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Technological properties of strains of *Enterococcus gallinarum* isolated from selected Nigerian traditional fermented foods

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ABSTRACT

Aims: Enterococci may be beneficial to health and can be used as starter cultures for fermented foods. Therefore, in this study Enterococci were identified from some Nigerian fermented foods, and their technological properties evaluated in order to gain a better understanding of their usefulness as starter cultures or probiotics.

Methodology and results: Five isolates from various Nigerian traditional fermented foods were identified as *Enterococcus gallinarum* based on phenotypic and genotypic tests. Technological properties were examined and antimicrobial investigations were carried out. Out of the five strains only *E. gallinarum* W211 and *E. gallinarum* T71 showed strong acidification, hydrophobicity, lipolysis, proteolysis and tolerance to bile salts. *E. gallinarum* W211 and *E. gallinarum* T71 did not produce biogenic amines while the other three strains produced tyramine from tyrosine. Only strains W211 and T71 produced bacteriocin(s), and these were active against 7 and 10 indicator organisms respectively out of 16. The bacteriocin from both strains had a molecular weight of 27KDa and the gene responsible was in each case identified as enterocin P. The bacteriocin was resistant to treatment with catalase, RNase and lysozyme, but activity was lost on treatment with α-chymotrypsin, trypsin, and proteinase K. Bacteriocin activity survived heating at 100 °C for 30 min.

Conclusion, significance and impact of study: Bacteriocinogenic *E. gallinarum* W211 and T71 have been selected for further characterization of their bacteriocin(s) because these strains exhibited useful probiotic and technological properties pointing to their suitability as candidates for starter cultures in fermentation process.

Keywords: acidification, bile tolerance, hydrophobicity, proteolysis and lipolysis

INTRODUCTION

Enterococcus species are members of the family of lactic acid bacteria (LAB) that is widely distributed in nature. They are used as starter cultures in food fermentation, because they produce unique flavours, and as probiotics due to their health-promoting activities. Some of them may have antimicrobial activity against pathogenic and spoilage microorganisms, thereby having potential applications in food preservation. Also, different probiotic supplements, not only for human but also for animals, include *Lactobacillus* and *Enterococcus* species in their food composition (Pollmann *et al.*, 2005; Tompkins *et al.*, 2008; Vankerckhoven *et al.*, 2008).

Lactic acid bacteria play a considerable role in the manufacturing and preservation of many fermented food products (Lozo *et al.*, 2007). They are also important in the control of undesirable microorganisms in the intestinal and urogenital tracts (Havenaar and Huis, 1992). In addition to indigenous LAB that colonizes the human gastrointestinal tract; several *Lactobacillus* strains from fermented food products have shown beneficial effects on gut health (Fuller, 1992). Apart from metabolic end products, some strains of *Lactobacillus* also secrete antimicrobial substances termed ''bacteriocins'' (Cotter *et al.*, 2005). Bacteriocins can be defined as ribosomally synthesized, bioactive peptides or proteins with a bactericidal or bacteriostatic effect on closely related species (Jack *et al.*, 1995). These peptides are of interest due to their potential application in food preservation

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since one of the distinctive characteristics of some enterocins is their strong listericidal effect, in particular those belonging to class IIa pediocin-like bacteriocins (Cleveland *et al.*, 2001).

The bacteriocins produced by *Enterococcus* species (the enterocins) show considerable diversity (Franz *et al.*, 2007). Based on their amino acid sequence similarity and their inhibitory spectra, nearly all of these bacteriocins have been included in the pediocin-like group (Nes *et al.*, 1996). Many bacteriocins are of interest because of their antimicrobial activity against food-borne pathogenic and spoilage bacteria in food systems (Giraffa, 2003; Moreno *et al.*, 2006). And, due to consumer concerns about chemical and irradiation preservation methods and the rising demand for minimally processed food products, alternative methods for enhancing safety and extending shelf life are needed. Bacteriocins are considered "natural" antimicrobials since many are produced by food-grade lactic acid bacteria, which are generally recognized as safe (Feng *et al.*, 2009). The inhibitory action of lactic acid bacteria is due to the accumulation of their main primary metabolites (lactic and acetic acids, ethanol and carbon dioxide) as well as to the production of other antimicrobial compounds, such as acetoin, hydrogen peroxide, diacetyl, formic acid, benzoic acids, and bacteriocins (Piard and Desmazeaud, 1991; 1992). The following enterocins from Enterococci have been characterized: enterocin (ent) A (Aymerich *et al.*, 1996), ent B (Casaus *et al.*, 1997), ent P (Cintas *et al.*, 1997), ent L50A and L50B (Cintas *et al.*, 1998), ent I – identical to ent L50A (Floriano *et al.*, 1998), ent Q (Cintas *et al.*, 2000), ent M – a new variant of ent P (Mareková and Lauková, 2002), ent V24 (Lauková *et al.*, 2003), ent esf100 (Ahmad *et al.*, 2004), ent M (Mareková *et al.*, 2007), ent E-760 (Line *et al.*, 2008), ent W (Sawa *et al.*, 2012), ent KP (Isleroglu *et al.*, 2012), ent RM6 (Huang *et al.*, 2013), ent TW21 (Chang *et al.*, 2013), ent HZ (Yildirim *et al.*, 2014) and so on.

The use of functional starter cultures in the food fermentation industry is being explored world-wide. They should possess at least one inherent functional property contributing to food safety and/or offer one or more technological, nutritional, organoleptic, or health benefit (Leroy and De Vuyst, 2004). Therefore, an increasing demand exists for new functional starter strains that have desirable effects on product characteristics. The search for starter cultures to be used for the production of functional foods still relies on screening a large number of isolates, preferably selected from natural and spontaneous fermented food products, for the desired metabolic activities and performance in small-scale food fermentations (Leroy and De Vuyst, 2004). In this study, the technological properties of some strains of *Enterococcus gallinarum* were evaluated with a view to determining their potential for application in the food industry as starter cultures and bio-preservatives.

