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Bacteriocin formation by dominant aerobic sporeformers isolated from traditional *maari*

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ABSTRACT

The antimicrobial activity of 8 *Bacillus* spp. and 2 *Lysinibacillus* spp. representing the predominant aerobic sporeformers during traditional *maari* fermentations, a traditional fermented baobab seeds product from Burkina Faso, was investigated. The antimicrobial activity was assessed against a total of 31 indicator organisms representing various Gram-negative and positive pathogens. The screening showed that 3 *Bacillus subtilis* strains (B3, B122 and B222) in particular had antimicrobial activity against some Gram-positive organisms and were selected for further studies. It was found that the antimicrobial substances produced were heat stable, in-sensitive to catalase, sensitive to protease and trypsin but resistant to the proteolytic action of papain and proteinase K and equally active at pH values ranging from 3 to 11. Bacteriocin secretion started in late exponential growth phase and maximum activity was detected during the stationary growth phase. The production of bacteriocin by *B. subtilis* B3, B122 and B222 was dependent on the aeration conditions. Maximum production of bacteriocin was observed under reduced aeration. Specific primers were used to screen isolates B3, B122 and B222 for genes involved in the synthesis of the bacteriocins subtilosin A, subtilin, sublancin and ericin. Amplicons of the expected sizes were detected for *iywB, sboA, sboX, albA* and *spaS* involved in the biosynthesis of subtilosin and subtilin, respectively. The translated nucleotide sequences had 100% identity to the YiwB, SboX and SboA amino acid sequences of the subtilosin A producing *B. subtilis* subsp. *subtilis* strain 168. Interestingly there was a 3 amino acid deletion at the N-terminal part of AlbA in B3, B122 and B222 that probably alters the activity of this enzyme. Analysis of the *spaS* gene sequences of B3, B122 and B222, encoding a subtilin precursor peptide, showed that the translated nucleotide sequence had 98% identity with the corresponding SpaS amino acid sequence of subtilin producing *B. subtilis* subsp. *spizizenii* strain ATCC6633.

1. Introduction

Fermentation, an old and economical method of producing and preserving food, is widely practiced in Africa (Mensah, 1997). *Maari* is a traditional condiment produced through a spontaneous, alkaline fermentation of baobab (*Adansonia digitata*) seeds. It is consumed in different regions of West African, including Burkina Faso, Mali, Benin and Nigeria (Parkouda et al., 2010). For traditional production of *maari*, seeds of *A. digitata* are boiled and fermented, resulting in a dark brown and pungent-smelling condiment (Parkouda et al., 2010) where a wide range of esters, organic acids, alcohols and ketones contribute to the flavour (Parkouda et al., 2011). The condiment is added to soups in many homes as a flavouring agent, but many urban and rural people also use it as a meat substitute. The fermentation of Baobab seeds into *maari* is dominated by *Bacillus* spp., especially *Bacillus subtilis*, with *Staphylococcus sciuri* and during the later stages of fermentation *Enterococcus faecium* playing a role as well (Parkouda et al., 2010).

However, the fermentation of baobab seeds into *maari* is spontaneous, uncontrolled and usually with varied fermentation times and temperatures, resulting in products inconsistent in quality attributes (Oguntoyinbo et al., 2007). In order to avoid growth of undesired microorganisms including pathogenic and spoilage microorganisms, starter cultures can be used. By the use of starter cultures it is possible to control fermentation of such condiments, avoiding growth of pathogenic and spoilage microorganisms, leading to a product of consistent taste and quality, as well as improved marketability (Ouoba et al., 2008).

Bacteriocins are potent antimicrobial peptides produced by bacteria in general targeting closely related organisms, and many are useful food preservatives (Cleveland et al., 2001). They inhibit target cells by forming pores in the membrane, depleting the transmembrane potential

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and/or the pH gradient, resulting in the leakage of cellular materials (Cleveland et al., 2001). Subtilin (Banerjee and Hansen, 1988), subtilosin A (Babasaki et al., 1985), ericin and sublancin (Abriouel et al., 2010) are bacteriocins produced by *B. subtilis*. Subtilin is structurally related to nisin, a known food preservative (Chan et al., 1993). Biosynthesis of subtilin by *B. subtilis* is dependent on the products of the genes *spaB, T, C, S, I, F, G, R* and *K* that are organized in a gene cluster (Entian and de Vos, 1996). Subtilosin A is a 35 amino acid bacteriocin originally isolated from *B. subtilis* (Babasaki et al., 1985) but also *Bacillus atrophaeus* (Stein et al., 2004) and *Bacillus amyloliquefaciens* (Sutyak et al., 2008) have been found to produce the bacteriocin. The production of mature subtilosin is based on the expression of the genes *sbo, albA, B, C, D, E, F* and *G* also organized in a gene cluster (Zheng et al., 1999). Ericin S (3442 Da) and ericin A (2986 Da) are two related lantibiotics produced by *B. subtilis* with strong similarities to subtilin (Stein et al., 2002). The ericin gene cluster contains two structural genes (*eriA, eriS*), and the open reading frames (ORFs) are closely related to corresponding genes of the subtilin cluster (Abriouel et al., 2010). Sublancin 168 (3878 Da) is a chromosomally encoded lantibiotic containing a single lanthionine linkage and two disulfide bridges. The operon responsible for sublancin production and immunity contains five genes: *sunA, sunT, bdbA, bdbB* and *sunI* (Abriouel et al., 2010). Subtilosin, subtilin, ericin and sublancin have been shown to be effective against various Gram-positive and Gram-negative pathogens such as *B. cereus, E. coli, L. monocytogenes* and *Staphylococcus aureus* (Liu and Hansen, 1993; Sutyak et al., 2008; Shelburne et al., 2007; Paik et al., 1998).

