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QUANTIFICATION OF ANTIMICROBIAL METABOLITES PRODUCED BY LACTIC ACID BACTERIA (LAB) ISOLATED FROM FERMENTED FOOD PRODUCTS

[1]Samira Arzika, [1]Musliu Abdulkadir, [2]Jemilu M. Ambrusa & [2]Idayat Shade Ijaiya
[1]Department of Science Laboratory Technology, College of Science and Technology
[2]Department of Preliminary Studies, College of Science and Technology
Waziri Umaru Federal Polytechnic, Birnin Kebbi, Kebbi State
Email: musille2003@yahoo.com

ABSTRACT

The lactic acid bacteria (LAB) used in this study was isolated from four fermented food products- ogi, yoghurt, cow and soya milk. Eight lactic acid bacteria (LAB) were isolated from all the samples. Lactobacillus fermentum had the highest percentage of occurrence (29%) while Lactobacillus casei had the least occurrence of 14%. The isolated LAB was tested for the production of various antimicrobial compounds such as lactic acid, diacetyl and hydrogen peroxide and quantitative estimation of these antimicrobial compounds was carried out. All the test isolates produced diacetyl with the peak of production after 24 hours of incubation. The highest yield of diacetyl was 3.01 g/L produced by Lactobacillus casei, highest yield of lactic acid was 2.25 g/L produced by L. Plantarum OGI1 while the highest yield of hydrogen peroxide was 2.24 g/L produced by L. Plantarum OGI1.

Keywords: Lactic Acid Bacteria, Lactobacillus Fermentum, Lactobacillus Casei, Antimicrobial Compounds, Diacetyl, Oxygen Proxide, Incubation.

INTRODUCTION

Lactic acid bacteria (LAB) consist of a number of bacterial genera within the phylum Formicates. The genera *Carnobacterium*, *Enterococcus*, Lactobacillus, *Lactococcus*, *Lactosphaera-*, *Leuconostoc*, *Melissococcus*, *Oenococcus, Pediococcus,* Streptococcus, *Tetragenococcus,* Vagococcus and Weissellaare recognized as LAB (Aly *et al.,* 2004) Lactic acid-producing Gram-positive bacteria but belonging to the phylum Actinobacteria are genera such *as Aerococcus, Microbacterium*, and Propionibacterium as well as *Bifidobacterium*. Members of LAB share the property of being Gram-positive bacteria that ferment carbohydrates into energy and lactic acid (De Vuyst and Vandamme,

1994). Depending on the organism, metabolic pathways differ when glucose is the main carbon source: *homo fermentative* bacteria such as *Lactococcus* and *Streptococcus* yield two lactates from one glucose molecule, whereas the *heterofermentative* (ie. *Leuconostoc* and *Weissella*) transform a glucose molecule into lactate, ethanol and carbon dioxide (Delphine *et al.*, 2011). In addition, LAB produces small organic compounds that give the aroma and flavor to the fermented product (Derek *et al.,* 2009).

The taxonomy of LAB based on comparative 16S ribosomal RNA (rRNA) sequencing analysis has revealed that some taxes generated on the basis on phenotypic features do not correspond with the phylogenetic relations. Molecular techniques, especially polymerase chain reaction (PCR) based methods, such asrep-PCR fingerprinting and restriction fragment length polymorphisms (RFLP) as well as pulse-field gel electrophoresis (PFGE), are regarded important for specific characterization and detection of LAB strains. Recently, culture-independent approaches have been applied for the detection of intestinal micro biota (Derek *et al.,* 2009). Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) analysis of faucal 16S rDNA gene and its Rrna amplicons have shown to be powerful approaches in determining and monitoring the bacterial community in faeces (Facklam and Elliot, 1995). The aims of the present study are to determined metabolites produced by lactic acid bacteria, isolated from fermented milk product, further they are used as potential starter culture in food fermentation.