MATERIALS AND METHODS

Identification of the bacterial strains

Five isolates from West African soft cheese (*wara*) and traditionally fermented vegetable condiments (*ugba*, *ogiri*, *okpehe*) were characterized phenotypically as *Enterococcus* species. The strains were grown in Slanetz and Bartley medium (Oxoid, Basingstoke, UK) at 37 °C for 24-48 h. They were presumptively identified by the following tests: observation of colonial characteristics and cell morphology, Gram staining, catalase, growth at 10 and 45 °C, growth in the presence of 6.5% NaCl and at pH 9.6 and fermentation of wide range of sugars. Genetic identification to species level was performed by 16S rRNA sequencing, as described by Harwood *et al.* (2004).

Determination of the technological properties of the strains

Survival of the strains at different pHs

The method of Tuncer (2009) was used with minor modifications to measure the survival of the strains at different pH levels. Briefly, 24 h old cultures of the strains were inoculated into 10 mL of fresh brain heart infusion broth and incubated for 24 h. The cultures were centrifuged at 1000 g for 10 min at 4 °C, the pellets were washed twice in sterile phosphate buffered saline (PBS, pH 7.2) and resuspended in 10 mL of PBS. For each bacterial suspension, 0.1 mL aliquots were added to a series of tubes containing 2 mL of sterile PBS at various pHs. The pH was adjusted to 1.0, 3.0, 7.0, and 9.0 using 0.5 M NaOH and HCl (2 M). After incubation for 0, 2 and 4 h, 0.1 mL samples from each tube were cultured on BHI agar plates and viable bacterial colonies counted.

Acidification ability

Acid production by the strains was tested by inoculating stationary cultures (1% v/v) into reconstituted skim milk (RSM, 1% w/v). Incubation was at 37 °C and pH changes were measured using a pH meter for 24 h at 6 h intervals (Tuncer, 2009).

Tolerance of Enterococcus gallinarum strains to bile salts of different concentrations

The ability of the strains to grow in the presence of bile was determined using the method of Vinderola and Reinheimer (2003). Overnight cultures were inoculated into tubes containing 10ml of sterile 2% BHI broth with 0.3, 0.5 or 1% (w/v) chicken bile (Sigma) and incubated at 37 °C for 24 h. Absorbance was measured at 560 nm and compared to a control culture (without bile salts). The results were expressed as the ratio (percentage) of growth ($OD_{560\ nm}$) in the presence of bile salts to that in the control.

Protease activity

The method of Moulay *et al.* (2006) with slight modifications was used to determine protease activity. Individual colonies were grown on 1% (w/v) skimmed milk agar plates at 37 °C for 24 h. A positive result was indicated by a zone of clearance around the bacterial colonies and the diameters of the zones were measured.

Lipase activity

Lipase activity was determined by growing the isolates on tributyrin agar supplemented with 10 mL/L (v/v) polyoxyethylene-(20)-hydrated castor oil. The inocula were incubated for 72 h at 37 °C and observed daily for halo formation around the growth. The widths of the halos (in mm) at the end of incubation were measured (Tuncer, 2009).

Decarboxylase activity

Decarboxylase activity was determined by the method of Kučerová *et al.* (2009) with some modifications. The ability to produce biogenic amines by decarboxylation of amino acids was tested on the medium designed by Bover-Cid and Holzapfel (1999), which contained either of the precursor amino acids; tyrosine or histidine. Overnight cultures of *Enterococcus* strains were streaked onto medium with tyrosine or histidine as published by Bover-Cid and Holzapfel (1999) and plates were incubated at 37 °C for 2-5 days. A purple colour in, and surrounding, the colonies indicated the production of biogenic amines from precursor amino acids.

Evaluation of the probiotic properties of the strains

Hydrophobicity

The method of Vinderola and Reinheimer (2003) with slight modifications was used for cell surface hydrophobicity testing. Cultures of the strains were harvested in the stationary phase by centrifugation at 12000 g for 6 min at 4 °C, washed twice in 50 mM K_2HPO_4 (pH 6.5) buffer and finally resuspended in the same buffer. The OD_{580nm} of each suspension was determined and 1.5 mL of each sample was added to an equal volume of n-hexadecane (organic phase) and thoroughly vortexed for two minutes. The phases were allowed to separate at room temperature for 30 min, after which 1.0 mL of the watery phase was removed and its OD_{580nm} was determined. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated from the formula: (OD_{580} reading 1 - OD_{580} reading 2 / OD_{580} reading 1) x 100 = % Hydrophobicity.

Determination of bacteriocin production and antimicrobial spectrum

The isolates were grown to early stationary phase in brain heart infusion (BHI) broth at 37 °C and centrifuged at 6700 g for 20 min, 4 °C. The supernatants were neutralized with 1 M NaOH and sterilized by passage through 0.22 µm-pore-size filters. The antimicrobial activity of the supernatants was determined by the agar well diffusion assay: 100 µL aliquots were placed in wells (3 mm diameter) cut in cooled soft BHI agar plates previously seeded with indicator microorganisms (10^5-10^6 CFU/mL). The indicator organisms (were obtained from the Culture Collection of the Department of Life Science and Biotechnology, Jadavpur University, Kolkata, India and sub-cultured on LB agar at 30 °C for 24 h. The indicator organisms used included *Bacillus subtilis* MTCC441, *Klebsiella pneumoniae* MTCC 618, *Escherichia coli* MTCC 739, *Shigella flexneri* MTCC 1457, *Lactococcus lactis* MTCC 3038, *Enterobacter cloacae* MTCC 509, *Staphylococcus aureus* MTCC 737, *Serratia marcescens* MTCC 06, *Micrococcus luteus* MTCC 106, *Proteus mirabilis* MTCC 425, *Pseudomonas aeruginosa* MTCC 1934, *Proteus vulgaris* MTCC 426 and *Bacillus cereus* MTCC 430. After 2 h at 4 °C, the plates were incubated at 30 °C to allow growth of the target organism; after 24 h, the diameters of any growth inhibition zones were measured. One arbitrary unit (AU) of bacteriocin activity was defined as the reciprocal of the highest dilution causing growth inhibition.

