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Full Length Research Paper

Microbiological analysis and screening of lactic acid bacteria from Tunisian date palm sap

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Date Palm sap is a fresh juice called "Legmi" widely produced and consumed in Southern Tunisia. The aim of this research was to study microbiological group's distribution present in 10 palm sap samples, and to select Lactic acid bacteria (LAB) with suitable properties for use as starter cultures in sap lactic fermentation. Microbiological analysis showed high load in fresh palm sap. Mesophilic aerobic bacteria varied from 6.07 to 8.57 log cfu/ml, coliforms ranged from 3 to 6.78 log cfu/ml, yeasts between 3 and 8.47 log cfu/ml and LAB from 5.36 to 8.47 log cfu/ml. Ten strains were chosen randomly to study the acidifying activity when growing in palm sap. These strains, tentatively identified by API 50CHL, were divided into two groups: *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Lactobacillus delbrueckii* subsp. *delbruckii*. All the strains were considered as fast acidifier since ∆pH was higher after 6 h of fermentation. The higher acidifying strains KH_3 (*L. delbrueckii*) and $5B_4$ (*L. mesenteroides)*, were used to inoculate sap in pure culture. During the fermentation, pH decreased from 6.94 and 6.36 for 5B4 and KH3, respectively, to about 4.0±0.1 after 24 h of fermentation for both strains. Total titrable acidity increase from 0.2 and 0.4% for 5B4 and KH3 respectively to around 3.0%. The application of these strains as starter cultures in the production of fermented palm sap could be investigated on further studies.

Key words: Date palm sap, legmi, lactic acid bacteria, starter culture.

INTRODUCTION

For a long time, traditional palm sap tapping has been a common practice. Sap is exuded from the trunk of the date palm tree during the tapping operation. It is refreshing beverage enjoyed by people in parts of Africa, Asia and South America. Palm sap can be converted by fermentation process into palm wine or vinegar (Mozingo, 1989). Fermented palm sap is referred to 'Toddy' in Sri Lanka, 'Tuba' in the Philippines and 'Tuak' in Indonesia (Lasekan and Abbas, 2010).

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The collection of sap from *Phoenix dactylifera L.* is a current practice in Tunisia. Date palm sap is directly consumed as a fresh juice, called 'Legmi' or used as an alcoholic beverage after spontaneous fermentation (Ben Thabet et al., 2010). "Legmi" is rapidly fermented by autochthonous microflora due to the availability of sugars (92–95% dry matter basis) (Atputharajah et al., 1986; Shamala and Sreekantiah, 1988; Ben Thabet et al., 2010). This endogenous microflora was composed essentially by yeasts, Lactic Acid Bacteria (LAB) and Acetic Acid Bacteria (AAC) (Stringini et al., 2009).

Many researchers have investigated the adding value to sap: Umerie et al. (2000) proposed the transformation of sap collected from oil palm (*Elaeis guineensis*) and wine palm trees (*Raphia hookeri*) for caramel production.

Ho et al. (2007) carried out palm sugar production from *Arenga pinnata* palm sap tree. Others researchers focused on the valorization of palm sap, oil palm and palm wine by adding industrial culture for producing ethanol, lactic acid and biomass (Jeyaseelan and Seevaratnam,1986; Kosugi et al., 2010). Some authors have suggested the use of autochthonous sap microbiota (mainly yeasts) as inoculum for the fermentation of coconut palm sap (*Cocos nucifera*) to palm wine (Wijesinghe and Samarajeewa, 1988). However, to the best of our knowledge no studies focused in the use of authchtonous lactic acid bacteria as starters for making lactic beverage from palm sap since that autochthonous strains always have better performances than allochthonous strains (Di Cagno et al., 2011). In fact, LAB showed important roles in the fermentation and preservation of a great variety of food products (Soomro et al., 2002; Mohamadou et al., 2010; Fowoyo and Ogunbanwo, 2011). In this way, the purpose of this work was (i) To determine the microbiological group's distribution of fresh palm sap and (ii) to select lactic acid bacteria starter on the basis of acidifying activity and to study their suitability for application as starter culture in the production of fermented beverage.

