Antimicrobial interactions of microbial species involved in the fermentation of cassava dough into agbelima with particular reference to the inhibitory effect of lactic acid bacteria on enteric pathogens

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Received 5 August 2002; received in revised form 3 February 2003; accepted 7 February 2003

Abstract

Lactic acid bacteria, \textit{Bacillus} species and yeasts are involved in the fermentation of cassava dough into agbelima. Microbial interactions within and between these groups of microorganisms were investigated in addition to the survival of five enteric pathogens inoculated into agbelima under various conditions. Nine out of 10 cultures of lactic acid bacteria isolated at the end of agbelima fermentation showed inhibitory effect against 10 cultures of lactic acid bacteria isolated at the start of fermentation. Only 3 out of 10 isolates of \textit{Bacillus subtilis} were inhibited by 10 isolates of lactic acid bacteria tested. No interactions were observed between yeasts and the lactic acid bacteria, whereas three of the \textit{Bacillus} isolates showed inhibitory effects against the yeasts. Twelve isolates of \textit{Lactobacillus plantarum} tested inhibited the growth of an isolate each of \textit{Lactobacillus fermentum} and \textit{Lactobacillus brevis} but none tested positive for bacteriocin production. The antimicrobial effect of the lactic acid bacteria was attributed to acid production. In fermenting cassava dough, enteric pathogens survived to different extents depending on pH and their sensitivity to acids. \textit{Vibrio cholerae} C-230, \textit{Salmonella typhimurium} 9 and \textit{Salmonella enteritidis} 226 were not detectable in 10 g of sample after 4 h when inoculated into the 48-h fermented product, agbelima, whereas \textit{Shigella dysenteriae} 2357T and \textit{Escherichia coli} D2188 were detectable up to 8 h in the product.

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\textit{Keywords:} Antimicrobial; Cassava; Agbelima; Lactic acid bacteria; Pathogens

1. Introduction

Agbelima, a smooth-textured sour cassava dough, is consumed extensively in Ghana and is usually prepared as a meal by cooking it together with fermented maize dough into a stiff porridge which is eaten with a stew. Agbelima is prepared by peeling cassava roots and grating them together with a traditional inoculum which is also prepared from cassava roots. The grated mash is packed into plastic sacks and allowed to ferment whilst weights are placed on top of the sacks to dewater the mash. The fermentation of agbelima has been reported to be both a souring and texture-degrading process involving \textit{Bacillus} spp., lactic acid bacteria, yeasts, and in some
cases, moulds (Amoa-Awua and Jakobsen, 1995; Amoa-Awua et al., 1996, 1997). The Bacillus species, moulds and some yeasts breakdown the texture of the coarse cassava mash into a smooth-textured dough through the production of cellulase enzymes which hydrolyse the cassava tissue. The lactic acid bacteria, on the other hand, cause a souring of the dough and are dominated by Lactobacillus plantarum, Lactobacillus brevis and Leuconostoc mesenteroides. In the fermentation process, the levels of some microbial species increase whilst others decrease. This gives an indication that antimicrobial interactions between the various species of microorganisms may be important in the fermentation of agbelima.

This work was carried out to investigate possible antimicrobial interactions that may occur within and between the lactic acid bacteria, Bacillus spp. and yeasts during the fermentation of inoculated cassava dough into agbelima and also the survival of some enteric pathogens when inoculated into agbelima.

2. Materials and methods

2.1. Materials

Samples of a traditional inoculum, kudeme, were obtained from a traditional cassava processor at Medie near Accra. The inoculum was prepared by the traditional processor as follows: A few cassava roots were peeled, washed and cut into chunks. The chunks were partially roasted in an open fire, wrapped with a piece of cloth and placed in a small basket to ferment for 3 days into the inoculum. The inoculum was used to prepare agbelima as follows: About 20 kg of cassava roots was peeled, washed and grated in a cassava grater together with 0.5 kg of the inoculum. The grated cassava mash was packed into a polythene sack, a weight placed on top and left to ferment for 3 days. During fermentation, samples of the dough were removed aseptically from the interior of the fermenting batch at 0, 24 and 48 h for analysis.

