

Full Length Research Paper

Evaluation of *Lactobacillus* spp. isolated from locally consumed probiotic food in Nsukka, Enugu State, Nigeria for antimicrobial activity utilizing agar well diffusion and pH tolerance tests

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Antimicrobial activity against entero-pathogens and tolerance to acid stress are crucial characters of probiotic bacteria. *Lactobacillus* spp. isolates were phenotypically characterized using colony observation, catalase test and Gram stain reaction. The pH (1.5, 2.5 and 3.5) tolerance of each isolate was evaluated at 0 and 4 h. The antibacterial activities of the isolates were tested against pathogenic strains of *Bacillus* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Staphylococcus aureus*. Among the isolates screened, 20 were Gram positive and catalase negative. In an agar well diffusion, isolate O₄ resulted in the highest inhibition zone diameter, 13 mm against *P. aeruginosa* while isolates OK₄, UK₁, P₂, P₃ and P₄ did not produce any inhibition zones against any of the pathogens tested. Isolate Y₁ showed the broadest inhibitory activity against the pathogens tested inhibiting all the pathogens tested except *S. typhimurium*. The pH tolerability studies showed that the isolates proliferated more at lower acidic pH: 1.5 > 2.5 > 3.5. Food products containing Ogiri, Ukpaka, Okpeye, Akamu and Yoghurt provides useful sources of probiotic bacteria.

Key words: Antimicrobial activity, *Lactobacillus* spp, pH tolerance, entero-pathogens.

INTRODUCTION

There is substantial concern that pharmaceutical industries are not able to develop novel and effective antibiotics at a rate sufficient to compete with the emergence of microbial resistance to antibiotics used in

the clinic. Thus, interest in the use of probiotic foods with beneficial microorganisms as an alternative to antibiotic therapy has geometrical increased within the past decades. The antibacterial properties and tolerance to

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low pH of lactic acid bacteria (LAB) is essential in their applicability as probiotics. To achieve a high concentration (10^9 to 10^{11} CFU/day) and to allow for their beneficial action, LAB must survive the low pH of the gastrointestinal tract (GIT), colonize it and synergistically dislodge pathogenic bacteria via production of defensive metabolites.

Probiotics are 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). Importantly, probiotics with probiotic grade must be devoid of any adverse effects (cytotoxicity, antibiotic resistance and hemolysis), and endowed with beneficial traits to inhibit pathogenic bacteria through different mechanisms as reported in different studies (Vieco-Saiz et al., 2019). Probiotics as well as their bacteriocins (produced by some probiotic organisms) are considered to substitute for antibiotics in the food and pharmaceutical sector. Lactic acid bacteria (LAB), commonly regarded as the major group of probiotic bacteria, are rods or cocci, facultative anaerobes, belong to the non-spore forming firmicutes groups with low Guanine (G) and cytosine (C) – G + C (< 50%) with members belonging to the genera - *Enterococci*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Berebon et al., 2019).

There are stringent selection criteria for the development of commercial probiotics. It is based on their unmistakable taxonomic identification, safety assessment, *in vitro* and *in vivo* functional characterization (FAO/WHO, 2006). Importantly, they should be tolerant to acids and bile salts, survival during passage through the gastrointestinal tract (GIT), ability to colonize the intestine, antagonistic properties against pathogenic microorganisms (Shokryazdan et al., 2017). Probiotics should be able to stimulate the immune system, degrade toxic substances and improve absorption of certain nutrients, accompanied by good technological properties (Grajek et al., 2016).

Stefanis et al. (2016) and Kaur et al. (2015) listed basic criteria that a microorganism should fulfill in order to be characterized as a probiotic among which is their ability to withstand and survive physiological stress of acidic pH within the GIT and possession of antimicrobial activity against potentially pathogenic bacteria. The pH tolerance of probiotics is well documented in the literatures (Sahadeva et al., 2011, Hassanzadazar et al., 2012). The aim of this study is to isolate potential probiotic isolates from locally fermented and ready to eat food and evaluate them for probiotic characters.

