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ORIGINAL ARTICLE

Metagenomic analysis of the bacterial microbiota linked to the traditional Algerian date product "Btana"

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Abstract In this study, using high throughput "pyrosequencing," we highlighted the bacterial diversity of the traditional Algerian date product "Btana" that is produced in southern Algeria using both direct (DBM) and indirect (UBM) methods. Metagenomic analysis yielded a total of 103,379 reads, with a 606 total operational taxonomic units (OTUs) detected. *Firmicutes* represented 84.79 % of the total pyrosequencing reads. Phylogenetic analysis revealed that the *Bacillales* represented 90.20 % ± 15.12 % of the total reads. Among the phylotypes detected, *Bacillus* was the dominant genus (39.53 %). While *Bacillus megaterium* was shared among all of the samples, its distribution varied widely. Non-metric multidimensional scaling (nMDS) analysis showed that UBM samples clustered together, and three main OTUs were found in these UBM samples: *Paenibacillus polymyxa*, *Paenibacillus xylanexedens*, and *Planomicrobium* JN082684. Correlation analysis showed no association between parameters of the samples (age, pH, water activity) and the specific microbiota.

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Introduction

Traditional food products play an important role in the diet of local populations in many countries around the world. These products are a major source of valuable nutrients, growth factors, and other health benefits, and are symbolic attributes of many ethnic groups as well (Schoustra et al. 2013). There has been recent continued interest in these foods, as evidenced by an increasing number of published papers and many coordinated national and international projects (Bonomo et al. 2010). Traditional African and Asian products are the most studied, owing to the rural populations who usually prepare vegetables (fruits and legumes), meat, and milk using well-known traditional methods passed down through generations (Chang et al. 2008; Obadina et al. 2009; Oguntoyinbo and Narbad 2012; Greppi et al. 2013). These practices ensure a stable food supply for local populations during lengthy food shortages. Btana is a traditional date product produced in southern Algeria, especially in the regions of Ziban (Biskra), Oued Righ, Adrar, and Beni Abbas. The product is both consumed at home and commercialized in the southern region of Algeria, and can be preserved for more than 2 years without any visible spoilage. In the past, this product was almost the only food available to honour voluntary workers, to welcome guests and to serve as subsistence during long periods of travel. Btana is normally served with fermented milk (leben), for a complete and high-energy meal. Btana is prepared by compressing dates (*Phoenix dactylifera L.*) in cotton or plastic sheets—or, in the past, in clean sheepskin, from which the word "Btana" is derived. In general, two methods are applied for Btana preparation (Supplementary Fig. S1). The direct

Btana method (DBM) is practiced in southeast Algeria (Ziban, Ghardaia, Oued Most pitfalls for microbial diversity studies arise from low DNA yield, low purity, and degradation, which lead to inaccurate information regarding diversity richness (Thakuria et al. 2008). DNA extraction has thus been highlighted as a limitation in culture-independent methods (Abriouel et al. 2006). Choosing an extraction technique often involves a trade-off between cost (materials and labor), optimal DNA yield, and the removal of substances that could interfere with polymerase chain reaction (PCR) (Sánchez-Hernández and Gaytán-Oyarzún 2006). Like other fruits, the date presents many challenges for DNA extraction due to its complex composition and the presence of a broad range of polysaccharides, carbohydrates, polyphenols, and fibers, and high salt content, which can interfere with enzymatic and chemical reactions during DNA extraction or PCR analysis (Boudries et al. 2007; Vayalil 2012). Here, we applied a modified CTAB (cetyltrimethylammonium bromide) protocol to extract bacterial DNA from the Btana product. DNA extracts were subjected to high-throughput pyrosequencing analysis to investigate the diversity of bacterial communities in direct (DBM) and indirect (UBM) Btana products.

Material and methods

Btana samples were collected from the Zibane, Elmeghier, and Adrar regions based on the method of preparation and potential zone of production. The Ziban and Elmeghier samples were produced by the DBM method, whereas samples from Adrar were produced by the UBM method. Ten grams of each sample was used for pH and water activity (aw) measurement (Abekhti et al. 2013). Next, 25-g samples were homogenized in sterile bags with 225 ml of alkaline phosphate buffer using a Stomacher apparatus. One milliliter of homogenate was used for counting the total microbial colony at 30 °C using plate count agar (PCA). Subsequently, 1.5 ml of each sample was centrifuged for 10 min at 14,000 rpm to harvest bacterial cells for DNA extraction.

