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Identification of Lactic acid Bacteria isolated from selected Nigerian Foods and Comparison of their Bacteriocins Activities

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Abstract: Three fermented food samples used for this study were fura, wara and nunu, after collection and inoculation in peptone water, these samples were kept in refrigerator at 5^{o}C for further analysis. After serial dilutions, 0.2 ml of 10^{-4} dilution factor from each sample was transferred into 20 ml of sterile De-Mann, Rogosa and Sharpe agar (MRS) medium, the plates were labeled as "F (fura), W (wara) and N (nunu)" respectively. All distinct and well isolated colonies were sub-cultured and examined for various morphological characteristics. Different species of *Lactobacillus*, *Leuconostoc* and *Lactococcus* isolated were subjected to biochemical tests to identify the species. Based on the morphological appearance three genuses of lactic acid bacteria from the samples were isolated. These bacteria were sub cultured in MRS broth and incubated at 32^{o}c, after 24hrs, they were centrifuged at 3000 rpm for 15 minutes. The supernatant containing the bacteriocins were collected. The antimicrobial assay was performed on six bacteria (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Klebsiella* spp, *Pseudomonas aeruginosa* and *Proteus mirabilis*) using agar well diffusion as described by Schillinger and Lucke (1989). After incubation, the plates were examined for zones of inhibition. Three replicates were made for each bacterium and the average activity was recorded. The results showed varied inhibitory effects of the bacteriocins on the clinical isolates, when these bacteriocins activity was compared with standard drug, (15µg Gentamicin) an increased activity was observed in gentamicin. In conclusion, the zones of inhibition observed in this research strongly suggest that bacteriocins from lactic acid bacteria are potent in treating diseases caused by these organisms

Keywords: *Acidophiline, bacteriocin, Bifidobacterium, bulgarican, heterofermenter, homofermenters, lactic acid bacteria (LAB), nisin.*

INTRODUCTION

Lactic acid bacteria (LAB) are groups of related bacteria that produce lactic acid as a result of carbohydrate fermentation [1]. These microbes are broadly used by man in the production of fermented food products, such as yogurt (*Streptococcus spp.* and *Lactobacillus spp*.), cheeses (*Lactococcus spp*.), sauerkraut (*Leuconostoc spp*.), sausage, vegetables and meats. LAB produce substances that have antagonistic activity against pathogenic and spoilage bacteria found in food products and can be used as starter cultures for cured meat products. LAB are Gram-positive, aerobic, micro-aerobic or facultative anaerobic microorganisms with variable metabolic characteristics, such as the production of diacetyl, hydrogen peroxide, lactic acid and others[2].

Most species have multiple requirements for amino acids and vitamins, thus LAB are generally abundant only in communities where these requirements can be provided[3]. They are often associated with animal oral cavities and intestines (e.g. *Enterococcus faecalis*) plant leaves (*Lactobacillus, Leuconostoc*) as well as decaying plant or animal matter such as rotting vegetables, feacal matter, compost, etc[1] [4]. LAB are used in the food industry for several reasons. Their growth lowers the carbohydrate content and the pH of foods they ferment due to lactic acid production, it is this acidification process which is one of the most desirable side effects of their growth. The pH may drop to 4, low enough to inhibit the growth of most other microorganisms including the most common human pathogens, thus allowing these foods prolong shelf life. The acidity also changes the texture of the foods due to precipitation of some proteins [4], and the biochemical conversions involved in growth enhance the flavour. The fermentation is self-limiting due to the sensitivity of LAB to such acidic pH. Most are free-living or undergo commensalism relationship where they act as normal flora of man and animals in the oral cavity, intestinal tract and vagina, where they play a beneficial role, though some are opportunistic pathogens [5].

