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Technological Properties of *Lactococcus lactis* subsp. *lactis* I23 Isolated from Nigerian Beef and Characterization of Bacteriocin Produced by It

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Author's contribution

This whole work was carried out by author OAO.

Original Research Article

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ABSTRACT

Aim: The present study reports the phenotypic and molecular characterization of *Lactococcus* strains isolated from Nigerian beef. The technological properties of selected strains were evaluated in order to test their suitability as candidates of starter cultures for food preservation.
Study Design: Experimental microbiological testing.
Place and Duration of Study: Microbiology Department, University of Ibadan, Nigeria and Division of Food Sciences, The University of Nottingham, UK; between January 2005 and February 2009.
Methodology: Phenotypic method was adopted in the initial characterization of the presumptive *Lactococcus* strains which were later characterized using pulsed field gel electrophoresis. The use of 16S rDNA sequencing was adopted in the full identification of selected strains. High performance liquid chromatography and Gas chromatography were employed in determining production of organic acids and diacetyl respectively by the strains of *Lactococcus.* Testing and identification of bacteriocin production by the strains were observed using *in vitro* and polymerase chain reaction methods. The antimicrobial activities of the strains were evaluated against known spoilage and pathogenic organisms of food origin.
Results: Three strains, *Lc. lactis* L11, *Lc. lactis* subsp. *lactis* I23 and *Lc. lactis* subsp.

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hordinae E91 showed better production of diacetyl and lactic acid than others; concentrations in excess of 31.15 µg/10^{-7}cfu and 17.56 g/10^{7}cfu at 18 and 24 h of incubation were recorded for the respective compounds. Result of their antimicrobial activities indicates that varying levels of inhibition were recorded against the spoilage/pathogenic organisms; however no inhibition was recorded against *Brochothrix thermosphacta* except by *Lc. lactis* subsp *lactis* I23 which was shown to encode gene for production of bacteriocin, nisin. The nucleotide sequence of the bacteriocin was deposited at the genBank under accession number EU667387.

Conclusion: The ability of the three *Lactococcus* strains to combat spoilage and pathogenic organisms could help promote safety in food processing and preservation in Nigeria; moreover, production of bacteriocin by *Lc. lactis* subsp. *lactis* I23 may confer added advantage during choice of candidacy for starter cultures in food biopreservation.

Keywords: Antimicrobial agents; spoilage and pathogenic organisms; inhibition; bacteriocin; safety; food biopreservation.

1. INTRODUCTION

Lactic acid bacteria (LAB) have been used for centuries in the fermentation of foods for improvement in flavour and texture, as well as for their ability to prevent the growth of pathogenic microorganisms in food products. It has been identified that the preservation effect of LAB results from the antimicrobial action of bacteriocins as well as metabolites, such as lactic acid and hydrogen peroxide, produced by them. As a result of their ability to produce many different antibacterial substances, LAB can inhibit the growth of several undesirable Gram-positive bacteria in the genera *Bacillus*, *Enterococcus*, *Listeria*, *Clostridium* and *Staphylococcus* [1]. LAB are also responsible for the fermentative processing and preservation of many food products including dairy, meat, vegetables and bakery products [2]. Preservation of fermented foods is due primarily to the conversion of sugars to organic acids with a concomitant lowering of the pH and removal of large amounts of carbohydrates as nutrient sources; the effects extend the shelf life and safety of the final product.

Bacteriocins are ribosomally synthesized antibacterial peptides produced by bacteria and are usually active against genetically closely related species [3]. They have been grouped into four classes based on structure and mode of action [4]. In general, bacteriocins are cationic peptide that display hydrophobic or amphiphilic properties and the bacterial membrane is in most cases the target for their activity.

The genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* have been identified as constituting part of LAB [5]. *Lactococcus* comprises five species including the well-known *Lactococcus lactis* containing three subspecies (*lactis*, *cremoris* and *hordniae*) mainly found in milk products. Isolations of species of the genus have been widely reported; howbeit from dairy sources. Species of the genus that have been isolated from meat sources include *Lc. piscium* [6], *Lc. lactis* subsp. *lactis* [7,8] and *Lc. lactis* WNC 20 [1]. However, information is scarce on evaluation of technological properties of strains the genus *Lactococcus* from beef, most especially bacteriocin production by *Lc. lactis.* Most reported studies have been carried out on the organism isolated from other sources, predominantly dairy [5,9,10].

The present study reports the phenotypic and molecular characterization of *Lactococcus* strains isolated from Nigerian beef. The technological properties of selected strains were also evaluated in order to test their suitability as candidates of starter cultures for food preservation.

2. MATERIALS AND METHODS

2.1 Source of Beef

The beef used for in this study was purchased from a retail market in Ibadan, Oyo State, Nigeria. The samples were conveyed on ice crystals from the point of purchase to the laboratory for isolation of presumptive isolates of *Lactococcus*.

2.2 Isolation of Presumptive *Lactococcus* Strains from beef

In order to promote growth of LAB, beef samples were immersed in 10% sterile sucrose solution about 7 min, after which samples were taken for isolation of *Lactococcus* [11]. Ten grams (10g) of sample was macerated in 90 ml sterile saline solution (1% w/v); the macerate was plated by spreading 1ml of it in sterile petri dishes containing M17 agar (Oxoid, UK) and then incubated at 30ºC for 24 h. Catalase negative colonies were randomly picked and subcultured repeatedly to obtain pure cultures. The cultures were subjected to Gram staining and microscopic examinations; only Gram positive cocci appearing in pairs or tetrads were stored on M17 agar slants as presumptive strains of *Lactococcus*.

2.3 Determination of Phenotypic Properties of Isolates

The physiological and biochemical characteristics of the presumptive *Lactococcus* isolates were determined according to the method of Stiles and Holzapfel [12]. Carbohydrate fermentation reactions were recorded by using the API 50CH (Biomérieux, France) according to Manufacturer's instruction; cultures grown in M17 broths at 30ºC for 24 h were used as inoculating suspensions. Results for API 50 CH were read at 48 h and 7 days.

