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Fermentation and antimicrobial characteristics of *Lactobacillus plantarum* and *Candida tropicalis* from Nigerian fermented maize (*akamu*)

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Abstract

This study investigated the ability of *Lactobacillus plantarum* strains (NGL5 and NGL7) and *Candida tropicalis* (NGY1) previously identified from *akamu*-a Nigerian fermented maize food with probiotic *L. plantarum* LpTx and *Saccharomyces boulardii* SB20 to ferment ground maize slurries based on pH, acidity, microbial biomass, levels of sugars and organic acids, and their antimicrobial activity against *Salmonella enterica* serovar Enteritidis NCTC 5188, *Escherichia coli* NCTC 11560, Bacillus cereus NCIMB 11925, *Staphylococcus aureus* NCTC 3750 and *Listeria monocytogenes* NCTC 7973 using an agar spot assay. *L. plantarum* strains either as single or mixed starter cultures with the yeasts had growth rates $\geq$0.15 h^{-1}, with pH significantly ($p \leq 0.05$) decreased to $\leq$3.93 after 12 h and then to $\leq$3.52 after 72 h and lactic acid >84 mmol L^{-1}. The yeasts had growth rates $\geq$0.18 h^{-1} but pH was $\geq$4.57 with lactic acid levels $\leq$20.23 mmol L^{-1} after 72 h in the single culture fermentation. There was no inhibition in modified MRS agar: 0.2% glucose and 0.2% glucose without Tween 80. Inhibition halos in MRS agar varied from 10.6 to 23.9 mm. *S. bourladii* was more inhibitory towards *L. monocytogenes* (8.6 mm) and *B. cereus* (5.4 mm) than was *C. tropicalis* (1.1 and 3.3 mm for *L. monocytogenes* NCTC 7973 and *B. cereus* NCIMB 11925 respectively) (0.9 mm) in malt extract agar. This study showed that *C. tropicalis* was less inhibitory to the pathogens while antimicrobial activities of the *L. plantarum* strains were mainly due to acidity and the *L. plantarum* strains either as single or mixed cultures with the yeasts demonstrated strong fermentation ability, with significant decrease in pH which is vital in the choice of starter for product safety.

Keywords: *L. plantarum*; *C. tropicalis*; Fermentation; Ground maize slurry; Acidity; Antimicrobial activities; Pathogens

1 Introduction

Fermentation is one of the oldest food preparation methods considered as safe and acceptable for improving the quality and safety of foods. Traditional fermentation technologies were based on a natural process, whereby wet foodstuffs undergo microbial degradation, and when the food is edible it was termed fermented (Lee, 2009). Over the years, the fermentation process has developed such that organic substrates are now converted into more desirable substances through

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the action of enzymes or microorganisms under controlled conditions to achieve several specific important functions (Towo, Matuschek, & Svanberg, 2006). In most African countries fermented cereal-based foods play an important role in the nutrition of infants and young children, as major infant complementary foods, and form an integral part of the main meal for adults. The traditional techniques of cereal food production depend on spontaneous or back-slop fermentation (the use of some portion of previously fermented product as inoculum to accelerate fermentation). Spontaneous fermentation of cereal-based foods is borne out of competitive activities of endogenous or contaminating microorganisms and its initiation may take 24 - 48 h. At the early stages of fermentation, contaminating microorganisms may increase slowly in number and compete for nutrients in order to produce metabolites (Holzapfel, 2002). The results are products of variable attributes in terms of quality and safety (Ouoba, Diawara, Jespersen, & Jakobsen, 2007). The growth of endogenous cultures of the substrate or those from the ingredients and equipment used in production may be inevitable, even with the introduction of starter cultures of the desired microorganism. It is therefore important that the selected microbial inoculant to facilitate the process should be adaptive to the substrate from the onset of fermentation so as to prevent the growth of unwanted microorganisms to a level that would impair the product's quality.

Lactic acid bacteria (LAB) and yeast occur as part of the natural microbial population in spontaneously fermented foods and as starter cultures in the food and beverage industry (Shetty & Jespersen, 2006). In a recent study by Obinna-Echem, Kuri, and Beal (2014), the LAB population of a selected Nigerian traditional fermented maize food called *akamu* was found to be dominated by strains of *Lactobacillus plantarum*, *L. fermentum*, *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus* while the identified yeasts included *Candida tropicalis, C. albicans, Clavispora lusitaniae* and *Saccharomyces paradoxus*. The presence of LAB and yeasts have also been associated with many other fermented maize doughs and porridges (Akinrele, 1970; Edema & Sanni, 2008; Omemu, Oyewole, & Bankole, 2007; Teniola & Odunfa, 2001). Amylolytic LAB breakdown starches to simple sugars which are favourable for LAB growth. The simple sugars are then converted to organic acids that create the acid environment which is known to improve product stability and safety. Although yeast's main function has been related to the production of alcohol and aroma compounds that impact significantly on food quality parameters such as taste, texture, odour and nutritive value (Aidoo, Nout, & Sarkar, 2006; Annan, Poll, Sefa-Dedeh, Plahar, & Jakobsen, 2003), Omemu et al. (2007) reported that yeasts associated with maize fermentation for *ogi* production produced several extracellular enzymes and the amylolytic activities of some of the yeasts were implicated in the breakdown of maize starch to simple sugars for other fermenting microorganisms.

Contamination of cereal-based complementary foods has been implicated in diarrhoeal episodes among infants and children <5 years in developing countries. Globally, diarrhoea causative agents are broad and include bacteria, viruses and parasitic agents. Pathogenic strains of *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Vibrio cholera* and *Campylobacter* spp. are the principal bacterial agents. The viral agents include Hepatitis A and E, Norovirus (calicivirus), Adenovirus (serotype 40/41), Astrovirus, Cytomegalovirus and Rotavirus. Protozoa such as *Giardia lamblia, Entamoeba histolytica, Cryptosporidium* spp. and Helminths such as *Strongyloides stercoralis, Angiostrongylus costaricensis, Schistosoma mansoni* and *S. japonicum* constitute the parasitic agents (Farthing et al., 2013; Motarjemi, 2002). The pathogenic bacteria and parasites are more prevalent in the developing countries than viruses (Farthing et al., 2013). Contamination of infant complementary foods may occur, through various sources, before, during and after food preparation. Fermentation of cereal-based foods has been reported to provide an antagonistic environment against pathogenic bacteria due to the production of organic acids that lower pH to a level inhibitory to some pathogenic organisms. However, some studies have been able to isolate relevant foodborne pathogens such as *Bacillus cereus, Clostridium perfringens, Aeromonas* spp., *Staphylococcus aureus* and some *E. coli* in fermented cereal foods

(Kunene, Hastings, & von Holy, 1999; Nyatoti, Mtero, & Rukure, 1997; Wakil, Onilude, Adetutu, & Ball, 2008).
Therefore this study was aimed at determining:

- the fermentation characteristics of *L. plantarum* strains (NGL5 and NGL7) and *Candida tropicalis* (NGY1) isolated from traditional *akamu* samples and their probiotic counterpart: *L. plantarum* Lptx and *Saccharomyces bourladii* SB20 for suitability as starter cultures in *akamu* fermentation based on pH, acidity and levels of sugars and organic acids; and
- the antimicrobial activity of *L. plantarum* strains and yeasts against five relevant foodborne pathogens: *Salmonella enterica* serovar Enteritidis NCTC 5188, *E. coli* NCTC 11560, *B. cereus* NCIMB 11925, *S. aureus* NCTC 3750 and *L. monocytogenes* NCTC 7973).

