

Isolation and screening of antimicrobial producing lactic acid bacteria from fermentating millet gruel

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The lactic acid bacteria counts, pH and titratable acidity of the fermenting millet gruel were determined using routine methods. The isolated and identified lactic acid bacterial isolates were *L. plantarum*, *L. fermentum*, *L. mesenteroides*, *L. jensenii*, *L. brevis*, *P. acidilactici* and *Lactobacillus* spp. These isolates were able to inhibit the growth of various indicator organisms; *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus licheniformis*, *Salmonella* spp., *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Proteus* spp., *Serratia* spp. and *Pseudomonas syringae* used in this investigation at varying degrees. Quantities of lactic acid, diacetyl and Hydrogen peroxide produced were also determined using standard methods. The pH of the growth medium was observed to have a significant effect on the production of these antimicrobial compounds by the respective lactic acid bacteria.

Keywords: Fermentation, lactic acid bacteria, indicator organisms, antimicrobial compounds.

INTRODUCTION

Millet is a staple food in many parts of Africa, Asia, Central America and the Arab countries of the Middle East and serves as the main source of beverage in some other countries (Kent, 1983). The grain is a superior food stuff containing at least 9% protein and a good balance of amino acids. It has more oil than maize and is a high-energy cereal. It has neither the tannins nor the other compounds that reduce digestibility in sorghum (Seenappa, 1987). The millets include species in several genera, mostly in the subfamily *Panicoideae*, of the grass family *Poaceae*. The most widely cultivated species in order of worldwide production are : Pearl millet (*Pennisetum glaucum*), Foxtail millet (*Setaria italica*), Proso millet (*Panicum miliaceum*) and Finger millet (*Eleusine coracana*) other types of millets include: Barnyard millet (*Echinochloa* spp.), Kodo millet (*Paspalum scrobiculatum*), Little millet (*Panicum sumatrense*), Guinea millet (*Brachiaria deflexa*) and Browntop millet (*Urochloa ramosa*) (Ihekoronye and Ngoddy, 1985). Millet grains are consumed in various forms which includes ; Tuwo (a thick porridge served with

soup), Fura (partially cooked millet flour pounded and rolled into balls), Masa (A deep-fried or water cooked snack served at occasions) Kunnu Zaki (A non alcoholic drink), kunu-tsamia (a non fermented meal) (Wakil *et al.*, 2004). Millet grains are usually fermented by steeping in water, after which they are wet milled, sieved through a screen mash and allowed to sediment and become sour.

Pearl millet was domesticated as a food crop in the tropical region of East Africa at least 4,000 years ago. Its use as a food grain has grown over the centuries, with an estimated 64 million acres of pearl millet being grown in Africa and India (this acreage is equivalent to the total U.S. corn crop) (Crawford, 1992). Pearl millet (*Pennisetum glaucum*) grown for grain has a growth habit similar to sorghum. Pearl millet is a warm season crop, planted in early summer when soils have warmed up and it reaches the stage of 50% flowering in about 60 to 70 days from planting. The flowers and seeds occur in a spike at the end of the stem or tillers, looking somewhat like a cattail or bullrush head. Including the grain head, the plant will typically be about 4 to 5 feet tall although height can vary from 3 to 6 feet depending on variety and growing conditions. The crop is primarily cross pollinated, and following pollination, it takes a flower about 30 more days to develop into a mature seed. Grain heads will mature a few weeks prior to leaf dry down, but seed

shatter is not usually a problem (Hoseney, 1988).

Fermentation processes play important roles in food technology in developing countries. In traditional fermentation processes, natural micro-organisms are employed in the preparation and preservation of different types of food. These processes add to the nutritive value of foods as well as enhancing flavor and other desirable qualities associated with digestibility and edibility (Khetarpaul and Chauhan, 1991). Fermentation extends the shelf life, adds a variety of flavors, and enhances the nutritional value of processed foods. In traditional fermented foods, the fermentation techniques are often characterized by the use of simple, non-sterile equipment, chance or natural inoculums, unregulated conditions, sensory fluctuations, poor durability, and unattractive packaging of the processed products. The production of fermented foods is still largely a traditional family art done in homes in a crude manner (Odunfa, 1985). The organisms involved in the fermentation of African foods are restricted to a few yeasts and bacteria. Cassava is fermented by lactic acid bacteria and some yeasts. The cereal for gruels and alcoholic beverages are fermented mainly by yeast and lactic acid bacteria. Organisms involved in fermentation of cereal include *Saccharomyces cerevisiae*, *Lactobacillus* spp, *Fusarium* spp, *Candida mycoderma*, *Penicillium* spp., *Corynebacterium*, *Aerobacter*, *Rhodoturula* and *Cephalosporium* (Akinrele, 1970).

