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Antimicrobial activities, toxinogenic potential and sensitivity to antibiotics of *Bacillus* strains isolated from Mbuja, an *Hibiscus sabdariffa* fermented seeds from Cameroon

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This study was carried out to screen for antimicrobial activities against common pathogenic and food spoilage bacteria, yeasts and molds, toxinogenic potential and sensitivity to antibiotics of 26 *Bacillus* strains (11 *Bacillus subtilis,* 5 *Bacillus megaterium,* 4 *Bacillus amyloliquefaciens,* 4 *Bacillus pumilus* and 2 *Bacillus thuringiensis*) isolated from different samples of *Hibiscus sabdariffa* fermented seeds (Mbuja). Antimicrobial assays were realised according to the cross-streak and the overlay methods. The main toxin and bacteriocin encoding genes were screened by PCR using specific primers and antibiotic resistance was assessed by the disc diffusion method. *Bacillus* species showed variable ability to inhibit bacterial and/or fungal species. The most antibacterial strains were *B. amyloliquefaciens* (S1 and S5) and *B. subtilis* (S12) whereas the most antifungal ones were *B. megaterium* S8 and S9. Subtilin and subtilosin A genes were detected in seven strains of *B. subtilis* but they were not associated with antimicrobial activities. All strains, except *B. thuringiensis,* did not contain toxin encoding genes and were sensitive to most tested antibiotics. This suggests that antimicrobial strains could be used in starter cultures for a controlled fermentation to produce *Mbuja* in order to better control the fermentation process of Mbuja and to increase consumer's safety.

Key words: *Bacillus*, antimicrobial activities, toxins, antibiotic resistance.

INTRODUCTION

The traditional condiment produced in Cameroon by fermenting *Hibiscus sabdariffa* seeds also known as

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Mbuja is commonly used in the diet of people from the Sahelian region, especially for those living in rural areas with low incomes. The condiment is mostly appreciated for its nutritional value and organoleptic properties and it is used to flavour soups and sauces (Mohamadou et al., 2009). Earlier studies revealed that Mbuja could contribute to consumers' health through its anti-oxidants and phenolic compounds (Mohamadou et al., 2007). *Bacillus* spp. mainly *Bacillus subtilis* and related genera are responsible for the fermentation (Mohamadou et al., 2013).

Like for many other traditional condiments, the production of Mbuja relies on spontaneous and uncontrolled fermentation that could impact the quality and safety of the products on one hand, and on the consumers' health on the other hand. Recent studies brought evidence that safety of fermented products could be significantly improved by selecting starter cultures with protective effects against most common pathogenic and spoilage bacteria, yeasts and molds (N'dir et al., 1994; Ouoba et al., 2007). Some *Bacillus* species produce antibiotics and antimicrobial compounds such as bacteriocins including subtilin and subtilosin synthesized by *B. subtilis* (Klein et al., 1992; Stein et al., 2004; Abriouel et al., 2011). On the other hand, *Bacillus cereus* and related species (*Bacillus thuringiensis* and *Bacillus anthracis*) are well-known food poisoning bacteria which produce either emetic heat-stable toxin or diarrheal enterotoxins (HBL, NHE and BcET) (Matarante et al., 2004). Another trait of *Bacillus cereus* and related species toxicity is their ability to secrete phospholipases, cell-lysing enzymes, including sphingomyelinase (sph) and phosphatidylinositol- and phosphatidylcholine-specific phospholipase (piplc) (Matarante et al., 2004). Furthermore, some studies reported that virulence factors were also present in other *Bacillus* species than *Bacillus cereus* (Kramer and Gilbert, 1989; Phelps and McKillip, 2002).

The aim of this study was to investigate the potential food preservation and contribution to food safety as criteria for selection of starter cultures to be used in controlled fermentation of *H. sabdariffa* seeds. Hence, the work intended to assess antimicrobial activity, toxinogenic potential and sensitivity to antibiotics of *Bacillus* strains isolated from Mbuja.

MATERIALS AND METHODS

Microorganisms

Eleven strains of *B. subtilis* (S2, S7, S12, S15, S16, S17, S18, S19, S20, S21 and SY), five strains of *Bacillus megaterium* (S3, S8, S9, S11 and S14), four strains of *Bacillus amyloliquefaciens* (S1, S5, S13 and SX), four strains of *Bacillus pumilus* (S4, S6, S22 and S23) and two strains of *B. thuringiensis* (S10 and SAc) maintained at the "Laboratoire Universitaire de Biodiversité et Ecologie Microbienne" (LUBEM) culture collection were investigated. These strains were previously isolated from different productions of Mbuja (fermented *H. sabdariffa* seeds) (Mohamadou et al., 2009) and identified with molecular and phenotypic methods (Mohamadou et al., 2013). Their antimicrobial activity was investigated against 6 indicators or pathogenic bacteria (*Bacillus cereus* ATCC 6464, *Listeria innocua* HPB13 [used as indicator for *L. monocytogenes*], *Pseudomonas aeruginosa* PAO1, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Escherichia coli* and *Staphylococcus aureus*). The last three pathogenic bacteria were clinical isolates obtained from the "CHRU" (Regional University University Hospital) of Brest. Five indicators of yeasts (*Rhodotorula mucilaginosa* UBOCC-A-202007; *Debaryomyces hansenii* CLIB197; *Saccharomyces cerevisiae* CLIB227; *Kluyveromyces marxianus* CLIB282; *Candida parapsilosis* CLIB214) and four molds (*Mucor plumbeus* CBS129.41; *Aspergillus niger* UBOCC-A-101073; *Aspergillus flavus* UBOCC-A-10826; and *Fusarium oxysporum* UBOCC-A-108079) commonly encountered in food spoilage in developing countries (Njongmeta et al., 2004; Djouldé et al., 2007; Yaouba et al., 2010) were also tested. Fungi were supplied by the Culture Collection of the University of Brest (UBOCC, Plouzané, France, http://www.univ-brest.fr/ubocc) and by the CLIB Yeast Culture Collection (CLIB, Thivernal-Grignon, France).

