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Isolation of Bacteriocin – Producing Lactic Acid Bacteria from *'Ugba'* and *'Okpiye'*, Two Locally Fermented Nigerian Food Condiments.

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ABSTRACT

In this work, 100 samples each of 'ugba' and 'okpiye' were evaluated for the presence of bacteriocin producing lactic acid bacteria. Thirty strains showing antibacterial activity against at least one of the indicator organisms were selected from a total of 752 colonies isolated from the condiments. Out of the 30, only five strains retained activity after the pH of the broth supernatant was adjusted to 6.5. When evaluated by the agar-well diffusion assay, the spectra of inhibitory activity showed that Staphylococcus aureus *was the most sensitive indicator organism tested, while* Listeria monocytogenes *was the most resistant. One strain (UG 2) was active against* Escherichia coli. *The assays using the cell-free supernatant of the cultures showed that the bacteriocins were completely inactivated by the proteolyses as well as by the chloroform treatment. In ethanol, the activity of the compounds was only partially modified. When incubated in a water bath at 80^0C for 30 min, no significant activity loss was recorded. The antimicrobial activity of the bacteriocins produced by the lactic acid bacteria has potential for use in biopreservation of condiments against the spoilage and food–borne pathogens.*

Key words: *'Ugba', 'Okpiye'*, Lactic acid bacteria, Bacteriocins, Inhibitory activity, Condiments, Cell free supernatant

INTRODUCTION

Ugba' and *'Okpiye'* (*okpei*) are two popular traditionally fermented food condiments in Nigeria. They are protein based and are used to complement the nutrient content of soups and sauces. They are sometimes substituted for the meat or fish. *'Ugba'* is obtained from the seeds of the African oil bean (*Pentaclethra macrophylla*) while *'Okpiye'* is prepared from the mesquite seeds (*Prosopis africana*). Both are products of natural fermentation. As a result, diverse groups of microorganisms including *Bacillus, Micrococcus, Leuconostoc, Staphylococcus, Enterobacteriaceae* and lactic acid bacteria (LAB) have been reported to play active roles in the process (Obeta 1983; Achi 1992; Njoku and Okemmadu 1998; Enujiugha 2009). They produce antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide and bacteriocins (Ogunbanwo et al. 2003) which are suspected to be associated with the preservation of many fermented food condiments in Nigeria. The preservation and safety are presently the two major challenges of the food industry because, huge economic losses are sustained yearly due to food spoilage while numerous consumers have been reported to develop adverse sensitivity reactions to chemical based preservatives. The LAB which are used widely as starter cultures for food fermentation are

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thought of as having the potential to bridge this gap (Budde et al. 2003; Delves-Broughton 2005). They produce antimicrobial compounds that show antagonism toward the closely related species. The LAB in *'ugba'* and *'okpiye'* enhance fermentation and also improve the product storage, quality and safety by preventing the spoilage microflora from deteriorating the food condiments and at the same time keeping off the pathogens such as *L. monocytogenes, S. aureus* and *E.coli* (Ogunshe and Olasugba 2008).

Bacteriocins are extracellularly produced primary compounds of bacterial ribosomal synthesis which have a relatively narrow spectrum of bactericidal activity (Caplice and Fitzgerald 1999). They are active against other bacteria despite varying greatly in the chemical nature and mode of action. Bacteriocins have important advantage over the classical antibiotics in being easily degraded by the digestive enzymes without the risk of disruption of normal tract ecology (Caplice and Fitzgerald 1999). Bacteriocin producing LAB have the 'generally recognized as safe' (GRAS) status and have been shown to strengthen the barrier function of the gut microflora as well as promote the non-specific enhancement of the immune system of man and animals (Tome et al. 2008).

