CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM TRADITIONALLY FERMENTED FOODS AGAINST SOME SELECTED FOOD PATHOGENS.

ABSTRACT

The antimicrobial compounds present in lactic acid bacteria isolated from some traditionally fermented foods, ogi, ugba and yogurt and their antimicrobial activity against some selected food pathogens was revealed in this research work. 15 food samples consisting 5 each of ogi, ugba and yogurt were screened for lactic acid bacteria. 9 organisms were isolated from the food samples and characterized both phenotypically and genotypically using PCR amplification and 16SrRNA DNA sequencing which revealed that 2 of the isolates were *Lactococcus spp*, 5 *Lactobacillus spp*, 1 *Bacillus spp* and 1 *Streptococcus spp*. The genotypic characterization revealed that the lactic acid bacteria isolates *Lactococcus lactissubsp lactis* 0711XYBLS,*Lactobacillus fermentum* CS19, *Lactococcus lactis* and *Lactobacillus fermentum* were closely related at 99% evolutionary distance. For the sole purpose of this research, *Lactococcus lactis subsp lactis* 0711XYBLS and *Lactobacillus fermentum* CS19 were utilized to determine their antimicrobial potentials against selected food pathogens. Antimicrobial metabolites, diacetyl, hydrogen peroxide and lactic acid were produced by the isolated lactic acid bacteria at varying concentrations. The selected latic acid bacteria isolates were tested against food pathogens and their clear zones of inhibitions were noted. *Lactococcus lactis subsp lactis* 0711XYBLS isolated from ogi showed the highest Gram positive and Gram negative food pathogens ranging from 18.67 ± 1.15 to 12.67 ± 0.57 and 16.33 ± 0.56 to 8.00 ± 6.08 respectively whereas *Lactobacillus fermentum* CS19 showed Antimicrobial activity for Gram positive food pathogens at 16.00 ± 1.00 to 8.67 ± 1.15 and 15.33 ± 1.15 to 8.67 ± 0.57 in Gram negative food pathogens. Between the pathogens, a significant difference was recorded at a significant level of $p<0.05$. However, both organisms portrayed a great deal of antimicrobial activity against the selected food pathogens and can serve as novel antimicrobial agents. This potentials can be harnessed in food industries on a large scale as biopresevatives instead of the use of chemical preservation which may pose detrimental health risk on its consumers.

KEYWORDS: Lactic acid bacteria, traditionally fermented foods, antibacterial activity, food pathogens, probiotics, food preservation

INTRODUCTION

Awareness on food safety and hygiene has increased over the years, hence, food poisoning and food borne-illnesses is a major public health concern in developing countries. Every year 40,000 people die due to food borne diseases (WHO, 2015) although the number of people that suffered from food borne diseases are higher in the under developed and developing countries, the number is nonetheless significantly large in the western countries, such as the USA (Mead *et al.*,1999). The common symptoms of these diseases include vomiting, bloating, stomach ache, flatulence, and excessive fluid discharge through feces. These diseases are due to wide range of pathogens which include bacteria, virus, protozoa, and parasites (Acheson, 1999). The popular ones are *Salmonella spp*, *Vibrio cholera, Campylobacter, Helicobacter pylori, Esherichia coli, Gardialambia* and many more (Newell *et al.*, 2010).

The pathogens come in contact with food during preparation and preserving time. Improper food preparation includes cooking with contaminated water, improperly washed vegetables, fruits and raw meat and fish with blood especially. Some of the pathogens die while cooking, whereas some stay alive and cause disease in the enteric and other parts of the body. Moreover, cooking for a short time also allows pathogens to grow and strive in the food products. Also food in restaurants and particularly food from road side are the most causes of food borne diseases (Rane, 2011).

In recent years, people have become more aware of the functions “probiotic organisms”, particularly lactic acid bacteria, play in fermented foods and the bioactive chemicals they generate. In contrast, lactic acid bacteria have several antibacterial properties in fermented foods. This is mainly due to the production of bacteriocins, organic acids, ethanol, Hydrogen peroxide, Diacetyl and reuterin (Cintas *et al.,* 2001). The most common anti-microbial agent produced by Lactic acid bacteria is bacteriocin (Deegana *et al.,* 2006).

Lactic acid bacteria play an important role in food industry by increasing nutritional values of food and food safety (Adeyemo and Onilude, 2013). The antimicrobials produced by Lactic acid bacteria have been used widely as bio-preservatives and shelf life extender and has found application in many industries and various commercial purposes. Bio-preservation refers to extended shelf life and enhanced safety of foods using microorganisms or their metabolites (Ross *et al.*, 2002). In fermentations when lactic acid bacteria is present, it not only promotes the sensory characteristics of the finished product, but also the safety of the microorganisms (Leroy *et al*., 2004). Lactic acid bacteria generate lactic- and acetic-acids, propionic, sorbic, benzoic-acids, hydrogen peroxide, diacetyl, ethanol, phenolic- and proteinaceous-compounds as well as antibacterial substances like bacteriocins.