CTAB Protocol (modified by Probeski et al. 1997)

The extraction buffer contained 100 mM Tris, pH 8, 1.4 M NaCl, 30 mM EDTA, pH 8, 2 % of polyvinylpyrrolidone (PVP) and 2 % of CTAB (Sigma-Aldrich Corp., St Louis, MO, USA), 40 mg/ml of lysozyme, 1 % (*v/v*) of b-mercaptoethanol, and 2 µl of bovine serum albumin (BSA). The buffer was preheated (56°C) and added (500 µl) to the pellet, then homogenized by vortexing for 5 s. Microtubes were then incubated at 56°C for 1 h. The lysis was followed by grinding, as described above. Then, 20 µl of Proteinase K was added to the pellet and incubated at 56°C for 1 h. The solution obtained was centrifuged (12,000 rpm) for 5 min. The supernatant was then cooled to room temperature, overlapped with 1 V isoamyl alcohol:chloroform (1:24) and mixed. The aqueous phase was recovered after centrifugation, treated with 0.5 V of 5 M NaCl, precipitated with 2 V of absolute cold 100 % ethanol (−20°C) and stored at −20°C for 10 min. Afterwards, the crude DNA pellet was recuperated by centrifugation (12,000 rpm for 5 min) and washed with 200 µl of cold 70 % ethanol, then resuspended in sterile RNA-free water.

DNA purification

The extracted DNA samples were purified with the genomic DNA-cleanup NucleoSpin kit (Macherey-Nagel GmbH & Co., Düren, Germany), according to the manufacturer's recommendations.

DNA recovery

The concentration and purity of extracted DNA samples were determined by the A260/A280 ratio, PCR amplification of 16S ribosomal RNA rRNA) gene, and agarose gel electrophoresis. Repeated measures analysis of variance (ANOVA) were used to compare the effect of purification on DNA quality and yield. Statistical analyses were performed using GraphPad Prism 5.01 software.

PCR reactions

For amplification of the 16S rRNA, 20-µl volumes each containing the following were used: 2 µl extracted DNA, 2 µl of dNTPs (2 mM), 1.6 µl of MgCl2 (25 mM), 2 µl of PCR buffer (10×), 0.8 µl of each primer (10 µM) 16S-1500 F: 5’- A-GTT-TGA-TCC-TGG-CTC-AG3’ (3968107, Tm = 60 °C; Eurogentec S.A., Seraing, Belgium) 16S-1500 REV: 5’-TAC-GGT-TAC-CTTGTTACGAC-3’ (3968108 Tm=58 °C; Eurogentec) and 0.2 µl of Taq DNA polymerase (2.5U, FastStart, Roche Diagnostics, Mannheim, Germany) and 2 µl of BSA (Invitrogen, Waltham, MA, USA). The PCR program was performed with a Mastercycler gradient (Eppendorf, Westbury, NY, USA) under the following conditions: initial denaturation at 94°C for 5 min, 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min.

16S rRNA gene library preparation for metagenomic analysis The gene library was constructed by amplifying the 16S rRNA gene from each Btana sample by targeting the V1-V3 regions with the primer pair E9-29 F and E514-430 R

(Brosius et al. 1981). Each primer was tagged with common titanium adapters (A or B) and sample-specific multiplex identifiers (MIDs) linked to the 5′ end of the each primer. PCR reactions were carried out according to Delcenserie et al. (2014) in a final volume of 100 μl containing 5 U of FastStart high-fidelity polymerase (Roche Diagnostics Belgium NV), 1× enzyme reaction buffer, 200 μM of dNTP mix (Eurogentec S.A., Seraing, Belgium), 0.2 μM of each primer, and 100 ng of template DNA. The PCR reactions were performed in a Mastercycler ep gradient apparatus (Eppendorf), with the following program: an initial denaturation for 15 min at 94°C, followed by 25 cycles of denaturation at 95°C for 40 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min, and a final extension step at 72°C for 7 min (Delcenserie et al. 2014). PCR products were then run on a 1 % agarose electrophoresis gel, and the DNA fragments were extracted and purified with the Wizard SV PCR purification kit (Promega Corporation, Belgium). Then they were quantified by a PicoGreen double-stranded DNA (dsDNA) assay with a fluorimeter (Isogen Life Science NV) and equal amounts of each of the 11 PCR products were pooled together and subsequently amplified by emulsion PCR.

