QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

ISOLATION OF ENDOGENOUS LACTIC ACID BACTERIA AND DEVELOPMENT OF SILAGE OF PALM TREES LEAVES FOR LIVESTOCK IN QATAR

BY

MUHAMMAD ZAID JAWAID

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COMMITTEE PAGE

The members of the Committee approve the Thesis of
Muhammad Zaid Jawaid defended on 28/11/2021.

Nabil Zouari
Thesis/Dissertation Supervisor

Mohammad Al-Ghouti
Co-Supervisor

Samir Jaoua
Committee Member

Talaat Abdelfattah Ahn Youssef
Committee Member

Approved:

Prof. Ahmed Elzatahry, Dean, College of Arts and Sciences

ABSTRACT

JAWAID, MUHAMMAD, Z., Masters: January : [2022], Environmental Sciences

Title: Isolation of Endogenous Lactic Acid Bacteria and Development of Silage of Palm Tree Leaves for Livestock in Qatar.

Supervisor of Thesis: Nabil Salem Zouari.

In Qatar, there are relatively low agricultural by-products available, mainly from palm trees for feeding livestock. Preservation of palm leaves is a problem due to harsh environmental conditions. Ensiling palm leaves with different lactic acid bacteria (LAB) was investigated in this study. Firstly, the characterization of palm tree leaves was performed, showing a high fiber content and low content of crude protein. Then, 87 LAB strains adapted to local environmental conditions in Qatar were isolated, identified and differentiated using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF MS), and the similarities were analyzed using principal component analysis (PCA) and dendrogram. LAB identified were from genus *Lactobacillus* and *Pediococcus.* Dairy feed was used to enrich the leaves silage with necessary nutrients. The pH, acidity, and bacterial growth (CFU) of the ensiled palm leaves at different feed/leave ratio were monitored for 7 weeks. The strain *Lactobacillus paracasei* SMZ20 and *Pediococcus acidilactici* SMZ41 were the most adapted to the medium and the weather conditions. *Lactobacillus paracasei* and *Pediococcus acidilactici* were found to be dominating their respectively inoculated silages. The low-cost media formulation to produce LAB was investigated using a factorial plan. The low-cost media with 5% feed mixed in water at low aeration had the most promising results in terms of CFU. This study demonstrated that the palm tree leaves can be ensiled and preserved for a longer period with mixing it with commercial feed and inoculating with local LAB.

DEDICATION

To all those who have been there supporting me and believing in me.

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So much of the effort put into this research work was a group effort, I would therefore like to express my appreciation to my mentor Prof. Nabil who knew exactly what can be achieved and how, my committee members; Prof Mohammad Al-Ghouti, Prof. Samir Jaoua, and Dr. Talaat Abdelfattah Ahm Youssef for their continuing support; Dr. Zulfa Al-Disi for always being there for guidance; the Department of Biological and Environmental Sciences for all the experiences and lessons; Dr. Yousaf Ashfaq for being extremely patient; and everyone who has made this journey possible

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CHAPTER 1: INTRODUCTION

Globally, the manufacturers of animal feeds focus on those, which are nutritionally balanced for all types of animals. However, they should satisfy the nutritional requirements of each animal. As an example, fibers are required for ruminants. The feed of livestock animals which is close to the natural feed, is composed of plant products, mostly grass, rapeseed, and alfalfa, etc., and plants that are grown especially for feeding the livestock (leaves, stem, and roots). In harsh environmental conditions the dairy animals, mostly cows are suffering due to the low production of grazing feed (Derara, 2020). Their production to sustain food security is then problematic. Indeed, for waste management, a quantity of the agriculture by-products is dumped in landfills directly. With high moisture content (>80%), improper dumping can cause environmental pollution resulting in greenhouse gas emissions, odors and pathogens generation, etc. (Ren et al., 2020). Therefore, there are need for economic and sustainable solutions to overcome this issue. The increase in efficient use of available feed resources is key for economic farming. The feeding system is actually called to increase the potential of the feeding resources.

In Qatar, there is relatively low agricultural by-products available for feeding livestock animals, especially dairy animals. However, palm trees provide a non-negligible source of abundant source of feed (green leaves, date pits, and not commercialized dates). All these by-products are seasonally produced (autumn) which raises the issue of their proper preservation as feed throughout the year. Due to the harsh environmental conditions in Qatar, the storage and preservation of these by-products are unfavorable. There are several ways to store and preserve fodder. The drying way, in which preservation is made possible by desiccation, either only under the action of the sun (natural drying) or supplemented by hot air produced by burners (barn drying)

leading to a moisture percentage of the fodder around 15% ensuring its stability (Boyer et al., 2019). However, drying the green biomass causes losses of its nutritional value due to precipitation of some nutrients, making them less available and losing their nutritional value, or due to the volatilization of some. The intermediate pathways such as haylage or round bale wrapping have been recently developed, for grassland plants. The wet way, called "silage", applies to grasses, maize, and possibly, to agro-food by-products such as beet pulp, brewer's grains, etc. is the best way to preserve the nutritional value of the green biomass and improve its quality by the production of acids and probiotics during the biological process of silage.

Palm trees by-products (not commercialized dates, green leaves, and pits) are already used to feed traditionally, local animals. Their preservation is not possible due to desiccation or microbial contamination. These strongly reduce their quality as animal feeds (Deffairi & Arhab, 2016). It is now necessary to develop biotechnological ways to preserve them and improve their potential as feed. Silage offers opportunities to preserve natural resources, which could serve for livestock feeding. Ensiling them would provide a solution to this problem, as cost-effective and preservation technique for the perishable biomass. Indeed, it is now considered that silage processes provide and alternate solutions to preserve green leaves of palm trees in Qatar since it is a reliable and cost-effective process of preserving the green components.

The total mixed ration (TMR) silage system is being adopted by many feeding systems worldwide. TMR silages can facilitate the feeding of the livestock all year long (CHEN et al., 2017). The silage practice basically involves the preservation of green components by using microorganisms, mostly lactic acid bacteria (LAB) in anaerobic conditions. LAB converts the water-soluble carbohydrates into acids, such as lactic acid and acetic acid. In doing so, the pH of the silage tends to drop and the growth of

undesirable microbes is inhibited, thus preserving the feed from spoilage (Yang et al., 2019).

Silage is done in desired proportions to meet the specific needs of the nutrients. Using fibrous residues with complete feeding is an optimal way to enhance the selected diet intake and with that increase the production performance of the animal. In this feeding system, ruminants have the ability to freely choose a uniform mixture that results in a more uniform rumen load and less fluctuation of ammonia release, which promotes more structured use of nitrogen. A stabilized and complete diet of ruminal fermentation improves the use of nutrients. In addition, this feed system allows the use of agricultural industrial by-products, crop residues and unconventional feeds in the ruminant quantity to maximize production and reduce feed costs, which is further appreciated (Zhang et al., 2020). However, more efforts need to be done in order to make this concept acceptable to farmers.

The main objective of this study is to contribute in enhancing the measures of food security in Qatar. The theme of "Local Food Production" is supported by this study and this research focuses on the characterization of the natural resources from Qatar and their potential to be preserved as feed for animals to be improved by different types of co-silage. The date palm can provide a development path for sustainable agriculture in areas with harsh environmental conditions, like that in Qatar, with many droughts and saline-affected regions. The composition of the date palm is highly dependent on the date variety. Therefore, the first objective of the study was to characterize green leaves, as the co-products and by-products from Qatari palm trees, which have a potential to be converted into silage for livestock feeding in Qatar. These could result in promising advancements in improving the yield and quality of feedings. The study also proposes new composition of silage based on local products of palm trees and local

lactic acid bacteria, which are adapted to local climate conditions and substrates. Mixing of a current feeding commercial product in the co-silage of the by-product of local agro-industry is also proposed.

This project will constitute a first study in Qatar on LAB and silage. The outcome of the study will be the construction of a local collection of LAB, which may be used as probiotics and highly adapted to silage processes of palm tree co-products and other agriculture by-products. The isolation, identification and characterization of the LAB were performed using the most recent, reliable cost-effective and rapid method of Matrix-Assisted Laser Desorption Ionization Time of Flight mass spectroscopy (MALDI TOF MS). The diversity of the LAB strains can be analyzed by the protein profile acquired by MALDI TOF MS. For the first time in literature, silage processes were analyzed, followed, and screened by coupling the microbial process to MALDI TOF MS analysis. The quality of the silage can also be evaluated by this proteomic technique, regarding the stability of the LAB and the contaminated microorganisms.

Then, this project will provide a process of production of LAB in Qatar. Low-cost media will be used to try to suggest a low-cost production on LAB.

Objectives/Significance:

1- Characterization of the green leaves by-products from Qatari palm trees and local farms, to evaluate their potential to be converted into silage for livestock feeding in Qatar. Mixtures of these raw materials with palm tree co-products and by-products, should represent an appropriate silage substrate for LAB.

2- Create a Qatari Collection of local Lactic acid bacteria (LAB), adapted to Qatari weather through a program of isolation, identification, characterization, screening, and production of LAB, useful for silage and other applications. This study will be performed by a combination of the

microbiological methods with the MALDI TOF proteomic technique

3- Formulation of silages composition, and evaluation of their potentials to support LAB growth and accumulation of acids: Investigation of silage processes at the laboratory scale and improvement of silage processes.

4- Optimization and evaluation of low-cost culture media for the production of LAB at the laboratory scale.

CHAPTER 2: LITERATURE REVIEW

2.1 General Introduction

The global requirement of animal products is constantly under pressure with the rise in the world population and intensifying pressure on the livestock industry to continuously increase productivity. This is a major concern since livestock production practices have a severe impact on the environment. The livestock industry is a major contributor of greenhouse gas emissions of about 14.5% and it uses a vast agriculture land of about 80% and agricultural water use is equal to 41% (Heinke et al., 2020). To overcome this issue, well-organized, integrated, and efficient farming practices are required for sustainability.

Globally, the manufacturers of animal nutritional products are producing feed that is nutritionally balanced for all types of animals. The animal nutrition industry is both an agro-supply and agro-food industry because the special feed of animals is produced from plants or their products such as cereals and oil-seed crops etc., and by-products of local agro-food industries. The feed of livestock animals is composed of plant products, containing mostly fodder such as grass, rapeseed, and alfalfa, etc., and plants that are grown especially for feeding the livestock, like leaves, stem, and roots (Derara, 2020). Among the two, green fodder is more appropriate for feeding livestock animals. The outcome of the agro-food industry is mainly food and nutrition certainty in a sustainable manner.

The definition of food security has widely differed throughout history and the change from increasing food production to improving the food has somewhat addressed the issue of food insecurity, with the focus being mainly on agriculture. Food security is necessary to ensure nutrition security, therefore these two concepts are interlinked with food security emphasizing the need for better nutrition in food and nutrition security addressing the nutritional concerns to achieve food security (El Bilali et al.,

2021). Attaining food security is not just to increase food production but rather to improve the food systems and functions in a sustainable manner. Recent debates have highlighted food security as part of sustainability. The sustainable development goal (SDG) focuses on ending hunger and achieving food and nutrition security as well as the promotion of sustainable agriculture (Vågsholm et al., 2020). The sustainable food supply with the future global challenges is the solution to exhausting natural resources. Harsh environmental regions tend to have problems with storage in dry or cold seasons. Feed cultivated and harvested in the rainy season tends to have better production in milk and meat in livestock. Preserving the feed in this season as feed-in form of silage may prevent nutrient deficits during harsh conditions. Silage processes provide a solution for the management of green and other palm trees by-products and co-products, it is a reliable and cost-effective technology and it can preserve and improve the green component quality as silage (da Silva et al., 2017).

On the other side, it is now admitted worldwide that animal nutrition manufacturers manufacture feeds that are nutritionally balanced for each type of animal. Cow feed is exclusively composed of plant products, mostly green fodder (grass, alfalfa, rapeseed, and others), and plants grown mainly to feed livestock (leaves, stems, and roots). Green fodders are more appreciated for feeding most livestock animals (da Silva et al., 2017). In Qatar, relatively low abundant agricultural by-products are useful to improved livestock feeding. They are mainly from palm trees (green leaves, date pits, and not commercialized dates) besides green parts after pruning plants. Even with a lower nutritional value, palm tree leaves should not be neglected, especially in times of fodder deficiency. In the Qatari climatic context, the periods of mass production of palm trees products and co-products are the autumn to winter periods. There is therefore an unavoidable need for storage. Hot weather is not

favorable to extend the preservation of palm leaves due to rapid desiccation. However, the climatic hazards and technical constraints are serious limits of the rational management of inputs (Aziz, 2020). Silage processes provide a reliable and cost-effective response to optimize annual management of the green and other palm trees co-products since it can preserve the green components and improve the quality as silage (Bayão et al., 2019). Silage is a method of preserving and improving wet agro-industrial by-products through anaerobic lactate fermentation (Ren et al., 2020). Silage of palm tree co-products especially leaves, pits, and not commercialized dates can be an interesting substitute for some imported products such as wheat bran in animal feed.

2.2 Silage practices

The practice of silage involves preserving and improving the wet agro-industrial by-products by anaerobic lactic acid fermentation. Ensiling a range of different agricultural products has been a common practice to achieve a common outcome, -i.e., increasing 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 compromising of one-third of the silages in the US (Grant & Adesogan, 2018).

Depending on the availability of dry matter content, there are several silage techniques, which are possible more or less, having different functions than one another. The technique has advantages and disadvantages that must be considered to know the risk and the means of implementation and ensuring effectiveness. Certain parameters need to respect that it does not affect the fat content in milk and beef production, as an example. The farmer must set production objectives (slaughter weight, age at sale, period of sale, etc.) considering his farming system and market conditions (Borreani et al., 2018). Using silage as the only feed in the Total Mixed Ratio (TMR) requires careful and consistency at the feeder, in addition to more advanced feed

monitoring. The factors of risks associated with a drop in milk fat levels and beef production are numerous and the interaction of these factors with each other makes the life of diet counselor's much more complicated when balancing TMR. Feeding and management of the herd are to be optimized to not affect milk and meat production (Wang et al., 2019). A good program is to identify which feeds are appropriate, how much, how and when should they be served. Getting the livestock to consume a large amount of food is the key to efficient milk and meat production. High dry matter intake results in high nutrient consumption and high milk yield (Cattani et al., 2017). Silage processes maintain high DM contents, starts the digestion of the feed and improve it by the accumulation of volatile fatty acids especially fatty acids, prebiotics and probiotics, such as fibers and lactic acid bacteria.

2.3 Silage, a fermentation technology

Silage is a method of preserving wet forage through anaerobic fermentation. This fermentation is characterized by rapid acidification caused by the production of organic acids, mainly lactic acid and acetic acid, produced by lactic acid bacteria (LAB) under anaerobic conditions (Yang et al., 2019). As a result, the pH tends to drop, and feed will be preserved from the deterioration of undesirable microorganisms. The success silage process is conditioned by the success of the four main steps that are described in Table 1.

Table 1: The four main phases of the silage process (Borreani et al., 2018)

Phases	Key events	Duration
Aerobic	Activity of aerobic microorganisms. Exhaustion of oxygen.	**Short (hours)**
Fermentation	Competition between anaerobic bacteria. Acidification LAB dominance in case of good fermentation	**Days**
Stabile storage	Low pH Inhibition of undesirable microorganisms Diminution of viable microorganisms	**Months**
Feed (feed -out)	Aerobic instability which causes: An increase of pH An increase in aerobic microorganisms Decrease of anaerobes	**Days**

As a rule, the higher the dry matter content, the more difficult the anaerobiosis, which is necessary to start the lactic fermentation. Silage has allowed industrialization of agriculture and dense breeding above ground. It has become an essential element of mixed farming systems of the twentieth century.

To achieve better quality, the dry matter content should not vary in silage, to preserve the optimal nutritional values. The high-quality production of silage is determined by:

i. The amount of soluble sugars converted into lactic and acetic acid by the LAB, naturally occurring in the feed.
ii. The growth and then the density of the probiotics (LAB)
iii. anaerobiosis and, consequently, acidification.

To ensure a high quality of the silage, several additives are permitted. These are preservatives to increase the acidification rate, stability, and shelf life of silage. They are one or more of the three types (Muck et al., 2018):

(i) Fermentation stimulant: Biological (selected lactic acid bacteria with or without soluble sugar sources) increasing lactic fermentation. These are selected strains of *Lactobacillus, Enterococcus,* and *Pediococcus*;

(ii) Nutrients: formic acid and various acid salts to enhance the acidification of the fodder artificially;

(iii) Fermentation inhibitors: bacteriostatic (sodium chloride and others) restraining the fermentation of bacteria and resumption of alcoholic development during the consumption of forage, as well as antifungal preservatives (sodium sorbate and others).

The conditions of anaerobiosis are only achieved late. In fact, when silage is fed for the first time, the conditions of the silo are aerobic (the silage contains oxygen). Aerobic bacteria produce heat when they convert carbohydrates and sugars into carbon dioxide and water using trapped oxygen. Once oxygen is consumed, the silage becomes anaerobic and conducive to the growth of anaerobic bacteria. These microorganisms transform carbohydrates and sugars into organic acids that help conserve silage. In addition, a certain amount of proteins is converted to amino acids, ammonia, and other non-protein nitrogen compounds (Yang et al., 2019). Some bacteria can break down cellulose and hemicellulose into several easily digestible sugars. Other bacteria break down simple sugars into lactic acid, acetic acid, and butyric acid. It is important that the bacteria which produce lactic acid and acetic acid multiply immediately after storing the feed. The production of acetic acid is desirable since ruminants alongside its participation in lowering pH, can use it. Lactic acid is the most preferred of all the acids of fermentation (Fabiszewska et al., 2019). In two or three weeks, the silage pH stabilizes between 4.0 and 5.0, and all bacterial and enzymatic activity ceases. In fact, silage reaches its required quality when the production of lactic acid predominates the

other acidic productions and the pH goes down 4 to 3.8. When ensiled product is consumed, livestock will also use lactic acid as a source of energy. Once the pH is stabilized, the risk of nutrient degradation disappears, allowing silage to be stored for longer periods, provided the air does not enter. No additional harmful processes can occur, until the silage is protected from oxygen (Liu et al., 2019).

2.4 Lactic Acid Bacteria (LAB) importance and role in silage

Silage preparations require microbial activity in anaerobic conditions mostly dominated by LAB, converting carbohydrates to organic acids, generally acetic acid and lactic acid, which decrease the pH and inhibits the growth of other microorganisms, while maintaining the nutritional value of the feed for a longer period of time in harsh environmental conditions in comparison to haymaking (Grant & Adesogan, 2018). The population of the LAB increases substantially during the harvesting and ensiling phase. Mostly used LAB in silages is from the genera "*Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Lactococcus,* and *Streptococcus"*. The LAB is classified into obligate homofermenters, facultative heterofermenters, and obligate fermenters based on their metabolism of sugar (Drouin et al., 2020). Obligate homofermenters produce lactic acid (LA) from sugars, facultative homofermenters produce LA and acetic acid (AC) with ethanol and obligate homofermenters produce LA, AC, CO_2 and ethanol.

Obligate homofermentative lactic acid bacteria are *Pediococcus damnosus* and *Lactobacillus acidophilus.* Facultative homofermentative LAB such as *Lactobacillus plantarum* and *Pediococcus acidilactici,* are usually used for inoculating silages for their rapid growth and instant release of LA and lowering the pH of the silage. However, silages inoculated with homofermentative lactic acid bacteria tend to have lower aerobic stability in comparison to heterofermentative lactic acid bacteria such as *Lactobacillus buchneri* (Calasso et al., 2020). The aerobic stability of a silage is

determined by observing the increase in pH of the silage in 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 the livestock. Yeast has been known to assimilate lactic acid when exposed to air. Chen et al., (2019) reported using weak organic acids with high anti-fungal properties such as propionic acid to tend to improve aerobic stability, but it has also been reported that propionic acid reduces the fermentation efficiency. Using homofermentative lactic acid bacteria with propionic acid has been reported to overcome the shortcoming of one another (CHEN et al., 2017).

2.5 Silage processing

In silage processes, there are associated environmental and health risks. Indeed, fodder silage is a method of biological preservation mostly compared to the processing of sauerkraut, nevertheless, the hygiene measures are not the same. Several risks related to ingestion of contaminated silage by animals are to be limited: Risk of intoxication with fungal or bacterial toxins, Risk of Listeria intake, Risk of toxic ethanol production for ruminants, and risk of excessive presence of butyric bacteria (Queiroz et al., 2018). The quality of the silage has also impacted the taste of milk or meat. The effect on cow's milk cheeses relates to the color of the dough and the quality of the taste. In general, the quality of the organoleptic characteristics of milk is improved by feeding silage (Kung et al., 2018).

There are several types of silage processes including: the vertical silo or silo tower (Figure 1-a), the horizontal silo or silo corridor (Figure 1-b), and the silo roll and the large pre-faded silage bales (Figure1-c).

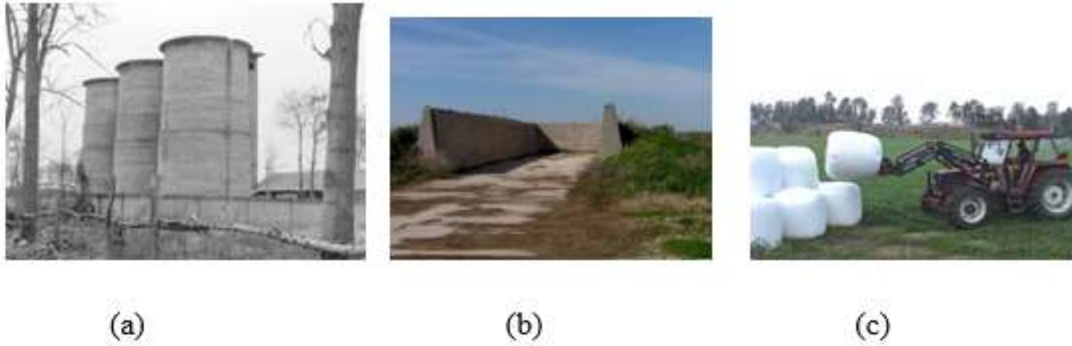

Figure 1: Several types of silage processes including (a) the horizontal silo; (b) the corridor silo and (c) the large pre-faded silage bales (Elferink et al., 2000)

2.6 Quality of silage

The duration of the first phase (aerobic) and the rate of decrease in pH are critical factors in determining silage quality. Good fermentation is characterized by a rapid drop in pH. This rapid acidification inhibits the growth of microorganisms such as *Clostridium*, *Enterobacteriaceae*, yeasts, and molds (Yang et al., 2019). It must be stable, allowing subsequent storage of silage for a long time.

2.7 Improved silage quality

Losses of silage quality are likely to occur during distribution for feed. They can even affect the quality of the milk, which can be contaminated by butyric spores produced by *Clostridium butyricum* leading to cheese-making problems (Borreani et al., 2019). This is why improving the quality of silage is a strategy to be studied for each type of silage, in several ways.