Partial purification of the bacteriocin

Ammonium sulphate was added to crude bacteriocin (produced by T71 and W211) to a final saturation of 70% with constant stirring at 4 °C for 3 h. The suspension was then centrifuged at 6700 g for 15 min 4 °C, and the the precipitate were resuspended in 0.05 M potassium phosphate buffer (pH 6.5) followed by dialysis at 4 °C against the same buffer (Banwo *et al.*, 2012). The dialyzed protein was applied to a DEAE-cellulose A-50 column pre-equilibrated with 0.05 M potassium phosphate buffer (pH 6.5) and washed with 3 volumes of equilibration buffer. Bound proteins were eluted with phosphate buffer of increasing molarity and decreasing pH. Fractions with high bacteriocin activity were pooled and concentrated in a lyophilizer (Rajaram, 2010). Protein was estimated using the method of Lowry (Lowry *et al.*, 1951).

Determination of the molecular weight of the bacteriocin

To estimate the molecular weight of the bacteriocin, the purified bacteriocin was analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Ready Gel; Bio-Rad, Laboratories, Inc., Hercules, CA), along with pre-stained molecular weight protein markers (Fermentas) (Schägger and Jagow, 1987). Duplicate aliquots (15 µL) of the bacteriocin preparation were loaded on the gel; after fractionation one lane was used

for molecular weight determination by fixing and staining with Coomassie Blue. The other one was assayed for antimicrobial activity; it was fixed consecutively in 20% (v/v) isopropanol and 10% (v/v) acetic acid, (30 min each), washed in distilled water, and kept in distilled water overnight at room temperature (~25 °C) (Schägger and Jagow, 1987). It was then overlaid with LB soft agar seeded with an overnight culture of *S. aureus* MTCC 737, incubated at 30 °C for 18 h and inspected for inhibition zones surrounding the protein bands (Du Toit, 2000).

Effect of heat, pH and enzymes on bacteriocin activity

Cell-free supernatants obtained from stationary phase cultures of T71 and W211 were used in these tests. Aliquots of supernatant were exposed to 40, 60, 80 and 100 °C for 30 min, 60 min and 90 min followed by immediate cooling on ice and testing for antimicrobial activity by the agar well diffusion method, as described by Jennes *et al*. (2000). The cell free supernatants were also adjusted to pH 2, 4, 6, 8, 10 and 12, then incubated for 4 h at room temperature and similarly assayed (Jennes *et al*., 2000).

Alpha-chymotrypsin (47 U/mg) was dissolved in 0.05 M Tris hydrochloride (pH 8.0) containing 0.01 M $CaCl_2$; lysozyme (400 U/mg) and trypsin (15,000 U/mg) were dissolved in 0.05 M Tris hydrochloride (pH 8.0); catalase (2,000 U/mg), in 10 mM potassium phosphate (pH 7.0); proteinase K (11.5 U/mg) in 1 N NaOH (pH 6.5) and RNase (≥70 U/mg). All enzymes were obtained from Sigma. The effects of lysozyme, catalase and RNase on the activity of bacteriocin were tested as described by Franz *et al*. (1996). The resistance of bacteriocin to proteolytic enzymes was determined by incubation of the bacteriocin samples (adjusted to pH 7.0) in the presence of Proteinase K, a-chymotrypsin and trypsin at 37 °C for 2 h.

Detection of bacteriocin genes by PCR

The presence of genes encoding enterocin P, enterocin A and L50 bacteriocins in the bacteriocin-positive strains was evaluated by PCR. Specific primers for each gene were used for the amplification. For enterocin P, forward primer was EntP F (5'-ATG AGA AAA AAA TTA TTT AGT TTA GCT CTT ATT GG-3') and reverse primer, EntP R (5'-TTA ATG TCC CAT ACC TGC CAA ACC AG-3'); for enterocin A the forward primer was EntA F (5'- ATG AAA CAT TTA AAA ATT TTG TCT ATT AAA G -3') and the reverse primer EntA R (5'- TTA GCA CTT CCC TGG AAT TGC TCC - 3'); for enterocin L50 the forward primer was EntL50 F (5'- ATG GGA GCA ATC GCA AAA TTA GTA GC- 3') and the reverse primer EntL50 R (5'- TTA ATG TCT TTT TAG CCA TTT TTC AAT- 3'). PCR was performed in a PCR SPRINT Thermal cycler (Thermo Electron Corporation, Japan) in 25 µL reaction mixtures containing 1X PCR buffer, 10 mM dNTPs, 25 mM $MgCl_2$, 1 unit of Taq DNA polymerase (Platinum Taq polymerase; Invitrogen, Carlsbad, California) and 100 pmol of each oligonucleotide primer. For enterocin P the cycling

conditions were: 5 min initial DNA denaturation at 95 °C followed by 35 consecutive cycles at 94 °C for 45 s; 64 °C for 30 s; 72 °C for 60 s and final extension at 72 °C for 10 min. For enterocin A, they were 5 min initial DNA denaturation at 95 °C followed by 35 consecutive cycles at 94 °C for 45 s; 59 °C for 30 s; 72 °C for 60 s, final extension of 72 °C for 10 min, and for enterocin L50, 5 min initial DNA denaturation at 95 °C followed by 35 consecutive cycles at 94 °C for 45 s; 65 °C for 30 s; 72 °C for 60 s, final extension of 72 °C for 10 min (Brandão *et al*., 2010). The PCR products were resolved by electrophoresis on 2% (w/v) agarose gels and analyzed with the Gel Doc 1000 documentation system (Bio-Rad Laboratories S.A., Madrid, Spain).