Preparation of *Bacillus* inocula

Bacillus strains cryopreserved in culture medium containing glycerol at 30% (v/v) were cultured on nutrient agar (NA) (AES Chemunex, Bruz, France) plates and incubated for 24 h at 37°C. The strains were then subcultured for 18 h at 37°C in 10 mL of trypton soy broth (TSB) (AES Chemunex, Bruz, France). These cultures were used for antimicrobial activities screening.

Preparation of fungal inocula

Molds' inocula were prepared by growing the molds on potato dextrose agar (PDA, AES Chemunex) slants at 25°C for 7 to 10 days until sporulation. The spores were then collected by vigorously shaking the slants with sterile peptone water (0.1%, w/v). Yeast cell inocula were prepared from cultures grown in M2Lev agar (20 g/L malt extract, 3 g/L yeast extract, and 15 g/L agar). Spores and yeasts concentrations were determined by microscopic Malassez cell counts and adjusted to 10^6 cells or spores/mL with sterile peptone water (0.1%).

Inhibition of indicators bacteria by cross-streak assay

The screening of inhibitory activity of *Bacillus* strains was realized according to the cross-streak assay described by Pugsley and Oudega (1987) with some modifications. Each *Bacillus* strain was picked from TSB and a single streak of this culture was applied both on a NA agar plate and on a glucose agar with bromocresol purple (BCP) plate using a 10 µL sterile loop. Cross-streak assay on BCP was aimed at verifying that inhibitory activity of *Bacillus* strains was not due to acid production. After incubating the plates for 18 h at 37°C, the densely overgrown streak was inactivated by applying chloroform (anhydrous, ≥ 99%, Fluka, Sigma-Aldrich, Saint-Quentin Fallavier, France) using a Pasteur-pipette to form a thin film covering the whole streak of bacteria. The Petri dish was left closed for 10 min to inactivate all living cells before they were opened for 10 min (under a hood) to evaporate the toxic chloroform. A streak of the tested pathogenic bacteria was then applied perpendicularly to the chloroform-inactivated *Bacillus* strain (Figure 1) to determine the inhibition activity. Standardized suspensions in 0.85% NaCl (Mc Farland Standard 1) of tested pathogen was applied with a 10 µL sterile loop as indicated by Zihler et al. (2009).

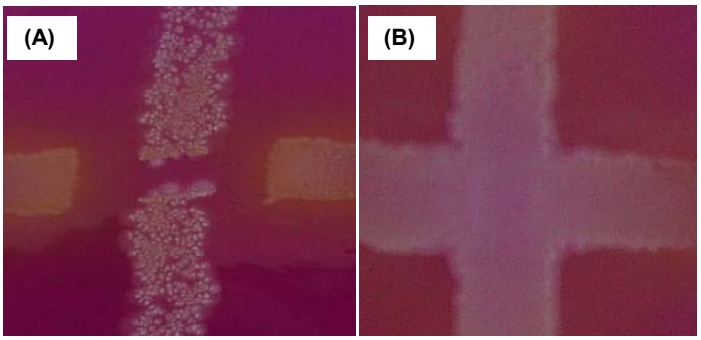

Figure 1. *In vitro* activity of *Bacillus* strains by Cross-streak with strong inhibition (A) and no inhibition on BCP agar (B).

Antifungal activity screening

The antifungal assay was performed by the overlay method described by Magnusson and Schnürer (2001). The method was realized using NA plates on which *Bacillus* strains were inoculated as a spot and incubated at 30°C. The plates were then overlaid with 10 mL of M2Lev soft agar (0.8% agar) containing 10^6 yeast cells or fungal spores per mL. The plates were examined qualitatively for clear zones of inhibition around the bacterial spots.

Total bacterial DNA extraction

The 26 *Bacillus* strains were grown for 18 h at 30°C on TSB (AES Chemunex). Tubes were centrifuged 10 min at 6000 *g* and the pellets were suspended in 0.5 mL of sterile physiological water (0.85% NaCl). DNA was extracted from suspended pellet and purified using the FastDNA® SPIN Kit (QBIOgene; MP Biomedicals, Solon, Ohio, USA) as recommended by the manufacturer's SPIN™ protocol. Pure DNA samples were frozen at -20°C until use.

