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Biodiversity of predominant *Bacillus* isolated from *afitin, iru* and *sonru* at different fermentation time

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ABSTRACT

Two hundred (200) presumptive isolates of *Bacillus* collected at different fermentation time from spontaneous fermented samples of *afitin, iru* and *sonru* produced in three different regions of Benin were identified at species and strains levels. ITS-PCR-RFLP revealed that 79% of the isolates were really identified as *Bacillus*, 5% as *Staphylococcus* and 16% as unidentified bacteria. The 16S rDNA sequencing showed that 74.7% of the *Bacillus* belonged to the *B. subtilis* group and 25.3% to the *B. cereus* group. Additional biochemical tests and API 50 CHB system applied to 50 isolates randomly selected from the *B. subtilis* group divided the latter into 80% typical *B. subtilis* and 20% typical *B. licheniformis*, which showed different PFGE-RFLP band patterns. Strains belonging to the *B. cereus* group were differentiated by PCR with specific primers BCW1F and BCW1R and specific enzymes *Eco*RI, *Sau*3AI and R*sa*I. All strains of the *B. cereus* group, except one, were found to be typical *B. cereus.* This work showed the predominance of *B. subtilis* in *afitin, iru* and *sonru* along the fermentation process.

Keywords: *Afitin, Iru, Sonru,* Fermentation, *Bacillus.*

INTRODUCTION

Afitin, *iru* and *sonru* are three traditional condiments produced by spontaneous fermentation of African locust bean (*Parkia biglobosa*) in different regions of Benin. The condiments, mainly consumed in Benin as flavouring agents for sauces, soups and other dishes, are becoming increasingly popular, not only in poor families of villages but also in urban areas (Azokpota, 2005). *Afitin* is produced by spreading cooked locust bean cotyledons on basket trays and wrapping with several layers of jute to maintain a hot atmosphere for about 18 h (Azokpota et al., 2006). The jute are removed and the fermented mash is left to cool at ambient temperature for about 6 h. *Iru* and *sonru* production involves the use of traditional additives and the fermentation lasts for about 48 h (Azokpota et al., 2006). Fermentation is known to be the crucial step during *afitin*, iru and *sonru* process (Gutierrez et al., 2000; Azokpota et al., 2006). The fermentation takes place naturally under uncontrollable conditions that often adversely affect the quality of the condiments generating misunderstandings and taboos among producers (Gutierrez et al., 2000).

Similar products like dawadawa (Nigeria), netetu (Senegal), soumbala (Burkina Faso), natto (Japan), *thaï thua-nao* (Thailand) have been fully studied (Odunfa, 1985; Antaï and Ibrahim, 1986; Diawara et al., 1992; N'Dir *et al.*, 1994; Wang and Fung, 1996; Beaumont, 2002). Generally, for these products, the fermentation lasts between 3 and

5 days and the microorganisms involved have been reported to be mainly *B. subtilis*, *B. pumilus* and *B. licheniformis or B. subtilis* variant *natto* (Allagheny et al., 1996; Beaumont, 2002; Ouoba et al., 2004). Furthermore *B. cereus* and *Staphylococcus* have also been found in traditional *dawadawa* and *netetu* as associate microorganisms (Odunfa and Oyewole, 1986; Odunfa, 1989; N'dir et al., 1994). Due to their role in the fermentation process and aroma production through their proteolytic and lipolytic activities, *B. subtilis*, *B. subtilis variant natto*, *B. pumilus* and *B. licheniformis* were reported to be good starter cultures to produce *dawadawa*, *soumbala*, *natto*, *thaï thua-nao* and others African locust or Soya bean–based condiments in controlled fermentation (Diawara et al. 1998; Beaumont, 2002; Ouoba et al., 2004).

In a preliminary study, *Bacillus spp* were found to be the dominant microorganisms in *afitin*, *iru* and *sonru* (Azokpota et al., 2006), suggesting that they could be targeted as possible starter cultures for the controlled fermentation of African locust bean to produce safe and reproducible quality of these condiments. It was reported that identification of microorganisms at species and strains level is a preliminary step for starter culture formulation (Ouoba et al., 2004). But to date, no detailed studies have been fully carried out to identify the *Bacillus* involved in *afitin*, *iru* and *sonru* at species or strains level. Actually, molecular genotyping techniques are considered to be effective and rapid tools for identification and characterization of *Bacillus* (O'Donnell et al., 1980; Marten et al., 2000; Miambi et al., 2003). These techniques include mainly restriction fragment length polymorphism of the PCR-amplified 16S-23S rDNA intergenic transcribed spacer (ITS-PCR-RFLP) for the grouping and typing of the isolates at species level (Jensen et al., 1993; Gütler and Stanisich, 1996; Joung and Coté, 2002), 16S rDNA sequencing for the description of phylogenetic relationships (Ash et al., 1992) and pulsed field gel electrophoresis (PFGE) for the differentiation of isolates at strain level (Liu and Chen, 1997; Mendo et al., 2000). In many cases, for better results, these biological molecular techniques are sometimes coupled with biochemical test and API systems (Ouoba et al., 2004). The aim of this work was to identify, at species and strain level, the predominant *Bacillus* isolated from the spontaneous fermented samples of *afitin*, *iru* and *sonru*, using molecular genotyping techniques, coupled with biochemical tests and API 50 CHB systems. Identification of *Bacillus* at species and strain level in *afitin*, *iru* and *sonru* is the first step of starter culture formulation to be used for the process of these condiments in controlled fermentation.