MATERIALS AND METHODS

Sap sampling

Palm-sap samples were collected from date palm *P. dactylifera* in early morning, from palm grove located in Southern Tunisia: Gabès, Medenine, Tataouine and Gbelli (April-May 2010). The local sap collection method was used. It consisted in cutting off the growing point of palm. The juice was collected from a shallow depression scooped out at the top (Barreveld, 1993). The samples (Fresh sap) were collected in sterile plastic containers and immediately stored in in an ice box (4°C) to avoid fermentation during transportation (60 min) to the laboratory of Arido-culture et Culture Oasiennes, Institut des Régions Arides (IRA), Medenine. The microbiological analysis of samples was determined within a day. Before analyzing, the sample was filtered by sheet cloth and kept at 4°C until analysis.

Microbial counts, LAB isolation and tentative identification of isolates

10 ml of palm sap was homogenized in 90 ml sterile salt peptone solution containing 0.1% bacteriological peptone and 0.9% NaCl as the 1:10 dilution. After serial dilution, Aerobic mesophilic bacteria were enumerated by pour plate on Plate Count Agar (PCA) incubated aerobically at 37°C for 3 days. Yeasts were counted on Sabouraud agar medium with chloramphenicol (500 µg/ml) (Sigma-Aldrich) to suppress bacteria growth and incubated at 30°C for 2 days. Total coliform were enumerated on Violet red Bile Lactose Agar (VRBA). Plates were incubated at 37°C for 2 days. LAB were enumerated on MRS agar (De Man, Rogosa and Sharpe) supplemented with cycloheximide 0.005% (Sigma-Aldrich), to inhibit yeast growth. Plates were incubated at 30°C for 2 days under aerobic conditions. All media were obtained from Oxoid. Microbial counts were analyzed in log scale (log cfu/ml).

Presumptive LAB was phenotypically characterized by Gram-staining, determination of morphology by phase-contrast microscopy, catalase activity. Only Gram-positive, catalase negative, non motile rod and cocci isolates strains were selected. The presence of catalase activity was assessed by the formation of gaz bubles after the suspension of bacterial cells in a droplet of 3% hydrogen peroxide on MRS. Stock cultures of the isolates were stored in MRS broth containing 15% glycerol (Merck) at -80°C.

Carbohydrate fermentation pattern of lactic acid bacteria used for sap fermentation were determined at least in duplicate using API 50CH®system (API system, BioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions (Amoa-Awwa et al., 2007). The results were recorded after 24 and 48 h of incubation at 30°C. The ultimate identification was ensured by API Labplus software provided by Bio-Mérieux.

Selection of starter cultures with acidifying activity

Fifty milliliters of sterilized palm sap (120°C; 15 min) was inoculated with 2% of culture at early stationary growth phase on MRS broth of the lactic acid bacteria strain. The palm sap was then incubated at 37°C. The pH was measured on aliquots after 6 h. The decrease in pH (ΔpH) was calculated as the difference between the values immediately after inoculation (t=0h) and the values at 6 h ($\Delta pH = pH_{at\ 6H} - pH_{zero\ time}$). Three trials of each strain were carried out.

Palm sap fermentation with selected starter cultures

Two LAB strains were selected as starter cultures for palm sap fermentation on the basis of acid production ability. Palm sap (100 ml) was sterilized by autoclaving at 120°C for 15 min and inoculated with selected starter (5%). Incubation was done at 37°C during 24 h. Samples were withdrawn periodically for pH and titrable acidity. pH is directly measured using pH meter HANNA instruments pH 210. For Total acidity, a few drop of 1% phenolphthalein was added to sample (10 ml). The homogenate was titrated with 0.1 N NaOH. Titrable acidity was expressed as percentages of acetic acid (Atputharajah et al., 1986).

RESULTS AND DISCUSSION

Microbial distribution on date palm sap

Figure 1 shows the mean variation in the population of microorganisms found in collected samples of sap from different palm grove areas. The sap of the palm tree has been shown to be a rich medium capable of supporting the growth of various types of micro-oganisms, as high numbers of aerobic mesophilic bacteria, lactic acid bacteria and yeasts. Aerobic mesophilic bacteria varied from 6.07 to 8.57 log cfu/ml. Yeasts ranged from 3 to 8.47 log cfu/ml. The microbial counts assessed on MRS media showed that there was a background microbial population of presumptive, autochthonous LAB of 5.36 to 8.88 log cfu/ml. Coliforms were also found in palm sap and ranged between 3 and 6.78 log cfu/ml. The presence of coliforms may be due to the contamination from environment and equipment used for tapping and from birds sheltering the palm tree.

