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LACTIC ACID BACTERIA AS BIOACTIVE POTENTIAL AGAINST SELECTED RESISTANCE *CANDIDA* SPECIES AND PATHOGENIC BACTERIA

Chiamaka Linda Mgbechidinma[1], Caleb Oladele Adegoke*[2] and Samuel Temitope Ogunbanwo[1]
[1]Department of Microbiology, University of Ibadan, Oyo State, Nigeria.
[2] Ogun State College of Health Technology Ilese Ijebu, Department of Medical Laboratory Technology P.M.B.
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Corresponding author: Caleb Oladele Adegoke

ABSTRACT

This research focused on the isolation and antagonistic action of Lactic Acid Bacteria (LAB) against certain antibiotics resistance disease causing bacteria and fungai. Antibiotic resistance is an increasing problem amid humans and animals in land-dwelling or marine environments hence making treatment of infections difficult. Antibiotic susceptibility test for bacteria pathogen was performed using the disc diffusion method while antifungal susceptibility and antimicrobial activity of LAB were carried out using agar well diffusion method. All the pathogenic bacteria used as indicator organisms were multiple antibiotics resistance and 100 percent resistance to gentamycin and pefloxacillin with the exception of *Staphlococcus aureus*. *Candida* species was 100 percent resistance to Ketoconazole, fluconazole and miconazole. Twenty-two LAB isolates were gotten from fermented milk and milk products. The isolates were identified as *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus delbrueckii, Leuconostoc mesenteriodes, Lactobacillus casei, Lactobacillus brevis, Lactobacillus acidophilus, Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus* and *Lactobacillus rhamnosus*. LAB produced lactic acid to varying concentrations, having its production peak (1.80g/L) at 48 h of incubation by *Lactobacillus plantarum. Lactobacillus fermentum*NU2 produced the highest quantity of diacetyl (2.80g/L) while *Lactobacillus acidophilus*GO8 and *Lactococcus lactis*GO9 produced the highest amount of hydrogen peroxide (0.030g/L) at 48 h of incubation. *Lactobacillus plantarum*GO16 inhibited *Bacillus cereus* while *Lactobacillus acidophilus*GO8 inhibited *Staphylococcus aureus* with 28 mm zone of inhibition. *Lactobacillus plantarum*NU1 and *Lactobacillus plantarum*GO16 inhibited *Candida albican* with 25 mm zone of inhibition. LAB can be used as probiotics in preventing infections caused by *Candida* species and pathogenic bacteria.

Keywords: Lactic Acid Bacteria, Fermented milk, antibiotics resistance, antagonistic activity, pathogens.

Introduction

The concept of Lactic acid bacteria (LAB) has been known to play a significant function and role in health upkeep and food manufacture (Emiliano *et al*., 2014). The search for food components with valuable bio-active properties inspired interest in lactic acid bacteria (LAB) with probiotic properties and their antimicrobial activity against pathogenic microorganisms (Hugo, 2006). Lactic acid bacteria (LAB) can produce antimicrobial substances such as bacteriocins, hydrogen peroxide, organic acids, and diacetyl with the tendency to antagonize the growth of pathogenic microorganisms (Sezer and Güven, 2009).

These microbes are naturally found in the environment and are present as normal flora in moist places like the intestinal system, mouth and vagina in human body (Adegoke *et al*., 2010; Ogunbanwo *et al.,* 2012). Recently, hospital-acquired microbial infections have emerged as an important public health problem causing serious morbidity and death (Chen *et al.,* 2006). Occurrence of transferable or infectious diseases caused by multidrug resistance pathogens require a complementary therapy due to the facts that commonly used antibiotics are no longer effective thus making treatment of such infection difficult (Adegoke and Ogunbanwo, 2017). CDC (2015), Pathogenic microorganisms are the major cause of transmitted diseases in human beings and animals.

Resistance do occur through one of these processes, natural resistance in certain types of bacteria such as *Salmonella typhi and shigella* species, genetic alteration or by one species acquiring resistance from another (Gerber *et al*., 2017). All classes of microbes can develop resistance for instance fungi develop antifungal resistance, viruses to antiviral, Protozoa to antiprotozoal and bacteria develop antibiotic resistance (*Levy, 2002).* Resistance can appear spontaneously because of random changes. However, extended use of antimicrobials appears to encourage selection for mutations which can render antimicrobials ineffective (Goossens *et al*., 2005). Penicillinase may have emerged as a defense mechanism for

bacteria in their habitats, such as the case of penicillinase-rich *Staphylococcus aureus*

Antibiotic resistance is an increasing occurrence among microorganisms in land-dwelling or aquatic environments (Martinez and Olivares 2012). In this regard, the extent of impurity of the environment, especially through water pollution and some sensitive area such as hospitals waste water and unprocessed urban wastes is growing and source of concern in community health scheme *(Marti et al., 2014)*. Antibiotics had been a serious toxic waste to the environment since their introduction through human waste, animals and the drug business *(Yezli and Li, 2012)*. The involvement of the drug company is so important that matches can be established between countries with peak rate of increasing antibiotic resistance and countries with major impression of pharmacological trade (Martinez and Olivares, 2012).

Hence, the focus of this work is based on finding a complementary therapy to the aforementioned problems with the use of antimicrobial compounds or metabolites produced by lactic acid bacteria against drug resistance pathogenic bacteria and Candida species.

Materials and Methods

Collection of Samples:

Raw milk including cow milk and goat milk as well as fermented milk products such as *Wara* and *Nunu* were purchased from Bodija markets in Ibadan, Oyo State, Nigeria. The fermented milk products were transported aseptically to the laboratory in a clean and sterile container for microbiological analysis. Pure cultures of bacterial and yeast pathogens were collected from culture collection center of Department of Medical Laboratory Science, Ogun State College of Health Technology, Ilese Ijebu.

