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Identification of bacterial strains isolated from the traditional date product "Btana" produced in south regions of Algeria.

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Abstract – Eleven samples of the traditional date product " Btana" prepared by direct (DBM) and undirect method (UBM) were analysed to characterize their bacterial diversity. The total aerobic count was ranged from 1,6 . 10^2 to 3,02 . 10^6 UFC/g. Two hundred isolates (200) were randomly selected, then 37 representative strains have been chosen for molecular identification. 16S rRNA gene sequencing revealed the presence of 17 species within 30.9% belonged to the main *Bacillus species; B. pumilus, B. endophytucis* and *B* amyloliquefaciens. The other identified species included *Staphylococcus (S. epidermidis S. pasteuri S capitis), Enterococcus* (*E. faecalis E. mundtii*, *E. faecium.* Within the minority represented species, we recorded the presence of *Paenibacillus (P.* xylanexedens; P. macerans), *Streptococcus (S. salivarius; S. lactis)*, *Lactobacillus sakei* and *Klebsiella pneumonia*e. Our results indicated that UBM is more selective for spore former bacilli contrary to DBM that shows more diversity in the bacterial flora with *Enterococcus* being the prevailing species. Enzymatic profil revealed a weak hydrolytic activity for the dominant *Bacillus* which exhibit instead a high lypolitic activity and other enzymes including proteases.

Key words: Btana, Date fruit, traditional food, bacterial diversity, food safety.

1. Introduction

Btana is a traditional date product obtained by processing dried date fruits. It is widely consumed in the rural regions of maghribian countries forming a significant part of their diets (Merzaia Blama et al, 2016). In Algeria, the product is largely consumed in the south region (Ziban, Oued Righ, Adrar and Beni Abbas) either by direct consumption or as a part of sweet dishes and cakes. Economically, Btana is considered as a form of valorisation of low quality cultivars that cannot reach the market standards as the noble cultivar "Deglet Nour". Traditionally, two methods are applied for Btana preparation (Abekhti et al., 2015). The direct Btana method is practiced in eastern south of Algeria (Ziban, Ghardaia, Oued righ and Oued souf). Soft date's varieties (especially Ghars cultivar) are directly amassed into bags by manual pressure, and then stored in dry conditions for one month to enhance past consistence. By contrast, undirect Btana method (UBM) is in use in western Algerian south, where hard varieties are often cultivated. The method involves, washing dates with fresh water to eliminate impurities then with boiled water to soften date fleshes. Thereafter, dates are left for drying for one night before they are amalgamated and mixed thoroughly. The past is then piled up in proper plastic bag or in linen bag or very originally into an old goat skin (Abekhti et al, 2013).

In normal conditions, Btana could be stored for almost two years without any noticeable alteration. In this sense, it is regarded as a natural preservation method that reduces postharvest loss linked to microbial and insect infestation. However, the safety of the product still needs to be confirmed by assessing the presence and the nature of bacterial contaminants that might originate from the processing practice. Therefore, the aim of this study is to assess the bacterial community associated with Btana and to reveal their diversity in the two types of Btana, using a combination of phenotypic and genotypic methods.

Materiel and methods

A total of 11 samples belonging to the date traditional product "Btana" were collected randomly from housekeeping and selected retailers over four regions in the south of Algeria (Sali, Elmeghier, Orlal, Tolga) and transported immediately in a cooler containing ice to the laboratory for analysis. Twenty five (25) g of each sample was suspended in 225 ml of buffered peptone water (BPW) and subsequently homogenized with a stomacher for 3 min (Cardamone et al., 2015). After serial dilutions, 1.0 ml of the appropriate dilution was poured on the appropriate medium (Kpikpi et al., 2014) . Plate Count Agar (Oxoid, Basingstoke, UK) was used for enumeration of total aerobic bacteria, after 72h of aerobic incubation at 30°C. MRS (Oxoid) and BHI agar (Merck) were instead incubated in anaerobiosis at 30°C using anaerobic jars with GENbox anaer (bioMérieux, Marcy-l'Etoile, France) for 48 h to enumerate LAB and total anaerobic bacteria respectively (Bao et al., 2012). Isolates of similar microscopic morphology were purified and grouped, then one representative isolate was selected for further examination (Tauler et al., 2016). Gram and catalase positive isolates were subsequently examined for spore-forming ability by heating BHI broth for 10 min at 80 °C and subsequent incubation at 30°C (Postollec et al., 2012). Other tests including; motility, nitrate utilisation, acetoine production (VP), citrate utilisation, were performed according to Guiraud (1998). All physiological tests were