2.7.1 Improvement by inoculation with LAB

The purpose of LAB inoculation in silage is to enhance the drop in pH by fermentation of water-soluble carbohydrates (WSC) to lactate. This rapid drop in pH makes it possible to conserve WSCs and to decrease proteolysis and deamination by inhibiting the long fermentation process (Yang et al., 2019).

2.7.2 Addition of enzymes

Treatment of feed with fibrolytic enzymes has been suggested to improve fiber digestibility and increase the WSC availability to serve as a substrate for LAB (Zhang et al., 2020).

2.7.3 Addition of certain preservatives

For a long time, the value of adding preservatives was judged through the quality of fermentation, that is to say, its impact on the pH, the NH_3 nitrogen compared to the total nitrogen, the acid content volatile fats that influence the actual nitrogen value, and the digestibility of the silage. In recent years, two additional actions have been sought: on the one hand, improving the air stability of the silage after opening the silos and on the other hand, reducing hygienic risks. Chen et al., (2019), sought to optimize formic acid preservatives by adding propionic, which is known to inhibit unwanted microorganisms, more effectively than formic acid. Borreani et al., (2019) studied the preservation quality and the number of spores of silages prepared from green fodder intentionally contaminated with *Clostridium* spp. and *Paenibacillus* spp. spores, since these spores remain in the milk and cheese after processing. The results showed a clear reduction in the number of spores in silages by good farm management and particular steps from the silage to milking stage.

2.8 Palm tree leaves as Silage

The severe environmental conditions generate challenges for the production of feed for the livestock. Semiarid and arid regions are mostly dominated by date palm trees. From approximately 105 million palm trees in the World, 62 million are located in the Middle East and North African region (Al-Khayri et al., 2015). Palm trees are one of the most valuable fruit tree in the Asian region, mainly the Middle East, due to their capacity to endure harsh arid environmental conditions. Date palm leaves have been used as a source of feed for the ruminants in these regions (Aziz, 2020). Khattab

& Abd El Tawab, (2018) reported that the nutritional value of palm tree leaves for feeding livestock is insufficient, although Echegaray et al., (2021) have 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 ethno-medicinal to improve human health. A single palm tree has roughly 20 kg of green leaves and after each harvesting large quantities of leaves are accumulated in the agriculture field. Ensiling palm tree leaves as a source of feed for the ruminants can be beneficial economically and agriculturally. Silage of palm tree co-products such as leaves, pits and other non-commercialized dates can represent an interesting substitute for the products that are imported such as wheat bran in animal feed. If the fermentation is properly performed, it is a source of prebiotic (lactic acid and fibers) and probiotics (lactic acid bacteria) (Pato et al., 2021).

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. For ensiling palm tree leaves, the characterization is essential to determine the fiber and lignocellulosic content, i.e., cellulose, hemicellulose, and lignin of the leaves. Nasser et al., (2016) determined the cellulose content of palm tree leaves by extracting them using 3% nitric acid solution in sintered glass crucible. The residue was then treated with 3% sodium hydroxide solution. The residue obtained was filtered, dried, and weighed. Hemicellulose was determined by extraction, using sulphuric acid, and boiling it in a sintered crucible glass. Mahdi et al., (2021) reported extraction of lignocellulosic content with 72% sulphuric acid and calcinated at 550°C. Initially, the leaflets were treated in hot water to extract the water-soluble compounds.

The palm tree leaves are low in moisture content ranging from 5 – 20% and high in dry matter concentration. Palm tree leaves have a low nitrogen content (crude

protein) ranging from 5 – 8% (Jonoobi et al., 2019). Palm tree leaves have high lignocellulosic composition mainly cellulose and lignin. Hence, palm tree leaves in silage could enhance the nutritive value and digestibility of the silage by mixing it with other feed and adding Cellulase and Fibrolytic enzymes (Khattab & Abd El Tawab, 2018).

2.9 Importance of fibers in nutrition in ruminants: basic principles

2.9.1 The rumen and its microorganisms with highly fibers-rich feed

The feed of the ruminants depends mostly on the digestion by the microbial fermentation, the idea of rumen digestion are assessed before discussing the needs for biotechnological innovations. The rumen is the most essential component of the digestive system of ruminants. It is home to concentrated and diversified populations of microorganisms. The microorganisms ferment the substances that are in the feed to produce mainly short-chain organic acids, methane, and carbon dioxide; the process provides the necessary requirements for the growth of microorganisms (Xing et al., 2020). The microbial assortment in the rumen is complicated and highly based on the diet. The microorganisms present in the rumen are bacteria, protozoa, and fungi that break down the fiber, sugars, and proteins in anaerobic conditions. Bacteria are the main microorganisms that initiate the fermentation of carbohydrates that are contained in the membrane of plant cells (Li et al., 2019). It has been recognized that, on the whole, the protozoa affect the rumen in a negative manner, especially when ruminants are fed fodder with little true protein. Protozoa are known to feed on bacteria and in doing so, in the rumen, the bacterial population tends to decrease and hence the protein availability of the ruminant. By doing so, the protein/energy ratio is lowered in the nutrients absorbed and the requirements of true proteins is increased. The existence of protozoa in the rumen increases the requirement of supplementary protein in the ration and, when the ration has less quantity of protein there is less efficiency in the use of

fodder for growth and milk production (Burk, 2018). In addition, protozoa can reduce the bacterial fermentation rate to digest fodder particles. Fiber digestion in the rumen is a complicated task and it requires a collaborative symbiosis of the microbes in rumen including protozoa. Researchers have observed different results in terms of digestibility, in some cases, there is no difference in digestibility and in other the digestibility tends to decrease in rations containing starch (Burk, 2018).

2.9.2 Fermentative efficiency in the rumen

Any nutrient deficiency for the microorganisms tends to reduce the ability of microbes to grow, resulting in a decrease in microbial biomass and, subsequently, digestibility and fodder supply, in particular of fibrous fodder. It is an essential priority that the ratio does not contain ant nutrient deficiency that hinders the growth of the microorganisms. It is essential that the growth of the microbes digest the fodder in the most efficient manner to transform the fodder into methane and Volatile Fatty Acids (VFA). Methane production initiates the production of acetate or butyrate, and there is an inverse relationship between methane and VFA production and microbial cells production (Li et al., 2018).

2.9.3 Conditions for efficient microbial growth in the rumen

The major limiting factor to microbial growth in the rumen is the ammonia in the digestive juice and deficiency of minerals, especially sulfur, phosphorus, magnesium, and some trace elements. The critical threshold of ammonia in the rumen must exceed long enough to make certain that the growth of the microorganisms and digestion is good and hence a large supply of fodder (Matthews et al., 2018). The optimal population of the microorganisms depends upon the protein/energy ratio of the nutrients that are absorbed (Li et al., 2019). Any deficiency in the nutrients will result in a low yield of microbes relative to VFA production and result in a low ratio, in nutrients absorbed, among protein (from microbes) and ATP (from VFAs). Silage is a

source a such an interesting bacteria as diet.

2.9.4 The consequences of the mode of digestion of ruminants

The fermentation in the rumen causes the formation of methane and heat in ruminants, one of the consequences of ruminant digestion. Furthermore, the proteins in the rumen are no longer sources of amino acids since they are hydrolyzed and the microbes suppress the amine function of the amino acids which compose them. General practice for feeding the ruminants in the tropical regions is to add a small amount of nutrients to the fodder to balance the nutrient deficiency. The anabolic efficiency of the nutrients for growth, gestation, lactation or labor is increased with the availability of proteins that are protected (Wang et al., 2018). Feeding with silage reduces these issues.

2.9.5 Quantitative aspects of fermentative digestion in the rumen

The quality of the fodder depends on the rate at which it is fermented and the nutrient balance in the fodder to ensure the growth of the rumen microorganisms. A certain quantity of the dry matter of fodder is transformed into VFAs, methane, and carbon dioxide, the rest being assimilated into microbial cells (Xing et al., 2020).

2.10 Use of probiotics in bovine production

Probiotics are living microorganisms that are added to the animal's diet provide beneficial effects by promoting a better microbial balance at the ruminal or intestinal level. The two most common types of microorganisms used in cattle production are bacteria and yeast. The use of probiotics has also demonstrated a high level of effectiveness as a growth promoter (Al- Dobaib and Mousa, 2009). Researchers, therefore, turned to the development of new avenues including that of probiotics. Among some which are beneficial for the animal, others are harmful and cause digestive disorders such as diarrhea which will adversely affect the health and growth of the animal. Most of the probiotics used are those that are beneficial such as *Lactobacillus* and bifidobacteria. An intake of probiotics promotes the health of the

animal by creating conditions unfavorable to the growth of pathogenic bacteria. Concretely it is observed 1) a reduction in the number of days the calves have diarrhea, 2) an increase in the gain of weight, and 3) reduced health costs. Several modes of action are suggested to explain this effect of probiotics: 1) competing for the same substrate food, probiotics deprive pathogens of essential elements for their growth, 2) the Most probiotic bacteria produce lactic acid which helps lower the pH intestinal and harms pathogens that are sensitive to acidity, 3) probiotic bacteria are able to adhere to the intestinal mucosa and thus create a barrier to the entry of pathogens in the system, 4) some probiotic bacteria produce antibacterial toxins that attack specific pathogens, 5) finally, it has been observed that certain strains of yeast bind to pathogenic bacteria and prevent their adhesion to the intestinal mucosa. Use of probiotics in adult ruminants showed following the addition of probiotics to the ration, an increase in the average of daily gain of around 2.5 to 5% on average and the index of consumption (kilos of gain /kilos of dry matter consumed) of the order of 2% according to Zommiti & Ferchichi, (2021). Probiotic bacteria would play a role in alleviating the symptoms of digestive disorders such as subacute or acute acidosis observed when steers undergo a drastic change in a ration from a ration rich in fodder to a ration rich in concentrated. In the event that the flora is not adapted to such a change or even with a flora adapted but a significant intake of carbohydrates, it happens that the acids resulting from ruminal fermentation are produced in an amount that exceeds the absorption capacity.

2.11 Microbial Biodiversity studies using MALDI TOF MS

Microbial identification and differentiation of LABs are essential in silage preparation in order 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. There is a constant demand to enhance the process of identification, being more reliable, cost-effective and less time consuming, not just in microbiology but in all fields of science (Seuylemezian

et al., 2018). Mass spectrometry discovered in the 1900s emerged as a revolutionary technique in identification and ever since it has been constantly developed to be more precise, cost-effective and instant. In mass spectrometry, chemical compounds are ionized and the mass to charge (m/z) ratio of charged molecules is measured. 'Matrix-assisted laser desorption ionization-time of flight mass spectrometry' (MALDI TOF MS) has been more advantageous than ESI MS in analyzing and interpretation the data more effortlessly. The process of identification being rapid, cost-effective, and less sample size requirement, has made MALDI TOF MS more acceptable by the microbiologists (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 characterization of strains at sub-species levels. MALDI-TOF MS is also used for the identification of antibiotics mechanisms and allergies by specific biochemical markers (Wieser et al., 2011). MALDI TOF MS in comparison to other microbial identification is a more attractive method due to its robust, cost effective and reliable approach and can prove to be a substitute to biochemical and molecular biology-based identification techniques which require complex removal of nucleic acids and polymerase chain reaction (PCR) or sequencing (Dec et al., 2014). The protein profile generated, by the software, is multivariate and the data is statistically processed to differentiate between the strains. Principle component analysis (PCA) is used to reduce the dimensions between the strains. PCA illustrates the spectrum in 2D and 3D dimensions showing the variance between the strains. The 2D coordinate system shows a higher percentage of variance in comparison to 3D. The strains with high similarities are grouped together in a cluster. The differentiation between the strains can be investigated by using the protein profile peaks

to establish dendrogram by using MALDI Biotyper Compass Explorer software. The dendrogram represents the similarities between the strains in the form of a graph, the strains with high similarity are grouped together and can be categorized as similar strains. PCA and dendrogram can illustrate the similarities between strains and can also highlight the differences between the identified strains graphically (Alsayegh et al., 2021).

CHAPTER 3: MATERIALS AND METHODS

3.1 Palm tree leaves sampling

The palm tree leaves samples were obtained from 5 farms in Qatar, Al-Waab farm, Umm Salal farm, Umm Birka farm, Mazzraty (NGA) farm, and Qatar University farm. The leaves were collected and transported to the laboratory at Qatar University. One entire brush from each of 5 palm trees was used and the green leaves of each were cut into small pieces (1 to 1.5 cm^2). The same procedure was performed on all lots. All the small pieces were homogenously mixed in a container, preserved anaerobically in dark and at 4°C until use. It was estimated based on personal communications with Qatar University Farm Engineers that a single palm tree has roughly 20 kg of green leaves and after each harvesting large quantities of leaves are accumulated in the agriculture field. The analyses were repeated in triplicates and the results are presented as the average of three determinations using three samples.

3.2 Determination of Dry matter and 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 days till a constant weight was achieved. After determining the dry matter of the samples, they were incinerated to determine the ash content at 550°C for 5 h. The same calculation is valid for both, dry matter and ash content using the AOAC methods (Tobaruela et al., 2018).

3.3 Determination of pH and Acidity

The pH of the palm tree leaves was determined by mixing 2 g of sample in 50 ml of distilled water and homogenized for 15 min (Tobaruela et al., 2018). The mixture was centrifuged at 2000xg for 15 min, the supernatant was used for the reading of pH using an electrode pH probe. For the determination of acidity, the solution was titrated with sodium hydroxide solution (0.1N) with phenolphthalein.

3.4 Determination of Protein Content

The protein content was determined by the standard Kjeldahl method (Goulding et al., 2020). The total protein conversion factor used is 6.25.

3.5 Determination of Fibers content (Hemicellulose, Cellulose and Lignin)

2 g of sample were dried at 103°C overnight and then hydrolyzed with 2.5 ml of sulfuric acid solution (12 mol /L) for one 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 (Tobaruela et al., 2018). The hydrolysate thus obtained was used to determine the fiber contents.

2 g of sample of the leaves were dried at 103°C till constant weight was achieved and then the dried leaves were crushed into fine particles and refluxed for 2 h with 300 ml of distilled water at 100°C. The leaves were filtered and dried. The dry weight was calculated for the determination of pectin and oligosaccharides. The dried residue was then refluxed for 2 h with 300 ml of 0.5 M H_2SO_4 at 100°C. The leaves were filtered and dried to determine the hemicellulose content. The dried residue was treated with 20 ml of 72% (v/v) of 0.5 M H_2SO_4 at 25°C for 4 h and then it was diluted with 300 ml of 0.5 M H_2SO_4 and refluxed for 4 h at 100°C. The dried residue after filtration was used to determine cellulose and lignin content (Pagarra et al., 2018).

3.6 Bacterial culture media

The isolation and identification of the LAB were performed on DE MAN, ROGOSA, and SHARPE medium (abbreviated MRS) and Luria-Bertani medium (LB). MRS medium was used, since it is a culture medium designed for the growth of *Lactobacilli*, the sodium acetate in the medium and the low pH prevents the growth of competing microorganisms and therefore the growth of *Lactobacilli* is promoted.

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; Polysorbate 80

(Tween)1; Agar 14 and pH is adjusted to 5.7.

LB medium is composed of (g/l): tryptone, 10; NaCl (10) and yeast extract (5). Solid LB media were obtained by adding 15 g/l agar before autoclaving.

3.7 Isolation of Lactic Acid Bacteria from Qatari samples.

The isolation of the LAB was carried out in enrichment cultures using MRS liquid medium and LB liquid media. The enriched cultures were used for the selection of LAB having the ability to grow in MRS medium at a pH of 5.7. For the isolation of the LAB from the Qatari environment, the followings were used:

1) Fresh palm leaves
2) Dairy feed from Mazzraty (composed of brans, grains, protein, minerals, and vitamins)
3) Spontaneous silages of,
    a. leaves
    b. feed
    c. Mixed leaves and feed (50% w/w)
4) Probiotics prescribed to humans (Trush et al., 2020)

1g of each sample was mixed in a final volume of 15 ml of MRS liquid or LB liquid or LB/MRS (50% v/v) in 50 ml sterile tubes and then incubated at 35°C for a period of 48 h. The exception was with probiotics, from which the isolation of LAB was performed by dissolving 1 tablet in 100 ml of milk and incubated for 24 h. Then, 1 ml of fermented milk was sampled and mixed in 15 ml of MRS liquid. After the incubation of all the cultures, 100 µl was taken from the tubes and plated on solid media for the growth of the bacterial isolates as colonies. The media used for the growth of the bacteria were LB and MRS. The growth was observed at both aerobic and anaerobic conditions both, the more preferred option was aerobic condition since we are isolating such LAB that is facultatively anaerobic. For anaerobic growth, the plates were placed

in a desiccator and a candle was lit inside to consume all the oxygen. Once the candle was blown, the desiccator was placed in the incubator. Separate colonies were selected randomly, since most of the colonies were highly similar, then cultured on MRS solid medium and then sub-cultured 5 successive times to get pure cultures and after that 1 separate colony was isolated, given a code, and used for identification and further studies.

3.8 Determination of growth of LAB and others during the silage (CFU)

Bacterial growth of the LAB was estimated by counting CFUs (Colony Forming Units). It was determined by serial dilutions, made from the culture to be analyzed. 1g from silage was taken and vortexed in 5 ml MRS liquid media in sterile tubes. 100 µl was taken from the tube and serial dilutions were done in 1.5 ml sterilized Eppendorf tubes. 100 µl of each dilution was spread in depth in solid MRS medium and incubated at 37 °C for 48 h.

3.9 Identification by MALDI-TOF MS

The LAB was identified through MALDI TOF MS. For the identification of the bacterial strains, the ribosomal proteins (highly conserved proteins within, genera, or even species and sub-species) are detected to distinguish between the strains and run through a database to identify the bacterial isolate. The database contains the data of the known and referenced bacteria, which will then be used for the identification of the unknown bacterial isolates. Based on the similarities between the mass spectra of the strain and the database, the results are expressed in the form of scores. The score demonstrates the accuracy of identifying the strain, the score ranges from 0 to 3, the bacteria is not identified if the score level is below 1.700, indicating that there is not enough similarity available in the database. The score between 1.700-2.000 indicates that the identification of the sample at the genus level. A score higher than 2.000 and below 2.300 identifies the sample at genus level and probable species level. Whereas

the score higher than 2.300 indicate the identification with high probability at species level indicating that the higher the score higher will be the accuracy of the results.

3.10 Principal Component Analysis (PCA) and strains clustering and dendrograms

MALDI Biotyper software was used to analyze the protein profile of the isolated strains and PCA clusters and dendrogram groups were performed to differentiate the strains and group the strains having similarity based on their protein profile. Species with high similarities are clustered and grouped together in the PCA and dendrogram and are illustrated in graphical forms.

3.11 Preparation of inoculum for silage

The cultures of the isolated strains were prepared in 50 ml sterile tubes using 15 ml of MRS liquid medium. LABs were selected based on their growth pattern, protein profile and score from the identification, to be inoculated in the silage. A single colony of the selected strains was vortexed in the 1.5 ml MRS liquid medium for 5 min. The optical density (OD) of the culture was measured at 600 nm with blank being the plain MRS liquid. Based on the result of the OD, the concentration of the inoculum was calculated by using Eq (1).

$$CV=CV \quad (1)$$

Where C = concentration of microorganisms, V= volume

The initial cell density in the inoculated cultures was 0.15.

3.12 Silage procedure

For the preparation of the silages, the palm tree leaves (cut into small pieces not exceeding 1 cm^2) were mixed with different feed ratios ranging from 0% feed to 50% (w/w), in order to monitor the effects of the different silages at constant moisture levels 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 inoculum, while the studied silages were inoculated with LAB using an initial approximate OD of 0.15 (Table 2).

Table 2: Composition of the silages and their controls

Control Silage (without inoculation)	0% feed 100% leaves	10% feed 90% leaves	20% feed 80% leaves	30% feed 70% leaves	40% feed 60% leaves	50% feed 50% leaves
Inoculated Silage	0% feed 100% leaves	10% feed 90% leaves	20% feed 80% leaves	30% feed 70% leaves	40% feed 60% leaves	50% feed 50% leaves

The mixture of palm tree leaves, and feed were homogenized with moisture and inoculum for the preparation of silage and then filled in the glass bottles to a point where almost all air is removed from the bottles to create anaerobic conditions. The bottles were tightly sealed and incubated at 37°C in dark. The pH, acidity and LAB growth using (CFU) were monitored on a weekly basis till the silage reached a stable state.

3.13 Optimization of the production of LAB as inoculum of the silage using experimental designs.

The optimization of the composition of the medium serving to produce LAB as inoculum to silage was performed by the application of the methodology of experimental designs. This methodology involves a first step of screening influencing factors and a second step of optimizing the level of each influencing factor. The composite plan, a widely used fractional factorial method, was adopted for the selection of culture media compounds influencing the production of LAB. The factors selected for the culture media were feed/leaves ratio, liquid MRS medium, and aeration at highest and lowest percentages, with 20% being high for both feed/leaves ratio and

MRS and low being 0% for MRS media and 5% for feeds/leaves. The aeration was adjusted with high and low revolutions per minute, with high being 200 rpm and low being 100 rpm.

The prepared cultures were covered with aluminum foil and autoclaved and then inoculated with the selected LAB strains and incubated at 35°C for one week. The pH, acidity, and CFU of the culture's media were determined.

3.14 Statistical analysis

Statistical analysis will be used to identify factors with a significant effect on LAB production in MRS or silage media. Depending on the number of influencing factors, a matrix of experiments will be established. The results obtained will be converted into a response surface to study the interactions between different factors and determine the optimal value of each variable. In addition, the construction of the experimental matrix, the statistical analysis and the analysis, of the results of the experiments will be carried out by the software NemrodW 6.0.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Characterization of Qatari Palm tree leaves

4.1.1 Introduction

The severe environmental conditions generate challenges for the production of feed for the livestock. Semiarid and arid regions are mostly dominated by date palm trees. From approximately 105 million palm trees in the World, 62 million are located in the Middle East and North African regions (Al-Khayri et al., 2015). Palm trees are one of the most valuable fruit tree in the Asian region, mainly the Middle East, due to their capacity to endure harsh arid environmental conditions. In Qatar, there are relatively low quantities of agricultural by-products, available for feeding the livestock animals. Those generated from palm trees are mainly green leaves, date pits, and dates that are not commercialized. They are abundant compared to all other agricultural by-products. Despite the fact that the nutritional value of these leaves and date pits is low, these should not be neglected, especially when there is fodder deficiency.

Qatar is ranked among the major date-producing countries in the world with 581,336 palm tree in an area of 2,469 ha (Al-Khayri et al., 2015). In Qatar, palm trees products and by-products are mass-produced in autumn to winter periods, due to the climate conditions. The storage of these products is therefore unavoidable. Also, the hot weather is not favorable for the preservation of palm leaves because of rapid desiccation. The climate conditions and technical constraints are the limiting factors for rational management of the inputs. Silage processes provide a solution for the management of the green leaves from palm trees. It is a reliable and cost-effective process and it can preserve and improve the quality of the green components as silage. For ensiling palm tree leaves, the characterization is essential to determine the fiber and lignocellulosic contents, i.e., cellulose, hemicellulose, and lignin of the fresh leaves. These are the main parameters, which are considered in feeding livestock.