Bacteriocin production is strain dependent and strongly influenced by factors such as the chemical composition and pH of the medium (Moita et al., 2005) and the incubation conditions, such as temperature and aeration (Amiali et al., 2001).

The aim of the present study was to screen strains representing the predominant aerobic sporeformers during *maari* fermentation for production of antimicrobial substances; to characterize these compounds and to investigate how the atmospheric conditions influence the production of these compounds.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Eight *Bacillus* and 2 *Lysinibacillus* spp. strains isolated from different productions of *maari* (Parkouda et al., 2010) were screened for their antimicrobial activity against 31 indicator bacteria representing Gram-positive and Gram-negative bacteria (Table 1). *B. subtilis* subsp. *spizizenii* DSM 15029 and *B. subtilis* subsp. *subtilis* DSM 10 (ATCC 6051), encoding subtilin and subtilosin respectively, were used as positive controls in polymerase chain reaction (PCR) experiments.

All the strains were maintained as stock cultures at −80 °C in Brain Heart Infusion (BHI) medium (Oxoid, England) with 20% (v/v) glycerol as cryoprotectant.

2.2. Preparation of inocula of Bacillus and Lysinibacillus spp. isolated from maari

For all the experiments, inocula of the dominant aerobic sporeformers isolated from traditional *maari* (*Bacillus* spp. and *Lysinibacillus* spp.) were prepared as follows: From BHI agar plates incubated for 24 h at 37 °C, the *Bacillus* and *Lysinibacillus* strains were subcultured for 18 h at 37 °C in 10 ml of BHI broth, pH 7. The cultures were centrifuged at 5000 *g* for 15 min and the pellet resuspended in 10 ml of sterile saline (8.5 g/l NaCl and 1.5 g/l bactopeptone (Difco, France), pH 7.0). The number of cells/ml was estimated by microscopy using a counting chamber (Neubauer, Wertheim, Germany) and dilutions were made in sterile saline to obtain a rate of inoculation of 10^5 cells/ml.

Table 1
Indicator bacteria, *Bacillus* spp. and their origins (references).

Indicator bacteria and *Bacillus* spp.		Codes	Origin, references
Indicator bacteria	*Bacillus cereus* MADM 1291	Bc11	Birthday cake (food poisoning), Brazil
	Bacillus cereus MADM 1561	Bc14	Cooked chicken (food poisoning), Brazil
	Bacillus cereus NVH391-98	Bc98	Food poisoning, kindly provided by INRA, France[a]
	Bacillus cereus 007525	Bc00	Stew (food poisoning)[b]
	Bacillus cereus F4810-72	BcF	Emetic food poisoning[c]
	Bacillus cereus NC7401	BcNC	Emetic food poisoning[d]
	Bacillus cereus Ba18H2	BcPA	Cereulide producer isolated from Sonru[e]
	Bacillus cereus C14	G1	Gergoush[g]
	Bacillus cereus F16	G2	Gergoush[g]
	Bacillus cereus L31	G3	Gergoush[g]
	Bacillus cereus W2	G4	Gergoush[g]
	Bacillus cereus W23	G5	Gergoush[g]
	Bacillus cereus F6H1	G6	Afitin[e]
	Bacillus cereus F6H3	G7	Cereulide producer, Afitin[e]
	Bacillus cereus F6H7	G8	Afitin[e]
	Bacillus cereus F12H6	G9	Afitin[e]
	Bacillus cereus Ba48H3	G10	Sonru[e]
	Listeria monocytogenes 057	Lm1	Culture collection of Copenhagen University
	Listeria monocytogenes L028	Lm2	Culture collection of Copenhagen University
	Listeria monocytogenes Scott A	Lm3	Culture collection of Copenhagen University
	Micrococcus luteus SKN 624	Ml	Culture collection of Copenhagen University
	Salmonella Infantis SKN 557	Si	Culture collection of Copenhagen University
	Salmonella Oranienburg SKN 1157	So	Human
	Salmonella Nigeria SKN 1160	Sn	Cocoa beans
	Salmonella Thompson SKN 565	Sth	Culture collection of Copenhagen University
	Salmonella Typhimurium SKN 533	St1	Culture collection of Copenhagen University
	Salmonella Typhimurium SKN 1152	St2	Human
	Salmonella Typhimurium SKN 1155	St3	Animal
	E. coli 81 nr. 149 SKN 541	Ec	Culture collection of Copenhagen University
	Yersinia enterocolitica 6A28 SKN 599	Ye1	Culture collection of Copenhagen University
	Yersinia enterocolitica 8A30 SKN 601	Ye2	Culture collection of Copenhagen University
Bacillus spp.	*Bacillus subtilis* B3	B3	Isolated from traditional *maari*[f]
	Bacillus pumilus B47	B47	Isolated from traditional *maari*[f]
	Bacillus subtilis B55	B55	Isolated from traditional *maari*[f]
	Bacillus subtilis B122	B122	Isolated from traditional *maari*[f]
	Bacillus subtilis B222	B222	Isolated from traditional *maari*[f]
	Bacillus circulans B288	B288	Isolated from traditional *maari*[f]
	Bacillus megaterium B317	B317	Isolated from traditional *maari*[f]
	Bacillus pumilus B360	B360	Isolated from traditional *maari*[f]
	Lysinibacillus sphaericus B371	B371	Isolated from traditional *maari*[f]
	Lysinibacillus sphaericus B455	B455	Isolated from traditional *maari*[f]

[a] Lund et al., 2000.
[b] Stenfors and Granum, 2001.
[c] Turnbull et al., 1979.
[d] Agata et al., 1994.
[e] Thorsen et al., 2010.
[f] Parkouda et al., 2010.
[g] Thorsen et al. (2011).