Plasmid curing in relation to bacteriocin production

Plasmid curing was conducted using different concentrations of ethidium bromide (2.5, 5.0, 6.5, 7.5 or 10 µg/mL) to determine if bacteriocin production was plasmid-linked. Overnight cultures of the isolates were inoculated in Brain Heart Infusion broth containing curing agents as above. Cultures were incubated at 37 °C for 48 h, transferred to fresh broth containing the next higher concentration of curing agent and incubated further. This subculture process was repeated several times. Cultures that survived the highest concentration of the curing agent were diluted, plated on BHI agar and incubated at 37 °C for 36-48 h. After replica plating, the colonies were overlaid with soft agar containing indicator organism, incubated once again for 12-15 h and checked for zones of growth inhibition. Colonies without inhibitory zones were considered to be cured. Some of these colonies were streaked on BHI agar and propagated in BHI broth. Plasmid DNA was isolated from them (Ruiz-Barba *et al*., 1991).

Plasmid isolation

Plasmids were isolated from both cured strains. Briefly, 1.5 mL of each overnight culture was centrifuged at 2834 g for 7 min at 4 °C, the supernatant was discarded and 100 µL of ice cold solution-1 (50 mM glucose,10 Mm EDTA, 25 mM Tris-Cl pH 8, lysozyme 4 mg/mL) was added. The mixture was vortexed and 3 µL RNase was added. It was kept at room temperature for 5 min; 200 µL of freshly prepared solution of 0.2 (N) NaOH and 1% sodium dodecyl sulphate (SDS) was added and the mixture was inverting 2-3 times, then incubated on ice for 5 min. Thereafter 150 µL of ice cold solution 3 (60 mL 5 M K- acetate, 11.5 mL Glacial acetic acid and 28.5 mL of water) was added. The tube was capped and inverted twice then incubated on ice for 3 min during which a white precipitate formed. After centrifugation at 7000 g for 10 min the supernatant was transferred to a fresh tube. An equal volume of isopropanol was added; following vortexing the suspension was allowed to stand on ice for 1 h. It was then centrifuged at 7000 g for 10 min; the supernatant was allowed to drain out and the tube was kept in an inverted position. 1 mL of 80% ethanol was

added and the tube was tilted up and down for 15 min, then centrifuged for 10 min at 7000 g; the supernatant was again drained out and the tubes were kept open to allow the ethanol to evaporate. This precipitate was then resuspended in 20 µL of elution buffer and any plasmids were analyzed by 0.7% agarose gel electrophoresis and staining with 0.5 µg/mL ethidium bromide. The MW of plasmids were estimated with the help of a calibration curve prepared using log MW (kb) of the standard molecular weight markers, λDNA BST E II digest (Sigma) run concurrently (Ruiz-Barba et al., 1991).

RESULTS

The presumptive Enterococci strains were characterized as gram-positive, catalase negative, oxidase negative, non-spore forming cocci (in singles and pairs) with the ability to grow in the presence of 6.5% NaCl at pH 9.6 and at 10 and 45 °C. The strains were able to hydrolyze esculin, pyrrolidonyl-β-naphthylamide, arginine but were unable to hydrolyze starch and were distinguished by their sugar fermentation pattern. As stated in the Materials and methods, further confirmation of identity was carried out by 16S rRNA sequencing. The sequences were deposited in the GenBank database and accession numbers assigned to each strain. The accession number assigned to Enterococcus gallinarum W211 (isolated from wara) is JF915769, E. gallinarum T71 (from okpehe) JF774411, E. gallinarum W184 (from wara) JN020631, E. gallinarum U82 (from ugba) JF774412 and E. gallinarum C103 (from ogiri) JF774410.

The ability of the strain to survive different pH was evaluated and it was found that all the strains were tolerant to an acidic environment as shown in Table 1. Initially E. gallinarum W211 had the highest $\log_{10}$ CFU/mL of 7.5 while the others where in the range of 7.3-7.4 at pH 1. After four hours, E. gallinarum W211 was able to survive with the highest $\log_{10}$ CFU/mL of 0.9, E. gallinarum T71 had 0.7. No colony forming unit was observed in the others. At pH 9, E. gallinarum W211 had the highest $\log_{10}$ CFU/mL of 7.6 while the others where in the range of 7.5-7.3 at zero h. After four hours, E. gallinarum W211 had the highest CFU/mL of 8.2 while the others where in the range of 8.0-8.1 at pH 9.

The acidification ability of the strains in reconstituted skimmed milk was tested by incubating them for 24 h and examining them at 6 h intervals as shown in Figure 1. E. gallinarum W211 reduced the pH of the medium from 5.8 at 6 h to 4.8 at 24 h while E. gallinarum T71 reduced the pH from 5.8 at 6 h to 4.7 at 24 h. Data on the bile tolerance of the strains is displayed in Table 2. E. gallinarum W211 had a plating efficiency of 89.2% and 41.2% at 0.3% and 1% bile concentration, while E. gallinarum W184 had the lowest plating efficiency of 76.3 and 28.6, respectively. However, all the selected strains showed significant tolerance to bile at 0.3%, 0.5% and 1%.

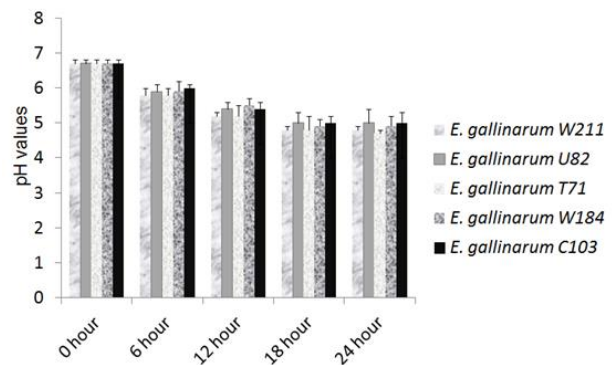Proteolytic and lipolytic activities were assessed from

Figure 1: Acidification ability of the *Enterococcus gallinarum* strains.

