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EFFICACY OF BACTERIOCIN PRODUCING LACTIC ACID BACTERIA ISOLATED FROM UGBA AND OKPIYE AGAINST SOME SELECTED BACTERIAL FOOD SPOILAGE AGENTS

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ABSTRACT
Numerous food products owe their production and characteristics to the fermentative activities of microorganisms. Bacteriocins are antimicrobial proteins produced by bacteria that kill or inhibit the growth of other related species. Lactic acid producing bacteria were isolated from Ugba and Okpiye using standard methods. The test organisms used were Staphylococcus aureus, Pseudomonas aeruginosa (VRFPA04) and Escherichia coli (ST2747) Fifty (50) samples of Ugba and Okpiye were collected and evaluated for the presence of bacteriocin lactic acid producers using a selective medium; De Mann Rogosa Sharpe (MRS) agar. A total of 288 isolates was isolated from the two traditional food condiments; 148 from ugba and 140 from Okpiye. It was observed that the total lactic acid bacterial count in Ugba ranges from 5.80 x 10^4 to 1.7 x 10^7 cfu/ml on MRS medium; while that of Okpiye ranges from 5.0 x 10^4 to 2.0 x 10^7 cfu/ml on MRS medium. Lactic acid bacterial count was higher in Ugba than in Okpiye considering the mode of storage in the market. The highest inhibitory activity was demonstrated against E. coli in Ugba sample while the least activity was demonstrated against P. aeuroginosa, also a significant high level of inhibition was shown against S. aureus while P. aeuroginosa and E. coli had little or no inhibitory activities recorded for Okpiye samples. The bacteriocin producers isolated from Ugba and Okpiye (U5, U6, OK1, OK2, OK3 and OK4) were identified as Lactobacillus sp and Lactococcus sp based on their morphological and biochemical characteristics. The antibacterial activity of the bacteriocins produced by the lactic acid bacteria has potential for use in biopreservation of condiments against food spoilage agents.
Keywords: Ugba, Okpiye, Bacteriocin, Lactic acid

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INTRODUCTION
The use of non-pathogenic microorganisms and/or their metabolites to improve microbiological safety and extend the shelf life of foods is defined as biopreservation (De Martinis *et al*., 2001) Contamination by pathogens is one of the concerns in food industry, they have been found to be the frequent cause of food borne diseases. 'Ugba' and 'Okpiye' 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 because of their protein content '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*

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and lactic acid bacteria (LAB) have been reported to play active roles in the process (Enujiugha, 2009).

Bacteriocins are antimicrobial proteins produced by bacteria that kill or inhibit the growth of other bacteria. Many lactic acid bacteria (LAB) produce a high diversity of different bacteriocins. LAB has a long history of application in fermented foods because of their beneficial characteristics. They cause rapid acidification of the raw materials through the production of organic acids mainly lactic acid. 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).

Microbial food spoilage and foodborne diseases over time have become a huge challenge to man, microbes competing with him for available resources in his environment. Recent studies have shown that some LAB possess beneficial Probiotics that has potential to combat gastrointestinal pathogenic bacteria such as *Helicobacter pylori*, *Escherichia coli* and *Salmonella* sp. Bacteriocins thus have an important advantage over the classical antibiotics used in being easily degraded by the digestive enzymes without the risk of disruption of normal tract ecology (Caplice and Fitzgerald 1999).

The aim of the study is to assess the antagonistic activity of bacteriocin produced by LAB isolated from Ugba and Okpiye against some food borne pathogens and the objectives were to isolate, characterize and screen Lactic acid producing bacteria from the Ugba and Okpiye, to evaluate bacteriocin produced by the isolated LAB and to assess their antagonistic capabilities from the inhibitory zone produced by each bacteriocin producing organism on selected food pathogenic microorganisms.