PCR detection of genes encoding the bacteriocins subtilin and subtilosin

PCR were performed to screen for the presence of subtilin and subtilosin genes, two bacteriocins frequently encountered in *Bacillus* species (Abriouel et al., 2011). All the *Bacillus* strains were tested. Primers designed by Sutyak et al. (2008) were used for targeting the subtilin gene (*spaS*), whereas for subtilosin A (*sboA*), primers were designed using Primer 3 software (Rozen and Skaletsky, 2000) on alignment of known sequence encoding the *sboA* gene from *B. subtilis* subsp. *spizizenii* (acc. n°BSUW23_18455) (Table 1). One microliter (95-105 ng) of genomic DNA from each *Bacillus* strains or *B. subtilis* LMG 8197 used as positive control were added to 24 µl of a mix PCR consisting of each primer (1 µM), dNTP (0.2 µM), $MgCl_2$ (1.5 µM), buffer (5 µL of 5× buffer), water (10 µL) and 0.5 µL of GoTaq polymerase (Promega, Charbonnières, France) to make a final volume of 25 µL. PCR was carried out using a PTC-100 programmable thermal controller (MJ Research, Waltham, Massachussetts, USA) according to the following conditions as described by Sutyak et al. (2008): denaturation for 30 s at 94°C, annealing for 30 s at 55°C (*spaS*) or 50°C (*sboA*) and elongation for 1 min at 65°C for a total of 30 cycles. PCR products were electrophoresed on agarose gel (1.5% w/v), stained with ethidium bromide and visualized under UV.

PCR detection of genes encoding a larvicidal protein (Cry1) in *Bacillus thuringiensis* strains

The presence of *Cry1* genes commonly encountered in *B. thuringiensis* and known to encode for proteins active against many insect orders including *Lepidoptera, Diptera, Coleoptera*, *Hymenoptera, Homoptera, Mallophaga*, and *Acari* (Cinar et al., 2007) was also investigated in the two *B. thuringiensis* strains (S10 and SAc) with primers (CJI-1 and CJI-2) designed by Céron et al. (1995) (Table 1). PCR mixtures were prepared using 1 µl of each primer (25 µM), 0.5 µl of dNTP (10 µM), 1.5 µl of $MgCl_2$ (25 µM), 5µl of 5× Green Buffer GoTaq® Flexi Buffer, 0.5 µl of GoTaq® DNA Polymerase (Promega), and 14.5 µl of water to make a final volume of 24 µL. Amplification was performed using 1 µl (about 100 ng) of genomic DNA from *Bacillus thuringiensis* strains and *B. subtilis* LMG 8197 (negative control) and 24 µl of PCR mixture in a PTC-100 programmable thermal controller (MJ Research) according to the following conditions defined by Bobrowski et al. (2001): initial denaturation at 95°C for 2 min followed by 30 cycles consisting of a denaturation step of 95°C for 1 min; annealing step of 52°C for 1 min, and an extension step of 72°C for 1 min and a final extension of 72°C for 5 min. PCR products were analyzed by electrophoresis, stained with ethidium bromide and visualized under UV.

PCR screening of genes encoding *B. cereus* enterotoxins and virulence factors

The genes encoding the major *B. cereus* enterotoxins and virulence factors were investigated by PCR in the 26 *Bacillus* strains. Parts of the following genes encoding hemolysin (*hbl-D/A,* 623 bp), non hemolytic enterotoxin (nheB, 769 bp), *B. cereus* enterotoxin T (*bceT,* 428 bp) and enterotoxin FM (*entFM,* 1269 bp) were screened. Virulence factors were also investigated with the targeting of genes coding for two phospholipases associated with cell lysis, sphingomyelinase (*sph* gene, 558 bp) and phosphatidylinositol-specific phospholipase C (*piplc* gene, 569 bp). Reference studies and used primers (Sigma-Aldrich) are listed in Table 1. PCR mixture of 25 µl contained 20 to 35 ng of genomic DNA (*Bacillus* strains and *Bacillus cereus* LMG 6923 used as positive control), 1 µM of each primer, 0.5 µM dNTP, 1.5 µM of $MgCl_2$, GoTaq® DNA Polymerase, 5× Green Buffer GoTaq® Flexi Buffer and distilled water. Amplification was realized according to Matarante et al. (2004) and consisted of an initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 25 s, 55°C for 45 s, and 72°C for 2 min and a final extension at 72°C for 5min. PCR was performed with a PTC-100 programmable thermal controller (MJ Research). PCR products were analyzed on 1.5% (w/v) agarose gel stained with ethidium bromide and visualized by UV.

Hemolytic activity of *Bacillus* strains

Hemolytic activity was determined for all strains on blood agar plates (AES Chemunex) containing 5% of sheep blood. *B. subtilis* LMG 8197 and *Bacillus cereus* LMG 6923 were used respectively as negative and positive controls. Five microliters of cells grown overnight in TSB were inoculated as spots on blood agar plates which were incubated at 30°C for 24 h. Positive strains produced clear zone of hemolysis around the colonies.

Antibiotic susceptibility of *Bacillus* strains

The antibiotic susceptibility screening of the *Bacillus* strains was carried out by the disc diffusion method (Biomérieux, Marcy l'Etoile, France) as indicated by the producer. A single colony of *Bacillus* strains grown on NA plate was diluted in sterile 0.85% NaCl

Table 1. Primers used for PCR detection of genes encoding toxins, virulence factors, antimicrobial peptides and insecticidal proteins.

