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A B S T R A C T

*Maari* is a spontaneously alkaline fermented food condiment made from baobab tree seeds. Due to the spontaneous nature of *maari* fermentations growth of the opportunistic human pathogen *Bacillus cereus* is occasionally observed. *Bacillus subtilis* strains are important for alkaline seed fermentations because of their enzymatic activities contributing to desirable texture, flavor and pH development. Some *B. subtilis* strains have antimicrobial properties against *B. cereus*. In the present work, three bacteriocin producing *B. subtilis* strains (*B3, B122 and B222*) isolated from *maari* were tested. The production of antimicrobial activity by the three strains was found to be greatly influenced by the substrate. All three *B. subtilis* strains produced antimicrobial activity against *B. cereus* NVH391-98 in BHI broth as determined by the agar well diffusion assay, whereas no antimicrobial activity was detected in whole cooked baobab seeds and in 10% (w/v) grinded baobab seeds. Incorporation of BHI with up to 5% (w/w) grinded baobab seeds enhanced the antimicrobial activity of *B. subtilis* compared with pure BHI in a strain dependent manner. Incorporation of BHI with 50% (w/w) baobab grinded seeds decreased the antimicrobial activity. Addition of the inorganic salts FeCl3, MgSO4 and MnSO4 has previously been reported to increase bacteriocin production of *B. subtilis*, but the addition of these salts to 10% (w/v) grinded baobab seed broth did not cause antimicrobial activity. Survival of *B. cereus* NVH391-98 in co-culture with *B. subtilis* was tested in BHI broth, 10% (w/v) grinded baobab seed based broth and during baobab seed fermentation to produce *maari*. *B. cereus* NVH391-98 grew well in all three substrates in mono-culture. All the 3 *B. subtilis* strains were able to decrease *B. cereus* NVH391-98 to levels below the detection limit (<10 CFU/ml) in BHI, but not in baobab seed based substrates, even though the outgrowth of *B. cereus* NVH391-98 was delayed by up to 40 h. In conclusion, production of antimicrobial activity by the investigated *B. subtilis* strains is highly substrate-specific and strain-specific. The three *B. subtilis* strains delayed but did not prevent *B. cereus* outgrowth during baobab seed fermentations. *Maari* is produced through mixed microbial population fermentations. *B. subtilis* co-starter culture candidates originating from *maari* which are able to prevent pathogen outgrowth remain to be identified.

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B. cereus is considered an opportunistic human pathogen with the potential for causing food borne illnesses such as vomiting through the production of cereulide, and diarrhea through the production of various enterotoxins (Stenfors Arnesen et al., 2008). Contrary to non-spore forming pathogenic bacteria, spore formers such as B. cereus are likely to survive the cooking process if in the spore state, when preparing dishes with maari.

There is an increased interest in developing starter cultures in order to control fermentation of various African condiments, to avoid growth of pathogenic and spoilage microorganisms, leading to a product of consistent taste and quality, as well as improved marketability (Ouoba et al., 2008). In addition to positively affect organoleptic properties of alkaline fermented seed products, indigenous B. subtilis strains have been shown to produce antimicrobial substances with activity towards a broad spectrum of microorganisms (Kaboré et al., 2012; Ouoba et al., 2007). B. subtilis strains could therefore potentially play an important role as protective starter cultures against pathogenic microorganisms, a role previously suggested for bacteriocin producing lactic acid bacteria (Cotter et al., 2005).

At present no starter cultures suitable for production of maari have been developed. However, in a previous study, the properties of dominant 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 antimicrobial substances with activity against the Gram positive pathogens L. monocytogenes and B. cereus (Kaboré et al., 2012). The genes involved in subtilin and subtilosin biosynthesis were detected in all the 3 B. subtilis isolates (Kaboré et al., 2012). The results which were obtained indicated that the three B. subtilis strains had the potential to serve as protective cultures for maari production (Kaboré et al., 2012).

With the goal of selecting strains of B. subtilis for development as protective starter cultures in maari production, this paper investigates the effect of baobab seed extracts and baobab seeds on the ability of B. subtilis strains B3, B122 and B222 to inhibit the growth of B. cereus.