2.2. Microbiological analysis

2.2.1. Isolation of bacteria and yeasts from agbelima

For all samples, 10 g of cassava dough was added to 90 ml sterile diluent containing 0.1% peptone (Difco 0118-17, Becton Dickinson and Co., Sparks, USA) and 0.85% NaCl with pH adjusted to 7.2 and homogenised in a stomacher (Lab Blender, Model 4001, Seward Medical, England) for 30 s at normal speed. Aerobic mesophiles were isolated on Plate Count Agar (PCA, Difco 0479-17-3, Difco Laboratories, Detroit, MI, USA) incubated at 30 °C for 3 days. Lactic acid bacteria were isolated on DeMan, Rogosa and Sharpe Agar (MRS, Merck 10660, Merck, Darmstadt, Germany) incubated anaerobically in an anaerobic jar with anaerocult A (Merck) at 30 °C for 5 days and yeasts on Malt Extract Agar (Merck 5398) containing 100 mg chloramphenicol (Sigma C-0378, Sigma, St. Louis, MO, USA) and 50 mg chlorotetacycline (Sigma C-4881) per litre and incubated at 25 °C for 5 days. Colonies from PCA were subcultured in Nutrient Broth (Merck 5443), MRS plates in MRS broth (Merck 10661) and MEA plates in Malt Extract Broth (Merck 5397). The cultures were purified by continual streaking on agar plates.

2.2.2. Characterisation and identification of isolates

Isolates on PCA that were Gram-positive, catalase-positive rods mostly bearing phase bright spores were suspected to be Bacillus species, and further tests were carried out to confirm their genus according to Claus and Berkeley (1986) and Parry et al. (1983). Tests carried out included morphological examination and biochemical tests. The biochemical tests included anaerobic growth; acid production from D-glucose, D-xylose, L-arabinose and D-mannitol; hydrolysis of casein, starch and gelatin; nitrate reduction; indole formation; and growth at pH 5.7 and 6.8, in 7% (w/v) NaCl and at 10, 30, 50 and 65 °C. The pattern of utilisation of the following sugars were also determined: cellobiose, fructose, galactose, lactose, maltose, melibiose, raffinose, salicin, sorbitol, melezitose and trehalose. Three millilitre slants containing 150 µl of 10% filter sterile solution of the carbohydrate and a basal medium containing g l⁻¹ distilled water: 1, diammonium hydrogen phosphate; 1, potassium chloride; 0.2, magnesium sulphate; 0.2, yeast extract and 15, agar; 0.006, brom cresol purple, pH 7.0, were inoculated with the test organism and incubated at 30 °C. A change in colour of the medium from purple to yellow indicated acid production.

Isolates on MRS were examined by colony and cell morphology, Gram reaction and catalase test. Gram-
positive, catalase-negative rods, coccobacilli and cocci were considered as lactic acid bacteria and examined further for gas production in MRS broth with Durham tube and also in MRS broth in which glucose was replaced with gluconate as sole carbon source, growth at 10 and 45 °C, growth at pH 4.4 and 9.6, growth in 6.5% and 18% (w/v) NaCl and Hugh and Leifson test (Hugh and Leifson, 1953). The species of Lactobacilli were identified by assaying cultures in API 50 CHL galleries (Bio Merieux, SA, France) using API 50 CHL medium (Bio Merieux). MEA isolates were examined by colony and cell morphology.

2.3. Antimicrobial studies

The inhibitory potential of lactic acid bacteria cultures and their supernatants were investigated using the Agar Well Assay method as described by Schillinger and Lücke (1989) and Olsen et al. (1995). The appropriate agar was poured into petri dishes and left to solidify and dry for 1–2 days. Circular wells were made in the agar using sterile cork borers. Ten cultures of lactic acid bacteria isolated from 48-h fermented cassava dough were each cultured in MRS broth (Merck 1.0661) at 30 °C for 20–24 h and 100 μl of the cultures transferred into the wells and left to diffuse into the agar for approximately 4–5 h. The wells were overlaid with about 10 ml of the appropriate soft agar (0.7% agar) containing the indicator strains. The indicator organisms were 10 cultures of lactic acid bacteria isolated at the start of agbelima fermentation, 10 cultures of Bacillus species isolated at different stages of agbelima fermentation and also 3 yeasts isolated during agbelima fermentation. The indicator lawns were prepared by adding 0.25 ml of 10⁻¹ dilution of an overnight culture of the indicator organism to 10 ml of either MRS agar (Merck 10660), Nutrient Agar (Merck 5450) or Malt Extract Agar (Merck 5398) for lactic acid bacteria, Bacillus spp. and yeasts, respectively. The plates were incubated overnight at 37 °C and clear zones around the wells were described as inhibitory reactions. Each experiment was carried out in duplicate.