the diameters of zones of clearance (in mm). E. gallinarum W211 had the highest proteolytic activity with a zone of inhibition of 16 mm while E. gallinarum W184 and E. gallinarum C103 had the smallest zones of inhibition of 12 mm each. E. gallinarum U82 did not show any proteolytic activity (Figure 2). Lipolytic activity was measured by the ability of the selected strains to degrade tributyrin under laboratory conditions. The diameter of the zones of clearance was observed to be 12 mm for E. gallinarum W211, T71 and C103 while the lowest diameter of 10 mm was observed for E. gallinarum W184 (Figure 2). The decarboxylase test showed that none of the strains decarboxylated histidine. However, E. gallinarum U82, W184 and C103 produced tyramine from tyrosine while E. gallinarum W211 and T71 did not. The hydrophobicity of the bacterial cell surfaces was evaluated and E. gallinarum W211 had the highest hydrophobicity of 40.9% while E. gallinarum W184 had the lowest value of 29.1%.

Two out of the five strains, E. gallinarum W211 and E. gallinarum T71, produced bacteriocin with inhibitory

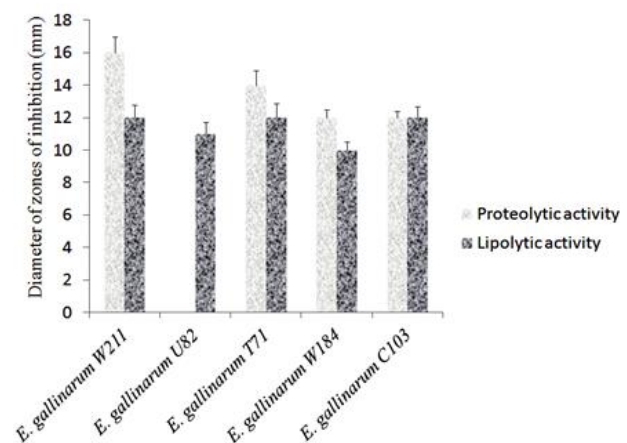**Figure 2:** Proteolytic and lipolytic activity of the *Enterococcus gallinarum* strains.

Table 1: Survival of the *Enterococcus gallinarum* strains at different pH levels.

Isolate	pH	Viable count (log $_{10}$ CFU/mL)		
		0 h	2 h	4 h
E. gallinarum W211	1	7.5 ± 0.10[a]	2.7± 0.20[d]	0.9 ± 0.10[c]
	3	7.4 ± 0.20[a]	4.3 ± 0.20[b]	4.0 ± 0.17[c]
	7	7.3 ± 0.10[a]	7.8 ± 0.10[a]	8.1 ± 0.20[b]
	9	7.6 ± 0.10[b]	8.0 ± 0.10[b]	8.2 ± 0.20[b]
E. gallinarum U82	1	7.4 ± 0.40[a]	1.4 ± 0.20[b]	ND
	3	7.2 ± 0.27[a]	3.8 ± 0.20[a]	2.8 ± 0.00[a]
	7	7.3 ± 0.06[a]	7.6 ± 0.20[a]	7.9 ± 0.20[a]
	9	7.4 ± 0.10[ab]	7.8 ± 0.20[a]	8.0 ± 0.17[a]
E. gallinarum T71	1	7.3 ± 0.10[a]	2.3 ± 0.17[c]	0.7 ± 0.20[b]
	3	7.3 ± 0.10[a]	4.8 ± 0.10[c]	3.9 ± 0.10[c]
	7	7.3 ± 0.10[a]	7.6 ± 0.10[a]	7.8 ± 0.10[a]
	9	7.5 ± 0.20[ab]	7.9 ± 0.10[ab]	8.1 ± 0.10[ab]
E. gallinarum W184	1	7.3 ± 0.10[a]	0.9 ± 0.10[a]	ND
	3	7.2 ± 0.10[a]	3.6 ± 0.20[a]	3.4 ± 0.20[b]
	7	7.3 ± 0.10[a]	7.6 ± 0.35[a]	7.8 ± 0.00[a]
	9	7.3 ± 0.06[a]	7.8 ± 0.20[a]	8.0 ± 0.10[a]
E. gallinarum C103	1	7.3 ± 0.06[a]	1.1± 0.17[ab]	ND
	3	7.4 ± 0.10[a]	3.6 ± 0.20[a]	3.2 ± 0.17[b]
	7	7.3 ± 0.20[a]	7.6 ± 0.20[a]	7.8 ± 0.17[a]
	9	7.3 ± 0.10[a]	7.8 ± 0.20[a]	8.1 ± 0.10[ab]

ND, Not detected; each value is a mean of 3 replicates ± standard deviation; values accompanied by identical superscript letters are not significantly different ($p \leq 0.05$).

Table 2: Tolerance of the *E. gallinarum* strains to different concentration of bile.

Isolates	Concentration of bile salt		
	0.3%	0.5%	1%
E. gallinarum W211	89.2 ± 0.20[d]	73.2 ± 0.20[d]	41.2 ± 0.20[e]
E. gallinarum U82	79.6 ± 0.20[b]	70.7 ± 0.20[c]	24.6 ± 0.20[a]
E. gallinarum T71	86.8 ± 0.20[c]	71.0 ± 0.06[c]	38.3 ± 0.20[d]
E. gallinarum W184	76.3 ± 0.52[a]	67.6 ± 0.35[b]	28.6 ± 0.20[b]
E. gallinarum C103	78.8 ± 0.20[b]	59.8 ± 0.53[a]	30.0 ± 0.10[c]

Each value is a mean of 3 replicates ± standard deviation; values accompanied by identical superscript letters are not significantly different ($p \leq 0.05$).

activity against both Gram positive and Gram negative bacteria. Bacteriocin from these two strains inhibited *Proteus vulgaris* MTCC 426, *Bacillus cereus* MTCC 430 and *Staphylococcus aureus* MTCC 737, *Aeromonas hydrophilia* MTCC 646, *Salmonella typhimurium*, MTCC 98, but not *Serratia marcescens* MTCC 86, *Bacillus subtilis* MTCC 441 and *Escherichia coli* MTCC 739. The greater spectrum of activity was observed for *E. gallinarum* T71 which inhibited ten of the sixteen indicator strains tested compared with seven for *E. gallinarum* W211 (Table 3). The molecular weight of the partially purified bacteriocins of the two strains was estimated to be 27 kDa (Figure 3).