carried out in BHI broth. In order to determine the pH range for growth, strains were grown at 30°C in pH: 4.5 and 9.6. For determining the range of growth temperature; strains were incubated at 8°C, 44°C and 55°C (Matsumura et al., 1974). Halotolerance and osmotolerance were tested in BHI broth supplemented with NaCl and sucrose at a final concentrations of 6.5% , 10% (w/v) and 10, 15 and 20 % (w/v) respectively. The ability of using various sugars was investigated in a classic gallery composed of 1% of the carbohydrate and red phenol base (Difco). Other Biochemical characteristics were carried out using the API 20 NE commercial system following the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France).

By consideration of all of the phenotypic traits, a representative strain was selected from each cluster and subsequently submitted to 16 RNA gene sequencing. For that, representative strains were grown overnight in brain heart infusion BHI and stored with 25% of glycerol at -20 C until being subjected to DNA extraction. DNA was extracted from fresh colonies using DNeasy Tissue Kit (Qiagen) according to the manufacturer recommendations. The purified DNA was eluted in a final volume of 50 µL of sterile RNA free water. The yield (ng) and purity of extracted genomic DNA (absorbance ratio at 260/280) were determined by Nano Drop® ND-1000 (Labtech International, UK) according to Van Tongeren (2011). The integrity of the extracted DNA was further checked by PCR amplification of 16S rRNA gene. Reactions were carried out using 20 µl volumes each containing: 2 µl extracted DNA, 2 µl of dNTPs (2mM), 1.6 µl of $MgCl_2$ (25 mM), 2 µl PCR buffer (10×), 0.8 µl of each primer (10 µM) 16S-1500 F: 5'-GAG- TTT-GAT-CMT-GGC-TCA-G-3' (Eurogentec, 3968107, Tm=60°C) 16S-1500 REV: 5'-TAC-GGT-TAC-CTT-GTT-ACG-AC-3' (Eurogentec, 3968108 Tm=58 °C.) and 0.2 µl of Taq DNA polymerase (2.5U, Fastastart, Roch, Germany). The PCR program was performed according to Abekhti et al (2017) with a Mastercycler gradient (Eppendorf, Westbury, NY) under the following conditions: Initial denaturation at 94 ºC for 5 min, 35 cycles of 94 ºC for 30 s, 56 ºC for 30 s and 72 ºC for 1 min with a final extension at 72 ºC for 5 min. PCR products were stained with Gel greenNucleic Acid Stain (Biotium, USA) and run in 0.8% agarose gel then visualized by UV transillumination system (Eastman Kodac Company, Scientific Imaging Systems, Rochester). For initial discrimination, an aliquot of DNA extract of representatives isolates was used as a template for Random Amplified Polymorphic DNA (RAPD) fingerprinting using the universal primer (GTG)5 : 5′-GTGGTGGTGGTGGTG-3′ according to Švec et al., (2010) and Švec et al. (2005). DNA amplicons of the distinct isolates were purified then transferred to GIGA centre (Liège, Belgium) for sequencing.

Sequence analysis and phylogenetic tree construction

The sequences obtained with forward and reverse primers from each strain were edited and merged into a single sequence covering about the entire 16S rRNA gene, then assembled using the CodonCode Aligner program (version 3.0.2) and compared to those present in the GenBank public database using the online BLAST program (La Scalia et al., 2016). Sequence data were aligned with the ClustalX package and a phylogenetic tree was drawn and visualized with the TreeView program (Parsaeimehr et al., 2019). The sequences of the 37 isolates are deposited in

the Genbank database under the accession numbers from MW718152 to MW718187.