However, some LAB strains can produce antimicrobial substances other than bacteriocins. Increased interest in developing natural antimicrobial agents that may help the safety of food items has risen in recent years due of their unique bacteriocin-like inhibitory substances (BLIS) capabilities. These antimicrobial compounds from lactic acid bacteria lack amino acid sequence characterization while possessing necessary bacteriocin capabilities. There has been much research on the bacteriocins produced generally regarded as safe (GRAS) lactic acid bacteria, since they may provide a useful new method of preventing pathogens in food.Perhaps preventing microbial contamination by natural and microbiotic agents might save food waste, which could in turn lead to reduced numbers of food poisoning cases. (Galvez *et al.*, 2008). Lactic acid bacteria have antagonistic effects on food borne pathogenic and spoilage microorganism's example inhibition of *Bacillus subtilis* which contaminates bread and causes spoilage survival of *Escherichia coli* 0157;

H7 in dairy products is a potential health hazard because of the link with dairy cattle and raw milk (Saad *et al.,* 2001).

Lactic acid bacteria constitute a group of genus that has the following common features: cocci, rods and a basic composition of DNA below 50mol% G + C. Most of them are Gram-positive, mesophilic, and can grow at temperatures between 5°C and 45°C, provided that they have access to oxygen. In addition, they are unable to oxidize or break down nitrates and don't produce indole or hydrogen sulfide. The group is made up of several different types of bacteria, such as *Lactobacillus, Lactococcus, Leuconostoc, Carnobacterium, Streptococcus, Enterococcus, Aerococcus, Bifidobacterium,* and *Pediococcus* (Doyle *et al.,* 2006). It is recognized that lactic acid bacteria serve several roles, such as antitumor activity, lowering cholesterol, reducing lactose intolerance, activation of the immune system, and keeping infections out (Reid, 2006).

Based on these functions, different kinds of Lactic acid bacteria have been developed as probiotics, and the market volume of probiotics has rapidly increased. In recent years Lactic acid bacteria have been playing important role in the food and feed fermentation and preservation either as the natural microflora or as starter culture added under controlled conditions. This is due to the fact that they have been recognized as GRAS (Generally Recognized as Safe) microorganisms (Juodeikiene *et al.,* 2012).

MATERIALS AND METHODS

TEST ORGANISMS

Test organisms procured from CESLAB Analytical laboratory, Umudike that were used for the study include; *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Bacillus cereus, Shigella sp* and *Pseudomonas aeruginosa*

COLLECTION OF FERMENTED FOOD SAMPLES

About 15 fermented food samples 5 each of ogi, yorgurt, and ugba were randomly obtained from 3 different markets in Umuahia metropolis, south east, Nigeria for the isolation of Lactic acid bacteria.

SAMPLE AND MEDIA PREPARATION

For the fermented food samples, 2.5 gram each were weighed, mashed/dissolved in 25ml of sterile water and a 10-fold serial dilution was conducted and samples were collected from tubes 10^{-4} and 10^{-7}. De Man Rogosa and Sharpe broth was prepared according to the manufacturer's specifications and autoclaved at 121^{o} C for 15 minutes and then allowed to cool before inoculation of samples.

ISOLATION OF LACTIC ACID BACTERIA FROM FERMENTED FOOD SAMPLES

A 2ml of each of the sample solutions was added to 10ml MRS broth individually and incubated for 24hours at 37^{o}C. From each of the MRS broth solutions 200ml was spread plated onto MRS agar plates and incubated further. The different bacterial colonies based on their morphology were selected in a way to ensure no two colonies displayed the same characteristic feature. The plates were sealed with parafin, refrigerated at 4^{o}C and sub cultured every two weeks to avoid degeneration of the bacterial colonies. (Maragkoudakis *et al*., 2006)

MORPHOLOGICAL AND MICROSCOPIC CHARACTERIZATION OF THE BACTERIA

Using sterile techniques, MRS plates were streaked to obtain discrete colonies and were incubated for 24 hours at 37^0C. After incubation, the bacterial colonies were evaluated for size, consistency, form, margin, elevation, gram reaction, cellular arrangement, and texture.

Gram staining

Using sterile technique, a drop of saline was placed on the slide and a small amount of a bacterial colony was transferred to slide using a sterile inoculating pin. A smear was made by evenly spreading the bacteria colony by means of a circular motion of the slide surface. The smear was then flooded with crystal violet and allowed to sit for a minute before rinsing under a running tap. Afterwards, was flooded again with the Grams iodine and allowed to sit also for a minute, followed by gentle wash off under a running tap. Again the smear was decolorized using 95% alcohol for 10 seconds before washing off. After decolourization, the smear was counter stained using a secondary dye known as safranin for 1 minute before rinsing. The slide was blot dried with bibulous paper and examined under oil immersion.