Target gene	Primer name	Primer sequence (5' - 3')	Amplicon size (bp)	Reference
Hbl-D/A	hblD-f	GGAGCGGTCGTTATTGTTGT	623	Matarante et al. (2004)
	hblA-r	GCCGTATCTCCATTGTTCGT		
nheB	nheB 1500S	CTATCAGCACTTATGGCAG	769	Granum et al. (1999)
	nheB 2269A	ACTCCTAGCGGTGTTCC		
bceT	ETF	TTACATTACCAGGACGTGCTT	428	Agata et al. (1995)
	ETR	TGTTTGTGATTGTAATTCAGG		
entFM	EntA	ATGAAAAAAGTAATTTGCAGG	1269	Asano et al. (1997)
	EntA	TTAGTATGCTTTTGTGTAACC		
Sph	Ph1	CGTGCCGATTTAATTGGGGC	558	Hisieh et al. (1999)
	Ph2	CAATGTTTTAAACATGGATGCG		
Piplc	PC105	CGCTATCAATGGACCATGG	569	Damgaard et al. (1996)
	PC106	GGACTATTCCATGCTGTACC		
Subtilin	spaSFwd	CAAAGTTCGATGATTTCGATTTGGATGT	125	Klein et al. (1992)
	spaSRev	GCAGTTACAAGTTAGTGTTTGAAGGAA		
Subtilosin	sboAf	ACAAAGGTTGTGCAACATGC	132	This study
	sboAr	TCCCCATAGACCGAATAGACC		
Cry1	CJI-1	TGTAGAAGAGGAAGTCTATCCA	280	Céron et al. (1995)
	CJI-2	TATCGGTTTCTGGGAAGTA		

Table 2. Interpretive criteria for MIC (mg/L) and MID (mm) by disc diffusion method.

Antibiotic	Minimal Inhibition Concentration (mg / L)		Minimal Inhibition Diameter (mm)	
	Sensitive	Resistant	Sensitive	Resistant
	≤	>	≥	<
Erythromycin	1	4	22	17
Vancomycin	4	8	17	X
Tetracyclin	4	8	19	17
Streptomycin	8	16	15	13
Kanamycin	8	16	17	15
Ampicillin	2	8	21	16
Trimethoprim-Sulfamethoxazol	2/38	8/152	16	10
Chloramphenicol	8	16	23	19

solution to obtain an Optical Density (OD) of Mc Farland Standard 0.5 (BioMérieux). Discs were purchased from BioMérieux and the antibiotics concentrations used were: erythromycin, 15 µg; vancomycin, 30 µg; tetracyclin, 30 µg; streptomycin, 10 µg; kanamycin, 30 µg; ampicillin, 10 µg; trimethoprim sulfamethoxazol, 1.25 µg + 23.75 µg and chloramphenicol, 30 µg. The bacterial suspension was applied to the surface of Mueller Hinton agar plates (AES Chemunex) using a sterile swab to obtain a homogenous bacterial film. The plates were then left to dry for 10 min before the disc were applied in the center of the plate. The plates were incubated at 37°C for 48 h. For tests strains susceptible to antibiotics, a clear area was observed around the disc. The diameters of inhibition areas were measured and interpreted according to the recommendations of the Antibiogram Committee of SFM, the French Society for Microbiology (2009) (Table 2).

RESULTS AND DISCUSSION

Antimicrobial activities of the *Bacillus* strains

The ability of the 26 *Bacillus* strains to inhibit indicators and pathogenic bacteria, yeasts and molds was investigated and reported in Tables 3 and 4. The antibacterial behaviour of *Bacillus* strains strongly varied between *Bacillus* strains and targets. *Bacillus* strains were both effective against Gram-positive and Gram-negative bacteria. *Bacillus cereus* ATCC6464 was inhibited by 14 strains, *Listeria innocua* HPB13 by 10 and *S. aureus* by 2 strains only. *E. coli* was the most sensitive

Table 3. Antimicrobial activity of the 26 *Bacillus* strains obtained with the cross-streak assay against 6 indicator bacteria.

Bacteria		Antibacterial activity						
Identitification	**Strain**	***Bacillus cereus* ATCC 6464**	***Listeria innocua* HPB13**	***Staphylococcus aureus***	***Pseudomonas aeruginosa* PAO1**	***Escherichia coli***	***Salmonella* Typhimurium**	**Inhibited species**
B. amyloliquefaciens	S1	+	+	+	-	+	-	4
	S5	++	++	-	-	++	-	3
	S13	+	++	-	-	+	-	3
	SX	+	++	-	-	+	-	3
B. thuringiensis	S10	+	-	-	-	-	-	1
	SAc	-	-	-	-	+	-	1
B. megaterium	S3	-	-	-	-	-	-	0
	S8	-	-	-	-	-	-	0
	S9	-	-	-	-	-	-	0
	S11	-	-	-	-	-	-	0
	S14	-	-	-	-	-	-	0
B. pumilus	S4	-	-	-	-	+	-	1
	S6	-	+	-	-	-	-	1
	S22	-	-	-	-	-	-	0
	S23	+	+	-	-	-	-	2
B. subtilis	S2	-	-	-	-	-	-	0
	S7	+	-	-	-	-	-	1
	S12	+	+	+	+	-	-	4
	S15	+	-	-	-	-	-	1
	S16	+	++	-	-	+	-	3
	S17	+	-	-	-	-	-	1
	S18	-	-	-	-	-	-	0
	S19	+	-	-	-	+	-	2
	S20	+	-	-	-	-	-	1
	S21	-	+	-	-	-	-	1
	SY	++	+	-	-	+	-	3

- , no inhibition; +, zone of inhibition between 6 and 8 mm; ++, zone of inhibition higher than 8 mm.

