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## Studies on the Probiotic Properties of Some *Lactobacillus* Species Isolated from Local Raw Cow Milk

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### ABSTRACT

The probiotic features and safety of *Lactobacillus* species from different foods in the advanced countries have been extensively studied. It is yet to be understood if the lactic acid bacteria that are involved in fermentation of some indigenous foods in Africa are probiotic. The probiotic characteristics of five *Lactobacillus* species isolated from local raw cow milk in Awka metropolis in Nigeria were evaluated by *in vitro* and *in vivo* methods. The *in vitro* tests used were: Tolerance to low pH and bovine bile, cell surface hydrophobicity and antimicrobial activities. Three strains showed good probiotic potentials *in vitro* and were selected for *in vivo* studies. Twenty albino rats were weighed and randomized into five groups in accordance with different treatments for each group. Three groups were administered orally with the three strains each (0.5 mL of  $10^8$ - $10^{10}$  CFU mL<sup>-1</sup> daily), while the two control groups received basal diet and sterilized skimmed milk, respectively, for six days. The blood samples were collected at the end of a ten-day post ingestion period and analysed for some blood biomarkers which included serum alanine aminotransferase, aspartate aminotransferase, serum cholesterol and haemoglobin. Results showed that three test groups had higher mean weights (203.90, 195.18 and 170.77 g) that were significantly different ( $p < 0.05$ ) from one of the control groups (119.77 g). The strains survived the gastrointestinal tract as evidenced by the *Lactobacilli* viable count (9.45-10.47 log CFU g<sup>-1</sup> faeces) in the test groups. The results of the blood biomarkers showed the three strains improved the health status of the albino rats in the test groups, to various degrees, compared to controls. Generally, *Lactobacillus* AC showed the best probiotic characteristics and could be safely used in probiotic formulations or in co-culture with other starter bacteria of fermented foods.

**Key words:** Probiotics, *Lactobacillus*, colonization factors, albino rats, biomarkers

### INTRODUCTION

The addition of microorganisms to the diet in order to provide health benefits beyond basic nutritive value is known as probiotics and commercially, the most important strains used are the lactic acid bacterial group (Vasiljevic and Shah, 2008). A strain is designated “probiotic” if it is able to survive low pH and bile salts of the stomach, adheres to intestinal cells and antagonizes pathogenic bacteria (Lin *et al.*, 2006). Beneficial bacteria could be contained in fermented foods, since dairy products appear to play roles as carriers of probiotics. These foods are well suited to promote the positive health image of probiotics (Heller, 2001). For a microorganism to develop any

beneficial effect on a host after ingestion, such a microorganism is expected to survive the intestinal tract and, at least, temporarily colonize the intestine (Iyer *et al.*, 2010). Some of the health benefits include alleviation of lactose intolerance (Gionchetti *et al.*, 2000), protection from pathogens (Corr *et al.*, 2007), anti-cholesterolemic effect and improvement of liver function (Kirpich *et al.*, 2008). The probiotic features and safety of *Lactobacillus* species from different food sources in the advanced countries have been extensively studied. This is not the case in a developing country like Nigeria where fresh, boiled or curdled milk is consumed daily by the rural population with little knowledge about its role in maintaining health or preventing diseases. The increasing need to determine the probiotic potential of strains of *Lactobacillus* from natural sources in order to improve human nutrition beyond basic nutritive value therefore forms the basis of the present study. It is yet to be understood if the lactic acid bacteria that are involved in fermentation of some indigenous foods in Africa are probiotic (Anukam, 2007). Modern 'high tech' probiotic diets are not available in developing countries but ancients had depended on foods such as raw milk to gain health benefits. For example, fresh, boiled or curdled milk is consumed by the rural population in Nigeria for daily nourishment and the Fulani women of Northern Nigeria appear to monopolize the local dairy production in the country (Walshe, 1991).

The aim of this study was to use *in vitro* and *in vivo* studies to evaluate the probiotic properties of *Lactobacillus* species isolated from the local raw cow milk. The strains were analyzed in order to obtain strains that are suitable for probiotic formulations for the local market.

## MATERIALS AND METHODS

**Source of isolates:** Raw milk samples were aseptically collected from certified healthy dairy cows at the local cattle market, serially diluted and cultured on de Mann Rogosa Sharpe (MRS) agar medium, incubated at 37°C for 48 h. Five *Lactobacillus* spp were isolated, characterized and identified based on their morphological and biochemical properties (Holt *et al.*, 1994). The five isolates were coded "*Lactobacillus* AA, AB, AC, AD and AE" respectively.

***In vitro* studies of the probiotic properties:** The isolated *Lactobacillus* species were selected for *in vitro* studies.