MATERIALS AND METHODS

Sample Collection

Twenty-five samples each of traditionally fermented 'ugba' and 'okpiye' were purchased from different selling points at Oja-oba and Ipata main markets in Ilorin, Kwara State - Nigeria. All the samples were stored at 4°C for a maximum of 24 hrs before the analysis. The Ugba samples were blended with an electric food blender and transported in sterile polythene bags to the laboratory.

Test Microorganisms

The food borne-pathogens used as indicator strains for testing the anti-bacterial activities of the LAB bacteriocins were *Staphylococcus aureus*, *Pseudomonas aeruginosa* (VRFPA04) and *Escherichia coli* (ST2747). They were obtained from the culture collection of the Microbiology Department of the University of Ilorin, Ilorin Kwara State. The organisms were sub-cultured for 24 hours before use.

Preparation of Culture Media

De Mann, Rogosa and Sharpe (MRS) Agar was used for the isolation of the lactic acid bacteria and Nutrient Agar (NA) was used to subculture the typed strains obtained before use.

De Mann, Rogosa and Sharpe Agar (MRS)

Exactly 15.85g of the commercially produced De Mann Rogosa and Sharpe Agar powder was weighed and dissolved in 250 ml distilled water, shaken and swirled thoroughly, the medium was then sterilized in the autoclave at 121°C for 15 minutes before pouring into the sterile Petri dishes. (Oyeleke and Manga, 2008).

Nutrient Agar

Seven (7) grams of the commercially produced Nutrient agar powder was weighed

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and dissolved in 250ml distilled water, shaken and swirled thoroughly and sterilized in the autoclave at 121°C for 15 minutes before pouring into the sterile Petri dishes. (Fawole and Oso, 2007)

Buffered Peptone water

Five (5) grams of the commercially produced buffered peptone water was dissolved in 250 ml distilled water, swirled thoroughly and sterilized in the autoclave at 121°C for 15 minutes before using as diluents in the homogenization of the samples (Fawole and Oso, 2007)

Ordinary Peptone Water

About 3.75g of the commercially produced ordinary peptone water was dissolved in 250 ml distilled water, swirled thoroughly and sterilized in the autoclave at 121°C for 15 minutes before using as diluents in the homogenization of the samples (Fawole and Oso, 2007)

Isolation of Lactic Acid Producing Bacteria (LAB)

Ten grams of each sample was grounded separately in a porcelain mortar and 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 – 3 mins before a 10-fold serial 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 evenly distributed using a sterile glass spreader. The plates were incubated anaerobically using the Gas Pack system (Oxoid) at 30 °C for 48 hrs. Halos developing around the colonies were regarded as an indication of organism's ability to solubilize $CaCO_3$ by acid production (Holuboya, 2004). 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 Fawole and Oso (2007). Pure cultures of Gram positive and catalase negative isolates were selected and stored on MRS agar slants at 4 °C for further utilization.

Tests for Detection and Enumeration or Test organisms

The three indicator strains for the anti-bacterial activities of the LAB bacteriocins; *Staphylococcus aureus*, *Pseudomonas aeruginosa* (VRFPA04) and *Escherichia coli* (ST2747) were subjected to simple laboratory tests for identification using traditional methods which include Culture characteristics on selective media, gram-staining and biochemical tests reactions such as Catalase test, Indole Production test, Citrate Utilization test and Sugar fermentation test respectively according to Bergey's manual of systematic Bacteriology (Holt *et al*.,1993)

Selective Media

The selective media used to culture *Staphylococcus aureus* was Baird-Parker medium. A loopful of the bacteria isolates was stricked on the already solidified Baird-Parker medium and incubated for 24 hours at 37˚C. After 24hours a black and shiny surface with narrow white margins surrounded by clear zones indicating the presence of *Staphylococcus aureus* was observed. The selective medium used for *Pseudomonas aeruginosa* (VRFPA04) and *Escherichia coli* (ST2747) were Malachite green Broth and Eosin-methylene blue respectively, a greenish metallic sheen in reflected light with blue black centre was observed on the plate of *Escherichia coli* and the *Pseudomonas aeruginosa* isolates retained the greenish coloration of the Malachite Green broth (MGB) after incubating for 48 hours at 37˚C.