Lactic acid bacteria are widely studied and used in the food industry[1, 6]. LAB are important because they can produce substances with pleasant sensory characteristics during meat processing [7]. Moreover, the action of various antimicrobial compounds produced during the fermentation process, such as lactic acid, acetic acid and propionic acid, may result in an unfavourable environment for the development of spoilage and pathogenic microorganisms[8]. These microorganisms, such as members of the families *Enterobacteriaceae* and *Pseudomonadaceae*[9], may occur in cured meat products when the hygienic conditions are poor during the manufacturing process. LAB are efficient in producing substances with inhibitory activity against microorganisms present in foods. Currently the major interest in using lactic acid bacteria is that the undesirable microorganisms in foods may be inhibited by one of the bacteriocin-producing LAB. Bacteriocins are microbial compounds of a proteic nature that have a bactericidal or bacteriostatic effect on other closely related species [10]. Many types of bacteriocins have been characterized and they have considerable potential for application in foods, aiming at the quality and safety of these foods [11]. According to Nascimento *et al*. (2008) [9], bacteriocins can be used in three different ways in fermented foods: *in situ* production by the addition of a bacteriocinogenic lactic culture, as a co-culture and by the direct addition of the bacteriocin.

Recent taxonomic revisions suggest that lactic acid bacteria comprise the following genera*: Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus,Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* [12]. Orla-jenson classified LAB according to morphologyl, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentration, acid or alkaline tolerance. Based on sugar fermentation patterns, two broad categories are known homofermenter and heterofermenter. Homofermenters are LAB that converts hexoses to produce almost quantitatively lactic acid, E.g. *Enterococcus faecalis, Lactobacillus acidophilus, Streptococcus bovis, Lactococci spp* and *Vagococci spp*, etc. Heterofermenters are LAB that converts sugars to lactic acid, ethanol and CO_2 they include *Lactobacillus brevis, Lactobacillus fermentum, Leuconostoc dextranicum, Weissella spp*, etc.

In 2008, Axelsson [13] highlighted a few pathogenic LAB for animals, most notably some members of the genus *Streptococcus*. In humans, *Streptococcus pyogenes* is a major cause of disease (strep throat, pneumonia, and other pyogenic infections, scarlet fever and other toxemias), *Streptococcus pneumoniae* causes lobar pneumonia, otitis media and meningitis; some *Viridans* and non-haemolytic oral *Streptococci* play a role in dental caries and may be an insidious cause of endocarditis [14]. Some strains of *Lactobacillus* spp. and other lactic acid bacteria possess potential therapeutic properties including anti-inflammatory and anti-cancer activities, as well as other features of interest. A study by researchers from the Beth Israel Deaconess Medical

Center and UCLA in 2009 demonstrated the protective effects of some strains of these bacteria for anti-tumor and anti-cancer effects. Dietary administration alleviates the risks of certain types of cancers and suppress colon tumor incidence in mice. For a few strains, oral administration effectively reduced DNA adduct formation, ameliorated DNA damage and prevented putative preneoplastic lesions such as aberrant crypt foci induced by chemical carcinogens in the gastrointestinal tract. Reports also indicated that some cultures administered to animals inhibited liver, colon, bladder, and mammary tumors, highlighting potential systemic effects of probiotics with anti-neoplastic activities[12] [15].

Lactobacilli can also be used to restore particular physiological balance such as in the vaginal eco-system. Their role is to physically protect the vaginal epithelium by building a thick layer separating the epithelium from pathogens[16] also to physiologically keep the balance of the vaginal ecosystem in maintaining the pH at 4.5 [17] and generating hydrogen peroxide against pathogens. *Lactobacilli* are highly tolerant to low pH and can easily maintain low pH and protect the vaginal eco-system from Gram-negative and Gram-positive bacteria [18]. Some *Lactobacillus* species have been associated with dental caries .The *Lactobacillus* count in saliva has been used as a "caries test" for many years. This is one of the arguments used in support of the use of fluoride in toothpaste and lozenges[19]. *Lactobacilli* characteristically cause existing carious lesions to progress, especially those in coronal caries.