MATERIALS AND METHODS

Preliminary identification

Two hundred (200) isolates of *Bacillus* were collected from samples of *afitin* (50), *iru* (70) and *sonru* (80) produced at three different occasions by two local producers and isolated at different fermentation time, as reported by Azokpota et al. (2006). The bacteria were enumerated, first, on Dextrose Tryptone Agar (DTA, Oxoid, CM 75, Basingstoke, Hampshire, England), as described earlier (Azokpota et al., 2006), and then purified by successive subculturing on the same culture medium. Preliminary identification was based upon colony morphology and appearance, Gram staining of cell and catalase reaction (Harrigan and McCance, 1976; Guiraud and Galzy, 1980; Sneath, 1986). During the study, purified cultures were grown and put on DTA agar slant at 5°C. The isolates were maintained at -80°C in 800 µl of Nutrient Broth (Difco 0003-17-8, Detroit, MI, USA) added to 400 µl of sterile glycerol 80 %.

Identification by ITS-PCR-RFLP, 16S rDNA sequencing and PFGE

Identification using ITS-PCR-RFLP and the 16S rDNA sequencing

The *Bacillus spp* were grouped and identified at species level, using ITS-PCR-RFLP, 16S rDNA sequencing, respectively, based upon genotyping as described by Ouoba et al. (2004).

Pulsed Field Gel Electrophoresis (PFGE) analysis

Identification at strain level was ensure using PFGE analysis according to the method of Ouoba et al. (2004) modified as followed: Cultures grown for 18 h in Nutrient Soya with shaking at 37 ^{o}C were used to prepare plugs cells; 600 µl of culture were centrifuged at 14000 x g for 2 min. One thousand microliters (1000 µl) of SE buffer were added to the

pellet and centrifuged at 14000 x g for 2 min. Six hundred microliters (600 µl) of SE buffer were added to the cells and 100 µl of the cell suspension were diluted before the OD_{600} was measured. The remaining 500 µl were centrifuged and the pellet was suspended in an amount in ml of SE buffer corresponding to OD_{600} and the suspension was dried at 90 ^{o}C for 5 min. The Bacterial suspension (90 µl) was mixed with 90 µl of 2 % Low-Melting agarose (Biolab, Richmond, Calif.) and cell pellets were digested with 500 µl of Lysozym/mutanolysin dilution ((Lysozym/ mutanolysin buffer (50 mM EDTA pH 8.5, 0.05 % N-laryl-sarcosin), 2mg/ml of lysozym and 3 U/ml mutanolysin) at 37 ^{o}C for 18 h. Formed plugs were also digested with 500 µl proteinase K dilution at 53 ^{o}C for 18 h. Plugs were washed with 1 ml of 50 mM EDTA with shaking and kept in 50 mM EDTA at 5 ^{o}C. A quarter (¼) of the plug was cut off and incubated for 30 min in 0.1 ml of NE buffer 4 (10x). The buffer was removed and 0.1 ml enzyme buffer added (12U/0.1ml *Apa*I) followed by incubation at 25 ^{o}C for 18 h. Two hundred milliliters (200 ml) of 1% agarose were boiled for 10 min in an autoclave (open valve) and cooled down to 50 ^{o}C. The agarose was added to the mould. Two liters (2 l) of 0.5 x TBE buffer were prepared and a few ml of 0.5 x TBE buffer was added in the wells before loading the samples. The agarose gel was run with CHEF-DRII Drive module (Biorad, Bie and Bernstein A-S) at 14 ^{o}C for 17 hours, followed by staining in ethidium bromide for 1 hour and washing with demineralized water with shaking for 2 hours before the gel was photographed.

Product was successively digested with three restriction enzymes, *Eco*RI (R0101S, New England *Biolabs*), *Rsa*I (R0177S, New England *Biolabs*) and *Sau*3AI (169S, New England *Biolabs*). In each case, gel electrophoresis was run as previously described, applying 7 µl of each restricted DNA

Differentiation of *B. subtilis* group

Fifty (50) isolates randomly selected from the closely related *B. subtilis* group have been differentiated. Anaerobic growth of the isolates was tested by inoculating tryptic soya broth (Difco, 0370-17-3) supplemented with 1% (w/v) glucose into tubes and incubating in a Gas Pack anaerobic system (BBL Gas Pack, MD 21152 USA) according to the manufacturer's instructions. Turbidity indicative of growth in the tubes was observed after 14 days at 37 and 55 °C. Aerobic cultures were used as control and incubated in the same conditions, as described by O'Donnell et al. (1980). Nutrient agar (Remel, 454182, Bie and Berntsen, Rødovre, Denmark) in plate dishes was inoculated with each isolate and incubated at 55 ^{o}C for 48 h to control for maximum temperature growth. Plate dishes incubated at 37 °C for 48 h were used as control. Propionate utilization was tested by inoculating slant tubes of nutrient agar containing sodium propionate (1 g), potassium chloride (1 g), magnesium sulphate monohydrate (1.2 g), diammonium hydrogen phosphate (0.5 g), agar (15 g), solution of phenol red 0.04% (20 ml) and distilled water (1000 ml) with each isolate and incubating at 37 and 55 °C for 14 days. The analysis was followed by the fermentation of carbohydrates using API 50 CHB galleries according to the manufacturer's instructions.