Pyrosequencing analysis Pyrosequencing was performed with the 454 GS Junior sequencer (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The sequencing reads from the different Btana samples were analysed using the MOTHUR package developed by Schloss et al. (2009). The sequences were trimmed and sorted according to their multiplex identifier (barcode). The Pyronoise algorithm (Quince et al. 2009) was used to remove reads of low quality, i.e., sequences shorter than 425 bps and containing more than 1 ambiguous base (Ns), and sequences with a homopolymeric track longer than 8 bases were also removed. The sequences were aligned with the SILVA reference database (Pruesse et al. 2007) followed by removing potential chimeric sequences using the chimera.slayer command (Haas et al. 2011) implemented in the MOTHUR package. Finally, the remaining sequences were clustered into OTUs using the nearest neighbour algorithm with a 0.03 distance cut off and taxonomically assigned by comparisons against the SILVA database using the BLASTN algorithm with an 80 %-homogeneity cut off.

Statistical analysis

OTU clusters were used to calculate the Shannon–Weaver diversity index (Shannon and Weaver 1963), the inverted Simpson's evenness index, the Chao1 and ACE estimates of richness (Chao and Bunge 2002), and to generate a rarefaction curve for each sample (Colwell and Coddington 1994). The estimated Good's coverage was also determined (Good 1953). The Btana parameters (age, aw, pH, PCA) were analysed to determine their correlation with community variation. An OTU-based comparison was performed using the Bray-Curtis distance between the micro flora to compare the overall bacterial communities of the Btana samples according to their origin and preparation methods (DBM or UBM). Results were then visualized by three axes non-metric multidimensional scaling (nMDS) plots (Schloss et al. 2009). We also assessed the relative contribution of the OTUs to the sample's distribution along the axes.

One-way ANOVA was performed to assess differences in the Btana parameters and differences in the biodiversity index. Nonparametric Kruskal–Wallis one-way ANOVA by rank was applied to verify differences in the relative abundance of the phylogenetic bacterial groups among the studied samples using XLSTAT 2007 software (Addinsoft, Brooklyn, NY, USA). Other statistical analyses were performed with MOTHUR, STAMP and R programs.

Results

Characteristics of the Btana

Btana samples were found to be acidic, with pH ranging between 4.67 to 6.15. The ANOVA one-way analysis ($P>0.05$) did not show any significant difference between the characteristics of the samples. However a negative correlation was observed between age and aw ($r=-0.65$, $P=0.05$). No association was found between the characteristics of the Btana and its preparation method; however, the lowest pH values were recorded for the Btana prepared by the UBM method in the Adrar region.

Information about the Btana samples, origin of sampling, age, aw, pH and microbial loads on PCA are summarized in Table 1.

The CTAB protocol recorded a DNA concentration ranging from 18.80 ng/μl to 437 ng/μl, which varied strongly among the samples ($P<0.05$). A great loss (55.65 %) of DNA yield was observed after purification, as determined by an ANOVA matched pairs test ($P<0.05$). The A260/A280 ratio was slightly increased after purification, leading to an improvement in amplification and signal intensity.

Taxonomic profile of the bacterial community (community composition)

Sequence analysis

A total of 103,379 reads were generated in a single pyrosequencing run from the 11 16S rRNA libraries. The quality control parameters yielded 44,852 high quality sequences which also included the plant chloroplast and the plant

Table 1 Btana sample parameters. PCA counts and DNA concentration

Samples	Origin	Age (months)	aw	pH	PCA (CFU/g)
B1	Ziban (DBM)	10	0.54	5.97	2.25 .103
B2	Ziban (DBM)	11	0.64	5.38	3.02 .106
B3	Ziban (DBM)	12	0.6	5.29	2.40 .102
B4	Ziban (DBM)	11	0.55	5.85	3.60 .102
B5	Ziban (DBM)	7	0.53	6.15	1.80 .103
B6	Ziban (DBM)	12	0.42	5.57	1.02 .106
B7	Ziban (DBM)	11	0.6	5.18	7 .102
B8	Lamghier (DBM)	24	0.425	4.88	1.60 .102
B9	Adrar (UBM)	3	0.65	5.83	1.75 .103
B10	Adrar (UBM)	15	0.49	4.67	3.02 .106
B11	Adrar (UBM)	8	0.548	4.77	3.03 .103

DBM direct Btana method, *UBM* undirected Btana method, *aw* water activity, *PCA* plate count agar

mitochondria sequences (12,670 sequences); these were all removed from the dataset analysis (Table 2).

Diversity analysis

Clustering of the remaining 32,182 bacterial sequences (average length 479 bps) at 97 % identity led to the identification of 606 OTUs. The rarefaction curves showed different patterns for the 11 samples, regardless of their origins (Fig. 1). The curves of the samples B3, B6, B7 (Zibane), B9, B10, and B11 (Adrar) flattened very soon after a small increase of the sequence's number, indicating that there were few OTUs in these samples. In contrast, the curves of the samples B1, B2, B4, B5 (Zibane) and B8 (Elmaghier) nver attained an asymptote phase, suggesting that the sequencing effort was too limited to obtain total diversity. On the other hand, Good's coverage at a similarity level of 97 % (Table 2) was very high in all samples (98.08 to 99.91 %), indicating the detection of most of the bacterial phylotypes, with the exception of sample B5, where we found a lower coverage (76 %).

