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Identification of acetic acid bacteria isolated from Tunisian palm sap

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Date palm sap (P*hoenix dactylifera* L.), called *Lagmi,* is a sugary substrate that readily ferments through the activity of native microflora that consists mainly of yeasts, lactic acid bacteria and acetic acid bacteria (AAB). The aim of this work was to perform a preliminary step in the isolation and identification of AAB species from different samples of palm sap collected in Southern Tunisia, in order to use them as a starter in vinegar production. AAB were isolated on GEY agar from the fresh palm sap (day (D) 0) and following spontaneous fermentation at room temperature after 3 (D3) and 7 days (D7). A preliminary phenotypic identification of 18 isolates was made by means of Gram, catalase, oxidase and ethanol oxidation to acetic acid, and then further to CO_2 and H_2O. Subsequently, genotypic identification was conducted by sequencing the gene coding for 16SrRNA and a phylogenetic analysis was obtained based on 16S rRNA sequences. Isolates were identified as *Acetobacter tropicalis* (11 strains), *Acetobacter pasteurianus* (3 strains), *Acetobacter senegalensis* (1 strain), *Acetobacter indonesiensis* (1 strain), *Acetobacter cerevisiaie* (1 strain), and *Gluconacetobacter liquefacienes* (1 strain). The application of selected strains as pure starter cultures for the production of vinegar from palm sap is under investigation in our laboratory.

Key words: Date palm sap, *Lagmi*, acetic acid bacteria (AAB), starter, vinegar.

INTRODUCTION

The date palm, *Phoenix dactylifera* L., belonging to the *Arecaceae* family, represents an important economic and ecological resource for many countries in North Africa and in the Arabian Gulf. In Southern Tunisia, date palm has a notable role since it constitutes the main source of revenue and economic base for the people living in the Tunisian Sahara (Ben Thabet et al., 2009; Ziadi et al., 2014; Hamza et al., 2015). Date palm sap, called "*Lagmi*" or "*Legmi*", is a clear juice obtained by tapping palm trees using a traditional local method. Sap exudes from the trunk of the date palm tree during the tapping operation. *Lagmi* is a popular juice appreciated for its sweet taste

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and its typical flavour. Palm sap is a nutritional and refreshing beverage enjoyed by people in parts of Africa, Asia and South America (Ben Thabet et al., 2009; Lasekan and Abbas, 2010; Salvi and Katewa, 2012; Girhepuje and Mondal, 2015). In Tunisia, the sap is collected throughout the spring over approximately four months (March-June) with a yield of 8 to 10 L per day and per palm (Barreveld, 1993). However *Lagmi* is rapidly fermented by autochthonous microflora due to the availability of sugars (*ca* 92–95% dry matter basis) (Ben Thabet et al., 2009). The endogenous microflora is composed essentially of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAC) (Stringini et al., 2009). The sap derived from other palm trees, like *Arenga pinnata*, *Cocos nucifera* and *Phoenix sylvestris*, is widely used in Asian countries for the production of palm sugar which is used in cakes, desserts and food coating mixed with drinks (Barreveld, 1993; Apriyantono et al., 2009; Ho et al., 2007). Palms such as *Borassus flabellifer, Metroxylan sago*, *Phoenix humilis, Raphia hookeri* and *Elaeis guineensis* are also tapped to collect the sap which is converted into an alcoholic beverage obtained from natural fermentation in Sri Lanka, India, the Philippines, Indonesia and in West Africa (Benin, Ivory Coast and Nigeria) (Atputharajah et al., 1986; Naidu and Misra, 1998; Umerie, 2000). Furthermore, palm wine may undergo further alcoholic fermentation by local distillers, who add sugar to the product and allow it to ferment for a further 4 to 7 days and then distilled to produce a potent gin, known by various names in West Africa, for example *akpeteshie* in Ghana (Amoa-Awua et al., 2007).

So far, to the authors' knowledge, no studies have been undertaken in Tunisia to evaluate date palm sap as a potential starting material for vinegar production. Indeed, the high sugar content of *Lagmi* is ideal for the growth of yeast resulting in alcohol formation. The alcohol can subsequently be converted by selected starter bacteria to a vinegar with a pleasing flavour and aroma. This study focused on the preliminary isolation and identification of acetic acid bacteria to be used as potential starters for making vinegar from palm sap since using defined starter cultures can lead to an efficient and well-controlled fermenting process.