2.3. Preparation of cell-free supernatant (CFS)

Each strain being investigated for antimicrobial properties was grown in 50 ml of BHI broth at 37 °C with agitation 120 rpm for

18 h. The cell-free supernatant (CFS) was obtained by centrifuging the culture (10,000 *g* for 30 min at 4 °C). The pH of the supernatant was adjusted to 7 with 1 N NaOH to exclude the antimicrobial effect of acid production, followed by filtration of the supernatant through a 0.45 μm pore-size filter.

2.4. Screening for antimicrobial activity under aerobic, microaerobic and anaerobic atmospheric conditions

An agar spot method was used to assess the production of antimicrobial compounds by 10 isolates representing the dominant aerobic sporeformers during fermentation of baobab seeds into traditional *maari* against 31 indicator bacteria. The agar spot test described by Ouoba et al. (2007) was carried out. A volume of 100 μl cell suspension of indicator microorganisms cultured for 18 h (about 10^7 CFU/ml) was mixed in 10 ml BHI agar in a Petri dish and allowed to solidify. Approximately 10^5 CFU/ml of the *Bacillus* spp. was spotted on the surface of BHI agar and the dish incubated at 37 °C for 24 h. The test was performed under aerobic, microaerobic (6% O_2, 12% CO_2) (Campygen, Oxoid) and anaerobic (<1% oxygen, 9–13% CO_2) (Anaerogen, Oxoid) atmospheric conditions. BHI broth was tested as a negative control on each plate. The presence of a clear zone around the spot indicating inhibition was measured and the results reported in mm. The experiment was performed in duplicate on three separate occasions.

The agar-well diffusion method described by Motta and Brandelli (2002) was used to investigate the antimicrobial effect of CFS from of the aerobic sporeformers.

For testing, 10 ml BHI agar inoculated with approximately 10^6 CFU/ml of indicator microorganism was mixed into a Petri dish and left to solidify. Wells were cut with a sterile cork borer (diameter: 6 mm) in the agar and 50 μl of CFS was added. The plates were incubated at 37 °C. The presence of a clear zone around the spot (24 h incubation) indicating inhibition was measured using a slide caliper and the results reported in millimeter. The experiment was performed in duplicate on three separate occasions.

2.5. Sensitivity of antimicrobial substance to catalase, proteolytic enzymes, heat and pH

The CFS from the cultures of B3, B122 and B222 was characterized with respect to thermal stability, pH sensitivity, sensitivity to catalase, and susceptibility to degradation by enzymes according to the method described by Cladera-Olivera et al. (2004). *Bacillus cereus* NVH391–98 was used as indicator organism in these and all subsequent experiments. The sensitivity after each treatment was determined using the agar-well diffusion method. In all assays, neutralized, untreated filtrates served as controls. Each assay was performed in duplicate.

Sensitivity to enzymes (all obtained from Sigma-Aldrich) was tested by treating CFS at 37 °C for 2 h with catalase (Product number C1345, bovine liver), papain from papaya latex (P2322), protease from *Streptomyces griseus* (P2325), proteinase K from *Tritirachium album* (P2548), and trypsin from bovine pancreas (P2328), each at a final concentration of 1 mg ml^{-1}. The activity remaining after enzyme digestion was determined by the agar-well diffusion method.

To determine the thermal stability, aliquots of each (CFS) were heated at 40 °C, 60 °C, 80 °C or 100 °C for 30 min, or autoclaved (121 °C) for 15 min; the aliquots of each (CFS) were also exposed to 4 °C for 2 weeks. The antimicrobial activity was then tested by the agar-well diffusion method.

For the determination of pH sensitivity the CFS was adjusted to pH 3, 4, 5, 6, 7, 8, 9, 10 and 11 with HCl or NaOH, incubated for 4 h at 37 °C and the activity remaining was determined by the agar-well diffusion method. BHI adjusted to pH 3, 4, 5, 6, 7, 8, 9, 10 and 11 served as control.

2.6. Estimation of size of the antimicrobial substance by SDS-PAGE

Proteins from 20 ml of CFS of *B. subtilis* strains B3, B122 and B222 were precipitated on ice by adding 50% w/v tricloroacetic acid (TCA) to reach a final concentration of 11% TCA. Proteins were pelleted by centrifugation at 15,000 *g* for 30 min at 4 °C and washed twice in 1/3 volume ice-cold acetone. Proteins were solubilized in 100 μl 10 mM MES pH 6 and 1 mM EDTA and the activity of the TCA precipitated proteins was tested by the well diffusion test against *B. cereus* BC98 as the indicator strain.

Proteins were separated on a 20% RunBlue SDS-page gel according to the manufacturer's protocol. The same volume from each strain was loaded on the gel with Invitrogens Mark 12 (LC5677) as standard. Following electrophoresis the gel was stained with Coomassie brilliant blue (0.1% Coomassie R-250, 40% ethanol, 10% acetic acid) for 1 h, destained 1 h (7.5% acetic acid, 10% ethanol), washed for 4 h in sterile distilled water to replace separation SDS and separation buffer in the gel. The gel was overlaid with 5 ml of soft BHI agar (0.7% w/v) inoculated with 10^5 cells/ml of the indicator bacteria *B. cereus* NVH391–98. The overlaid gel was incubated at 37 °C for 24 h and the inhibition zone was detected (Bizani et al., 2005).

For enhanced protein detection silver staining of a corresponding gel was preformed. In brief; proteins were fixed to the gel for 1 h (40% ethanol, 10% acetic acid) followed by three 30 s washes. The gel was sensitized for 5 min (0.02% sodium thiosulfate) before staining for 1 h with cold staining solution (0.1% silver nitrate, 0.02% formaldehyde). The gel was rinsed 3 times 1 min before development in 3% sodium carbonate, 0.05% formaldehyde staining was terminated when bands appeared by 5% acetic acid.