2. Materials and methods

2.1. Bacterial strains and culture conditions

B. subtilis B3, B122 and B222 (Kaboré et al., 2012; Parkouda et al., 2010), B. cereus NVH391-98 (Lund et al., 2000) (used as indicator microorganism), B. subtilis subsp. spizizenii DSM 15029 and B. subtilis subsp. subtilis DSM 10 (used as positive controls in bacteriocin production experiments) were maintained as stock cultures at −80 °C in Brain Heart Infusion (BHI) medium (Oxoid, Basingstoke, UK) with 20% (v/v) glycerol as a cryoprotectant. The strains were subcultured in 10 ml BHI broth at 37 °C for 24 h and subsequently streaked on BHI agar plates (18 h at 37 °C) before use.

Inocula used for antimicrobial production and co-culture experiments were prepared as follows: from BHI agar plates incubated for 18 h at 37 °C, the cells were sub-cultured for 18 h at 37 °C in 10 ml of BHI broth, pH 7. The cultures were then centrifuged at 5000 g (4 °C) for 15 min, the supernatant removed and the pellet re-suspended in 10 ml of sterile saline solution containing 8.5 g/l NaCl and 1.5 g/l bactopeptone (Difco, France), pH 7.0. Cell count was determined by use of a counting chamber (Neubauer, Wertheim, Germany) and dilutions were made in sterile saline solution to obtain approximately 7 log_{10} CFU/ml.

2.2. Effect of baobab seed extract on the production of antimicrobial substances by B. subtilis

Baobab seeds are the raw material for maari production and are prepared by an initial cooking process after which a spontaneous fermentation involving B. subtilis occurs (Parkouda et al., 2010). The following experiments were conducted to test the effect of baobab seed extracts on the production of antimicrobial substances by B. subtilis. Baobab seeds, collected in Burkina Faso, were ground using a crusher (Bosch MKM 6003). The ground seeds were then added in different amounts to the culture medium BHI (Oxoid) in 100 ml conical flasks. The following amounts were used: BHI control (no ground seeds); BHI containing 0.05% (w/w) ground seeds; BHI containing 0.5% (w/w) ground seeds; BHI containing 5% (w/w) ground seeds; BHI containing 50% (w/w) ground seeds. In addition, flasks containing 5 g of ground seeds and 5 g of whole seeds respectively were set up. Distilled water (50 ml) was added to each flask. The flasks of each mixture were cooked at temperatures of 95–98 °C for 7 h according to the protocol for preparation of baobab seeds for maari fermentation (Compaoré, 2009), with addition of distilled water to maintain a final volume of 50 ml after 7 h of cooking. The different mixtures were inoculated with B. subtilis to obtain a final population of 6 log_{10} CFU/ml, mimicking the levels which would be used for a starter culture, and incubated at 37 °C, at 120 rpm. Antimicrobial activity production of B. subtilis strains B3, B122 and B222 has previously been shown to be present from early stationary phase (Kaboré et al., 2012). The samples were therefore collected at 0, 10 and 20 h for pH (PHM 210, Radiometer, Copenhagen, Denmark), viable count (CFU/ml) (on BHI agar) and determination of antimicrobial activity of cell free supernatants (CFS) against B. cereus NVH391-98. The CFS were prepared 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, followed by filtration through a 0.45 μm pore-size filter (Q-Max™ PES, Frisenette, Knebel, Denmark).

2.3. Assay of antimicrobial activity

The agar-well diffusion method described by Motta and Brandelli (2002) was used to investigate the antimicrobial effect of the cell free supernatants (CFS). Briefly, 10 ml BHI agar inoculated with approximately 6 log_{10} CFU/ml of B. cereus NVH391-98 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 prepared as described above 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 were reported in mm. All experiments were performed in duplicate on three separate occasions.