MATERIALS AND METHODS

Sample collection

Palm sap samples were collected from different oases located in four regions of Southern Tunisia (Kebili, Gabès, Medenine and Tataouine). Four different varieties of *Lagmi* were surveyed: Kinta, El Hammouri, Deglet Noor and Alligue. Sample collection was performed in the early morning. The extraction of the sap was carried out according to the practices used by local collectors (Barreveld, 1993). The samples were collected in Stomacher sterile bags and transferred in ice boxes (4°C) for analysis at the Laboratoire d'Ecologie et de Technologie Microbienne, Institut National des Sciences Appliquées et de Technologie (Tunis). A total of 12 palm sap samples of different varieties were collected in the same month, from different oases. Microorganisms were enumerated on arrival in laboratory (D0), after 3 days (D3), and after 7 days (D7) (Table 1). Counts were carried out in three replicates.

Microbiological analysis

The palm sap sample was shaken by hand in the Stomacher bag and 10 ml 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 mesophilics bacteria (AMB) were enumerated by pour plate on Plate Count Agar (PCA, Oxoid, Basingstoke, UK) incubated aerobically at 32°C for 48 h.

Lactic acid bacteria (LAB) were enumerated on MRS agar (Oxoid) supplemented with cycloheximide 0.005% (Sigma-Aldrich, St. Louis, USA), to inhibit yeast growth. Plates were incubated at 30°C for 3 days under anaerobic conditions. Yeasts (YEs) were counted on Sabouraud agar medium (Oxoid) with chloramphenicol (500 µg/ml) (Sigma- Aldrich) to suppress bacteria growth and incubated at 25°C for 3 days. Acetic acid bacteria (AAB) were isolated by spread plate on GEY agar, composed of 2% D-glucose; 0.8% yeast extract; 0.5% ethanol; 0.5% peptone; 0.3% $CaCO_3$ and 1.5% agar (Yamada et al., 1999). The medium was supplemented with 10 mg ml^{-1} of cycloheximide (Sigma–Aldrich) made up in 50% ethanol and 20 ml l^{-1} of penicillin (Sigma–Aldrich) prepared from a 0.25% stock solution to inhibit the growth of yeasts and lactic acid bacteria, respectively. GEY agar plates were incubated under aerobic conditions at 30°C for up to 1 week. Following incubation, the number of colony forming unit (cfu) was recorded, followed by morphological characterization and counts of each colony type obtained. Isolates originating from GEY agar plates were maintained on agar slants of AG medium as described by Katsura et al. (2001).

Isolation and putative identification of AAB

Representative colonies (65 strains) chosen at random were subcultured from GEY agar by streaking repeatedly on the same substrate until pure cultures were obtained. Only isolates which were able to produce clear halos around the colonies were further characterized. Selected isolates that were Gram negative, catalase positive and oxidase negative were examined by their ability to grow on differential agar including YPM, modified Carr medium, yeast extract-ethanol broth containing bromocresol purple for over-oxidation of ethanol (Carr, 1968; Du Toit and Lambrechts, 2002) and GYPG medium to examine morphological and cultural characteristics of the isolates. The YPM medium contained: g l^{-1} distilled water; 5, yeast extract (Oxoid); 3, peptone (Oxoid); 25, mannitol (Merck), and 12, agar (Oxoid). The modified Carr medium contained: gl^{-1} distilled water; 30, yeast extract (Merck) and 20, ethanol. For over-oxidation of ethanol, the broth medium contained: g l^{-1} distilled water; 30, yeast extract (Oxoid); 20, ethanol and 0.022 g of bromocresol blue with an inverted Durham tube to trap the CO_2 generated by over-oxidation of ethanol. GYPG medium was composed of 1.0% D-glucose, 1.0% glycerol, 0.5% yeast extract, 1.0% peptone and 1.5% agar. The sixty-five (65) acetic acid bacterial isolates were reduced further to 18 isolates, representatives of all sampling zones and times, based on physiological and morphological similarities.

Molecular identification: DNA extraction and PCR

DNA extraction was conducted from a 72 h preculture of each isolated strain, using the Ultraclean Microbial DNA isolation kit (MoBio, Carlsbad, Ca - USA) following the supplier's instruction.

Table 1. Microbial counts of palm sap collected from four different oasis in south of Tunisia (results are mean values from three replicates).