Determination of enzymatic activity

In order to check the starch hydrolysis ability, isolates were spread in bold lines on inorganic salts starch agar then incubated during overnight. After visible growth, the agar surface was flooded with lugol iodine to monitor the eventual formation of visible translucent halo around the growth lines resulting from starch hydrolysis (Vera *et al.,* 2012). Casein hydrolysis was determined on streak-inoculated agar containing 6 ml of liquefied agar and 2 ml sterile skimmed milk. Haemolysis activity was examined by growing the isolates on 5 % horse blood using Columbia Blood Agar base (Oxoid), at 37 °C for 24 h. Further enzymatic activities were assessed using the semiquantitative APIZYM (bioMérieux) system (Wawron *et al.,* 2011). For that reason, 65 µl culture suspension corresponding to 5 McFarland standard were inoculated in each of the 20 reaction well in the APIZYM strip and incubated for 4 h at 37°C. Enzymatic activities were recorded after addition of the APIZYM reagents A and B according to the manufacturer's instructions. The intensity of the colour was measured on a scale from 0 to 5 by comparing the colour developed within 5 min to the API-ZYM colour reaction chart.

Results

Bacteria associated with Btana samples:

The results of bacterial enumeration are summarized in Table 1 and are represented as colony forming units per gram (CFU/g mean ± SD). According to the results, aerobic count is ranged from 1,6 . 10^2 to 3,02 . 10^6 UFC/g while the highest number was recorded for Orlal samples (10^6 ±1.6.10^5 ufc/g), followed by those from Sali (3,4 .10^5 ± 6.10^4). However, low loads were observed in Tolga (1,5. 10^3±10^3) and Elmeghier samples (1,6 x 10^2 ufc/g). According to the variance F test, no significant difference was stated in the microbial load with the two Btana methods (DBM, UBM). (P>0.05) which means that load variation is independent of origin and preparation method.

The anaerobic count ranges between 0 and 3.10^5 cfu/g whereas a few LAB were detected on MRS medium. In order to identify the major bacterial flora present in Btana, two hundred isolates (200) were randomly selected from the observed colonies, subcultured, purified and checked microscopically. The isolates with similar characteristics were grouped together in four groups (Table 2): Group 1: rod shaped, Gram and catalase positive, mobile and forming endospores isolates; were presumptively identified as *Bacillus* strains. Group 2: Gram and catalase-positive cocci isolates were presumptively identified as *Staphylococci* (Table 3). Group 3: Cocci shaped, Gram-positive, catalase negative isolates were considered as presumptive *Enterococcus* (Table 3). The fourth group included minor forms representing Gram positive, catalase negative bacteria (presumptive LAB) and Gram negative bacteria. So far the homogeneity of the species belonging to Group 2 and 3 was verified with RAPD technique mentioned above. In total, 37 representative isolates were picked up from clusters and subjected to molecular identification by 16S rRNA gene sequencing.

Within the representative isolates, 17 species have been identified according to GENBANK database (Fig). 13 of the selected isolates are belonging to *Bacillus* genus : *B. pumilis B. endophytucis B. cereus*, *B. nealsonii* and *B. amyloliquefaciens.* Other species are belonging to *Staphylococcus (S.*