4.1.2 Physical and chemical characterization of fresh Palm Tree Leaves

In order to evaluate the palm tree leaves for transforming them into silage for feeding the ruminants, the first step was to evaluate the composition in terms of fibers besides the conventional parameters, mentioned in Table 3. In fact, the samples analyzed during this work were obtained as follows: One entire brush from each of 5 palm trees was used and the green leaves of each of them were cut into small pieces (1 to 1.5 cm^2). The same procedure was performed for 5 lots distributed around Doha. All the small pieces were homogenously mixed in a container, preserved anaerobically in dark and at 4°C until use. It was estimated based on personal communications with Qatar University Farm Engineers that a single palm tree has roughly 20 kg of green leaves and after each harvesting, large quantities of leaves are accumulated in the agriculture field. The analyses were repeated in triplicates and the results are presented as the average of three determinations using three samples.

Table 3: Characterization of Palm Tree Leaves. The results are presented as average weight percentages.

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

The palm tree leaves were found to contain high dry matter content being of 63.95% and low in ash content with 4.34%. Similar results are reported by Aziz (2019) in terms of dry matter being 62.94%. The crude proteins content was found to be low

(8.72%) similar to the results reported by Jonoobi et al. (2019). The palm tree leaves are rich in total fibers (85.5%). The high percentages of cellulose (22.07%) and lignin (39.6%) show the strength of the fibers in the palm tree leaves. The inorganic content of the palm tree leaves is 4.34%, determined by the ash content. The organic content in dry matter was 59.61%. However, the leaves contain 52.5% of cellulose, hemicellulose, lignin, and proteins. Mirmehdi et al. (2014) reported similar results with palm leaves from Iran in terms of hemicellulose and lignin being of 12.8% and 32.3% with a higher concentration of cellulose only (40.21%). Similarly, Nasser et al. (2016) reported higher cellulose composition of date palm tree leaves from Saudi Arabia with 47.14%, with hemicellulose and lignin being close to the currents registered in Qatari Palm trees, being 16.13% hemicellulose and 36.73% lignin. AL-Oqla & Sapuan, (2014) used palm trees from Malaysia and reported low cellulose and lignin contents of 20% and 23% with high hemicellulose content of at 55%, low ash content of 1.1%. These variations in the composition of the fiber contents could be mainly due to the variety of the plants used, their age, and environmental conditions (AL-Oqla, 2020).

4.1.3 Conclusion

The high fiber contents in the palm tree leaves are expected with a high percentage of lignin and low crude protein content. This composition reduces the digestibility and nutritional value of palm tree leaves, to be used as feed for the ruminant. However, Khattab & Abd El Tawab (2018) suggested that the nutritional value and digestibility of the palm tree leaves can be enhanced with the addition of fibrolytic enzymes or by the addition of nutrients, or by mixing with other feed sources for increasing the ruminal fermentation. Ensiling palm tree leaves as a source of feed for the ruminants can also be beneficial economically and agriculturally. In addition, palm tree leaves are a good source of bioactive compounds and have been linked to

ethno-medicinal properties to improve human health (Mirmehdi et al., 2014). This may be beneficial also to ruminants, which will be fed with rations containing silage of palm tree leaves. Silage is a biological process, which preserves the nutritional quality of the feedstock.

4.2 Isolation and Identification of Lactic Acid Bacteria (LAB)

4.2.1 Introduction

Identification of LABs is essential in silage preparation to analyze the outcomes of the fermentation process such as the decrease in pH and increase in acidity due to the release of acids by LAB. The isolation is generally performed using enrichment culturing mode with the well-known MRS medium. The MRS medium (deMan, Rogosa, Sharpe) is used for culturing *Lactobacillus*. The selectivity of the medium is only ensured by its pH. The medium acidified to pH 5.7 makes it possible to count *Lactobacillus* cells and mesophilic lactic acid bacteria. It is advisable to use a more selective medium for heavily contaminated samples. There is a constant demand to enhance the process of identification, being more reliable, cost-effective and less time-consuming, not just in microbiology but also in all fields of science. MALDI TOF MS in comparison to other microbial identification is a more attractive method due to its robustness, cost-effective and reliability and can prove to be a substitute to biochemical and molecular biology-based identification techniques, which require complex extraction of nucleic acids and perform polymerase chain reaction or sequencing (Seuylemezian et al., 2018). The process of identification being rapid and less sample size required, have made MALDI TOF MS more acceptable by the microbiologists. MALDI TOF MS has proven to be reliable based on numerous studies and different uses not only in microbial identification but also in detection of pathogens in food and water, identification of pathogens in blood, and epidemiological studies due to the characterization of strains at sub-species levels (Singhal et al., 2015). MALDI TOF MS

has also been used for the identification of antibiotics mechanisms and allergies by specific biochemical markers (Wieser et al., 2011).

An objective of this research is to isolate and identify the LAB adapted to the local harsh environmental conditions in Qatar through a program of isolation. MALDI TOF MS has been adopted for the identification and differentiation of different LAB isolates from silages in this research.

4.2.2 Isolation and identification of LAB

The isolation of the LAB was carried out in enrichment cultures using MRS liquid medium and LB liquid medium. The enriched cultures were used for the selection of LAB having the ability to grow in MRS medium at a pH of 5.7. For the isolation of the LAB from Qatari environments, the following were used: Fresh palm leaves, Animal feed, spontaneous silages of palm tree leaves or feed only, or a 50% mixture of leaves and feed. The study also used a commercial probiotic to humans, to compare the commercial LAB used as probiotic to the LAB existing in the Qatari Environment. Initially, the isolation was performed at aerobic conditions, since the more preferred option was to isolate aerobic or facultative LAB strains. Then, it was extended to the anaerobic conditions, ensured in hermetically jars in which a candle was lighted and then shutdown is an indicator of anaerobic conditions after the complete use of the available oxygen in the jar.

The growth of the isolated microorganisms from leaves and mixed silage was higher at aerobic conditions, but the strains isolated from probiotics showed better growth at anaerobic conditions. The isolation of LAB from leaves, feed, and spontaneous silages was performed in anaerobic and aerobic conditions to identify the microorganisms that will grow in both conditions. The results of the identification of aerobic and anaerobic strains were different.

The identification of the LAB isolates from different sources on MRS agar medium was performed by MALDI TOF MS (Table 4). Eighty-seven LAB isolates were identified and shown belonging to eight different species. The strains isolated from palm tree leaves are all belonging to the genera *Staphylococcus*, with five *Staphylococcus epidermidis* and two *Staphylococcus gallinarum.* Eight LAB strains were isolated from probiotics in anaerobic conditions since the growth of the isolates was not detectable at aerobic conditions. 3 LAB strains were isolated from leaves silage at aerobic conditions. 76 LAB strains were isolated from mixed silage performed with spontaneous silage (50% feed and 50% leaves), both at aerobic and anaerobic conditions, 22 grew in anaerobic conditions and 57 grew in aerobic conditions. The objective of this study was to study the diversity in the silage on the basis of growth conditions of the isolated LAB strains.

The identification of the 87 strains using MALDI TOF MS is shown in Table 4 with the score and the source of each isolated strain and the conditions in which the isolation was performed. The MALDI TOF MS scores of the isolated strains are illustrated in Figure 2. 6 strains with score greater than 2.3, depicting high probable species identification. 37 strains with score greater than 2 and less than 2.3, depicting identification at genus level and probable species level. 44 strains were identified with score greater than 1.7 and less than 2.0, indicating the identification of the strains at genus level.

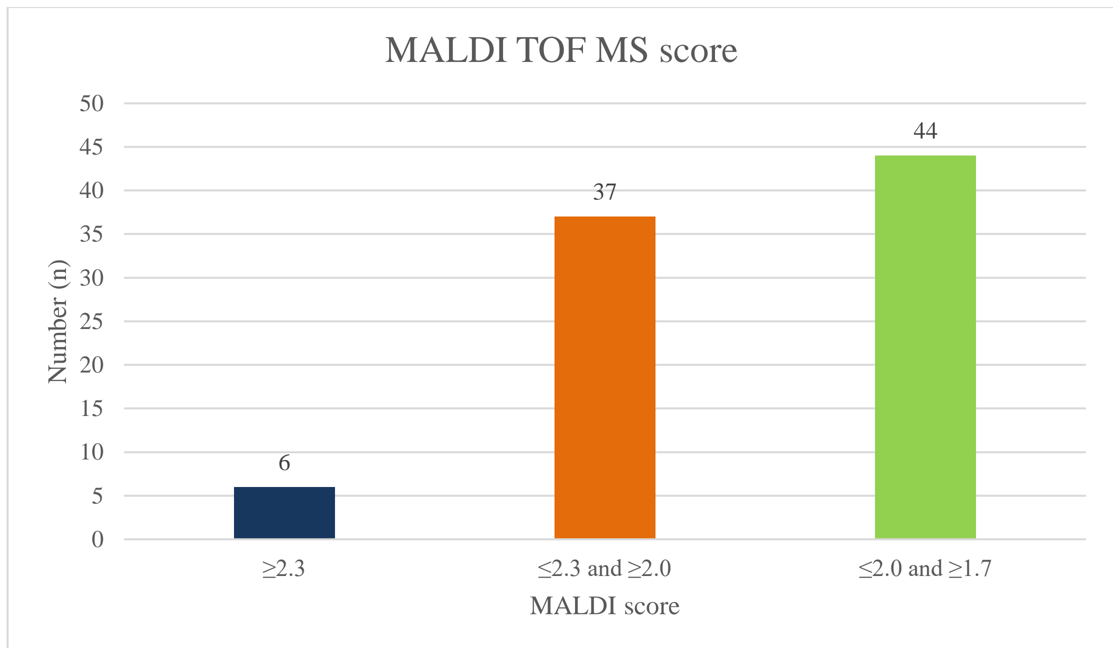

Figure 2: MALDI TOF MS scores of the isolated lactic acis bacteria

The strains showing better growth in anaerobic conditions were 31 and the isolates growing better at aerobic conditions were 57. The 31 strains that showed good growth in anaerobic conditions (Figure 3) were 13 strains of *Lactobacillus paracasei*, 5 strains of *Lactobacillus oris*, 7 strains of *Lactobacillus vaginalis,* 5 strains of *Lactobacillus plantarum* and 1 strain of *Pediococcus acidilactici.* The 57 strains that showed good growth in aerobic conditions (Figure 4) were 7 strains of *Lactobacillus plantarum*, 1 strain of *Lactobacillus jhonsonii* and *Lactobacillus pentosus* each, 16 strains of *Lactobacillus farciminis* and 32 strains of *Pediococcus acidilactici*. As a first notice, it is likely that *Pediococcus acidilactici* is more adapted to co-silage of palm tree leaves and the commercial feed, followed by *Lactobacillus farciminis* and then *Lactobacillus plantarum*, these LABs are the most abundant.

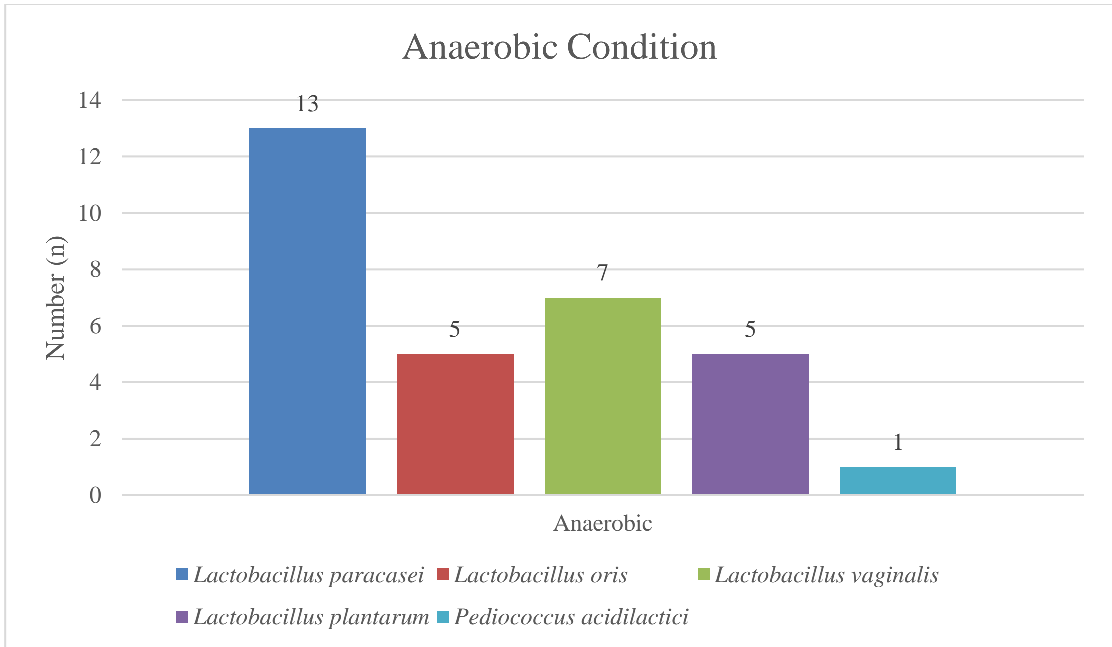

Figure 3: Lactic acid bacteria strains grown in anaerobic conditions

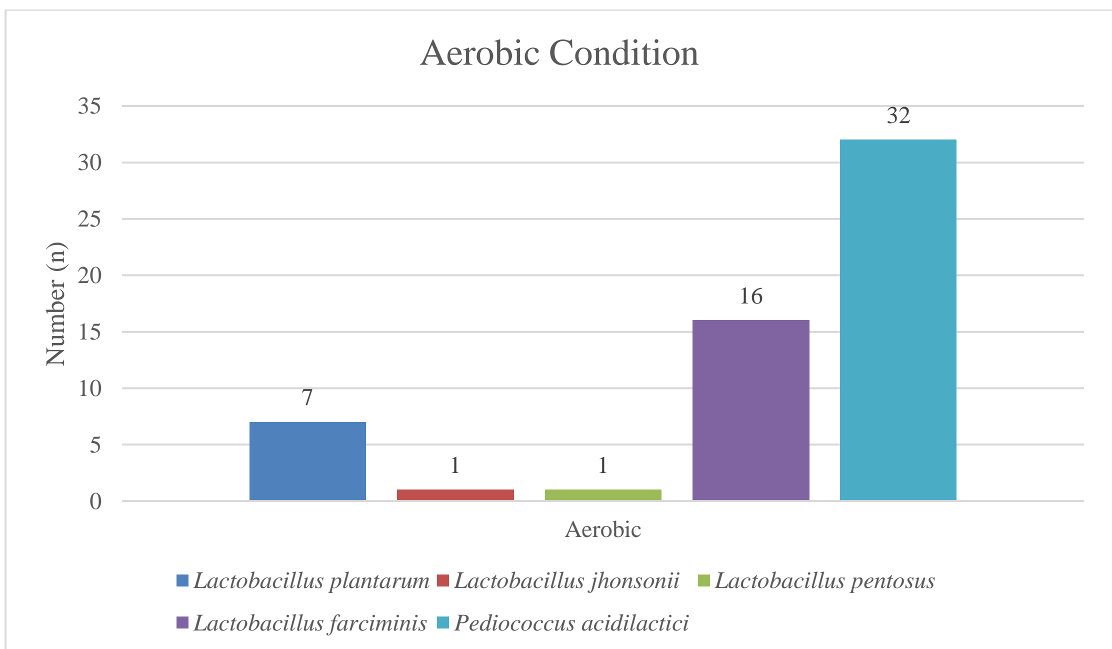

Figure 4: Lactic acid bacteria strains grown in aerobic conditions

Table 4: Identification of LAB isolated from different sources using MALDI TOF MS

Source	Strain Code	Identification by MALDI TOF MS	MALDI TOF MS Score	Code for PCA Analysis
		Aerobic growth conditions		
Leaves	SMZ2	*Staphylococcus gallinarum*	1.7	-
	SMZ7	*Staphylococcus epidermidis*	1.79	-
	SMZ8	*Staphylococcus epidermidis*	1.92	-
	SMZ11	*Staphylococcus epidermidis*	2.06	-
	SMZ12	*Staphylococcus epidermidis*	1.98	-
	SMZ13	*Staphylococcus epidermidis*	2	-
	SMZ4	*Staphylococcus gallinarum*	1.95	-
Leaves Silage	SMZ92	*Pediococcus acidilactici*	2.15	12
	SMZ43	*Pediococcus acidilactici*	1.86	10
	SMZ46	*Lactobacillus plantarum*	2.22	11
Mixed Silage	SMZ47	*Lactobacillus johnsonii*	2.22	9
	SMZ62	*Lactobacillus farciminis*	1.82	27
	SMZ63	*Lactobacillus farciminis*	1.78	28
	SMZ97	*Pediococcus acidilactici*	2.21	61
	SMZ53	*Lactobacillus plantarum*	1.84	13
	SMZ54	*Pediococcus acidilactici*	1.78	19
	SMZ48	*Pediococcus acidilactici*	1.72	15
	SMZ49	*Pediococcus acidilactici*	1.7	16
	SMZ55	*Pediococcus acidilactici*	1.84	20
	SMZ56	*Lactobacillus farciminis*	1.72	21
	SMZ57	*Lactobacillus farciminis*	1.7	22
	SMZ52	*Pediococcus acidilactici*	2.0	14
	SMZ51	*Pediococcus acidilactici*	2.03	18
	SMZ75	*Pediococcus acidilactici*	1.82	38
	SMZ58	*Lactobacillus farciminis*	1.79	23
	SMZ59	*Lactobacillus farciminis*	1.75	24
	SMZ60	*Lactobacillus farciminis*	1.79	25
	SMZ61	*Lactobacillus farciminis*	1.80	26
	SMZ64	*Lactobacillus farciminis*	1.80	29
	SMZ65	*Lactobacillus farciminis*	1.81	30
	SMZ66	*Pediococcus acidilactici*	2	31
	SMZ67	*Pediococcus acidilactici*	2.1	85
	SMZ68	*Pediococcus acidilactici*	1.9	32
	SMZ69	*Lactobacillus farciminis*	1.76	33
	SMZ70	*Lactobacillus farciminis*	1.76	34
	SMZ71	*Lactobacillus farciminis*	1.79	35
	SMZ72	*Lactobacillus farciminis*	1.81	36

Source	Strain Code	Identification by MALDI TOF MS	MALDI TOF MS Score	Code for PCA Analysis
	SMZ73	*Lactobacillus farciminis*	1.8	37
	SMZ74	*Lactobacillus pentosus*	1.7	41
	SMZ77	*Pediococcus acidilactici*	2	40
	SMZ41	*Pediococcus acidilactici*	2.32	56
	SMZ94	*Pediococcus acidilactici*	2.1	58
	SMZ95	*Pediococcus acidilactici*	2.18	59
	SMZ93	*Pediococcus acidilactici*	2.07	57
	SMZ96	*Pediococcus acidilactici*	2.07	60
	SMZ98	*Pediococcus acidilactici*	2.2	62
	SMZ99	*Pediococcus acidilactici*	2.3	63
	SMZ100	*Pediococcus acidilactici*	2.21	64
	SMZ101	*Pediococcus acidilactici*	1.92	65
	SMZ102	*Lactobacillus plantarum*	1.98	66
	SMZ19	*Lactobacillus plantarum*	2.09	67
	SMZ104	*Lactobacillus plantarum*	1.99	68
	SMZ105	*Lactobacillus plantarum*	1.98	69
	SMZ106	*Lactobacillus plantarum*	2.1	70
	SMZ107	*Pediococcus acidilactici*	2.04	71
	SMZ108	*Pediococcus acidilactici*	1.83	72
	SMZ109	*Pediococcus acidilactici*	2.07	73
	SMZ112	*Pediococcus acidilactici*	2.12	76
	SMZ110	*Pediococcus acidilactici*	1.86	74
	SMZ111	*Pediococcus acidilactici*	2.13	75
	SMZ113	*Pediococcus acidilactici*	1.89	86
	SMZ114	*Pediococcus acidilactici*	2.15	87
	SMZ122	*Lactobacillus farciminis*	1.73	84
	SMZ76	*Pediococcus acidilactici*	1.9	39
		Anaerobic growth conditions		
Probiotics	SMZ103	*Lactobacillus plantarum*	1.78	1
	SMZ34	*Lactobacillus plantarum*	2.26	5
	SMZ20	*Lactobacillus paracasei*	2.34	3
	SMZ39	*Lactobacillus plantarum*	2.34	6
	SMZ24	*Lactobacillus paracasei*	1.85	4
	SMZ15	*Lactobacillus paracasei*	2.11	2
	SMZ25	*Lactobacillus plantarum*	2.13	8
	SMZ37	*Lactobacillus plantarum*	2.01	7
Mixed silage	SMZ77	*Pediococcus acidilactici*	2	40
	SMZ50	*Lactobacillus paracasei*	2.20	17
	SMZ82	*Lactobacillus oris*	1.91	46

Source	Strain Code	Identification by MALDI TOF MS	MALDI TOF MS Score	Code for PCA Analysis
Mixed silage	SMZ78	*Lactobacillus paracasei*	2.36	42
	SMZ79	*Lactobacillus paracasei*	2.28	43
	SMZ80	*Lactobacillus oris*	1.85	44
	SMZ81	*Lactobacillus oris*	1.99	45
	SMZ83	*Lactobacillus oris*	2.11	47
	SMZ84	*Lactobacillus oris*	2.07	48
	SMZ85	*Lactobacillus vaginalis*	1.98	49
	SMZ86	*Lactobacillus vaginalis*	2.17	50
	SMZ87	*Lactobacillus vaginalis*	2.16	51
	SMZ88	*Lactobacillus vaginalis*	2.12	52
	SMZ89	*Lactobacillus vaginalis*	2.04	53
	SMZ90	*Lactobacillus vaginalis*	2.03	54
	SMZ91	*Lactobacillus vaginalis*	2.02	55
	SMZ115	*Lactobacillus paracasei*	2.17	77
	SMZ116	*Lactobacillus paracasei*	2.09	78
	SMZ117	*Lactobacillus paracasei*	2.29	79
	SMZ120	*Lactobacillus paracasei*	2.34	82
	SMZ118	*Lactobacillus paracasei*	2.28	80
	SMZ119	*Lactobacillus paracasei*	2.32	81
	SMZ121	*Lactobacillus paracasei*	2.3	83

Lactobacillus plantarum is a facultative homofermentative lactic acid bacterium. It is widely used as a lactic acid bacterium in silages due to its quality of being a powerful lactic acid fermenter and high acid tolerant (Corsetti & Valmorri, 2011). *Lactobacillus paracasei* is also a homofermentative LAB and has been reported to have the ability to hydrolyze starch into simple carbohydrates, glucose and lactic acid. This species can survive at acidic pH down to pH 2.0 making its high resistance in the digestive tract. *Lactobacillus paracasei* in feed can increase carbohydrates and proteins metabolism and enhance the performance of the organisms (Gobbetti & Minervini, 2014). *Lactobacillus farciminis* is a probiotic reported to be used as stress suppresser, due to the spontaneous release of nitric oxide in the colonial lumen which

has anti-inflammatory effects. *Lactobacillus farciminis* in feed can prevent stress-inducing hypersensitivity in animals (Ait-Belgnaoui, 2005). *Lactobacillus johnsonii* is commonly found in the intestines of humans and animals with many other microorganisms. *Lactobacillus johnsonii* is characterized as the "acidophilus complex" of the *Lactobacillus* genus, involved in probiotic activities and is beneficial for the health and wellbeing of humans and animals. *Lactobacillus johnsonii* is also characterized as efficient for pathogen inhibition and immunomodulation (Pridmore et al., 2004). *Lactobacillus pentosus* is a facultative homofermentative LAB and a probiotic, most commonly isolated from the gastrointestinal tract and it exhibits good growth and survival capacities in such aforementioned conditions. *Lactobacillus pentosus* has the ability to combat pathogenic bacteria by the fermentation of several prebiotics and lactose. *Lactobacillus pentosus* is highly dependent on its ecological niche (Abriouel et al., 2017). *Lactobacillus vaginalis* is commonly sound in the uterus of dairy animals. It plays an important role in maintaining a healthy vaginal ecosystem by acting as a first defense against pathogenic bacteria by producing acids such as acetic and lactic acid and other antibacterial molecules. They act as the first immune response against invading pathogens (Gärtner et al., 2015). *Lactobacillus oris* is commonly found in the milk and oral cavity of humans and animals. *Lactobacillus oris* has a high pH tolerance and can survive at pH 1.0, and therefore can survive in the gastrointestinal tract. *Lactobacillus oris* has been reported to exhibit a consistent reduction of pathogenic bacteria in the inhibitory zones (Afrin et al., 2021). *Pediococcus acidilactici* is facultative homofermentative LAB and is mostly used as silage inoculants, with high similarities to the genus *Lactobacilli*, phenotypically and genotypically, therefore widely used in food products with other LABs (Crow & Curry, 2002).