Culture media used:

De man, Rogosa, Sharpe (MRS) agar and broth were used for growth and isolation of lactic acid bacteria (LAB), Yeast extract agar (YEA) for the subculture of *Candida* species, Nutrient agar (NA) for the subculture of pathogenic bacteria and Muller-Hinton agar (MHA) for the antimicrobial screening against selected pathogenic organisms.

Sterilization method:

All glass wares used in this research were washed thoroughly with detergent, rinsed with water and then sterilized in an autoclave at 121 ^{o}C for 15 mins. The work bench was disinfected with 70% ethanol and inoculating loops were sterilized by flaming before and after use.

Preparation of media:

The culture media; de man, Rogosa, Sharpe agar (Oxoid, Hampshire, England), Nutrient agar, Yeast extract agar, Muller-Hinton agar and peptone water used were all prepared in Erlenmeyer flasks according to the manufacturer's instructions and sterilized in an autoclave at 121 ^{o}C for 15 mins.

Isolation of Lactic Acid Bacteria:

Serial dilutions were made for all the fermented milk samples. 9 mL of distilled water was dispensed into sterile test tubes and was sterilized at 121 ^{o}C for 15 minutes in an autoclave. The raw milk (cow and goat milk) was subjected to fermentation at various hours (6, 12, 18 and 24) hours. The *Wara, Nunu* and fermented raw milk at various time intervals were used for the serial dilution. 1mL/1g of the sample was pipette into a sterile test tube containing 9 ml of sterile distilled water and shaken vigorously to obtained dilusion 10^{-1}. This process was repeated until dilution 10^{-6} was obtained. Subsequently, 1 mL of 10^{-2}, 10^{-4} and 10^{-6} diluents were introduced into a well labeled sterile petri dishes respectively, after which sterilized and cooled MRS agar (de Man-Rogosa-Sharpe; Oxoid, Hampshire, England) was poured into the plates aseptically. The plates were carefully swirled in clockwise and anticlockwise directions to ensure uniformity and even distribution of inoculums throughout the growth medium. Control plates in which the inoculums were not introduced were also prepared in order to detect any form of contamination from the media used. The inoculated plates were then allowed to set and solidify on a flat surface, before been inverted and incubated at 37 °C for 24-48 h anaerobically. After which the plates were observed and representative colonies were noted and sub-cultured (Harrigan and MaCane, 1966).

Purification of culture and preservation:

Morphologically distinct colonies on the plates were selected and streaked on solidified MRS agar to obtain pure culture of LAB. Pure culture of LAB were transferred to slant in McCartney bottles, allowed to grow for 24 hour at 37 ^{o}C and stored in the refrigerator at 4 °C.

Identification of isolates:

Isolated LAB was characterized based on their morphological, biochemical and physiological properties. Twenty-four old cultures were used in carrying out all procedures for the characterization of isolates except where otherwise stated (Sneath, 1986).

Quantitative estimation of metabolites:

Quantitative estimation of metabolites was carried out by preparing MRS broth, dispensing into bottles, sterilized and allowed to cool to about 50 ^{o}C. Lactic acid bacteria was inoculated into the broth and incubated anaerobically. Quantitative estimation was done at 24 hours interval for all the isolates.

Quantitative Estimation of Lactic Acid:

The quantity of lactic acid produced by antimicrobial producing isolates at 24 h, 48 h, 72 h and 96h was determined by transferring 25 mL of cell free broth cultures of test organisms into100 ml flasks. This was titrated with 0.25 mol l^{-1} NaOH and 1 mL of phenolphthalein indicator (0.5% in 5% alcohol). The titratable acidity was calculated as lactic acid (% w/v). Each milliliter of 1 N NaOH is equivalent to 90.08 mg of lactic acid. The titratable acidity was then calculated according to A.O.A.C. (1995).

$$\text{Titratable acidity (\% Lactic acid)} = \frac{\text{mL NaOH} \times \text{M NaOH} \times \text{M.E} \times 100}{\text{Volume of sample used (mL)}}$$

Where:

mL NaOH = Volume of NaOH used

N NaOH = Normality (Molarity) of NaOH Solution

M.E = Equivalence factor (90.08/mg)

Quantitative Estimation of Diacetyl:

Diacetyl production at 24 h, 48 h, 72 h and 96 h was determined by transferring 25 mL of cell free broth cultures of test organisms into 100 mL flasks. Hydroxylamine solution (7.5 mL) of 1 molar was added to the flask and to a similar flask for residual titration. Both flasks were titrated with 0.1 M HCl to a greenish yellow end point using bromothymol blue as indicator. The equivalence factor of HCl to diacetyl is 21.52 mg. The concentration of diacetyl produced was calculated using the A.O.A.C. (1995).

$$AK = \frac{(b\text{-}s)\,(100E)}{W}$$

Where Ak = % of diacetyl

b- s = volume of HCl used

E = equivalence factor (21.52/mg)

W = volume of sample (broth)

100 = constant

Quantitative Estimation of Hydrogen Peroxide:

Twenty ml of dilute sulphuric acid was added to 25 mL of the supernatant and titration was carried out with 0.1 M potassium permanganate which is equivalent to 1.7 mg of hydrogen peroxide. A decolourisation of the sample was regarded as the end point (A.O.A.C. 1995).

$$H_2O_2 + 2KMnO_4 + 3H_2SO_4 \longrightarrow K_2SO_4 + 4H_2O + O_2$$

$$H_2O_2 \text{ concentration} = \frac{\text{ml } KMnO_4 \times NKMnO_4 \times \text{M.E} \times 100}{\text{mL } H_2SO_4 \times \text{Volume of sample}}$$

