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Short communication

Identification of *Bacillus* species occurring in Kantong, an acid fermented seed condiment produced in Ghana

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ABSTRACT

Kantong is a condiment produced in Ghana by the spontaneous fermentation of kapok tree (*Ceiba pentandra*) seeds with cassava flour as an additive. Fermentation is over a 48 h period followed by a drying and a kneading process. Although lactic acid bacteria (LAB) have previously been identified other micro-organisms may also be involved in the fermentation process. In this study we examined the occurrence of aerobic endospore-forming bacteria (AEB) in raw materials, during fermentation and in the final product at 2 production sites in Northern Ghana. Total aerobic mesophilic bacterial counts increased from 5.4 ± 0.1 $\log_{10}$ CFU/g in the raw materials to 8.9 ± 0.1 $\log_{10}$ CFU/g in the final products, with the AEB accounting for between 23% and 80% of the total aerobic mesophilic (TAM) counts. A total of 196 AEB were identified at a species/subspecies level by the use of phenotypic tests and genotypic methods including M13-PCR typing, 16S rRNA and *gyrA* gene sequencing. *Bacillus subtilis* subsp. *subtilis* (63% of the AEB), *Bacillus safensis* (26% of the AEB) and *Bacillus amyloliquefaciens* subsp. *plantarum*/*Bacillus methylotrophicus* (9% of the AEB) were the predominant *Bacillus* species during fermentation and in the final products. *B. amyloliquefaciens*/*B. methylotrophicus* originated from cassava flour, *B. safensis* from seeds and cassava flour, while the origin of *B. subtilis* was less clear. *Brevibacillus agri* and *Peanibacillus* spp. occurred sporadically. Further investigations are required to elucidate the role of AEB occurring in high numbers, in the fermentation of Kantong.

1. Introduction

Kantong is a traditional acid fermented food condiment produced from the crushed and fermented seeds of kapok (*Ceiba pentandra*). It is indigenous to the Dagomba socio-cultural groups of Northern Ghana. The main production process includes cleaning the seeds, dehulling by crushing and sifting to obtain a fine flour, and addition of approximately 10% fermented cassava (*Manihot esculentum*) flour, and water to form a thick paste. Fermentation is over a 48 h period. In the first 12 to 24 h the mixture is covered in a bowl, and then opened and dried in the sun for the next 12 h; it is then covered for the final 12 h. The fermentation process is followed by a drying and kneading process (Fig. 1) (Kpikpi et al., 2009, 2010). Kantong is used in soups as a thickener, as well as flavor and taste enhancer. It is additionally used in the diet of recuperating individuals, as it is considered to aid quick recovery according to local producers.

Very little is known about the microorganisms and their role in Kantong fermentation. Growth of lactic acid bacteria (LAB) has been observed, and *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Leuconostoc mesenteroides* and *Pediococcus acidilactici* were identified as the dominant LAB species (Kpikpi et al., 2010). Kapok seeds, which constitute 90% of the raw materials of Kantong production, have been reported to consist of approx. 28% crude protein, 25% crude fiber, 7% starch, 4.5% sugars as well as fat, ash etc. (Narahari and Rajini, 2003). The seeds further contain anti-nutritional factors as tannins and trypsin inhibitor activity (Narahari and Rajini, 2003). The cassava flour which constitutes 10% of the raw material in Kantong production contains carbohydrates which can be utilized by LAB for growth (Kpikpi et al., 2010). Kpikpi et al. (2009) observed an increase in amylase, protease and lipase activity during Kantong fermentation, as well as an increase in levels of free amino acids, but a decrease in fat and total fiber. A combination of seed and microbial enzyme activities is likely to contribute to the observed changes. Apart from producing organic acids leading to a decrease in pH, it is possible that the LAB species of Kantong could play a role in starch degradation (Agati et al., 1998), proteolysis (Moslehishad et al., 2013), lipolysis and hydrolysis of tannins (Matthews et al., 2006). LAB have been reported to be unable to degrade plant polysaccharides as cellulose (Amoa-Awua et al., 2007; Morais et al., 2013) and xylan (Crittenden et al., 2002; Matthews et al., 2006; Morais et al., 2013), although exceptions do exist (Madhukumar and Muralikrishna, 2012; Matthews et al., 2006). Therefore, the observed changes in chemical compositions and enzymatic activities in Kantong productions (Kpikpi et al., 2009) suggest

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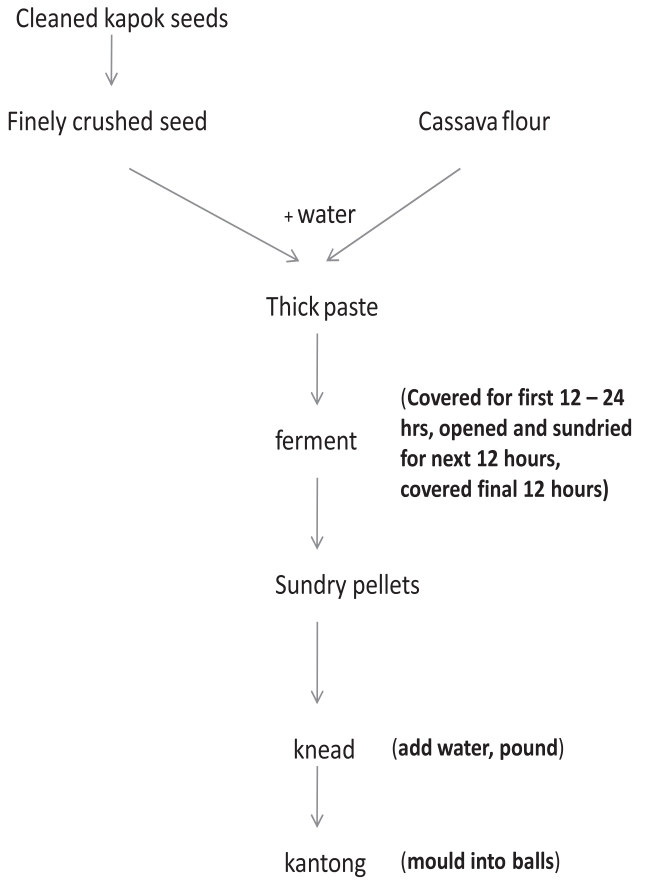

