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Use of Randomly Amplified Polymorphic DNA (RAPD) for Characterization of Dominating Lactic Acid Bacteria in Maize Dough

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Abstract

In previous studies *Lactobacillus fermentum/reuteri* was described as the dominant bacteria in the spontaneous fermentation of maize for the production of „kenkey" in Ghana. The identification methods used were not able to distinguish between these phenotypically very similar species. The present work describes the use of Random Amplified Polymorphic DNA (RAPD) for characterization of 28 isolates of dominant lactobacilli from the previous studies of maize fermentation. Cluster analysis of the RAPD profiles obtained showed the presence of two main clusters. Cluster 1 included all of the so called *Lactobacillus fermentum/reuteri* isolates whereas Cluster 2 comprised the remaining *Lactobacillus spp.* The two distinct clusters emerged at the similarity level of 60%. All isolates in Cluster 1 showed similarity in their RAPD profile to the reference strains of *Lactobacillus fermentum* included in the study. These isolates having two distinct bands of approximately 557bp for the primers used, were divided into three subclusters (subspecies) indicating that several strains are involved in the fermentation and remain dominant through out the process. The present investigation has demonstrated that *L. fermentum* and not *L. reuteri* is the dominant *Lactobacillus sp.* of the fermentation.

Introduction

Fermented maize dough is the starting material from which several products are prepared. It contributes to the staple diets of the people of the southern and coastal belt of Ghana and other West African countries. The process involves steeping of maize for 24 to 48 h, followed by milling.The milled maize is reconstituted with water to form a stiff dough which is packed in fermenting troughs and left to ferment for 48h-72h.

The microbiology of the fermentation process has been extensively studied and found to be lactic acid type with the dominating lactic acid bacteria identified as *L. fermentum* or *L. reuteri.* (Halm *et al.* 1993).

Lactobacilli form a diverse and important genus that contains a lot of beneficial microorganisms used as starter cultures in the fermentation industries such as the dairy, meat and vegetable industry. It is also an important group which exhibits several antimicrobial properties as well as production of anti-microbial agents which are effective against a wide range of spoilage and pathogenic organisms. These anti-microbial properties have been confirmed with strains of lactic acid bacteria isolated from fermented maize dough (Mensah *et al*, 1991, Olsen *et al*, 1995).

It is important that the *Lactobacillus* species isolated from food products are classified to both species and subspecies level in order to relate them to some of the technological properties. Traditional methods of characterization have been found to be inadequate in the discrimination of *L. fermentum* and *L. reuteri* in previous studies conducted by Halm *et al*, (1993) due to the similarity in their phenotype.

DNA-based technique offers a more precise and rapid method of identification. This paper reports preliminary investigations in the use of a DNA-based technique for the identification to subspecies level of *Lactobacillus spp.* isolated from fermented maize dough. The immediate objective of the present study is to adopt a method that is able to discriminate between *L. fermentum* and *L. reuteri* and to characterize the dominant *Lactobacillus* spp. in this fermented product to subspecies level on the principle of Randomly Amplified Polymorphic DNA (RAPD). The ultimate objective is to create a basis for selection and identification of starter culture strains from the fermentation process and also to provide means of characterizing strains for patenting.

Materials and method

Cultures

Fifty-one strains of microorganisms of lactobacilli isolated from fermented maize dough and fermented cassava products and previously identified by conventional methods (Halm *et al*, 1993) and some reference strains of *Lactobacillus fermentum* and *Lactobacillus reuteri* as shown in Table 1 were used. Cultures where grown overnight at 37°C in De Man, Rogosa & Sharpe (MRS) broth (Merck, Darmstadt, Germany).