Now-a-days consumers are concerned about the synthetic chemicals used as preservatives in food, and there is resulting trend towards less processed food. These untreated foods can harbor dangerous pathogens which can multiply under refrigeration and without oxygen. A solution to this dilemma is the use of antimicrobial metabolites of fermentative microorganisms. Many antimicrobial chemicals have been in use for a long time without any known adverse effects. Many of the organic compounds which have stirred interest are antimicrobial metabolites of bacteria used to produce, or associated with fermented foods (Ray and Daeschel, 1992). Hence fermentative microorganisms are being researched to improve the use as biopreservative which offers a possibility for the development of effective natural preservation method for use in food industries. Already nisin which is a bacteriocin produced by lactic acid bacteria has been in use for many years as a biopreservative.

The potential application of such antimicrobial substances as consumer friendly biopreservatives in either the form of protective cultures or as additives is significant besides being less potentially toxic or carcinogenic than current antimicrobial agents. Lactic acid bacteria and their byproducts have been shown to be more effective and flexible in several applications, these antimicrobial substances are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in various foods. This research is vital in the

sense that functional properties of antimicrobial producing microorganisms improve preservative effect and add flavor and taste. Isolation and screening of microorganisms from naturally occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes (Vandenberg, 1993). These isolated strains can positively have impact on their use as starter cultures for traditional fermented foods, with a view to improving the hygiene and safety of fermented food so produced. Therefore the objectives of this study are to isolate and characterize antimicrobial producing lactic acid bacteria present in fermenting pearl millet gruel, and to determine the activity of the antimicrobial produced.

MATERIALS AND METHODS

Collection of samples

Samples of pearl millet (*Pennisetum glaucum*) used for this study were obtained from 'Bodija' market Ibadan, Oyo state, Nigeria. The millet was purchased in clean polythene bags and brought to the microbiology laboratory of the department of Botany and Microbiology, University of Ibadan, Ibadan and kept in the refrigerator for further analysis.

Sample treatment and isolation

The millet grains were cleaned and washed using distilled water and then sundried. Two hundred grams of pearl millet was weighed and dry milled using a clean surface sterilized domestic blender. The blended millet powder was mixed with one (1) liter of sterile distilled water and allowed to ferment spontaneously for 72 hours. 1ml of fermenting gruel and steep water was aseptically removed from the fermenting vessel after shaking for serial dilution at 0hr, 24hrs, 48hrs and 72hrs, and plated out in the MRS agar medium and pure cultures of organisms obtained by streaking were stored on slants for further work.

Characterization of the lactic acid bacteria isolates

The lactic acid bacteria isolated from the pearl millet fermenting gruel were characterized and identified according to methods stated by Bergey's Manual of Systematic Bacteriology (Sneath, 1986).

Determination of pH

The pH change of the fermenting gruel and steeping water was monitored daily using a pH meter. Ten

milliliters of the fermenting gruel was aseptically removed and its pH was determined using a pH meter equipped with a glass electrode.

Titrateable acidity

The titrateable acidity (expressed as percentage lactic acid) was determined by shaking the fermenting vessel to enable mixing of steep water and fermenting gruel as described by A.O.A.C. (1980).

Screening for antimicrobial producing lactic acid bacteria

Indicator organisms used were *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus licheniformis*, *Salmonella* spp., *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Proteus* spp., *Serratia* spp. and *Pseudomonas syringae* collected from the postgraduate laboratory of the department of Botany and Microbiology, University of Ibadan. The organisms were stored in nutrient broth and day old cultures were used to test all isolates for antimicrobial activity and further study was done on organisms which showed positive antimicrobial activity.

