

Isolation of lactic acid bacteria from kantong, a condiment produced from the fermentation of kapok (*Ceiba pentandra*) seeds and cassava (*Manihot esculentum*) flour

Elmer Nayra Kpikpi ^{a, b}, Richard L. K. Glover ^a, Victoria Pearl Dzogbefia ^b, Dennis Sandris Nielsen ^c Mogens Jakobsen ^c.

^a Department of Applied Biology, Faculty of Applied Sciences, UDS P.O. Box 1350 Tamale N/R, Ghana

^b Department of Biochemistry and Biotechnology Faculty of Biosciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

^c Department of Food Science, Food Microbiology, Centre for Advanced Food Studies (LMC), Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30 1958, Frederiksberg C, Denmark

elmer_kpikpi@yahoo.com

Abstract: Kantong, a traditional food condiment of the people of Northern Ghana, is produced by fermentation of *Ceiba pentandra* seeds and cassava flour. Knowledge of the microbiology of the fermentation process will be useful in its technological improvement and starter culture development. There was a drop in the initial pH from 6.9 before fermentation to 4.9 after fermentation with change in color of the product from grayish to dark brown as well as the development of a more desirable flavor. Lactic acid bacteria (LAB) with counts between 10^6 and 10^9 cfu/g were isolated on MRS agar and subjected to Gram, catalase and oxidase tests. The LAB were further identified by biochemical and genotypic methods using rep-PCR, (GTG)₅ primer, 16S rRNA gene sequencing and carbohydrate assimilation profiling. A total of 331 Lactic acid bacteria were isolated of which 47% were *Lactobacillus plantarum*, 18% *Lactobacillus fermentum*, 8% *Leuconostoc mesenteroides*, 12% *Pediococcus acidilactici* and 15% as *Lactobacillus brevis*. [Report and Opinion 2010;2(8):1-7]. (ISSN: 1553-9873).

Key words: fermentation; condiment; lactic acid bacteria; *Ceiba pentandra*

1. Introduction

In the Northern parts of Ghana, seeds of *Parkia biglobosa* (locust bean), *Arachis hypogea* (groundnut), *Glycine max* (cotton), and *Ceiba pentandra* (silk cotton, kapok) among others, are fermented and used as condiments. Dishes in these areas are quite simple especially with the rural folk, so simple that there could be the danger of little or no protein content. Due to poverty and/or traditional beliefs, meat, fish and eggs are not included in the diets of children. Hence the importance of fermented condiments which when added in substantial quantity to soups and stews serve as a source of protein.

'Kantong' is a fermented oil seed cake primarily prepared from the seeds of *Ceiba pentandra* (silk cotton, kapok) and is eaten predominantly by the Dagombas of Northern Ghana and among the Hausa's of Northern Nigeria. The seeds are dehulled by pounding in a mortar and sifted, the seed coat is discarded and the fine seed flour is mixed with cassava flour in the ratio 5:1. Water is added to this

mixture to make a thick paste which is then fermented for 2 days (approx 48 hours). The 48 hour fermentation involves an alternation of anaerobic and aerobic fermentation (the paste is covered for 24 hours after preparation and opened the next 12 hours in the sun for drying, it is then turned and covered another 12 hours). After the fermentation process, it is made into small lumps, dried, pounded and molded into balls. It is then ready for consumption. The process of fermenting *Ceiba pentandra* seeds and cassava flour to produce kantong has not been investigated microbiologically previously; but according to Kpikpi 2006, it is most likely an acidic fermentation involving lactic acid bacteria.

Amplification of repetitive elements in the genome using PCR (rep PCR) has proven to be an excellent tool for reliable and rapid grouping of lactic acid bacteria (Gevers et al., 2000; Kostinek et al., 2007; Nielsen et al., 2007). In a previous study (Kpikpi et al. 2008), the enzymatic process of kantong production was reported. A thorough

knowledge of the microorganisms involved in the process will however provide a means of improving the process technologically as well as an opportunity for starter culture development to standardize the fermentation process. The Lactic acid bacteria involved in the production of kantong were therefore investigated using morphological, biochemical as well as molecular approaches to identify the predominant species and forms the subject of this report.

