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Phenotypic grouping, genotypic characterization of lactic acid bacteria and Ochratoxin A (OTA) production by moulds isolated from cocoa beans

BY

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REPORT OF RESEARCH INTO THE PHENOTYPIC GROUPING, GENOTYPIC CHARACTERIZATION OF MICROORGANISMS AND OCHRATOXIN A (OTA) PRODUCTION BY MOULDS ISOLATED FROM COCOA BEANS

BY

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SUMMARY

A total number of 196 strains of lactic acid bacteria isolated from Nigerian fermented cocoa beans were grouped phenotypically (by means of their reactions to gram stain, gas production, catalase, diaminopimelic acid content, sugar fermentation and growth at different temperatures). The lactic acid bacteria strains consisted of 40 obligately heterofermentative lactobacilli that produced gas from glucose and were DL-lactate; 110 facultatively hetero/obligately homofermentative lactobacilli that did not produce gas from glucose and were DL-lactate; one facultatively hetero/obligately homofermentative lactobacillus that did not produce gas from glucose but was D-lactate; one facultatively hetero/obligately homofermentative lactobacillus that produced gas from glucose and was D-lactate; 36 presumptive pediococci, cocci in tetrads that did not produce gas from glucose and was DL-lactate and also 8 presumptive enterococci that was L-lactate with one presumptive pediococcus which was L-lactate. The cluster of 110 obligately homo/facultatively heterofermentative rods were observed to comprise of 107 facultative heterofermentative strains belonging to the *L. plantarum* group that fermented arabinose, ribose or xylose, produced DL-lactate and possessed diaminopimelic acid (mDAP) in the cell wall and 3 obligately homofermentative rods which did not ferment the sugars, produced DL-lactate and also possessed mDAP. All pediococci isolates (100%) fermented arabinose, galactose, ribose and xylose; 0 % fermented lactose and maltose while 2.7 % fermented melezitose. Genotypic characterization was by Rep-Polymerase Chain Reaction (Rep-PCR) and sequencing. Mould isolates, namely *Aspergillus ochraceus*, *A. niger* and *Penicillium nordicum* grown on YES, Coffee and Cocoa media and analysed for ochratoxin A (OTA) production by HPTLC method showed YES and Coffee media supported higher OTA production than Cocoa media for all the mould isolates; with the highest OTA production by *Aspergillus ochraceus*. *A. niger* produced the least amounts in all the three media.

TITLE: **Phenotypic grouping, genotypic characterization of lactic acid bacteria and Ochratoxin A production by moulds isolated from cocoa beans**

1. INTRODUCTION

The work carried out under the overall supervision of Prof. (Dr.) W. H. Holzapfel on the identification of microbial isolates from cocoa beans was part of the COCOQUAL project on *Developing biochemical and molecular markers as indices for determining quality assurance in the primary processing of cocoa in West Africa*.
The core laboratory research activities involving lactic acid bacteria and moulds were carried out by Dr. Louis Ban-Koffi of Centre National de Recherche Agronomique (CNRA) of Cote d'Ivoire and Dr. Margaret Ottah Atikpo of the CSIR-Food Research Institute in Accra, Ghana; while Dr. Nduka Dike of the Federal Institute of Industrial Research (FIIRO), Nigeria observed the activities.
Supervision of the work involved Dr. Charles Franz and Ms. Melanie Kostinek (lactic acid bacteria); Dr. Faeber and Mr. Alexander Hanak (OTA-producing moulds).
All the lactic acid bacteria isolates were obtained from heap and tray fermentation in Nigeria.

2. ACTIVITIES CARRIED OUT

2.1 Lactic acid bacteria

These comprised of *Lactobacillus* (110 samples) and *Pediococcus* strains (37).

- Phenotypic grouping – gram stain, gas production, catalase, diaminopimelic acid determination, sugar fermentation tests, growth at different temperatures, use of Bionumeric computer programme to group isolates.
- Genotypic characterization of samples – DNA extraction, Rep-Polymerase Chain Reaction (PCR) for Pediococci and sequencing.

2.2 Moulds

- Ochratoxin A (OTA) production by mould isolates from cocoa beans were compared with known OTA producers i.e. *Aspergillus ochraceus*, *Aspergillus niger* and *Penicillium nordicum* grown and monitored in Yeast Extract Agar (YES), Coffee Agar and Cocoa Agar.