Differentiation of *B. cereus* group

The method described by Manzano et al. (2003) and modified as follows was used to differentiate *B. cereus* group. The DNA template for PCR was the same as that used for the PCR analysis for the 16S-23S rDNA ITS-PCR-RFLP. Amplification took place in 50 µl reaction mixture containing 5 µl of PCR buffer (10 x), 5 µl of $MgCl_2$ (25 mM), 1 µl of forward primer BCFW1 (150 pmol/µl) (5'-GTT TCT GGT GGT TTA CAT GG -3'), 1µl of reverse primer BCRW1 (147 pmol/µ) (5'-GGT GGA ATT AAA TCA TAC GTT G-3') (DNA Technology Ahrus, Denmark), 0.25 µl of Taq-Polymerase (1.25UI) (Amersham Pharmacia Biotech, USA), 20.75 µl of autoclaved MilliQ water (Millipore) and 1µl of DNA template. Amplification conditions consisted of 95 °C denaturation for 5 min, 35 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 7 min before cooling at 4 °C in a PCR thermocycler (Trio-Thermoblock, Biometra, Göttingen, Germany). The PCR product was successively digested with three restriction enzymes, *Eco*RI (R0101S, New England *Biolabs*), *Rsa*I (R0177S, New England *Biolabs*) and *Sau*3AI (169S, New England

Biolabs). In each case, gel electrophoresis was run as previously described, applying 7 µl of each restricted DNA.

RESULTS AND DISCUSSION

At the genus level, phenotypic characterization of the isolates obtained from *afitin*, *iru* and *sonru* showed typical colony and cell morphology that identified isolates as belonging to the genus *Bacillus*, according to Harrigan and McCance (1976), Guiraud and Galzy (1980) and Sneath (1986). ITS-PCR-RFLP fingerprinting of the isolates resulted in four representative groups (Fig.1). *Bacillus* of group I were homogeneous, showing the same type of three-band pattern, and represented 60.7% of the *Bacillus* isolates. They were found in all the three condiments. Those of group II showed two bands and represented 17.7% of the *Bacillus* isolates. They were found only in *afitin* and *sonru*. Those of group III showed four bands, the feature distinguishing them from those of group I being an additional major band of 400 bp. They represented about 14% of the *Bacillus* isolates and were found in all the three condiments. Those of group IV had only one band and represented 7.6% of the *Bacillus* isolates. They were found only in *afitin* and *iru*. Twenty percent (20%) of the bacteria isolated from *afitin* (5% of the total isolates) differed completely from those of the above four groups. They formed a fifth group and were characterized by 2 bands, one of 140 and the other of 1200 bp (results not shown). Sixteen percent (16%) of the total isolates, all from *iru* and *sonru*, showed no PCR bands. Distribution by group and by condiment of the *Bacillus* identified by PCR-RFLP and 16S rDNA sequencing are shown in Table 1.

Table 1 Main groups of *Bacillus spp.* identified in *afitin*, *iru* and *sonru* by PCR-RFLP and 16S rDNA sequencing

PCR[1] groups	ITS-PCR[2] size (bp)	16S rDNA[3] sequencing	Distribution of *B. subtilis* group among the condiments (%)		
			Afitin	***Iru***	***Sonru***
I	140; 200; 340	*B. subtilis* group	92	90.7	61.5
III	140; 200; 340; 400	*B. subtilis* group	8	9.3	38.5
			Distribution of *B. cereus* group among the condiments (%)		
			Afitin	***Iru***	***Sonru***
II	140; 200	*B. cereus* group	60	0	100
IV	250	*B. cereus* group	40	100	0

[1]: Polymerase Chain Reaction ; [2]: Intergenic Transcribed Spacer- Polymerase Chain Reaction; bp (base pair): International Standard Unit in Molecular biology; [3]: International Standard abbreviation used in Molecular biology to design the position of a sequence gene related to a DNA (Desoxyribose Nucleic Acid).

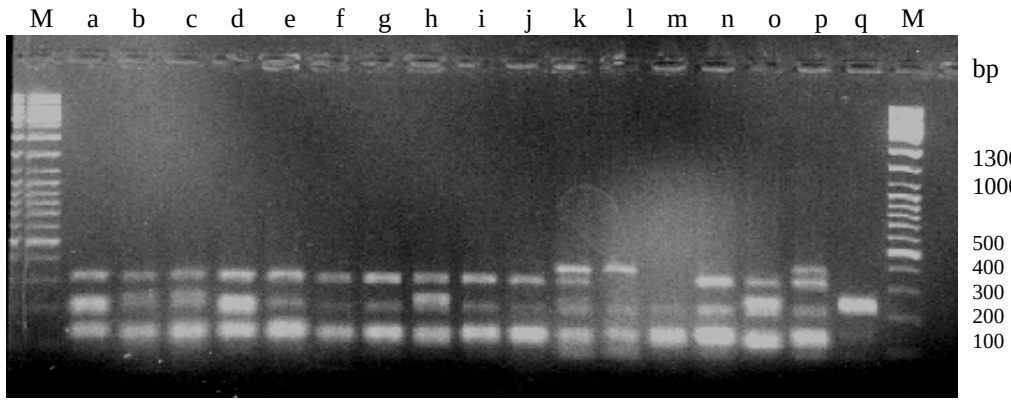


Fig. 1: ITS-PCR-RFLP electrophoretic profile of the main groups of the bacteria isolated from *afitin, iru and sonru.* The fragment size products were amplified with 16S-1500F and 23S-32R primers on 2% agarose gel. Lanes a, b, c, d, e, f, g, h, i, j, n, o: group I (96 isolates); lane m: group II (28 isolates); lanes k, l, p: group III (22 isolates) and lane q: group IV (12 isolates). Lane M: DNA size marker (Gene Ruler ™ DNA Ladder mix SM 0332, MBI Fermentas).

At the species level, the sequences of representative isolates from the four groups were aligned with those in the Genbank database. Groups I and III were identified as belonging to the closely related *B. subtilis* group, which comprises *B. subtilis* and *B. licheniformis* with percent identity varying from 97 to 99%. They represent 74.7% of the total *Bacillus* isolated from the three condiments and 62.5%, 90% and 67.2% of those isolated from *afitin*, *iru* and *sonru* respectively.