4.2.3 Conclusion

LAB is abundantly available in spontaneous silages with a high percentage and variety of species belonging to the genus *Lactobacilli*. A high number of species from the genus *Pediococcus* have also been identified alongside *Lactobacilli* in the spontaneous silage. The high occurrence of LAB found in the spontaneous silage of the palm tree leaves mixed with animal feed is a good indication that ensiling palm leaves can support the growth of LAB and produce a good quality of silage. However, other bacteria of the genus *Staphylococcus* are also present in the leaves. In the case of preparing silages using palm tree leaves, there is a possibility that these bacteria can be found in the final silage and may also dominate the silage instead of the LAB and degrade the quality of the silage or the rapid decrease of pH during silage preparation which can kill these pathogens with time and maintain the quality of the silage.

4.3 Differentiation of the isolated strains

4.3.1 Introduction

The differentiation of the isolated LAB strains and the relationship among them was determined by combining MALDI TOF MS and PCA analysis. Indeed, it is absolutely important to establish the relationship and the similarity between the isolates because several isolates may be the same. However, the MALDI TOF MS technique allowed obtaining the proteins profile of each isolate. The PCA analysis helps in differentiating closely related isolates and is illustrated graphically. The peaks generated by the protein profile of the strains were used to prepare the PCA graph by using a default algorithm. This method illustrates the results in the form of clustered groups that help in differentiating the isolates with similar characteristics. The results are represented in the 3D coordinate system (PC1, PC2 and PC3).

4.3.2 Diversity study among the isolated LABs

The PCA codes used in the clustering diagram are shown in Table 4 and the

results of the PCA analysis are shown in Fig 5-A.

The PCA results illustrated large biodiversity among the strains at the proteins profiles level. The total variance of the 10 PCA's is shown in Fig 5-B. The three principal components PC1, PC2, and PC3, carry 28%, 13%, and 11% of variability among the strains, combining a total of 52% variability. The three principal components were used to create 5 cluster groups of LAB isolates. The distance between the clusters shows the variation at the group level and the distance within the cluster between the strains shows the difference in proteins profile at the strain level. Cluster 1, which has negative correlation with PC1 and a positive correlation with PC2 and PC3, includes 14 *Lactobacillus farciminis* isolates (SMZ56, SMZ57, SMZ58, SMZ59, SMZ60, SMZ61, SMZ62, SMZ63, SMZ69, SMZ71, SMZ72, SMZ73, and SMZ122). Cluster 2 which has a positive correlation with PC1 and PC2 and negative correlation with PC3, includes 9 isolates of *Lactobacillus paracasei* (SMZ20, SMZ50, SMZ79, SMZ115, SMZ116, SMZ117, SMZ119, SMZ120, and SMZ121) and one isolate of *Lactobacillus farciminis* (SMZ70). Cluster 3 which has a positive correlation with PC1 and PC3 and negative correlation with PC2, includes one strain of *Lactobacillus paracasei* (SMZ78), 3 strains of *Lactobacillus plantarum* (SMZ102, SMZ104, and SMZ105), 2 strains of *Lactobacillus farciminis* (SMZ64 and SMZ65) and all the 20 strains of *Pediococcus acidilactici* (SMZ41, SMZ43, SMZ48, SMZ49, SMZ51, SMZ52, SMZ55, SMZ66, SMZ67, SMZ68, SMZ76, SMZ77, SMZ92, SMZ93, SMZ94, SMZ95, SMZ96, SMZ97, SMZ98 and SMZ99). Cluster 4 which has a negative correlation with all three components is composed of the isolate *Lactobacillus johnsonii* (SMZ47), 9 isolates of *Lactobacillus plantarum* (SMZ19, SMZ25, SMZ34, SMZ39, SMZ37, SMZ46, SMZ53, SMZ103, and SMZ 106) and 3 isolates of *Lactobacillus paracasei* (SMZ15, SMZ24 and SMZ118). Cluster 5 having a negative correlation with all the components has 1

isolate of *Lactobacillus pentosus* (SMZ74), all the 5 isolates of *Lactobacillus oris* (SMZ80, SMZ81, SMZ82, SMZ83 and SMZ84) and all the 7 isolates of *Lactobacillus vaginalis* (SMZ85, SMZ86, SMZ87, SMZ88, SMZ89, SMZ90 and SMZ91).

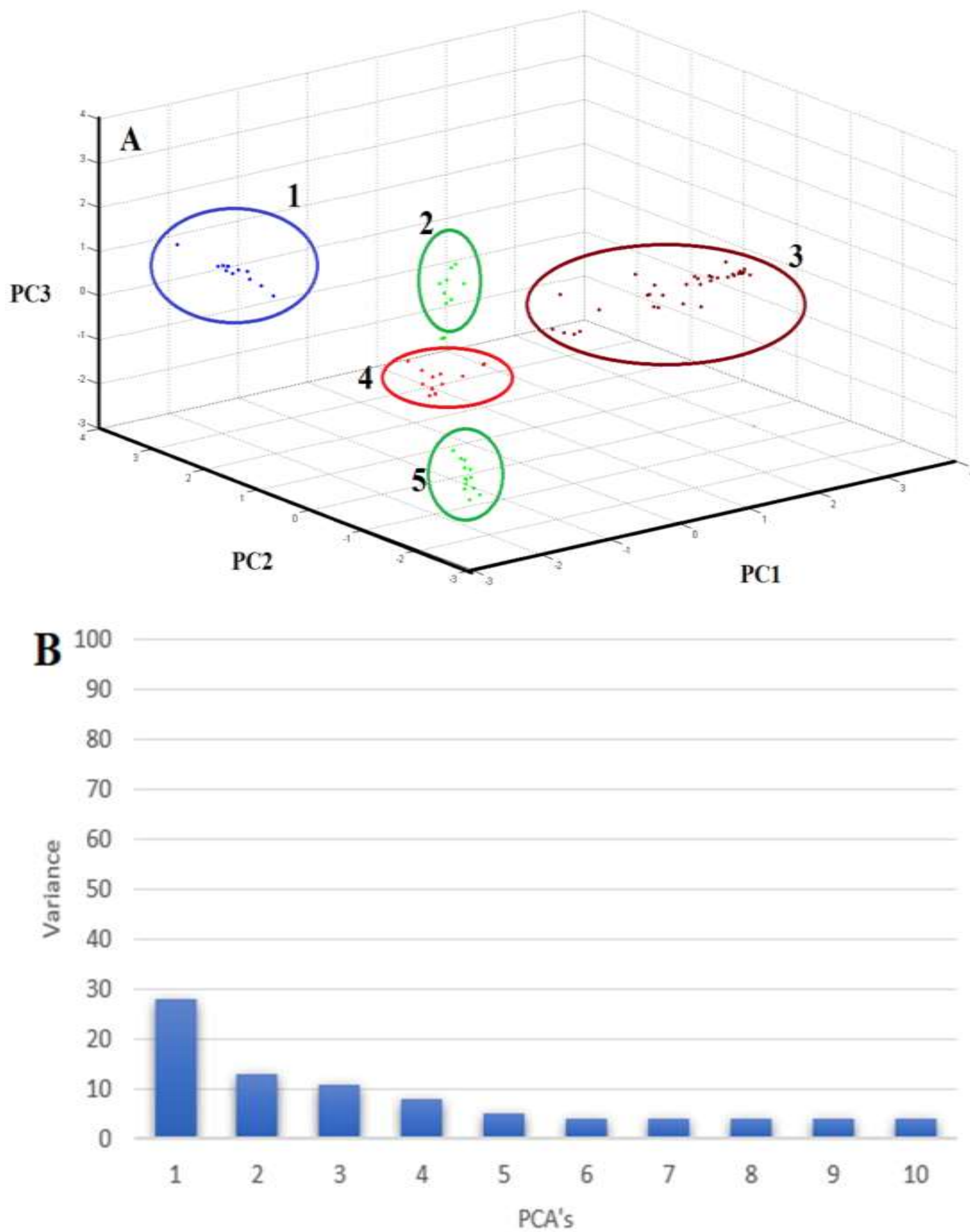

Figure 5: Classification of LAB strains using PCA (A) and Percentage of variance explained (B).

It can be observed that the same species of LAB are grouped together in the same clusters such as *Lactobacillus farciminis* in Cluster 1, *Lactobacillus paracasei* in

Cluster 2 and *Lactobacillus plantarum* in Cluster 4. The most diversified cluster group was Cluster 3 with 4 different LABs, *Lactobacillus farciminis*, *Lactobacillus paracasei*, *Lactobacillus plantarum* and *Pediococcus acidilactici* showing high similarities of their protein profiles. Cluster 5 formed of 3 different LAB species: *Lactobacillus pentosus*, *Lactobacillus oris* and *Lactobacillus vaginalis* shows high similarities between them.

The PCA results led to the creation of the corresponding dendrogram as phyllo-proteomic tree. The isolated strains were grouped based on their protein expression profile as illustrated in Fig 6. The isolated strains were categorized into five distinct groups on the basis of their similarity. Eleven *Lactobacillus farciminis* (SMZ57, SMZ58, SMZ60, SMZ61, SMZ62, SMZ63, SMZ70, SMZ71, SMZ72, SMZ73 and SMZ122) and *Pediococcus acidilactici* (SMZ68) were grouped together in group I. Twelve *Lactobacillus paracasei* (SMZ15, SMZ20, SMZ24, SMZ50, SMZ79, SMZ115, SMZ116, SMZ117, SMZ118, SMZ119, SMZ120 and SMZ121) and one *Pediococcus acidilactici* (SMZ75) were grouped in group II. One *Lactobacillus pentosus* (SMZ74), five *Lactobacillus oris* (SMZ80, SMZ81, SMZ82, SMZ83 and SMZ84) and seven *Lactobacillus vaginalis* (SMZ85, SMZ86, SMZ87, SMZ88, SMZ89, SMZ90 and SMZ91) were grouped in group III. Eight *Lactobacillus plantarum* (SMZ19, SMZ25, SMZ34, SMZ37, SMZ39, SMZ46, SMZ53 and SMZ106) and single strains of *Pediococcus acidilactici* (SMZ108) and *Lactobacillus johnsonii* (SMZ47) were grouped in group IV. Two *Pediococcus acidilactici* (SMZ109 and SMZ112) and *Lactobacillus plantarum* (SMZ103) are grouped in group V. Twenty-four *Pediococcus acidilactici* (SMZ41, SMZ43, SMZ49, SMZ51, SMZ52, SMZ54, SMZ55, SMZ66, SMZ67, SMZ76, SMZ77, SMZ92, SMZ93, SMZ94, SMZ96, SMZ97, SMZ98, SMZ99, SMZ100, SMZ101, SMZ107, SMZ110, SMZ111, SMZ113), three *Lactobacillus farciminis* (SMZ56, SMZ59 and SMZ69), three *Lactobacillus plantarum*

(SMZ102, SMZ104 and SMZ105) and *Lactobacillus paracasei* (SMZ78) were grouped in group VI.

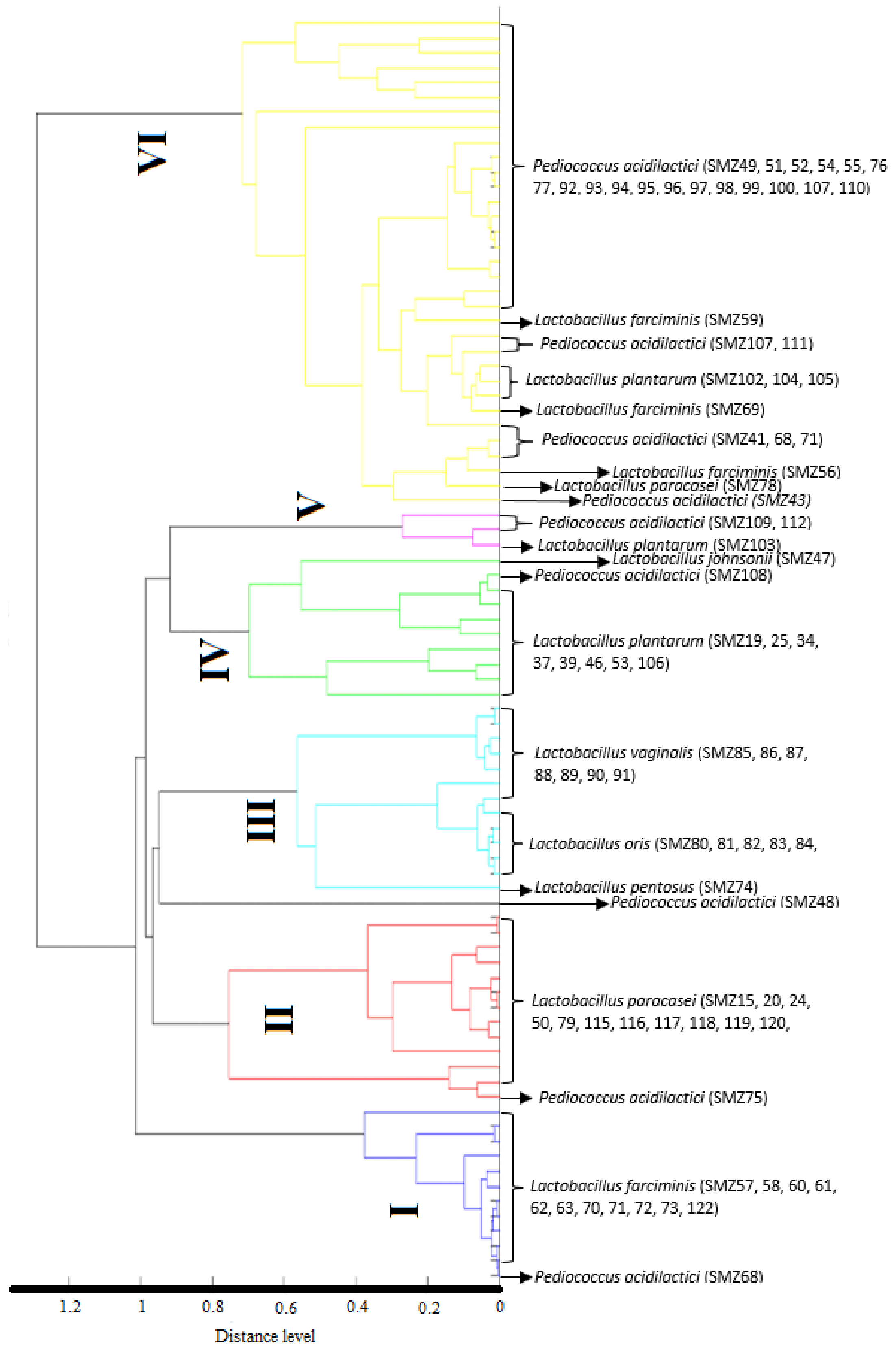

Figure 6: Dendrogram illustrating the clustering of 87 strains of LAB identified by MALDI-TOF MS.

The results of PCA and dendrogram showed similar results in terms of a grouping of the LABs. *Lactobacillus oris*, *Lactobacillus vaginalis* and *Lactobacillus pentosus* have shown similarities in both PCA and dendrogram graphs being in the same clusters and groups, based on the high similarities of their proteins profile. *Lactobacillus farciminis* has been grouped in a single cluster and group in PCA and dendrogram showing that the isolated strains of this species are similar to one another. Similarly, *Lactobacillus paracasei* have also been grouped in the same cluster and group showing high similarity among the isolates of this species. *Lactobacillus plantarum* and *Lactobacillus johnsonii* have shown similarity in both PCA and dendrogram, being in the same cluster and group. *Lactobacillus plantarum* and *Lactobacillus johnsonii* have been reported to have similar environmental conditions and also resides in the gastrointestinal tract with the same characteristics of being tolerant to acids and bile salts and being able to secrete antimicrobial compounds and restricting the growth of pathogens (Tiani et al., 2017). The places of the isolated strains of *Pediococcus acidilactici* in both PCA and dendrogram graphs, show their high similarity in addition to some other LAB as well, such as *Lactobacillus farciminis*, *Lactobacillus paracasei* and *Lactobacillus plantarum*. Phenotypically the LAB has a very large variability and some species or strains are most closely similar to one another. *Pediococcus* and *Lactobacillus* in description have similar growth characteristics and transformation patterns, Bosma et al., (2017) reported the similarities between *Pediococcus acidilactici*, *Lactobacillus plantarum* and *Lactobacillus paracasei* based on their growth pattern, transformation and phylloproteomic tree (similar to this research).

Based on the results of the identification, *Pediococcus acidilactici* isolates have shown similarities to *Lactobacilli* species. The isolate SMZ68 has been grouped with

Lactobacillus farciminis in both the PCA cluster and dendrogram group. The isolate SMZ75 has been grouped with *Lactobacillus paracasei* in both PCA and dendrogram. Similarly, the isolate SMZ108 has been found in the same group of PCA and dendrogram with *Lactobacillus plantarum*. Cluster 3 in PCA with high numbers of *Pediococcus acidilactici* has shown similarities to the same *Lactobacilli* species being in the same cluster and similar results can be observed in the dendrogram graph as well. Santos et al., (2019) reported a study in which LAB strains belonging to *Lactobacilli* and *Pediococcus* genera were identified and compared on the basis of similarities. The results indicated a high level of similarity between *Pediococcus* and *Lactobacillus* genera and these genera form a separate group from other LAB based on phylogenetic (Puntillo et al., 2020). Similar results of the similarity of the genera *Lactobacillus* and *Pediococcus* were reported by Säde & Björkroth (2019), due to their close phylogenetic relationship. *Lactobacillus* and *Pediococcus* share characteristics based on the intergenic spacer region (ISR) that is found between the 16S and 23S rRNA genes. The ISR exhibits a powerful tool for the differentiation between bacterial genera.

4.3.3 Conclusion

The use of the proteins profile in the identification and differentiation of the LAB isolates is an informative approach. MALDI TOF MS can prove to be precise, quick and cost-effective and reliable, applicable to LAB. The accuracy of this technique, however, is reliant on the database of the strains in the reference spectral database (Abdel Samad et al., 2020). In this study, the similarities and the differences between a large number of 87 LAB strains allowed the selection of different isolates for the silage process of palm trees leaves. This approach is shown very interesting to be used also to follow up the occurrence of LAB strains dominating the silage and to investigate their variations during the process. It will also help in selecting the most

appropriate strain which can dominate all the others during the silage.

4.4 Alternatives for the ensiling the palm tree leaves

4.4.1 Introduction

LAB are abundantly used in the food preservation and induced silage of non-conventional agricultural by-products. The 4 main used genera of LAB are; *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus*. Based on several studies, *Lactobacillus* and *Pediococcus* are the most found and used genera in silage. The most dominant species are *Lactobacillus plantarum* and *Pediococcus acidilactici* (Drouin et al., 2020). *Lactobacillus plantarum* is the species, which is the most widely used for inoculating many silages, such as wheat, sorghum, lucerne, and maize (Grant & Adesogan, 2018). *Pediococcus acidilactici* has proven to have a short lag phase and therefore starting the fermentation process more robustly than other LAB. Due to its instant growth, *Pediococcus acidilactici* is known to dominate the initial stages of the silage and making the conditions favorable for the growth of other LABs (Fugaban et al., 2021). *Pediococcus acidilactici* is abundantly used for ensiling with *Lactobacillus plantarum*. *Lactobacillus paracasei* have been reported to maintain lower contents of NH_3-N in the silage and therefore protecting the feed proteins from degradation. This is due to the rapid growth of *Lactobacillus paracasei*, inhibiting thus, the growth of aerobic microorganisms. *Lactobacillus paracasei* has also been known to exhibit great performance in producing water-soluble carbohydrates and prevention of proteolysis (LIU et al., 2016). *Lactobacillus paracasei*, with its efficient proteolysis prevention, maintains the proteins content in the feed and enhance the quality of the silage.

4.4.2 Selection of LAB for silage

Based on this analysis of the importance of several LAB species in silage, considering the identification, and clustering of the LAB isolated in this study, 4 strains were selected for the inoculation of the silage based on the palm trees leaves (Table 5).

In addition, a preliminary study performed in our laboratory classified the strains based on their growth rates at aerobic and anaerobic conditions (results not shown). Two strains of *Lactobacillus plantarum* were selected as isolated from different sources. The strains *Lactobacillus plantarum* SMZ103 were isolated from probiotics and *Lactobacillus plantarum* SMZ46 isolated from Leaves silage. In addition, the strain *Pediococcus acidilactici* SMZ41 was selected as having the shortest lag phase of growth on MRS solid medium, compared to the other LAB isolates of our collection. Colonies start to appear after overnight incubation, while the other LAB strains start to show colonies after 24h at least. The rapidity of growth of the inoculated LAB strains in the initial phase depletes the residual oxygen rapidly and creates rapidly the anaerobic conditions appropriate for the fermentation. Finally, *Lactobacillus paracasei* SMZ20 was selected as the fourth strain to be inoculated in the silage, since isolated from the commercial probiotics. The inocula of the strains *Lactobacillus paracasei* SMZ20 and *Lactobacillus plantarum* SMZ103 were prepared at anaerobic conditions since they did not show good growth at aerobic conditions, whereas those of the strains *Pediococcus acidilactici* SMZ41 and *Lactobacillus plantarum* SMZ46 were prepared at the aerobic conditions. The selected strains registered MALDI TOF scores, higher than 2.000, indicating their identification at the genus level and probable species level (Score 2.000 – 2.299). In addition, the selected strains were shown enough different since they belong to distinct groups of the PCA and dendrogram analyses (Table 5).