Where:

mL $KMnO_4$ = volume of $KMnO_4$

N $KMnO_4$ = Normality of $KMnO_4$

mL H_2SO_4 = Volume of H_2SO_4used

M.E = Equivalence factor (1.701/mg)

Antibacteria susceptibility test:

Antibiotic susceptibility test for each bacteria pathogen was performed using the disc diffusion method. Actively growing culture (0.1 mL) containing 1 x 10^6 cfu/mL of each bacterium pathogen used was introduced into Petri dishes and 20 mL of molten agar added. The antibiotic sensitivity discs (Abtek Biological Ltd) consisting of different antibiotics namely: Septrin (30 µg), Chloramphenicol (30 µg), Sparfloxacin (10 µg), Ciprofloxacin (10 µg), Amoxacillin (30 µg), Augmentin (30 µg), Gentamicin (10 µg), Pefloxacin (30 µg), Tarivid (10 µg) and Streptomycin (30 µg) were placed on the solidified agar surface. The plates were incubated aerobically at 37 °C for 24 h. After this period, the diameter of the zone of inhibition of each disc was measured (Norrby, 1992). The zone of inhibition was corresponded to the antibiotic activity of each disc.

Antifungal Susceptibility:

Well diffusion antifungal susceptibility assay was carry out by the method described by Magaldi *et al.* (2004). Sabouraud dextrose agar (Oxoid) (SDA) plates with actively growing culture (0.1 mL) containing 1 x 10^6 cfu/mL inoculum size were assayed in the wells made using sterilized cork borer, to which, antifungal agents amphotericin B (100 U), ketoconazole (20 μg/mL), itraconazole

(20μg/mL), fluconazole (20 μg/mL) and miconazole (20 μg/mL) were loaded. The plates were incubated at 37 °C for 48 hours and the zone of inhibition was recorded.

Antimicrobial activity of LAB against bacteria pathogens:

The antimicrobial activities of the LAB isolates against test organisms such as *Bacillus subtills, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Salmonella typhi, Shigella dysentry* and *Staphylococcus aureus* was carried out using the agar well diffusion as described by Ravi et al. (2013). Pure cultures of each of the isolate was grown in 10mls of MRS broth and incubated anaerobically at 37 °C for 72 hours. Each of the incubated broth culture was centrifuged at 4,000 rpm for 20 minutes at 4 ^{0}C to obtain cell free supernatants (CFS). Twenty-four old cell suspension of the broth culture of the pathogenic bacteria was adjusted to 0.5 McFarland turbidity standards and used to seed 20mls Muller-Hinton agar using sterile swab stick on Petri dishes. The Muller-Hinton agar plates were allowed to dry, and four wells, each with a diameter of 7mm were bored on each plate using a sterile cork borer and 0.1 mL of CFS of LAB was pipetted into the bored wells and labeled accordingly. The plates were incubated at 37 ^{o}C for 24 hours and were examined for clear zones of inhibition around the wells. The diameter of the zones of inhibition around each well was measured in millimeters and recorded.

Antimicrobial activity of LAB against fungal isolates:

Antifungal effect of Lactic acid bacteria isolated from fermented milk products against *Candida albicans, Candida glabrata, Candida krusei, Candida tropicalis* and *Candida pseudotropicalis* was examined using agar well diffusion method (Toba *et al.,* 1991). The lactic acid bacteria were incubated in MRS liquid culture medium (BD, USA) under anaerobic conditions at 37^{o}C for 24 h. The cultures were then centrifuged at 10 000 rpm for 15 mins. The supernatant was recovered with an injector and filtered through 0.45 µ pore filter (Millipore, Molsheim, France) (Toba *et al*., 1991). The *Candida* species were activated in yeast extract agar and subcultured into yeast extract broth. The turbidity of the test broth was compared with that of 0.5 McFarland standard tubes and used as an inoculum. In all media, 7 mm wells were bored and 0.1mL supernatant added into the bored wells. The plates were kept at room temperature for 2 h for the diffusion of the supernatant into the agar and left to incubate at 37 ^{o}C for 24 h. Then, the zones around the wells were measured in mm (Toba *et al*., 1991).

Results

A total of twenty two lactic acid bacteria isolates were obtained from the fermented milk products. These organisms were identified as Lactobacillus helveticus, Lactobacillus casei Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus delbrueckii, Leuconostoc mesenteriodesLactobacillus plantarum, Lactobacillus brevis, Lactococcus lacti Streptococcus thermophilius and *Lactobacillus rhamnosus*. The isolates were characterized based on their microscopic, morphological, physiological and biochemical tests were also considered such as Gram's stain, catalase, oxidase, citrate utilization and sugar fermentation. All the isolates were Gram positive rod, *cocci* to *coccobacilli*, catalase negative and non-spore formers. All the isolates showed circular colonies which are opaque with colors varying from white to cream on MRS agar plate. Their surfaces were smooth and glistering with sizes either punctiform, small, or medium. Percentage of occurrence of LAB isolates from fermented milk products was represented in figure 1. Goat milk had 45.45% followed by Cow milk which has 27.27%. Nono and Wara had equal percentage of 13.64.