Oasis	D_0[a]				D_3				D_7			
	AMB[b]	LAB[c]	Yes[d]	AAB[e]	AMB	LAB	YEs	AAB	AMB	LAB	YEs	AAB
Kebeli	5.3×10^6	1.9×10^5	1.5×10^6	Nd[f]	7.9×10^8	6.3×10^7	8.6×10^7	3.4×10^4	9.6×10^8	5.1×10^5	3.8×10^5	6.3×10^6
Gabes	2.2×10^7	3.9×10^4	3.9×10^5	Nd	8.1×10^8	3.3×10^7	7.9×10^7	4.5×10^4	8.3×10^8	7.3×10^5	4.9×10^5	7.3×10^6
Medenine	8.8×10^7	5.6×10^5	6.8×10^6	Nd	5.1×10^8	6.6×10^7	8.7×10^7	6.4×10^4	9.1×10^8	5.2×10^5	1.3×10^5	6.5×10^6
Tataouine	9.7×10^7	6.9×10^5	7.7×10^6	Nd	6.3×10^8	7.5×10^7	9.2×10^6	5.3×10^3	8.9×10^8	6.4×10^5	1.5×10^5	9.1×10^6

[a]Counts at arrival of samples in laboratory (D0); after three days (D3) and after seven days (D7); [b]AMB, Aerobic mesophilic bacteria; [c]LAB, Lactic acid bacteria; [d] Yes, Yeasts; [e]AAB, Acetic acid bacteria; [f]Nd, not determined

DNA quality was checked by 1.2% agarose gel electrophoresis in TAE 1X buffer (TAE 1X: 1 mM Na_2EDTA, 40 mM Tris-acetate, pH 7.6), stained with GelRed (Biotium, Hayward, Ca - USA). PCR amplification of the 16S rDNA was carried out with primers BSF8 (5'-AGAG-TTTGATCCTGGCTCAG-3') and BSR 1541 (5'AAGGAGGTGATCCAGCCGCA-3') (Wilmotte et al., 1993). Primers were synthesized by Sigma Proligo (Hamburg, Germany). PCR reaction was conducted by using Taq PCR Core Kit (Qiagen, Milan Italy). Each reaction (final volume of 25 µl) consisted of: 1X buffer, 0.4 µmol l^{-1} dNTP, 0.6 µmol l^{-1} of each primer, 1.25 U of Taq DNA polymerase, 20-40 ng of template DNA and sterile deionised water until final volume. PCR reactions were carried out in a MyCycler thermal cycler (Bio-Rad, Hecules, Ca - USA) according to the following conditions: an initial denaturation cycle at 94°C for 4 min, then 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min and a final extension cycle at 72°C for 5 min. Positive control (DNA from *Lactobacillus plantarum*), negative control (DNA from *Saccharomyces cerevisiae*) and no template control were added in the PCR assay. PCR products were checked on 1.2% agarose gel electrophoresis in TAE buffer (1X) stained with GelRed (Biotium). The molecular weight of the PCR products was estimated by comparing a molecular weight marker (1 KB DNA ladder, Promega, Milan - Italy). The PCR reaction product was purified using the Qia-quick PCR Purification Kit (QIAGEN) and quantified on 1.2% agarose gel.

Molecular identification: 16S rDNA sequencing and phylogenetic analysis

The purified PCR products were sequenced by using ABI PRISM Big Dye Terminator Kit (Applied Biosystems, Foster City, Ca - USA). Sequences were assembled and analyzed aided by Bioedit software (Hall, 1999). Assembled sequences were compared with the entries of the Ribosomal Database Project (RDP Release 10, Update 29; http:// rdp.cme.msu.edu/) and with the sequences available in the Genbank database using the Basic Local Alignment Search Tool version 2.2.27 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analyses were conducted using MEGA version 6.0 (Tamura et al., 2007). Sequences were deposited into the Genbank nucleotide sequence database under accession numbers from KX424628 to -645.

RESULTS AND DISCUSSION

Microbial counts in palm sap

Table 1 shows the variation in the population of microorganisms found in samples of sap collected from different palm oasis in Southern Tunisia. Aerobic mesophilic bacteria varied from 5.3×10^6 to 9.7×10^7 cfu ml^{-1} at Day 0 (when the samples were examined immediately after their arrival at laboratory), from 5.1×10^8 to 8.1×10^8 cfu ml^{-1} at Day 3, and they remained almost at the same concentration (10^8) at Day 7. The microbial counts assessed on MRS medium (LAB) showed a drastic increase from 3.9×10^4 (in sample from Gabès oasis at D0) to 7.5×10^7 cfu ml^{-1} (in sample from Tataouine at D3). After this increase in numbers, the LAB population dipped slightly at D7. Yeasts ranged from 3.9×10^5 (Gabès, D0) to 4.9×10^5 (Gabès, D7). All these counts, even if slightly lower, are in agreement with those reported by Ziadi et al. (2011). Apart from few reports that show physicochemical characteristics of Tunisian palm sap (Ben Thabet, 2007, 2009) and the microbiological analysis carried out by Ziadi et al. (2011), there are no other information in literature on the microflora of Tunisian date palm sap "*Lagmi*".