To our knowledge, there is dearth of information on the microflora of Tunisian date palm sap "Legmi". Only few reports show physicochemical characteristics of Tunisian

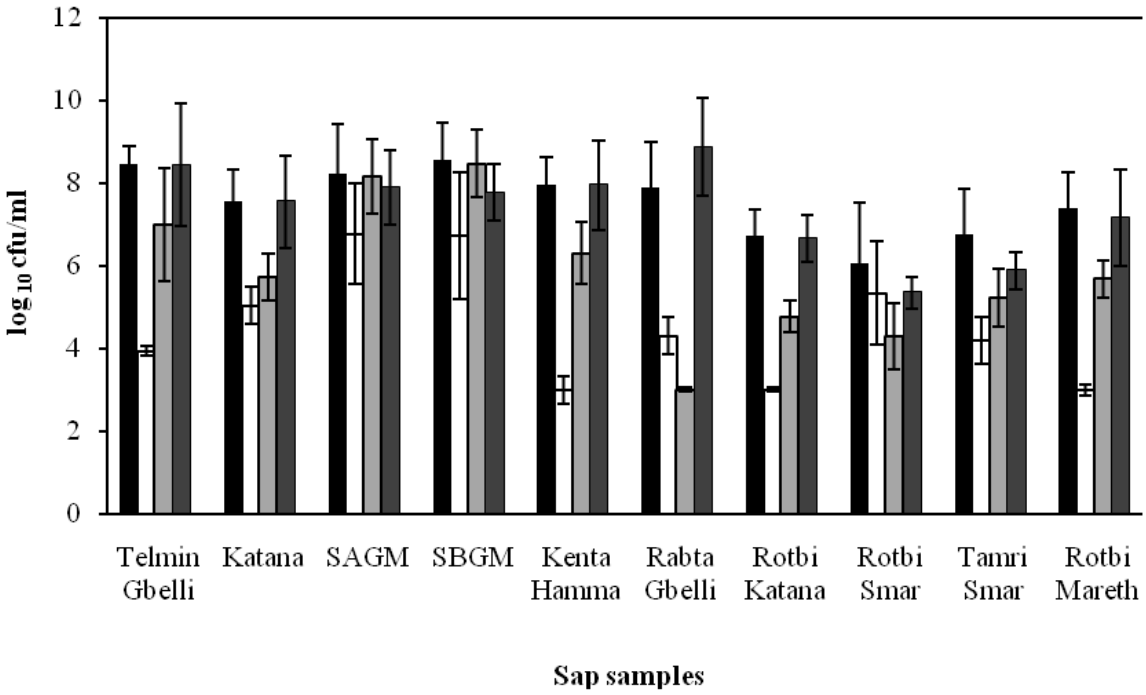

Figure 1. Counts (Mean $\log_{10}$) of different microbial groups of palm sap collected in palm grove from southern Tunisian: (■) Total Aerobic Bacteria, (□) Total Coliforms, (■) Yeasts and (■) Lactic Acid Bacteria. (Results are mean values from three replications ± standard deviations).

palm sap (Ben Thabet et al., 2007; 2009; 2010). However, many reports were investigated on palm wine obtained by sap fermentation of the palmae family such as the oil palm, coconut palm, coconut palm, date palm, Nipa palm, Kithul palm and Raffia palm (Jayatissa et al., 1978; Sanni and Lonner, 1993; Ejiofor et al., 1994; Ayogu 1999; Nwachukwu et al., 2006; Stringini et al., 2009). Studies over the years have been devoted to the isolation and identification of yeasts responsible for palm wine fermentation.

Stringini et al. (2009) reported microbial population of the tapped palm wine from Cameroon before fermentation, where they show presence of yeasts and LAB at about 6 and 7 log cfu/ml respectively. Our data found in fresh sap was generally in similar way as reported by Stringini et al. (2009). However, it is worth noting the lower amounts of LAB population in Rotbi-Smar and Tamri-Smar sap samples (about 5 log cfu/ml). Amoa-Awua et al. (2007) reported LAB load from 5 to 9 log cfu/ml; yeasts from 5 to 7 log cfu/ml and aerobic mesophilic from 7 to 9 log cfu/ml in 15 samples of palm sap collected within 3 h of tapping. According to Shamala and Sreekantiah, (1988) microflora present in fresh sap and palm wine toddy in India were almost similar involved LAB and yeasts, except that Acetic Acid Bacteria were found only in the latter (when levels of alcohol had become substantial).