The sequencing results agreed with the ITS-PCR-RFLP analysis: it has been seen that bacteria of groups I and III differentiated by ITS-PCR-RFLP were identified by 16S rDNA sequencing as belonging to the *B. subtilis* group which represents the dominant species in *afitin*, *iru* and *sonru*. The bacteria of groups II and IV were similarly identified by 16S rDNA as belonging to the *B. cereus* group at species level**.**

It was not possible to distinguish *B. subtilis* from *B. licheniformis* or *B. cereus* from *B. thuringiensis* by 16S rDNA sequencing. Both groups have been reported to be so closely related that they could not easily be distinguished by sequencing the 16S rDNA (Rainey et al., 1994; White et al., 1993; Daffonchio et al., 1998).

Utilization of propionate by the strains as an organic substrate was illustrated by the production of red color in the tubes (Guiraud and Galzy, 1980; O'Donnell et al., 1980; Sneath, 1986). On this basis, a total of 80% (40/50) of the isolates randomly selected from the closely related *B. subtilis* group didn't growth neither at 55 °C, nor in anaerobic conditions and didn't use propionic acid as substrate. Consequently, they were identified as typical *B. subtilis* (Table 2), whereas the remaining 20% (10/50) which was grown at 55 °C in anaerobic conditions and used propionic acid as substrate was identified as typical *B. licheniformis* (Table 2). The biochemical properties of the 50 investigated *Bacillus* agreed with the descriptions of O'Donnel et al. (1980) and Odunfa and Oyewole (1986). In both cases, these results were identical to the results of the fermentation of carbohydrates by the investigated isolates using API 50 CHB systems (results not shown).

Similarly, fermented locust bean products such as *dawadawa*, *soumbala* and *netetu* are dominated by *B. subtilis* (Odunfa and Oyewole, 1986, Ouoba et al., 2004; N'dir et al., 1994). For an example of comparison, as for *afitin*, *iru* or *sonru*, the *B. subtilis* strains identified by the ITS-PCR-RFLP in *soumbala* were characterized by three bands (Ouoba et al., 2004). However, a difference was observed concerning the molecular sizes of band patterns of *B. subtilis* isolated in the two types of condiment. It is quite remarkable that the molecular size of band patterns of *B. subtilis* from *soumbala* were relatively higher than those of *B. subtilis* identified in *afitin*, *iru* or *sonru* (Ouoba et al., 2004) which means that the latter moved more quickly in the agarose gel than *B. subtilis* isolated from *soumbala*. This difference could confer to the strains specific characteristics to be investigated. Even more species including *B. pumilus and B. sphaericus* have been identified in *soumbala* (Ouoba et al., 2004).

At the strains level, the PFGE fingerprints of 20 *Bacillus* strains including 15 *B. subtilis* and five *B. licheniformis* are shown in Fig. 2. From this figure, the PFGE patterns were heterogeneous and some differences were observed between the isolates, although *B. subtilis* strains from *afitin* were identical, with the same band patterns around 25 kbp (Fig. 2: lanes c, d, e and f). Among the other strains, some *B. subtilis* from *iru* (Fig. 2: lanes g and i) showed band patterns very similar to those from *afitin*, which have a molecular weight of about 50 kbp, while others (Fig. 2: lanes m and n) showed very different ones. Two *B. subtilis* strains from *sonru* (Fig. 2: lanes o and p) have the same profile but are totally different from those from *afitin* and *iru*, as they have additional bands with a molecular size of about 194 kbp. Strains of *B. licheniformis* from both *iru* (lanes h and j) and *sonru* (lanes r, s, and t) showed similar band patterns. A little difference is in a *B. licheniformis* strain from *sonru* (lane t), the band intensity of which appears weak. Three strains of *B. subtilis* including two from *afitin* (Fig. 2: lanes a, b) and one from *iru* (lane k) didn't show clear band patterns by PFGE.

These results show the diversity among the isolates. It was possible to discriminate between *B. subtilis* strains from the same

Table 2. Biochemical differentiation of 50 isolates randomly selected from the *Bacillus subtilis* group (*B. subtilis* and *B. licheniformis*)

No.	Code strains	Growth at 37°C	Growth at 55°C	Anaerobic growth	Utilization of propionate
			***B. subtilis* (80%)**		
	Afitin				
1	F0H1	+	-	-	-
2	F0H2	+	-	-	-
3	F0H7	+	-	-	-
4	F6H4	+	-	-	-
5	F6H9	+	-	-	-
6	F6H10	+	-	-	-
7	F12H7	+	-	-	-
8	F12H8	+	-	-	-
9	F12H9	+	-	-	-
10	F18H5	+	-	-	-
11	F18H6	+	-	-	-
12	F18H10	+	-	-	-
13	F24H3	+	-	-	-
14	F24H9	+	-	-	-
15	F24H10	+	-	-	-
	Iru				
16	Y0H2	+	-	-	-
17	Y0H3	+	-	-	-
18	Y6H2	+	-	-	-
19	Y6H4	+	-	-	-
20	Y12H1	+	-	-	-
21	Y12H2	+	-	-	-
22	Y12H4	+	-	-	-
23	Y18H4	+	-	-	-
24	Y18H5	+	-	-	-
25	Y18H6	+	-	-	-
26	Y18H10	+	-	-	-
27	Y24H5	+	-	-	-
28	Y24H9	+	-	-	-
29	Y24H10	+	-	-	-
	Sonru				
30	Ba0H5	+	-	-	-
31	Ba0H10	+	-	-	-
32	Ba0H11	+	-	-	-
33	Ba12H14	+	-	-	-
34	Ba12H15	+	-	-	-
35	Ba12H16	+	-	-	-
36	Ba18H12	+	-	-	-
37	Ba18H13	+	-	-	-
38	Ba18H14	+	-	-	-
39	Ba18H16	+	-	-	-
40	Ba24H5	+	-	-	-
			***B. licheniformis* (20%)**		
	iru				
1	Y36H4	+	+	+	+
2	Y36H5	+	+	+	+
3	Y36H9	+	+	+	+
4	Y36H10	+	+	+	+
5	Y48H5	+	+	+	+
6	Y48H9	+	+	+	+
	sonru				
7	Ba24H9	+	+	+	+
8	Ba24H10	+	+	+	+
9	Ba24H14	+	+	+	+
10	Ba48H5	+	+	+	+

