Microbiological quality during storage of pork *balangu* inoculated with *nisin* producing *Lactococcus lactis* subsp. *lactis*

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Abstract

Introduction. The aim of this study was to characterize *Lactococcus* spp. isolates from pork *balangu* and use suitable strain in the biopreservation of the product.

Materials and methods. Isolation of *Lactococcus* isolates was carried out using traditional methods while their presumptive identities were obtained using phenotypic method involving biochemical reactions. Their full identification was obtained by the use of 16S rDNA sequencing. Production of antimicrobial agents was done by the use of reported methods. The abilities of suitable isolate(s) to control spoilage and pathogenic organism of food origin were examined.

Results and discussion. Thirty four *Lactococcus* spp. were isolated from pork *balangu*; their phenotypic characterization revealed they were composed of seven groups, members of which shared similar biochemical reactions. The phenotypic identities of seven isolates of the *Lactococcus* isolates, including one from each group, were confirmed by their 16S rDNA sequencing as *L. raffinolactis*, *L. garviae*, *L. lactis*subsp. *cremoris*, *L. lactis* subsp. *hordniae*, *L. lactis* subsp. *lactis*, *L. plantarum* and *L. piscium*. Among them *L. lactis* subsp. *lactis* produced higher concentration of lactic acid (21.45 g/10^7 CFU) than others, and also produced *nisin* (~608 bp), active against the spoilage organism of meat *Brochothrix thermosphacta*. Inoculation of *nisin* producing *L. lactis* subsp. *lactis* in pork *balangu* showed 4 log reductions in the counts of *Listeria monocytogenes* and *B. thermosphacta* from their initial 6 log in the meat product *in situ*.

Conclusion. The *nisin* producing *L. lactis* subsp. *lactis* could be used in the shelf life extension of pork *balangu* beyond the day of production. The *Lactococcus* strain could therefore be applied as biopreservative culture to promote safety of meat products in West Africa and other parts of the world.

Introduction

In Nigeria, meat is normally cut into various thin slabs, spiced and grilled over glowing charcoals, resulting in products which are called by various names, including *suya*, *tsire*, *balangu* etc, depending on the specific method adopted during preparation. One of such popular grilled meat product is *balangu*, and may be produced from the muscle of cow, mutton, lamb, chicken or other animals, although preparation from beef is common. *Balangu* is a traditional Nigerian meat product, commonly prepared from raw meat, especially beef with the addition of various spices and cooked by grilling. It is usually eaten as delicacies and has associated sensory characteristics that play important role in its acceptance by consumers [1]. The meat product is consumed by many people as delicacies, mostly during leisure. Generally, producers of grilled meat products in Nigeria do not normally exhaust their sales on the day of production, thereby leaving remains until the second day or beyond. Unfortunately due to poor storage facilities, the remains are kept at ambient temperature of approximately 30°C. This temperature may encourage spoilage by opportunistic microorganisms and have adverse effect on the physicochemical properties of the product due to deterioration by spoilage organisms. This has resulted in product's rejection by customers, although such has not been reported in the literature due to limited research studies [1].

Food security, the availability of food and its accessibility to people in safe forms, has been an important concern in most developing countries where food preservation techniques have been very inadequate [2]. In Nigeria, this has adverse effects on grilled meat products, especially *balangu*, where losses have been experienced due to poor storage facilities[1]. Biopreservation refers to the extension of the shelf-life and improvement of the safety of foods using microorganisms and/ or their metabolites [3]. One of the common biological agents used in the biopreservation of food is lactic acid bacteria (LAB). LAB have a GRAS (generally regarded as safe) status and have been widely used as starters in the industrial preservation of meats [4]. LAB can secrete many antimicrobial substances with presumptive antimicrobial effect in foods that could be exploited in preventing many foodborne pathogens and spoilage organisms [5]. LAB are used to ensure safety, preserve food quality, develop characteristic new flavours, and to improve the nutritional qualities of food [6]. Many LAB cultures like *Leuconostoc* spp., *Lactococcus* spp., *Pediococcus* spp., and *Lactobacillus* spp. are being used in meat processing and few of these cultures could produce bacteriocins, a useful factor which has been noted to enhance their choice as starters [7]. The ability of LAB to inhibit the growth of undesirable bacteria has been reported and inhibition may be due to the production of organic acids, hydrogen peroxide, carbon dioxide, acetaldehyde, diacetyl or bacteriocins [3,8].

Some strains of LAB have been identified and used as starters in the preservation of some meat products [5,8,9]. There is limited research studies on the Nigerian traditional grilled meat product *balangu* and more efforts are required to protect consumers and ensure safe availability of the product in promoting food security in Nigeria. The only known study was reported by Olaoye [1] who investigated some physicochemical factors of *balangu* as possible indicators of spoilage during storage. The present study therefore reports on characterization of *Lactococcus* isolates from pork *balangu* and the use of a *nisin* producing *L. lactis* subsp. *lactis* in the biopreservation of the product.

Materials and methods

Source of materials. The pork meat used in this study was obtained from a Nigerian retail shop in the city of Nottingham, UK. The meat samples were conveyed over ice crystals to the laboratory in clean polyvinyl chloride (PVC) bags and stored briefly at -5°C (Haier Thermocool, BD-66A, Westgate Ealing, London, UK) before use. Other materials used which included ground red pepper (*Capsicum* sp.), ginger (*Zingiber officinalis*), groundnut (*Arachis hypogaea*), salt and groundnut oil were all purchased from the same source.