Okafer (1978) has reported that types and number of microorganisms encountered in palm wine fermentations vary widely and sometimes even from tree to tree. These variations of microbial content may come also from the quality of the raw material (genetic and metabolite characteristic of palm tree, and palm grove), environment factors (temperature) and harvesting conditions (ustensils used during the taping of felled palm trees, personal hygiene and sanitary equipement). In general, the methods of palm wine tapping and collection of palm sap influence the microbial content of the sap (Ayernor and Matthews, 1971; Amoa-Awwa et al., 2007; Naknean et al., 2010).

Identification and screening starter for sap lactic fermentation

Ten strains of lactic acid bacteria, isolated from fresh palm sap, were chosen according to the difference in cell morphology to study the acidifying activity. These strains were identified using API galleries. Results of carbohydrates fermentations are illustrated in Table 1. The strains are divided in two groups. The first ones were dominated by regular rods (KH_4, KH_3, KH_5) which fermented mostly ribose, salicin and gluconate and were tentatively identified as *L. delbrueckii* subsp. *delbruckii*. A second type of LAB identified was lenticular to coccoid in shape ($5B_4$, RS_2, K_7, K_6, TS_1, $2B_2$, $6B_1$). They fermented mostly, L-arabinose, ribose, xylose, mannitol, Acetyl-D-mannoside, amygdaline, salicin, D-cellobiose, maltose,

Table 1. Fermentation profiles of lactic acid bacteria used for palm sap fermentation.

Strains	5B4	KH4	KH3	KH5	RS2	K7	K6	TS1	2B2	6B1
L-arabinose	+	-	-	+	-	+	-	+	+	-
D-ribose	+	+	-	+	-	+	-	+	+	+
D-xylose	+	-	-	+	-	+	+	+	+	-
D-galactose	+	-	-	-	-	+	-	+	-	-
D-mannitol	-	-	-	-	-	+	+	+	-	-
A-Y1-d-mannoside	-	-	-	-	-	+	-	-	-	-
Acétyle-D-mannoside	+	-	-	+	+	+	+	+	+	+
Amygdaline	+	-	-	+	-	+	-	+	+	-
Arbutine	+	+	-	-	-	+	-	+	+	+
Salicine	+	+	+	+	-	+	+	+	+	+
D-cellobiose	+	-	-	+	-	+	-	+	+	-
D-maltose	+	-	-	+	+	+	+	+	+	+
D-lactose	+	-	-	-	-	+	-	+	+	-
D-melibiose	+	-	-	+	-	+	-	+	+	-
D-raffinose	-	-	-	+	-	-	-	+	-	-
Gentibiose	+	-	-	+	-	+	-	+	-	-
D-turanose	+	-	-	+	+	+	+	+	+	+
Gluconate	-	+	-	+	-	-	-	-	-	-
2-Ketogluconate	-	-	-	+	-	-	-	-	-	-
5-Ketogluconate	-	-	-	+	-	-	-	-	-	-
Identification	*Leuconostoc mesenteroides* sp. *mesenteroides*	*Lb. delbruckii* sp. *delbruckii*	*Lb. delbruckii* sp. *delbruckii*	*Lb. delbruckii* sp. *delbruckii*	*Leuconostoc mesenteroides* sp. *mesenteroides*	*Leuconostoc mesenteroides* sp. *mesenteroides*	*Leuconostoc mesenteroides* sp. *mesenteroides*	*Leuconostoc mesenteroides* sp. *mesenteroides*	*Leuconostoc mesenteroides* sp. *mesenteroides*	*Leuconostoc mesenteroides* sp. *mesenteroides*

+: positive, w: weakly positive, - : negative after 48 h of incubation at 37°C.
All strains fermented: Glucose, Fructose, Mannose, N-acytil Glucosamine, Esculin, D-trehalose. None fermented: Glycerol, Erythritol, D-arabinose, D-xylose, Analytol, B-méthyl-D-xylose, L-sorbose, L-rhamnose, Dulcitol, Inositol, D-sorbitol, Inuline, D-melezitose, Starch, Glycogen, Xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol and L-arabitol.