3. MATERIALS AND METHODS

3.1 Phenotypic Characterization of lactic acid bacteria

i. *Gram stain*

Growth of sample was macerated in a drop of distilled water on a sterile slide, and fixed with heat. Gentian violet was poured on it and left for 1 min before rinsing with water. Lugol was poured on and left to stand for 1 min. before a second rinsing with distilled

water. Acid alcohol was used to rinse the slide followed by water rinsing. Saffranin was added for 1 min and rinsed with water before fixing the slide by heating. Immersion oil was put on the dry slide before viewing under x 100 magnification.

ii. ***Gas production***
Broth cultures of the organism was incubated up to 5 days at 30 °C with Durham tubes inserted. The production of gas in the tubes indicated a positive reaction.

iii. ***Catalase test***
Catalase test was done by placing bacterial growth taken from plates unto sterile slides and adding a few drops of 3 % H_2O_2 to it. The presence of bubbles indicated the bacterium was catalase positive.

iv. ***Lactate test***
An overnight growth culture (1.8 ml) of the lactic acid bacteria was pipetted into an eppendorf tube. After centrifuging at 7,500 g for 6 min in a Biofuge pico (Haraeus) centrifuge, 1.0 ml of the supernatant was pipetted into another eppendorf tube and frozen.

A lactate buffer solution was prepared from a mixture of 7.5 g Glycylglycine buffer (Merck 1.04233), L-Glutamic acid (Merck, 1.00291), 40.0 ml distilled water, 4.6 ml of 10 N NaOH. The volume was made up to 60.0 ml with distilled water and the pH maintained at 10.

Nicotinamid-adenin-dinucleotide (NAD) solution was prepared by dissolving 420 mg in 12.0 ml MilliQ water.

A 1:50 dilution of the sample supernatant was made with MilliQ water. Sterile cuvettes were filled with 50.0 µl of the diluted sample, 500.0 µl lactate buffer, 100 µl NAD, 10 µl GPT and 700.0 µl MilliQ water and thoroughly mixed. A reference cuvette was prepared with using 750.0 µl MilliQ water, 500.0 µl lactate buffer, 100.0 µl NAD and 10 µl GPT. The cuvettes were left to stand for 5 minutes and readings taken in a spectrophotometer. Then 10 µl L-LDH was added to the eppendorf tubes and left to stand for 20 min. before readings taken. Then 25 µl D-LDH was added to each tube which were left to stand for 30 min before the final reading taken.

v. ***Diaminopimelic acid (mDAP) detection in cell wall***
A 1.5 ml overnight broth culture taken in an eppendorf tube was centrifuged at 13,000g for 5 min. The pellets were suspended in 0.2 ml of 4M HCl and hydrolyzed overnight at 100°C using a thermoblock. The HCl was then removed by drying with a stream of Nitrogen gas maintained at 40 – 50°C. The dry residue was then hydrolyzed with 20 µl MilliQ water and 2.0 x 2 µl spotted onto a 20 x 20 cm thin layer plate (Merck Art 1.05577, Darmstadt, Germany). A 2.0 µl volume of standard diaminopimelic acid (5 mg/ml water) was used as the reference spot. The chromatogram was then developed in the solvent comprising Methanol:pyridine:water:10M HCl in the ratio 320:40:70:10 in volume terms. The chromatogram was then air-dried at room temperature before being sprayed with acidic ninhydrin (Merck 1.06705), and then heated for 5 minutes at 100°C

in a hot air oven. DAP was characterized by its low R_f value and olive green colour which changed to yellow.

vi. ***Sugar fermentation test***
Sugar solutions were prepared by dissolving 0.25 g of the sugar in 10 ml distilled water. This was filtered using a sterile filter of 0.2 µm. For the lactobacilli, three sugars (arabinose, ribose, xylose) were used. For the pediococci, seven sugars (arabinose, galactose, lactose, melezitose, ribose and xylose) were investigated.

A litre of minimal medium with the following composition was prepared: 10.0 g Peptone, 4.0 g Yeast Extract Agar, 1.0 ml Tween 80, 2.0 g K_2HPO_4, 5.0 g Sodium acetate, 2.0 g Sodium Hydrogen phosphate, 0.2 g $MgSO_4$, 0.05 g $MnSO_4$, 0.04 g Chlorophenol red indicator. The pH of the medium was adjusted to 6.2, and sterilized.

The sugar fermentation test was carried out by centrifuging 1.0 ml of growth culture in eppendorf tubes. The pellets were resuspended in 1.0 ml of minimal medium. Then 20 µl of this was diluted in 1.0 ml minimal medium. A 25.0 µl volume of sugar solution was pipetted into microtitre plates and 100.0 µl of the diluted culture was added. The plates were incubated at 30°C for an initial 24 h and read. The plates with weak positive results were then reincubated for another 24 hours.
A positive reaction was shown by changes in the Chlorophenol red indicator turning yellow.

vii. ***Growth test***
Growth at different temperatures of 10, 15 and 45°C were investigated by inoculating 9.0 ml MRS broth with 100 µl of overnight growth culture maintained at 30°C. The tubes were then incubated at the various temperatures for 4 days with daily observation for growth.

viii. ***Morphology***
The surface of glass slide was sterilized by flaming. A 10.0 µl growth culture was pipetted unto the slide and cover slips placed over it. A drop of emersion oil was applied and viewed under a microscope at x 100 magnification.

ix. ***Isomerization***
The different lactic acid configurations (L, D or DL-lactate) of lactobacilli were indicated by calculated percentage compositions i.e. > 90 % was equated to L or D-lactate, while < 90 % was DL-lactate, showing a mix of the two isomerases.

x. ***Bionumeric computer programme***
This programme was used to group the lactic acid bacteria using all the isolated phenotypic characteristics investigated.