Bacterial strains and culture conditions. The bacteria cultures, used in challenge experiments against the starter culture (nisin producing *Lactococcus lactis* subsp. *lactis*) in the pork meat product *in situ*, included *Brochothrix thermosphacta* NCIMB 10018 (STAA, oxoid; 30°C), *Listeria monocytogenes* NCTC 11994 (OX, oxoid; 30 °C) and *Salmonella typhimurium* (XLD, oxoid; 30°C) which were obtained from culture collection unit of Food Microbiology, Division of Food Sciences, University of Nottingham, UK. Frozen cultures were maintained in BHI broth media (Oxoid, UK) containing 20% glycerol at -80 °C [10].

Isolation of Lactococcus from pork balangu. Isolation of *Lactococcus* isolates was done according to a modification of the method of Onilude *et al.* [8]. A weighed quantity (10 g) of fresh pork meat was immersed in a sterile 10% (w/v) sucrose solution for about 7 min to stimulate LAB growth [11]. This was then homogenized in 90 ml sterile distilled water (SDW) in standard stomacher bags (BA 6141, Seward, UK), using a Seward stomacher (model 400 circulator, P/4/518, 50-60Hz, UK). The resultant homogenate was made into 10 fold dilutions, 1 ml of suitable dilution was measured into sterile petri dishes after which 10-15 ml of molten deMann Rogosa and Sharpe, MRS (~45 °C; Oxoid UK) agar was carefully poured while swirling gently. The plates were allowed to cool and then incubated in micro-anaerobic jars (18 – 24 h, 37 °C). Upon observation for growth, Gram positive, catalase and oxidase negative colonies were picked for sub-culturing on sterile MRS agar. Streaking of single colonies was done repeatedly to obtain pure cultures which were examined microscopically for cell appearing as cocci in pairs or short chains, which are characteristic *Lactococcus* [12]. Pure cultures of presumptive *Lactococcus* isolates were kept for characterization and identification.

Phenotypic characterization and identification of the Lactococcus isolates. Seven presumptive isolates of *Lactococcus* were selected based on their biochemical reactions were characterized using API 20 Strep kit (API Systems, Biomerieux Sa, France). Presumptive identification of the isolates was done using the results obtained in the API website (www.apiweb.biomerieux.com).

Full identification of the presumptive Lactococcus isolates. Confirmation of full identities of the presumptive *Lactococcus* isolates was carried out using PCR-16S rDNA . DNA was extracted by a modification of the boiling method described by Suwanjinda *et al.* [13]. PCR amplification was carried out with specific primers targeting approx. 390 bp of the 16S rDNA (V4 regions) in the *Lactococcus* isolates. This was in a 50µl reaction volume containing 1.25 units of *Taq* DNA polymerase (ABgene, Thermo Fischer, UK), 2.5mM magnesium chloride (Promega, Southampton, Southamptonshire, UK), 0.2mM dNTPs (Promega), 0.1µl of each reverse 5'-CCGTCAATTCCTTTGAGTTT-3' and forward primer 5'- CAGCAGCCGCGGTAATAC-3' [14], 5µl PCR buffer and 5µl of DNA template. Volume was made up with SDW.

Electrophoresis of the PCR products was performed on the Bio-Rad Contour-Clamped Homogenous Electric Field (CHEF) DRII electrophoresis cell (Hemel Hempstead, UK). Agarose gel (Biogene Kimbolton Cambs, UK), 1.5% (w/v), stained with 0.5µg/ml ethidium bromide, was used in 1 X TAE (Tris-Acetate EDTA) buffer at 84 volts for 1.5 – 2 h. A 100 bp molecular size marker was as ladder.

Sequencing of 16S rDNA genes was done by resolving 40 µl of the PCR products in 1% Agarose gel. Amplicons were excised from gel, purified using the Wizard PCR Preps DNA Purification System (Promega) and sent to Germany (MGW-Biotech, Germany) for sequencing. The nucleotide sequences were used in the GenBank database using BLAST at the website (http://www.ncbi.nlm.nih.gov/blast/) to determine the closest known relatives of the 16S rDNA gene sequences.

Selection of starter culture. The *Lactococcus* isolates were tested for abilities to produce organic acids (lactic acid and acetic acid), which is characteristic of the fermentative ability of LAB in lowering pH of food samples and thereby creating unconducive environment for the growth of many spoilage/pathogenic organisms. Possible production of bacteriocin by the isolates was also evaluated in *in vitro* assay against sensitive indicator organism.

Production of organic acids was tested using the method of high performance liquid chromatography (HPLC) described by Olaoye and Onilude [15].

The modified method described by Suwanjinda *et al.* [13], involving overlaying of M17 plates, containing live colonies of *Lactococcus*, with indicator organism (*Brochothrix thermosphacta* NCIMB 10018) was used to detect antagonism of the *Lactococcus* isolates. Serial dilutions were made and used to obtain plates containing 10-50 colonies of *Lactococcus* on M17 agar; the plates were carefully overlaid with test indicator strain (50 µl of an overnight culture in 10 ml of BHI broth containing 0.7% agar). Plates were allowed to solidify and then incubated at 30°C for 24 h. The plates were examined for presence of zones of inhibition around the *Lactococcus* colonies.

The paper disc assay method [16] was also used detect antimicrobial activity of the *Lactococcus* isolates against *Brochothrix thermosphacta* NCIMB 10018. The *Lactococcus* isolates were grown in M17 broth for 24 h at 30 °C. The cultures were then centrifuged at 5000 × *g* for 15 min (Centrifuge Falcon 6/300 series, CFC Free, UK) and the cell free supernatants (CFS) were collected for use in antimicrobial assay.

A sterile filter paper disc (Whatman AA, 6mm, Fisher Scientific, UK) was soaked in CFS for 30 min, and then applied on plates previously seeded with BHI broth (with 0.7% agar) containing 50 µl of indicator organisms. The plates were incubated overnight at 30 °C for 24 h and then observed for zones of inhibition. Clear zones extending for 1 mm or more were considered as positive for inhibition [17].

