



## **Preliminary Studies on Isolation, Bile Tolerance and Antibigram of Potential Probiotics (Probiotics) from Locally Fermented Food Products at Beach Market, Nsukka Metropolis, Enugu State, Nigeria**

**D. P. Berebon<sup>1\*</sup>, K. C. Ofokansi<sup>2</sup>, A. A. Attama<sup>2</sup>, C. O. Eze<sup>1</sup>, R. C. Onwusoba<sup>1</sup> and I. C. Ugwoke<sup>1</sup>**

<sup>1</sup>Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 41001, Enugu State, Nigeria.

<sup>2</sup>Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 41001, Enugu State, Nigeria.

### **Authors' contributions**

*This work was carried out in collaboration among all authors. Authors KCO and AAA are PhD supervisors of DPB and co-designed the study with author DPB. Author ICU assisted author DPB to perform some of the laboratory experiments. Author DPB wrote the protocol and the first draft of the manuscript. Author DPB managed the analyses of the study. Authors DPB, ICU, COE and RCO managed the literature searches. Authors KCO, AAA and COE proofread the manuscript. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Aims:** The study aims to isolate, evaluate bile tolerance and antibiogram studies of potential probiotics (*Lactobacillus spp*) from locally fermented Food Products (Akamu, Aqua Rafa<sup>®</sup> Yoghurt, Ogiri, Okpeye) and Kunu at Beach Market, Nsukka.

**Study Design:** A ten - fold serial dilution and spread plate method using De Man, Rogosa and Sharpe (MRS) medium was adopted for isolation of potential Probiotics.

**Place and Duration of Study:** Department of Pharmaceutical Microbiology and Biotechnology,

\*Corresponding author: E-mail: [dinebari.berebon@unn.edu.ng](mailto:dinebari.berebon@unn.edu.ng), [dberebon@gmail.com](mailto:dberebon@gmail.com);

Faculty of Pharmaceutical Sciences, University of Nigeria Nsukka, between March - September, 2018.

**Methodology:** Only catalase negative and Gram positive isolates characteristic of lactobacilli were used. Bile tolerance test was performed by monitoring the bacterial growth at different Bile salt concentrations (0.2%, 0.3% and 2%). The antibiogram of the isolates was assessed using the Kirby-Bauer disc diffusion method against commercial antibiotic discs of ampicillin, vancomycin, gentamycin, ciprofloxacin, methicillin and erythromycin.

**Results:** All the 18 screened isolates were tolerant to bile salt at 0.2 % and 0.3 % with inhibition of growth at 2 % bile concentration. All isolates were observed to be resistant to methicillin (100 %) but very sensitive to gentamycin (11%) and ciprofloxacin (22%) respectively. The isolates showed intermediate resistance to other antibiotics: vancomycin (33%), erythromycin (33%) and ampicillin (44%). The decreasing pattern of resistance was thus: methicillin > ampicillin > vancomycin and erythromycin > ciprofloxacin > gentamycin. Isolates from Yoghurt (66.67%) and Ogiri (53.33%) provided most of the resistant isolates. Methicillin would provide best antagonist potential as all the isolates exhibited very high level of resistance (100 %).

**Conclusion:** These results suggest that all the eighteen potential *Lactobacillus spp* strain show potential for probiotic applications and the locally fermented food products are rich sources of probiotics.

**Keywords:** Bile tolerance; antibiogram; *Lactobacillus spp*; probiotics; locally fermented food.

## 1. INTRODUCTION

Probiotics are live microorganisms, which when administered in adequate doses, confer health benefit on the host [1]. However, it is recommended that any probiotic strain used in food matrix should generally be regarded as safe (GRAS) devoid of potentially transferable antibiotic resistant traits. There is now an increasing demand for probiotic food/products that have the capacity to enhance health, beyond providing basic nutrition, since humans are now aware of the correlation between diet, lifestyle and good health [2]. Lactic acid bacteria (LAB) commonly regarded as the major group of probiotic bacteria are rods or cocci, facultative anaerobes, non-spore forming firmicutes groups with low Guanine (G) and cytosine (C) – G + C (< 50%) members belonging to the genera - *Enterococci*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. LAB may ferment sugars primarily into lactic acid in homo-fermentative pathways or lactic acid, acetic acid, ethanol and CO<sub>2</sub> via hetero-fermentative pathways based on presence of aldolase enzyme in homofermentative LAB which are absent in the heterofermentative LAB.