2.4. Effect of minerals on the production of antimicrobial compounds by B. subtilis

Inorganic salts have previously been shown to enhance the production of bacteriocins by B. subtilis (Hammami et al., 2009; Tabbene and Slimene, 2009). The following experiments were done to test the effect of mineral salts on the production of antimicrobial compounds by B. subtilis. Baobab seed extracts (10% (w/v)) were prepared by cooking 5 g of ground baobab seeds in 50 ml of water at temperatures of 95–98 °C for 7 h followed by cooling at 37 °C. Evaporation of water during cooking was compensated by adjusting the volume with sterile MilliQ water to 50 ml. Inorganic salts were individually added to the baobab seed extracts at final concentrations of 0.2 g/l MgSO_{4}; 0.15 mg/l MnSO_{4}; and 0.135 g/l FeCl_{3}, according to the method described by Tabbene and Slimene (2009). The pH of the supplemented baobab seed extracts (50 ml) was then adjusted to 7 and sterile filtered into 100 ml sterile conical flasks. These flasks were inoculated with B. subtilis, and incubated and culture supernatants were assayed for antimicrobial activity as described above (Section 2.2). The experiments were performed in duplicate on three separate occasions.
2.5. Survival of B. cereus in co-culture with B. subtilis in BHI broth, baobab seed extracts and whole baobab seeds

Starter cultures may prevent pathogen outgrowth, through the production of antimicrobial compounds and simply through competitive exclusion. Whole baobab seeds (50 g) were cooked together with ash (72 g ash per 2 kg of seeds) for 7 h in 100 ml conical flasks according to the method described by Parkouda et al. (2010). Baobab seed extracts (50 ml, 10% (w/v) grinded seeds) were prepared as described in Section 2.4. BHI-broth (50 ml) was also prepared. B. subtilis was inoculated into the two mentioned substrates at a final rate of 6 log₁₀ CFU/ml, either without or with the addition of B. cereus NVH391-98 (final rate 3 log₁₀ CFU/ml). Incubation was set at 37 °C, at 120 rpm, and the samples were collected at 0, 5, 10, 20, 30 and 40 h for the determination of pH and total viable counts using BHI agar and brilliance B. cereus agar (Oxoid) as described by Røssland et al. (2005). The examined B. subtilis strains were unable to grow on brilliance B. cereus agar, while B. cereus showed greenish-blue colony growth. The counts for B. subtilis were thus obtained by subtracting the counts on brilliance B. cereus agar from the total counts on BHI-agar. The experiments were performed in duplicate on three separate occasions.

3. Results

3.1. Influence of baobab seed extract on the production of antimicrobial compounds by B. subtilis B3, B122 and B222

Table 1 shows the production of antimicrobial activity against B. cereus NVH391-98 by B. subtilis B3, B122 and B222 (isolated from traditional maami), B. subtilis subsp. subtilis DSM 10 and B. subtilis subsp. spizizenii DSM 15029 (reference strains producing bacteriocin) after culturing in baobab seeds as well as in BHI containing different amounts of baobab seed extract. As shown in Table 1, strain B222 produced maximum antimicrobial activity against B. cereus NVH391-98 when grown in pure BHI and in BHI containing 0.05% to 5% (w/w) grinded baobab seeds, while strains B3 and B122 produced maximum antimicrobial activity in BHI containing 5% (w/w) grinded seeds. B. subtilis subsp. spizizenii DSM 15029 was the only strain able to produce antimicrobial activity in 10% (w/v) grinded seeds and cooked baobab seeds. All the B. subtilis cultures grew from approximately 6–6.5 log₁₀ CFU/ml at inoculation (0 h) to between 7.5 and 8.7 log₁₀ CFU/ml at 10 h and remained approximately at this level until 20 h (results not shown). The pH at time 0 h varied between 6.8 and 7.3, and at 10 h between 5.4 and 7.0, while at 20 h the pH varied between 5.7 and 7.9 dependent on the substrate and the strain (results not shown).

3.2. Effect of minerals on antibacterial activity

No antimicrobial activity of the CFS produced by B. subtilis B3, B122 and B222 inoculated in grinded baobab seed broth supplemented with Fe²⁺, Mg²⁺ or Mn²⁺ was observed. Growth of B3, B122 and B222 was similar with and without the added minerals (Fe²⁺, Mg²⁺ and Mn²⁺) to the substrate (results not shown).