Antimicrobial activities of the respective lactic acid bacteria were screened by the agar-well diffusion method (Tagg and McGiven, 1971). 20ml of Mueller-Hinton agar (Becton Dickinson, USA) inoculated with overnight culture (10^6 cfu/ml) of an indicator strain was overlaid on an agar plate. After cooling, wells (10 mm diameter) were punched in the agar plate using sterile cork borer and filled with 100 µl of test organisms. After incubation for 24hrs, the diameters of the inhibition zone were measured (Rammelsberg and Radler, 1990).

Production of antimicrobial metabolites by the lactic acid bacteria

Quantitative Estimation of Lactic Acid:—The quantity of lactic acid produced by antimicrobial producing isolates at 24hrs, 48hrs, 72hrs and 96hrs was determined by transferring 25ml of broth cultures of test organisms into 100 ml flasks. This was titrated with 0.1M NaOH and 1 ml of phenolphthalein indicator (0.5 % in 5 % alcohol). The titrateable acidity was calculated as lactic acid (% w/v). Each milliliter of 1 N NaOH is equivalent to 90.08 mg of lactic acid. The titrateable acidity was then calculated as stated in A.O.A.C (1980).

$$\% \text{ acidity} = \frac{\text{ml NaOH} \times \text{MNaOH} \times \text{M.E}}{\text{volume of sample}} \times 100$$

Where; ml NaOH = volume of NaOH used

N NaOH = molarity of NaOH

M.E = equivalence factor (90.08/mg)

Quantitative Estimation of Diacetyl

Diacetyl production at 24hrs, 48hrs, 72hrs and 96hrs was determined by transferring 25ml of 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. (1980).

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

Where Ak = % of diacetyl

b- s = volume of HCl used

E = equivalence factor (21.52/mg)

W = volume of broth

100 = constant

Quantitative Estimation of Hydrogen Peroxide

Hydrogen peroxide production at 24hrs, 48hrs, 72hrs and 96hrs was determined by measuring 25 ml of broth cultures of the test organisms into a 100 ml flask. To this was added 25 ml of freshly prepared 0.1M H₂SO₄. This was then titrated with 0.1M potassium permanganate (KMnO₄). Each milliliter of 0.1 N KMnO₄ is equivalent to 1.701 mg of H₂O₂. A de-colorization of the sample was regarded as the end point. The volume of H₂O₂ produced was then calculated (A.O.A.C; 1980).

$$H_2O_2 \text{ produced} = \frac{\text{ml KMnO}_4 \times N \text{ KMnO}_4 \times \text{M.E}}{\text{ml H}_2\text{SO}_4 \times \text{volume of sample used}} \times 100$$

Where ML KMnO₄ = volume of KMnO₄

NKMNO₄ = Normality of KMnO₄

ml H₂SO₄ = Volume of H₂SO₄ used

M.E = Equivalence factor (1.701/mg)

Production of Crude Bacteriocin from Isolates

Lactic acid bacteria isolates were propagated in 1000 ml MRS broth (pH 7.0, glucose, 0.25% w/v, peptone, 0.5% w/v) for 48 hrs at 28±2°C under microaerophilic conditions. For extraction of bacteriocin, a cell-free solution was obtained by centrifuging cultures which had been placed in the freezer for 1hr at 4,000 rpm for 20 min. The culture was adjusted to pH 7.0 by means of 1M NaOH to exclude the antimicrobial effect of organic acid, followed by filtration of the supernatant through whatman filter paper no1. The supernatant was dialysed for 24 h at

Table 1. Lactic count of microorganism and acidity obtained during fermentation of pearl millet

Fermentation Time (hrs)	Microbial Counts (cfu/ml)	pH	TTA
0	ND	6.1	0.54
24	1.4×10^6	4.4	2.9
48	4.2×10^6	3.9	3.42
72	1.7×10^8	3.7	4.05

MRS - De Mann Rogosa and Sharpe Agar

TTA- Total Titratable Acidity

4°C (Schillinger and Lucke, 1989).