Primary criteria for screening potential probiotics such as bile tolerance, resistance to acids, mucosal adhesion, and antibiogram studies have been researched extensively and well documented in literature [3,4,5]. A potential probiotic must exhibit excellent tolerability to high acidity in the stomach and the high concentration

of bile components in the proximal intestine of the host [6].

Extensive research has been done on novel potential probiotic strains found mostly in our locally fermented foods that specifically confer health benefits on consumption of these foods. These benefits include control of blood cholesterol, *H. pylori* infection, suppression of growth and invasion of pathogenic bacteria, immunomodulatory activity etc. [7].

A deeper understanding of antibiotic resistance against these probiotic organisms will lead to development of more effective therapeutic co-administration protocols. Resistance of these organisms may be intrinsic (absence of target, low affinity to target, low permeability, efflux mechanism) or acquired (modification of target, efflux mechanism) and inactivation of drug [8].

Exposure of probiotic bacteria to bile salts causes disruptions of cellular homeostasis causing dissociation of lipid bilayer and integral protein of their cell membranes, resulting in bacterial content leakage and finally death of cell [9]. Thus, survivability of probiotics within the gastrointestinal tract (GIT) depends largely on their bile salt tolerance. Bile salt hydrolyses the fatty and lipid content of the bacterial cell membrane. Strompfova and Laukova [10] proposed that resistance against bile salt is the second important criterion for the colonization and metabolic activity of probiotic bacteria in the small intestine of the host.

For effective antibiotic – probiotic adjuvant therapy, resistant not susceptible strains of the probiotics to the antibiotics is preferred to achieve synergistic effect. This is because susceptible probiotics would be destroyed by the antibiotics it is combined with. Though probiotic strain carrying inducible antibiotics resistant strains may appear susceptible in - vitro they have potential to develop resistance upon in - vivo selection by the appropriate antibiotics.

In the Eastern part of Nigeria, locally fermented food products such as Akamu (pap), Aqua Rafa<sup>®</sup> Yoghurt, Ogiri, Okpeye and Kunu readily provide the bulk of ready - to - eat - food (RTEF). Traditional fermented dairy foods are good reserves for the various genus of *Enterococci*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* as probiotics. *Lactobacilli* are the most prevalent genus isolated from dairy and pharmaceutical products [11]. The aim of this study was to provide a preliminary study on isolation, bile tolerance and antibiogram of potential probiotics (*Lactobacillus spp*) from locally fermented food products at Beach Market, Nsukka, Enugu State, Nigeria.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

All chemicals used for the study were of analytical grades and included ethanol (70%), 3% hydrogen peroxide, Gentian violet, Lugo's iodine, safranin, oil immersion, distilled waters, bile salt (sodium taurocholate) and phosphate buffered saline (PBS).

### 2.2 Food Samples and Media Used

Five types of ready – to – eat food (RTEF) samples were procured for the isolation of potential probiotic strains used in this work: Kunu (K), locally fermented foods including: Ogiri (O), Okpeye (OK), Akamu [Pap] (P) and Yoghurt (Y) (Aqua Rafa<sup>®</sup> yoghurt). A total of fifty (50) food samples comprising ten food samples each were randomly procured. The media used include De Man, Rogosa and Sharpe (MRS) agar (TM Media, India), MRS broth (TM Media, India) and Muller Hinton agar (TM Media, India). The MRS is a selective medium for isolation of lactic acid bacteria (LAB).

### 2.3 Antibiotic Disk Used

Vancomycin (VAN), Methicillin (MET),  
Gentamycin (GEN), Erythromycin (ERY),

Ciprofloxacin (CIP), and Ampicillin (AMP). All antibiotic disk was procured from Hi - Media.

### 2.4 Sample Collection

Yoghurt and some locally fermented food products including Kunu, Ogiri, Okpeye and Akamu (Pap) were bought fresh from Beach market in Nsukka between March – June, 2018 and transported under safe and aseptic condition in an icebox (- 4 °C) to the Department of Pharmaceutical Microbiology and Biotechnology laboratory of the University of Nigeria, Nsukka where further studies were performed immediately.