The occurrence of LAB species isolated from different fermented milk products is showed in Table 1. Six different species of lactic acid bacteria were isolated from both the Cow milk and ten different species of lactic acid bacteria were isolated from Goat milk. Isolates from fermented cow milk were *Lactobacillus acidophilus, Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus plantarum, Lactococcus lactis* and *Lactobacillus brevis* while from fermented Goat milk were *Lactobacillus rhamnosus, Leuconostoc mesenteriodes Streptococcus thermophillus, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus delbrueckii, Lactococcus lactis, Lactobacillus helveticus, Lactobacillus brevis* and *Lactobacillus casei.* Equal number of species of Lactic acid bacteria was also isolated from *Nono* and *Wara*. These are *Leuconostoc mesenteriodes, Lactobacillus plantarum, Lactobacillus delbrueckii* from *Wara* and *Lactobacillus casei Lactobacillus*

plantarum, and*. Lactobacillus fermentum,* are from *Nono.*

Table 2 shows the antibiotic sensitivity pattern of selected pathogenic bacteria. Most of the pathogenic bacteria used in this research work were resistant to Gentamycin with 9 mm zone of inhibition, septrin (10 mm), Chloraphenicol (11 mm), ciprofloxacin (14 mm), amoxyllin (15 mm), and oxaxyllin (18 mm). The organisms were sensitive to augmenting and streptomycin with 21 mm and 23 mm zone of inhibition respectively. *Shigella dysentery* was the least resistant to amoxyllin with 7 mm zone of inhibition and *Bacillus subtills* was most sensitive to streptomycin with 23 mm zone of inhibition.

Antifungal susceptibility pattern of some selected pathogenic Candida species was shown in Table 3. Candida krusei was the least resistant to fluconazole with 8 mm zone of inhibition and Candida ablicans most sensitive to miconazole with 25 mm zone of inhibition followed by amphotericin B against Candida ablicans with 20 mm zone of inhibition. Candida ablicans was resistant to ketoconazole (7 mm), Fluconazone (12 mm) and intraconazole (18 mm), closely followed by Candida glabrata which was resistant to all antifungal drugs used except amphotericin B with 20 mm zone of inhibition.

The Lactic acid bacteria obtained from fermented milk products; wara, goat milk, nono and cow milk were quantified for the production of antimicrobial compounds such as lactic acid, hydrogen peroxide and diacetyl. Most of the isolates had their highest production of lactic acid, diacetyl and hydrogen peroxide at 48 hours of incubation time with few exceptions observed by Streptococcus thermophilus (GO10) during lactic acid production as well as Lactobacillus fermentum (NU2) and Lactobacillus rhamnosus (GO14) during the production of hydrogen peroxide.

The highest quantity (1.8 g/L) of lactic acid was produced by *Lactobacillus plantarum* (GO16). However, the quantity of lactic acid produced by the LAB isolates increased with an increase in the incubation time. After which the quantity of lactic acid produced decreased with an increase in incubation time as shown in figure 2.

Lactobacillus fermentum (NU2) had the highest production of diacetyl (2.80 g/L) at 48 hours of incubation, while the least production was 1.08 g/L by *Lactococcus lactis* (GO9) and *Streptococcus thermophilus* (GO10) at 96 hours and 24 hours of incubation time respectively as represented in figure 3.

However, figure 4 shows the quantity of hydrogen peroxide produced by LAB. *Lactobacillus acidophilus* (GO8) and *Lactobacillus lactis* (GO9) produced the highest quantity of hydrogen peroxide (0.030 g/L) at 48 hours of incubation while the least (0.004 g/L) production was by *Streptococcus thermophilus* (GO10) at 96 hours of incubation time.

Antagonistic activity of lactic acid bacteria against selected pathogenic bacteria such as *Bacillus subtills, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Salmonella typhi, Shigella dysentry* and *Staphylococcus aureus* are shown in Table 4. Metabolites produced by *Lactobacillus plantarum* (GO16) inhibited *Bacillus cereus* while metabolites produced by *Lactobacillus acidophilus* (GO8) inhibited *Staphylococcus aureus* with 28 mm as their highest zone of inhibition. *Bacillus cereus* was least inhibited by metabolites produced by *Lactobacillus delbrueckii* (NU3) and *Streptococcus thermophilus* (GO10) with 14 mm zone of inhibition. More so, *Staphylococcus aureus* was least inhibited by metabolites produced by *Lactobacillus casei* (CH6) with 15 mm zone of inhibition. *Bacillus subtills, Escherichia coli* and *Salmonella typhi* were best inhibited by metabolites produced by *Lactobacillus fermentum* (NU2), *Lactobacillus plantarum* (GO16) and *Lactobacillus plantarum* (GO16) respectively with 27 mm zone of inhibition each. Metabolites produced by *Lactococcus lactis* (GO9) showed antagonistic activity against *Shigella dysentry* with 25 mm zone of inhibition. Moreover, *Klebsiella pneumoniae* and *Listeria monocytogenes* were inhibited by metabolites produced by *Lactobacillus acidophilus* (GO8) and *Lactobacillus plantarum* (GO16) respectively with 25 mm as their best zone of inhibition.

The zone of inhibition of cell-free supernatants (CFS) obtained from lactic acid bacteria isolates against the pathogenic Candida species such as Candida albicans, Candida glabrata, Candida krusei, Candida tropicalis and Candida pseudotropicalis are shown in Table 5. The antagonistic activity of CFS produced by *Lactobacillus plantarum* (NU1) and *Lactobacillus plantarum* (GO16) against *Candida albicans* showed the widest (25 mm) zone of inhibition while CFS

produced by *Streptocoocus thermophilus* (GO10) had the least (10 mm) zone of inhibition against *Candida tropicalis*. CFS produced by *Lactobacillus fermentum* (NU2) inhibited *Candida glabrata* with 20 mm zone of inhibiton. However, CFS produced by *Lactobacillus plantarum* (NU1) and *Lactobacillus plantarum* (GO16) inhibited *Candida krusei* with 17 mm zone of inhibition. Nevertheless, none of the CFS produced by LAB isolates had inhibitory effect on *Candida pseudotropicalis.*