While many scientific reports are available on *'ugba'* and '*okpiye'*, such studies have hitherto addressed mainly the microorganisms associated with the fermentation processes of the seeds as well as systems for optimization of the fermentation conditions (Obeta 1983; Achi 1992; Odibo et al. 1992). Equally, research on the biochemical changes during the fermentation as well as the proximate composition and properties of the seeds have also received modest scientific attention (Sanni and Onilude 1997; Achi and Okereka 1999; Aremu et al. 2007 and Odibo et al. 2008). There is presently, paucity of scientific information on the ecological contribution of the LAB and bacteriocins for the safety and biopreservation of the food condiments. The objective of this study therefore was to isolate and identify bacteriocin producing LAB from *'ugba'* and *'okpiye'* as well as evaluate the stability and activity of the bacteriocins against the common food pathogens.

MATERIALS AND METHODS

Sampling

One hundred samples each of traditionally fermented *'ugba'* and *'okpiye'* were purchased from different selling points at Ogige main market, Nsukka – Nigeria. All the samples were stored at 3 ± 1^0C for a maximum of 24 h before the analysis.

Isolation of Lactic acid Producing Bacteria (LAB)

Ten gram of each sample was homogenized separately in a porcelain mortar and then were transferred into 250 ml Erlenmeyer flask containing 90 ml of buffered peptone water (BPW, Oxoid) as diluent. The samples were mixed thoroughly by agitation for 2 – 3min before serial 10-fold dilution was made in 0.1 % peptone water. For the isolation of the LAB, 0.1 ml supernatant of appropriate dilution was inoculated into de Mann, Rogosa and Sharpe (MRS) agar plates containing 0.6 % $CaCO_3$ (May & Baker, Ltd., England) and distributed properly with a sterile glass spreader. The plates were incubated anaerobically at 30 ^{0}C for 24 – 48 h. Anaerobiosis was confirmed with a BBL anaerobic indicator. Halos developing around the colonies were regarded as an indication of colony's ability to solubilize $CaCO_3$ by acid production (Vanos and Cox 1986). The cultures were purified successively on the MRS agar plates and then investigated to determine their Gram staining and catalase status according to the assay methods of Harrigan and McCance (1976). Gram positive and catalase negative isolates were selected and stored on the MRS agar slants at 4 ^{0}C for use in further study.

Test Microorganisms

The food-borne pathogens chosen as indicator strains for testing the antibacterial activity of the LAB bacteriocins were *Staphylococcus aureus* (ATCC 12600), *Listeria monocytogenes* (ATCC 15313) and *Escherichia coli* (ATCC 11775). They were obtained from the culture collection of the Microbiology Department of the University of Nigeria, Nsukka. The organisms were subcultured in Tryptone Yeast Extract Glucose broth (TYG, Oxoid Ltd, UK) for 24 h before use.

Bacteriocin Production by the Isolated LAB

Bacteriocin production by the LAB was performed by inoculating the LAB isolates into 6.0 ml MRS medium and incubating at 30 ^{0}C. Cell free supernatants (CFS) were collected by centrifugation (5000 g, 15 min at 4 ^{0}C) of the overnight broth cultures. The pH of the CFS was

adjusted to 6.5 with 4 N NaOH to eliminate the effect of organic acids. Possible inhibition by the hydrogen peroxide was also removed by the addition of catalase (Sigma Aldrich, Germany) at a final concentration of 1.0 mg/ml at 30 ^{0}C for 1 h.

Sensitivity of the Bacteriocin-like Compounds to Various Treatments

The CFS were divided into 2.0 ml portions before subjecting them to treatments in organic solvents (chloroform and ethanol), proteolytic action (pepsin and trypsin, Sigma Aldrich, Germany) and heat in order to evaluate the effect of the different factors on the activity and stability of bacteriocin. The solvents and enzymes were filter sterilized through Millex GV 0.22 µm filters (Millipore SA., France) before adding to the CFS. The controls consisted of distilled water and untreated CFS in 0.1 M phosphate buffer. The samples and controls were incubated at 30 ^{0}C for 2 h before the remaining bacteriocin activity was determined by the agar-well diffusion (AWD) assay against the indicator strains.