**Tolerance to acidity:** This was carried out as described by Khali (2009).

**Bile tolerance:** This was carried out according to Vinderola *et al.* (2008).

**Cell surface hydrophobicity assay:** This was carried out according to Vinderola and Reinheimer (2003).

**Antibiotic susceptibility test:** The antibiotic disk susceptibility test was done according to Kirby-Bauer method. The *Lactobacillus* strains were screened for possible resistance against 10 commonly used antibiotics which included: amoxicillin, ampicillin, azithromycin, chloramphenicol, ciprofloxacin, clindamycin, lincomycin, rifampicin, septrin and tetracycline. The assay was carried out using multiple discs on the same plate to eliminate differential effects from growth time and temperature. MRS agar was used since the isolates failed to thrive on Mueller Hinton agar which is the conventional medium for the test.

**Antimicrobial activity of the isolates:** The agar well-diffusion assay was used. For preparation of plates containing pathogens, nutrient agar was used for *E. coli* 1 and *E. coli* 2, *Pseudomonas* and *Staphylococcus* species. Nutrient agar supplemented with lactose was used for *Streptococcus* sourced from commercial yoghurt, MRS agar for *Lactobacillus* sourced from commercial yoghurt and sabouraud dextrose agar for *Candida* sp. Tests were carried out according to Vinderola *et al.* (2008).

**In vivo studies of the probiotic features:** Three of the five *Lactobacillus* species, being studied, were selected to undergo *in vivo* studies. *Lactobacillus* AC, AD and AE were chosen because of the probiotic potentials exhibited in the *in vitro* studies.

**Experimental design for in vivo feeding trial:** Twenty albino rats of 3-4 weeks old were procured from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The rats were weighed and grouped into five according to treatments to be given. They were fed *ad libitum* on basal diet and water for fifteen days before treatment. The rats were grouped during the treatment period (feeding period) as follows:

- Group BD:** Fed on basal diet alone through out the period (Control 1)
- Group BM:** Fed on basal diet and administered orally with 0.5 mL of sterile skimmed milk daily (Control 2)
- Group BL1:** Were fed daily with the basal diet and orally dosed with 0.5 mL of skimmed milk fermented by *Lactobacillus* AC
- Group BL2:** Were nourished daily with the basal diet and 0.5 mL skimmed milk fermented by *Lactobacillus* AD
- Group BL3:** Fed daily on the basal diet and administered orally with 0.5 mL of skimmed milk fermented by *Lactobacillus* AE

These treatments were carried out for six days and a post-feeding period of ten days was observed. The fermented skimmed milk was prepared daily by inoculating sterile skimmed milk (10% w/v and autoclaved at 110°C for 10 min) with each test strain of *Lactobacillus* and incubated microaerophilically for 18 h at 37°C. The bacterial number in fermented milk was between  $10^8$ - $10^{10}$  CFU mL<sup>-1</sup>. Animals were housed in plastic cages and monitored for any abnormality in behaviours and general health status. Individual weight of the rats was monitored once a week and the mean weight per week was calculated. Faecal samples were aseptically taken from each group on weekly basis during acclimatization period, sixth day in the feeding period and seventh day of the post-feeding period. At the end of the ten-day post-feeding period, the rats were anaesthetized by mild chloroform inhalation and blood samples were taken from the heart. The blood samples were collected into plain plastic bottles and EDTA bottles for analysis of some serum biomarkers and haemoglobin estimation, respectively.

**Analyses of rat droppings:** Faecal samples (1 g from each group) were homogenized in peptone water and serially diluted. The diluted homogenates were plated on MRS agar for the enumeration of *Lactobacilli* and on Mac Conkey agar for the enumeration of enterobacteria. This was carried out to estimate the number of *Lactobacilli* and enterobacteria during acclimatization period, feeding and post-feeding periods in order to confirm that the *Lactobacillus* species, administered to each group, were able to survive the stress in the gastrointestinal tract.

Lactase deficiency test was also conducted with faecal samples collected on the 4th day of the post-feeding period. Heat-resistant test tubes were labeled accordingly (positive control, negative control, BD, BM, BL1, BL2 and BL3). Benedict's reagent (2.5 mL) was dispensed into each tube. After which 0.2 mL of lactase control solution was dispensed into the positive control tube, 0.2 mL of distilled water was added to the negative control tube, while 8 drops of freshly passed fluid droppings (1 g of faeces homogenized in 3 mL of water) was dispensed into tubes labeled BD, BM, BL1, BL2 and BL3 in accordance with the source of the samples. The contents of the tubes were mixed and placed in a beaker of boiling water for 5 min. The tubes were then removed and the solution therein examined for colour changes and precipitate. Based on the colour changes and precipitate, the lactose concentrations were reported.

**Determination of serum alanine aminotransferase (ALT) level:** This test was carried out to determine the effect of the three *Lactobacillus* species on the rat's liver function. The Reitman-Frankel method was used as described by El-Maghraby *et al.* (2010).

**Determination of serum aspartate aminotransferase (AST) level:** This test was done to also investigate the influence of the administered *Lactobacilli* on liver function. The Reitman-Frankel method was also used like that of ALT activity, except that reagent 1 specific for AST activity assay was used (El-Maghraby *et al.*, 2010).