DNA template preparation

Cells from 1ml overnight cultures in MRS broth were collected by centrifugation (10,000 x g for 2 mins) and washed twice with 1ml sterile Milli Q water (Milli-Q Plus, Ultra pure Water System, Millipore, Molsheim, France). Cells were then resuspended in 100 l sterile Milli Q water by vigorous mixing. The cell walls of the cells were disrupted by vigorous shaking with glass beads (0.2mm diam) in each tube using an eppendorf mixer (IKA-VIBRAX-VXR, Janke & Kunkel, Germany) for 1h at 4°C. Disrupted cells were centrifuged at 10,000rpm for 5 minutes and 1 l of supernatant fluid was used as the source of template for the PCR reaction.

PCR amplification of (RAPD) DNA fragments

One l of crude DNA extract (supernatant) was used in the PCR reaction which was carried out in a Thermal Cycler (2400 Perkin Elmer, Norwalk USA). Each sample (50 l total volume) was amplified in a reaction mixture containing 0.2mM each of dNTP (Perkin Elmer N808-0007), 2 M of primer(ACGCGCCCT), 0.5 l (50ug/ml) of Tag polymerase and 5 l OF 10 x PCR buffer + Mg^{+} (Boehringer Mannheim 1146173) as recommended by the manufacturer. The primer mentioned was used because it had been successfully applied and previously described by Johansson *et al*, (1995). The reaction mixture was cycled through the following temperature profile according to Johansson *et al*, (1995): 94 °C, 45 s; 30 °C, 20 s; 72 °C, 60

s; for 4 cycles followed by : 94 °C, 5 s; 36 °C, 30 s; 72 °C, 30 s for 26 cycles. The PCR reaction was terminated at 75 °C for 10 min and thereafter cooled to 4 °C.

Gel electrophoresis

Gel electrophoresis was run by applying 20 l of sample to submerged horizontal 1.5% agarose (Type III. High EEO Sigma A-6138) slab gels (Bio-Rad DNA sub cell™, Bio-Rad Lab. Inc. USA). Gels were run at 100V for 2.5hr in TB electrophoresis buffer (45mM Tris-base (Sigma T-8524), 89mM boric acid (Sigma B-6768), 2.5mM EDTA (Sigma E-5134), pH 8.3) without cooling. A DNA molecular marker VI (0.5 g) (Boehringer Mannheim 1218611) was used as a standard. Gels were stained in ethidium bromide (0.3ug/ml) for 5mins and thereafter washed for 10mins and visualized at 302nm with UV transilluminator and photographed.

Reading of band patterns and numerical analysis

Band patterns on photo-negatives were scanned and data collected by using the LKB 2400 Gelscan XL Program (Pharmacia LKB Biotechnology AB, Sweden) and then normalized and further processed by using the Gelcompar 3.0 Program (Applied Maths. Kortirjk, Belgium). Cluster analysis was generated using the Gelcompar 3.0 Program.

Table1:
Lactobacillus spp. used in the study

Strain		Orgin
Lactobacillus fermentum		
	B7-23, A7-11, A4-4, B4-3, A4-19, A7-15, A4-2	Fermented maize dough, 24h
*Group D	B4-10	"
*Group I	B4-8	"
*Group E	A7-9	"
*Group E	B7-2	"
	A3-19, A3-14, A8-9, B8-13, B8-5, B10-12	Fermented maize dough, 48 h
	A9-19, A6-7, A6-11, B9-19, A6-33, B9-10, A6-23, B6-6, B6-4	Fermented maize dough, 72 h
*Group E	A9-18	"
Lactobacillus buchneri		
	A1-24	Fermented Gari (cassava), 24h
Lactobacillus plantarum		
	A1-17, B1-6, B1-9, A1-6, A1-24, B11-6, B11-1	"
	A2-16, B2-1	Agbelima (cassava dough), 48 h
Lactobacillus buchneri		
	B2-2	"
Lactobacillus fermentum		
	8145, 6902^T, 11441, 8902, 8900, 8899	**LMG, Belgium
Lactobacillus reuteri		
	9213^T	"
Lactobacillus casei		ATTC 7469
Lactobacillus plantarum		ATTC 8014
Lactobacillus leichmann		ATTC 7830

* Group D, E and I were previousely identified as probably *L. fermentum*.