2 Materials and Methods

2.1 Ground maize and fermentation slurry

Ground organic maize (L1530) was obtained from the Health Food Shop, Rickard Lane's, Plymouth City Centre, UK. About 50±0.01 g of the flour was weighed into cellophane bags, sealed and irradiated with ^{60}Co at 25.88 ± 0.79 kGy (Becton, Dickinson and Company, Plymouth, UK). The slurry for fermentation was thereafter prepared by adding 100 mL of sterile distilled water containing the microbial inoculant. To verify the sterility of the irradiated sample, some un-inoculated slurries were kept in an incubator (Swallow Incubators, LTE Scientific Ltd, Green field, Oldham, UK) at 30°C without agitation. The slurries after 0 to 7 days were checked for microbial growth by streak plating on Nutrient agar plates followed by incubation at 30 and 37 °C for 3 - 7 days. The sterility of the irradiated samples was confirmed when no microbial growth was observed in the incubated plates. Although maize grains for *akamu* production would not ordinarily be irradiated, irradiation was used in this study to ensure that the characteristics being evaluated were that of the inoculated starter cultures.

2.2 Microbial inoculant

LAB and yeasts

The LAB and yeast cultures used were *L. plantarum* strains (NGL5 and NGL7) and *C. tropicalis* (NGY1), previously identified from a traditional *akamu* sample (M3) obtained from Rivers State, Nigeria using PCR and sequencing analysis by Obinna-Echem et al. (2014) and their probiotic counterpart: *L. plantrum* LpTx isolated from a probiotic food supplement capsule obtained from the Health Food Shop in Plymouth City Centre, UK, and *S. boulardii* SB20 from Lalleman Levucell cultures, Nottingham, UK.

Pathogens

Antimicrobial activities, using the agar spot method, were assayed against five foodborne pathogens: *B. cereus* NCIMB 11925, *E. coli* NCTC 11560, *L. monocytogenes* NCTC 7973, *Salmonella enterica* serovar Enteritidis NCTC 5188, and *S. aureus* NCTC 3750 obtained from stock cultures in the microbiological laboratory of Plymouth University, UK.

2.3 Inoculant preparation

Generally, the microorganisms were cultivated by streaking on the appropriate agar media, with incubation at the organisms' optimum growth conditions as follows: the *L. plantarum* strains were cultivated on de Man, Rogosa and Sharpe (MRS) agar and incubated at 37°C for 24 h; the yeasts were cultivated on malt extract agar and incubated at 25°C for 48 h; and the pathogens were cultivated on Nutrient agar and incubated at 37°C for 24 h, except for *B. cereus* that was incubated at 30°C for 24 h. MRS, Malt extract and Nutrient broths were used for broth cultures of the LAB, yeasts and the pathogens respectively. A distinct colony of the respective microorganisms from the agar plate culture was inoculated into 10 mL of the appropriate broth media and incubated at 37°C without agitation for 18 – 20

h for the *L. plantarum* strains and the pathogens except for *B. cereus* that was incubated at 30 °C while the yeasts were incubated at 25°C without agitation for 40 h. Cultures were harvested by centrifugation (Hettich Zentrifugen Rotina 46 S, Tuttlingen, Germany) at 4000 x *g* for 10 min and washed twice in phosphate buffered saline (PBS) (pH 7.3±0.2) and re-suspended in PBS such that 1 mL of inoculum produced 10^9, 10^7 and 10^6 CFU mL^{-1} for the LAB, the yeasts and the pathogens respectively. The media and the diluent used were obtained from Oxoid Limited (Basingstoke, Hampshire, UK).

2.4 Fermentation characteristics

Inoculation and fermentation of ground maize slurry

The single culture fermentation utilized 1 mL of the microbial inoculum added to 100 mL of sterile distilled water that was thoroughly mixed with 50±0.01 g irradiated ground maize while the mixed culture fermentation used an equal volume (1 mL) of a strain of the *L. plantarum* and the specified yeast. 18 ml of inoculated slurries were dispensed into sterile transparent 50 mL plastic pots with lids and incubated aerobically at 30°C without agitation for 72 h. Using 1 mL graduated sterile disposable plastic pipettes, samples were aseptically withdrawn after 0, 6, 12, 24, 48, and 72 h of fermentation for determination of pH, titratable acidity, microbial count, organic acids and sugars. Samples for determination of organic acids and sugars were preserved in eppendorff tubes at -80°C until needed. Un-inoculated samples served as controls.

Determination of pH and Titratable acidity (TTA) of the fermentation

The pH of 1 mL of sample in 10 mL of sterile distilled water was determined with a pH meter (Accumet^R AB10, Fisher Scientific, Loughborough, UK). The TTA was determined titrimetrically as described by Obinna-Echem et al. (2014).

Determination of organic acids and sugars

Reagents and standards

The chemicals used were either of analytical or high performance liquid chromatography (HPLC) grade. Concentrated H_2SO_4, ethanol and methanol were purchased from Fisher Scientific, Loughborough, UK. The standard sugars (glucose, fructose, sucrose and maltose) and organic acids (acetic, butyric, lactic and propionic acid) were supplied by Sigma Aldrich, Gillingham Dorset, UK. Appropriate concentrations of reagents and standards were obtained by dissolving or making-up the appropriate weight or volume in deionised water purified through a Milli-Q system (Millipore Corp., Bredford, MA, USA).

Instrumentation

The organic acid composition (citric, acetic, lactic, propionic and butyric acids) of the fermented ground maize slurry was analysed using ultra high performance liquid chromatography (UHPLC), Dionex-Ultimate 3000 UHPLC+Focused (Dionex Softron GmbH, Germering, Germany). The ultra high performance liquid chromatography (UHPLC) was equipped with WPS-3000 autosampler, LPG-3400SD pump, TCC-3000 column compartment, RI-101 refractive index detector and MWD-3000 Ultra violet detector.

Sample preparation

Samples of fermented ground maize slurries (1.5 mL) stored frozen in eppendorf tubes were allowed to thaw at room temperature. The samples were homogenised on a Multi-mixer (Fisher Scientific, Loughborough, UK) for 10 s and then centrifuged at 13000 x *g* for 20 min (Sanyo-MSE MSB010.CX2.5 Micro Centaur Centrifuge, Alconbury, UK). 20 μL of 7% H_2SO_4 was added to 100 μL of the sample supernatant, in 400 μL of Milli-Q water. Addition of the mineral acid, according to Niven, Beal, and Brooks (2004), was to shift the acid equilibrium of weak acids to the protonated form so as to help maintain the column functionality. The prepared samples were thoroughly mixed by vortexing for 5 s, filtered through a MF-millipore microfiltre (0.22 μm pore

size) into vials, sealed with a crimp cap (11mm, Rubber/PTFE, Fisher Scientific, Loughborough, UK) and stored at -80°C until needed for analysis.