Antimicrobial metabolites secreted by LAB mitigate the proliferation of pathogens within the GIT. The preservative potentials of LAB in food matrix is attributed to the production of antimicrobial metabolites including organic acids and bacteriocins

(Macaluso et al., 2016). Hawaz (2014) reported the antimicrobial activities of LAB against pathogenic bacteria such as: *Bacillus* sp., *Escherichia* sp., *Pseudomonas* sp., *Salmonella* sp. and *Staphylococcus* sp.

LAB-probiotics can reduce the spread of pathogenic bacteria by mechanisms involving production of inhibitory compounds and competitive exclusion (Vieco-Saiz et al., 2019). The antimicrobial effect of LAB may be due to the production of antimicrobial peptides (AMPs) or small organic molecules such as organic acids, ethanol, diacetyl, carbon dioxide, hydrogen peroxide and smaller peptides, that is, bacteriocins (Liao and Nyachoti, 2017). Several bacteriocins have been shown to act in synergy with conventional antibiotics (Cavera et al., 2015, Wolska et al., 2012), thus reducing bactericidal concentrations and reduction in their undesirable side-effects while some produced by Gram positive bacteria are active against viruses (Ben Lagha et al., 2017)

Different techniques had been reported for assaying the antimicrobial activity of LAB. Some examples that were previously reported are delayed antagonism, disc diffusion assay, spot on lawn or agar overlay (immersion bioautography), well diffusion assay and paper disc methods (Soomro et al., 2007; Macaluso et al., 2016; Balouiri et al., 2016; Oliveira et al., 2017), critical dilution assays (Barbosa et al., 2016), flip-streak method (Lewus and Montville, 1991). The aim of this study was to isolate, evaluate the antimicrobial activity and pH tolerance of LAB isolated from locally consumed probiotic food in Nsukka, Enugu State, Nigeria.

MATERIALS AND METHODS

Chemicals and reagents

A 70% ethanol, 3% hydrogen peroxide, dilute sodium hydroxide and dilute HCl solution were prepared from their stock solutions. Gentian violet, Lugol's iodine, safranin, immersion oil, were obtained from their manufacturers and prepared as needed. All chemicals used for the study were of analytical grade.

Food samples and pathogenic organisms

Food samples

Food samples included processed Ogiri (*Sesamum indicum* L.), Okpeye (Iron plant - *Prosopis africana* endosperm), Pap or Akamu (*Zea mays* steep liquor), Ukpaka (Oil bean - *Pentaclethra macrophylla*) and Yoghurt (Aqua Rapha®) were used. The samples were coded as: O (Ogiri), OK (Opkeye), P (Pap), U (Ukpaka) and Y (Yoghurt) respectively.

Pathogens

Pathogens included *Bacillus* *Pseudomonas aeruginosa*,

Salmonella sp, *typhimurium*, *Staphylococcus aureus* and *Escherichia coli*. All pathogenic isolates were obtained from the Department of Pharmaceutical Microbiology and Biotechnology laboratory, UNN.

Media used

Media used in this study includes De Man Rogosa and Sharpe (MRS) agar, De Man, Rogosa and Sharpe (MRS) broth, Nutrient agar.

Collection of food samples

A total of 60 samples comprising of 12 samples per food type (Okpeye, Ogiri, Pap, Ukpaka and Yoghurt) were randomly procured between May and June, 2018 from Ikpa market in Nsukka L.G.A, Enugu State, Nigeria. Samples were transferred in an icebox (-4°C) to the laboratory using sterile containers.

Isolation of potential lactic acid bacteria

A tenfold serial dilution and a spread plate method was used as reported in previous study (Berebon et al., 2019). Based on visual examination a distinct colony was picked with a sterile wire loop and transferring aseptically into a sterile MRS agar plate by quadrant streak method. The plates were incubated anaerobically at 37°C for 24 to 48 h. Each isolate was tested for presence of catalase production. Only isolates which tested negative for catalase production were selected and stocked on MRS slants in a bijou bottle for further studies.

Colony characteristics of lactic acid bacteria

Each colony was observed and the following features were determined: Margin, size, surface, elevation, form recorded. A total of 20 isolates with LAB morphologies on MRS agar plates were selected and coded as: O₁, O₂, O₃, O₄; OK₁, OK₂, OK₃, OK₄; P₁, P₂, P₃, P₄; UK₁, UK₂, UK₃, UK₄ and Y₁, Y₂, Y₃, Y₄.