Bacteriocin Extraction and Characterization

Bacteriocin production by the LAB was performed by inoculating the LAB isolates

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into 6.0 ml MRS broth medium and incubated anaerobically using the gas generating pack system (Oxoid) at 30˚C. After incubation, the broth was adjusted to pH 6.5 by adding 4N NaOH to eliminate the effect of organic acids. Cell free supernatants (CFS) were collected and centrifuged at (4500rpm, 20 min at 4˚C) from the overnight broth cultures and then used as raw bacteriocin to evaluate antibacterial activities using the agar well diffusion method.

Antibacterial activity of the Bacteriocins

A modification of the agar-well diffusion (AWD) method was employed in the assay. A loopful of the indicator strains (*Staphylococcus aureus*, *Pseudomonas aeruginosa* (VRFPA04) and *Escherichia coli ST2747*) were inoculated separately into 6.0ml nutrient broth and incubated at 37^0C for 24hrs. One hundred milliliters (100 ml) nutrient agar were prepared in six 250 ml Erlenmeyer flasks and before settling, 1.0ml of each indicator strain at a concentration of 10^8Cfu/ml were separately added to each medium, swirled gently to mix and then dispensed into sterile Petri dishes. The plates were allowed to solidify under a laminar airflow. Thereafter, 8 milimeters diameter wells were made in each of the plates using a sterile cork borer. A 0.1ml of the CFS was introduced into the different wells and left for 1hr in a refrigerator allowing the diffusion of the metabolite into the agar before incubating anaerobically using the gas generating kit (Oxoid) at 30^0C.The plates were examined for the development of translucent halos in the bacterial lawns surrounding the wells after 24 hours incubation. A direct comparison was made between the zones of inhibition produced by the different strains. The percentage bacteriocin activity was calculated from the diameter of zones of inhibition measured after each treatment relative to the halos produced from the positive control against each target organism (Balouiri *et al*., 2016)

Statistical Analysis

Data was subjected to Analysis of Variation (ANOVA) to determine the significant difference of the mean values of bacterial variables present in the samples collected. Turkey's test was used to differentiate between means and ssignificance was declared at $p<0.05$.

RESULTS

Enumeration of Lactic Acid Bacteria in Ugba and Okpiye Samples

Lactic acid bacterial count was higher in Ugba than in Okpiye samples considering the mode of storage in the market (Table 1).

Cellular and Colonial Morphology of Bacterial isolates

Bacteria isolated from Ugba and Okpiye with typical characteristics such as white color, small with entire margin on MRS media were picked as shown in (Table 2).

Biochemical Characterization of Isolates

Based on their biochemical characteristics they were suspected to be of the *Lactobacillus* sp and *Lactococcus* sp respectively. The isolates (Ok1- Ok6 and U1, U2) were Gram positive, indole negative and catalase negative. Citrate was not utilized. The bacteria strains isolated from the two traditionally fermented condiments were capable of fermenting sugars namely Glucose, Lactose and Maltose (Table 3)

The Inhibitory activities produced by each bacteriocin-like inhibitory substance (BLIS) of Lactic acid bacteria

The crude bacteriocin extracted from the isolates (Ok4, Ok6, U1 and U2) showed inhibitory activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* (VRFPA04) and *Escherichia coli ST2747* (Table 4).

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Statistical Analysis using analysis of variation method

Based on the Analysis of Variation (ANOVA) method used to determine the significant difference of the mean values of bacterial variables present in the samples collected, the following were the statement of Hypothesis: There is no significant difference in the lactic acid bacterial count in Ugba samples.

DISCUSSION

Bacteriocins producing lactic acid bacteria isolated from Ugba and Okpiye could ensure the safety and extend the shelf life of these two traditionally fermented food condiments. The isolates from Ugba and Okpiye were confirmed as *Lactobacillus* and *Lactococcus* based on their morphological and biochemical characteristics. Similarly, a *L. plantarum* SIK-83 strain, which was presumed to be *L. lactis* strain, produced a nisin like bacteriocin, when isolated from fermented carrots as claimed by Jacquelyn (2012).