**Determination of serum cholesterol level:** Liebermann-Burchard reaction method was used. Haemoglobin estimation: This was done with the cyanmethaemoglobin technique which is the standard method. Uncoagulated rat's blood (0.02 mL) was diluted in 4 mL of Drabkin's fluid. The samples were read spectrophotometrically at 540 nm wave length.

**Statistical analysis:** Results were expressed as Mean $\pm$ SD deviation (SD) for each group. The data from *in vivo* studies were processed using one-way analysis of Variance (ANOVA). The level of significance was set at  $p < 0.05$ ; difference between means was checked using a two tailed student's t-test.

## RESULTS

Several *Lactobacillus* isolates were obtained from the raw cow milk samples. Only five were considered. The strains were tentatively assigned to "*Lactobacillus*" and assigned alphabets to differentiate the strains.

Figure 1-3 show initial and final absorbance of the isolates grown in MRS broth at pH of 4.0, 3.0 and 2.5 for 3 h. All isolates survived pH of 4.0 since their final absorbance/culture turbidity value appreciated significantly (Fig. 1). However, this survival was reduced at pH 3.0 (Fig. 2) with *Lactobacillus* AC surviving the most. At pH of 2.5, all test strains (except *Lactobacillus* AA) survived with significant change in culture turbidity as evidenced in Fig. 3. *Lactobacillus* AC was the most acid tolerant compared to other isolates, as supported by the 300% increase in the final culture turbidity value relative to the initial value. Generally, the survival of the isolates appreciated with increase in pH.

Table 1 show results obtained from the study of resistance to fresh bovine bile among the isolates. It is interesting to note that *Lactobacillus* AC, AD and AE with 0.0 mm each, were highly resistant to the bile used in this study. While *Lactobacillus* AB was the most sensitive with 25.0 mm inhibition zone.

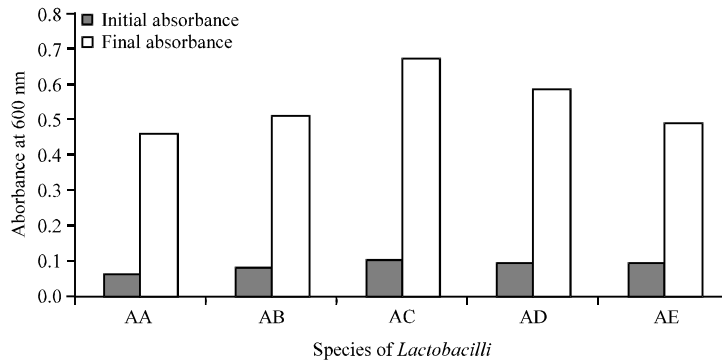


Fig. 1: Absorbance of the five strains exposed to pH of 4.0 in de Mann Rogosa Sharpe broth for 3 h

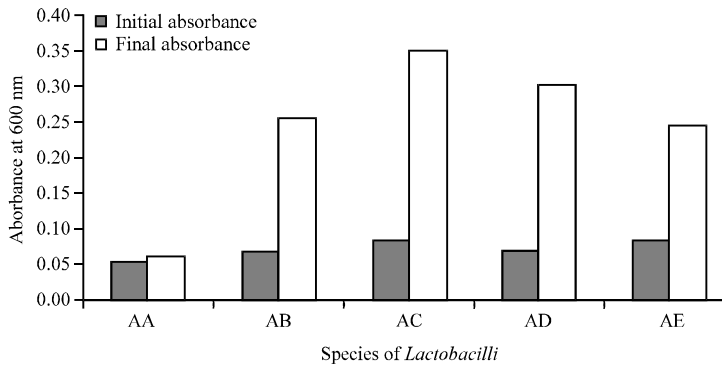


Fig. 2: Absorbance of the five strains exposed to pH of 3.0 in de Mann Rogosa Sharpe broth for 3 h

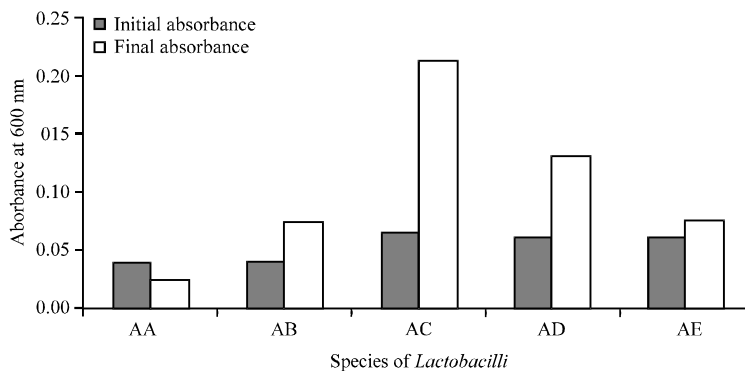


Fig. 3: Absorbance of the five strains exposed to pH of 2.5 in de Mann Rogosa Sharpe broth for 3 h

Results of the cell surface hydrophobicity which reflects the adherence potential of the strains to intestinal mucosa is shown in Table 2. The strains showed variable degree of hydrophobicity which ranged from 2.5-40.3% and *Lactobacillus* AD exhibited the highest level of hydrophobicity.

