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Molecular characterisation and antimicrobial activity of bacteria associated with submerged lactic acid cassava fermentation

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ABSTRACT

Molecular identification of microorganisms associated with submerged cassava fermentation was carried out and isolates of lactic acid bacteria (LAB) were examined for antimicrobial activity, including ability to produce antimicrobial peptides as a first step to define starter cultures for controlled cassava fermentations. A total of 75 isolates, including 41 LAB, 31 aerobic bacteria (AB) and three anaerobic bacteria were isolated from unfermented and fermenting cassava roots, cassava leaves and fermented cassava dough and identified by a combination of phenotypic tests and sequencing of 16S rRNA, *rpoA*, *rpoB* and *pheS* genes. Microbial diversity at interspecies and intraspecies level was screened by, respectively, PCR amplification of the 16S-23S rDNA intergenic transcribed spacer (ITS-PCR) and repetitive sequence based PCR (rep-PCR). Antimicrobial activity of LAB cultures and supernatants against indicator bacteria; *Escherichia coli*, *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium), *Bacillus cereus* and *Staphylococcus aureus* was studied using agar diffusion tests. Furthermore, inactivation of indicator bacteria was investigated in both liquid medium and during controlled cassava fermentation. Results revealed a diversity of bacterial genera, species and subspecies associated with submerged cassava fermentation. DNA sequencing enabled identification of LAB isolates as *Lactobacillus plantarum*, *Weissella confusa*, *Weissella paramesenteroides*, *Lactobacillus rhamnosus*, *Lactobacillus hilgardii*, *Lactobacillus paracasei*, *Leuconostoc mesenteroides*, *Enterococcus faecium*, *Enterococcus casseliflavus*, and *Pediococcus acidilactici*. *Lactobacillus* spp. were the predominant LAB and were present in all cassava samples studied. Aerobic bacteria were predominantly *Bacillus* spp., including *Bacillus subtilis, Bacillus amyloliquefaciens* and *B. cereus*. Other species identified included *Staphylococcus pasteuri* and *Clostridium beijerinkii*. Cells, supernatants and cell free supernatants (CFS) of selected LAB isolates were able to inhibit both Gram positive and Gram negative pathogenic bacteria. LAB isolates inactivated all indicator organisms during controlled cassava fermentations, with a 4–6 log reduction after 48 h fermentation. The antimicrobial effect of the LAB was attributed to acid production.

1. Introduction

Cassava (*Manihot esculenta*, Crantz) ranks third after rice and maize as a source of calories in tropical countries (FAO, 2008). Fermentation is an important processing technique used to reduce toxicity and prevent post harvest deterioration of the roots. Cassava fermentation processes have been broadly categorised into two main types; 'solid state' as in *gari* production, where cassava is grated prior to fermentation and constantly watered during the process, and the 'submerged' (retting) process as in *fufu* or *lafun* production, where the tubers are cut into chunks and soaked in water for the duration of the fermentation, during which both acidification and softening of the roots take place (Moorthy & Matthew, 1998; Oyewole, 1995).

Although the microbiology of various indigenous fermented cassava obtained from solid state fermentations such as *agbelima*, *attieke* (Ghana, Cote d'Ivoire) and *gari*, (Nigeria, Benin), has been extensively studied (Amoa-Awua, Appoh, & Jakobsen, 1996; Assanvo, Agbo, Behi, Coulin, & Farah, 2006; Kostinek et al., 2005), microorganisms associated with submerged fermentations have been studied only to a limited extent. Bacteria associated with the submerged fermentation process have been reported to include

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Lactobacillus spp., *Leuconostoc* spp., *Bacillus* spp. and *Corynebacterium* spp. (Nwachukwu & Edwards, 1987; Oyewole & Odunfa, 1988). These studies have however mainly relied on phenotypic characterisation of the microorganisms involved, which can be both inconclusive and misleading unless confirmed using genotypic techniques. It is therefore important to identify microorganisms associated with submerged cassava fermentation using molecular methods, in addition to phenotyping.

Comparative analysis of sequences obtained from amplification of the 16S rRNA gene are commonly used in bacterial identification; however 16S rRNA sequences are not always able to discriminate between closely related species and sequencing of other genes such as *rpoA*, *pheS* and *rpoB* have been suggested as more reliable alternatives (Koo et al., 2003; Naser et al., 2007; Santos & Ochman, 2004).

Lactic acid bacteria (LAB) responsible for cassava fermentation may be able to produce antimicrobial compounds inhibitory to growth of both spoilage and pathogenic organisms, contributing to stability and safety of the fermented product (Holzapfel, 2002). In Africa, cassava fermentation is mainly a traditional technology carried out locally in households, often occurring as a spontaneous process, although in some cases traditional inocula are used to initiate fermentation. The use of unspecified procedures complicates control of the fermentation process. Such uncontrolled fermentations lead to variation of stability, nutritional and hygienic quality of the final product. The use of well-defined starter cultures for controlled fermentations is vital for the optimisation of the fermentation process, to provide a product of consistent organoleptic quality and safety (Sanni, 1993).

The aim of this study was to identify the predominant bacteria associated with submerged lactic fermentation of cassava, cassava leaves and cassava dough using a combination of phenotypic and genotypic methods and additionally, to investigate the antimicrobial activity of LAB isolates against pathogenic bacteria as a criterion for selection of starter cultures for controlled cassava fermentations.