HPLC conditions and data analysis

The chromatographic separation was achieved with an elution phase of degased 5 mmol L^{-1} of H2SO4 in a PL Hi-Plex H Guard column 50 x 7.7 mm (Agilent Technologies, Waghaeusel-Wiesental, Germany) at a column temperature of 25°C. The eluent was pumped at a flow rate of 0.5 μL min^{-1} within a pressure limit of 5 -100 bars. The injected sample volume was 20 μL. The sugars and organic acids were detected using Refractive Index and Ultra Violet detectors respectively. The retention times of sugar and organic acid standards were noted and used in the identification of the sample peaks. All data obtained was processed using Chromeleon® 7.1 Chromatography Data System Software (Dionex Softron GmbH, Germering, Germany). Calibration curves for each sugar and organic acid were obtained from four different concentrations (100, 50, 25 and 12.5 mmol L^{-1}) of the standards stock solutions.

2.5 Fermentation characteristics

Ten-fold dilutions of 1 mL of sample slurries in 9 mL of PBS were prepared and plated out using the drop method by Miles, Misra, and Irwin (1938) on MRS agar supplemented with 0.01% cyclohexamide for the *L. plantarum* strains and on Rose Bengal Chloramphenicol Agar (RBCA) containing 0.01% (w/v) chloramphenicol selective supplement for yeasts. The plates were incubated aerobically at 37°C for 24 - 48 h and 25°C for 48 h for the *L. plantarum* strains and yeasts respectively. The number of colony forming units per millilitre was computed as eq. 1:

$$CFUmL^{-1} = CFU/V_s \quad (1)$$

Where CFU represents the average number of colonies for a dilution X Dilution factor and V_s is the volume of the sample plated.

2.6 Antimicrobial activities

Antimicrobial activities were determined by the agar spot test, with some modification to the method described by Majhenic, Lorberg, and Rogelj (2007). The *L. plantarum* strains were grown both in MRS and modified MRS agar: MRS with 0.2% glucose (MRS.2) and MRS with 0.2% glucose and without Tween 80 (MRS.2-T80). Acid production was checked by the reduction of glucose while the removal of Tween 80 was to enhance any production of antimicrobial agents. Two microlitres of washed and re-suspended 18 h *L. plantarum* cells (c.10^9 CFU mL^{-1}) were spotted on the agar plates and incubated both at 37°C for 24 h. Malt extract agar plates were spotted with 10 μL of washed and re-suspended 48 h yeast cells and incubated at 25°C for 48 h. Afterwards, plates were overlaid with 10 mL of soft Nutrient agar (7.5 g L^{-1}) and inoculated with 33 μL of cell suspension containing 10^6 CFU mL^{-1} of the respective five foodborne pathogens. The overlaid plates were incubated at the optimal growth temperature for the respective pathogens: 30°C for *B. cereus* and 37°C for the others. After 24 - 48 h of incubation, the plates were observed for inhibition zones and the diameter (mm) of inhibition halos were measured at 3 - 4 different angles using an analogue sliding Vernier Calliper 120 mm (Skill Tech 5 in, Frankfurt, Germany) and the average reading was taken.

2.7 Statistical analysis

Data obtained was statistically analysed using Minitab (Release 16.0) Statistical Software English (Minitab Inc. UK). Statistical differences were evaluated by analysis of variance (ANOVA), under a general linear model, and Tukey pairwise comparisons at the 95% confidence level. The growth rates of the microorganisms were obtained using the microbial model (DMFit version 2.0) by Baranyi and Roberts (1994).

3 Results and Discussion

3.1 Fermentation characteristics

pH and Titratable acidity (TTA)

The effect of fermentation with the *L. plantarum* strains, *Sacch. Bourlardii* SB20 and *C. tropicalis* on pH and TTA are presented in Figure 1 and Figure 2 respectively. A decrease in pH has been established as an important parameter for assessing how fast a process will reach conditions (pH <4.5) which can inhibit the growth of pathogenic organisms (Nguyen et al., 2007). The pH of the *L. plantarum* single ($\leq$3.43) or mixed culture fermentation with the yeasts ($\leq$3.52) were such that would not permit the survival of most unwanted microorganisms, while the decrease in pH of the yeast single culture fermentation was $\geq$4.57. Progression in fermentation of cereal and cereal-legume mixtures has often been marked with an increase in microbial numbers, concomitant with a decrease in pH and an increase in TTA (Mugula, Narvhus, & Sorhaug, 2003; Teniola & Odunfa, 2002; Wakil et al., 2008). This was observed with the *L. plantarum* strains, either as a single or mixed starter cultures with the yeasts. The pH (5.59 - 5.23) and TTA (0.09 - 0.24%) of the control samples did not change significantly ($p \leq 0.05$). However, this was expected as the maize flour samples were irradiated and sterile distilled water was used in the fermentation. Lactic acid was mostly responsible for the lowering of pH in the fermentations and there was no detectable level of microorganisms in the control for any notable change. The TTA ($\leq$0.33%) of the yeast single culture fermentation was significantly ($p \leq 0.05$) lower than that of the *L. plantarum* single ($\geq$1.05%) and in the mixed culture fermentation ($\leq$0.90%) but did not show any significant difference from that of the control. This may also be attributed to the little or no lactic acid production by the yeasts.

Levels of lactic acid, ethanol and sugars

Table 1 shows the lactic acid levels of the fermentation. Bacteria produce organic acids from the fermentation of carbohydrate and over time decreased the amount of sugars: glucose, fructose and maltose (Table 2, Table 3 and Table 4 respectively) detected in the fermentation by the selected *L. plantarum* strains. The initial glucose level increased within the first 6 h of fermentation while the maltose and fructose levels were depleted. This may imply the breakdown of complex carbohydrate first to the simple sugar: glucose before its utilization. This may also explain the higher lactic acid levels in the samples as compared to the initial glucose levels since homofermenters like *L. plantarum* are capable of producing 2 mol of lactic acid from 1 mol of glucose (Reddy, Altaf, Naveena, Venkateshwar, & Kumar, 2008). Although, glucose levels of $\geq$26.88 mmol L^{-1} were detected in the *L. plantarum* single culture fermentation after 72 h, it was completely depleted after 24 h in the yeast single and mixed culture fermentation with the *L. plantarum* strains. One would logically think that the lactic acid levels in fermentation, where all the glucose has been utilized, would be significantly greater. However, the lactic acid concentration of the single *L. plantarum* culture fermentation was significantly ($p \leq 0.05$) greater than the yeast mixed culture fermentation, with particular reference to the fermentation with *S. boulardii*. This indicates competition for glucose in the mixed culture fermentation with the yeasts and its metabolism to the respective fermentative microorganism's metabolic by product. The *L. plantarum* strains in the presence of more fermentable glucose after 72 h could survive longer in the single culture fermentation and thus prolong the keeping quality of the product.