epidermidis, S. pasteuri, S. capitis); *Enterococcus (E. mundtii, E. faecalis, E. faecium, E. lactis)* and five (05) other minor species namely: *Paenibacillus macerans*, *P. xylanexedens*, *Streptococci salivarius*, *Lactobacillus sakei* and *Klebsiella pneumonia*. Samples from Orlal (DBM) showed the highest number of species (11 species), followed by UBM sample from Sali (7 species) and Tolga (6 species). Whereas DBM samples from Elmeghier were dominated by one species (*E mundtii*). *B. pumilus* was the most prevalent representative rod species. It was detected in DBM and UBM in Sali, Orlal and Tolga samples. Remarquably, *B. endophyticus* strains were exclusively found in Ziban samples (DBM: Orlal and Tolga). Whereas *B nelsonii* was identified in some of Orlal samples. Some confusion rises from the identification of *B. cereus (*HP1 and LB1). The ambiguity stemmed from the genetic relationships between *B. cereus* and *B. thuringiensis* which are belonging to the *B cereus* phylogenic group composed of *B. cereus, B. mycoides, B. thuringiensis* and *B. anthracis (*Zheng et al., 2017; Peruca Ana Paula et al., 2008). Biochemical and phenotypic features helped to orient the identification in the absence of other molecular identifiers like housekeeping genes (Ko et al., 2014). The distinction was finally made by the absence of parasporal bodies which characterises *B. cereus* according to Postollec *et al.,* (2010); Thorsen *et al.,* (2011) and Ahaotu *et al.,* (2013). Beside, the isolates HP1 and LB1 present both further key features of *B. cereus* including haemolysis, lecithin hydrolysis, and the inability to ferment mannitol (Thorsen *et al.,* 2011). On the other hand, the cluster assigned to *B. amyliquefaciens* displays the phenotypic traits of the species including potent amylase and urease activities comparing to the other isolates (Singh et al., 2017). Regarding the cocci group, three species; *Staphylococcus*, *Enterococcus* and *Streptococcus* were identified. *Staphylococcus* is the prevalent cocci genus in both DBM (Orlal) and UBM (Sali) samples accounting for four (04) species (*S. epidermidis , S. pasteuri, S. capitis, S. hominis*),whereas the predominant species was *S. epidermidis* (6 isolates). Obviously, it is stated that the Orlal samples (DBM) exhibited the greater diversity in species, and have representative of almost all isolated cocci species with an exception for *S. pasteuri* found exclusively in UBM Sali samples. The identity of *S. pasteuri* was confirmed by the yellow pigment and the high urease activity. These two features are decisive to separate *S. pasteuri* from *S. warneri* which are both very close genetically (Bergeron et al., 2011; Bannerman e al., 2006; Behme et al., 1996). On the other hand, RAPD patterns (Fig. 2, Fig3) are similar for *Staphylococcus* strains sharing the same origin. Interestingly, the pattern observed for *S. epidermidis* strains (AP3, ER5, GP15) originating from DBM Orlal samples was distinct from the RAPD pattern of the strain *(AP17)* isolated from UBM Sali. However, an exception was stated for *Staphyococcus pasteuri* strains within which the strain"DR2" displays a distinct RAPD pattern.

Regarding *Enterococcus* strains, they were represented by four species namely *E mundtii*, *E faecium, E, faecalis* and *E lactis*. The species are more prone in DBM samples comparing to UBM in which *E faecium* was the only identified *Enterococcus*. *E. mundtii* strains were mainly encountered in Orlal DBM samples. Whereas, all the identified *Enterococcus faecalis* were isolated from (Lemeghiar and Tolga samples.. Regarding RAPD fingerprinting, *E mundtii* isolates and the oher enterococcus have a similar RAPD patterns

within the same species with a little variation (JM1 isolate belonging to *E. mundtii* (91%) with a very low homogeneity score).

The enzymatic activity checked by API-ZYM strip revealed great differences at the species and the strain's level. In general, a high activity in esterase, lipase, and naphtol-AS-BI- phosphohydrolase was recorded for almost all rod species. So far, *Klebsiella pneuminae* (HP3) presented the strongest enzymatic profile shown by production of 12 enzymes. Their high rate production was recorded for esterase, naphtol-AS-BI-phosphohydrolase, α-galactosidase, and N-acetyl-glucosaminidase. On the other hand an important glycosyl hydrolase activity was recorded for *Paenibacillus macerans* (FP1) and *B. pumilis* (EP1) that showed in addition a strong N-acetyl--glucosaminidase acivity. By far, α-galactosidase activity were shared by the majority of *Bacillus* strains excepting *B. endophyticus* and *B safensis* that instead had β-glucuronidase and β-galactosidase activities respectively. By far *Paenibacillus* spp showed an exclusive β-glucosidase activity and mowever, none of the *bacillus* isolates had α-glucosidase, alkaline phosphatase or chymotrypisn activities excepting, *B cereus* strains that produced most of enzymes, including trypisn enzyme.