2.4 Pulsed Field Gel Electrophoresis (PFGE) of the LAB Isolates

Genomic DNA was prepared *in situ* in agarose blocks using a modified method of [13]. For each isolate a colony was inoculated in 10 ml BHI broth and was incubated at 30ºC overnight under soft agitation (100 rpm). One milliliter of the broth was centrifuged at 13,000 rpm (Biofuge *pico* Heraeus D-37520 O sterode) for 1 min. The cells were re-suspended twice in 1.5ml sterile TN buffer (10 mM Tris-HCl and 1 M NaCl) at 4ºC and centrifuged at the same rpm. Further re-suspension of the cells in 250 µl TN buffer was done to ensure a bacterial suspension absorbance (A_{600nm}) of about 1.7. The suspension was mixed with 250 µl of 1% (w/v) molten pulsed field certified agarose, PFCA (BIO- RAD) solution in TN buffer. Three hundred microliters (300 µl) of this was dispensed into reusable perspex plug moulds (BIO- RAD) and allowed to set for 20 min at room temperature. The plugs were then transferred into 5 ml capacity bijou bottles containing 3 ml of lysis buffer (10 gl^{-l} lysozyme, pH 7.6) and incubated at 37ºC for 16 h. The lysis buffer was removed and the plugs washed trice in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) buffer at room temperature for 30 min. The plugs were incubated in proteinase-K (Sigma P-2308) buffer solution (1% N-lauroyl-sarcosine sodium, proteinase-K 1 gl^{-l}, pH 9) at 55ºC under soft agitation for 24 h. The plugs were washed for 30 min trice with TE buffer at room temperature. To inactivate

proteinase-K, 3 ml of 100 mM phenylmethylsulfonyl fluoride was added to each plug and further incubated for 1 h under soft agitation (100 rpm). It was then rinsed for 30 min trice with TE buffer and stored at 4ºC in the same buffer until use.

Digestion of the plugs was done with *Sma* I restriction enzyme (Promega WI53711-5399 USA). Each plug was cut into slices of about 2 mm each using sterile scalpels and were rinsed in 500 µl of sterile deionised water (SDW) for 15 min. The plug slices were then rinsed in 100 µl of 1 X sure cut buffer J, after the SDW has been removed. The slices of each plug were collected into new eppendorf tubes containing 100 µl of 1 X buffer J, 0.2 µg acetylated BSA and 40 U *Sma* I and then incubated for 4 h at 25ºC. After incubation, the plug slices were stored in 0.5 X TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1.25 mM EDTA) at pH 8.

Electrophoresis of the restriction digests was performed on the Bio-Rad Contour-Clamped Homogenous Electric Field (CHEF) DRII electrophoresis cell, using a 1% (w/v) PFCA. A 50-1000 kb DNA ladder (Sigma D-2416) was used as a molecular size marker. Pulsed time was ramped from 4 to 45 s for 16 h. The gel was stained in deionised water (DO) containing 0.5µg/ml ethidium bromide for 1 h and destained in DO water for 30 min. The DNA bands were then visualised using a UV transilluminator with 313 nm emission and photographed using Fuji Film Imaging system FT1-500.

2.5 Technological Properties of *Lactococcus* Strains

Evaluations of organic acids (lactic and acetic acids) and diacetyl produced by the *Lactococcus* strains were carried out using high performance liquid chromatography and Gas chromatography respectively, according to the methods described by Olaoye and Onilude [14]. Concentrations of organic acids were normalized as g/10^7 CFU and that of diacetyl as µg/10^7 CFU.

For the determination of acidification abilities of the strains, the isolates were initially grown in M17 broth and then in sterile reconstituted skim milk supplemented with yeast extract (3 g/l) and glucose (2 g/l) for two successive subcultures. Sterile reconstituted skimmilk (100 ml) was inoculated with 1% (v/v) of a 24 h activated culture and pH changes were determined using pH meters (glass electrode, HANNA instruments, Padova, Italy) during incubation at 30ºC for 48 h.

2.6 Antimicrobial Activities of the *Lactococcus* Strains against Spoilage/Pathogenic Organisms

The *Lactococcus* strains were grown in M17 broth for 24 h at 30ºC. The cultures were centrifuged at 5000 × *g* for 15 min (Centrifuge Falcon 6/300 series, CFC Free, UK) and the cell free supernatants (CFS) were then collected for use in antimicrobial assay.

The antimicrobial activities of the strains were tested against selected known spoilage and pathogenic organisms of foods, including *Salmonella* Typhimurium, *Escherichia coli* NCTC 86, *Yersinia enterocolitica* NCTC 10460, *Pseudomonas fluorescens*, *Klebsiella pneumoniae* U11468, *Listeria monocytogenes* NCTC 11994, *Bacillus cereus* NCTC 21113, *Brochothrix thermosphacta* NCIMB 10018 and *Pseudomonas aeruginosa* NCIMB 10848. These organisms were obtained from the Food Microbiology laboratory, University of Nottingham, UK.

The modified method described by Suwanjinda [15], involving overlaying of M17 plates containing live colonies of LAB strains with indicator organisms was used to detect antagonism of the *Lactococcus* strains. Serial dilutions were used to obtain plates containing 10-50 colonies of *Lactococcus* on M17 agar; the plates were carefully overlaid with test indicator strains (50µl of an overnight culture in 10ml of BHI or Nutrient broth containing 0.7% agar). Plates were allowed to solidify and then incubated at 30 or 37ºC, depending on indicator strain, for 24 h. The plates were then examined for presence of zones of inhibition around the *Lactococcus* colonies.

The paper disc assay method [16] was used for measuring zones of inhibition. 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 and Nutrient broth (with 0.7% agar) containing 50 µl of indicator organisms (in BHI or nutrient broth). The plates were incubated overnight at 30 or 37ºC for 24 h and zones of inhibition were measured. Clear zones extending for 1mm or more were considered as positive for inhibition [17].