Counts and phenotypic identification of acetic acid bacteria

All AAB were absent on Day0, but were isolated in all samples on the third day (D3) showing a concentration between 5.3×10^3 (Tataouine oasis) and 6.4×10^4 cfu ml^{-1} (Medenine oasis) (Table 1). The bacteria increased in number of about 2 log orders of magnitude in all oases on D7. Overall, the largest group of microorganisms (not considering aerobic mesophilic bacteria) at D0 and D3 was represented by lactic acid bacteria and yeasts, whereas at D7 acetic acid bacteria became the dominant group. A similar trend was observed by Pereira et al. (2012) in a different

Table 2. Alignments of *16S rDNA* sequences grouped by closest sequence matches via the EMBL database.

Strain	Type strain	Closest relative Acession number of 16SrRNA ref seq.	Similarity (%)
1 Mc	*A. tropicalis*	NR_036881.1	99
1 RSb			99
2 RSb			99
1 HSb			99
2 HSb			99
1DG.b			100
1 DSb			99
1 RCb			99
3 KH.b			99
1 KH.b			99
2 KH.b			99
1 SRc	*A. pasteurianus*	NR_102925.1	100
1 RMc			99
1 HSc			99
1 RCc	*A. senegalensis*	NR_043252.1	100
2 HSc	*A. cerevisiae*	NR025512.1	99
1HTb	*A. indonesiensis*	NR_113847.1	99
3 HSb	*G. liquefaciens*	NR026132.1	99

eco-system (cocoa fermentation) where the simultaneous growth of the yeasts and LAB, was gradually replaced by the AAB. AAB have been ascribed to the phenomenon of viable but non-culturable state (VBNC) (Millet and Lonvaud-Funel, 2000) where the population of AAB is very often underestimated by the inability to culture the population on growth media. This phenomenon, along with the fact that AAB are difficult to isolate and cultivate on artificial medium, despite the great number of growth media proposed (Bartowsky and Henschke, 2008), could explain their absence on D 0 in the present work.

Acetic acid bacteria isolated (*n*=18) from palm sap were all Gram-negative, rod-shaped, catalase-positive, oxidase negative and were able to dissolve the calcium carbonate on the GEY agar plates, due to acid production during growth.

However, isolates which were considered to be *Acetobacter* species redeposited the calcium carbonate after a few days. The colonies of the isolates were circular, raised or convex with entire edges, and white to cream on GYPG agar plates. Growth of these isolates on modified Carr medium with bromocresol blue and Durham tube resulted in a change in colour from purple to yellow, and on further incubation reverted to purple. They also produced gas in the Durham tubes. These results showed that the isolates were able to oxidize ethanol to acetic acid and then further to CO_2 and H_2O showing that they probably belong to *Acetobacter* genus (Du Toit and Lambrechts, 2002; Sengun and Karabiyikli, 2011), *Acetobacter* genus is currently located in the *Acetobacteraceae* family of the *α-proteobacteria* class (Komagata et al., 2014).

Genotypic identification and phylogenetic analysis

Comparison of 16S rRNA gene sequence of the strains with the total nucleotide collection in the EMBL-EBI nucleotide database was used to assign the bacterial name with ≥99% similarity. The AAB species identified in palm sap samples were mainly distributed within two genera, namely *Acetobacter* and *Gluconoacetobacter* (Table 2).

In particular, sequences of 11 strains (61%) were matched with the highest omology (>98%) with *Acetobacter tropicalis*. Three strains (17%) closely matched (>99%) with *Acetobacter pasteurianus* sequences. One strain (5% each) matched with *Acetobacter indonesiensis* (1HTb), *Acetobacter senegalensis* (1RCc), *Acetobacter cerevisiaie* (2HSc), and *Gluconacetobacter* (*Ga*) *liquefaciens* (3HSb). The two predominant groups were thus identified as *A. tropicalis* and *A. pasteurianus*. The phylogenetic tree as illustrated in Figure 1 was constructed from evolutionary distance by using the neighbour joining method.