+: Positive reaction; - : Negative reaction

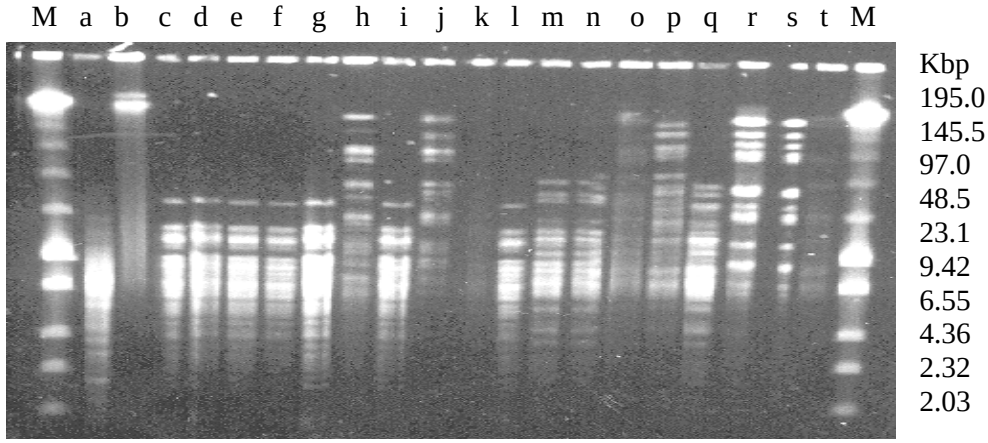


Fig. 2: Pulse-Field Gel Electrophoretic profile of *B. subtilis* group on 2 % Low-Melting agarose (Biolab, Richmond, Calif.). Lane M :DNA size marker (Low Range PFG marker 350S, new England, *Biolabs*); lanes c, d, e, f, g, i, l: B1; lanes h, j and p: B2; lanes m and n: B3; lanes r and s: B4 ; Lanes a, b, k: B5 ; lane o: B6; lane q: B7 lane t: B8.

condiment as well as from different condiments. In addition some *B. subtilis* strains isolated from *afitin* and *sonru* had the same patterns. A clear difference was also observed between *B. licheniformis* strains identified in *iru* and in *sonru* (Fig. 2). Furthermore, the fact that *B. licheniformis* was found only in *iru* and *sonru* at the latter stage of the fermentation (between 24 and 48 h could be partially explained by the length or by the nature of the fermentation. It was reported that *afitin* process involved a short fermentation (24 h), whereas the fermentation of African locust beans to produce *iru* and *sonru* occurred over 48 h. In addition, *iru* and *sonru* process involves the use of traditional additive called *yanyanku* by local producers, contrary to *afitin* (Azokpota et al., 2006).

Bacillus of groups II and IV belong to the *B. cereus* group and comprise *B. cereus* and *B. thuringiensis* at a similarity of 98 or 99%. They represent 25.3% of the total Bacillus and 37.5%, 10% and 32.7% of those isolated from *afitin*, *iru* and *sonru*, respectively. The PCR products of the 40 isolates of the *B. cereus* group digested with *Eco*RI, *Sau*3AI and R*saI* endonucleases were 350, 200 and 300 bp, respectively (Fig. 3). The digested PCR fragment size corresponds in each case to that of *B. cereus*, when compared to data from the literature (Manzano et al., 2003).On this basis all the isolates of the *B. cereus* group, except one, were identified as typical *B. cereus*. Generally, *B. cereus* strains were also found in many African fermented locust bean condiments (Ouoba et al., 2004) and were reported to be potentially enterotoxic (Granum et al., 1996; Choma and Granum, 2002). However, to date, no foodborne diseases have been reported in Benin due to the consumption of *afitin*, *iru* or *sonru*. Overall, clinical investigations are needed to confirm whether some *B. cereus* strains identified in *afitin*, *iru* and *sonru* are toxigenic and whether they represent a potential hazard for consumers.

The details results of identification by PCR-ITS-RFLP of the presumptive *Bacillus spp* isolated from the three condiments at different fermentation time are shown in Fig. 4. A total of 33.3% of *B. cereus* strains in *afitin* were found during the first 6 h of fermentation (Fig. 4a). Three of the six *B. cereus* strains identified in *iru* were isolated at the start of fermentation (Fig. 4b). In contrast, no *B. cereus* was found in *sonru* during the same period of fermentation (Fig. 4c), suggesting that the process environment which varies from one condiment to another, might influence the growth and distribution of *B. cereus* during fermentation process. Furthermore, 16% of the isolates obtained from *iru* and *sonru* could not be identified (Fig. 4: b and c) because of the absence of PCR bands, a phenomenon that we were unable to explain. The large number of *B. subtilis* strains isolated from *afitin*, *iru* and