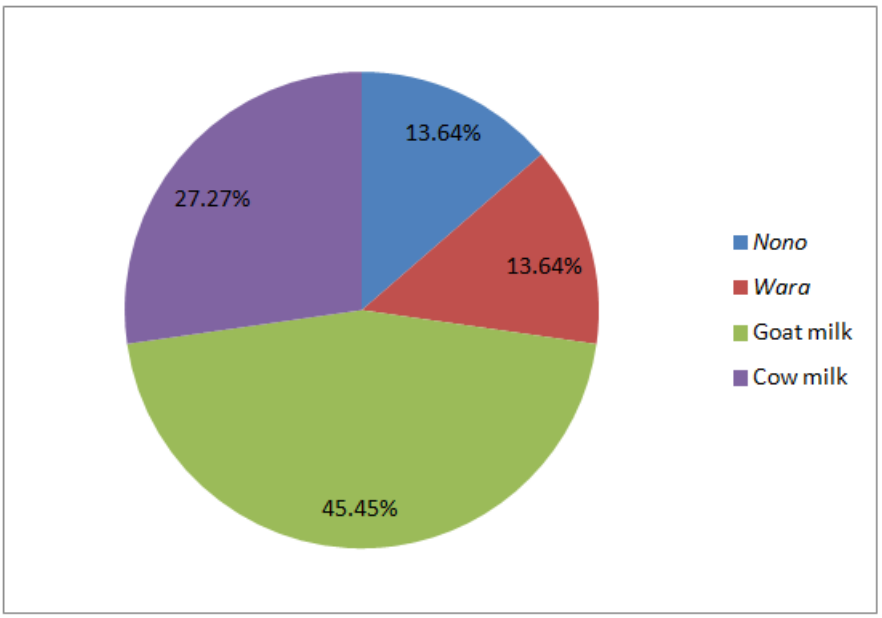

Figure 1: Percentage occurrence of LAB isolates from Fermented Milk and milk Products

Table 1: Occurrence of LAB species isolated from Fermented Milk and milk Products

Sources	Probable LAB Isolates
Nono	*Lactobacillus plantarum, Lactobacillus fermentum , Lactobacillus delbrueckii*
Wara	*Lactobacillus plantarum, Lactobacillus casei, Leuconostoc mesenteriodes*
Fermented Goat milk	*Lactobacillus plantarum, Lactobacillus acidophilus, Lactococcus lactis, Lactobacillus delbrueckii, Streptococcus thermophillus, Lactobacillus helveticus, Lactobacillus rhamnosus, Leuconostoc mesenteriodes, Lactobacillus brevis, Lactobacillus casei.*
Fermented Cow milk	*Lactobacillus plantarum , Lactobacillus fermentum , Lactobacillus delbrueckii, Lactobacillus acidophilus, Lactococcus lactis, Lactobacillus brevis*

Table 2: Antibiotic sensitivity profile of selected pathogenic bacteria

Indicator organisms	SXT	CH	SP	CPX	AM	AU	CN	PEF	OFX	S
Bacillus subtills	R(10)	R(14)	R(11)	R(14)	R(15)	S(21)	R(09)	S(19)	R(18)	S(23)
Bacillus cereus	S(19)	S(18)	S(09)	S(10)	S(18)	S(19)	R(10)	S(20)	R(12)	R(12)
Escherichial coli	R(16)	R(11)	R(16)	S(21)	R(11)	S(21)	R(13)	S(17)	R(17)	S(20)
Klebsiella pneumonia	R(14)	S(17)	S(18)	R(12)	R(13)	R(11)	R(15)	S(21)	R(11)	R(11)
Listera monocytogens	S(23)	R(09)	S(20)	R(15)	R(10)	S(19)	R(08)	S(21)	R(11)	R(09)
Salmonella typhi	R(09)	R(08)	S(16)	R(13)	R(10)	S(21)	R(10)	R(12)	R(14)	S(14)
Shigella dysentery	R(12)	R(11)	S(21)	S(21)	R(07)	S(20)	R(16)	S(21)	R(10)	R(13)
Staphlococcus aureus	R(14)	S(18)	R(15)	R(14)	S(19)	S(23)	R(07)	S(24)	S(21)	R(11)
Proteus mirabilis	S(21)	S(22)	R(13)	R(11)	R(14)	S(19)	R(09)	S(22)	R(19)	R(14)

Key: SXT**;** Septrin (30µg); CH; Chloramphenicol (30µg); SP; Sparfloxacin (10µg)
CPX; Ciprofloxacin (10µg); AM; Amoxycillin (30µg); AU; Augmentin (30µg); CN; Gentamicin (10µg); PEF; Pefloxacin (30µg); OFX; Tarivid (10µg); S; Streptomycin (30µg)
S: Susceptible I: Intermediate and R: Resistance.

Table 3: Antifungal susceptibility (mm) pattern of some selected *Candida* species

Indicator organisms	Ketoconazole	Miconazole	Intraconazole	Fluconazole	Amphotericin B
Candida ablican	R(07)	S(25)	R(18)	R(12)	S(20)
Candida glabrata	R(09)	R(14)	R(17)	R(12)	S(14)
Candida krusei	R(11)	R(10)	R(11)	R(08)	R(13)
Candida tropicalis	R(14)	R(11)	R(09)	R(12)	R(09)
Candida pseudotropicalis	R(12)	R(13)	R(09)	R(09)	R(11)