2. Materials and Methods

2.1 Enumeration and isolation of Lactic Acid Bacteria

A total of 331 lactic acid bacteria (LAB) were isolated from kantong production sites in two villages in the Northern region of Ghana. Samples were taken aseptically from various stages (0hr, 24hr, 48hr of fermentation and final product) of kantong production, serially diluted and cultured anaerobically on MRS agar at 30° C for 48 hrs. They were phenotypically identified on the basis of colony and cell morphology, in addition to Gram, oxidase and catalase reactions at the UDS/DANIDA MICROBIOLOGY LAB, Navrongo Ghana. These isolates then underwent genotypic tests. They were maintained at 4°C on De Man, Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany).

2.2 Phenotyping

Initial typing of the representative isolates was based on colony and cell morphology, Gram, catalase and oxidase reactions. Carbohydrate fermentation of isolates was investigated using API 50CHL (BioMerieux, Marcy-L'Étoile, France) galleries

2.3 Genotyping

2.3.1 Extraction of DNA

Each isolate was grown anaerobically on MRS agar for 48 hr at 30°C. An isolated colony was suspended in 1ml of autoclaved Milli – Q water in a microfuge tube, centrifuged for 1 min at 12000g and the supernatant discarded. DNA was isolated from the pellet using the InstaGene matrix (Bio-Rad, Hercules, CA, USA) following the instructions of the manufacturer. The resulting supernatant was used as DNA template for PCR.

2.3.2 rep – PCR

The amplification of the repetitive DNA elements of isolates was carried out in 25µl of reaction mixture containing 1.5µl of DNA template, 2.5µl PCR – buffer(x10), 4µl of dNTP (1.25mM), 1.5µl MgCl₂ 25mM, 4µl of primer GTG5;(5pmol/µl) (5' - GTGGTGGTGGTGGTG - 3'), 0.25µl of Formamide, 0.25µl of BSA (Bovine Serum Albumin, 0.1mg/ml), 10.8µl of autoclaved Milli Q water and 1.5µl of Taq polymerase.

The amplification was performed with 30 PCR cycles in a thermocycler (Trio – Thermoblock, Biometra, Germany). The cycling program was started with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 45°C for 30s and elongation at 65°C for 8 min. The PCR was ended with a final extension at 65°C for 16 min and the amplified product cooled at 4°C

The DNA fragments were separated by applying 10µl of each PCR product with 2µl of loading dye to 1.5% agarose gel. A 1kb DNA marker (GeneRuler DNA ladder, Fermentas) was included as standard for the calculation of the fragments. The gel was run in 1x TBE buffer (108g Trisbase/l, 55g boric acid/l and 40 ml of 0.5 M EDTA, pH 8.0) for 5 hours at 120V. The gel was then stained with ethidium bromide for 20 min, washed with distilled water and photographed under UV illuminator using a digital camera. Cluster analysis of gels was carried out using BioNumerics Version 2.5 software (Applied Maths, SINT – MARTENS – LATEM, Belgium) based on the Pearson Coefficient and the Unweighted Pair Group Method using Arithmetic averages (UPGMA).

2.3.3 Sequencing of 16S rRNA

PCR reaction was carried out by mixing 1µl of extracted DNA with a mixture containing 5µl PCR – buffer, 8µl dNTP (1.25mM), 3µl of MgCl₂(25mM), 1µl of primer 0011F, 1µl of primer 1510r, 0.5µl formamide, 0.25µl Taq polymerase and 30.25µl of autoclaved Milli Q water. The amplification was carried out in 30 PCR cycles, first denaturation at 94°C for 5 min then 30 cycles at 99°C for 90s, 52°C for 30s and 72°C for 90s. The final extension was carried out at 72°C for 7 min and the product cooled at 4°C.

The PCR product was purified using QIAquick purification Kit (Qiagen, Germany). The sequencing was performed and a database search was carried out in GenBank database using BLAST programme.

2.4 Determination of pH.

10g of sample was homogenized in 90ml of sterile distilled water. It was left to stand for a while at room temperature and then pH was taken with a pH meter (JENWAY 3310, Jenway Ltd. UK). The pH meter was calibrated against standard buffer solutions at pH 4.0 and 7.0.

3. Results

Samples of the product were taken in the morning between the hours of 7am (during fermentation period) and 11 am (for the final

product) from Klunyevilla and Bulpela, two villages in the Northern region where kantong is produced on commercial basis. The pH of the product ranged between 6.9 before fermentation and after fermentation reduced to 4.9 as previously reported (Kpikpi *et al.* 2008)

Based on their phenotypic characteristics (Table 1), 331 isolates were preliminarily identified as lactic acid bacteria which were Gram positive and catalase and oxidase negative.

