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Full Length Research Paper

Characterization of some atypical lactic acid bacteria associated with the fermentation of *Hibiscus sabdariffa* seeds

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The fermentation of most African seeds into traditional condiments is known to be carried out by *Bacillus* species and other genera. In the Mbuja (condiment), produced by fermentation of *Hibiscus sabdariffa* seeds, lactic acid bacteria appear to play a secondary but important role alongside *Bacillus*. In the present study, the diversity of eight lactic acid bacteria isolated during the manufacturing process of Mbuja was studied. Morphological, cultural, physiological and biochemical characteristics of the strains were assessed. Some lactic acid bacteria showed unspecific physiological characters and were not easily identifable. These isolates were identified using 16S rDNA sequencing. Both lactic cocci and rods were isolated. Very good identifications were obtained for *Lactobacillus brevis* (98%) and *Leuconostoc mesenteroides dextranicum* (99%) using API 5OCHL. Three isolates showed variable but non-specific biochemical and growth properties and were identified as *Pediococcus pentosaceus* by 16S rDNA. However, the phylogenetic analysis revealed variability with its close relative, probably explaining the atypical expression of phenotypic characters.

Key words: Lactic acid bacteria, atypical strains, phenotyping, DNA sequencing.

INTRODUCTION

The interest in traditional fermented products from Africa has been highlighted for their potential to meet nutritional requirements of people. They appear to be the cheapest means for poor people to meet their required needs in most nutrients. Recent studies in Africa indicate that traditionally fermented condiments are important sources of amino acids, proteins, fatty acids (Yagoub et al., 2004). The nutritional value of such products is believed to be due to the fermentation process and metabolic activities of the associated micro-organisms. However, the production of traditional condiments in Africa relies on spontaneous fermentation with uncontrolled processes and hazardous starter. More emphasis has since been put on the mastering of starter cultures for use in a more standardised process. Results obtained by different authors who investigated the micro flora associated with the fermentation of African seeds revealed the predominance of *Bacillus* spp. (Sanni et al., 2000; Sakyi-Dawson, 2001; Oguntoyinbo et al., 2003).

Studies on African traditional condiments did not so far attribute a great role to lactic acid bacteria, despite their known importance in the development of flavors in the processes of fermentation. The presence of fermentative lactic acid bacteria is of major importance to the intrinsic properties of foods (Soomro et al., 2002). Health benefits are also attributed to food fermented by lactic acid bacteria. Evidence and role of lactic acid bacteria in

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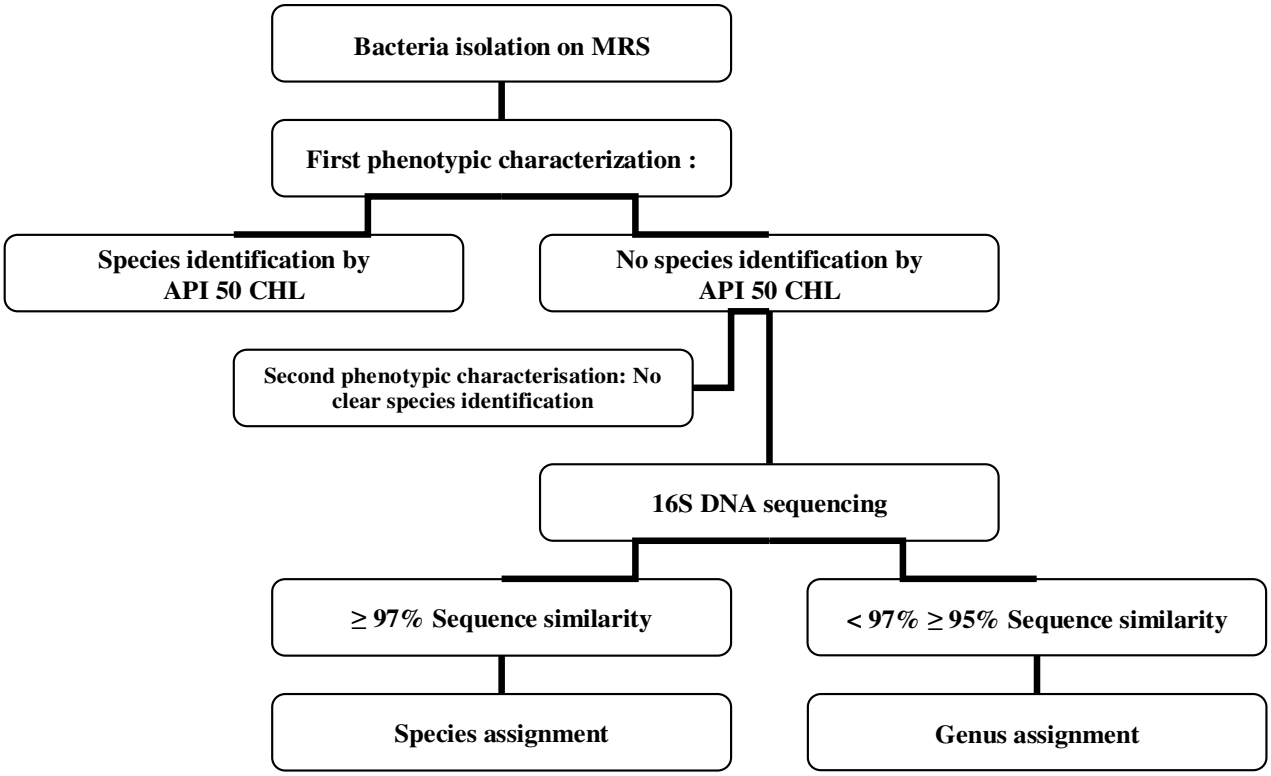