The species richness (OTU richness) observed in rarefaction curves was consistent with the diversity indices. The Chao1 richness index differed significantly among the DBM samples ($P<0.05$) but not among the UBM samples ($P>0.05$). The Shannon diversity (H) ranged between 0.07 and 4.77. The Simpson index revealed a significant divergence in the DBM samples ($P<0.05$). The B1, B4, and B5 communities were remarkably evenly diverse (0.11, 0.04, and 0.01, respectively), but samples B3, B6 and B7 were dominated by a few phylotypes (0.62, 0.98) containing rare bacteria.

Bacterial microflora composition of the Btana

Taxonomic assignment of the pyrosequencing reads from the Btana samples showed good resolution at all taxonomic

Table 2 Summary of metagenomic statistics of 11 Btana samples

Sample	Number of bacterial 16S rRNA sequences	Number of OTUs	Singleton OTUs	Estimated OTU richness		Shannon	Simpson	ESC (%)
				Chao1	ACE			
B1	2949	172	28	187	178	3.57	0.11	99.15
B2	2753	108	39	151	183	2.39	0.21	98.55
B3	3675	17	5	20	21	0.8	0.62	99.86
B4	1706	176	66	259	246	3.89	0.04	96.13
B5	517	203	122	386	603	4.77	0.01	76.74
B6	3422	12	5	15	16	0.07	0.98	99.85
B7	3169	9	3	11	15	0.35	0.86	99.91
B8	3123	155	59	202	223	2.47	0.22	98.08
B9	3480	24	6	28	30	1.83	0.2	99.8
B10	4320	16	5	26	22	1.34	0.35	99.88
B11	3068	18	3	20	22	1.74	0.23	99.87

ESC estimated sample coverage (Good's coverage)

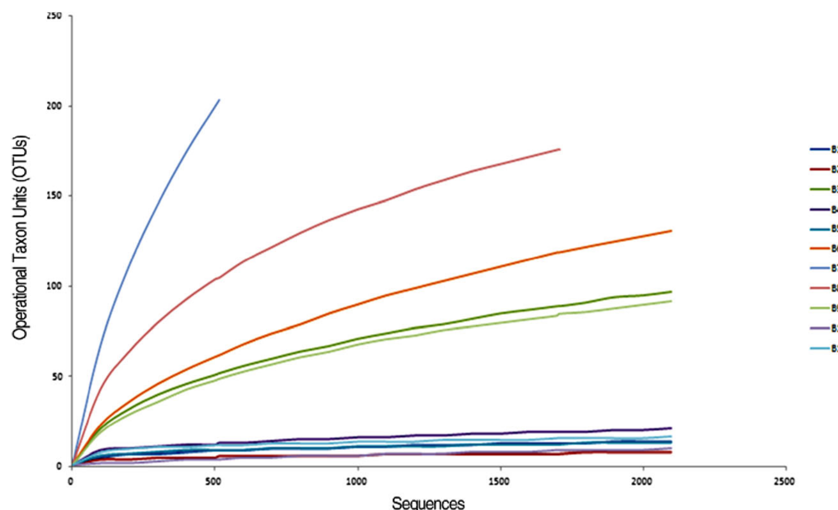

Fig. 1 Rarefaction analysis of V1/V3 pyrosequencing reads of the bacterial 16S rRNA gene from 11 Btana samples. Rarefaction curves were constructed with a similarity cut-off value of 97 sequences.

levels, and classified the indentified OTU within 8 phyla (Fig. 2). These phyla contained diverse taxonomic lineages represented by 19 classes, 33 orders, 86 families, 201 genera, and 606 species. However, only 6 OTUs had relative abundance as high as 5 % of the total reads, and represented more than 63 % of the total population. The subdominant subgroup (1–5 %) included 8 OTUs that represented 17.37 % of the total population, while those of the rare OTUs accounted for 592 OTUs (97.85 % of total OTUs), but surprisingly, represented a relative abundance of 19.85 %. We noted that of the 606 detected OTUs, 459 OTUs (75.87 % of the total species) were detected only once in the studied samples, with 224 singleton OTUs.

