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The Potentials of Lime (*Citrus aurantifolia*) for Improving Traditional Corn Fermentation for Probiotic Lactic Acid Bacterial Proliferation

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Abstract: Lactic Acid Bacteria (LAB) as probiotic organisms can be sourced from fermented dairy and vegetable products. The problem in Nigeria is their affordability to the greater populace, as the common sources are relatively expensive. The study was aimed at sourcing for a low cost meal rich in probiotic LAB by utilizing raw lime juice for the fermentation of corn. Washed yellow corn variety (6%) was fermented using prepared lime solution (pH 5.1-5.5). The lime-fermented corn was wet-milled, sieved and the resulting substrate was enumerated for LAB and coliform on DeMan Rogosa Sharpe' agar and MacConkey agar plates respectively. The predominant LAB were characterized as *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus acidophilus*. At their predetermined optima growth periods (18 h, 22 h and 24 h), they were evaluated for antimicrobial potentials against *Salmonella typhi*, *Shigella dysenteriae* and *Candida albicans*. Their resistance to acid, bile and antibiotics was determined. The LAB ($\geq 3.80 \times 10^8 \pm 0.12$ cfu/ml) recovered from lime-treated samples were significantly ($p < 0.05$) higher than that from untreated samples ($\leq 5.70 \times 10^6 \pm 0.03$ cfu/ml). The pH (≤ 5.3) and coliform counts ($\leq 0.50 \times 10^1 \pm 0.13$) were also reduced in the treated sample. Although their antibiotic resistance was very low, the test LAB especially *L. acidophilus* demonstrated efficient probiotic properties by inhibiting the test pathogens, maintaining >78% acid stability at pH 3 and withstanding up to 20% bile concentration at 51.4% survival rate. These results reveal that low cost lime-fermented corn is a rich source for probiotic LAB and thus can be recommended as a food supplement.

Key words: Lime, corn fermentation, probiotics, lactic acid bacteria

INTRODUCTION

Fermentation of various food stuffs by Lactic Acid Bacteria (LAB) is one of the oldest forms of bio-preservation practiced by mankind. During the past 20 years, much of the research on LAB focused on dairy Lactococci but now investigations include different LAB involved in a wide variety of fermentation processes (Soomro *et al.*, 2002).

The LAB have demonstrated a number of desirable properties that are beneficial to mankind. Their potential benefits range from the prevention of harmful bacterial growth, antibiotic-associated diarrhea, colon cancer to immune support and management of lactose intolerance in the body (Anukam *et al.*, 2008; Ouweland *et al.*, 2002; Brady *et al.*, 2000). Based on these probiotic potentials, they are sometimes recommended by doctors and more frequently by nutritionists after a course of antibiotic to booster the body immune systems.

The crucial issue in this regard is the availability and affordability of the functional LAB for use. Much attention has been directed towards the use of dairy products such as yogurt or kefir and other fermented vegetables such as sauerkraut which are not readily affordable by

the greater populace. There is therefore considerable need to extend the range of foods harboring probiotic organisms from dairy foods to infant formulae, baby foods, fruit juice and cereal based-products (Lee and Salminen, 1995). These functional foods may not contain only probiotic LAB culture but also the prebiotic substrates that favor their growth. The fermented drink "Fyos (Nutricia)" for instance, is a combination of probiotic culture of *Lactobacillus casei* and prebiotic oligo-fructose (Soomro *et al.*, 2002). The traditional fermented corn ("Akamu") which serves as a weaning food in Nigeria has also been associated with LAB (George-Okafor *et al.*, 2007; Achi, 2005; Amusa *et al.*, 2005).

Akamu, as a LAB food supplement is likely to be effective in the treatment and prevention of acute diarrhea and dental caries in children, as observed by most supplements (infant formulae) (Weizman *et al.*, 2005). Although affordable by the populace, the limitation for its wide use has been traced to its unpleasant odor and the health risk posed by the presence of pathogenic microflora (Chukwuemeka *et al.*, 2006; Adeyemi and Beckley, 1986).

The application of sterility measure during the corn steeping had a positive impact on reducing the problem (George-Okafor *et al.*, 2007). The critical issue, however, is the adoption of such practice by the commercial producers (local producers). Hence, there is need to seek for alternative measure for improvement, not only for odor reduction but also for probiotic enrichment, since antimicrobial properties of fermented foods seem to be their most interesting quality. The study therefore, was aimed at utilizing raw lime for the 72 h-corn fermentation with a view of creating suitable environment (e.g. low pH, vitamins) for probiotic LAB proliferation and subsequent inhibition of pathogenic flora.