Based on this analysis, it was observed that phylogenetic tree reflects the results obtained in Table 2. Particularly, 13 of isolated strains (72%) from different oasis and at different fermentation time, belong to the *A. aceti* cluster and 3 (17%) *A. pasteurianus* cluster. The relative low diversity of species isolated could be due to the selective effect of fermentation process, that allowed recovering only the strains that actively take part on the fermentations of palm sap. Thus, isolated strains, selected by the process itself can be all considered a promising representative of AAB that could be used as

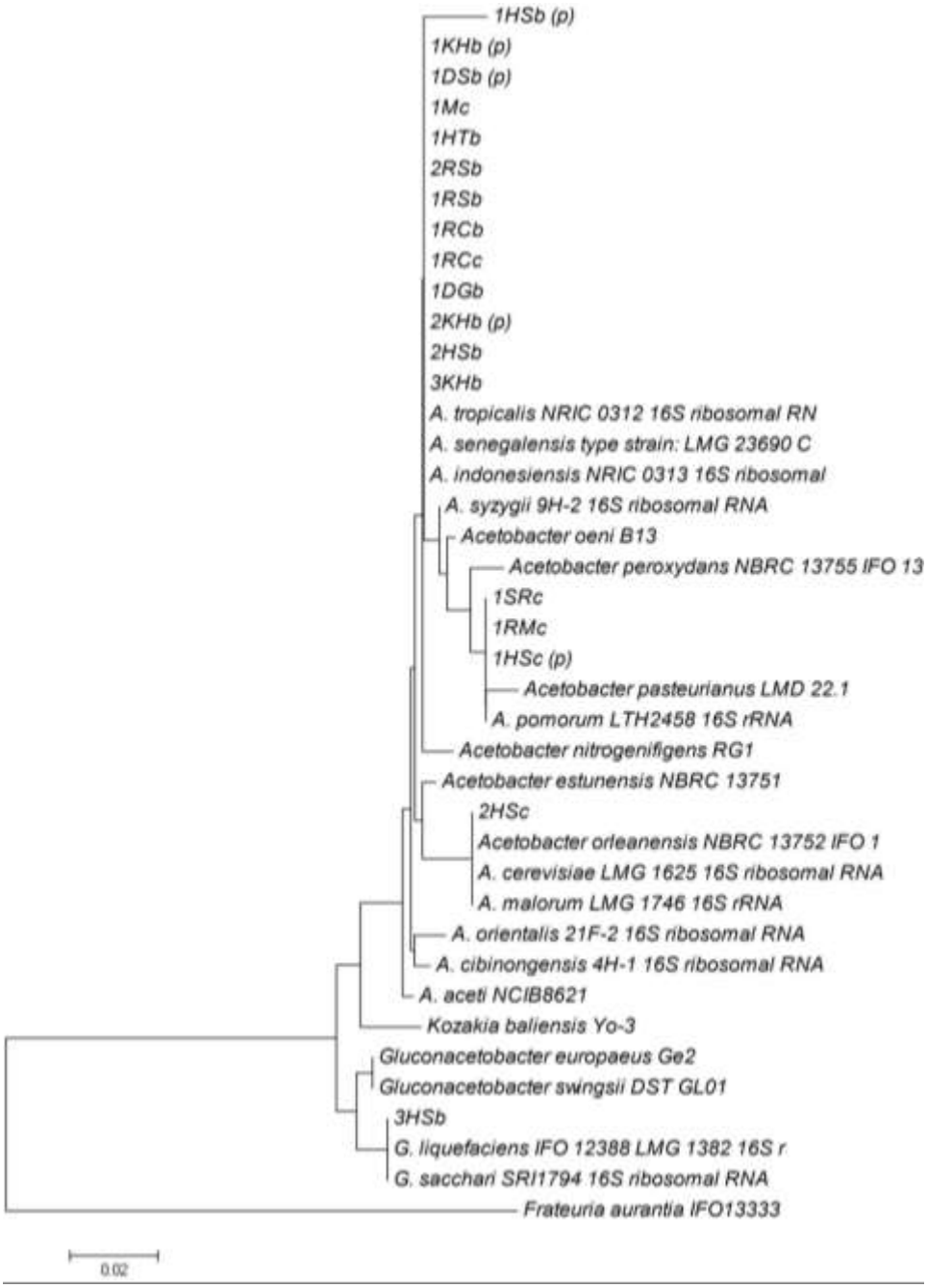

Figure 1. Phylogenetic tree based on 16S rDNA gene sequences for Tunisian isolates assigned to the genus *Acetobacter.* The phylogenetic tree was constructed by the neighbor-joining method. The type strain of *Frateuria aurantia* IFO13333 was used as an outgroup.