BIOCHEMICAL CHARACTERIZATION OF THE BACTERIA

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen. Most of the methods were done according to Bergys manual of determinative bacteriology

a. **Catalase test**: This test was performed in order to determine the ability of isolated cultures to degrade hydrogen peroxide by producing the enzyme catalase. The test was carried out using slide method. Culture from a typical colony was placed onto a clean grease free glass slide using an inoculating needle. A drop of 3% hydrogen per oxide

solution was added to the culture and closely observed for the evolution of bubbles (Puniya *et al.,* 2014).

b. **Citrate utilization test**: The isolates were inoculated in Simmons Citrate agar incubated at 37^{o}C for 24 hours. The colour of the agar was observed (Wang *et al.,* 2008)

c. **Indole test**: This test was used to identify whether the isolate had the tryptophanse enzyme which helps to hydrolyze the amino acid tryptophan. A sterile wire loop was used to transfer the organism onto a test tube containing 5ml of peptone water and incubated for 48 hours at a temperature of 35^{0}C. Thereafter 0.5ml of Kovac reagent was added to the test tubes and allowed to stand for 15 minutes. The production of a rose spank colour indicated a positive result.

d. **Methly red test**: The test organism was introduced into a test tube containing 5ml of prepared peptone water and incubated for 48hours at 35^{0}C. After incubation, 0.5ml of methyl red was added to the test tubes and allowed to stand for 15 minutes. A production of a red colour indicated a positive result. (Holt *et al.,* 2004)

e. **Vogues-proskauer test**: This test was used to determine the ability of the test organism to produce non-acidic 2, 3-butandiol as a fermentation product from glucose. The inoculum from the pure culture was aseptically transferred into a sterile tube of Methyl-red-Vogues-Poskauer (MRVP) broth and then incubated at 35 — 37^{o}C for 24 hours. After incubation, 5 drops of Barrits A solution was added to the tube and gently shaken to get a uniformed mixture. Thereafter, another 5 drops of Barrits B solution was added before shaking the tube allowing for maximum oxygen exposure. Afterward the mixture was allowed to stand for 30 minutes and then it was observed for the development of a red colour which indicates a positive result (Holt *et al.,* 2004).

f. **Oxidase test**: This test was used to identify if the organism had the ability to produce the enzyme cytochrome oxidase. Kovacs oxidase test (Cytochrome oxidase activity). According to the method recommended by Kovacs (1956) the organisms were freshly grown on nutrient agar slants. A platinum loop was used to pick a bit of inoculum and made a compact smear on a filter paper moistened with 2-3 drops of a 1% solution of tetramethyl-p-phenylene diamine dihydrochloride (TPDD). A positive result was recorded when the smear turned violet within 10 seconds, indicating the formation of indophenol (Ref).

SCREENING OF LACTIC ACID BACTERIA

Isolates were screened to obtain Lactic Acid bacteria based on Gram staining, catalase test using 3% hydrogen peroxide and growing isolates in agar medium MRS with 0.3% $CaCO_3$.Isolates were selected and incubated anaerobically at 37^{o}C for 48 hours (Maragkoudakrs *et al., 2006*).

GENOTYPIC CHARACTERIZATION OF ISOLATES

1. **DNA extraction using the boiling method**

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were heated at 95^{0}C for 20 min. after re-suspending in 500µl of normal saline. At 14000rpm, the bacterial suspension heated was cooled on ice and spun for 3 min. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20^{o}C for other downstream reactions.

2. **DNA quantification**

Nanodrop 1000 spectrophotometer was used for the extraction of genomic DNA quantified. By double clicking on the Nanodrop icon, the software of the equipment was lunched. The

equipment was initialized with 2ul of sterile distilled water and blanked using normal saline. Onto the lower pedestal, two microlitre of the extracted DNA was loaded. To contact the extracted DNA on the lower pedestal, the upper pedestal was brought down. By clicking on the “measure” button, the DNA concentration was measured.

3. **16SrRNA amplification**

The 16SrRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3 primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The following was in the PCR mix; the Master mix (X2 Dream taq) supplied by Inqaba, South Africa (DNTPs, MgCl and taq polymerase), the extracted DNA as template and the primers at a concentration of 0.5µM. The conditions for PCR were as follows: Initial denaturation (95ºC for 5 minutes), denaturation (95ºC for 30 seconds), annealing (52ºC for 30 seconds), extension (72ºC for 30 seconds) for 35 cycles and final extension (72ºC for 5 minutes). On a 1% agarose gel at 130V for 30 minutes, the product was resolved and visualized on a blue light trans-illuminator.