Among the OTUs discovered, Firmicutes represented more than 84.79 %. Proteobacteria represented the next most dominant phyla (10.61 %), followed by Actinobacteria (3.01 %) and Bacteroidetes (1.14 %). Firmicutes members averaged 91.90 % ±11.77 % in the DBM samples (B1, B2, B3, B6, B7) and 87.42 % ±17.12 % in the UBM samples. In contrast, two DBM samples (B4, B5) contained only 46.60 % and 26.63 % of Firmicutes, respectively. Community comparison indicates that, of the 11 samples studied, only two (B6, B8) belonging to DBM were not dominated by Bacillales. The other samples were highly dominated by Bacillales (average 90.20 % ±15.12 %). Supplementary Table S1 shows the most representative OTUs detected in the Btana samples (OTUs that recorded at least a 0.1 % proportion of total reads in at least one sample), and demonstrate that the *Bacillus* genus is the most abundant (39.53 %), accounting for a total of 56 OTUs (results not shown). Community comparison indicated that of the 11 samples studied, only two (B6, B8) belonging to DBM were not dominated by Bacillales. The other samples were highly dominated by Bacillales (average 90.20 % ±15.12 %). Supplementary Table S1 shows the most representative OTUs detected in the Btana samples (OTUs that recorded at least a 0.1 % proportion of total reads in at least one sample), and demonstrates that the *Bacillus* genus was the most abundant (39.53 %), accounting for a total of 56 OTUs (results not shown). *Bacillus megaterium* was detected in all 11 samples although its distribution varied widely, and it was dominant in two Ziban DBM samples, B3 (77.06*Terribacillus aidingensis* was by far the predominant species in DBM sample B1, and was also identified in most other samples (0.03–29.34 %). *Staphylococcus*

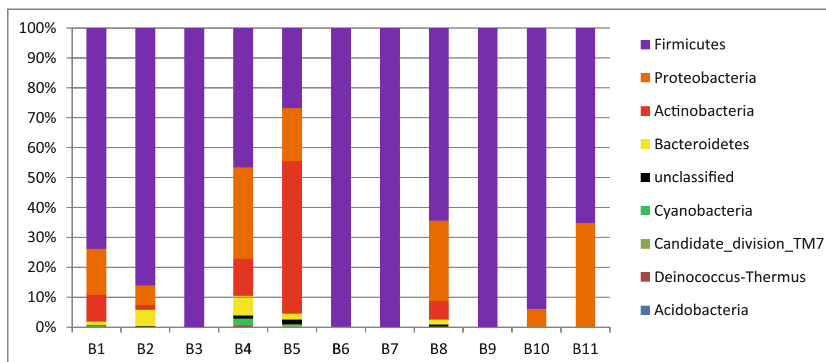

Fig. 2 Diversity of major bacterial phyla in the 11 Btana samples

species were found in a few of the DBM and UBM samples. *S. epidermidis* was dominant in DBM sample B2 (41.37 %), while *Staphylococcus equorum* subsp. *equorum* was mainly detected in B11 (9.62 %).

Diversity analysis indicated that *Enterococcus* was the third most frequent genus detected (16.04 % of total OTUs). *Enterococcus mundtii* was the most represented species (60.69 % in the *Enterococcus* genus) and was dominant (99.1 %) in DBM sample B6. In addition, DBM samples B2 and B7 and UBM sample B11 contained a relative load of *Enterococcus mundtii*.

Clostridiales phylotypes were primarily detected in Ziban and Elmeghier DBM samples.

Within the Proteobacteria phylum, high levels were recorded in UBM sample B11 (34.78 %) and DBM sample B4 (30.58 %).

The Bacteroidetes were detected only in DBM samples, mainly in sample B5, which was also dominated by Actinobacteria (50.69 %). In parallel, some *Corynebacterium* species were found with a dominant frequency in the same sample.

Comparison of the bacterial microflora of Btana from the various regions

A Venn diagram (Fig. 3) was generated based on the origin of the samples, which showed seven OTUs (*Enterococcus sp, Bacillus megaterium, Comamonas sp. HM.AF10, Staphylococcus epidermidis*, *Paracoccus* sp., *Staphylococcus equorum* subsp. *equorum*, and *Acidovorax JF925025*) shared between DBM and UBM, accounting for 36.90 % of the total sequences. Besides overlap of the species, the sequence incidence of the OTUs clearly showed that few OTUs were abundant, meaning that OTU overlap was less important. Moreover, the species were most often shared within the Btana type (DBM, UBM) rather than between the Btana origins. The Adrar samples produced by UBM (B9, B10 and B11) shared seven species: *Bacillus safensis, Bacillus cereus, Bacillus megaterium, Paenibacillus polymyxa, Paenibacillus xylanexedens, Terribacillus aidingensis* and *Planomicrobium JN082684.* There was a low level of specific OTUs for each sample.