### 2.5 Sample Preparation and Isolation of Lactic Acid Bacteria (LAB)

Processing of the various fermented food substrate and isolation of LAB was carried out as described elsewhere [12]. Serial dilutions (10 - fold) of each sample using 0.85 % w/v of sterile normal saline as diluent was carried out. Aliquots (0.1 ml) of 10<sup>-3</sup> and 10<sup>-6</sup> folds of each of the food samples were transferred into already prepared Petri dishes and spread over the plate using a glass spreader. The inoculated plates were incubated in an anaerobic jar at 37 °C for 24 - 48 h. The plates were observed for development and appearance of colonies. Each probiotic isolate was purified by repeated streak plate method on MRS agar and incubated at 37°C for 24 - 48 h. The purified isolates were then transferred to MRS agar slants and then maintained in refrigerator at 4°C till further analysis.

### 2.6 Colonial and Morphological Characteristics of Isolates

Colonial and morphological characteristics of the isolated strains such as their form, color, elevation, margin, and surface characteristics were observed visually and recorded accordingly. Isolates showing phenotypic characters similar to *Lactobacillus* species on MRS agar media were selected for further experiments.

### 2.7 Gram Staining Reaction of Isolates

The test was performed as described by Dubey and Maheshwari [13]. The test was performed to study the Gram reaction and morphology of the isolates. Only the Gram positive bacteria were selected for further studies.

## 2.8 Catalase Test for Isolates

The method of Cheesbrough [14] was adopted. The test is used to detect the presence of catalase, which catalyzes the breakdown of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to water and oxygen. A 3% hydrogen peroxide was dropped on a clean glass slide, using a sterile wire loop. A little amount of the isolate was mixed with the drop and observed for presence or absence of effervescence. Only isolates that do not cause effervescence (catalase negative) were selected for further studies.

## 2.9 Bile Salt Tolerance Test (BSTT)

Isolates were cultured overnight in MRS broth. Subsequently, 0.2, 0.3 and 2% w/v of different concentrations of bile (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) were prepared using phosphate buffered saline (PBS) as diluent. Aliquot (0.1ml) of the broth culture suspension adjusted to 0.5 McFarland standard was inoculated into the test tubes containing 10 ml each of the bile salt while the test tube not inoculated served as control. Growth was

monitored at 0 hour and 4 h by increased absorbance at a wavelength of 600 nm using a spectrophotometer (Spectrumlab725s, England) blanked with the control.

## 2.10 Antibiotics Sensitivity (Antibiogram) Test

The antibiotic sensitivity (antibiogram) of isolated LAB was assessed using antibiotic disc diffusion method on Muller Hinton agar plates. Broth cultures of LAB was prepared using MRS broth and adjusted to 0.5 McFarland standards. The freshly grown overnight isolated potential *Lactobacillus spp.* cultures were spread on Muller Hinton agar plates using sterile swab sticks. The antibiotic discs were placed on the surface of Muller Hinton agar plates and the plates were incubated at 37 °C for 24 - 48 h. Antibiogram of the resistance phenotypes was assessed using Vancomycin (VAN), Methicillin (MET), Gentamycin (GEN), Erythromycin (ERY), Ciprofloxacin (CIP), and Ampicillin (AMP). The inhibition zones diameters (IZD) were measured and the results were expressed as sensitive (S) and resistant (R) according to CLSI [15].

## 3. RESULTS AND DISCUSSION

### 3.1 Isolation and Characterization of Isolates

Table 1: Colony Morphology, Gram Reaction and Catalase Test

Probiotic	Form	Color	Elevation	Margin	Surface	Gram reaction	Catalase test
K-13	Circular	Milky	Convex	Entire	Smooth	+	Negative
K-15	Circular	Milky	Convex	Entire	Smooth	+	Negative
O-2	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
O-5	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
O-6	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
O-13	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
O-15	Circular	Milky	Convex	Entire	Smooth	+	Negative
OK-3	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
OK-4	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
OK-23	Punctiform	Milky	Convex	Entire	Smooth	+	Negative
P-1	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
P-2	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
P-5	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
P-7	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
P-8	Circular	Milky	Convex	Entire	Smooth	+	Negative
Y-7	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
Y-11	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative
Y-12	Circular (L)	Milky	Convex	Entire	Smooth	+	Negative

Note: Kunu - K, Ogiri - O, Okpeye - OK, Pap (Akamu) - P and Yoghurt -Y; L- Large, + positive