Table 1: Survival of the five strains in fresh bovine bile

Isolates	Zone of inhibition (mm)
<i>Lactobacillus</i> AA	20
<i>Lactobacillus</i> AB	25
<i>Lactobacillus</i> AC	0
<i>Lactobacillus</i> AD	0
<i>Lactobacillus</i> AE	0

Table 2: Cell surface hydrophobicity of the strains

Isolates	Hydrophobicity (%)
<i>Lactobacillus</i> AA	2.5
<i>Lactobacillus</i> AB	36.6
<i>Lactobacillus</i> AC	8.6
<i>Lactobacillus</i> AD	40.3
<i>Lactobacillus</i> AE	35.1

Table 3: Antibiotic susceptibility profiles of the *Lactobacillus* strains as obtained by disk diffusion method

Antibiotics	<sup>a</sup> AA	AB	AC	AD	AE
Amoxicillin	0	0	10	0	0
Ampicillin	0	0	0	0	0
Azithromycin	10	14	0	0	0
Chloramphenicol	15	10	0	0	0
Ciprofloxacin	0	0	0	0	0
Clindamycin	24	19	20	22	25
Lincomycin	20	20	6	9	13
Rifampicin	10	18	0	0	0
Seprtin	0	10	0	0	5
Tetracycline	6	9	0	0	0

<sup>a</sup>*Lactobacillus* isolates, \*Measured in millimeter

Table 4: Antimicrobial activity of the CFS obtained from the isolates

Test organisms	<sup>a</sup> AA	AB	AC	AD	AE
<i>Candida</i> sp.	0	0	0	17	0
<i>Escherichia coli</i> 1	0	0	19	0	0
<i>E. coli</i> 2	0	0	0	0	0
<i>Lactobacillus</i> sp. <sup>b</sup>	0	0	0	0	0
<i>Pseudomonas</i> sp.	0	0	0	0	0
<i>Staphylococcus</i> sp.	0	0	16	0	16
<i>Streptococcus</i> sp. <sup>b</sup>	0	0	0	0	0

\*Diameter measured in millimeter. <sup>a</sup>*Lactobacillus* isolates <sup>b</sup>Isolates from commercial yoghurt

All strains were sensitive to clindamycin and lincomycin which are lincosamide antibiotics as shown in Table 3. Nevertheless, *Lactobacillus* AC, AD and AE were resistant to most of the antibiotics used in this study. The zones of inhibition ranged from 5.0-25 mm. *Lactobacillus* AB was sensitive to 70% of the antibiotics used, while *Lactobacillus* AD was susceptible to only 20% of the antibiotics as shown in Table 3.

Table 4 shows that the Cell Free Supernatants (CFS) of the isolates generally had poor antimicrobial activity towards pathogenic bacteria. CFS from *Lactobacillus* AC and AE inhibited *Staphylococcus* sp., with 16.0 mm as shown in Table 4. *Lactobacillus* AA and AB failed

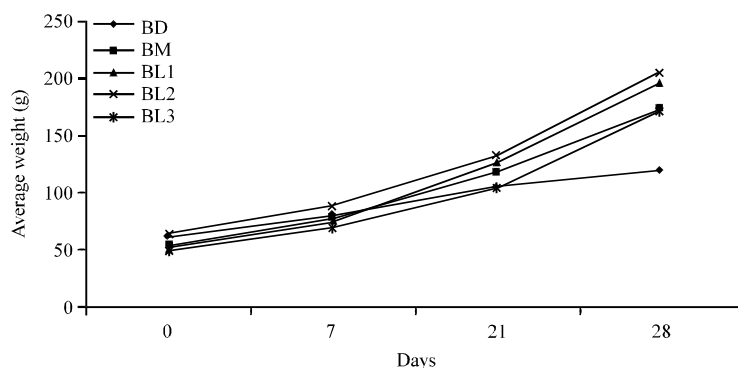


Fig. 4: Average weights of the rats at various periods. Mean value of each group. BD: Rats placed on basal diet alone, BM: Rats placed on basal diet and sterile milk, BL1: Rats placed on basal diet and *Lactobacillus* AC, BL2: Rats placed on basal diet and *Lactobacillus* AD, BL3: Rats on basal diet and *Lactobacillus* AE. Days 0-15: Acclimatization period. Days 16-21: Feeding or treatment period. Days 22-28: Post feeding period

to inhibit any of the bacteria tested. *Candida* was only inhibited by *Lactobacillus* AD. No inhibitory activity was detected against *Lactobacillus* and *Streptococcus* species sourced from commercial yoghurt.

The average weights of the rats per group at various periods are shown in Fig. 4. The initial weight of the rats on the average ranged from 50.49-65.28 g. On day 7, group BL2 had the highest weight (88.29 g) followed by group BD (80.06 g). However, by day 21 (at the end of the treatment period), group BD lost its second position to group BL1. Considering day 28, group BL2 had the highest weight gain (203.90 g) followed by group BL1 (195.18 g). The control group (BD) had the lowest weight gain (119.77 g) despite initial weight of 61.68 g. The weight of the rats in the test groups were significantly higher ( $p < 0.05$ ) than control group BD, while the weight for group BL3 was not significantly higher ( $p < 0.05$ ) than control group BM.