The highest inhibitory activity of bacteriocin obtained from LAB isolates from Ugba samples was demonstrated against *E. coli* while the least activity was demonstrated against *P. aeuroginosa,*. This could be as a result of the efficient efflux system of *P. aeruginosa.* LAB isolates from Okpiye samples also had a significant high level of inhibition shown against *S. aureus* no inhibition was recorded against *P. aeuroginosa* and *E.coli.*. *Lactococcus sp* exhibited the broadest host range. The bacteriocins of all isolates inhibited the growth of at least 1 or 2 of the indicator strain. The inhibitory effect demonstrated by this isolate against these bacteria is an indication of possessing antibacterial activity (Quereda *et al*., 2016)

Based on this, an analysis of variation was carried out on the samples collected; the result stated that since F-tabulated (4.53) is greater than F-calculated (0.52), therefore the null hypothesis would be accepted and conclude that there is no significant difference in the number of bacterial isolates from Ugba collected while since F-tabulated (4.76) is less than F-calculated (27.45), therefore the null hypothesis would be rejected and conclude that there is a significant presence of bacterial isolates in the Ugba collected.

These result shows the potential usefulness of these bacteriocins justifying a more in depth investigation for their identification and application as food bio preservatives. These Bacteriocin productions was strongly dependent on pH and temperature according to Torodov and Dicks (2004) and since bacteriocin is considered as natural products, they might have good acceptance from customers who start to demand for more natural and safer food products. Some legal drawbacks that must however be considered for the application of novel bacteriocins are safety factors in foods and feeds as well as a continued research, since up to date only a few have been officially approved for use in foods (Parada *et al*., 2007).

CONCLUSION

This study concluded that the bacteriocin produced by U1, U2, Ok1, Ok2, Ok3 and Ok4 has demonstrated that inhibitory effects against bacterial pathogenic microorganisms are present in these traditionally fermented condiments (Ugba and Okpiye). Like Lacticin 3147 the bacteriocin produced by these isolates in the present study also has the enormous potential for food applications as bio preservatives against food spoilage microorganisms under careful supervision. Several factors are responsible for the viability of bacteriocins produced from each bacterium, and they are the temperature, pH, Water activity of the diluent e.t.c. for the bacteriocin isolated to be effective, all this factors must be in place.

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Appendices

Table 1: Heterotrophic plate count of Ugba and Okpiye samples

Samples/Lactic acid bacteria count

Samples	UGBA (cfu/ml)	OKPIYE (cfu/ml)
1	5.80 x 10^4	5.00 x 10^4
2	4.20 x 10^5	4.20 x 10^5
3	3.10 x 10^6	2.80 x 10^6
4	1.70 x 10^7	2.00 x 10^7

Table 2: Colonial and Cellular characterization of isolates

Samples/ Bacterial Isolates		Colonial Morphology						Cellular Morphology		
		Edge/Form	Elevation	Colour	Surface	Consistency	Optical Characterization	Gram Staining	Shape	Arrangement
UGBA	U1	Circular	Raised	Creamy white	Smooth	Viscid	Translucent	+	Cocci	Bi and Tri
	U2	Circular	Raised	White	Smooth	Viscid	Translucent	+	Rod	Chains
	U3	Circular	Raised	White	Rough	Butyrous	Opaque	+	Rod	Single
	U4	Circular	Raised	Creamy white	Rough	Butyrous	Opaque	+	Rod	Cluster
	U5	Circular	Raised	White	Rough	Butyrous	Opaque	+	Rod	Single
	U6	Circular	Raised	White	Rough	Butyrous	Opaque	+	Rod	Single
	U7	Circular	Raised	Creamy white	Rough	Butyrous	Opaque	+	Rod	Single
OKPIYE	Ok1	Circular	Raised	Creamy white	Glistering smooth	Viscid	Translucent	+	Cocci	Interlock cluster