3.2 Genotypic characterization of *lactic acid bacteria*

i. *Extraction of Genomic DNA*

Total genomic DNA was extracted with the guanidium extraction method of Pitcher *et al.*, 1989 as modified by Björkroth and Korkeala, 1996.

A 5.0 ml overnight culture was centrifuged at 7,500 rpm for 5 min, and the supernatant discarded. The pellets were washed with 1.5 ml of 1 x TE buffer (10mM Tris HCl, 1mM EDTA, pH 8.0), 0.5 % NaCl. The solution was vortexed and into an eppendorf tube and centrifuged at 7,500 rpm for 5 min. Digestion was effected with 100 µl TERMLS solution and then incubated at 37 °C for 1 h. Cell lysis and protein denaturation was carried out by adding 500 µl of GES solution (5M Guanidium thiocyanate, 100mM EDTA, 0.5% Sarkosyl). The tubes were cooled on ice and gently shaken to mix. A 250 µl volume of 7.5 M Ammonium acetate was added, shaken to mix and then cooled on ice for 10 min. A ratio of 24 : 1 Chloroform-pentanol mixture (250 µl) was added, mixed and then centrifuged at 15,000 rpm for 10 min. The supernatant was pipetted into an eppendorf tube, 460 µl ice-cold Isopropanol was added and centrifuged at 15,000 rpm for 10 min. The pellets obtained were washed twice with 400 µl of 70 % Ethanol and then centrifuged each time at 15,000 rpm for 5 min. the pellets were dried for 5 min in a vacuum oven to obtain white pellets. About 110 – 400 µl Tris HCl, pH 8.0 was added and the tube tapped gently to mix the contents before incubation in a water bath at 50 °C for 15 – 20 min. The supernatant was pipetted into sterile eppendorf tubes. The optical density of the diluted DNA in MilliQ water (1:10) was measured spectrophotometrically at 260 nm.

ii. *PCR and Sequencing*

Rep-PCR was carried out for the Pediococci. A master mix of volume of 40 µl in each tube was prepared from 5µl Primer GTG 5, 5 µl of 3 mM $MgCl_2$ Buffer, 2 µl Dimethyl Sulfoxide, 8 µl dNTP-Mix, 0.3 µl Taq Polymerase and 19.7 µl MilliQ. To this 10 µl DNA was added to get a final total volume of 50 µl in each PCR tube. The PCR run was programmed at 95 °C for 7 min, 30 x 90 °C for 30 sec, 40 °C for 1 min, 65 °C for 8 min, 1 x 65 °C for 16 min and 6 °C for 6.5 h.

The PCR products were then run on agarose gel and then photographed.

4. Ochratoxin production of mould isolates from cocoa beans

i. *Preparation of cocoa and coffee bean powder*

The respective beans were placed in liquid nitrogen to harden and then ground to fine powder. These were placed in polyethylene bags and sealed. The bags were then sterilized by irradiation at.

ii. *Preparation of spore solution*

Spore solution was made from 4.5 g NaCl, 0.5 g Tween 80, 0.5 g Agar agar dissolved in 500 ml distilled water.

iii. *Moulds used*
Penicillium nordicum BFE 487, *Aspergillus ochraceus* BFE 635, *Aspergillus niger* BFE 632

iv. *Preparation of agar media*:

YES agar
20.0 g Yeast Extract, 150.0 g Saccharose, 15.0 g Agar agar, 1000 ml distilled water, pH 6.5.

Cocoa agar
400.0 g of sterile cocoa powder was dissolved in sterile solution of 15.0 g Agar agar in 1000 ml distilled water.

Coffee agar
400.0 g of sterile coffee powder was dissolved in sterile solution of 15.0 g Agar agar in 1000 ml distilled water.

v. *Inoculation of mould spores on agar media*
Spore solution (10 ml) was placed into a sterile conical flask. About 200 µl of the spore solution was pipetted unto the surface of mould growth on agar medium. A sterile loop was used to scrape the surface and pipetted back into the conical flask. This process was repeated until a uniform spore suspension was obtained. About 200 µl of the suspension was placed unto sterile agar media of YES, Cocoa, Coffee and spread evenly with a sterile spreader. Plates with *A. ochraceus* were incubated at 30°C while those with *A. niger* and *P. nordicum* suspensions were incubated at 25°C.

vi. *Sampling*
Cultured mould isolates on the respective agar media were sampled with a 10 mm cork borer and two plugs of growth transferred into each of duplicate eppendorf tubes. These were placed in a freezer to harden. A sterile spatula was then used to break the plugs and macerate the samples.