Fig. 1. Flow chart of traditional production of Kantong.

growth and metabolic activities of other microorganisms besides those of the LAB. Studies on acidic food fermentations produced by mixed microbial populations have reported high levels of *Bacillus* spp. alongside LAB, and e.g. yeasts (Amoa-Awua and Jakobsen, 1995; Sherfi and Hamad, 2001). Interestingly, it has been reported that *Bacillus* spp. positively contribute to cassava fermentation by producing cellulase activity necessary for softening of the cassava tissue (Amoa-Awua and Jakobsen, 1995). LAB, *Clostridium* and *Bacillus* spp. co-exist during acid fermentation to produce the Sudanese bread snack Gergoush made from milk and different legumes. The suggested role of the *Bacillus* spp. is to produce amylases, hydrolyzing starch to sugars assimilable to the fermenting microorganisms (Sherfi and Hamad, 2001). Likewise *Bacillus* spp. could grow and make biochemical changes to Kantong fermentations.

The aim of this study was to identify the *Bacillus* spp. present in raw materials (kapok seed flour and cassava flour) as well as at different stages of fermentation and in the dried and final product Kantong. Samples were taken from two traditional production sites in Northern Ghana, and the *Bacillus* spp. were identified by the use of phenotypic and genotypic methods. The results obtained will provide a basis for evaluating the role of *Bacillus* spp. in Kantong fermentation.

2. Materials and methods

2.1. Sampling

Sampling was carried out at two production sites in Nyankpala, a village in Northern Ghana. Samples were taken aseptically from the raw materials (kapok seed flour and cassava flour in a ratio of approximately 9:1), various stages of the fermentation process (0 h, 24 h and 48 h), and from the final product. The samples were transported on ice to the laboratory for pH measurement (Basic 20 Crison Instruments, Allela, Spain) and microbiological analysis within 24 h. The temperature at each sampling stage during fermentation was measured at the production site.

2.2. Total aerobic mesophilic (TAM) counts, and isolation and purification of aerobic endospore-forming bacteria (AEB)

Ten grams of sample was homogenized in 90 ml sterile diluents (1.0% (w/v) peptone (Difco, Detroit, MI, USA), 0.85% (w/v) NaCl, pH 7.0) at normal speed for 30 s with a stomacher (Bagmixer, Interscience, Saint Nom France). Tenfold serial dilutions were made for each sample and 1.0 ml of the appropriate dilution was cultured in plate count agar (Merck, Darmstadt, Germany) by the use of the pour plate technique and incubated for 24 h at 30 °C for enumeration of TAM. Twenty colonies from a segment of the plates representing >15% of the colonies on plates from the appropriate dilutions were further purified by successive streaking on nutrient agar (Merck). For long term maintenance, isolates were stored at −80 °C in nutrient broth containing 20% (v/v) glycerol.

Cell morphology, motility, presence of endospores, Gram test and catalase test were determined as described by Agbobatinkpo et al. (2013).

2.3. DNA isolation and genotypic characterization of isolates

A total of 196 AEB isolates were analyzed in the present study. DNA extraction was described by Compaore et al. (2013). M13-PCR typing and grouping of the obtained profiles were performed as described by Agbobatinkpo et al. (2013). Previously, as part of a study on probiotic additives for animal feed 56 of the 196 isolates obtained in the present study were partly identified by the use of 16S rRNA gene sequencing. Five of these isolates were identified to subspecies level by *rpoB* and *gyrB* gene sequencing (Larsen et al., 2013). In the present study identification at group level by 16S rRNA and at a species/subspecies level by *gyrA* gene sequencing was performed as described by Agbobatinkpo et al. (2013). The obtained *gyrA* gene sequences as well as *gyrA* gene sequences retrieved from the GenBank database were used to construct a phylogenetic tree by the maximum-likelihood method as described by Seiler et al. (2013).

2.4. Phenotypic characterization

Kantong isolates related to *B. amyloliquefaciens* were examined for salt tolerance (0.0%, 7.0%, 10.0%, 14.0% (w/v) NaCl), growth at different pH values (2.0, 3.0, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0), acid production from carbohydrates (Table 2) at 37 °C according to recommendations of Schleifer (2009). Growth at different temperatures (45 °C, 50 °C and 55 °C) was performed using nutrient agar slants (Schleifer, 2009). The type strains (indicated by a T) *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7^T and *Bacillus siamensis* DSM25261^T (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany) and *B. methylotrophicus* NCCB100236^T (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) were used as reference strains.

Isolates closely related to *Paenibacillus polymyxa* were tested for salt tolerance (2.0%, 5.0%, 7.0%, 10.0% (w/v) NaCl), growth at different temperatures (7 °C, 10 °C, 30 °C, 40 °C, 50 °C, 65 °C) and pH values (5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0) as described by Aguilera et al. (2001). The type strains *Paenibacillus polymyxa* DSM36^T, *Paenibacillus peoriae* DSM8320^T, and *Paenibacillus jamilae* DSM13815^T (DSMZ) were used as reference strains.