2.3.1. Test for possible bacteriocin production using bacteriocin indicator organisms

Volumes of 100 μl of overnight cultures of isolates of L. plantarum were transferred separately into MRS agar wells and overlaid with 10 ml of MRS soft agar containing Lactobacillus sake or Listeria monocytogenes obtained from the Department of Veterinary Microbiology, Royal Veterinary and Agricultural University, Denmark, as bacteriocin indicator organisms and incubated anaerobically at 37 °C for 24 h and examined for inhibitory reactions.

2.3.2. Test for possible bacteriocin production using culture supernatants digested with proteolytic enzymes

For each of the 12 isolates of L. plantarum tested, 10 ml of a 24-h culture of the isolate was centrifuged at 3000 × g for 15 min at 4 °C and the clear supernatant filtered using a 0.20-μm sterile filter (Sartorius Minisart, Gottingen, Germany). Cell-free extract (100 μl) was digested separately with Proteinase K (Merck EC 3.4.21.19, Merck) and Pronase E (Sigma P-6911, Sigma) at a concentration of 1 mg ml⁻¹ at 30 °C for 2 h and tested against the bacteriocin indicator strains, L. sake and L. monocytogenes using the Agar Well Diffusion Assay method and incubated anaerobically at 37 °C for 24 h.

2.3.3. Test for hydrogen peroxide as antimicrobial property

Supernatants (100 μl) of the 12 isolates of L. plantarum were each treated with catalase enzyme (Catalase, Sigma C6665) which had been sterilised by filtering through 0.2 μm filter (Sartorius Minisart). The final concentration of the enzyme in the supernatants was 1 mg ml⁻¹ and treatment was for 2 h. The supernatants with catalase were tested against indicator strains using the Agar Well Diffusion Assay method and incubated at 37 °C for 24 h.

2.3.4. Test for acid production as antimicrobial property

Cell-free extracts of L. plantarum isolates were neutralised by adding 0.1 M NaOH to the supernatants to raise their pH to 6.8 ± 0.1. Both neutralised and non-neutralised supernatants were sterilised by filtering through 0.20 μm filter (Sartorius Minisart). The neutralised and non-neutralised supernatants were tested separately against indicator strains of L. sake and L. monocytogenes using the Agar Well Diffusion Assay method.
2.3.5. Acid production by isolates of *L. plantarum* in batch culture

The rate of acid production in 10% sterile cassava dough slurry by four different isolates of *L. plantarum* was investigated by cultivating in a 2-l fermentor (Biostat B., B. Braun, Biotech International, Melsungen, Germany). The selected isolates were *L. plantarum* LAB 6, LAB 9 and LP 11, which showed the greatest ability to inhibit other organisms, and *L. plantarum* LP 3, which showed the least ability to inhibit other organisms based on the number of positive reactions and diameter of inhibitory zones in the antimicrobial interactions. Freshly grated cassava dough was sterilised by irradiating with 15 kG of ionising radiation at the Ghana Atomic Energy Commission in Accra. A 10% solution of the grated cassava was prepared using sterile water in a 2-l fermentor which had been sterilised by autoclaving. The substrate was inoculated with the *L. plantarum* culture at a concentration of 10^6 cfu ml^{-1} and the batch fermentor run for 30 h at 32 °C with a stirrer speed of 50 rpm without controlling the pH. Samples of the fermenting medium were collected at 2–3 h intervals and the titratable acidity determined. For determination of titratable acidity, 10 ml of sample was made up to 250 ml by addition of distilled water. Diluted sample (100 ml) was filtered and titrated against 0.1 M NaOH using phenolphthalein as indicator. One millilitre of 0.1 M NaOH was taken as equivalent to 9.008 × 10^{-3} g lactic acid.