The viable counts of lactobacilli and enterobacteria in the faecal samples of the rats are presented in Fig. 5 and 6, respectively. The viable count of lactobacilli ranged from 5.05 log CFU g<sup>-1</sup> of faeces to 5.91 log CFU g<sup>-1</sup> of faeces sampled on days 7 and 14 (Fig. 5). On the 21st day, the lactobacilli count increased in the test groups more than the control groups: which is consistent with the different treatments each group received. Rats in group BL1 maintained the highest count of lactobacilli even up to the 28th day followed by BL3. On the other hand, the viable count of enterobacteria varied among the groups. During the acclimatization period, viable count of enterobacteria ranged from 5.46-6.22 log CFU g<sup>-1</sup> of faeces (Fig. 6). Generally, there was a decrease in viable count of enterobacteria among the test groups on the 28th day compared to other periods, except for group BL3 that had an increase in viable count during the post feeding period. The trend of viable count of enterobacteria in Group BD through out all periods can be easily distinguished compared to other groups.

Table 5 shows the levels of sugar in the faecal samples obtained from different groups. The appearance of the solution aided in estimating the concentration of sugar (lactose) in the faeces. The faecal samples from group BM contained the highest concentration of lactose (about 1.0 g%) compared to other groups. The faecal samples of group BL1 contained trace concentration of lactose, while that of group BL2 had no concentration of lactose.



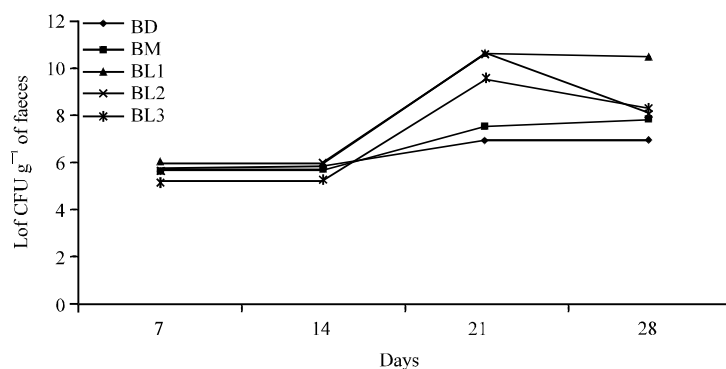


Fig. 5: Viable count of lactobacilli in faecal samples of the rats at various periods. Mean value of each group. BD: Rats placed on basal diet alone, BM: Rats placed on basal diet and sterilized milk, BL1: Rats placed on basal diet and *Lactobacillus* AC, BL 2: Rats placed on basal diet and *Lactobacillus* AD, BL 3: Rats placed on basal diet and *Lactobacillus* AE. Days 0-15: Acclimatization period. Days 16-21: Feeding or treatment period. Days 22-28: Post feeding period

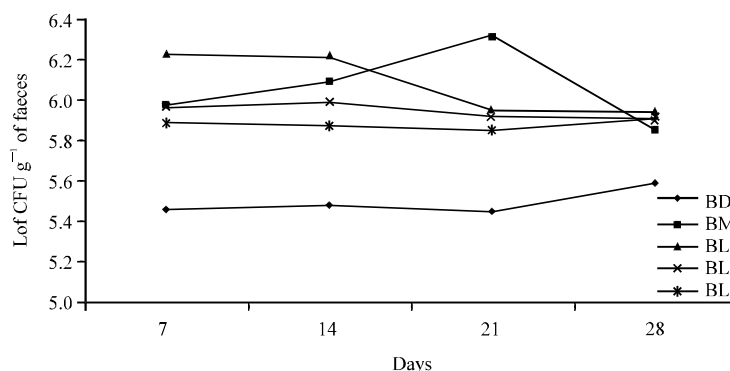


Fig. 6: Viable count of enterobacteria in faecal samples of the rats at various periods. Mean value for each group. BD: Rats placed on basal diet alone, BM: Rats placed on basal diet and sterilized milk, BL1: Rats placed on basal diet and *Lactobacillus* AC, BL 2: Rats placed on basal diet and *Lactobacillus* AD, BL 3: Rats placed on basal diet and *Lactobacillus* AE. Days 0-15: Acclimatization period. Days 16-21: Feeding or treatment period. Days 22-28: Post feeding period

Table 5: Sugar levels in the faecal samples from each group

Group	Appearance of solution	Sugar conc. (%)
BD	Green with precipitate	About 0.5 g
BM	Light brown and cloudy	About 1.0 g
BL1	Green and no precipitate	Trace
BL2	Deep blue and cloudy	Nil
BL3	Green with slight precipitate	About 0.5 g

BD: Rats placed on basal diet alone, BM: Rats placed on basal diet and sterilized milk, BL1: Rats placed on basal diet and *Lactobacillus* AC, BL 2: Rats placed on basal diet and *Lactobacillus* AD, BL 3: Rats placed on basal diet and *Lactobacillus* AE