4. **Sequencing**

On a 3510 ABI sequencer, sequencing was done using the BigDye Terminator kit by Inqaba Biotechnological, Pretoria South Africa. At a final volume of 10µl, comprising the following components; a 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp, the sequencing was carried out. The condition for sequencing were as follows; 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

5. **Phylogenetic analysis**

Bioinformatics algorithm Trace edit was used to edit the obtained sequences, from the National Center for Biotechnology Information (NCBI) data base using BLASTN, similar sequences were downloaded. MAFFT were used to align the sequences. Neighbor-Joining method in MEGA 6.0 was used to infer the evolutionary history (Saitou and Nei, 1987). To represent the evolutionary history of the taxa analyzed, the bootstrap consensus tree inferred from 500 replicates was taken. The evolutionary distances were computed using the Jukes-Cantor method (Saitou and Nei, 1987).

DETERMINATION OF LACTIC ACID PRODUCED BY LACTIC ACID BACTERIA ISOLATES

Estimation of lactic acid was determined by titration of 25 ml of broth cultures of the test organism (24hours old) with 0.1N NaOH and three drops of phenolphthalein (1% w/v) as indicator. To the sample, the NaOH solution was added slowly until a pink colour appeared. Each ml of 0.1 N NaOH was equivalent to 90.08mg of lactic acid (Association of Official Analytical Chemist Methods, 2000). The formula below was used to calculate the lactic acid;

$$\text{Titratable acidity} = \frac{\text{volume [ml] of NaOH} \times \text{Normality of NaOH} \times \text{Lactic acid equivalent}}{\text{Volume of sample}}$$

Normality of NaOH=0.1

Lactic acid equivalent = 90.08 mg

Volume of sample = 25 mL

DETERMINATION OF HYDROGEN PEROXIDE PRODUCTION BY LACTIC ACID BACTERIA ISOLATES

Twenty millilitres of 0.1M H_2SO_4 was added to 25ml of the 24hours old MRS broth cultures of the test isolates. A 0.1N potassium permanganate was used to carry out titration. Each ml of 0.1M H_2SO_4 was equivalent to 1.70mg of Hydrogen peroxide and decolourization of the sample was regarded as end point. Concentration of hydrogen peroxide was calculated using the formula below:

$$\text{Hydrogen perioxide concentration} = \frac{\text{mlKMnOxNKMnO x M.E}}{\text{ml } H_2SO_4 \text{ x Volume of sample}} \times 100$$

ml $KMnO_4$=Volume of $KMnO_4$

N $KMNO_4$=Normality of $KMnO_4$

ml H2SO4=Volume of H_2SO_4

M.E=Equivalence factor of H_2O_2=1.701 mg

DETERMINATION OF DIACETYL PRODUCED BY THE LACTIC ACID BACTERIA ISOLATES

The amount of diacetyl produced by the Lactic acid bacteria isolates was estimated following the method of (AOAC, 2000). To 25 ml of 24 hours old MRS broth culture of the test isolates in conical flasks was added, 7.5 ml hydroxyl amine (0.1M) solution which served as substrate for residual titration. A 0.1N HCl was used to titrate the content of the flasks to a green-yellow end-point using bromophenol blue as indicator. A 21.52 mg of diacetyl is equivalent to each 1.00 ml of 0.1N HCl (AOAC, 2000). The formula below was used to calculate diacetyl;

$$\boldsymbol{AK} = (B - S)\,(100 - E)/W$$

AK= diacetyl Percentage

B= during titration of the sample, ml of 0.1N HCl consumed

E=Equivalent factor of diacetyl=21.52 mg to 1 mL of 0.1N HCl

W=Volume of sample used

S= during titration of 7.5 mL Hydroxyl amine, volume of ml 0.1N HCl consumed

EVALUATING ANTIMICROBIAL PROPERTIES OF DIFFERENT BACTERIAL STRAINS

Agar well diffusion test

Colonies of each isolates were picked and stabbed with cork borer on MRS agar medium and incubated anaerobically for 24 hours at 37°C. The plateswere later overlaid with 10ml of Nutrient soft agar (0.5%). The overlaid agar was seeded with 10^4cfu/ml of the spoilage bacteria and tested for sensitivity (*Bacillus cereus, Escherichia coli, Staphylococcus aureus and Salmonella typhi*). After incubation for 18- 24 h at 37 °C the plates were checked for zone of inhibition (Ngene *at al*., 2019).

STATISTICAL ANALYSIS

Data generated were subjected to Analysis of Variance (ANOVA), while least significance difference (LSD) at $p<0.05$ was used for statistical test of significance.

RESULTS

The table 1 below portrays the cultural, microscopic and biochemical attributes of the isolated organism ranging from their cellular arrangement, shape, margins, Gram reaction, elevation, encapsulation, spore formation and production of various enzymes during its biochemical characterization.

The obtained 16SrRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16SrRNA of the isolate B1 and B3 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16SrRNA of the isolates B1, B2, B3 and B7 within the Lactobacillus, and *Lactococcus spp* respectively and revealed a closely relatedness *Lactococcus lactis subsp lactis* 0711XYBLS *Lactobacillus fermentum* strain CS19, *Lactobacillus fermentum* and *Lactococcus lactis* than other bacterial species respectively (Fig.1 and Fig. 2).