In addition to lactic acid, the other organic acids investigated in this study were citric, acetic, propionic and butyric acids (data not shown). Although, citric, acetic, pyruvic, succinic, formic and propionic acids had been identified in different LAB fermented cereal foods (Banigo & Muller, 1972; Gobbetti & Corsetti, 1997; Muyanja, Narvhus, Treimo, & Langsrud, 2003), the *L. plantarum* strains in this study produced mainly lactic acid. Although, the fructose and maltose levels of the control sample were fairly constant (Table 3 and Table 4 respectively), the observed significant increase in glucose level could be attributed to the hydrolysis of the maize starch to its basic component as

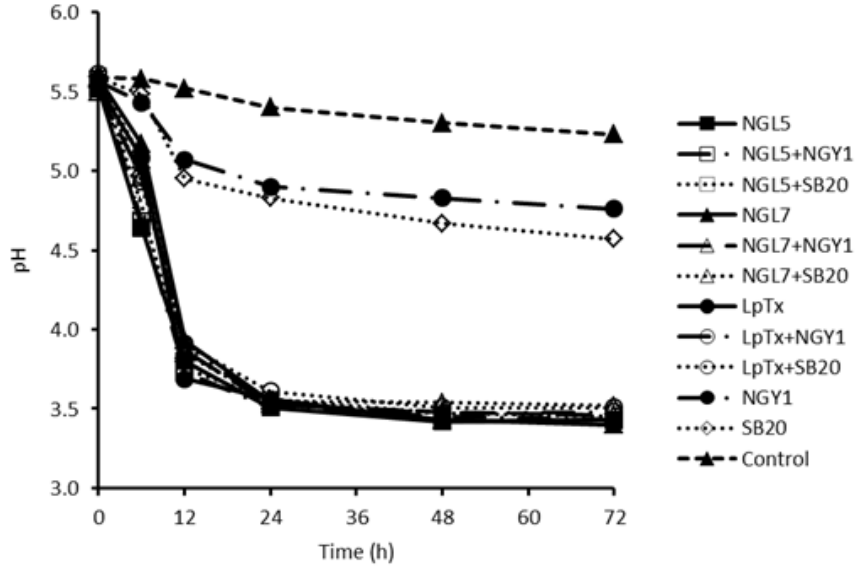

Figure 1: pH of ground maize slurry fermented with single or mixed cultures of *Lactobacillus plantarum* strains (NGL5, NGL7 and LpTx) and yeasts (*Candida tropicalis* -NGY1 and *Saccharomyces boulardii* SB20)

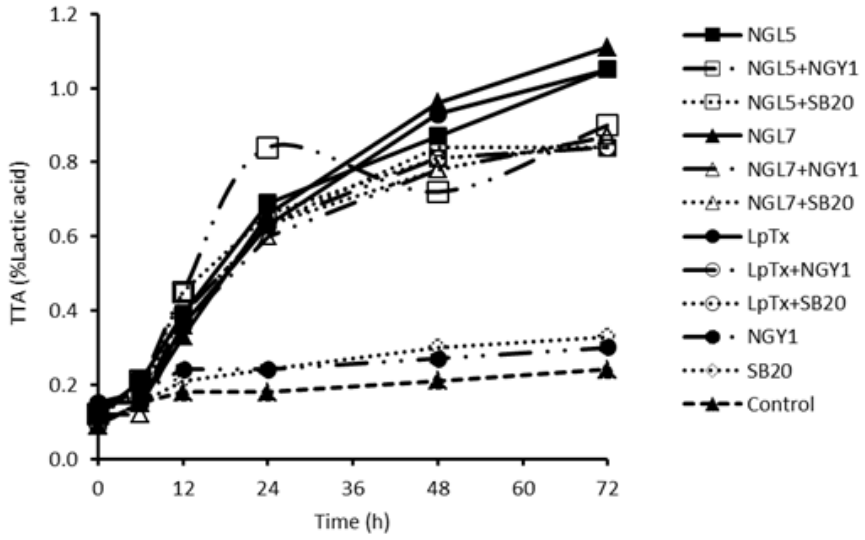

Figure 2: Titratable acidity (%Lactic acid) of ground maize slurry fermented with single or mixed cultures of *Lactobacillus plantarum* strains (NGL5, NGL7 and LpTx) and yeasts (*Candida tropicalis* - NGY1 and *Saccharomyces boulardii* SB20)

glucose by the endogenous grain amylases under the wet conditions.

The levels of ethanol detected in the fermentation are presented in Table 5. There was no ethanol detected in the samples fermented by the single and the mixed cultures of *L. plantarum* strains and *C. tropicalis* except for LpTx+NGY1 which had an ethanol level of 14.8 mmol L^{-1} after 24 h. Ethanol production in the yeast single culture fermentation was significantly ($p \leq 0.05$) higher than the mixed culture fermentation. The highest ethanol level was detected in *S. boulardii* SB20 single culture fermentation. Higher ethanol levels were also detected in the mixed culture fermentation with the *L. plantarum* strains. Ethanol production may not be needed in the fermentation for infant and children feeding. The absence and/or very low level of ethanol production in the *L. plantarum* strains and *C. tropicalis* mixed culture fermentation was indicative of the dominant activities of the LAB in the fermentation, as well as a contamination free process. The role of yeast in the flavour of fermented maize products has been reported by Annan et al. (2003), Mugula et al. (2003) and Omemu et al. (2007). Therefore, the characteristic of *C. tropicalis* to grow in the fermentation, with little or no ethanol production, could be important in *akamu* production as the product flavour is an important sensory property. The ability of yeast to utilise certain bacterial metabolites as carbon sources (Akinrele, 1970; Leroi & Pidoux, 1993) and the competition for utilizable sugars could be contributory to the reduction in the lactic acid content of the mixed culture fermentation.

Microbial growth

The viable counts of the *L. plantarum* strains and yeasts: *C. tropicalis* and *S. boulardii* SB20 in the fermentations are respectively shown in Figure 3 and Figure 4, while their growth model parameters are presented in Table 6 and Table 7 respectively. A typical pattern of microbial growth in a closed system was obtained. The main phases of growth observed were the log, decelerating and the stationary phases. The viable counts reached a maximum of approximately 10^9 CFU mL^{-1} after 24 h and then declined before reaching fairly constant values towards the end of fermentation, thus depicting the decelerating and the stationary phases. Although, glucose levels >26 mmol L^{-1} were detected after the peak viable count and towards the end of fermentation, the *L. plantarum* strains in a close fermentation system for 72 h may not be able to continue to grow exponentially with the accumulation of lactic acid and other less abundant inhibitor products of cell metabolism. Furthermore, the exhaustion of utilisable carbon dioxide (Sezonov, Joseleau-Petit, & D'Ari, 2007) in the fermentation pots (18 mL in 50 mL), where cells are increasing in number, may also explain entry into the stationary phase of growth, although *L. plantarum* strains isolated from fermented maize porridges have been found to withstand low pH (2.5) (Kalui, Mathara, Kutima, Kiiyukia, & Wongo, 2009). The absence of lag time could be influenced by several factors such as the inoculum quantity, the environmental conditions of the original and the new growth medium, the growth stage of the microorganisms during the time of inoculation (Ginovart, Prats, Portell, & Silbert, 2011; Swinnen, Bernaerts, Dens, Geeraerd, & Van Impe, 2004) and probably the investigation period. The *L. plantarum* strains and the yeasts were suspended in PBS at an active growth stage and inoculated into the maize slurry environment that had enough nutrients to support their growth. Also, enumeration after 6 h of fermentation would have given enough time for any adaptation by the inoculants. This adaptive nature is important for early and efficient production of the desired metabolic end product.

