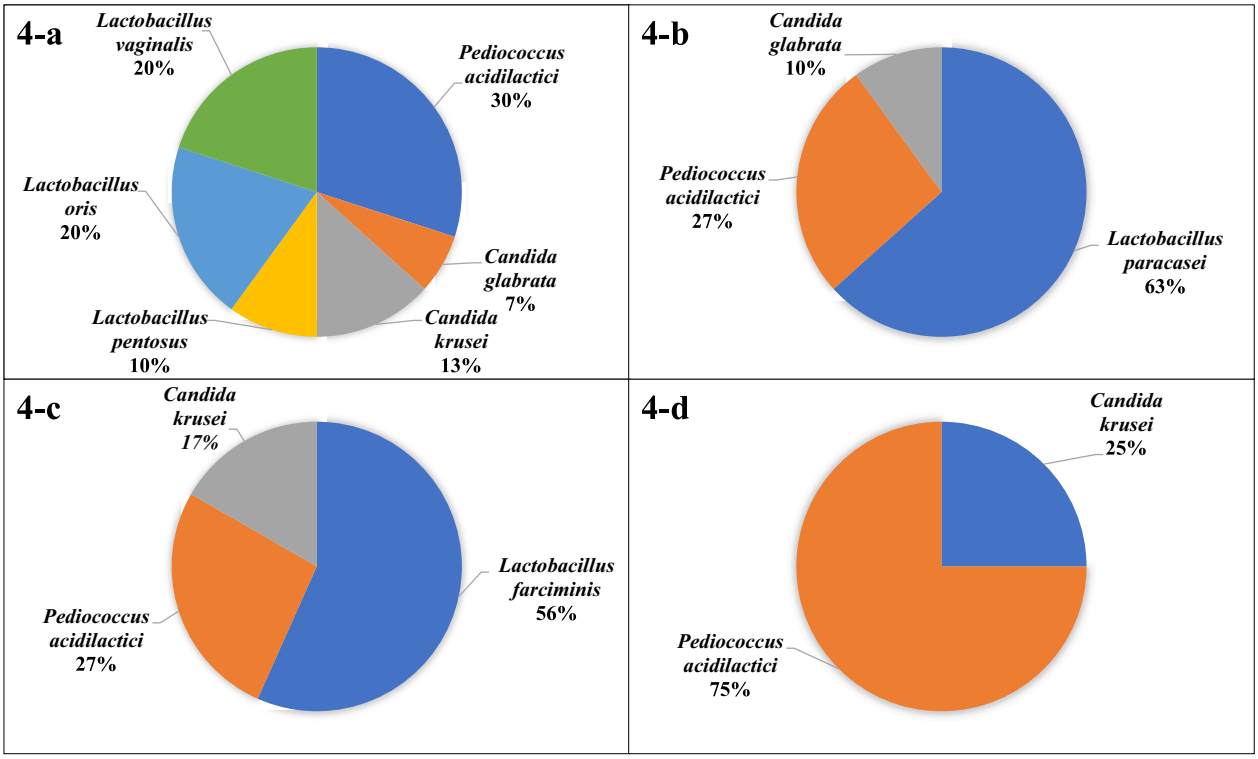

Fig. 4. Dominating species in 10 % feed/leaves silages in (4-a) Inoculant 1 (*L.plantarum* SMZ103), (4-b) Inoculant 2 (*L. paracasei* SMZ20), (4-c) Inoculant 3 (*L. plantarum* SMZ46) and (4-d) Inoculant 4 (*P. acidilactici* SMZ41).