To test for possible production of bacteriocin, the CFS was neutralized to pH 6.5 using NaOH to eliminate activity of organic acids. To also get rid of influence of hydrogen peroxide, the neutralized CFS was treated with 300 units/ml of horseradish peroxidase (Sigma-Aldrich); this gives a crude bacteriocin used in bacteriocin assay [18] against the sensitive indicator organism (*Brochothrix thermosphacta* NCIMB 10018) using the paper disc method as previously described.

The *Lactococcus* isolates, that their CFS displayed antagonism against the sensitive indicator organism *B. thermosphacta* NCIMB 10018, were tested for presence of bacteriocin (*nisin*) encoding genes by PCR. DNA templates were obtained using the extraction method of Suwanjinda *et al.* [13]. PCR reactions were same as for 16S rDNA. The primers used include 5’-CTATGAAGTTGCGACGCATCA-3’ (Forward) and 5’-

CATGCCACTGATACCCAAGT-3' (Reverse), targeting the 608 bp of the *nisin* operon in *Lactococcus* (Suwanjinda *et al.*, 2007). The same methods as used for PCR amplification conditions, resolving and visualization of PCR products, sequencing and identification of 16S rDNA genes were used for the *nisin* gene(s).

Preparation of pork balangu. The traditional technique was mimicked for preparation of pork *balangu* with little modification [1]. Fresh pork meat was rinsed in SDW and then cut into chunks (25 x 16 x 1.2 cm), each weighing about 75±2.3g. They were allowed to drain for 15 min in clean perforated plastic containers. The entire surface of each of the pork chunks was covered with mixed ground spices, consisting of red pepper (23%), groundnut powder (52%), ginger (20%) and salt (5%). The spiced pork chunks were then spread on wire gauze and grilled over glowing charcoals at 100-120°C for 30-45 min. They were intermittently turned to ensure even cooking while groundnut oil was sprinkled on them at regular intervals during the grilling process to simulate the traditional technique of avoiding burning or charring [8].

Inoculation of pork balangu with bacteriocin producing Lactococcus lactis subsp. lactis as starter culture. The *nisin* producing *Lactococcus lactis subsp. lactis* culture was subcultured three times in M17 broth at 30^{0}C for 24 h. Filter sterilised glucose solution (~ 5% w/v final concentration) was applied to the pork *balangu* pieces [19] and then placed separately in aluminium foils. Two sets (Ing-lc and N-Ing-lc) of the pork meat were inoculated with the *Lactococcus* culture to about 10^6 cfu/g of meat, wrapped in sterile transparent bags and incubated at 30°C for 5 days. Un-inoculated samples (Ing-cont and N-Ing-cont) served as control. The ability of the *Lactococcus lactis subsp. lactis* to control *Ls. monocytogenes*, *Salmonella typhimurium* and *Brochothrix thermosphacta* was tested in challenge experiments by inoculating them separately on different sterile pork *balangu* samples (Ing-lc and N-Ing-lc) at 10^6 cfu/g prior to inoculation with *nisin* producing *Lactococcus* isolate. The pathogenic/spoilage organism were previously grown for 24 h in BHI broth (Oxoid) before their application on the meat product.

Microbial enumeration of pork balangu during storage. Pork *balangu* samples (10 g) were homogenized in standard stomacher bag (BA 6141, Seward, West Sussex, UK) containing 90 ml maximum recovery diluent (MRD) for 3 min at 230 rpm, using a Seward stomacher (model 400 circulator, P/4/518, 50-60Hz, UK). One millilitre (1 ml) of homogenate was serially diluted in 9 ml of MRD to obtain 10 fold dilutions; 1 ml of appropriate dilutions were spread or pour plated in replicates on selected agar media. The media used included deMan Rogosa Sharpe (MRS, Oxoid) for LAB, incubated at 30 °C for 48 h; Rose Bengal Chloramphenicol Agar (RBCA, Oxoid) for yeast and moulds at 25 °C for 72 h; Mannitol salt phenol red agar, MSPRA (Sigma-Aldrich, St. Louis, Missouri, USA) for *Staphylococcus* at 37 °C for 24 h; MacConkey Agar (Oxoid) for coliforms at 37 °C for 24 h; Violet red bile glucose agar (VRBGA, Oxoid) for *Enterobacteriaceae*, at 30 °C for 48 h; Xylose lysine deoxycholate (XLD, Oxoid) for *S. typhimurium* at 37°C for 24 h; Oxford formulation (Oxoid) for *Ls. monocytogenes* at 30 °C for 48 h; and STAA agar (Oxoid) for *B. thermosphacta* at 30 °C for 24-48 h.. Emerging colonies were counted and the results expressed in colony forming units per gram (cfu/g) of meat.

pH measurement. pH of the pork *balangu* samples was monitored during storage by taking 10 g of sample and homogenized in standard stomacher bags (BA 6141, Seward, UK) containing 100 ml SDW, using a Seward stomacher (model 400 circulator, P/4/518,

50-60Hz, Leighton Buzzard,UK). The pH was then measured by a pH meter (pH 212 Microprocessor, Hanna Instruments, USA) using the method of Marugg *et al.* [20].

Statistical analysis. The effect of storage time on the respective pork *balangu* samples was evaluated by subjecting the data obtained on each day of storage to analysis of variance using the statistic software, Design expert (version 6.0.6; Stat-Ease Inc., East Hennepin Ave, Minneapolis, MN). Significant differences were determined at $p<0.05$.