MATERIALS AND METHODS

Preparation of raw lime solution: A freshly plugged lime fruits were used for the study. The fruits were thoroughly washed with tap water. Thereafter, the skin and the seeds were removed prior to mechanical homogenization. The homogenized substrate was filtered by pressure with sterile muslin cloth to obtain the 'must'. The 'must' (pH 2.0-3.0) was diluted with tap water (pH 8.0) and the resulting lime solution of pH 5.1-5.5 (optimum for most LAB) (Jay, 1992) served as the fermentation medium.

Fermentation process: A well sorted and washed yellow corn variety (6%) was steeped in a steel-made fermenter (2-liter capacity), containing the fermentation medium (1000 ml). The fermentation medium was maintained at 28-38°C during steeping (Wollowski *et al.*, 2001).

Fermentation was carried out for 72 h with intermittent mechanical agitation and 48 h constant change of lime solution at 12 h intervals. The fermented corn was wet-milled (Disc Afriton Mill Model 10-2A, India) and sieved under pressure with muslin cloth to obtain the filtrate which served as the substrate for the recovery of LAB. The control sample was the filtrate from the corn steeped with only tap water (pH 8.0) and fermented under similar condition.

Bacterial strains recovery: The test and control samples were serially diluted with sterile water and 1% of each type was inoculated into DeMan Rogosa Sharpe' (MRS) medium and MacConkey broth respectively and incubated at 37°C/24 h. Thereafter, 0.1 ml of each culture was sub cultured in triplicates into the agar form of the same medium under the same experimental conditions. The pH of the fermented liquor was determined using pH meter (Model PHM 92, Copenhagen). The grown microbiomes were estimated using Quebec Colony Counter. The mean LAB counts from the two samples were compared and the significant difference at $p < 0.05$ was tested using Analysis of Variance (ANOVA).

The dominant strains of LAB were identified by morphological and biochemical tests as described by Holt (1994) and Collins *et al.* (1991). They were subjected to the following assays for proof of their probiotic characteristics for *in vivo* application.

Acid tolerance assay: A modified method of Brizuela *et al.* (2001) was adopted. Each LAB isolate (10%) was incubated under reduced O₂ content, with agitation (150 rpm) at 37°C in MRS broth adjusted with 1 N HCl for pH 2.0 and 3.0 respectively. At every 6 h interval for 24 h, the viable cells were estimated spectrophotometrically (LKB) at 600 nm. The cell density (%) was expressed in relation to the mass of the control sample (Unadjusted MRS) which was taken as 100%.

Bile tolerance assay: Freshly processed bile from a young rat gall bladder was used. The bile tolerance capacity of the isolates was tested as described for the acid except that the MRS was supplemented with the bile (5-25%) and incubated only for 24 h. The MRS medium not supplemented with the bile was the control.

Antibiotic resistance assay: The antibiotics used were Erythromycin (10 µg/ml) and Chloramphenicol (10 µg/ml) as stipulated by Denou *et al.* (2008). The prepared antibiotic solutions (1%v/v) were respectively incorporated into the MRS broth and similar conditions were applied for inoculation and incubation as earlier described. The medium (MRS) without the antibiotics was the control.

Time-course determination on LAB growth rates: The young cultures (10%) of the identified LAB (*Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus fermentum*) were respectively grown in freshly prepared MRS broth for 30 h. At every 2 h, a culture of each type was pooled and the viable cell density determined as earlier described. The cell density at 0 h served as the control. The time corresponding to the highest cell density was recorded as the optimum.

Assay for antimicrobial potentials: *Salmonella typhi*, *Shigella dysenteriae* and *Candida albicans* obtained from University of Nigeria Teaching Hospital, Ituku-Ozalla, Enugu, were the test pathogens utilized for the assay. A loopful of each LAB culture (10%), was streaked vertically in triplicates on Nutrient agar plates and pre-incubated at 37°C for 18 h (for *L. fermentum*), 22 h (for *L. plantarum*) and 24 h (for *L. acidophilus*). Thereafter, equal concentrations (10%) of each the test pathogens were cross-streaked and incubated for 24-48 h for haloes development around the contact points. The diameters of the haloes were measured and the mean results obtained.