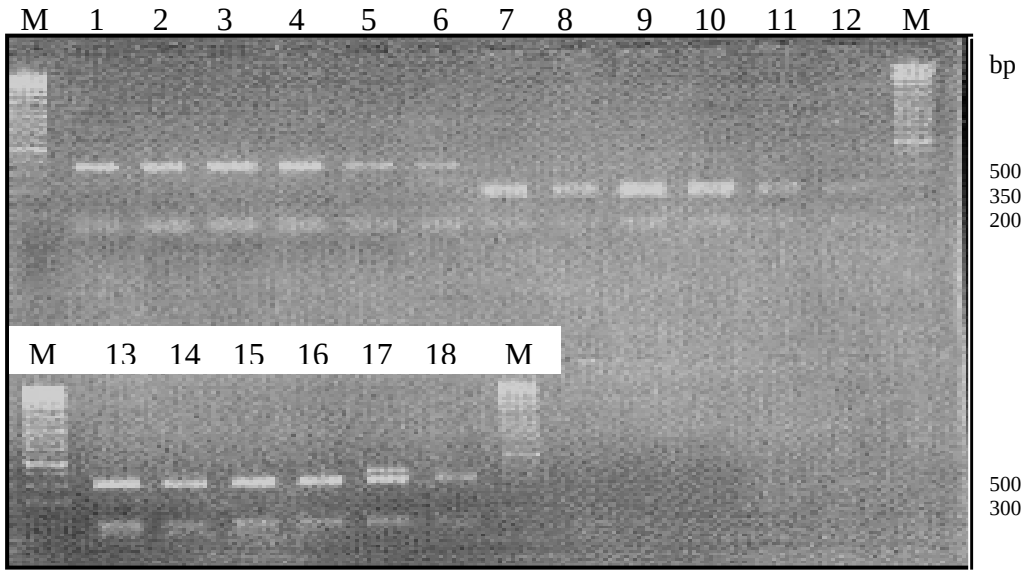


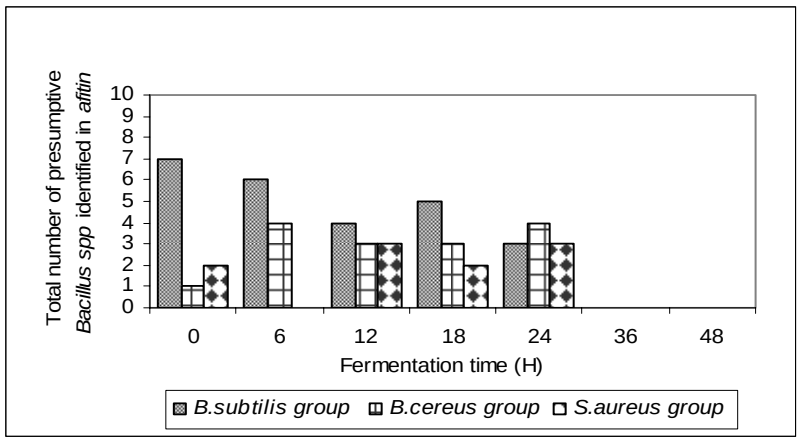
Fig. 3: PCR–RFLP of *B. cereus* group. Digestion was done with *Eco*R1 (lanes 1-6); *Sau*3A1 (lanes 7-12) and *Rsa*1 (lanes 13-18). MWM: molecular weight marker 100 bp (Sigma, Aldrich).

sonru (74.7% of the total *Bacillus spp.*) and the fact that they were broadly distributed among the three condiments according to the fermentation time demonstrate their abundance and diversity in the three products.

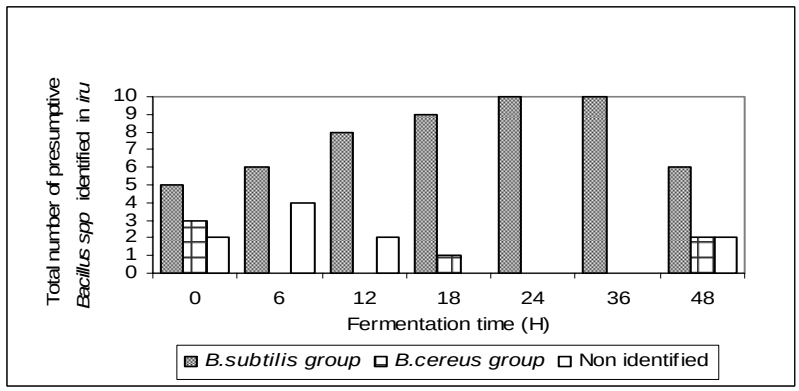
Five percent (5%) of the total isolates were identified as belonging to the *Staphylococcus aureus* group with percent identity of 94%. They were found only in *afitin.* They correspond to bacteria with ITS-PCR-RFLP fingerprints with two bands, one of 140 and the other of 1200 bp.

Globally, except for the minor group of *Staphylococcus*, the isolates from *afitin*, *iru* and *sonru* were identified as belonging to the genus *Bacillus*. The present findings can be generalized to any of alkaline fermentation of African locust or Soya bean using similar *afitin*, *iru* and *sonru* process. Several authors reported that *Bacillus spp* were generally the dominant microorganisms involved in the fermentation of African locust or Soya bean to produce different African or Asian traditional condiments as *dawadawa*, *soumbala*, *netetu*, *natto* or *kinema* (Odunfa and Oyewole, 1986; Aderibigbe and Odunfa, 1990; Diawara and al., 1992; Diawara and al., 1998; Kiers et al., 2000; Beaumont., 2002). The presence of the *Staphylococcus* in *afitin* may be opportunistic and these strains can be found in any of the three condiments, as demonstrated recent investigations which have revealed their presence also in *iru* and *sonru.* However, the presence of *Staphylococcus* in a fermented African locust bean product is not surprising, as *Staphylococcus* species have frequently been found in *netetu* (N'dir et al., 1994) and *dawadawa* (Odunfa, 1989; Omafuvbe et al., 2000). In addition, *Bacillus pumilus* which are absent in *afitin*, *iru* and *sonru* have been identified in *soumbala*. But, the particularity of the present study on *afitin*, *iru* and *sonru* resides in the investigation of a high number of *Bacillus spp* occurring in the three condiments according to the fermentation processes. For each process, *Bacillus spp* have been isolated at different fermentation time leading to a variety of isolates with potential specific characteristics to be used as starter culture. Physico-chemical characterization of *Bacillus spp* isolated from *afitin*, *iru* and *sonru* was then necessary in the perspective to get new information to be compared to the well-known characteristics of *Bacillus spp* identified in *soumbala*, *dawadawa*, *netetu* or *kinema* and for starter culture formulation to be used for *afitin*, *iru* and *sonru* process.