BACTERIOCIN

Bacteriocins are microbial compounds of a proteic nature that have a bactericidal or bacteriostatic effect on other closely related species [20],[21]. Many types of bacteriocins have been characterized and they have considerable potential for application in foods, aiming at the quality and safety of these foods [22]. Some bacteriocins produced by LAB, such as nisin, inhibit not only closely related species but are also effective against food-borne pathogens and many other Gram-positive spoilage microorganisms [23]. For this reason, bacteriocins have attracted considerable interest for use as natural food preservatives in recent years, which have led to the discovery of an ever increasing potential source of these protein inhibitors. Bacteriocin of *Lactobacillus plantarum* isolated from the traditional pearl millet based African fermented food “ben saalga” was tested for inhibition of food poisoning and pathogenic bacteria in MRS broth and in malted millet flour slurry. The bacteriocin completely eliminated *Bacillus cereus, Escherichia coli* O157:H7 and *Salmonella enterica* cells within 48hrs incubation at 22–30°C. A much lower inhibition was observed at 15 °C. The inhibitory effect of bacteriocin on the above-mentioned target bacteria was corroborated in the malted millet flour slurry, reducing viable cell counts below detection levels after 8hrs storage for *B. cereus* or after 24 hrs for *S. enterica* and 48 hrs for *E. coli.*

Since bacteriocins are obtained from foods which normally contain LAB, they have unknowingly been consumed for centuries. A study of 40 Wide-type strains of *Lactococcus lactic* showed that 35 produced nisin [16]. Nisin is the only bacteriocin with GRAS (Generally Regarded as Safe) status for use in specific foods and this was awarded as a result of a history of 25 years of safe use in many European countries and was further supported by the accumulated data indicating its non-toxic, non-allergenic nature. Other bacteriocins without GRAS status will require pre-market approval. Therefore, bacteriocinogenic starters especially when used in natural fermentation will most likely afford the best opportunities for the application of bacteriocins in near future. The target of bacteriocins is the cytoplasmic membrane and because of the protective barrier provided by the Lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria, they are generally active against Gram-positive cells [24],[25]. In the context of fermentation, important targets include spoilers such as species of Clostridium and foodborne pathogens including *Listeria monocytogenes*, *Staphylococcus spp*. Clostridium, Enterococcus, and *Bacillus spp*. The permeability of Gram-negative bacteria can be increased by sub lethal injury including that which can occur when using ultrahigh hydrostatic pressure (UHP) and pulsed electric field (PEF) as non-thermal methods of preservation [26]. In addition, disruption of the integrity of the outer membrane through the use of food grade chelating agents such as ethylenediamine tetraacetic acid (EDTA) and citrate which bind magnesium ions in the LPS layer can increase the effectiveness of bacteriocins against Gram-negative bacteria[27],[28]. Many bacteriocins are most active at low pH [29] and there is evidence that bacteriocinogenic strains can be readily isolated from fresh and fermented foods[27]. Strains may naturally produce more than one bacteriocin and heterologous expression of bacteriocins has been demonstrated in constructed strains[30]. Protein engineering has led to the development of nisin derivatives with altered

antimicrobial activities or greater solubility at pH 6 than the wild-type nisin[31]. An advantage of bacteriocins over classical antibiotics is that digestive enzymes destroy them.

MATERIALS AND METHOD

Collection of samples

The sample of fura used for this study was obtained from the Fulani village behind crown estate, Okada, Edo State, Nigeria while the Wara and Nunu were collected directly from the Fulani's living in neighboring village around Ore town in Ondo State Nigeria. To maintain its originality, peptone water was prepared in the sterile bottle to collect the samples and immediately kept in the laboratory refrigerator at 5^oC for further analysis.

Serial dilution of the samples

Stock solutions of 10% of fermented fura, nunu and wara was prepared by dissolving 1gram each of the samples in 10ml of sterile peptone water in a test tube, this was further serially diluted to obtain concentrations of 10^{-4}, 0.2 ml of 10^{-4} dilution from each sample was transferred into 20 ml of sterile DeMann Rogosa and Sharpe agar (MRS), it was thoroughly mixed before pour plating. The plates of wara, fura and nunu labeled as 'W, F and N' respectively were allowed to set and incubated anaerobically at 32^oC for 48hrs, the different colonies of the bacteria observed.

Isolation and identification of the bacteria: Distinct and well isolated colonies were sub-cultured and examined by Gram staining for various sizes, shapes, colours and texture, a series of tests such as catalase, oxidase, indole, motility, Nitrate reduction and sugar fermentation were also carried out to identify the bacteria.