Determination of the effect of initial pH on the production of antimicrobial substances

One hundred milliliters of composed MRS Broth was adjusted to initial pH values of 4.5, 5.5, 6.5 and 7.5, using 1M hydrochloric acid or 1M NaOH. Each medium was inoculated (1% v/v) with an overnight culture of antimicrobial producing organisms and incubated at 30°C for 48hr; the quantity of antimicrobials produced was estimated using the method of A.O.A.C (1980) as described above for the quantification of antimicrobial substances.

RESULTS

Table 1 shows the plate count obtained on the De Mann Rogosa and Sharpe (MRS) for Lactic acid bacteria count. Counts were recorded in colony forming units per ml (cfu/ml). No growth was detected on MRS agar at 0 hrs but microbial counts increased from 1.4×10^6 cfu/ml at 24hr to 1.7×10^8 cfu/ml by 72 hrs showing a rapid increase in the population of Lactic acid bacteria. The table also shows the pH and Total Titratable Acidity (TTA) of the fermenting gruel with respect to fermentation time. The pH decreased progressively from 0 – 72hr from 6.1- 3.7 while TTA increased from 0.54 at 0hrs to 4.05 at 72hrs. A total of twenty six (26) Lactic acid bacteria were identified on MRS agar. LAB isolates were screened for antimicrobial activity against selected indicator organisms and results are shown in Table 2. The zones of inhibition were the diameter of the circle formed as a result of the inhibitory activity of isolates against indicator organisms excluding the diameter of the cork borer used (10mm). The zones of inhibition ranged from 5mm - 18mm in diameter. The highest inhibitions (18mm) were from isolate FL9 against *P. aeruginosa* and *S. aureus*, isolate FL19 against *P. aeruginosa* and *S. aureus*, isolate FL14 against *S. aureus* and isolate FL 20 against *P. fluorescens* while lowest inhibition (5mm) was by isolate FL18 against *Salmonella* species. *Pseudomonas fluorescens* shows the highest susceptibility to LAB

isolates while *Salmonella* species showed the least susceptibility. 22 of the 26 LAB isolates showed antimicrobial activity; Isolates FL1, FL3, FL5 and FL22 did not show antimicrobial activity against any of the indicator organisms.

Lactic acid bacteria that showed antimicrobial activity were further characterized using various physiological and biochemical tests including growth at different pH, Gram reaction, catalase test, growth in different salt concentrations etc. Bases on these results and the results of the biochemical and physiological characterization, the antimicrobial producing LAB were identified as *L. plantarum*, *L. fermentum*, *L. jensenii*, *Lactobacillus* species, *L. mesenteriodes*, *L. brevis* and *Pediococcus acidilactici*. The percentage of occurrence is shown in figure 1, which shows that *L. plantarum* was dominant in occurrence with 45% occurrence, *L. fermentum* showed 18.2% occurrence, *L. jensenii*, *Lactobacillus* species and *L. mesenteriodes* all had 9.1% occurrence, *L. brevis* and *Pediococcus acidilactici* showed 4.5% occurrence.

Table 3 shows the quantity of secondary metabolites responsible for antimicrobial activity of the LAB isolates. The secondary metabolites detected were Hydrogen peroxide, Lactic acid and Diacetyl. Production of bacteriocins was not detected. The antimicrobial secondary metabolites were quantified by titration and the highest hydrogen peroxide production at 48hrs (0.034g/l) was by *L. mesenteriodes* (FL 15) while the lowest quantity (0.013g/l) was produced by *L. brevis* (FL18) and *P. acidilactici* (FL 6). The highest diacetyl production at 48hrs (2.80g/l) was by *L. fermentum* FL 4 and *L. plantarum* (FL 24) while the lowest (1.92g/l) was by *L. plantarum* (FL10). The highest lactic acid production at 48 hrs (1.80g/l) was from *L. plantarum* (FL9) while the lowest production at 48hrs (0.63g/l) was by *L. brevis* (FL18). The relationship between incubation time and the production of secondary metabolism was also observed with the highest quantity of antimicrobial substances produced at 48 hours for most of the isolates while there was a general decline at 72 hrs. The effect of initial pH of the medium used to incubate the isolates on production of antimicrobial substances is shown on table 4. The pH of the MRS medium was adjusted to 4.5, 5.5, 6.5 and 7.5 respectively and inoculated. Quantification of