2.7. PCR detection of subtilin, subtilosin, sublancin and ericin encoding genes

DNA was extracted from cultures of *B. subtilis* (DSM 10, DSM 15029, B3, B122 and B222) according to the method described by Hansen and Hendriksen (2001). In brief, the cultures were grown on Luria-Bertani (LB) agar (Bertani, 1951) for 18 h at 25 °C. One colony of the culture was suspended in 250 μl of 1× TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and lysed at 100 °C for 10 min. The lysate was centrifuged at 15,000 ×*g* for 10 min and the supernatant used for PCR amplification.

Primers used for amplification of genes involved in *B. subtilis* subtilin, sublancin, subtilosin A and ericin biosynthesis are listed in Table 3. Primers were designed using the Primer3 (v. 0.4.0) software (Rozen and Skaletsky, 2000). The PCR reaction (25 μl) contained 2× PCR Master Mix/DreamTaq Green (12.5 μl) (Fermentas, Germany), sterile milli Q water (9.5 μl), 1 μl of each primer (10 pmol/μl) and 1 μl DNA (100 ng/μl). PCR amplification was conducted under the following cycle parameters: 95 °C for 3 min, then 30 cycles of denaturation at 95 °C for 30 s, annealing 58 °C for 30 s and elongation 72 °C for 1 min, followed by 10 min at 72 °C.

PCR products were sequenced (Macrogen Inc., South Korea) using the forward and reverse primers listed in Table 3, manually corrected using Chromas (vers. 2.33) software and aligned using ClustalW2. DNA sequences were blasted against available GenBank sequences using the Basic Local Alignment Search Tool (Altschul et al., 1997).

2.8. Kinetics of bacteriocin production of the B. subtilis B3, B122 and B222

Overnight cultures of *B. subtilis* B3, B122 and B222 were separately inoculated into 10 ml BHI broth and incubated at 37 °C under shaking at 120 rpm. When the culture reached an optical density at 600 nm (OD_{600}) = 1, 50 μl was transferred to 100 ml Erlenmeyer flasks containing 50 ml of BHI broth. Cultures were then incubated at 37 °C under shaking at 120 rpm and 2 ml aseptically removed every hour.

After centrifugation at 10,000 *g* for 30 min, supernatants were adjusted to pH 7 and filtered through a 0.45 μM filter (Xie et al., 2009). Antimicrobial activity of the supernatants was evaluated as described above by the agar-well diffusion method with *B. cereus* NVH391–98 as indicator strain. At the same time the optical density (Spectrophotomer Shimadzu, Kyoto, Japan) of the culture was measured at 600 nm and the pH recorded (PHM 210, Radiometer, Denmark). In parallel, samples were taken hourly to determine CFU/ml by plating on BHI agar.

2.9. Effect of aeration on bacteriocin production and growth of *B. subtilis* B3, B122 and B222

In order to investigate the effect of aeration conditions on growth and bacteriocin production of *B. subtilis* B3, B122 and B222, 25 ml, 50 ml and 75 ml of BHI broth (pH 7) in 100 ml loosely cotton plugged Erlenmeyer flasks were inoculated separately with the suspensions of each strain cultured for 18 h at a rate of 10^6 CFU/ml and incubated at 37 °C under shaking conditions (120 RPM). The same experiment was performed under static conditions. Optical density, pH, CFU/ml and inhibition zone were determined as described earlier for the well assay after 10 h and 20 h of incubation using *B. cereus* NVH391–98 as the indicator strain.

2.10. Nucleotide accession numbers

Nucleotide sequence data reported have been assigned GenBank accession numbers JN118835-JN118839.

3. Results

3.1. Screening for antimicrobial activity under aerobic, microaerophilic and anaerobic conditions

As shown in Table 2, the ability of the tested isolates to inhibit the indicators varied according to the isolate, the indicator microorganisms and incubation conditions. All tested *Bacillus* and *Lysinibacillus* spp. strains grew under anaerobic conditions but no inhibition of indicator organisms was observed in this atmosphere (Table 2). None of the strains tested were able to inhibit Gram-negative bacteria regardless of atmospheric conditions (Table 2 and results not shown). Except for *B. circulans* B288, and *Lysinibacillus sphaericus* B371, all the tested *Bacillus* spp. isolates (*B. megaterium, B. pumilus, B. circulans* and *B. subtilis*) and *Lysinibacillus sphaericus* B455 were able to inhibit growth of at least one indicator bacterium. The largest spectrum of inhibition was observed for *B. subtilis* strains B3, B122 and B222 showing activity against several strains of *L. monocytogenes* and *B. cereus*. Generally, the ability to inhibit the indicators was more pronounced under microaerophilic conditions compared to atmospheric conditions (Table 2). Strain B222 was found to inhibit more *B. cereus* strains as compared to B3 and B122 in aerobic condition. Based on their inhibitory spectrum, *B. subtilis* B3, B122 and B222 were selected for further investigation.

3.2. Sensitivity of antimicrobial substance to catalase, proteolytic enzymes, heat and pH

The results obtained for cell free, neutralized, enzymatically digested supernatants of B3, B122 and B222 showed that the inhibitory

Table 2
Antimicrobial activity spectrum of dominant aerobic sporeformers isolated from traditional *maari* grown on BHI agar for 24 h at 37 °C under aerobic, microaerophilic and anaerobic conditions: inhibition zone (mm) exhibited against the indicator bacteria.