vii. *OTA extraction*
An extraction solution prepared from a mixture of 1.0 ml Dichloromethane and 1 % Acetic acid was added to the macerated plugs. It was placed in a mixer for 30 minutes, then transferred into an ultrasonic water bath for 5 minutes. The samples were then centrifuged for 10 min at 8,000 g. The solids were removed and the supernatant dried under nitrogen gas in an evaporating system at 30°C. Then 500 µl of Methanol was added to each sample. The samples were dried with nitrogen gas and resuspended in 500 µl Methanol before placing in an ultrasonic water bath for 5 min. The samples were centrifuged at 7,000 g for 30 seconds. Pooled samples of 500 µl from each duplicate eppendorf tubes were then aseptically pipetted into vials and stored in the freezer for HPTLC analysis.

viii. *HPTLC analysis*

Samples were analyzed for ochratoxin production using HPTLC machine (CAMAG TLC Scanner 3 and CAMAG Sprayer system) and computer programmed software. The HPTLC plates were pre-heated at 70°C for between 10 – 30 min before use. For each plate, a calibration curve was prepared.

The mobile phase was prepared in the ratio of 1 part Formic acid: 30 parts Ethylacetate: 60 parts Toluene.

The concentration of the standard OTA used was 4.74 ppm.

5. RESULTS

Lactic acid bacteria isolates from heap and tray fermented cocoa beans

Phenotypic characterization of 196 strains of lactic acid bacteria (Fig. 1) isolated from tray and heap fermented cocoa beans indicated 40 obligately heterofermentative lactobacilli that produced gas from glucose (DL-lactate); 110 facultatively hetero/obligately homofermentative lactobacilli that did not produce gas from glucose (DL-lactate); 1 facultatively hetero/obligately homofermentative lactobacillus that did not produce gas from glucose (D-lactate); 36 presumptive pediococci which did not produce gas from glucose (DL-lactate); 1 presumptive pediococcus (L-lactate) and 8 presumptive enterococci (L-lactate).

Cluster analysis of 110 facultatively hetero/obligately homofermentative lactobacilli were observed to comprise of 107 facultatively heterofermentative strains belonging to the *L. plantarum*-group which fermented arabinose, ribose or xylose, produced DL-lactate and possessed Diaminopimelic acid (mDAP) in the cell wall (Fig 2). There were 3 obligately homofermentative rods which did not ferment arabinose, ribose or xylose, produced DL-lactate and also possessed mDAP in the cell wall (Fig. 2).

The characterization of 107 *Lactobacillus plantarum* isolates from cocoa beans during fermentation is as shown in Fig. 3.

Figure 4 shows the sugar fermentation profile of Pediococci isolated from cocoa beans during heap and tray fermentation. The sugars investigated were arabinose, galactose, lactose, melezitose, maltose, ribose and xylose. Out of a total of 37 isolates, none (0 %) fermented lactose and maltose. All isolates (100 %) fermented arabinose, galactose, ribose and xylose. Only one isolate (2.7 %) fermented melezitose (Fig. 4).