fermentation starters. Of note is the fact that the 2HS strain is in the same subcluster that regroup *A. orleanensis* and *A. cerevisiaie*. These two later species are very closely related (Cleenwerck and De Vos, 2008). Lisdiyanti et al. (2006) studying the diversity of AAB in Indonesia, Thailand and the Philippines**,** found *A. pasteurianus* only in fermented foods such as palm vinegar, palm wine, rice wine, pickles, etc., while *A. tropicalis* was isolated from fermented foods (palm wine and rice wine), fruits (lime, orange, guava, coconut), and coconut juice. In addition, Ndoye et al. (2007) isolated two strains of *A. tropicalis*, and *A.pasteurianus* with ability to grow at temperature around 40°C. These thermotolerant strains were proposed for the industrial production of vinegar in hot and tropical countries, as they considerably reduce the costs associated with the

use of cooling water. Nielsen et al. (2007) also showed that *A. pasteurianus* and *A. tropicalis*, were part of predominant acetic acid bacteria during the fermentation of cocoa from Ghana. Du Toit and Lambrechts (2002) showed that *A. pasteurianus* was the dominant species in the middle (Day 6), and at the end of fermentation of wine (day eleven), due to its high tolerance to ethanol. Similarly, in the present work, it was observed that all strains identified as *A. pasteurianus*, were isolated after 1 week of spontaneous fermentation. Strains identified as *A. tropicalis*, except of 1Mc strain (Tale 2) were isolated also after one week (D 7) of spontaneous fermentation. *A. senegalensis* and *A. indonensiensis* were isolated by Jia Wu et al. (2012) among the non-*A. pasteurianus* strains in the acetic acid fermentation of traditional Shanxi Chinese vinegar. Hidalgo et al. (2013) used an *A. cerevisiae* strain to produce vinegar from blueberry as one method to preserve this seasonal fruit and allow extended consumption. Lastly, *G. liquefaciens,* as in this work, has been isolated from palm, sugar cane and coconut juice by Seearunruangchai et al. (2004). Some species of genus *Gluconacetobacter* have been described mainly at the end of acetification in wine the vinegar process, when acetic acid concentration is high (Seearunruangchai et al., 2004; Vegas et al., 2010) It has been proposed that *A. pasteurianus* pioneers the production of vinegar, and is followed by *Gluconacetobacter* spp. which may be more resistant to acetic acid and less resistant to ethanol (Gullo et al., 2009; Vegas et al, 2010).

Overall, in the present study, AAB isolates belong to *Acetobacter* and *Gluconacetobacter*, 2 separated cluster. *Acetobacter* spp. and *Gluconacetobacter s*pp. have been isolated from various sources around the world and they are the primary species used in vinegar fermentation due to their strong ability to oxidize ethanol and to tolerate high acetic acid concentrations (Gullo et al., 2014). They produce acetic acid from ethanol by two sequential oxidation reactions involving alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Adachi et al., 2003; Perumpuli et al., 2014). In our laboratory, further studies are in progress for a complete characterization of the technological potential of the isolated strains (measurement of ethanol and lactic acid oxidizing power, amount of acetic acid produced, and tolerance to acid, heat, acetic acid, and ethanol) to establish their usefulness in industrial application.

Conclusions

To the authors' knowledge, no studies have focused on the use of acetic acid bacteria as starters for making vinegar from fresh palm sap. The results indicate that *Lagmi* collected in Southern Tunisia revealed the presence of AAB from the third day onwards. *Acetobacter* strains were isolated and identified based on the phenotypic characteristics (at genus level) and molecular aspects (at species level). Strains were identified belonging 11 type strain *A. tropicalis*, 3 type strain *A. pasteurianus* , and 1 type strain each *A. senegalensis, A. cerevisiae, A. indonesiensis* and *Gluconacetobacter liquefaciens.* This data suggest that 16S rRNA gene sequence allows differentiation of species or group/species and represents a tool for a rapid and cost effective preliminary profiling of AAB genera. The molecular technique can also be useful to highlight the phylogenetically closely related species. This first knowledge of the acetic acid bacteria will serve as a guide in selecting starter for the production of vinegar from fresh palm sap.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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