Incubation conditions	Strains[a]	Indicator bacteria[a]															[b]
		Bc 00	Bc11	Bc14	BC98	BcF	BcNC	BcPA	Lm1	Lm2	Lm3	G1	G2	G3	G5	G7	
Aerobic conditions	B3	−	−	−	+	−	−	−	−	−	−	+	−	−	+	+	−
	B47	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
	B55	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
	B122	+	−	−	+	+	+	−	−	−	−	+	−	−	+	−	−
	B222	+	−	+	+	−	−	−	+	−	+	+	+	++	−	++	−
	B288	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B317	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
	B360	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−
	B371	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B455	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Microaerophilic conditions	B3	−	+	+	+	−	++	+	++	+	++	+	−	−	+	+	−
	B47	+	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−
	B55	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−
	B122	+	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
	B222	+++	++	+	+++	−	−	−	++	+	++	+	+	++	−	++	−
	B288	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B317	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
	B360	−	−	−	+	++	−	−	−	−	−	−	−	−	−	−	−
	B371	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B455	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Anaerobic conditions	B3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B47	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B55	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B122	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B222	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B288	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B317	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B360	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B371	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B455	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

−: no inhibition; +: 0<Inhibition Zone (IZ)<3 mm; ++: 3 mm≤IZ<5 mm; +++: IZ≥5 mm.
[a] Refers to strain abbreviations in Table 1.
[b] No inhibition was observed against the indicators M1, Si, Sn, So, St1, St2, St3, Sth, Ec, Ye1, Ye2, G4, G6, G8, G9 and G10.

activity was lost by treatment with protease and trypsin. However, little or no change in the size of the inhibitory zone was observed when the same filtrates were treated with papain, proteinase K and catalase. The inhibitory activity was not influenced by temperatures from 40 °C to 100 °C for 30 min and 121 °C for 15 min. There was no loss of inhibitory activity when stored for up 14 days at refrigerated temperature. The antimicrobial substance was active over a wide range of pH values, as full activity was retained at pH 3–11.

3.3. Estimation of size of the antimicrobial compound from B. subtilis B3, B122 and B222 by SDS-PAGE

Following electrophoresis, several proteins were detected in the CFS as seen in Fig. 1B.

By overlaying the gel with BHI containing *B. cereus* NVH391–98 as indicator an apparent single protein band associated with the antimicrobial activity of the 3 investigated *B. subtilis* strains was revealed as seen in Fig. 1A. The band associated with antimicrobial activity had an apparent molecular mass of approximately 3 kDa (Fig. 1A). For each *B. subtilis* strain, the same band was observed in culture supernatants sampled after 10 and 15 h of growth on BHI at 37 °C (results not shown).

3.4. PCR detection of bacteriocin biosynthesis genes

PCR was used to screen for genes involved in the biosynthesis of four antimicrobial substances reported to be produced by *B. subtilis* (Stein, 2005) namely subtilin, subtilosin, sublancin and ericin. Amplicons of the expected sizes (152 bp) were detected for *spaS* encoding the subtilin precursor peptide SpaS, whereas for subtilosin related genes (*ywiB, sboA, spoX, albA*) the amplicons were slightly smaller than expected being approximately 500 bp versus 536 bp (sbo-alb primers) and approximately 1170 versus 1200 bp (osboP primers) (Table 3). Unspecific products were observed with the ericin primers, while no PCR products were obtained with the sublancin primers (results not shown).

Analysis of the *ywiB, sboA, sboX* and *albA* gene sequences of B3, B122, and B222 showed high similarity between the three strains (1–2 bp difference out of 1071 bp). The translated nucleotide sequences of strains B3, B122, B222 showed 100% identity to the gene products YiwB (partial sequence), SboX (complete sequence) and SboA (complete sequence) of the subtilosin A producing *B. subtilis* subsp. *subtilis* strain 168 (GenBank reference sequences: NP_391615, YP_054594 and NP_391616). Interestingly there was a 3 amino acid deletion at the N-terminal part of AlbA (partial sequence) in B3, B122 and B222 as compared to in strain 168 (otherwise 99.2% identity to GenBank reference sequence NP_391617). Analysis of the partial *spaS* gene sequences of B3, B122 and B222 showed 100% identity between the three strains (152 bp). The translated nucleotide sequence showed 98.0% identity with the corresponding SpaS amino acid sequence of the subtilin producing strain *B. subtilis* subsp. *spizizenii* ATCC 6633 (GenBank no. AAA22778).

3.5. Production of antimicrobial substance during growth of B. subtilis B3, B122 and B222 in BHI broth

In order to study the production of antimicrobial activity during *B. subtilis* B3, B122 and B222 growth, the inhibitory activity against *B. cereus* NVH391-98 was evaluated at different time intervals (Fig. 2).

Growth reached early stationary phase within 3–4 h of incubation for B3, B122 and B222 (Fig. 2A, B and C). Antimicrobial substance production was observed from around 4–5 h, and maximum antibacterial activity was observed from 7 h to 12 h for B3 while for B122 and B222 maximum activity was observed after 15 h of incubation. After an initial slight decrease, pH values rose to more than 8 for all strains.

3.6. Effect of aeration on antimicrobial substance production and cell growth

Environmental factors strongly influence the formation of antibacterial substances by bacteria. The effect of aeration on production of compounds with antimicrobial activity by *B. subtilis* B3, B122 and B222 was studied by varying the volume of the growth medium where aliquots of 25, 50 and 75 ml were inoculated with each test strain (B3, B122 and B222) (Table 4). No antimicrobial effect was produced by B3 and B122 under static conditions whereas B222 exhibited a slight inhibitory activity after 20 h of growth in 25 and 50 ml (Table 4). Under shaking conditions, *B. subtilis* B3, B122 and B222 exhibited antimicrobial activity against the indicator when inoculated in all 3 different volumes of BHI broth (25, 50 and 75 ml). However, stronger activity was observed at reduced aeration (50 ml BHI under shaking conditions) compared to low aeration (75 ml BHI under shaking conditions) and high aeration (25 ml BHI under shaking conditions). *B. subtilis* B3, B122 and B222 grew to similar cell