Test Bacteria

The test organisms used in this study were *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp, *Pseudomonas aeruginosa* and *Proteus mirabilis* and were provided by University College Hospital (UCH), Ibadan, they were grown on differential and selective media. Distinct and well isolated colonies were sub-cultured. Microscopy was performed for various sizes, shapes, colours and texture, a series of biochemical tests such as catalase, oxidase, indole, MRVP, citrate and urease to identify the organisms

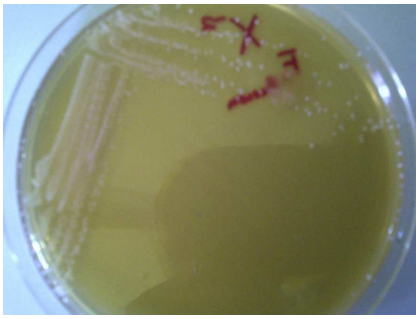
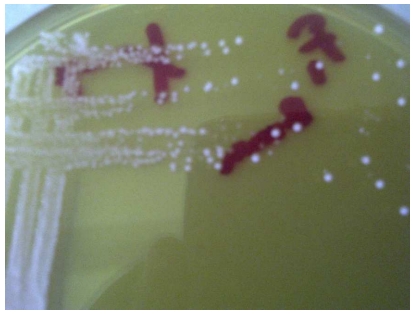
Detection of antimicrobial activity

Eight test-tubes of 5ml each of MRS broth were prepared and inoculated with eight isolates obtained from the three samples labeled as W1, W2, W3, F1, F2, N1, N2 and N3. After 24hrs, they were centrifuged at 3000 rpm for 15 minutes, the more dense organelles settle at the bottom of the test tube while the supernatant that contains the bacteriocin forms the upper layer. The antimicrobial assay was performed using the agar well diffusion described by Schillinger and Lucke (1989) [32].The bacteria were seeded into the molten agar poured in plates and left to set, 0.2ml of bacteriocin obtained was introduced into the wells bored with 5 mm cork borer. The plates were incubated right side up at 32^0C for 48hrs after which the plates were examined for zones of inhibition. Three replicates were made for each bacteria and the average activity was recorded. The antimicrobial activity of the bacteriocins was compared with 15µg of Gentamicin.

RESULTS

Description of bacterial growth on plates

There were various distinct colonial morphologies ranging from cream to white colonies, the microbes are small, smooth and grainy-looking colonies as shown below-

Plates showing different colonies

Table 1: showing various tests determining morphological and biochemical activities

Isolate	Gram stain	Catalase Test	Coagulase Test	Indole Test	Oxidase Test	Spore Test	Motility Test	Growth at 4% NaCl
W1	+	-	-	-	-	-	-	+
W2	+	-	-	-	-	-	-	+
W3	+	-	-	-	-	-	-	+
F1	+	-	-	-	-	-	-	+
F2	+	-	-	-	-	-	-	+
N1	+	-	-	-	-	-	-	+
N2	+	-	-	-	-	-	-	+
N3	+	-	-	-	-	-	-	+

A total of 8 isolates were obtained based on their colonial, morphological and biochemical activities

Table 2: morphological characteristics

Isolates	**Color**	**Shapes**	**Morphology**
W1	white	rods	Clustered chains
W2	Dirty-white	cocci	Clustered straight
W3	Dirty-white	cocci	Clustered
F1	Cream	cocci	Thick, spherical, chain
F2	white	rods	Thick, short rods, chain
N1	white	rods	Chains in groups
N2	cream	cocci	Long cocci chains
N3	white	rods	Thick but short bacilli

KEY- W1 *Lactobacillus spp*, **W2** *Leuconostoc spp*, **W3** *Lactococcus spp*, **F1** *Leuconostoc spp*, **F2** *Lactobacillius spp*, **N1** *Lactobacillus spp*, **N2** *Leuconostoc spp*