ANTIMICROBIAL SUBSTANCE PRODUCE BY LAB

Lactic acid bacteria (LAB) are a group of gram-positive bacteria including the general *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* and *Streptococcus.* The general description of the bacteria included in the group is gram-positive, non-spore forming, cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. Lactic acid bacteria are nutritionally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acids and vitamins. Recent taxonomic revisions of these genera suggest that the lactic acid bacteria comprise the following: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella*. The classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, and configuration of the lactic acid produced, ability to grow at high salt

concentrations, and acid or alkaline tolerance. The lactic acid bacteria can be mainly divided into two groups based on the end-products formed during the fermentation of glucose. Homofermentative lactic acid bacteria such as *Pediococcus, Streptococcus, Lactococcus* and some lactobacilli produce lactic acid as the major or sole end-product of glucose fermentation. Homofermentative lactic acid bacteria use the Embden-Meyerhof-Parnas pathway to generate two moles of lactate per mole of glucose and derive approximately twice as much energy per mole of glucose as heterofermentative lactic acid bacteria. Heterofermentative lactic acid bacteria such as *Weissella* and *Leuconostoc* and some lactobacilli produce equimolar amounts of lactate, CO_2 and ethanol from glucose via the hexose monophosphate or pentose pathway. Foods distinctive flavors, textures, and aromas while preventing spoilage, extending shelf-life, and inhibiting pathogenic organisms. The preservative action of starter culture in food and beverage systems is attributed to the combined action of a range of antimicrobial metabolites produced during the fermentation process. These include many organic acids such as lactic, acetic and propionic acids produced as end products which provide an acidic environment unfavourable for the growth of many pathogenic and spoilage microorganisms.

Acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions. They have a very broad mode of action and inhibit both gram-positive and gram-negative bacteria as well as yeast and moulds. One good example is propionic acid produced by propionic acid bacteria, which has formed the basis for some biopreservative products, given its antimicrobial action against microorganisms including yeast and moulds. Microgard is a Food and Drug Administration (FAD)-approved fermentate produced by *Propionibacterium freudenreichii* subsp. *shermanii* which contains propionic acid and is used in an estimated 30% of the cottage cheese manufactured in the United State. In addition to acids, starter strains can produce a range of other antimicrobial metabolites such as ethanol from the heterofermentative pathway, H_2O_2 produced during aerobic growth and diacetyl which is generated from excess pyruvate coming from citrate.

In particular, H_2O_2 can have a strong oxidizing effect on membrane lipids and cellular proteins and is produced using such enzymes as the flavo protein oxidoreductases NADH peroxidase, NADH oxidase and glycerophosphate oxidase. Obviously, each antimicrobial compound produced during fermentation

provides an additional hurdle for pathogens and spoilage bacteria to overcome before they can survive and/or proliferate in a food or beverage, from time of manufacture to time of consumption. Since any microorganism may produce a number of inhibitory substances, its antimicrobial potential is defined by the collective action of its metabolic products on undesirable bacteria (García-Ruiz *et al.,* 2011). Other examples of secondary metabolites produced by lactic acid bacteria which have antagonistic activity include the compound reuterin and the recently discovered antibiotic reuterocyclin, both of which are produced by strains of *Lactobacillus reuteri*. Reuterin is an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of hydroxy-propionaldehyde. It has broad spectrum of activity and inhibits fungi, protozoa and a wide range of bacteria including both gram-positive and gram-negative bacteria. This compound is produced by stationary phase cultures during anaerobic growth on a mixture of glucose and glycerol or glyceraldehydes.