Gram-negative bacteria to *Bacillus* strains with nine inhibitions whereas *P. aeruginosa* PAO1 was inhibited with one strain only and *S. typhimurium* was not inhibited. The broadest spectrum of inhibition was shown by *B. amyloliquefaciens* S1 and *B. subtilis* S12 (with 4 sensitive targets) while the strongest effects were observed with *B. amyloliquefaciens* S5. *Bacillus cereus* showed weak inhibition ability and no *B. megaterium* was able to inhibit the tested bacteria.

As for bacteria, the ability of tested *Bacillus* spp. to inhibit fungal targets varied with *Bacillus* strains and tested fungi. However, the antifungal *Bacillus* species (*B. megaterium, B. thuringiensis* and *B. pumilus*) differed from the antibacterial ones (*B. amyloliquefaciens* and *B. subtilis*) (Table 4). Both molds and yeasts were inhibited. The most sensitive molds were *Fusarium oxysporum* UBOCC-A-108079 (sensitive to 9 *Bacillus* isolates), followed by *Aspergillus flavus* UBOCC-A-10826 and *Mucor plumbeus* CBS129.41 (3 *Bacillus*) while *Debaromyces hansenii* CLIB197 and *Kluyveromyces*

Table 4. Antimicrobial activity of the 26 *Bacillus* strains obtained with the spot test assay against 9 indicator fungi.

Bacteria	Code	Antifungal activity									Inhibited species
		Yeast						Mold			
		Rhodotorula mucilaginosa UBOCC-A-202007	*Debaryomyces hansenii* CLIB197	*Saccharomyces cerevisiae* CLIB 227	*Kluyveromyces marxianus* CLIB282	*Candida parapsilosis* CLIB214	*Mucor plumbeus* CBS129.41	*Aspergillus niger* UBOCC-A-101073	*Aspergillus flavus* UBOCC-A-10826	*Fusarium oxysporum* UBOCC-A-108079	
B. amyloliquefaciens	S1	-	-	-	-	-	-	-	-	-	0
	S5	-	-	-	-	-	-	-	+++	+	2
	S13	-	-	-	-	-	-	-	-	-	0
	SX	-	-	-	-	-	-	-	-	-	0
B. thuringiensis	S10	-	-	-	-	-	-	-	+	+	2
	SAc	-	-	-	+	+	+	+	-	+	5
B. megaterium	S3	++	-	+++	-	-	-	-	-	-	2
	S8	+	+++	+++	+++	++	-	-	-	+++	6
	S9	-	+++	+++	+++	+	-	-	-	+++	5
	S11	-	-	-	-	-	+	-	-	-	1
	S14	-	-	-	-	-	-	-	-	-	0
B. pumilus	S4	-	-	-	-	-	-	-	-	+	1
	S6	-	-	-	-	-	-	-	-	+	1
	S22	-	-	-	-	-	-	-	-	+++	1
	S23	-	-	-	+++	-	+++	-	+	+++	4
B. subtilis	S2	-	-	-	-	-	-	-	-	-	0
	S7	-	-	-	-	-	-	-	-	-	0
	S12	-	+	-	-	-	-	-	-	-	1
	S15	-	+	-	-	-	-	+	-	-	2
	S16	-	-	-	-	-	-	-	-	-	0
	S17	-	-	-	-	-	-	-	-	-	0
	S18	-	-	-	-	-	-	-	-	-	0
	S19	-	-	-	-	-	-	-	-	-	0
	S20	-	-	-	-	-	-	-	-	-	0
	S21	-	-	-	-	-	-	-	-	-	0
	SY	-	-	-	-	-	-	-	-	-	0

- , no inhibition; +: inhibition diameter between 4 and 6 mm; ++: inhibition diameter between 8 and 12 mm; +++: inhibition diameter higher than 12 mm.

marxianus CLIB282 were the most sensitive yeasts (inhibited with 4 *Bacillus* strains) followed by *Candida parapsilosis* CLIB214 and *Saccharomyces cerevisiae* CLIB227 (3 isolates). *Aspergillus niger* UBOCC-A-10826 and *Rhodotorula mucilaginosa* UBOCC-A-202007 were the most resistant fungi (inhibited by only 2 *Bacillus*). *B. megaterium* S8 (inactive against bacteria) showed the broadest activity spectrum and the strongest activity against six fungi (the 5 tested yeasts plus *Fusarium oxysporum* UBOCC-A-108079) followed by *B. megaterium* S9 (strong inhibition against 4 yeasts and *Fusarium oxysporum* UBOCC-A-108079). *B. thuringiensis*

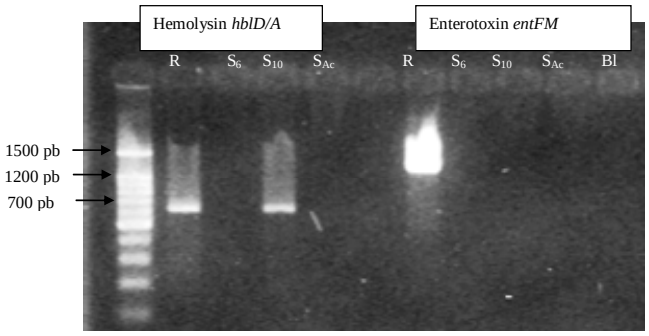

Figure 2. PCR gel of hemolysin *hbl D/A* (623 pb) and Enterotoxin FM *entFM* (1269 pb) genes. R is the reference strain of *Bacillus cereus* LMG 6923 used as a positive control, and B1 is the reference strain of *Bacillus subtilis* LMG 8197 used as a negative control.