a) Afitin



b) *Iru*



c) *Sonru*

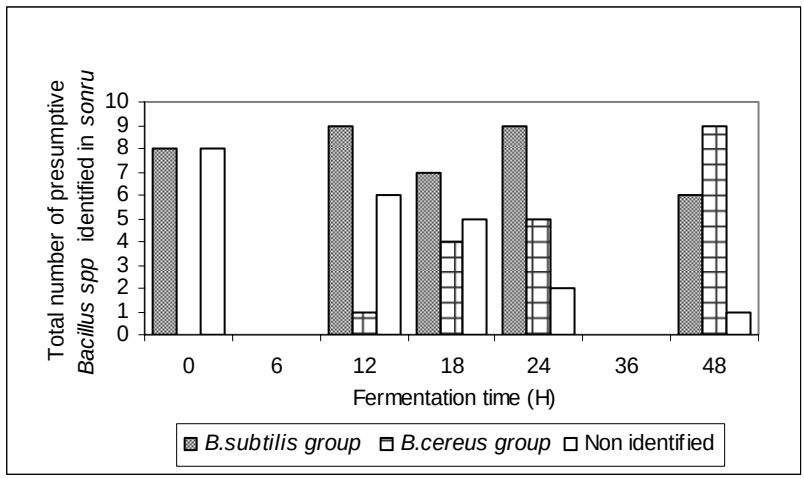


Fig. 4: Distribution of the number of *bacteria* identified in *afitin*, *iru* and *sonru* by PCR-ITS-RFLP at different fermentation time.

Conclusion

The identification of the predominant microorganisms in the fermented *afitin*, *iru* and *sonru* was performed in the present work for the first time. Diversity was observed at species level of *Bacillus* (primarily in *B. subtilis* but also in *B. licheniformis* and *B. cereus*) as well as at strain level. *Staphylococcus spp* were initially identified in *afitin*, but recent investigations have revealed their presence also in *iru* and *sonru*. *B. subtilis* and *B. licheniformis* were differentiated by 16S rDNA sequencing and by additional biochemical testing and API 50 CHB system, illustrating the possibility of combining genotyping and phenotypic analyses to distinguish closely related *B. subtilis* and *B. licheniformis* species.

For further investigation, the identified *Bacillus spp* are potential target to be used as starters culture to produce *afitin*, *iru* and *sonru* in controlled fermentation. First of all, there is a need to deeply characterize these strains, since the final selection of strains to be used as starter culture will depend on individual performance under practical conditions, proteolytic or lipolytic activity and production of a desirable aroma.

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REFERENCES

Aderibigbe EY, Odunfa SA. 1990. Growth and extracellular enzyme production by strains of *Bacillus* species isolated from fermenting locust beans, *iru*. *Journal of Applied Bacteriology*, **69**: 662-671.

Allagheny N, Obanu ZA, Campbell-Platt G, Owens JD. 1996. Control of ammonia during *Bacillus subtilis* fermentation of legumes. *International Journal of Food Microbiology*, **29**: 321-333.

Antaï SP, Ibrahim MH. 1986. Microorganisms associated with African locust bean (*Parkia biglobosa*. *Filicoida Welw*) fermentation for "dawadawa" production. *Journal of Applied Bacteriology*, **61**: 145-148.

Ash C, Farrow JAE, Wallbanks S, Collins MD. 1992. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit ribosomal RNA sequences. *Letters of Applied Microbiology*, **13**: 202-206.

Azokpota P. 2005. Etude de la dynamique physico-chimique et microbiologique de la fermentation des graines de néré pour la production de l'*afitin*, de l'*iru* et du *sonru.* Ph.D. Thesis. Université d'Abomey-Calavi- Université Royale Agricole et Vétérinaire de Copenhague au Danemark, 172 p.

Azokpota P, Hounhouigan DJ, Nago MC. 2006. Microbiological and chemical changes during the fermentation of African locust bean (*Parkia biglobosa)* to produce *afitin*, *iru* and *sonru*, three traditional condiments produced in Benin. *International Journal of Food Microbiology*, **107**: 304-309.

Beaumont M. 2002. Flavoring composition prepared by fermentation with *Bacillus* spp. *International Journal of Food Microbiology*, **75**: 189-196.

Choma C, Granum PE. 2002. The enterotoxin T (BcET) from *Bacillus cereus* can probably contribute to food poisoning. *FEMS Microbiology Letters*, **217**: 115-119.

Daffonchio D, Borin S, Frova G, Gallo R, Mori E, Fani R, Sorlini C. 1998. 16S-23S rDNA internal transcribed spacers as molecular markers for the species of the 16-rRNA group I of the genus *Bacillus*, *FEMS Microbiology Letters*, **163**: 229-236.

Diawara B, Sawadogo L, Kabore IZ. 1992. Contribution à l'étude des procédés traditionnels de fabrication du *soumbala* au Burkina Faso. Aspects biochimiques, microbiologiques et technologiques. *Sciences et Techniques*, **20**: 5-14

Diawara B, Sawadogo L, Amoa-Awua WKA, Jakobsen M. 1998. *Quality system for the production of soumbala*. The HACCP System. Taastrup: Waitro.