More recently, the first antibiotic produced by lactic acid bacteria was discovered. Reuterocyclin is a negatively charged, highly hydrophobic antagonist, and structural elucidation revealed it to be a novel tetramic acid. The spectrum of inhibition of the antibiotic is confined to gram-positive bacteria including *Lactobacillus* spp., *Bacillus subtilis, Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus* and *Listeria innocua.* Interestingly, inhibition of *Escherichia coli* and *Salmonella* is observed under conditions that disrupt the outer membrane, including truncated lipopolysaccharides (LPS), low pH and high salt concentrations. Since it is well known that nisin can kill gram-negative bacteria under conditions which disturb the outer membrane, it is likely that there are similarities in the mode of action of nisin and this novel antibiotic (García-Ruiz *et al.,* 2011).

METHODOLOGY

Collection of Sample

The raw cow milk were purchased from local farmers and allowed to ferment spontaneously, soya beans were purchased from Birnin Kebbi central market, sort out and processed for soya, milk preparation, fermenting ogi was also collected from household and a sample of soy milk and yogurt was purchased from super market at gesse phase 1.

Media Preparation

DeMann Rogosa and Sharpe medium (MRS) for the growth of Lactic acid bacteria, the medium was aseptically prepared using manufactures instructions and standard operating procedure.

Preparation of Sample

The collected samples were allowed to fermented 24hrs.

ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA TO GENUS LEVEL

0.1ml of each sample was aseptically added into 10ml of sterile distilled water. These were then homogenized and serially diluted to 10^4; 1ml of the diluent was poured plated on the man, Rogosa and Sharpe (MRS) agar, respectively. Plates were then incubated at 37^0C for 48hrs. The representative colonies were then selected randomly and sub-cultured on MRS agar culture plates. The stock isolates were stored at 4°c for subsequent identification. The bacteria were characterized by microscopic morphological examination and by conventional biochemical tests. Such as Gram staining, catalase activity, gas production from glucose, Harrigen and Maccance (1966) and Roissart and Luguet (2008) while the identification work will be done according to the method described in Bergey's Manual (Baird-Parker, 19974).

Colony Morphology

The cultural characteristics and the cellular morphology of the isolates were determined on the MRS Agar plates.

Smear Making and Gram Staining

The inoculating loop was sterilized to red hot in a flame then allowed to cool and was used to place a drop of water on a clean glass slide. The loop was re-sterilized and then used to pick a small portion of the 24 hours old growth culture of the test organism and then was spread on the slide to give a thin homogenous film or smear. Then the loop was sterilized and kept aseptically. The smear was labeled appropriately, allowed to air dry and then heat fixed. The fixed smear was flooded with crystal violet stain for 60 seconds (primary dye). Then washed off rapidly with distilled water, the water on the slide was tip off and then covered with lugol's iodine for 60 seconds (mordent). The stain was decolourized with acetone/ethanol and washed off immediately with distilled water then counter stained with safranin for 60 seconds (secondary dye) and washed off with distilled water. The back of the slide was wipe cleaned

with cotton wool and was allowed to air dried. They were examined under the microscope for its reaction with gram's reagents, purple colour is positive, while red or pink is negative to Gram's reaction (Barrrow and Feltman, 1993).

Biochemical Test

A. Catalase Test

A drop of 3% freshly prepared hydrogen pereoxide was put on a clean slide, sterile inoculating wire loop was used to pick pure isolates of 18 to 24 hours of incubation from agar plates and smear. Evolution of gas as white froth indicates a catalase positive reaction. Absence of this froth indicates a negative reaction (Barrow and Feltham, 1993).

$2H_2O_2 \rightarrow 2H_2O+O_2$

B. Oxidase Test

Filter paper was soaked in 1% aqueous tetramethyl-p-phenylenediaminedihyrochloride (oxidase reagent). Flamed platinum wire loop was used to collect 24 hours culture and was nibbled on the filter paper. The appearance of a deep purple colour within 10 seconds indicates positive reaction, while the absence of deep purple colour indicates negative reaction (Barrow and Feltham, 1993).

IDENTIFICATION OF LACTIC ACID BACTERIA TO THE SPECIES LEVEL

After their microscopic examination, gram positive, oxidase and catalase negative *lactobacillus* were tested for their sugar fermentation pattern, production of ammonia from arginine in addition to their ability of growth at 15^0C and 45^0 C according to Harrigan and Maccance (1966).