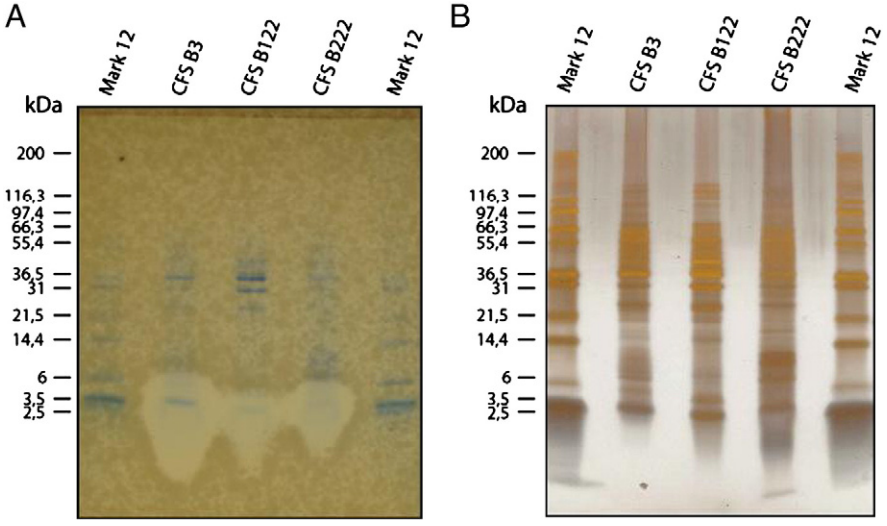

Fig. 1. SDS-PAGE gel of precipitated proteins from cell-free supernatants of B3, B122 and B222. A): Direct detection of antimicrobial activity by overlaying the gel with soft BHI agar containing the indicator organism *Bacillus cereus* BC98. Inhibition zones were observed after 24 h incubation at 37 °C. B): Silver staining of a corresponding gel for increased protein detection.

Table 3
Primers used for PCR detection of bacteriocin biosynthesis genes from *Bacillus subtilis* strains, B3, B122 and B222 isolated from *maari*.

Bacteriocin	Genes	Primers	Sequences (5′→3′)	Expected PCR product size/detected	Accesion no. Primer pos.[b]	Reference
Subtilosin	*ywiB* *sboA* *sboX* *albA*	osboP1N[a]	5′-CCTCATGACCAGGACTTCGCCTT-3′	1200 bp/yes	AL009126 3835545–3835567→ 3836764–3836745←	Present study/Zheng et al. (1999)
		osboP2N[a]	5′-CGGTGCCGAGCGCTTCAGGT-3′			
	spoA *sboX* *albA*	sbo-alb_F	5′-CATGCTCGATCGGAGCCGCT-3′	536 bp/yes	AL009126 3836098–3836117→ 3836633–3836614←	Present study
Subtilin	*spaS*	sbo-alb_R	5′-CGGCTGGCCCGGTTTCCAAG-3′			Sutyak et al. (2008)
		spaS_Fwd	5′-CAAAGTTCGATGATTTCGATTTGGATGT-3′	152 bp/yes		
Sublancin	*sunT*	spaS_Rev	5′-GCAGTTACAAGTTAGTGTTTGAAGGAA-3′			Chung et al. (2008)
		SUNT-F1	5′-GCTTTGTTAGAAGGGGAGGAAT-3′	974 bp/no		Present study
		SUNT-R1	5′-CTTGTCCCAACCCATAGGATAA-3′			
Ericin	*eriC* *eriSa* *eriSb*	Eric_F	5′-TCAACTGACCGGGCAGGAGC-3′	1440 bp/no	AF233755 6427–6446→ 7866–7843←	
		Eric_R	5′-AAGTATTTGGCCTACAGCGACTCG-3′			

[a] Based on primers developed by Zheng et al., 1999, the original primers were osboP1 : CCTCATGACCAGGACTTCGCCTTCGCTTACTTT, and osboP2 CGGTGCCGAGCGCTTCAGG-TAAGCTTTCCAAA .
[b] →upper-strand sequence; ← lower-strand sequence.

counts regardless of incubation under static or shaking conditions. The pH was higher (between 7.6 and 8.6) in the 25 ml as compared to incubation in 50 ml (between 6.8 and 8.1) and 75 ml (between 6.7 and 7.0) at 20 h of incubation under shaking conditions. The pH was between 6.3 and 7.5 when antimicrobial activity was detected (shaking conditions).

4. Discussion

The screening for antimicrobial activity of aerobic sporeformers isolated during fermentation of baobab seeds into *maari*, showed that the ability of the tested isolates to inhibit the indicators varied according to the isolate, the indicator microorganisms and incubation conditions. No inhibition was observed under anaerobic conditions. None of the strains tested were able to inhibit Gram-negative bacteria regardless of atmospheric conditions which is in agreement with results reported by He et al. (2006) and Hyronimus et al. (1998). Except for *B. circulans* B288, and *Lysinibacillus sphaericus* B371, all the tested *Bacillus* spp. isolates and *Lysinibacillus sphaericus* B455 were able to inhibit growth of at least one indicator bacteria. Among all the indicator strains tested, *B. cereus* NVH391–98 was found to be the most sensitive indicator, being inhibited by most of the selected strains. The largest spectrum of inhibition was observed for *B. subtilis* strains B3, B122 and B222 inhibiting cereulide and diarrheal toxin producing *B. cereus*, an etiologic agent of emetic and diarrheal syndromes (Stenfors et al., 2008) as well as the food-borne pathogen *Listeria monocytogenes*. *B. cereus* is often isolated from African fermented foods, and occasionally occurs in high numbers of up to 10^9 CFU/g (Padonou et al., 2009; Parkouda et al., 2009, 2010). Inhibition of opportunistic human pathogens such as *B. cereus* is of considerable interest to enhance the hygienic quality and safety of *maari* and other African fermented condiments. These results suggest that the use of starter culture preparations containing B3, B122 and B222 might prevent the occurrence of pathogenic bacteria during the fermentation of Baobab seeds leading to a safe product.