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	Ok2	Circular	Raised	Creamy white	Glistering smooth	Viscid	Translucent	+	Cocci	Interlock cluster
	Ok3	Circular	Raised	Creamy white	Glistering smooth	Viscid	Translucent	+	Cocci	Interlock cluster
	Ok4	Circular	Raised	Creamy white	Glistering smooth	Viscid	Translucent	+	Cocci	Interlock cluster
	Ok5	Circular	Raised	Creamy white	Glistering smooth	Viscid	Translucent	+	Rod	Chains
	Ok6	Circular	Raised	Creamy white	Glistering smooth	Viscid	Translucent	+	Rod	Single

Table 3: Biochemical characterization of isolates from Ugba and Okpiye Samples

Sample		**Biochemical Tests**						**Probable microorganism**
		Catalase	Indole	Citrate	**Sugar Fermentation**			
					Maltose	**Lactose**	**Glucose**	
UGBA	U1	-	-	-	A	A	A	*Lactococcus* sp
	U2	-	-	-	A	A	A + G	*Lactobacillus* sp
	U3	+	-	-	A	A	A	NLAB
	U4	+	-	-	A	A	A	NLAB
	U5	+	-	-	A	A	A	NLAB
	U6	+	-	-	A	A	A	NLAB
	U7	+	-	-	A	A	A	NLAB
OKPIYE	Ok1	-	-	-	A	A	A	*Lactococcus* sp
	Ok2	-	-	-	A	A	A	*Lactococcus* sp
	Ok3	-	-	-	A	A	A	*Lactococcus* sp
	Ok4	-	-	-	A	A	A	*Lactococcus* sp
	Ok5	-	-	-	A	A	A	*Lactobacillus* sp
	Ok6	-	-	-	A	A	A + G	*Lactobacillus* sp

Key: R; Rod, **C;** Cocci, **A;** Acid Production, **G;** Gas Production, (+) Positive reaction, (-) Negative reaction, (d) delayed reaction, **NLAB,** Non-lactic-acid bacteria.

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Table 4: Antibacterial activity of bacteriocin (from Ugba) against test organisms

Lab Isolates	Test Organisms/	Zone of Inhibition(mm)	
	S. aureus	*Pseudomonas aeruginosa*	*E. coli*
U1a	24.00	12.00	42.00
U1b	22.00	7.00	35.00
U2a	30.00	8.00	35.00
U2b	20.00	17.00	32.00

U1 – U2 Lab Isolates from Ugba samples

Table 5: Antibacterial activity of bacteriocin (from Okpiye) against test organisms

LAB Isolate	Test Organisms/	Zone of inhibition(mm)	
	S.aureus	*Pseudomonas aeruginosa*	*E. coli*
Ok4a	20.00	0	0
Ok4b	20.00	0	0
Ok6a	37.00	0	0
Ok6b	22.00	0	0

OK4 and OK6 Lab Isolates from Okpiye samples

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Table 6: Analysis of Variance

Samples	*S.aureus*	*P. aeroginosa*	*E.coli*	Row total	Row mean
U1a	24	12	42	78	24
U1b	22	7	35	64	21.3
U2a	30	8	35	73	24.3
U2b	20	17	32	69	23
Column total	96	44	144	284	
Column mean	24	11	36		

Table 7: Analysis of Variance

Source of Variation	Sum of Sequence	D.F	Mean sum of Sequence	F-Cal	F-tab
Ugba (U)	35.34	a-1	35.34/3	11.78/22.78	4.53
		4-1=3	= 11.78	=0.52	
Bacteria (B)	1250.67	b-1	1250.67/2	625.34/22.78	4.76
		3-1=2	= 625.34	= 27.45	
Error €	136.66	(a-1)(b-1)	136.66/6		
		(4-10(3-1)	=22.78		
		3x2=6			
Total	1422.67				