2.3.6. Survival of enteric pathogens in agbelima

The ability of different enteric pathogens to survive in agbelima under different conditions was studied using three different systems. The enteric pathogens used were *Salmonella typhimurium* 9, *Salmonella enteritidis* 226, *Shigella dysenteriae* 2357T, *Escherichia coli* D2188 serotype 0157:K:7 (VTEC) and *Vibrio cholerae* C-230, all obtained from the State Serum Institute, Copenhagen, Denmark. The three systems investigated were (i) freshly grated cassava dough containing inoculum; (ii) 48-h fermented cassava dough; and (iii) fermented cassava dough, the pH of which had been adjusted/neutralised to pH 5.7 using 0.1 NaOH.

Pure cultures of each pathogen were plated on Nutrient Agar and incubated at 37 °C for 24 h. The culture was washed off by pouring 9 ml of Salt Peptone Solution (SPS, Difco) onto the agar plate and the cell suspension withdrawn with a sterile syringe. The pathogens were each inoculated into the agbelima sample at a concentration of 10^6–10^7 cfu g^{-1}. Ten grams of each sample was collected at intervals and the population of surviving pathogens enumerated by spread plate on SSI-Enteric medium (Statens Serum Institut, Copenhagen, Denmark) (Blom et al., 1999).

3. Results and discussions

3.1. Isolation and identification of microorganisms

*Bacillus* species were isolated from Plate Count Agar plates as Gram-positive catalase-positive rods which often bore spores when observed under phase-contrast microscopy. Most of the isolates had small cells with centrally placed spores and produced acid from glucose, arabinose, xylose and mannitol, hydrolysed starch and casein, grew at pH 5.7 and 6.8 in 7% (w/v) NaCl and at 30 °C. The most frequently occurring type of isolate fermented glucose, cellobiose, fructose, maltose, melibiose, raffinose, salicin, sorbitol, trehalose, arabinose, xylose and mannitol but failed to utilise galactose, lactose and melezitose. Based on this pattern of carbohydrate utilisation (Claus and Berkeley, 1986) and by comparison with known *Bacillus* cultures previously identified from agbelima by Amoa-Awua and Jakobsen (1995), the dominating *Bacillus* species was assumed to be *Bacillus subtilis*. Isolates of *B. subtilis* were used for the antimicrobial studies.

Gram-positive, catalase-negative regular rods, very short rods and coccoids isolated anaerobically on MRS agar were examined by assaying cultures in API 50 CHL galleries. The most frequently occurring isolates were regular rods, which metabolised glucose fermentatively in Hugh and Leifson medium, were non-oxidative and produced gas from glucose but not gluconate, hence, facultatively heterofermentative. This lactobacillus species could neither grow at 45 °C nor in 6.5% (w/v) NaCl. In the API galleries, they fermented L-arabinose, ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, α-methyl-D-mannoside, N-acetyl glucosamine, amygdaline, arbutin, esculin, salicin, cellobiose, maltose, lactose,
melibiose, saccharose, trehalose, melezitose, D-raffinose, β-gentiobiose, D-turanose and gluconate. This
dominant species was identified as *L. plantarum* using
the API 50 CHL database and is in agreement with the
findings of Amoa-Awua et al. (1996) who reported *L.*
plantarum as the predominant lactic acid bacteria in
agbelima fermentation. The second group of isolates
identified as *L. brevis* was composed of very short
rods which produced gas from both glucose and
 gluconate, hence, obligately heterofermentative. They
did not grow in 6.5% (w/v) NaCl and were able to
ferment L-arabinose, ribose, D-xylene, galactose, D-
glucose, D-fructose, maltose, melibiose, saccharose, D-
raffinose and gluconate. The third group of isolates
identified as *Lactobacillus fermentum* metabolised
glucose fermentatively in Hugh and Leifson medium
and was obligately heterofermentative. They were
able to grow at 45 °C and fermented ribose, D-xylene,
galactose, D-glucose, D-fructose, D-mannose, maltose,
lactose, melibiose, saccharose, D-raffinose and gluco-

3.2. Antimicrobial interactions during agbelima
fermentation

3.2.1. Antimicrobial interactions between lactic acid
bacteria present at the start and end of fermentation

Widespread inhibitory reactions were observed
between lactic acid bacteria isolated at the start and
end (0 and 48 h) of agbelima fermentation. All lactic
acid bacteria isolated at the end of fermentation were
able to inhibit all isolates from the start of fermenta-
tion with the exception of LAB S2 and LAB S6 (Table 1). This was expected since those isolated at
the end of fermentation had been able to survive
microbial competition throughout the fermentation
process. In the case of LAB S6, four of the isolates
from the end of fermentation were not able to
prevent its growth. In maize dough fermentation,
Olsen et al. (1995) found that lactic acid bacteria
isolated from an advanced stage of fermentation
showed strong inhibition against cultures isolated
from the early stages of processing.