Table 1: Bacterial growth profile from 72 h-lime-fermented and non-lime-fermented corn

Sample no.	Lime-fermented-corn			Non-lime-fermented-corn		
	pH	LAB counts ($\times 10^8$ cfu ml ⁻¹)	Coliform counts ($\times 10^1$ cfu ml ⁻¹)	pH	LAB counts ($\times 10^6$ cfu ml ⁻¹)	Coliform counts ($\times 10^3$ cfu ml ⁻¹)
1	4.9	3.80±012	0.50±003	6.5	4.50±021	1.14±003
2	5.0	4.20±033	0.40±003	6.2	5.70±030	2.90±001
3	5.0	4.70±001	0.40±013	6.7	3.80±001	7.80±014
4	5.2	5.80±005	0.50±020	6.3	5.10±005	3.40±004
5	5.3	5.30±011	0.30±015	6.6	2.60±017	5.30±011

LAB: Lactic Acid Bacteria

RESULTS AND DISCUSSION

Bacterial recovery: The growth of LAB in MRS medium inoculated with lime-fermented corn filtrate was higher ($\geq 3.80 \times 10^8 \pm 0.12$ cfu/ml) than that recovered from non-lime-fermented corn filtrate (Table 1). The growth difference and the changes in pH after the fermentation (72 h) was significant ($p < 0.05$). The significant difference in the LAB counts is an indication that lime supported the profuse growth of LAB. The support could be linked to both the acidic nature of the lime (which aided in the maintenance of adequate pH level for the LAB growth) and the supply of vitamins (B6 and C) and minerals which are essential for the metabolic activities of LAB (Okafor, 1987). The lime utilization could also be responsible for the very low pH of the lime-fermented liquor (Table 1), since LAB metabolic activities always result in high yields of acids which reduce the pH (Akpapunam and Sefa-Dedeh, 1995). Coliform count which is a measure of the safety of a product was significantly ($p < 0.05$) low in the lime-fermented corn. This is an additional advantage on the utilization of lime for corn fermentation.

The LAB yield recovered from our study was higher than that (5.0×10^5 cfu/g) recovered from Nixtamalized maize prepared by boiling whole maize in 1% lime solution for 30 min prior to fermentation (Sefa-Dedeh *et al.*, 2004). The variation could be attributed to the different fermentation methods utilized.

The dominant LAB isolates were identified to be *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Lactobacillus acidophilus*. These organisms especially *L. plantarum* have been severally isolated from spontaneous fermented cereal-based meals (George-Okafor *et al.*, 2007; Edema and Sanni, 2006; Sefa-Dedeh *et al.*, 2004).

Growth rate profile of LAB isolates: Specific growth rates of the three dominant isolates are shown in Fig. 1. *L. acidophilus* exhibited the highest growth rate but at a longer time. It achieved its optimum growth at 24 h while *L. fermentum* and *L. plantarum* demonstrated their optima growths at 18 h and 22 h respectively. The observed variation in their optima growth rates could be explained from their varying physiological characteristics as LAB grow and thrive with the production and activity of their desirable enzymes (Herrero *et al.*, 1996).

The LAB optima growth periods were determined in order to expose the LAB to the test pathogens when

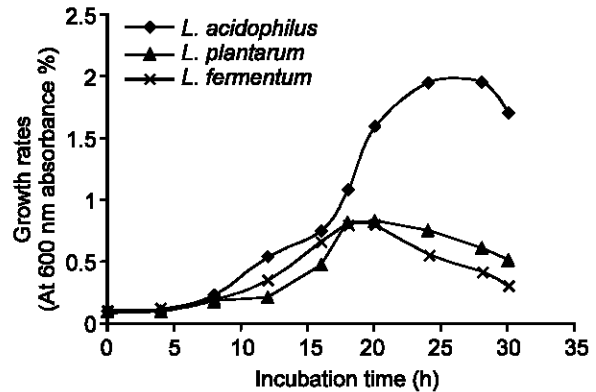


Fig. 1: Time-course on the growth rates of the lactic acid bacterial isolates

some or most of their antimicrobial products must have been produced. For instance, most bacteriocins (natural antibiotics) being secondary metabolites, are produced almost at the end of the log phase or at the onset of the stationary phase (Okafor, 1987).

Antimicrobial sensitivity: The antimicrobial potentials of the three isolates are stipulated in Table 2. *L. acidophilus* demonstrated the highest antimicrobial activity against *Salmonella typhi*, *Shigella dysenteriae* and *Candida albicans* ($> 6.2 \pm 0.02$ mm), followed by *L. plantarum* ($> 3.4 \pm 0.04$ mm). *L. fermentum* exhibited only activity against *Candida albicans* (8.1 ± 0.02 mm).