2.7 Antimicrobial Activity of the Crude Bacteriocin

The CFS was pH neutralized to 6.5 using NaOH to obtain cell-free neutralized supernatant (CFNS). The CFNS was treated with 300 units/ml of horseradish peroxidase (Sigma-Aldrich) to obtain crude bacteriocin (CB) which was used in bacteriocin assay [18], using the overlay and paper disc methods described earlier.

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2.8 Detection of Bacteriocin (nisin) Encoding gene(s) in the *Lactococcus* Strains by Polymerase Chain Reaction (PCR)

DNA templates were obtained from strains using the extraction method of Suwanjinda *et al.* [15]. DNA was similarly extracted from a known bacteriocin producing type strain *Lc. lactis* NCTC 8586 (obtained from National Collection of Type Cultures, Salisbury, UK) as positive control. PCR reactions were set up in a total volume of 50µl containing 1.25 units of *Taq* DNA polymerase (ABgene, Thermofischer, UK), 2.5mM magnesium chloride (Promega, UK), 0.2mM dNTPs (Promega, UK), 0.1µl of each reverse and forward primer, 5µl PCR buffer and 5µl of DNA template. Volume was made up with sterile distilled water.

The primers used include 5'-CTATGAAGTTGCGACGCATCA-3' (Forward) and 5'-CATGCCACTGATACCCAAGT-3' (Reverse), targeting the 608 bp of the nisin operon in *Lactococcus* strains [15]. PCR amplification conditions consist of initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 30 s, extension at 72ºC for 45 s, and a final extension at 72ºC for 5 min. PCR products were resolved by gel electrophoresis, using Agarose gel (1.5%), stained with of 0.5µg/ml ethidium bromide, in 1xTAE buffer at 84 volts for 1.5 h. Visualization was by UV transilluminator with 313 nm emission and photographed using Fuji Film Imaging system FT1-500.

2.9 Identification of Bacteriocin (*Nisin*) Encoding Gene(S)

Sequencing of bacteriocin (*nisin*) genes was done by resolving 40 µl of the PCR products in 1% agarose gel. Amplicons were carefully excised from gel and purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) and sent to Germany (MGW-Biotech, Germany) for sequencing. The nucleotide sequences were used in the

GenBank database website (http://www.ncbi.nlm.nih.gov/blast/) to determine the closest known relatives for identification of nisin gene(s).

2.10 Full Identification of *Lactococcus* Strains by 16S rDNA Sequencing

The 16S rDNA V3 regions of three *Lactococcus* strains, shown to produce considerable quantities of antimicrobial agents, were amplified using the forward, 5'-CCTACGGGAGGCAGCAG-3' and reverse, 5'-ATTACCGCGGCTGCTGG-3', primers [19]. PCR reactions were set up in a total volume of 50µl containing 1.25 units of *Taq* DNA polymerase (ABgene, Thermofischer, UK), 2.5mM magnesium chloride (Promega), 0.2mM dNTPs (Promega), 0.1µl (~ 10 pmol) of each reverse and forward primer, 5µl PCR buffer and 5µl of DNA template and made up to volume with sterile deionised water. The forward primer and reverse primer used were 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-CAAGGCATCCACCGT-3' respectively. PCR amplification conditions consisted of initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 30 s, extension at 72ºC for 45 s, and a final extension at 72ºC for 5 min. PCR products were resolved by gel electrophoresis, using an agarose gel (1.5%; Biogene), stained with of 0.5µg/ml ethidium bromide, in 1xTAE buffer at 84 volts for 1.5 - 2 h. Molecular size markers used were 100bp and 1kb (Promega G210A). The DNA bands were then visualised using a UV transilluminator with 313 nm emission and photographed using Fuji Film Imaging system FT1-500. The amplicons obtained were excised from the gel, purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) and sent to Germany (MGW-Biotech, Germany) for sequencing. The nucleotide sequences were subjected to searches in the GenBank database using BLAST at the website, http://www.ncbi.nlm.nih.gov/blast/, to determine the closest known relatives for full identification of isolates.

2.11 Partial Characterization of Crude Bacteriocin

The CB of *Lactococcus lactis* subsp. *lactis* I23 was partially characterized. The effects of proteinase enzymes, pH and temperatures were tested on the on it. *Brochothrix thermosphacta* NCIMB 10018 was used as sensitive strain for detection of the bacteriocin produced by the *Lactococcus* strain.

2.11.1 Effect of enzymes on bacteriocin activity

To determine the nature of any remaining antimicrobial activity in the crude bacteriocin, the CFNS was treated with proteinase K, pronase, papain, pepsin and trypsin, catalase and α-amylase enzymes. Antimicrobial activity was monitored by using the paper disc assay method [16].

2.11.2 Effect of temperature

The effect of temperature on bacteriocin activity was tested by incubating CFNS at 4, 30, 37, 55, 70, and 100ºC for up to 120 min. Antimicrobial activity was again tested using the disc assay method.

2.12 Statistical Analysis

Experiments carried out were done in three replicates. The data obtained were subjected to one-way analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test; significant differences were expressed at $P<0.05$. The SPSS statistic programme, version 10.01 was used [20].

3. RESULTS AND DISCUSSION

In the present report, thirty four presumptive strains of the genus *Lactococcus* were isolated from beef (Table 1), with the aim of screening for candidates that may be of technological importance in combating spoilage and pathogenic organisms in the preservation of meat and other food products. The biochemical profiles of these strains were obtained and used for their phenotypic identification. The strains were phenotypically identified as *Lc. piscium* (8 strains), *Lc. raffinolactis* (5), *Lc. lactis* subsp. *cremoris* (1), *Lc. garviae* (5), *Lc. lactis* subsp. *lactis* (3), *Lc. lactis* subsp. *diacetylactis* (2), *Lc. plantarum* (4), *Lc. lactis* subsp. hordinae (3) and *Lc.* spp (3). Three strains could not be identified to the species level by their biochemical profiles. Identification of different species of the genus *Lactococcus* from beef in this study could be of interest as there has been scarce report on their isolation from beef. Among other researchers who have reported isolation of species of the genus from meat sources, Sakala et al. [6] isolated *Lc. piscium* from beef while isolation of *Lc. lactis* subsp. *lactis* was obtained from sausages by Conter et al. [7] and El Malti and Amarouch [8]. Also, *Lc. lactis* WNC 20 was reported to have been isolated from *nham*, a traditionally Thai fermented sausage, by Noonpakdee et al. [1]. However, information is limited on evaluation of technological properties of strains of *Lactococcus* from beef.