The microbial growth model estimated the growth rate of the individual microorganisms in the fermentation. Although variations were observed among the viable counts of the microorganisms with time, the mixed culture fermentation did not have any significant ($p \leq 0.05$) influence on the growth rates of the *L. plantarum* strains and yeasts except for *S. boulardii* SB20 in mixed fermentation with NGL5. This indicates the ability of the microorganisms to coexist in the fermentation, where each carries out its metabolic activities irrespective of the presence of the other. The growth of *S. boulardii* SB20 and *C. tropicalis* in the mixed culture fermentation, with *L. plantarum* strains at a low pH $\leq$3.61, also

Table 1: Lactic acid levels (mmol L^{-1}) of ground maize slurries fermented with single and mixed cultures of *Lactobacillus plantarum* strains and yeasts

Sample	Time (h)					
	0*	6	12	24	48	72
NGL5	5.99±0.13	16.01±0.12^{a}	43.70±0.83^{a}	77.51±2.46^{abc}	101.94±1.90^{a}	109.24±1.44^{ab}
NGL5+NGY1	5.99±0.00	14.13±0.49^{abc}	44.96±1.18^{a}	77.54±2.25^{abc}	90.81±9.35^{abc}	100.05±11.96^{abc}
NGL5+SB20	6.11±0.06	15.14±0.69^{ab}	41.47±14.48^{a}	73.89±5.51^{abc}	91.66±3.51^{abc}	86.92±3.01^{c}
NGL7	6.00±0.25	9.98±0.24de	40.52±14.66^{a}	82.93±13.66^{a}	103.50±5.23^{a}	120.09±3.39^{a}
NGL7+NGY1	6.86±2.76	11.66±0.36^{bcd}	41.49±0.03^{a}	72.39±4.02^{abc}	82.27±13.26^{bc}	97.30±11.18^{ab}
NGL7+SB20	6.04±0.21	10.65±0.50^{cd}	37.21±2.15^{a}	63.88±5.43^{c}	72.74±1.63^{c}	84.57±6.34^{c}
LpTx	4.69±0.23	11.24±0.42^{cd}	36.98±0.43^{a}	73.98±2.05^{abc}	100.51±0.50^{ab}	110.63±6.12^{abc}
LpTx+NGY1	6.10±0.27	10.69±0.63^{cd}	37.63±2.42^{a}	75.68±3.62^{abc}	90.48±11.61^{abc}	94.50±12.82^{ab}
LpTx+SB20	5.45±0.93	8.42±4.05^{def}	37.72±1.81^{a}	66.84±0.25^{bc}	80.77±8.55^{c}	89.56±6.79^{bc}
SB20	5.89±0.39	5.29±0.62^{fg}	10.69±0.28^{b}	17.16±1.80^{d}	20.23±3.81^{d}	19.21±2.62^{d}
NGY1	5.40±0.14	6.75±0.20^{efg}	8.90±0.22^{b}	13.89±0.32^{d}	17.97±1.93^{d}	17.80±3.38^{d}
Control	4.77±0.19	4.75±0.28^{g}	3.88±0.18	2.86±0.63	5.85±0.57	6.53±0.52

* Values do not differ significantly
Values that share the same superscript in the same column do not differ significantly (p≤0.05). N=3±SD
NGL5 & NGL7 - Nigerian fermented maize *L. plantarum* strains and LpTx - commercial probiotic *L. plantarum* strain
NGY1 & SB20 - Nigerian fermented maize *C. tropicalis* and probiotic *S. boulardii* SB20 respectively

Table 2: Glucose levels (mmol L^{-1}) of ground maize slurries fermented with single and mixed cultures of *Lactobacillus plantarum* strains and yeasts

Sample	Time (h)					
	0	6	12	24	48	72
NGL5	15.52±0.91^{efg}	41.50±0.46^{bc}	43.14±0.94^{ab}	37.28±1.55^{b}	31.81±1.26^{a}	29.63±0.30^{a}
NGL5+NGY1	19.34±0.00^{cde}	43.97±0.32^{bc}	24.05±0.96^{def}	2.74±1.39^{c}	-	-
NGL5+SB20	27.69±1.20^{a}	67.94±2.67^{a}	14.52±5.93^{f}	-	-	-
NGL7	15.24±0.87^{efg}	42.16±1.99^{bc}	41.23±8.48^{bc}	46.05±8.23^{a}	32.08±2.5^{a}	29.83±1.59^{a}
NGL7+NGY1	17.62±0.86^{cdef}	45.43±2.14^{b}	33.02±0.50^{cd}	2.75±1.10^{c}	-	-
NGL7+SB20	25.25±1.41^{ab}	60.34±2.17^{a}	26.89±1.46^{de}	-	-	-
LpTx	14.35±0.50^{fg}	39.14±1.24^{bc}	46.06±1.18^{ab}	40.05±1.29^{ab}	31.88±1.74^{a}	26.88±2.04^{a}
LpTx+NGY1	21.00±1.96^{bc}	40.59±4.20^{bc}	33.09±1.18^{cd}	3.20±0.54^{c}	-	-
LpTx+SB20	20.73±4.15^{cd}	31.47±13.87^{c}	22.36±0.94^{ef}	-	-	-
SB20	12.26±0.61^{g}	32.41±0.92^{c}	18.95±0.51^{ef}	-	-	-
NGY1	13.69±0.79^{fg}	36.72±1.32^{bc}	23.39±2.16^{def}	-	-	-
Control	16.56±0.26^{defg}	39.57±0.64^{bc}	52.48±1.06^{a}	65.75±0.67	80.12±8.86	87.59±9.40

Values that share the same superscript in the same column do not differ significantly (p≤0.05). N=3±SD
NGL5 & NGL7 - Nigerian fermented maize *L. plantarum* strains and LpTx - commercial probiotic *L. plantarum* strain
NGY1 & SB20 - Nigerian fermented maize *C. tropicalis* and probiotic *S. boulardii* SB20 respectively

Table 3: Fructose levels (mmol L^{-1}) of ground maize slurries fermented with single and mixed cultures of *Lactobacillus plantarum* strains and yeasts