Table 5: Selected lactic acid bacteria for silage inoculation

Source	Strain	Specie	Score	PCA and dendrogram group	Growing Conditions
Probiotics	SMZ103	*Lactobacillus plantarum*	2.34	Group V	Anaerobic
Leaves Silage	SMZ46	*Lactobacillus plantarum*	2.22	Group IV	Aerobic
Mixed Silage	SMZ41	*Pediococcus acidilactici*	2.15	Group VI	Aerobic
Probiotics	SMZ20	*Lactobacillus paracasei*	2.11	Group II	Anaerobic

4.4.3 Co-silage of palm trees leaves with animal feed

The inoculum, which should be optimal to inoculate the silage, plays an important role in the growth profile of the inoculated LAB strain and its interaction with the endogenous bacteria. Here, a preliminary work performed in our laboratory showed that the liquid culture of the LAB is optimal in a 50 ml-falcon tube containing 15 liquid MRS medium, inoculated with a suspension of vegetative cells giving a final OD of 0.15 at 600 nm. The suspension should be prepared by re-suspending 1 isolated colony from an overnight incubated MRS plate, in 1.5 ml MRS. If more suspension volume is needed, the same procedure is applied using a 15 ml MRS and the equivalent of 10 colonies. Silages were prepared in 500 ml bottles with a total volume of 550 ml. A volume of approximately 4.5 ml inoculum was used to inoculate the silage, based on the OD of the inoculum. The initial OD_{600nm} in the silage is estimated by calculation for an initial value of 0.15. Different feed/leaves ratios were used (0%, 10%, 20%, 30%, 40% and 50%). Based on preliminary work, the addition of 100 ml distilled water to every 550 ml of piled up mixture is appropriate, to prevent any lixiviate generation in the bottom of the silage. The lixiviate is a source of liquid fermentation, while the

objective is to perform a solid fermentation. The silage was performed by incubation at 37°C, in the dark. This temperature was chosen to be close to the average temperature in Qatar. The pH, acidity, and CFU were measured every week in order to monitor the growth of the LAB populations and the evolution of the silage. Table 6 shows the composition of the silages.

Table 6: Composition of the silages prepared

Inoculated strains	**Ratio Feed/Leaves (%) (w/w)**					
Control (without inoculation)	0	10	20	30	40	50
Inoculum 1 (*Lactobacillus plantarum* SMZ103 from probiotics)	0	10	20	30	40	50
Inoculum 2 (*Lactobacillus paracasei* SMZ 20 from probiotics)	0	10	20	30	40	50
Inoculum 3 (*Lactobacillus plantarum* SMZ 46 from silage)	0	10	20	30	40	50
Inoculum 4 (*Pediococcus acidilactici* SMZ 41 from silage)	0	10	20	30	40	50

4.4.3.1 Evolution of pH in the silages

The main factor of the success of silage is the rapid drop of its pH to inhibit the growth of the non-desired bacteria. Figure 7 shows the evolution of the pH in the silages. The control silages, without LAB incubation, showed that in absence of any inoculated strain, the increase in the feed/leaf's ratio accelerates the drop of the pH following a pH drop profile similar to what is expected with the silage process. The pH of the silage performed with 0% feed dropped from 6.28 to 5.37 while that in 50% ratio, it dropped down to 4.14 and be stable after week 3 incubation. These results clearly show that the endogenous acid-forming microorganisms in the feed and the leaves are performant to drop the pH and maintain a potential higher stability of the mixture from pathogenic microorganisms, which cannot grow and tolerate low pH. In addition, the

pH of the silage performed with 10% feed dropped from 6.22 to 4.42 reaching stability after week 4 but with a trend of drop more accelerated than that of the 0% feed-silage. The same situation was almost encountered with 20% feed-silage. Starting a ratio of 30%, the drop is fast and the pH stabilized at week 3.

The trends of drop in pH in the silages performed with inoculant 1 (*Lactobacillus plantarum* SMZ103), were almost similar to the control. The drop in pH for the silage with 100% leaves dropped from 6.18 to 5.56 and for the silages with 50% leaves, the pH dropped from 6.2 to 4 and the pH stabilized at week 4. In the silages with inoculant 1, the highest drop (from 6.23 to 3.98) was observed with 60% leaves and the pH became stable after week 4. Similar trends of pH drop were observed in 10% and 20% feed silages with the pH dropping from 6.24 and 6.29 to 4.24 and 4.22 respectively, both silages pH were stabilized after week 4. The drop in pH for silage containing 30% feed/leaves ratio was from 6.14 to 4.12 with the pH being stable after week 4.

Using the Inoculant 2 (*Lactobacillus paracasei* SMZ20), the pH decreases with time and is more accentuated with the increase in feed/leaves ratio. The lowest decrease in pH was observed in the silage with 100% leaves, the pH dropped from 6.14 to 5.39 and the highest drop in pH was observed with 50% feed/leaves, it dropped from 6.31 to 4.2 and the pH is stable after week 5. The drop in pH with 10% and 20% feed/leaves ratios were similar, the pH dropped from 6.19 and 6.27 to 4.5 and 4.46, and both silages pH were stabilized after week 5. With 30% feed/leaves, the drop in pH was from 6.15 to 4.26 and the silage's pH stabilized after week 5 and for the 40% feed silage, the pH dropped from 6.22 to 4.22, very close to that of 50% feed/leaves ratio and the pH of silage with 40% feed stabilized after week 4.

The trends of drop in pH in the silages performed with inoculant 3

(*Lactobacillus plantarum* SMZ46), were similar to those of the other silages. The drop in pH for the silage with 100% leaves was from 6.17 to 5.06. For the silages of 50% feed/leaves ratio, the pH dropped from 6.21 to 3.85, which was the highest drop observed among all the silages inoculated with the selected LAB, and the drop in pH was stabilized after week 4. For the silages performed with 40% feed and 60% leaves, the pH dropped from 6.23 to 4.08 and the pH became stable after week 4. Similar trends of pH drop were observed in 10% feed silage (from 6.19 to 4.29), the silage's pH was stabilized after week 4. The drop in pH for the silage containing 20% feed, was from 6.26 to 4.14 with the pH being stable after week 4. For 30% feed-silage, the drop in pH was from 6.18 to 4.11 and the silage's pH was stabilized after a week. In the 40% feed-silage, the pH dropped from 6.22 to 4.22, stabilized after week 4.

Finally, the silages performed with the inoculant 4 (*Pediococcus acidilactici* SMZ41) also followed the same trend of drop in pH as that of other silages. The pH decreases with time and the drop is accentuated with the increase of feed ratios. The lowest decrease in pH was observed in the silage performed with 100% leaves, the pH dropped from 6.28 to 5.47 and the highest drop in pH was observed with 50% feed, it dropped from 6.17 to 3.97 and the pH being stable after week 5. The drop in pH for 10% and 20% feed/leaves ratios was similar (from 6.18 and 6.13 to 4.15 and 4.12 respectively). Both silages' pH were stabilized after week 4. For 30% feed-silage, the drop in pH was from 6.11 to 4.09 and the silage's pH stabilized after week 5. Using 40% feed, the pH dropped from 6.15 to 4.08 and the pH stabilized after week 4.

These results indicate that with high feed/leaves ratios, the pH tends to decrease at a faster rate to reach stability earlier in comparison to low feed/leaves silages, regardless of the inoculation. The pH in the silages inoculated with LAB tends to have a higher and quicker drop in pH than control indicating high initial microbial activity.

Pediococcus acidilactici has been reported to initiate the microbial activity in silage at a faster rate than other LAB (Alhaag et al., 2019), and this can be observed in this study as well, that the silages inoculated with *Pediococcus acidilactici* SMZ 41 showed the fastest drop in pH in comparison to the other silages in just after 1-week incubation.

The drop in pH indicates the microbial activity of LAB and release of acids. Even without any inoculation, in control silages, the pH dropped for 10% feed/leaves ratio-silages to 4.42, indicating that silages of palm tree leave with just 10% feed concentration and 90% leaves can provide the nutrients for the LAB to grow and lower the pH. With the increase in feed ratios, the pH tends to drop to lower values. The lowest pH was observed with inoculant 3 silages inoculated with *Lactobacillus plantarum* SMZ 46 isolated from spontaneous mixed silage. The fastest drop in pH was observed in inoculant 4 silages inoculated with *Pediococcus acidilactici* SMZ 41 isolated from spontaneous mixed silage. Based on previous studies, *Pediococcus acidilactici* has been known to drop the initial pH of the silages at a faster rate, making the conditions more favorable for other LABs.

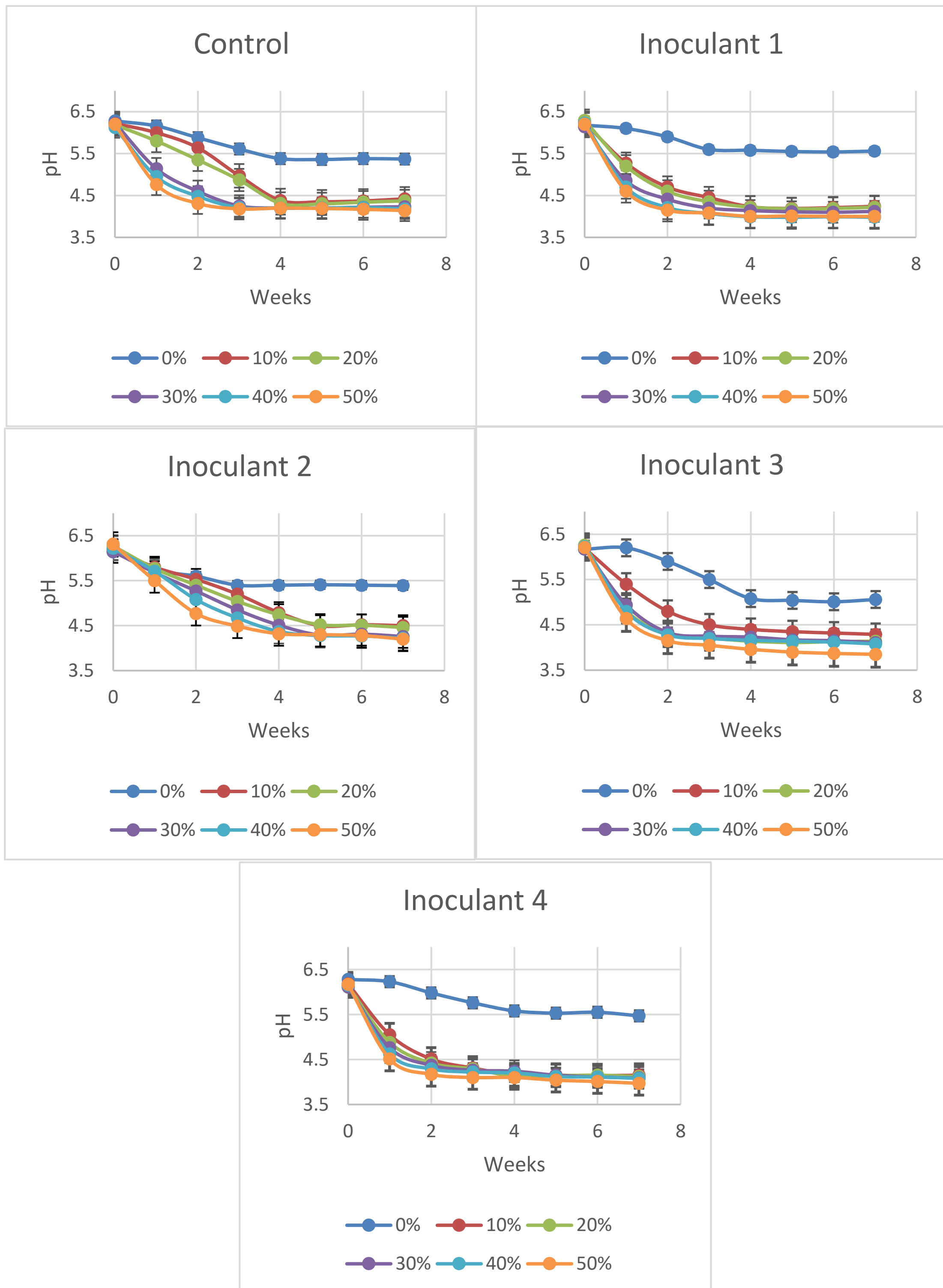

Figure 7: pH of the silages. Control Silage (without inoculation), Inoculant 1 Silages (*Lactobacillus plantarum* SMZ103), Inoculant 2 (*Lactobacillus paracasei* SMZ20), Inoculant 3 (*Lactobacillus plantarum* SMZ46), Inoculant 4 (*Pediococcus acidilactici* SMZ41).

4.4.3.2 Evolution of acidity in the silages

The acidity is estimated as the equivalent of acidity per kilogram of fresh silage. All the control silages showed that with the increase in feed/leaves ratios, the acidity of the silages tend to increase with time compared to the silage performed without feed (Figure 8). The acidity of the silages with 100% leaves remained steady at 0.025 equivalent Acidity/kg, throughout the weeks and the highest acidity was observed for the silage performed with 50% feed/leaves ratio showing a 0.225 equivalent Acidity/kg and reached stability after week 3. The same trends were observed for the other control silages. The acidity of the silage realized with a 10% feed/leave ratio increased from 0.025 to 0.075 equivalent Acidity/kg and the acidity of the silage stabilized after week 4. The acidity of the silage performed with a 20% feed/leaves ratio increased from 0.025 to 0.1 equivalent Acidity/kg and the acidity stabilized after week 5. For the silage containing 30% feed, the acidity increased from 0.05 to 0.2 equivalent Acidity/g and reached stability after week 2. The acidity of the silage containing 40% feed increased from 0.05 to 0.2 and reached stability after the first week, this silage was the fastest to be stabilized among the others. Here, considering the stability of the acidity after 1 week of incubation may be considered as a result of interaction between several endogenous microorganisms and the inoculated strain. This is in comparison to the stabilization of acidy after 3 weeks in the 50% feed/leaves silage. The ratio feed/leaves is also an important factor in enhancing the growth and interaction between the existing bacteria in the silage.

The acidity of the inoculant 1 (*Lactobacillus plantarum* SMZ103) silages increased with the increase of the feed/leave ratios and with time. For the silages performed with 100% leaves, the acidity remained the same, showing the highest increase in acidity in silage realized with a 50% feed/leave ratio in which the acidity

increased from 0.075 to 0.225 equivalent Acidity/kg in a period of one week and then stabilized throughout the weeks. The acidity for the silage performed with 10% feed increased from 0.025 to 0.1 equivalent Acidity/kg and the acidity was stable after week 3. For the silage containing 20% feed, the acidity increased from 0.025 to 0.125 equivalent Acidity/kg and became stable after week 4. The acidity for silage performed with 30% feed increased from 0.05 to 0.15 and stabilized from week 2 onwards. The increase in acidity in 40% feed silage was from 0.05 to 0.225 equivalent Acidity/kg with stabilization after week 4. Interestingly observed, the same acidity of 0.225 equivalent Acidity/kg was determined in 40% and 50% feed silages. But this acidity was obtained after 1-week incubation in 40% feed silage while 4 weeks in 40% feed-silage. This observation was also clear when studying the evolution of pH in the silages. The acidity increased as the feed/leave ratio in the inoculant 2 (*Lactobacillus paracasei* SMZ20) silages increases with time. The acidity in the silage performed with 100% leaves remained stable at 0.025 equivalent Acidity/kg. The highest increase was observed in the silage performed with the highest percentage of feed/leaves ratio (50%), in which the acidity increased from 0.075 to 0.2 equivalent Acidity/kg and became stabilized, after week 5. The 10% and 20% feed/leave ratios gave similar results with the acidity increasing from 0.025 to 0.075 equivalent Acidity/kg for both the silages and became stable after week 4. The silage containing 30% and 40% silages showed an acidity which increased from 0.05 to 0.15 equivalent Acidity/kg for both silages and which stabilized after week 4.

Similar trends of increase in acidity were observed using the inoculant 3 (*Lactobacillus plantarum* SMZ41). The biggest increase among all the silages was observed with 50% feed, in which the acidity increased from 0.075 to 0.3 equivalent Acidity/kg and became stable after week 3. The 10% feed-silage's acidity increased

from 0.025 to 0.075 equivalent Acidity/kg and for the 20% feed-silage, the pH increased from 0.025 to 0.15 equivalent Acidity/kg, both stabilized after week 3. The silage performed with 30% feed/leaves ratio registered an acidity increase from 0.05 to 0.2 equivalent Acidity/kg and that with 40% feed/leaves from 0.05 to 0.225 equivalent Acidity/kg. The acidity in both silages reached stability after week 3.

Finally, using the inoculant 4 (*Pediococcus acidilactici* SMZ46) the trend of acidity increase was also the same as for the other strains. The acidity increased as the ratio of feed in the silage increases and with time. The acidity of the silage performed with leaves only remained stable at 0.025 equivalent Acidity/kg. The biggest increase was observed in the silage performed with the highest feed/leaves ratio being of 50%, since the acidity increased from 0.075 to 0.25 equivalent Acidity/kg and became stable after week 3. The 10% feed-silage's acidity increased from 0.025 to 0.1 equivalent Acidity/kg. In the 20% feed-silage, the acidity increased from 0.025 to 0.175 equivalent Acidity/kg while in 30% feed-silage, the acidity increased from 0.05 to 0.2 equivalent Acidity/kg. The acidity of all the three silages (10%, 20% and 30% feed/leaves) reached stability after week 2. In the 40% feed-silage, the acidity increased from 0.05 to 0.225 equivalent Acidity/kg and acidity stability reached after week 3.

The trend of the increase in acidity has been the same for all the inoculated and control silages. The highest increase in acidity was observed in inoculant 3 silages inoculated with *Lactobacillus plantarum* SMZ103. The increase of the acidity by *Pediococcus acidilactici* SMZ46 was observed to be the fastest.

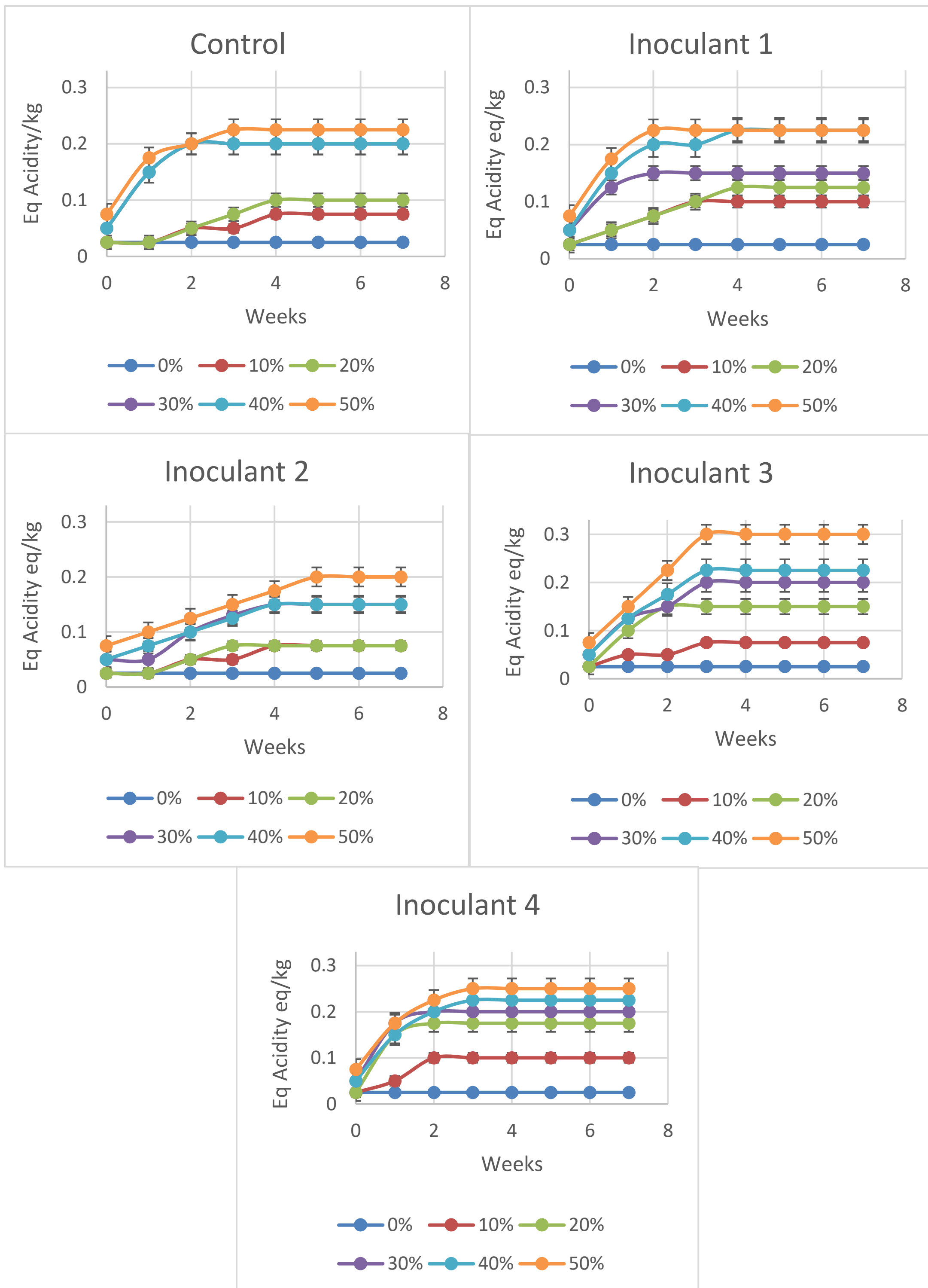

Figure 8: Acidity of the silages. Control Silage (without inoculation), Inoculant 1 Silages (*Lactobacillus plantarum* SMZ103), Inoculant 2 (*Lactobacillus paracasei* SMZ20), Inoculant 3 (*Lactobacillus plantarum* SMZ46), Inoculant 4 (*Pediococcus acidilactici* SMZ41).

4.4.3.3 Evolution of LAB growth

The lactic acid bacteria growth was determined by taking the logarithm value of the number of the colony-forming unit (CFU), formed in the MRS medium. The trends of evolution for the growth were similar to that of acidity evolution with a notable increase with the increase of the feed/leaf's ratio in the silage. This is expected since the growth of the LAB is normally increased with the nutrients provided by the feed, as illustrated in Figure 9. For the control silages performed without any inoculant, growth was observed till week 3 and after that the silages showed stability in terms of LAB density. The silage with 100% leaves reached 5.3 log (CFU/g), initially starting from 2 log (CFU/g). The silage performed with 50% feed and 50% leaves, showed the highest density of LAB growth of 7.2 log (CFU/g) and reached stability after week 4. The CFU of the silage containing 10% feed, increased from 2.3 to 5.5 log (CFU/g) stabilized after week 3. The CFU of 20% feed-silage increased from 3 to 6.3 log (CFU/g) and reached stability after week 4. The silage containing 30% feed, showed LAB growth increasing from 3.3 to 6.69 log (CFU/g) which was stable after week 4. The LAB growth of silage performed with 40% feed increased from 3.7 to 7.2 log (CFU/g) and was stable after week 4.