KEY: R — resistance and S – Sensitivity

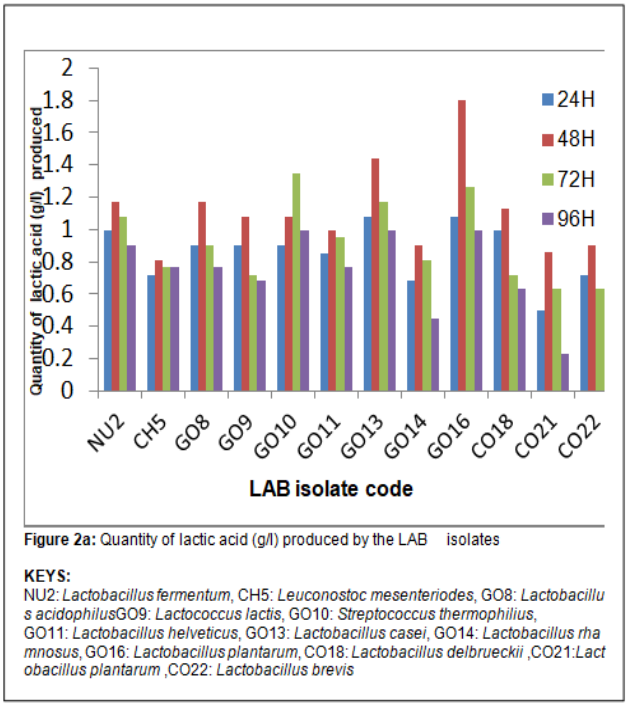

Figure 2a: Quantity of lactic acid (g/l) produced by the LAB isolates

KEYS:
NU2: *Lactobacillus fermentum*, CH5: *Leuconostoc mesenteriodes*, GO8: *Lactobacillus acidophilus*GO9: *Lactococcus lactis*, GO10: *Streptococcus thermophilius*, GO11: *Lactobacillus helveticus*, GO13: *Lactobacillus casei*, GO14: *Lactobacillus rhamnosus*, GO16: *Lactobacillus plantarum*, CO18: *Lactobacillus delbrueckii* ,CO21:*Lactobacillus plantarum* ,CO22: *Lactobacillus brevis*

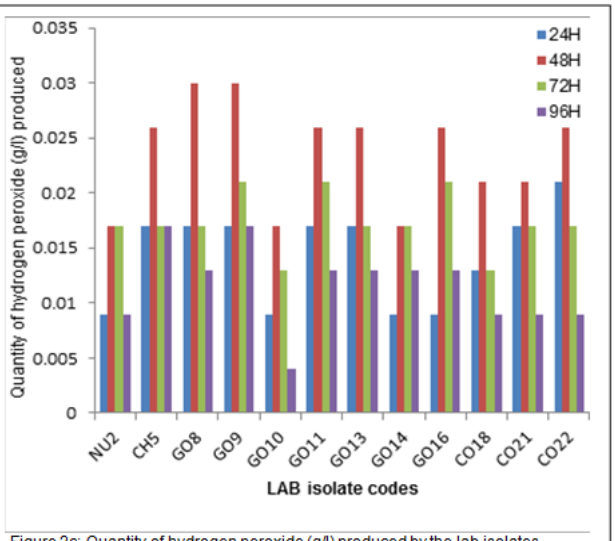

Figure 2c: Quantity of hydrogen peroxide (g/l) produced by the lab isolates

KEYS:
NU2: Lactobacillus fermentum, CH5: Leuconostoc mesenteriodes, GO8: Lactobacillus acidophilusGO9: Lactococcus lactis, GO10: Streptococcus thermophilius GO11: Lactobacillus helveticus, GO13: Lactobacillus casei, GO14: Lactobacillus rhamnosus, GO16: Lactobacillus plantarum, CO18: Lactobacillus delbrueckii , CO21: Lactobacillus plantarum , CO22: Lactobacillus

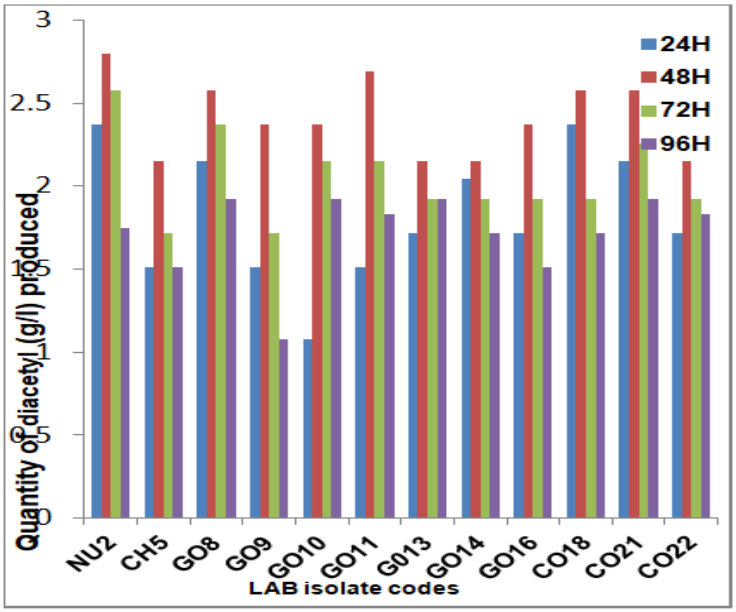

Figure 2b: Quantity of diacetyl (g/l) produced by the LAB isolates

KEYS:
NU2: *Lactobacillus fermentum*, CH5: *Leuconostoc mesenteriodes*, GO8: *Lactobacillus acidophilus*GO9: *Lactococcus lactis*, GO10: *Streptococcus thermophilius*,GO11: *Lactobacillus helveticus*, GO13: *Lactobacillus casei*, GO14: *Lactobacillus rhamnosus*, GO16 : *Lactobacillus plantarum* CO18: *Lactobacillus delbrueckii*, CO21: *Lactobacillus plantarum*, CO22: *Lactobacillus*

Table 4: Antagonistic activity of Lactic acid bacteria (mm) against selected pathogenic bacteria