### 3.2 Bile Tolerance Test (BTT)

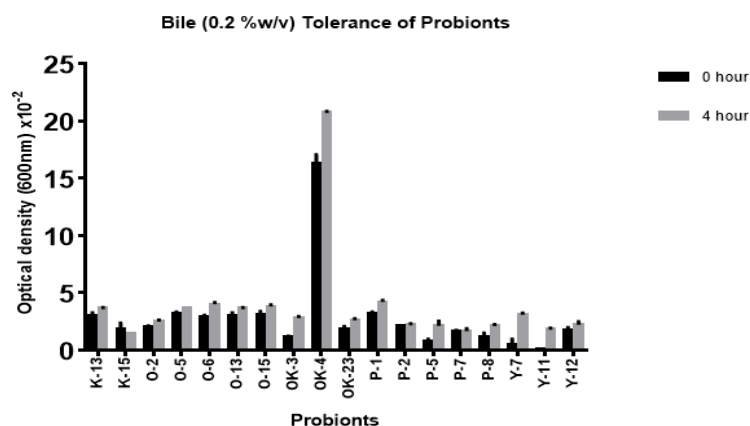


Fig. 1. Tolerance of probiotics to bile salt (0.2 % w/v). kunu - K, yoghurt -Y, okpeye - OK, pap (akamu) - P, ogiri – O.

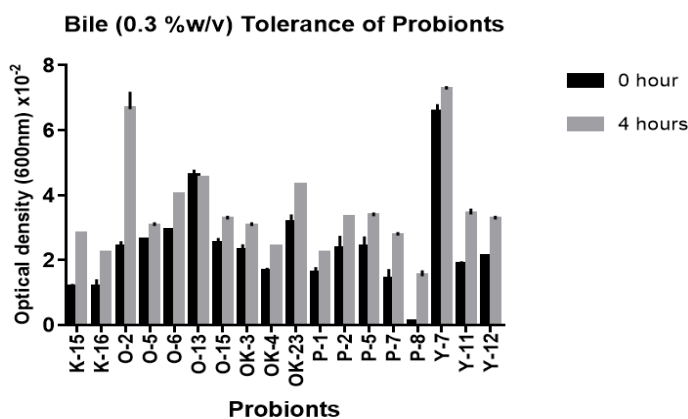


Fig. 2. Tolerance of probiotics to bile salt (0.3% w/v). kunu - K, yoghurt -Y, okpeye - OK, pap (akamu) - P, ogiri – O.

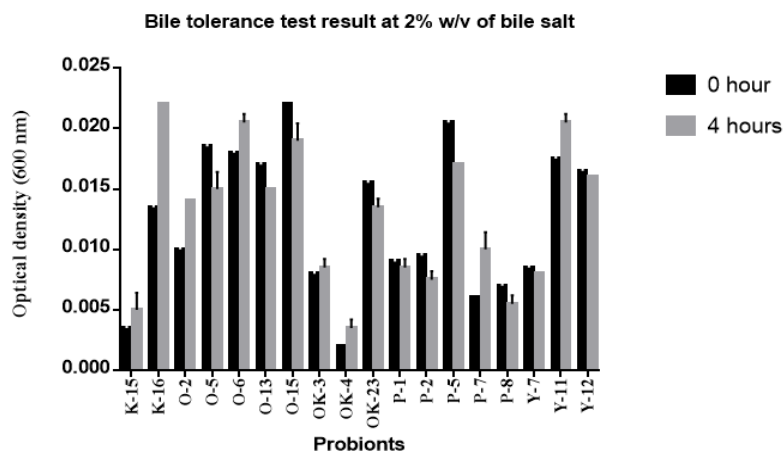


Fig. 3. Tolerance of probiotics to bile salt (2%w/v). kunu - K, yoghurt -Y, okpeye - OK, pap (akamu) - P, ogiri – O.

### 3.3 Antibiotics Sensitivity (Antibiogram)



Fig. 4. Antibiogram of selected probiotics (a - c): a. O-15 (isolate No.15 from ogiri); b. K-15 (isolate No.15 from kunu); P-7 (isolate No.7 from pap - akamu); \*each isolate was done in duplicate.