Results and discussion

Thirty four presumptive isolates of *Lacococcus* were isolated from pork *balangu*, with the objective of selecting suitable isolate for use as starter culture in biopreservation of the meat product. The biochemical characteristics of the *Lactococcus* isolates (Table 1) indicate the presence of seven groups, with each member having similar biochemical reactions. The members of the groups were Gram positive cocci, appearing in pairs or short chains which are characteristic of the genus *Lactococcus* [12]. The phenotypic (biochemical) characteristics of the isolates were used in their phenotypic identification with particular reference to the Bergey's manual of determinative bacteriology [12]. The isolates of each group were therefore phenotypically identified as *Lactococcus piscium*, *L. garviae*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *hordniae*, *L. lactis* subsp. *lactis*, *L. plantarum* and *L. raffinolactis* respectively. Their full identifications were obtained by sequencing of their 16S rDNA genes (V4 region) that were amplified by PCR (Figure 1); PCR products of approximately 400 bp in size were obtained. The 16S rDNA nucleotide sequences were used in the genBank database to obtain their closest relatives. The *Lactococcus* isolates showed between 99 and 100% homologies to the respective closest relatives, thus giving acceptable full identifications [21].

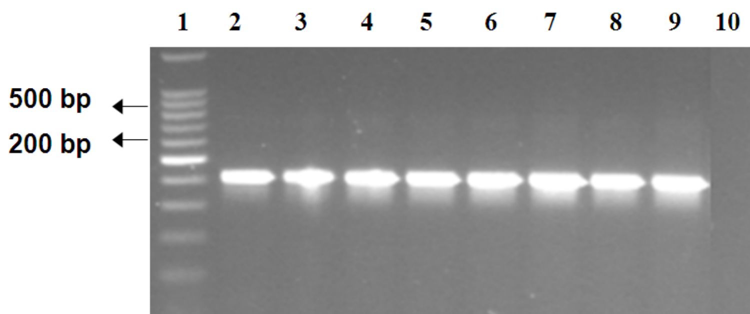

Figure 1. PCR amplification of 16S rDNA -V4 region of the phenotypically identified *Lactococcus* isolates from pork *balangu*

1 – 100 bp DNA marker; 2, -presumptive *Lactococcus piscium*; 3 3 presumptive *L. garviae*; 4 – presumptive *L. lactis* subsp. *cremoris*; 5 – presumptive *L. lactis* subsp. *hordniae*; 6 – presumptive *L. lactis* subsp. *lactis*; 7 – presumptive *L. plantarum*; 8 – presumptive *L. raffinolactis*; 9 – positive control – *L. lactis* NCIMB 4918; 10 – Negative control – sterile deionized water.

Table 1. Phenotypic/biochemical characteristics and presumptive identities of *Lactococcus* isolates from PFGE clusters

Cell shape	cocci in pairs or short chains	cocci in pairs or short chains	cocci in pairs or short chains	cocci in pairs or short chains	cocci in pairs or short chains	cocci in pairs or short chains	cocci in pairs or short chains
Grams reaction	+	+	+	+	+	+	+
Acid production from:							
Amygdalin	+	+	-	-	-	+	-
Galactose	+	+	+	-	+	-	+
Lactose	+	-	+	-	+	-	-
Maltose	+	+	-	-	+	+	+
Melibiose	+	-	-	-	-	-	+
Melezitose	+	-	-	-	-	+	+
a-Methyl-D-glucoside	+	-	-	-	+	-	-
a-Methyl-D-mannoside	+	-	-	-	-	-	-
D-Raffinose	+	-	-	-	-	-	+
Sucrose	+	-	-	+	-	+	+
Trehalose	+	+	-	+	+	+	+
D-Turanose	+	-	-	-	-	+	+
D-Xylose	+	-	-	-	+	-	+
Growth at 0°C	+	-	-	-	-	-	-
Growth at 40°C	-	+	-	-	+	-	-
Growth 4% NaCl	-	+	-	-	+	+	-
Production of:							
APPA[a]	+	+	+	+	+	-	+
Arginine Dehydrolase	-	+	-	+	+	-	-
a-Galactosidase	+	+	-	-	-	-	+
b-Galactosidase	+	+	+	+	+	-	+
b-Mannosidase	+	-	-	-	-	-	-
PYRA[b]	-	+	-	-	-	-	-
Hippurate hydrolysis	-	-	+	-	+	-	-
Probable identity	*L. piscium* (5)	*L. garviae* (8)	*L. lactis* subsp. *cremoris* (5)	*L. lactis* subsp. *hordniae* (6)	*L. lactis* subsp. *lactis* (3)	*L. plantarum* (3)	*L. raffinolactis* (4)

+, positive; - , negative; APPA, Alanyl-phenylalanyl-proline arylamidase; PYRA, Pyrrolidonyl arylamidas

Among the *Lactococcus* isolates, only the neutralized supernatant of *L. lactis* subsp. *lactis* was shown to display antagonism against a *nisin* sensitive indicator organism *B. thermosphacta* in an *in vitro* assay. The broth medium was neutralized in order to eliminate the presence of organic acids which may interfere with the antimicrobial assay [13]. Additionally, the broth supernatant was treated with peroxidase enzyme to get rid of hydrogen peroxide that may also interfere in the assay [16]. The antimicrobial activity recorded against the indicator organism by the neutralized broth supernatant of *L. lactis* subsp. *lactis* suggested the presence of bacteriocin encoding gene in the isolate. The positive PCR amplification of the bacteriocin (*nisin*) gene of about 608 bp in the *Lactococcus* isolate with specific primer was used to confirm this finding, this was similar to those reported in earlier investigations [22,23]. Beside production of *nisin* by *L. lactis* subsp. *lactis*, its ability to produce considerable quantities of organic acids, especially lactic acid, was considered in its choice as starter culture for pork *balangu*. During preliminary experiments, *L. lactis* subsp. *lactis* produced higher quantity of lactic acid (21.45 g/10^7 CFU) than others, giving it an advantage as starter culture for the pork meat product. The ability of LAB to produce lactic acid that contributes to reduction in the pH of food products is one of the very important factor in their antagonistic activities against spoilage and pathogenic organism [5,23,24]. The lowering of pH due to production of organic acids can also cause characteristic changes in flavour and texture of meat products; however, this does not normally have any adverse effect on the sensory appeal of the product [11,25].