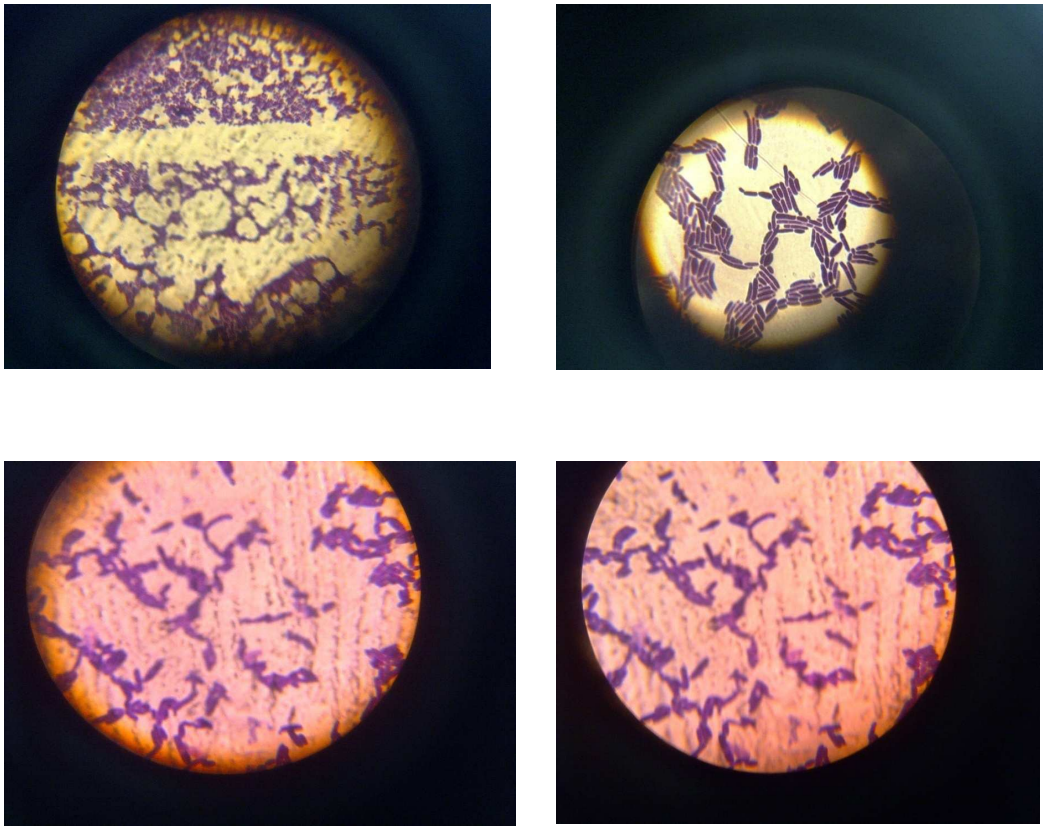


Plate 2: Microscopical Morphology of some of the isolates

Table 3: result of sugar fermentation

	ISOLATES							
sugars	**W1**	**W2**	**W3**	**F1**	**F2**	**NI**	**N2**	**N3**
Gelatin	-	-	-	-	-	-	-	
glycerol	-	-	-	-	-	-	-	-
ribose	-	+	+	+	+	-	+	-
D-xylose	-	+	+	-	+	-	+	-
D-glucose	+	+	+	+	+	+	-	+
D-fructose	+	+	+	+	+	+	+	+
D-mannose	+	+	+	-	+	-	+	+
N-A.glucosamine	+	+	-	-	+	-	-	-
Amygdalin	-	-	+	+	+	-	-	-
Salicine	+	+	+	-	+	-	+	-
Manitol	-	-	-	+	+	-	-	-
Maltose	+	-	+	+	+	-	-	+
Lactose	-	-	-	+	+	+	-	+
Galactose	+	+	+	+	+	-	-	-
Trehalose	+	+	-	+	+	-	+	-
Saccharose	+	-	+	-	+	-	-	+

Table 4: The results of susceptibility tests of various bacteriocins extracted from LAB

	ZONE OF INHIBITION(mm)								
Test organisms	**W1**	**W2**	**W3**	**F1**	**F2**	**N1**	**N2**	**N3**	**CN (**30µg**)**
Bacillus subtilis	11	-	-	-	10	-	-	14	5.5
Staphylococcus aureus	14	12	9	-	9	12	13	12	8
Escherichia coli	-	10	-	-	12	10	-	12	7.5
Klebsiella spp	12	12	-	9	-	9	12	10	5
Pseudomonas aeruginosa	-	-	-	-	10	-	-	-	6.8
Proteus mirabilis	10	11	-	-	13	12	13	11	7

N.B: Values represent mean of duplicates.