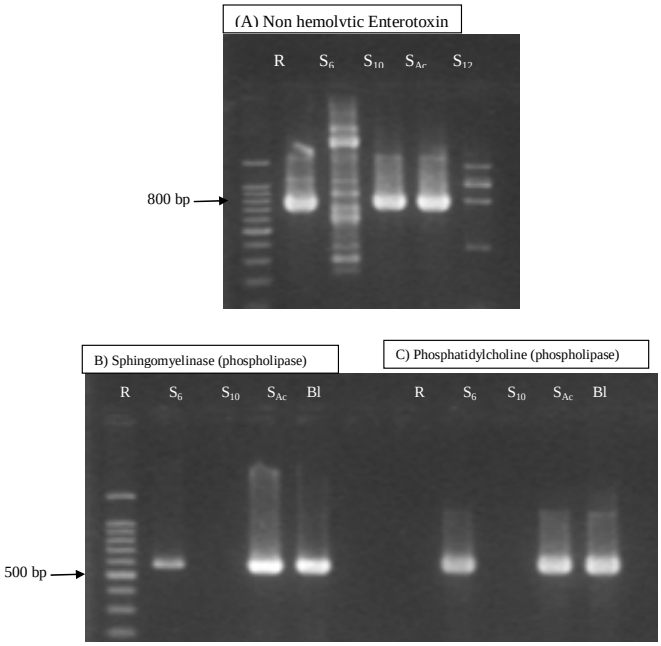

Figure 3. PCR gel of A) non hemolytic enterotoxin *nheB* gene (769pb), B) sphingomyelinase *sph* gene (558 bp) and C) phosphatidylinositol-specific phospholipase C *piplc* gene (569 bp). R is the reference strain of *Bacillus cereus* LMG 6923 used as a positive control, B1 is the reference strain of *Bacillus subtilis* LMG 8197 used as a negative control

showed a moderate but broad activity against 5 fungi while *B. pumilus* S23 showed lower antifungal ability (4 fungi inhibited). At the whole, *B. amyloliquefaciens* and *B. subtilis* isolates exhibited very
weak ability to inhibit both yeasts and molds.

PCR screening for bacteriocins and larvicidal proteins genes

The 26 *Bacillus* strains were assayed for the presence of two bacteriocins (subtilin and subtilosin) genes generally present in *B. subtilis* and related species. The PCR investigation revealed the presence of *spaS* (subtilin gene) in *B. subtilis* S2 only. Subtilosin A gene (*sboA*) was detected in *B. subtilis* S2, S7, S15, S17, S18, S19 and S20. Seven strains out of 11 *B. subtilis* contained either *spaS* or *sboA*. The screening of these bacteriocins encoding genes was negative for all other *Bacillus* species. The *Cry1* gene, encoding for a protein (Cry1) active against many insects was not detected in any of the 26 tested *Bacillus* strains including *B. thuringiensis* S10 and SAc.

PCR screening for toxins encoding genes

The presence of virulence genes was assessed for the 26 *Bacillus* strains by PCR screening (Figures 2 and 3). The presence of some genes of the enterotoxins responsible for diarrheal poisoning was confirmed for two strains identified as *B. thuringiensis.* Indeed, non hemolytic enterotoxin (*nhE*) genes were amplified by the specific primers in *B. thuringiensis* S10 and SAc while hemolysin gene (*hbl-D/A*) was detected only in SAc. However, *Bacillus cereus* enterotoxin (BcET) and enterotoxin FM genes were not amplified in all strains including the two *B. thuringiensis.* Two *Bacillus cereus* enzymes encoding genes were also screened: sphingomyelinase (*Sph*) and Phosphatidylinositol-specific phospholipase C (Piplc). These haemolytic and cell membrane hydrolytic enzymes genes were amplified in the 2 *B. thuringiensis* S10 and SAc. No other *Bacillus* species contained *Sph* and *Piplc* genes.

Hemolytic activity

Hemolytic activity was tested for the 26 *Bacillus* strains. Only the two *B. thuringiensis* strains (S10 and SAc) exhibited haemolytic activity on 5% sheep blood agar plates.

Antibiotics susceptibility

Relative susceptibilities to eight antibiotics were determined for 26 *Bacillus* strains (Table 5). The 26 strains were susceptible to erythromycin, vancomycin and streptomycin. Cases of resistance to antibiotics by at least one species were obtained for the six other antibiotics tested. Indeed, 18% of *B. subtilis* strains (strains S21 and SY) were resistant to tetracyclin; 25% of *B. pumilus* (S22) to kanamycin; 100% of *B. thuringiensis* (S10 and SAc) to ampicillin; 25% of *B. pumilus* (S22) to trimethoprim sulfamethoxazol; 25% of *B. amyloliquefaciens* (SX), 50% of *B. thuringiensis* (SAc), 40% of *B. megaterium* (S8 and S11) and 18% of *B. subtilis* (S19 and SY) were resistant to chloramphenicol.

Table 5. Antibiotic susceptibility (percentage) of the 26 *Bacillus* strains against nine tested antibiotics.