Sugar Fermentation

MRS broth with phenol red indicator and inverted Durham's tubes was used to confirm glucose fermentation by lactic acid bacteria and the other sugar used were as substitute in MRS broth without glucose and meat extract that is, the sugars that were tested for were use in place of glucose (Ashe and Paul, 2010). Acid production is shown by a change in the colour of the indicator, phenol red change to yellow and gas production is shown in the Durham tubes. No change in colour (red) indicates no acid nor gas production (Barrow and Feltham, 1993).

Physiological Tests

Physiological tests were conducted as described by Schlinger and Lucke (1989). The isolates were tested for growth at different pH level, different temperature and different salt concentration. Suspected LAB were tested at pH of 3, 4, 5 and 6 and growth temperature of 15°C and 45°C.

QUANTITATIVE DETERMINATION OF ANTIMICROBIAL COMPOUND PRODUCED BY LACTIC ACID BACTERIA

For these measurements the test organisms were grown in MRS broth for 72h and centrifuged at 4000g for 30 min.

Quantitative Estimation of Lactic Acid Production

The production of lactic acid was determined by transferring 25ml of supernatant fluid of test organisms into 100ml flasks. This was titrated with 0.1M NaOH and 1ml of phenolphthalein indicator (0.5 in 5% alcohols). The titratable acidity was calculated as lactic acid % w/v (Ogunbanwo, 2005). Each millimetre of 0.1M 4aOH is equivalent to 90.08mg of Lactic acid. The titratable acidity was then calculated as stated in A.O.A.C (1980) as;

$$\text{Titratable acidity} = \frac{\text{MI NaOH} * \text{N NaOH} * \text{M.E} * 100}{\text{Volume of Sample Used}}$$

Where: MI = Volume of NaOH used, N NaOH = molarity of NaOH solution, M.E = Equivalent factor.

Quantitative Estimation of Hydrogen Peroxide Production

20mL of dilute H_2SO_4 acid was added to 25mL of the supernatant fluid of the test organism. Titration was carried out with 0.1M potassium permanganate (KMnO4). Each mL of 0.1M. Potassium permanganate is equivalent to 1.79mg of Hydrogen peroxide solution. Decolourization of the sample was regarded as the end point. The volume of H_2O_2 produced was then calculated (A.O.A.C, 1990) as;

$$H_2O_2 \text{ produced} = \frac{\text{ml } KMnO_4 * \text{N } KMnO_4 * \text{M.E} * 100}{\text{Ml } H_2SO_4 * \text{volume of sample}}$$

Where; ml $KMnO_4$ = Volume of $KMnO_4$ used, N $KMnO_4$, ml H_2SO_4 = Volume of H_2SO_4 added, M.E = Equivalent factor.

Quantitative Estimation of Diacetyl Production

Diacetyl production was determined by transferring 25mL of the supernatant fluid of the test organisms into 100mL flasks. Hydroxylamine solution (7.5 mL) of 1M was added to the flask and to a similar flask for residual titration. Both were titrated with 0.1M HCL to a greenish yellow end point using bromophenol

blue as indicator (Ogunbanwo, 2005) the equivalent factor of HCL to diacetyl is 21.52mg. The concentration of diacetyl produced was calculated using the A.O.A.C (1980) as;

$$Ak = \frac{(b - s)(100E)}{W}$$

Where; Ak = percentage of diacetyl, b = No of 0.1mL HCL consumed in titration of sample, E= Equivalent factor, W = volume of sample

RESULTS AND DISCUSSION

Results

Table 4.1: Biochemical Characterization of Isolates from Fermenting Yogurt, Soya and Cow Milk and Ogi