The table 2 below represent the production level of metabolites by the isolates. Some of the metabolites include lactic acid, hydrogen peroxide (H_2O_2) and diacetyl (mg/ml). The first column in table 2 represents the production of the metabolites lactic acid by the isolates during titration in a reaction involving a solution of the organism and a standard base (NaOH). From the result *Lactococcus lactis* subsp. *lactics* 0711XYBLS showed highest production of lactic acid with the highest titratable acidity value of 15.84 + 0.36 mg/ml. The second column in table 2 depicts organism's ability to produce hydrogen peroxide in a reaction involving a solution of the organism and a standard base ($KMnO_4$) and acid (H_2SO_4). In this case the values of hydrogen peroxide recovered in the case of the reaction was highest in *Lactococcus lactis* subsp. *lactis*

0711XYBLS having the value of 0.32+0.02mg/ml. The third column in table 2 represents the ability of the organism to produce diacetyl low molecular mass antimicrobial in a reaction involving a solution of the organism and an acid (HCl) and hydroxylamine. The result shows that *Lactococcus lactis* subsp. *lactis* 0711XYBLS also possessed the highest value of 0.74+0.02.

Table 3 below portrays the results obtained as regards the antimicrobial activity of the isolates against some selected food pathogens. From the table 3 below, it was shown that *Lactococcus lactis* subsp. *lactis* 0711XYBLS portrayed the highest antimicrobial activity in all selected food pathogens with the highest zones of inhibition being observed in *Bacillus cereus, Salmonella typhi, Escherichia coli* and *Staphylococcus aureus.* The presence of these high zones of inhibition is a clear indication that these isolates possess antimicrobial properties against the selected food pathogens.

Table 1. Phenotypic characterization of isolates

Characteristic features/Isolates	B1	B2	B3	B4	B5	B6	B7	B8	B9
Cell shape	cocci	rod	Rod	Rod	short rod	Rod	Cocci	rod	Cocci
Form/Margin	smooth/entire	smoth/entire	smooth/irregular	sooth/entire	smooth/irregular	sooth/entire	smooth/entire	smooth/entire	smooth/entire
Elevation	Raised	Flat	slightly rasied	Flat	Flat	Flat	raised	raised	Convex
Colour	creamy white	Creamy	Creamy	Creamy	off white	Creamy	creamy	creamy white	creamy grey
Motility	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
Endospore formation	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve
Encapsulatio	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	--ve

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Calatase	**-ve**	**-ve**	**-ve**	**-ve**	**+ve**	**-ve**	**-ve**	**-ve**	**-ve**
Gram reaction	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
Citrate utilization	**-ve**	**-ve**	**-ve**	**-ve**	**+ve**	**-ve**	**-ve**	**-ve**	**-ve**
Methy red	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**
Indole	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**
Oxidase production	**-ve**	**-ve**	**-ve**	**-ve**	**+ve**	**-ve**	**-ve**	**-ve**	**-ve**
Vogues-proskauer	**-ve**	**-ve**	**-vve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**
Presumptive organims	***Lactococcus spp.***	***Lactobacillus spp.***	***Lactobacillus spp.***	***Lactobacillus spp.***	***Bacillus spp.***	***Lactobacillus spp.***	***Lactococcus spp.***	***Lactobacillus spp.***	***Streptococcus spp.***

Key: B represents the isolate, +ve represents positve, -ve represents negative

L B1 B2 B3 B7

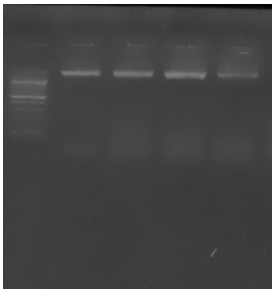

Figure 1: Agarose gel electrophoresis of the 16SrRNA gene of some selected bacterial isolates. LanesB1-B7 represent the16SrRNA gene bands (1500bp), LaneL represents the 100bp molecular ladder.