3.2.2. Antimicrobial interactions between lactic acid
bacteria, *Bacillus* species and yeast isolates

There was less interaction between the lactic acid
bacteria and *Bacillus* species isolated from age-
lima. Three out of the 10 isolates of *B. subtilis* were
inhibited by more than 7 of the 10 lactic acid
bacteria isolated from the end of fermentation
(Table 2). However, only 2 of the *Bacillus* isolates
could not be inhibited by any of the lactic acid
bacteria.

None of the lactic acid bacteria isolates were able
to inhibit any of the yeast cultures (results not
shown). Several authors have reported the coexis-
tence and positive interactions involving yeasts and
lactic acid bacteria in different African fermented
foods (Jerpersen et al., 1994; Hounhouigan et al.,

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Table 1
The antimicrobial interactions within the lactic acid bacteria population in agbelima

<table>
<thead>
<tr>
<th>Lactic acid bacteria isolated at the end of fermentation (test isolates)</th>
<th>LAB S1</th>
<th>LAB S2</th>
<th>LAB S3</th>
<th>LAB S4</th>
<th>LAB S5</th>
<th>LAB S6</th>
<th>LAB S7</th>
<th>LAB S8</th>
<th>LAB S9</th>
<th>LAB S10</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB 1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>LAB 2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>LAB 3</td>
<td>+</td>
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<td>LAB 4</td>
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<td>LAB 5</td>
<td>+</td>
<td>-</td>
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<tr>
<td>LAB 6</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+</td>
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<td>++</td>
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<td>++</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>LAB 8</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>LAB 9</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++</td>
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<td>+</td>
<td>++</td>
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<tr>
<td>LAB 10</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- : no inhibition.
+ : 5–8 mm inhibition zone.
++: >8 mm inhibition zone (very strong inhibition).
The stimulating effect of yeasts on lactic acid bacteria has been attributed to the provision of some compounds such as soluble nitrogenous compounds, B-vitamins, CO₂, pyruvate, propionate, acetate and succinate (Nout, 1991; Leroi and Pidoux, 1993). Vollmar and Meuser (1992) have also shown that yeasts multiplication is associated with an increase in acid formation of particular lactic acid bacteria in fermented products.

Of the 10 isolates of *B. subtilis*, only 3 cultures inhibited growth of all 3 yeast isolates tested (results not shown).

### 3.2.3. Antimicrobial interactions between *L. plantarum* and other species of lactobacilli, *B. subtilis* and yeasts

Each of the 10 selected isolates of *L. plantarum* showed inhibitory reaction against an isolate of *L. fermentum*. With respect to an isolate of *L. brevis* tested, 8 out of the 10 selected isolates of *L. plantarum* showed inhibitory reaction (Table 3). This ability of the isolates of *L. plantarum* to inhibit some of the other lactobacilli present in agbelima could explain the dominance of *L. plantarum* in agbelima fermentation.

There was very little interaction between *L. plantarum* and *B. subtilis*. Out of 120 antimicrobial tests carried out between 12 isolates of *L. plantarum* and 10 isolates of *B. subtilis*, only 15 inhibitory tests were positive (results not shown). None of the 12 isolates of *L. plantarum* exhibited inhibitory activity against any of the 3 yeast isolates tested.