LAB isolates	Indicator organisms / Zone of Inhibition (mm)							
	Bacillus subtills	***Bacillus cereus***	***Escherichia coli***	***Klebsiella pneumonia***	***Listeria monocytogenes***	***Salmonella typhi***	***Shigeella dysentry***	***Staphylococcus aureus***
Lactobacillus plantarum	-	-	23	-	20	25	20	20
Lactobacillus fermentum	27	25	20	20	26	23	24	24
Lactobacillus delbrueckii	12	14	20	-	22	16	18	27
Lactobacillus plantarum	23	-	22	20	25	25	20	22
Leuconostoc mesenteriodes	-	-	20	22	23	21	23	18
Lactobacillus casei	-	22	22	-	-	-	23	15
Lactobacillus brevis	-	15	14	-	-	-	20	25
Lactobacillus acidophilus	16	21	16	25	-	-	21	28
Lactococcus lactis	-	17	23	-	24	-	26	27
Streptococcus thermophilius	-	14	10	20	17	18	25	25
Lactobacillus. Helveticus	-	27	-	24	27	22	-	20
Lactobacillus delbrueckii	-	20	23	-	20	24	-	-
Lactobacillus casei	20	-	18	-	-	22	16	-
Lactobacillus rhamnosus	-	-	25	-	-	26	24	-
Lactobacillus fermentum	-	22	24	22	18	-	14	-
Lactobacillus plantarum	20	28	27	-	-	27	-	26
Lactococcus lactis	15	15	-	-	23	25	-	16
Lactobacillus delbrueckii	17	-	-	-	16	18	-	16
Lactobacillus fermentum	17	-	20	20	18	-	25	-
Lactobacillus acidophilus	16	-	23	-	-	22	16	18
Lactobacillus plantarum	24	23	26	-	-	24	20	20
Lactobacillus brevis	-	16	14	17	25	24	20	22

Key: No inhibition

Discussion

Lactic acid bacteria: Lactobacillus fermentum, Lactobacillus plantarum Lactobacillus casei, Lactobacillus delbrueckii, Leuconostoc mesenteriod es, Lactobacillus brevis, Lactobacillus acidophilus, Lactococcus lactis, Streptococcus thermophilius, Lac tobacillus helveticus, Lactobacillus delbrueckii and Lactobacillus rhamnosus isolated from milk products such as nono, wara, fermented goat milk and cow milk were identified with reference to Bergey's Manual of Systematic Bacteriology (Sneath et al., 1994). Varngm (2002) reported suitable environment as an important feature of LAB, even though nutritionally demanding. They are able to inhabit a wide range of environments which was the reason why they all found in the acidified milk products as aligned with this research work.

Most of the selected pathogenic bacteria and fungai isolated in this study were multidrug resistant to antibiotics. Resistance to antibiotic could be due to a transferred of gene between bacteria in a horizontal fashion either by conjugation or transduction or by transformation (Adegoke et al., 2010). Therefore, a gene for antibiotic resistance grown from natural selection may be due to many antibiotic resistance genes located on plasmid that assists their transfer (Aubry-Damon and Courvalin, 2003).

Resistances to antibiotics do occur through natural mutation and change (Ochei and Kolhathar, 2000). It may also occur when bacteria continue to multiply at a rapeutically achievable concentration (Roland, 1984). The proportion of resistance by pathogens was quite remarkable in this study. The findings in this research work were similar with what was observed by Lamikanra and Okeke. (1997) in which investigation was carried out on inadequate drug supply and poor drug prescriptions on Tuberculosis. The data obtained in this work confirmed indiscriminate use of antibiotics as reported by Hart and Kariuki (1998); and Okeke et al. (1999).

As a result of multiple-drug resistance, there is a serious implication for the physical manifestations of infections caused by pathogenic microbes. Almost all the antibiotics used in this study are low-priced and are broadly used even without obtaining instructions from accredited health organization; oral ingestion of these drugs are known to provide discriminating pressure eventually leading to a higher level of bacteria and fungi resistant (Levin et al., 1997).

The highest amount of antimicrobial substances was produced by LAB at 48 hours and few at 72 hours, this aligned with the findings of Adegoke *et al.* (2010), Afolabi *et al.* (2008) and Ogunbanwo *et al.* (2004) where they obtained a similar result for *Lactobacillus plantarum* isolated from fufu, a native fermented cassava product. Hence, the presence of LAB in the different habitat is suggestive of their worldwide nature.

Lactobacillus fermentum (NU2) produced the highest amount of diacetyl while *Lactococcus lactis* (GO9) and *Streptococcus thermophilus* (GO10) produced the lowest. The production peak of diacetyl by all the tested isolates was at 48 h of incubation after which deterioration set in. The antimicrobial properties of diacetyl abundant in the scientific journals (Jay et al., 1983). Pathogenic bacteria require exposure to a concentration of approximately 200 mg/kg of the compound to be inhibited (Jay et al., 1983).

Lactobacillus fermentum (NU2) produced the highest quantity of diacetyl while the lowest production of diacetyl was from *Lactococcus lactis* (GO9) and *Streptococcus thermophilus* (GO10). The greatest production of diacetyl by all the tested isolates was at 48 h of incubation after which decline set in. The antimicrobial properties of diacetyl are well-documented (Jay et al., 1983). Gram-negative bacteria require exposure to a concentration of approximately 200 mg/kg of the compound to be inhibited (Jay et al., 1983).

The highest amount of hydrogen peroxide was at 48 h of incubation from *Lactobacillus acidophilus* (GO8) and *Lactococcus lactis* (GO9). Berthier (1993) reported the detection of hydrogen peroxide producing LAB which is often sought because of their antimicrobial activity. Hydrogen peroxide has been used as a well-known antimicrobial since its discovery in 1818 and is utilized as a direct antimicrobial in dairy products and also for the purification of wrapping materials and other surfaces coming into contact with food (Sommers and Sheen, 2015).