Detection of Antimicrobial Activity of the Bacteriocin

A modification of the agar-well diffusion (AWD) method was employed in this assay. Firstly, loopful of the indicator strains – *S. aureus*, *L. monocytogenes* and *E. coli* were inoculated separately into 6.0 ml TYG broth and incubated at 37 ^{0}C for 24 h. Then, TYG agar (100 ml) were prepared in six 250 ml Erlenmeyer flasks and just before the setting, 1.0 ml each of the indicator strains at a concentration of 10^8 CFU/ml were separately added into each medium, swirled gently to mix and then dispensed into sterile petri-dishes. The plates were left to solidify under a laminar airflow. Thereafter, the wells (8 mm diameter) were made in each of the plates using a sterile cork borer. A 0.1 ml of the CFS was introduced into the different wells and left for 1 h in a refrigerator to allow the diffusion of the metabolite into the agar before incubating at 30 ^{0}C. The plates were examined for the development of translucent halos in the bacterial lawn surrounding the wells after 24 h incubation. A direct comparison was made between the diameters of the halos (zones of clearing) produced by the different strains. The percentage bacteriocin activity was calculated from the diameter of zones of clearing measured after each treatment relative to the halos produced from the positive control (untreated CFS) against each target organism.

RESULTS

Strains and Cultures

A total of 752 colonies was isolated from both the condiments – 472 from *'ugba'* and 280 from *'okpiye'*. The colonies were successively screened for the antibacterial activity against the indicator strains. Thirty colonies which showed reasonable inhibitory action against at least one of the indicator organisms were selected. The CFS of only five colonies composed of *Lactobacillus* spp. (Table 1) retained their activity after the pH was adjusted to 6.5. All the five isolates showed good activity against *S. aureus* and *L. monocytogenes* (Tables 3 and 4). However, only one isolate (UG 2) was active against *E. coli* (Table 2).

Table 1 - Characteristics of isolated LAB strains.

Source	Strain	Tentative Identification	Gram Reaction	Shape	Catalase Reaction
Ugba	UG1	*Lactobacillus* spp.	+	Straight rods	—
	UG2	*Lactobacillus* spp.	+	Straight rods	—
Okpiye	OK 1	*Lactobacillus* spp.	+	Straight rods	—
	OK 2	*Lactobacillus* spp.	+	Straight rods	—
	OK 3	*Lactobacillus* spp.	+	Straight rods	—

Table 2 - Loss of antimicrobial activity of isolates CFS against *E. coli* ATCC 11775 after different treatments.

Source	Isolate	Chloroform (25% $^v/_v$)	Ethanol (25% $^v/_v$)	Pepsin (1mg/ml)	Trypsin (1mg/ml)	Heat 80^{0}C/30 min
Ugba	UG1	NA	NA	NA	NA	NA
	UG2	100	92	100	100	17
Okpiye	OK1	NA	NA	NA	NA	NA
	OK2	NA	NA	NA	NA	NA
	OK3	NA	NA	NA	NA	NA

NA means no activity was recorded against the test bacterium.

Table 3 - Loss of antimicrobial activity of isolates CFS against *S. aureus* ATCC 12600 after different treatments.

Source	Isolate	Chloroform (25% $^{v}/_{v}$)	Ethanol (25% $^{v}/_{v}$)	Pepsin (1mg/ml)	Trypsin (1mg/ml)	Heat 80^{0}C/30 min
Ugba	UG 1	100	89	100	100	12
	UG 2	100	74	100	100	07
Okpiye	QK 1	100	82	100	100	15
	QK 2	100	87	100	100	09
	QK 3	100	71	100	100	11

Table 4 - Loss of antimicrobial activity of isolates CFS against *L. monocytogenes* ATCC 15313 after different treatments.