Bacteria	Number of strains	Antibiotics							
		E	VA	TE	S	K	A	SXT	C
B. amyloliquefaciens	4	100	100	100	100	100	100	100	**75**
B. thuringiensis	2	100	100	100	100	100	**0**	100	**50**
B. megaterium	5	100	100	100	100	100	100	100	**60**
B. pumilus	4	100	100	100	100	**75**	100	**75**	100
B. subtilis	11	100	100	**82**	100	100	100	100	**82**

E: Erythromycin; S: streptomycin; SXT: trimethoprim sulfamethoxazol; VA: vancomycin; K: kanamycin; TE: tetracyclin; A: ampicillin; C: chloramphenicol.

The most important antibiotic resistances for all species were observed in chloramphenicol. The most susceptible species were *B. megaterium* with resistance (40%) to only one antibiotic (chloramphenicol).

This study was carried out to investigate the potential food preservation and contribution to food safety as criteria for selection of starter cultures to be used in controlled fermentation of *Hibiscus sabdariffa* seeds. Both bacteria and fungi (yeasts and molds) are important food spoilage or pathogens in different food systems in developing countries. Some microorganisms evaluated in this study are regularly cited in food toxiinfections cases in Cameroon. Therefore, there is a need for affordable and safe methods to inhibit bacterial and fungal growth in fermented foods. This work documents the control of food spoilage and pathogenic bacteria and fungi by 26 *Bacillus* strains belonging to 5 species and their potential use as starters with a protective potential for a controlled and safe fermentation process. The strains showed different antibacterial and antifungal profiles between and within identified species. *Bacillus* strains active against bacteria were not effective against fungi.

With regard to antibacterial activities, *B. subtilis* and related species (*B. amyloliquefaciens*) induced different degree of inhibition depending on the tested species, independently of their Gram staining. Differences in antibacterial properties observed between closely related species were also observed in their phenotypic characteristics and could be explained by their genetic diversity (Mohamadou et al., 2013). The most antibacterial species were *B. amyloliquefaciens* followed by *B. subtilis* whereas *B. megaterium* induced no inhibition of bacteria and *B. pumilus* only two. *Bacillus* strains mainly inhibited, in a decreasing order, *B. cereus, L. innocua* (used as a model for *L. monocytogenes*), *S. aureus* and *E. coli,* which are among the most common foodborne pathogenic bacteria in Cameroon (Njongmeta et al., 2004; Djouldé et al., 2007). The inactivation tests showed that *B. amyloliquefaciens* S1 and S5 and *B. subtilis* S12 exhibited the broadest activity against the most common pathogens studied. *B. amyloliquefaciens* S1 and S5 inhibited Gram positive (*B. cereus* ATCC6464, *L. innocua* HPB13 and *S. aureus*) and Gram negative (*E. coli* and *P. aeruginosa* PAO1) bacteria. However, that none of the 26 strains was able to inhibit *Salmonella* Typhimurium, a leading and endemic cause of bloodstream infection in sub-Saharan Africa (Morpeth et al., 2009) is disappointing.

The pathways of antibacterial activities were partially screened. The cross-streak test on BCP indicated that the *Bacillus* species did not inhibit bacteria indicators through acid production, because the medium did not turn yellow as a sign of acidification. PCR screening of *spaS* and *sboA* genes showed that seven *B. subtilis* strains were susceptible to produce known bacteriocins: subtilin and/or subtilosin A. The production of these 2 antibacterial peptides is well documented and has been reported for *B. subtilis* and other related species like *B. amyloliquefaciens* (Klein et al., 1992; Stein et al., 2004; Sutyak et al., 2008). Subtilin, a cationic pentacyclic antimicrobial peptide is a lantibiotic that shows antimicrobial activity against a broad spectrum of Gram-positive bacteria (Abriouel et al., 2011). Subtilosin A shows a bactericidal activity against Gram-positive and Gram-negative bacteria (Shelburne et al., 2007; Sutyak et al., 2008). However, none of the most antibacterial strains (S1, S5 and S12) appears equipped to produce neither subtilin nor subtilosin A. These results suggest that *B. subtilis* and *B. amyloliquefaciens* strains isolated from Mbuja probably produced other antibacterial molecules active against Gram-positive and Gram-negative bacteria or they possess divergent sequences.

Molds and yeasts may also be pathogenic (through their toxins, like aflatoxins) and important food spoilage organisms in Cameroon (Mbiapo et al., 1989; Djouldé et al., 2007). *Bacillus* isolates were able to inhibit *in vitro* fungal growth. Both molds and yeasts were sensitive to representatives of the studied species. The most important antifungal activities were reported for *B. megaterium* strains while *B. subtilis* proved to be very weak fungi inhibitors. The strain *B. megaterium* S8 showed the broadest antifungal spectrum but inhibited mostly yeasts. In contrast, *B. amyloliquefaciens* S5 inactivated only two fungi, *A. flavus* and *F. oxysporum,* but they are among the most common food spoilage and poisoning fungi encountered in developing countries

(Yaouba et al., 2010).

As said earlier, none of the *Bacillus* strains produced sufficient organic acid to inhibit studied indicators. In addition, most fungi are weakly sensitive to organic acid. That *B. amyloliquefaciens* inhibits fungi is not surprising since Yoshida et al. (2001) earlier reported antibacterial and antifungal activity of this species through bacteriocin-like inhibitory substances (BLIS). But to our knowledge, the available literature has not yet reported the antifungal activity of *B. megaterium,* mostly known as a producer of broad spectrum bacteriocin active against food spoilage bacteria (Khalil et al., 2009). This study describes for the first time strains of *B. megaterium* active against fungi but not against bacteria.