As a first conclusion, it is clear that the initial and the final CFU in the silages performed with inoculation, increased with the increase of the feed/leaf's ratio. This means that the feed is a source of LAB which also inoculates the silages.

The CFU in the silages performed with inoculate 1 (*Lactobacillus plantarum* SMZ103*)* increased with time and with the increase in the feed ratio. The lowest increase of LAB growth was registered with 100% leaves-silage, in which it increased from 2.4 to 5.1 log (CFU/g). The highest increase of CFU (from 4 to 7.11 log (CFU/g) was observed with the 50% feed-silage and was stable after week 5. The CFU in silage

performed with a 10% feed/leaves ratio increased from 3.4 to 6.1 log (CFU/g) and stabilized after week 5. The silages containing 20% and 30% feed, showed similar LAB growth evolution with initial CFU of almost 3.9 log (CFU/g) and final after 5 weeks of 6.4 log (CFU/g) for 20% feed-silage and 6.54 log (CFU/g) for 30%-feed silage. The CFU for the silage containing 40% feed/leaves ratio increased from 4 to 6.7 log (CFU/g) and the silage was stable after week 5.

The CFU in the silages of inoculate 2 (*Lactobacillus paracasei* SMZ20) increased with time and with the increase of feed ratios. The lowest increase of LAB growth was obtained with 100% leaves-silage (from 2.3 to 5.5 log (CFU/g)). The highest increase of CFU was observed in 50% feed-silage, (from 3.6 to 7.56 log (CFU/g)) and the silage's CFU was stable after week 5. In the silage performed with 10% feed, the CFU increased from 2.3 to 6.3 log (CFU/g) and the cell density was stable after week 5. The CFU of the silage containing 20% feed increased from 2.7 to 6.8 log (CFU/g) and the silage was stable after week 4. The silages performed with 30% and 40% showed a similar trend in terms of LAB growth since the CFU increased from 3.07 log (CFU/g) for 30% and 3.3 log (CFU/g) for 40% to 7.4 and 7.45 log (CFU/g) respectively. At both conditions, the CFU reached stability after week 5.

The CFU in the silages performed with the inoculate 3 (*Lactobacillus plantarum* SMZ46) increased with time and with the increase of feed/leaves ratios. The lowest increase of bacterial growth was in 100% leaves-silage (from 2.3 to 5.84 log (CFU/g)). The highest increase of CFU was observed with a 50% feed/leaves ratio (from 3.84 to 7 log (CFU/g)) and the silage was stable after week 4. The CFU of silage with 10% feed increased from 3.3 to 6.47 log (CFU/g) to be stable after week 6. The silages containing 20% and 30% feed/leaves ratios showed similar LAB growth evolution (from 3.47 and 3.6 log (CFU/g) to 6.4 log (CFU/g) and 6.47 respectively). Both cell

counts were stable after week 6. The CFU for the silage containing 40% feed increased from 3.69 to 6.66 log (CFU/g) in week 7, but the highest CFU was observed in week 4 with 6.77 log (CFU/g) and the silage was stable after week 5.

The bacterial growth of the silages of inoculate 4 (*Pediococcus acidilactici* SMZ41) increased with time and with the increase in feed ratios. The lowest increase in LAB growth was registered with 100% leaves-silage (from 2.47 to 5.9 log (CFU/g)). Interestingly, this inoculated strain allowed the highest LAB growth observed in 100% leaves-silages compared to all the other inoculates. The highest increase in CFU was observed with 50% feed-silage, in which the CFU increased from 4 to 7.7 log (CFU/g) and the silage's CFU was stable after week 3. For the silage performed with 10% feed, the CFU increased from 2.3 to 6 log (CFU/g) and the CFU was stable after week 5. The CFU of the silage containing 20% feed, increased from 3 to 6.27 log (CFU/g) and the CFU was stable after week 6. In the silage containing 30% feed, the bacterial growth increased from 3.47 to 7.2 log (CFU/g) and the CFU reached stability after week 3. The bacterial growth of silage containing 40% feed increased from 3.69 to 7.4 log (CFU/g) and the CFU was stable after week 3. The results of bacterial growth showed that the biomass density in the silage is higher and reached faster, by increasing the feed/leaves ratios.

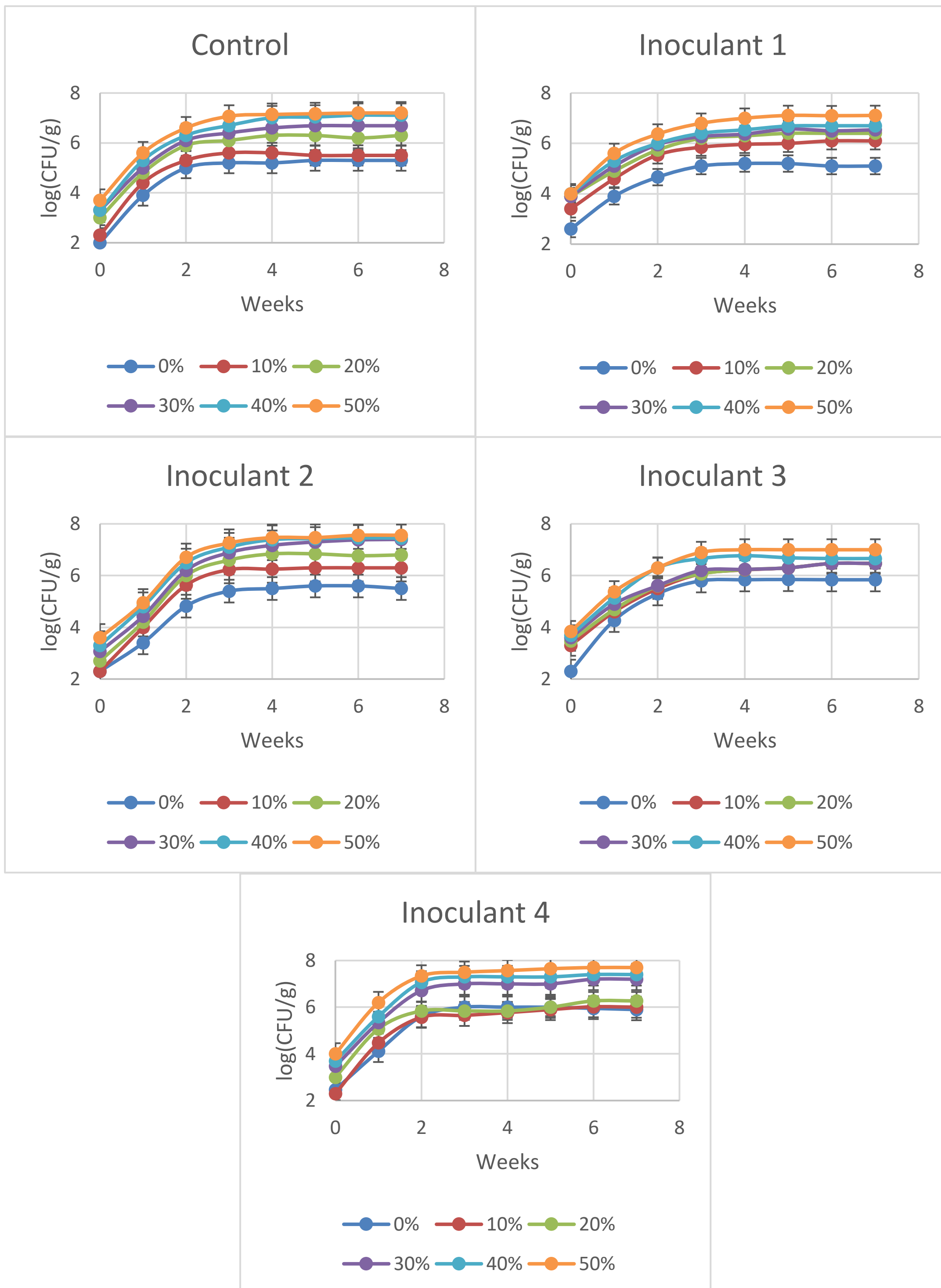

Figure 9: CFU of the silages. Control Silage (without inoculation), Inoculant 1 Silages (*Lactobacillus plantarum* SMZ103), Inoculant 2 (*Lactobacillus paracasei* SMZ20), Inoculant 3 (*Lactobacillus plantarum* SMZ46), Inoculant 4 (*Pediococcus acidilactici* SMZ41).

4.4.3.4 Conclusion

The trends of the increase in LAB growth were similar for all the silages with the highest CFU observed in 50% feed-silage inoculated with *Lactobacillus paracasei* SMZ20 isolated from commercial probiotics. Since the silages need to be prepared for feeding livestock, their enrichment with feed is appropriate. Considering silages performed with 10% feed and 90% leaves, the highest CFU was observed in silages inoculated with *Lactobacillus plantarum* SMZ46 originally isolated from spontaneous mixed silage. The final cell density corresponded to 6.47 log (CFU/g). However, the fastest increase of CFU has observed with inoculant 4 silages, inoculated with *Pediococcus acidilactici* SMZ41 isolated from spontaneous mixed silage.

4.5 Identification of dominant LAB species during palm tree ensilaging

Based on the results of the previous sections, the necessity to inoculate the silages is clear as well as the incubation during a period of almost 7 weeks to reach the stability of pH, acidity, and CFU. 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 bacteria that can form colonies in MRS solid plates. These CFU are expected to be relatives to LAB. However, the evolution and the balance between the LAB in the silage cannot be evaluated only by CFU determination because the colonies in the MRS plates are highly similar. Also, 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 evolution of the LAB populations in the silage, the identification of the dominating species was performed with MALDI TOF MS. In addition, since 10% of feed silages showed interesting evolution of the three parameters (pH, acidity, and CFU), it was selected to study the interaction between the LAB in a so low feed/leaves ratio. For comparison, the richest silage (50% feed/leaves ratio) was considered in this study and also to evaluate the possibility to ensure the

dominance of the inoculated LAB strain in a rich medium. At least 30 colonies were used at each condition, for the identification and study of the distribution of the bacteria which formed colonies on MRS. Indeed, the liquid MRS harvested after suspending the silages was diluted 10,000 times and 100 microliters were spread on the plates. The number of obtained CFUs was between 30 and 150 colonies. At least 30 colonies were selected randomly from the plates. From the total count, almost 50% of the colonies were identified to determine the dominant species.

The dominant species in 10% feed-silage of inoculant 1 (*Lactobacillus plantarum* SMZ103) are shown in Figure 10. The total colonies count was 53 and 30 random colonies were selected and identified. *Pediococcus acidilactici* was found to be represented by 30% of the colonies, *Lactobacillus pentosus* only 10% while 20% of the colonies were *Candida sp*. The results show that there are no prominently dominating species rather a mixture of lactic acid bacteria comprising of 80% and 20% *Candida sp*. in silage. However, it is to note that none of the colonies was of *Lactobacillus plantarum*. This means that the inoculated strain *Lactobacillus plantarum* SMZ103 disappeared or became a minority, not detectable at the used dilution. The endogenous LAB (*Pediococcus acidilactici*) dominated the inoculated LAB strain SMZ103 of *Lactobacillus plantarum*. *Pediococcus acidilactici* seems to be the most adapted LAB. Regarding the 10% feed-silage inoculated with *Lactobacillus paracasei* SMZ20, isolated from probiotics (Figure 11), there were 54 colonies on the plate and 30 random colonies were selected for the identification. The results show that *Lactobacillus paracasei* was represented by 63% of the used colonies. *Pediococcus acidilactici* represented 27% and *Candida sp.* represented 10%. The results show that the inoculated species of *Lactobacillus paracasei* SMZ20 was dominating the silage and the endogenous *Pediococcus 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 inoculate 3 (*Lactobacillus plantarum* SMZ46, isolated from mixed feed and leaves silage) provided a total colony count of 58 and 30 random colonies were selected for the identification (Figure 12). After identification, 56% colonies were of *Lactobacillus farciminis*, 27% were of *Pediococcus acidilactici* and 17% of *Candida sp.* This result clearly shows that the silage is being dominated by the endogenous strains of *Lactobacillus farciminis*, and *Pediococcus acidilactici*. Using the 10% feed-silage with inoculant 4, which is the strain *Pediococcus acidilactici* SMZ41, isolated from mixed feed and leaves silage (Figure 13), there were 63 colonies on the plate and 28 random colonies were identified. The results clearly show that *Pediococcus acidilactici* was dominating with 75% of the colonies, while *Candida krusei* was represented with 25%. None of the other endogenous LAB was detected at the dilution employed in the plating. It is to note, that all the previous results showed that the endogenous *Pediococcus acidilactici* was able to compete with all the other inocula.

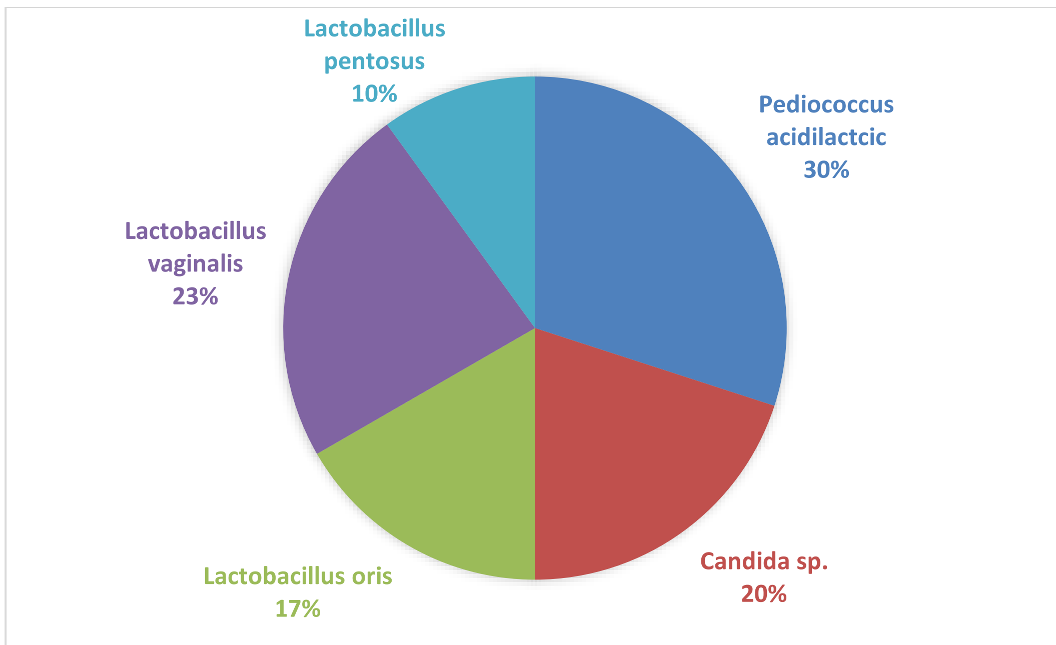

Figure 10: Dominating species in Inoculum 1 (*Lactobacillus plantarum* SMZ103 isolated from probiotics) 10% feed/leaves silages

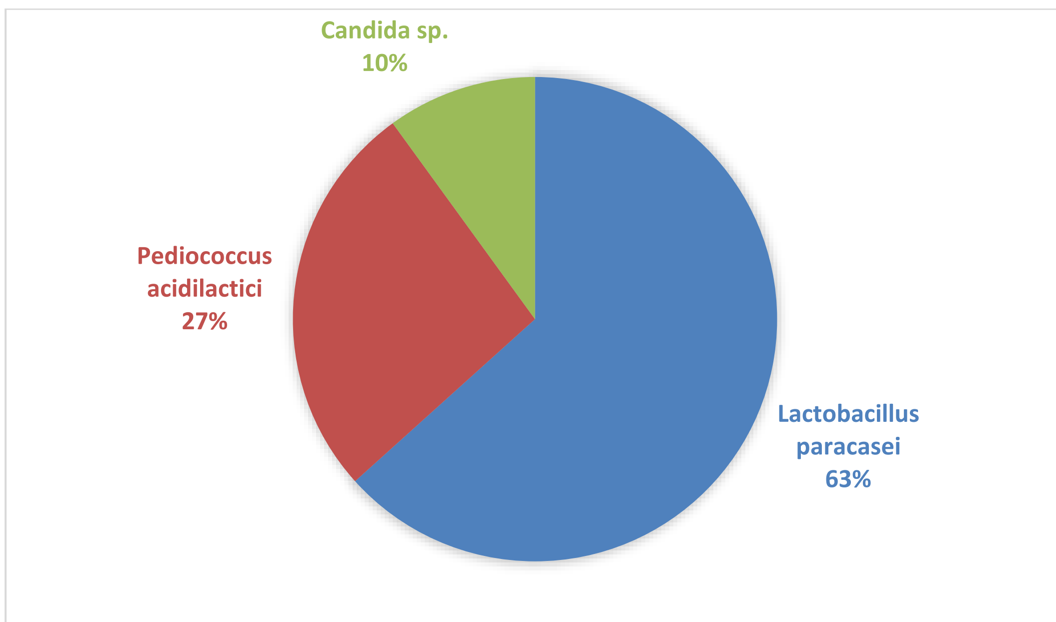

Figure 11: Dominating species in Inoculumt 2 (*Lactobacillus paracasei* SMZ20 isolated from probiotics) 10% feed/leaves silages

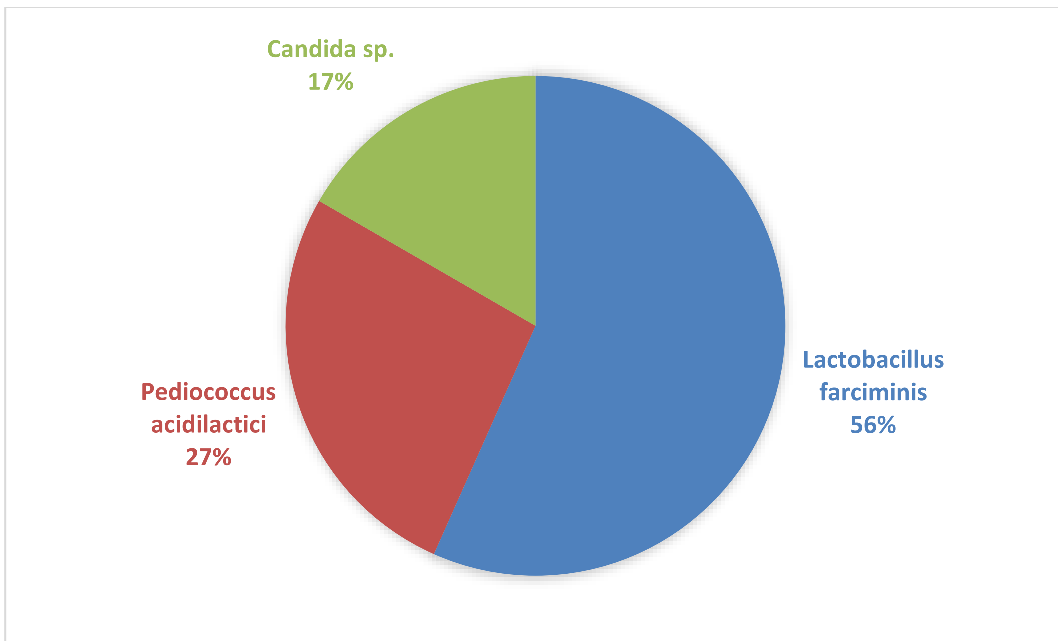

Figure 12:Dominating species in Inoculum 3 (*Lactobacillus plantarum* SMZ46 isolated from silage) 10% feed/leaves silages

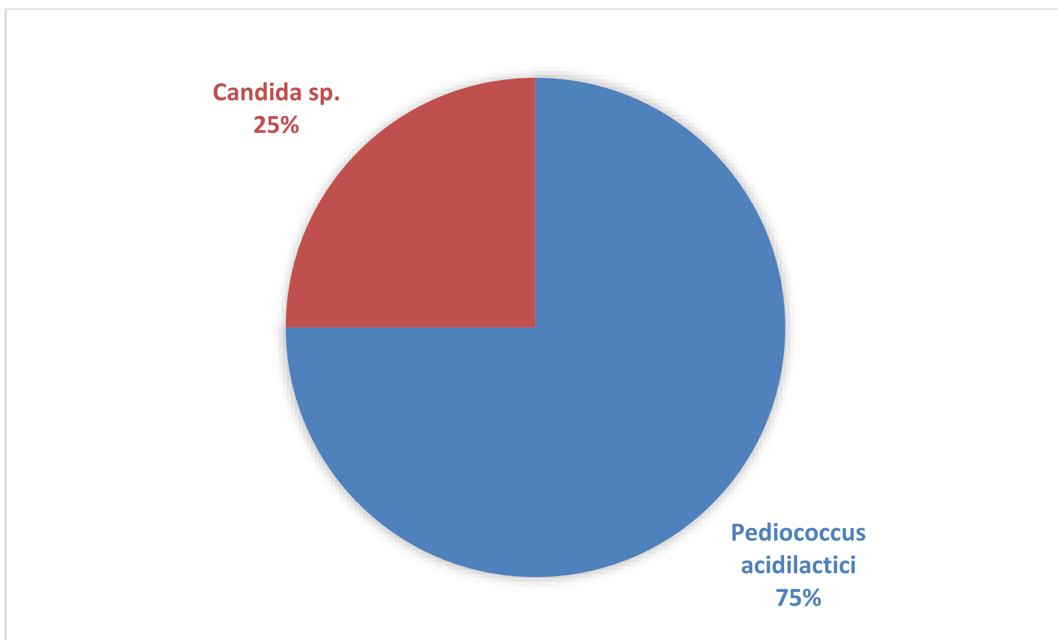

Figure 13: Dominating species in Inoculum 4 (*Pediococcus acidilactici* SMZ41 isolated from silage) 10% feed/leaves silages

The inoculant 1 (*Lactobacillus plantarum* SMZ103), initially isolated from probiotics was used as inoculum in 50% feed-silage. The total plate count was 103 colonies among them 45 colonies were randomly selected, and the corresponding LABs were identified (Figure 14). *Lactobacillus plantarum* was represented in 51% of the identified colonies, while *Pediococcus acidilactici* was found to correspond to 22% and *Candida sp*. to 27%. The results show that *Lactobacillus plantarum* was almost the dominating species in the silage of 50% feed/leaves. Using the 50% feed-silage with inoculant 2 (*Lactobacillus paracasei* SMZ20), also isolated from probiotics, there were 114 colonies on the plate and 50 of them were randomly selected for identification (Figure 15). The results indicate that *Lactobacillus paracasei* corresponded to 38% of the colonies and *Pediococcus acidilactici* to 62%. This shows that the studied silage was dominated by *Pediococcus acidilactici*. None of the colonies was formed by a yeast. The 50% feed-silage performed with the inoculate 3 (*Lactobacillus plantarum* SMZ46, isolated from mixed feed and leaves silage) had a total plate count of 158 colonies from which 60 were randomly selected for the identification (Figure 16). 20% of the colonies were of *Pediococcus acidilactici* and 77% of *Candida sp*. with 1 colony for *Vegococcus fluralis* and 1 for *Staphylococcus warneri*. This indicates that the silage was being dominated by *Candida sp*. Using the 50% feed-silage with inoculant 4 which is *Pediococcus acidilactici* SMZ41, initially isolated from mixed feed and leaves silage, there were 183 colonies on the plate and 74 colonies were identified (Figure 17). The results indicate that *Pediococcus acidilactici* was represented with 72% of the identified colonies, *Candida species* with 21%, *Staphylococcus epidermidis* with 3 colonies and *Staphylococcus capitis* with 2 colonies. The silages performed with the inoculant 4 *Pediococcus acidilactici* dominated the silage (72%).