3.3. Survival of B. cereus NVH391-98 in co-culture with B. subtilis B3, B122 and B222 in BHI broth

Growth of B. cereus NVH391-98 in mono- and co-culture with B. subtilis B3, B122 and B222 was investigated in BHI broth and results are shown in Fig. 1. When inoculated as mono-culture, B. cereus NVH391-98 reached stationary growth phase within 5 h of incubation. When co-cultured together with B. subtilis, B. cereus NVH391-98 decreased to below the detection limit within 5–10 h of incubation, while B. subtilis grew to 7–8 log₁₀ (CFU/ml) (results not shown). The antagonistic effect of B3, B122 and B222 against B. cereus NVH391-98 was similar. During incubation the pH increased from 7 to between 7.2 and 7.7 depending on the bacterial composition (results not shown).

3.4. Survival of B. cereus in baobab seed extracts

Growth of B. cereus NVH391-98 as mono-culture and co-cultured with B. subtilis B3, B122 or B222 was investigated in 10% (w/v) grinded baobab seed extracts as shown in Fig. 2. B. cereus NVH391-98 reached the stationary growth phase (around 8.9 log₁₀ CFU/ml) within 5 h when grown as mono-culture. Growth of B. cereus NVH391-98 was retarded when co-cultured with B. subtilis B3, B122 and B222 and it took

![Fig. 1](image-url) Viable counts of B. cereus NVH391-98 in BHI-broth during growth at 37 °C for 40 h in mono-culture (NVH391-98) and in co-culture with B3 (NVH391-98/B3), B122 (NVH391-98/B122), and B222 (NVH391-98/B222). Each point represents the mean ± SEM of three independent experiments. Detection limit: 10 CFU/ml.
B. subtilis co-culture with B. subtilis B. cereus 8.5 log₁₀ CFU/ml (B122) and 8.2 log₁₀ CFU/ml (B222) as seen from Fig. 2. Each point represents the mean ± SEM of three independent experiments. Detection limit: 10 CFU/ml.

40 h to reach counts of 7.8 log₁₀ CFU/ml (co-cultured with B. subtilis B3), 8.5 log₁₀ CFU/ml (B122) and 8.2 log₁₀ CFU/ml (B222) as seen from Fig. 2. Growth of B3, B122 and B222 was not inhibited by B. cereus NVH391-98 as compared to when the strains were grown in mono-culture (results not shown). The pH of the broth decreased from 6.9 to between 5.5 and 6.3 during the first 20 h, and then increased to between 6.7 and 7.4 depending on the bacterial composition (results not shown).

3.5. Survival of B. cereus in cooked baobab seeds

Growth of B. cereus NVH391-98 as mono-culture and co-cultured with B. subtilis B3, B122 or B222 was investigated in cooked baobab seeds as shown in Fig. 3. B. cereus NVH391-98 reached stationary phase (around 8.5 log₁₀ CFU/g) after 10 h when inoculated alone. In co-culture with B. subtilis B3 and B222, the growth of B. cereus NVH391-98 was somewhat retarded taking 20 (when co-cultured with B. subtilis B3) to 40 h (co-cultured with B. subtilis B222) to reach counts of between 8.3 and 8.5 log₁₀ CFU/g. B. subtilis B122 did not retard growth of B. cereus NVH391-98 when co-cultured in cooked baobab seeds.

Growth of B3, B122 and B222 was not inhibited by B. cereus NVH391-98 as compared to when the strains were grown in mono-culture (results not shown). The pH of the seeds at 40 h ranged between 6.3 and 7.5, depending on the bacterial composition (results not shown).