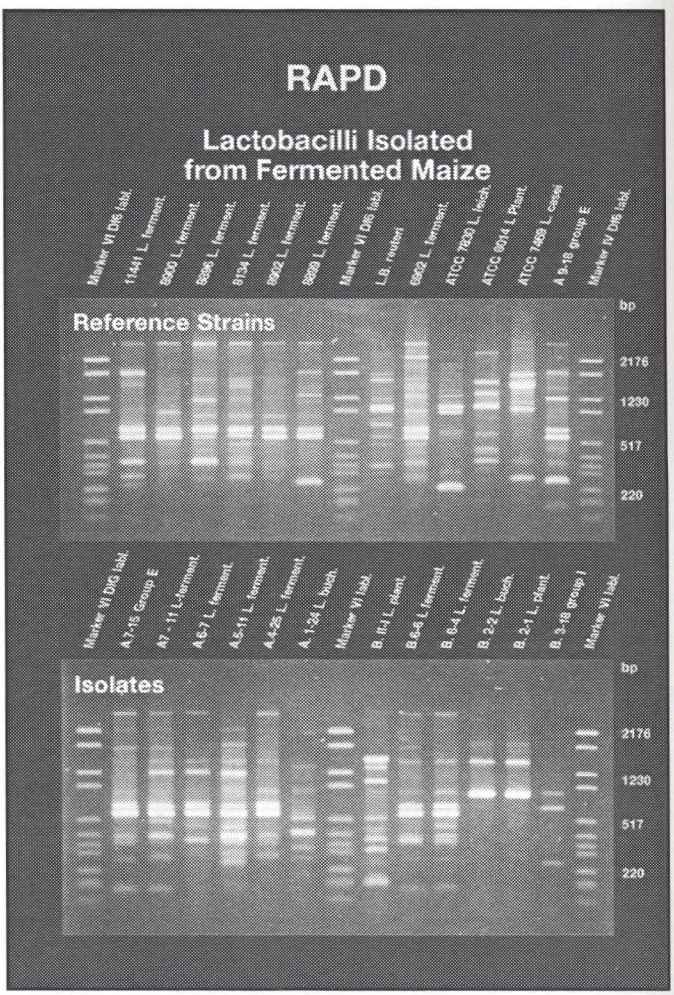
** LMG: Laboratorium voor Microbiologie, Gent, Belgium.

Results

Several species of *Lactobacillus* were analyzed including reference strains from the culture collection bank LMG (Laboratorium voor Microbiologie), Belgium, other species of *Lactobacillus* was included for comparison and to ensure the specificity of the method. However emphasis was put on the strains of *Lactobacillus fermentum* or *Lactobacillus reuteri* isolated from fermented maize dough due to the difficulty in differentiation between the two as shown in previous studies (Halm *et al*, 1993). All the strains were tested in duplicate at different occasions to ensure reproducibility of the method.

The results of the gel electrophoresis (Fig. 1) showed marked differences between the *L. reuteri* (ref. and type strain) and the rest of the strains designated *L. fermentum* from maize dough. In addition the *L. fermentum* strains from maize dough and *L. fermentum* (ref. strains) all showed similar PCR product profiles with a pronounced band at 557bp. This band does not exist on the *L. reuteri* RAPD profile. Moreover, this band also does not exist in the other species of *Lactobacillus* included in the study.

Fig. 1 :
RAPD profiles of some *Lactobacillus* spp. from fermented maize dough and some reference stains of *L. fermentum*



The results of the cluster analysis is presented in a dendrogram shown in Figure 2. There were two main clusters present. Cluster 1 included all *L. fermentum* strains and all these strains had two distinct bands of approximately 557bp.