Figure 1. General flow diagram of the methodology of bacterial identification.

enhancing the immune system and preventing the occurrence of chronic diseases were brought by some investigators (Cebra, 1999; Chowdhury, 2002). The use of such bacteria in traditional fermented foods such as Mbuja is therefore advantageous to the consumer's health and nutritional status.

Mbuja is a traditional condiment in Cameroon produced by cooking and fermenting the seeds of *Hibiscus sabdariffa* and used in seasoning vegetable sauces and as meat substitute. In previous studies, we showed that bacterial fermentation of *H. sabdariffa* seeds was associated with the increase of antioxidant value of Mbuja. *Bacillus* spp. and lactic acid bacteria were the main starters, the former being more important than the latter (Mohamadou et al., 2007). In another study a sensory analysis of Mbuja from different producers and localities clearly indicated that consumers preferred samples with high concentrations of *Bacillus* spp. but also lactic acid bacteria (Mohamadou et al., 2009). The tasters preferred most flavour and taste of samples. *Bacillus species* isolated from Mbuja were characterized and identify in a study in press (Mohamadou et al, 2010).

The aim of the present work is hence to characterise the lactic acid bacteria isolated during the spontaneous fermentation of *H.sabdariffa* seeds by both physiological and molecular techniques, for their use in starter formulation in controlled fermentation of the seeds.

MATERIALS AND METHODS

Lactic bacteria isolation

The strains were isolated from fermenting cooked *H. sabdariffa* seeds at different dates using the procedure of inoculation on MRS agar plates. Ten fold dilutions of samples mashed using a MIX 1 STOMACHER (AES Laboratory) were prepared in trypton-salt (9.5 g of trypton-salt diluted in 1 litre). Plates were incubated under anaerobic conditions at 30°C for 48 h for enumeration of lactic acid bacteria. Colonies of different shapes were isolated and their purity checked by streaking on MRS agar.

Phenotypic characterisation and identification of bacteria

The characterization of isolated lactic acid bacteria was carried out in two phases (Figure 1). The identification to genus was based on key characteristics and tests for lactic acid bacteria performed on the whole isolates: microscopic observation (morphology and cell arrangement) after Gram staining, catalase test, anaerobic growth (growth in VF medium, 5 days incubation at 30°C), and fermentation (assessed in MRS broth with inverted Durham tubes, 3 days incubation at 30°C). The identification to the species level was carried out using the API 50CH galleries and API 50CHL medium (BioMerieux, France) according to the manufacturer's instructions. Results were analysed and interpreted using the APILAB software (BioMerieux).

Some cocci grew well on MRS but also on BEA medium, a specific medium for *Enterococcus*. Hence, the strains were grown on other selective media for *Enterococcus* (Litsky, Slanetz media and blood agar with 40% bile) and incubated (40%) at 37°C for 24

Table 1. Sequences and target positions of the primers.

Name	Sequence (5'>3')	Target (Position[1])
w18	GAGTTTGATCMTGGCTCAG	Gene rRNA16S (F9)
po2	GCGTGTGTACAAGACCC	Gene rRNA 16S (R1401)

[1]The position corresponds the extremity 5' of the primer in reference to 16S rRNA (Brosius et al., 1981); F and R designate respectively forward primer and reverse primer.

to 48 h. Additional characteristic tests for *Enterococcus* were carried out: Growth in 6.5% NaCl (BSF medium with salt, 37°C for 24 h), heat resistance (BSF medium, 60°C for 30 min), growth on 0.1% methylene blue milk and hydrolysis of esculin on esculin agar were evaluated for the 3 strains (Holt et al., 1994). Haemolysis was assessed on blood agar as well growth on Barnes medium and reduction of TTC. The fermentation of different sugars was evaluated using API 50 CH galleries. The culture media were purchased from AES laboratory (France).