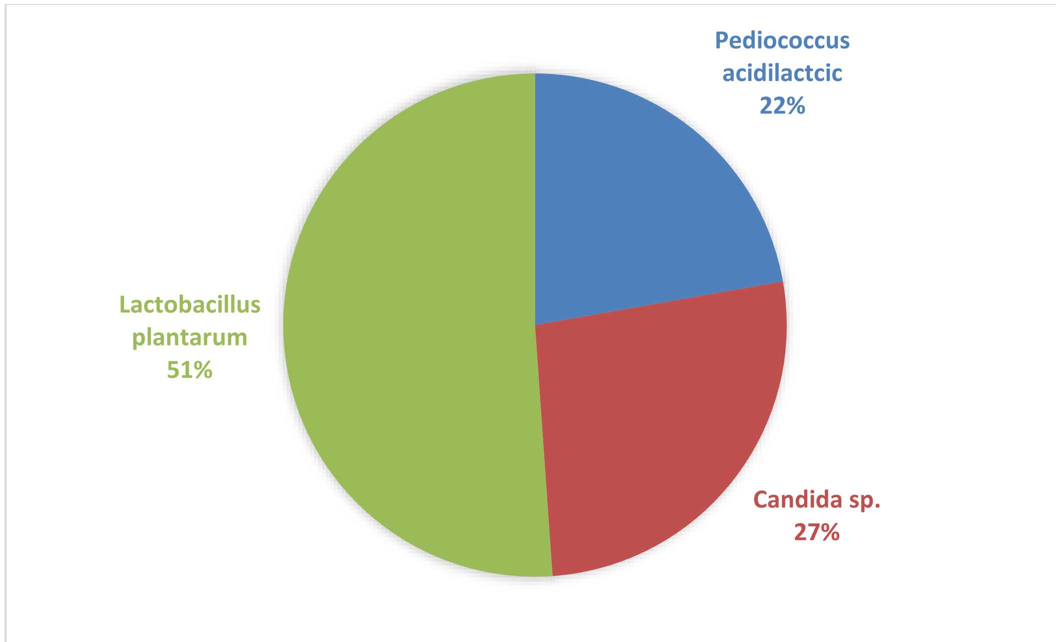

Figure 14: Dominating species in Inoculum 1 (Lactobacillus plantarum SMZ103 isolated from probiotics) 50% feed/leaves silages

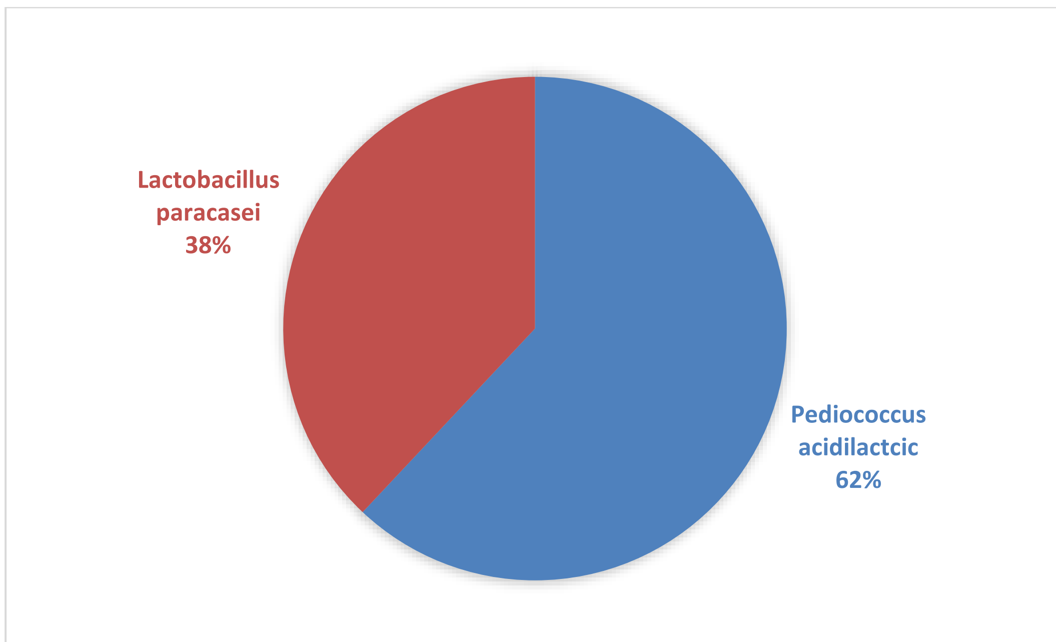

Figure 15: Dominating species in Inoculum 2 (Lactobacillus paracasei SMZ20 isolated from probiotics) 50% feed/leaves silages

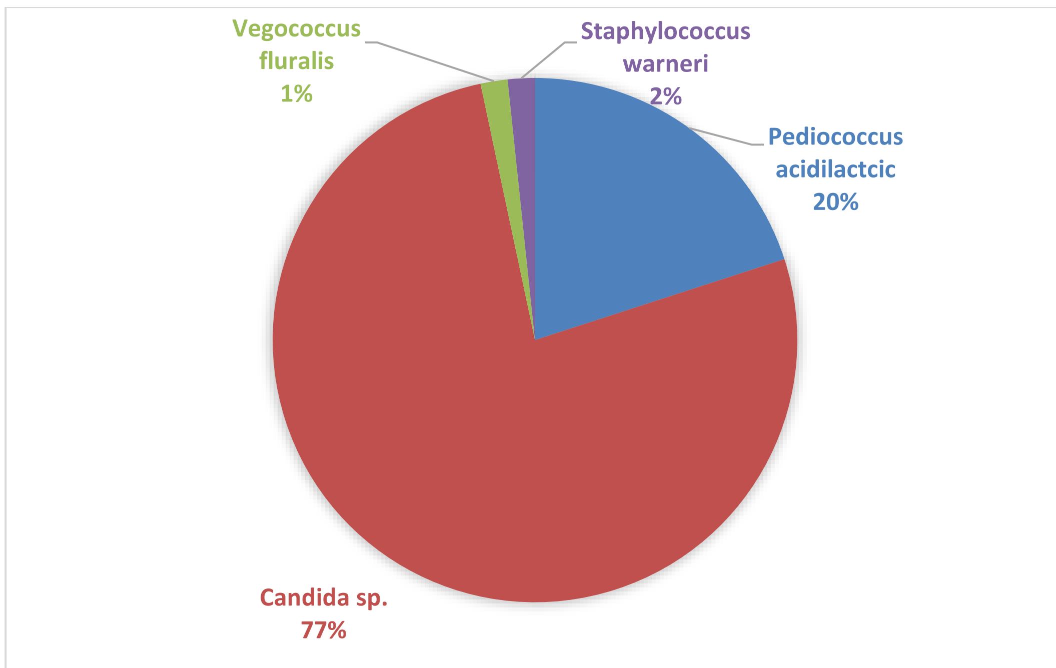

Figure 16: Dominating species in Inoculum 3 (Lactobacillus plantarum SMZ46 isolated from silage) 50% feed/leaves silages

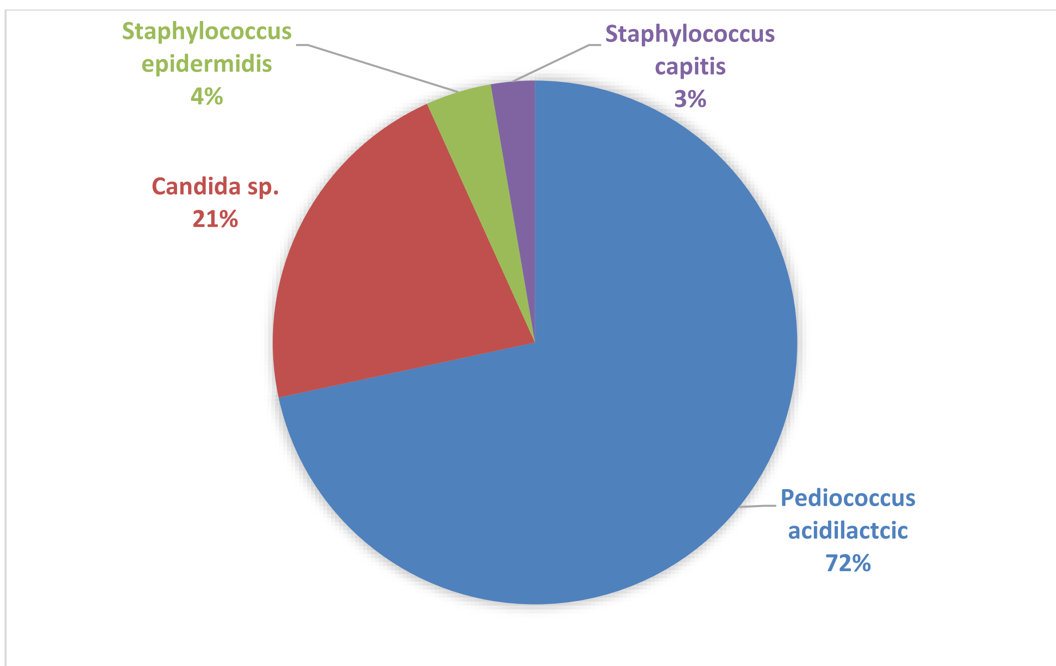

Figure 17: Dominating species in Inoculum 4 (Pediococcus acidilactici SMZ41 isolated from silage) 50% feed/leaves silages

The figures above clearly show that *Lactobacillus plantarum* does not dominate the silage with a low concentration of feed (10% feed/leaves silages), as it disappeared among other represented LAB. These silages of *Lactobacillus* have shown growth of other LAB such as *Lactobacillus pentosus, Lactobacillus oris*, *Lactobacillus vaginalis and Lactobacillus farciminis,* indicating that with inoculation of *Lactobacillus plantarum* the silage is dominated by other LAB. However, *Lactobacillus plantarum* was observed representing almost 50% of the colonies in silages containing 50% feed/leaves. *Lactobacillus paracasei* has shown dominancy in both 10% and 50% feed silages with slightly less in 50% feed silage where *Pediococcus acidilactici* was also well represented. *Pediococcus acidilactici* has been the most dominant species in the silages with even high dominancy in silages inoculated with other LAB since *Pediococcus acidilactici* has been known to dominate the silage in the initial stages of fermentation till the pH drops below 4. Once the pH drops below 4, other LAB start to dominate the silage. Mostly, the pH of the silages was above 4 since the presence of *Pediococcus acidilactici* can be observed in most of the silages (Alhaag et al., 2019).

4.6 Ensilaging of palm tree leaves and animal feed inoculated with a mixture of *Pedicoccus acidilactici* and *Lactobacillus paracasei*

4.6.1 Evolution of the silage characteristics (pH, acidity and bacterial growth)

The results of the identification of the dominating LAB in 10% feed/leaves and 50% feed/leaves silages, showed that *Pediococcus acidilactici* and *Lactobacillus paracasei* are able to dominate the other LAB in the silages. Considering these results, the idea was to perform silages inoculated with both *Lactobacillus paracasei* SMZ20 and *Pediococcus acidilactici* SMZ41, to investigate the competition among both strains at different feed/leaves ratios. The results are shown in Fig. 7.

The silages followed the same patterns in terms of pH, acidity, and CFU as of the silages prepared in the previous sections of this work. For the pH, there is a decrease with time and which is more accentuated if feed/leaves ratios increased. A slight decrease in pH was observed in the silage performed with 100% leaves (from 6.35 to 6.2) and the highest drop in pH was observed with 50% feed/leaves silage (from 6.31 to 4.03 being stable after week 2). The drop in pH for 10% feed-silage was from 6.32 to 4.61, stabilized after week 2. The pH of the silage with 20% feed-silage dropped from 6.3 to 4.45 and stabilized after week 2. For 30% feed/leaves silage, the drop in pH was from 6.29 to 4.33, stabilized after week 2. Using 40% feed-silage, the pH dropped from 6.32 to 4.1, very close to that of 50% feed-silage and the pH of silage stabilized after week 2.

The acidity increased as the concentration of feed in the silage increases. The acidity of the silage with 100% leaves remained the same at 0.025 equivalent Acidity/kg. The biggest increase was observed in the silage with the highest percentage of feed concentration (50% feed/leaves), in which the acidity increased from 0.05 to 0.125 equivalent Acidity/kg and be stable after week 3. The 10% feed/leaves silage showed an increase of acidity from 0.025 to 0.05 equivalent Acidity/kg and stability after week 1. For the silage performed with 20% feed, the acidity increased from 0.025 to 0.075 equivalent Acidity/kg and be stable after week 1. The silage containing 30% feed, showed an increase of acidity from 0.025 to 0.1 equivalent Acidity/kg and reached stability after week 2. The acidity of the silage containing 40% feed/leaves increased from 0.025 to 0.125 and was stable after week 3.

The CFU of the silages inoculated with *Lactobacillus paracasei* SMZ20 and *Pediococcus acidilactici* SMZ46 increased with time and with the increase of feed/leaves ratios. The lowest increase in bacterial growth was in the 100% leaves-

silage (from 2.81 to 5.7 log (CFU/g)) and the highest increase in CFU was observed in 50% feed/leaves silage (from 3.3 to 7.26 log (CFU/g)). In fact, the highest CFU was observed in week 3 (7.77 log (CFU/g)) and then there was a decline in CFU till it reaches stability (7.26 log (CFU/g)) after week 4. In the 10% feed/leaves silage, the CFU increased from 2.69 to 5.81 log (CFU/g), with the highest CFU observed in week 4 (6.29 log (CFU/g)) which declined to stability after week 4. The CFU in the 20% feed/leaves silage increased from 3 to 6.2 log (CFU/g), however, the highest CFU was observed in week 3 (6.51 log (CFU/g)) which stabilized after week 4. The silages performed with 30% feed/leaves showed an increase of CFU (from 3.1 to 7 log (CFU/g)), with the highest CFU observed in week 2 (7.36 log (CFU/g)) which slightly decreased and stabilized after week 5. The silage was performed with 40% feed/leaves, the CFU increased from 3.3 to 7.26 log (CFU/g), but the highest CFU was observed in week 3 (7.6 log (CFU/g)) and then declined till it reach stability after week 4.

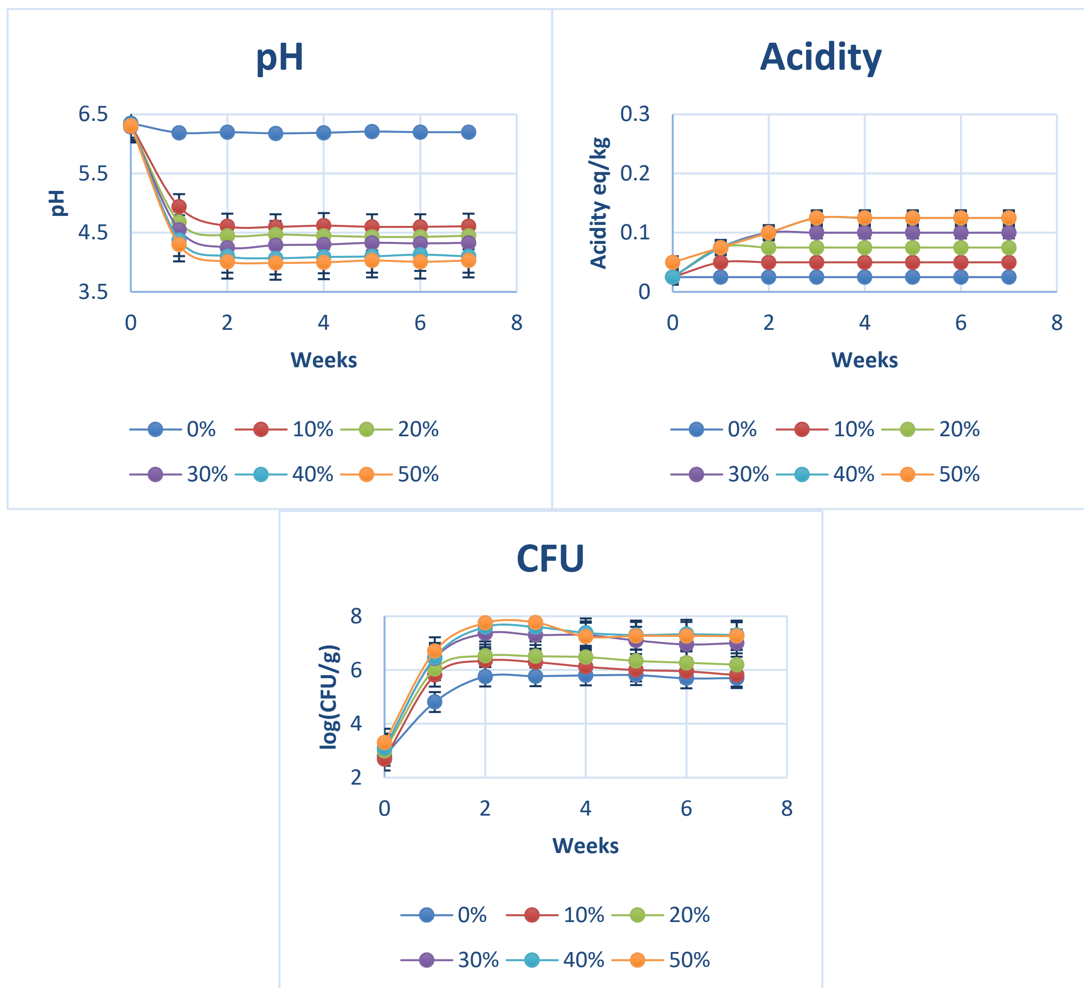

Figure 18: pH, Acidity and CFU of inoculum 5 (*Lactobacillus paracasei* SMZ 20 + *Pediococcus acidilactici* SMZ 41) silage.

All the studied silages followed the same pattern in terms of pH, acidity, and bacterial growth, that with the increase in feed/leaves ratio, the pH tends to drop faster and lower and acidity and bacterial tend to increase. The instant drop in pH from week 1 can be due to the presence of *Pediococcus acidilactici* SMZ 41 inoculated in the silage.

4.6.2 Evolution of *Lactobacillus paracasei* and *Pediococcus acidilactici* growth and dominance in the silage

The dominant species were identified by MALDI TOF MS performed on

randomly selected colonies representing 50% or more of the colonies obtained on MRS plates after dilution of silages, after 7 weeks of ensiling. The results of the identification of the dominant species in 10% are shown in Figure 19 inoculated with *Lactobacillus paracasei* and *Pediococcus acidilactici*. The total plate count in 10% feed/leaves silage was 13 and all the colonies were subjected to identification by MALDI TOF MS. 77% of the colonies (10 out of 13) were of *Lactobacillus paracasei* and 23% (3 out of 13) were of *Pediococcus acidilactici*. The results show that the silage is being dominated by the inoculated species of *Lactobacillus paracasei* and *Pediococcus acidilactici* making the LAB in the silage to be 100%. However, it seems that such conditions (10% feed/leaves silage) *Lactobacillus paracasei* dominated *Pediococcus acidilactici*.

In the 50% feed/leaves silage, the colonies count was 80 from which 37 colonies were randomly selected for identification (Figure 20). The identified species in the silage showed that 49% of the colonies were of *Lactobacillus paracasei*, 22% of *Pediococcus acidilactici*, 16% of *Candida glabrata* and 13% of *Staphylococcus epidermidis*. The results show that the inoculated *Lactobacillus paracasei* is dominating the silage and to some extend by *Pediococcus acidilactici* making the LAB count 71% of the total colonies.

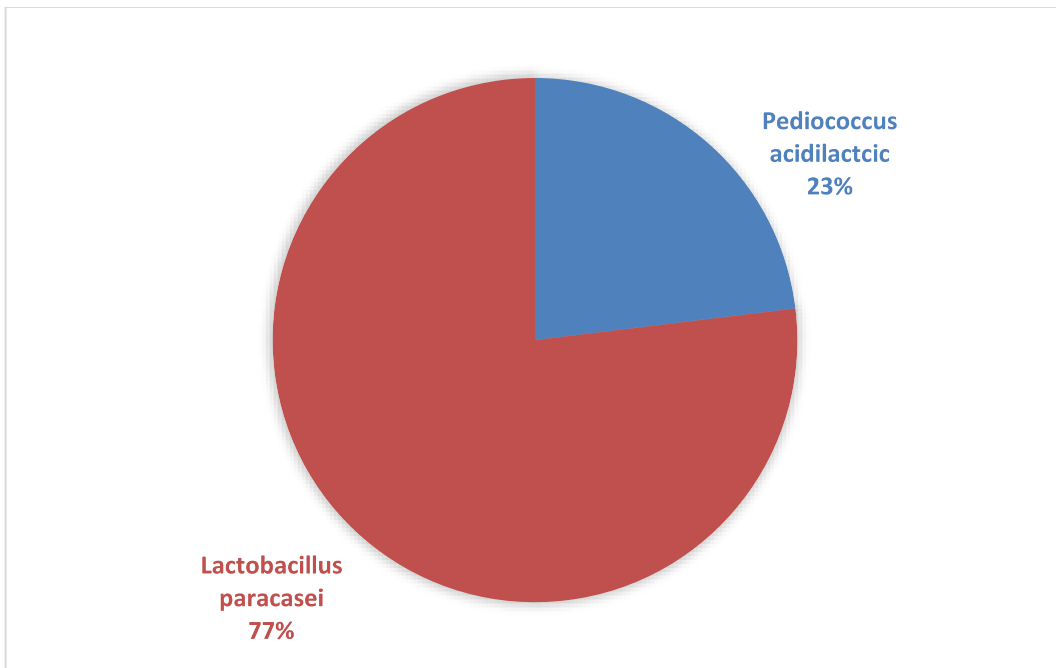

Figure 19: Dominating species in Inoculum 5 (*Lactobacillus paracasei* SMZ20 and *Pediococcus acidilactici* SMZ41) 10% feed/leaves silages

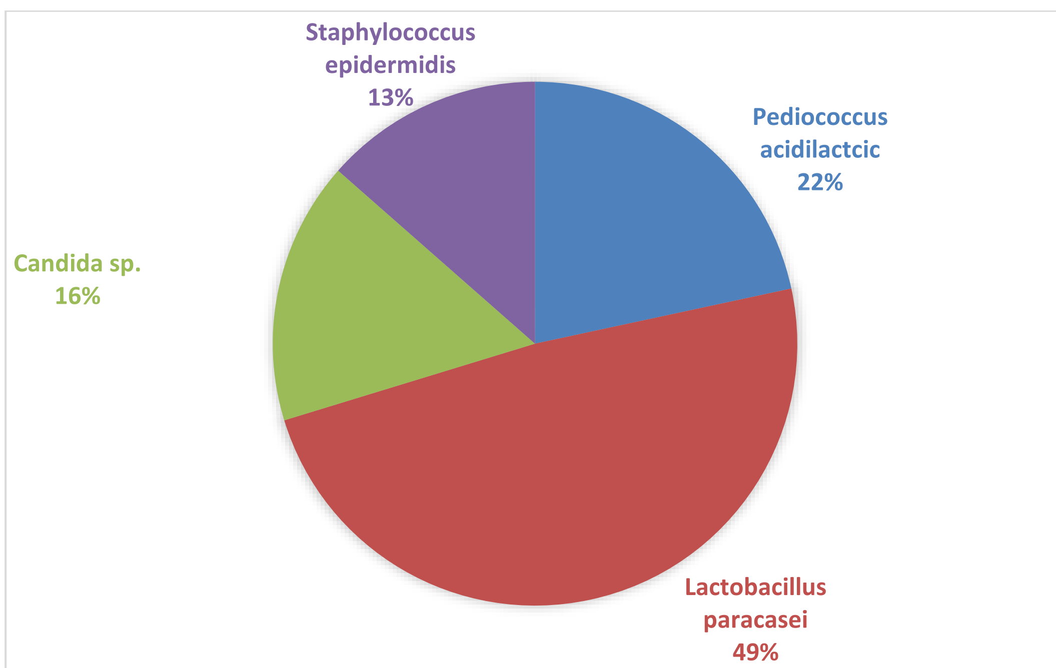

Figure 20: Dominating species in Inoculum 5 (*Lactobacillus paracasei* SMZ20 and *Pediococcus acidilactici* SMZ41) 50% feed/leaves silages

4.6.3 Conclusion

The results of the co-inoculation clearly showed that the dominance by *Lactobacillus paracasei* and *Pediococcus acidilactici* was similar to that in the silages inoculated separately by each of the two strains. Interestingly, in the low feed/leave ratio (poor nutrients content), the co-inoculation of the 10% feed/leaves silages provided a 100% dominance of both LABs without other LAB and yeast. However, in the richer medium (50% feed/leaves silage) both inoculated LAB represented 70.27% of the total colonies. The two strains of *Lactobacillus paracasei* and *Pediococcus acidilactici* have shown dominancy in the ensiling process of palm tree leaves mixed with 10% feed.