Table 1: Phenotypic characteristics and preliminary identification of isolates

Group	Representative isolate	Colony morphology / diameter (mm)	Cell morphology	Catalase	Oxidase	Gram reaction	Preliminary Identification
1	A1, H11, V3	White, shinny, margins entire, 0.5 – 1mm in diameter.	Short/long rods, single and chained	-	-	+	<i>Lactobacillus brevis</i> *
2	B14, N1, U13	Cream, smooth, shinny, margins entire, 0.5 – 2mm in diameter.	Short rods, single / paired	-	-	+	<i>Lactobacillus plantarum</i> *
3	P10, Q1	Pale yellow, shinny, smooth and margins entire, 0.5 – 1mm in diameter.	Cocobacilli , single / paired.	-	-	+	<i>Lactobacillus pentosus</i> *
4	P6	Dirty white, margins entire, 1 – 2 mm in diameter	Long rods, single, paired / chained.	-	-	+	CNBI
5	X21	White, shinny, margins entire, 1 – 2mm in diameter.	Short rods, single, paired / chained.	-	-	+	CNBI
6	J6	White, shinny, margins entire, 0.5 – 1mm in diameter.	Cocci, single / paired, tetrads.	-	-	+	CNBI
7	X3	White, shinny, margins entire, 0.5 – 1mm in diameter	Short rods, single / paired	-	-	+	CNBI
	O6, J9						

- *API 50CHL identification, CNBI - could not be identified.

Rep – PCR profiling of all 331 isolates preliminarily identified as LAB showed bands which were aligned according to their DNA weight (Plate1). This allowed easy grouping of isolates with similar bands. Fourteen isolates were then selected, based on the grouping, and further purified and sequenced. The 16S rRNA gene sequencing led to the identification of 5 species of lactic acid bacteria: *Lactobacillus plantarum*, *Lactobacillus fermentum*,

Leuconostoc mesenteroides, *Pediococcus acidilactici* and *Lactobacillus brevis*. The 16S rRNA gene sequencing gave a more definitive identification of the predominant lactic acid bacteria, based on their genetic code, up to the species and subspecies level. In all, eight (8) different groups were derived and identified variously with similarities between 98% and 99%. (Table 2)

Table 2: Identification of lactic acid bacteria by 16SrRNA sequencing

Group	Representative isolate	Number of isolates	16S rRNA sequencing identification	% similarities	% population
1	A1, H11	53	<i>Lactobacillus plantarum</i>	98%	16
2	P10, Q1, V3	66	<i>Lactobacillus plantarum</i>	99%	20
3	X21	36	<i>Lactobacillus plantarum</i>	99%	11
4	B14, N1	36	<i>Lactobacillus fermentum</i>	99%	11
5	J6, U13	24	<i>Lactobacillus fermentum</i>	98%	7
6	P6	27	<i>Leuconostoc mesenteroides</i>	99%	8
7	X3	40	<i>Pediococcus acidilactici</i>	98%	12
8	O6, J9	49	<i>Lactobacillus brevis</i>	98%	15

API50 CHL analysis of isolates identified representative groups (Table1). Group 1 isolates were identified as *Lactobacillus brevis*, Group 2 isolates as *Lactobacillus plantarum*, and Group 3 isolates as *Lactobacillus pentosus*. Isolates in Groups 4 – 7 could not be identified in API galleries. The identification of Lactic acid bacteria by API galleries was based on their ability or inability to utilize the 49 different sugars. This however did not give a conclusive identification of all lactic acid bacteria present.

4. Discussion

The process of fermenting *Ceiba pentandra* seeds to produce 'kantong' was studied to determine the predominant microorganisms involved in the fermentation process. The predominant microorganisms were identified and characterized by traditional microbiological methods as well as molecular biology based methods. Lactic acid bacteria were found to be the predominant microorganisms occurring at various stages of the production of 'kantong'. This suggests that fermentation of silk cotton seeds and cassava flour to