4. Discussion

Bacteriocin production by B. subtilis is strain dependent and strongly influenced by factors such as medium composition, temperature, pH and incubation conditions (Chevanc et al., 1986; Kaboré et al., 2012; Moita et al., 2005). In our previous study (Kaboré et al., 2012), we observed that the production of antimicrobial substances by B. subtilis B3, B122 and B222 in BHI broth was highly dependent on aeration, with maximum bacteriocin production observed under reduced aeration (small headspace, but with agitation). Limited or no bacteriocin production was observed under static conditions (25–75 ml broth) and under anaerobic conditions. In the present study we investigated the effect of different baobab seed extracts and baobab seeds on the ability of B. subtilis B3, B122 and B222 to inhibit the growth of the opportunistic human pathogen B. cereus. This pathogen may occur in high levels in maaari productions (Parkouda et al., 2010). The effects on the inhibitory ability of the B. subtilis strains were investigated in model systems using substrates and temperature incubation conditions mimicking parts of maaari production (Parkouda et al., 2010). B. subtilis B3, B122 and B222 were originally isolated from maaari (Parkouda et al., 2010), and it was therefore not surprising that these strains grew well in 10% (w/v) grinded baobab seed based broth and cooked baobab seeds in the present study. However, in order to obtain antimicrobial activity production by the strains, the baobab seeds had to be incorporated with various levels of BHI. Full antimicrobial activity of CFS was observed when B. subtilis B222 was grown in pure BHI broth, in BHI containing 0.05% to 5% (w/w) grinded seeds, while slight antimicrobial activity was produced during growth in BHI containing 50% (w/v) grinded seeds. In comparison strains B3 and B122 produced the highest activity in BHI containing 5% (w/v) grinded seeds. Similar to our observations, in the study by Motta and Brandelli (2008), antimicrobial activity production by a Bacillus species was substrate dependent, being produced in BHI broth, soybean protein, peptone and trypticase soy broth, but not in fish meal and whey. Based on dry weight values, baobab whole seeds are reported to contain 5.2–56.8% carbohydrates, 9–33.3% lipids and 14.4–36.7% protein (Chadare et al., 2009). According to Nascimento et al. (2010), the low antimicrobial activity in natural substrates could be due to interactions of the antimicrobial compounds with substrate components, such as fat.

In the present study, a strain and subspecies variation was observed. Thus B. subtilis subsp. spizizenii DSM15029 produced strong antimicrobial activity when grown in 10% (w/v) grinded baobab seeds as well as in whole baobab seeds as the only strain of the 5 investigated strains. At the same time this strain produced weaker antibacterial effect against B. cereus NVH391-98 as compared with e.g. B. subtilis B222 when grown in BHI containing 5% (w/v) grinded seeds. A single B. subtilis strain may produce more than one antimicrobial compound (Stein et al., 2004). B. subtilis B3, B122 and B222 were previously shown to harbor genes for both subtilin and subtilosin biosyntheses (Kaboré et al., 2012). In the present study we used B. subtilis subsp. subtilis ATCC6051 (DSM 10), known to produce subtilosin (Stein et al., 2004) and B. subtilis subsp. spizizenii DSM15029 known to produce the subtilin like lantibiotic, entianin (Fuchs et al., 2011) as control strains for bacteriocin production. Even though the antimicrobial spectrum of the strains was similar (against B. cereus) between the strains/subspecies investigated, the observed differences in substrate dependency could be due to differences in the types of bacteriocin produced, as well as differences in the regulatory mechanisms involved in their production.
Cations and the addition of the inorganic salts such as FeCl₃, MgSO₄ and MnSO₄ have previously been found to enhance the production of bacteriocins by B. subtilis (Hammami et al., 2009; Tabbene and Slimene, 2009), but the addition of these salts to 10% (w/v) grinded baobab seed extracts did not induce production of antimicrobial compounds active against B. cereus NVH391-98 by any of B. subtilis B3, B122 and B222.

The inhibition of B. cereus NVH391-98 in co-cultures in BHI was expected as CFS from B. subtilis B3, B122 and B222 grown in pure BHI broth was inhibitory in the agar well diffusion assay. Even though no antimicrobial activity was observed in cooked seeds and in 10% (w/v) grinded baobab seed based broth, the growth of B. cereus NVH391-98 was delayed. Furthermore, B. subtilis B3 and B222 were found to be more efficient than B122 in inhibiting B. cereus NVH391-98 growth in whole baobab seeds. Similar results for no antimicrobial activity production were obtained when the indigenous B. subtilis strains were co-cultured with B. cereus NVH391-98 in African locust beans, which are used to produce a fermented product similar in taste, color and smell to maari (results not shown). In this substrate, however, B. subtilis B122 was the most efficient inhibitor of B. cereus NVH391-98 (results not shown). In general, our results show that production of antimicrobial compounds and inhibition of B. cereus by B. subtilis are strongly influenced by the substrate in a strain dependent manner.