The observed antimicrobial potentials of *L. acidophilus* and *L. plantarum* are similar to other reports (Wang *et al.*, 2004; McCarthy *et al.*, 2003; Simakachorn *et al.*, 2000). However, limited information exists on the antimicrobial potentials of *L. fermentum* against the test pathogens and the susceptibility of *Shigella dysenteriae* towards *L. acidophilus* and *L. plantarum* which were observed in this study.

The susceptibility of the test pathogens to *L. acidophilus* and *L. plantarum* could be linked to stronger antimicrobial substances produced by them. For instance, *L. acidophilus* has been implicated for the production of extracellular substance which inhibited several enteropathogens (*in vivo* and *in vitro*), including *Salmonella enterica* var *typhimurium* (Coconnier *et al.*, 2000). It inhibited the adhesion of *Salmonella typhimurium* by secreting cell-surface proteins

Table 2: Antimicrobial potentials of the LAB isolates

LAB isolates	Zones of inhibition of the test pathogens (mm)		
	<i>Salmonella typhi</i>	<i>Shigella dysenteriae</i>	<i>Candida albicans</i>
<i>Lactobacillus acidophilus</i>	12.2±001	6.2±002	18.6±030
<i>Lactobacillus plantarum</i>	6.6±002	3.4±004	10.4±010
<i>Lactobacillus fermentum</i>	-	-	8.1±020

(Gueimonde *et al.*, 2006). The antimicrobial activity of *L. plantarum* could be due to the presence of plantaricin which has been reported to be lethal to most microorganisms including *Enterococcus faecalis* (Knoll *et al.*, 2008). On the other hand, the resistance of *Salmonella typhi* and *Shigella dysenteriae* towards *L. fermentum* could be explained by the presence of strong acidic polysaccharide outer layer of their cell walls which tend to protect the organisms against any bactericidal actions (Cruickshank *et al.*, 1980). It could also be as a result of the development of a great variety of toxic substances that include several acids, ammonia and digestive enzymes.

In vitro acid and bile resistance: *L. acidophilus*, *L. plantarum* and *L. fermentum* showed unrelated resistance patterns over exposure to acids (Fig. 2 and 3). Although the acid treatment reduced the Lactobacilli viable counts, yet *L. acidophilus* was able to achieve 43% and 81.2% stability at pH 2 and 3 for 18 h, while *L. fermentum* which exhibited the least resistance, maintained about 24% and 51% stability within the first 12 h. At 24 h acid exposure (pH 2), only *L. acidophilus* could withstand the harsh acidity which is similar to the strong acid level of an empty stomach (fasted condition). The acid tolerance of *L. acidophilus* could be through mediation by membrane ATPase as described by Lorca and Font de Valdez (2001).

Generally, the viable counts of the 3 *Lactobacillus* sp., were higher at pH 3 than at pH 2 (Fig. 2 and 3); an indication that their survival will be higher in non-fasted stomach condition (*in vivo*) than the fasted one (pH of 1 to 2). This observation is similar to the report that the decreased acidity following the consumption of food favored the increase in the viable cells of Bifidobacterium and Lactobacillus such that their resistance was significantly high (Saxelin *et al.*, 1999; Hull *et al.*, 1996). It is then important that the probiotic microorganisms are able to reach the Gastrointestinal Tract (GIT) and remain viable there for 4 h or more (Conway *et al.*, 1987).

The relatively reduced viable counts observed by the isolates in this study is contrary to the result obtained for *Lactobacillus* strain LB-12 which achieved small cell concentration increments at pH 3 under 24 h exposure (Brizuela *et al.*, 2001). However, our result is similar to the acid stability profiles exhibited by *Lactobacillus* strain B/103-1-5 at pH 3 and other *Lactobacillus* and *Bifidobacterium* species at pH 4.0 (Brizuela *et al.*, 2001; Charteris *et al.*, 1998).