Over the years, Lactic acid bacteria had been taken to be safe organisms which produce antimicrobial substances that initiate bactericidal to pathogenic microbes (Sexline *et al.,* 1996; Bromberg *et al*.,

2005). All the LAB isolates obtained in this study showed antimicrobial activity against pathogenic bacteria and yeast. This is similar to the work carried out by Adeniyi *et al*. (2006) on the antimicrobial activities of lactic acid bacteria isolated from diary food (fermented) against organisms associated with urinary tract infection. Sexline et al. *(*1996) also carried out isolation and screening of antibacterial producing lactic acid bacteria from fermentation of millet gruel. The ability of LAB to show antimicrobial activity against other microorganisms is well documented. Afolabi et al. (2008) showed that antimicrobial producing microorganisms had the tendency to inhibit the growth of pathogenic bacteria. The LAB isolates were able to inhibit the selected indicator organisms at different degrees. The ability to inhibit other organisms is due to the fact that LAB produces antimicrobial compounds which are injurious to the pathogenic organisms depending on the concentration or quantity produced.

In this study, the antagonistic activity of lactic acid bacteria against selected pathogenic bacteria such as *Bacillus subtills, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Salmonella typhi, Shigella dysentry* and *Staphylococcus aureus* was observed. *Lactobacillus plantarum* (GO16) inhibited *Bacillus cereus* while *Lactobacillus acidophilus* (GO8) inhibited *Staphylococcus aureus* with the highest zone of inhibition while *Lactobacillus delbrueckii* (NU3) showed the lowest zone of inhibition against *Bacillus subtilis*. Such antimicrobial activity were also demonstrated in the works of Adesokan *et al*. (2008) where LAB species inhibited *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, Escherichia coli* and *Proteus vulgaris.* The antagonistic activity of *Lactobacillus plantarum* against *S. aureus*, *B. substilis, S. typhii* and *E. coli* has been reported by Obadina *et al*. *(*2006). More so, the antibacterial substances produced by LAB that can inhibit pathogenic bacteria of possible contaminants in fermented products had been reported (Raccah *et al*., 1979; Smith and Palumbo, 1983; Cintas *et al.,* 1998). Daeschel (1993) described the tendency of LAB to yield lactic acid which reduced the pH of the fermenting medium discouraging the survival of spoilage and foodborne bacteria. This is responsible for the improved stability and safety of the microbiological food. The acidity could also leads to the curdling of the final product which is typical of fermented grains and vegetables.

The hydrogen peroxide produced by LAB helped the activity of antimicrobial and in some cases a pioneer for the production of other effective antimicrobial compounds such as hydroxyl (OH^-) radicals and super oxide (O_2^-) (Condon, 1983; Thomas and Pera, 1983). The antimicrobial action of hydrogen peroxide may result from the sulfhydryl groups' oxidation thus leading to the destruction of a number of enzymes, and from the penetration of membrane lipids thereby increasing membrane penetration (Kong and Davison, 2000).

Lactic acid bacteria produced diacetyl mainly such strains include *Leuconostoc, Lactococcus, Pediococcus* and *Lactobacillus* which exhibited strong inhibitory action against pathogenic bacteria (Schnurer and Magnusson, 2005). They are able to perform this role because of the production of diacetyl, which contribute to the distinctive aroma and taste of many foods, particularly products obtained from animals and their antimicrobial effect has been linked to diacetyl production (Jay, 1996).

Scientific investigations have shown that management with antifungal drugs usually lead to strains of fungi that are resistant (Hampton, 2008). Multi drug resistance fungi are caused by the increased expression of genes that expresses nonspecific drug-efflux pumps which is of the family ABC transporter proteins (Balzi *et al*., 1987). Early investigations have shown the antimicrobial activities of LAB against fungal pathogens which are in support with this work as *Candida albican* had the highest zone of inhibition (25 mm) by *Lactobacillus plantarum* (NU1) and *Lactobacillus plantarum* (GO16). Ronnqvist *et al.* (2007) reported that *L. fermentum* showed activity against *C. glabrata* and *C. albicans*. No activity was demonstrated against *C. pseudotropicalis* which confirms the work of Gulahmadov *et al*. (2009). Research work on the activity of LAB antifungal showed that the production of substances that inhibited fungal occurred among many different species although; species of the genus *Lactobacillus* were described in the majority of studies (Sathe *et al.,* 2007).

Most studies of the effects of weak acids on fungal growth have established that a certain pH is necessary for the inhibitory action, whereby the

acid is undissociated, leading to diffusion across the membrane (Adegoke *et al.,* 2010). Diacetyl is important for the organoleptic quality of food products. Diacetyl is known to be effective against yeasts and molds (Jay, 1996). Apart from the actual inhibition of fungal growth, LAB can also specifically inhibit production of mycotoxins (Gourama and Bullerman, 1997) or immobilize mycotoxins through binding to their surface.

However, in this research work, while comparing the antibacterial action of lactic acid bacteria on pathogenic bacteria and fungi, it was revealed that the activity shown on the two agents were similar to a variable grade of activity with the antimicrobial effect on lactic acid bacteria showing a greater zone of inhibition against pathogenic bacteria than pathogenic *Candida* species. The inhibitory activity of the LAB isolates has been credited to the production of hydrogen peroxide, diacetyl and lactic acid (Ogunbanwo, 2005; Adeniyi *et al.,* 2006; Sathe *et al*., 2007; Adesokan *et al*., 2008; Afolabi *et al.,* 2008).

Conclusion:

The inhibitory activity of Lactic acid bacteria against pathogenic bacteria and *Candida* species could be attributed to the production of Lactic acid, hydrogen peroxide and diacetyl and could be used as probiotics in the complementary therapy for the prevention of illnesses/ infections caused by these pathogens. As these pathogenic microorganisms have developed resistance to most of the commercially available antibiotics, thereby making treatment difficult. Lactic acid bacteria are strongly recommended because it is cheap and the possibility of the microorganisms developing resistance to it is very remote because it has not been reported to have any adverse side effect.

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