Investigation of genomic diversity of the *Lactococcus* strains isolated in this study was carried out by employing pulse field gel electrophoresis (PFGE) with the use of *Sma*1 restriction enzyme. This was used to obtain discriminations or similarities between the various strains. The analysis of the resulting bands of DNA segments generated a dendrogram represented in Fig. 1. The first cluster consisted of strains with phenotypic identity of *Lc. garvieae* (44%) while clusters II, III & IV comprised of *Lc. piscium* (23.5%), *Lc. raffinolactis* (14.7%) and *Lc. lactis* subsp. *lactis* (8.8%) respectively. Other clusters identified in the dendrogram are V, VI & VIII which consisted of strains with respective phenotypic identities of *Lc. lactis* subsp. *diacetylactis* (5.9%), *Lc. lactis* subsp. *hordiniae* (8.8%) and *Lc. plantarum* (12%). Phenotypic identities to species level of strains consisted in cluster VII (*Lc.* spp.; 8.8%) could not be obtained by their biochemical profiles. One strain *Lc. lactis* subsp. *cremoris* (L11), consisting of 2.9% of the strains tested, did not belong to any of the eight clusters identified from the PFGE dendrogram.

Table 1. Phenotypic profiles of the *Lactococcus* strains

Characteristics	Strains							
	Lc. piscium	***Lc. piscium***	***Lc. garviae***	***Lc. lactis* subsp. *cremoris***	***L. lactis* subsp. *hordniae***	***Lc. Lactis* subsp. *lactis***	***Lc. plantarum***	***Lc. raffinolactis***
Acid production from								
Amygdalin	+	+	+	-	-	-	+	-
Galactose	+	+	+	+	-	+	-	+
Lactose	+	+	-	+	-	+	-	-
Maltose	+	+	+	-	-	+	+	+
Melibiose	+	+	-	-	-	-	-	+
Melezitose	+	+	-	-	-	-	+	+
a-Methyl-D-glucoside	+	+	-	-	-	+	-	-
a-Methyl-D-mannoside	+	+	-	-	-	-	-	-
D-Raf®nose	+	+	-	-	-	-	-	+
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	-	-	-	+	-	+	-	-

+, positive; - , negative; a, Alanyl-phenylalanyl-proline arylamidase; b, Pyrrolidonyl arylamidase (pyroglutamic acid arylamidase)

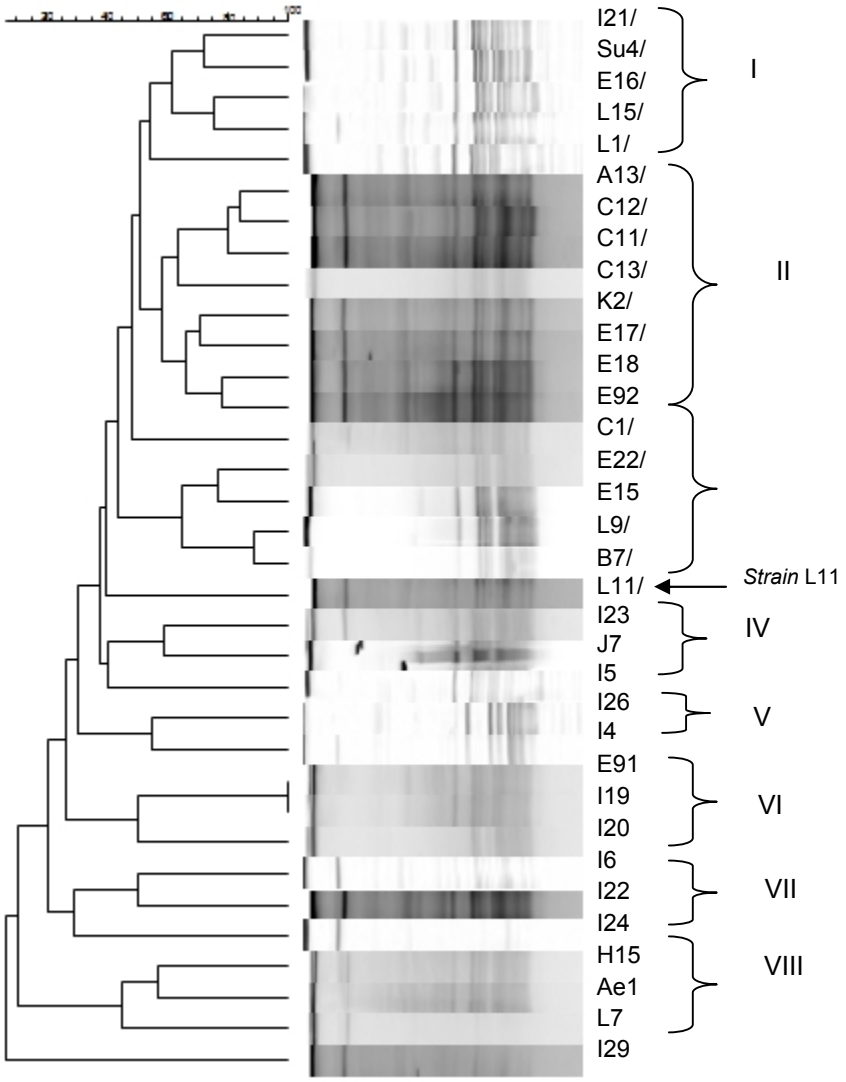

Fig. 1. Dendrogram obtained by PFGE pattern of *Lactococcus* strains after digestion with Sma1 restriction enzyme