The effects of heat, pH and enzymes on bacteriocin activity were investigated and recorded in AU/mL. Both bacteriocin preparations were heat stable at 100 °C for 30 min (1600 AU/mL) and activity thereafter declined (Table 4). Data on pH stability are also given in Table 6. The inhibitory activity of the bacteriocin produced by *E. gallinarum* T71 was stable at pH 2 to 6 and declined to 800 AU/mL at pH 8 to 10. However no activity was observed at pH 12. The activity observed for *E. gallinarum* W211 was stable at pH 2 to 8 and declined to 1600 AU/mL at pH 10 to 12 (Table 4). The sensitivity of the bacteriocin to various enzymes was also examined (Table 4).
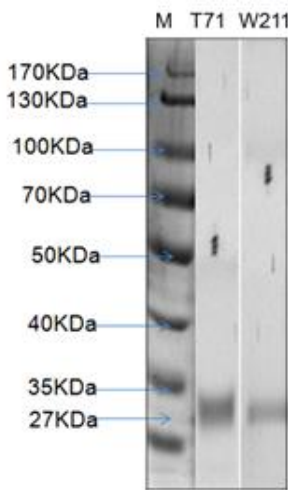
Figure 3: SDS PAGE showing molecular weight of the bacteriocins from *E. gallinarum* T71 and *E. gallinarum* W211.

Figure 4: Agarose gel Electrophoresis of the PCR product obtained from the amplification of enterocin P gene (216bp) using specific primers.

The activity of the bacteriocins from the two strains were lost after treatment with α-chymotrypsin, trypsin, and proteinase K but were stable to treatment with catalase, RNase and lysozyme.

We screened for the structural genes encoding bacteriocin in the two strains by amplifying their respective DNAs with primers specific for enterocin P, A and L50 genes (see Methods). Enterocin P gene amplified as shown by the PCR products obtained in both cases (Figure 4) whereas enterocin A and L50 genes were not amplified. Plasmid curing showed that the gene for bacteriocin production was plasmid-encoded because, inhibitory activity was completely lost in both strains after curing with ethidium bromide. The result of the plasmid profiles showed that the number of fragments present in a single strain was about six in number. The fragments range from 2000 bp – > 5000 bp.

DISCUSSION

Phenotypic and genotypic characterization was employed in the identification of the *E. gallinarum* strains reported by Oladipo *et al.* (2013). Most bacteria do not survive at low pH and the severe acidic conditions of the crop, proventriculus and gizzard could have an adverse effect on the bacteria. Thus, it has been suggested that microbial cultures to be used as growth promoters should be screened for their resistance to acidity (Conway *et al.*, 1987). The strains in this study were tolerant to acidic environments, with *E. gallinarum* W211 and *E. gallinarum* T71 being the most tolerant. Gastric juice at a pH of approximately 2.0 is secreted each day in the stomach (Charteris *et al.*, 1998a), which causes the destruction of most microorganisms ingested (Kimoto *et al.*, 2000). Hence, resistance to human gastric transit is an important selection criterion for probiotic microorganisms (Charteris *et al.*, 1998b).
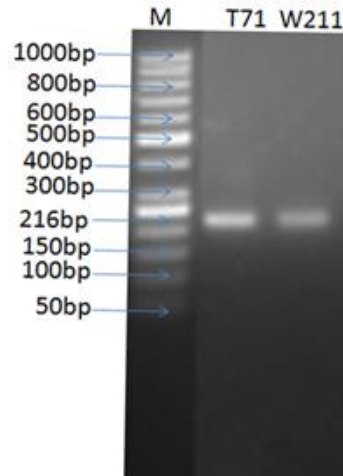
Considerable work has been reported on acid production by *Enterococcus* species in milk. The rate of acid development is a critical factor in milk fermentation (De Vuyst, 2000). Rapid acidification of the raw materials prevents growth of undesirable microorganisms and is also essential for the aroma, texture and flavour of the end product (De Vuyst, 2000). *E. gallinarum* W211 and *E. gallinarum* T71 demonstrated good acidification ability based on their ability to reduce the pH of the medium to 4.8 and 4.7 respectively after 24 h, while *E. gallinarum* U82, *E. gallinarum* W184 and *E. gallinarum* C103 had moderate acidifying ability. Giraffa (2003) reported that Enterococci, in general, exhibit low milk acidifying ability.

Resistance to bile is an important characteristic that enables *Lactobacillus* to survive and grow in the intestinal tract (Jin *et al.*, 1998). The relevant physiological concentrations of human bile range from 0.3% (Dunne *et al.*, 1999) to 0.5% (Zavaglia *et al.*, 1998). Therefore it is generally considered necessary to evaluate the ability of potential probiotic bacteria to resist the effects of bile acids (Collins *et al.*, 1998, Dunne *et al.*, 1999). The five strains in this study varied in their tolerance to bile salts; *E. gallinarum* W211 and *E. gallinarum* T71 exhibited better tolerance to bile than the other strains. Bile entering the duodenal section of the small intestine has been found to reduce the survival of bacteria. Jin *et al.* (1998) reported that this is probably due to the fact that all bacteria have cell membranes consisting of lipids and fatty acids and these are very susceptible to destruction by bile salts. Hence, resistance to bile is considered an important property for selection of probiotic bacteria and a prerequisite for colonization and metabolic activity of the strains in the small intestine of the host (Liong and Shah, 2005; Strompfová and Lauková, 2007).

Table 3: Antimicrobial activity of the bacteriocin producing *Enterococcus gallinarum* strains against indicator organisms.