Isolate Code	Gram Staining	Catalase	Oxidase	Genera
MC	+bacilli	Negative	Negative	Lactic acid bacteria
YU2P	+bacilli	Negative	Negative	Lactic acid bacteria
YU2C	+bacilli	Negative	Negative	Lactic acid bacteria
SOYA	+bacilli	Negative	Negative	Lactic acid bacteria
SOYA 1	+bacilli	Negative	Negative	Lactic acid bacteria
SOYA 2	+bacilli	Negative	Negative	Lactic acid bacteria
OGI 1	+bacilli	Negative	Negative	Lactic acid bacteria
OGI 2	+bacilli	Negative	Negative	Lactic acid bacteria

Table 4.2: Physiological Reactions and Carbohydrate Fermentation Pattern of Lactic Acid Bacteria Isolated From fermented Milk Samples

Isolate code	Growth at 15°C	Growth at 45°C	Growth at pH 3.9	Growth at pH 8.5	Growth at pH 9.6	Growth at 4.5% NaCl	Growth at 6.5% NaCl	Growth at 8% NaCl	Arginine Hydrolysis	Fermentation Type	Xylose	Raffinose	Sorbitol	Salicin	Maltose	Mannitol	Arabinose	Ribose	Lactose	Sucrose	Inositol	Glucose	Fructose	Trehalose	Melibiose	Probable Identity
MC	+	+	+	+	+	+	+	-	-	Hetero	+	+	-	+	+	-	+	+	+	+	-	+	+	+	+	*Leuconostoc mesenteroides*
YU2P	-	+	+	+	-	+	+	-	+	Hetero	-	+	-	-	+	-	-	+	+	+	+	+	+	-	+	*Lactobacillus fermentum*
YU2C	-	+	+	+	-	+	+		-	Hetero	-	+	-	-	+	-	-	+	+	+	+	+	+	-	+	*Lactobacillus casei*
SOYA	-	-	+	+	-		+	-	-	Hetero	-	+	-	+	+	-	+	+	+	-	-	+	+	-	-	*Lactobacillus acidophilus*
OGI1	+	-	+	-	-	+	+	-	-	Hetero	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	*Lactobacillus plantarum*
SOYA1	-	-	+	+	-		+	-	-	Hetero	-	+	-	+	+	-	+	+	+	-	-	+	+	-	-	*Lactobacillus acidophilus*
OGI2	+	-	+	-	-	+	+	-	-	Hetero	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	*Lactobacillus plantarum*
SOYA2	-	+	+	+	-	+	+	-	+	Hetero	-	+	-	-	+	-	-	+	+	+	+	+	+	-	+	*Lactobacillus fermentum*

Key: Soya = isolates from soya milk; MC = isolates from milk; Yu = isolates from yoghurt; Ogi = isolates from ogi; + = positive, - = negative