4.7 Study of the nutritional and cultural requirements for the production of LAB inocula

4.7.1 Introduction

The food security in developed countries is established by legislation that protect the economy via the protection of the marketed products. The developing countries need to develop food security at a faster rate to facilitate the local food demands. The livestock industry is highly dependent on food safety and security due to its constant and high demand for feed sources. The industry, depending on the region, is highly susceptible to environmental conditions and microbial infections in livestock and further affecting human health. Various drugs and antibiotics are used for the control of pathogens, but these products are being banned by the European Union since these antibiotics promote the emergence of resistant strains (BERISVIL et al., 2020). An alternate solution needs to be provided to overcome this issue. The use of natural biological techniques to improve the quality of the feed of the livestock can be a beneficial tool. The use of probiotics is well reported to control food-borne pathogens. Probiotics can limit the growth of the bacterial population and is an ingestible food

ingredient. LAB is the most abundantly used probiotics in the feed of livestock. Currently, the commercial media used for the growth of LAB is MRS media, which contains macro and micronutrients to enhance bacterial growth (Almansour et al., 2019). The use of MRS media on large scale for LAB growth is not economical. The use of LAB in the agro-food industry will largely depend on the media cost and supporting mass bacterial production. One of the objectives of this study was to prepare low-cost media for the growth of LAB at a laboratory scale. This work has to study the nutritional and cultural requirements to produce LAB based on the selected LAB which showed their interest to be applied for the silage of palm tree leaves.

The cultural media prepared for the optimum media for growing LAB contained feed, which is one of the components of the silage. MRS liquid medium is highly used for the fast growth of LAB. Aeration is the most important parameter in growth. It should be optimized for the obligate aerobic LAB and should be low for the facultative anaerobic LAB. Here, the aeration was considered to be high if the culture medium is highly agitated in the shaker set at 200 rpm and considered low with an agitation of 100 rpm. A fractional factorial method was used for the experimentation as shown in Table 7. It is a 2^3 factorial plan experimental design, with three parameters (Volume of MRS, % Feed and agitation rate of the flasks). Preliminary work was performed using the three parameters, after which the 2 limits were fixed. The pH of the media was adjusted at 5.7 before sterilization. The prepared media were inoculated separately with the 4 selected strains of LAB: *Lactobacillus plantarum* SMZ 103, *Lactobacillus paracasei* SMZ 20, *Pediococcus acidilactici* SMZ 41 and *Lactobacillus plantarum* SMZ 46. The inoculum was similar to all the strains (at time zero, 0.15 $OD_{600\ nm}$). The cultures were incubated in a shaker set at 35 °C for 1 week.

Table 7: 2^3 factorial plan experimental design. The MRS values are the percentage of the MRS liquid in the final volume of the culture. The feed was represented as % (w/v) and aeration represented by agitation speed of the cultures.

MRS	Feed	Aeration
0% (-)	5% (-)	100 rpm (-)
0% (-)	5% (-)	200 rpm (+)
0% (-)	20% (+)	100 rpm (-)
0% (-)	20% (+)	200 rpm (+)
20% (+)	5% (-)	100 rpm (-)
20% (+)	5% (-)	200 rpm (+)
20% (+)	20% (+)	100 rpm (-)
20% (+)	20% (+)	200 rpm (+)

4.7.2 Study of the nutritional and cultural requirements of the strain *Lactobacillus plantarum* SMZ103

The results of the evolution of pH, acidity, and growth in the cultural media inoculated with *Lactobacillus plantarum* SMZ103 are shown in Table 8. A drop in pH was observed for all the cultures, after 1 week of incubation. The pH of the culture containing 0% MRS and 5% feed and incubated at 100 rpm dropped from 6.2 to 4.73. It is to be noticed that in the same culture agitated with 200 rpm (higher aeration), the pH also dropped down to 4.72. The pH of the cultures prepared with 20% feed dropped down to 5.12 when incubated at 100 rpm and to 5.39 at 200 rpm agitation. The pH of the cultures containing 20% MRS and agitated at 100 rpm dropped to 4.49 and when agitated at 200 rpm the pH dropped to 5.12 only. The pH of the cultures containing 20% feed and 20% MRS and incubated at 100 rpm dropped to 3.97 while dropped to 4.15 at 200 rpm agitation.

The acidity of the culture containing 0% MRS and 5% feed and incubated at 100 rpm was 0.0008 equivalent acidity/L while it was 0.0008 equivalent acidity/L when the culture was incubated at 200 rpm. The acidity of the culture prepared with 20% feed

was 0.007 equivalent acidity/L at 100 rpm and 0.0044 equivalent acidity/L at 200 rpm. In the culture containing 20% MRS, the acidity was 0.0057 equivalent acidity/L at 100 rpm, and 0.0034 equivalent acidity/L at 200 rpm. The acidity of the culture containing 20% feed and 20% MRS was 0.0148 equivalent acidity/L at 100 rpm and 0.012 equivalent acidity/L at 200 rpm.

The growth of the LAB (CFU) in the culture containing 0% MRS and 5% feed was 6.89 log (CFU/ml) at 100 rpm and 6.31 log (CFU/ml) at 200 rpm. The growth in the culture prepared with 20% feed was 8.48 log (CFU/ml) at 100 rpm and 7.48 log (CFU/ml) at 200 rpm. The culture containing 20% MRS was 8.48 log (CFU/ml) at 100 rpm, the CFU and 6.9 log (CFU/ml) at 200 rpm. The CFU of the culture containing 20% feed and 20% MRS was 7.27 log (CFU/ml) at 100 rpm and 6.2 log (CFU/ml) at 200 rpm.

Table 8: pH, Acidity and CFU of the cultural media inoculated with *Lactobacillus plantarum* SMZ103 isolated from probiotics.

Factors	**pH**	**Acidity (equivalent/L)**	**log (CFU/ml)**
Water – MRS – Feed (100 rpm)	4.71±0.02	0.0008±0.0002	6.89±0.03
Water – MRS – Feed (200 rpm)	4.72±0.02	0.0008±0.0002	6.31±0.04
Feed + Water – MRS (100 rpm)	5.12±0.02	0.0070±0.0003	8.48±0.02
Feed + Water – MRS (200 rpm)	5.39±0.03	0.0044±0.0002	7.48±0.02
MRS + Water – Feed (100 rpm)	4.49±0.05	0.0057±0.0004	8.48±0.02
MRS + Water – Feed (200 rpm)	5.12±0.02	0.0034±0.0002	6.90±0.02
Feed + Water + MRS (100 rpm)	3.97±0.05	0.0148±0.0003	7.27±0.02
Feed + Water + MRS (200 rpm)	4.15±0.03	0.0120±0.0002	6.20±0.05

4.7.3 Study of the nutritional and cultural requirements of the strain *Lactobacillus paracasei* SMZ20

The results of the evolution of pH, acidity and bacterial growth of the cultural

media inoculated with *Lactobacillus paracasei* SMZ 20 are shown in Table 9. A drop in pH was observed for all the cultures after 1-week incubation. The pH of the culture containing 0% MRS and 5% feed incubated at 100 rpm dropped from 6.2 to 4.73 and for the same culture at 200 rpm agitation, the pH dropped to 4.7. The pH of the culture prepared with 20% feed at 100 rpm dropped to 5.2 and the same culture's pH dropped to 5.42 at 200 rpm agitation. The culture prepared to contain 20% MRS at 100 rpm, the pH dropped to 4.73 and at 200 rpm the culture's pH dropped to 5.32. The pH of the culture comprising of 20% feed and 20% MRS at 100 rpm dropped to 3.98 and at 200 rpm the same culture's pH dropped to 4.12.

The acidity of the culture containing 0% MRS and 5% feed at 100 rpm was 0.001 equivalent acidity/L and it was 0.0008 equivalent acidity/L when the culture was incubated at 200 rpm. The acidity of the culture prepared with 20% feed at 100 rpm was 0.0062 equivalent acidity/L and the same culture's acidity was 0.0048 at 200 rpm agitation. The culture was prepared with 20% MRS at 100 rpm, the acidity was 0.0057 equivalent acidity/L and at 200 rpm the culture's acidity was 0.0039 equivalent acidity/L. The acidity of the culture comprising of 20% feed and 20% MRS at 100 rpm was 0.015 equivalent acidity/L and at 200 rpm the same culture's acidity 0.0133 equivalent acidity/L. There was an increase in the growth of the LAB (CFU) in the cultures. The growth of the culture with 0% MRS and 5% feed at 100 rpm was 6.91 log (CFU/ml) and for the same culture at 200 rpm the CFU was 5.03 log (CFU/ml). The growth of the culture prepared with 20% feed at 100 rpm was 8.12 log (CFU/ml) and 6.34 log (CFU/ml) at 200 rpm. In the culture with 20% MRS at 100 rpm, the growth was 8.5 log (CFU/ml) and at 200 rpm the culture's growth was 6.89 log (CFU/ml). The growth of the culture with 20% feed and 20% MRS at 100rpm was 7.2 log (CFU/ml) and 6.52 log (CFU/ml) at 200 rpm.

Table 9: pH, Acidity and CFU of the cultural media inoculated with *Lactobacillus paracasei* SMZ20 isolated from probiotics.

Factors	pH	Acidity (equivalent/L)	log (CFU/ml)
Water – MRS – Feed (100 rpm)	4.73±0.02	0.0010±0.0002	6.91±0.02
Water – MRS – Feed (200 rpm)	4.70±0.02	0.0008±0.0002	5.03±0.02
Feed + Water – MRS (100 rpm)	5.20±0.03	0.0062±0.0004	8.12±0.03
Feed + Water – MRS (200 rpm)	5.42±0.05	0.0048±0.0002	6.34±0.05
MRS + Water – Feed (100 rpm)	4.73±0.04	0.0057±0.0003	8.50±0.02
MRS + Water – Feed (200 rpm)	5.32±0.02	0.0039±0.0002	6.89±0.03
Feed + Water + MRS (100 rpm)	3.98±0.05	0.0150±0.0003	7.20±0.04
Water – MRS – Feed (100 rpm)	4.12±0.03	0.0133±0.0002	6.52±0.04

4.7.4 Study of the nutritional and cultural requirements of the strain *Pediococcus acidilactci* SMZ41

The results of the evolution of pH, acidity and growth of the cultural media inoculated with *Pediococcus acidilactici* SMZ 41 are shown in Table 10. A drop in pH was observed for all the media after 1-week incubation. The pH of the culture containing 0% MRS and 5% feed incubated at 100 rpm dropped from 6.2 to 4.73 and for the same culture at 200 rpm the pH dropped to 4.7. The pH of the culture prepared with 20% feed at 100 rpm dropped to 4.57 and to 5.23 at 200 rpm. The culture was prepared with 20% MRS at 100 rpm, the pH dropped to 4.46 and at 200 rpm the culture's pH dropped to 5.7. The pH of the culture comprising of 20% feed and 20% MRS at 100 rpm dropped to 4.05 and at 200 rpm the pH was 4.36.

The acidity of the culture containing 0% MRS and 5% feed at 100 rpm was 0.001 equivalent acidity/L and was 0.001 equivalent acidity/L at 200 rpm agitation. The acidity of the culture prepared with 20% feed at 100 rpm was 0.0067 equivalent acidity/L and was 0.0046 at 200 rpm. In the culture containing 20% MRS at 100 rpm, the acidity was 0.0065 equivalent acidity/L and at 200 rpm it was 0.0028 equivalent acidity/L. The acidity of the culture with 20% feed and 20% MRS at 100 rpm was

0.0153 equivalent acidity/L and at 200 rpm the acidity was 0.0132 equivalent acidity/L. There was an increase in the bacterial growth (CFU) of the LAB. The LAB growth of the culture containing 0% MRS and 5% feed at 100 rpm was 6.91 log (CFU/ml) and at 200 rpm it was 5.03 log (CFU/ml). The growth in the culture prepared with 20% feed/leaves at 100 rpm was 8.5 log (CFU/ml) and the culture's growth was 7.34 log (CFU/ml) at 200 rpm. In the culture containing 20% MRS at 100 rpm, the growth was 8.43 log (CFU/ml) and at 200 rpm it was 6.68 log (CFU/ml). The growth of the culture with 20% feed and 20% MRS at 100 rpm was 7.27 log (CFU/ml) and at 200 rpm it was 6.3 log (CFU/ml).

Table 10: pH, Acidity and CFU of the cultural media inoculated with *Lactobacillus plantarum* SMZ103 isolated from silage

Factors	**pH**	**Acidity (equivalent/L)**	**log (CFU/ml)**
Water – MRS – Feed (100 rpm)	4.73±0.02	0.0010±0.0002	6.91±0.02
Water – MRS – Feed (200 rpm)	4.7±0.02	0.0010±0.0002	5.03±0.02
Feed + Water – MRS (100 rpm)	4.57±0.04	0.0067±0.0003	8.50±0.05
Feed + Water – MRS (200 rpm)	5.23±0.03	0.0046±0.0002	7.34±0.04
MRS + Water – Feed (100 rpm)	4.96±0.05	0.0065±0.0004	8.43±0.05
MRS + Water – Feed (200 rpm)	5.70±0.04	0.0028±0.0002	6.68±0.03
Feed + Water + MRS (100 rpm)	4.05±0.03	0.0153±0.0002	7.27±0.04
Water – MRS – Feed (100 rpm)	4.36±0.02	0.0132±0.0002	6.30±0.02

4.7.5 Study of the nutritional and cultural requirements of the strain *Lactobacillus plantarum* SMZ46

The results of the evolution of pH, Acidity and CFU of the cultural media inoculated with *Lactobacillus plantarum* SMZ46 are shown in Table 11. A drop in pH was observed for all the media after 1 week. The pH of the culture containing 0% MRS and 5% feed at 100 rpm dropped from 6.2 to 4.73 and at 200 rpm the pH dropped to 4.72. The pH of the culture prepared with 20% feed at 100 rpm dropped to 4.82 and to 5.25 at 200 rpm agitation. The pH of culture prepared with 20% MRS incubated at 100

rpm agitation dropped to 4.35 and at 200 rpm agitation, it dropped to 5.29. The pH of the culture with of 20% feed and 20% MRS at 100 rpm dropped to 4.02 and at 200 rpm the pH was 4.43.

The acidity of the culture containing 0% MRS and 5% feed at 100 rpm was 0.001 equivalent acidity/L and at 200 rpm the acidity was 0.001 equivalent acidity/L. The acidity of the culture prepared with 20% feed at 100 rpm was 0.0055 equivalent acidity/L and 0.0041 at 200 rpm agitation. The culture prepared with 20% MRS incubated at 100 rpm, the acidity was 0.0047 equivalent acidity/L and at 200 rpm it was 0.0026 equivalent acidity/L. The acidity of the culture with 20% feed and 20% MRS at 100 rpm agitation was 0.0153 equivalent acidity/L and at 200 rpm the acidity was 0.0123 equivalent acidity/L.

There was an increase in the bacterial growth (CFU) of the LAB of the cultures. The growth of the culture containing 0% MRS and 5% feed at 100 rpm was 6.43 log (CFU/ml) and at 200 rpm the growth was 6.43 log (CFU/ml). The bacterial growth of the culture prepared with 20% feed incubated at 100 rpm was 8.36 log (CFU/ml) and 7.12 log (CFU/ml) at 200 rpm. The culture prepared with 20% MRS at 100 rpm, the growth was 8.5 log (CFU/ml) and at 200 rpm it was 7.84 log (CFU/ml). The growth of the culture containing 20% feed and 20% MRS at 100 rpm was 7.27 log (CFU/ml) and at 200 rpm agitation, it was 6.1 log (CFU/ml).

Table 11: pH, Acidity and CFU of the cultural media inoculated with Pediococcus acidilactici SMZ46 isolated from silage.

Factors	pH	Acidity (equivalent/L)	log (CFU/ml)
Water – MRS – Feed (100 rpm)	4.73±0.015	0.0010±0.0002	6.43±0.02
Water – MRS – Feed (200 rpm)	4.72±0.015	0.0010±0.0002	6.43±0.05
Feed + Water – MRS (100 rpm)	4.82±0.02	0.0055±0.0004	8.36±0.03
Feed + Water – MRS (200 rpm)	5.25±0.02	0.0041±0.0002	7.12±0.02
MRS + Water – Feed (100 rpm)	4.35±0.05	0.0047±0.0004	8.50±0.02
MRS + Water – Feed (200 rpm)	5.29±0.04	0.0026±0.0002	7.84±0.02
Feed + Water + MRS (100 rpm)	4.02±0.025	0.0153±0.0002	7.27±0.02
Water – MRS – Feed (100 rpm)	4.43±0.02	0.0123±0.0002	6.10±0.05

4.7.6 Conclusion

Similar patterns in terms of pH, acidity and CFU evolution were observed for all the cultures that were prepared with LAB inoculation. The drop in pH was more in low agitated cultures (100 rpm) than in culture with high agitation (200 rpm). Similarly, the acidity and bacterial growth were higher in low agitated cultures than high agitated cultures, indicating that the growth of LAB is enhanced in less agitated conditions. The main objective was to enhance the growth of LAB and feed with water at low agitation and MRS with water at low agitation has proven to provide the optimum conditions for the growth of LAB. Comparing these two-culture media, MRS culture is more expensive than feed culture, and therefore feed culture with water at low agitation has proven to be the most cost-effective among all the inoculated cultural media.

CONCLUSION

The lignocellulosic content of the Qatari palm tree leaves were depicting high strength in terms of fiber and low in crude protein content. The composition in the palm tree leaves reduces their digestibility and nutritional value to be used as direct feed. The isolation program of local lactic acid bacteria (LAB) allowed the construction of a local collection of 87 isolates. All the isolates were identified by matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI TOF MS) showing that the isolates belong to the genus *Lactobacillus* from different sources, with most of the isolated strains were from spontaneous mixed silages of palm leaves and animal feed. The principal component analysis (PCA) and dendrogram using the MALDI TOF MS proteins profiles of all the LAB isolates allowed the categorization of the isolates into 5 main groups, with high similarities within those belonging to the same group. This allowed the selection of 4 isolates for the ensiling process of the palm trees leaves. The silage was performed using different feed/leaves ratios, to supplement the silage with the necessary nutrients to the LAB. The trends of the drop in pH and increase in acidity and bacterial growth (CFU) were similar for all the silages with the lowest pH and highest acidity and CFU being of silages with 50% feed and 50% palm leaves. However, the ensiling of the palm tree leaves using 10% feed, was showing a drop of pH and increase in acidity and cell growth allowing us to conclude that this feed/leaves can be appropriate. Among the inoculated silages *Lactobacillus paracasei* SMZ20 isolated from probiotics and *Pediococcus acidilactici* SMZ41 isolated from spontaneous silage, had the most promising results with the lowest pH and highest acidity and CFU. *Pediococcus acidilactici* had the fastest drop in pH and an increase in acidity and CFU among all the silages. The dominant species in the silages were identified using MALDI TOF MS and *Lactobacillus paracasei* and *Pediococcus*

acidlactici were shown to be dominating, in their respective inoculated silages in high percentages. The results of the silages of co-inoculation of *Lactobacillus paracasei* and *Pediococcus acidilactici* showed the same trends in terms of pH, acidity and CFU, as of their separately inoculated silages. Both these species were also found to be the ones dominating the co-inoculated silages.

In order, to investigate the low-cost production of LAB inoculant for ensiling palm trees leaves, a factorial plan experimentation design (2^3 factorial plan) was implemented for the most affecting factors (% feed, % MRS and aeration). The cultural media in the 8 experiments showed similar trends in terms of pH, acidity and CFU. The bacterial growth was observed to be high in low agitated cultures. The main objective was to develop a low-cost culture media to promote LAB growth. The most promising results were of the culture media with animal 5% feed mixed in water at low aeration. This study demonstrated that even with the nutritional value of palm leaves being low, it can be enhanced by mixing with animal feed at a ratio of 10% only (Feed/leaves) and the silage will be dominated by the very interesting local LAB, *Lactobacillus paracasei* and *Pediococcus acidilactici*. These strains were shown to grow easily in 5% feed with low aeration to produce inoculant which may be used to inoculate the silage of the 10% feed/leaves during a period of 7 weeks.

Ensiling palm leaves, mixing with feed, and inoculating with LAB prove to be promising option for the preservation of the palm leaves for a longer period and enhance their nutritional value with the addition of feed. For the first time, the silage was optimized and assisted by the proteomic technique of MALDI TOF allowing to follow up the microbial population dynamics.

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