Table 2. Antibiotics sensitivity test result using antibiotic discs

PROBIOTIC ISOLATE	INHIBITION ZONE DIAMETER (mm)					
	VAN (30µg)	MET (5 µg)	GEN (10 µg)	ERY (15 µg)	CIP (5 µg)	AMP (25 µg)
OK-3	55 (S)	8 (R)	55 (S)	0 (R)	0 (R)	0 (R)
OK-4	40 (S)	12 (R)	45 (S)	40 (S)	40 (S)	42 (S)
OK-23	12 (S)	10 (R)	35 (S)	30 (S)	41 (S)	32 (S)
Y-7	0 (R)	0 (R)	0 (R)	10 (R)	9 (R)	15 (R)
Y-11	8 (R)	11 (R)	40 (S)	0 (R)	0 (R)	0 (R)
Y-12	11 (S)	12 (R)	35 (S)	40 (S)	30 (S)	31 (S)
P-1	12 (S)	12 (R)	31 (S)	42 (S)	26 (S)	35 (S)
P-2	15 (S)	8 (R)	35 (S)	45 (S)	28 (S)	40 (S)
P-5	12 (S)	8 (R)	38 (S)	45 (S)	30 (S)	36 (S)
P-7	23 (S)	9 (R)	30 (S)	30 (S)	40 (S)	27 (S)
P-8	0 (R)	0 (R)	0 (R)	31 (S)	33 (S)	40 (S)
O-2	8 (R)	8 (R)	27 (S)	32 (S)	42 (S)	25 (S)
O-5	9 (R)	9 (R)	29 (S)	40 (S)	20 (S)	21 (R)
O-6	7 (R)	8 (R)	17 (S)	12 (R)	10 (R)	14 (R)
O-13	23 (S)	9 (R)	27 (S)	0 (R)	20 (S)	0 (R)
O-15	21 (S)	11 (R)	16 (S)	9 (R)	18 (S)	12 (R)
K-15	19 (S)	10 (R)	22 (S)	22 (S)	25 (S)	18 (R)
K-16	13 (S)	12 (R)	18 (S)	39 (S)	28 (S)	30 (S)

Note: R= Resistance S = Susceptible [22, 15, 23]. VAN (Vancomycin), MET (Methicillin), GEN (Gentamycin), ERY (Erythromycin), CIP (Ciprofloxacin) and AMP (Ampicillin).

Table 3: Percentage (%) of antibiotic resistance of 18 probiotic isolates

Antibiotics	Number of resistant isolates	Percentage of resistance (%)
Vancomycin	6	33.33
Methicillin	18	100
Gentamycin	2	11.11
Erythromycin	6	33.33
Ciprofloxacin	4	22.22
Ampicillin	8	44.44

Note: 0 – 30 (low); 31 – 50 (moderate); 51 – 69 (high) and 71 – 100 (very high)

All the potential probiotic colonies isolated on MRS agar plates were morphologically characterized (Table 1) based on their microscopic examination and colony characteristics obtained along with their Gram reaction and catalase test. Phenotypic characters similar to Lactobacilli such as circular form, creamy milk color, convex shape with margins entire and surface smooth were observed. Importantly, the MRS agar is a selective medium for the isolation of Lactobacilli. Almost all the isolates tested negative and positive for catalase and Gram reaction respectively. This is in accordance with previous studies [7,17,18]. Catalase is an enzyme produced by aerobic organisms. It breaks down hydrogen peroxide to water and oxygen. Bacteria that produce catalase enzyme will produce oxygen bubbles when placed in hydrogen peroxide. Lactic acid bacteria (LAB) being facultative anaerobes do not produce catalase enzyme perhaps due to lack of the porphyrin and cytochrome enzyme. This explain why lactic acid bacteria (LAB) though tolerant to less oxygen tension, cannot obtain their energy via electron transport cycle but by substrate level phosphorylation. Thus, this test is used to distinguish them from other bacteria. Normal saline (0.85%) was used as diluents for the isolation of the probiotics since it is isotonic to body fluids and the organisms can tolerate it at a concentration of about 1.0 - 6.5% [18].

The results in Figs. 1 and 2 showed that bile at 0.2 - 0.3% w/v had little or no effect on the inhibition of growth of almost all the isolates tested with the exception of K-15 that showed slight inhibition after 4 h. Resistance to bile is an important characteristics of probiotic bacteria which enables them to survive and grow in the gastrointestinal tract (GIT) without being destroyed. The bile concentration of human GIT varies with a mean concentration of 0.3 % w/v and a staying time of 4 h [19]. A concentration of 0.15 - 0.3 % of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use [20]. According to the findings of this work, all the isolates showed growth at 0.2 - 0.3% bile concentration. These finding suggests that these isolates have the potential to functionally and effectively survive in the GIT, to perform essential role in specific and non-specific host immune defense mechanisms. However, there was inhibition of growth at 2%. This is in accordance with similar studies [17] that showed growth at 0.05 - 0.3% bile. Another

work showed growth of up to 0.2 % bile with inhibition at 0.4 % bile concentration [21].