B. subtilis isolates have been reported to produce proteinacious antibiotics such as subtilin, subtilosin, sublancin (Paik et al., 1998; Stein et al., 2004), which are mostly active against Gram-positive bacteria (Ouoba et al., 2007). The antimicrobial substances produced by B3, B122 and B222 were heat stable and active over a range of pH values, with full activity retained after an hour at 100 °C and across the pH range of 3–10. These observations are in agreement with the results reported by He et al. (2006) and Hyronimus et al. (1998) and increase the likelihood of these antimicrobial compounds being suitable for food preservation purposes (Sutyak et al., 2008). The inhibitory activity of *B. subtilis* B3, B122 and B222 CFS was not influenced by catalase treatment and exposure to papain and proteinase K but the activity was lost completely after protease and trypsin treatment, thereby revealing their proteinaceous nature. Stein et al. (2002) found that subtilin was resistant to proteinase K whereas ericin S was resistant to trypsin and pepsin. Subtilosin activity has been reported to be completely lost in the presence of pepsin and proteinase K, and significantly decreased by trypsin and chymotrypsin (Sutyak et al., 2008). Using SDS-PAGE it was estimated that the apparent molecular masses of the antimicrobial substances produced by B3, B122 and B222 were approximately 3 kDa. Considering these properties, the antimicrobial substances were characterized as bacteriocin-like substances (BLS) (Hyronimus et al., 1998). The fact that the BLS retained their activity after exposure to papain and proteinase K, indicates that the BLS are cyclic peptides and therefore more resistant to protease hydrolysis (Bizani and Brandelli, 2002). The BLS were detected from early stationary growth phase and maximum activity was observed some hours into the stationary growth phase. The decrease followed by the disappearance of the antagonistic activity during late stationary phase may be attributed to adsorption to producer cells or to degradation by proteolytic enzymes from the cells (Parente and Ricciardi, 1999).

The antimicrobial activity of partially purified proteins from the CFS was associated with a single protein band with an apparent molecular mass of about 3 kDa (Fig. 1: A and B). The same band was observed in CFS obtained after 10 and 15 h of growth indicating that the observed antibacterial activity in the late stationary growth phase corresponded to the same bacteriocin and is characterized by a very good physical stability.

B. subtilis B3, B122 and B222 were shown by PCR with specific primers (Table 3) to harbour genes of the subtilosin operon, as well as *spaS* from the subtilin operon. The genes *eriC* and *sunT* involved in ericin and sublancin synthesis respectively were not detected in any of the 3 strains. Subtilin is a bacteriocin belonging to the class I bacteriocins or lantibiotics and has been described as a nisin analog (Klaenhammer, 1993). Several *B. subtilis* species, including the closely related species *B. atrophaeus* and *B. amyloliquefaciens* have been shown to produce subtilosin (Stein et al., 2004; Sutyak et al., 2008). The molecular mass of the band exerting antimicrobial activity in the SDS gels was similar to the molecular weight of subtilosin (3.40 kDa; Marx et al., 2001) and subtilin (3.32 kDa; Stein, 2008). The inhibition of *L. monocytogenes* and *B. cereus* by subtilosin (Sutyak et al., 2008) and subtilin (Liu and Hansen, 1993) has

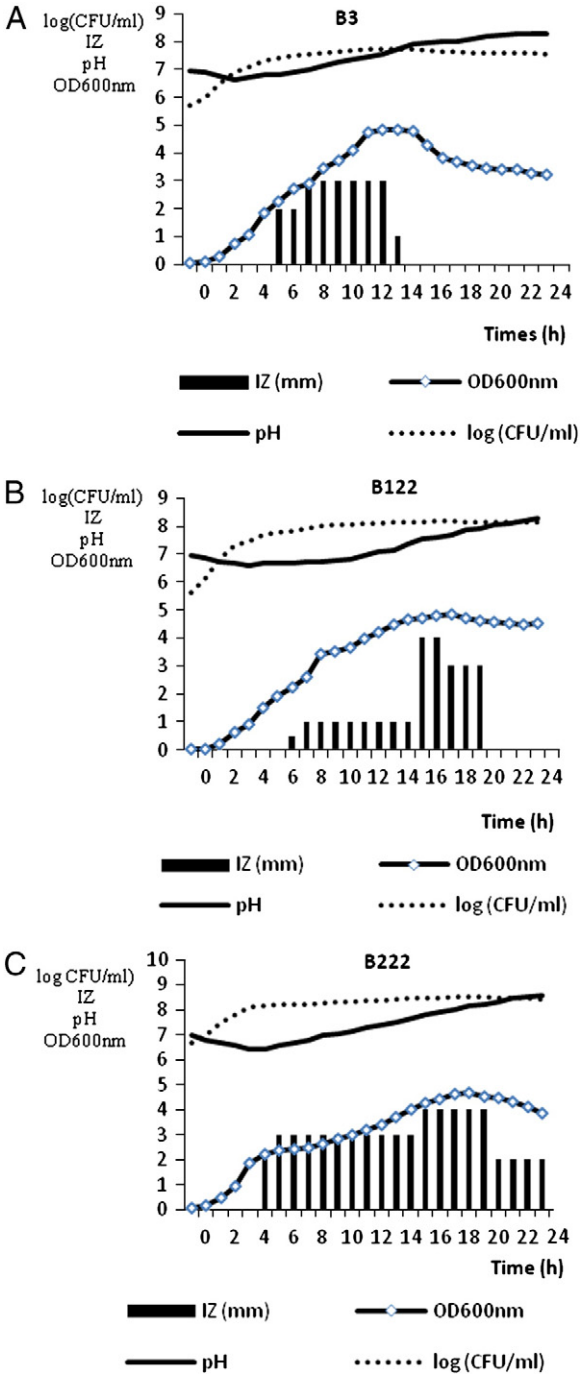

Fig. 2. Kinetics of bacteriocin production during the growth of *Bacillus subtilis* B3 (panel A), B122 (panel B) and B222 (panel C) in BHI broth at 37 °C for 24 h. Optical density at 600 nm (OD600nm), Inhibition Zone against *Bacillus cereus* NVH391-98 (IZ in mm), pH and CFU/ml was registered hourly. Each point represents the average of 2 repeated measurements from 2 independently replicated experiments with a standard deviation less than 5% about the mean.

previously been reported underlining the potential of subtilin and subtilosin A producers to form part of a starter culture consortium producing safer foods.