Table 2. Diameter of zones of inhibition (mm) of indicator organisms by lab isolates

Isolates	<i>S. aureus</i>	<i>E. coli</i>	<i>Proteus</i> spp.	<i>P. aeruginosa</i>	<i>P. flourescens</i>	<i>B. lichieniformis</i>	<i>B.cereus</i>	<i>Serratia</i> spp.	<i>Salmonella</i> spp.	<i>P. syringae</i>
FL 1	-	-	-	-	-	-	-	-	-	-
FL 2	16	-	-	11	8	17	-	-	-	16
FL 3	-	-	-	-	-	-	-	-	-	-
FL 4	-	-	-	16	7	18	-	11	-	14
FL 5	-	-	-	-	-	-	-	-	-	-
FL6	-	-	-	10	11	-	14	-	-	11
FL 7	14	12	-	14	12	-	-	12	-	-
FL 8	-	8	-	10	7	-	7	6	-	-
FL 9	18	14	-	18	16	14	-	-	10	-
FL 10	17	-	-	-	15	14	-	10	-	14
FL 11	15	17	-	17	14	-	15	-	-	18
FL 12	16	-	-	13	12	-	-	-	10	16
FL 13	-	-	-	-	16	18	14	-	16	-
FL 14	18	16	-	14	15	16	-	12	-	17
FL 15	12	-	-	17	14	10	10	10	-	13
FL 16	18	12	-	-	14	14	11	10	-	-
FL 17	14	12	-	-	12	10	-	8	14	12
FL 18	10	8	-	-	12	-	-	7	5	10
FL 19	18	-	-	17	16	-	-	13	10	15
FL 20	16	12	-	12	18	14	-	12	-	18
FL 21	13	11	-	9	12	-	-	8	-	-
FL 22	-	-	-	-	-	-	-	-	-	-
FL 23	15	12	-	-	10	-	14	-	-	-
FL 24	-	-	-	13	15	14	12	13	-	-
FL 25	12	10	-	-	11	11	-	8	-	12
FL 26	14	-	-	10	13	13	-	10	-	12

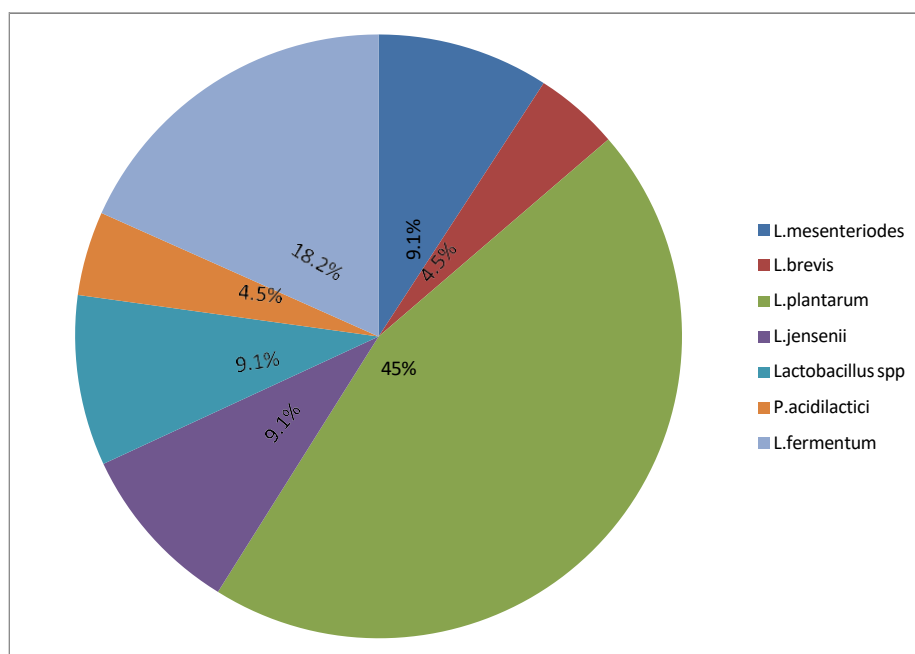


Figure 1. Frequency of occurrence of LAB species (in percentage) isolated from the fermenting millet gruel