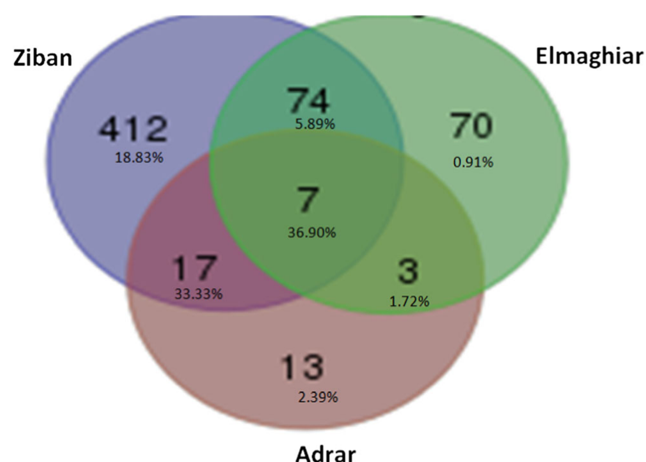

Fig. 3 Venn diagram showing OTU distribution in the Btana samples across regions

Seventy four (74) OTUs overlapped between DBM samples (Ziban and Elmeghier samples), with two common species: *Bacillus malacitensis* and *Bacillus megaterium*. However, the sequence incidence of the shared OTUs was very low (5.89 %). Elmeghier sample B8 shared a total of 81 OTUs with Ziban samples, which may be partially explained by the close distance (90 km) between the two regions (Elmaghier and Ziban).

The weighted UniFrac test, using phylogenetic distances between randomly selected sequences, confirmed that the bacterial communities from the DBM and UBM samples were significantly different ($P<0.001$The nMDS (Fig. 4) showed that the DBM bacterial communities were randomly scattered, with no specific influence of sample origin. In contrast, UBM samples clustered together along the first ordination axis (nMDS1). Three OTUs primarily contributed to the spatial distribution of the UBM samples: *Paenibacillus polymyxa* ($r=0.81$, $P<0.01$), *Paenibacillus xylanexedens* ($r=0.55$, $P<0.01$), and *Planomicrobium JN082684* ($r=0.42$, $P<0.05$). Some DBM samples (B2 and B4, B3 and B7) were very close to each other in the plot, suggesting similarities in bacterial community composition. Nevertheless, they differ from the other DBM samples belonging to the same region (B1, B5,

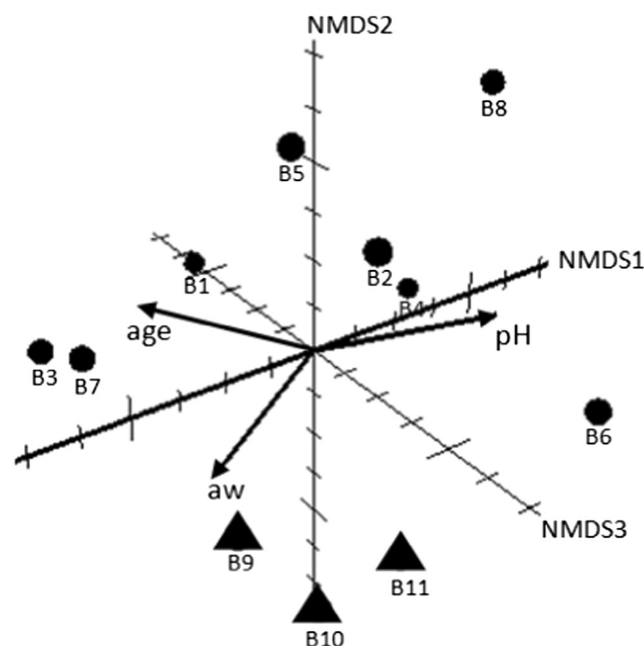

Fig. 4 Non-metric multidimensional scaling plot (nMDS) based on Bray-Curtis dissimilarity matrixes of the Btana bacterial communities (DBM samples, *black triangle*; UBM samples, black circle)

B6) and from B8 (Elmeghier) prepared by the same method. The OTU network, not available in MOTHUR, was performed in the QIIME pipeline (Caporaso et al. 2010), which gives a diagram (Supplementary Fig. S2) in which each node represented either a sample or a bacterial OTU. Connections were drawn between samples and OTUs, with edge weights defined as the number of sequences from each OTU that occurred in each sample (De Filippis et al. 2013). Networks were visualized using Cytoscape 3.0. (Shannon et al., 2003). Network analysis (Fig. 6) shows a mild clustering of UBM samples; however, three DBM samples shared a number of OTUs with the UBM samples. Each Btana method had a distinct microbial core, and the number of shared species within the Btana method was very high. The number of OTUs shared among the two methods was very low, where all samples represented some unique OTUs.