Source	Isolate	Chloroform (25% $^{v}/_{v}$)	Ethanol (25% $^{v}/_{v}$)	Pepsin (1mg/ml)	Trypsin (1mg/ml)	Heat 80^{0}C/30 min
Ugba	UG 1	100	92	100	100	13
	UG 2	100	70	100	100	11
Okpiye	QK 1	100	90	100	100	10
	QK 2	100	81	100	100	12
	QK 3	100	78	100	100	17

Effect of Different Treatments on Antimicrobial Activity of CFS

The sensitivity of the bacteriocin-like compounds to various treatments is shown in Tables 2 – 4. The activity of the CFS was completely lost by the treatment with chloroform, pepsin or trypsin when they were challenged with the Gram positive indicators. A considerable loss of activity was recorded after the treatment with ethanol. Strain OK 3 had the lowest level of activity loss (71 %) while UG 1 had the highest (89 %) when the CFS was challenged with *S. aureus*. On the other hand, the CFS from strain UG2 was least affected by the ethanol treatment (70 %), while OK 1 and UG 1 were the most compromised when challenged with *L. monocytogenes.* The antibacterial activity of the CFS remained stable even after exposure to a temperature as high as 80 ^{0}C. Results showed that the activity loss was negligible. When the indicator strains were challenged with the heat treated CFS, the highest recorded activity loss as determined in the assays involving *S. aureus* varied between 7 – 15% while a corresponding range of 10 – 17% was recorded when *L. monocytogenes* was used as the test organism. In the tests conducted against *E. coli*, the percentage loss of activity of the CFS was 17% from strain UG 2.

DISCUSSION

Out of the 752 colonies that were isolated, only five that were confirmed as bacteriocin producing were selected and used in the study. Several factors might have influenced such low number of isolation. According to Sezer and Guven (2009), the culture medium, incubation conditions, target microorganisms as well as the sensitivity of methods used in determining the antimicrobial activity are important factors. The antibacterial activity of the centrifuged culture supernatants are thought to be due to the presence of bacteriocins since the potency of the culture metabolite was neither lost by the addition of catalase nor diminished by the pH adjustment. Thus, the recorded inhibition levels were not due to interference by hydrogen peroxide nor lactic acid produced by the culture in the supernatant fluid. The bacteriocins produced were completely inactivated by the pepsin and trypsin as well as by the chloroform treatment. Such susceptibility to proteolytic degradation is a confirmatory characteristic of proteinaceous compounds. According to Tatsajieu et al. (2009), the LAB are well-known for their production of protein with antimicrobial properties, known as bacteriocins. Fricourt et al. (1994) and Piard and Desmazeand, (1992) reported that many of the bactericidal agents synthesized by the LAB were bacteriocins with a proteinaceous active moiety while others were non–protein agents. The loss of activity of the bacteriocins due to the treatment in organic solvents suggested that the antimicrobial agent might have a lipid moiety in their chemical composition. Studies by Saad and Nadra (1993), and lately by Bromberg et al. (2004) corroborated this view.

The bacteriocins produced in the CFS were remarkable by its quality of heat stability. After 30 min at 80 ^{0}C, about 83 – 93% of their antimicrobial potency was retained. The bacteriocin produced by the UG 2 was considered the most heat stable as only 7% loss of activity was recorded. Several reports on the ability of bacteriocins to withstand the high temperature conditions have been published (Nettles and Barefoot, 1993; Deraz et al. 2005 and Campos et al. 2006). At temperatures as high as 121 ^{0}C, bacteriocins have been shown to retain activity and stability (Ogunbanwo et al. 2003). This property of high temperature tolerance may be important in the food industry where some food preparation procedures involve a heating step (Ogunbanwo et al. 2003). The bacteriocin produced by the UG 2 had some interesting characteristic that could justify its further study. The most striking being its activity against a Gram negative bacterium. Bacteriocins are known to harbor the narrow antibacterial spectra of activity (Caplice and Fitzgerald 1999) although some have been reported to show broad activity spectrum.

Studies on the activity of bacteriocins against the target strains usually take place in laboratory media not in the foods. As a result, several intrinsic factors such as water activity could bring about reduction in the activity of bacteriocins. Further insight into this using a system that effectively replicates a food microenvironment (probably solid-state culture) could be imperative in order to study why high *in-vitro* bacteriocin activity did not always corresponded to high activity in *in-vivo* environment. That way the use of bacteriocin producing microorganisms to preserve the food products would become easily optimized and readily adaptable.

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