During preliminary investigation, all the strains of the *Lactococcus* were evaluated for production of various antimicrobial agents including diacetyl, lactic and acetic acid. Three of the strains were observed to produce reduced concentration of acetic acid and enhanced concentrations of lactic acid and diacetyl than others; these strains were therefore chosen for further investigation in terms of technological properties. The values of antimicrobial agents recorded for the three strains are presented in Table 2. There was no detection of the organic acids (lactic and acetic) at 6 h of incubation. Production of the antimicrobial agents was generally observed to increase with incubation period. Similar findings were reported by Olaoye et al. [21] and Olaoye and Onilude [14] where increase in concentrations of the antimicrobial agents was observed with incubation time when *Pediococcus* strains were grown in M17 medium. In the present report, the highest production of lactic acid, acetic acid

and diacetyl was observed for *Lc. lactis* subsp. *hordiniae* E91 at varying periods of incubation. Diacetyl production reached peak at 24 h while that of acetic and lactic acids attained peak at 24 and 30 h respectively; production of the compounds declined after these periods. In a report of technological properties of *Lc. lactis* strains isolated from milk sources [22], production of lactic acid was noticed to increase with time of incubation. A similar finding was reported by Sakala et al. [6] on *Lactococcus* isolates obtained from meat.

Table 2. Organic acids and diacetyl production by the *Lactococcus* strains

IP (h)	*Lactococcus* strains		
	***Lc. lactis* L11**	***Lc. lactis* subsp. lactis I23**	***Lc. lactis* subsp. hordinae E91**
***Lactic acid* (g/10^7 cfu)**			
6	not detected	not detected	not detected
12	6.71 (±0.05)	7.5.53 (±0.12)	4.63 (±0.19)
18	15.35 (±0.12)	16.62 (±0.16)	11.00 (±0.19)
24	17.56 (±0.12)	18.99 (±0.14)	22.37 (±0.14)
30	21.67 (±0.15)	22.51 (±0.12)	28.89 (±0.12)
36	15.39 (±0.02)	20.88 (±0.11)	24.45 (±0.23)
42	22.21 (±0.01)	28.02 (±0.22)	26.33 (±0.27)
48	13.12 (±0.03)	19.33 (±0.31)	22.86 (±0.33)
***Acetic acid* (g/10^7 cfu)**			
6	not detected	not detected	not detected
12	1.31 (±0.12)	1.87 (±0.53)	1.72 (±0.12)
18	3.58 (±0.04)	2.95 (±0.03)	5.78 (±0.06)
24	3.54 (±0.07)	4.22 (±0.03)	10.40 (±0.03)
30	7.56 (±0.01)	10.17 (±0.05)	6.89 (±0.06)
36	5.46 (±0.02)	4.46 (±0.04)	3.89 (±0.02)
42	7.41 (±0.43)	15.19 (±0.26)	7.79 (±0.43)
48	4.04 (±0.19)	7.56 (±0.13)	6.56 (±0.31)
***Diacetyl* (µg/10^7 cfu)**			
6	12.72 (±0.77)	10.31 (±0.07)	7.92 (±0.57))
12	19.02 (±0.88)	15.82 (±0.72)	23.18 (±0.36)
18	35.29 (±0.03)	31.15 (±0.07)	41.13 (±0.04)
24	37.25 (±0.05)	34.83 (±0.04)	55.89 (±0.28)
30	29.33 (±0.04)	27.14 (±0.16)	30.69 (±0.05)
36	26.15 (±0.07)	35.16 (±0.10)	26.54 (±0.03)
42	24.09 (±0.09)	23.39 (±0.14)	19.72 (±0.06)
48	22.02 (±0.01)	18.36 (±0.03)	15.91 (±0.10)

IP, Incubation period; h, hour

Enhanced production of lactic acid and diacetyl has been noted to impart positively on the technological properties of candidate organisms being proposed for use as starter cultures of food preservation [14]. Acetic acid production by lactic acid bacteria (LAB) should be at reduced levels as the compound has been implicated in contributing to pungent off flavours in foods [23]. The LAB usually implicated in producing acetic acid at levels that may impart negatively on the flavour quality of food are heterofermentative which include *Leuconostoc*, some strains of *Lactobacillus* among others [24]. It should be noted that the strains under investigation belong to the genus *Lactococcus* which has been categorised as homofermentative [6]. It is therefore not surprising that higher concentrations of lactic acid were observed than acetic acid by the three strains of *Lactococcus* under investigation.

Homofermentative LAB have been favoured over their heterofermentative counterparts in the choice of candidacy for starter cultures in food processing [25]. The heterofermentative strains of LAB have been reported as not suitable for food preservation because of the formation of large amounts of carbon dioxide which leads to holes of different sizes in the product [26].

pH of growth medium of the different *Pediococcus* strains generally decreased with incubation period and values were lower than 4 from 24 h (Fig. 3). At 18 h, the lowest value 3.86 was recorded for *Lc. lactis* subsp. *lactis* I23 among the strains. Lowering of pH with progressive increase in incubation period could obviously be attributed to production of organic acids by the *Pediococcus* isolates.

Full identifications of the three phenotypic strains of *Lactococcus*, E91, I23 and L11, were obtained by 16S rDNA sequencing (Table 3); they were each shown to have 99% identities with *Lc. lactis* subsp. *hordiniae* (Accession no EF694031.1), *Lc. lactis* subsp. *lactis* (EF589778.1) and *Lc. lactis* subsp. *cremoris* (AM490365.1) respectively. Their phenotypic identities were thus validated by the molecular method.