Table 6: Levels of some blood biomarkers after the treatment period

Group	ALT (IU L <sup>-1</sup> )	AST (IU L <sup>-1</sup> )	Chol (mmol L <sup>-1</sup> )	Hb (%)
BD	37±1.82 <sup>c</sup>	49±1.82 <sup>b</sup>	1.60±0.06 <sup>bc</sup>	54±2.65 <sup>a</sup>
BM	26±3.91 <sup>bc</sup>	45±3.56 <sup>b</sup>	1.38±0.01 <sup>b</sup>	66±2.58 <sup>ab</sup>
BL1	19±3.16 <sup>b</sup>	27±1.64 <sup>ab</sup>	1.17±0.01 <sup>ab</sup>	73±3.61 <sup>b</sup>
BL2	21±2.58 <sup>b</sup>	31±6.05 <sup>ab</sup>	0.86±0.03 <sup>a</sup>	51±3.56 <sup>a</sup>
BL3	13±0.81 <sup>a</sup>	20±2.83 <sup>a</sup>	2.16±0.16 <sup>c</sup>	79±3.32

<sup>c</sup>Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Cholesterol (Chol), Haemoglobin (Hb). Two means marked by different superscripts along the columns are significantly different (p<0.05)

Moreover, the levels of some blood biomarkers of the rats are presented in Table 6. Alanine aminotransferase (ALT) activity of the rats in the control group BD was the highest (37±1.82 IU L<sup>-1</sup>) compared to other groups while the lowest was that of group BL3 (13±0.81 IU L<sup>-1</sup>). The mean levels of ALT in the test groups were significantly different (p<0.05) from control groups.

Similarly, group BD had the highest mean level of aspartate aminotransferase activity (AST) (49±1.82 IU L<sup>-1</sup>) followed closely by group BM (45±3.56 IU L<sup>-1</sup>) as shown in Table 6 but the difference between the mean levels of AST of the two control groups was not significant (p<0.05). The group of rats administered with *Lactobacillus* AE (group BL3) exhibited the lowest mean AST activity and was significantly different (p<0.05) from control groups.

Determination of serum cholesterol level of the rats revealed that the group of rats administered with *Lactobacillus* AD (group BL2) had the lowest mean level of serum cholesterol (0.86±0.03 mmol L<sup>-1</sup>) compared to other groups and was significantly different (p<0.05) from control groups. However, the reverse was the case with group BL3 which had increased level of cholesterol (2.16±0.16 mmol L<sup>-1</sup>) compared to the control group (Table 6).

The haemoglobin levels ranged from 51-79% as shown in Table 6. Group BL3 had the highest mean level of haemoglobin, while group BL2 had the lowest mean level. The mean level of group BL2 was not significantly different (p<0.05) from control group BD. The mean level of the control group BD (54%) helps to appreciate the effect of the *Lactobacillus* species on the rats in the test groups.

## DISCUSSION

The presence of *Lactobacillus* species in raw milk of different mammals has been reported by several authors (Oyetayo *et al.*, 2003; Khedid *et al.*, 2009; Martin *et al.*, 2010). The five strains, used in this study, differed in their resistance to acidity. At pH of 2.5, *Lactobacillus* AC and *Lactobacillus* AD retained viability within the 3 h of exposure as evidenced in their final absorbance values. The incubation time of 3 h chosen for this acid tolerance assay is ideal since it mimics the residence time in the human stomach (Olejnik *et al.*, 2005; Khali, 2009). *Lactobacillus* AB and AE exhibited low viability while *Lactobacillus* AA lost its viability at pH of 2.5 but all isolates showed increasing viability as pH increased to 4.0. This is in accordance with the fact that several notable lactobacilli have been found to retain viability when exposed to pH values of 2.5-4.0 but displayed loss of viability at lower values (Conway *et al.*, 1987; Dunne *et al.*, 1999; Maragkoudakis *et al.*, 2006, 2009). It will be fair to note that humans secrete approximately 2.5 L of gastric juice each day which generates a fasting pH level of 1.5, increasing to 3.0 and even up to 5.0 during food intake (Cotter and Hill, 2003; Lebeer *et al.*, 2008; Karasu *et al.*, 2010). On this note, survival of ingested bacteria in the stomach may obviously be influenced by the buffering

capacity of food components (Schillinger *et al.*, 2005) such as milk. Vinderola *et al.* (2000) demonstrated that an Argentinian fresh cheese conferred high protection to probiotic bacteria against simulated gastric acid solution when compared to naked cells. In the present study, survival of all isolates, except *Lactobacillus* AA, at pH of 2.5 implied good degree of acid tolerance and good assurance of survival in the upper gastrointestinal tract. Studying the tolerance of *Lactobacillus* strains over a range of low pH may also assist in choosing foods that will serve as carriers for delivering these strains into the gastrointestinal tract.