Discussion

Bacterial analysis showed a dominance of the *Bacillus* species. It is the case for a large range of traditional fermented products *(*Chettri ***et al., 2015).*** Some authors explain the recurrent presence of *Bacillus* species in plant origin food by the ubiquitous nature of their long-life spores that sustain a various growth conditions (Reglinski and Walters, 2009). However, usually *Bacillus* dominance is only stated in rich protein traditional food that undergoes an alkaline fermentation and proteins hydrolysis resulting in ammonia and a high pH (Agbobatinkpo et al., 2013). Thus, and as far as Btana is a protein poor product with a low pH, the growth of *Bacillus* species would not be possible in such conditions and their presence is due mainly to environmental contamination by the spread of their spore. In addition, some concerns might be raised of the spontaneous aspect of the fermentation of Btana like the nature of the microbiological risk following the detection of *Bacillus cereus* (Ahaotu et al., 2013; Thorsen et al., 2011). By far, the risk is moderate as well as our previous study (Abekhti et al., 2014) have revealed that Btana fermentation is due mainly to yeasts activity. Our finding is supported by the insignificant growth of the isolated bacillus in date extract medium. In other hand, the presence of bacillus species in Btana is due mainly to the low microbial quality of the date fruits used in Btana preparation which were usually contaminated by bacillus telluric microflora (Bellaouchi et al., 2017). However it is difficult to imagine that the growth of *B. cereus* detected in some Btana samples (Tolga and Orlal samples) is possible in such product owing to its poor protein content which is necessary to the development of *Bacillus* (Odunfa, 1985). Further some authors argued that a bacterial load of 10^3–10^5 CFU/g is necessary to get the infectious dose and the health concern that would be caused by this bacterium according to European recommendations (EFSA, 2005; Thorsen *et al.,* 2011). On the other hand, the many *Staphylococci* species detected, are mostly common inhabitants of human skin and mucous membranes of individuals manipulating food and animals, and are able to contaminate raw products and processed foods if these are not handled properly (Cunha *et al.,* 2006). By far, it has been

reported that *S. hominis* and *S pasteuri* (which belongs to the *S. epidermidis* group) are not frequently associated with food staff but rather prevailing in human samples (Irlinger, 2008). Kloos and Schleifer (1975b) found that *Staphylococcus hominis* is prevalent on human skin and it is usually found in second or equal number with *S. epidermidis*. Regarding *S. capitis;* Euzéby (2003) reported that it is commonly associated with the skin of humans and the hides of warm-blooded animals and recently it was found abundantly in the head where sebaceous glands are plentiful (Nizet and Bradley, 2011). However, recently *S. hominis* has been isolated from fish fermented product (Majumdar and Gupta, 2020). This suggests that it is more prevailing in water foodstuff, and their presence in UBM might be originated from water used during Btana preparation. Previous study demonstrated the permanent presence of *Staphylococcus spp* in high osmophilic food like sugar thick juice (Justé *et al.,* 2008). It was also reported that *Staphylococcus* could grow at a_w as low as 0.83 because of the presence of solutes in the medium that contribute to sucrose tolerance of *Staphyococcus* species (Samelis *et al.,* 1998). Interestingly, dates have a high proline content which could enhance osmotolerance of *Staphylococcus* strains and contribute to their intracellular homeostasis (Robert et al., 2000). Seemingly, *Enterococcus* spp are commonly involved in food compromising issues mainly in vegetables, and plant material. Detection of *Enterococcus* in Btana indicates often an ancient fecal contamination for they can persist for long period in the environment (Suhogusoff et al., 2013). However, besides their animal and humans gut origin, the bacteria is ubiquitous that can be even found on plants and in soil. Some authors suggest that the identity of species might help in discerning the origin, of *Enterococcus*. *So wherase E. faecalis* and *E. faecium* presence is more linked to human fecal contamination, other species like *Enterococcus mundtii* is an indicator of environment contamination (Boehm et al., 2014). Some Btana were peeled in low hygienic quality container, and some are produced from dates fruits obtained in their original packages which usually facilitates contamination. The other species detected include *Paenibacillus macerans*, *P. Xylanexedens* which are frequently isolated from soil, rhizosphere, water but they were reported as safe whereas, *Streptococci salivarius* and *Lactobacillus sakei* are common human commensals originating from vegetables and *fruits as well* (Martinović et al., 2020). Likewise, *K. pneumoniae* is a common contaminant of fresh fruits and vegetables and is often involved in several human infections, and considered as a potential public health threat. The species had been previously identified in our prior study, and recently, was reported in the contamination of Omani dates, (Elkharoussi et al., 2016).