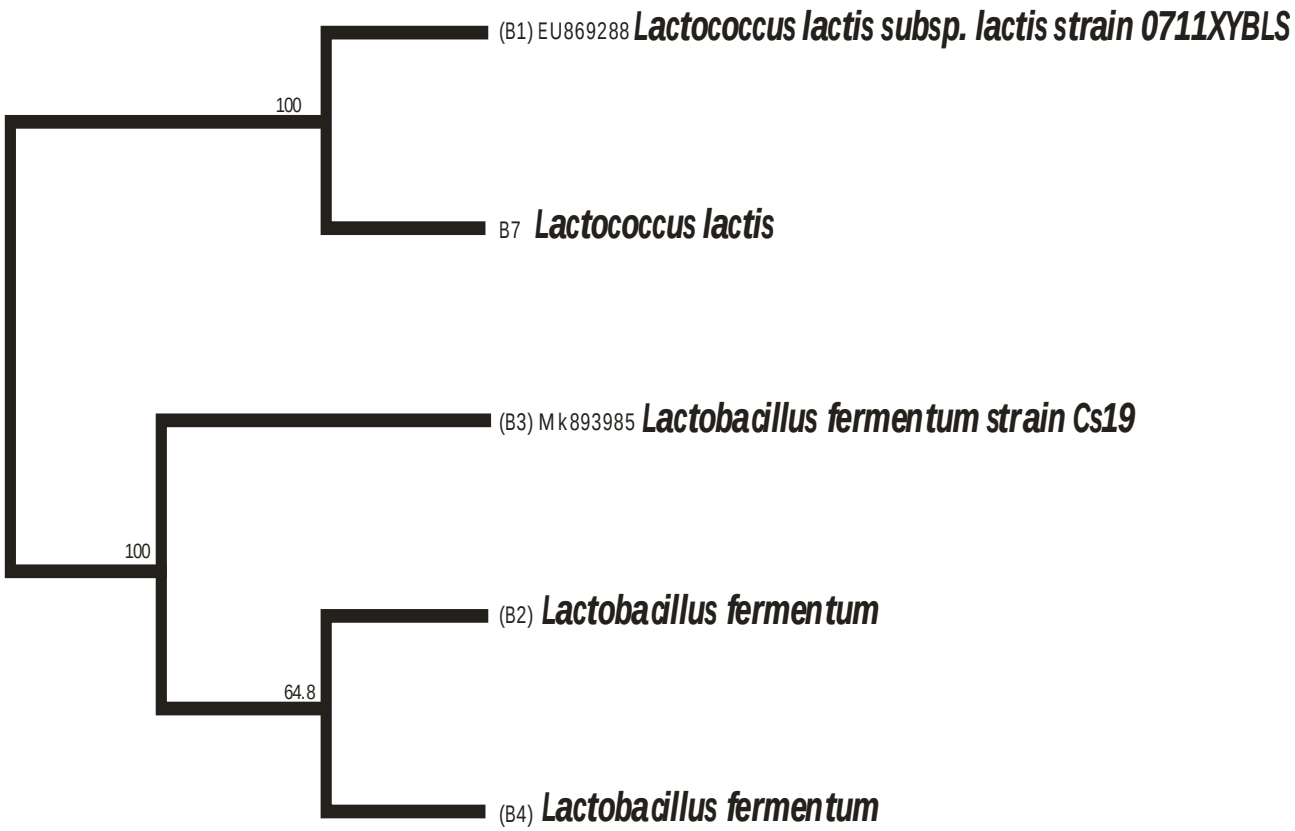

Fig 2. Phylogenetic tree showing the evolutionary distance between the bacterial isolates.

Table 2. Metabolites production by isolates

Isolates	**Lactic acid (mg/ml)**	**H_2O_2 (mg/ml)**	**Diacetyl (mg//ml)**
Lactococcus lactis **subsp. lactis 0711XYBLS**	**15.84 ± 0..36**	**0.32 ± 0.02**	**0.74±0.02**
***Lactobacillus fermentum* CS19**	**13.52 ± 0.78**	**0.29 ± 0.02**	**0.69 ± 0.00**

Table 3. Antimicrobial activity of Lactic acid bacteria isolates against selected food pathogens

Isolates	*Bacillus cereus*	*Pseudomonas aeroginoa*	*Salmonella typhi*	*Shigella spp*	*Escherichia coli*	*Staphylococcus aureus*
Lactococcus lactis subsp. lactis 0711XYYBLS	18.67 ± 1.115	8.00 ± 6.08	16.33 ± 0.57	9.67 ± 1.52	12.67 ± 0.57	12.67 ± 0.57
Lactobacillus fermentum CS19	16.00 ± 1.00	9.33 ± 0.57	15.33 ± 1.15	8.67 ± 0.57	11.00 ± 1.00	8.67 ± 1.15

Discussion

This research work revealed that some strains of Lactic Acid bacteria isolated from ogi, akpu and ugba and have antimicrobial effect against some selected food pathogens. Two lactic acid bacteria isolated from the various food sources were tested for their antimicrobial potentials against the selected food pathogens.

Lactococcus lactis subsp. *lactis* 0711XYYBLS and *Lactobacillus fermentum* CS19 isolated during this research produced lactic acid, diacetyl and hydrogen peroxide at varying degrees. This observation supports the research findings of Ogunbanwo *et al.* (2004) who reported that Lactic Acid bacteria produced the antimicrobial bacteriocin against some pathogens. Their importance is associated mainly with their safe metabolic activity while growing in foods and utilizing available Lactic Acid bacteria sugar for the production of organic acids and other metabolites. Their common occurrence in foods and feeds coupled with their long-lived use contributes to their natural acceptance as GRAS (Generally Recognized as Safe) for human consumption (Muruzovic *et al.*, 2018).