Sample	Time (h)					
	0	6	12	24	48	72
NGL5	9.23 ± 0.30^{bcd}	8.04 ± 0.34^{ab}	3.54 ± 0.22^{d}	-	-	-
NGL5+NGY1	10.72 ± 0.00^{ab}	9.93 ± 0.92^{ab}	-	-	-	-
NGL5+SB20	10.30 ± 0.30^{abc}	10.44 ± 0.53^{a}	-	-	-	-
NGL7	9.32 ± 0.47^{bcd}	9.22 ± 0.42^{ab}	5.41 ± 2.13^{cd}	-	-	-
NGL7+NGY1	12.12 ± 1.82^{a}	9.93 ± 0.60^{ab}	6.02 ± 0.09^{bc}	-	-	-
NGL7+SB20	10.14 ± 0.26^{abc}	9.56 ± 0.12^{ab}	-	-	-	-
LpTx	8.62 ± 0.26^{bcd}	8.29 ± 0.23^{ab}	5.85 ± 0.05^{bc}	-	-	-
LpTx+NGY1	10.20 ± 0.54^{abc}	9.42 ± 0.73^{ab}	5.00 ± 0.31^{cd}	-	-	-
LpTx+SB20	9.10 ± 1.85^{bcd}	7.20 ± 3.22^{b}	4.17 ± 0.25^{cd}	-	-	-
SB20	8.33 ± 0.54^{cd}	7.94 ± 0.61^{ab}	3.81 ± 0.63^{d}	-	-	-
NGY1	8.12 ± 0.30^{cd}	8.29 ± 0.60^{ab}	4.94 ± 0.04^{cd}	-	-	-
Control	8.91 ± 0.11^{bcd}	7.67 ± 0.30^{ab}	8.94 ± 0.06^{a}	8.74±0.39	9.03±1.25	9.46±0.73

Values that share the same superscript in the same column do not differ significantly ($p\leq0.05$). N=3±SD
NGL5 & NGL7 - Nigerian fermented maize *L. plantarum* strains and LpTx - commercial probiotic *L. plantarum* strain
NGY1 & SB20 - Nigerian fermented maize *C. tropicalis* and probiotic *S. boulardii* SB20 respectively

Table 4: Maltose levels (mmol L^{-1}) of ground maize slurries fermented with single and mixed cultures of *Lactobacillus plantarum* strains and yeasts

Sample	Time (h)					
	0	6	12	24	48	72
NGL5	4.00 ± 0.15^{ab}	2.88 ± 0.13^{cde}	-	-	-	-
NGL5+NGY1	3.56 ± 0.00^{b}	2.42 ± 0.07^{de}	-	-	-	-
NGL5+SB20	4.03 ± 0.19^{ab}	2.56 ± 0.18^{de}	-	-	-	-
NGL7	4.15 ± 0.32^{ab}	3.69 ± 0.13^{abcde}	-	-	-	-
NGL7+NGY1	3.90 ± 0.17^{ab}	2.92 ± 0.10^{cde}	-	-	-	-
NGL7+SB20	4.83 ± 0.47^{a}	3.62 ± 1.14^{abcde}	-	-	-	-
LpTx	4.54 ± 0.38^{ab}	3.71 ± 0.26^{abcde}	-	-	-	-
LpTx+NGY1	4.29 ± 0.18^{ab}	3.13 ± 0.29^{bcde}	-	-	-	-
LpTx+SB20	4.14 ± 0.80^{ab}	2.27 ± 1.16^{e}	-	-	-	-
SB20	4.39 ± 0.27^{ab}	4.29 ± 0.14^{abc}	-	-	-	-
NGY1	4.28 ± 0.31^{ab}	4.65 ± 0.29^{a}	2.12 ± 0.18^{b}	-	-	-
Control	4.78 ± 0.16^{ab}	4.46 ± 0.26^{ab}	4.30 ± 0.07^{a}	4.06±0.35	3.92±0.36	4.13±0.52

Values that share the same superscript in the same column do not differ significantly ($p\leq0.05$). N=3±SD
NGL5 & NGL7 - Nigerian fermented maize *L. plantarum* strains and LpTx - commercial probiotic *L. plantarum* strain
NGY1 & SB20 - Nigerian fermented maize *C. tropicalis* and probiotic *S. boulardii* SB20 respectively

Table 5: Maltose levels (mmol L^{-1}) of ground maize slurries fermented with single and mixed cultures of *Lactobacillus plantarum* strains and yeasts

Sample	Time (h)			
	12	24	48	72
NGL5+SB20	-	18.31 ± 5.23^{gh}	14.32 ± 2.12^{ghi}	39.10 ± 3.70^{ef}
NGL7+SB20	-	9.92 ± 0.52^{ghi}	18.83 ± 1.37^{gh}	53.20 ± 10.54^{e}
LpTx+NGY1	-	14.8 ± 1.06^{ghi}	-	-
LpTx+SB20	9.24 ± 1.18^{ghi}	16.87 ± 2.03^{gh}	10.25 ± 2.54^{ghi}	52.90 ± 12.95^{e}
SB20	42.30 ± 8.64^{e}	91.37 ± 6.39^{c}	127.79 ± 26.5^{b}	194.00 ± 24.7^{a}
NGY1	-	24.40 ± 1.72^{fg}	74.39 ± 3.75^{d}	77.50 ± 4.14^{cd}

Values that share the same superscript do not differ significantly ($p\leq0.05$). N=3±SD
In all the samples no ethanol was detected after 0 and 6 h of fermentation
The NGL5, NGL5+NGY1, NGL7, NGL7+NGY1, LpTx and the control samples had no detectable ethanol levels
NGL5 & NGL7 - Nigerian fermented maize *L. plantarum* strains and LpTx - commercial probiotic *L. plantarum* strain
NGY1 & SB20 - Nigerian fermented maize *C. tropicalis* and probiotic *S. boulardii* SB20 respectively

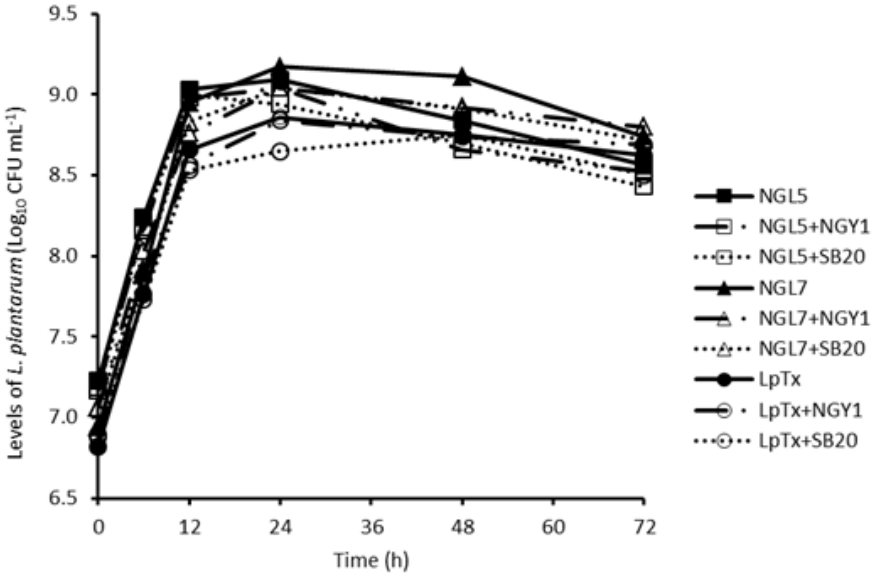

Figure 3: Levels (Log_{10} CFU $mL^{-1)}$ of *Lactobacillus plantarum* strains (NGL5, NGL7 and LpTx) in single or mixed culture fermentation of ground maize slurry with yeasts (*Candida tropicalis* - NGY1 and *Saccharomyces boulardii* SB20).