Resistance to bile is considered another colonization factor for probiotic strains, given that the liver excretes about 1.0 L of bile each day into the small intestine (Begley *et al.*, 2005). The effect of bile salts on bacterial cultures is much more detrimental than low pH (Olejnik *et al.*, 2005). *Lactobacillus* AA and AB were strongly inhibited by the fresh bovine bile used in this study. *Lactobacillus* AC, AD and AE exhibited resistance towards the bile. The mechanism of bile salt resistance could be the ability to deconjugate bile salts. Such feature has been related also to the capacity to remove cholesterol from the intestinal environment (Begley *et al.*, 2005), reduce serum cholesterol (Corzo and Gilliland, 1999) and also to produce a detergent-shock protein that enables lactobacilli to survive exposure to bile (De Smet *et al.*, 1995).

Adhesion is the first step necessary for colonization (Alander *et al.*, 1997). It is a desirable characteristic of probiotic strains. The adhesion of microorganisms to intestinal epithelial cells involves several mechanisms (Savage, 1992), of which cell surface hydrophobicity is one of the physico-chemical properties that facilitates the first contact between the microorganism and the host cells. This initial interaction precedes the subsequent adhesion process mediated by mechanisms involving cell surface proteins and lipoteichoic acids (Granato *et al.*, 1999; Rojas *et al.*, 2002; Schillinger *et al.*, 2005). Thus, in this study, the five strains of *Lactobacillus* were evaluated for hydrophobicity towards a hydrocarbon (xylene). The strains showed variable degree of hydrophobicity with an average of 24.6%. This aligns with the result of the study by Vinderola *et al.* (2008) which revealed that most of the isolated *Lactobacillus* strains exhibited hydrophobicity values of less than 40%. One of the explanations for the variation in hydrophobicity among strains is that adhesion depends on the surface properties of the organism (De Ambrosini *et al.*, 1998). The low hydrophobicity of *Lactobacillus* AA and AC may not imply low adhering potential when compared with the value reported by Schillinger *et al.* (2005) using a different hydrocarbon which recorded 2% hydrophobicity for *Lactobacillus acidophilus* BFE 719 and this strain was eventually able to bind to mucus-secreting cell lines by 40%. Therefore, the level of hydrophobicity of *Lactobacillus* AC, AD and AE could connote good adhering capacity to epithelial cells.

Antibiotic susceptibility profile has been outlined as an important safety criterion that is considered when novel starter cultures and probiotics are selected. This is to avoid possible transfer of antibiotic resistance genes to pathogens in food or intestine (Mattila-Sandholm *et al.*, 1999; FAO/WHO, 2002). *Lactobacillus* AC, AD and AE were resistant to most of the antibiotics used but sensitive to clindamycin and lincomycin: which are lincosamide antibiotics. *Lactobacillus* AA and AB were sensitive to most antibiotics, while being resistant to cell-wall-synthesis-inhibitor antibiotics. One of the important properties of probiotic strains is their safety for human consumption without harbouring acquired and transmissible antibiotics resistance genes (Zhou *et al.*, 2005). The high rate of resistance towards antibiotics recorded in this study is not far from expectation since lactobacilli are known to be naturally resistant towards several antibiotics (Ronka *et al.*, 2003). Such resistance feature is usually intrinsic and non-transmissible

(Curragh and Collins, 1992). The administration of antibiotics often causes disturbances in the normal microbiota of the intestine (Lindberg *et al.*, 2004). That *Lactobacillus* AC, AD and AE were resistant to most antibiotics tested, may imply competitive advantage over antibiotics-sensitive strains and should benefit patients whose normal intestinal microbiota have become disturbed (Erdogrul and Erbilir, 2006). On the other hand, genetic analysis of these strains is required to find out whether the resistance genes are plasmid borne (transmissible) or not.

The antimicrobial effect of lactic acid bacteria against pathogens is mainly by the production of lactic acid, acetic acid, bacteriocins, diacetyl, hydrogen peroxide, aldehydes and other compounds (Erdogrul and Erbilir, 2006). Generally, the antimicrobial activities of the five *Lactobacillus* isolates were low. That *Lactobacillus* AC inhibited *E. coli* 1 and *Staphylococcus* lends more weight to the findings of Oyetayo *et al.* (2003) which reported inhibitory activities among the two *Lactobacillus* species isolated from raw milk. In another study, three *Lactobacillus* strains were found to produce hydrogen peroxide but could not exert any inhibitory effect (Aroutcheva *et al.*, 2001). The *Lactobacillus* isolates used in this study could not inhibit the growth of the two starter bacteria from commercial yoghurt as shown. This may permit their use in co-culture with the conventional starter bacteria for yoghurt. No single probiotic strain is expected to possess all the beneficial properties suggested for probiotics (Ronka *et al.*, 2003).

The body weights of the rats corresponding to the different treatments differed considerably at all periods (Fig. 4). At the end of the study, group BL2 had the highest weight gain followed by Group BL1 and BL3 compared to control groups. This result aligns with other findings in studies that used broiler chickens (Islam *et al.*, 2004; Singh *et al.*, 1999). This could be because of the ability of the isolates to improve digestion and absorption of nutrients in the intestine of the rats.