B. cereus NVH391-98 alone was able to grow to more than 8 log₁₀ CFU/ml in BHI and in cooked whole seeds. A similar trend was reported for B. cereus (Ouoba et al., 2007; Thorsen et al., 2011) in fermented African locust beans. This implies that, on its own, B. cereus NVH391-98 could grow to potentially unsafe levels. Ouoba et al. (2007) reported that B. cereus as well as other indicators (Micrococcus luteus, Staphylococcus aureus and Escherichia coli) was completely inhibited by B. subtilis strains B7 and B15 (now identified as B. amylophilofaciens, unpublished data) during African locust bean fermentations to produce soumblu. In comparison, in the present study, the investigated strains B. subtilis B3, B122 and B222 were only able to retard but not completely inhibit growth of B. cereus NVH391-98 when co-cultured in 10% (w/v) grinded baobab seeds, and during cooked baobab seed fermentations.

It is indeed possible that the ability of B. subtilis B3, B122 and B222 to retard growth of B. cereus NVH391-98 when co-cultured in baobab seed based broth, cooked baobab seeds and African locust beans is due to production of antimicrobial compounds, but it is also possible that the observed inhibition may be due to competition for nutrients (Røssland et al., 2005). The B. subtilis strains were inoculated in higher levels (6 log₁₀ CFU/ml) than B. cereus NVH391-98 (3 log₁₀ CFU/ml) in the present co-cultures. It is likely that rapid growth of a large population of B. subtilis could restrict the growth of other organisms simply by uptake of the most readily assimilable nutrients and co-factors or even by physical occupation of the available space (Røssland et al., 2005).

Results of the present study show that B3, B122 and B222 are probably not suitable as protective cultures against B. cereus on their own. In our previous study antimicrobial activity production by B3, B122 and B222 was tested against seventeen different strains of B. cereus of different origin and toxigenic potentials, and B. cereus NVH391-98 was the most sensitive strain (Kaboré et al., 2012). The results of the inability to inhibit B. cereus growth in 100% grinded seeds and whole seeds obtained in the present study with strain NVH391-98 therefore most likely apply to other B. cereus strains too. Maari however is produced by a mixed microbial fermentation (Parkouda et al., 2010), wherefore other co-starter cultures of other genera with protective properties could be considered, and the B. subtilis strains could be considered for organoleptic reasons. To our knowledge, the present study is the first to investigate the effect of indigenous bacteriocin producing B. subtilis maari strains (Kaboré et al., 2012; Parkouda et al., 2010) on the growth of an opportunistic pathogenic species (B. cereus) which is known to occur during baobab tree seeds and African locust bean fermentations (Azokpota et al., 2007; Parkouda et al., 2009, 2010).

5. Conclusion

The findings of the present study lead to conclude that the production of bacteriocin by B. subtilis B3, B122 and B222 isolated from traditional maari is strongly dependent on the substrate. The production was further strain dependent, as maximum activity was observed in BHI containing 5% (w/w) grinded baobab seeds for B. subtilis strains B3 and B122, while strain B222 produced full activity in 100% BHI and in BHI containing 0.05% to 5% (w/w) grinded seeds. No activity was observed in 10% (w/v) grinded and in whole cooked baobab seeds for any of the three strains B3, B122 and B222. Inhibition of growth of B. cereus NVH391-98 by B. subtilis B3, B122 and B222 during baobab tree seed was highly strain dependent. Based on the results, the investigated indigenous strains are not suitable as protective cultures, although they still may be suitable as starters contributing to desirable organoleptic properties. As maari is produced by a mixed microbial fermentation other protective co-starter cultures could be considered.

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