Preparation of standard inoculum

Each potential probiotic isolate was inoculated into 10 ml MRS broth in test tubes. The cultures were incubated for 24 h at 37°C. These cultures were used as the standard inoculum for further experiments.

pH tolerance test

The pH of duplicate tubes of 10 ml of MRS broth was adjusted to pH 1.5, 2.5 and 3.5 using 1 M HCl and 1 M NaOH and autoclaved at 121°C for 15 min. A 100 µl of each probiotic culture was added to each tube and adjusted to 0.5 McFarland standard. The absorbance readings of each isolate was taken at 0 and 4 h using UV/Visible spectrophotometer (Spectrumlab 725s, England) at λ_{max} of 600 nm. Sterile MRS broth pH 7.0 (control) was used as blank.

Antimicrobial activity assay

The antibacterial activity of the LAB isolates against pathogenic pathogens was cultured overnight in Brain Heart Infusion agar. A 100 µl of the pathogenic bacteria (adjusted to 0.5 McFarland

standards using sterile saline) were spread on nutrient agar plates and the 100 µl of LAB were added to wells and allowed to diffuse at room temperature into the agar. Subsequently, the inoculated plates were incubated at 37°C for 24 to 48 h. The antimicrobial activity of each probiotic strains was evaluated by measuring the inhibition zone diameter (IZD) around probiotic growth.

RESULTS AND DISCUSSION

Table 1 shows the colonial morphologies of the potential probiotic bacteria isolates belonging to the genus *Lactobacillus* with small circular colonies, Gram positive, non-motile anaerobic and catalase negative.

The survival patterns of each isolate at different acidic pH conditions of 1.5, 2.5 and 3.5 (Figures 1 to 3) indicated that the various potential *Lactobacilli* survived more generally at low acidic condition with the general decrease in survival at pH: 1.5 > 2.5 > 3.5. This observation corroborated the findings of Tokatli et al. (2015) that 35 to 85 % and 33 to 64 % strains of *L. plantarum* and *L. brevis* survived at pH 2.5 for 4 h. However, Succi et al. (2017) noted that *L. plantarum* only exhibited a slight growth at pH: 3.5 and 4.0 but not at 3.0. The isolates are acid tolerant, a property characteristic of probiotic bacteria. Taken together, these results show that pH tolerance is strain - dependent among *Lactobacillus* spp. At pH 1.5, all the probiotic isolates showed increase in growth after 4 h except Y₄ which had remarkable reduction in growth turbidity. Other workers have reported survivability of *Lactobacillus* spp. at acidic pH (Succi et al., 2017; Ngov et al., 2014; Miller et al., 2011). Cotter and Hill (2003) and De Angelis and Gobbetti (2011) reported that acidotolerant observation in lactococci may be due to their innate pH homeostatic system such as: Arginine deiminase (ADI), H⁺-ATPase proton pump, and the glutamate decarboxylase gene (GAD) which stabilizes the acid stress.

At pH 2.5, only isolate O₁ showed growth reduction with time while at pH 3.5 all the isolates showed increase in growth with time. According to Sahadeva et al. (2011), the survival pattern of the probiotic isolates at acidic pH conditions is very important because it determines the choice of probiotics used in management of gastrointestinal infections. Isolates that tolerate high acidic condition are good agents used in the management of infections of susceptible bacterial infections of the gastrointestinal tract such as: Diarrhea, peptic ulcer, colitis and salmonellosis (Vantsawa et al., 2017).

The antibacterial activity (Figure 4) of each of the isolates against *Bacillus* sp., *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Escherichia coli* show that isolate - O₄ had the widest inhibition zone diameter (IZD) of 13 mm against *P. aeruginosa* while isolates: OK₄, UK₁, P₂, P₃, and P₄ did not inhibit any of the pathogens tested. Isolate - Y₁ had the broadest activity inhibiting all the pathogens except

Table 1. Preliminary colony characteristics, catalase test and gram reaction of LAB isolates.