produce 'kantong' is basically lactic. Also during fermentation the pH of the product dropped from 6.9 to 4.9 in the final product. Although different studies (Odunfa, 1985; Ikenebomeh, 1989) have shown that fermentation of proteinaceous oil seeds are mainly alkaline fermentations with *Bacillus sp* being the predominant microorganisms involved in the fermentation process, this study showed that the fermentation of silk cotton seeds into 'kantong' is most likely an acidic fermentation. The cassava flour serves as a source of carbohydrate, which is the main substrate for lactic acid bacteria, thus enhancing their growth in the fermenting product. Various studies have shown the predominance of lactic acid bacteria in various fermented products processed from cassava (Ngaba and Lee, 1979; Sefa – Dedeh, 1995; Amoa – Awua and Jakobsen, 1995). *Lactobacillus*, *Leuconostoc* and *Streptococcus* species have also been isolated from spontaneously fermenting cassava marsh (Okafor and Uzuegbu, 1987). *Lactobacillus plantarum* was also found to be the dominant species of lactic acid bacteria during the fermentation of cassava into agbelima, with *Leuconostoc mesenteroides* and *Lactobacillus brevis* also occurring in reasonable numbers (Amoa – Awua et al, 1996).

Phenotyping of lactic acid bacteria using morphological, physiological and biochemical (API50 CHL) characteristics was useful only in identifying three large groups of lactic acid bacteria; *Lactobacillus brevis*, *Lactobacillus plantarum* and *Lactobacillus pentosus* (Table 2). It was rather subjective and non – definitive as isolates with minor differences in colony and cell characteristics as well as physiology were lumped together under same genera. The identification system used thus did not give 100% accuracy of identity.

Genotyping is known to give a surer identity of organisms. In recent years it has become accepted by microbial taxonomists that nucleic acid hybridization and sequencing studies provide the best available methods for designation of species and determining relationships between different organisms (Dellagio et al, 1975). Different studies have shown that genotyping 16S rRNA sequencing analysis is a more reliable method of identifying and characterizing the genera of lactic acid bacteria to species, subspecies and strain levels (Collins et al 1990; Olsen et al, 1991)

Thus the genotyping using 16S rRNA sequencing placed the isolates into eight (8) groups, made up of 5 species, based on their similarities.

Groups 1, 2 and 3 were seen to be *Lactobacillus plantarum* with similarities of 98%, 99% and 99% respectively (Table2). This could be due to the fact that 16S rRNA sequencing gave identification even to the subspecies and strain levels, hence the difference in similarities was likely to be because of the presence of different strains of the same species. This also applies to group 4 and 5 which were identified as *Lactobacillus fermentum* with similarities of 99% and 98% respectively.

Group 6 had a 99% similarity to *Leuconostoc mesenteroides* with Group 7 and Group 8 having a 98% similarity to *Pediococcus acidilactici* and *Lactobacillus brevis* respectively.

The predominant LAB during 'kantong' production was *Lactobacillus plantarum* which made up 47% of the lactic acid bacteria population and were isolated from various stages of 'kantong' production. They can also be isolated from diary products, sauerkraut, pickled vegetables and cowdung and numerous fermented products of African origin. (Wood and Holzapfel, 1995).

The second most abundant LAB was *Lactobacillus fermentum* which made up 18% of total LAB population (Table 3). They are obligate heterofermenters (Wood and Holzapfel, 1995). Making up 15% of LAB population was *Lactobacillus brevis* (Table 2), they are obligate heterofermenters. Kantong is produced at the local homestead where the cattle are also kept and this could be partly responsible for the presence of *Lactobacillus brevis* in 'kantong'.

12% of the total LAB population was *Pediococcus acidilactici* which are mainly associated with fermenting plant material (Wood and Holzapfel, 1995). Bhowmick and Marth (1990b) showed that all strains of *Pediococcus acidilactici* and *P. pentosaceus* produce proteases, dipeptidases and aminopeptidases and so could play a role in proteolysis during kantong production.

The last 8% of total LAB population were *Leuconostoc mesenteroides*. They are non – proteolytic (Garvie, 1967b). They play a role in changing the organoleptic quality and texture of fermented food products (Wood and Holzapfel, 1995).

5. Conclusion

The present study has shown, for the first time, the isolation of lactic acid bacteria from kantong as well as the characterization of the LAB species from kantong using genotypic methods.

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Correspondence to:

Elmer Nayra Kpikpi

Dept of Applied Biology, FAS

University for Development Studies

P.O.Box 24 Navrongo Upper East Region Ghana.

West Africa

Telephone: +233 242 867971

email: elmer_kpikpi@yahoo.com

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