Enzymatic profile was monitored to assess the eventual functionality of the isolates in Btana production and the development of its characteristic features. This study was excluded to the rod species only as far as they are more prevailing in the samples and because of their established activity in many traditional fermented foods. Overall a weak hydrolytic activity was assessed for the dominant *Bacillus* which exhibit instead more lypolitic activity and other enzymes including proteases and to a less extent osidases. Even more it is not possible to verify if the identified bacillus were in dormant state under sporal form before cultivation. The harsh condition

of Btana including the osmotic stress might hamper the vegetative development of the bacillus strains. Otherwise, the only glucosyl hydrolase activities frequently encountered were α-galactosidase and β-glucosidase activity. Nevertheless these activities are poorly expressed by some species and are controlled by the presence of specific substrates like a-galactosidase enzyme that is linked to lactose availability. This suggests that most of the identified species are contaminant with no real role during preparation or storage period.

Conclusion

Identification of isolates in this study demonstrated that spore former bacteria (bacilli) are the main bacterial flora prevailed in the traditional date product Btana. Data analysis showed the presence of distinct species in both types of Btana but a high count was found in UBM samples. The occurrence of *Bacillus spp* and also the subsequent domination observed in some samples is likely due to the ubiquitous presence of airborne bacilli spores. The high bacterial count indicates that the hygienic conditions of Btana are not satisfactory and should be improved Further the selective pressure induced by the high sugar content of Btana product reflects the natural selection of the bacilli spore former bacteria which would presumably achieved during Btana preservation. By far, preliminary results indicate UBM are more selective of spore former bacilli contrary to DBM that show more diversity in the bacterial flora with a prevalence of *Enterococcus* and *Staphylococcus* species. Nevertheless, additional data are required for the other microbial flora (yeast and fungi) that could carry a functional role during data preservation.

11. References

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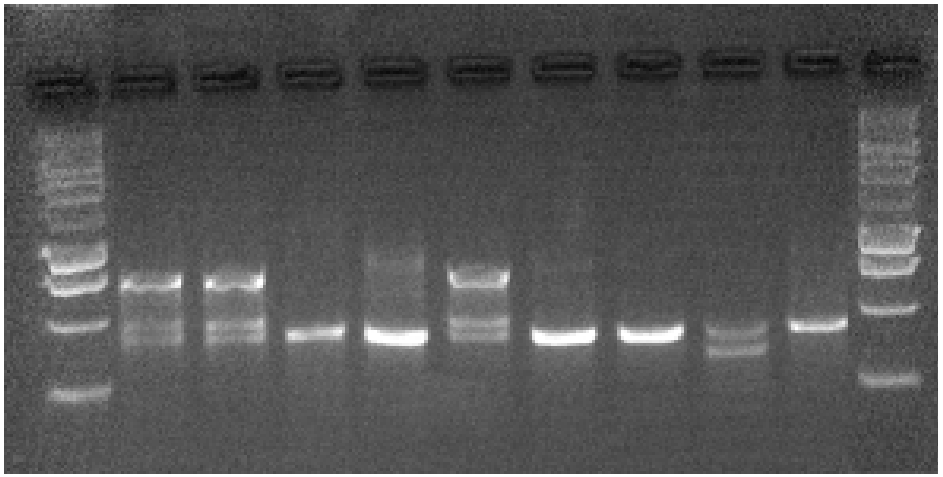

Fig.1. RAPD fingerprint amplification product of *Staphylococcus* isolates using the universal primer (GTG)5 : 5′-GTGGTGGTGGTGGTG-3′primer 5 ′ - AAGCCTCGTC-3 ′ . ML: molecular ladder; Ap3(*S. epidermidis*) ; Er5(*S. epidermidis*) ; Gp15(*S. epidermidis*),Fp5(*S capitis*); Ap17 (*S. epidermidis*); Ap1(*S pasteuri*); Er3(*S pasteuri*); Dr2 (*S pasteuri*); Jm2 (*Staphylococcus hominis).*