Isolate	Form (shape)	Color	Elevation	Margin	Surface	Gram reaction	Catalase test
OK ₁	Circular	Milky	Convex	Entire	Smooth	Positive	Negative
OK ₂	Circular	Milky	convex	Entire	Smooth	positive	Negative
OK ₃	Circular	Milky	convex	Entire	Smooth	positive	Negative
OK ₄	Circular	Milky	convex	Entire	Smooth	positive	Negative
UK ₁	Circular	White	convex	Entire	Smooth	positive	Negative
UK ₂	Circular	White	convex	Entire	Smooth	positive	Negative
UK ₃	Circular	White	convex	Entire	Smooth	positive	Negative
UK ₄	Circular	White	convex	Entire	Smooth	positive	Negative
P ₁	Circular	Milky	convex	Entire	Smooth	positive	Negative
P ₂	Circular	Milky	convex	Entire	Smooth	positive	Negative
P ₃	Circular	Milky	convex	Entire	Smooth	positive	Negative
P ₄	Circular	Milky	convex	Entire	Smooth	positive	Negative
Y ₁	Circular	Milky	convex	Entire	Smooth	positive	Negative
Y ₂	Circular	White	Flat	undulate	Smooth	positive	Negative
Y ₃	Circular	White	Flat	Entire	Smooth	positive	Negative
Y ₄	Circular	White	Flat	Entire	Smooth	positive	Negative
O ₁	Circular	Milky	convex	Entire	Smooth	positive	Negative
O ₂	Circular	Milky	convex	Entire	Smooth	positive	Negative
O ₃	Circular	Milky	convex	Entire	Smooth	positive	Negative
O ₄	Circular	Milky	convex	Entire	Smooth	positive	Negative

OK, Okpeye; UK, Ukpaka; P, Pap or Akamu; Y, Yoghurt O, Ogiri.