Indicator organisms	Strains No	*E. gallinarum* W211	*E. gallinarum* U82	*E. gallinarum* T71	*E. gallinarum* W184	*E. gallinarum* C103
Serratia marcescens	MTCC 86	ND	ND	ND	6.0 ± 0.22	ND
Micrococcus luteus	MTCC 106	ND	ND	11.0 ± 0.20	ND	ND
Proteus mirabilis	MTCC 425	ND	ND	11.4 ± 0.11	ND	ND
Proteus vulgaris	MTCC 426	9.0 ± 0.20	ND	13.0 ± 0.20	ND	ND
Bacillus cereus	MTCC 430	10.1 ± 0.21	ND	10.4 ± 0.13	ND	ND
Bacillus subtilis	MTCC441	ND	ND	ND	ND	ND
Klebsiella pneumoniae	MTCC 618	ND	ND	9.2 ± 0.21	ND	ND
Escherichia coli	MTCC 739	ND	ND	ND	ND	ND
Shigella flexneri	MTCC 1457	ND	ND	12.2 ± 0.14	ND	ND
Lactococcus lactis	MTCC 3038	ND	ND	ND	ND	ND
Enterobacter cloacae	MTCC 509	8.0 ± 0.10	ND	ND	ND	ND
Staphylococcus aureus	MTCC 737	11.0 ± 0.12	ND	11.0 ± 0.10	ND	ND
Pseudomonas aeruginosa	MTCC 1934	8.0 ± 0.20	ND	ND	ND	ND
Aeromonas hydrophilia	MTCC 646	8.0 ± 0.13	ND	14.0 ± 0.20	ND	ND
Salmonella typhimurium	MTCC 98	9.2 ± 0.11	ND	11.0 ± 0.14	ND	ND
Bacillus pumilus	MTCC 1607	ND	ND	12.1 ± 0.21	ND	ND

Table 4: Effect of heat, pH and enzymes on bacteriocin produced by *Enterococcus gallinarum* strains.

	Activity Unit (AU/mL)	
Treatments	*E. gallinarum* T71	*E. gallinarum* W211
Control	1600	3200
Enzymes		
α-Chymotrypsin	ND	ND
Trypsin	ND	ND
Proteinase K	ND	ND
Catalase	1600	3200
RNase	1600	3200
Lysozyme	1600	3200
pH		
2	1600	3200
4	1600	3200
6	1600	3200
8	800	3200
10	800	1600
12	ND	1600
Temperature (min)		
40 °C (30)	1600	3200
40 °C (60)	1600	3200
40 °C (90)	1600	3200
60 °C (30)	1600	3200
60 °C (60)	1600	3200
60 °C (90)	1600	3200
80 °C (30)	1600	3200
80 °C (60)	1600	3200
80 °C (90)	1600	3200
100 °C (30)	1600	3200
100 °C (60)	800	1600
100 °C (90)	800	1600

Altogether four of the five strains had proteolytic activity while all five strains exhibited lipolytic activities. Proteolysis and lipolysis as technological relevant properties are usually assessed to evaluate strains suitable as starter cultures in fermentation due to their contribution to flavour development (Martin *et al*., 2006). This further confirms the suitability of these strains as starter culture in fermentation processes.

Amino acid decarboxylation can have an important energetic role in nutritionally poor environments. In fact, bacterial decarboxylation systems can translocate charge across the cytoplasmatic membrane, influencing the membrane potential (Konings *et al*., 1997). Enterococci can toxify food through production of biogenic amines from the decarboxylation of amino acids (Kučerová, 2009). None of the strains in this study decarboxylated histidine. However, *E. gallinarum* U82, *E. gallinarum* W184 and *E. gallinarum* C103 produced tyramine from tyrosine; the tyrosine decarboxylase negative strains were *E. gallinarum* W211 and *E. gallinarum* T71. These results are in agreement with Giraffa *et al*. (1997) who reported that the lactic acid bacteria known to possess significant tyrosine decarboxylase activity in cheeses are Enterococci. Three out of the five strains in this study showed tyrosine decarboxylase activities.

Bacterial adhesion has been interpreted in terms of hydrophobicity. Some authors have also indicated an influence of the electrical charges of bacteria and solid surfaces on adhesion (Van-Loosdrecht *et al*., 1987). Hydrophobicity is directly related to the concentration of carbon in hydrocarbon form and inversely related to oxygen concentration or to the nitrogen/phosphate ratio (Mozes *et al*., 1988). The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue (Kiely and Olson, 2000). This property could confer a competitive advantage, which is important for bacterial maintenance in the human gastrointestinal tract. Greater hydrophobicity of cells is postulated to generate greater attractive forces and higher levels of adhesion (Naidu *et al*., 1999). *Enterococcus gallinarum* W184 and *E. gallinarum* C103 (with 29.1 and 30.0% hydrophobicity respectively) showed weak hydrophobicity. However, *E. gallinarum* W211, *E. gallinarum* U82 and *E. gallinarum* T71 showed moderate hydrophobicity with 40.9, 31.0 and 36.8% hydrophobicity respectively, which may indicate that these bacteria are better-suited for adhering to intestinal cells in vivo than the other strains. None of the strains showed the strong hydrophobicity that is considered an advantage in the colonisation of mucosal surfaces (Ljungh and Wadstöm, 1982).

The bacteriocins produced by *E. gallinarum* W211 and *E. gallinarum* T71 had inhibitory activity against both Gram positive and Gram negative organisms even though Gram negative bacteria are considered resistant to the many bacteriocins from Enterococci species. However some reports are in agreement with this finding in that certain lactic acid bacterial bacteriocins, especially the class 2 bacteriocin, pediocin, can inhibit a limited range of Gram negative bacteria (Cintas *et al*., 2001, De Kwaadsteniet, 2005 and Moreno *et al*., 2005). The mechanism of bacteriocin action on both Gram positive and negative bacteria has been reported to be the adsorption of bacteriocin on cell surface, thereby inducing pore formation in their membranes resulting in leakage of cell electrolytes and ultimately cell death (Klaenhammer, 1988; Enan *et al.*, 1996; Alvarez-Cisneros *et al*., 2011; Enan *et al*., 2014).