Molecular identification by 16S DNA sequencing of atypical strains

Genomic DNA extraction

Total DNA was extracted directly from pure cultures of cells. Cell lysis was achieved by heat shocks as described by Cavalcanti et al. (2007) with some modifications and ultrasounds. Young cultures were suspended in 300 µl of sterile physiological water and frozen at -20°C for 24 h before being heated to 80°C for 10 min in dry conditions using a ThermoStat plus (Eppendorf). Subsequent treatment with ultrasound for 10 min was done on suspensions to extract genomic DNA.

PCR amplification of the 16S rDNA

The 16S of the rDNA were specifically amplified by Polymerase Chain Reaction (PCR) using universal bacterial primers (Table 1) according to Renault et al. (2007). The PCR reagents used in preparing the Mix-PCR comprised 0.5 µM of each primer, 1X *Taq* Titanium™ DNA (BD Biosciences, Clontech laboratories), 200 µM of each dNTP, 1X of *Taq* Titanium PCR buffer and sterile distilled water to obtain a final volume of 27 µl. This Mix-PCR was added to 3 µl of DNA extract. The PCR was carried out using a PTC-100 programmable thermal controller (MJ research, Inc.) with the following experimental conditions: an initial denaturation of 2 min at 94°C followed by 25 amplification cycles including 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min elongation at 72°C. A final elongation of 10 min. at 72°C was carried out. The PCR products were checked on agarose gels (1% W/V, Promega, USA) stained with ethidium bromide (0.5 mg/l).

16S rDNA sequencing

The 16S rDNA sequencing was done on 10 µl of PCR product using the Montage µPCR (Millipore) kit as described by Renault et al. (2007). The reaction of sequence was carried out by a GeneAmp® PCR System 9700 thermocycler (Applied Biosystem) according the following conditions: An initial denaturation for 5 min at 96°C; 50 cycles of amplification including 30s denaturation at 96°C, 30s annealing at 57°C and 4 min elongation at 60°C; a final elongation of 10 min. at 72°C. The primers used were w18 and po2 (Table 1). Each 5 µl of the reaction volume contained 1µl of DNA; 1µl of primer; 0.5 µl of Big Dye Terminator V 3.1; 0.75 µl of 5X buffer and 1.75 µl of distilled water. Sequencing was done on an automatic sequencer, Applied Biosystems 3130xl Genetic Analyser (GIS Ouest Genopole-GENOMER, Roscoff, France).

Strain Identification

The sequence obtained was compared with 16S rDNA sequences available in GenBank (www.ncbi.nlm.nih.gov/blast) using the BLAST programme (Camacho et al., 2009). A phylogenetic tree was constructed to show the genetic relationship between the sequence and the closest relatives by the method of neighb our joining included in the Mega 4.0 software (Tamura et al., 2007).

RESULTS

General characteristics

Eight Gram-positive and catalase-negative strains with different shapes on MRS were isolated (Table 2). Both rods and cocci constituted the lactic acid flora. Microscopic observation revealed pairs and very few tetrads of homofermentative cocci (Lb1, Lb3, and Lb6) on one hand and small chains of heterofermentative cocci (Lb7) on the other hand. The rods isolated (Lb2, Lb4, Lb5 and Lb8) were heterofermentative. Based on the characteristics listed in Table 2, the isolates were assigned to the genera *Lactobacillus*, *Enterococcus* or *Pediococcus* and *Leuconostoc.* For the latter, the ability to produce gas was an important characteristic for distinguishing the *Leuconostoc* (Garvie, 1984).

Identification to species

Very good to excellent identification was obtained for five strains by API 50CHL: Lb2, Lb4, Lb5 and Lb8 were identified as *Lactobacillus brevis* (98% level of identification) while Lb7 was *Leuconostoc mesenteroides dextranicum* (99.9% level of identification).

Three cocci were not clearly identified. Their cellular arrangement (mainly in pairs and very few irregular tetrads) was in favour of *Enterococcus* or *Pediococcus*. The three strains were therefore cultured on BEA medium (specific for *Enterococcus*) on which their growth was fair good. Attempts to confirm their affiliation to the

Table 2. General characteristics lactic acid bacteria strains isolated.