The specific microbial organisms that were evaluated in the meat samples during storage exhibited their defined colony characteristics on respective media; LAB as whitish or milky; coliforms as pinkish or reddish; Enterobacteriaceae as pink; *Staphylococcus* had red or yellow colourations around them; *Ls. monocytogenes* as black; *Salmonella typhimurium* appeared as red with black centre colourations; and *B. thermosphacta* appeared as straw coloured colonies. The specific organisms emerging from respective growth media were also confirmed by PCR-16S rDNA, and their nucleotide sequences used in the genBank database to know the closest relatives.

There was no detection of coliforms in the pork *balangu* samples until after 72 h of storage (Figure 2). Lower counts of coliforms were recorded in the starter culture inoculated samples (SCIS) compared to the uninoculated control samples (UCS), suggesting possible inhibitory action by the starter culture. Kalalou *et al.* [11 reported the reduction of coliform counts during storage of a Moroccan fermented meat product inoculated with LAB. In similar studies, Olaoye *et al.* [5] and Olaoye and Onilude [25] reported reduction of coliforms in fresh meat inoculated with LAB starter cultures. The researchers concluded that the use of LAB as starter culture was moderately effective for the control of coliforms. Reduction of coliforms by the LAB cultures could be attributed to production of antimicrobial agents by the latter [15,26]. The non-detection of coliforms in the early period of storage in the *balangu* samples could be due to the lethal action of heat on them during the grilling process.

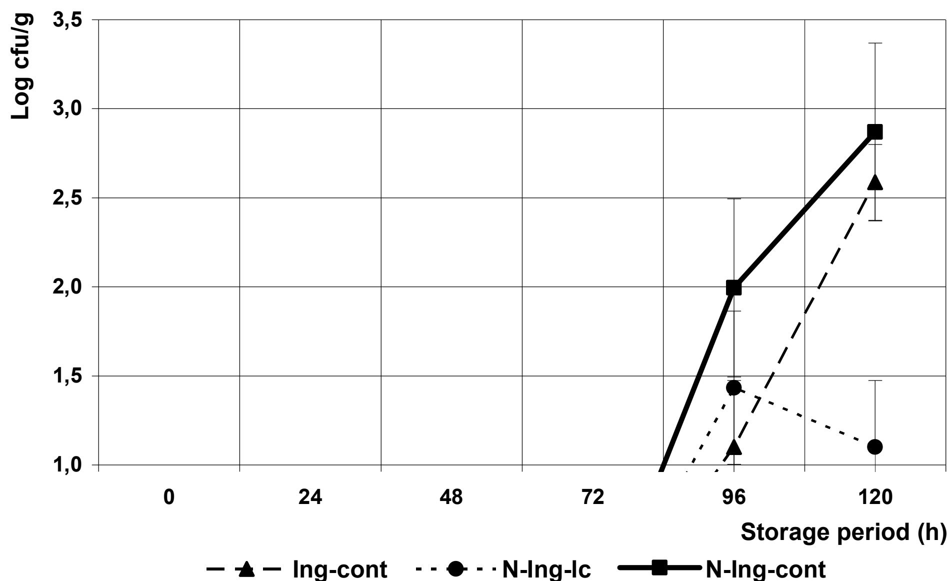

Figure 2. Counts of Coliforms in pork *balangu* during storage
Ing-lc, pork *balangu* containing spices and inoculated with *L. lactis* subsp. *lactis*; Ing-cont, pork *balangu* containing spices and uninoculated with starter culture; N-Ing-lc, pork *balangu* containing no spices and inoculated with *L. lactis* subsp. *lactis*; N-Ing-cont, pork *balangu* containing no spices and uninoculated with starter culture.

From the results of counts of total bacteria (CTB), a decrease was recorded in the SCIS compared UCS (Figure 3). While CTB decreased from the initial count of about 3.81 at 0 h to 2.84 at 48 h in the Ing-lc samples, an increase from 3.79 to 7.88 was recorded for the Ing-cont samples over same period. In all pork *balangu* samples, CTB was comparatively lower in the SCIS than UCS, indicating the effect starter culture on the microflora associated with the meat product. The effect could possibly be due to production of antimicrobial agents by the *Lactococcus* starter culture. Similar findings have been reported on the reduction of CTB in meat products treated with cultures of LAB [9,19,27]. Yeast and moulds were not detected in the Ing-lc samples during storage; however, counts (log count/g) of 3.45 and 4.82 and above were recorded from 72 h in the N-Ing-lc and N-Ing-cont respectively (Figure 4). Counts were generally lower in the SCIS compared to UCS ($p<0.05$); increment of 3-5 log was observed in the latter samples compared to 0–2 log recorded for the former. Casaburi *et al.* [28] observed a reduction in yeast and mould counts of Italian style sausages on storage after inoculation with LAB starter culture. In another study, Erkmen [27] made similar observation in a Turkish sausage treated with LAB. Similarly, Olaoye [9] reported reduction in the counts of yeast and moulds in a Nigerian stick meat *tsire* inoculated with cultures of LAB during storage. The researchers concluded that the LAB cultures reduced the growth of yeast and moulds in the meat products.