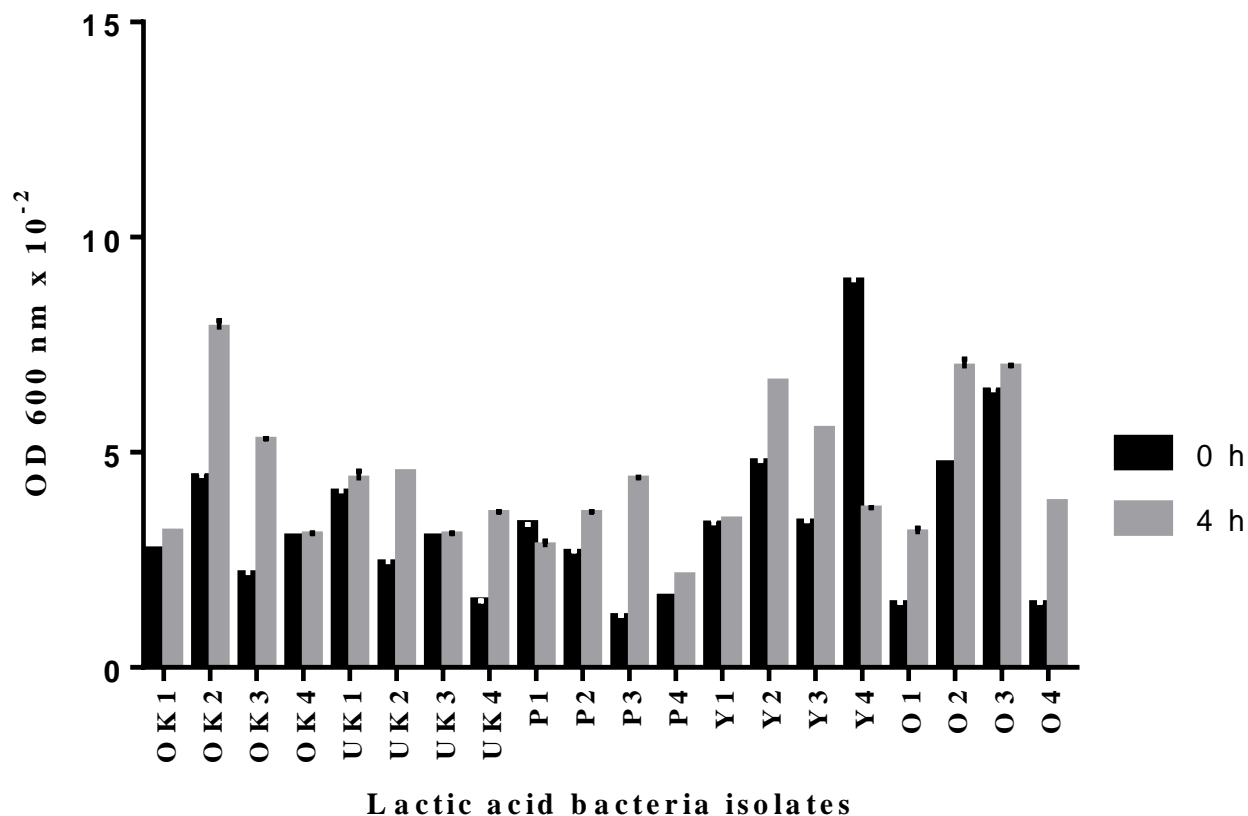


Figure 1. Tolerance of LAB at pH 1.5. OK, Okpeye; UK, Ukpaka; P, Pap or Akamu; Y, Yoghurt O, Ogiri.

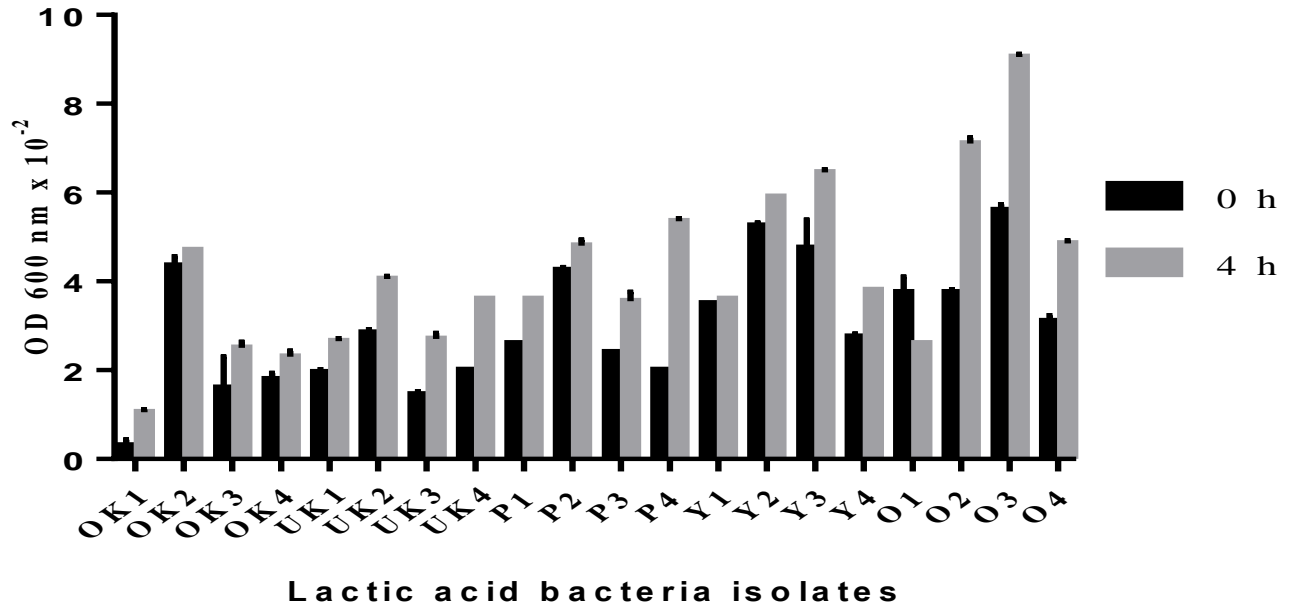


Figure 2. Tolerance of LAB isolates at pH 2.5. OK, Okpeye; UK, Ukpaka; P, Pap or Akamu; Y, Yoghurt O, Ogiri.