Characteristic	STRAINS							
	Lb1	**Lb2**	**Lb3**	**Lb4**	**Lb5**	**Lb6**	**Lb7**	**Lb8**
Gram and cell arrangement	Cocci G+ pairs and few irregular tetrads	Rods ChainsG+	Cocci G+ pairs and few irregular tetrads	Rods Chains G+	Rods ChainsG+	Cocci G+ pairs and few irregular tetrads	Cocci pairs and chains G+	Rods ChainsG+
Respiration	AA	AA	AA	AA	AA	AA	AA	AA
Catalase	-	-	-	-	-	-	-	-
Fermentation type	Ho	He	Ho	He	He	Ho	He	He

AA: Facultative anaerobes, Ho: Homofermentative, He: Heterofermentative.

group and the species of *Enterococcus* showed variable responses to some specific tests (Table 3). However, the three strains had similar characteristics and certainly represented the same species. Hence, a clear identification of these strains could not be achieved until molecular typing is carried out.

PCR amplification of the 16S rDNA showed that the sequences from Lb1, Lb3 and Lb6 had the same size (Figure 2). A sequence of 1497 bp was obtained by 16S rDNA sequencing. Comparison with GenBank database strains showed a similarity of 99% identity with the sequence of 16S rDNA of *Pediococcus pentosaceus* LM2632, *P. pentosaceus* SL4, *P. pentosaceus* DSM 20336 and *P. pentosaceus* R095 (Figure 3). Therefore Lb3 was identified as *P.pentosaceus* despite physiological proximity with *Enterococcus.*

DISCUSSION AND CONCLUSION

The present study revealed the occurrence of three lactic acid bacteria species in fermenting seeds of *H. sabdariffa*. Phenotypic tests helped in identifying more than half of the isolates. Very good percentages of identification (98 and 99.9%) were obtained for *Lactobacillus brevis* and *Leuconostoc mesenteroides dextranicum* respectively using biochemical characterisation by API system. Generally, the species were in agreement with the characteristics described in the Bergey's manual of determinative bacteriology (Holt et al., 1994). Denis et al. (1988) earlier noted the presence of *L. mesenteroides dextranicum* mainly in vegetables and milks. Both *L. brevis* and *L. dextranicum* are heterofermentative lactic acid bacteria and as such their role in aroma production could participate in the development of the characteristic aroma of Mbuja, a condiment used mainly as seasoning agent.

The three other lactic acid bacteria, Lb1, Lb3 and Lb6, were homofermentative and could therefore be associated with the production of lactic acid and contribute to the acidification of the product. Their identification by API 50CHL was uncertain. Failure to identify them using the API system made it necessary to carry out further investigations. Excellent growth on BEA medium was in favour of their affiliation to *Enterococcus* spp. and required further specific tests described in Bergey's manual of determinative bacteriology (Holt et al., 1994). Their shape, mostly made of pairs and very few irregular tetrads, was ambiguous. Growth on specific media such as Litsky, Slantetz, and blood agar with 40% bile was positive for the three isolates. The strains were haemolytic, grew on potassium telluride agar, and reduced TTC. These tests were also in favour of the affiliation to *Enterococcus* spp. However, other key characteristic tests (growth at 6.5% NaCl and heat resistance, 30 min at 60°C) were negative, bringing doubt on their belonging to genus *Enterococcus.* Nevertheless, the 3 lactic cocci provided identical responses to tests on specific medium as well as to fermentation of sugars. Misidentification of other lactic acid bacteria from non dairy product has already been reported using biochemical tests. *Enterococcus* was thus confused with *Lactococcus* (Elliott and Facklam, 1996; Svec and Sedlacek, 2008).

16S rDNA sequencing drew more light on the identity of these strains. Lb3 was identified as *P. pentosaceus.* However, phylogenetic analysis showed that Lb3 does not constitute a strong branch with any of its closest relative in the Blast (Figure 3). This strain presented a certain genetic diversity with clones of the same species. This might explain the difficulty to identify Lb3 phenotipically due to the various expressions of the biochemical properties of this *P. pentosaceus* which resembled *Enterococcus.* This strain was not often isolated in most traditional condiments produced by fermentation of proteinaceous seeds

Table 3. Characterisation of Lb1, Lb3 and Lb6.