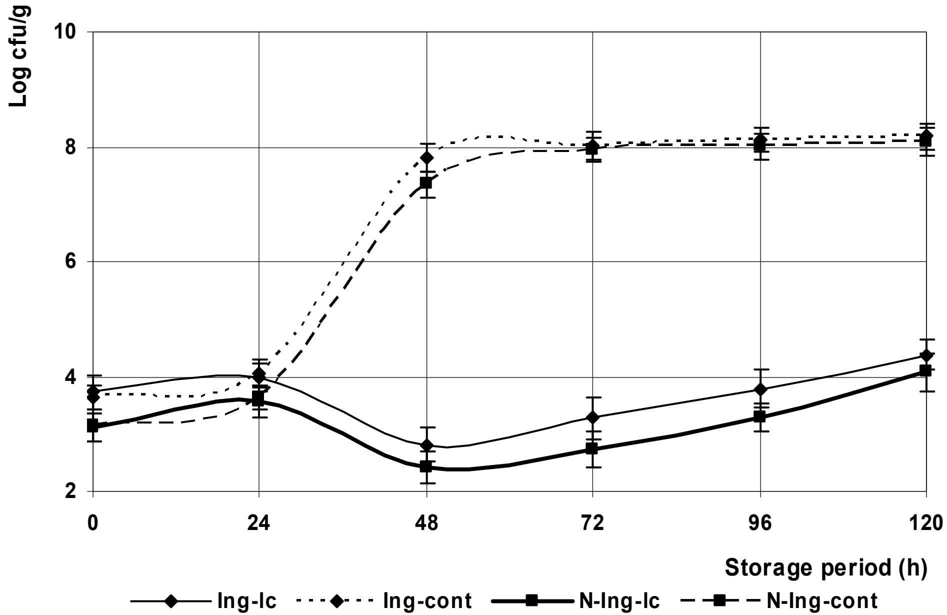

Figure 3. Counts of total bacteria in pork *balangu* during storage

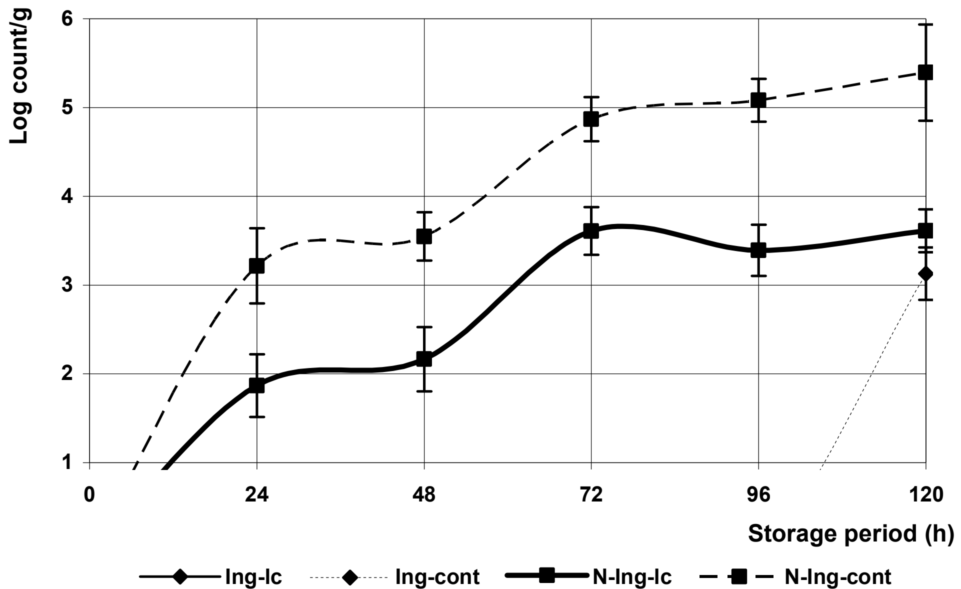

Figure 4. Counts of yeast and moulds in pork *balangu* during storage
Ing-lc, pork *balangu* containing spices and inoculated with *L. lactis* subsp. *lactis*; Ing-cont, pork *balangu* containing spices and uninoculated with starter culture; N-Ing-lc, pork *balangu* containing no spices and inoculated with *L. lactis* subsp. *lactis*; N-Ing-cont, pork *balangu* containing no spices and uninoculated with starter culture.

Figure 5 presents the *Ls. monocytogenes* counts in the pork *balangu* samples during storage. Prior to storage, the meat product was inoculated with approximately 6 log cfu/g of the pathogenic organism to challenge the starter culture. Counts of *Ls. monocytogenes* decreased by about 1.6 and 4 log at 24 h and 48 h respectively in the Ing-lc samples, and at 72 h were below 2 log. On the contrary, an increase of up to 5 log was recorded in the UCS, i.e. Ing-cont and N-Ing-cont samples. Counts increased from 7.07 at 0 h to 11.78 at 120 h in Ing-cont, while similar increase from 6.79 to 11.18 was recorded in N-Ing-cont during same period. Sensitivity of *Ls. monocytogenes*, an important foodborne pathogen, to *nisin* produced by *Lactococcus lactis* subsp. *lactis* was reported by Cintas *et al*. [29]. The risk of *Ls. monocytogenes* in causing serious threat to food safety and health of consumers has been noted [30]. The pathogen has also been known to contaminate meat and meat products during slaughter, processing and production while it can also persist and grow at low pH values, at low water activity and at refrigeration temperatures [15]. Hence the need for its control, especially by the use of biopreservative agents is of ultimate importance in order to safeguard public health [31]. The control of *Ls. monocytogenes* recorded in the *tsire* samples by the *Lactococcus* culture is therefore very important, as no previous report is available on its control in the Nigerian traditional meat product *balangu*.