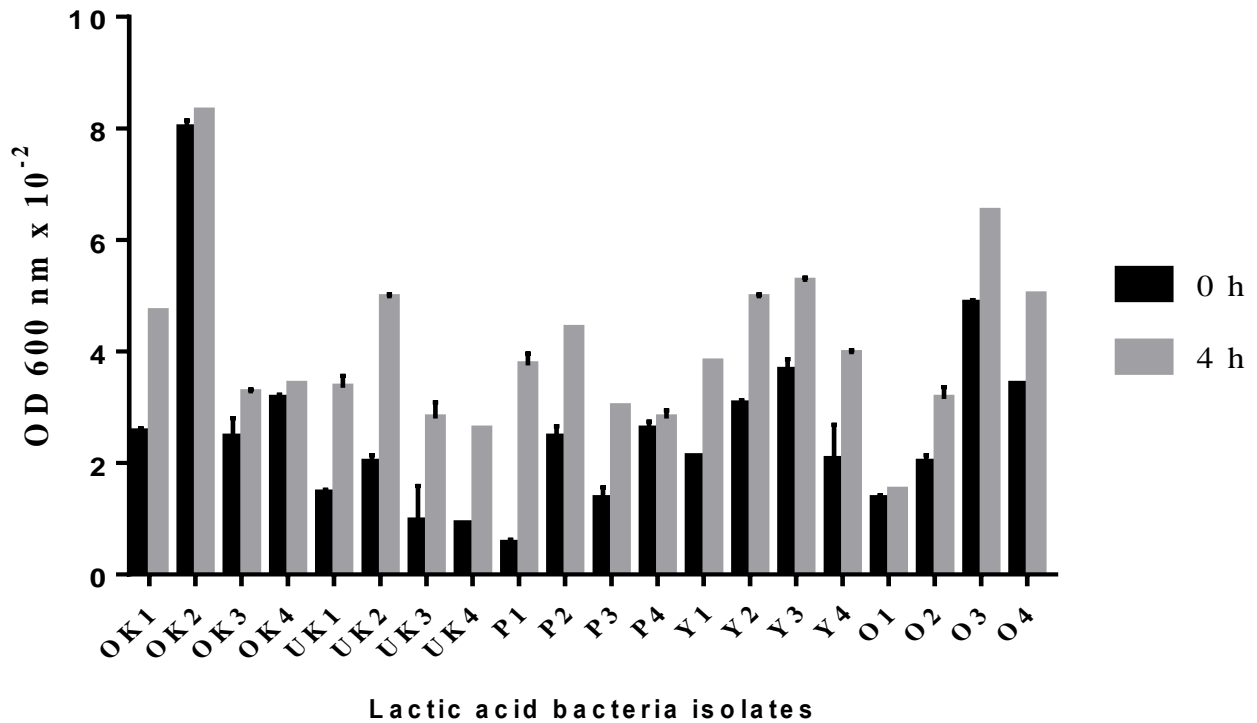


Figure 3. Tolerance of LAB at pH 3.5. OK, Okpeye; UK, Ukpaka; P, Pap or Akamu; Y, Yoghurt O, Ogiri.

S. typhimurium. The cause for antipathogenic activity of isolates O₄ and Y₁ against *Bacillus* sp., *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Escherichia coli* is not known and requires

further study. It might be due to formation of either bacteriocins, organic acids (acetic acid, lactic acid) or hydrogen peroxide all having desirable properties as sustainable alternatives to antibiotics. The results of the