Fig. 1. Phenotypic characterization of 196 strains of lactic acid bacteria

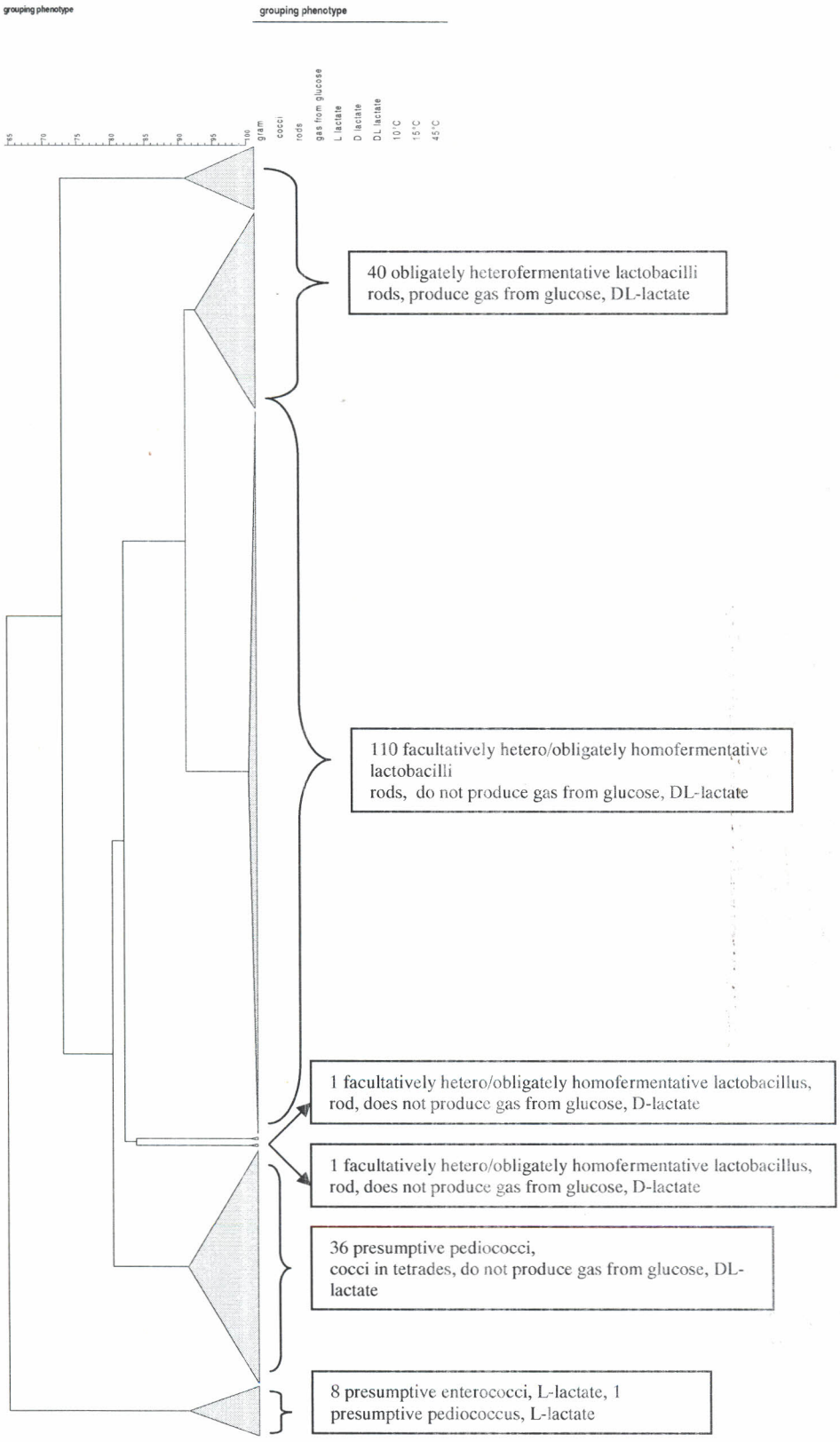


Fig. 2. Cluster of 110 obligately homo/facultatively heterofermentative rods