Table 3. Identification of *Lactococcus* strains by 16S rDNA V3 sequences in the genBank database

LAB strain	length (bp)	Closest organism relative	% identity	GenBank accession no
E91	150	*Lactococcus lactis* subsp. *hordniae*	99	EF694031.1
I23	149	*Lactococcus lactis* subsp. *lactis*	99	EF589778.1
L11	150	*Lactococcus lactis* subsp. *cremoris*	99	AM490365.1

Based on the production of considerable concentrations of antimicrobial agents by the three *Lactococcus* strains, evaluation of their antimicrobial activities against known pathogenic and spoilage organisms of food origins was studied (Table 4). The results show that all indicator organisms, with the exception of *Brochothrix thermosphacta*, were sensitive to the growth media in which the *Lactococcus* strains had been grown, obviously due to secretion of antimicrobial agents by the latter. However, when the media were adjusted to pH 6.5 and treated with peroxidase enzyme to eliminate the effect of organic acids and hydrogen peroxide respectively, antimicrobial activity was lost against the indicator organisms. However, an exception was observed with *Lc. lactis* subsp. *lactis* I23 which maintained activity against *Bro. thermosphacta*, suggesting the presence of a specific bacteriocin in the former. To confirm this finding, the presence of the gene encoding for a bacteriocin, nisin, was tested in the *Lc. lactis* subsp. *lactis* I23 strain by PCR with the use of specific primers. A positive result showing presence gene encoding for nisin production was obtained (Fig. 2); the amplicon was about 610 bp in size. Similarly, an amplicon of 608 bp was recorded for the gene encoding for nisin production in a strain of *Lc. lactis* in a study reported by Suwanjinda *et al.* (2007); thus justifying the result of the present finding.

Antimicrobial activities of LAB against pathogenic and spoilage organisms have been observed by other researchers. Ammor et al. [27] reported that *Lc. garvieae* displayed antagonism against *Staphylococcus aureus*. Antagonisms by LAB against species of *Salmonella*, *Bacillus*, *Shigella* and *Pseudomonas* were also reported in another study [5]. Moreover, Mirhossini et al. [9] were able to detect antagonism by a strain of *Lc. lactis* against *Listeria monocytogenes* and *Bacillus cereus*. Antagonisms by LAB against most spoilage and pathogenic organisms have been attributed to the production of organic acids, hydrogen

peroxides, diacetyl and bacteriocin [27,23,14]. However, some spoilage organisms may not be sensitive to low pH that is usually associated by production of organic acids by LAB. This was the case in the present finding where no antimicrobial activity was recorded against *Bro. thermosphacta*, except with the bacteriocin producing *Lc. lactis* subsp. *lactis* I23. The ineffectiveness of organic acid production by the LAB strains against *Bro. thermosphacta* could be due to the ability of this organism to produce organic acids; hence it is able to survive at low pH [28]. Similar observations were made by other researchers where growth of *Bro. thermosphacta* strains was not affected by organic acid production by LAB, except in strains encoding for genes for production of bacteriocins to which the spoilage organism is sensitive [29,30].

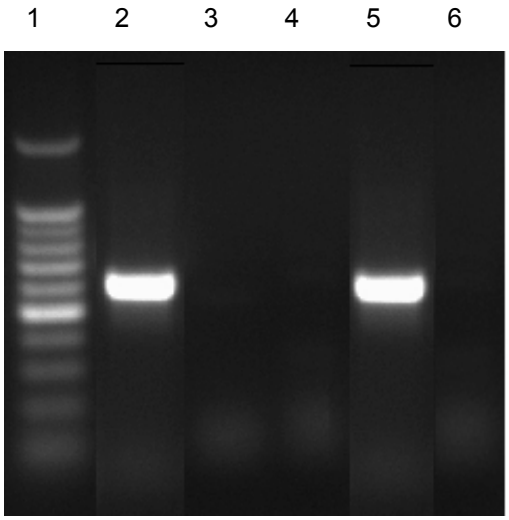

Fig. 2. PCR Amplification of n is genes in *Lactococcus* strains

Lanes 1, 100bp DNA marker; 2, Positive control (Lactococcus lactis NCTC 8586); 3, Negative control; 4, Lc. subsp. cremoris L11; 5, Lc. lactis subsp. lactis I23; 6, Lc. lactis subsp. hordinae E91

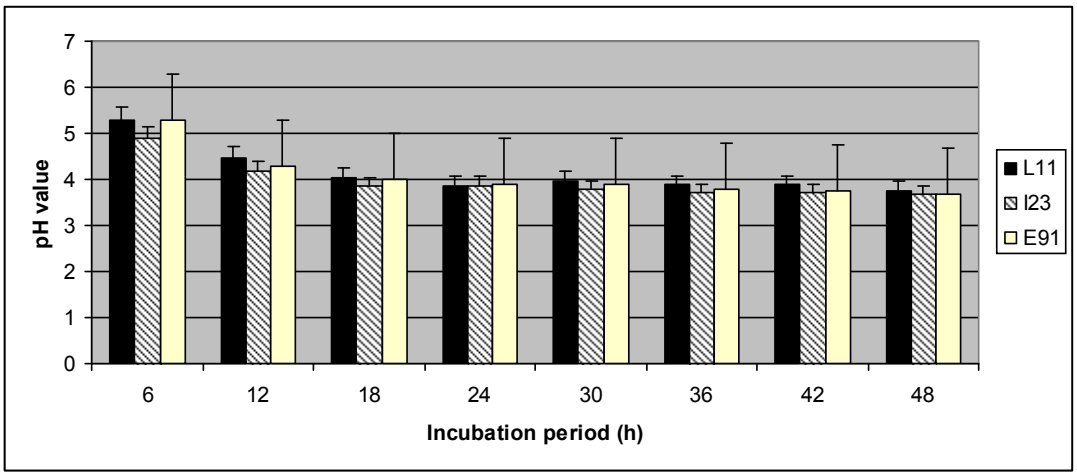

Fig. 3. pH of the *Lactococcus* strains during growth

L11, Lc. subsp cremoris L11;I23, Lc. lactis subsp. lactis I23; E91, Lc. lactis subsp hordinae E91