Discussion

The modified CTAB method yielded much better DNA than the other methods (results not shown). Two steps may have been largely responsible for these efficiencies: treatment with the high-concentration salt solution (5 M NaCl), which separated polysaccharides from the cell extract; and BSA added during amplification, which acted as an antioxidant to eliminate residual phenols responsible for PCR inhibition (Yang et al. 2007). However, amplification of some samples was inhibited before dilution (results not shown), indicating that PCR inhibitors were not totally eliminated. The DNA quality was further enhanced by purification, leading to amplification of all DNA extracts.

The DNA extracted from the 11 Btana samples was subjected to pyrosequencing-based analysis of the 16S rRNA genes, which is a powerful and useful tool for studying the microbial diversity of a variety of traditional food items (Oguntoyinbo et al. 2007; Chao et al. 2009). To our knowledge, this is the first study investigating the microbial diversity of the traditional date product Btana using high-throughput pyrosequencing techniques. For this, a thorough study of the bacterial community was performed to gain full insight into the microbiota of this product. The high resolution of pyrosequencing was proven by the detection of several bacterial groups consistent with dominant and minor populations. Results revealed that *Bacilli* (*Bacillaceae* and *Paenibacillaceae*) were broadly dominant in all samples, although not all had the same sample-specific species. *Bacillus* and *Bacillus*-like bacteria were dominant in eight samples UBM, whereas *Enterococcus* and *Staphylococcus* were predominant in three DBM samples (Ziban and Elmeghier). Other major groups of bacteria were detected in the Btana as well, including *Paenibacillus, Planomicrobium*, and *Terribacillus.*

As is evident, the sole difference observed between the two Btana microbial communities is the dominance of former spore bacteria in the UBM samples, which could be due to many factors in the treatment of the date fruit during Btana preparation. Adrar Btana prepared through boiled water treatment (UBM) exhibited a common dominance of three species (*Paenibacillus polymyxa*, *Paenibacillus xylanexedens* and *Planomicrobium JN082684*). On the other hand, although water is added during Btana preparation, although the final aw was not higher than the DBM samples, obviously because in the Adrar region, citizens use dried date varieties like Hmira, Takarboutch and Tgaza for Btana preparation instead of the soft variety (Ghars) used in DBM (Charlery De La Masseliere 2004; Boulal et al. 2010). On the other hand, a date's chemical composition, environmental and seasonal conditions (wet or dry) and preservation period could also define the final bacterial diversity of the Btana product. Likewise, data analysis shows that bacterial communities of UBM samples are less diversified. Treatment with high-temperature water during the softening step probably eliminates a substantial portion of vegetative bacteria. In contrast, during DBM, dates are not submitted to any microbial destructive treatment, whereby the method lacks the bacterial selection effect and the initial microbiota can persist during preservation. Also, the great geographical distance between the Adrar region and the Ziban and Elmeghier regions (about 1300 km) may account for the difference among the microbial communities present in the Btana. Thus, we conclude that boiled water treatment reduces most of the vegetative bacteria, while, on the other hand, some species like *Terribacillus aidingensis*, *Bacillus malacitensis*, *Bacillus safensis*, *Acidovorax JF925025,* and *Comamonas* sp. *HM AF10* were detected in almost all Btana samples regardless of its type, indicating a possible adaptation to the Btana product. In the cases of species being found unequally in the Btana product, it is most likely attributable to environmental contaminants. Generally, Btana is prepared in open air places that facilitate catching of such types of bacteria. Raw dates can also function as a potent vehicle for some plant-originated bacteria such as *Enterococcus mundtii* (Collins et al. 1986) and the human skin bacteria *Staphylococcus epidermidis* that might be transferred to Btana during manual. The common features of these bacteria are their environmental origin, facultative anaerobic mode of nutrition and osmotolerant ability (De Vos et al. 2009). Bacterial microflora was found on the outer surface of most dates because of it being spread by wind, rain, and water flux, etc. Whereas, amalgamation of dates during Btana preparation enhances microbial distribution and further modifies its parameters such as oxygen availability, pH and aw, on other hand, the development of strictly aerobic bacteria in Btana would have been inhibited because of the low oxygen availability which affects their tolerance to non-ideal pH and aw conditions (Mossell 1975). Hence, the majority of the observed species in microflora of the Btana are facultative anaerobes. Unlike the majority of fermented