D-turanose. They were tentatively identified as *L. mesenteroides* subsp. *mesenteroides*. Earlier studies has been reported the presence of the *Lactobacillus* and *Leuconostoc* genera in coconut palm sap (Atputharajah et al., 1986). Shamala and Sreekantiah (1988) isolated the following species from date palm wine in India: *Leuconostoc dextranicum*, *Micrococcus sp., Pediococcus* sp., *Bacillus* sp. and *Sarcina* sp. Later, Amoa-Awwa et al. (2007) isolated *Lactobacillus plantarum* and *L. mesenteroides* as the dominated lactic acid bacteria from palm wine in Ghana. In fact, as reported by Rivera-Espinoza and Gallardo-Navarro (2010), Mohamadou et al. (2010) strains of species belonging to *Lactobacillus* and *Leuconostoc* (*L. mesenteroides*) are the most common bacteria in natural vegetable.

In order to select a strain starter culture for lactic fermentation of palm sap, the strains were characterized on the basis of acid production ability. Figure 2 shows the acidifying activity ($\Delta pH = pH_{at}$

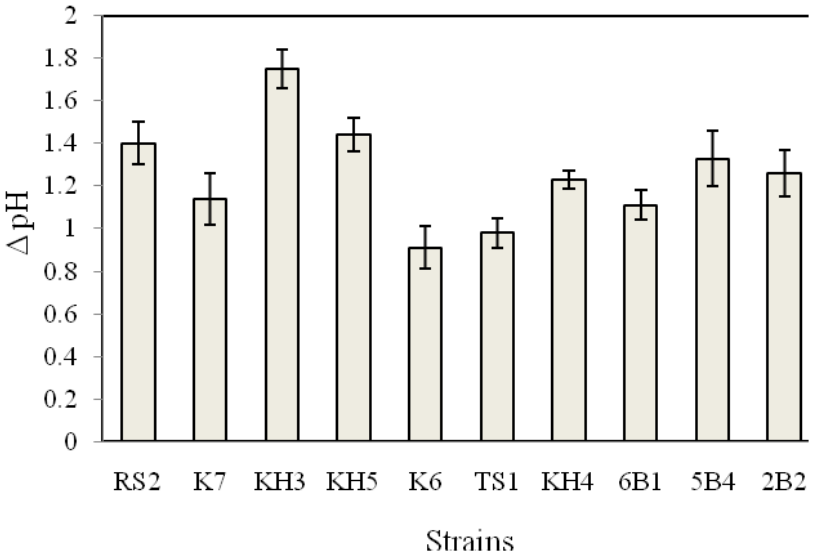

Figure 2. Change in pH (Δ pH) after 6h of fermentation of date palm sap by lactic acid bacteria.

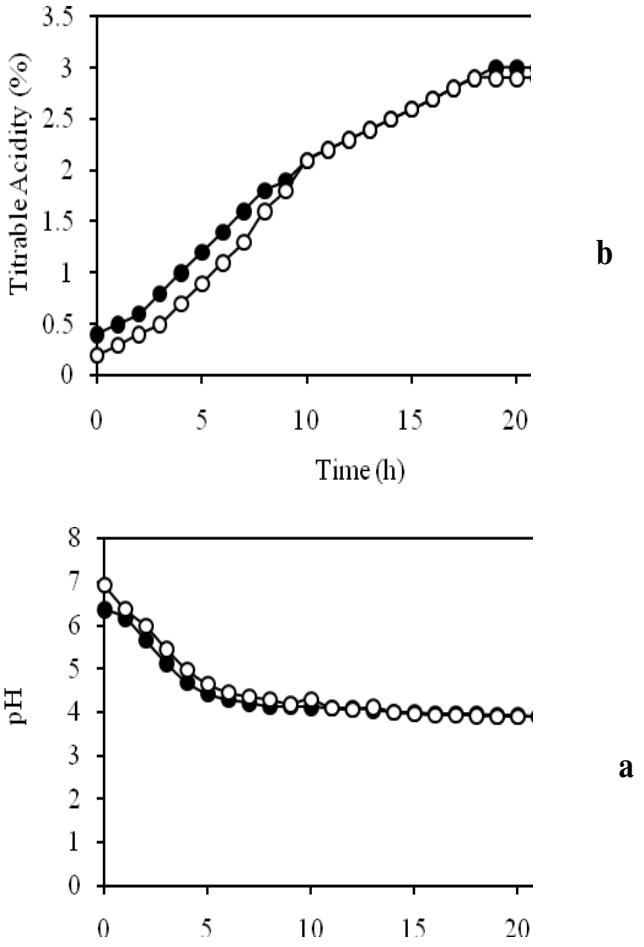

Figure 3. Evolution of pH (a) and titrable acidity (%) (b) during fermentation of date palm sap started with *Lactobacillus delbrueckii* KH_3 (●) and *Leuconostoc mesenteriodes* $5B_4$ (○).