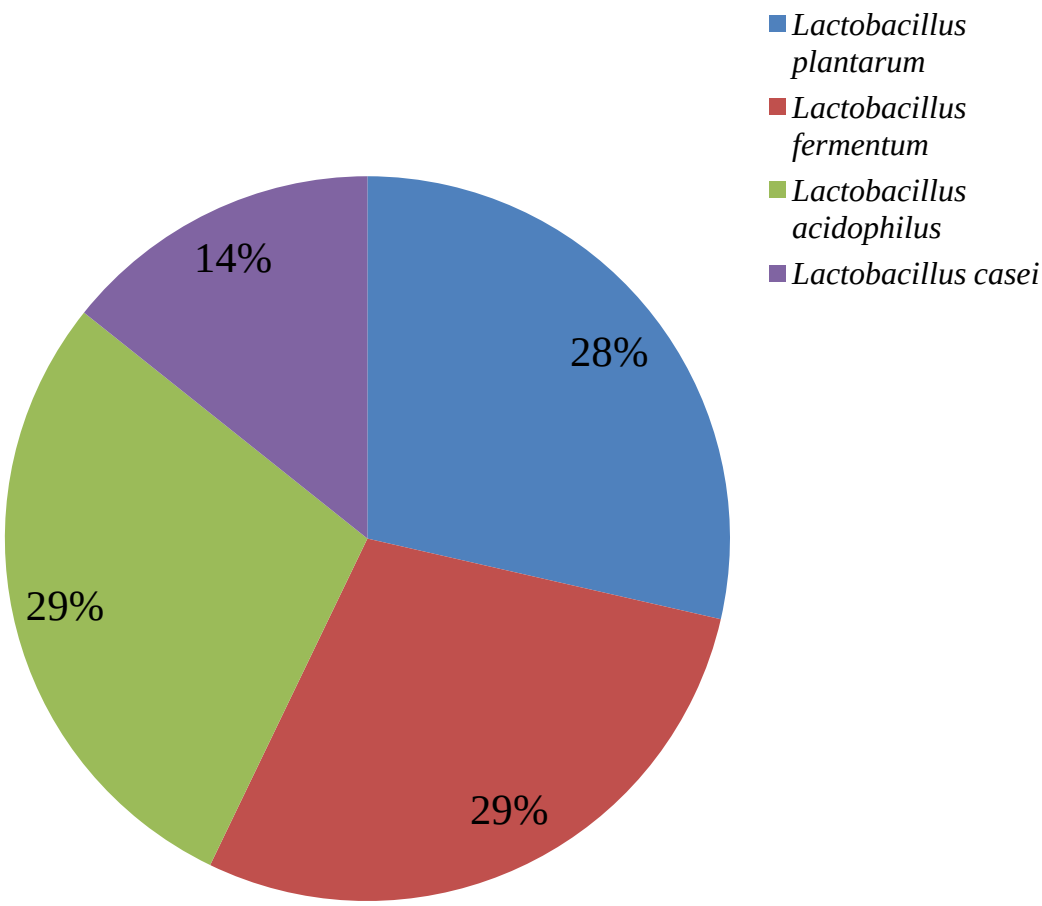

Fig. 4.1: Percentage occurrence of lactic acid bacterial isolated from fermenting cow milk, yogurt, ogi and soya milk sample.

Table 4.3: Quantity of Diacetyl Produced by Isolates of Lactic Acid Bacteria (g/l)

Isolates	Fermentation Time (hours)/Diacetyl Concentration (g/L)		
	8	16	24
Lactobacillus fermentum YU2P	0.49	1.32	1.49
Lactobacillus fermentum SOYA2	0.52	1.44	2.90
Leuconostoc mesenteroides MC	0.42	1.31	1.78
Lactobacillus acidophilus SOYA	0.43	1.23	1.90
Lactobacillus acidophilus SOYA1	0.49	1.32	2.3
Lactobacillus plantarum OGI1	0.45	1.88	1.83
Lactobacillus plantarum OGI2	0.52	1.34	1.93
Lactobacillus casei YU2C	0.49	1.49	3.01

Table 4.4: Quantity of Lactic Acid Produced by Isolates of Lactic Acid Bacteria (g/l)

Isolates	Fermentation Time (hours)/Lactic acid Concentration (g/L)		
	8	16	24
Lactobacillus fermentum YU2P	0.41	1.33	1.48
Lactobacillus fermentum SOYA2	1.22	1.84	2.26
Leuconostoc mesenteroides MC	0.41	1.22	1.35
Lactobacillus acidophilus SOYA	0.49	1.53	1.71
Lactobacillus acidophilus SOYA1	0.40	0.82	1.17
Lactobacillus plantarum OGI1	1.87	1.97	1.71
Lactobacillus plantarum OGI2	0.97	1.37	2.25
Lactobacillus casei YU2C	0.46	1.23	1.62

Table 4.5: Quantity of Hydrogen Pereoxide (H_2O_2) Produced by Isolates of Lactic Acid Bacteria (g/l)