The pork *balangu* wase also inoculated with approximately 10^6 cfu/g of *Salmonella typhimurium* to challenge the starter culture in the meat product. The graph (Figure 6) shows that there was about 3 log reduction in the counts of *S. typhimurium* in the SCIS samples during storage. An increase of approximately 4 log was however recorded in UCS. The antagonism recorded against *S. typhimurium,* a foodborne pathogen, may be attributed to production of antimicrobial agents, such as organic acids and hydrogen peroxide, by the *Lactococcus* starter culture. The *nisin* produced by the starter culture may not contribute to the antagonism of the pathogen because bacteriocins of LAB have been reported to be ineffective against gram negative organisms [18,29].Furthermore, *Brochothrix thermosphacta*, a meat spoilage organism, was inoculated at 10^6 cfu/g of pork *balangu* to challenge the *nisin* producing *L. lactis* subsp. *lactis* used as tarter culture during storage. Result indicates that there was reduction of about 4 log in the Ing-lc sample at 24 h, after which period the spoilage organism was no more detected throughout storage (Figure 7). A similar observation was noted in the N-Ing-lc sample, where 3 and 4 log reductions were recorded at 24 and 48 h of storage respectively. There was however an increase of 4 log of the spoilage organism in the UCS. The *nisin* produced by the *L. lactis* subsp. *lactis* may have contributed mainly to the inhibition of the spoilage organism, as the indicator organism is known to be sensitive to the bacteriocin. In a related study, Ercolini *et al.* [32] demonstrated effective antimicrobial activity of purified *nisin* against *B. thermosphacta* in meat during storage. Hence, the antagonistic activity of *Lactococcus* starter culture against this spoilage organism in the present study could be very significant towards curtailing spoilage and extending shelf life of pork *balangu* in Nigeria.

The result of pH monitored in the pork *balangu* samples showed that values in SCIS were lower than 5 from day 2 of storage, unlike in the UCS where values of above 6 were recorded (Figure 8). The decrease in pH values of SCIS may be due to production of organic acids by the starter culture. Lowering of pH in food products inoculated with lactic acid bacteria has been recorded by other researchers [1,5,33] and this has been noted as an important factor in the control of undesirable microorganisms in food [34]. The reduction in pH in starter treated samples may thus contribute to the lower counts recorded in those samples than in their uninoculated control counterparts.

Statistically, results generally indicate that there were significant differences ($p<0.05$) between pork *balangu* samples inoculated with *nisin* producing *Lactococcus lactis* subsp. *lactis* and the uninoculated control samples.