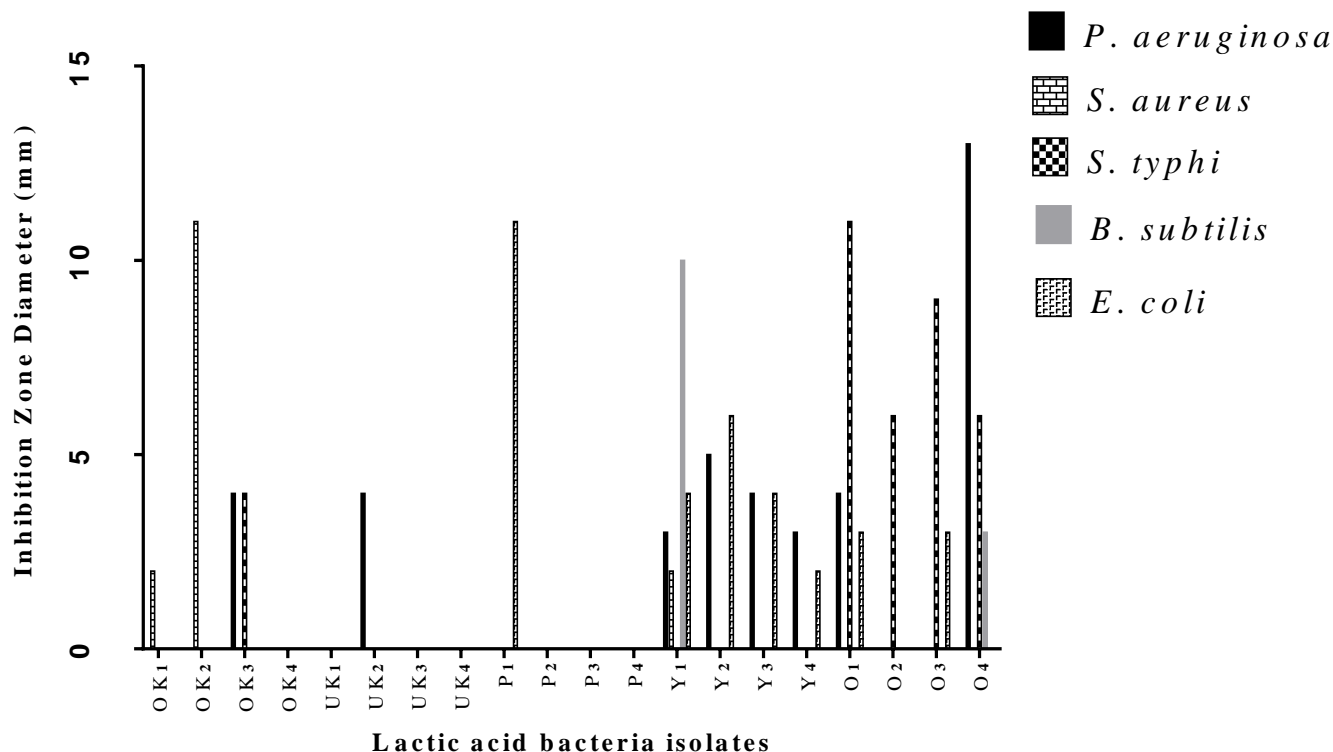


Figure 4. Antibacterial activity of LAB isolates. OK, Okpeye; UK, Ukpaka; P, Pap or Akamu; Y, Yoghurt O, Ogiri.

antimicrobial activity of Lactobacilli against enteropathogens are corroborated by another author who reported their *in vitro* inhibitory activity on *Bacillus* sp., *P. aeruginosa*, *Staph. aureus*, *E. coli* and *S. typhimurium* (Abubakr, 2018). Among the pathogens tested, *Bacillus* sp. had the highest resistance against the probiotic isolates being sensitive to only two isolates. The high resistance profile recorded for *Bacillus* sp. may be attributed to their tolerance to bile and acidic pH concentrations, biofilms formation potentials, versatile intrinsic ability to produce protease and lipases that are stable at high temperature. *E. coli* and *P. aeruginosa* showed highest sensitivity with more than eight isolates inhibiting their growth.

Conclusion

Potential *Lactobacillus* species were isolated from the following fermented food products: Ukpaka, Ogiri, Okpeye, Pap and Yoghurt from Ikpa market in Nsukka metropolis. The isolates had morphological characteristics of known features of *Lactobacillus* bacteria. The *in vitro* antibacterial activity of the isolates showed that some had ability to inhibit selected pathogenic organisms, an indication of their potential relevance in therapeutic treatment of infectious diseases. Survival of these probiotics at acidic pH

condition validate them to be potentially useful either as adjuvant or active ingredients in preparations that are targeted to the stomach and upper duodenal regions of the gastrointestinal tract (GIT). Further investigations and molecular characterization of the novel potential probiotic isolates and identification to their species level for subsequent pharmaceutical applications are in progress.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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