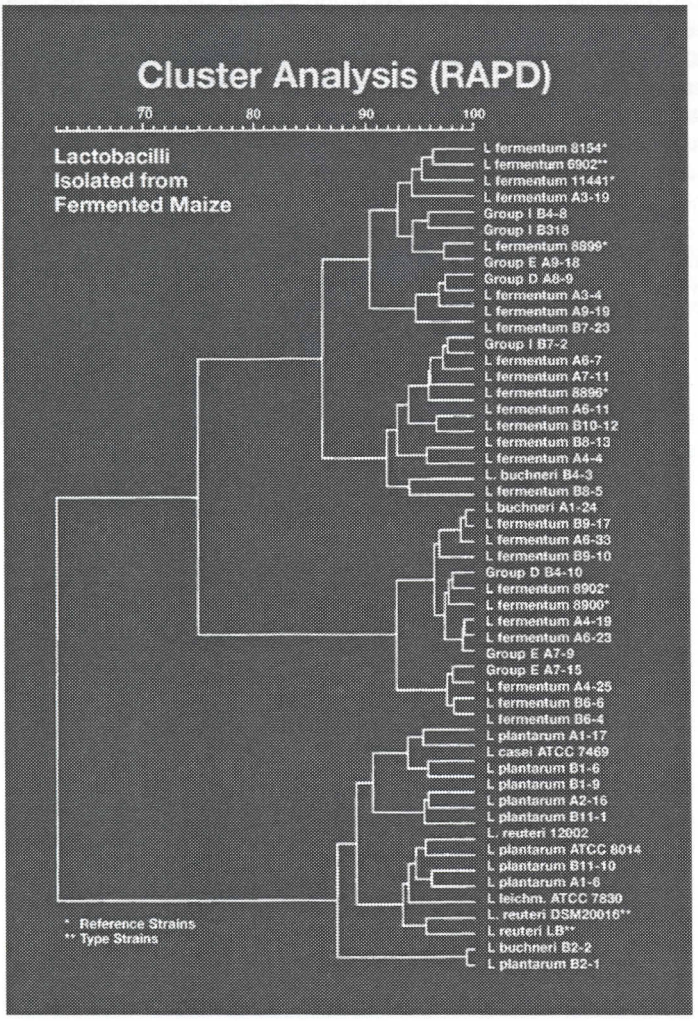


Fig. 2:
Dendrogram showing tne clustering of Lactobacillus spp. from fermented maize dough and fermented cassava products

Cluster 2 included all the *Lactobacillus* species other than *L. fermentum.* The two distinct clusters, 1 & 2, can also be seen merging at the similarity level of 60%.

Cluster 1 consists of 3 sub-clusters emerging at a similarity level of 75%, 85% and 90% respectively. Within the 3 subclusters of cluster 1 could be seen various sub-groups of each sub-cluster. It can also be seen that the 3 sub-cluster 1 each had a reference strain of *L. fermentum.* Cluster 2 contained *L. reuteri, L. planterum and L. buchneri* etc and also made up of 3 main subclusters merging at a similarity of 87%, 89% and 90% respectively.

Discussion

Crude cell extract was the source of template for the PCR reaction and satisfactory results were obtained, the similarity in the duplicate runs of results confirmed the reproducibility of the method. The primer used which had been previously described by Johansson *et al*, (1995) gave a large number of bands and therefore was able to discriminate between *L. fermentum* and other *Lactobacillus spp.*

Several specific bands could be observed within species as well as subspecies specific bands within the *L. fermentum* strains.

The fact that the *L. fermentum* reference strains could be found in subclusters indicated the presence of subspecies within the species and evidences in strain variation. Although the primer used in this investigation could discriminate between *L. fermentum* and *L. reuteri* and subspecies level within the species *L. fermentum*, it was not satisfactory for other *Lactobacillus sp.* So that it was difficult to explain the merging of *L. plantarum* and *L. reuteri*. The clustering of two different species together at high similarity percentage could only be due to unacceptable species designation (Johnansen et al, 1995). Also the success of cluster analysis in general, is highly influenced by primers and reference strains of a particular species and a large amount of reference samples improves the performance of the clustering. To further characterize these strains techniques such as restriction endonuclease analysis and DNA:DNA hybridization needs to be investigated.

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