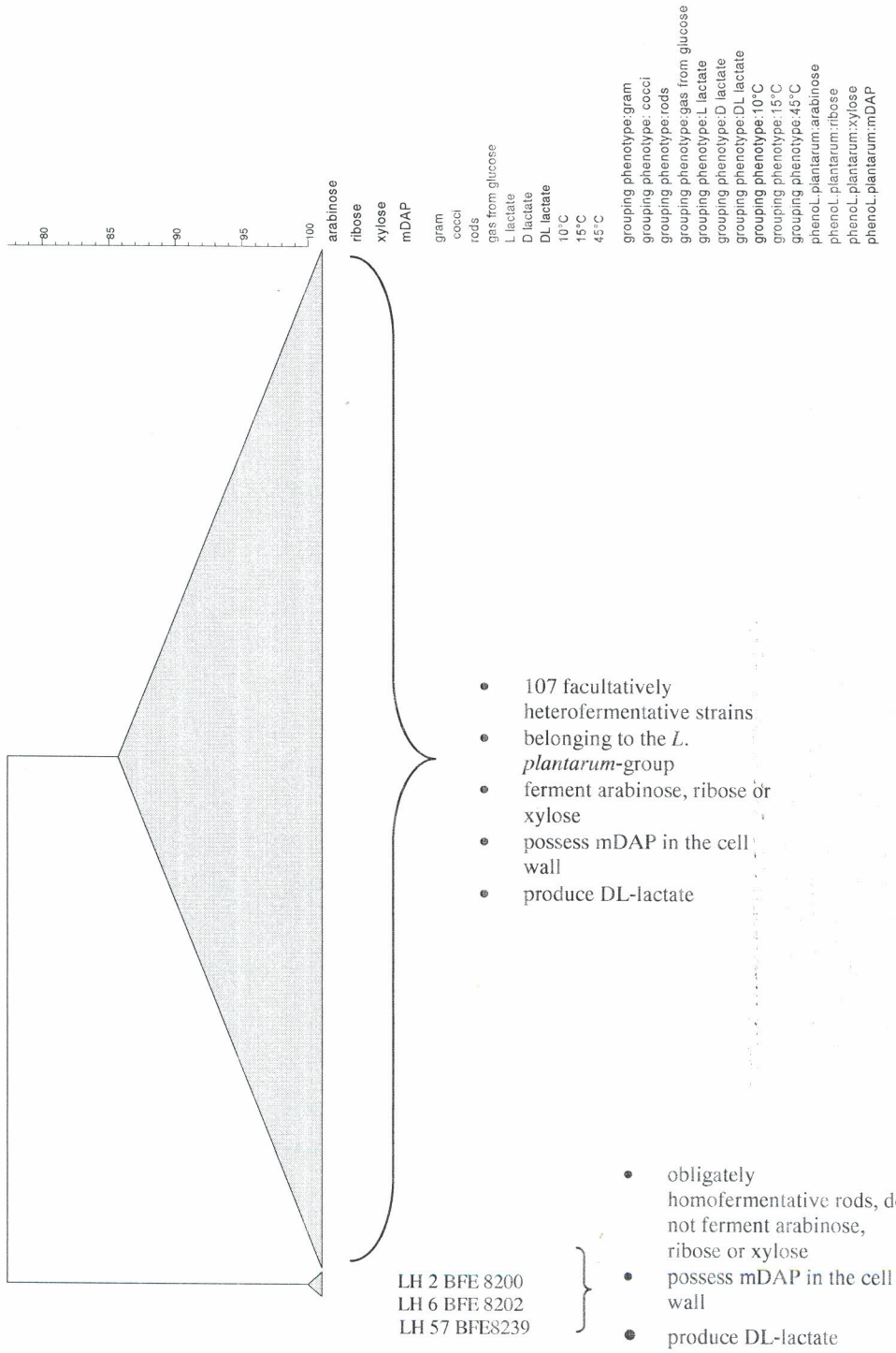


Fig. 3. *Lactobacillus plantarum* (107 strains)

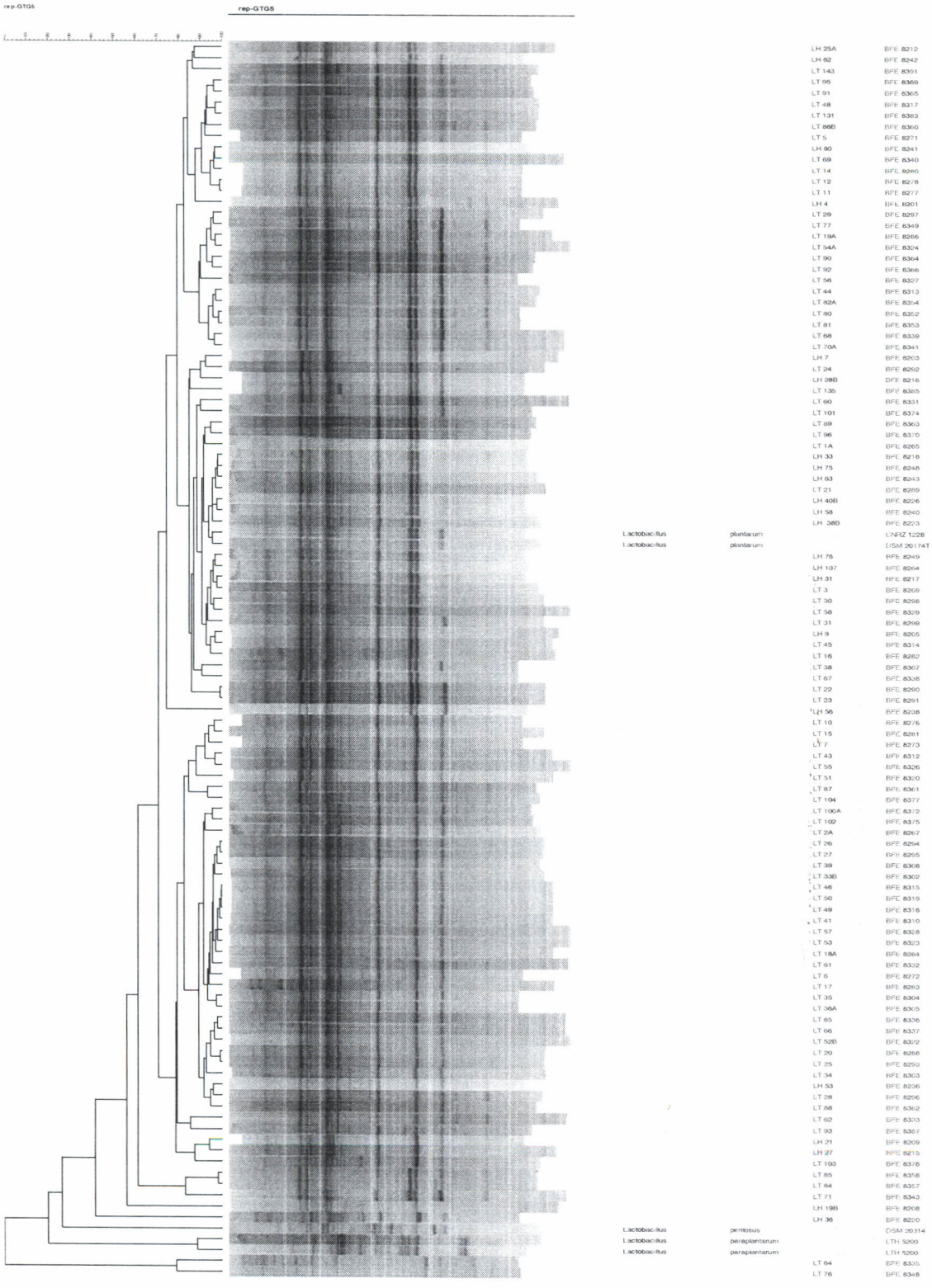


Fig. 4. Sugar fermentation of Pediococci isolated from cocoa beans during heap and tray fermentation