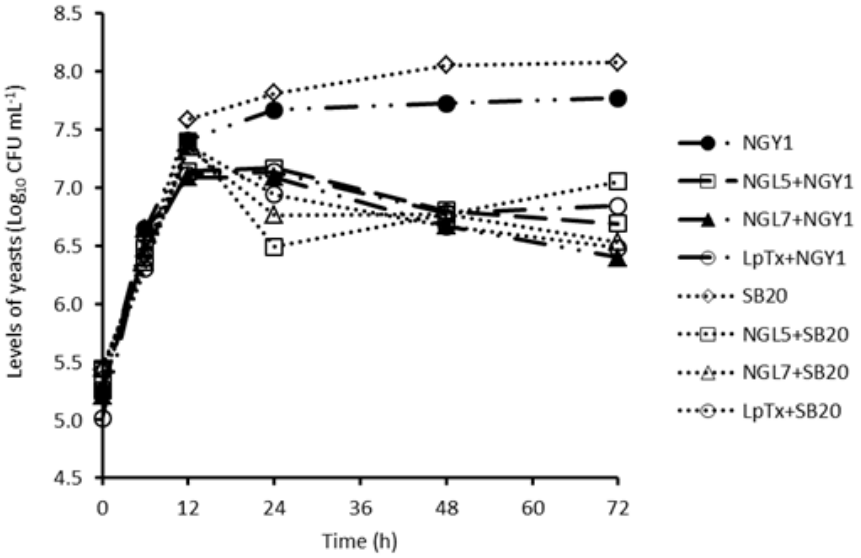

Figure 4: Levels (Log_{10} CFU mL^{-1}) of *Candida tropicalis* (NGY1) and *Saccharomyces boulardii* SB20 in single or mixed culture fermentation of ground maize slurry with *Lactobacillus plantarum* strains (NGL5, NGL7 and LpTx)

confirms the ability of these yeasts to tolerate the acid environment and suggests that they could be involved in some way in the cereal fermentation. The likely mechanism for the tolerance of a Candida species at low pH to lactic acid was explained by Halm, Hornbaek, Arneborg, Sefa-Dedeh, and Jespersen (2004), while Omemu et al. (2007) documented the role of yeast in cereal and cereal-legume fermentation.

The Se(fit) indicated the total deviation of the observed values (viable counts) from the estimated model fit while the R^2 statistics further elucidated how successful the fits were. Some curves were fitted well whilst others were not. The yeast single culture fermentation curve fit was 98%, while the decrease in viable count after 12 and 48 h for *S. boulardii* SB20 and *C. tropicalis* respectively in the mixed culture fermentation affected the fit of their growth model curves, with *C. tropicalis* having <89% and *S. boulardii* SB20 in mixed culture fermentation with NGL7 and LpTx having 77 and 70% respectively.

3.2 Antimicrobial activity

Table 6 shows the diameter of inhibition halos for *L. plantrum* strains and yeasts against the five relevant foodborne pathogens. The diameter of inhibition halos in the MRS agar varied from 10.6±0.2 to 20.7±0.6 mm for *L. monocytogenes* NCTC 7973 and S. Enteritidis NCTC 5188 respectively. The inhibitory activity of the *L. plantarum* strains in the MRS agar against *B. cereus* NCIMB 11925, *E. coli* NCTC 11560, S. Enteritidis NCTC 5188, *L. monocytogenes* NCTC 7973 and *S. aureus* NCTC 3750 was mainly due to acidity as there was no inhibition recorded in the modified MRS media. This was in line with the report of Trias, Baneras, Badosa, and Montesinos (2008) that *L. plantarum* strains were without significant activity against *L. monocytogenes* and *S. aureus* in MRS, with 90% reduced glucose. *S. boulardii* antagonistic ability had mainly been observed in vitro and demonstrated that the prevention of C. difficile and V. cholerae associated infection is through the production of an antimicrobial proteolytic enzyme (Murphy & Kavanagh, 1999; Tasteyre, Barc, Karjalainen,

Table 6: Growth model parameters of *Lactobacillus plantarum* strains (NGL5, NGL7 and LpTx) in ground maize slurries fermented by single or mixed starter cultures with *Candida tropicalis* (NGY1) or *Saccharomyces boulardii* SB20

Curve	[a] Experimental data		Modelling parameters				
			[b] Primary parameters			[c] Statistic	
	yDatMin	yDatMax	rate	y0	yEnd	se(fit)	R^2
NGL5	7.23 ± 0.08^{a}	9.09 ± 0.05^{ab}	0.17 ± 0.01^{abc}	7.22 ± 0.08^{a}	8.88 ± 0.05^{bc}	0.24 ± 0.05^{ab}	0.88 ± 0.06^{bc}
NGL5+SB20	7.17 ± 0.03^{ab}	9.00 ± 0.03^{b}	0.17 ± 0.01^{abc}	7.17 ± 0.03^{ab}	8.77 ± 0.02^{cde}	0.26 ± 0.01^{a}	0.85 ± 0.01^{c}
NGL5+NGY1	7.19 ± 0.03^{ab}	9.04 ± 0.02^{b}	0.17 ± 0.00^{abc}	7.18 ± 0.03^{ab}	8.80 ± 0.02^{bcd}	0.26 ± 0.03^{a}	0.86 ± 0.03^{c}
NGL7	6.94 ± 0.04^{cde}	9.17 ± 0.07^{a}	0.18 ± 0.01^{ab}	6.91 ± 0.07^{cde}	9.02 ± 0.05^{a}	0.20 ± 0.06^{abcd}	0.95 ± 0.03^{ab}
NGL7+SB20	7.06 ± 0.05^{bcd}	9.04 ± 0.03^{b}	0.16 ± 0.00^{bc}	7.03 ± 0.04^{bcd}	8.90 ± 0.01^{b}	0.15 ± 0.02^{abcd}	0.96 ± 0.01^{a}
NGL7+NGY1	7.06 ± 0.04^{bc}	9.04 ± 0.02^{b}	0.15 ± 0.00^{c}	7.08 ± 0.05^{abc}	8.91 ± 0.04^{ab}	0.10 ± 0.02^{cd}	0.98 ± 0.01^{a}
LpTx	6.82 ± 0.09^{e}	8.86 ± 0.04^{c}	0.16 ± 0.01^{bc}	6.81 ± 0.10^{e}	8.75 ± 0.03^{de}	0.10 ± 0.04^{cd}	0.98 ± 0.01^{a}
LpTx+SB20	6.89 ± 0.04^{e}	8.75 ± 0.05^{c}	0.15 ± 0.01^{c}	6.87 ± 0.04^{de}	8.63 ± 0.04^{f}	0.13 ± 0.05^{bcd}	0.97 ± 0.02^{a}
LpTx+NGY1	6.88 ± 0.09	8.86 ± 0.04^{c}	0.16 ± 0.00^{bc}	6.89 ± 0.10^{de}	8.74 ± 0.06^{def}	0.10 ± 0.07^{cd}	0.98 ± 0.03^{a}