$_{6H}$ –pH $_{zero\ time}$) of the strain after 6 h of incubation at 37°C in sterilized palm sap. The decrease in pH ranged from 0.91 to 1.75 at 6h. The isolates belonging to *L. delbrueckii* KH_3, KH_5 and $5B_4$ had the highest acid production after 6h of incubation. While *L. mesenteroides* K_6, RS_2 and TS_1 had the lowest values of decrease in pH. For reasons of food safety, a fast reduction in pH of the fermenting substrate is desirable, as it serves to inhibit the growth of especially the gram-negative, acid-sensitive food-borne pathogens or spoilage bacteria (Holzapfel, 1997). In order to compare the acidifying activity between strains, further work was conducted to follow the pH and titrable acidity during palm sap fermentation. The fast acid-producing strains of both genera were chosen: KH_3 and $5B_4$ were chosen as starter cultures.

Application of the starter for palm sap fermentation

Changes that occur in the pH and Total acidity of fermented palm sap inoculated by the strains KH_3 (*L. delbrueckii*) and $5B_4$ (*L. mesenteroides*) are shown in Figure 3. The pH of the sap decreases from 6.8 to 4.3 after 9 h of fermentation (Figure 3a). After, pH remains constant about 4.0±0.1 until the end of fermentation for both strains. This decrease is due to the rapid growth of the starter cultures. Acidification, due to the production of organics acids is an important metabolic activity. Productions of organic acids during fermentation to achieve a pH value lower than 5.0 is effective in preventing rope spoilage.

Titrable acidity increases from 0.4 and 0.2% for KH_3 and and $5B_4$ strains, respectively to around 3% at the 19th h of fermentation. Then, total acidity values remain constant until the end of fermentation (Figure 3b).

As reported by Ben Thabet et al. (2007; 2009) composition of fresh sap from date palm revealed that sugars are the major components (92–95% dry matter basis) with the dominance of sucrose. Therefore, Lactic acid bacteria (KH_3 or $5B_4$) can convert sucrose into glucose and fructose by invertase and finally to organic acids and alcohols in palm sap (Naknean et al., 2010). The occurrence of invertase in palm sap was due to its present naturally and also synthesized by micro-organisms. It is generally known that the primary sources of invertase are yeasts such as *Saccharomyces cerevisae*, *Saccharomyces carlsbergensis* and fungi such as *Aspergillus oryzae* and *Aspergillus niger* (Takano, 2005). Moreover, an increase in total acidity and decrease in pH are also responsible for the inversion reaction (Naknean et al., 2010).

We noticed that during sap fermentation the sap color has been changed to milky white. In fact, according Lasekan et al. (2007) lactic acid bacteria were responsible for the consistency and soluble white color of palm sap through their production of gum likely dextrans in the early stage of fermentation in the beverage. This product changes the consistency and the color from transparent to whitish. In addition, a heavy suspension of yeast and bacteria also gave a milky-white appearance (Lasekan et al., 2007).

Conclusion

The results indicate that the palm sap collected in southern Tunisia revealed a diversity of microflora includeing yeasts, coliforms and lactic acid bacteria. 10 species of lactic acid bacteria dominated by regular rods and lenticular in shape were tentatively identified by determining their pattern of carbohydrate fermentation in API 50 CHL galleries. *L. delbrueckii* and *Leuconostoc mesenteriodes* have been identified as the dominant lactic acid bacteria. Fermentation of palm sap with the selected autochthonous *L. delbrueckii* KH_3 and *Leuconostoc mesenteriodes* $5B_4$ favored a fast acidification. This first knowledge of the microflora associated with fresh palm sap will serve as a guide in the design of lactic beverage and to select starter for further application, the focus of ongoing research in our laboratory. This work presents an initial step, but further studies were needed for a complete characterization of the technological properties of the starter culture and the organoleptic properties of the fermented beverage.

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