Lactococcus lactis subsp. *Lactis* 0711XYYBLS and *Lactobacillus fermentum* CS19 were used because a form of screening was carried out using some technological properties such as production of metabolites and production of antimicrobials against selected pathogens and enzyme production in abundance. The production of antimicrobial compounds by these Lactic Acid bacteria strains implies that they can serve as a source of novel preservative products in food industries. This is in agreement with an earlier report by Adeyemo *et al*. (2018). Considering the increasing importance of Lactic Acid bacteria as antibiotics alternative, the knowledge of the antimicrobial activity of Lactic Acid bacteria species especially *Lactococcus*

lactis subsp. *Lactis* 0711XYYBLS in particular is of high significance. The antimicrobial activity of *Lactococcus lactis* subsp. *Lactis* 0711XYYBLS and *Lactobacillus fermentum* CS19 shows that it can be used as a food preservative to reduce contaminants in food. There are three mechanisms that could explain the antimicrobial activity of Lactic Acid bacteria especially *Lactococcus lactis* subsp. *Lactis* 0711XYYBLS and *Lactobacillus fermentum* CS19; the production of bacteriocins; the yield of organic acids and other inhibitory substances such as ethanol, carbon dioxide and hydrogen peroxide; and the competition for nutrients (Muruzovic *et al.,* 2018). These cannot be overemphasized.

The Lactic Acid bacteria strains isolated were tested against food pathogens and the zones of inhibition were observed. *Lactococcus lactis* subsp. *Lactis* 0711XYYBLS showed the highest zones of inhibition on Gram positive and Gram negative bacteria from food sources this was followed by *Lactobacillus fermentum* CS19. The report of these findings is similar to the work reported by Rodríguez *et al.* (2009) who reported that antimicrobial compounds such as phenyl-lactic acid and lactic acid were effective against many Gram-negative and Gram-positive pathogenic bacteria such as *Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, and Enterococcus faecalis*. The antagonistic activity of Lactic Acid bacteria metabolites against the spoilage bacteria also agrees with the findings of Vasiee *et al.* (2020).

The inhibitory effect caused by *Lactococcus lactis* subsp. *lactis* 0711XYYBLS and *Lactobacillus fermentum* CS19 can be considered that the Lactic acid bacteria produced organic acids, especially lactic and acetic acids, exerting a strong inhibitory effect on Gram-negative and positive bacteria (Muruzovic *et al.,* 2018). They stated further that the low pH observed during the fermentation of selected food sources may not be the sole reason for the observed inhibition

effects. It could however be an important condition for the passage of organic acids through the membrane to the intracellular environment, where they will accumulate and exert inhibitory activity (Patel *et al.,* 2012). The lowering of the pH of fermented foods by Lactic acid bacteria to below 4 through acid production inhibits the growth of pathogenic microorganisms which can cause food spoilage, food contamination and food poisoning. Lactic acid bacteria because of their potential use as natural antimicrobial agents have been used to enhance the safety of food products. Most chemical preservatives used in processed foods have been found to contribute to health hazards among consumers when used in high doses (Badis *et al.,* 2004). Some preservatives have also been reported by Food and Drug Administration (FDA) to produce allergic reactions (Timbo *et al.,* 2004).

The increase in the use of chemical preservatives has resulted to the need for finding a safer alternatives in food preservation. The application of Lactic acid bacteria with the simultaneous control of factors that affect microbial growth can help to minimize food spoilage. The selection and addition of novel isolates of Lactic acid bacteria may be the key to reducing the use of chemical preservatives, enhancing/ improving nutrients and extending the shelf life of food products (Muhialdin *et al.,* 2013).

Lactococcus lactis subsp. *lactis* 0711XYYBLS and *Lactobacillus fermentum* CS19 are effective against a variety of bacterial pathogens and some food —borne microorganisms; they can serve as alternative antimicrobial agents and food preservatives against the corresponding food borne pathogens. Instead of the chemical preservatives that we use which have some side effects, as bio-preservatives, this potential can be harnessed by the food industries on a large scale.

CONCLUSION

The increase in food poisoning and contamination is the major challenge in developing countries. However, this research study was focused on identifying possible organic antimicrobial metabolites of microbial origin but could serve as biopreservatives. Lactic acid, diacetyl and hydrogen peroxide were some of the bioactive compound produced by *Lactococcus lactis* subsp. *Lactis* 0711XYYBLS and *Lactobacillus fermentum* CS19 all of which showed high antimicrobial activity against some selected food pathogens which are common among humans. However, the promising result of this study suggest the important role the antimicrobial substances produced by their isolate as source of novel antimicrobial agents in food industries as well as good source of natural biopreservative compounds to improve the safety and shelf life of foods in food industries.