The identity of the genes involved in the biosynthesis of subtilin and subtilosin A was confirmed by sequencing of related amplicons. Sequencing showed that both subtilin and subtilosin A encoding genes were found in *B. subtilis* B3, B122 and B222. The sequenced fragments had 100% identity with the subtilosin A gene (*sboA*) from *B. subtilis* subsp. *subtilis* strain 168 and 96.2% identity with the subtilin

Table 4
Effect of aeration on bacteriocin production and cell growth at 37 °C (neutralized cell-free supernatant from *B. subtilis* B3, B122 and B222 was tested against *Bacillus cereus* NVH391-98) using agar-well diffusion assay; see text for details).

Bacillus strains	Volume of BHI broth	Incubation time	Static conditions			Shaking conditions, 120 rpm		
			pH	$\log_{10}$ (CFU/ml)	IZ (mm)	pH	$\log_{10}$ (CFU/ml)	IZ (mm)
Bacillus subtilis B3	25 ml BHI	0 h	6.9	6.1	0	6.9	6.0	0
		10 h	7.0	8.7	0	7.2	8.8	0
		20 h	7.4	8.7	0	7.6	8.9	0
	50 ml BHI	0 h	6.9	6.1	0	6.9	6.1	0
		10 h	6.8	8.7	0	6.7	8.7	3
		20 h	6.9	8.6	0	7.7	8.8	0
	75 ml BHI	0 h	6.9	6.3	0	6.9	6.3	0
		10 h	6.7	8.3	0	6.6	8.3	2
		20 h	6.8	7.4	0	7.0	8.6	0
Bacillus subtilis B122	25 ml BHI	0 h	6.9	6.2	0	6.9	6.2	0
		10 h	6.9	8.2	0	7.5	8.3	1
		20 h	7.1	8.2	0	8.6	8.7	0
	50 ml BHI	0 h	6.9	6.3	0	6.9	6.3	0
		10 h	6.8	8.5	0	6.7	8.7	2
		20 h	7.0	8.1	0	8.1	8.7	0
	75 ml BHI	0 h	6.9	6.2	0	6.9	6.2	0
		10 h	6.7	8.1	0	6.3	8.4	1
		20 h	6.8	8.7	0	6.7	8.8	0
Bacillus subtilis B222	25 ml BHI	0 h	6.9	6.7	0	6.9	6.7	0
		10 h	6.7	8.4	0	7.0	8.6	4
		20 h	7.4	8.5	0,5	8.4	8.8	2
	50 ml BHI	0 h	6.9	6.7	0	6.9	6.7	0
		10 h	6.6	8.1	0	6.7	8.3	1,5
		20 h	7.2	8.0	1	6.8	8.8	5
	75 ml BHI	0 h	6.9	6.7	0	6.9	6.7	0
		10 h	6.5	8.0	0	6.6	8.3	0
		20 h	6.9	8.0	0	6.8	8.6	3

gene (*spaS*) from *B. subtilis* subsp. *spizizeni* strain ATCC6633. In the present study, an approximately 34 bp deletion (corresponding to positions 3836300–3836333 in *B. subtilis* subsp. *subtilis* 168; GenBank: AL009126.3) was observed within the subtilosin A gene cluster of *B. subtilis* B3, B122 and B222. The missing part partly belongs to the gene *albA* (located at 3836323–3837669). The first gene of the *alb* operon, *albA* (*ywiA*) (Kunst et al., 1997), encodes a protein with significant homology to those that function in cofactor heme, Pyrroloquinoline Quinone (PQQ), and molybopterin cofactor synthesis (Zheng et al., 1999). The product of *albA* (*ywiA*) is believed to be necessary for pro-subtilosin modification (Zheng et al., 1999). Thus the deletion of this part might modify the activity of the final subtilosin A. Alternatively, the small deletion might also inactivate the ability of strains B3, B122 and B222 to produce subtilosin A with biological activity.

The stability at high temperatures of the antimicrobial compound/compounds produced by B3, B122 and B22 is similar to that observed for both subtilin and subtilosin A (Sutyak et al., 2008; Mannanov and Sattarova, 2001). Similar to what have been reported for subtilin (Kuboi et al., 1994) and subtilosin (Stein et al., 2004), the inhibitory substances from B3, B122 and B222 were detected from early stationary growth phase. Nakano et al. (2000) reported that subtilosin transcription was increased under oxygen-limited and anaerobic conditions. For the strains investigated in the present study maximum production of bacteriocin was observed under reduced aeration (50–75 ml BHI broth, 120 rpm) and microaerophilic condition (agar system), while generally no production was observed in static conditions (25–75 ml broth) and under anaerobic (agar system) conditions.

5. Conclusion

The predominant aerobic sporeformers isolated from traditional *maari* were investigated and the results revealed that 3 *B. subtilis* strains (B3, B122 and B222) were able to produce bacteriocins with activity against Gram-positive pathogens such as *L. monocytogenes*

and *B. cereus*. Aeration plays an important role on cell growth and production of antimicrobial activity by the selected strains as higher bacteriocin production was observed under reduced aeration for the 3 *B. subtilis* strains (B3, B122 and B222). The genes involved in subtilin and subtilosin biosynthesis were detected in all the 3 *Bacillus subtilis* isolates. The ability to produce a bacteriocin with activity against *B. cereus* makes the investigated strains promising candidates to form part of a starter culture consortium inhibiting growth of pathogens and spoilage bacteria during production of *maari*.

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