2. Materials and methods

2.1. Cassava fermentation and microbiological sampling

Cassava roots were obtained from Burkina Faso and sampled aseptically within 24 h of receipt in the United Kingdom. Cassava roots purchased in the UK were also examined. The roots were cut into pieces of approximately 10 g, peeled and submerged in sterile distilled water for up to 48 h at 37 °C to allow fermentation to take place. Samples (10 g) were homogenised in 90 ml sterile Maximum Recovery Diluent (MRD, Oxoid, CM0733, Basingstoke, UK) using a paddle-type blender (AJ Seward, Colworth 400, London, UK) for 1 min. Ten-fold dilutions were prepared in MRD and 100 μl were plated in duplicate on deMan, Rogosa and Sharpe agar (MRS; Oxoid CM0361), Plate Count Agar (PCA; Oxoid CM0463) and Reinforced Clostridial Agar (RCA; Oxoid CM0151). Plates were incubated aerobically (PCA) for recovery of aerobic bacteria and anaerobically (MRS, RCA) using Gas Kits (Oxoid BR0038) for 48 h at 37 °C for the isolation of LAB and other anaerobic bacteria. Ten gram samples of fermented cassava dough and cassava leaves from Burkina Faso were also examined in the same way.

Predominant organisms were separately subcultured in Nutrient Broth (NB; Oxoid, CM0001), MRS broth (MRSB; Oxoid, CM0359), Reinforced Clostridial Medium (RCM; Oxoid, CM0149) as appropriate and streaked several times on agar to obtain pure cultures. For long-term storage, all microorganisms were maintained on cryobeads (Microbank™, Pro-lab Diagnostics, UK) at −20 °C.

2.2. Phenotypic characterisation

After purification, isolates on MRS, PCA and RCM were examined for Gram (Sigma, 77730, Dorset, UK) oxidase and catalase reactions, and sporulation as appropriate. In the catalase test, one drop of 30% (v/v) hydrogen peroxide (H3410, Sigma) was added to a colony on a glass slide. Immediate release of oxygen was taken as a positive reaction. For the oxidase test, filter paper (Whatman No. 4, Whatman Plc., Kent, UK) was saturated with N,N,N′,N′-Tetramethyl-p-phenylenediamine dihydrochloride solution (87890, Sigma). A sterile glass Pasteur pipette was used to take a portion of a colony, which was smeared on the saturated filter paper. A change in the colour on the filter paper from colourless to dark blue in less than 20 s was taken as a positive reaction and no colour change, or a change after 20 s, as a negative reaction. In addition, presumptive LAB isolates on MRS agar were examined for gas production in MRS broth supplemented with 1% (w/v) glucose. Carbohydrate fermentation profiles were determined using the API50CHB kits for aerobic bacteria and API50CHL kits for LAB, according to manufacturer's instructions (Biomerieux, 50410, Basingstoke, UK). Results were recorded visually and tentative identification was obtained after analysis using APIWeb software (Biomerieux).

2.3. Genotypic identification

2.3.1. Typing of bacteria by 16S − 23S rDNA internal transcribed spacer PCR (ITS-PCR) and rep PCR

Each isolate was streaked on the agar from which it had been isolated and incubated at 37 °C for 24–48 h. DNA from a single colony was extracted with InstaGene matrix (BioRad, 732-6030, Hertfordshire, UK) according to manufacturer's instructions. Isolates were grouped at species level based on genetic fingerprinting obtained from amplification of the 16S − 23S ITS region. Primers S-D-Bact-1494-a-S-20 (5′-GTCGTAACAAGGTAGCCGTA-3′: 10 pmol μl^{-1}), and L-D-Bact-0035-a-A-15 (5′-CAAGGCATCCACCGT-3′: 10 pmol μl^{-1}) were used to direct the amplification under the following conditions: 94 °C for 1 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min using Taq polymerase (5U; N808-0161, Applied Biosystems, Warrington, UK). The PCR was ended with a final extension at 72 °C for 7 min (Ouoba, Parkouda, Diawara, Scotti, & Varnam, 2008). Isolates were further discriminated at intraspecies level by rep-PCR using the primer GTG5 (5′-GTG GTG GTG GTG GTG-3′; 5 pmol μl^{-1}) under the following conditions: 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 1 min, elongation at 65 °C for 8 min and final extension at 65 °C for 16 min (Ouoba et al., 2008). Amplified PCR products were separated by 1.5% (w/v) agarose gel electrophoresis at 120 V for 1 h and 2 h respectively for ITS and rep-PCR products. Gels were stained with ethidium bromide and documented using the Gel Doc It Imaging System (M-26X, UVP, Cambridge UK). The DNA profiles were observed and all bacteria showing the same profile were clustered in the same group. Profiles were analysed using the Bionumerics system (Bio-Numerics 2.50, UPGMA Pearson Correlation, Applied Maths, Sint-Martens-Latem, Belgium).

2.3.2. Identification of bacteria by 16S rRNA, pheS, rpoA and rpoB gene sequencing

Primers, pA (5′-AGA-GTT-TGA-TCC-TGC-CTC-AG-3′; 100 pmol μl^{-1}) and pE (5′-CCG-TCA-ATT-CCT-TTG-AGT-TT-3′; 100 pmol μl^{-1}) based on conserved regions of the 16S rRNA gene were used to direct the amplification. The procedure consisted of: denaturation at 95 °C for 5 min, then 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min followed by a final extension at 72 °C for 5 min (Ouoba et al., 2008). Positive PCR products were confirmed by