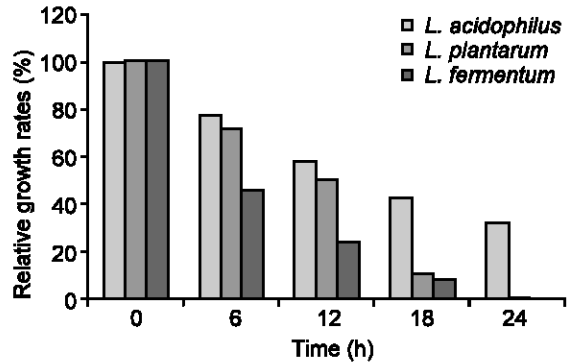


Fig. 2: Acid tolerance (pH 2) pattern of LAB

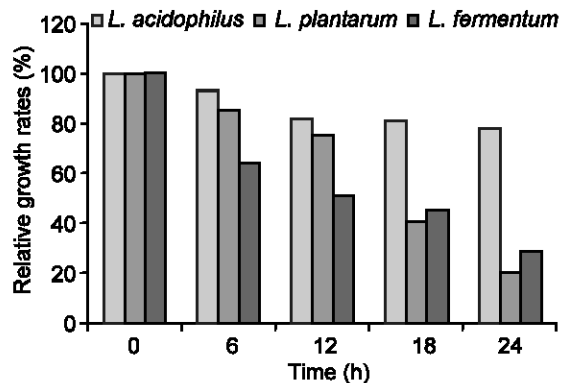


Fig. 3: In vitro acid effect (pH 3) on LAB growth

Table 3: Effect of bile concentrations on LAB growth

Bile Concn. (%)	Relative growth rates (%)		
	<i>L. acidophilus</i>	<i>L. plantarum</i>	<i>L. fermentum</i>
0	100.0	100.00	100.00
5	83.4	60.10	55.40
10	83.3	44.70	14.90
15	65.9	0.94	0.05
20	51.4	-	-
25	41.8	-	-

The resistance pattern of our LAB isolates to 5-25% of bile concentration (Table 3) is similar to that of acid stability profile. As the bile concentration increased, the viable cell concentration decreased. However, *L. acidophilus* was still able to maintain 51.4% stability at 20% bile concentration which was found to be within the normal range in human GIT (Charteris *et al.*, 1998). The observed result is encouraging as most LAB were reported to be susceptible to bovine and porcine bile

Table 4: Resistant pattern of LAB to test antibiotics

Relative growth rates (%)						
Time (h)	<i>L. acidophilus</i>		<i>L. plantarum</i>		<i>L. fermentum</i>	
	Ery	Chlo	Ery	Chlo	Ery	Chlo
0	100.00	100.00	100.00	100.00	100.00	100.00
6	53.46	48.74	45.35	32.94	21.30	24.1
12	18.85	16.51	14.31	8.21	0.50	-
18	3.27	0.57	1.03	-	-	-
24	-	-	-	-	-	-

Ery = Erythromycin; Chlo = Chloramphenicol

in vitro but resistant to human bile (Dunne *et al.*, 2001). The *L. acidophilus* bile resistance appeared to be mediated by bile salt hydrolysis which usually results in the precipitation of cholesterol (Ahn *et al.*, 2003; Ashar and Prajapathi, 1998). This reaction could be responsible for the decrease of serum cholesterol in patients treated with probiotics (Ashar and Prajapathi, 2000). The inability of *L. fermentum* and *L. plantarum* to withstand the bile concentration at 20-25% could be due to the absence or limited number of strong genes encoding bile salt hydrolysis which were identified in *L. acidophilus* (McAuliffe *et al.*, 2005).

Influence of antibiotics on the LAB isolates: The inhibitory effects of antibiotic treatment on the isolates were more than that observed for acid and bile treatment. Their resistance varies with time (Table 4). At prolonged exposure (24 h), no growth was detected especially with Chloramphenicol which exerted more cidal effect than Erythromycin. The observed cidal effect of the test antibiotics on the investigated LAB, especially at prolonged time complies with the report that the prolonged use of antibiotics has the effect of destroying LAB from digestive system and as such stimulates the development and predominance of harmful microorganisms (Hull *et al.*, 1996). We then hypothesized that LAB may not have possessed strong antibiotic resistant encoded genes which Hull *et al.* (1996) associated with different kinds of enterobacteria. Our result on antibiotic resistance differs from that reported by Mattila-Sandholm *et al.* (1999) and Brizuela *et al.* (2001), which stipulated that many *Lactobacillus* strains were able to survive in the presence of antibiotics. However, the *in vivo* antibiotic assay with mice (Denou *et al.*, 2008), produced results similar to that of the present study. The implication of this result is the proper avoidance of indiscriminate or prolonged use of antibiotics for effective protection of probiotic LAB in the digestive system.

Conclusion: The results obtained in relation to the assayed parameters gave an insight that lime which is easily affordable richly improves the fermented corn for use as a dietary probiotic supplement. Its in-take will

enrich the digestive system with adequate LAB for proper body functioning as it has mixed population of LAB with different antimicrobial characteristics. It will also improve the safety and storage stability of the product. Hence, it will be important to utilize it properly during corn fermentation for increase in LAB yields that have probiotic potentials.

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