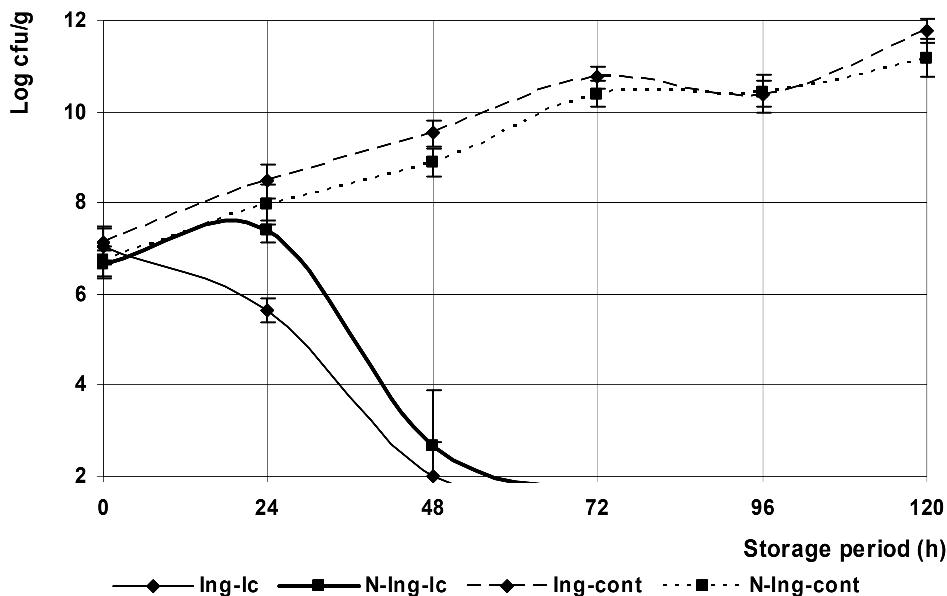

Figure 5. Counts of *Listeria monocytogenes* in pork *balangu* during storage

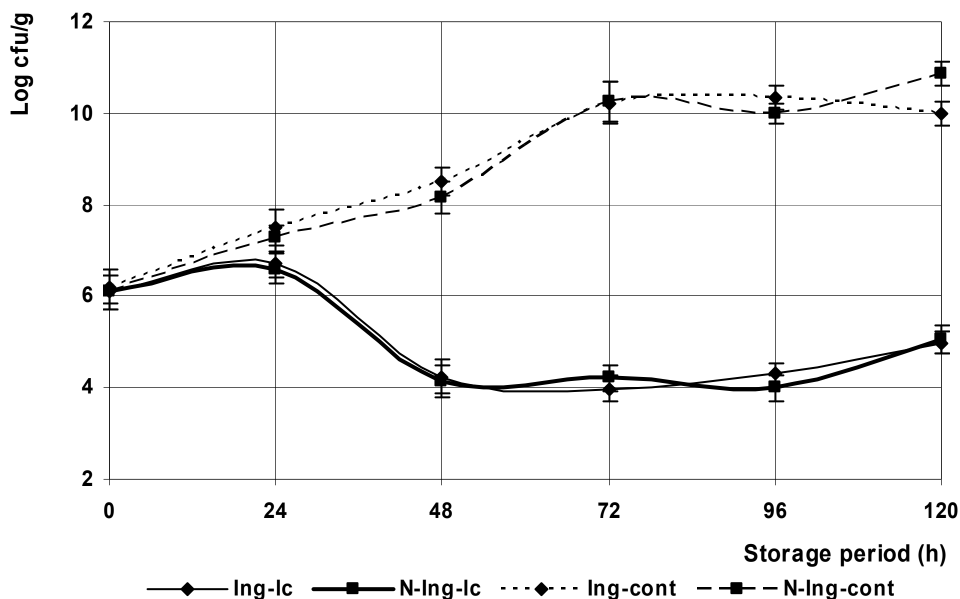

Figure 6. Counts of *Salmonella typhimurium* in pork *balangu* during storage
Ing-lc, pork *balangu* containing spices and inoculated with *L. lactis* subsp. *lactis*; Ing-cont, pork *balangu* containing spices and uninoculated with starter culture; N-Ing-lc, pork *balangu* containing no spices and inoculated with *L. lactis* subsp. *lactis*; N-Ing-cont, pork *balangu* containing no spices and uninoculated with starter culture.

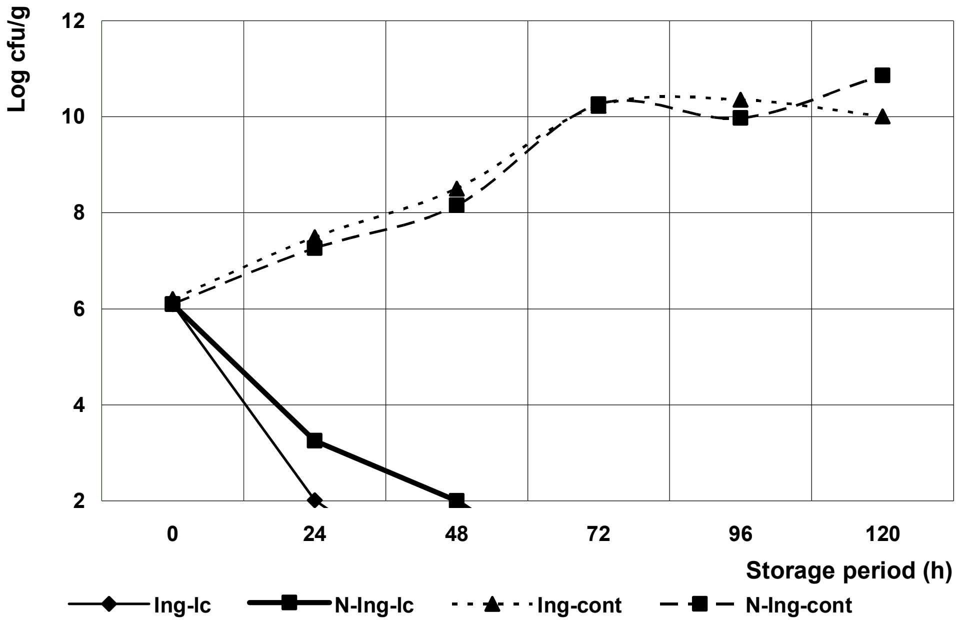

Figure 7. Counts of *Brochothrix thermosphacta* in pork *balangu* during storage

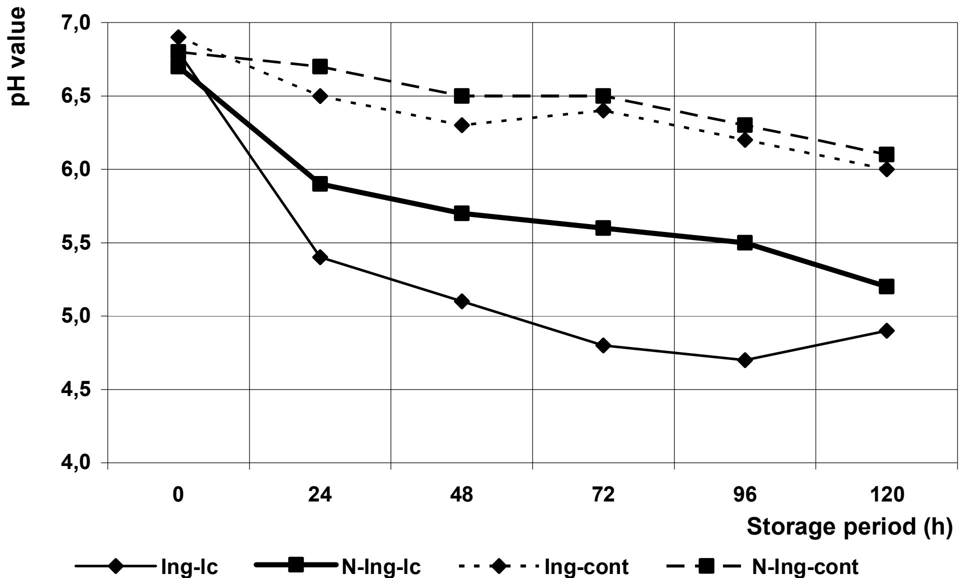

Figure 8. pH patterns of pork *balangu* during storage

Ing-lc, pork *balangu* containing spices and inoculated with *L. lactis* subsp. *lactis*; Ing-cont, pork *balangu* containing spices and uninoculated with starter culture; N-Ing-lc, pork *balangu* containing no spices and inoculated with *L. lactis* subsp. *lactis*; N-Ing-cont, pork *balangu* containing no spices and uninoculated with starter culture.

Conclusion

The *nisin* producing *Lactococcus lactis* subsp. *lactis* used as starter culture in pork *balangu* demonstrated effective control of spoilage and pathogenic organisms in the meat product. Of most important is the control recorded against the pathogens *Ls. monocytogenes* and *S. typhimurium*, and spoilage organism *B. thermosphacta* which are commonly associated with meat products. The findings of this study may therefore be every useful in the preservation of the product, as possible transformation into practical applications could constitute an important approach for improving safety and availability.

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