3.2.4. Mechanism of *L. plantarum* inhibitory activity

The culture supernatants of the 10 selected isolates of *L. plantarum* from the end of agbelima fermentation were tested against *L. sake* and *L. monocytogenes* as possible indicators of bacteriocin production. With the exception of *L. plantarum* LP 3, which was unable to inhibit the *L. monocytogenes*, all isolates showed inhibitory reactions against *L. sake* and *L. monocytogenes* (results not shown). However, after neutralisation of the culture supernatants, no such inhibitory reactions were observed for any of the cultures (results not shown). The addition of catalase and the proteolytic enzymes, Proteinase K and Pronase E to the supernatants did not have an effect on their inhibitory activity against the indicator organisms. It was concluded that the inhibitory action of the *L. plantarum* isolates was due to acids produced rather than the production of bacteriocins, hydrogen peroxide or any other antimicrobial compounds. Five isolates of *L. fermentum* and one isolate of *L. brevis* were also tested against the bacteriocin indicator organisms but they showed only weak or no inhibitory activity.

### Table 3

The inhibitory effect of *L. plantarum* against other species of lactic acid bacteria isolated from cassava dough (agbelima)

<table>
<thead>
<tr>
<th>Lactobacillus plantarum (test isolates)</th>
<th>Indicator isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactobacillus</td>
</tr>
<tr>
<td></td>
<td>fermentum</td>
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<tr>
<td></td>
<td><em>L. monocytogenes</em></td>
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<tr>
<td><em>L. plantarum</em> LP 1</td>
<td>++</td>
</tr>
<tr>
<td><em>L. plantarum</em> LP 2</td>
<td>+</td>
</tr>
<tr>
<td><em>L. plantarum</em> LP 3</td>
<td>+</td>
</tr>
<tr>
<td><em>L. plantarum</em> LP 5</td>
<td>+</td>
</tr>
<tr>
<td><em>L. plantarum</em> LP 6</td>
<td>++</td>
</tr>
<tr>
<td><em>L. plantarum</em> LP 11</td>
<td>++</td>
</tr>
<tr>
<td><em>L. plantarum</em> B2-17</td>
<td>+</td>
</tr>
<tr>
<td><em>L. plantarum</em> B2-14</td>
<td>+</td>
</tr>
<tr>
<td><em>L. plantarum</em> B2-4</td>
<td>+</td>
</tr>
<tr>
<td><em>L. plantarum</em> B0-1</td>
<td>+</td>
</tr>
<tr>
<td><em>L. plantarum</em> LAB 6</td>
<td>++</td>
</tr>
<tr>
<td><em>L. plantarum</em> LAB 9</td>
<td>++</td>
</tr>
</tbody>
</table>

− : no inhibition.

+: 5–8 mm inhibition zone.

++: >8 mm inhibition zone (very strong inhibition).

1997; Oyewole and Odunfa, 1990). The stimulating effect of yeasts on lactic acid bacteria has been attributed to the provision of some compounds such as soluble nitrogenous compounds, B-vitamins, CO₂, pyruvate, propionate, acetate and succinate (Nout, 1991; Leroi and Pidoux, 1993). Vollmar and Meuser (1992) have also shown that yeasts multiplication is associated with an increase in acid formation of particular lactic acid bacteria in fermented products.

Of the 10 isolates of *B. subtilis*, only 3 cultures inhibited growth of all 3 yeast isolates tested (results not shown).
3.2.5. Rate of acid production by isolates of *L. plantarum*

The production of acid by four different isolates of *L. plantarum* when grown separately in a 10% cassava dough slurry in batch culture is shown in Fig. 1. Acid production in the cassava medium was faster and higher by isolate LAB 9 and slower and lower by isolate LP 3. Productions of acid by LAB 6 and LP 11 were similar and fell between the two ranges. The pattern in acid production of the four isolates is in conformity with the observations in their antimicrobial activities. In all the antimicrobial interactions involving these isolates, LAB 9 showed the greatest number of inhibitory reactions, whereas LP 3 showed the least number of inhibitory reactions. The diameter of inhibitory zones also tended to be larger with LAB 9 and lower with LP 3. These results tend to confirm that the inhibitory activities of these lactic acid bacteria were due to the production of acid.

3.3. Enteric pathogens

3.3.1. Survival of enteric pathogens inoculated into cassava dough at the start of fermentation

The survival of five enteric pathogens which were inoculated into freshly grated cassava dough at the start of fermentation at a concentration of about $10^6$–$10^7$ cfu g$^{-1}$ is shown in Fig. 2. All five pathogens, *S. typhimurium* 9, *S. enteritidis* 226, *S. dysenteriae* 235 7T, *E. coli* D2188 and *V. cholerae* C-230, were no longer detectable in 10 g of sample in the fermenting dough after 24 h. The pH at 24 h had dropped from 5.7 to 4.1.