Although no history of food intoxication by Mbuja was recorded (Mohamadou et al., 2009), the presence of at least one member of the *B. cereus* group (a well known poisoning organism) and the rare but possible production of toxin by non-cereus strains (From et al., 2005) led us to investigate the toxinogenic potential of the 26 isolates. Virulence factors and toxin encoding genes usually present in *B. cereus* were not detected in 24 strains of *B. subtilis, B. amyloliquefaciens, B. megaterium* and *B. pumilus.* However, positive PCR results were obtained for *hbL*, *nhE*, *spH* and *Piplc* genes but not for *bceT* and *entFM* genes on the two *B. thuringiensis* strains tested. These interesting results confirm the very low risk of food-borne disease due to other *Bacillus* species than *B. cereus* closest relatives and support their use as starter in a controlled fermentation process to produce a safe Mbuja. The present results go in the same line as those obtained by Matarante et al. (2004) who reported the absence of *B. cereus* toxin encoding genes in *B. subtilis* and *B. pumilus* isolated in industrial and artisanal cured sausages in Italy. When haemolytic activity was tested, only the two *B. thuringiensis* produced halos indicating a strong haemolytic power common in *B. cereus* and close related species.

This study suggests that *B. thuringiensis* strains S10 and SAc should be excluded in starter formulation to produce a controlled Mbuja. Nevertheless, another interesting use of *B. thuringiensis* is their potential to protect food crops from insects, pending on their ability to produce Cry1 proteins. These proteins are insecticidal on certain insects but not toxic to other insect, plants and animal. Due to its selective and specific action, the Cry1 protein has been use as efficient biological insecticide and an alternative to chemical insecticide (Bobrowski et al., 2001). *B. thuringiensis* tested in the present work were analysed for the presence of *Cry1* gene. No amplification product could be detected by PCR for these 2 strains. Recently, Bozlagan et al. (2010) investigated the presence of *Cry1* gene in 60 *B. thuringiensis* strains from agricultural fields and their bioactivity against larvae. These authors showed that only 17 isolates carried the *Cry1* gene, indicating that some *B. thuringiensis* may not have this gene. However, the absence of *Cry1* gene is not indicative of the total absence of insecticidal activity (Bobrowski et al., 2001).

Despite the absence of toxin genes in most of the species (except for *B. thuringiensis*), the interest in antibiotic resistance of the *Bacillus* strains could be justified by their possible side effects on certain Mbuja consumers. Indeed the immune-compromised consumers, in the context of general outbreak of HIV/AIDS in developing countries, may face increased risks of opportunistic infections. In addition possible antibiotic resistance gene transfer between *Bacillus* spp. and the intestinal microbiota on one hand and between *Bacillus* spp. and pathogenic bacteria on the other hand must be considered. All the strains displayed diversity in their susceptibility and resistance to the 8 antibiotics tested. The most effective antibiotics were erythromycin, vancomycin and streptomycin. Most species were resistant to chloramphenicol. Resistance to antibiotics was strain-dependent. Hence, *B. subtilis,* the main fermenting species isolated in Mbuja, exhibited 18% to 64% resistance to tetracyclin and chloramphenicol while all *B. thuringiensis* were resistant to ampicillin and chloramphenicol. Similar diverse susceptibilities were reported for these species and their close relatives by other studies in foods, environmental and clinical samples (Aslim et al., 2002; Schlegelova et al., 2003; Dautle et al., 2004; Luna et al., 2007; Adewumi et al., 2009; Chaves et al., 2011). At the whole, most of the strains were sensitive to different classes of antibiotics.

The most active *Bacillus* spp. against pathogenic and food spoilage micro-organisms were either antibacterial or antifungal. They were totally exempted with toxins genes and were sensitive to different antibiotics. A previous paper (Mohamadou et al., 2007) stated that *B. amyloliquefaciens* (S1 and S5) were highly amylolytic and proteolytic while *B. subtilis* S12 and *B. megaterium* S8 and S9 were highly to moderately proteolytic. These traits are important technological properties in fermenting proteinaceous *Hibiscus sabdariffa* seeds to produce Mbuja. These strains could therefore present a double significant advantage on the nutritional value and safety of Mbuja.

This work was carried out to contribute in selecting starter cultures for controlled production of Mbuja by assessing safety and potential risk of *Bacillus* strains. In the development of the starter culture, strains with important antimicrobial activities and sensitive to a maximum of antibiotics should be encouraged while toxinogenic and antibiotic-resistant strains should be discouraged. In this respect, this study raised the interest of five strains. *B. subtilis* S12 and *B. amyloliquefaciens* S1 were not toxinogenic, active against most pathogenic bacteria tested and were sensitive to all antibiotics. *B. megaterium* S9 was also not toxinogenic, sensitive to all antibiotics and active against 5 fungi. *B. amyloliquefaciens* S5 with strong activity against bacteria and two important pathogenic molds should also be

considered. Fortunately, these strains showed good technological properties. It will be suitable to undertake *Hibiscus sabdariffa* seeds fermentations based on the combination of these species to test their technological as well as their protective properties against a wider panel of pathogenic strains within Mbuja.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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