The administered *Lactobacillus* AC, AD and AE survived the gastrointestinal tracts of the albino rats during the feeding period: this confirms the earlier observation in this study (Fig. 3) of their tolerance to pH 2.5. *Lactobacillus* AD-treated group (BL2) could not maintain this colonization up to the 28th day as the viable count of lactobacilli plummeted to a range similar to that of control group BM. This seems important because survival of the intestinal transit and at least transient colonization are the main preconditions for microorganisms to be of any beneficial effect after consumption (Iyer *et al.*, 2010). The activities of the *Lactobacillus* species ingested can be inferred by monitoring the viable count of enterobacteria. Considering the 21st and 28th days, there were significant decreases in the count of enterobacteria among the two test groups (BL1 and BL2) compared to control groups which had increase. There are reports indicating that several probiotic agents are able to inhibit the adherence of pathogenic bacteria to intestinal epithelial cells through their ability to increase the production of intestinal mucins (Mack *et al.*, 1999; Servin and Coconnier, 2003). Group BL3 had an increase in enterobacteria count on the 28th day which is the single datum at variance with that of Oyetayo *et al.* (2003). The ability of *Lactobacillus* AC and AD to reduce the count of enterobacteria considerably, indicates good probiotic property. Such reduction in bacterial overgrowth will prevent bacterial translocation which manifests in liver disease (Gratz *et al.*, 2010).

The faecal samples of rats in group BL2 had no concentration of lactose sugar while group BL1 contained trace concentration of sugar. This may imply good degree of expression of intracellular  $\beta$ -galactosidase (lactase) enzyme by the *Lactobacillus* AC and AD which was not the case with the indigenous lactobacilli of group BM rats. The significance of this result could be appreciated by the fact that the decline of the intestinal  $\beta$ -galactosidase (which digest lactose) activity is a common feature of the maturing intestine (Vasiljevic and Shah, 2008). Thus, lactase deficiency is unlikely in groups BL1 and BL2.

Alanine aminotransferase and aspartate aminotransferase are liver function parameters. Their increase in the general circulation of the body system implies liver dysfunction. Alanine aminotransferase (ALT) is more specific in indicating liver problems (Denniston *et al.*, 2004; Cheesbrough, 2005). The activities of these two enzymes were low in all test groups (BL1, BL2 and BL3) compared to the two control groups. This may imply better liver function as deduced from similar findings in a recent pilot study that used alcoholic subjects (Kirpich *et al.*, 2008) and broiler chickens (Islam *et al.*, 2004). The longstanding practice of using lactulose in the treatment of liver encephalopathy, suggests involvement of gut microflora in the management of chronic liver disease (Loguercio *et al.*, 2002). *Lactobacillus* AC, AD and AE were all able to improve liver function in albino rats better than those in the control groups. The mechanism could be through modification of intestinal barrier function and prevention of bacterial translocation, since patients with liver cirrhosis have imbalanced intestinal microflora with increased aerobes, anaerobes and decreased lactic acid bacterial count in stool (Zhao *et al.*, 2004).

*Lactobacillus* AC and AD reduced the serum cholesterol levels in groups BL1 and BL2 compared to controls. This may not be surprising since the isolates survived fresh bovine bile. In the present study, *Lactobacillus* AC and AD reduced the serum cholesterol levels of rats in groups BL1 and BL2 by at least 17.9% compared to control groups. The increased level of cholesterol observed in group BL3 could be as a result of variations in the baseline levels of serum cholesterol of the rats within this group.

In view of the high weight gain of the albino rats in the test groups, the haemoglobin levels of the rats were determined. The levels of haemoglobin significantly increased only in groups BL1 and BL3 compared to control groups. This could be due to increased nutrient absorption. Similar result was also obtained in a study with *Streptococcus thermophilus* strains (Iyer *et al.*, 2010). The increase in haemoglobin recorded in this study could suggest reduced risk of anaemia in susceptible individuals.

In conclusion, this study began with five strains of *Lactobacillus* and three strains were found by both *in vitro* and *in vivo* studies to possess probiotic features, even though to variable extents: which supports observations that *Lactobacillus* spp from the same ecological niche express different probiotic properties that are strain specific. The results of *in vivo* studies showed consistent relationships between ingestion of the *Lactobacillus* isolates and the health status of the albino rats.

## CONCLUSION

The strain with the best probiotic properties, among the strains investigated, was found to be *Lactobacillus* AC and could be considered a valuable addition to the field of probiotics. The results obtained from this study provide evidence for the presence of probiotic *Lactobacillus* species in local cow milk in Awka, Nigeria and have also shown preliminary indication of their health benefits and safety. The conclusion is that basic research methods possible in developing countries like Nigeria, could determine the probiotic potentials of strains of *Lactobacillus* from dairy sources to improve human health. The success of which can lead Nigeria to becoming a known producer of probiotic milk in Africa thereby leading to wealth creation. However, further studies are required before commercial application.

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