3.3.2. Survival of enteric pathogens inoculated into 48-h fermented cassava dough (final product)

The enteric pathogens were also inoculated into cassava dough which had already been fermented for 48 h and had a pH of 3.9 which dropped to pH 3.8 within 8 h. *S. typhimurium* 9, *S. enteritidis* 226 and *V. cholerae* C-230 could not be detected in 10 g of sample after 4 h, whereas *S. dysenteriae* 2357T and *E. coli* D2188 could not be detected after 8 h (results not shown). The pH of the fermented dough after 8 h was 3.8. If agbelima is thus contaminated by any of these pathogens and the dough that is usually kept for a couple of days is not used for food preparation within 8 h, it is likely to be free of the pathogen.

3.3.3. Survival of enteric pathogens in neutralised fermented cassava dough

To confirm that the reduction in numbers of the enteric pathogens in agbelima was due to the low pH/high acidity, the pathogens were also inoculated into fermented cassava dough after the pH had been adjusted from 4.0 to 5.7, that is, the original pH of

![Fig. 1. Production of acid in 10% cassava slurry by *L. plantarum* cultures isolated from agbelima.](image-url)
the dough before fermentation. With the exception of *V. cholerae* C-230, the other pathogens were detectable in 10 g of sample after 24 h (Fig. 3). After 48 h, *S. enteritidis* 226 and *S. typhimurium* 9 could also not be detected, whereas *S. dysenteriae* 2357T and *E. coli* D2188 could still be detected in 10 g of the neutralised dough sample. The reduction in numbers of pathogens inoculated into agbelima during fermentation could therefore be attributed to acid production/low pH because raising the pH of fermented cassava dough prolonged the survival of the pathogens in the sample. The surviving pathogens were not evaluated after 48 h.

![Fig. 2. Survival of enteric pathogens in fermenting cassava dough (agbelima).](image)

![Fig. 3. Survival of enteric pathogens inoculated into fermented cassava dough with pH adjusted to 5.7.](image)
3.3.4. Survival of enteric pathogens in agbelima

Variations in the survival of the different enteric pathogens in fermented cassava dough have been confirmed by the results of this work. *V. cholerae* C-230 and the *Salmonella* species were the most sensitive of the pathogens studied and could not survive for more than 4 h when inoculated into the fermented dough, agbelima. The *Shigella* and *E. coli* strains, however, survived for 8 h in agbelima.

A number of investigations on the survival of enteric pathogens in some traditional African fermented foods have shown that the sensitivity of pathogens to pH differs (Lorri and Svanberg, 1991; Mbugua and Njenga, 1991; Mensah et al., 1990). Several studies have shown that in particular, veroxin-producing *E. coli* can be very acid tolerant (Conner and Kotrola, 1995; Diez-Gonzalez and Russell, 1997).

4. Conclusion

In this work, the fermentation of cassava dough into agbelima has been shown to involve interactions within the lactic acid bacteria population with the dominant species, *L. plantarum*, being able to inhibit the growth of some of the other species including *L. fermentum* and *L. brevis*. Some of the lactic acid bacteria are also able to inhibit growth of some *B. subtilis* isolates, but with respect to yeasts, no such inhibitions were observed, and this is attributable to synergistic association between lactic acid bacteria and yeasts. In fermenting cassava dough, enteric pathogens survive to different extents depending on pH and their sensitivity to acids. *V. cholerae* C-230, *S. typhimurium* 9 and *S. enteritidis* were not detectable in 10 g of sample after 4 h when inoculated into agbelima, whereas *S. dysenteriae* 2357T and *E. coli* D2188 could not be detected after 8 h. The antimicrobial properties of *L. plantarum* isolates were attributed mainly to the production of acid.

Acknowledgements

This work was funded by The Danish International Development Assistance (DANIDA) under the collaborative research project of the CSIR-Food Research Institute, Accra, ‘Capability Building for Research Into Traditional Fermented Food Processing in West Africa’. The authors are grateful to Mr. John Anlobe of the CSIR-Food Research Institute for his assistance in carrying out the work.

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