Table 3: Quantity of lactic acid, diacetyl and hydrogen peroxide (g/l) produced by the lab isolates at different incubation times

Isolates	Lactic acid (g/l)				Diacetyl (g/l)				Hydrogen peroxide (g/l)			
	24hrs	48hr	72hr	96hr	24hrs	48hrs	72hrs	96hr	24hrs	48hrs	72hrs	96hrs
FL 2	0.63	1.26	0.72	0.45	1.83	2.69	2.15	1.92	0.013	0.021	0.013	0.009
FL 4	0.99	1.17	1.08	0.9	2.37	2.80	2.58	1.75	0.009	0.017	0.017	0.009
FL 6	0.23	1.08	0.68	0.45	2.15	2.69	2.37	1.92	0.009	0.013	0.017	0.009
FL 7	0.72	1.35	1.08	0.99	1.08	2.15	1.72	1.08	0.017	0.026	0.017	0.009
FL 8	0.72	0.81	0.77	0.77	1.51	2.15	1.72	1.51	0.017	0.026	0.017	0.017
FL 9	1.17	1.80	0.72	0.63	2.15	2.58	1.92	1.51	0.017	0.026	0.021	0.009
FL 10	1.08	1.26	1.35	0.77	1.72	1.92	1.92	1.08	0.017	0.026	0.021	0.017
FL 11	0.9	1.17	0.9	0.77	2.15	2.58	2.37	1.92	0.017	0.030	0.017	0.013
FL 12	0.9	1.08	0.72	0.68	1.51	2.37	1.72	1.08	0.017	0.030	0.021	0.017
FL 13	0.9	1.08	1.35	0.99	1.08	2.37	2.15	1.92	0.009	0.017	0.013	0.004
FL 14	0.85	0.99	0.95	0.77	1.51	2.69	2.15	1.83	0.017	0.026	0.021	0.013
FL 15	0.81	1.26	1.17	1.08	2.15	2.37	1.72	1.51	0.026	0.034	0.013	0.009
FL 16	1.08	1.44	1.17	0.99	1.72	2.15	1.92	1.92	0.026	0.026	0.017	0.013
FL 17	0.68	0.9	0.81	0.45	2.04	2.15	1.92	1.72	0.009	0.017	0.017	0.013
FL 18	0.45	0.63	0.45	0.23	1.92	2.15	2.04	1.83	0.009	0.013	0.013	0.004
FL 19	1.08	1.44	1.26	0.99	1.72	2.37	1.92	1.51	0.009	0.026	0.021	0.013
FL 20	0.9	0.99	0.95	0.68	1.72	2.58	1.92	1.72	0.013	0.026	0.017	0.009
FL 21	0.99	1.13	0.72	0.63	2.37	2.58	1.92	1.72	0.013	0.021	0.013	0.009
FL 23	1.12	1.35	1.35	1.08	2.04	2.15	1.72	1.51	0.009	0.017	0.013	0.009
FL 24	0.5	0.86	0.63	0.23	1.92	2.80	2.58	2.15	0.009	0.026	0.017	0.013
FL 25	0.63	0.9	0.81	0.68	2.15	2.58	2.26	1.92	0.017	0.021	0.017	0.009
FL 26	0.72	0.9	0.63	0.63	1.72	2.15	1.92	1.83	0.021	0.026	0.017	0.009