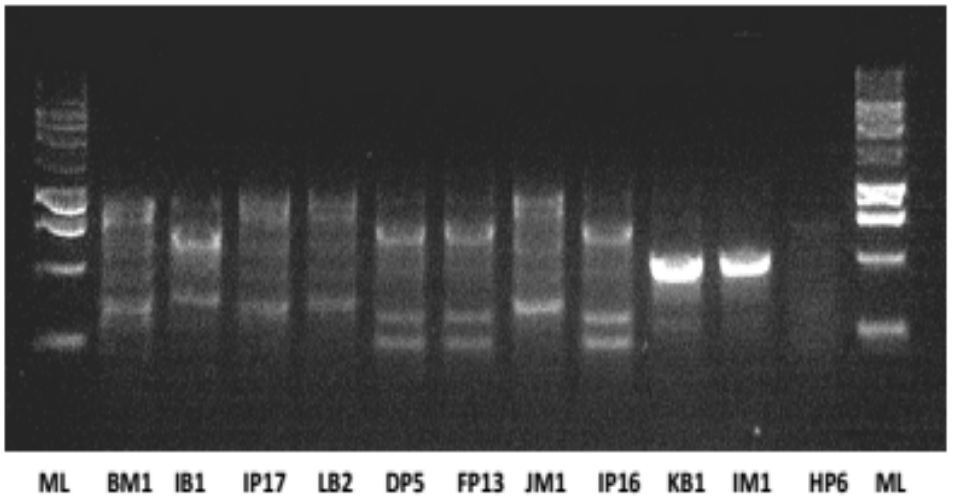

Fig. 2. RAPD fingerprint amplification product of *StrEnterococccus* isolates using the universal primer (GTG)5 : 5′-GTGGTGGTGGTGGTG-3′primer 5 ′ - AAGCCTCGTC-3 ′ ML: molecular ladder; BM1 (*E. mundtii*);IB1(E mundtii) IP17 (E. mundtii);LB2(E. mundtii); DP5(E. faecium); FP13(E. faecium); JM1(E. mundtii) IP16(E. faecium); KB1(E faecalis); IM1(E. faecalis); Hp6(E. lactis)

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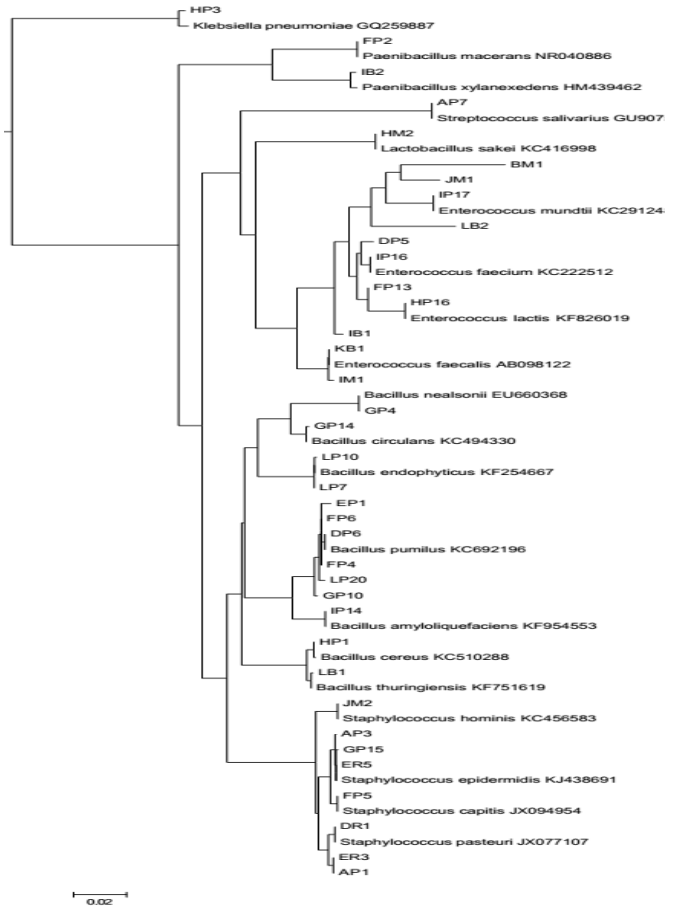

Fig. 3 .Phylogenetic tree of the bacterial strains isolated from Btana product prepared according to the neighbour joining methods using BLAST logarithm of th

Availab

Table .1

Table.1 : The microbial counts recorded for the Btana samples.