Table 4. Antimicrobial activities of the presumptive *Lactococcus* strains

Pathogenic/Spoilage organisms	*Lactococcus* strains					
	PA		PB		PC	
	b	a	b	a	b	a
Staphylococcus aureus	+	-	+	-	+	-
Salmonella typhimurium	+	-	+	-	+	-
Escherichia coli NCTC 86	+	-	+	-	+	-
Yersinia enterocolitica NCTC 10460	+	-	+	-	+	-
Pseudomonas aeruginosa NCIMB 10848	+	-	+	-	+	-
Klebsiella pneumoniae U11468	+	-	+	-	+	-
Listeria monocytogenes NCTC 11994	+	-	+	-	+	-
Brochothrix thermosphacta NCIMB 10018	-	-	+	+	-	-
Bacillus cereus NCTC 21113	+	-	+	-	+	-
Pseudomonas fluorescens	+	-	+	-	+	-

+, positive antimicrobial activity; -, negative antimicrobial activity; b, activity before pH adjustment of growth medium and peroxidase treatment; a, activity after pH adjustment of growth medium and peroxidase treatment; PA, Lc. subsp. cremoris L11; PB, Lc. lactis subsp. lactis I23; PC, Lc. lactis subsp. hordinae E91

After successful PCR amplification of the gene encoding for production of nisin in *Lc. lactis* subsp. *lactis* I23, the full identification of the bacteriocin was obtained by sequencing of the isolated and purified amplicon. The nucleotide sequences showed that the bacteriocin had 99% similarity to that of *Lactococcus lactis* nisin operon in the genBank database (Table 5). The nucleotide sequence of the gene encoding for nisin production in *Lc. lactis* subsp. *lactis* I23 was deposited with genBank database under the accession number EU667387.

From the result of effect of heat on the *nisin* of *Lc. lactis* subsp. *lactis* I23 (Table 6), the bacteriocin was heat stable up to 55^{o}C without any loss of activity; however, some activity was lost at 70^{o}C and above. At 120^{o}C, the bacteriocin lost above 75% of its activity. This result is supported by the findings of Murtaza et al. [10] on bacteriocin produced by a strain of *Lc. lactis* that was isolated from milk. Evaluation of the effects of proteolytic and amylolytic enzymes on the bacteriocin of *Lc. lactis* subsp. *lactis* I23 revealed that the it was proteinaceous as a result of its complete loss of antimicrobial activity after treatment with proteolytic enzymes (Table 7). This corroborates reports that bacteriocins are antimicrobial proteinaceous compounds [31,3]. The amylolytic enzymes, however, did not have any effect on the antimicrobial activity of the bacetriocin, thus confirming the proteinaceous nature of the nisin produced by *Lc. lactis* strain in this study. In support of this finding, a similar finding was reported by Yildirim and Johnson [31] on the bacteriocin produced by strains of *Lc. lactis*.

Bacteriocins have been reported to act against a relatively narrow range of microorganisms especially those related to the producer organism [28]; those produced by LAB have been found to be predominantly active against Gram positive bacteria. This is applicable to the present study where nisin of *Lc. lactis* subsp. *lactis* I23 was antagonistic against *Bro. thermosphacta*. *Bro. thermosphacta* is an important spoilage organism of meat and the ability of the *Lc. lactis* subsp. *lactis* I23 to antagonise it could be very useful in processing and preservation of meat products.

Table 5. Identification nisin of *Lactococcus lactis* subsp. *lactis* I23 in the genBank database

LAB Strain	length (bp)	Closest organism relative	% Identity	GenBank Accession no
I23	519	*Lactococcus lactis* nisin operon	99	gb|L11061.1

Table 6. Effect of Temperature (ºC) on nisin produced by *Lactococcus lactis* subsp. *lactis* I23

Temp (ºC)		Time (minutes)				
		0	**30**	**60**	**90**	**120**
4	zi	5.5	5.5	5.5	4.5	4.5
	% loss	0	0	0	18.2	18.2
30	zi	5.5	5.5	5.5	5.5	5.5
	% loss	0	0	0	0	0
37	zi	5.5	5.5	5.5	5.5	5.5
	% loss	0	0	0	0	0
55	zi	5.5	5.5	5.5	5.5	5.5
	% loss	0	0	0	0	0
70	zi	5.5	5.0	4.5	4.5	4.5
	% loss	0	9.1	18.2	18.2	18.2
100	zi	5.5	4.5	3.0	3.0	1.5
	% loss	0	18.2	54.5	54.5	72.7

zi, zone of inhibition; % loss, percentage loss

Table 7. Effect of Enzymes on nisin produced by *Lactococcus lactis* subsp *lactis* I23

***Enzyme* (1.0mg/ml)**	**zi**	**% loss**
Proteinase	0	100
Papain	0	100
Pronase	0	100
Pepsin	0	100
Trypsin	0	100
α-Amylase	5.0	0
Catalase	5.0	0
Control	5.0	0