[a] yDatMin and yDatMax: Initial and maximum viable count

[b] Rate: potential maximum growth rate (h^{-1}); y0: initial point of the sigmoid curve; yEnd: upper asymptote of the sigmoid curve. There was no Lag time observed.

[c] Se(fit) Standard error of fit which is the estimated standard deviation of the observed independent values; R^2: Adjusted R-square statistics of the fitting

The curvature parameters (mCurv and nCurv) at the beginning and end of the linear phase were 10 and 0 respectively except for NGL5+SB20 with mCurv of 0

Values that share the same superscript in the same column do not differ significantly ($p \leq 0.05$). N=3±SD

NGL5 & NGL7 - Nigerian fermented maize *L. plantarum* strains and LpTx - commercial probiotic *L. plantarum* strain

NGY1 & SB20 - Nigerian fermented maize *C. tropicalis* and probiotic *S. boulardii* SB20 respectively

Table 7: Growth model parameters of *Candida tropicalis* (NGY1) and *Saccharomyces boulardii* (SB20) in ground maize slurries fermented by the single or mixed starter cultures with *Lactobacillus plantarum* strains (NGL5, NGL7 and LpTx)

Curve	[a] Experimental data		Modelling parameters				
			[b] Primary parameters			[c] Statistic	
	yDatMin	yDatMax	rate	y0	yEnd	se(fit)	R^2
NGY1	5.25 ± 0.04^{bc}	7.79 ± 0.06^{b}	0.22 ± 0.04^{abc}	5.28 ± 0.06^{ab}	7.69 ± 0.08^{a}	0.12 ± 0.04^{b}	0.98 ± 0.01^{a}
NGL5+NGY1	5.26 ± 0.03^{bc}	7.18 ± 0.09^{de}	0.21 ± 0.01^{abc}	5.26 ± 0.03^{ab}	6.95 ± 0.09^{a}	0.27 ± 0.03^{ab}	0.86 ± 0.04^{ab}
NGL7+NGY1	5.21 ± 0.01^{c}	7.09 ± 0.02^{e}	0.26 ± 0.04^{ab}	5.21 ± 0.01^{ab}	6.81 ± 0.02^{a}	0.34 ± 0.06^{a}	0.76 ± 0.08^{ab}
LpTx+NGY1	5.02 ± 0.08	7.20 ± 0.05^{de}	0.27 ± 0.05^{a}	5.01 ± 0.08^{b}	6.97 ± 0.11^{a}	0.27 ± 0.10^{ab}	0.88 ± 0.07^{ab}
SB20	5.43 ± 0.02^{a}	8.08 ± 0.06^{a}	0.18 ± 0.00^{bc}	5.39 ± 0.02^{a}	7.98 ± 0.03^{a}	0.14 ± 0.02^{b}	0.98 ± 0.00^{a}
NGL5+SB20	5.44 ± 0.04^{a}	7.49 ± 0.17^{c}	0.01 ± 0.01	6.26 ± 0.09	0.00 ± 0.00	0.66 ± 0.03	0.83 ± 0.22^{ab}
NGL7+SB20	5.45 ± 0.03^{a}	7.35 ± 0.04^{cd}	0.16 ± 0.00^{c}	5.44 ± 0.03^{a}	6.85 ± 0.02^{a}	0.36 ± 0.01^{a}	0.77 ± 0.00^{b}
LpTx+SB20	5.35 ± 0.02^{ab}	7.41 ± 0.08^{cd}	0.14 ± 0.05^{c}	5.47 ± 0.26^{a}	4.55 ± 3.94^{a}	0.43 ± 0.12^{a}	0.70 ± 0.03^{b}

[a] yDatMin and yDatMax: Initial and maximum viable count

[b] Rate: potential maximum growth rate (h^{-1}); y0: initial point of the sigmoid curve; yEnd: upper asymptote of the sigmoid curve. There was no Lag time observed.

[c] Se(fit) Standard error of fit which is the estimated standard deviation of the observed independent values; R^2: Adjusted R-square statistics of the fitting

The curvature parameters (mCurv and nCurv) at the beginning and end of the linear phase were 10 and 0 respectively except for NGL5+SB20 with mCurv of 0

Values that share the same superscript in the same column do not differ significantly ($p \leq 0.05$). N=3±SD

NGL5 & NGL7 - Nigerian fermented maize *L. plantarum* strains and LpTx - commercial probiotic *L. plantarum* strain

NGY1 & SB20 - Nigerian fermented maize *C. tropicalis* and probiotic *S. boulardii* SB20 respectively

Bourlioux, & Collignon, 2002). Pathogen inhibition on agar plates by yeasts in this present study is quite unique as reports of such are scarce in the literature. *S. bourladii* SB20 was more inhibitory towards *L. monocytogenes* NCTC 7973 and *B. cereus* NCIMB 11925 than *C. tropicalis* in malt extract agar was. *S. aureus* NCTC 3750 was resistant to both yeasts. The inhibitory mechanisms, whilst unclear, could be attributed mainly to ethanol production. *S. boulardii* SB20 in this study had maximum lactic acid and ethanol concentrations of 20.23 and 194 mmol L^{-1} respectively during the fermentation of sterile ground maize slurry. However, the review by Shetty and Jespersen (2006) indicated that the antagonistic property of *S. cerevisiae* was mostly due to the polysaccharide part of the cell wall that is involved in surface binding of pathogenic toxins. *C. tropicalis* (NGY1) in this fermentation experiment did not produce a significant amount of ethanol, neither was there a significant decrease in pH for antimicrobial activity. It was not surprising, therefore, that *C. tropicalis* (NGY1) had no inhibitory effect against the pathogen.

4 Conclusions

The *L. plantarum* strains, either as single or mixed starter cultures with yeasts, demonstrated strong fermentation ability, with significant reduction in pH, and were inhibitory against foodborne pathogens which is vital in the use of selected organisms as starters for the safety of products. Although, *C. tropicalis* was less inhibitory to the pathogens, its ability to grow in the fermentation with little or no ethanol production could be preferred in its use as a mixed starter culture with the LAB in the production of infant complimentary foods. The study also demonstrated that the ground maize slurry could act as a vehicle to relay probiotics in *akamu* production.

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