Granum PE, Andersson A, Gayther C, Giffel M, Larsen H, Lund T, O'Sullivan K. 1996. Evidence for a further enterotoxin complex produced by *Bacillus cereus*. *FEMS Microbiology Letters*, **217**: 115-119.

Guiraud J, Galzy P. 1980. *L'analyse microbiologique dans les industries alimentaires* (edn De l'Usine Nouvelle). Collection Génie Alimentaire: Paris; 89-91

Gutierrez ML, Maizi P, Nago CM, Hounhouigan J. 2000. *Production et commercialisation de l´afitin dans la region d´Abomey-Bohicon au Bénin*. CERNA, CNEARC, CIRAD Librairie de CIRAD; 124p.

Gütler V, Stanisich A. 1996. New approaches to typing and identification of bacteria using the 16S -23S rDNA spacer region. *Microbiology*, **142**: 3-16.

Harrigan WF, McCance ME. 1976. *Laboratory methods in Foods and Dairy Microbiology*. Acad. Press: London; 389p.

Jensen MA, Webster JA, Straus N. 1993. Rapid identification of bacteria on the basis of polymerase reaction-amplified ribosomal DNA polymorphism. *Applied and Environmental Microbiology*, **59**: 945-952.

Joung KB, Coté JC. 2002. Evaluations of ribosomal RNA gene restriction patterns for the classification of *Bacillus* species and related genera. *Journal of Applied Microbiology*, **92**: 97-108.

Kiers JL, Van laeken AEA, Rombouts FM. 2000. *In vitro* digestibility of Bacillus fermented Soya beans. *International Journal of Food Microbiology*, **60**: 163-169.

Liu PY-F, Chen S-L. 1997. Use of Pulse-Fields Gel Electrophoresis to investigate a pseudo-Outbreak of *B. cereus* in a Pediatric Unit. *Journal of Clinical Microbiology*, **35**: 1533-1535.

Manzano M, Cocolin L, Cantoni C, Comi G. 2003. *Bacillus cereus, Bacillus thuringiensis* and *Bacillus mycoides* differentiation using a PCR-RE technique. *International Journal of Food Microbiology*, **81**: 249-254.

Marten P, Smalla K, Berg G. 2000. Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to *Bacillus subtilis. Journal of Applied Microbiology*, **89**: 463-471.

Mendo ALV, Henriques SI, Correia CMA, Duarte MCJ. 2000. Genetic Characterization of a New Thermotolerant *Bacillus licheniformis* Strain. *Current Microbiology*, **40**: 137-139.

Miambi E, Guyot J-P, Ampe F. 2003. Identification, isolation and quantification of representative bacteria from fermented cassava dough using an integrate approach of culture-dependent and culture-independent methods. *International Journal of Food Microbiology*, **82**: 111-120

N'Dir B, Hbid C, Cornélius C, Roblain D, Jacques P, Vanhentenryck F, Diop M, Thonart P. 1994. Propriétés antifongiques de la microflore sporulée du *nététu. Cahiers Agricultures* , **3**: 23-30.

Odunfa SA. 1985. Biochemical changes in fermenting African locust beans (*Parkia biglobosa*) during "*iru*" fermentation. *Journal of Food Technology*, **20**: 295-303.

Odunfa SA. 1989. Nutritional characteristics of *Staphylococcus* sp. From fermenting African locust bean (*Parkia biglobosa*). *Die Nahrung Microbiology*, **5**: 195-200.

Odunfa SA, Oyewole BO. 1986. Identification of *Bacillus* species from "*iru*, a fermented African locust bean (*Parkia biglobosa*) product. *Journal Basic Microbiology*, **26**: 101-108.

O´Donnell AG, Norris, JR, Berkeley, RCW, Clauss D, Kaneko T, Logan NA, Nozaki, R. 1980. Characterization of *Bacillus subtilis*, *Bacillus pumilis*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* by Pyrolysis Gas-Liquid Chromatography, Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridisation, Biochemical Tests and API Systems. *International Journal of Systematic Bacteriology*, **30**: 448-459.

Omafuvbe BO, Shonukan OO, Abiose SH. 2000. Microbiological and biochemical changes in the traditional fermentation of

soybean for ´soy-daddawa´Nigeria food condiment. *Food Microbiology*, **17**: 469-474

Ouoba LI, Diawara B, Amoa-Awua WK, Traoré AS, Moller PL. 2004. Genotyping of starter cultures of *B. subtilis* and *B. pumilus* for fermentation of African locust bean (*Parkia biglobosa*) to produce *Soumbala*. *International Journal of Food Microbiology*, **90**(2): 197-205.

Rainey FA, Fritze D, Stackebrandt E. 1994. The phylogenetic diversity of thermophilic members of the genus *Bacillus* as revealed by 16S rDNA analysis. *FEMS Microbiology Letters*, **115**: 205-212.

Sneath PHA. 1986. Endospore-forming Gram-Positive Rods and Cocci. In *Bergey´s Manual of Systematic Bacteriology* (2*)*, Mair NS, Sharpe ME, Holt JG (eds). Waverly Press, Inc.; 1104 -1137.

Wang J, Fung DYC. 1996. Alkaline-Fermented Foods: A review with Emphasis on Pidan Fermentation. *Critical Reviews in Microbiology*, **22**(2): 101-138.

White D, Sharp RJ, Priest FG. 1993. A polyphasic taxonomic study of thermophilic bacilli from a wide geographical area. Antonie Van Leeuwenhoek; *International Journal of General Molecular Microbiology*, **64**: 357-386.