The bacteriocins produced by *E. gallinarum* W211 and *E. gallinarum* T71 were studied further due to the unique technological properties of the two strains harbouring them, as well as their broad-spectrum of antimicrobial activity. The effects of heat, pH and enzymes on bacteriocin activity were investigated and the bacteriocins from *E. gallinarum* W211 and *E. gallinarum* T71 were found to be heat stable based on the fact that the activity was resistant to heating at 100 °C for 30 min but declined thereafter. Many procedures of food preparation involve a heating step; temperature stability is therefore, important if the bacteriocins are to be used as a food preservatives. This is in agreement with the previous work of Ogunbanwo *et al.* (2003).

The two bacteriocins from *E. gallinarum* W211 and *E. gallinarum* T71 were also found to have antimicrobial activity at pH 2 to 8 and 2 to 6 respectively; this suggests that the activity of these bacteriocins may be pH dependent and may be adapted to function at low pH since inactivation occurred at high pH. This also agrees with previous work of Ogunbanwo *et al.* (2003).

There was a loss of activity when these bacteriocins were treated with α-chymotrypsin, trypsin and proteinase K but they were stable when treated with catalase, RNase and lysozyme; this may be an indication of the proteinaceous nature of these bacteriocins (De Kwaadsteniet, 2005). This result is also supported by the work of Ogunbanwo *et al.* (2003) who reported stability of bacteriocin produced by *Lactobacillus* species after treatment with these enzymes.

Interest in Enterococci has increased in recent decades, and most *in vitro* studies deal with the ability of Enterococci to produce bacteriocins (spectrum of inhibitory activity, physicochemical properties, application in food products etc.). However, there is little information on the distribution of genes encoding enterocins in strains from different sources (Strompfová *et al*., 2008). According to the results of this study, there is at least one enterocin gene in each of the bactericin--producing strains; this is in agreement with Strompfová *et al*. (2008) who reported the presence of enterocin structural genes to be widespread among Enterococcal isolates. The widespread enterocin gene distribution may be due to the remarkable ability of Enterococci to donate and receive genetic material between strains and between genera (eg. between Staphylococci and Enterococci) (Murray *et al*., 1986). This probably accounts for the similar sequences of putatively exchanged material (eg. enterolysin A and enterocin L50 have sequence similarity to staphylococcal lysostaphin and staphylococcal peptide toxins, respectively) (Cintas *et al*., 1998; Nilsen *et al*., 2003). Well-studied gene transfer mechanisms in

Enterococci include conjugative (pheromone responsive with high-frequency of transfer) and non-conjugative plasmids, as well as conjugative transposons (Clewell, 1990).

Concerning the occurrence of bacteriocin genes in the tested *E. gallinarum* strains, the gene encoding Ent P was found in the two strains, while those of EntA and EntL50 (EntL50A and EntL50B), were absent. According to the classification scheme of Franz *et al.* (2007), EntP and EntA are class II.1 (pediocin-like) bacteriocins, which have a very effective anti-listerial activity. Although previous studies suggest that EntB (class II.3 bacteriocin) acts synergistically with EntA (Casaus *et al.*, 1997), Brandão *et al.* (2010) reported that *entB* was only present in strains containing *entA* and that some isolates harbored *entA* but not *entB*. Our results revealed the presence of one gene per strain suggesting that these strains are unlikely to produce more than one bacteriocin, although other studies have described enterococcal strains that produce multiple bacteriocins (Casaus *et al.*, 1997; Cintas *et al.*, 1998, 2000; Poeta *et al.*, 2007). Nevertheless, it should be noted that not all enterocin genes are expressed simultaneously in the multiple bacteriocin-producing strains (Casaus *et al.* 1997; Cintas *et al.* 1998; Criado *et al.*, 2006). Moreover, several authors have described the existence in Enterococci of silent bacteriocin genes which may become activated by external factors, such as the conditions found in the gastrointestinal tract, effects of bacterial synergism, the balance of microorganisms in the intestinal microbiota, as well as the presence and persistence of large numbers of viable Enterococci (Eaton and Gasson, 2001; Poeta *et al.*, 2007). The structural gene encoding enterocin P gene was detected in *E. gallinarum* W211 and T71 strains but there was a slight difference in the physiological characteristics/phenotypes of the two bacteriocins. The two bacteriocins showed the same responses to temperature and enzymes but there was a difference in their activity units (AU/mL). They also differed in their spectrum of activity and reaction to different pH levels. The difference in the properties of the bacteriocin produced by the two strains may be, as Strompfová *et al.* (2008) reported, that detection of the enterocin structural genes does not necessarily mean production of the corresponding enterocin, or it may be due to the presence of a silent gene in one or the two stains.

Plasmid curing trials revealed that the gene for bacteriocin production in each strain was plasmid- and not chromosome-encoded because exposure to ethidium bromide led to the loss of inhibitory activity in both strains. This result is in agreement with the work of Gálvez *et al.* (2007) who reported that bacteriocins are generally encoded by plasmids. This makes it easy to manipulate their numbers to increase yield. The result of our plasmid profiling showed that the two strains appear to be similar in both number of plasmids harboured and molecular weight of the plasmids.

CONCLUSION

In conclusion, two of the *Enterococcus gallinarium* strains had good technological and probiotic properties because of their lipolysis, proteolysis, tolerance to bile and low pH, safety and hydrophobic properties. The bacteriocin produced by the two strains had inhibitory activities against food pathogens and spoilage micro-organisms. The structural gene encoding enterocin P was detected in both *E. gallinarum* W211 and T71 but the properties of the bacteriocins produced by these two strains were different. These two strains have been selected for further characterization of their bacteriocin(s) for their possible use as starter cultures in food fermentation processes.

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