foods dominated by Lactobacillus (LAB), *Bacillus sps*. were implicated in fermentation of traditional foods based on leaves, seeds and seafood in Africa (*Okpehe*: Oguntoyinbo et al. 2007; *Hawaijar*: Jeyaram et al. 2008; *Jeotgal*: Guan et al. 2011). Moreover, *Bacillus* species were also isolated from similarly high osmopressure food items like honey (Iurlina and Fritz 2005). Similarly, Btana has high osmopressure resulting from the high sugar content in dates (Vayalil 2012). Grant (2004) revealed that high-sugar foods (with low aw) are mainly dominated by xerophilic fungi and osmophilic yeasts. However, the presence of halotolerant bacteria in these foods was often reported, although the osmotic stress imposed by cations (salts) and uncharged organic solutes (like sucrose) is not the same (Williams and Hallsworth 2009). Recently, a halotolerant bacterium *Tetragenococcus halophilus* was found to be dominant (>99 %) in a high-sugar "thick juice" (Justé et al. 2008). Thus, it is expected that one would find many halotolerant bacteria in Btana samples, such as: *Corynebacterium*, *Acinetobacter*, *Acidovorax, Propionibacterium, Staphylococcus* and *Peptostreptococcus* (De Vos et al. 2009). Moreover, osmotolerance is linked with the availability of osmoprotectants in the surrounding medium to help bacteria to maintain the internal homeostasis (Williams and Hallsworth 2009). Interestingly, it was reported that dates are very rich in proline (12–369 mg/100 g), choline (6.30–9.90 mg/100 g), betaine (0.4 mg/100 g), and also contain a high level of potassium (703–1130 mg/100 g) (Favier et al. 1993; Al-Shahib and Marshall 2002; El-Sohaimy and Hafez 2010; Kchaou et al. 2013). The uptake of the aforementioned compounds plays a key role in cellular adaptation to a hypertonic environment, thus permitting some bacterial groups to survive the osmotic conditions of the Btana (Vilhelmson and Miller 2002). However, our molecular approach based on 16 s RNA genes as the template does not exclusively select active bacteria, as dead and damaged cell can. Also, the presence of vegetative aerobic bacteria such as *Kocuria*, *Acinetobacter GQ096364*, *Acinetobacter junii* and *Acidovorax JF925025* should be investigated if no recent contamination from a secondary source is expected. Generally, Btana is prepared in open air places that facilitate catching of such types of bacteria. Raw dates could also represent a potent vehicle of some plant-originated bacteria such as *Enterococcus mundtii* (Collins et al. 1986). Also, *Staphylococcus epidermidis* inhabiting human skin may be transferred to Btana during manual preparation.

The detection of a low number of pathogenic bacteria were reported in some samples (B1: *Staphylococcus aureus*; B2: *Clostridium* GQ158426, *Bacillus cereus*; B3: *C. botulinum*; B4 and B5: *Kocuria palustris*) underlines the need for stricter hygiene during Btana preparation. Also, further investigation is needed to examine the viability of these bacteria because pyrosequencing analysis is a semi-quantitative technique that does not verify the viability of the detected microorganisms, in particular, for the spore bacteria (Amend et al. 2010). Moreover, we should use specific nucleic acid extraction tools such as those targeting RNA or cDNA, that exclusively select active microbial populations (De Pasquale et al. 2014).

Conclusions

It is well established that high-quality NGS analysis for the accurate study of microbial diversity requires optimal DNA extraction. Here, using the classic CTAB method, we have succeeded in isolating a good DNA yield suitable for further molecular analysis from the traditional date product Btana. Results obtained from the 16S rRNA-targeted metagenomic analysis give us a more detailed view of the bacterial communities in Btana and also show high prevalence of the spore-forming *Bacillus* and its relatives. This study also revealed that the culturing-dependent methods are still important for studying the viable organisms, and are appropriate for investigating bacterial adaptation to osmotic stresses. Preparation of Btana influences the microbial diversity, owing to hot water treatment applied during the UBM that leads to a substantial reduction in the bacterial load in the Btana product. Moreover, Btana produced by the DBM is more susceptible to *Enterococcus* and *Staphylococcus* growth in comparison with the UBM. It is noteworthy to point out that this investigation was performed on random samples; therefore, future studies should standardize the Btana characteristics for more accurate comparison to avoid variation associated with the sampling plan. Beside this, more attention should be paid to bacterial functionality and to the other microbial groups (yeasts) in development of the product characteristics.

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