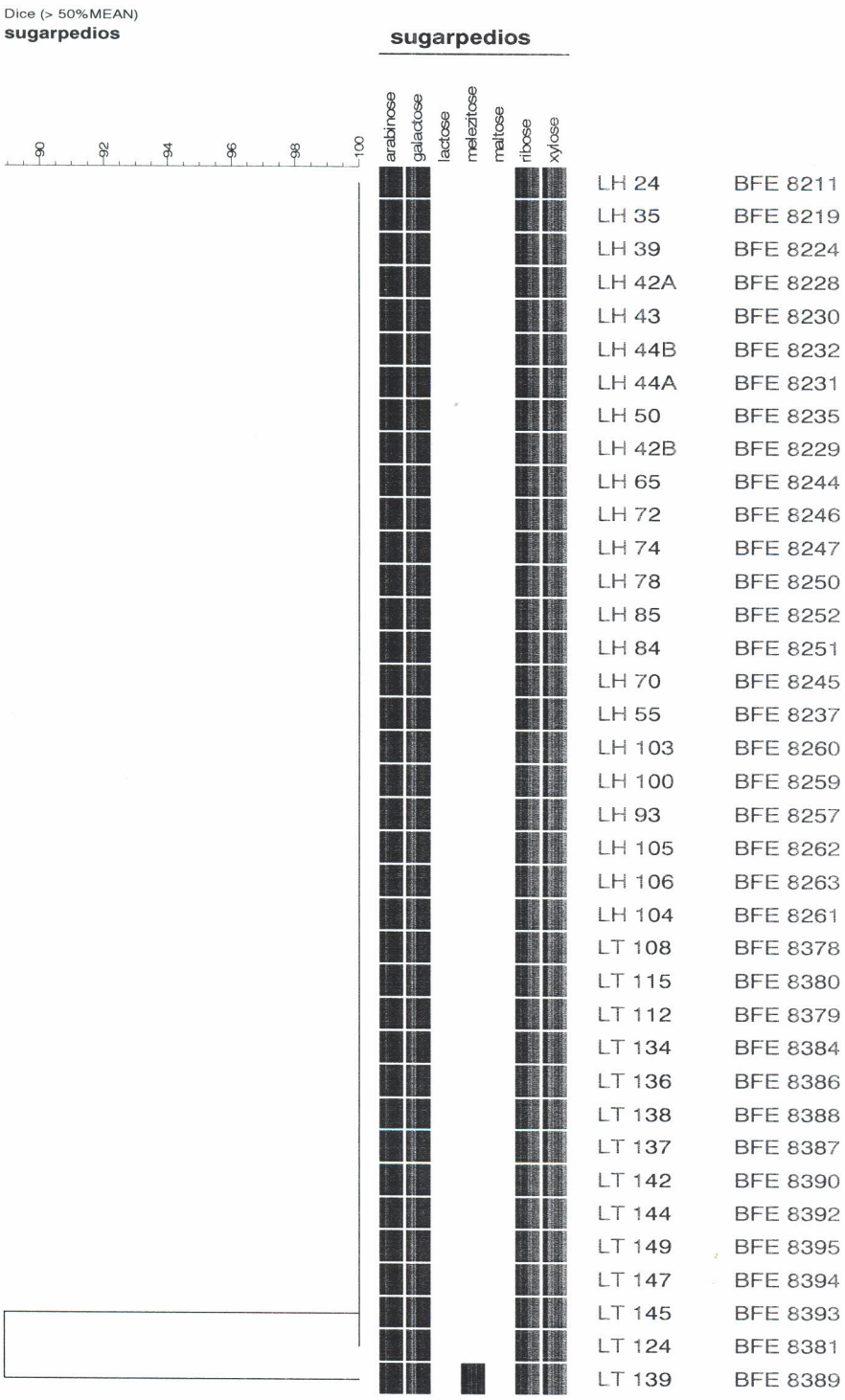


Fig.5. **Ochratoxin A producing capacity of mould isolates from heap and tray fermented cocoa beans**

Fig.5a. **Ochratoxin A (OTA) production by *Aspergillus ochraceus* in YES, Coffee and Cocoa media**

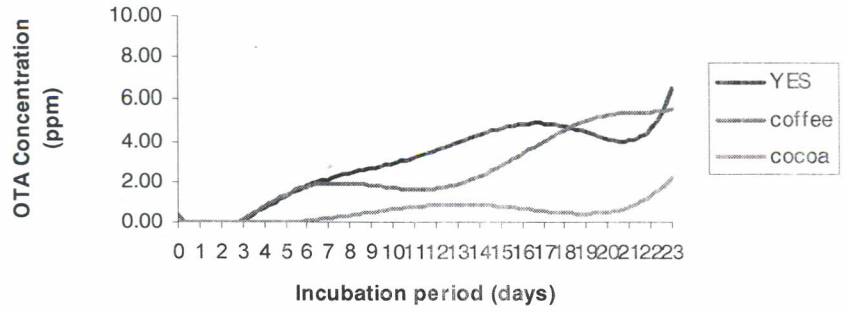


Fig.5b. **Ochratoxin A (OTA) production by *A. niger* in YES, Coffee and Cocoa media**