KEY

CN Gentamicin -- means no inhibition
W1 *Lactobacillus spp*, **W2** *Leuconostoc spp*, **W3** *Lactococcus spp*, **F1** *Leuconostoc spp*, **F2** *Lactobacillius spp*, **N1** *Lactobacillus spp*, **N2** *Leuconostoc spp*

DISCUSSION

In the current research, strains of lactic acid bacteria showing antimicrobial activity to one or more test organisms were isolated. A very low number of LAB were detected from each of the three samples. Products manufactured without the addition of starter cultures are often more susceptible to spoilage and to pathogenic microorganisms. LAB and their final products of metabolism act as bio-preservatives increasing the shelf-life of foods[4, 33], and reducing the risks of foodborne diseases [8],[34]. Thus the presence LAB may confer desirable qualities and increase the safety of fermented products [1] ,[35].

The eight colonies of lactic acid bacteria isolated from fermented samples were spherical and colour ranging from cream to white with an average diameter of 2.0 mm. The isolates *Lactobacillus*, *Leuconostoc* and *Lactococcus* are Gram positive, catalase negative and grew at 4% NaCl. Table 4 above shows zones of inhibition to the various bacteriocins and this indicates the efficacy of these proteins on the test bacteria used. *B.subtilis* was only sensitive to bacteriocin of *Lactobacillus spp* isolated from wara, fura and nunu and resistant to the other bacteriocins. In all the bacteria used, *S aureus* was seen to be most susceptible to the bacteriocins, there were zones of inhibition ranging from 9-14mm except the bacteriocin of *Lactococcus spp* from wara and *Leuconostoc spp* from fura that *Staphylococcus aureus* and *Klebsiella* spp were resistant to respectively. *E.coli* on the other hand was resistant to bacteriocins of *Lactobacillus* and *Lactococcus* from wara, *Leuconostoc* from wara and nunu respectively but susceptible to the other bacteriocins with varied zones of inhibition. The bacteriocins of *Lactococcus* isolated from wara and *Lactobacillus* from fura do not have any antimicrobial effect on *Klebsiella spp*, while other bacteriocins were potent. *Pseudomonas aeruginosa* was resistant to all the bacteriocins but susceptible to bacteriocin of *Lactobacillus spp* from fura with zone of inhibition of 10mm. Bacteriocins of *Lactococcus spp* from wara and *Leuconostoc spp* from fura did not have any activity on *Proteus mirabilis* but this bacterium was resistant to bacteriocins of *Lactobacillus spp* isolated from wara, fura and nunu, including *Leuconostoc* from wara and nunu. From these result it was observed that bacteriocins of *Lactococcus spp* from wara and *Leuconostoc spp* from fura were least active against the test organisms, while bacteriocins of *Lactobacilli* from fura and nunu were most potent on the organisms. Gentamicin used as positive control at the concentration of 15µg had antimicrobial activities against all the bacteria used with varied zones of inhibition.

CONCLUSION

The bacteriocins from the various LAB demonstrated a promising and potent antimicrobial activities which can be harnessed for medical and commercial purpose especially in the treatment of diseases caused by pathogenic organisms like urinary tract infection, diarrhea, meningitis in infants, pulmonary tract infections, burns, wounds, blood infections, skin infections (e.g. boils, respiratory disease (e.g. sinusitis), and food poisoning. [36]. LAB have displayed numerous antimicrobial activities through the production of bacteriocins and other compounds, such as ethanol, H_2O_2, organic acids, diacetyl, reuterin, [37] etc. Several bacteriocins with industrial potential have been purified and characterized. Application of bacteriocin-producing starter cultures in fermented foods has been studied during *in vitro* laboratory fermentations as well as on pilot-scale level. The promising results of these studies underlined the important role that bacteriocinogenic lactic acid bacteria may play medically and in food industries.

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