zi, zone of inhibition; % loss, percentage loss

4. CONCLUSION

In conclusion, the three strains of *Pediococcus* under investigation seem to possess good technological properties, especially in terms of production of antimicrobial agents; hence they may be useful candidates of starter cultures for food biopreservation. However, *Lc. lactis* subsp. *hordinae* E91 showed better production of diacetyl and lactic acids that others, and this may confer an added advantage during choice of candidacy for food processing and preservation. Furthermore, the ability of *Lc. lactis* subsp. *lactis* I23 to produce bacteriocin active against the spoilage organism *Bro. thermosphacta* will be of immense importance towards achieving safety in food preservation.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Noonpakdee W, Santivarangkna C, Jumriangrit P, Sonomoto K, Panyim S. Isolation of nisin-producing *Lactococcus lactis* WNC 20 strain from nham, a traditional Thai fermented sausage. International Journal of Food Microbiology. 2003;81:137-145.
2. Nagalakshmi PK, Sumathi R, Kanimozhi K, Sivakumar T. Isolation of bacteriocin nisin producing *Lactococcus lactis* from dairy products. J. Acad. Indus. Res. 2013;1:627-630.
3. Todorov SD, Dicks LMT. Bacteriocin production by *Pediococcus pentosaceus* isolated from marula (*Scerocarya birrea*). International Journal of Food Microbiology. 2009;132:117-126.
4. Heng NCK, Wescombre PA, Burton JP, Jack RW, Tagg JR. In: Riley M.A, Chavan, M.A. (Eds.), The Diversity of Bacteriocins in Gram-Positive Bacteria Bacteriocins: Ecology and Evolution. Springer-Verlag, Berlin Heidelberg; 2007.
5. Mohammed SSD, Balogu TV, Yunusa A, Aliyu H. Antimicrobial Efficiency of Purified and Characterized Bacteriocins Produced by Lactobacillus bulgaricus Y34 and *Lactococcus lactis* N22 Isolated from Fermented Milk Products. International Journal of Advanced Research. 2013;1:63-70.
6. Sakala RM, Hayashidani H, Kato Y, Kaneuchi C, Ogawa M. Isolation and characterization of *Lactococcus piscium* strains from vacuum-packaged refrigerated beef. Journal of Applied Microbiology. 2002;92:173-179.
7. Conter M, Muscariello T, Zanardi E, Ghidini S, Vergara A, Campanini G, Ianieri A.1. Characterization of lactic acid bacteria isolated from an Italian dry fermented sausage. Ann. Fac. Medic. Vet. di Parma. 2005;25:167-174.
8. El Malti J, Amarouch H. Microbiological and physicochemical characterization of the natural fermented camel meat sausage. African Journal of Biotechnology. 2009;8:4199-4206.
9. Mirhossini M, Nahvi I, Kermanshahi RK, Tavassoli M. Isolation of bacteriocin producing *Lactococcus lactis* strain from dairy products. International Journal of Dairy Sciences. 2006;1:51-56.
10. Murtaza MA, Shahid M, Hafiz I, Mueen-ud-Din G. Characterization of Bacteriocin Produced by *Lactococcus lactis* ssp. Lactis. International Conference on Ecological, Environmental and Biological Sciences (ICEEBS'2012). 2012;7-8.
11. Onilude AA, Sanni AI, Olaoye OA, Ogunbanwo ST. Influence of lactic cultures on the quality attributes of tsire, a West African stick meat. World Journal of Microbiology & Biotechnology. 2002;18: 615–619.
12. Stiles ME, Holzapfel WH. Lactic acid bacteria of foods and their current taxonomy. International Journal of Food Microbiology. 1997;36:1–29.
13. Moore MA, Datta AR. DNA Fingerprinting of *Listeria monocytogenes* strains by pulse field gel electrophoresis. Food Microbiology. 1994;11:31-38.

14. Olaoye OA, Onilude AA. Quantitative estimation of antimicrobials produced by Lactic Acid Bacteria isolated from Nigerian beef bacteria. International Food Research Journal. 2011;18: 1104-1110.
15. Suwanjinda D, Eames C, Panbangred W. Screening of Lactic Acid Bacteria for Bacteriocins by Microbiological and PCR Methods. Biochemistry and Molecular Biology Education. 2007;35:364–369.
16. Gurira OZ, Buys EM. Characterization and antimicrobial activity of *Pediococcus* species isolated from South African farm-style cheese. Food Microbiology. 2005;22:159-168.
17. Noordiana N, Fatimah AB, Mun AS. Antibacterial agents produced by lactic acid bacteria isolated from Threadfin Salmon and Grass Shrimp. International Food Research Journal. 2013;20:117-124.
18. Albano H, Todorov SD, Reenen CA, Hogg T, Dicks LMT, Teixeira P. Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. International Journal of Food Microbiology. 2007;116: 239-247.
19. Ercolini D, Moschetti G, Blaiotta G, Coppola S. Behavior of Variable V3 Region from 16S rDNA of Lactic Acid Bacteria in Denaturing Gradient Gel Electrophoresis. Current Microbiology. 2001;42:199–202
20. SPSS. SPSS 10.01 for Windows. USA: SPSS; 1999.
21. Olaoye OA, Onilude AA, Dodd CER. Identification of *Pediococcus* spp. From Beef and Evaluation of Their Lactic Acid Production in Varying Concentrations of Different Carbon Sources. Advances in Natural and Applied Sciences. 2008;2:197-207.
22. Ozkalp C, Ozden B, Tuncer Y, Sanlibaba P, Akcelik M. Technological characterization of wild-type Lactococcus lactis strains isolated from raw milk and traditional fermented milk products in Turkey. Lait. 2007;87: 521-534.
23. Ammor, M.S., Mayo, B. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. Meat Science. 2007;76: 138-146.
24. Björkroth J, Holzapfel W. Genera Leuconostoc, Oenococcus and Weissella. In: M. Dworkin (ed.), The prokaryotes: a handbook on the biology of bacteria: Firmicutes, Cyanobacteria, vol. 4, 3rd ed. Springer-Verlag, New York, NY. 2006;267-300.
25. Aymerich T, Martín B, Garriga M, Vidal-Carou MC, Bover-Cid S, Hugas M. Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. Journal of Applied Microbiology. 2006;100:40-49.
26. Buckenhuskes HJ. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiology Reviews. 1993;12: 253-272.
27. Ammor S, Tauveron G, Dufour E, Chevallier I. Antimicrobial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility 1-Screening and characterization of the antimicrobial compounds. Food Control. 2006;17:454-461.
28. Jones RJ, Husseinb HM, Zagorecc M, Brightwella G, Tag JR. Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. Food Microbiology. 2008;25:228–234.
29. Maragkoudakis PA, Mountzouris KC, Psyrras D, Cremonese S, Fischer J, Cantor MD, Tsakalidou E. Functional properties of novel protective lactic acid bacteria and application in raw chicken meat against *Listeria monocytogenes* and Salmonella enteritidis. International Journal of Food Microbiology. 2009;130:219–226.

30. Gribble A, Mills J, Brightwell G. The spoilage characteristics of Brochothrix thermosphacta and two psychrotolerant Enterobacteriacae in vacuum packed lamb and the comparison between high and low pH cuts. Meat Science. 2014;97:83–92.
31. Yildirim Z, Johnson MG. Detection and characterization of bacteriocin produced by *Lactococcus lactis* subsp. cremoris R isolated from radish. Letters in Applied Microbiology. 1998;26:297-304.

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