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APPENDICIES

Appendix 1: Statistical analysis results of antimicrobial activity of isolates and production of metabolites

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Antimicrobial activity against *Bacillus cereus*	*Lactococcuslactis subsplactis* 0711XYBLS	3	18.67	1.155	.667	15.80	21.54	18	20
	Lactobacillus fermentum CS19	3	16.00	1.000	.577	13.52	18.48	15	17
	Total	6	17.33	1.751	.715	15.50	19.17	15	20
Antimicrobial activity against *Pseudomonas aeroginosa*	*Lactococcuslactis subsplactis* 0711XYBLS	3	8.00	6.083	3.512	-7.11	23.11	1	12
	Lactobacillus fermentum CS19	3	9.33	.577	.333	7.90	10.77	9	10
	Total	6	8.67	3.933	1.606	4.54	12.79	1	12

Antimicrobial activity against *Salmonella typhi*	*Lactococcuslactis subsplactis* 0711XYBLS	3	16.33	.577	.333	14.90	17.77	16	17
	Lactobacillus fermentum CS19	3	15.33	1.155	.667	12.46	18.20	14	16
	Total	6	15.83	.983	.401	14.80	16.87	14	17
Antimicrobial activity against *Shigella spp*	*Lactococcuslactis subsplactis* 0711XYBLS	3	9.67	1.528	.882	5.87	13.46	8	11
	Lactobacillus fermentum CS19	3	8.67	.577	.333	7.23	10.10	8	9
	Total	6	9.17	1.169	.477	7.94	10.39	8	11
Antimicrobial activity against *Escherichia coli*	*Lactococcuslactis subsplactis* 0711XYBLS	3	12.67	.577	.333	11.23	14.10	12	13
	Lactobacillus fermentum CS19	3	11.00	1.000	.577	8.52	13.48	10	12
	Total	6	11.83	1.169	.477	10.61	13.06	10	13
Antimicrobial activity against *Staphylococcus aureus*	*Lactococcuslactis subsplactis* 0711XYBLS	3	12.67	.577	.333	11.23	14.10	12	13

	Lactobacillus fermentum CS19	3	8.67	1.155	.667	5.80	11.54	8	10
	Total	6	10.67	2.338	.955	8.21	13.12	8	13
Lactic acid production	*Lactococcuslactis subsplactis* 0711XYBLS	3	15.8400	.36000	.20785	14.9457	16.7343	15.48	16.20
	Lactobacillus fermentum CS19	3	13.5233	.78399	.45263	11.5758	15.4709	12.68	14.23
	Total	6	14.6817	1.38122	.56388	13.2322	16.1312	12.68	16.20
Hydrogen peroxide production	*Lactococcuslactis subsplactis* 0711XYBLS	3	.3200	.02000	.01155	.2703	.3697	.30	.34
	Lactobacillus fermentum CS19	3	.2933	.02309	.01333	.2360	.3507	.28	.32
	Total	6	.3067	.02422	.00989	.2812	.3321	.28	.34
Diacetyl production	*Lactococcuslactis subsplactis* 0711XYBLS	3	.7467	.02309	.01333	.6893	.8040	.72	.76
	Lactobacillus fermentum CS19	3	.6967	.00577	.00333	.6823	.7110	.69	.70
	Total	6	.7217	.03125	.01276	.6889	.7545	.69	.76

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
Antimicrobial activity agains *Bacillus cereus*	Between Groups	10.667	1	10.667	9.143	.039
	Within Groups	4.667	4	1.167		
	Total	15.333	5			
Antimicrobial activity against *Pseudomonas aeroginosa*	Between Groups	2.667	1	2.667	.143	.725
	Within Groups	74.667	4	18.667		
	Total	77.333	5			
Antimicrobial activity against *Salmonella typhi*	Between Groups	1.500	1	1.500	1.800	.251
	Within Groups	3.333	4	.833		
	Total	4.833	5			
Antimicrobial activity against *Shigella spp*	Between Groups	1.500	1	1.500	1.125	.349
	Within Groups	5.333	4	1.333		
	Total	6.833	5			
Antimicrobial activity against *Escherichia coli*	Between Groups	4.167	1	4.167	6.250	.067
	Within Groups	2.667	4	.667		

	Total	6.833	5			
Antimicrobial activity against *Staphylococcus aureus*	Between Groups	24.000	1	24.000	28.800	.006
	Within Groups	3.333	4	.833		
	Total	27.333	5			
Lactic acid production	Between Groups	8.050	1	8.050	21.634	.010
	Within Groups	1.488	4	.372		
	Total	9.539	5			
Hydrogen peroxide production	Between Groups	.001	1	.001	2.286	.205
	Within Groups	.002	4	.000		
	Total	.003	5			
Diacetyl production	Between Groups	.004	1	.004	13.235	.022
	Within Groups	.001	4	.000		
	Total	.005	5			