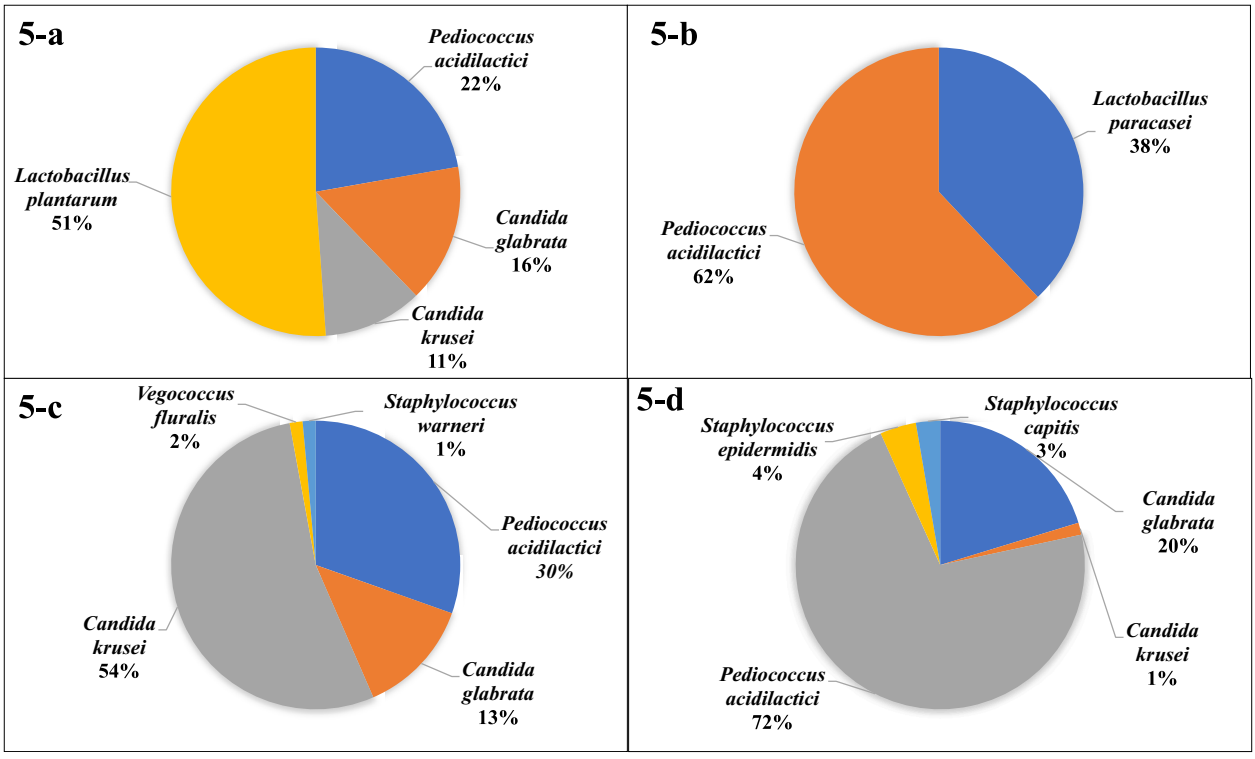

Fig. 5. Dominating species in 50 % feed/leaves silages in (5-a) Inoculant 1 (*L. plantarum* SMZ103), (5-b) Inoculant 2 (*L. paracasei* SMZ20), (5-c) Inoculant 3 (*L. plantarum* SMZ46) and (5-d) Inoculant 4 (*P. acidilactici* SMZ41).

silages was mostly above 4 as *P. acidilactici* was not observed in most of the silages. This study shows that using *P. acidilactici* to inoculate silages performed with palm tree leaves is recommended to achieve better acidity and LAB population serving as probiotics for ruminants.

4. Conclusions

The study clearly demonstrated that the palm tree leaves contain high lignin and low proteins contents. While the composition is not favorable for feeding ruminants with these leaves because of their low digestibility and low nutrients supply, however, the findings showed that they can be the basis of feed for the ruminants. Indeed, the silage of palm tree leaves is possible if appropriate LAB are used. The spontaneous silage caused growth of yeast and diverse LAB populations, which did not contribute to the fast stability of the silage. It was then clear that mixing the palm tree leaves with 10 % animal feed is beneficial to support the growth and dominance of the selected LAB inoculated into the silage. Among the appropriate LAB strains isolated from the local harsh conditions, *L. paracasei* SMZ20 from mixed feed/leaves silage and *P. acidilactici* SMZ41 from spontaneous leaves silage were the most dominating LAB. In addition, it was shown that if the silage is not inoculated with *P. acidilactici*, the drop of pH cannot be below 4, causing less growth of the LAB. These finding were obtained by the study of the LAB population dynamics, which was performed by MALDI-TOF MS. This is the first reported work in which the combination of the bioprocess of silage with the molecular technique was useful to conduct a silage of high and safe quality. This approach is useful to select the appropriate LAB inoculum, which discards totally yeast and fungi growth and the pathogenic microorganisms.

Ensiling palm leaves mixed with animal feed, and inoculated with selected LAB of L. *paracasei* and *P. acidilactici* is proven to be a promising option for the valorization of the palm leaves by preservation for a longer period and improvement of their nutritional value.

Author declaration template

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work. [OR] We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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CRediT authorship contribution statement

Muhammad Zaid Jawaid: Contributed in performing the experiments and in interpretation and writing of the manuscript.

Mohammad Yousaf Ashfaq: Contributed in performing the experiments and in interpretation and writing of the manuscript.

Mohammad Al-Ghouti: Contributed in experiments design and writing of the manuscript.

Nabil Zouari: Designed the project, contributed in experiments design and results interpretation and writing of the manuscript.

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Declaration of competing interest

None.

Data availability

Data will be made available on request.

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