Isolates	Fermentation Time (hours)/H_2O_2 Concentration (g/L)		
	8	16	24
Lactobacillus fermentum YU2P	0.43	1.20	1.57
Lactobacillus fermentum SOYA2	0.92	1.24	1.36
Leuconostoc mesenteroides MC	0.45	0.92	1.11
Lactobacillus acidophilus SOYA	0.59	1.33	1.84
Lactobacillus acidophilus SOYA1	0.48	1.22	1.34
Lactobacillus plantarum OGI1	1.17	2.27	2.24
Lactobacillus plantarum OGI2	0.47	0.97	1.34
Lactobacillus casei YU2C	0.46	1.23	0.90

DISCUSSION

The result for the biochemical test of isolated bacteria is shown in Table 4.1, eight isolates where obtained, the lactic acid bacteria isolated from all the fermented samples were catalase and oxidase negative, which is a general characteristic of all lactic acid bacteria. All of the isolates were positive rods. This result agrees with the description of Kandler and Weiss (1986) for lactic producing bacteria and confirmed with Bergey's manual of systematic bacteriology (Baird-Parker, 1974). Table 4.2 shows the physiological reactions and carbohydrate fermentation pattern of lactic acid bacteria isolated from fermented food samples. All the isolated lactic acid bacteria were hetero-fermentative organism. *Lactobacillus fermentum* had the highest percentage of occurrence (29%) followed by *Lactobacillus platarum* (28%) while *Lactobacillus casei* had the least occurrence of 14% (Fig. 4.1).

In this study, the isolated LAB produced antimicrobial compounds to a varying degree. *Lactobacillus plantarum* OGI1 recorded the highest yield of lactic acid (2.25 g/l) after 24 h of growth in MRS broth while *Lactobacillus acidophilus* had the lowest value of 0.40 g/l. Although lactic acid production increased with time, it was observed that the production peak was reached at 24h of growth for all the test isolates (table 4.4). The highest concentration of lactic acid produced by *L. plantarum* agreed with the findings of Ogunbanwo, (2005). The increase in the production of lactic acid with time has been attributed to lower pH, which permit the growth of LAB to the detriment of the competing organism (Kandler and Weiss, 1986). Lactic acid bacteria are known to inhibit the growth of unrelated organisms in a mixed culture. The antimicrobial effects of lactic acid and acetic acid have been extensively reviewed (Ogunbanwo *et al.,* 2004). The effect is due to the un-dissociated form of the acids, which can penetrate the membrane and liberate hydrogen ions in the neutral cytoplasm, thus leading to inhibition of vital cell functions (Corlett and Brown, 1980).

All the test isolates produced diacetyl with the peak of production after 24 hours of incubation. However, *Lactobacillus casei* YU2C which produced 3.01 g/l at 24h had the highest yield of diacetyl, while the least producer was *Lactobacillus fermentum* YU2P (table 4.3). Diacetyl is important for the organoleptic quality of fermented products, such as cottage, cheese, butter and fermented cream (Boumerdassi *et al.*, 1997). The antimicrobial properties of diacetyl are well-documented (Jay *et al.*, 1983). All the organisms also produced hydrogen peroxide to a varying degree. After 24h incubation, *Lactobacillus*

plantarum OGI1 had the highest yield of 2.24 g/l, whereas *Leuconostoc mensenteroides* MC had the lowest, 1.11 g/l (table 4.5). Berthier, (1993) reported the detection of hydrogen peroxide producing LAB, which are often searched for because of their antibacterial activity. Collins and Aramak (1980) reported on the inhibition of *Pseudomonas fragi* and *Staphylococcus aureus* by hydrogen peroxide by certain LAB strains, which can contribute to their inhibitory activity against other microorganisms, including food–borne pathogens (Ogunbanwo, 2005).

CONCLUSION

The LAB isolated from fermented foods in this study produced different antimicrobial compounds the quantity varies with time. These compounds may be used to combact the growth of pathogenic microorganisms in the food industry.

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