Characteristics		Lb1	Lb3	Lb6
Growth on *Enteroccus* selective media	Litsky	+	+	+
	Slanetz	+	+	+
	Bile Esculin Azide	+	+	+
	Blood agar with 40% bile	+	+	+
Enteroccus characters	Growth at 6.5% NaCl	-	-	-
	Heat resistance, 30 min. at 60°C	-	-	-
	Growth on methylene blue milk 0.1%	±	±	±
	Hydrolysis of esculin	+	+	+
	Hemolysis	-	-	-
	Aspect of colonies and reduction of TTC on Barnes medium	+ pink	+ pink	+ pink
	Resistance to tellurite	-	-	-
Differentiation of species and fermentation of sugars	Fermentation of mannitol	+	+	+
	sorbitol	-	-	-
	D-xylose	+	+	+
	L-Rhamnose	-	-	-
	Lactose	-	-	-
	Melibiose	-	-	-
	Raffinose	-	-	-
	Melezitose	-	-	-
	Glycerol	-	-	-
	Adonitol	-	-	-
	Sorbitol	-	-	-
	Raffinose	-	-	-

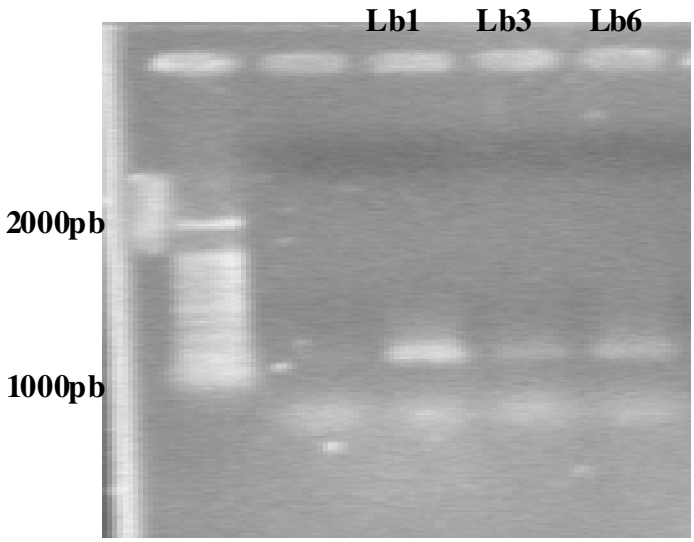

Figure 2. Agarose gel of the PCR products from Lb1, Lb3 and Lb6 DNA extracts.

seeds such as “iru pete”, “iru woro”, “netetu”, “soumbala”, “daddawa” (Oyeyiola, 1988; Ogbadu and Okagbue, 1988; Ndir et al.,1997; Sarkar et al., 2002). However, studies carried out on different product like cheese revealed the importance of *P. Pentosaceus* in the development of aroma (Tzanetakis et al., 1991).

The present study demonstrates that biochemical tests are not sufficiently reliable methods for identification of some lactic acid bacteria isolated in Mbuja. *Pediococcus* can be confused with *Enterococcus* due to their growth on specific *Enterococcus* media as well as positive tests used for identification of *Enterococcus* spp.. *Pediococcus* can grow in proteinaceous seeds and develop specific biochemical characters leading to misidentification using classical tests. Molecular tools, like 16S rDNA sequencing used in this study should be a reliable means to differentiate between *Enterococcus* and *Pediococcus.* However, the complementary use of both biochemical and molecular methods help in identifying lactic acid bacteria and in choosing those with interesting properties

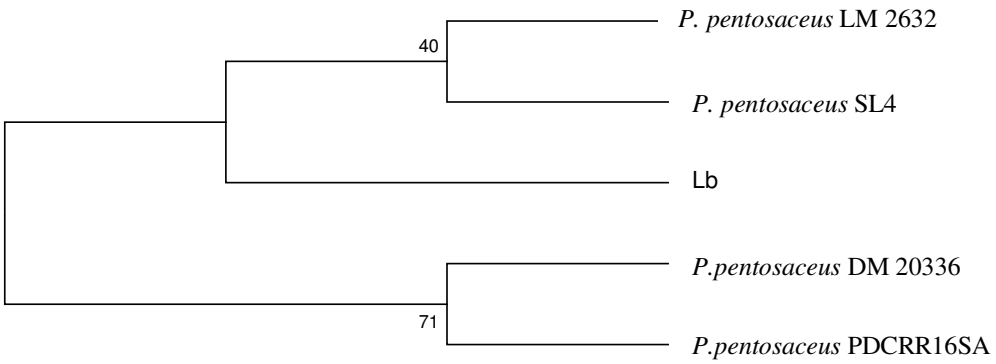

Figure 3. Phylogenetic distance between the 16S rDNA of Lb3 and closest relatives in the Blast by Neighbour Joining. (Values on the branches represent the percentage of bootstrap).

for use in starter formulation for controlled fermentation of the seeds. In this respect, *P. Pentosaceus* could be included in the starter for a better aroma production in the final condiment and longer preservation of the product since the bacteria is homofermentative and virtually produces more lactic acid.

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