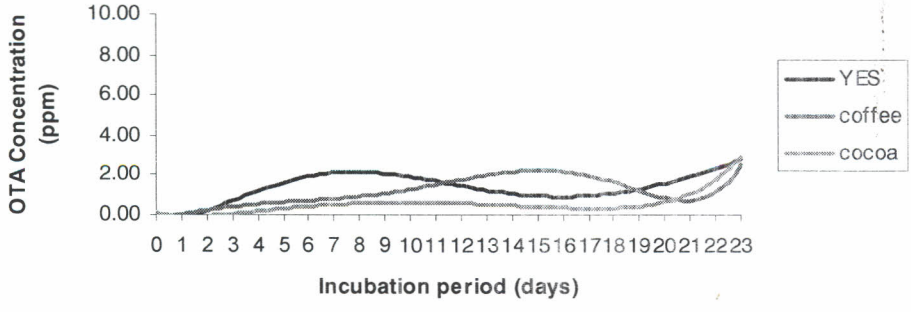
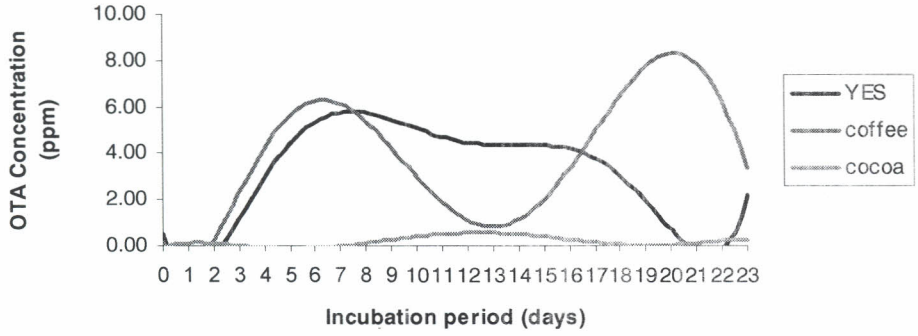


Fig. 6. ***Ochratoxin A (OTA) production by P. nordicum in YES, Coffee and Cocoa media***



OTA production by *Aspergillus ochraceus* in YES and Coffee media were high and more than observed in the Cocoa media. Maximum levels of OTA produced during the growth of *A. ochraceus* in YES, Coffee and Cocoa media were 6.23, 8.09 and 2.29 ppm and occurred on day 23, 19 and 11 respectively.

A. niger produced low levels of OTA in all the three media. However, YES and Coffee media were observed to support higher OTA production than Cocoa media. Maximum OTA concentration observed in YES media was 3.12 ppm on day 22. For Coffee media, 3.60 ppm occurred on day 15 while in Cocoa media 3.10 ppm was observed on day 23.

The production of OTA by *P. nordicum* also showed the same trend with higher levels observed using YES and Coffee media, as compared with Cocoa media.

Maximum levels of OTA produced during the growth of *P. nordicum* in YES, Coffee and Cocoa media were 8.16, 8.96 and 0.84 ppm and occurred on day 7, 19 and 12 respectively.

6. CONCLUSIONS

The techniques acquired from this study were very useful and would serve as a spring board for useful work on isolates from other fermentation systems in Ghana, taking into account the wide range of fermented foods in the country.

7. RECOMMENDATIONS

It is recommended that acquisition of High Performance Thin Layer Chromatography (HPTLC) equipment by FRI to effectively utilize the knowledge acquired from the training would be appreciated. This would also serve as a center of knowledge in analyzing mould species that produce Ochratoxin A. The HPTLC is more robust, fast and easier to use than the HPLC.

Collaborative research work involving European partners enhances the total work output of the African partners. It is therefore imperative that European partners are involved in future research to facilitate easy access or availability of equipment and training opportunities for African partners on a project.

It is anticipated that further work to be carried out in Ghana would involve working in close collaboration with the European partners in order to organize training courses for dissemination of the knowledge acquired. This would help to train laboratory technicians and other researchers from food processing industries in Ghana. Such facilities if available could be used to establish a center of excellence in this field for the African subregion.

8. CONSTRAINTS

Accomodation was reasonably good and close to the Institute for comfort, however per diem for sustenance and general upkeep were delayed such that I had to depend on Prof. Holzapfel to feed. Nevertheless, the fatherly nature of Prof. Holzapfel and his benevolence made life easy in Karlsruhe as he and his wife were more than parents to the visiting researchers.

9. ACKNOWLEDGEMENT

I wish to acknowledge Dr. C. Franz, Ms. M. Kostinek and Mr. A. Hanak for the excellent collaboration.

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