Samples type and origin	Aerobic count	Anaerobic count	MRS count
A (Orlal), DBM	$2.2x\ 10^3$	$3\ x10^2$	20
B (Orlal), DBM	$3\ x\ 10^6$	$3\ x10^5$	$1.7\ x10^5$
D (Orlal),DBM	$3\ x10^6$	$7\ x\ 10^3$	$1.1\ x\ 10^2$
E (Sali), UBM	$1.7\ x\ 10^3$	$8\ x\ 10^3$	10
F (Sali), UBM	$1\ x\ 10^6$	ND	ND
G (Orlal), DBM	$2.4\ x\ 10^2$	$2\ x\ 10^2$	$1.9\ x\ 10^2$
H (Orlal), DBM	$3\ x\ 10^2$	ND	20
I(Orlal), DBM	$1.8\ x\ 10^3$	$3.1\ x\ 10^2$	30
J (Sali),UBM	$3\ x\ 10^3$	30	10
K (Lemghier), DBM	$1.\ 6\ x\ 10^2$	$4.7\ x\ 10^4$	$1\ x\ 10^3$
L (Tolga), DBM	$7\ x\ 10^2$	30	50

Table 2 Phenotipical clustering of the selected rods strains.

Phenotipical group	Clust 1	Clust 2	Clust 3	Clust 4	Clust 5	Clust 6	
Representatives Strains(N°) / Main Tests	EP1(4) FP4(2) FP6(1) LP20 (7) DP6(3), GP10	LP7 (4) JP3 (3) LP10(1)	IP14, IP10	GP4 GP14 (5)	HP1, LB1 (2)	IB2(1)	FP2 (1)
ANA	D	-	+	-	+	-	-
NO3	-	-		-	+	+	+
Spore	+	+	+	+	+	+	+
STAR	-	-	++	-	+	+	+
CASE	+	-	+	-	+	-	-
NACL 6.5%	+	+	+	-	-	-	-
NACL 10%	D	D	-	-	-	-	-
pH 9,6	+	+	-	+	+	+	-
pH 4,5	+	+	-	+	+	-	+

Growth at 44°c	+	+	+	+	+	-	+
55°c	-	-	-	-	-	-	+
B-hemolyse	+	-	-	-	++	-	+
VP	+	-	+	+	+	-	+
Cit	-	-	-	-	-	-	-
lactose	-	-	+	+	+	+	+
Acidfrom							
glucose	+	+	+	+	+	+	+
Urée	D	-	+	+	+	-	-
rhamnose	D	+	-	-	-	+	+
manithol	+	+	+	-	+	+	+
sorbitol	+	-	+		+	+	-
lecithin	ND	ND	ND	+	+	ND	ND
16 s RNA BLAST hit	B. pumilis	B. endophytucis	B. amyloliquefaciens	B. nealsonii /circulans	B. cereus	Paenibacillus xylanexedens	P. macerans,

Table. 3 : phenotipical clustering of the selected cocci strains*.

Phenotipical group	Clus 1 (7)	Clu 2 (11)	Clu 3 (12)	Clu 4 (4)	Clus 5(12)	Clus 7
Representatives Strains (numbers) / **Tests**	AP1(2), DR1(3), ER3(2)	AP3(4), ap16(3), Ap17, ap2(1),GP15, ER5(2) JM2	BM1 (8), IB1 (2), IP17(2)	IP16(1), KB1(2), IM1	DP6(1), Hp6* (lactis) (1), Fp13(4), JM1(1), LB2(3), AP7(2)	FP5(3)
pigments	+	-	+	-	-	-
G 45°C	+	+	+	+	+	+
pH 9,6	+	+	+	+	+	+
6,5% NaCl	+	+	+	+	+	+
10% NaCl	+	+	-	-	+	+
60°C/30mi	+	+	+	+	+	+
Manith	+	-	+	+	+	+
catalase	+	+	-	-	-	+
CAS	-	+	+	+	+	-
NIT	+	+	-	-	-	+
Esculine	-	-	+	+	+	-
VP	+	+	+	+	+	+

Gasfrom glucose	-	-	-	-	-	-
hemolyse	-	-	-	-	-	-
16 s RNA BLAST hit	*S. pasteuri*	*S. epidermidis/S hominis*	*E. mundtii*	*E. faecalis*	*E. faecium*	*S capitis*

***other identified species are** *Streptococci salivarius*, *Lactobacillus sakei* and *Klebsiella pneumonia*.