Table 4. The effect of initial ph on production of antimicrobial substances

Isolate	Lactic acid (g/l)					Diacetyl (g/l)			Hydrogen Peroxide (g/l)			
pH	4.5	5.5	6.5	7.5	4.5	5.5	6.5	7.5	4.5	5.5	6.5	7.5
FL 2	1.35	1.53	1.35	1.22	1.72	2.15	1.72	1.29	0.043	0.068	0.051	0.043
FL 4	1.35	1.53	1.22	1.13	1.51	4.30	1.29	1.61	0.043	0.060	0.047	0.043
FL 6	1.80	1.94	1.17	1.67	1.83	3.44	1.18	1.08	0.060	0.068	0.051	0.051
FL 7	1.71	1.98	1.85	1.44	1.18	3.02	1.08	0.86	0.043	0.060	0.043	0.043
FL 8	1.80	2.03	1.44	1.35	1.08	2.80	0.86	0.75	0.043	0.068	0.043	0.047
FL 9	1.98	2.34	2.16	1.90	1.51	3.23	1.18	0.97	0.043	0.043	0.060	0.043
FL 10	1.53	1.89	1.35	1.35	1.51	1.72	1.29	1.08	0.060	0.068	0.047	0.043
FL 11	1.35	1.35	1.22	1.08	1.72	2.37	1.08	1.08	0.034	0.055	0.043	0.034
FL 12	1.62	2.16	1.53	1.17	1.51	1.83	1.18	1.29	0.026	0.051	0.043	0.043
FL 13	1.89	2.25	1.53	1.53	1.94	3.23	1.72	0.86	0.026	0.043	0.060	0.043
FL 14	1.76	1.80	1.71	1.53	1.72	2.37	1.08	1.08	0.047	0.068	0.060	0.026
FL 15	1.17	1.62	1.53	1.35	1.29	3.02	0.97	0.75	0.047	0.068	0.060	0.055
FL 16	1.80	2.21	1.84	1.35	1.29	1.94	1.94	1.08	0.026	0.034	0.051	0.061
FL 17	1.71	2.03	1.53	1.35	1.51	1.94	1.29	1.08	0.026	0.060	0.043	0.047
FL 18	0.99	1.62	0.89	0.54	1.51	2.58	0.65	0.86	0.026	0.043	0.034	0.026
FL 19	1.80	2.25	1.98	1.49	1.29	3.02	1.72	0.97	0.026	0.047	0.026	0.034
FL 20	1.49	1.85	1.44	1.08	1.29	1.83	1.51	1.08	0.055	0.060	0.060	0.061
FL 21	1.53	2.16	1.35	1.35	1.40	1.72	0.43	1.29	0.026	0.043	0.026	0.043
FL 23	1.71	1.98	1.67	1.53	1.40	2.15	1.08	0.86	0.043	0.060	0.060	0.043
FL 24	1.58	1.89	1.35	1.26	3.02	4.08	2.80	1.29	0.043	0.060	0.051	0.051
FL 25	1.62	2.16	1.49	0.45	1.51	2.15	1.94	0.86	0.038	0.055	0.051	0.047
FL 26	1.71	2.16	1.62	1.83	1.53	2.58	1.08	1.08	0.038	0.047	0.043	0.043

antimicrobial substances produced showed that production of antimicrobial compounds was more favored by acidic pH with maximum performance at pH 5.5 and antimicrobial production reduced as pH increased above 5.5.

DISCUSSION

Various species of lactic acid bacteria (LAB) were isolated from the fermentation process. The changes in pH and titratable acidity observed in this study have been reported to be characteristic of the fermentation of carbohydrate rich plant material which is in line with findings of Odunfa and Adeyele (1985) and Sanni and Oso (1988). The decrease in pH and increase in TTA is due to the activity of Lactic acid bacteria producing acid primarily lactic acid which is a common characteristic of fermenting wet mash (Raimbault, 1995). The dominance of *L. plantarum* (45% occurrence) among the Lactic acid bacteria in the fermentation process is in line with the results of Sanni *et al.* (1999) who reported the dominance of *L. plantarum* among *Lactobacillus* spp. isolated from *ogi*, an indigenous fermented food.

The LAB isolates were able to inhibit the selected indicator organisms to varying degrees. The ability to inhibit other organisms is due to the fact that LAB produces substances which are injurious to the indicator organisms depending on the concentration or quantity produced. These substances serve as a competitive advantage to LAB when in mixed culture especially during fermentation hence the dominance of LAB during fermentation of cereals and vegetables. The ability of LAB to show antimicrobial activity against other microorganisms is well documented. For example Afolabi *et al.* (2008) showed that antimicrobial producing microorganisms had the ability to inhibit the growth of other bacteria which included both gram negative and gram positive bacteria. Such antimicrobial activity were also demonstrated in the works of other researchers such as Adesokan *et al.* (2008) where LAB species were tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Escherichia coli* and *Proteus vulgaris*.