Antibiogram (Antibiotic sensitivity test) of 18 isolates were evaluated using Kirby-Bauer (disc diffusion) method as described elsewhere [22,15,23] and shown on Table 2. No definitive breakpoint has been established for lactic acid bacteria, thus susceptibility or resistance to antibiotics is usually determined by MIC values with the proposed breakpoints [24]. The isolates showed more of susceptibility and intermediate resistance than resistance. Methicillin had the highest preponderance of activity and gentamycin showing less activity with almost all the isolates. The decreasing pattern of resistance among the isolates is methicillin > ampicillin > vancomycin > erythromycin > ciprofloxacin > gentamycin. Koppula and Bhukya [16] observed that LAB isolates were resistant to ampicillin and vancomycin which was in agreement with results obtained in this work; however, they observed isolates were also resistant to gentamycin which is at variance from observation in the present work. This may be due to presence of specific enzyme in the isolates which can cause antibiotics resistance. Hoque et al. [25] observed that *Lactobacillus spp* is sensitive to erythromycin, gentamycin, vancomycin, ciprofloxacin, methicillin and resistant to ampicillin. These results are in agreement with results of the present work except that all the isolates were totally resistant to methicillin and vancomycin. Intrinsic resistant to vancomycin in heterofermentative lactobacillus species has been reported decades ago by other author [26]. The resistance of these probiotics to vancomycin is intrinsic, due to irreversible replacement of the normal dipeptide D-alanyl-D-alanine with D-alanyl-D-lactate in their peptidoglycan [27,28]. Intrinsic resistance to vancomycin was earlier also confirmed for *L. paracasei*, *L. salivarius* and *L. plantarum* (MIC  $\geq$  32  $\mu$ g/L) [29], *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *L. rhamnosus* [30].

These findings show that results of antibiotic resistance vary from study to study probably due to difference in experimental conditions. Lack of current standard methods for antibiotic susceptibility testing of LAB, despite several microdilution methods been used which account for why some LAB strains previously reported to harbor tetracycline or erythromycin resistant patterns, were found to be negative for *tet* (M) or *erm* (B) genes respectively [11].

Table 3 showed the percentage (%) of antibiotic resistance of 18 potential probiotic isolates. Isolates from the different locally fermented products and Yoghurt showed resistance as follows: Kunu (K) = 25 % (3/12), Ogiri (O) = 53.33 % (16/30), Okpeye (OK) = 33.33 % (6/18), Pap (P) = 23.33 % (7/30) and Yoghurt (Y) = 66.67 % (12/18). Isolates from Yoghurt and Ogiri were more resistant to the antibiotics generally.

The isolates were observed to be more sensitive to gentamycin and ciprofloxacin confirming their least antimicrobial potential in combinations (antibiotic – probiotic adjuvant therapy) with the drugs. Susceptible probiotics provide least antimicrobial potential in combination (synbiotics) with the antibiotics. This is because most of the probiotics would eventually be destroyed by the antibiotics. It is advisable to use resistant probiotic strains in various drug combinations (antibiotic – probiotic adjuvant therapy) instead of sensitive ones in order to bring their best antimicrobial effect. Thus, methicillin would be a better combination in formulation for any of the isolates. However, there is need to evaluate the resistant genes carried by these isolates to avoid transfer to pathogenic organisms which has the potential to increase antibiotic resistance menace.

#### 4. CONCLUSION

Antibiogram of probiotics (majorly lactobacillus spp) sourced from Nsukka locality was prepared against the frequently used antibiotics. Most of the isolates were found to be resistant to methicillin with intermediate resistance to vancomycin, erythromycin and ampicillin. Almost all the isolates were susceptible to gentamycin and ciprofloxacin. This study has revealed the sensitivity patterns (antibiogram) of probiotic isolates found in the local foods. This would be useful when deciding on isolates to be combined with antibiotics since isolate resistant to an antibiotic is best used in combination to bring about the best synergistic potential in any given commercial probiotic preparations. Methicillin would be the best antibiotic for combination with any of the isolates. Further studies will focus on the molecular identification of the different isolates up to the strain (species) level, molecular determination of resistant determinants (genes) in isolates found to be resistant to antibiotics, determination of acid and bile resistance of probiotics in vivo or in vitro by a more complex model and determination of the minimum inhibitory concentration (MIC) for selected antibiotics.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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