Obadina *et al.* (2005), also showed the antagonistic effects of *Lactobacillus plantarum* on *S. typhii*, *S. aureus*, *E. coli* and *B. subtilis*. Raccach *et al.* (1979), Smith and Palumbo (1983), Cintas *et al.* (1998) have demonstrated that the antimicrobial compounds produced by LAB can inhibit the growth of pathogenic bacteria of possible contaminants in fermented products. Puttalingamma *et al.* (2006) showed the ability of LAB to inhibit pathogenic bacteria using the agar well method of Tag *et al.* (1976). The LAB isolates showing antimicrobial activity were discovered to produce antimicrobial substances like Lactic acid, hydrogen peroxide and diacetyl. This shows that the ability to inhibit other

organisms was directly related to the ability of these organisms to produce these substances. The highest quantity of antimicrobial substances was produced after 48 hours this is in line with the results of Adesokan *et al.* (2008) and Afolabi *et al.* (2008). The highest lactic acid production at 48 hrs was from *L. plantarum* (FL9) while the lowest production at 48hrs was by *L. brevis* (FL18). Ogunbanwo *et al.* (2004) also obtained a similar result for *L. plantarum* isolated from *fufu*, a traditional fermented cassava product. Daeschel (1993) reported the ability of LAB to produce Lactic acid thereby reducing the pH of the fermenting medium. The lactic acid produced serves to reduce the pH of the medium thereby making it acidic which is not conducive for the survival of spoilage bacteria which may have found their way into the fermenting vessel during spontaneous fermentation as lactic acid is a natural preservative that inhibits putrefying bacteria and is responsible for the improved microbiological stability and safety of the food. The acidity also leads to the souring of the final product which is characteristic of fermented grains and vegetables. The hydrogen peroxide produced adds to the antimicrobial activity of LAB and in some cases be a precursor for the production of other potent antimicrobial compounds such as super oxide (O_2^-) and hydroxyl (OH^-) radicals (Condon, 1987; Thomas and Pera, 1983). The antimicrobial effect of hydrogen peroxide may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus the increased membrane permeability (Kong and Davison, 1980). Nettles (1993) reported that hydrogen peroxide accumulates in cultures of *Lactobacillus*, *Leuconostoc* and *Pediococcus* species.

Diacetyl which is mainly produced by Lactic Acid Bacteria including strains of *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Lactobacillus* is effective against bacteria (Schnurer and Magnusson, 2005). In fermented foods, LAB plays an important role in the rheological and flavoring quality of the fermented product (Sozzi and Pirovano, 1993). They are able to perform this role because of the production of diacetyl, which contribute to the typical flavor and taste of many foods, particularly dairy products and their antagonistic activity has been partly traced to the antimicrobial properties of diacetyl (Jay *et al.*, 1983).

The pH of the culture medium was seen to affect the production of antimicrobial substances by LAB. Varying the initial pH of the medium and allowing the same incubation time for all the isolates showed that there was a difference in the quantity of antimicrobial substances produced. The acidic range of pH was seen to favor production of antimicrobial substance. The maximum production of antimicrobial substances occurred at pH 5.5 this is in line with the results of Afolabi *et al.* (2008). The optimum pH for the growth of LAB is 5.5 and at this pH the quantity of antimicrobial substances produced was highest. This shows that the quantity of antimicrobial

substances produced is related to the ability of the isolates to proliferate in the culture medium.

CONCLUSION

The potential application of the antimicrobial substances as consumer friendly bio-preservatives either in the form of protective culture or as additives will be significant besides being less potentially toxic or carcinogenic than current antimicrobial agent. Lactic acid bacteria and their by-products have been shown to be more effective and flexible in several applications. Most inhibitory substances produced by microorganisms are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in various foods. These isolated strains can positively have impact on use of starter cultures for traditional fermented foods, with a view to improving the shelf life, nutritional values and safety of fermented food so produced hence these organisms are recommended for use as starters and as biopreservatives

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